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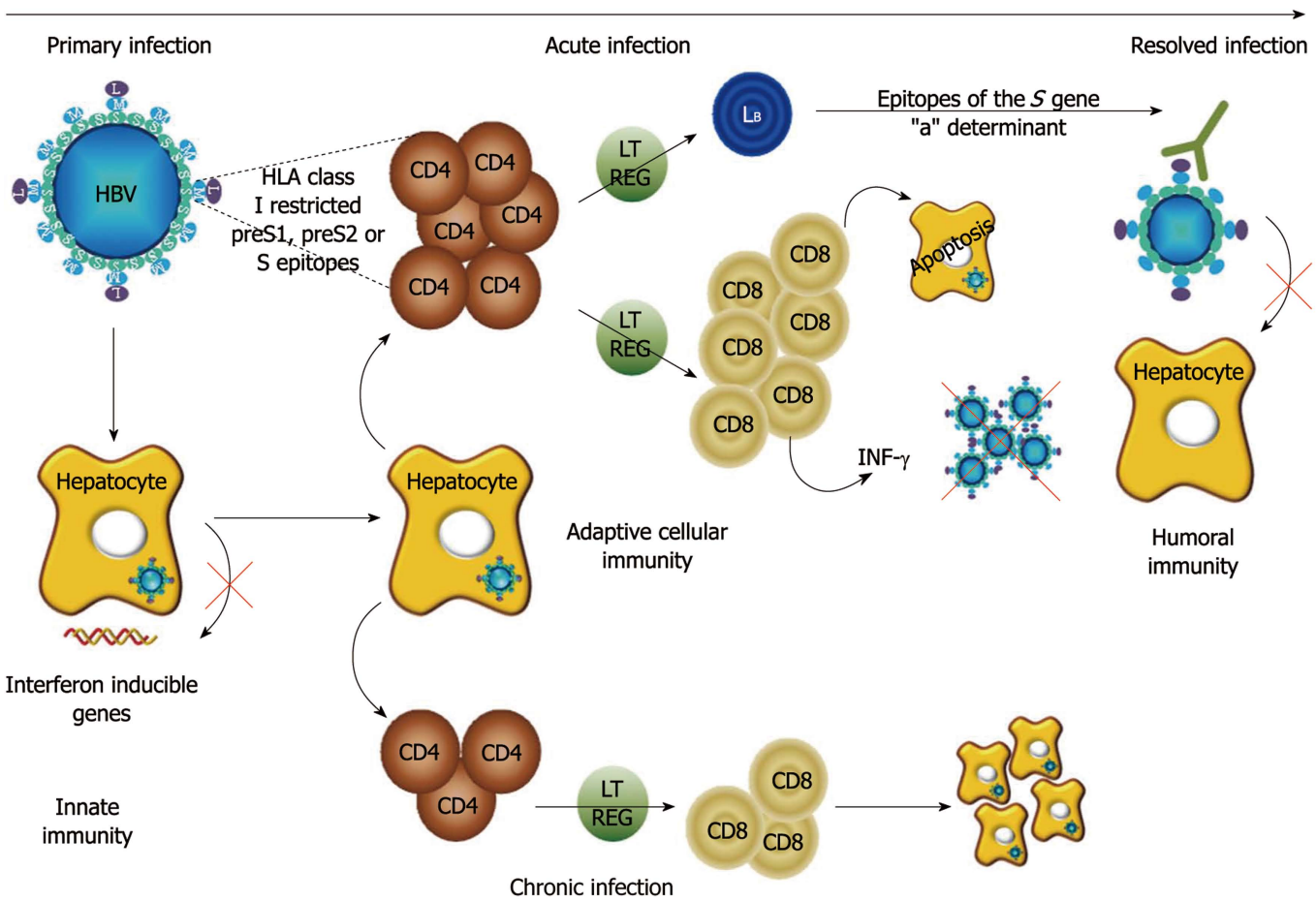

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Contents

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EDITORIAL

Serrated pathway: Alternative route to colorectal cancer

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Abstract

Serrated polyps have been an area of intense focus for gastroenterologists over the past several years. Contrary to what was thought before, a growing body of literature indicates that these polyps can be precursors of colorectal cancer (CRC). Most of these lesions, particularly those in the proximal colon, have so far been under-recognized and missed during colonoscopy, qualifying these lesions to be the main cause of interval cancers. It is estimated that 10%-20% of CRCs evolve through this alternative, serrated pathway, with a distinct genetic and epigenetic profile. Aberrant DNA methylation plays a central role in the development of this CRC subtype. This characteristic molecular background is reflected in a unique pathological and clinical manifestation different from cancers arising *via* the traditional pathway. In this review we would like to highlight morphological, molecular and clinical features of this emerging pathway that are essential for gastroenterologists and may influence their everyday practice.

Key words: Serrated pathway; DNA methylation; Hyperplastic polyps; Serrated adenomas; Colorectal cancer; Endoscopic surveillance

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INTRODUCTION

It is being increasingly recognized that colorectal cancer (CRC) is not a single disease, but rather a heterogeneous disorder including a collection of many distinct diseases with diverse molecular background and clinicopathological manifestations. According to the adenoma-carcinoma sequence proposed by Vogelstein *et al*[1] adenomatous polyps have long been considered as the sole preneoplastic lesions leading to CRC. On the other hand, hyperplastic polyps (HP) often found in the distal colon, until recently have been considered innocuous lesions, despite some contradictory opinions[2,3]. This common view has recently been challenged, as it turned out that these polyps along with other similar lesions commonly termed "serrated polyps" can be precursors to CRC[4,5].

The aim of this article is to provide a thorough clinicopathologic overview of this emerging pathway in colorectal carcinogenesis and help to understand how this accumulating data can be translated into clinical management strategies and better clinical outcomes.

CLASSIFICATION OF SERRATED POLYPS

General features of serrated polyps

The term "serrated polyp" contains a wide variety of colonic lesions and broadly refers to HP and different serrated adenomas. The main histological feature of serrated polyps is the infolding of the crypt epithelium[5], that is represented as a serrated or saw-toothed appearance in longitudinal section and a stellate or starlike appearance on cross section (Figure 1). The molecular basis for this histological feature has been attributed to decreased apoptosis[6-8] that is caused by the activated

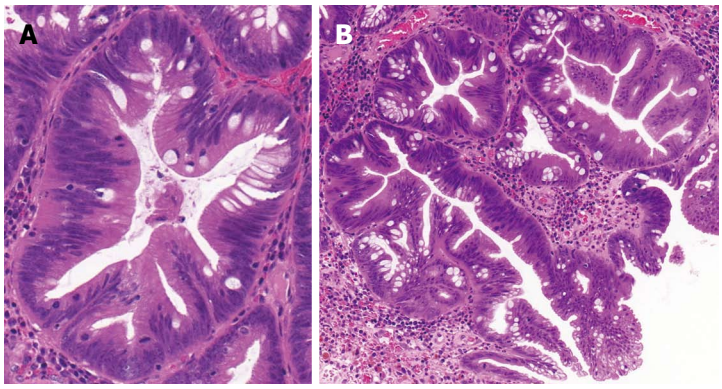

Figure 1 Microscopic features of serrated polyps. A: On cross section serrated crypt shows a stellate or starlike appearance; B: In longitudinal section a characteristic serrated or saw-toothed appearance can be seen.

mitogen activated protein kinase (MAPK)-ERK pathway that is induced by either *BRAF* or *KRAS* mutation (Figure 2). Inhibited apoptosis leads to the accumulation of non-proliferating cells. Serration is a general characteristics of this pathway from HP all the way to serrated adenocarcinoma (SAC)[9].

Hyperplastic (non-dysplastic) aberrant crypt foci

The earliest known microscopical precursors to CRC are mucosal abnormalities termed aberrant crypt foci (ACF). ACF can be further subclassified into two categories: dysplastic and hyperplastic[10,11] (also called as heteroplastic or non-dysplastic). Dysplastic ACFs, often termed as microadenomas, have been associated with sporadic adenomas arising *via* the traditional pathway[12,13]. Hyperplastic ACF may be serrated or non-serrated[11]. They are very frequent; almost every individual over 50 has at least one ACF in the distal colorectum[14]. Serrated hyperplastic ACF has a higher frequency of *BRAF* mutations, than non-serrated ACF, whereas non-serrated ACF has a higher frequency of *KRAS* mutations, than serrated ACF[11,15]. This finding supports the idea that these lesions are potentially initiating step on the serrated pathway to CRC[15], however their high frequency imply that only a small fragment progresses to HP or more advanced lesions of the serrated pathway[9]. *BRAF* and *KRAS* (mutually exclusive) mutations induce the activation of the MAPK-ERK pathway leading to decreased apoptosis and an initial burst of MAPK-ERK-dependent proliferation, leading to the formation of hyperplastic crypts. This uncontrolled proliferation is counteracted by a protective phenomenon called oncogene-induced senescence that is driven by telomere attrition, that triggers the induction of tumor suppressors including *p16*[16] or *IGFBP7*[17] (insulin-like growth factor binding protein 7), similarly as it was described in melanocytes. Hyperplastic crypts may remain dormant for prolonged periods due to the induction of crypt senescence[8] (Figure 2).

HP

HPs are the most common (80%-90%) and the best described serrated polyps. They occur most frequently in the distal colon and the rectum; they are usually slightly elevated, diminutive polyps, less than 5 mm in size. Key morphological features include elongated crypts with serration limited to the upper half of the crypt, with lack of cytologic or architectural dysplasia. These alterations can be seen only in the upper third or only on the surface of the crypts[18]. The proliferative zone may be expanded, but usually confined to the crypt base. The nuclei are small, uniform and basally placed[18], the cytoplasm is eosinophilic. If surface epithelium is not present for histological evaluation, a thickened basal membrane and muscularis mucosae with short smooth muscle extensions into the basal part of the mucosa ("comb-like" appearance) can be helpful hints to identify HP[19] (Table 1).

HPs usually occur a decade earlier (in the fifth and the sixth decade) than adenomatous polyps[20]. Several risk factors have been linked with the prevalence of serrated polyps including cigarette smoking, alcohol consumption, obesity and low-folate intake[20,21], whereas regular nonsteroidal anti-inflammatory drug use, hormone replacement therapy, and high calcium intake have been associated with reduced risk[20]. It is of note that besides smoking, all other factors have also been linked to adenoma formation *via* the traditional pathway[9,20]. This observation gained further importance when it was discovered that smoking is only a strong risk factor for those CRCs that exhibit a unique molecular phenotype [CpG island methylator phenotype (CIMP)] linked to sessile serrated pathway[22,23].

Subclassification of HP

Based on the epithelial mucin content, Torlakovic *et al*[19] histologically subclassified HPs into three categories: goblet cell-rich, microvesicular, and mucin-poor.

Microvesicular hyperplastic polyp (MVHP), also called type 2 HP, is the most common type and the typical representation of HPs encountered in the distal colon. It is characterized by large microvesicular mucin-containing epithelial cells in the upper half of the crypt, reduced goblet cells compared to normal colonic mucosa, and goblet cell abnormalities. MVHP shows prominent serration mostly in the upper half of the crypt and it has a large proliferative zone, which may take up the basal half of the mucosa. Nuclear stratification is present, but it is not prominent. The overall architecture is slightly distorted and minimal to mild crypt dilatation is present. Almost all MVHPs are slightly thicker than surrounding normal colonic mucosa. At the molecular level, MVHPs

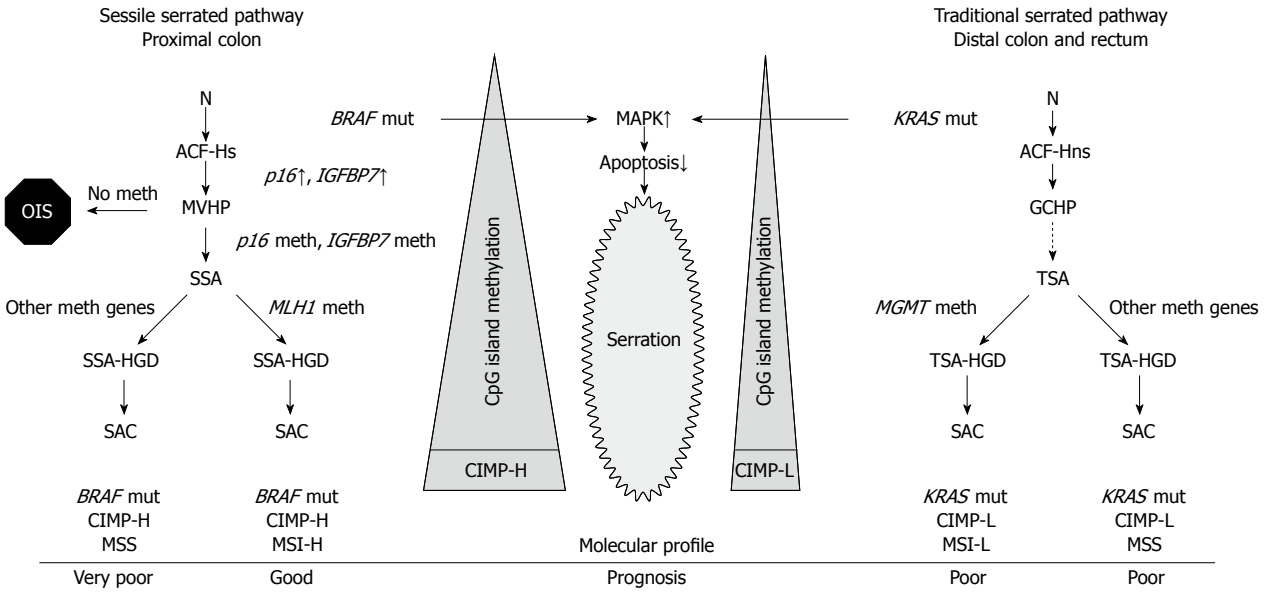

Figure 2 Schematic representation of the sessile and traditional serrated pathways. N: Normal mucosa; ACF-Hs: Serrated hyperplastic-type aberrant crypt focus; ACF-Hns: Non-serrated hyperplastic-type aberrant crypt focus; MVHP: Microvesicular hyperplastic polyp; OIS: Oncogene-induced senescence; GCHP: Goblet cell-rich hyperplastic polyp; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; SSA-HGD: Sessile serrated adenoma with high grade dysplasia; TSA-HGD: Traditional serrated adenoma with high grade dysplasia; SAC: Serrated adenocarcinoma; *IGFBP7*: Insulin-like growth factor-binding protein 7; MAPK: Mitogen activated protein kinase-ERK pathway; *MLH1*: MutL homolog 1; *MGMT*: O-6-methylguanine-DNA methyltransferase; CIMP-H: CpG island methylator phenotype-high; CIMP-L: CpG island methylator phenotype-low; MSI-H: High-level microsatellite instability; MSI-L: Low-level microsatellite instability; MSS: Microsatellite stable.

commonly (80%) exhibit *BRAF* V600E mutation[24], where valine is substituted for glutamic acid. As it was discussed above, this induces MAPK-ERK pathway that is followed by oncogene-induced senescence that includes overexpression of growth control genes *p16* and *IGFBP7* holding the cells in a dormant state. Aberrant CpG island methylation of the promoter region of *p16* and *IGFBP7* bypasses this dormant state[8] and drives MVHPs further to the next stage of serrated polyp progression, namely sessile serrated adenoma[9]. CpG island methylation is more pronounced in proximal MVHPs than in those located distally[25,26] (Figure 2).

Goblet cell-rich hyperplastic polyp (GCHP), also known as type 1 HP, is most commonly found in the distal colon, and is probably the most under-recognized variant. As its name implies this subtype is abundant of large, mature, distended goblet cells in the upper half of enlarged crypts and surface epithelial cells. Surface serrations are less prominent than in MVHP. Nuclear atypia is generally not present, but nuclei are slightly enlarged. *KRAS* mutations (in codon 12 and 13) were detected in almost half of these lesions[24,27], whereas *BRAF* mutations were rarely detected[27]. Successor lesions of GCHPs were rarely observed and it is open question whether they are self-limiting[9,24] or progress to advanced *KRAS*-mutated serrated polyps, presumably traditional serrated adenomas (TSA)[28] (Figure 2).

The mucin-poor hyperplastic polyp is the rarest form, almost absent of goblet cells. It has prominent nuclear atypia, hyperchromatic nuclei, lack of mucin, therefore it is considered to be a reactive version of MVHP with unknown clinical significance[29].

This distinction among these HP variants is primarily of theoretical importance, and an area of academic interest with little or no clinical importance at the moment. However, the distinction between HPs and more advanced lesions is of cardinal clinical importance[28] (Table 1).

PRECURSOR LESIONS TO SERRATED ADENOCARCINOMA

Serrated adenomas

In their landmark paper from 1990, Longacre and Fenoglio-Preiser[30] retrospectively overviewed 18 000 colorectal polyps and identified 110 (0.6%) as serrated adenomas. In 2003, Torlakovic *et al*[19] further divided serrated adenomas into two categories, TSA (those originally described by Longacre and Fenoglio-Preiser) and a new group identified as sessile serrated adenomas (SSAs), lesions with a serrated morphology without cytologic dysplasia.

Sessile serrated adenoma

SSAs are thought to be the second most common form of serrated polyps representing about 20% of all serrated polyps[18,19,31], however more recent studies have shown decreased prevalence (3%-8%)[32,33]. As mentioned above, before 2003 SSAs were labeled as "HP"[34]. It was demonstrated in a recent case series that according to the new WHO classification for serrated colonic polyps[35] a considerable proportion of HPs (especially those greater than 5 mm) were reclassified as SSAs[36].

Still today it is hard to distinguish SSAs from HPs, as

there are only subtle differences, and SSAs lack the typical features (such as cytologic dysplasia) of traditional adenomas. SSAs tend to locate in the proximal colon, but they can also be encountered in the distal colon (Table 1).

The microscopical features of SSAs were first described by Torlakovic and Snover[37] in their landmark paper in 1996, then the term was reintroduced in 2003[19,31]. Microscopically, most characteristic features include horizontal crypt extensions (inverted T- or L- shape) at the crypt bases, crypt branching, crypt invaginations and inverted crypts beneath the mucosal muscle layer (pseudoinvasion), mature goblet cells at the crypt bases, dilation in the lower crypts, serration throughout the crypt length, extending into the lower third of the crypt as well[18,38]. The proliferation zone can extend to the basis of the crypt. SSAs can exhibit mild nuclear atypia, but always lack cytologic dysplasia.

Endoscopically, SSAs are flat or slightly elevated, malleable lesions with irregular borders and may be covered with a thin layer of yellowish mucus giving them a pale appearance[28,38]. They are usually larger than 5 mm in diameter. Their surface is smooth or granular[28], sometimes resembling a prominent mucosal fold. These features altogether make it difficult to detect and remove completely with conventional white-light endoscope, therefore advanced, image-enhanced endoscopy techniques including traditional or virtual magnifying chromoendoscopy are needed. Chromoendoscopy is an image-enhanced endoscopic technique that highlights differences in colonic mucosa based on structural patterns, so-called "pit patterns". In a recent magnifying endoscopy study a new Type Ⅱ open-shape pit pattern (Type Ⅱ-O) was described and shown highly predictive of SSAs (with a sensitivity of 65.5% and a specificity of 97.3%). Progression of SSAs to more advanced lesions was associated with additional morphological changes, including the Type Ⅲ, Ⅳ and Ⅴ pit patterns[39] (Table 1).

On a molecular level, SSAs exhibit *BRAF* mutation and high level of CpG island methylation, supporting the hypothesis that they represent an intermediate stage between MVHPs and sporadic CIMP-H cancers. It was shown that SSAs can progress to dysplasia (SSA with high grade dysplasia, SSA-HGD) and then to CIMP-H cancers. Methylation and consequential loss of expression of *MLH1* (a major DNA mismatch repair gene) is thought to drive this transformation. Impaired mismatch repair leads to high level of microsatellite instability (MSI-H) (Figure 2). It was hypothesized that this malignant progression can occur at faster rate than that observed in the lesions emerging *via* the traditional pathway[40]. This is based on the observation that SACs are more prevalent than SSA-HGD[31,32,38]. The exact time of progression from SSA to SAC is unknown. In a recent case report an untreated SSA was described to transform into an early submucosal invasive cancer in a period of 8 months[41]. These data further underline the need for improved detection of these lesions.

Traditional serrated adenoma

TSAs were first described by Longacre and Fenoglio-Presier[30] in 1990. As mentioned above, until 2003 they were termed serrated adenomas when Torlakovic *et al*[19] divided serrated adenomas to SSA and TSA. In 2008 the same group further characterized these lesions[42]. TSAs represent the rarest subtype of serrated lesions, with a frequency of 1%-6%[18]. Similar to the majority of serrated lesions (unlike SSAs), TSAs have a predilection for the distal colon and the rectum. Macroscopically they resemble traditional adenomas, as having a pedunculated, polypoid appearance. Cytologic dysplasia (90% low-grade and 10% high-grade[18]), as their name implies, is a major feature of TSAs (Table 1).

In 2008, Torlakovic *et al*[42] proposed ectopic crypt formation as decisive morphological criterion for diagnosing TSAs. Ectopic crypts are newly formed aberrant crypts that lost their anchoring to the underlying muscular layer of mucosa[18]. Other characteristics include diffuse eosinophilic cytoplasm, mucosal bridges and protrusions resembling tennis-racquets[42]. On a molecular level, TSAs are frequently *KRAS* mutants; however they can exhibit *BRAF* mutation, and also lack both mutations.

Filiform serrated adenoma is an unusual, less aggressive variant of traditional serrated adenoma, with morphological features similar to TSA[43]. Unlike TSA, filiform SA is composed predominantly of prominent, thin, elongated filiform projections lined by neoplastic epithelium with a serrated contour[44].

Mixed polyps

Mixed polyps are combinations of traditional adenomas and serrated lesions. They are postulated to be the result of collision tumors[45] and successors of SSAs. It is not encouraged to use this term as it does not disclose the preinvasive nature of these lesions[18]. Still, "mixed polyp" is a widely used term and when used it is recommended to describe the components of these lesions (*e.g.*, TSA and traditional adenoma *etc.*)[18].

SERRATED ADENOCARCINOMA

Morphologic features

SAC, a special subtype of colorectal adenocarcinomas morphologically and histochemically resembling serrated polyps, was first described by Jass and Smith in 1992[46]. The relationship between serrated adenomas and SAC were further confirmed by Mäkinen *et al*[47], then histological characteristics of SAC were described[48] and reviewed by the same group[38]. Based on these seminal reports, most important diagnostic criteria of SAC include epithelial serrations, eosinophilic and abundant cytoplasm with vesicular nuclei, chromatin condensation and lack of necrosis. SAC was further classified into three major growth patterns. The most common (70%) serrated pattern contains mature, abundant, mucus-producing epithelium with well-preserved polarity, very similar to serrated

polyps[38]. The mucinous pattern (43%) strongly overlaps with the first group, and is characterized by eosinophilic papillary rods (93%) and eosinophilic cell balls floating in the mucus[38]. The least common (7%) trabecular pattern is a feature of poorly differentiated SACs, where serrated structures are absent and cancer cells grow in a trabecular pattern, but still these cases show eosinophilic epithelium with vesicular nuclei, uncharacteristic of poorly differentiated traditional CRCs[38].

Molecular features

Gene expression profiling study by Laiho *et al*[49] provided molecular evidence that SAC is a biologically distinct subclass of CRC. Comparison of SAC and conventional CRCs revealed 201 differentially expressed genes. Three potential candidates were identified that can be involved in the oncogenesis of SAC: Ephrin receptor B2 (*EPHB2*), hypoxia-inducible factor 1-alpha (*HIF1a*) and patched (*PTCH*) appeared as genes important for the oncogenesis of serrated CRC. *EPHB2* and *PTCH* expression are decreased in SAC compared to conventional CRC. On the other hand, constitutive overexpression of *HIF1a*, a major proangiogenic factor, can be the cause of infrequent necrosis seen in SAC[50]. Activating mutations of oncogenic *BRAF* and *KRAS* are common findings in SAC[26]. As mentioned above, these mutually exclusive mutations induce MAPK-ERK pathway that leads to the inhibition of apoptosis resulting in serrated appearance. Accumulation of CpG island methylation in the promoter regions and consequential silencing in key proapoptotic and tumor suppressor genes, such as *p16* or *IGFBP7* sets up a vicious circle. It is well established that methylation of *MLH1* leading to MSI-H phenotype (closely linked with the sessile serrated pathway) and methylation of *MGMT* leading to MSI-L are the main inducing factors in the malignant progression of serrated adenomas (Figure 2).

It is generally thought that CpG island hypermethylation is confined to the serrated pathway (CIMP)[51,52], however a recent study showed that sporadic CRCs and precursors arising *via* the traditional adenoma-carcinoma pathway also have a characteristic DNA methylation pattern different from those evolving through the serrated pathway[53].

Clinical characteristics

SAC predominantly locates to cecum (52%) and rectum (33%)[47]. It is estimated that 16% of proximal CRCs are SACs, whereas this proportion in the distal colon is only 6%[48]. It is hypothesized that proximal SACs (mostly MSI-H) arise from SSAs and distal SACs (MSI-L and MSS) originate from TSAs[38] (Figure 2). While serrated adenomas are more common in males, SACs are almost twice (1.9 : 1) as common in females, than in males[48]. The higher risk of malignant progression of serrated adenomas to SAC in (elderly) women was explained with postmenopausal estrogen deficiency and decreased folate level, however this needs to be further investigated[38].

The prognosis of SAC seems to be defined by its molecular profile (Figure 2). *BRAF*-mutated, microsatellite-stable cancers in the proximal colon confer a very poor survival[54] with adverse histological features such as lymphatic and perineural invasion and high tumor budding[55]. On the other hand, *BRAF*-mutated cancers with MSI-H phenotype (sporadic MSI-H CRCs) have a favorable prognosis[56].

SERRATED POLYPOSIS: A GENETIC PREDISPOSITION SYNDROME

Serrated polyposis, formerly called hyperplastic polyposis, is a rare form of intestinal polyposis, initially described in 1970 by Goldman *et al*[2]. Current diagnostic criteria, manifested in 2010[35], include (1) at least five serrated polyps proximal to the sigmoid colon with two or more of these being > 10 mm; (2) any number of serrated polyps proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis; and (3) > 20 serrated polyps of any size distributed throughout the colon (not all in the rectum).

Although serrated polyposis provided the first evidences for the malignant potential of serrated polyps, it is still one of the most under-recognized and poorly understood intestinal polyposis syndrome. This is probably due to its rarity (1 in 3000)[57], but also to the phenotypic plasticity[58] and overlapping clinical phenotypes within this disorder[59]. However, based on clinical observations including earlier onset of CRC, multiple cancers, increased individual and familial risk, accumulating evidence indicates that serrated polyposis is a genetic predisposition syndrome to CRC and probably confers also an increased risk for some extracolonic cancers[60].

IMPLICATIONS FOR MANAGEMENT OF SERRATED POLYPS

It is imperative to detect and completely remove serrated lesions, as majority of these lesions tend to progress, and contribute to the development of interval cancers. Data on the natural history of serrated polyps is limited, only retrospective studies with small sample size[61] are currently available. High risk serrated polyps are frequently flat and associated with synchronous advanced colorectal neoplasms[62]. Magnifying chromoendoscopy can facilitate to differentiate between serrated polyps, but it is still difficult to distinguish between SSA and typical HP[63]. Both endoscopists and pathologists should know the most important features in order to detect and diagnose these lesions (Table 1).

To date, no consensus guidelines exist on the management of serrated polyps, but new guidelines including recommendations for management and follow-up of serrated polyps are expected to be available in the near future. With the exception of small and diminutive HPs in the rectosigmoid, that confer no malignant potential,

Table 1 Most important features of serrated polyps[18,19,38,39,62]

	Sessile serrated adenoma	Traditional serrated adenoma	Hyperplastic polyps
Location	Proximal	Distal	Distal
Macroscopic characteristics	Sessile, flat, covered with mucus, poorly defined borders	Protruding, pedunculated	Flat
Color	Normochromatic, pale	Reddish	Pale
Size	> 5 mm	> 5 mm	< 5 mm
Molecular features	*BRAF* mt	*KRAS* mt	
Histological characteristics	Dilated, branched serrated crypts at the bottom	Prominent crypt serration, ectopic crypt formation	Serrations at the top
Pit pattern	Open-shape (type II-O)	Fern or pinecone-like	Starlike (type II)
Precursor	MVHP	GCHP	ACF
Malignant potential	+++	++	-
CIMP status	CIMP-H	CIMP-L	
MSI status	MSI-H or MSS	MSI-L or MSS	MSS
Gender predominance	Female	Male	Male
Dysplasia	Absent	Present	Absent
Ectopic crypt formation	Absent	Present	Absent

MVHP: Microvesicular hyperplastic polyp; GCHP: Goblet cell-rich hyperplastic polyp; ACF: Aberrant crypt focus; CIMP-H: CpG island methylator phenotype-high; CIMP-L: CpG island methylator phenotype-low; MSI-H: High-level microsatellite instability; MSI-L: Low-level microsatellite instability; MSS: Microsatellite stable.

Table 2 Colonoscopic management and surveillance strategies for serrated polyps based on experts' opinion and current literature[28,34,51,63]

Serrated polyp	Intervention	Surveillance interval
Hyperplastic polyp in the rectosigmoid	No surveillance recommended	Screening colonoscopy at 10 yr
SSA without dysplasia	Endoscopic resection (EMR)	< 3 lesions, < 1 cm: 5 yr ⩾ 3 lesions, ⩾ 1 cm: 3 yr
SSA with dysplasia	Endoscopic resection (EMR)	Complete: 2-6 mo Incomplete: Segmental colectomy
TSA	Endoscopic resection (cold-snare)	Complete: 3 yr Incomplete: Segmental colectomy
Serrated polyposis	Endoscopic resection	Follow-up colonoscopy at 6-12 mo Children: screening at an age 10 yr younger than index case
Serrated polyposis in first-degree relative	Screening at an age 10 yr younger	Follow-up colonoscopy at 12 mo

SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; EMR: Endoscopic mucosal resection.

all other serrated polyps should be endoscopically removed. If endoscopic resection cannot be implemented, then segmental colectomy is advised[51]. There is no consensus among experts on the optimal post-polypectomy surveillance intervals, however because of lack of data and presumed faster progression rate a more intensive surveillance is recommended (Table 2).

Due to their sessile nature, SSAs are difficult to detect and remove endoscopically. For the removal of flat SSAs endoscopic mucosal resection (EMR) is the method of choice[34]. It is recommended to use a chromoendoscopy contrast dye (either onto the surface or injected submucosally) to define the border of the lesion, lifting it, then snare removing *in toto* or in multiple sessions[28,34]. It is important to note that because of the thin wall of proximal colon (where SSAs typically locate[65]), this difficult technique is even more challenging, as one has take the complications of EMR (bleeding, perforation, incomplete resection) into account and it is advised to use argon plasma coagulation to reduce the risk of complications and recurrence[34,66].

CONCLUSION

It is getting generally accepted that CRC is a heterogeneous disease. Serrated pathway is serrated pathway is a rapidly evolving concept in colorectal carcinogenesis and it is postulated that 10%-20% of CRCs arise *via* this alternative pathway. In the past two decades since its original description our knowledge of morphologic and molecular alterations of serrated lesions has greatly expanded and these lesions are getting increasingly recognized. A major challenge is how to translate these new findings into clinical practice and how to determine appropriate surveillance intervals in order to avoid interval cancers. Further investigation is needed to better characterize natural history, optimize management and improve clinical outcomes.

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REVIEW

Controversial role of toll-like receptors in acute pancreatitis

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Abstract

Acute pancreatitis (AP) is a common clinical condition with an incidence of about 300 or more patients per million annually. About 10%-15% of patients will develop severe acute pancreatitis (SAP) and of those, 10%-30% may die due to SAP-associated complications. Despite the improvements done in the diagnosis and management of AP, the mortality rate has not significantly declined during the last decades. Toll-like receptors (TLRs) are pattern-recognition receptors that seem to play a major role in the development of numerous diseases, which make these molecules attractive as potential therapeutic targets. TLRs are involved in the development of the systemic inflammatory response syndrome, a potentially lethal complication in SAP. In the present review, we explore the current knowledge about the role of different TLRs that have been described associated with AP. The main candidate for targeting seems to be TLR4, which recognizes numerous damage-associated molecular patterns related to AP. TLR2 has also been linked with AP, but there are only limited studies that exclusively studied its role in AP. There is also data suggesting that TLR9 may play a role in AP.

Key words: Acute pancreatitis; Severe acute pancreatitis; Pathophysiological mechanism; Toll-like receptors; Intervention

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INTRODUCTION

A central characteristic of the innate immune system is its capability to identify constitutive and conserved products of microbial metabolism. Several metabolic pathways are unique for invading microorganisms and absent in host cells. Being essential for survival, they are even highly conserved among a given class of microorganisms. A classical example is lipopolysaccharide (LPS), a molecule made by *Gram-negative bacteria*, but not by eukaryotic cells.

Molecules found in microorganisms, but not in host cells, can serve as molecular signatures that can be recognised by the innate immune system, starting an immunological response against the invasion and if necessary, assisting in the activation of the adaptive immune system[1]. As these molecules are conserved molecular patterns, they are called pathogen-associated molecular patterns (PAMPs) and interact with pattern-recognition receptors (PRRs), which are receptors of the innate immune system.

PAMPs are marvellous targets for innate immune recognition, as they are only produced by microbes, are invariant between microorganisms of a given class and are essential for microbial survival. Therefore, PRRs representes a vital component of the immune system, which probably developed early during evolution, as PRRs are found in all mammals, invertebrates and even in plants. PRRs are expressed on the cell surface or in intracellular

compartments, but they can be secreted into the blood stream and tissue fluids as well[2]. However, the role of PRRs in immune recognition is not solely associated to PAMPs, since PRRs in addition can recognize alarmins[3]. Alarmins are endogenous molecules released into the extracellular compartment by activated or necrotic cells in response to stress or tissue damage[4]. Even extracellular matrix molecules are alarmins when up-regulated upon injury or degraded following tissue damage[5]. Alarmins and PAMPs constitute damage-associated molecular patterns (DAMPs), which are the main targets of PRRs[3,4].

The relative recent discovery of a main PRR family, toll-like receptors (TLRs), has vastly increased our comprehension of the physiological and pathophysiological role of the innate immune system. While mice have twelve TLRs (TLR1 to TLR9 and TLR11 to TLR13), humans express only ten functional TLRs (TLR1 to TLR10) and ligands have been identified for all human TLRs, except for TLR10[3]. A selection of PAMPs and alarmins recognized by human TLRs are shown in Tables 1 and 2.

TLRs are type I transmembrane glycoproteins composed of an extracellular N-terminal that contains leucine-rich repeats, a single transmembrane domain and an intracellular C-terminal tail known as the Toll/IL-1 receptor (TIR)[6]. When TLRs form heterodimers or homodimers, an activating signal is started and TIR-TIR dimers are involved in the recruitment of five known signalling adaptor molecules: Myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIRAP inducing interferon β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile-alpha and Armadillo containing motif protein[7-9].

Once the adaptor molecules are recruited, TLRs can activate two major intracellular signalling pathways. All TLRs except TLR3 can activate a MyD88-dependent pathway, in which IL-1R-associated kinases (IRAK), TNF receptor-associated factor 6 (TRAF-6) and mitogen-activated kinases are involved[5]. This pathway results in the transcription of pro-inflammatory genes through the activation of nuclear factor κβ (NFκB) and/or the activation of activating protein 1[9]. An alternative, non MyD88-dependent pathway can be activated by TLR3 and TLR4. In the TRIF pathway, the activation of interferon-regulated factors (IRF) *via* TRIF leads to the synthesis of interferon (IFN)[5].

As TLRs recognize several PAMPs and DAMPs, their involvement in the pathophysiology of several diseases has become a major research field[8,10]. Moreover, recent studies have reported the importance of NFκB pathways in the development of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS)[11,12]. However, NFκB pathways are not restricted to TLRs, which converts the elucidation of the role of TLRs in SIRS and MODS into a tremendous challenging task.

SIRS is a nonspecific condition that can be caused by infection, ischemia, trauma, and inflammation, or by the combination of several insults. Considered by many as a self-defense mechanism, SIRS results in a complex inflammatory cascade that involve humoral and cellular responses, complement, and cytokines cascades. Severe complications depend on the underlying etiology and the magnitude of the inflammatory response, and may include *e.g.*, single or multiple organ failure[13].

Table 1 Human Toll-like receptors: Localization, known pathogen-associated molecular patterns and their producing microorganisms

	Localization	PAMP	Origin of PAMP
TLR1	Plasma membrane	Soluble factors	*N. meningitidis*
		Triacyl lipopeptides	*Bacteria, mycobacteria*
TLR2	Plasma membrane	Glycoinositolphospholipids	*Trypanosoma cruzi*
		Glycolipids	*Treponema maltophilum*
		Haemagglutinin	*Virus*
		Lipoarabinomannan	*Mycobacteria*
		Lipoprotein/lipopeptides	*Various pathogens*
		Lipoteichoic acid	*Gram-positive bacteria*
		Peptidoglycan	*Gram-positive bacteria*
		Phenol-soluble modulin	*S. epidermidis*
		Porins	*Neisseria*
		Zymosan	*Fungi*
TLR3	Endosome	Double-stranded RNA	*Virus*
TLR4	Plasma membrane	Envelope protein	*Mouse-mammary tumour virus*
		Fusion protein	*Respiratory syncytial virus*
		Heat-shock protein 60	*Chlamydia pneumoniae*
		Lipopolysaccharide	*Gram-negative bacteria*
		Taxol	*Plants*
TLR5	Plasma membrane	Flagellin	*Bacteria*
TLR6	Plasma membrane	Diacyl lipopeptides	*Mycoplasma*
		Lipoteichoic acid	*Gram-positive bacteria*
		Zymosan	*Fungi*
TLR7	Endosome	Single-stranded RNA	*Virus*
TLR8	Endosome	Single-stranded RNA	*Virus*
TLR9	Endosome	DNA (CpG)	*Bacteria, virus*
		Haemozoin	*Plasmodium spp.*
			Rhodnius spp.
			Schistosoma spp.
TLR10	Endosome	Not determined	Not determined

Modified after Akira *et al*[7]. TLR: Toll-like receptor; PAMP: Pathogen-associated molecular patterns.

Acute pancreatitis (AP) is one common cause of SIRS and MODS. AP occurs with an incidence of about 300 or more patients per million annually[14,15], reported to have increased during the last decades[16,17]. Since the principal risk factors for AP are gallstones and excessive alcohol intake[18,19], plausible the augmented incidence reflecting the overweight epidemic observed in the Western World[20-22].

Most cases of AP are mild and self-limiting, with only brief need of clinical support[14,15]. However, about 10%-15% of patients will develop severe acute pancreatitis (SAP) and of those, 10%-30% may die due to SAP-associated complications (Table 3)[23]. Complications may be local or extrapancreatic and in up to one third, the pancreatic injury leads to pancreatic necrosis, acute fluid collections and pseudocyst formation. SIRS may develop early during the course of SAP and cause *e.g.*, adult respiratory dysfunction syndrome,

Table 2 Human toll-like receptors and a selection of known alarmins

	Proteins, peptides	Fatty acids, lipoproteins	Proteoglycans, glycosaminoglycans
TLR1	β-defensin-3		
TLR2	Antiphospholipid antibodies β-defensin-3	Serum amyloid A	Biglycan
	Eosinophil-derived neurotoxin		Hyaluronic acid fragments
	Heat-shock protein 60, 70, Gp96		Versiglycan
	High-mobility group protein B1 (HMGB1)		
	HMGB1-nucleosome complexes		
	Surfactant protein A, D		
TLR4	Antiphospholipid antibodies	Oxidised low-density protein	Biglycan
	β-defensin-2	Saturated fatty acids	Heparan sulphate fragment
	Fibrinogen	Serum amyloid A	Hyaluronic acid fragment
	Fibronectin (extra domain-A)		
	High-mobility group protein B1		
	Heat-shock protein 60, 70, 72, 22, Gp96		
	Lactoferrin MRP8, MRP14		
	Neutrophil elastase		
	Surfactant protein A, D		
	Tenascin-C (fibrinogen-like globe)		

Modified after Paccinini *et al*[5]. TLR: Toll-like receptor.

Table 3 Complications of severe acute pancreatitis

Pancreatic	Systemic
Abscess	Acute kidney failure
Fat necrosis	Acute liver failure
Hemorrhages	Adult respiratory distress syndrome
Infected necrosis	Disseminated intravascular coagulation
Pseudocyst formation	Encephalopathy
Sterile necrosis	Gut ischemia
	Hypocalcemia
	Paralytic ileus
	Shock

Modified after Baddeley *et al*[24].

acute renal and liver failure[24]. SIRS in association with SAP may also result in MODS, which carries a 40% mortality rate[15]. A model for the course of AP is shown (Figure 1).

Despite the improvements done in the diagnosis and management of SAP, the rate of mortality has only marginally declined during the last decades[24]. This is of particular concern, considering that AP patient numbers has been persistently increasing. In order to decrease the mortality rates, it is imperative to find new therapeutic strategies that target the underlying pathophysiological mechanism of SAP.

TLRs seem to play a major role in the development of numerous diseases[5,10], which make these molecules attractive as potential therapeutic targets. Since SIRS is a potentially lethal complication in AP, and TLRs are involved in the development of SIRS[25]; it is plausible that TLRs play a major role in severe acute pancreatitis.

In the present paper, we aim to explore the current knowledge about the role of different TLRs that have been reported associated with AP. In addition, future therapeutic strategies will be discussed.

TLR2-FAR AWAY FROM THE ANSWER

TLR2 is expressed on the plasma membrane of a large diversity of cells, including monocytes and macrophages, dendritic cells, polymorphonuclear leukocytes, B cells, T cells and microglia. This PRR recognises a wide range of DAMPs (Tables 1 and 2) and forms heterodimers with TLR1, TLR6 or TLR10[10]. Usually associated to the innate immune response against *Gram-positive bacteria* (several of its ligands origin in these microorganisms), TLR2 signals through a MyD88-dependent pathway (Figure 2A). CD14 is a protein found either in soluble form or anchored into the plasma membrane by a glycosylphosphatidylinositol tail. It has been reported that CD14 is required for the activation of TLR2-TLR6 dimers, since it facilitates the transport of specific ligands, such as peptidoglycan or lipoteichoic acid. Additionally, CD36, a membrane protein found in lipids rafts, seems to be important in the activation of TLR2-TLR6 pathways. However, it is unknown how CD36 enhances the formation of TL2-TLR6 dimers. CD44 is another membrane protein that has been associated to TLR2 and appears to enhance TLR2-mediated pro-inflammatory response[26]. However, the mechanisms are still unknown. There are also reports suggesting that CD44 may interact with TLR2, down-regulating TLR2-mediated inflammation[27,28].

Upon dimerization with TLR1, TLR6 or TLR10, TIR-TIR dimers recruit TIRAP, which is needed for the further recruitment of MyD88. MyD88 interacts with IRAK-4, which phosphorylates IRAK-1 and IRAK-2. IRAK-1 and IRAK-2 then activate TRAF-6, which in turn activates TGF-β activated kinase-1 (TAK-1). TAK-1 phosphorylates I-κ-β kinase β (IKKβ), which together with IKKα phosphorylate I-κ-β kinase α (IKKα). Upon phosphorylation IκBα becomes inactive, allowing the nuclear translocation of NFκB, with subsequent production of several cytokines and molecules involved in immune responses (Table 4)[29].

Besides its role in infectious diseases, TLR2 has also been reported involved in several non-infectious disorders, including atherosclerosis, asthma, renal disease, systemic lupus erythematosus and even sporadic colorectal cancer[30-34]. Though, the role of TLR2 in AP has only been evaluated in a limited number of studies.

Pancreatic damage

The TLR2mRNA expression was found to be increased

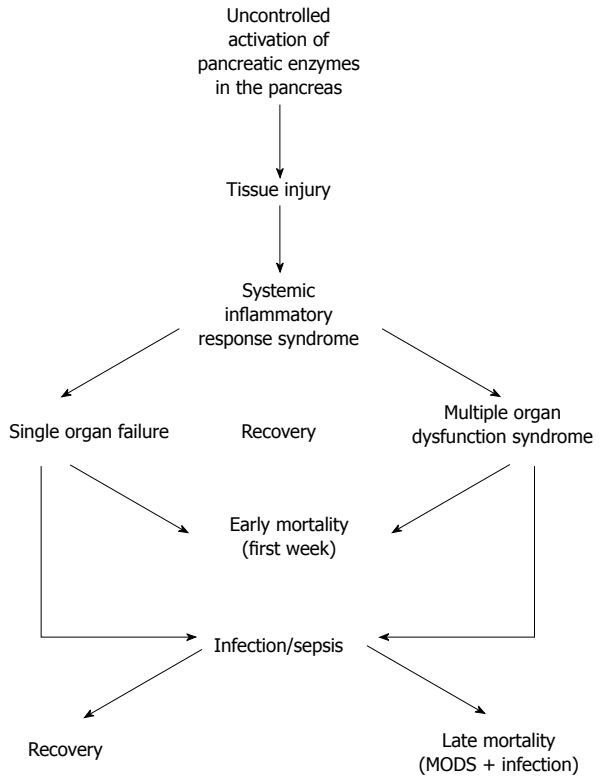

Figure 1 Systemic inflammatory response syndrome in acute pancreatitis.

in the pancreas in cerulein-induced AP in rats[35]. Furthermore, TLR2 levels in the gland were also increased, as well as TNF-α, intracellular adhesion molecule 1 and IL-6. TLR2mRNA overexpression seemed to be caused by peroxisome proliferator-activated receptor-α (PPAR-α), since WY14643, a synthetic PPAR-α antagonist, decreased TLR2 levels in treated animals. Even if the observed TLR2mRNA overexpression was associated with PPAR-α, it is uncertain how important TLR2 was in the development of inflammation and tissue damage. Possibly, hyaluronic acid fragments found in the injured pancreas could be a major source of TLR2 upregulation[36]. Small molecular weight hyaluronic acid fragments seem to need TLR2 in order to stimulate the pro-inflammatory state in mouse macrophages[37]. Heat shock proteins may leak into the extracellular compartment after necrotic cell death in AP, interact with CD14/TLR2 and induce the production of inflammatory cytokines (especially TNF-α)[38].

Lung injury

Acute lung injury (ALI) is possibly the most serious complication associated to SAP, since it accounts for most deaths in untreated patients and in hospitalised patients that die during the first week after the onset of AP[39].

In 2005, Wu *et al*[40] described that TLR2mRNA overexpression in the lungs in a sodium taurocholate-based AP rat model was coupled to elevated TNF-α levels and lower nitric oxide (NO) levels, when compared to controls. Moreover, L-Arg administration decreased TLR2mRNA expression and pulmonary TNF-α levels. Since L-Arg stimulates the production of NO, it is difficult to evaluate if the anti-inflammatory response was caused by NO itself or by a L-Arg-mediated TLR2 down-regulation. Chloroquine showed similar effects (both TLR2mRNA and TNF-α were decreased) but the lung injury was not significantly ameliorated. Chloroquine is a well-known anti-malaria drug that prevents against endosomal acidification[41] and is used to study the role of intracellular TLRs[42].

The authors concluded that SAP-associated ALI was coupled to higher TLR2 levels in the lungs and that reduced NO levels were in part responsible for the grade of inflammation, since NO has known anti-inflammatory properties. However, how NO levels are associated with TLR2 and its potential therapeutic use in AP, is still unknown.

Matsumura *et al*[43] reported that the expression of TLR2mRNA decreased in pulmonary macrophages in deoxycholate-induced AP in rats. Macrophages were obtained through bronchoalveolar lavage fluid (6 h after induction) and the cells were exposed to lipoteichoic acid. No change in TLR2mRNA was initially observed, but after six hours TLR2mRNA expression significantly decreased in macrophages. Additionally, the production of TNF-α after lipoteichoic acid stimulation was reduced. An important observation made was the increment of bacterial translocation (cultured from mesenteric lymph nodes) 18 h after AP-induction, indicating that impaired TLR2mRNA expression in pulmonary macrophages could partially be responsible for the development of ALI in SAP complicated by sepsis. It is widely accepted that $TLR2^{-/-}$ mice are vulnerable to infections[44,45]. Pulmonary macrophages have been pointed out as major players in ALI in SAP, as monocyte chemoattractant protein-1 and TNF-α are released by these cells[46]. Hence, diminished TLR2mRNA expression in pulmonary macrophages under AP is doubtfully important for the development of ALI when TLR2-related PAMPs are absent.

Liver involvement

Liver failure is a major complication in AP. Besides, there is increasing data suggesting that activated Kupffer cells mediates the inflammatory response and pulmonary damage seen in SAP[47].

Xiong *et al*[48] showed that hepatic TLR2mRNA expression was increased in AP-induced mice. The incremented TL2mRNA expression was observed 3 h after the induction, peaking at 12 h. Likewise, severe damage in the liver and SIRS ensued in the animals. However, in order to strengthen the immunological reaction, LPS was injected as well, leading to a TLR4mRNA expression increment in the liver. Thus, it is difficult to establish if SAP-related SIRS is associated with an increase in TLR2 or TLR4. Possibly, a combined increment of both TLRs account for the observed severity. Zhang *et al*[49] obtained similar results in a taurocholate-based rat model. Besides, decreased hepatic TLR2mRNA expression, chloroquine

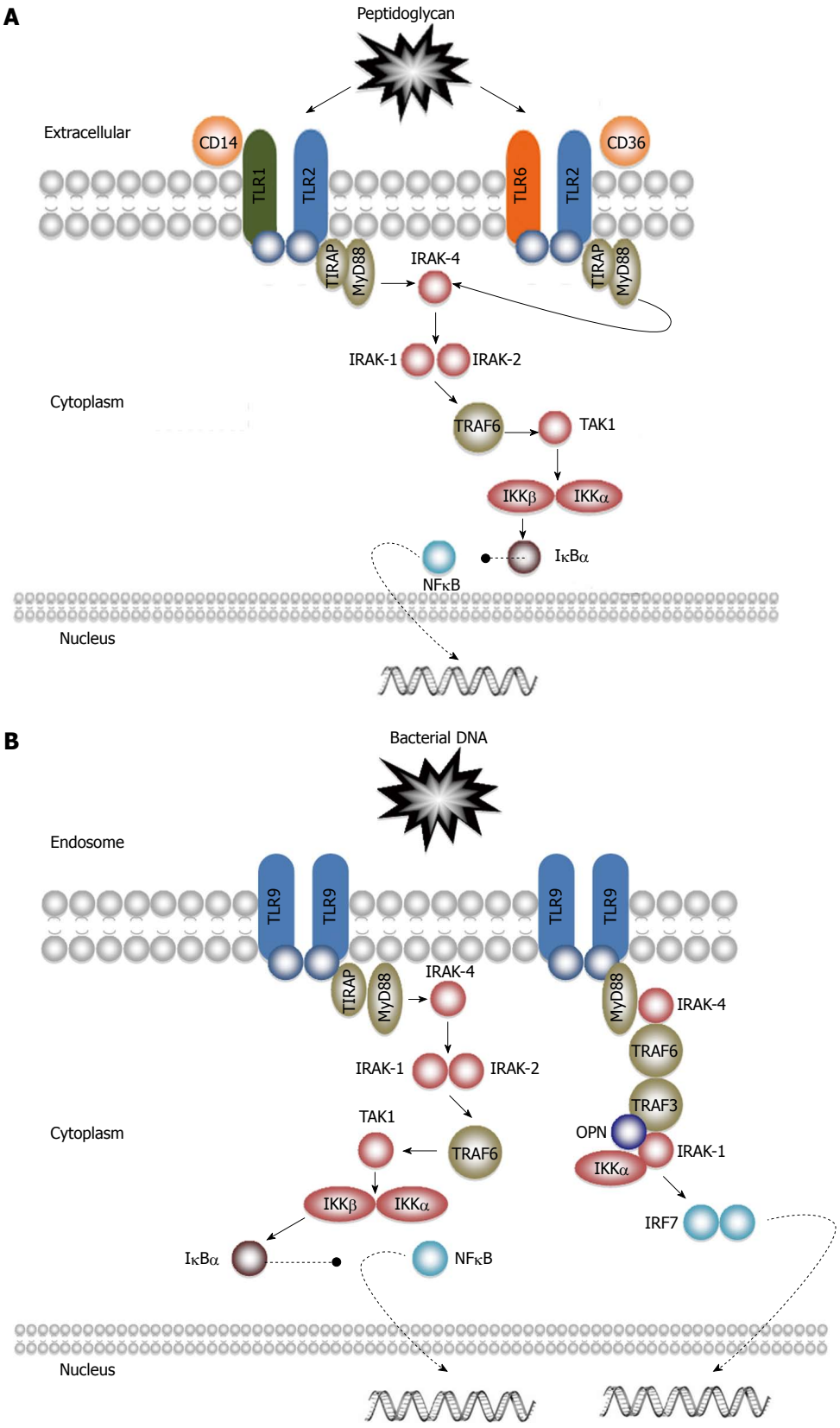

Figure 2 Toll-like receptor 2 and receptor 9 signalling pathways. A: Receptor 2; B: Receptor 9.

or L-Arg administration attenuated the liver injury and decreased TNF-α levels. Increased pro-inflammatory cytokines and chemokines are released following overexpression of TLR2mRNA in the liver of untreated animals. As previously shown in the lungs[40], NO levels also decreased in the liver. This is of special interest since reduced NO levels correlated with diminished secretion of anti-inflammatory mediators.

Table 4 A selection of genes regulated by nuclear factor κB

Acute phase proteins	C-reactive protein
	Complement factor Bf
	Complement factor C3
	TNFβ
Adhesion molecules	E-selectin
	Intracellular cell adhesion molecule 1
	Vascular cell adhesion molecule 1
Chemokines	IL8
	Monocyte chemoattractant protein-1
	Chemokine C-C motif ligand 5 (also known as RANTES)
Cytokines	IL2, 6, 12
	ILβ
	IL1β
	TNFβ
	TNFα
Growth factors	Granulocyte colony stimulating factor
	Granulocyte-macrophage colony stimulating factor
	Macrophage colony stimulating factor

Modified after Christman *et al*[11]. TLR: Toll-like receptor; TNF: Tumour necrosis factor; IL: Interleukin.

Even if several studies associate TLR2 to AP, it is still debated if TLR2 plays a pathophysiological role. Awla *et al*[50] reported that the progression of SAP was not significantly different in TLR2$^{-/-}$ mice when compared with wild-type mice. No significant differences were observed concerning pancreatic tissue damage, chemokine formation and neutrophil recruitment in taurocholate-induced AP.

TLR2 and AP in humans

In humans, microsatellite polymorphism in intron 2 in the human *TLR2* gene was associated with an increased risk for AP in Japan[51]. For this experiment, DNA was harvested from 202 patients of which 80 were diagnosed with SAP. When compared to healthy Japanese controls, AP patients showed significantly increased polymorphism rates in *TLR2* genes. Since the same polymorphism has been associated with susceptibility to colorectal cancer, tuberculosis, rheumatoid arthritis and sarcoidosis[34,52-54]; it is conceivable that TLR2 signalling is altered if intron 2 is changed. However, it remains to be elucidated if the relationship between changed *TLR2* genes and the risk for AP is just limited to Japanese subjects, or if the results can be extrapolated to other populations. Other studies have shown that gene-related vulnerability for AP in a country could not be demonstrated in other populations[55,56].

A very interesting observation was made by Szabo *et al*[57]. Monocytes were harvested from healthy volunteers before alcohol consumption and 24 h thereafter. The cells showed a decreased pro-inflammatory profile. Furthermore, *in vitro* acute alcohol stimulation of monocytes activated the pro-inflammatory state and decreased IL-production when TLR2 and TLR4 ligands were added. However, if only TLR2 ligands were added, no significant changes were observed. Therefore, acute alcohol stimulation appears to inhibit TLR2 expression in monocytes, worsening by this way the innate immune response against microorganisms that produce TLR2 specific ligands. Acute alcohol stimulation seems to be a double edged sword, since it could induce both anti and pro-inflammatory responses depending on which TLRs are involved.

In summary, TLR2 seems to be up regulated in the pancreas, lungs and liver in experimental AP animals. Moreover, TLR2-deficiency or inhibition (chloroquine or L-Arg) seems to ameliorate ALI in SAP.

How the increased TLR2 levels in vital organs are associated with SAP is still unknown. It is plausible that the overexpression of TLR2 in the pancreas, lungs and liver, amplifies the inflammatory response observed in SIRS when DAMPs released in SAP are recognised.

Despite an association found in Japan, the relationship between modified human *TLR2* genes and AP has not been reported in other populations. Even if TLR2 seems to be relevant for the development of ALI in SAP, further research has to be done before this important PRR can be considered as a potential therapeutic target in AP.

TLR4-THE MAIN CANDIDATE?

TLR4 was the first TLR identified and is widely expressed on the plasma membrane of various immune cells, including macrophages and dendritic cells[58]. TLR4 recognises several DAMPs, including LPS, fibrinogen, and various heat shock proteins (Tables 1 and 2). Upon activation, TLR4 forms homodimers or heterodimers with TLR6. The TLR4 recognition of many DAMPs demands several accessory molecules. As for TLR2, CD14 is needed for binding LPS to TLR4 dimers. Another vital protein for LPS recognition is myeloid differentiation protein-2[3].

In the same matter as other TLRs, except TLR3, TLR4 signals through a MyD88-dependent pathway, leading to the activation of NFκB (see previous section). Besides, TLR4 can signal *via* a TRIF-dependent pathway. Once activated, TIR-TIR dimers recruit TRIF and TRAM. TRIF then activates I-κ-β kinase epsilon (IKKε), which binds to TANK-binding kinase 1 (TBK1). Finally, IKKε-TBK1 phosphorylates and activates IRF3, culminating in the transcription of IFN-α and IFN-β (Figure 3)[26].

TLR4 was initially mainly studied because of its involvement in Gram-negative bacterial infection, but has in recent years also been associated with an increasing number of diseases. Many reports suggest its involvement in atherosclerosis, liver disease, obesity, cardiac disease, and renal disease, among others[59-63].

Bacteria are important microorganisms in AP. In acute haemorrhagic-necrotizing pancreatitis, intestinal bacterial translocation into the injured/necrotic pancreas or the systemic circulation, may lead to SIRS, ALI and MODS[16-19]. As TLR4 is related to LPS and a wide range of alarmins released during inflammation and tissue damage, its role in AP appears to be highly possible.

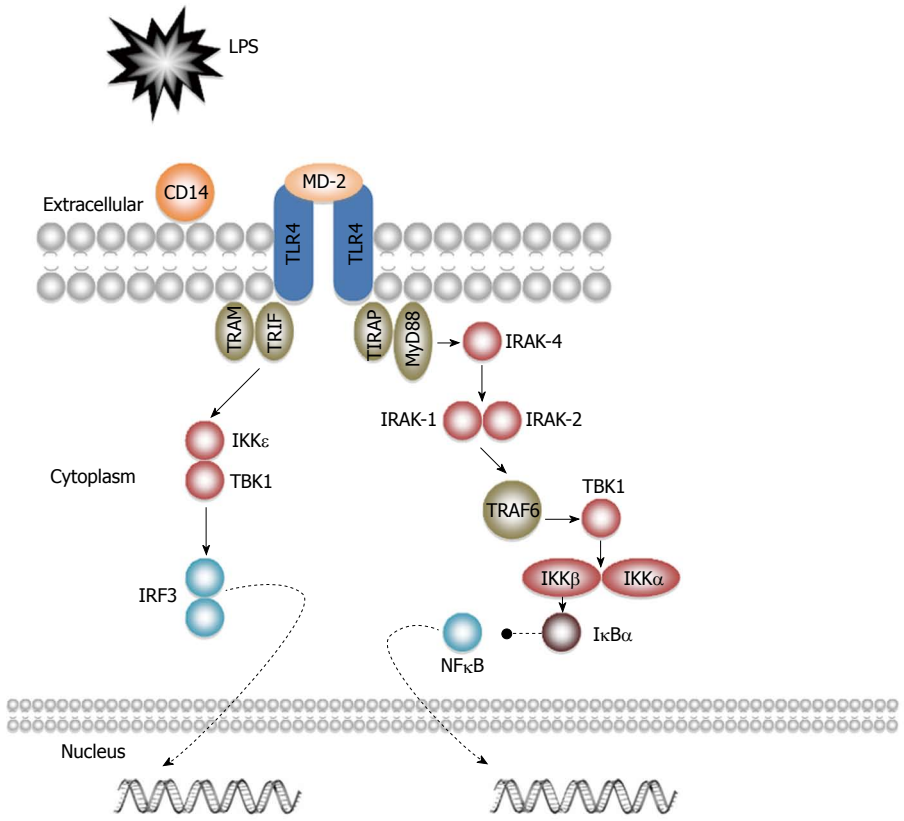

Figure 3 Toll-like receptor 4 signalling pathways.

Pancreatic damage

As for TLR2, overexpressed TLR4mRNA was also found in the pancreas in rats with cerulein-induced AP[35]. Li *et al*[64,65] reported (in the same animal model) that in both healthy and AP-induced animals, TLR4 was expressed mainly in the epithelium of the pancreatic duct and the pancreatic microcirculation. Even if some staining was observed in endocrine islets, no TLR4 protein was detected in the acinar cells. Likewise, TLR4 mRNA overexpression was observed, peaking after 1 h and returning to base levels after 4 h. In taurocholate-induced AP in TLR4$^{-/-}$ mice[50], serum amylase and myeloperoxidase levels in the pancreas decreased as compared to wild-type induced mice. Additionally, acinar cell necrosis, oedema, and haemorrhage significantly reduced. Interestingly, pancreas and serum levels of CXCL2, a chemokine, released by monocytes and macrophages, recruiting neutrophils, were strongly diminished.

Opposite results were presented by Ding *et al*[66] using the same AP model. No significant changes in serum amylase levels and pancreatic histological scores were observed between TLR4$^{-/-}$ and the controls. Curiously, TLR4$^{-/-}$ mice showed decreased levels of pro-apoptotic proteins in the pancreas 2 h after induction, but not after 4 h. TLR4 can increase the apoptotic rate in different cells[67]. As some studies suggest that apoptosis is favourable in AP[68,69], it is feasible that TLR4 is involved both in the beginning and in the resolution of AP.

Wang *et al*[70] showed that the administration of anti-CD14 antibody prior to cerulein-induction and LPS challenge in mice, reduced the severity of pancreatic injury, decreased pancreatic myeloperoxidase activity and down-regulated the secretion of pro-inflammatory cytokines.

TLR4 signalling pathways have been investigated in AP. Ding *et al*[71] showed in TLR4$^{-/-}$ mice that IRAK-4 could play a role in AP, independently of TLR4. In this experiment, lacking TLR4-mediated response did not result in increased IRAK-4 levels as was expected. This suggest that TLRs other than TLR4 may also play a role in AP, as the activation of TLR2 and TLR9 also results in decreased IRAK-4 in macrophages[72]. The hypothesis is supported by a report in which TRAF6 mediated inflammation in AP in TLR4$^{-/-}$ mice[73]. The author concluded that TLR4 may not be exclusively required for initiating AP, but its signal pathway may be of importance.

However, the association between alarmins released and TLR4-mediated inflammation in AP appears to be robust. In mononuclear inflammatory cells, the enzyme pancreatic elastase seems to activate NFκB, inducing TNF-α secretion[74,75]. Hietaranta *et al*[76] found that when human myeloid cells were exposed to porcine elastase, increased expression of NFκB and AP1 was achieved. The effects of elastase seem to be mediated by TLR4, since the blocking of TLR4 with a specific neutralizing antibody strongly prevented the expression of pro-inflammatory factors in elastase-exposed cells. Premature elastase activation has been reported in AP, implying its role in the disease[77]. Hence, TLR4-mediated inflammation may be important in AP. Blocking elastase might attenuate the severity of AP.

The degradation of matrix components appears to be connected with the pathophysiology of AP. Reports indicate that low molecular weight polysaccharides from degraded hyaluronan activates dendritic cells through TLR4[78]. Besides, dendritic cells might protect the pancreas against cell stress in AP[79]. Blycans and oligosaccharides of hyaluronan seem to signal *via* TLR4 and induce the production and secretion of great quantities of TNF-α and macrophage inflammatory protein-2 in macrophages[80]. Elastase and trypsin, both involved in the initiation of AP, cleaves heparin sulphate (HS) from cell surfaces and extra cellular matrix *in vitro*[81]. This, probably may in turn lead to free endogenous HS in damaged tissues in AP. Johnson *et al*[25] demonstrated that the rapid degradation of HS in mice causes a TLR4-mediated SIRS-like reaction in mice. When soluble HS was injected intraperitoneally, almost all wild type, but no TLR4-deficient mice, perish. This reaction was HS specific since molecules structurally similar to HS did not stimulate TLR4 and HS was not contaminated with LPS. Moreover, wild type, but not TLR4-deficient, mice had increased TNF-α serum levels 1 h after administration. Continuing in this line, Axelsson *et al*[82] confirmed that HS is involved in the initiation of AP in the rat.

Akbarshahi *et al*[83] later showed that the HS-induced TLR4-dependent immune response in the murine pancreas is IRF3-mediated. When TLR4$^{-/-}$ or MyD88$^{-/-}$ mice were challenged with HS or LPS, myeloperoxidase activity was annulled in the pancreas. The same pattern with HS was observed in IRF3$^{-/-}$ mice. However, LPS administration initiated a strong immune response in these animals. This outcome could explain preceding reports about discrepancies between TLR4-deficiency and decreased TRAF6 and IRAK-4 levels in AP[71,73]. Additionally, in this experiment, pre-treatment with the TLR4 antagonist eritoran inhibited the otherwise occurring increase in myeloperoxidase production in HS-treated wild type mouse pancreas. Eritoran is a synthetic Lipid A analogue that bind to TLR4/myeloid differentiation protein-2, thereby competing with Lipid A, resulting in inhibited LPS-mediated immune response[84].

Possibly, the inhibition of TLR4, elastase or HS, alone or in combination, could ameliorate pancreatic damage in AP, thus considerably reducing the risk for developing SAP.

Lung injury

As the role of TLR4 in AP has been investigated previously together with TLR2, similar results have been reported (see TLR2 section). Briefly, TLR4mRNA expression was increased in the lungs of AP-induced rats; leading to increased TNF-α levels combined with decreased NO levels. Chloroquine and L-Arg decreased TLR4mRNA expression and attenuated the lung injury[40]. Moreover, pulmonary macrophages decreased the TLR4mRNA expression, which caused decreased LPS-induced TNF-α levels and predisposition for intestinal bacterial translocation[41].

Pastor *et al*[85] concluded that TLR4 may not play a role in AP-associated ALI, but it may participate in pulmonary injury mediated by endotoxemia. In a cerulein-induced pancreatitis model in TLR4$^{-/-}$ mice there were no significant changes in serum amylase levels, pancreatic myeloperoxidase activity, and pancreatic oedema and acinar necrosis when compared to wild type mice. Additionally, cerulein-related pulmonary damage did not decrease in TLR4$^{-/-}$. Although, when cerulein was combined with LPS, a significant decrease in pulmonary damage was reported. Thus, TLR4 would only be of importance in AP-related ALI when the disease is worsened by sepsis.

Sharif *et al*[86] obtained pole opposed results. TLR4$^{-/-}$ mice showed decreased serum amylase activity and pancreatic damage (oedema, myeloperoxidase activity, necrosis). Importantly, myeloperoxidase activity in the lungs also decreased, indicating reduced neutrophil sequestration. Furthermore, when AP was induced in CD14$^{-/-}$ mice, pancreatic and pulmonary damage were reduced as previously observed in TLR4$^{-/-}$ animals. Since TLR4/CD14 are very important for LPS-mediated TLR4 activation, TLR4 appears to be involved in the development of ALI in AP, even when endotoxemia is absent. Still, the subject is controversial, since ALI can ensure LPS-mediated TLR4/CD11b activation in CD14$^{-/-}$ mice[87].

Matsuda *et al*[88] proposed that the TLR4-associated inflammatory response in AP complicated by endotoxemia is mediated by macrophage migration inhibitory protein (MIF). Increased MIF expression in the lungs was observed after cerulein/LPS administration in mice. MIF levels were coupled to ALI and increased TLR4 expression in the lungs. Moreover, AP-induced MIF$^{-/-}$ mice showed lower TLR4 expression than in wild type mice; and anti-MIF antibody administration greatly suppressed the pulmonary expression of TLR4. It is important to stress that MIF can mediate inflammation independently of TLR4. For instance, the induction of cytosolic phospholipase A_2, an enzyme that has been linked to ALI, may be MIF-mediated[89,90].

The role of TLR4 in AP-associated ALI is debated. While it is generally accepted that TLR4 plays a role in the course of ALI when endotoxemia ensues in AP; most reports are divided between those that suggests TLR4 as an important player (even in the absence of endotoxemia) and those that minimize its role. Fortunately, this discrepancy may lead to new experiments that focuses not only in LPS but also in the wide range of alarmins related to AP, ALI and TLR4. For instance, neuropeptide substance P, which has pro-inflammatory properties that increase vascular permeability and is correlated to AP and ALI[91]; has also been associated to the up regulation of TLR4 in AP[92]. Moreover, extracellular heat shock protein 70 can induce SIRS-like immune responses in cerulein-challenged mice[93]. The action of this protein appears to be mediated by TLR4, as TLR4$^{-/-}$ mice did not showed the same outcome.

Liver, kidneys and intestine

Similarly to TLR2, TLR4mRNA expression is increased

in the liver during AP-associated SIRS[48,49]. Briefly, after AP-induction, higher levels of liver enzymes and TNF-α were observed. Hepatic NO levels decreased. All these changes appeared to be related to the TLR4mRNA overexpression in the liver. The administration of chloroquine and L-Arg decreased TLR4mRNA expression, thus reducing the liver damage.

Peng *et al*[94] showed that the deletion of TLR4 attenuated liver injury in AP. AP was induced in mice by choline-deficient ethionine diet. Besides the expected augmentation of TLR4 mRNA in mice liver, an increment in protein kinase C-zeta (PKCζ) was detected. However, induced TLR4$^{-/-}$ mice showed less apoptosis in hepatic cells and the hepatic PKCζmRNA expression was clearly reduced. PKCζ activates NFκB, which is essential for the production of cytokines in Kupffer cells[95]. Consequently, TLR4-deficiency protects the liver in AP through down-regulation of PKCζ, resulting in less inflammation and hepatic cell apoptosis.

Sawa *et al*[96] showed that in SAP, TLR4 expression is not only increased in the liver, but also in the kidneys and small intestine. Closed duodenal loop operation was performed in mice. The increased expression of TLR4 occurred 4 h after the ligation and returned to baseline after 12 h. Curiously, TLR4-deficient mice showed the same tendency and histological analyses of the liver and kidney did not show any differences between the different groups. However, apoptosis was seen in the liver and kidney of ligated TLR4-deficient, but not in wild type, mice. Additionally, Gram-negative bacterial translocation into the pancreas occurred in higher rates in TLR4-deficient animals. The translocation ensued 12 h after induction, but not before. Even Gram-positive bacterial translocation was registered, but the difference between the groups was not significant. Bacterial translocation highly correlated to fluctuations in TLR4 expression. According to the authors, as MODS develops in the early or the late phase of SAP, the early inhibition and late stimulation of TLR4 could result in a milder clinical course, since it could prevent MODS and bacterial translocation. Yet, results from another experiment in mice challenges this hypothesis[97]. van Westerloo *et al* showed that the immune response against Escherichia coli in AP-induced TLR4$^{-/-}$ mice was not different from that observed in controls. Nevertheless, there is a chance that mice developed the compensatory anti-inflammatory response syndrome (CARS), which is generally (but not exclusively) related to SIRS and sepsis[98]. CARS is a systemic deactivation of the immune system, that can ensue after SIRS and sepsis in order to restore homeostasis[99]. Thus, CARS could explain why LPS-mediated TLR4-activation was not observed in TLR4$^{+/+}$ mice after bacterial challenge in AP.

TLR4 and AP in humans

Despite controversy, there is an increasing amount of data suggesting that TLR4 plays a role in experimental AP in rodents. However, in humans, the role of TLR4 in AP patients has been investigated mainly indirectly *via* DNA analysis or immune cells taken from blood.

In a Chinese study 310 patients were diagnosed with AP according to the Atlanta severity classification[100,101]. Pancreatic necrosis was recognized in 115 patients and in 37 of those, TLR4 mutation (*896G allele*) was identified. When compared to AP patients without TLR4 mutation (*896A allele*), patients with mutated TLR4 had an increased morbidity following Gram-negative infection. When compared to healthy volunteers (n = 80), TLR4 mutation frequency was significantly higher in patients with pancreatic necrotic infection. Thus, according to this study, TLR4 *Asp299Gly* polymorphism appears to be associated with an increased risk for pancreatic necrotic infection in AP. However, this study may have limited value as all patients were recruited from one hospital located in southwestern China, thus questioning its representative value for the whole Chinese population. In a later study, Zhang *et al*[102] could not find any significant difference in the occurrence of *Asp299Gly* polymorphism between 238 Chinese AP patients and 121 healthy volunteers.

DNA samples were harvested from 92 AP patients in a Hungarian medical centre[103]. Not only TLR4 *Asp299Gly* polymorphism was considered, but also as *Thr399Ile* polymorphism was taken into account. No significant differences were detected between AP (n = 42) and SAP patients (n = 50) in any polymorphism analysed. Likewise, no differences were found between the 92 patients and healthy controls (n = 200). Perhaps, the strongest data against the involvement of TLR4 mutations in AP comes from an intercontinental study reported by Guenther *et al*[104]. No significant differences could be found between 521 AP patients (343 from Germany and 178 from United States) and healthy controls (128 Germans and. 265 Americans, respectively) in the incidence of *Asp299Gly* and/or *Thr399Ile* polymorphism. Thus, current knowledge indicates that the investigated TLR4 polymorphisms are not important for the development and clinical course of AP, even if these mutation have been linked to impairment of LPS-induced TLR4-mediated immune response in humans[105].

In vitro acute alcohol stimulation seems to inhibit TLR4 expression in human monocytes[57]. When LPS was added, a decreased secretion of pro-inflammatory cytokines was observed. Intriguingly, the effect was abolished if LPS and TLR2 ligands were added at the same time to the cells. Li *et al*[106] showed that the expression of TLR4 in human peripheral blood mononuclear cells in mild AP, raises in the beginning of the disease to return to baseline levels after a week, when the patients recovered. As TNF-α and IL-6 showed the same pattern, the authors concluded that TLR4 might play an important role in the pathogenesis of AP.

In summary, there is strong data indicating that TLR4 plays an important role in experimental AP in rodents. Nevertheless, contrary results have been published. While most studies emphasize the role of TLR4 in the pancreas in AP, its role in AP-associated ALI is controversial. There is some evidence linking TLR4 levels and tissue

damage in the liver in AP. Likewise, the general opinion is that TLR4 is very important in the development of endotoxemia in AP.

In humans, DNA mutations in *TLR4* genes do not seem to be of importance for the development or the clinical course of AP, but TLR4 expression changes in monocytes and macrophages may be of significance. Unfortunately, the latter is only based on a very limited number of studies. There are some promising reports about the use of herbs from traditional Chinese medicine in AP that may act through TLR4 inhibition[107-109]. *Salvia miltiorrhizae* (also known as danshen) appears to inhibit the binding of LPS to TLR4 in the rat liver in SAP, which may reduce bacterial translocation and liver injury. Moreover, treatment with *emodin* and/or *baicalin* reduced serum levels of amylase, IL-6 and TNF-α in AP-induced rats. Pancreatic damage and ALI were also ameliorated. The effect of *emodin* and *baicalin* appears to be mediated by TLR4, since decreased TLR4mRNA expression and protein levels were found in the pancreas and lungs in treated animals. Its implication in experimental AP in rodents makes TLR4 a potentially very promising therapeutic target in AP, its utility though to be demonstrated in humans in future studies.

TLR9-UNEXPLORED IMPLICATIONS

As for TLR2 and TLR4, TLR9 is expressed intracellularly (endosomes) in several immune cells, including B cells and dendritic cells[110]. TLR9 recognises unmethylated *CpG* dinucleotides found in *bacteria* and *virus*[111,112]. *CpG* motifs are rare in vertebrate DNA (less 1% in human genome), but they are common in bacterial DNA. Besides, if present in vertebrate DNA, *CpG* motifs use to be methylated. *CpG* DNA directly stimulates B cells, macrophages and dendritic cells to secrete cytokines[113]. TLR9 can also recognize haemozoin, which is a disposal product formed from the digestion of erythrocytes by some parasites (*e.g.*, *Plasmodium spp.*)[114].

Upon activation, TLR9 signals through a MyD88-dependent pathway, leading to the production of pro-inflammatory cytokines (see previous sections). This pathway is mainly observed in macrophages, B cells, conventional dendritic cells and plasmacytoid dendritic cells. However, in plasmacytoid dendritic cells, TLR9 can induce the production of type I IFN through a different pathway. After the recruitment of Myd88 and IRAK-4, these interact with TRAF6, TRAF3, IRAK-1, IKKα and osteopontin. IRAK-1 and IKKα phosphorylate and activate IRF7, culminating in the production of type I IFN (Figure 2B)[26]. Apart from its role in bacterial, viral or malaria infection; TLR9 has been associated with SLE and cancer[115,116].

TLR9 and AP

To date, there are only two publications in which the role of TLR9 in AP has been investigated. Zeng *et al*[117] reported that TLR9 was expressed in rat pancreas in cerulein-induced AP. TLR9 staining was detected both in the pancreas from AP-induced rats, as in controls. The epithelium of the pancreatic duct and pancreatic microcirculation were the main sites for TLR9 staining in AP-induced animals. Controls showed staining in the vasculature, but not in the pancreatic duct. Moreover, no staining was detected in pancreatic acinar cells in either group. Interestingly, a similar pattern of staining has been reported for TLR4 in AP[64]. TLR9mRNA expression was increased after 30 min, peaked at 1 h, and remained high for the first eight hours after cerulein challenge.

In a more extensive study, Hoque *et al*[118] found that TLR9 is involved in pancreatic acinar cell death in AP-induced mice. After cerulein-administration, TLR9$^{-/-}$ mice had less oedema, leukocyte infiltration and IL-1βmRNA expression in the pancreas. Even if several cell types express TLR9 in the pancreas, bone marrow-derived CD45^{+} cells (mainly macrophages) had the highest expression level. Furthermore, the action of a TLR9 antagonist (IRS954) was evaluated. TLR9$^{+/+}$ animals had IRS954 administered 1 h before inducing AP by cerulein. Pancreatic oedema, leukocyte infiltration and pancreatic cell apoptosis were ameliorated. Moreover, pancreatic pro-IL-1β elevation was also reduced, as serum amylase levels. These results were observed at 1 h after AP-induction.

The authors speculated if IRS954 might have a therapeutic value. Interestingly, the administration of IRS954 after cerulein challenge reduced pancreatic oedema, leukocyte infiltration and pancreatic cell apoptosis. Similar results were observed when another model for AP was used. Pre-treatment with IRS954 at 1 h after AP induction through taurolithocholic acid 3-sulphate challenge in TLR9$^{+/+}$, reduced serum amylase elevation, pancreatic necrosis and inflammatory cell infiltration in mice lungs. Additionally, *in vitro* experiments were performed. When isolated peritoneal macrophages were exposed to pancreatic homogenate and DNA, significant NFκB activation was observed. Interestingly, pre-treatment with IRS954 reduced NFκB activation to baseline levels.

IRS954 appears to be a good candidate for further investigation. Like TLR2 and TLR4, TLR9 may be important when AP is aggravated by sepsis. Reports indicate that during sepsis, TLR9 is expressed both in murine and human adrenal glands and its stimulation leads to corticosterone release and inflammatory response[119]. Macrophages and NK-cells in the liver mediates liver toxicity and express high levels of TLR9 in murine peritonitis[120]. Chloroquine inhibits TLR9 and may prevent sepsis-induced acute kidney injury in mice[121]. Additionally, TLR9$^{-/-}$ mice have reduced mortality in polymicrobial sepsis[122].

Still, the number of studies is very limited. Hence, it is impossible to conclude if TLR9 plays an important role in AP with a clinical course complicated with our without sepsis.

Closing remarks

An increasing number of publications suggest that TLRs

Table 5 Toll-like receptors and acute pancreatitis

	Observed change	Tissue/cell	Ref.
TLR2	Increased mRNA expression	Pancreas	[35]
		Lungs	[40]
		Liver	[48,49]
	Increased protein levels	Pancreas	[35]
		Liver	[48]
	Decreased mRNA expression	Pulmonary macrophages	[43]
TLR4	Increased mRNA expression	Pancreas	[35,64,73,92]
		Lungs	[40,88]
		Liver	[48,94,96]
		Kidney	[96]
	Increased protein levels	Small intestine	[96]
		Pancreas	[35,92]
		Lungs	[88]
		Liver	[48,49]
		Intestine	[65]
		Blood monocytes	[106]
	Decreased mRNA expression	Pulmonary macrophages	[43]
TLR9	Increased mRNA expression	Pancreas	[117,118]
	Increased protein levels	Pancreas	[118]

TLR: Toll-like receptor.

Table 6 Toll-like receptor intervention in acute pancreatitis

	Substance	Ref.
TLR2	Chloroquine	[40,49]
	L-Arg	[40,49]
	WY14643	[35]
TLR4	Anti-MIF antibody	[88]
	Baicalin	[107]
	Chloroquine	[40,49]
	Emodin	[107]
	Eritoran	[83]
	L-Arg	[40,49]
	Salvia miltiorrhizae	[108,109]
TLR9	IRS954	[118]

TLR: Toll-like receptor; MIF: Migration inhibitory protein.

are involved in the pathophysiology of several important medical conditions. In acute pancreatitis, TLRs appear to play a role, but a general consensus has not been achieved, since contradictory results have been presented.

The main candidate for targeting seems to be TLR4, which recognizes numerous DAMPs associated to AP[123,124]. TLR2 has also been linked to AP, but there are few studies that exclusively has studied its role in AP. There is data suggesting that TLR9 also can play a role in AP. The associations found between TLRs and AP, as possible novel types of therapy are presented (Tables 5 and 6). There is also some evidence indicating that TLR4 and TLR3 may be involved in chronic pancreatitis[125,126]. This is of particular importance since chronic pancreatitis may develop following repeated AP episodes, as chronic pancreatitis is to some extent a risk factor for pancreatic cancer[127].

CONCLUSION

To our knowledge, this is the first time the current understanding of the role of TLRs in acute pancreatitis is summarized. Of course, further research and elucidation of involved mechanisms is warranted, hopefully stimulated by the present review giving an update and state-of-the-art concerning the role of TLRs in acute pancreatitis and its potential future clinical implications.

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Prophecy about post-endoscopic retrograde cholangiopancreatography pancreatitis: From divination to science

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Abstract

One unresolved issue of endoscopic retrograde cholangiopancreatography (ERCP) is post-ERCP pancreatitis (PEP), which occurs in up to 40% of patients. Identification of risk factors for PEP is especially important in the field of ERCP practice because it may assist physicians in taking protective measures in situations with high risk. A decade ago, Freeman *et al* meticulously evaluated a large number of potentially relevant risk factors for PEP, which can be divided into patient-related and procedure-related issues. In this commentary, we summarize this classic article and reevaluate the risk factors for PEP from the current point of view. This is followed by assessment of strategies for prevention of PEP that can be divided into mechanical and pharmacologic methods.

Key words: Endoscopic retrograde cholangiopancreatography; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Risk factor; Prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis; Pancreatic stents; Nonsteroidal antiinflammatory drugs

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COMMENTARY ON HOT TOPICS

One unresolved issue of endoscopic retrograde cholangiopancreatography (ERCP) is post-ERCP pancreatitis (PEP), which occurs after 1% to 40% of procedures[1-4]. This variable frequency of PEP is due to a number of factors, including the definition used, the patient population, the type of maneuvers, the type and duration of patient follow-up, and the personnel performing the ERCP[2,3,5]. Postulated mechanisms for pancreatitis after ERCP include mechanical, chemical, hydrostatic, enzymatic, microbiologic, and thermal disruptions[1,2]. A widely used consensus definition for PEP is: (1) new or worsened abdominal pain; (2) new or prolongation of hospitalization for at least 2 d; and (3) serum amylase three-fold or more above the upper limit of normal, measured more than 24 h after the procedure[6,7].

Prediction of PEP is of utmost importance in the field of ERCP practice. Although PEP is sometimes inevitable and can occur in the best of hands, identification of its risk factors may assist physicians in taking protective measures in situations with high a priori risk[8,9]. This article is intended as a commentary on the classic article "Risk factors for post-ERCP pancreatitis: a prospective, multicenter study" by Freeman *et al*[1] published in *Gastro-*

intestinal Endoscopy, a landmark study in the field of PEP, and it also provides strategies for the prevention of PEP.

In the classic article[1], comprehensive evaluation of the risk factors for PEP were performed by 26 endoscopists from 11 centers in the United States (6 private practices and 5 university-affiliated teaching hospitals), who collaborated in a painstaking prospective study of almost 2000 procedures[10]. The authors of the classic article meticulously determined eye-opening numbers (n = 32) of potentially relevant risk factors for PEP and provided vigilant analysis.

Risk factors for PEP

In the classic article[1], the authors classified risk factors into clearly demarcated patient-related and procedure-related factors. This allowed them to demonstrate that the risk of PEP is determined as much by patient characteristics as by endoscopic technique or maneuvers. Stratification of patients into low-risk or high-risk categories for PEP is important in order to ensure that gastroenterologists remove borderline indications of ERCP in high-risk patients and that adequate pre-procedure information is provided to the patient[5].

Patient-related risk factors: Of the 16 patient-related risk factors identified, in the classic article, 5 variables were statistically significant by multivariate analysis: history of PEP, suspected sphincter of Oddi dysfunction, female gender, normal serum bilirubin, and absence of chronic pancreatitis. Although a few other factors, such as young age and absence of common bile duct stones, were only significant in univariate analysis, subsequent multivariate studies by other groups or further meta-analysis showed that young age, non-dilated extrahepatic ducts, and absence of common bile duct stones are factors that may increase the risk for PEP (Table 1)[3,5,6,11,12]. A recent multivariate study, published in abstract form, suggested that obesity and hyperlipidemia were independent risk factors for PEP based on a nationwide database analysis[13]. Interestingly, some of the patient-related risk factors, such as normal serum bilirubin, non-dilated extrahepatic ducts, and absence of common bile duct stones, are poor indicators of ERCP. This may attest to the statement by Cotton[10,14] that "ERCP is most dangerous for those who need it least".

Procedure-related risk factors: Of the 16 procedure-related risk factors identified, in the classic article, 4 variables were significant by multivariate analysis: biliary balloon sphincter dilation, moderate-to-difficult cannulation, pancreatic sphincterotomy, and pancreatic contrast injections. Subsequent multivariate studies by other groups or further meta-analysis showed that precut sphincterotomy, ampullectomy, pancreatic brush cytology, failure to clear bile duct stones, and involvement of trainees are all factors that may increase the risk for PEP (Table 2)[3,5,6,11,12].

Although pancreatic stent placement was associated with risk of pancreatitis by univariate analysis, no independent contribution of pancreatic stent placement was evident in the multivariate analysis because pancreatic stents were primarily placed in patients with multiple other independent risk factors for PEP. Actually, pancreatic stent placement can reduce the risk of PEP in a number of settings, so that prophylactic pancreatic stent placement has become a standard of care for reducing PEP in high-risk cases[15-17].

The classic article also indicated the importance of the general similarity of the overall risk of pancreatitis for diagnostic and therapeutic ERCP and showed that the performance of biliary sphincterotomy did not appear to add significant independent risk of pancreatitis to ERCP[1]. The article emphasized that these observations point not to the safety of sphincterotomy, but rather to the risk of diagnostic ERCP[1]. Despite the general similarity of the overall risk of PEP for diagnostic and therapeutic ERCP, certain high risk procedures such as pancreatic sphincterotomy, pancreatic brush cytology, and ampullectomy may increase the risk for PEP. Therefore, we should pay attention to the type and complexity of maneuvers when comparing the incidence of PEP between studies.

Interactive effect of patient-related and procedure-related risk factors: The striking message of the classic article was that patient-related and procedure-related risk factors are cumulative and perhaps even synergistic[1,10]. Subsequent studies confirmed that patients with multiple factors had an extremely high chance (up to 40%) of developing PEP[5,17-20]. The typical very high risk patient is a young to middle-aged woman with recurrent abdominal pain, normal serum bilirubin, no biliary obstruction, and difficult cannulation. The combinations of patient-related and procedure-related risk factors allow reliable prediction of the possibility that an individual patient will develop PEP[17]. Furthermore, this cumulative effect may influence the severity as well as the incidence of PEP. According to the literature[1,21-23], nearly all patients who developed severe or fatal pancreatitis after ERCP had multiple risk factors. Gastroenterologists are now more able to predict PEP.

Field of vision after a decade from the classic article: Even from the current point of view, the classic article investigated most of the potentially relevant risk factors for PEP, dividing these into patient-related and procedure-related issues. Over the past decade, subsequent studies have usually confirmed or just slightly altered the significance of these risk factors. The confirmation of the risk of diagnostic ERCP has focused the role of ERCP into an exclusively therapeutic modality with the advent of other diagnostic modalities[22]. Confirmed risk factors for PEP have also enabled the conception of several strategies for the prevention of PEP in actual practice[3,5,17,22,24,25].

Strategy for prevention of PEP

The most important goal of recognizing the risk factors for PEP is the development of a strategy for prevention

Table 1 Comparison of patient-related risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis by multivariate analysis in the classic article and current knowledge by meta-analysis or multivariate studies

Risk factors in the classic article	Current knowledge[1]
Significant in multivariate analysis	High risk factors
Suspected sphincter of Oddi dysfunction	Suspected sphincter of Oddi dysfunction
Female gender	Female gender
History of post-ERCP pancreatitis	Previous pancreatitis
Normal serum bilirubin	Normal serum bilirubin
Absence of chronic pancreatitis	Young age
Significant only in univariate analysis	Possible risk factors
Pancreas divisum	Non-dilated extrahepatic ducts
Recurrent abdominal pain	Absence of chronic pancreatitis
History of acute pancreatitis of any etiology	Absence of definite common bile duct stone
Cholangiogram normal	Obesity[2]
Pancreatogram normal	
Age < 55 yr	
Prior cholecystectomy	
Absence of definite common bile duct stone	
Not significant	Not related
Previous sphincterotomy	Pancreas divisum
Distal common bile duct diameter ≤ 5 mm	Allergy to contrast media
Prior failed ERCP	Prior failed ERCP

[1]Current knowledge is based on recent guidelines by American Society for Gastrointestinal Endoscopy[6] and European Society of Gastrointestinal Endoscopy[5], and relevant articles[3,22]; [2]Based on recent multivariate analysis in abstract form[13]. ERCP: Endoscopic retrograde cholangiopancreatography.

Table 2 Comparison of procedure-related risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis by multivariate analysis in the classic article and current knowledge by meta-analysis or multivariate studies

Risk factors in the classic article	Current knowledge[1]
Significant in multivariate analysis	High risk factors
Difficult cannulation	Difficult or failed cannulation
Balloon dilation of biliary sphincter	Balloon dilation of biliary sphincter
Pancreatic sphincterotomy	Pancreatic sphincterotomy
≥ 1 pancreatic contrast injections	Pancreatic duct injection
	Precut sphincterotomy
	Failed attempts at placing pancreatic duct stent
Significant only in univariate analysis	Possible risk factors
Sphincter of Oddi manometry	Ampullectomy
Pancreatic stent placement	Pancreatic acinarization
Minor papilla cannulation	Pancreatic brush cytology
Precut (access) papillotomy	Failure to clear bile duct stones
≥ 1 pancreatic deep wire pass/cannulation	Involvement of trainee during ERCP
Endoscopist performing > 2 ERCP/wk	
Not significant	Not related
Acinarization of pancreas	Sphincter of Oddi manometry (using aspirated catheter)
Biliary sphincterotomy	Biliary sphincterotomy
Intramural contrast injection	Intramural contrast injection
Pancreatic stricture dilation by any method	Prior failed ERCP
Pancreatic duct tissue sampling by any method	Therapeutic *vs* diagnostic
Training fellow involved	

[1]Current knowledge is based on recent guidelines by American Society for Gastrointestinal Endoscopy[6] and European Society of Gastrointestinal Endoscopy[5], and relevant articles[3,22]. ERCP: Endoscopic retrograde cholangiopancreatography.

of PEP. A practical strategy appears to be the combination of careful patient selection - which means avoiding inappropriate ERCP in high-risk patients - and selection of appropriate preventive measures (Table 3)[2,6].

Patient selection: Gastroenterologists should take much more care in the selection of patients for ERCP. The clinical role of ERCP has diminished substantially with the advance of relevant diagnostic and therapeutic modalities[6,9,26,27]. For diagnostic purposes, a plethora of relevant diagnostic procedures now are available, such as magnetic resonance cholangiopancreatography and endoscopic ultrasonography (EUS), which may obviate the need for ERCP or better focus its application[9]. For therapeutic purposes, minimally invasive surgeries now show considerable improvements in safety and outcomes[9]. Thus, balancing the benefit with risk is a prerequisite for determining the indication for ERCP. If the potential risk

Table 3 Clinical pearls to help avoid post-endoscopic retrograde cholangiopancreatography pancreatitis

Remember that ERCP is the most dangerous endoscopic procedure that can be associated with bad outcomes
Instead of diagnostic ERCP, use alternative imaging techniques such as magnetic resonance cholangiopancreatography or EUS, especially in high-risk patients
Rectal NSAIDs before or after ERCP procedure can be a simple measure to prevent PEP
Tailor a variety of cannulation techniques to the individual risk profile and the papillary anatomy of the patient
In cases of difficult cannulation, early precut or fistulotomy technique with a pancreatic stent (performed by an expert endoscopist) can decrease the risk of PEP
Quit the ERCP procedure earlier in high-risk patients if success is not achieved quickly. After a failed ERCP, alternative therapeutic methods such as percutaneous or EUS-guided approaches can be considered
In high risk patients, make sure that a prophylactic pancreatic stent is placed. In cases with equivocal risk at the end of the procedure, a prophylactic pancreatic stent can eliminate the fear of PEP

ERCP: Endoscopic retrograde cholangiopancreatography; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis; EUS: Endoscopic ultrasonography; NSAIDs: Non-steroidal anti-inflammatory drugs.

is much greater than the possible benefit, gastroenterologists should seek other diagnostic and/or therapeutic modalities or refer their high-risk patients to tertiary centers that have more experience and other tools.

Once a decision for ERCP is made, the gastroenterologist should reassess the risk profile of the patient and apply several mechanical and pharmacological interventions in order to reduce the likelihood of PEP[2]. In addition, an adequate consent process is very important to ensure that patients and their relatives understand that ERCP is potentially dangerous[14].

Mechanical prevention: Careful endoscopic techniques in cannulation and therapy are naturally important, but these are not sufficient to prevent PEP in high-risk patients[17]. Several modifications in endoscopic technique have been identified that can reduce the risk of PEP. (1) Prophylactic placement of pancreatic duct stents**:** The prophylactic placement of pancreatic duct stents improves the drainage of the manipulated pancreatic duct. Otherwise, this might be impaired by mechanical injury to the pancreatic sphincter from catheter and guidewire manipulation, and from thermal injury caused by biliary and pancreatic sphincterotomy[17]. The general consensus in the literature is that prophylactic pancreatic stents can reduce the risk of PEP in high-risk populations, such as those undergoing ampullectomy, pancreatic sphincterotomy, sphincter of Oddi manometry, precut sphincterotomy, pancreatic brush cytology, difficult cannulation, and pancreatic duct injection[3,8,17,28,29]. Recent meta-analyses of high-risk populations demonstrated that prophylactic pancreatic stents reduced the incidence of PEP from 18.6% to 5.6%[28,29]. An elegant cost-effectiveness analysis also suggested that prophylactic pancreatic stents in high-risk patients were a cost-effective strategy[30]. Actually, the routine use of pancreatic stents in high-risk cases has reduced the incidence and severity of PEP to a more acceptable level in advanced centers, allaying the fear of ERCP in high risk settings[17,31]; (2) Wire-guided cannulation: Compared with conventional use of contrast injection, wire-guided cannulation may avoid inadvertent injection of contrast into the pancreatic duct and decrease the risk of PEP[3,8]. Several meta-analyses have demonstrated a greater success of biliary cannulation and a lowered risk of PEP[21,32], but recent studies have shown that guidewire manipulation of the pancreatic duct for guidewire biliary cannulation is an another independent risk factor for PEP[33-35]. Prophylactic pancreatic stents might be recommended after pancreatic-guidewire assisted biliary cannulation to reduce the incidence of PEP[33]; and (3) Early precut biliary sphincterotomy and fistulotomy in cases of difficult cannulation: Although precut biliary sphincterotomy is an independent risk factor for PEP, prolonged cannulation attempts using standard techniques may also impart a higher risk for PEP than does the precut biliary sphincterotomy itself[5,36-39]. Early precut technique can be considered in cases of difficult biliary cannulation by advanced endoscopists with expertise in various cannulation techniques[5]. Fistulotomy is a variation of the precut needle-knife techniques that creates a direct bilio-enteric fistula by making an incision at the upper end of the ampullary region of the major papilla[40,41]. This technique has a potential advantage in that it evades the pancreatic orifice, which is the probable site of initiation of the cascade of PEP. Several studies have reported a lower incidence of PEP (up to 0%) in patients who underwent fistulotomy, with or without the prophylactic pancreatic stent[40-43]. Fistulotomy, however, may be more feasible in patients with bulging papillae and clear landmarks than in patients with tiny or diminutive papillae[40, 44]. For sparing the pancreatic orifice, reported investigational techniques include suprapapillary puncture with a needle-tip catheter, blunt dissection using a cotton swab, and EUS-guided suprapapillary puncture[39,45-47].

Pharmacologic prevention: Numerous trials have been attempted with many kinds of pharmacologic agents in order to reduce the risk of PEP. Pharmacologic agents, based on various theoretical benefits, have included nitroglycerin, ceftazidime, somatostatin, octreotide, gabexate, ulinastatin, nafamostat, antioxidants, allopurinol, glucocorticoid, non-steroidal anti-inflammatory drugs (NSAIDs), and *etc.*[5,24,48-52]. Early studies suggested that protease inhibitors such as gabexate or nafamostat may decrease the incidence of PEP[53,54]. At present, the dismally universal finding is that a strategy of pharmacologic prevention that proves effective in a few trials ultimately yields largely disappointing results over the long term

Table 4 Unresolved issues with prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis

The ideal design of a prophylactic pancreatic stent
Cannulation technique to lower incidence of PEP, tailored to the shape of the major papilla
The ideal pharmacologic agent
Comparison of rectal NSAIDs *vs* pancreatic stent placement *vs* combination in high risk patients
The route (rectal or intravenous) and the timing (before or after ERCP) of NSAIDs administration

ERCP: Endoscopic retrograde cholangiopancreatography; PEP: Post-ERCP pancreatitis; NSAIDs: Non-steroidal anti-inflammatory drugs.

when adopted in routine clinical practice[55,56]. Endoscopists in the ERCP field appear to believe that mechanical techniques such as pancreatic stents, much more than pharmacologic prophylaxis, play a key role in the prevention of PEP[55].

Despite a current climate of skepticism regarding the efficacy of any prophylactic medication for PEP, evidence for the efficacy of rectal NSAIDs in reducing PEP continues to accumulate[15,57-60]. Rectal NSAIDs are particularly attractive because of their low cost, easy administration, and known favorable risk profiles.[15] In addition to several meta-analyses[15,58,59], a recent well-designed randomized controlled trial beautifully showed the effect of rectal indomethacin in preventing PEP in 602 high risk patients (9.2% in the indomethacin group *vs* 16.9% in the placebo group, $P = 0.005$)[57]. However, only time will tell whether rectal NSAIDs can significantly reduce PEP.

Selection of preventive measures: According to the European Society of Gastrointestinal Endoscopy guidelines, periprocedural rectal administration of NSAIDs is recommended for low-risk ERCPs, whereas prophylactic pancreatic stent placement should be strongly considered for high-risk ERCPs[5]. However, the combined use of NSAIDs and prophylactic pancreatic stent placement might further reduce the rate of PEP in high-risk patients[61]. Therefore, rectal administration of NSAIDs for all ERCPs and prophylactic pancreatic stent placement for high-risk ERCPs might be more practical. A further practical strategy might be rectal administration of NSAIDs for patient-related high risk and prophylactic pancreatic stent placement for procedure-related high risk. However, the possibility remains that rectal NSAIDs may obviate the need for prophylactic pancreatic stent placement[61]. Studies to compare the effectiveness of rectal NSAIDs and pancreatic stent placement in high risk patients are warranted.

Future prospects for research

Although PEP has benefited from evolved understanding, there is still room for continuing research. In the future, the individual incidence of PEP should be accurately calculated according to the previously listed patient-related and procedure-related risk factors for PEP. In addition, the complexity of ERCP procedures[62,63] should be incorporated into the calculation of the individual incidence of PEP. Regarding the prevention of PEP, many issues still remain to be resolved (Table 4).

In conclusion, more than a decade has passed since the publication of the classic article that revealed the risk factors for PEP. Subsequent multivariate analyses have confirmed a number of risk factors for PEP that can be divided into patient-related and procedure-related issues. Prophylactic pancreatic stent placement has become a standard of care in high-risk patients and rectal NSAIDs have become a potential candidate as an ideal pharmacologic agent for preventing PEP. However, PEP is still the most frequent and most feared complication of ERCP. In the past decade, indications of ERCP have become more stringent owing to the development of other diagnostic and therapeutic modalities. To minimize PEP and maximize benefits[9], ERCP should be done for the best indications, while recognizing accurate risks to the individual and using meticulous endoscopic techniques with optimal preventive measures.

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TOPIC HIGHLIGHT

Luis Bujanda, PhD, Professor, *Series Editor*

Alcohol consumption on pancreatic diseases

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Abstract

Although the association between alcohol and pancreatic diseases has been recognized for a long time, the impact of alcohol consumption on pancreatitis and pancreatic cancer (PC) remains poorly defined. Nowadays there is not consensus about the epidemiology and the beverage type, dose and duration of alcohol consumption causing these diseases. The objective of this study was to review the epidemiology described in the literature for pancreatic diseases as a consequence of alcoholic behavior trying to understand the association between dose, type and frequency of alcohol consumption and risk of pancreatitis and PC. The majority of the studies conclude that high alcohol intake was associated with a higher risk of pancreatitis (around 2.5%-3% between heavy drinkers and 1.3% between non drinkers). About 70% of pancreatitis are due to chronic heavy alcohol consumption. Although this incidence rate differs between countries, it is clear that the risk of developing pancreatitis increases with increasing doses of alcohol and the average of alcohol consumption vary since 80 to 150 g/d for 10-15 years. With regard to PC, the role of alcohol consumption remains less clear, and low to moderate alcohol consumption do not appear to be associated with PC risk, and only chronic heavy drinking increase the risk compared with lightly drinkers. In a population of 10%-15% of heavy drinkers, 2%-5% of all PC cases could be attributed to alcohol consumption. However, as only a minority (less than 10% for pancreatitis and 5% for PC) of heavily drinkers develops these pancreatic diseases, there are other predisposing factors besides alcohol involved. Genetic variability and environmental exposures such as smoking and diet modify the risk and should be considered for further investigations.

Key words: Acute pancreatitis; Chronic pancreatitis; Alcohol consumption; Alcohol metabolism; Genetic variability; Pancreatic cancer; Risk

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INTRODUCTION

Alcohol causes different diseases and they are considered an important medical and social burden on society. Alcohol has been identified as a leading risk factor for death and disability globally, accounting for 3.8% of death and 4.6% of disability adjusted life years lost in 2004[1].

Pancreas is one of the most important organs adversely affected by alcohol consumption. An association between alcohol abuse and pancreatic injury was reported by Friedreich[2] as early as 1878. Freidreich recognized an

association of alcohol abuse with chronic pancreatic injure.

Since Friedreich's initial observation, many studies have confirmed that excessive alcohol intake is associated with pancreatic damage. Nowadays it is widely recognized that pancreatic injure due to alcohol consumption ranges from isolated episodes of acute pancreatitis (AP) to chronic manifestations that with time could move to pancreatic cancer (PC). However, there is not a consensus in the epidemiology and it is no clear how different drinks or dose of alcohol affect to the development of pancreatic diseases and finally, how drinking trigger pancreatic injury only in a minority of alcoholics.

Here, we review different studies and meta-analysis that have been published in the last years relating alcohol and pancreatic diseases as AP, chronic pancreatitis (CP) and PC. We summarize the effects of alcohol consumption in these diseases and at the end we present the possible mechanisms that have been proposed as a cause of this pancreatic pathogenesis.

Alcohol consumption measures

The incidence and prevalence of alcoholic pancreatic diseases are difficult to ascertain with precision, in part because of the variable considerations of drinking behavior and alcohol intakes measures.

Most of the studies in cohorts measures alcohol intake from wine, beer, and spirits separately. The final value of the daily alcohol intake is expressed in grams for each beverage based on the frequency of consumption, the alcohol content of the beverage, and the average quantity consumed. The United States Department of Agriculture established that alcoholic beverages are 12.8 g for 335 mL of alcohol for a bottle of beer (12-oz, 5% alcohol), 11.0 g for 118 mL (4-oz, 12% alcohol) glass of wine, and 14.0 g for 44 mL (1.5 oz, 40% alcohol) of 80-proof liquor.

The definition of a "standard drinker" differs between countries[3,4]: in United States it is 14 to 15 g, in Great Britain is 8 g and 19.75 g of alcohol in Japan. In addition to the amount to alcohol in one drink, the cut points to define "moderate" and "heavy" drinking also vary. In general, and to simplify a "standard drink" can be considered equal to 10 g of alcohol. A glass of wine, beer or half a glass of liquor would equivalente a beverage. For example in United States the standards are as following: moderate drinking is for women less than 2 drinks per day and less than 3 drinks for men; Heavy drinking is more than 7 drinks per week or 3 per occasion and more than 14 drinks per week or 4 per occasion; Bingo drinkers is 4 or more drinks in a row for women and more than 5 for men[5].

PANCREATITIS

Alcoholic pancreatitis is a potentially fatal illness that may be short term (*i.e.*, acute) or long term (*i.e.*, chronic). The relationship between acute and CP is complex. Symptoms are shared by acute and CP and include abdominal pain and interference with normal pancreatic functions. Although the prevalence of alcoholic pancreatitis in the population is unknown, clinicians usually agree that both acute and chronic alcoholic pancreatitis are responsible for a significant amount of illness and death in the world. However, the proportion of cases of pancreatitis attributed to alcohol varies widely among countries and even among different studies in the same country.

There are not universally accepted criteria to assign alcohol as an etiology of patient's pancreatitis but experts defines that varying from consumption of over 50 to 80 g (4-7 drinks/d) with or without a minimum drinking duration[6-9]. An international consensus defined alcoholic CP based on typical clinical history, threshold alcohol consumption (80 g or more of alcohol for a few years in males and less in females) and morphological evidence of CP on imaging studies or histology[10].

Besides the alcohol intake, other list of etiologies have been described as factors that could increase the risk for pancreatitis as gallstones, cystic fibrosis, hyperlipidemia, hyperparathyroidism, pancreas divisum or traumas, infections, genetic factors and autoimmune disease[11,12].

There is only a few numbers of epidemiological studies on the quantitative aspects of risk estimations for pancreatitis in relation to alcohol. In 2004, Corrao *et al*[13] conducted a meta-analysis of case-control and cohort studies published between 1966 and 1995 that assessed the association between alcohol consumption and CP. Then, Irving *et al*[14] in a review and meta-analysis published in 2009 analyzed studies published between 1980 and 2008 and assessed the association between alcohol consumption and the risk of pancreatitis. They concluded that the threshold between alcohol consumption and pancreatitis is 4 drinks daily.

However, about 70% of pancreatitis cases are believed to be attributable to chronic, heavy alcohol consumption but this percentage differs between countries[13,15,16]. Autopsies done on alcohol abusers have shown that up to 75% of them present CP[17]. On another hand, as less than 10% of consumers of alcohol in excess develop CP, other factors modify ethanol toxicity *in vivo*[16].

ACUTE PANCREATITIS

Historically it has been well-known that the risk of developing AP increases with increasing doses of alcohol[18] and with the duration of alcohol abuse[19], although epidemiological studies have shown that only a minority of heavy drinkers develop evident pancreatitis episodes[20,21]. This correlation shows again that besides of alcohol effects, additional factors are involved in AP develop.

Alcohol is the second most common cause of AC after gallstones[22]. The clinical features at the time of initial presentation are abdominal pain, jaundice, distal common bile duct obstruction and exocrine or endocrine insufficiency. Generally, the onset of alcoholic pancreatitis occurs in the 4th decade of males with an average of alcohol consumption around 150 g/d for a period of

Table 1 Alcoholic pancreatitis epidemiology in the different countries

Ref.	Country	Alcoholic etiology
Layer *et al*[7]	United States	56%
Coté *et al*[31]	United States	45%
Yadav *et al*[32]	United States	51%
Lin *et al*[36]	Japan	56%
Cavallini *et al*[6]	Italy	43%
Frulloni *et al*[37]	Italy	79%
Lankisch *et al*[9]	Germany	78%
Nøjgaard *et al*[29]	Denmark	44%
Díte *et al*[33]	Czech Republic	60%
Ammann *et al*[38]	Switzerland	71%
Robles-Díaz *et al*[39]	Mexico	68%
Dani *et al*[40]	Brazil	90%
Marks *et al*[41]	South Africa	80%
Balakrishnan *et al*[42]	India	33%

10-15 years[23]. Initially patients present acute abdominal pain, elevated serum levels of pancreatic enzymes and evidence of pancreatic damage in imaging studies.

These acute toxic effects of alcohol on the pancreas are considered AP but the progression of acute episodes (potentially reversible) leads to chronic disease with irreversible changes in the pancreas.

Traditionally, AP has been classed as fundamentally different from CP as the first one is characterized by restoration of normal pancreatic histology after full clinical recovery[24]. However, acute, recurrent acute and CP are now regarded as a disease continuum[25,26]. For this reason, the majority of the epidemiologic studies of alcoholic pancreatitis are referred to CP.

There is no data in the literature showing whether the heavy alcohol intake (> 80 g/d) in abstainers or moderately drinkers is a risk factor for AP. Also it is unknown whether this risk would be the same for all alcoholic beverages (wine, beer or spirits).

CHRONIC PANCREATITIS

CP is an inflammatory disorder of the pancreas typically associated with heavy alcohol consumption. Clinical features of CP consist of abdominal pain, recurrent attacks of clinical AP, and exocrine and/or endocrine insufficiency[27].

Alcohol is the most common cause for CP[22]. Clinical and experimental studies analyzing alcoholic CP development have concluded that alcoholic pancreatitis begins as a acute process that progresses to chronic irreversible pancreatic damage as a consequence of repeated acute attacks[23,28].

The overall survival in patients with alcoholic pancreatitis is significantly lower compared with the background population, but most of the patients die from causes unrelated to pancreatitis[29,30].

Dufour *et al*[16] concluded that CP development is proportional to the dose and duration of alcohol consumption (minimum, 6-12 years of approximately 80 g of alcohol per day).

The incidence rates of CP and the proportion of cases attributed directly to alcohol etiology differ based on geographic distribution (see Table 1).

In United States alcohol etiology accounts around 50%-55% depending on the study. Layer *et al*[7] published a study with patients obtained since 1976 to 1982 and they found that 56% were heavy drinkers (> 50 g/d) and present alcoholic etiology. Coté *et al*[31] published their results from a multicenter study (2000-2006) in 2010 concluding that alcohol contributes to CP in 45% of the patients. In 2011, Yadav *et al*[32] published a population-based study with patients from Mayo Clinic in Olmsted County, MN concluding that 51% presented alcoholic CP. In these studies patients with alcohol consumption of > 50 g/d were defined as alcoholic CP.

In general this percentage is lower than Europeans series[6,9,33,34] or other regions[35] (67%-89%) but similar to those from Japan (56%)[36].

In European series there is some variability between countries. For example in Italy the described percentage was 43%-79%[6,37], Germany 78%[9], Denmark 44%[29], Czech Republic 60%[33] and Switzerland 71%[38].

The series published in Mexico show 68%[39] of patients with alcoholic etiology, while alcohol is the responsible factor in 90%[40] of patients in Brazil, 80%[41] in South Africa and 33%[42] in India.

Taken together the data presented above, independently of the region it has been shown that 50% or more of AP or CP are associated with alcohol consumption.

Besides of the geographic distribution, CP due to alcohol consumption could be different based on the race but not on the sex. Lowenfels *et al*[43] determined that at equal levels of consumption the rates of alcoholic pancreatitis are similar for males and females although patients with alcoholic pancreatitis are more likely males. This is due to the overrepresentation of males among patients with alcoholic pancreatitis, showing a higher prevalence of alcohol consumption than sex-based differences in susceptibility.

Also, regardless to the race it was initially described by Lowenfels *et al*[30] and then confirmed by other authors[44-46] that blacks have two to three folds higher rates of alcoholic pancreatitis. Other authors have described that polymorphisms associated to specific populations and races are associated with functional differences in alcohol metabolizing enzymes leading to variation in pancreas damage[47].

However, detailed reasons for geographic or racial differences in susceptibility to alcoholic pancreatitis are still unknown and most likely are because differences in alcohol intake influenced by habits and genetic susceptibility. Further investigations are needed to confirm that or look in detail for cofactors associated to alcohol consumption.

Based on alcohol consumption, Kristiansen *et al*[48] estimated that the risk of any pancreatitis among non drinkers (abstainers) is 1.33% while it is among 2.5% (1.6% acute and 1.6% chronic) in heavy drinkers (> 35 drinks/wk or > 5 drinks/d). Lowenfels *et al*[30] concluded that the risk of pancreatitis among heavy drinkers (> 5

drinks/d) is about 2%-3%.

Also, some studies have been made to evaluate whether the type of beverage (beer, wine or spirits) or the frequency of drinking (daily, almost daily, weekly or monthly) is associate with the risk of pancreatitis[48]. This study concluded that consumption (> 14 drinks/wk) of beer but not wine or spirits increase the risk of pancreatitis. However this analysis was limited by the number of cases at high levels of consumption. These authors did not find an independent association between the frequency of consumption and risk. In addition some experimental studies carried out in animals conclude that prolonged ethanol feeding does not induce CP although causes little histological changes and variation in pancreatic enzymes[49,50].

Although it has been well established that pancreatic inflammation appears to increase the risk of PC[51] and it could be that alcoholic pancreatitis could leads also to PC, future epidemiological studies are needed to analyze this correlation in population.

Some studies have indicated that CP can result in type 2 diabetes, and with a time (up to 20 years) 2%-4% of these pancreatitis cases will develop PC[52]. Also, long-standing type 2 diabetes is a risk factor for PC[53]

Even in heavy alcoholics, CP and alcoholic cirrhosis seldom occur together in the same patient[54,55], despite a few contradictory reports. The mechanisms or factors determining this dichotomy of some alcoholics developing pancreatitis and others developing liver cirrhosis have not been adequately explained. Whether this can be explained by the quantity, duration, and pattern of drinking, or by other cofactors, tobacco smoking, genetic predispositions or dietary factors is debatable.

Veena *et al*[56] showed that a longer duration of use and bigger amounts of alcohol consumption are necessary before cirrhosis disease compared with CP.

PANCREATIC CANCER

Looking at the risk factors of PC, cigarette smoking represent one of the most important contributing lifestyle risk factor accounting around 20% of the patients. Other factors include CP, diabetes, *Helicobacter pilory* infection, obesity, family history of PC and also heavy alcohol consumption[57].

While the association between alcohol abuse and pancreatitis is well established the association between alcohol consumption and PC is less clear and remains controversial.

Historical studies in different cohorts have shown that patients with CP have an increased risk of PC; the excess risk was observed in men and women[52].

Epidemiological data on alcohol and PC are difficult to interpret due to several reasons as: the small sample size (and limited power to detect a possible weak effect of a rare exposure), variability in dose and time of alcohol exposure and other causes influencing (genetic or environmental).

Since 2009 three pooled data analysis and one meta-analysis have been performed. The first pooled study published in 2009 was adjusted for age, smoking status, diabetes, weight, food intake and time of exposure. The control group was no drinkers (0 g ethanol/d). The authors showed a moderate effect of heavy drinking (> 30 g ethanol/d or approximately more than 2-3 alcoholic beverages/d) in women but not in men although the difference in the results by gender was not statistically significant. No associations were observed for the different types of beverage (beer, wine or liquor). This finding is consistent with a modest increase in risk of PC for alcohol intakes of at least 30 g/d[58].

The second pooled analysis[59] was performed using PanScan study. Analysis was adjusted for age, study, race, smoking status, diabetes and body mass index. The control group was a very light drinkers (> 0-4.99 g ethanol/d) for the total alcohol intake and non-drinkers (0 ethanol/d) for the beverage type group. The author concluded that heavily consumption of alcohol from liquor (> 45 g ethanol/d) was associated with PC in men but not in women and not in men and women combined.

The third pooled analysis[60] included a big number of PC cases. Analysis was adjusted for age, sex, race, area, smoking status, education, body mass index and diabetes. The study concluded that heavy drinkers (> 9 drinks/d) have a moderately increased risk of PC compared with lightly drinkers (< 1 drink/d).

The meta-analysis of alcohol consumption and PC includes 21 case-control studies and 11 cohort studies published since 2009[61]. The results obtained indicate that heavy drinkers (> 3 drinks/d) but not moderate or low drinkers, have an increased risk of PC. This result is valid for both men and women.

In summary, these analysis and meta-analysis suggest that heavy drinkers (>30-40 g ethanol/day or > 3 drinks/d) can result in an increased risk of PC. The authors conclude that in a population of 10%-15% of heavy drinkers, 2%-5% of all PC cases could be attributed to alcohol consumption.

Most of the data published in the literature indicate that alcohol drinking at the levels typically consumed by the general population is probably not a risk factor for PC, however heavy alcohol drinking may be related to PC risk[62]. As an extreme case, Gupta *et al*[63] conclude that men binge drinkers (> 5 drinks/episode or > 70g ethanol/episode) leads to a 3.5-fold increased risk of PC and this risk is higher with the numbers of drinks per binge episode. This effect is greater in current smokers than in former or never smokers.

In contrast, most previous epidemiological studies could not demonstrate an excess risk for PC associated with moderate alcohol intake. Velema *et al*[64] did not find sufficient evidence for a causal relationship. Ye *et al*[65] concluded that the excess risk for PC among alcoholics was small, and may be totally attributable to a mix of causes, for example also increased by smoking.

Some of these studies demonstrated no significant

elevation in risk of PC was observed among alcohol consumers less than 55 years old; however, no similar elevation in risk was observed among those 55 or more years old[66].

In conclusion, there is several difficulties to evaluate the association between alcohol and PC and in epidemiological studies the small sample size has probably contributed to the problem of limited power to detect a possible weak effect of a rare exposure (*i.e.*, chronic heavy alcohol drinking). Also causes like exposure measurement error, limited number of cases under study and competing causes have made difficult study the potential effects of alcohol consumption on the risk of PC[67].

Compared with the general population, alcoholic drinkers without alcoholic CP or alcoholic liver cirrhosis have a modest 40% excess risk to develop PC. Even patients diagnosed with alcoholic CP or liver cirrhosis, have only two folds risk for PC. In addition, it has been shown that non-alcoholic CP patients have a markedly greater excess risk for PC, and this risk is higher than that among alcoholic CP patients[65]. These data suggest that in some circumstances, pancreatitis may be an early manifestation of an as yet undiagnosed PC. Also Kudo *et al*[68] concluded that alcohol drinking was not significant risk factor for developing PC in CP patients. So far, no studies have demonstrated that alcoholic pancreatitis leads to an increased risk of PC compared with non-alcoholic pancreatitis.

Taking these data together, although the role of alcohol consumption in PC remains unclear, it appears that low to moderate alcohol consumption is not associated with PC risk, but chronic heavy drinking (and perhaps prolonged bingering, although available data are confusing) may increase risk of PC[58,59,61,63]. Heavy alcohol consumption may increase PC risk by potentiating the effects of other risk factors such as tobacco smoking, poor nutrition, and inflammatory pathways related to CP, but also may have independent genetic and epigenetic effects.

Overall alcohol intake is only one dimension of drinking behavior. Other considerations such as spacing of drinking occasions, quantity of alcohol consumed, type of alcoholic beverages, ways in which beverages are mixed and commodities consumed in conjunction with alcoholic beverages as salt[69], coffee or tobacco or food[70] are important of pancreatic disease and were described many years ago.

POTENTIAL MECHANISMS OF ALCOHOL CONSUMPTION AND PANCREATIC DISEASES

Over the last decades there have been numerous efforts to elucidate the mechanisms by which alcohol damages the pancreas.

The injurious effects of ethanol on the pancreas are mediated through different mechanisms[71] as (1) sensitization of acinar cells to cholecystokinin (CCK) inducing premature activation of zymogens[72]; (2) potentiation of the effect of CCK on the activation of transcription factors, nuclear factor-κB and activating protein-1[73,74]; (3) generation of toxic metabolites such as acetaldehyde and fatty acid ethyl esters; (4) sensitization of the pancreas to the toxic effects of coxsackievirus B3[75]; and (5) activation of pancreatic stellate cells by acetaldehyde and oxidative stress and subsequent increased production of collagen and other matrix proteins[76].

Chronic alcohol exposure leads to impaired exocytosis mediated by acetaldehyde-induced microtubular dysfunction and apical cytoskeleton reorganization in acinar cells, with a subsequent accumulation of intracellular enzymes[77]. In addition, alcohol decreases the stability of zymogen and lysosomal membranes and enhancers acinar cell sensibility to CCK further increasing susceptibility to pathological enzyme activation[78,79]. Some theories also show that physiologically ethanol leads to the formation of protein secretory plugs that obstruct pancreatic ducts, spam of the sphincter of Oddi or decreased tone of the sphincter causing reflux[80-82].

Ethanol and its major metabolite, acetaldehyde, are classified by the International Agency for Research on Cancer as group 1 carcinogens[83].

Alcohol metabolism depends on enzymes that transform ethanol. Genes for these modifying enzymes have specific polymorphisms that differ between subjects and races leading to differences in susceptibility to alcohol effects and alcohol dependence[47].

Although the liver is the major ethanol-metabolizing organ in the body, the pancreas can metabolize alcohol both via oxidative and non-oxidative pathways.

The oxidative pathway is catalyzed by the enzyme alcohol dehydrogenase (ADH) and the cytochrome P450 and produces the metabolite acetaldehyde. Finally, the oxidative alcohol metabolism results in the generation of oxygen species (ROS)[84] and a depletion of the ROS scavenger glutathione[85]. The increased ROS production (which damage DNA and proteins) and a reduction of proteins that eliminate this ROS (glutathione and enzymes related) lead to oxidant stress and resultant damage in tissue. This stress could be responsible for induce alcoholic pancreatitis as has been demonstrated by several models[86-88].

But in pancreas, the non-oxidative pathways may be more important than oxidative metabolism, generating fatty acid ethyl esters (FAEE) by fatty acid ethyl ester synthases (FAEE synthases)[89]. It has been shown that pancreas exhibits higher FAEE synthase activity than liver[90] and FAEEs accumulation have been observed in human and rat pancreas after alcohol intake[91-93].

The products of alcohol oxidation (acetaldehyde and ROS) and of non-oxidative metabolism have been reported to cause acinar cell injury. Acetaldehyde cause morphological changes in rat and dog's pancreas[94] and it has been showed that inhibits CKK-simululated cinar cell secretion[95]. Also, several studies have demonstrated that alcohol intake causes oxidant stress within the pan-

creas[86-88] which may play a role in the alcohol-induced destabilization of zymogen granules and lysosomes. In addition, alcohol oxidation contributes to acinar damage altering the intracellular redox state (a reduced NAD/NADH ratio and increased lactate/piruvate ratio). Other results obtained in isolated mouse pancreatic acinar cells suggest that FAEEs leads to mitochondrial damage, loss of ATP and rise in cytosolic free calcium, which leads to acinar cell toxicity[96]. Other authors have shown that acute application of ethanol at clinically relevant concentrations (1-50 nmol/L) of isolated acinar cells resulted in calcium influx due to the production of oxidative metabolites of alcohol[97]. Together, these data show that the role of alcohol metabolites in acinar cell damage could be due to aberrant calcium signals[98]. FAEEs can elevate Calcium greater that ethanol alone. In addition, FAEEs and their products, fatty acids induces necrosis in acinar cells and this process could be avoided by calcium chelation[99].

These physiological changes leads to the pathobiology found in alcoholic pancreatitis including acute and chronic inflammation, elimination of parenchymal cells of the pancreas by a deregulation of apoptosis/necrosis and/or modification in cell proliferation[49]. The hypothesis called "necrosis-fibrosis sequence" shows these pathological changes where in the early episodes of pancreatitis, patients present focal necrosis and mild fibrosis while patients evaluated years later of the onset of symptoms presents fibrosis and calcifications but not necrosis[100].

But the fact that only a minority of heavily drinkers develops pancreatitis or PC indicates that other susceptibility factors as lipid tolerance, smoking or hereditary factors play an important role. In the last decades, genetic susceptibility has been considered between the factors that contribute mainly to the development to alcoholic pancreatic diseases.

One study showed an association between a polymorphism of the gene for one FAEE synthase enzymes, carboxylester lipase and risk of developing alcoholic pancreatitis[101].

In addition, the G191R variant in the anionic trypsinogen gene *PRSS2*, has been shown to result in a form of trypsin that is easily degraded, is more infrequent in alcoholic pancreatitis patients compared with healthy controls[102].

Other studies have demonstrated that mutation N34S in *SPINK1* gene is found in 5%-5.8% of patients with pancreatitis compared with 1% in healthy controls[103,104]. But still the functional consequences of this mutation are unknown.

One of the enzymes that have been also related to alcoholism and drug dependence for decades is ADH. Li *et al*[105] performed a recent meta-analyses and confirmed strong associations of the *ADH1B* and *ALDH2* genes with alcoholism and alcohol-related medical diseases[106]. Recently, Celorrio *et al*[107] demonstrated that some specific polymorphism in the genes *TH*, *ADH1B* increase the risk to develop diseases a consequence of excessive consumption of alcohol.

Although it is clear that alcohol consumption is genetically influenced, but characterized by incomplete penetrance, phenocopies, heterogeneity, and polygenic inheritance.

In conclusion, nowadays it appears clear that alcohol consumption is the first or second most common cause of pancreatitis. Based on the different epidemiology studies published in the literature the percentage of pancreatitis cases attributable to alcohol abuse vary since 30% to 90% between countries. A statistical association has been shown with a threshold of $\geqslant$ 5 drinks per day with a dose of alcohol $\geqslant$ 50 g/d.

But despite that excessive alcohol consumption is primarily responsible for most cases of pancreatitis, alcohol intake alone is not sufficient to lead to this disease, as less than 10% of heavily drinkers develop pancreatitis.

Regarding to PC, the role of alcohol consumption remains less clear, and low to moderate alcohol consumption do not appear to be associated with PC risk, but only chronic heavy drinking increase the risk compared with lightly drinkers.

Genetic variability and environmental exposures such as smoking and diet could act synergistically with regard to pancreatitis and PC and should be considered for further investigations. Probably heavy alcohol consumption may increase pancreatic disease risk most likely potentiating the effects of these other risk factors, but also may have independent genetic and epigenetic effects.

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Use of antibiotics in the treatment of Crohn's disease

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Abstract

Many data coming from animal models and clinical observations support an involvement of intestinal microbiota in the pathogenesis of Crohn's disease (CD). It is hypothesized in fact, that the development of chronic intestinal inflammation is caused by an abnormal immune response to normal flora in genetically susceptible hosts. The involvement of bacteria in CD inflammation has provided the rationale for including antibiotics in the therapeutic armamentarium. However, randomized controlled trials have failed to demonstrate an efficacy of these drugs in patients with active uncomplicated CD, even if a subgroup of patients with colonic location seems to get benefit from antibiotics. Nitroimidazole compounds have been shown to be efficacious in decreasing CD recurrence rates in operated patients, and the use of metronidazole and ciprofloxacin is recommended in perianal disease. However, the appearance of systemic side effects limits antibiotic long-term employment necessary for treating a chronic relapsing disease. Rifaximin, characterized by an excellent safety profile, has provided promising results in inducing remission of CD.

Key words: Antibiotics; Crohn's disease; Gut microbiota; Mycobacteria

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ROLE OF GUT MICROBIOTA IN CROHN'S DISEASE

Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by an altered composition of the intestinal commensal bacteria (dysbiosis)[1,2]. Dysbiosis is considered to have a preeminent role in CD pathogenesis by inducing an abnormal immune response in genetically susceptible individuals[3-7].

Intestinal sites commonly affected by CD lesion, in fact, are those with the highest bacterial concentration, such as colon, terminal ileum, especially after the loss of the ileocecal valve, and upstream from strictures. The luminal content is necessary for causing intestinal inflammation, and CD lesions do not appear when it is diverted from the gut, whereas restoration of bowel continuity or infusion of faecal material into the bypassed intestine rapidly results in recurrence of the disease[8-10]. In genetically engineered rodent models susceptible to spontaneous colitis, inflammation does not occur when the animals are raised in germ-free conditions[11]. Patients with CD have an altered composition of gut microbiota with increased concentrations of invasive bacteria, especially *Escherichia coli* (*E. coli*) and a decreased number of protective bifidobacteria, lactobacilli and the more recently studied *Faecalibacterium prausnitzii,* that has been shown to have anti-inflammatory properties[12-15]. Genetic susceptibly in a subgroup of patients with CD is related to polymorphisms in the *NOD2/CARD15* gene, suggesting that individuals with mutations in *NOD2* present defective intestinal immune responses to gut microbiota[16-20]. About 20% of CD patients are homozygous for *NOD2* variants and they may have an increased susceptibility to CD localized at the ileum[21].

Moreover, a loss of immunologic tolerance to the

commensal flora has been observed in patients with active IBD[22].

Experimental studies in animal models have shown that broad-spectrum antibiotics are efficacious in almost all models of acute and chronic colitis and ileitis[23]. However, they have only a transient benefit in HLA-B27 transgenic rats[24]. Broad-spectrum antibiotics can both prevent and treat experimental colitis of rodent models, while metronidazole and ciprofloxacin can only prevent the onset of colitis, but not reverse the established disease[25,26].

ANTIBIOTIC TREATMENT IN CROHN'S DISEASE

These and other data, confirming the role of the gut microbiota in the pathogenesis of CD, provide the rationale for a therapeutic manipulation of the intestinal flora through the use of antibiotics and probiotics[27-30].

Some of the suggested mechanisms of action for antibiotics in CD are the ability to reduce luminal and mucosa adherent bacteria concentrations, to eliminate in a selective way aggressive bacterial species, to decrease bacterial tissue invasion and translocation. Additionally, some antibiotics also exert a potential immunosuppressive action.

The role of antibiotics as primary or adjunctive treatment of active, uncomplicated CD has not been clearly demonstrated and their use is controversial. A recent meta-analysis suggests that antibiotics may be effective as primary therapy of CD[31], but guidelines do not recommend their use except for the treatment of septic complications of CD, symptoms attributable to bacterial overgrowth, or perianal disease[32].

ANTIBIOTICS FOR INDUCTION AND MAINTENANCE OF DISEASE REMISSION

The similarity of CD to tuberculous enteritis and Johne's disease of ruminants, caused by *Mycobacterium Avium* subspecies *Paratuberculosis* (MAP), and the isolation of atypical Mycobacteria from blood and tissue of CD subjects, have lead to evaluate the efficacy of anti-mycobacterial drugs in these patients[33-37]. However, the results of the randomized controlled trials performed with antibiotics active against atypical Mycobacteria for obtaining and maintaining CD remission have been conflicting.

A meta-analysis that considered eight trials employing different associations of anti-mycobacterial drugs showed that these drugs seem to be ineffective for inducing remission without a course of steroid therapy[38].

In the largest study 213 Australian patients were randomized to receive a combination of clarithromycin plus rifabutin and clofazimine, antibiotics active against MAP, or placebo for up 2 years, in addition to a 16-week course of corticosteroids[39]. The results showed a significant benefit only at 16 wk, when the antibiotic combination was added to steroids, confirming the data founded by the meta-analysis, and suggesting that the short-term advantage could be related to a generic antibacterial effect. Therefore, this study does not support a significant role for MAP in CD pathogenesis, although several objections to this conclusion have been raised[40-44]. At the present time the mycobacterial hypothesis cannot be completely ruled out, and it continues to be plausible that an infectious agent could start the inflammatory process[45].

Pathogenic adherent and invasive *E. coli* have been detected in Crohn's ileal and colonic tissue[46-49]. This bacterium can invade and replicate within macrophages, inducing the secretion of large quantities of tumor necrosis factor[50]. Clarithromycin is a broad spectrum macrolide antibiotic that can penetrate into macrophages, and may therefore be effective in eradicating the bacteria. However, a study comparing clarithromycin 1 gr to placebo for 3 mo in patients with active CD, was stopped because of poor efficacy[51].

Metronidazole, which is active against anaerobic bacteria and some parasites, and ciprofloxacin, particularly active against *E. coli* and *Enterobacteriacee*, are the most frequent studied and used antibiotics. Several randomized clinical trials have been performed employing metronidazole and/or ciprofloxacin for induction of CD remission.

The results of the trials have indicated that metronidazole is efficacious in active Crohn's colitis and ileo-colitis, but not in small bowel location[52-55]. Five randomized controlled studies evaluating the efficacy of ciprofloxacin, alone or in association with metronidazole, in patients with active CD, have shown uncertain results[56-60].

Patients with colonic involvement get more benefit from antibiotics, probably because of the high concentration of bacteria in the colon.

The efficacy of antibiotics in CD seems to be related to a prolonged therapy, that is frequently burdened by an elevated number of systemic adverse events (AEs). In particular, there is concern about the *Clostridium difficile* infection caused by antibiotics, especially fluoroquinolones such as ciprofloxacin[61]. *Clostridium difficile* infection can induce CD relapse and gastroenterologists in charge of CD patients must be aware of this serious complication. In 2007 two retrospective studies demonstrated a dramatically increased incidence of *Clostridium difficile* infection in patients with IBD, who appeared to be more susceptible to this infection than non IBD-patients[61,62].

The minimally absorbed antibiotic rifaximin, which is active against gram-positive and gram-negative bacteria, has been shown to be effective in active CD patients. In an exploratory study, a gastro resistant formulation of rifaximin [extended intestinal release (EIR)] (rifaximin-EIR) at a dose of 800 mg twice daily for 12 wk reported significantly higher rates of remission and response compared to placebo, in a subgroup of patients with mild to moderate CD and an elevated value of C-reactive protein[63].

In a recent, larger study 402 patients with moderately active CD were randomized to receive rifaximin-EIR 400, 800, 1200 mg or placebo twice daily for 12 wk[64]. The

results showed that rifaximin-EIR 800 mg twice daily was significantly superior to placebo in inducing remission, defined as a Crohn's Disease Activity Index (CDAI) score < 150, after 12 wk of therapy (62 % *vs* 43%). The effect was maintained throughout a subsequent 12-wk follow-up period in 65% of patients. Remission rates in patients treated with 400 and 1200 mg twice daily (54 % and 47 %, respectively) showed a trend towards but without reaching the statistical significance in comparison with placebo. The lack of a dose-response relationship was probably caused by the higher percentage of subjects who discontinued the treatment due to AEs in the 1200 mg twice daily group. Median CRP values over time showed no statistically significant differences between treatment groups. The most frequent AEs were of gastrointestinal origin, either determined by an underlying disease, with consequent CDAI score increase and treatment failure, or to rifaximin related side effects. Also in this study colonic location appeared to be associated with a higher response to the antibiotic therapy. Overall, the safety profile of rifaximin-EIR was good, indicating that rifaximin could be administered for a long period of time. However, *Clostridium difficile* infection was reported in a single patient with rifaximin 800 mg twice daily 20 d after the end of the treatment period. Rifaximin has been successfully employed for the treatment of CDI in metronidazole resistant patients[65], however it is probable that rare clones of rifaximin-unresponsive *Clostridium* can develop[66].

ANTIBIOTICS FOR PREVENTION OF POSTOPERATIVE RECURRENCE

Prevention of recurrence after intestinal resection is one of the major aims in the treatment of CD, and antibiotics have been used in this setting in 3 randomized placebo-controlled studies. The rationale for the employment of antibiotics is that bacteria are strongly suspected to be the main reason for the recurrence of lesions. In the first trial, metronidazole, at a dosage of 20 mg/kg per day for a 3-mo period, significantly decreased the incidence of early severe endoscopic recurrence, and also seemed to delay the symptomatic recurrence at 1 year, but it was associated with a high percentage of side effects[67]. The same authors have later performed a 12 mo placebo-controlled trial employing ornidazole, which has the same bacterial spectrum with a better safety profile. Ornidazole at a dose of 1 g/d proved significantly to reduce the clinical recurrence rate at 1 year, but more than 30% of patients in the antibiotic group discontinued the therapy because of side effects[68]. More recently, D' Haens *et al*[69]compared metronidazole (250 mg 3 times per day) given for 3 mo plus azathioprine for 12 mo to metronidazole alone in 81 operated CD patients at high risk of recurrence. This drug combination uses metronidazole as bridge therapy, given the slowness of azathioprine activity. At 3 mo after surgery severe endoscopic recurrence occurred in 34.3% of patients in the metronidazole/azathioprine group and in 52.6% of patients in the metronidazole/placebo group (P = 0.11). At month 12, severe endoscopic recurrence was observed in 43.7% of patients in the metronidazole/azathioprine group and in 69.0% of patients in the metronidazole/placebo group (P = 0.048). The study treatment was well tolerated and only 3 patients discontinued the therapy in the first 3 mo because of side effects, probably ascribable to metronidazole. The authors concluded that the combined treatment seems to be recommendable to CD patients with an elevated risk for postoperative recurrence. Recurrence prevention requires a long-term treatment, and this is burdened by a high number of AEs. Given its high safety profile, rifaximin, provided that its efficacy is completely demonstrated, should be employed for long term recurrence prevention.

ANTIBIOTICS FOR TREATMENT OF PERIANAL DISEASE

Antibiotics are widely employed for treatment of perianal CD, alone or as adjuvant therapy, and, despite the lack of controlled trials, European Crohn's and Colitis Organisation consensus statements recommend them in simple and complex fistulising perianal disease[70].

Most of the studies of perianal disease treated with antibiotics are, in fact, uncontrolled with a small sample size. In these studies, metronidazole and ciprofloxacin used alone or in combination have proved to induce a decrease of fistula drainage, but rarely induce closure. Moreover, symptoms tend to recur after suspending the treatment[71-73]. AEs resulting from prolonged use of antibiotics may, however, limit their use.

Recently, a randomized, placebo-controlled pilot study evaluating ciprofloxacin or metronidazole for the treatment of perianal fistulas in 25 CD patients failed to demonstrate a significant benefit of either antibiotic treatment over placebo in the cessation of drainage[74]. However the study was probably too small to permit detecting differences between treatment arms.

In conclusion, the different antibiotic regimens evaluated in the randomized controlled studies, the limited number of patient enrolled, the heterogeneity between the trials, and the uncertain results have led to the conclusion that antibiotics cannot be recommended for treatment of active CD, except for septic complications, symptoms attributable to bacterial overgrowth, or perianal disease. In addition, their efficacy could be limited by the prolonged therapy usually required for treating CD. However, there seems to be a subgroup of patients with colonic disease who can respond to these medications, likely due to the differences in gut microbiota between ileal and colonic location. Nitroimidazole antibiotics seem to be effective in decreasing both endoscopic and clinical recurrence rates after surgery, but their long-term use is complicated by an elevated number of AEs.

Treatment of patients with mild and moderate CD with rifaximin seems promising, but further larger studies are needed.

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MINIREVIEWS

Clinical impact of hepatitis B and C virus envelope glycoproteins

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Abstract

Chronic infection by either hepatitis B virus (HBV) or hepatitis C virus (HCV) share epidemiological characteristics with risks for development of severe complications such as liver cirrhosis and hepatocellular carcinoma. HBV and HCV also share a high genetic variability. Among highly variable regions, viral genes encoding surface proteins (hepatitis B surface antigen, E1/E2 HCV glycoproteins) play key roles in the stimulation of the host-related immune response and viral entry into hepatocytes. Specific segments of HBV envelope proteins (preS1, "a" determinant) are crucial in the entry process into permissive cells. HCV entry is a complex multistep process involving multiple cell cofactors (glycosaminoglycans, low density lipoprotein receptor, SR-B1, CD81, claudin-1, occludin, EGFR, EphA2) in the interaction with HCV E1/E2 envelope glycoproteins. *In vitro* both viruses can be controlled by antibody-mediated neutralization targeting viral envelope, also essential in preventing HBV infection *in vivo* as observed through successful vaccination using HBs antigen. But preventive vaccination and/or therapeutic pressure can influence HBV and HCV variability. For HBV, the patterns of antiviral drug resistance in chronic hepatitis are complex and the original *pol*/*S* gene overlap has to be taken into account. Treatment-induced HBV mutations in pol could indeed generate S mutants with subsequent modified antigenicity or increased cancer induction. Variability of HBV and HCV envelope proteins combining high exposure to selective pressures and crucial functional roles require investigation in the context of diagnostic, vaccination and treatment tools. In this editorial a synthesis is performed of HBV and HCV envelope properties at the entry step and as antigenic proteins, and the subsequent clinical impact.

Key words: Hepatitis B; Hepatitis C; Viral envelope glycoproteins; Clinical outcome

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INTRODUCTION

Approximately 400 million people are currently living with chronic infection due to hepatitis B virus (HBV) and the number is approaching 160 million for chronic hepatitis C virus (HCV)-related infection[1]. Besides sharing many epidemiological features, HBV and HCV can both provoke chronic infections after an acute phase, due to a failure of humoral and cellular immune host responses[2-5].

HBV and HCV also share some biological similarities, especially the high frequency of mutations occuring dur-

ing viral replication. As a result both viruses can develop mutations leading to drug failure, especially for drugs with low potency and a low genetic barrier to resistance such as with anti-HBV lamivudine monotherapy. It is expected this will be less likely during treatment by entecavir or tenofovir which exhibit potent anti-HBV activity and high genetic barriers to resistance.

Viral dynamics are rapid with daily production of $10^{12\text{-}13}$ virions for HBV and 10^{12} for HCV. The half-life of free viral particles is short, less than 4 h for HBV[6,7] and 2-3 h for HCV[8]. Although both HBV and HCV can produce consistently high levels of virus in serum, they do so using viral templates with very different life-spans. The template for HBV replication, covalently closed circular DNA (cccDNA), resides in the nucleus of infected hepatocytes and is very stable in chronic infection, likely persisting for months[9], if not longer. On the other hand the intracytoplasmic minus strand RNA HCV template only persists for a few hours. Correspondingly HBV eradication is more difficult and only occurs after several years of viral suppression by therapy. Another characteristic of HBV is that its genome consists of overlapping reading frames: as a consequence, mutation rates to functional virus are lower for HBV compared to HCV which does not contain any overlapping gene. The counterpart of this molecular HBV characteristic is that changes at one position may affect the structure and function of more than one viral protein: the polymerase gene overlaps with the envelope gene, and consequently mutations have yet been published in polymerase gene under treatment pressure can produce mutations in the envelope *S* gene[10].

Despite the availability of an effective vaccine against HBV, new infections are still highly frequent worldwide. Hence, a careful examination of circulating viral strains must be considered in order to possibly adapt the antigenic vaccine content. To date however no effective vaccine is available against HCV. For both viruses, surface envelope glycoproteins are the external antigens first encountered by host-related immune responses during primary infection. These proteins contain both highly variable and conserved regions, the latter providing possible targets for preventive immunization and/or complementary therapeutic approaches.

The aim of this editorial is to give an overview of the current knowledge on hepatitis B and hepatitis C envelope glycoproteins, however with a particular emphasis on HBV. Structural and functional data will be described on the role of these proteins in the viral entry step into hepatocytes and in the stimulation of host-related responses and clinical impacts for HBV will also be described.

GENERAL PRESENTATION OF HBV ENVELOPE GLYCOPROTEINS AND ANIMAL/CELL CULTURES SYSTEMS SUITABLE FOR THEIR INVESTIGATION

For more than 20 years, primary human hepatocytes (PHH) from surgically excised liver specimens have been the only available *in vitro* HBV infection model. The transfection of hepatocyte-related cells (based on primary hepatocytes or hepatic cell lines which are easier to handle) with replication-competent HBV genomes allowed the production of secreted infectious virions. Regardless of the difficulty in maintaining these *in vitro* models, PHH cultures have for a long time been the only way to study viral infectivity. Due to poor efficiency of HBV infection in this model, virus amplification required the use of dimethylsulfoxide (DMSO) and of 4% polyethylene glycol during cultivation and infection, respectively. Primary hepatocyte cultures from *Tupaia belangeri* can also be infected with HBV, as efficiently as PHH cultures, but with fewer restrictions. Among human hepatic cell lines, the optimal system for various experiments was shown to be HepaRG cells, recently established from a liver tumor, and which become susceptible to HBV upon treatment with DMSO and hydrocortisone[11,12] (Table 1).

Many discoveries about the viral replication cycle, persistence and clearance have been obtained using animal models such as chimpanzee, gibbon or tupaia infected with HBV[13,14]. Moreover, humanized uPA/SCID mice have been recently used to study HBV/hepatitis D virus coinfection and represent an interesting model allowing us to investigate new antiviral drugs[15]. Experimental systems have also been provided by other *Hepadnaviridae* such as the duck HBV transmissible to many duck species or the woodchuck hepatitis virus, restricted to the North-Eastern American woodchuck[13].

Hepadnaviruses are characterized by specific liver tropism and high species specificity, restricting *in vivo* infection to their natural host or closely related species and contributing to the difficulty in developing suitable models of HBV infection[11].

HBV envelope glycoproteins are key viral elements in the viral cycle and in the stimulation of host-related immunity. Within virions, HBV surface envelope antigen (HBsAg) includes three viral surface glycoproteins, named large, medium, and small proteins (LHBs, MHBs and SHBs). They exhibit various distributions either in virions or in subviral particles. Their expression is directed by one stop codon and three start codons in a single open reading frame for translation into preS1, preS2 and S proteins[16]. The preS1 protein [108 or 119 amino acids (aa) depending on the genotype] is present only in LHBs; the preS2 protein (55 aa) is present in LHBs and MHBs; and the S protein (226 aa) is shared by LHBs, MHBs and SHBs[11] (Figure 1).

The three HBV surface preS1, preS2 and S proteins have different functions. Within the preS1 domain, amino acids 3-77 of the Large protein are essential for infectivity as is the myristoylation of glycine at position 2. The preS2-domain is present in both M- and L-proteins but because of the cytosolic orientation of the preS1-domain in the L-protein, it is N-glycosylated only in the M-protein. Although antibodies against the N-terminal part of preS2 can inhibit HBV infection *in vitro*, the Medium protein of HBV is believed to play an accessory

Table 1 Cell culture systems and *in vitro* models developed to study hepatitis B virus and hepatitis C virus envelope glycoproteins

	In vitro model/cell culture system	Step of the viral cycle	Benefits and major findings	Drawbacks
HBV	Primary hepatocyte cultures PHH PTH	Replication	No need for DMSO and hydrocortisone for PTH system	Not easy to handle Cells cannot be propagated *in vitro* Addition of growth factors
	Hepatic cell lines HepG2 HepaRG	Binding and infection (HBV and HDV)	Specific binding and uptake Cellular determinants of hepatocyte differentiation and their influence on HBV infection	No productive infection Addition of DMSO and hydrocortisone
HCV	Recombinant E2 glycoprotein: Truncated soluble form of recombinant E2 glycoprotein	Entry process	Identification of two major receptors CD81 and SR-BI Interaction with heparan sulfate proteoglycans	Different behavior from E1 E2 heterodimers Binding to various cell lines different from hepatocytes
	VLPs: Self assembly of HCV structural proteins produced in insect or mammalian cells using a recombinant virus	Entry process	E1–E2 heterodimers at virion surface Cell attachment Attractive vaccine candidate	Difference in glycosylation status Difficult to prepare Non replicative
	HCVpp: Unmodified HCV envelope glycoproteins assembled onto retroviral or lentiviral core particles	Entry process	Study of infectivity and neutralization	Only the very early steps of viral cycle No association with lipoproteins No budding at the ER
	HCVcc: Transfection of one HCV strain sequence (JFH1) from a Japanese patient with fulminant hepatitis, in Huh 7 cell line.	Entire life cycle	Entry process +++ Replication Virus production Screening of antiviral molecules	Restricted to Huh-7 cell line Restricted to JFH1 non structural proteins sequence

HBV: Hepatitis B virus; HCV: Hepatitis C virus; VLP: Virus-like particles; HCVpp: HCV pseudotype particles; HCVcc: Cell culture derived HCV; HDV: Hepatitis D virus; DMSO: Dimethyl sulfoxide; SR-BI: scavenger receptor class B type I ; ER: Endoplasmic reticulum; PHH: Primary human hepatocytes; PTH: Primary *Tupaia belangeri* hepatocytes; DMSO: Dimethylsulfoxide.

role in infectivity. While the preS-domains form linear epitopes, the S-domain forms multiprotein complexes using inter- and intramolecular disulfide bonds with the eight Cys residues within the antigenic loop. A crucial function of the S-domain is viral morphogenesis, but the first transmembrane sequence also plays a role in viral entry into hepatocytes[11]. Moreover, two different transmembrane topologies have been described for the L surface protein: L chains with an internal N-terminal preS1 part are required in virion morphogenesis, whereas the L molecules which expose their preS1 domain on the viral particle surface probably link to a putative virus receptor and could determine the species specificity and viral tropism[16]. In addition to the preS1 domain, a second infectivity determinant, located in the antigenic loop of the S domain, is also required for infectivity[17].

HBsAg plays a central role in stimulating and, because of its variability, also oppositely thwarting the host-related immune response. The main antigenic part of HBsAg, namely the "a" determinant which is the core part of the major hydrophilic region (amino acid residues 99-169 of HBsAg), is a target of neutralizing antibodies. Immunogenic T cell epitopes are also present in the HBs Ag : P1 (aa 16-33) and P4 (aa 213-226) are the dominant epitopes in vaccine immunization[18].

INTERACTION WITH PUTATIVE CELLULAR RECEPTOR(S)

HBV envelope glycoproteins are the key components contributing to the attachment to the hepatocyte plasma membrane, thus defining organ and species specificity of HBV. Viral tropism is essentially restricted to the liver[14]. Although there is considerable evidence that a HBV receptor exists on hepatocyte membranes, it still remains to be identified. Convergent data place the receptor-binding site in the preS1 segment of HBV envelope proteins that play a key role at the early entry step for further viral infectivity, as described above. The preS1 (21-47) region of the envelope protein was demonstrated to be the dominant binding site to hepatoblastoma cell line (HepG2). More recently, the region consisting of amino acids 9-18 of the preS1 domain was identified as a conserved crucial attachment site while amino acids 28-48 as an accessory binding site[19]. The receptor for the preS1 peptide seems to be a single major peptide of molecular weight 31 kD[20]. The asialoglycoprotein receptor was described as playing a role in the attachment process[21], and numerous other cellular partners have been suggested to also contribute, such as heparin or annexin V, earlier referred to as Endonexin Ⅱ[22-26]. Interestingly, heparan sulfate proteoglycans emerged as potential major components in the HBV entry step[27]. However HBV entry and different steps of this process are not characterized in detail, while it is substantially known for HCV.

HOST-RELATED IMMUNE RESPONSES

During viral infection, immunological reactivity usually begins with an innate response in infected cells, where viral replication induces activation of intracellular an-

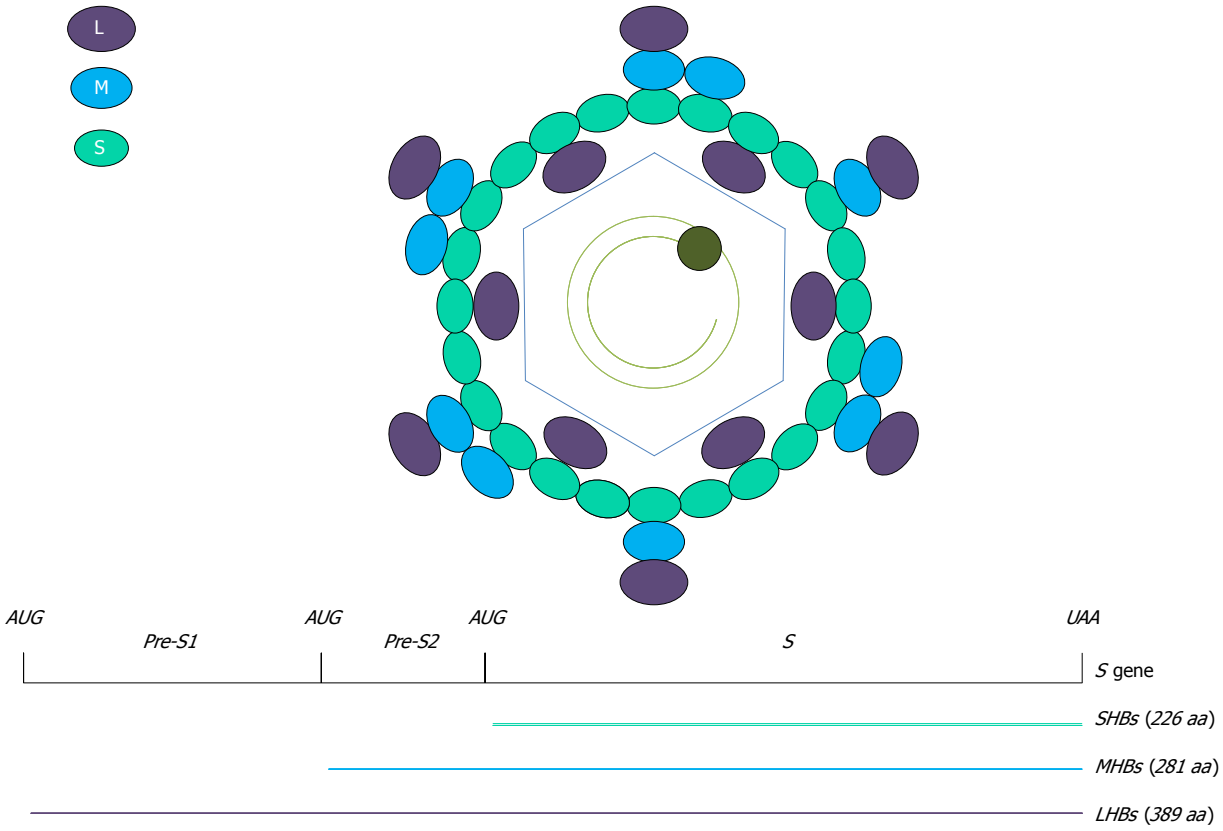

Figure 1 Schematic representation of hepatitis B virus envelope proteins and genome. Hepatitis B virus (HBV) surface envelope antigen includes three viral surface glycoproteins, named large, medium, and small proteins (LHBs, MHBs and SHBs). Their expression is directed by the *S* gene including three start codons and one stop codon in a single open reading frame.

tiviral mechanisms through transcription of interferon (IFN) inducible genes. However, in acutely HBV infected chimpanzees no specific pattern of cellular genes expression was observed during widespread expansion of HBV in the liver, revealing an absence of innate response by infected cells[28]. Interestingly, this could be thwarted by using IFN-α which has been described to inhibit HBV transcription and replication in cell culture and in humanized mice by targeting the nuclear cccDNA[29]. The adaptive immune response is responsible for viral clearance as well as HBV-related diseases pathogenesis. In patients with acute hepatitis, the T cell response is vigorous and multispecific. The CD8+ T cell response, under the control of CD4+ T cells, acts on viral clearance by triggering direct hepatocyte apoptosis and by secreting IFN-γ which inhibits HBV replication. In chronically infected patients, a weak and focused T cell response is observed (Figure 2). This seems less related to sufficient numbers of CD8+ T cells, but more to their functional alteration[30]. Human leukocyte antigen (HLA) class I and HLA class Ⅱ restricted T-cell epitopes have been recently reviewed in Desmond 2008[31]. Several HBV epitopes recognized by CD4+ T cells were described in the surface protein, including $S2_{109-134}$, $S_{200-214}$ and $S_{337-357}$, with $S_{179-194}$, considered as an immunodominant HLA-DR1-restricted epitope[32].

Regulatory T (Treg) lymphocytes, known to suppress the activation, differentiation and proliferation of immune cells, could be major actors in the inadequate immune responses observed during chronic HBV infection. The Treg response protects the host by limiting liver immunopathology while at the same time favouring the virus by inhibiting a protective T-cell response. The timing of the Treg response is vital; it needs to be not too late otherwise resulting in the excessive destruction of hepatocytes by effector T-cells, and not too early which would favour the establishment of chronic infection by blocking antiviral T-cell activity[33]. As suggested by Manigold *et al*[33], a higher frequency of Treg cells has been described in patients with chronic infection and correlated with greater HBV DNA concentration in serum.

The humoral immune response is based on anti-envelope protective antibodies. On the one hand, antibodies complex with free particles, removing them from circulation and on the other hand they prevent HBV attachment to hepatocytes, thus playing a critical role in viral clearance. Both viral and host factors can delay or inhibit antibody-mediated neutralization of HBV. As it will be further described, viral variability contributes to the failure of the humoral response. Other factors such as HIV coinfection[34] or immunosuppression from a variety of causes can also negatively modulate humoral reactivity[35]. Additionally, the development of an immune response, usually characterized by the strong appearance

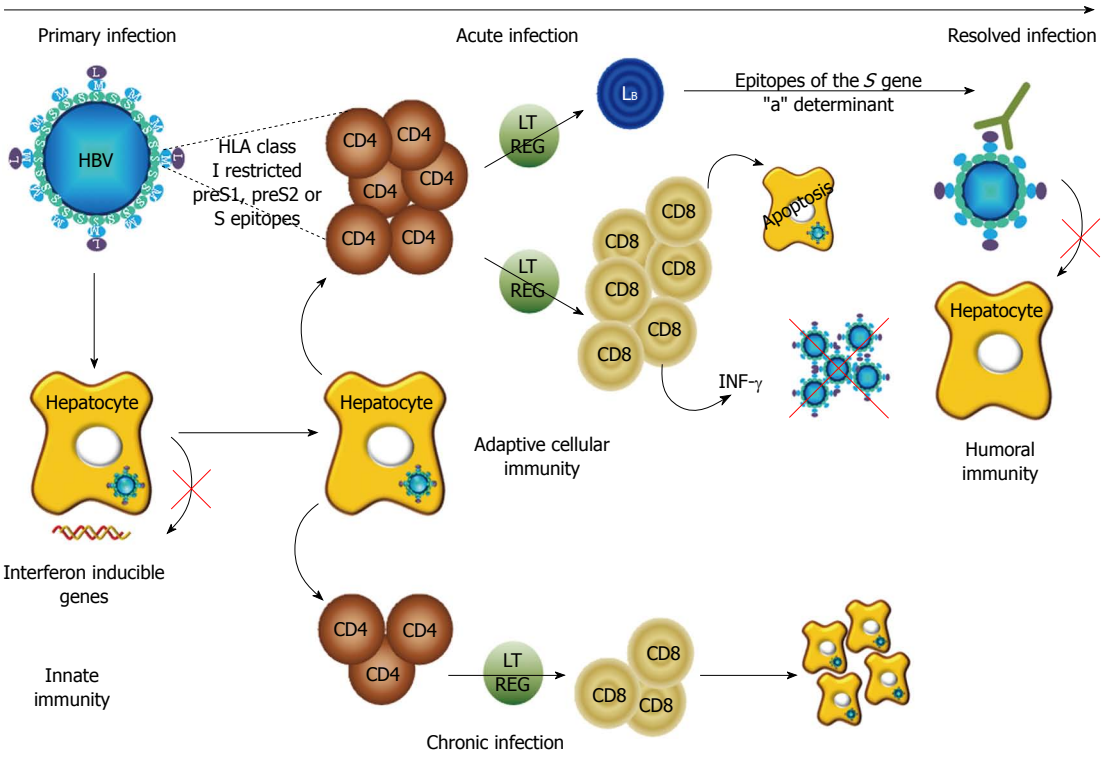

Figure 2 Role of the hepatitis B surface protein in the immune response to hepatitis B infection. Hepatitis B virus (HBV) primary infection does not induce activation of intracellular antiviral mechanisms through transcription of interferon inducible genes. After acute infection, the evolution to resolved hepatitis B is promoted by a vigorous and multispecific T cell response (CD4+ and CD8+ T cell) through hepatocyte apoptosis and interferon (IFN)-γ secretion, while evolution to chronicity is characterized by a weak and focused T cell response. Regulatory T cells (Treg) cells (LT REG) limit the function of effector T cells preventing excessive auto-destructive disease. A protective immunity is due in part to humoral and cellular anti-envelope response, preventing HBV attachment to hepatocytes, thus playing a critical role in prevention of infection after classical vaccination and in viral clearance in resolved hepatitis. HLA: Human leukocyte antigen.

of anti-HBs antibodies, may be delayed by social stress, as identified during HBV vaccination of students under exam stress[36].

GENERAL PRESENTATION OF HCV ENVELOPE GLYCOPROTEINS AND RELEVANT ANIMAL/CELL CULTURE MODELS

HCV envelope glycoprotein E1 and E2 represent crucial elements in the viral life cycle. Indeed, they are involved in virus cellular entry and are also targeted by host immune components. Thus characterization of these two proteins is essential to better understand virus-host interactions.

E1 and E2 are transmembrane glycoproteins containing an important N terminal ectodomain and a C terminal hydrophobic anchor domain. The E2 protein displays an amphipathic helix that forms a stem region, linking the ectodomain and the transmembrane region. The two glycoproteins form a non-covalent heterodimer at the viral particle surface, interacting with each other during virus entry into target cells. E1 and E2 are both stabilized by numerous disulfide bonds. Krey *et al*[37] described the connectivity of the E2 disulfide bonds and suggested that E2 could display a tertiary structure similar to that of class II fusion proteins, and containing three domains termed DI, DII and DIII. Their model reveals the distribution of E2 amino acids among the different domains. E1 and E2 are highly glycosylated proteins and glycosylation is needed for correct glycoprotein processing, folding and/or cellular entry.

The E2 glycoprotein contains three hypervariable regions, termed hypervariable region (HVR) 1, HVR2 and HVR3 and conformational binding sites to certain cellular co-receptors (CD81 binding site as an example). E1 and E2 glycoproteins are targets of both humoral and cellular immune responses generated during HCV infection and several specific epitopes have been described in E1 and E2.

Our knowledge of the HCV viral cycle and of the structural and functional characteristics of envelope glycoproteins has been hampered by the lack of reliable *in vivo* and *in vitro* models. Chimpanzee infection has long been the only available animal model, but with many limitations including ethical considerations, high cost, and clinical differences with human HCV infection. Other small animal models, such as humanized mice, are in development to overcome these problems.

Some *in vitro* models, such as soluble glycoproteins, virus like particles, and HCV pseudoparticles, have been developed that allow investigation of the viral entry process. Despite their ability to duplicate the early steps

of HCV entry, none of these models support the entire HCV replication cycle or efficient production of infectious particles. In this regard an important breakthrough was achieved with the cell culture system HCVcc, which is capable of producing infectious particles. The most useful *in vitro* models that allow the study of HCV glycoproteins are detailed in Table 1.

INTERACTIONS OF HCV ENVELOPE GLYCOPROTEINS WITH CELLULAR RECEPTORS

The main target cells of HCV are hepatocytes. HCV enters into target cells in a complex multistep process involving the following entry host factors: tetraspanin CD81, scavenger receptor class B member I (SR-BI), Heparan Sulfate and the tight junction proteins Claudin-1 and Occludin. Other partners in HCV entry were recently characterized, such as EGFR and EphA2[38].

Highly sulfated heparan sulfate structures are cell surface factors mediating initial virus attachment prior to virus interaction with CD81 and SR-BI, which directly interact with HCV envelope glycoproteins. SR-BI may define the attachment of the virion in the form of virolipoparticles to the cell surface and that then favours particle interactions with CD81 and Claudin coreceptors with a role of EGFR and EphA2 activated kinases in the CD81-Claudin binary complex. CD81 and Claudin-1 are essential for the clathrin dependant particle internalization, and the engagement of CD81 promotes internalization. CD81 associates with HCV and mediates entry *via* Claudin-1 and occludin complexes. Claudin-1/CD81 complexes seem to localize at the basolateral surface of polarized hepatoma cells. After uncoating and internalization by clathrin-dependent endocytosis, the viral genome is released into the cytoplasm leading to its translation and replication.

INTERPLAY BETWEEN HCV ENVELOPE GLYCOPROTEINS AND THE HOST IMMUNE RESPONSE

Persistent viral infection can be explained by the fact that HCV escapes both innate and adaptative immune responses or interferes with host defense mechanisms. Despite broadly reactive neutralizing and multispecific T cell responses generated during chronic infection, in most cases viral clearance is not achieved.

HCV RNA can be detected in patient serum between one and two weeks following infection whereas the appearance of HCV specific T-lymphocytes and antibodies is delayed up to several weeks after infection. In the early phase of infection neutralizing antibodies are rapidly induced by patients who subsequently clear the virus or control viral infection whereas they are absent or of low quantity in patients who progress to chronic HCV infection[39]. Both cellular and humoral responses fail to control HCV infection during the very early phase of infection. If viral clearance is characterized by a broad vigorous and persistent T cell response, cellular immunity is weak, narrow and transient in patients chronically infected (Figure 3). Some mutations occurring in epitopes targeted by CD4+ or CD8+ T lymphocytes can show a strong inhibition of immune reactivity, such as HCV E1 226-240 and HCV E2 436-450[3]. Patients who do not clear the virus present high quantity and even cross-neutralizing antibodies during the chronic phase, but these antibodies are unable to control HCV infection.

The failure of the humoral response is mainly due to the rapid onset of mutations in HVR1 of E2 and in other epitopes of the E1 and E2 proteins. However, HVR1, the most variable region of the HCV genome, is one of the targets of the neutralizing immune response. HVR1 may function as an "immunological decoy", stimulating an ineffective immune response for viral clearance but inducing selection of viral variants[40].

The E2-CD81 interaction has been shown to modulate B and T cell function. During HCV infection, the E2-CD81 interaction induces hypermutation of the heavy chain immunoglobulin in B cells. Hypermutation may lower the affinity and specificity of HCV-specific antibodies, enabling HCV to escape immune surveillance. Additionally, engagement of CD81 on natural killer cells by the E2 protein has been shown to inhibit the antiviral function of NK cells early in the infection process.

GENES AND PROTEINS VARIABILITY, ESPECIALLY IN VIRAL ENVELOPE GLYCOPROTEINS

Despite the compact arrangement of its DNA genome and the overlapping open reading frames that limit genomic plasticity, HBV is evidently diverse on a global scale as indicated by the eight referenced genotypes (A-H). HCV is also characterized by high genetic variability given that six (and probably a seventh) major genotypes have been described, each including several subtypes. Intragenomic variability of HBV and HCV is further reflected in the quasispecies distribution defined by the genetic heterogeneity of the viral pool found in any infected subject at a given time point[41,42]. For both viruses, this genetic variability is a consequence firstly of the high spontaneous error rate associated with the lack of a proofreading mechanism of viral polymerases, and secondly of the structural and/or functional constraints exerted on the virus by host immunity, immunoprophylaxis or antiviral therapy pressure.

Due to the structural constraints of its genomic organization, the emergence of HBV mutants occurs more slowly than for HCV. Since the preS1 and preS2 domains overlap with the region of the polymerase gene encoding for the spacer domain, deletions in preS can occur without affecting the enzymatic properties of HBV

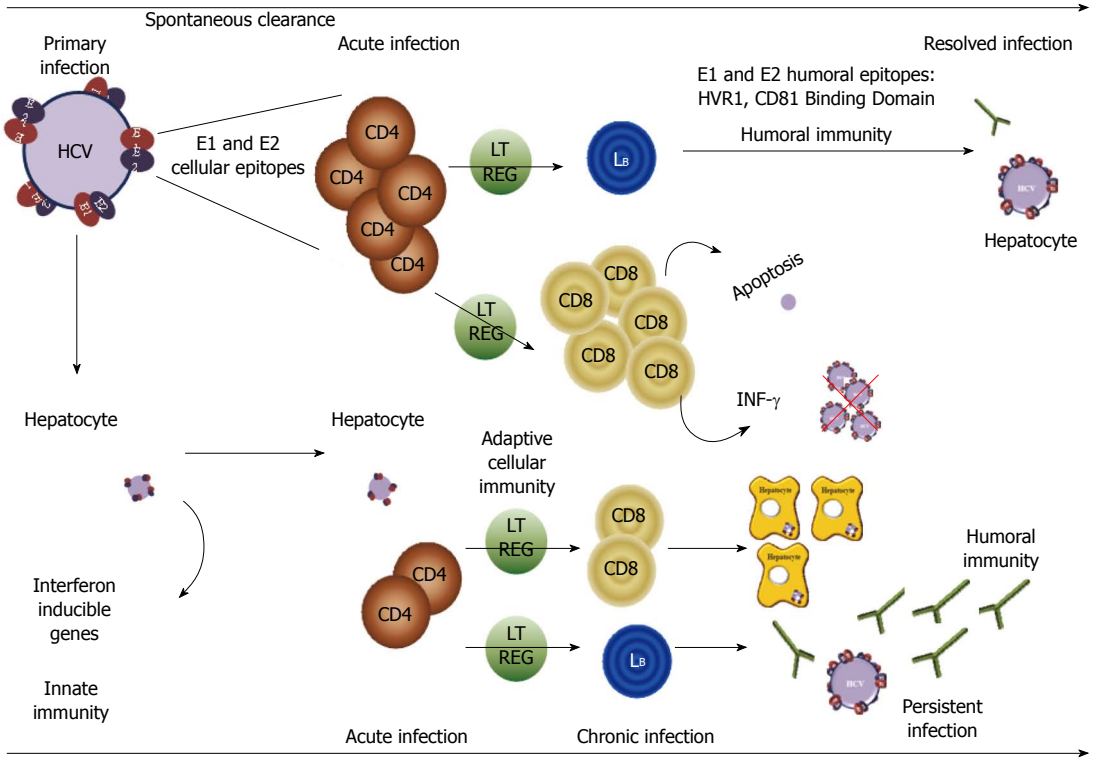

Figure 3 Role of hepatitis C glycoproteins in the immune response to hepatitis C infection. Following hepatitis C virus (HCV) infection, innate host response is initiated by hepatocytes producing an antiviral state by stimulating interferon inducible genes expression. In case of spontaneous clearance or resolved infection, a strong cellular response, due to CD4+ and CD8+ T lymphocytes (denoted as CD4 and CD8 in the figure), is observed in the early phase of infection. Moreover Regulatory T cells (Treg) cells (LT REG) limit the function of effector T cells. Then neutralizing antibodies are rapidly expressed. In most cases, the host adaptative immunity is unable to control HCV infection evolving to a chronic phase. The cellular response (CD4+ and CD8+ T lymphocytes and B lymphocytes, mentioned as L_B in the figure) is weak and transient. The appearance of neutralizing antibodies is delayed and the humoral response not efficient. IFN: Interferon.

polymerase. However, although preS1 deleted mutants are able to replicate they usually need a helper virus to infect new hepatocytes[43]. The surface S gene seems to vary less than the preS1 and preS2 regions[44]. Functional mutations that have been described in the HBV envelope genome consist of preS1 and preS2 deletions, preS2 start codon mutations, and C-terminally truncated or "a" determinant mutated S proteins. These viral variants exhibit modified replication capacities and antigenic characteristics; on the other hand they may be resistant to antiviral therapies and/or contribute to escape from host-related immune surveillance. They can also alter the sensitivity of diagnostic immunoassays. In HepG2 cells, replication of preS/S variants leads to decreased HBsAg secretion, retention of envelope proteins in the endoplasmic reticulum, less efficient virion secretion and higher amounts of cccDNA in the nucleus. In patients with HBV-related chronic liver disease, the emergence of preS/S variants appears to provoke the loss of correlation between HBV DNA replication and HBsAg synthesis/secretion[45].

Briefly for HCV, the HCV genome contains both highly conserved and highly variable regions. The most variable regions are located in NS5A and in parts of the E2 glycoprotein (HVR)[46,47]. Some mutations have been described in E2 showing an impact on HCV infectivity and/or its accessibility to antibodies or cellular response mediated by CD4+ and CD8+ T cells[3,48-51].

PREVENTIVE OPTIONS AND THE RISK OF ESCAPE MUTANTS

Vaccination using a recombinant HBV surface antigen is the most important prophylactic measure available for the prevention of new HBV infections. Immunoprophylaxis with vaccine or immunoglobulins is also recommended after exposure to blood from HBV-infected patients, in new born children from HBV-infected mothers, and to prevent recurrent HBV infection in patients receiving liver transplants for end-stage hepatitis B liver disease.

HBV surface gene variants can be selected under immune pressure after preventive immunization. This is illustrated by report of several surveys in Taiwan describing an increase in the prevalence of *S* gene "a" determinant mutants during a vaccination campaign[52]. In HBV DNA positive children from four sequential surveys in Taiwan, the prevalence of hepatitis B surface gene "a" determinant mutants increased from 7.8% before the vaccination program, to 19.6%, 28.1% and 23.1% at five, 10 and 15 years after the immunization period. However, lower infectivity of the G145R mutant virus, the use of a recombinant vaccine, and mutant loss with older age seem to decrease the "a" mutant prevalence in an immunized population over time[53]. In Europe and North America, mutations in the HBV *S* gene are mostly observed in infants born from HBV-infected mothers,

in a small proportion of occult HBV infections, and in liver transplant recipients[54]. Among variants which were described, the Arg-145 and Arg-129 mutants show the lowest binding ability to monoclonal antibodies. In addition, variants epitopes in HBs antigen induced decreased or a lack of T-cell reactivity[18]. As an illustration, HBV replication was detected in vaccinated chimpanzees challenged with an HBV strain containing polymerase/envelope overlapping mutations[55].

The considerable global diversity of HCV has hampered the development of any successful vaccine. Consequently, neither vaccine, nor specific immunoglobulins are currently available for clinical practice. Current HCV vaccine research therefore follows two main pathways: (1) prophylactic; and (2) therapeutic to increase virological response to antiviral treatment and to reduce the duration of therapy. Although numerous approaches have been developed only a few of these have progressed to human trials, especially for potential therapeutic vaccines. HCV envelope proteins that induce antibodies against conserved domain involved in cell binding are considered as a promising target for vaccine research[56]. However, several mechanisms make antibody-mediated neutralization difficult: interference by interfering antibodies, glycans or lipids, the quasispecies distribution of HCV, and difficult to access cell-to-cell transfer[57].

TREATMENT AND RISK OF ESCAPE MUTANTS

The treatment of chronic hepatitis B aims to reduce liver cell inflammation, prevent the progression to cirrhosis and hepatocellular carcinoma through the suppression of viral replication, and ultimately to clear the infection. HBV treatments use the immunomodulatory agents IFN-α or its pegylated form as well as direct-acting antiviral molecules. IFN-α binding to type 1 IFN receptors activates an anti-viral state in cells by inducing stimulation of *IFN* genes. IFN also stimulates the cellular immune response and production of IFN-γ by CD4+ and CD8+ T cells[58]. On the other hand, nucleos(t)ide analogues block HBV polymerase functions[59]. A sustained virological response to antiviral therapy is defined by plasma HBV DNA < 2000 IU/mL six months after discontinuation of therapy, while a complete response is defined by the disappearance of both HBV DNA and HBsAg with or without appearance of anti-HBs antibodies[58]. During treatment various escape mutants can be detected. Well-characterized mutations in the reverse transcriptase (rt) gene have been described (*e.g.*, rtM204V for lamivudine and rtN236T for adefovir). HBV resistance can develop after a single mutation with some agents or only after multiple mutations for others. Entecavir exhibits low rates of resistance, with fewer than 1% of patients on monotherapy developing resistant mutants after 5 years of treatment[59], if patients strictly respect treatment recommendations. Similarly tenofovir has a very high genetic barrier. Due to the structure of the HBV genome, mutations in the rt domains can affect the amino acid sequence of other HBV proteins, as demonstrated for the rtM204V + rtV173L + rtL180M mutations that lead to envelope changes behaving as mutants escaping vaccination *in vitro*[59]. In addition, studies analyzing HBV quasispecies demonstrated that resistance mutations to nucleos(t)ide analogues can cause amino acid changes within epitopes of the HBV preS/S or core antigen leading to changes in HBV immunogenicity[60].

Treatment of HCV chronic infection consists of a combination of pegylated IFN-α and ribavirin, and this combination remains the basis of gold standard treatments including the use of anti-protease molecules. The rate of sustained virological response (undetectable HCV viral load six months after the end of the therapy, detection threshold at 12-15 IU/mL) depends on the genotype of HCV with lower rates for genotype 1 and is correlated to pre-treatment HCV load and several host-related factors. Both IFN and ribavirin have broad spectrum non specific antiviral activity without direct action on viral genomes[46]. IFN produce an antiviral state by the up-regulation of IFN stimulated genes, leading to the expression of several antiviral proteins including 2', 5' oligoadenylate synthase, and the Mx protein. Four putative mechanisms of action have been proposed to explain the effect of ribavirin on HCV infection: modulation of the host adaptive antiviral response, HCV RNA mutagenesis, action on the intracellular GTP pool necessary for RNA synthesis and inhibition of HCV polymerase. Thus resistance to treatment is a complex mechanism involving host- and virus-related features.

Several HCV proteins (E2, core, NS5A, NS5B) are suspected to be linked with resistance mechanisms to antiviral therapy[46,61,62]. In relation to HCV envelope glycoproteins, treatment outcome can be influenced by amino acid substitutions within CD81 binding sites and HVR2 of E2[63] as well as theoretically through mutations in the PKR/eIF-2α phosphorylation homology domain of E2[61]. A lower HVR1 heterogeneity among viral variants in an individual before antiviral therapy seems to be associated with higher rates of virological response to IFN-based treatment[46,64]. HCV genetic variability in regions such as E2 HVR1 has been associated with antiviral treatment failure[46,65]. In a clinical context, Aurora *et al*[66] described frequent covarying amino acids positions, most often located in E1 and E2, that linked to response to treatment.

CONCLUSION

For both HBV and HCV, viral envelope glycoproteins play a key role in viral entry into hepatocytes and are exposed to host-related immune responses. Even though data that fully elucidates HBV entry into permissive cells are still lacking, specific segments of HBV envelope proteins (preS1, "a" determinant) are instrumental in the process. For HCV, numerous experimental results have recently demonstrated that viral entry is a com-

plex multistep process involving multiple cell cofactors (glycosaminoglycans, low density lipoprotein receptor, SR-B1, CD81, claudin-1, occludin, EGFR, EphA2) in the interaction with HCV E1/E2 envelope glycoproteins. *In vitro* both viruses can be controlled by antibody-mediated neutralization targeting viral envelope. Neutralizing antibodies are also essential in preventing HBV infection *in vivo* as is well characterized through successful vaccination using HBs antigen.

Several factors, such as preventive vaccination and/or therapeutic pressure, can influence HBV and HCV variability. Moreover, for HBV, the patterns of antiviral drug resistance in chronic hepatitis are complex and the original pol/S gene overlap has to be taken into account in the analysis of public health significance of emerging genomic mutations in infected populations. Treatment-induced HBV mutations in pol could indeed generate S mutants with subsequent modified antigenicity or pathogenesis such as increased cancer induction[10].

Variability of HBV and HCV envelope proteins combining high exposure to selective pressures and crucial functional roles require investigation for the adaptation of diagnostic, vaccination and treatment tools. In this context, improved therapy for chronic HCV infection has emerged from a better understanding of the viral replication cycle, with HCV entry providing a large number of therapeutic targets[67,68]. Besides IFN-α and nucleos(t)idic analogues, complementary therapies for chronic HBV infection are desperately needed and the HBV entry process, when better characterized, can offer potential therapeutic opportunities.

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MINIREVIEWS

Burning mouth syndrome

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Abstract

Burning mouth syndrome is a debilitating medical condition affecting nearly 1.3 million of Americans. Its common features include a burning painful sensation in the mouth, often associated with dysgeusia and xerostomia, despite normal salivation. Classically, symptoms are better in the morning, worsen during the day and typically subside at night. Its etiology is largely multifactorial, and associated medical conditions may include gastrointestinal, urogenital, psychiatric, neurologic and metabolic disorders, as well as drug reactions. BMS has clear predisposition to peri-/post menopausal females. Its pathophysiology has not been fully elucidated and involves peripheral and central neuropathic pathways. Clinical diagnosis relies on careful history taking, physical examination and laboratory analysis. Treatment is often tedious and is aimed at correction of underlying medical conditions, supportive therapy, and behavioral feedback. Drug therapy with alpha lipoic acid, clonazepam, capsaicin, and antidepressants may provide symptom relief. Psychotherapy may be helpful. Short term follow up data is promising, however, long term prognosis with treatment is lacking. BMS remains an important medical condition which often places a recognizable burden on the patient and health care system and requires appropriate recognition and treatment.

Key words: Burning mouth syndrome; Glossodynia; Glossopyrosis; Burning tongue

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INTRODUCTION

Burning mouth syndrome (BMS) is a chronic pain disorder characterized by burning, stinging, and/or itching of the oral cavity in the absence of any organic disease. It lasts at least 4 to 6 mo and most often involves the tongue with or without extension to the lips and oral mucosa[1,2]. BMS can be accompanied by dysgeusia (distortion in sense of taste) and subjective xerostomia (dry mouth). Its onset is spontaneous and the syndrome has a clear predisposition to peri-/postmenopausal women. Its secondary form has been associated with a variety of conditions including thyroid disease, psychiatric illnesses, oral infections, drug use, dental treatment, vitamin/mineral deficiencies, and others[3,4].

First described in mid nineteenth century, this condition was further characterized in the early twentieth century by Butlin and Oppenheim as glossodynia[5]. Over the ensuing years, BMS has been referred to as glossopyrosis, oral dysesthesia, sore tongue, stomatodynia, and stomatopyrosis[6]. It was first categorized as a distinct disease in 2004 by the International Headache Society, which defined primary BMS as "an intraoral burning sensation for which no medical or dental cause can be found." Current diagnostic criteria consist of daily persistent pain in the mouth with normal oral mucosa after exclusion of local and systemic diseases[7]. Its etiology is thought to be multifactorial, involving various local, sys-

temic, and/or psychogenic causes. Female gender, perimenopause, depression and anxiety, Parkinson's disease, and chronic medical conditions including gastrointestinal and urogenital diseases are risk factors for developing BMS[1,8-11].

BMS is an important clinical condition with aggravating symptoms, directly and indirectly impacting the quality of life, which often places a recognizable burden on the patient and health care system. Clinical consultations with a practicing gastroenterologist are common practice in today's medicine for the patients with BMS. This review focuses on various aspects of BMS, including its epidemiology, pathophysiology, etiology, clinical presentation, differential diagnosis, classification, clinical diagnosis, current treatment, and general prognosis.

EPIDEMIOLOGY

The estimated prevalence of BMS in the general population varies widely in the literature. Tammiala-Salonen *et al*[12] reported a rate of 15% of burning mouth symptoms in Finnish adult population, though half of the patients had visible oral mucosal lesions. In a cross-sectional analysis of over 1000 randomly selected Swedish patients from Public Dental Health Service registers, 3.7% of subjects were diagnosed with BMS after reporting burning mouth symptoms and undergoing a subsequent physical examination[8]. In contrast, Lipton *et al*[13] reported a prevalence of 0.7% based solely on self-reported symptoms from over 45 000 American households. Haberland *et al*[14] noted that 10% of new patients observed in his practice were diagnosed with BMS. Most recently, a large retrospective study of over 3000 Brazilian patients referred to an oral pathology service reported a prevalence of about 1%[1]. These highly variable rates are attributed to the wide age disparities of the examined population (typically, prevalence of BMS dramatically increases with age) as well as previous lack of universally accepted diagnostic criteria for BMS. Although further studies are needed with satisfactory criteria to determine the true prevalence of BMS, this data illustrates that the disease is an important medical condition that may be often encountered in the clinical practice.

BMS has a clear predisposition to gender and age. Women are 2.5 to 7 times more commonly affected than men[1,8,10]. Furthermore, up to 90% of female patients with BMS are perimenopausal women with typical onset from 3 prior- to 12 years post- the beginning of menopause[9]. BMS may affect any age group, with patients age ranging from 27 to 87 years of age, and a reported mean age of 61 years. Recent analysis by the same group showed an increased likelihood of gastrointestinal and urogenital disease in patients with BMS, with estimated odds ratio of 3.5 and 2.9, respectively, compared to control subjects. Patients with BMS had a statistically higher intake of medications for gastric disease compared to control group as well[1].

ANATOMY

The oral cavity is primary responsible for the ingestion and mastication of food. Its anatomical boundaries include the lips anteriorly, the cheeks laterally, the oropharynx posteriorly, the palate superiorly, and the floor of the mouth inferiorly[15]. The oral cavity contains several structures, including upper and lower dentition, the tongue, salivary glands, and mucosal glands[16]. It is lined by non-keratinized stratified squamous epithelium, which is moistened by secretions from various salivary glands[17].

The neuroanatomy of the oral cavity, particularly somatosensory innervation, has been implicated in the pathophysiology of BMS. The maxillary (V2) and mandibular (V3) branches of the trigeminal nerve supply most of the somatosensory innervation in the oral cavity, with some contribution from the glossopharyngeal nerve (cranial nerve Ⅸ). In regards to the tongue, the most commonly affected area in BMS, the lingual branch of V3 innervates the anterior two-thirds of the tongue while the glossopharyngeal nerve innervates the posterior third of the tongue. Together, they innervate the receptors on the papillae of the tongue, which are sensitive to mechanical, thermal, and tactile stimuli[17].

Alterations in taste and quantity of salivation are commonly reported in BMS. The chorda tympani branch of the facial nerve (Ⅶ) supplies chemoreceptors for taste in the anterior two-thirds of the tongue. The glossopharyngeal nerve (Ⅸ) provides taste sensation for the posterior third of the tongue. There are also taste receptors on the soft palate supplied by the greater superficial petrosal nerve branch of Ⅶ and on the larynx from the superior laryngeal nerve of the vagus nerve (Ⅹ). The salivary reflex begins with afferent inputs from taste and mechanoreceptors in the mouth that reach the brainstem salivatory centers. Parasympathetic and sympathetic fibers then supply the efferent fibers that act on the salivary glands[15].

PATHOPHYSIOLOGY

The pathophysiology of BMS has not been fully elucidated. Various studies have shown significant differences in thermal and nociception thresholds of patient with BMS compared to control subjects[18,19]. Thus, a neuropathic mechanism for BMS is currently favored. However, controversy remains over whether a peripheral or central dysfunction is responsible for BMS.

Evidence in the literature links BMS to a peripheral neuropathy. Superficial biopsies of the anterolateral tongue from BMS patients showed a significantly lower density of epithelial and subpapillary nerve fibers than controls. Morphologic changes were consistent with axonal degeneration. This supports a trigeminal small-fiber sensory neuropathy or axonopathy[20]. Moreover, Borelli *et al*[21] found increased levels of nerve growth factor, a neuropeptide vital to nociceptive function in adults, in the saliva of BMS subjects. Other histopathologic studies of patients with BMS have shown increased density

of TRPV1 ion channels and $P2X_3$ receptors on scattered nerve fibers, a finding previously linked to hypersensitivity and neuropathic pain symptoms in various models of human pain conditions[22]. Additionally, dysfunction of the chorda tympani branch of the facial nerve may be involved in the pathogenesis of BMS. Patients with BMS will report improved symptoms with eating, suggesting that stimulation of the gustatory system decreases pain sensation. Finally, increased excitability or inhibition of the trigeminal system has been implicated as patients with BMS have greater alterations in blink reflexes compared to normal subjects[23-28].

However, recent evidence indicates that dysfunction in the central nervous system can also cause BMS. Albuquerque *et al*[29] showed that BMS patients process thermal and pain stimulation in the brain differently than pain-free individuals as demonstrated by functional magnetic resonance imaging of the thalamus. Additionally, the dysregulation of the nigrostriatal dopaminergic system has been implicated in BMS[30]. Patients with Parkinson's disease are reportedly five times more likely to have BMS than the general population[31]. Finally, hospital anxiety and depression scores were significantly higher in the patients with central BMS[22].

As evident from these studies, the pathophysiology of BMS is highly complex, likely involving neural pathways at different levels of neuraxis. A recent double blind, randomized cross-over study of postmenopausal women showed heterogeneity of the response of BMS symptoms to lingual nerve block with lidocaine. If fact, it may lead to an effective increase, decrease, or an unchanged burning pain in patients, an effect attributed to variation in central, peripheral, or combined neurological pathways in pathogenesis of BMS[32]. The symptoms of BMS can result from subclinical insults at various points in the nervous system, presenting with instant or gradual bilateral distribution. Recent classification suggests a possible overlap of three distinct subclasses in BMS: a peripheral oral small fiber neuropathy (50%-60% of cases), subclinical major trigeminal neuropathy (20%-25%), and central deficiency in dopaminergic top-down inhibition (20%-40%)[23].

ETIOLOGY

The exact etiology of BMS remains imprecise and is likely multifactorial, including neuropsychiatric, endocrine, immunologic, nutritional, infectious, and iatrogenic causes. The disorder has been associated with several psychiatric diseases[9,33-35]. Depression or anxiety occurs in more than 50% of BMS patients, with depression predominating[34]. Personality disorders are also linked to BMS, affecting 86% of sufferers compared to 24% of normal individuals, with significant predilection to Cluster A disorders[36]. Most recently, a cross-sectional controlled study showed that BMS patients have a significantly higher frequency of past or present major depressive disorder, general anxiety disorder, hypochondria, and cancerophobia[37]. Although psychiatric disease was initially considered as a primary cause of BMS, it is now considered a concurrent or secondary factor as there is no definite correlation between the onset of BMS and stressful events and many other causes of BMS have been identified[9,33,35,38,39].

As described previously, BMS most commonly affects perimenopausal women, a finding that is attributed to dryness of mucosal membranes from age-related reduction in estrogen and progesterone levels and increased frequency of psychological disorders in middle-aged and elderly women[40]. Woda *et al*[41] has suggested that the fall in neuroprotective gonadal and adrenal steroids during menopause leads to a concomitant decrease in neuroactive steroids, leading to degeneration of oral mucosal small nerve fibers and brain areas involved in oral somatic sensations. These changes can become irreversible, resulting in burning pain and associated symptoms. Gao *et al*[42] showed that peri-/post-menopausal patients suffering from BMS may have lower levels of estradiol and increased levels of follicle stimulating hormone compared to healthy controls. Other endocrine conditions implicated in BMS may include diabetes mellitus and hypothyroidism[8].

Evidence also exists for an immunologic etiology. Allergic reactions have been demonstrated in BMS patients to dietary antigens. These include sorbic acid, cinnamon, nicotinic acid, propylene glycol, and benzoic acid[43,44]. Other allergens identified by patch testing are dental metals such as zinc, cobalt, mercury, gold, and palladium[45]. Sodium lauryl sulfate, a detergent in toothpaste known to cause dry mouth, may also be involved in the development of BMS[46]. Finally, autoimmune connective tissue disorders such as Sjogren's syndrome and systemic lupus erythematosus, are also associated with BMS[47].

BMS has also been linked to nutritional deficiencies including vitamins B1, B2, B6 and B12 as well as folic acid[2]. Most recently, zinc deficiency was shown to be a possible cause of BMS, with patients reporting improved symptoms after zinc replacement therapy[48].

A potential relationship between smoking and development of BMS has been described, with an estimated odd ratio of 12.6 in a recent study[43].

Certain oral infections have been implicated in BMS, particularly candidiasis. Patients with BMS have a higher intraoral prevalence of *Candida* species and coliforms like *Enterobacter* and *Klebsiella*[49,50]. Although this finding may be related to xerostomia and prosthetic dental wearing, a possible infectious origin of BMS is suggested by reports of remission after oral antifungal therapy[18].

Drug-associated BMS has also been reported in the literature. ACE inhibitors and angiotensin receptor blockers may trigger development of BMS, possibly due to increased levels of kallikrein in the saliva of BMS patients leading to increased inflammation in the oral cavity[9,51-53]. Nevirapine and efavirenz have been reported to cause BMS *via* an unknown mechanism[54,55]. Levodopa may play a role in the development of BMS in patients with Parkinson disease[31]. Finally, a case report of topiramate causing burning mouth-like symptoms has been de-

scribed, with symptom resolution upon discontinuation of the medication[56].

CLINICAL PRESENTATION

A typical patient with BMS is a peri- or post-menopausal woman with various medical comorbidities who complains of the classic triad of unremitting oral mucosal burning pain associated with dysgeusia and xerostomia in nearly two thirds of the cases with no visible disease in the oral mucosa for 4-6 mo duration. Clinical presentations may vary as some patients can be oligosymptomatic (pain and dysgeusia or xerostomia) or monosymptomatic (pain only)[10]. In general, 63% of patients report accompanying dry mouth, 60% bitter/metallic taste, and 35% altered taste perception[9]. The pain is described as burning, scalding, tingling, or numbness. It is of moderate to severe intensity and can decrease during eating. It is commonly bilateral and most often involves the tongue followed by the palate and lower lip. In contrast, the buccal mucosa and floor of the mouth are rarely affected[9]. The onset is spontaneous, though some BMS patients report antecedent dental procedures, initiation of medications, or other illnesses[10,57]. Xerostomia may be subjective however some patients have demonstrated alterations in saliva quantity and quality[10]. Vertical visual analogue scale (VAS, 0-10 cm)[58] is commonly used to describe pain intensity in BMS.

Review of systems may be remarkable for headache, chronic fatigue, gastrointestinal and urogenital symptoms, insomnia, mood changes, irritability, anxiety, and depression[4,57]. Other observed clinical conditions may include gastroesophageal reflux disease, hypertension, hematological disorders, nutritional deficiencies, diabetes mellitus, thyroid disorders, Parkinson's disease, Sjogren's syndrome and other autoimmune diseases[51,59]. Finally, parafunctional habits such as lip and cheek biting, bruxism, tooth grinding and clenching, tongue thrusting, and lip licking are observed with BMS[10].

Physical examination and laboratory analysis are classically unremarkable in primary BMS. However, they can be abnormal in secondary BMS. Oral findings potentially include areas of erythema, geographic tongue, candidiasis, atrophic glossitis, lichen planus, and xerostomia. Laboratory evaluation may reveal positive fungal oral cultures, elevated fasting blood sugar, decreased levels of vitamin B1, B2, B6, B12, folate, iron, and zinc, abnormal thyroid function studies, and positive serum autoantibodies[4,49].

MIMICKERS OF BMS

BMS presents with a main complaint of an intraoral sensation of burning, tingling, or stinging and sometimes accompanied by taste disturbances or dry mouth. The mimickers of BMS may include stomatitis, atypical facial pain, atypical odontalgia, idiopathic facial arthromyalgia, pemphigoid, pemphigus, neoplastic lesions in the oral cavity, acoustic neuroma, denture design or tooth restoration failures, herpes simplex or herpes zoster, trauma to lingual or mandibular nerves after dental surgery[10,57]. Detailed history and physical exam is crucial to differentiate above medical conditions.

CLASSIFICATION

Two classification schemes have been proposed based on either etiology or clinical symptoms. When classifying by etiology, primary BMS is the idiopathic form for which organic causes cannot be identified while secondary BMS results from local or systemic pathological conditions[10]. The other scheme divides BMS cases into three types based on diurnal fluctuations of symptoms. Patients with type 1 BMS (35%) are symptom-free upon awakening with worsening symptoms throughout the day and variable symptoms at night. Type 2 BMS (55%) is defined by continuous symptoms in the day but none at night. Patients with type 3 BMS (10%) have intermittent symptoms interspersed with symptom-free days[34,60]. Type 1 BMS is linked to nutritional deficiencies and diabetes, type 2 to chronic anxiety, and type 3 to dietary or prosthetic allergies[2,18,44,61].

CLINICAL DIAGNOSIS

The diagnosis of BMS remains challenging as diagnostic criteria are not sufficiently defined or universally accepted, several confounding diagnoses exist, and the clinical picture is often variable. Scala *et al*[10] proposed the following fundamental criteria: (1) daily and deep bilateral burning sensation of the oral mucosa; (2) burning sensation for at least 4 to 6 mo; (3) constant intensity, or increasing intensity during the day; (4) no worsening but possible improvement on eating or drinking; and (5) no interference with sleep. Additional supportive criteria are, (1) dysgeusia and/or xerostomia; (2) sensory or chemosensory alterations; and (3) mood changes or psychopathological alterations.

Since primary BMS is a diagnosis of exclusion, thorough investigation for local and systemic factors associated with secondary BMS is essential. Careful review of recent mood disturbances, dietary habits, history of dental procedures, use of dental prosthetics, nutritional deficiencies, and changes in medication is necessary in the evaluation of BMS. Physical examination primarily consists of detailed study of the oral cavity, including dental inspection. Laboratory analyses must include hematological assessment of nutritional deficiencies, blood glucose levels, autoimmune markers, estrogen and progesterone concentrations, patch testing for specific allergies[10]. Measurement of salivary flow rates should be employed[62].

Additional studies may warrant oral cultures and scrapings to evaluate for a bacterial or fungal origin of symptoms. Tongue biopsy is not required if the tongue appears normal on clinical exam and is only indicated when a particular lesion is visualized. In general, the diagnosis of BMS remains a major challenge, requiring extensive clinical and laboratory evaluation with a particular atten-

tion to details of patient's history and physical exam.

TREATMENT

The first step in management is contingent on the specific type of BMS, primary versus secondary. The goal of therapy for secondary BMS should initially be directed at treating the causative local or systemic disease and withdrawing offending medications (such as ACE inhibitors). This etiology-directed therapy typically yields a good response. The cure for primary BMS, however, remains elusive despite attempts with different classes of medication. The variable response rate to medical therapy is likely due to the multifactorial pathophysiology of idiopathic BMS, including irreversible processes. Treatment is aimed at management this disease as a type of chronic neuropathy. Investigated strategies include benzodiazepines, antidepressants, topical capsaicin, alpha-lipoic acid, hormone replacement therapy, anticonvulsants, biofeedback technique to modify parafunctional habits, and psychosocial therapies.

Early studies by Italian researchers supported the use of alpha lipoic acid (ALA, a potent antioxidant) in 600 mg daily dose over two months in patients with BMS[63]. This data was not replicated by subsequent analysis in Brazilian patients[64], possibly due to the multivitamin compound of the ALA supplements or longer duration of the original therapy. Interestingly, same group suggested added benefit of ALA in patients with thyroid disease[65].

Recent randomized double blind placebo controlled trial showed that use of gabapentin alone (300 mg daily) or in combination with ALA (600 mg daily) was beneficial in reducing symptoms in 50% and 70% of patients with BMS, respectively, compared to placebo (15%)[66]. In line with the observed predisposition of BMS to peri-/post- menopausal women, a hormone replacement therapy may be intuitive, but short of initial promise with local oral administration, it is largely not effective when given systemically[67] possibly due to the irreversible nature of the neuropathic changes.

Clonazepam lozenges (oral dissolution of 1-mg tablets for 3 min with subsequent expectoration three times a day) are beneficial in patients with predominantly peripheral BMS. A double blind, randomized controlled study of topical clonazepam reported a reduction in pain intensity in 66% of patients after 2 wk and 29% after 6 mo[68]. Similar clinical benefit was replicated in follow up studies[33].

Short term use of oral Chlordiazepoxide (5-10 mg three times a day) to treat BMS has been reported, however, its long term effects were not significant[69].

Efficacy of capsaicin, a desensitizer of receptors for neurogenic inflammation, has been evaluated in several studies. Systemic capsaicin 0.25% capsules three times daily showed dramatic improvement (93%) in patients with severe BMS (VAS scale 8-10) at 1 mo[70]. Side effects including gastric pains in 32% of the patients were cumulative, and may preclude long term use of this medication. Local capsaicin rinse may be beneficial in treating BMS, with reported improvement of symptoms in over 75% of the patients after 8 wk of therapy without significant side effects[71].

Use of proton pump inhibitors (PPI), although clearly increased in the patients with BMS[1], may not necessarily be of benefit. Recent study measuring oropharyngeal pH found no significant correlation between laryngopharyngeal reflux and intraoral burning sensation in the examined population[72]. Anecdotal evidence exists in case reports of symptom relief with twice daily omeprazole in patients with endoscopy proven gastroesophageal reflux disease[73]. Whether there is a casual relationship between use of PPI and clinical primary BMS, an observation phenomenon, a true response, or simply a failed therapy remains to be established. Treatment of secondary BMS in the setting of gastroesophageal reflux disease is, however, warranted. Larger studies are needed to address potential benefit (or lack of it) of PPI in treatment of BMS.

Medical management of BMS may also include antidepressants and antipsychotics. In a prospective, open-label, noncomparative study of selective serotonin reuptake inhibitor (SSRI) paroxetine, 80% of patients experienced overall pain reduction including 36% of patients who reported complete remission after 12 wk of incremental treatment. Both treatment effects and adverse events were found to be dose dependent[74]. However, prophylactic use of domperidone for nausea in selected study patients may have had a potential confounding effect on the results. Another single-blinded non-placebo controlled study comparing paroxetine (20 mg daily), sertraline (50 mg daily), and an atypical antipsychotic amisulpride (50 mg daily) demonstrated a nearly 70% improvement in VAS score after 8 wk of therapy in each of the three groups[75]. A group from Spain reported complete remission in all female patients after 8 wk of amisulpride use, an effect lasting up to 24 wk with continuous administration of the medication[76].

Additionally, modifications of parafunctional habits may offer some symptom relief. Tongue protectors (worn 15 min three times a day) were shown to significantly improve pain scales in BMS patients after two months of treatment, however sample sizes were small and placebo effect could have been introduced[77].

Finally, psychiatric interventions show great promise in treating patients with BMS. Weekly one-hour sessions of cognitive behavioral therapy lasting for 12-15 wk significantly reduced BMS symptoms in all study patients compared to placebo control group, with an estimated 27% of patients remaining symptom-free at 6 mo follow up (none in placebo group)[78]. Weekly group psychotherapy administered for three consecutive months achieved symptom improvement in 70% of the patients[52]. Femiano *et al*[79] noted a statistically significant symptom improvement with cognitive psychotherapy (40%), alpha lipoic acid (81%), combination therapy (90%) compared to pill placebo control group (13%) of patients with BMS.

Anecdotal use of diphenhydramine/pectin swish and spit elixir[18] as well as oral pramipexole in BMS patients with Parkinson's disease have been reported[57].

Perceived variation in the results of the published data may stem from lack of effective differentiation between predominantly peripheral and predominantly central pathways in the pathogenesis of BMS. Therefore, improvement in future clinical diagnosis and discrimination between such groups of patients may improve clinical response rate to targeted local and systemic therapies.

For now, BMS remains a challenging medical condition to treat, and further research is required to determine the true efficacy of current management strategies for patients with this disorder. Future blinded randomized control trials with large sample size are necessary to provide new insight for use of various treatment modalities in BMS.

PROGNOSIS

Although the short term follow up studies may show potential symptomatic improvement with treatment in patients with BMS, the long-term outcomes for BMS remain unclear. Early observational report by Gilpin[5] in 1936 may provide a closer look at the natural history of the disease that follows a "rule of 3's": up to one third of cases would enter spontaneous remission, another third would show moderate improvement, and finally the last third would show no improvement or even worsening of the symptoms. Prospective clinical and pharmaceutical advances may have significantly changed the landscape of BMS, as recent study showed nearly 10% of spontaneous remission, 26% of moderate improvement, 37% of no significant change, and finally 26% of worsening of symptoms in patients receiving no therapy with an estimated follow up of at least 18 mo. Therapy may be effective in 29% of the patients, with 56% reporting no changes, and 15% admitting worsening of the pain[62]. In perspective, complete understanding of the etiology and pathogenesis is imperative to the development of novel and efficacious therapeutic strategies, and will guide overall prognosis of the disease in the future.

CONCLUSION

BMS is a relatively common chronic intraoral pain disorder classically characterized by intractable burning that may be associated with dysgeusia and xerostomia. Its pathogenesis relates to complex interlay of central and/or peripheral neural pain pathways. Etiology of BMS is multifactorial and a secondary form of BMS should be diligently sought for and treated. Multidisciplinary approach, including medical and psychosocial therapy may be effective in symptom relief in patients with BMS, however, further studies are necessary to establish long term prognosis. BMS remains an important medical condition which often places a significant burden on the patient and health care system, and requires diligent recognition and treatment.

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ORIGINAL ARTICLE

Desferrioxamine in warm reperfusion media decreases liver injury aggravated by cold storage

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Abstract

AIM: To evaluate whether desferrioxamine decreases ischemia and perfusion injury aggravated by cold storage (CS) in a rat liver perfusion model.

METHODS: Isolated rat livers were kept in CS in University of Wisconsin Solution for 20 h at 4 ℃, then exposed to 25 min of warm ischemia (WI) at 37 ℃ followed by 2 h of warm perfusion (WP) at 37 ℃ with oxygenated (95% oxygen and 5% carbon dioxide) Krebs-Henseleit buffer. Desferrioxamine (DFO), an iron chelator, was added at different stages of storage, ischemia and perfusion: in CS only, in WI only, in WP only, in WI and perfusion, or in all stages. Effluent samples were collected after CS and after WI. Perfusate samples and bile were collected every 30 min (0, 0.5, 1, 1.5 and 2 h) during liver perfusion. Cellular injury was assessed by the determination of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in the effluent and perfusate samples. Total iron was analysed in the perfusate samples. After WP, the liver was collected for the determination of liver swelling (wet to dry ratio) and liver morphological examination (hematoxylin and eosin staining).

RESULTS: Increased CS time caused increased liver dysfunction during WP. After 2 h of WP, liver injury was indicated by increased release of AST (0.5 h CS: 9.4 ± 2.2 U/g liver *vs* 20 h CS: 45.9 ± 10.8 U/g liver, $P < 0.05$) and LDH (0.5 h CS: 59 ± 14 U/g liver *vs* 20 h CS: 297 ± 71 U/g liver, $P < 0.05$). There was an associated increase in iron release into the perfusate (0.5 h CS: 0.11 ± 0.03 μmoL/g liver *vs* 20 h CS: 0.58 ± 0.10 μmoL/g liver, $P < 0.05$) and reduction in bile flow (0.5 h CS: 194 ± 12 μL/g *vs* 20 h CS: 71 ± 8 μL/g liver, $P < 0.05$). When DFO was added during WI and WP following 20 h of CS, release of iron into the perfusate was decreased (DFO absent 0.58 ± 0.10 μmoL/g liver *vs* DFO present 0.31 ± 0.06 μmoL/g liver, $P < 0.05$), and liver function substantially improved with decreased release of AST (DFO absent 45.9 ± 10.8 U/g liver *vs* DFO present 8.1 ± 0.9 U/g liver, $P < 0.05$) and LDH (DFO absent 297 ± 71 U/g liver *vs* DFO present 56 ± 7 U/g liver, $P < 0.05$), and increased bile flow (DFO absent 71 ± 8 μL/g liver *vs* DFO present 237 ± 36 μL/g liver, $P < 0.05$). DFO was also shown to improve liver morphology after WP. Cellular injury (the release of LDH and AST) was significantly reduced with the addition of

DFO in CS medium but to a lesser extent compared to the addition of DFO in WP or WI and perfusion. There was no effect on liver swelling or bile flow when DFO was only added to the CS medium.

CONCLUSION: DFO added during WI and perfusion decreased liver perfusion injury aggravated by extended CS.

Key words: Iron chelation; Ischemia and perfusion injury; Liver; Organ preservation; Rat

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INTRODUCTION

Hypothermia is used extensively to preserve many types of donor organs for transplantation and protects organs, in part, by reduced requirements for metabolic energy[1]. Donor livers are flushed and then stored at 4 ℃ in specialized storage solutions (*e.g.*, University Wisconsin solution). However, preservation injury can cause primary graft dysfunction and increased morbidity and mortality in liver transplant recipients[2]. When cold storage (CS) time is extended beyond 10 h, there is a significant increase in primary graft dysfunction and long term biliary complications following liver transplantation[3-6].

The mechanisms responsible for donor liver dysfunction after preservation are not fully understood but one important potential contributor to liver damage is redox active iron. Redox active iron has been identified as a contributor to isolated hepatocyte death and liver damage during hypothermia[7-10]. Redox active iron can react with hydrogen peroxide and initiate radical reactions which damage cellular macromolecules, such as DNA, proteins and membrane lipids. Chelation of iron by specialised proteins usually prevents the generation of radical species *in vivo*. However, when cells[7,9,11] or organs[12-14] are cooled there is an increase in redox active iron and this increase has been linked to cell death during hypothermia[7,9,10].

One approach to abrogating the activity of redox active iron is to chelate free iron with iron chelators such as desferrioxamine (DFO)[7,9,15]. When DFO was added to CS media there was reduced hepatocyte and endothelial cell death during CS, and liver damage on re-perfusion[7,11,14,16]. However, it is unlikely that DFO is taken up intracellularly by liver cells during storage at 4 ℃[7,17,18], so intracellular redox active iron has the potential to predispose the donor liver to injury during implantation [warm ischemia (WI)] and subsequent re-perfusion[14]. DFO enters tissue by endocytosis at 37 ℃ and therefore the presence of DFO during WI and re-perfusion may offer additional protection from CS injury. To test this hypothesis, isolated rat livers were perfused after CS, with DFO added to the preservation media during CS, WI and WP. This strategy significantly protected livers from CS damage.

MATERIALS AND METHODS

Materials

Krebs-Henseleit buffer (KHB), DFO and reduced glutathione were purchased from SIGMA and Aldrich, Sydney, Australia. Hartmann's solution was obtained from Baxter Health Care Pty Ltd, Old Toongabbie, Australia, University of Wisconsin solution (UWS) from Bristol-Myers Squibb Company, New York, United States, gentamicin from Pfizer, West Ryde, Australia and Actrapid human insulin from Novo Nordisk Pharmaceuticals Pty Ltd, Baulkham Hills, Australia. Dexamethasone sodium phosphate was from DBL, Rowville, Australia.

Liver isolation, CS and WI

The use of animals was approved by the Animal Ethics Committee of the University of Western Australia. All animals received food and water before graft retrieval. Adult male rats (Strain PVG, average weight 272 ± 5 g, n = 35) were anesthetized with halothane. The gastroduodenal vein, splenic vein, right renal artery, right and left adrenal veins were ligated and the bile duct was cannulated. The liver was flushed with 20 mL of cold Hartmann's solution (control) or UWS with supplements (reduced glutathione, dexamethasone, insulin and gentamicin, pH 7.35-7.4 on ice) and heparin (5 U/mL) *via* the aorta. The liver was excised and placed in UWS with supplements for 20 h at 2-4 ℃. After CS the suprahepatic vena cava (outlet) and portal vein (inlet) were cannulated, and the liver was connected to a rat liver perfusion system while it was in the CS medium. In the control group, after the liver was excised, the liver was connected to a rat liver perfusion system while it was in cold saline for up to 0.5 h (CS0.5 h). The liver was then transferred to the perfusion chamber (37 ℃) and flushed with 20 mL of Hartmann's solution and heparin (5 U/mL). The first 6.5 mL of effluent and the CS medium were collected. To simulate WI during liver implantation, the liver was covered with damp gauze and left at 37 ℃ chamber for 25 min.

Liver WP

After WI, the liver was perfused using a water-jacketed perfusion system (Radnoti Glass Technology, Inc., Monrovia, United States) with oxygenated (95% oxygen and 5% carbon dioxide) KHB at 20 mL/min at 37 ℃ and the first 6.5 mL of perfusate was collected. After 7.5 min of perfusion, the perfusate was re-circulated and filtered through a pre-filter and filter (0.8 μm/0.2 μm, 32 mm OD, Pall Life Sciences, Australia) and the liver was then perfused for 2 h. During liver perfusion, perfusate (1.5 mL) and bile were collected at 0, 0.5, 1, 1.5 and 2 h and

Table 1 Details of experimental groups

Experimental group	Number per group	CS time (h) in UWS	Addition of DFO		
			CS	WI	WP
CS0.5 h	5	-	-	-	-
CS20 h	5	20	-	-	-
DFO (CS)	5	20	DFO	-	-
DFO (WI)	5	20	-	DFO	-
DFO (WP)	5	20	-	-	DFO
DFO (WIP)	5	20	-	DFO	DFO
DFO (CS WIP)	5	20	DFO	DFO	DFO

DFO: Desferrioxamine; CS: Cold storage; UWS: University of Wisconsin solution; WI: Warm ischemia; WP: Warm perfusion; WIP: Warm ischemia and perfusion.

kept on ice. Perfusates were centrifuged at 10 000 *g* for 10 min at 4 ℃ and the supernatant was analysed for lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and total iron.

After 2 h of perfusion, the liver was pat-dried and weighed. Tissue (approximately 0.5 g) was dried at 80 ℃ for 72 h for the calculation of wet to dry ratio and a portion of tissue was snap frozen in liquid nitrogen for subsequent analysis. The remaining liver was fixed in 10% formaldehyde for histopathological examination.

Assays

Perfusate LDH levels were measured by the method of Passonneau[19]. AST enzyme activity was measured by automated biochemical analyser (Hitachi 917, Roche Diagnostics). Total iron levels were measured by Inductively Coupled Plasma-Mass Spectrometry (Varian 820, California, United States) with aqueous standards used for the calibration curve. LDH, AST and total iron were expressed relative to dry liver weight.

Liver histopathology

Liver sections were stained with hematoxylin and eosin and then examined independently by a pathologist (B DeBoer) and a hepatologist (GP Jeffrey). A scale for morphological classification of hepatic injury was used as previously described[20]. The 9 point scale was: (1) normal rectangular structure; (2) round hepatocytes with an increase of sinusoidal spaces; (3) vacuolization in zone 3; (4) vacuolization in zone 2; (5) vacuolization in zone 1; (6) vacuolization and nuclear pyknosis in zone 3; (7) vacuolization and nuclear pyknosis in zone 2; (8) vacuolization and nuclear pyknosis in zone 1; and (9) necrosis.

DFO addition to media

There were seven experimental groups and each group consisted of 5 rats. DFO (1 mmol/L) was tested in five experimental groups with different combinations of DFO during CS, WI, WP and perfusion (WIP), or CS, WI and perfusion (CS WIP) (Table 1). The required amount of DFO was weighed and added to the media prior to use and the concentration (1 mmol/L) was based on our previous isolated rat hepatocyte study[7].

Statistical analysis

All analyses were carried out using R 2.10.1[21], using the package base and agricolae[22]. A one-way between ANOVA was used to compare the outcome of each measurement (the levels of LDH, AST, liver wet to dry ratio and bile flow) and treatment condition (Table 1). Least Square Difference (LSD) was used for Post Hoc testing. Differences are considered to be significant if $P < 0.05$. Data is reported as mean ± SE.

RESULTS

Effect of extended CS

The degree to which liver damage occurred during CS, WI and WP was measured by the release of LDH and AST. Liver damage was evident immediately after extended CS. The LDH level was 7.9 ± 0.6 U/g liver and the AST level was 1.9 ± 0.1 U/g liver after 20 h of CS compared to 0.7 ± 0.1 U/g liver and 0.1 ± 0.0 U/g liver in the controls (CS0.5 in Figure 1). Increased damage was also evident after WI, with 9 fold higher LDH and 8 fold higher AST release in extended CS, relative to the controls (CS0.5 in Figure 1). The bulk (more than 95%) of LDH and AST release occurred during WP of livers subjected to extended CS (Figure 1). After 2 h of perfusion, LDH levels were 297 ± 71 U/g liver and AST levels were 45.9 ± 10.8 U/g liver in livers subjected to 20 h of CS compared to 59 ± 14 U/g liver and 9.4 ± 2.2 U/g liver respectively in livers of the control group. Twenty hours of CS resulted in a 14% increase in liver swelling and a 63% decrease in bile flow (Figure 1). Taken together, these data indicate that 20 h of CS caused a detrimental effect on liver function during subsequent WI and WP.

Protective effect of DFO

DFO was first added to CS medium only. There was no significant improvement in LDH levels or AST levels during WI. However during WP there was a 40% reduction of LDH and AST levels after 2 h of perfusion compared to livers that had not been exposed to DFO during CS (Figure 2). There was no effect on liver swelling or bile flow (Figure 2).

To test if either redox active iron was not completely chelated by DFO during CS, or additional redox active iron release occurred during WI and WP, DFO was added to solutions during all stages of the experiment. This resulted in a significant reduction of AST levels from 6.7 ± 1.3 U/g liver to 3.4 ± 0.5 U/g liver compared with no DFO during WI ($P < 0.05$). LDH levels were not significantly affected (DFO absent 39.9 ± 7.9 U/g liver *vs* DFO present 24.4 ± 2.5 U/g liver). In contrast, after 2 h of WP there was a large reduction of AST levels and LDH levels from 45.9 ± 10.8 U/g liver to 8.1 ± 0.9 U/g liver and 297 ± 71 U/g liver to 56 ± 7 U/g liver respectively when compared to no DFO (Figure 2). These levels were similar to the controls (CS0.5 in Figure 1). Consistent DFO effects were observed with improved liver swelling, decreased from 4.01 ± 0.17 to 3.37 ± 0.05 (wet weight to dry weight ratio) and bile flow increased from 71 ± 8

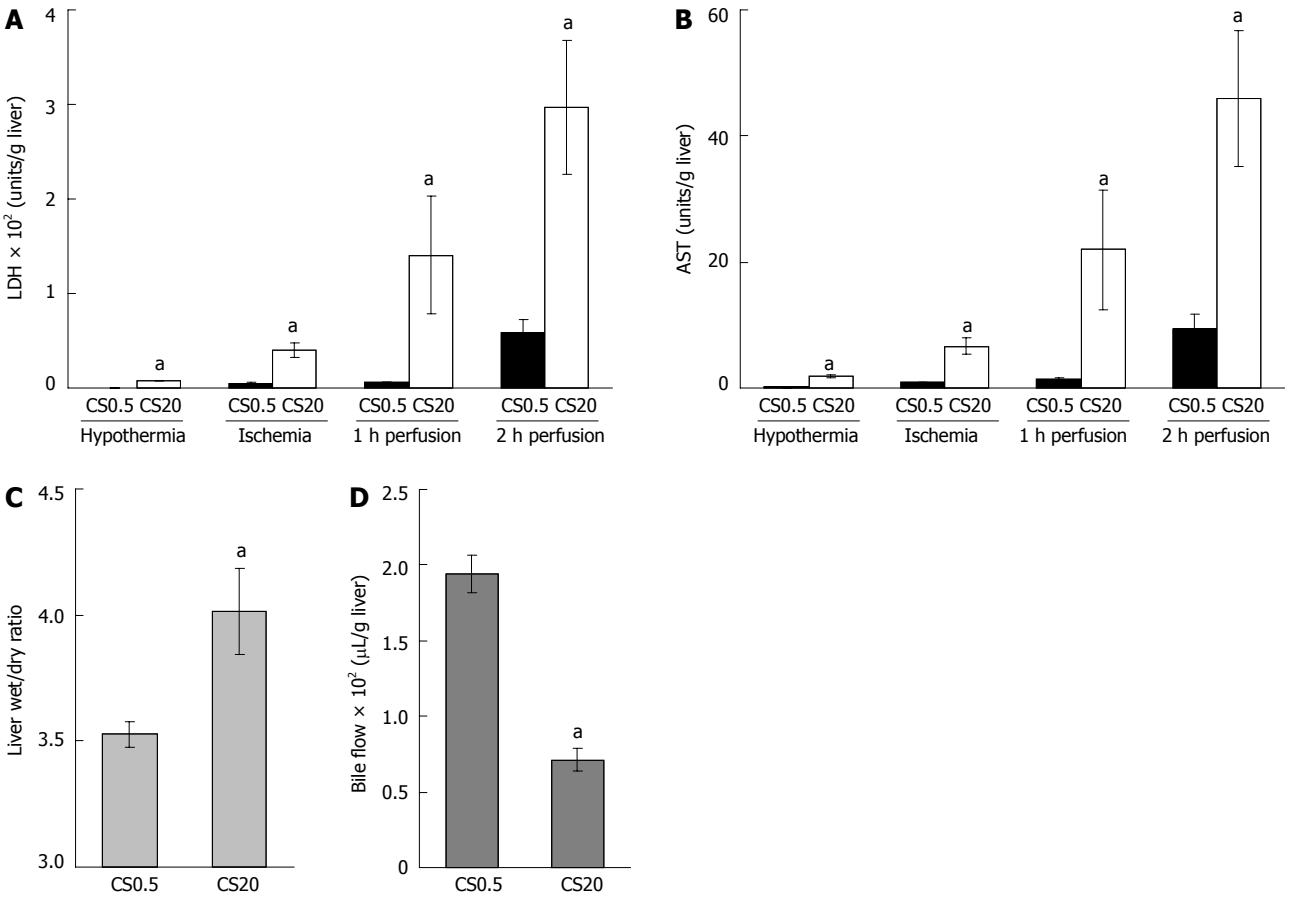

Figure 1 Liver function following cold storage, warm ischemia and perfusion. A, B: Liver damage was assessed by measuring lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in media from control livers after 0.5 h of cold storage (CS0.5) and from the livers after 20 h of extended cold storage (CS20). LDH and AST were measured in media flushed from livers following cold storage, in media flushed from livers following 25 min of warm ischemia and in media at 1 and 2 h of warm perfusion; C, D: Liver health was assessed by liver swelling (liver wet to dry ratio) after 2 h of perfusion and bile flow over 2 h of perfusion. Significantly different from the controls (mean ± SE, $n = 5$, $^aP < 0.05$).

μL/g liver to 237 ± 36 μL/g liver (Figure 2).

To test the possibility that DFO had its predominant protective effect during WI and/or WP, DFO was added to either WI or WP media alone. DFO had no effect when added during WI alone, however there was a protective effect when it was added to perfusion medium alone (Figure 2). There was a reduction of LDH from 297 ± 71 U/g liver to 72 ± 15 U/g liver and AST from 45.9 ± 10.8 U/g liver to 13.4 ± 2.2 U/g liver compared to livers not treated with DFO (Figure 2). Furthermore, liver swelling was prevented and bile flow was maintained (Figure 2). Of note, the protective effect was comparable to that observed when DFO was present during all three stages of the experiment and this is consistent with the concept that redox active iron continued to be a major cause of liver damage during WI and WP following extended CS.

To further assess the protective effect of DFO, liver morphology was examined following 2 h of WP and assessed using a nine point scale ranging from 1 (normal structure) to 9 (necrosis) (Figure 3). Relative to the controls (CS0.5 h, morphological classification of 3.0 ± 0.1, mean ± SE, $n = 4$), extended CS resulted in extensive vacuolization and marked pyknosis (morphological classification of 5.5 ± 0.9, mean ± SE, $n = 4$, $P < 0.05$). DFO was largely effective in preventing changes to liver morphology (morphological classification of 3.0 ± 0.4, mean ± SE, $n = 4$, $P < 0.05$ relative to no DFO).

Iron release during CS and perfusion

The effectiveness of DFO in preventing liver damage indicated redox active iron was likely a major contributor to liver damage. To further investigate this, iron release was measured following WI and during perfusion. Following WI and 20 h of CS there was 0.15 ± 0.02 μmoL iron/g liver compared with 0.02 ± 0.01 μmoL iron/g liver in the controls (CS0.5 in Figure 4, $P < 0.05$). After 2 h of WP, iron release after 20 h of CS was 0.58 ± 0.10 μmoL iron/g liver which was significantly higher than 0.11 ± 0.03 μmoL/g liver in the controls (CS0.5 in Figure 4, $P < 0.05$). Iron concentrations were significantly correlated with LDH release and AST release during WI (Figure 5). During WP, the concentration of iron was also correlated with LDH release and AST release (Figure 5). The significant correlation between iron and the damage markers LDH and AST was consistent with the hypothesis that redox active iron was causing liver damage.

The presence of DFO altered iron concentrations in

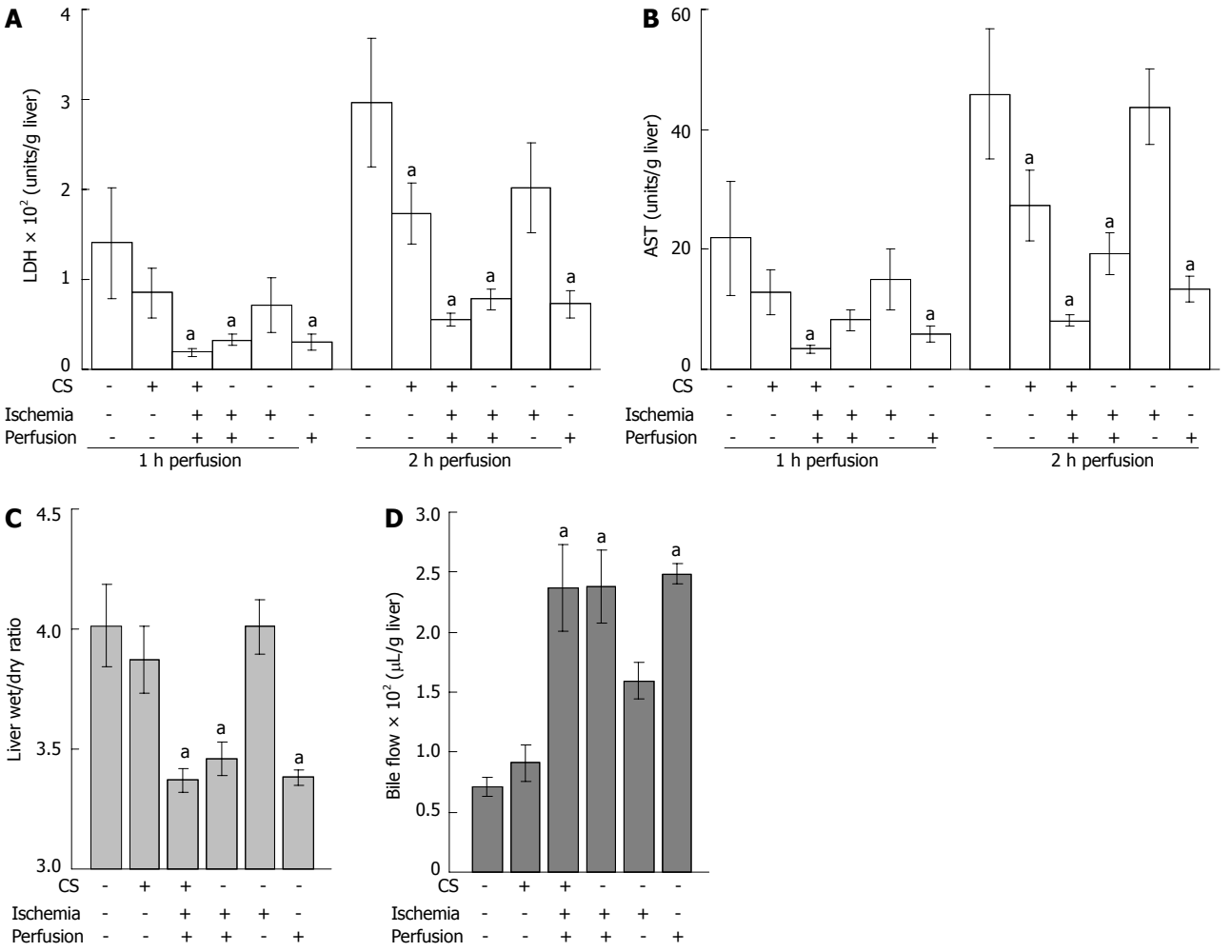

Figure 2 The effect of desferrioxamine added during cold storage, warm ischemia and/or perfusion on liver function following 20 h of cold storage. A,B: Desferrioxamine was added (indicated by +) to cold storage (CS), warm ischemia and perfusion media. Liver damage was assessed by measuring lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in media from livers during perfusion at 1 and 2 h; C, D: Liver health was assessed by liver swelling (liver wet to dry ratio) after 2 h of perfusion and bile flow over 2 h of perfusion. Data for extended (20 h) CS are shown. Significantly different from 20 h of CS (mean ± SE, $n = 5$, $^{a}P < 0.05$).

WP media. When DFO was added during CS, WI and perfusion, iron concentrations were significantly decreased from 0.58 ± 0.10 μmoL/g liver (no DFO) to 0.31 ± 0.06 μmoL/g liver (with DFO, $P < 0.05$) (Figure 4). There was also a significant relationship between the concentration of iron and liver damage during both WI and WP (Figure 5). However, the slope of the line describing the relationship was significantly lower in the presence of DFO compared to the absence of DFO during WP (Figure 5). Consequently, for the same concentration of iron, there was less damage in the presence of DFO relative to absence of DFO. One explanation for this observation may be that by chelating iron during WI or perfusion, DFO prevented ongoing liver damage caused by redox active iron.

DISCUSSION

It is well established that extended CS causes increased liver damage and that the presence of iron chelators in storage media can substantially protect hepatocytes and livers from damage during cold incubation or storage[9,14,23,24]. In this study, extended CS also aggravated liver damage during the simulated transplantation protocol involving WI (mimicking implantation) and WP. The presence of DFO during CS only partial decreased the subsequent vulnerability of livers to the simulated transplantation protocol. The novel finding of this study was that the presence of DFO during WI and WP could substantially decrease the vulnerability of livers to extended CS.

Liver damage during WP was related to events occurring during CS, such that extended CS sensitised the liver to subsequent WI and perfusion injury. Redox active iron has been identified as a contributor to hepatocyte death[10] and liver damage during CS[7,9,10]. Data from this study provided evidence that redox active iron was involved in sensitising the liver during extended CS, as the presence of DFO during CS did partially protect against subsequent liver damage. There is evidence that DFO is not able to cross membranes during incubation at 4 ℃[7,18], so by what mechanism did DFO partially protect the liver in this present model? Iron is present in UWS solu-

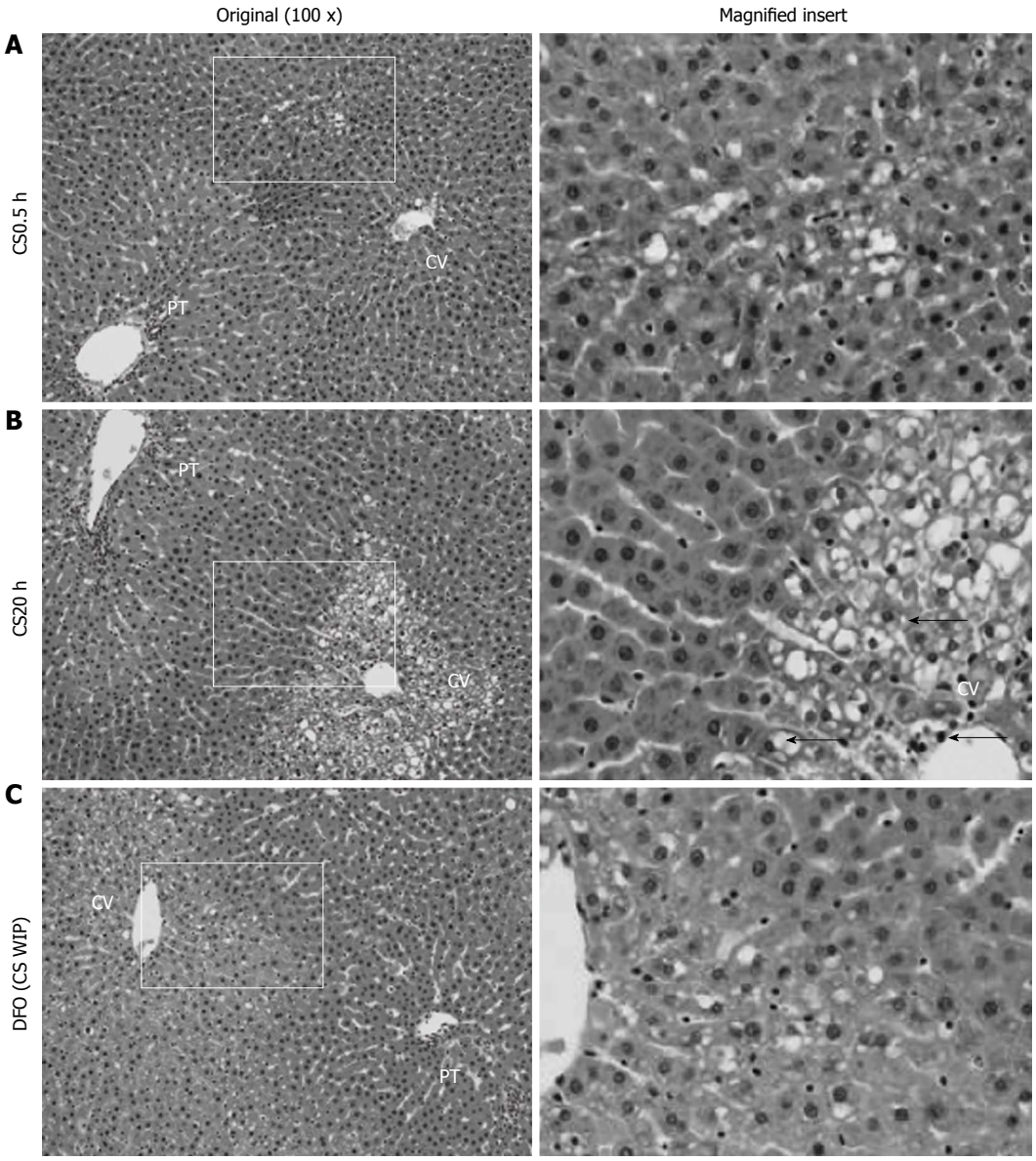

Figure 3 Histopathological appearance of livers following cold storage, warm ischemia and perfusion and the effect of desferrioxamine. A: The control liver shows some early vacuolization in zone 2 following 0.5 h of cold storage (CS0.5) (magnified insert); B: Following 20 h of cold storage (CS20), the liver shows marked vacuolization and nuclear pyknosis in zones 3 as indicated by arrows (←, magnified insert); C: Following 20 h of CS with desferrioxamine (DFO) in CS, warm ischemia and perfusion media (CS WIP), the liver shows mild vacuolization in zone 3 (magnified insert). Hematoxylin and eosin staining and the original magnification was 100 ×. PT: Portal tract; CV: Central vein.

tion as measured in our laboratory (4.43 ± 0.05 μmoL/L, n = 4) and in another study[25], so the protective effect of DFO added during CS could be a result of chelation of extracellular iron. It has also been suggested that DFO is able to reduce intracellular iron by draining cytosolic iron to the extracellular medium[26]. Consistent with this concept, we have previously shown that chelation of extracellular iron protects hepatocytes during cold incubation[7]. Therefore, during extended CS, extracellular iron could have contributed to the sensitisation of livers to subsequent WI and perfusion injury.

Intracellular redox active iron could have also contributed to the sensitization of livers to subsequent WI and perfusion injury. Intracellular redox active iron has been shown to increase during cold incubation/storage in isolated hepatocytes[9,11] and whole liver[14]. Additionally, we have linked increased intracellular redox active iron to hepatocyte cell death during extended cold incubation[7]. In previous experiments not involving CS, redox active iron has been implicated in renal ischemia and perfusion injury[12,27] and ischemia and perfusion injury in livers[28]. Therefore, an increase in the concentration of intracellular redox active iron caused by the extended CS would exacerbate an ongoing cycle of redox active iron release during WI and WP. This explanation would account for the added effectiveness of DFO during WI and WP.

The findings of this study have implications for liver transplantation. There has been a focus on protecting livers during CS by developing various cryoprotective media or improving liver function prior to transplant us-

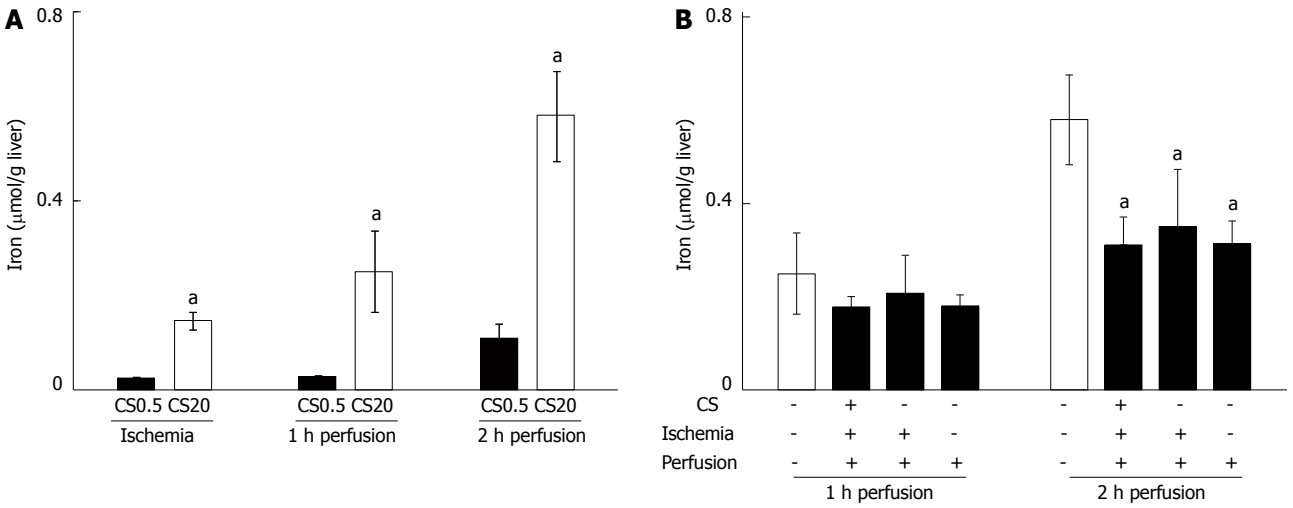

Figure 4 Iron release during warm ischemia and perfusion and effect of desferrioxamine on iron release. A: Iron release was measured in media after warm ischemia (WI) and at 1 and 2 h of perfusion from the control livers after 0.5 h of cold storage (CS0.5) and from the livers after 20 h of extended cold storage (CS20). Significantly different from the controls (mean ± SE, $n = 5$, $^{a}P < 0.05$); B: Iron release was measured in media at 1 and 2 h of perfusion from livers following 20 h of cold storage (CS). Addition of desferrioxamine (DFO) to CS, WI and perfusion media is indicated by (+). Significantly different from 20 h of CS with no DFO (mean ± SE, $n = 5$, $^{a}P < 0.05$).

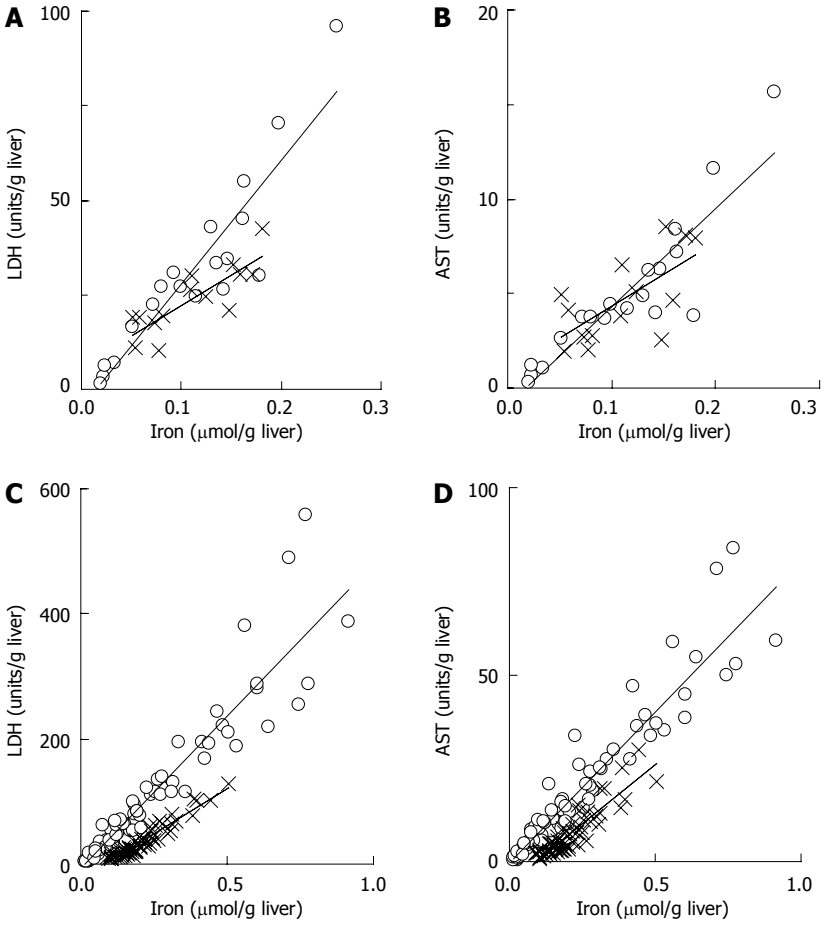

Figure 5 Relationship between presence of iron and liver damage. Following ischemia: A: The relationships between lactate dehydrogenase (LDH) and iron in the absence of desferrioxamine (DFO) (circles) and the presence of DFO (crosses) were described by y = 327x - 5 ($n = 19$, $r^2 = 0.84$) and y = 162x + 6 ($n = 14$, $r^2 = 0.69$) respectively; B: The relationships between aspartate aminotransferase (AST) and iron in the absence of DFO (circles) and the presence of DFO (crosses) were described by y = 52x - 0.9 ($n = 19$, $r^2 = 0.81$) and y = 33x + 1 ($n = 14$, $r^2 = 0.44$); C: During perfusion, samples collected at 0.5 h, 1 h, 1.5 h and 2 h were analysed. The relationships between LDH and iron in the absence of DFO (circles) and the presence of DFO (crosses) were described by y = 487x - 8.1 ($n = 76$, $r^2 = 0.88$) and y = 291x - 24.4 ($n = 56$, $r^2 = 0.91$) respectively; D: The relationships between AST and iron in the absence of DFO (circles) and the presence of DFO (crosses) were described by y = 81x - 0.5 ($n = 76$, $r^2 = 0.90$) and y = 62x - 5.6 ($n = 56$, $r^2 = 0.80$) respectively. All correlation coefficients were significant ($P < 0.05$), and all slopes for media containing iron were significantly different ($P < 0.05$) from the equivalent treatment without DFO.

ing perfusion systems. This data indicates there may be a further opportunity to enhance liver preservation during transplantation and following transplantation by chelating redox active iron. This concept will need further testing in liver transplant models as the *in vitro* model used in this study does not fully encapsulate the complexity of the recipients' response to the transplant procedure and drug intervention. From a clinical application perspective it is worth noting that DFO is already approved for a variety of clinical applications such as treating iron overload patients[29,30].

COMMENTS

Background

Cold donor organ preservation techniques were developed to reduce cellular metabolic activity and maintain cellular viability in donor organs. However, livers stored beyond about 12 h are generally considered to be unsuitable for transplantation.

Research frontiers

Extending the time of cold storage (CS) increases the susceptibility of livers to ischemia and reperfusion injury during transplantation. Preventing ischemia and reperfusion injury would permit extended times of CS.

Innovations and breakthroughs

Desferrioxamine (DFO), an iron chelator, is often included in CS media to prevent oxidative stress caused by redox active iron. The novel finding of this study was that including DFO during warm ischemia and warm perfusion could substantially decrease the vulnerability of rat livers to an extended time (20 h) of CS.

Applications

This study shows that it is possible to extend the time in which livers can be kept in CS. Extended CS times would improve the numbers of livers available for transplant.

Peer review

This is an experimental study of correct metodologia. The bibliography is sorted correctly.

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ORIGINAL ARTICLE

Interleukin-17 plays a critical role in the acute rejection of intestinal transplantation

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Abstract

AIM: To investigate the role of interleukin (IL)-17 in small bowel allograft rejection.

METHODS: We detected the expression of helper T cell 17 (Th17) cells in biopsy specimens from 3 cases of living small bowel transplantation in our department through immunofluorescence stain. We then established a rat heterotopic small bowel transplantation model. The rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after small bowel transplantation. The degrees of transplantation rejection in rat intestine graft were examined through hematoxylin eosin (HE) stain, and the expression of Th17 cells in rat intestine graft were detected through immunofluorescence stain. In addition, the recipient rats undergoing intestinal transplantation were administrated with mouse-anti-rat IL-17 monoclonal antibody (mAb), and the survival of rats was analyzed. The recipient rats which received mouse-anti-rat IL-17 mAb treatment were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after small bowel transplantation. The degrees of transplantation rejection and the expression of Th17 cells in rat intestine graft were detected through HE and immunofluorescence stain. The expression of IL-17, IL-1β, tumor necroses factor receptor-α (TNF-α), IL-6, and IL-8 in the intestine graft or serum were also detected.

RESULTS: The expressions of Th17 cells ran parallel with the degree of acute rejection in human intestine grafts. The intestine graft rejection of rats was aggravated with prolonged duration after intestinal transplantation, and the expressions of Th17 cells were also correlated with the degree of acute rejection in rat intestine grafts. Administration of mouse-anti-rat IL-17 mAb prolonged the survival of rats after small bowel transplantation ($P < 0.001$). Furthermore, we found that the administration of mouse-anti-rat IL-17 mAb significantly decreased the intensity of $CD4^+IL\text{-}17^+$ Th17 cells in intestine grafts on the 2nd, 3rd, 5th, and the 7th d (97.22 ± 4.05 *vs* 12.45 ± 2.02 on the 7th d, $P < 0.0001$), and suppressed the severity of acute rejection. The expression of IL-17 in the intestine graft declined after mouse-anti-rat IL-17 mAb administration on the 2nd, 3rd,

5th, and the 7th d (0.88 ± 0.03 *vs* 0.35 ± 0.02 on the 7th d, $P < 0.0001$). We also detected the IL-17 serum level and found that the IL-17 level reduced from the 1st d to the 7th d (6.52 ± 0.18 ng/mL *vs* 2.04 ± 0.15 ng/mL on the 7th d, $P < 0.0001$). No significant difference in the level of IL-17 mRNA in the intestine graft was identified between the two groups. The levels of IL-1β, TNF-α, IL-6, and IL-8 mRNA in the intestine graft after the administration of mouse-anti-rat IL-17 mAb were also tested. We found that on the 3rd, 5th, and 7th d after intestinal transplantation, administration of mouse-anti-rat IL-17 mAb significantly inhibited the levels of IL-1β (12.11 ± 1.16 *vs* 1.27 ± 0.15 on the 7th d, $P < 0.001$), TNF-α (27.37 ± 2.60 *vs* 1.06 ± 0.26 on the 7th d, $P < 0.001$), IL-6 (21.43 ± 1.79 *vs* 1.90 ± 0.32 on the 7th d, $P < 0.001$), and IL-8 (20.44 ± 1.44 *vs* 1.34 ± 0.20 on the 7th d, $P < 0.001$) mRNA in the intestine graft.

CONCLUSION: IL-17 may act as a promising and potent target for inhibiting acute rejection after small bowel transplantation.

Key words: Interleukin-17; Helper T cell 17; Small bowel transplantation; Acute rejection; Monoclonal antibody

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INTRODUCTION

Small bowel transplantation is a prevailing therapy for short bowel syndrome[1-3]. However, compared with liver[4,5], kidney[6,7], and heart transplantation[8,9], small bowel transplantation has less satisfying effects. The small bowel and mesentery involve redundant lymphoid tissue, which are organs with high immunogenicity prone to inducing severe transplantation rejection. FK506 (Tacrolimus) may inhibit the activation of T cells through suppressing the production of rejection related cytokines, calcium-dependent phosphatase calcineurin, and JNK/p38 pathways[10,11]. FK506 can prevent the aggregation of lymphocytes in the early rejection and chemotaxis of inflammatory cells. But the usage of FK506 after clinical small bowel transplantation may result in severe side effects, such as renal toxicity and neurotoxicity[12].

Recently, the helper T cell (Th)1/Th2 paradigm has been expanded, following the discovery of a third subset of effecter T helper cells that produce interleukin (IL)-17 (Th17) and exhibit effecter functions[13-15]. On the basis of these studies, investigators proposed that IL-17-producing T cells serve as a distinct T helper cell subset, which are called Th17 cells[16-18]. The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells[19]. Th17 cells, as potent inducers of tissue inflammation, have a proven association with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions[20,21].

In the present study, we reveal that Th17 cells and IL-17 cytokine are expressed in the intestine graft after small bowel transplantation. Furthermore, we found that the level of Th17 cells ran parallel with the degree of rejection. The data clearly indicates that Th17 cells and IL-17 cytokine may play an important role in transplant rejection.

MATERIALS AND METHODS

Patients and specimens

This study was approved by the Ethics Committee of the Fourth Military Medical University. Biopsy specimens embedded in paraffin from 3 cases of living-related small bowel transplantation were collected from Xijing Hospital of Digestive Diseases, Fourth Military Medical University (FMMU) from 1999 to 2003. All clinical information was available. The sections were stained for pathological examination. The brief clinical characteristics of these patients are listed as follows: Patient No. 1 was an 18-year-old boy with a 40 cm intestine who received a 150 cm segment of distal ileum from his father; Patient No. 2 was a 17-year-old boy with a 8 cm intestine who received a 170-cm graft of distal ileum from his father and; Patient No. 3 was a 15-year-old boy with a 10 cm intestine who received a 160-cm graft of distal ileum from his mother. All the recipients underwent different degrees of graft rejection after operation. The first two patients survived, while the third died from acute graft rejection and severe infection.

Mice

Forty inbred male F344/NCrl BR and forty LEW/Crl rats (age: 8 to 12 wk old, weight: 180 to 230 g) were purchased from Vital River Lab Animal Technology Co., Ltd (Beijing, China). Animals were maintained in specific pathogen-free conditions. All animal experiments were approved by the Animal Experiment Administration Commission of FMMU.

Small bowel transplantation

Donors and recipients were intraperitoneally anesthetized with pentobarbital (5 g/L). The small intestine 5 cm distal to the ligament of Treitz was harvested from the donor rats and 20 cm of isolated jejunum, along with mesenteric blood vessels, was prepared for transplantation. The left kidney of each recipient was removed and the infrarenal abdominal aorta isolated. An end-to-side vascular anastomosis was performed between the recipient and donor's abdominal aortae. The cuffed portal vein was inserted into the left renal vein. The two ends of the small bowel graft were constructed as separate stomas through the left abdominal wall. Detailed procedures were followed as previously

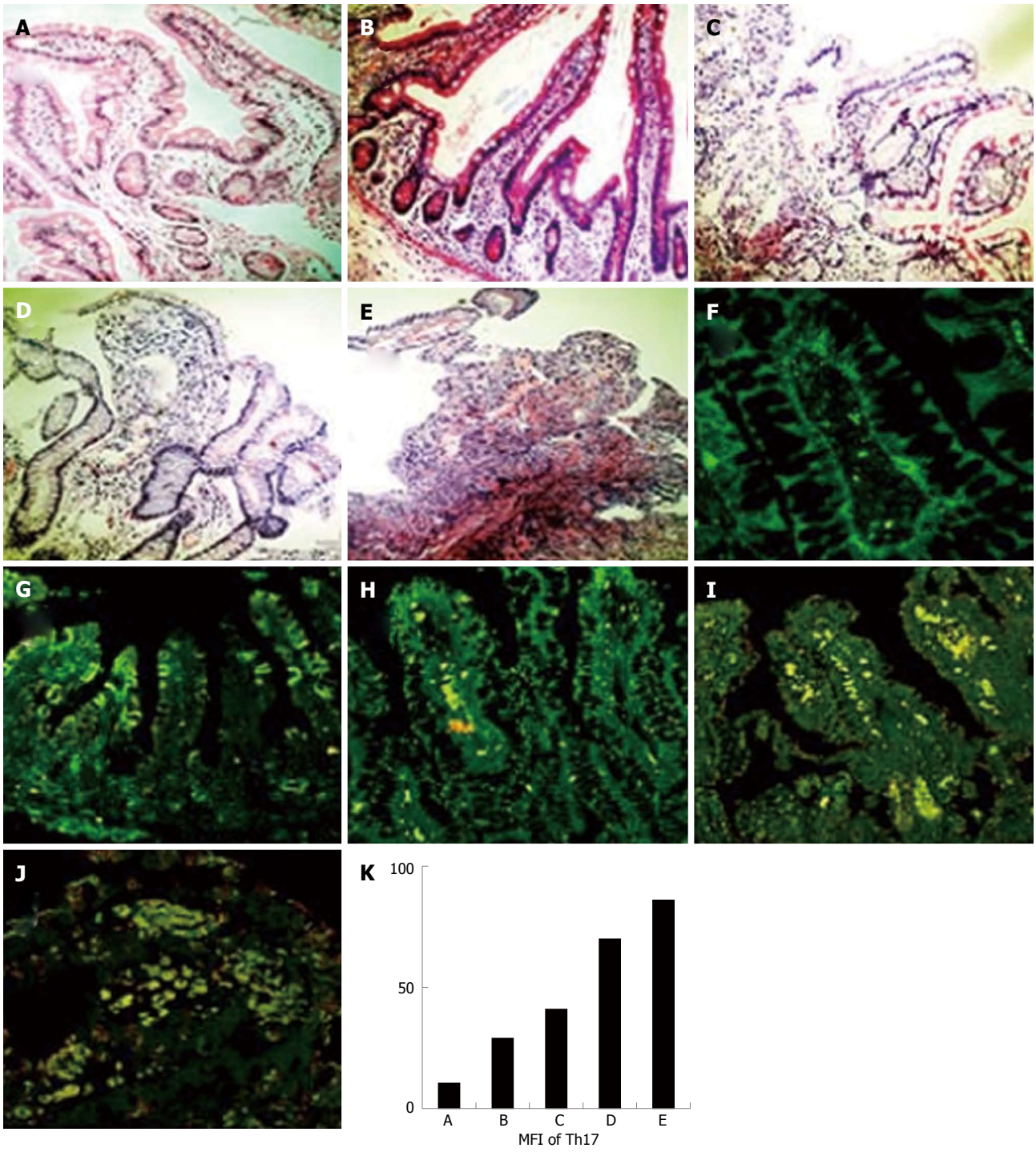

Figure 1 Expression levels of helper T cell 17 cells in intestine grafts paralleled with the degree of rejection in human recipients. The paraffin-embedded human intestine tissues after small bowel transplantation were sectioned and stained with hematoxylin eosin (HE), anti-rat-interleukin-17, and anti-rat-CD4 fluorescence antibodies (× 400). A-E: HE staining of intestine graft; F-J: Helper T cell 17 (Th17) immunofluorescence staining of intestine graft; A, F: No acute rejection; B, G: Suspicious acute rejection; C, H: Mild acute rejection; D, I: Moderate acute rejection; E, J: Severe acute rejection; K: Mean fluorescence intensity (MFI) of Th17 expression of intestine graft.

described[22]. In treatment experiments, the recipient rats in the control group were administrated with mouse-immunoglobulin G (IgG), while the recipient rats in the anti-IL-17 monoclonal antibody (mAb) group were administrated intravenously with 200 μg mouse-anti-rat IL-17 mAb[23] daily during the operation, and on the 1st, 2nd, 3rd, 5th, and 7th d afterwards.

Histology

Specimens were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin, following standard methods

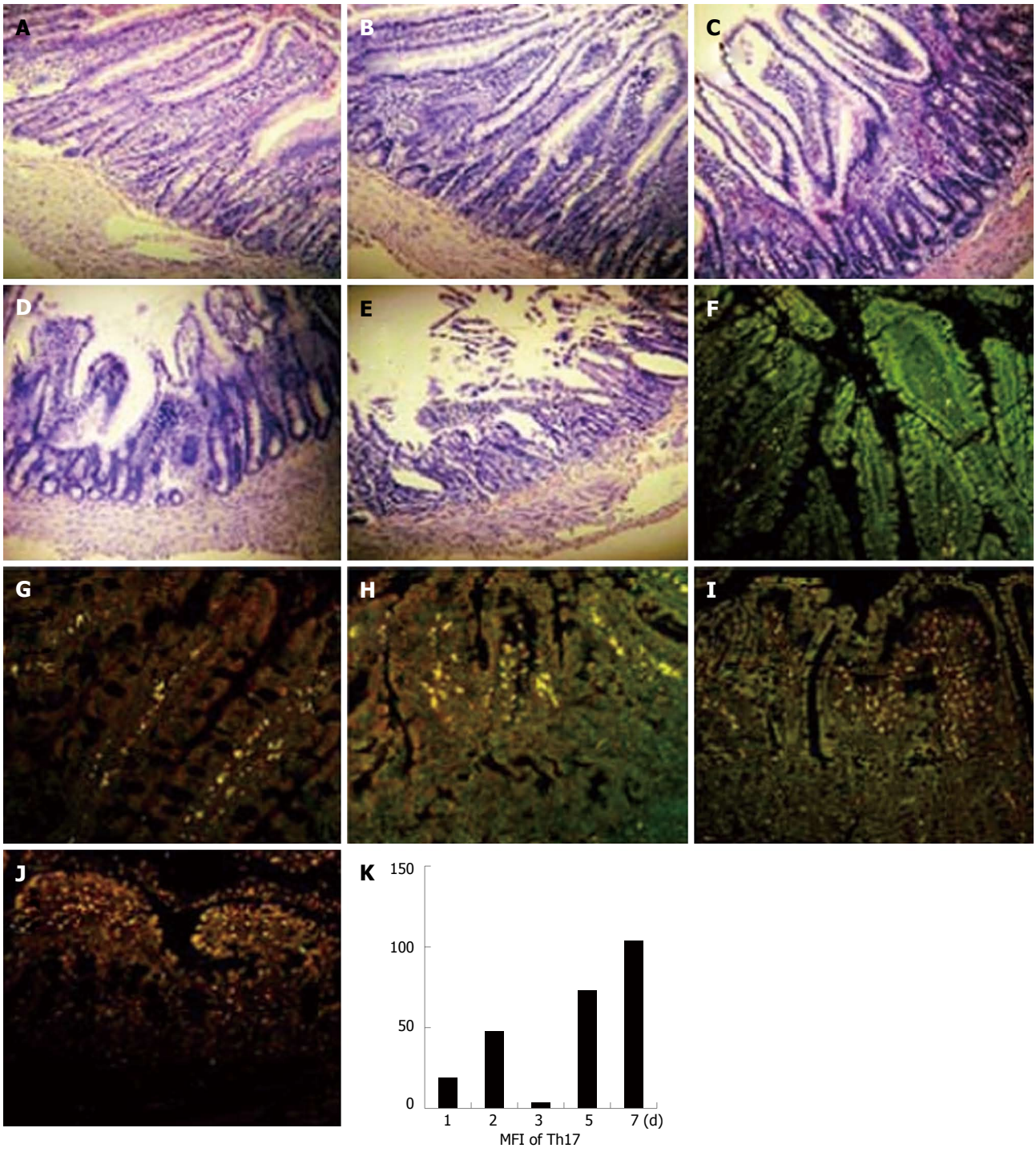

Figure 2 Expression levels of helper T cell 17 cells in intestine grafts paralleled with the degree of rejection in rat recipients. The intestines from inbred F344 were transplanted to LEW rats. The recipient rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after transplantation. The grafts were removed, fixed, and embedded. The sections were stained with hematoxylin eosin (HE), anti-rat-interleukin-17, and anti-rat-CD4 fluorescence antibodies (× 400). A-E: HE staining of intestine graft; F-J: Helper T cell 17 (Th17) immunofluorescence staining of intestine graft; A, F: 1 d after transplantation; B, G: 2 d after transplantation; C, H: 3 d after transplantation; D, I: 5 d after transplantation; E, J: 7 d after transplantation; K: Mean fluorescence intensity (MFI) of Th17 expression of intestine graft.

for routine morphological analysis.

Diagnostic criteria for transplant rejection of human and rat small bowel transplantation

The biopsy specimens of human and rat intestine grafts were stained with hematoxylin eosin (HE), and analyzed by two independent pathologists. The histological degrees for acute intestine graft rejection were divided into four grades: indeterminate for acute rejection, mild acute rejection, moderate acute rejection, and severe acute rejection. The details of the diagnostic criteria for acute intestine graft rejection have been described previously[24].

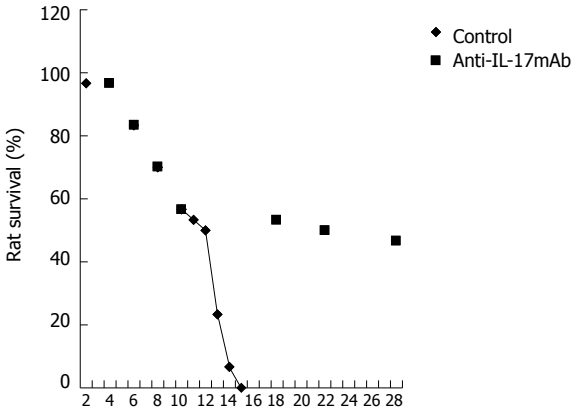

Figure 3 Administration of anti-interleukin-17 monoclonal antibody prolonged the survival of rats after small bowel transplantation. The intestines from inbred F344 were transplanted to LEW rats. The recipient rats were randomly divided into control and anti-interleukin (IL)-17 monoclonal antibody (mAb) treated groups. The survival of recipient rats was analyzed.

Immunofluorescence

The paraffin-embedded human intestine mucosa specimens were sectioned at 4 μm thickness. Immunofluorescence was performed by standard procedures, with rabbit-anti-human IL-17 polyclonal antibody (1:50 dilution; Santa Cruz Biotechnology) and mouse-anti-human CD4 mAb (1:100 dilution; Santa Cruz Biotechnology) as the primary antibodies. Goat-anti-rabbit IgG-TR (1:50 dilution; Santa Cruz Biotechnology) and goat-anti-mouse IgG-FITC (1:50 dilution; Santa Cruz Biotechnology) were taken as secondary antibodies. We did not compare the staining for IL-17 before and after transplantation, but only selected the paraffin-embedded human intestine mucosa specimens at different transplantation rejection degrees in order to detect the expressions of Th17 cells.

The negative control sections were used in our immunofluorescence method. We took phosphate buffered solution (PBS) instead of the primary antibodies as a negative control, and adopted rat cardiac allograft specimen sections as a positive control (data not shown). The methods of the immunofluorescence stain of Th17 in our present study were in accordance with our previous report[25].

Western blotting

The total proteins of the intestine graft were extracted and determined according to the manufacturer's manuals. Proteins were electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane. The membrane was incubated with rabbit anti-rat IL-17 polyclonal antibody (1:500 dilutions). After three washes for 15 min in PBS-T, the membrane was incubated with the HRP-conjugated goat-anti-rabbit IgG antibody (1:2000 dilutions). Blots were developed by using an enhanced chemiluminescence system (ECL, Amersham, Little Chalfort, United Kingdom). β-actin was considered as an internal control.

Enzyme-linked immunosorbent assay

Blood from the heart was extracted when the rat was sacrificed. Serum was collected and adopted in order to detect the level of IL-17 with a rat IL-17 enzyme-linked immunosorbent assay kit (ELISA) (Rapidbio, America) by standard procedures. $A_{450\ nm}$ was recorded by a spectrophotometer, and was compared between groups.

Quantitative real time polymerase chain reaction

Total RNA was extracted from rat intestine grafts with a homogenizer by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Complementary DNA was prepared with a reverse-transcription kit from TOYOBO (Osaka, Japan). Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed by using a kit (SYBR Premix EX Taq, Takara) and the ABI PRISM 7500 real-time PCR system, with β-actin as an internal control. Primers used in real-time PCR were as follows, β-actin, forward: CATCCGTAAAGACCTCTATG CCAAC, reverse: ATGGAGCCACCGATCCACA; IL-17, forward: GGGAAGTTGGACCACCACAT, reverse: TTCTCCACCCGGAAAGTGAA; IL-1β, forward: CTTCAAATCTCACAGCAGCATCTCG, reverse: TCCACGGGCAAGACATAGGTAGC, tumor necroses factor receptor-α (TNF-α), forward: CTGTGCC TCAGCCTCTTCTCATTC, reverse: TTGGGA ACTTCTCCTCCTTGTTGG; IL-6, forward: GACTGATGTTGTTGACAGCCACTGC, reverse: TAGCCACTCCTTCTGT GACTCTAACT, IL-8, forward: GCCAACAC AGAAATTATTGTAAAGCTT, reverse: CCTCTGCACCCAGT TTTCCTT.

Statistical analysis

Statistical analysis was performed with the SPSS 16.0 program. Results were expressed as means ± SD. Comparisons between groups were made by the unpaired Student's *t*-test. For survival studies, Kaplan-Meier survival curves were generated and a statistical analysis was performed via the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression levels of Th17 cells in intestine graft ran parallel with the degree of rejection.

To examine the expression of Th17 cells on a human intestine graft, biopsy specimens embedded in paraffin from 4 cases of living small bowel transplantation in our department were collected and stained with HE (Figure 1A-E) and antibodies against IL-17 and CD4 (Figure 1F-J). The density of IL-17$^+$CD4$^+$ Th17 cells was calculated and analyzed by Image-Pro-Plus 5.1 software. As shown in Figure 1K, the density of IL-17$^+$CD4$^+$ Th17 cells increased when the rejection degrees aggravated.

In order to investigate whether Th17 cells exist in rat intestine grafts after transplantation, intestines from F344/NCrl BR were grafted to LEW/Crl rats; the recipi-

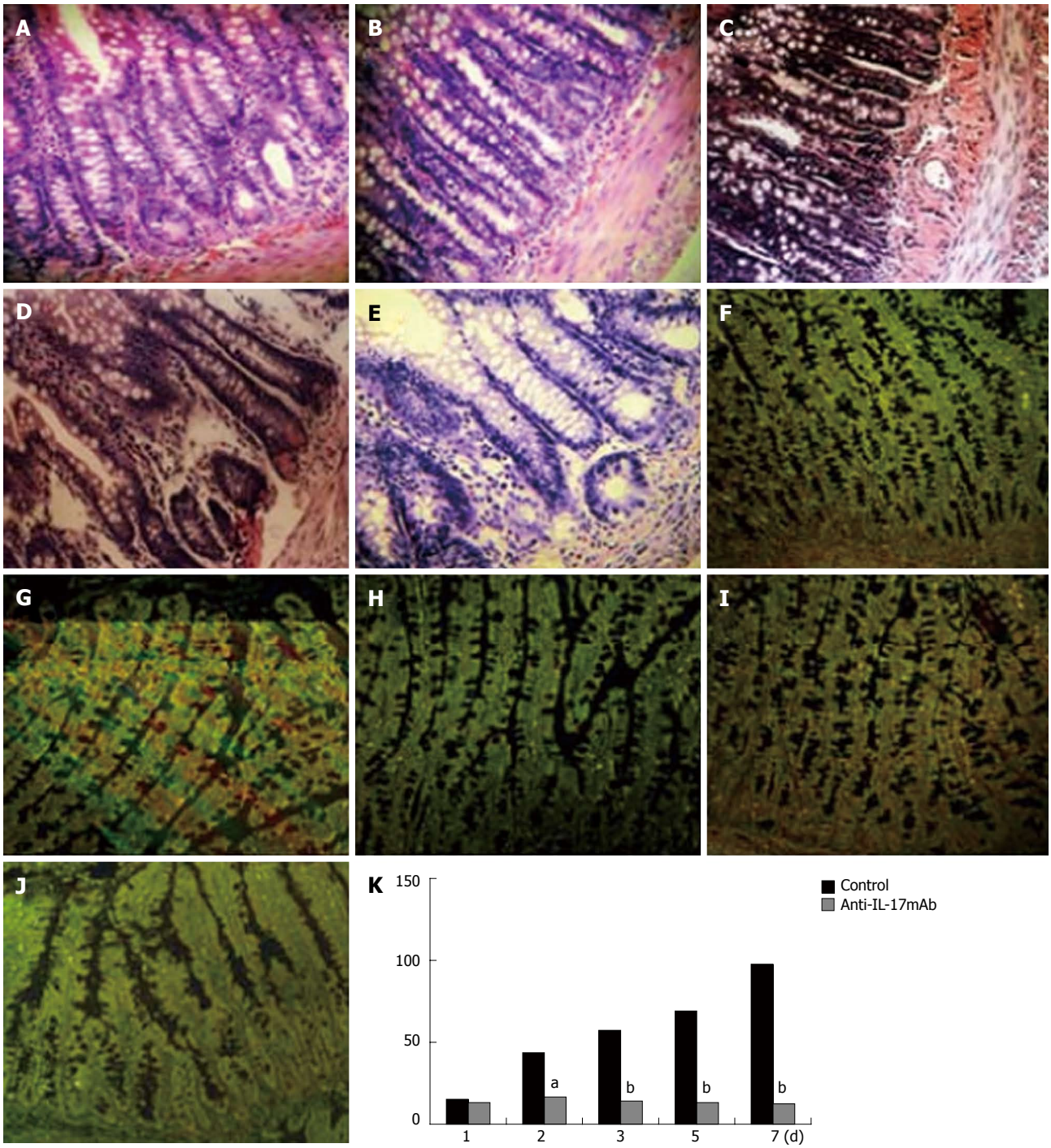

Figure 4 Administration of anti-interleukin-17 monoclonal antibody decreased the expression levels of helper T cell 17 cells in intestine grafts in rat recipients. The intestines from inbred F344 were transplanted to LEW rats. The recipient rats were randomly divided into control and anti-interleukin (IL)-17 monoclonal antibody (mAb) treated groups. The rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after transplantation. The grafts were removed, fixed, and embedded. The sections were stained with hematoxylin eosin (HE), anti-rat-IL-17, and anti-rat-CD4 fluorescence antibodies (× 400). A-E: HE staining of intestine graft; F-J: Helper T cell (Th) 17 immunofluorescence staining of intestine graft; A, F: 1 d after transplantation; B, G: 2 d after transplantation; C, H: 3 d after transplantation; D, I: 5 d after transplantation; E, J: 7 d after transplantation; K: Mean fluorescence intensity of Th17 expression of intestine graft. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

ent rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after transplantation, and the expression of Th17 cells in rat intestine grafts were analyzed (Figure 2A-J). In accordance with the findings in human intestine grafts, we found that Th17 cells were located in rat intestine grafts and the degree of Th17 cells corresponded to the severity of transplant rejection (Figure 2K). Taken together, Th17 cells did exist in intestine grafts, and the levels of Th17 cells might relate to the transplant rejection degrees in both human and rat recipients.

Administration of anti-IL-17 mAb prolonged the survival of rat post-transplantation

Since IL-17 may play a role in small bowel transplantation rejections, we hypothesized that IL-17 could be considered as a potential target for the treatment of graft rejec-

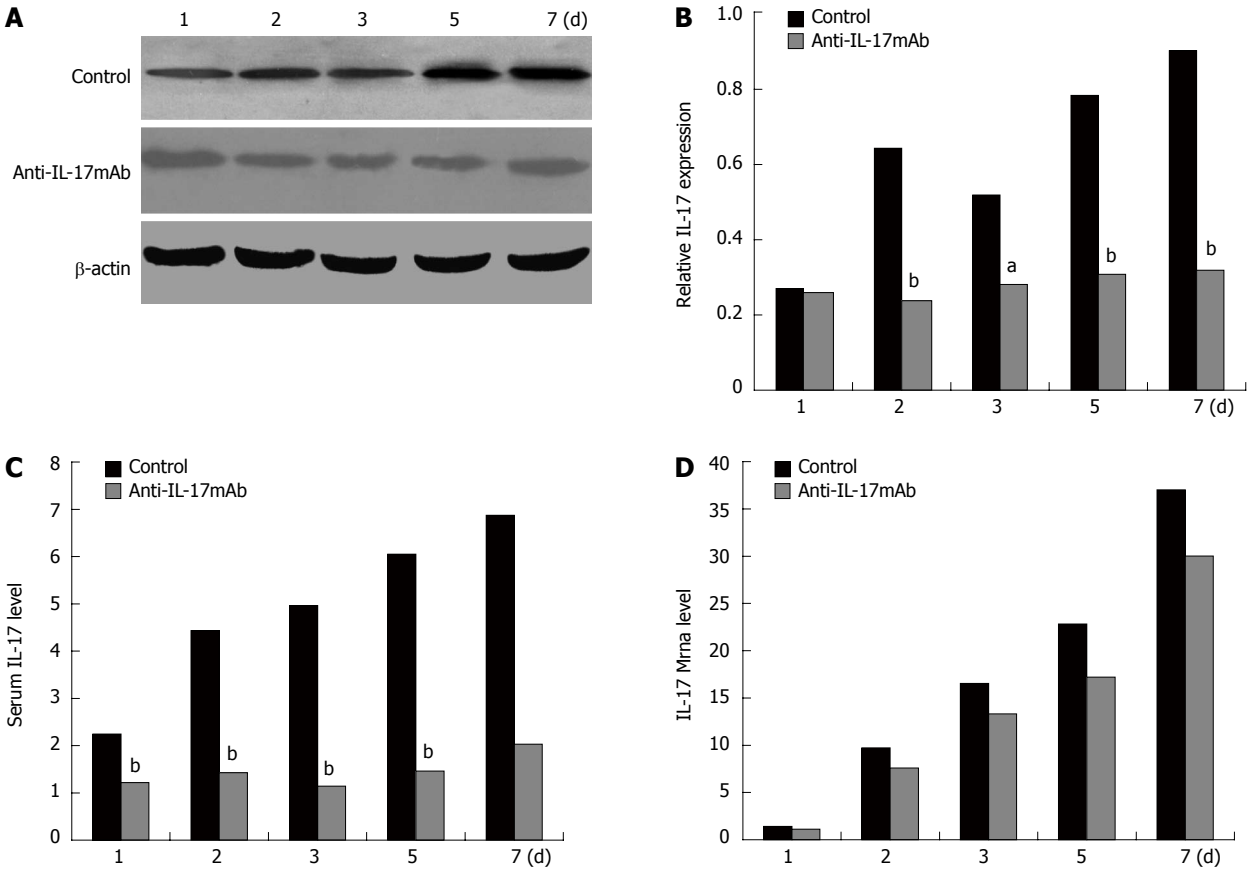

Figure 5 Expression of interleukin-17 in rat intestine and serum after transplantation. The intestines from inbred F344 were transplanted to LEW rats. The recipient rats were randomly divided into control and anti-interleukin (IL)-17 monoclonal antibody (mAb) treated groups. The rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after transplantation. A: Western blot analysis of graft proteins of different groups. β-actin were considered as an internal control; B: The bands were scanned and the protein expressions were analyzed with Band Scan software; C: Blood from the heart was extracted and the serum IL-17 levels was detected by enzyme-linked immunosorbent assay; D: The mRNA of IL-17 was detected by quantitative reverse transcriptase polymerase chain reaction. β-actin was considered as an internal control. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

tion. In order to further demonstrate our hypothesis, the recipients were administrated with mouse-anti-rat IL-17 monoclonal antibody intravenously during, and after, small bowel transplantation, and the survival of recipients were analyzed. We found that administration of anti-IL-17 mAb could significantly prolong the survival of rats after small bowel transplantation (Figure 3).

Anti-IL-17 mAb administration inhibited transplant rejection of intestine graft

We further analyzed the effect of anti-IL-17 administration on graft rejections. The administration of anti-IL-17 mAb was proved to effectively inhibit transplant rejection of intestinal grafts in rats post-transplantation by HE staining (Figure 4A-E). Furthermore, we found that the expression of Th17 cells in intestine grafts also dramatically declined (Figure 4F-K), indicating that anti-IL-17 mAb could possibly prolong the survival of recipient rats by inhibiting transplant rejection.

Anti-IL-17 mAb administration decreased IL-17 expressions in rat

During and after allotransplantation, recipient rats were administrated with anti-IL-17 mAb. The recipient rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after transplantation. The expressions of IL-17 in intestine grafts and serums were further detected by western blot and ELISA (Figure 5). In the control group which only received saline administration, the levels of IL-17 protein in the intestine graft and serum were found to have increased post-allotransplantation and run parallel with transplant rejection degrees. In the anti-IL-17 mAb administration group, the expression of IL-17 could also be detected, but the degrees were remarkably lower than the control group. The levels of IL-17 mRNA in the intestine graft in two groups were not significantly different. Therefore, the administration of anti-IL-17 mAb could decrease the IL-17 level in intestine grafts and serum.

Anti-IL-17 mAb administration decreased expressions of related cytokines

As shown in Figure 6, the expressions of IL-1β, IL-6, IL-8, and TNF-α mRNA of intestine grafts in the control group were found to increase post-allotransplantation and correlate to transplant rejection degrees. Administration of anti-IL-17 mAb could dramatically decrease the expression of mRNA levels of these cytokines in intestine grafts, compared with the control group.

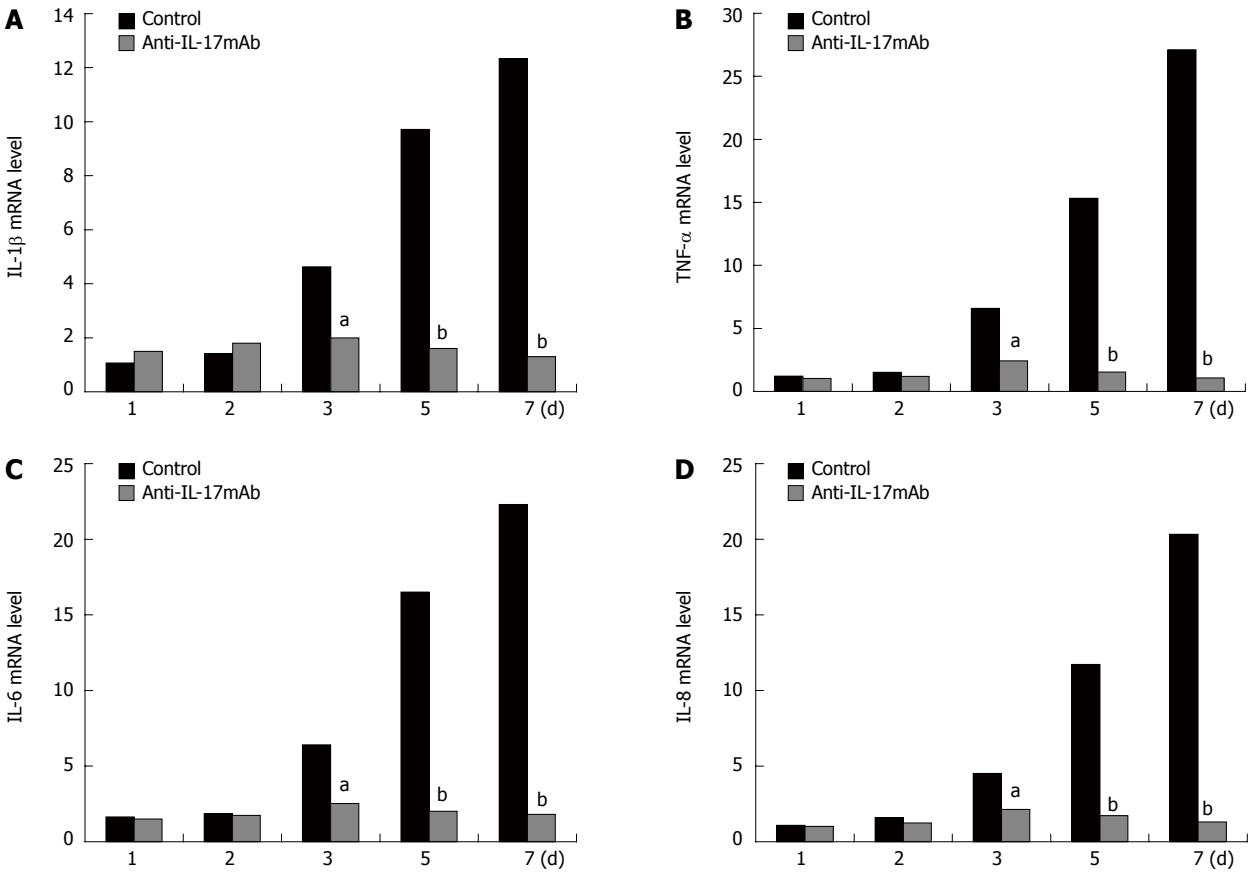

Figure 6 mRNA level of interleukin-1β (A), tumor necrosis factor-α (B), interleukin-6 (C), and interleukin-8 (D) were analyzed by reverse transcriptase polymerase chain reaction. The intestines from inbred F344 were transplanted to LEW rats. The recipient rats were randomly divided into control and anti-interleukin (IL)-17 mAb treated groups. The rats were sacrificed on the 1st, 2nd, 3rd, 5th, and 7th d after transplantation. The mRNA was extracted and quantitative reverse transcriptase polymerase chain reaction was performed. β-actin was considered as an internal control. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

DISCUSSION

Th17, known as a distinct lineage of helper T cells from Th1 and Th2, has both a defense role against microbe infection and a pathogenic role in several autoimmune diseases[26]. Th17 cells have been shown to be implicated in allograft rejection of solid organs, such as lung and cardiac transplantation[25,27,28]. Vanaudenaerde *et al*[27] observed that IL-17 expression increased in bronchoalveolar lavages in patients with acute lung transplantation rejection. The disease-promoting role of Th17 in cardiac allograft rejection was also confirmed, especially in the absence of a Th1 response[25]. Th17 cells not only function in host-versus-graft disease, but also participate in graft-versus-host disease[29-31]. So far, the relationship between Th17 cells and small bowel transplantation has been unclear. In this study, we demonstrated that Th17 cells participated in the development of human and rat small bowel transplantation rejection.

Here we found that Th17 cells existed in intestine grafts with different degrees of acute rejection. Furthermore, we indicated that the density of Th17 cells increased when the rejection degree aggravated, suggesting that it might be related to the transplant rejection degrees in human recipients. Th17 cells were also found to be located in rat intestine grafts, and the degree of Th17 cells correlated with transplant rejection degrees, which was in accordance with the findings in human intestine grafts. These demonstrate that Th17/IL-17 may participate and play a critical role in the graft rejections of small bowel transplantation.

IL-17, secreted by Th17 cells, is a highly inflammatory cytokine with robust effects on stromal cells in many tissues[32,33]. Hsieh *et al*[34] reported that IL-17 could serve as a predictive parameter for borderline subclinical renal allograft rejection in the future. Itoh *et al*[35] found that IL-17-deficient recipient mice had decreased allograft inflammatory cell recruitment, and demonstrated that IL-17 contributed to the pathogenesis of chronic allograft rejection. In order to demonstrate the key role of IL-17 in the graft rejection of small bowel transplantation, we utilized mouse-anti-rat IL-17 mAb to treat recipient rats undergoing small bowel transplantation for the first time. Surprisingly, after administration of anti-IL-17 mAb, the acute rejection degrees of recipient rats significantly decreased compared to the control group. The levels of Th17 cells that had infiltrated the intestine graft during anti-IL-17 mAb administration also fell greatly. Administration with anti-IL-17 mAb could extend the survival time of rats undergoing small bowel transplantation. To sum up, IL-17 cytokine could probably be taken as a potent target to treat acute rejection in small bowel

transplantation.

The differentiation factors (transforming growth factor beta plus IL-6 or IL-21), the growth and stabilization factor (IL-23), and the transcription factors (signal transducer and activator of transcription 3, related orphan receptor-γt (RORγt), and RORα) were involved in the development of Th17 cells. Mice reconstituted with the bone marrow of RORγt deficient mice showed an impaired Th17 differentiation. The combinations of IL-1β plus IL-6[36] or IL-1β plus IL-23[37] were proposed to be the differentiation factors for human Th17 cells. We found that the expression of IL-1β and IL-6 was significantly decreased after anti-IL-17 mAb administration. Therefore, anti-IL-17 mAb might suppress the expression of IL-1β and IL-6 in rat intestine grafts, and then inhibit the development and activation of Th17 cells. TNF is also induced by IL-17 cytokine. Its expression was found to be reduced after anti-IL-17 mAb administration. The migration and infiltration of inflammatory cells into intestine grafts requires the expression of chemokines. Chemokine (C-X-C motif) ligand 8 (IL-8), a target of IL-17, is involved in transplant rejections. Compared to the control group, the expression of IL-8 was found to be obviously decreased in intestine grafts treated with anti-IL-17 mAb. This suggests that anti-IL-17 mAb administration might suppress the migration and infiltration of Th17 cells into intestine grafts by decreasing the expression of chemokines.

In conclusion, we have illustrated that Th17 cells might play an important role in human and rat small bowel acute transplantation rejection. The administration of anti-IL-17 mAb could significantly suppress the acute rejection degree of rat intestine grafts and prolong the survival time of recipient rats. IL-17 could be considered a promising and potent target for inhibiting acute rejection after small bowel transplantation.

COMMENTS

Background

Small bowel transplantation is a widespread therapy for short bowel syndrome. However, the efficacy of small bowel transplantation is not satisfactory due to severe transplantation rejection. Although administration of FK506 may inhibit the activation of T cells, prevent the aggregation of lymphocytes in early rejection, and restrain the chemotaxis of inflammatory cells, its administration after human small bowel transplantation may cause severe side effects such as renal toxicity and neurotoxicity.

Research frontiers

The helper T cell (Th) 17 cell, as a distinct lineage of helper T cells from Th1 and Th2, has a proven implication in the allograft rejection of solid organs, such as lung and cardiac transplantation. Interleukin (IL)-17 expression was increased in bronchoalveolar lavages in patients with acute lung transplantation rejection. The disease-promoting role of Th17 in cardiac allograft rejection was also confirmed. Th17 cells are involved not only in host-versus-graft disease, but graft-versus-host disease as well.

Innovations and breakthroughs

So far, the relationship between Th17 cells and small bowel transplantation has been unclear. Authors demonstrated the presence of Th17 cells in the intestine grafts of humans and rats, and further found that the expression levels of Th17 cells in intestine grafts correlated with the degree of rejection. The authors hypothesized that Th17/IL-17 might play a critical role in small bowel transplantation rejection and could be regarded as a potential target for the treatment of graft rejection. They then treated recipient rats with mouse-anti-rat IL-17 monoclonal antibody (mAb) and found that its administration could significantly prolong the survival of rats after small bowel transplantation. Furthermore, the authors found that the expression of Th17 cells in intestine grafts also dramatically declined, indicating that mouse-anti-rat IL-17 mAb may prolong the survival of recipient rats by inhibiting transplant rejections. In the present study, the authors demonstrated that Th17 cells participated in the development of human and rat small bowel transplantation rejection.

Applications

The administration of mouse-anti-rat IL-17 mAb could significantly suppress the acute rejection degree of rat intestine grafts and prolong the survival time of recipient rats. IL-17 could be considered as a promising and potent target for inhibiting acute rejection after small bowel transplantation.

Terminology

Small bowel transplantation: an operation to replace a diseased or shortened small bowel with a healthy bowel from a donor and a valuable therapy for short bowel syndrome. Transplant rejection: immune system attacks between the transplant recipient and the transplanted organ or tissue, which include host-versus-graft disease and graft-versus-host disease. Th17 cell: known as a distinct lineage of helper T cells from Th1 and Th2 that has both a defense role against microbe infection and a pathogenic role in several autoimmune diseases.

Peer review

This is an investigation of the role of IL-17 in acute intestinal transplantation rejection and the administration of anti-IL-17 monoclonal antibodies for the suppression of IL-17 production by Th17 cells in the intestine graft and, as a result, suppression of acute rejection of the intestine graft in both human and rat models. It is a very good work and I hope it is applicable in human subjects undergoing intestinal transplantation.

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ORIGINAL ARTICLE

Comparison of different methods of intestinal obstruction in a rat model

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Abstract

AIM: To investigate different methods of creating incomplete intestinal obstruction in a rat model and to compare their electrophysiologic, morphologic and histologic characteristics.

METHODS: Rat ileum was partially obstructed by the respective application of: braided silk (penetrated the mesentery and surrounded intestine); half ligation (penetrated directly and ligated 1/2 cross-section of the intestine); wide pipe (6 mm in width, surrounded the intestine); narrow pipe (2 mm in width, surrounded the intestine). A control was also included (no obstruction). Various behavioral and electrophysiologic variables, as well as morphologic and immunohistochemical observations were recorded by blinded investigators at different time points (12, 24, 48, 72 h), including daily general condition, ileal wet weight and circumference, macromorphous and micromorphous intestine, bowel movement capability *in vivo* and *in vitro*, slow wave and neural electrical activity, and the number of c-Kit positive interstitial cells of Cajal (ICC).

RESULTS: Despite being of a similar general condition, these methods resulted in different levels of obstruction in each group compared with the control at different time points (12, 24, 48, 72 h). However, these fields of the wide pipe rat showed significantly differences when compared with the other three obstructed groups at 12 to 72 h, including macroscopic and histological presentation, intestinal transit ratio and contractility, circumference and wet weight, amplitude and frequency of nerve electrical discharge and slow wave, and ICC numbers (all $P < 0.01$).

CONCLUSION: The wide pipe rat method is significantly more reliable and stable than the other methods of obstruction, demonstrating that use of the wide pipe method can be a useful model of incomplete intestinal obstruction.

Key words: Intestinal obstruction; Model; Comparative study; Electrophysiology; Morphology; Interstitial cells of Cajal

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INTRODUCTION

Intestinal obstruction (IO) is one of the most common diseases in abdominal surgery. It can slowly lead to changes in intestinal structure and function, and in extreme cases it can be life-threatening[1,2]. Incomplete intestinal obstruction is the initial phase of intestinal obstruction and is the clinical pathological basis for other types of intestinal obstruction. In addition, when considering the impairment associated with incomplete intestinal obstruction, exploring its diagnosis and treatment has important clinical significance.

Animal models of incomplete intestinal obstruction are important in the investigation of the pathogenic mechanisms involved in human intestinal obstruction, and play an important role in identifying and testing novel interventions. To date, animal models of incomplete intestinal obstruction have included mice, rats, rabbits, pigs and approximately six other types of animals[3-5]. No particular model parallels the complex nature of human intestinal obstruction. The drawback of using animal models is that the basis for the high rate of incomplete intestinal obstruction is not known.

Therefore, it is necessary to establish a reliable animal model to study the pathogenesis of intestinal obstruction, and to have a good experimental model to allow the exploration, research and ultimate treatment of intestinal obstruction. Different types of materials to induce obstruction are currently available. The prosthetic valve has been used since 1996[5]. This was followed by half ligation using braided silk, which was developed in 2004 and has been available since 2008. One report describes the changes elicited by ligating the intestines outside the body[4]. A 24-Fr latex T-tube, cut to form a pipe, was used in China to establish a model of intestinal obstruction[6].

With regard to species selection, the anatomy and physiology of primates are similar to humans, but due to ethical and economic limitations this model is not widely used. Whichever method or species is selected, the ideal animal model should be similar and comparable to the human disease, demonstrate repeatability, be economic and easy to use[7]. In this study, rats were selected and three different materials were used to establish intestinal obstruction.

Based on the above factors, the purpose of the present experimental study was to compare the characteristics of four methods of incomplete intestinal obstruction in the rat model. In addition, we also obtained electrophysiologic, morphologic and histologic information on each method which may have clinical relevance.

MATERIALS AND METHODS

Animals

Healthy adult female and male Sprague-Dawley rats, weighing between 180 and 280 g, were provided by the Laboratory Animal Center [Licence No. SCXK (CHUAN) 2006-10; Sichuan University, Chengdu, China], and were individually housed at 22 ℃ in a 12-h light/dark cycle, with free access to tap water and a standard pellet diet (Laboratory Animal Center, Sichuan University, Chengdu, China) for at least 1 wk before the start of the experiments.

Surgical procedure

One hundred and fifty rats were randomly divided into five groups of thirty in each group. 12 h prior to each study, the animals were fasted but had free access to water, and were anesthetized with 1.5% pentobarbital sodium (30 mg/kg body weight, intraperitoneal; Beijing Chemical Reagent, batch number, 090205; Beijing, China). In this study, a loop of intestine was exposed, and partially obstructed using the respective material. Three different materials were used to obstruct the intestine. A round needle with braided silk No. 0 (Pudong Jinhuan Medical Products, batch number, 11L02028; Shanghai, China) penetrated the mesentery, a polyethylene pipe (2 mm exterior diameter) was placed at the side of the intestine and a ligature was formed with the braided silk, the pipe was then pulled out smoothly from the ligation. This group was known as the braided silk group. Braided silk No. 3-0 (Pudong Jinhuan Medical Products, batch number, 11L02028; Shanghai, China) was used to directly penetrated the intestine and ligate 1/2 cross-section of the intestine. This group was known as the half ligation group. A 24-Fr latex T-tube (Nantong Angel Medical Instruments, batch number, 20110304; Jiangsu, China) was cut to form a pipe 10 mm in length and 6 mm in width which surrounded the intestine. This group was known as the wide pipe group. In another group of rats, a narrow pipe 10 mm in length and 2 mm in width was used to obstruct the intestine. This group was known as the narrow pipe group. The rings were located in the ileum 20 mm oral to the ileocecal sphincter, and this was followed by closure of the abdomen. All materials were placed with the cut side between the lateral extensions of the mesenteric vascular bed to avoid vascular injury[3]. The control group underwent a similar procedure, but the rings used in these animals were 10 mm in length and did not result in occlusion of the ileum.

Postoperative care

After surgery, the animals were treated with an intraperitoneal injection of a 2 mL mixture of glucose (9 mg/mL; Shuanghe Pharmaceutical Industry, batch number,

Table 1 Criteria for scoring macromorphous intestinal damage

Feature	Description	Score
Adhesions	None	0
	Slight, ileum can be separated from other tissues with little effort	1
	Involved in a number of intestinal loops	2
Stenosis	None	0
	Slight	2
	Severe, proximal intestinal distention	3
Ulceration	None	0
	Damage extended to < 1 cm along the length at one site	1
	Damage extended to < 1 cm along the length at two sites	2
	Damage extended to > 1 cm along the length at one siteor multiple lesions	3
Bowel wall thickness	< 1 mm	0
	1-3 mm	1
	> 3 mm	2
Total score		10

Table 2 Criteria for scoring micromorphous intestinal damage

Feature	Description	Score
Ulceration	None	0
	Ulceration < 3 mm, no hyperemia or focal hyperemia, no ulceration	1
	Ulceration < 3 mm, with focal hyperemia or ulceration > 3 mm, without hyperemia	2
	Ulceration > 3 mm or hyperemia	3
Inflammation	None	0
	Slight	1
	Moderate	2
	Severe	3
Fibrogenesis	None	0
	Slight	1
	Severe	2
Extent of involved bowel wall	None	0
	Submucosa	1
	Muscular layer and even serosa	2
Total score		10

100829 6K; Anhui, China) and sodium chloride (50 mg/mL; Shuanghe Pharmaceutical Industry, batch number, 100825 1E; Anhui, China) in a ratio of 1:1 (vol/vol), and 0.1 mL potassium chloride (100 mg/mL; Jiaozuo Pharmaceutical Industry, batch number, 11031641; Tianjin, China). The animals were then allowed to recover on a heated blanket. The rats were allowed to fast 2 h before the last administration, and were rapidly euthanized with pentobarbital sodium at 12, 24, 48, and 72 h, respectively. The abdomen was then cut open and the test was started.

Daily general condition

After surgery, the rats were housed in individual cages. Each day at approximately 9 AM, body weight and food intake were recorded, and feces were collected from each cage. The number of feces pellets was counted and color was observed. Feces from each rat were then lyophilized and weighed to determine fecal dry weight. The total number and dry weight of the fecal pellets were calculated. The mental status and death of rats were recorded.

Measurement of ileum circumference and wet weight

Following sacrifice of the rats, a 3 cm fresh intestinal segment was obtained, the intestinal contents were removed using Tyrode's solution, the segment was then dried with filter paper, and ileal weight was recorded. The ileal wet weight index was calculated using the fasting body weight (kg) divided by the weight (g) of the 3 cm intestinal segment. A bowel segment (10 cm) cut longitudinally along the opposite side of the mesenteric edge was obtained, and its circumference was measured at three sites, and the average was recorded.

Morphological studies

The ileum was opened by an incision along the mesenteric border and laid flat. Tissue damage was assessed and was scored on the 3 cm intestinal segment which was taken 5 cm oral to the ileus, using the criteria outlined in Tables 1 and 2 adapted from Butzner *et al*[8]. Each section was then graded by two blinded observers. Scoring was based on a number of features which were scored from 0 to 10 depending on the presence and severity of visible ileal damage, which consisted of the extent and severity of adhesions, stenosis, ulceration, and bowel wall thickness. A score of 0 to 10 was used to describe the total scores of ulceration, inflammation, fibrogenesis and extent of bowel wall involved. Finally, the extent of histological intestinal mucosal damage was evaluated according to the grading system of Chiu *et al*[9].

Hematoxylin and eosin staining

Histological analysis of intestinal damage was carried out on pieces of muscle taken the same distance from the site of occlusion adjacent to the tissue regions used for electrophysiology. The intestinal tissues in Tyrode's solution were pinned. Connective tissues were removed, cut and pinned into a rectangular shape (1 cm × 2 cm, width and length). The ileal tissues were then fixed in 4% paraformaldehyde for 6 h at room temperature (RT). Two longitudinal sections, 1 cm in length and 2 mm thick, were taken from each fixed tissue segment. These sections were rinsed for 2 h in running water, dehydrated through a graded series of alcohols and embedded in paraffin using the Intelligent Program-controlled Automatic Hydroextractor of Biological Organization (Xiaogan Taiwei Science and Technology Industry, Hubei, China) and a tissue embedding console system (Jinhua Kedi Instrumental Equipment, Zhejiang, China). All paraffin-embedded tissue blocks were sectioned at 5 μm thickness using a microtome (Microsystems Nussloch; Leica Instruments, Shanghai, China), and slides were then prepared. The paraffin was removed from the slides by treatment with xylene. Sections were rehydrated with an ethanol series and stained with hematoxylin and eosin

and examined by light microscopy.

Bowel movement capability analysis

After the rats had fasted for 4 h, all groups received 3% activated charcoal suspension (Sigma, Aldrich, United States) in saline (1.5 mL/rat, *po*) as a charcoal meal. One hour after administration of the marker, the animals were anesthetized with an intraperitoneal injection (2 mL/kg) of pentobarbital sodium, and the small intestine from the pylorus sphincter to the ileocecal sphincter was removed. The intestinal transit ratio was calculated using the percentage of the small intestine length divided by the length the charcoal had travelled.

After the animals had been killed, the small intestine was exposed by a mid-line abdominal incision, and a 3 cm tubular segment of intestine was obtained 1 cm oral to the ileus. The bowel was opened along the mesenteric border and the intestinal contents were rinsed with Tyrode's solution. Segments from a 2 cm piece of bowel were cut and the mucosa was removed by sharp dissection. Strips of muscle (20 mm × 5 mm) were pinned to the hook and contractions were measured isometrically under a resting tension of 0.5-1 g in a 20 mL tissue chamber (Chengdu Technology and Market, Sichuan, China) containing Tyrode's solution for at least 30 min. A force transducer coupled to the BL-420F Biological Function Experimental System (Chengdu Technology and Market, Sichuan, China) was used to record the contractility of the ileum. The settings for the tension pattern was: scanning speed, 2.56 s/div, power gain, 2000, time constant, 5 s, and high-frequency filtering, 3 Hz. The sustained record time was up to 30 min, followed by 30 s of recorded data. Frequency and amplitude were recorded and calculated.

Measurement of neural electrical activity

Along the central line of the neck, a longitudinal cut open to the skin, layer by layer separating the muscles, fascia, and exposing the carotid sheath was made. After separating the vagus nerve, the nerve was immersed in 37 ℃ warm paraffin (Jiangcheng Lipid Chemical Industry, batch number, 100101; Jilin, China) and insulated with the surrounding tissue to prevent the nerve drying during recording. The recording electrodes were hooked to the vagus nerve, the reference electrode was placed on the skin fold, and the electrode input was connected to the BL-420F Biological Function Experimental System. The settings for the neural discharge pattern were: scanning speed, 80 ms/div, power gain, 10 000, time constant, 0.1 s, and high-frequency filtering, 1 KHz. As mentioned above, both frequency and amplitude were recorded.

Determination of slow wave

Ileal slow waves were measured using electrophysiology. A pair of self-made silver electrodes (5 mm in length, 0.1 mm in diameter) was implanted into the seromuscular layer of the intestine. The distance between the electrodes was about 5 mm and they were 10-20 mm away from the ring. The reference electrode was placed on the skin fold, the terminal was wired and connected to the BL-420F Biological Function Experimental System. A layer of paraffin gauze was used to cover the exposed ileum. The settings for the slow electrical signals pattern were: scanning speed, 1.28 s/div, power gain, 2000, time constant, 5 s, and high frequency filtering, 30 Hz. The sustained record time was up to 30 min, followed by 30 s of recorded data. Ten periods were then randomly intercepted using the modified Tomita method[10], and both frequency and amplitude were recorded, calculated and compared (as described earlier).

Immunohistochemistry

The expression of c-Kit in ileal tissue was assessed by immunohistochemistry. Specimens were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin, as described previously. A mixed liquid suspension containing 5 mL garlic juice and fresh egg white was added to 100 mL glycerol, which was then used to coat a glass slide. The tissues were then deparaffinized in xylene and rehydrated in a descending ethanol series. After dewaxing and rehydration, antigen retrieval was performed using high pressure for 2 min, and then blocked by incubation in 3% H_2O_2 for 15 min at 37 ℃ to ablate endogenous peroxidase. The sections were washed in phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4), and then incubated with goat anti-c-Kit specific polyclonal antibody (sc-1494, 1:100, Santa Cruz, Biotechnology, United States) for 1 h at 37 ℃ in a wormwall incubator (Huyueming Instrument, Guangzhou, China). The slides were washed in PBS (5 × 3 min), and then incubated with a horseradish-peroxidase-conjugated rabbit anti-goat IgG(H+L) (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 40 min at 37 ℃, rinsed (5 × 3 min) in PBS, and the sections were colored with 3,3-diaminobenzidine, kept at RT in the dark for 30 s. Following that, the slides were counterstained with hematoxylin, differentiated with 5 g/L hydrochloric ethanol, 1:400 ammonia recurrented blue, sequential ethanol dehydration, and then clearing and mounting with neutral resin. In the negative control the same steps as previously described were carried out, but the primary antibody was replaced by PBS. The positive expression of c-Kit in interstitial cells of Cajal (ICC) was shown by dotted dark brown cells with a nucleus or individual processes of dark brown rods.

Statistical analysis

All data were presented as means ± SE. P values < 0.01 were considered statistically significant. Each intestinal segment was taken from individual animals. The electrical parameters of each segment were analyzed: (1) frequency; (2) amplitude; and (3) contraction capability (contraction frequency multiplied by amplitude). A one-way ANOVA

test was used to compare parameters and the Kruskal Wallis test was used to compare parameters displaying abnormal distribution among the groups. Tukey's Honestly Significant Difference (HSD) test was used to detect the group causing variation when comparing qualitative data, and the Mann Whitney *U* test was used to detect the group causing variation. χ^2 or Fisher's exact test was used to compare qualitative data. All analyses were performed using SPSS 13.0 for Windows (SPSS Inc, United States). Adobe Photoshop 8.0 (Adobe, Mountain View, CA, United States) was used to construct and display the figures made from digital photos.

RESULTS

Clinical

Body weight, food intake, feces, mental status as well as mortality were recorded every 12 h. Of the average values in the control group, body weight, food intake, feces, mental status and death showed a wider band change from 12 to 24 h, this index showed a narrower band change from 24 to 72 h. Mean body weight, food intake, feces, mental status and death in the four different obstructed groups showed significant changes with persistent loss or gain ($P < 0.01$). The general condition of the rats is shown in Table 3.

Macroscopic and histological evaluation

The rings were placed on the ileum 20 mm oral to the ileocecal sphincter, which resulted in visible intestinal damage 12 to 72 h after surgery. In the control rats, no marked gross morphological changes in the intestine were observed (Figure 1A). However, morphological observation of the intestines revealed adhesions, stenosis, ulceration and bowel wall thickness of the rings to varying degrees in the rats with obstruction. In addition, flushing, tumefaction of intestinal mucosa, hyperemia and hemorrhage of bowel wall and stasis of bowel contents from the proximal region to the site of the ring were noted (Figure 1B-E). Figure 1F shows analytical data of the total scores of intestinal damage following macroscopic evaluation in obstructed and control rats.

The histological findings in ileal tissues are presented in Figure 2. No marked changes were observed in the control rats (Figure 2A). Histological observation of the intestines showed some features (including ulceration, inflammation, fibrogenesis and extent of involved bowel wall) of obstruction to varying degrees. As shown in Figure 2B-E, ulceration in the mucosa and submucosa, mucosal edema and inflammation, vascular congestion with focal hemorrhage from the mucosa to the muscular layer and even the serosa, occasional fibrogenesis, and infiltration of polymorphonuclear cells, plasma cells and neutrophils were observed. Figure 2F shows analytical data of the total scores of intestinal damage following histological evaluation and the scores of histological mucosal damage in obstructed and control rats. In the obstructed intestine, there was a marked increase in the thickness of the muscle layers. However, no apparent inflammatory changes, such as marked leucocyte infiltration of the tunica muscularis, were observed.

Circumference and wet weight of the intestine

After removing the intestine, 3 cm dilated intestinal segments were blotted with filter paper and weighed on an analytical balance. The sections were cut longitudinally and the circumference was measured at three sites. The average values for the ileal wet weight (Figure 3A) and ileal circumference (Figure 3B) in the dilated portion of the obstruction rat tissue were significantly larger ($P < 0.01$) than those in the corresponding portion of the controls.

Ileal contractility

We examined the contractile response of the obstructed and control rats, and the study was evaluated in terms of absolute force (mN per mg tissue wet weight). We also examined spontaneous contractile activity of the intestinal segments using isometric force measurements. Figure 4A-B shows the typical spontaneous contractile activity of the dilated intestine isolated from obstructed and control rats, which demonstrated that the slow form of the intestinal segments from control rats was regular; in contrast, the slow form of the intestinal segments from the obstructed rats at 12 to 72 h was attenuated and irregular to varying degrees. Analytical data of the intestinal transit ratio were obtained at four different time points and are shown in Figure 4C.

Autonomic nerve electrical discharge

Typical autonomic electrical activity of the vagus nerve was determined in obstructed and control rats *in vivo*, which indicated that the slow form of the vagus nerve autonomic electrical discharge in control rats was normal (Figure 5A); however, the slow form in obstructed rats at 12 to 72 h was attenuated and irregular to varying degrees (Figure 5B-C). At various time points, the obstructed group showed significant differences in nerve electrical discharge frequency and amplitude compared with the control group ($P < 0.01$). When the four groups were compared, the differences in the wide pipe group appeared to be most obvious, followed by the braided silk group, the half ligation and the narrow pipe group.

Ileum myoelectricity

The myoelectric changes in the ileum of each rat were determined (Figure 6A). The intestinal segments from the control rats did not show significant differences at the various time points, and the slow form was regular. In contrast, the obstructed rats showed significant reductions in vagus nerve electrical discharge frequency and amplitude ($P < 0.01$), the reduction in the wide pipe group was most obvious, followed by the braided silk group, the half ligation and the narrow pipe group. The

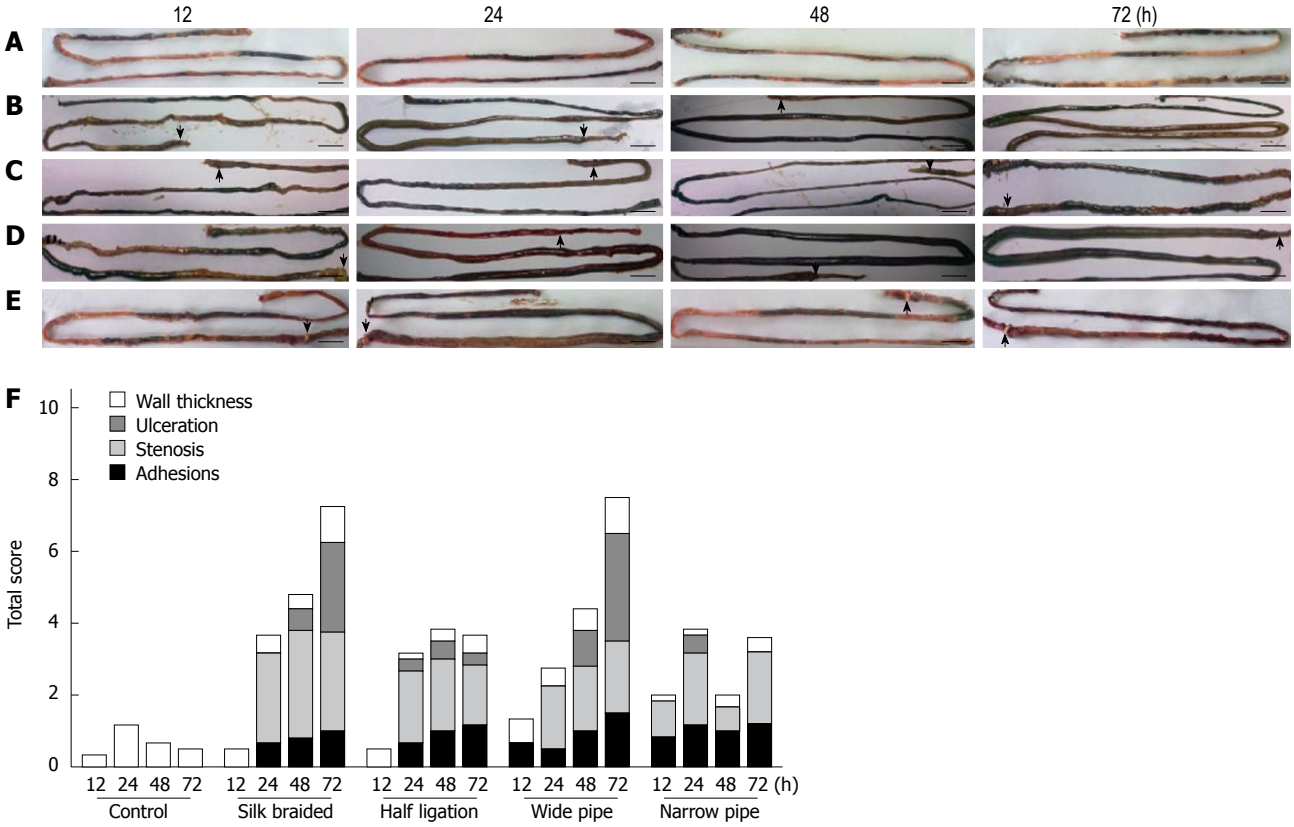

Figure 1 Dissection of the entire intestine from the pyloric antrum to the ileocecal valve in the obstructed and control rats. A: Control; B: Braided silk; C: Half ligation; D: Wide pipe; E: Narrow pipe. Rats were killed at different time points after surgery; F: The morphological score of changes in the extent and severity of adhesions, stenosis, ulceration and bowel wall thickness. The arrow indicates the portion of obstructed intestine bearing a ring (scale bar: 1 cm).

waveform of the dilated intestinal segment at 12 to 72 h was irregular to varying degrees and the appearance of a notching wave resembled a sine curve. The amplitude of the slow waves in the different groups was compared and is shown in Figure 6C. Amplitude and frequency values of the slow waves were significantly different in the obstructed group compared with the control group (both $P < 0.01$). Slow-wave amplitude was significantly reduced ($P < 0.01$) at all sites tested after surgery compared with the control rats. Frequency was reduced only at 72 h at the obstructed sites (Figure 6B).

ICC networks in the intestine

ICC networks are widely distributed within the submucosal (ICC-SM), intramuscular (ICC-IM, ICC-DMP) and inter-muscular layers (ICC-MY)[11,12]. Interestingly, the ICC-MY play an important role in the myenteric plexus and are characteristic of intestinal ICC. The control rats exhibited a dense network in the ICC-MY region of intestinal tissue from 12 to 72 h (Figure 7A), whereas the ICC-MY appeared to be greatly disrupted or absent in the obstructed rat tissue (Figure 7B-E). Analysis of c-Kit-like immunopositive cells in the obstructed rats showed that the immunopositive cells were significantly decreased compared with control animals.

DISCUSSION

An animal model is a reliable method of studying disease mechanisms and to test the effectiveness of a variety of therapeutic measures. Although the pathogenesis of intestinal obstruction is known, complete simulation of the pathology to establish an animal model of incomplete intestinal obstruction is difficult. In recent years, a number of studies have been published, such as that conducted by Chang *et al*[3], where the authors established incomplete intestinal obstruction in a mouse model by placing a polyethylene clip onto the intestine. The morphological and electrophysiological changes which occurred proximal to the site of the clip were investigated. Won *et al*[13] used a silicon tube to partially obstruct the ileum, and observed changes in intestinal contractility associated with the immunologically activated components.

In the present study, all rings were successfully placed to produce incomplete obstruction of the ileum. 12 to 72 h after obstruction, daily behavior did not significantly change in the control group, however, different daily behaviors were seen in the rats with intestinal obstruction (Table 3). In the obstructed rats, a significant reduction in body weight was observed ($P < 0.01$), which was most obvious in the narrow pipe group followed by the

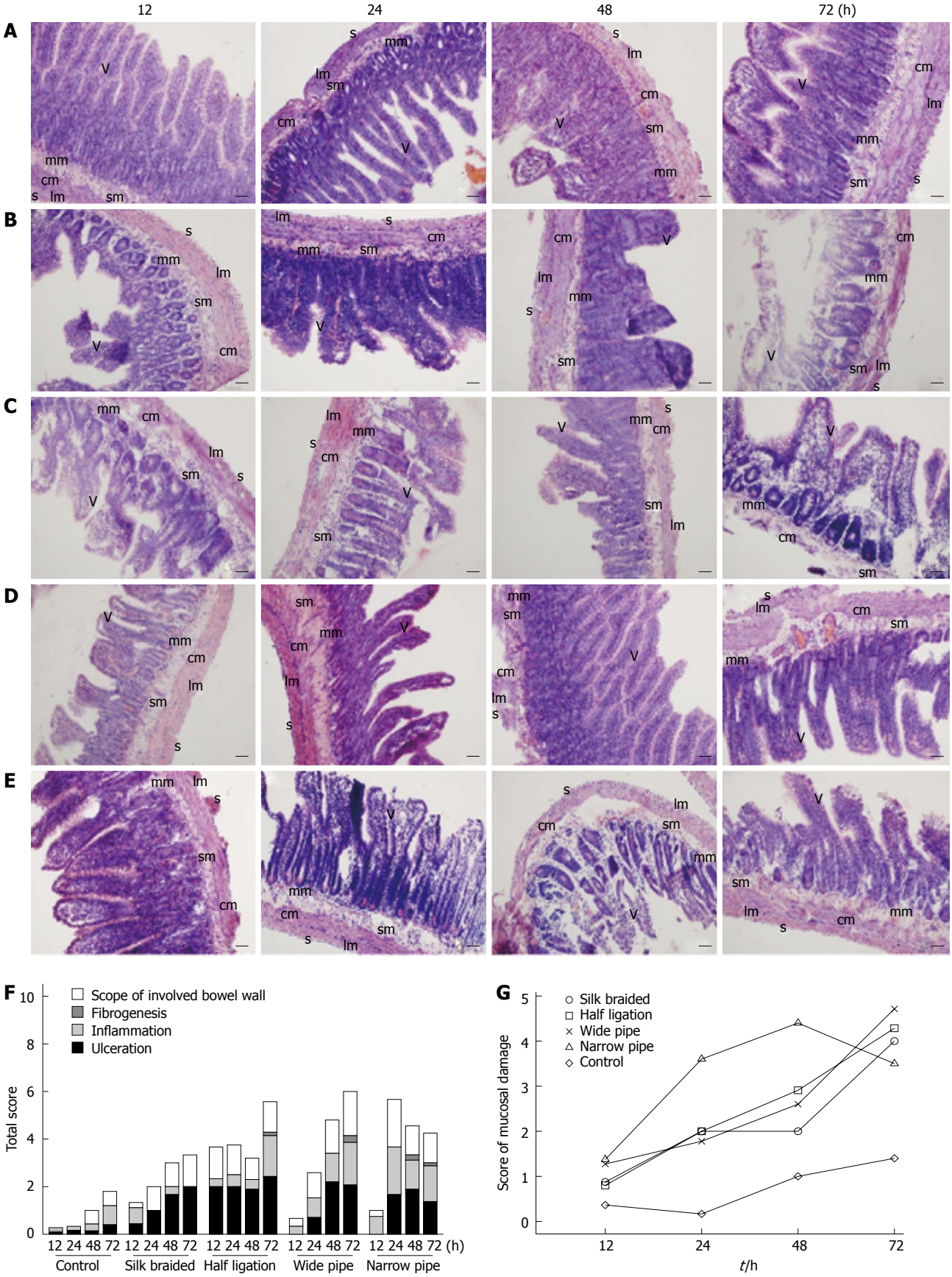

Figure 2 Cross-section of the ileum in obstructed and control rat tissues stained with hematoxylin-eosin. A: Control; B: Braided silk; C: Half ligation; D: Wide pipe; E: Narrow pipe. Rats were killed at different time points after surgery; F: The histopathological score of changes in the extent and severity of ulceration, inflammation, fibrogenesis and scope of involved bowel wall; G: Analytical data of the total scores of intestinal damage following histological mucosal damage in obstructed and control rats. (scale bar: 20 μm).

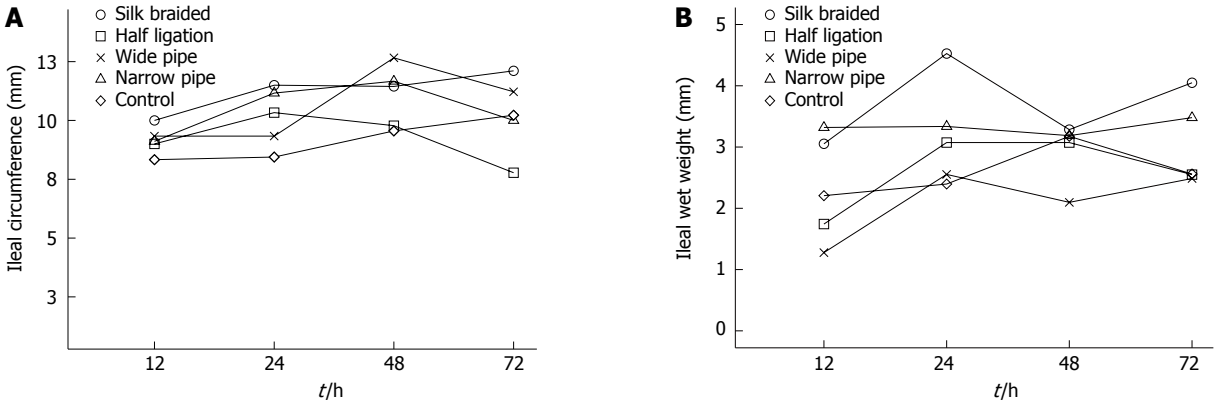

Figure 3 Average ileal wet weight and circumference in the obstructed and control rats. A: The average values for the ileal circumference in the dilated portion of the obstruction rat tissue were significantly larger than those in the corresponding portion of the controls ($P < 0.01$); B: In the obstructed intestine, there was a marked increase in the wet weight due to thickening of the muscle layers. When the groups were compared, these increases were most obvious in the braided silk group, followed by the wide pipe, the narrow pipe group and the half ligation group.

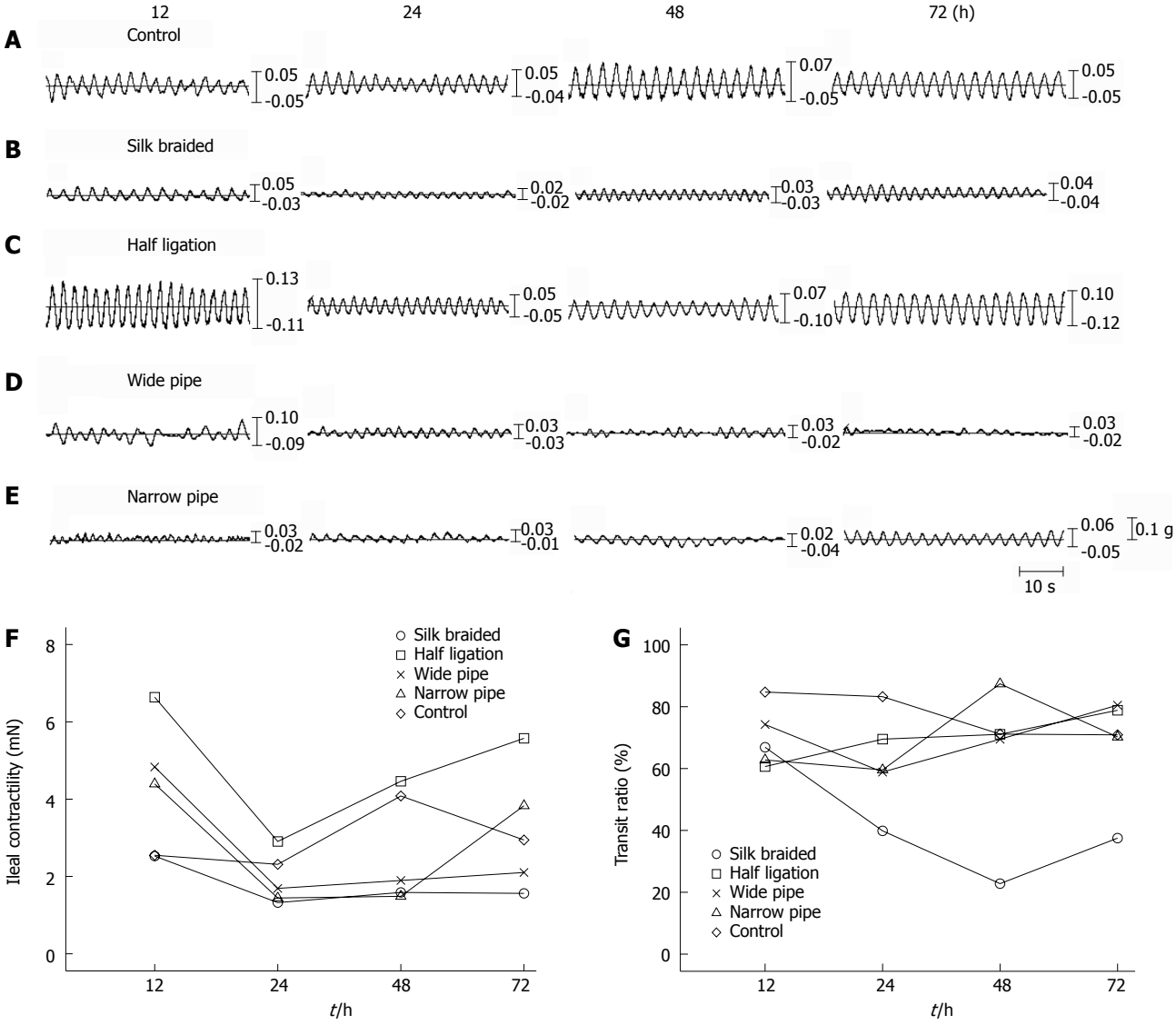

Figure 4 Changes in spontaneous rhythmic contractions. Original traces of rhythmic contractions in obstructed and control ileum. A: Control; B: Braided silk; C: Half ligation; D: Wide pipe; E: Narrow pipe. The data illustrate typical traces of spontaneous contractions at different time points; F: Data showing ileal contractility of spontaneous rhythmic motility; G: Data of intestinal transit ratio showing movement capability *in vivo*.

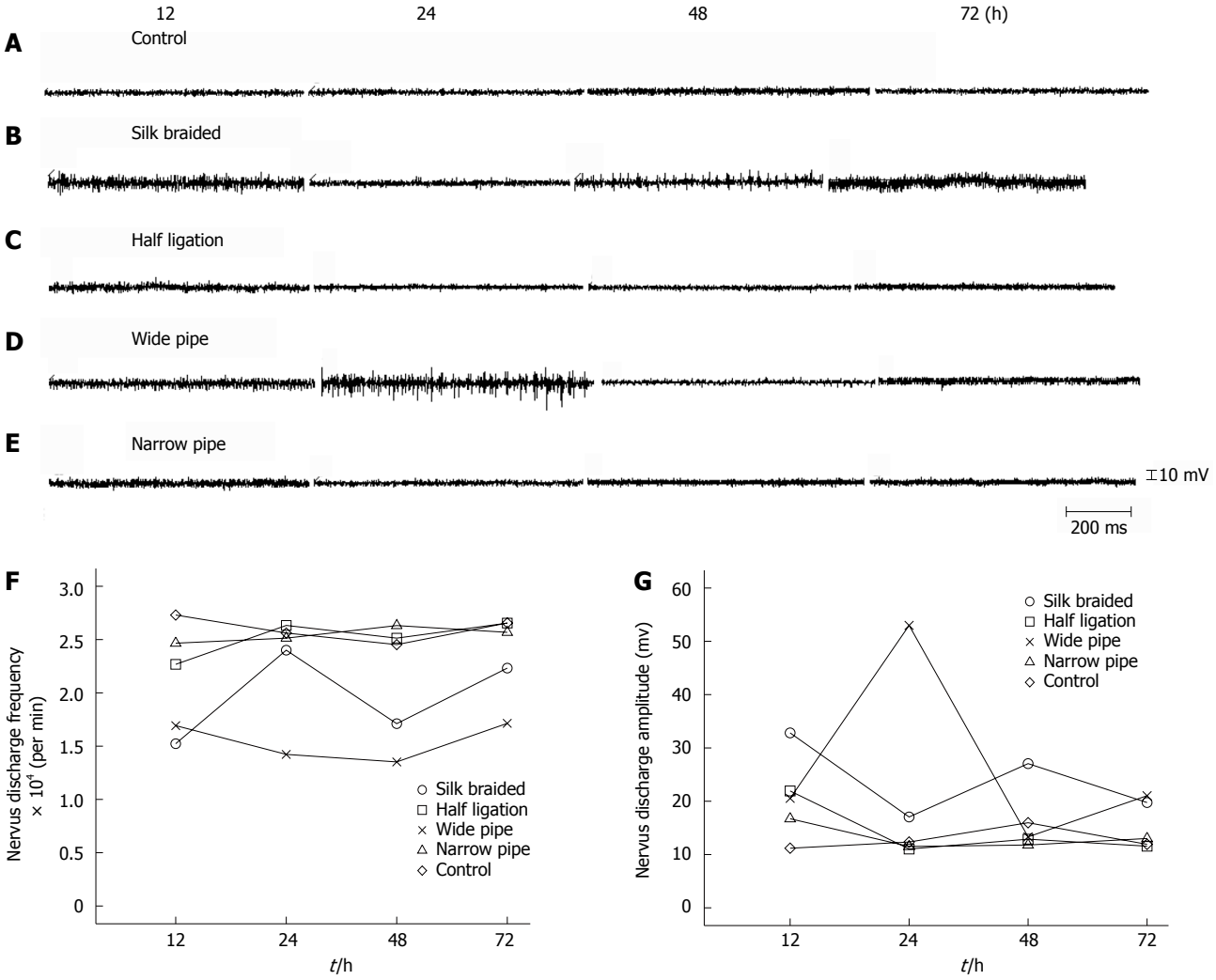

Figure 5 Changes in autonomic nerve electrical discharge. Original traces of the vagus nerve in the obstructed and control ileum. A: Control; B: Braided silk; C: Half ligation; D: Wide pipe; E: Narrow pipe. The data illustrate typical traces of autonomic electrical discharge at different time points; F: Data of frequency of vagus electrical discharge showing autonomic nerve electrical activity; G: Data of amplitude of vagus electrical discharge showing autonomic nerve electrical activity.

half ligation group, the braided silk group and then the wide pipe group which showed a slight decrease at 12 h, but gradually increased from 24 to 72 h. With regard to food intake and feces, these increased in the narrow pipe and half ligation groups compared with the controls. In contrast, the braided silk and wide pipe groups showed significant changes with varying degrees of weight loss or gain ($P < 0.01$). The mental status of the controls, narrow pipe and half ligation groups was good or moderate, however, the braided silk and wide pipe groups displayed the opposite status. Although not statistically significant, the number of deaths was lowest in the control rats. Deaths due to obstruction were higher in the controls, followed by the narrow pipe group the braided silk group, the half ligation group and the wide pipe group. These findings show that the braided silk and wide pipe established the incomplete intestinal obstruction model more successfully, with fewer deaths, than the other models.

Consistent with the findings in other reports[3-6,13-15], the changes in the extent and severity of adhesions, stenosis, ulceration and bowel wall thickness in the obstructed and control rats were assessed using the total scores of intestinal damage following macroscopic evaluation (Figure 1F). The controls (Figure 1A) showed slight intestinal wall thickness and no evidence of intestinal damage. In contrast, obstructed rats showed flushing, tumefaction of intestinal mucosa, hyperemia and hemorrhage of bowel wall and stasis of bowel contents to varying degrees from the proximal region to the site of the ring compared with control rats (Figure 1B-E). Statistically significant differences in the scores of intestinal damage were observed in obstructed rats compared with the controls ($P < 0.01$). Intestinal damage due to the braided silk and narrow pipe was serious as time passed, especially stenosis and ulceration. The half ligation and wide pipe groups showed medium damage at 12 to 72 h.

Some studies reported that intestinal wet weight and circumference were able to reflect the degree of dilation and increased weight of the intestine[15-16], therefore, we

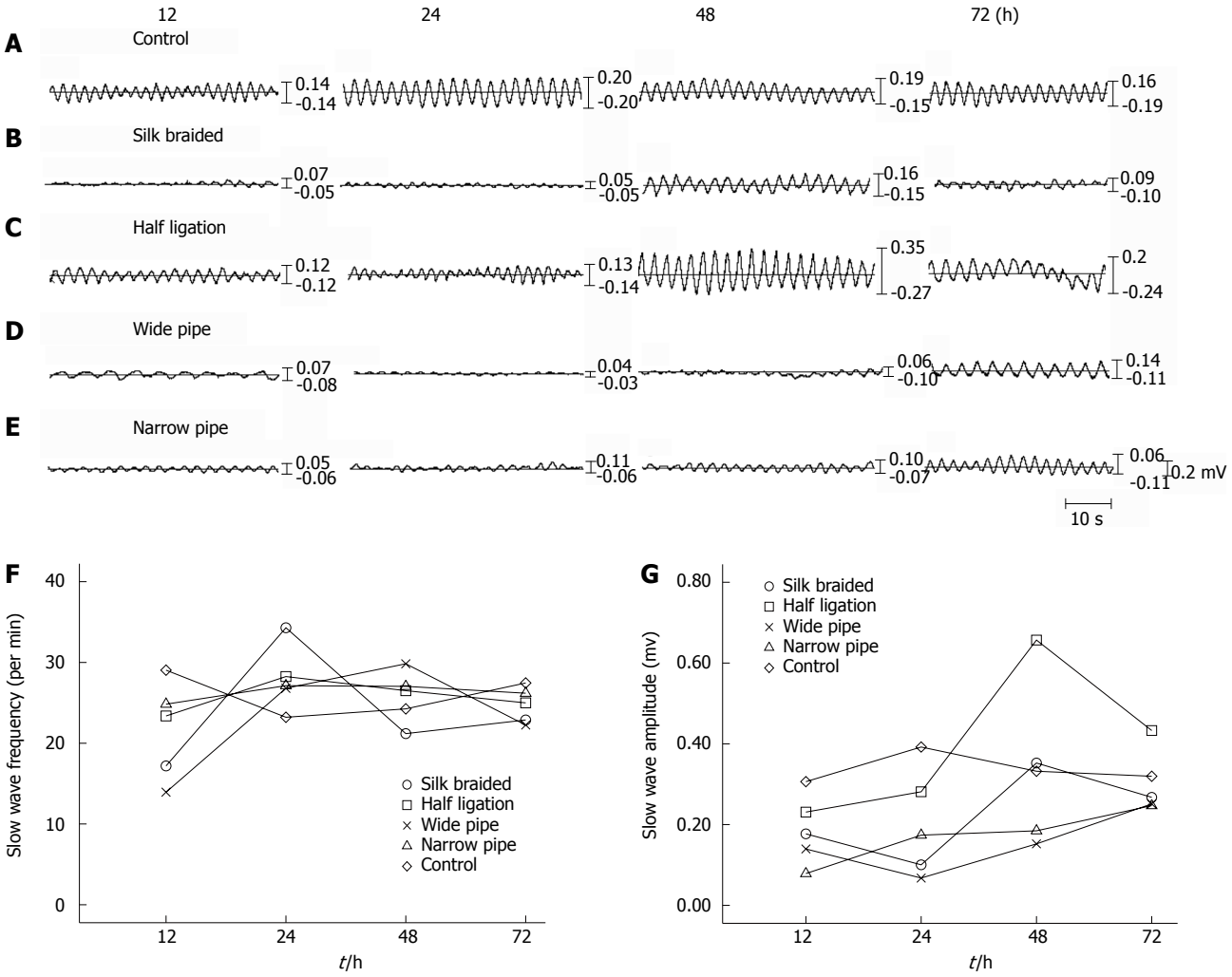

Figure 6 Change in pacemaker activity in the ileum. Typical intracellular electrical activity (slow waves) recorded in the small intestine of control rats and at 12-72 h at the site of intestinal obstruction. A: Control; B: Braided silk; C: Half ligation; D: Wide pipe; E: Narrow pipe; F: Data on slow wave frequency in normal functioning tissue away from the obstruction site; G: Slow-wave amplitude was markedly reduced near the site of obstruction and increased in amplitude in normal functioning tissue away from the obstruction site.

measured the wet weight and circumference of ileum 3 cm distal to the obstruction. In the *in vivo* study, ileal wet weights of the braided silk tissue in the dilated portion were significantly greater ($P < 0.01$) when compared with the controls (Figure 3B). The magnitude of this increase was reduced in the narrow pipe group. In contrast, other obstructed rats showed a decrease compared with those in the corresponding portion of the controls. The ileal circumference showed a negligible increase in both the obstructed and control groups (Figure 3A).

In accordance with the above findings, we speculated that the braided silk and wide pipe models would be useful in the clinic. Therefore, we evaluated the photomicrographs of tissues stained with hematoxylin and eosin (Figure 2), and found that all rats with intestinal obstruction showed ulceration of the mucosa and submucosa, and vascular congestion with focal hemorrhage in the entire tissue layer. In addition, mucosal edema and inflammation were found in the wide pipe and narrow pipe groups, and occasional fibrogenesis in the half ligation, wide pipe and narrow pipe groups at later time periods. Compared with the control rats (Figure 2A), the dilated intestinal region of obstructed rats (Figure 2B-E) showed infiltration of polymorphonuclear cells, plasma cells and neutrophils. A comparison of these scores in the obstructed rats showed that they were significantly different from each other ($P < 0.01$), and the scores were increased in obstructed rats compared with control rats (Figure 2F). Therefore, the braided silk and wide pipe models may better simulate intestinal obstruction found in the clinic.

In the *in vitro* experiments, the braided silk and wide pipe groups demonstrated reduced ileal contractility compared with the controls, the narrow pipe group demonstrated a moderate reduction, and the half ligation group demonstrated an increase in ileal contractility (Figure 4A-B). We observed that the basic spontaneous mechanical contraction activities of dilated intestine *in vivo* and *in vitro* were significantly reduced ($P < 0.01$). The transit ratio in obstructed rats was less than that in con-

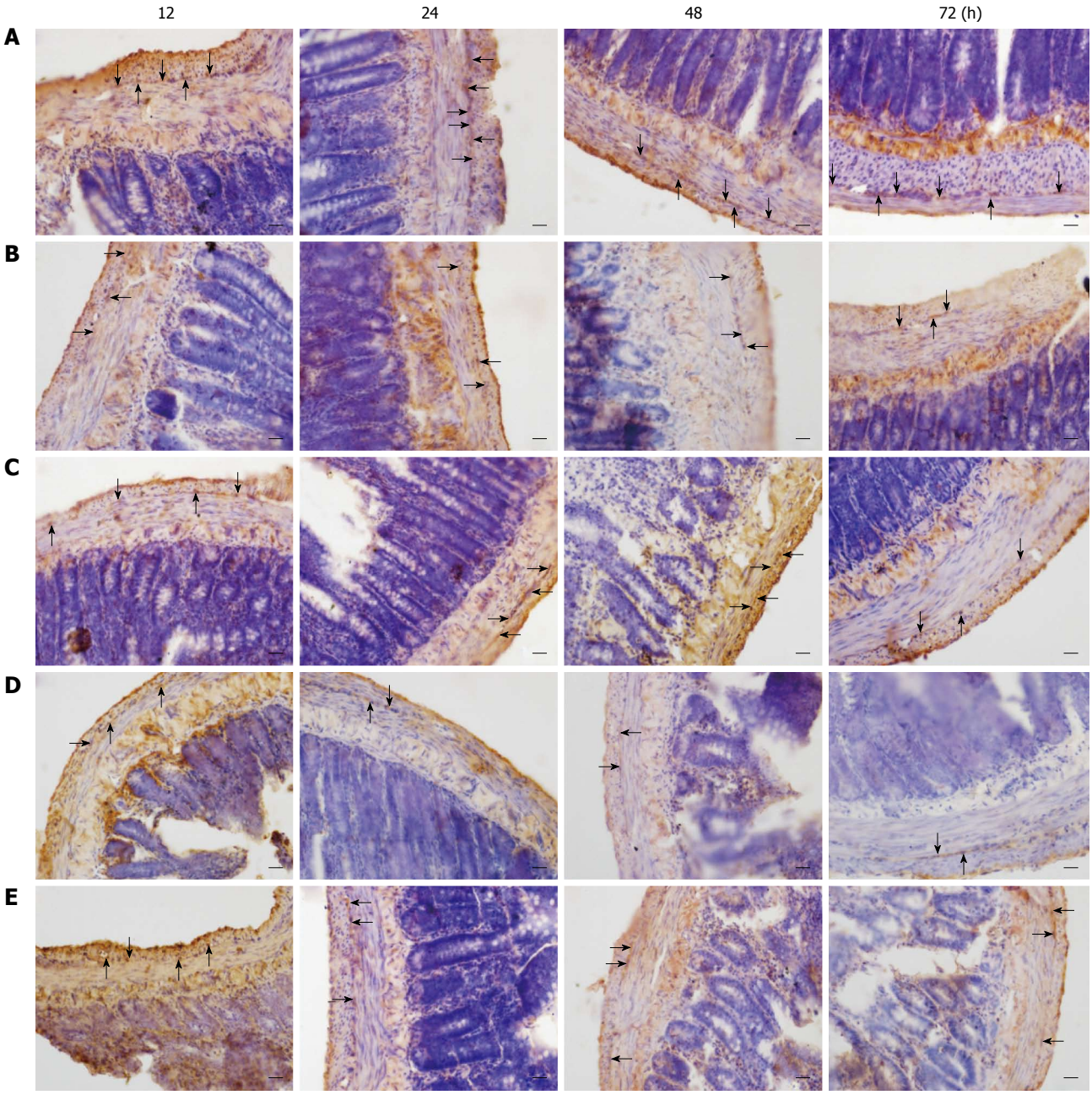

Figure 7 Immunohistochemistry of c-Kit positive interstitial cells of Cajal (arrow) in whole mount preparations of ileum in the obstructed and control rats. A: Control; B: Braided silk; C: Half ligation; D: Wide pipe; E: Narrow pipe. Rats were killed at different time points after surgery. Each panel shows c-Kit positive interstitial cells of Cajal at different times (scale bar: 20 μm).

trols, especially in the braided silk group, and the other obstructed groups showed a smaller reduction in the transit ratio. In contrast to the *in vitro* approach, these *in vivo* studies showed that the incomplete intestinal obstruction models using braided silk and wide pipe were better than the other models, particularly with regard to influence on intestinal movement capability (Figure 4C). These findings are similar to those of Huizinga *et al*[17] who demonstrated that there was a close relationship between intestinal transit and myogenic mechanisms, and indicated that electrical and mechanical activities can influence each other.

Cheng *et al*[18] reported that intestinal motility is an autonomic rhythmic activity under normal circumstances, which is regulated by nerves, hormones and other factors. The autonomic nervous system plays an important role in the regulation of intestinal motility rhythm. External innervation of the intestinal tract is mainly by the vagus nerve, and the sympathetic nerves play a role in balance and coordination[19,20]. In the present study, we compared nerve electrophysiological activity in different models of intestinal obstruction in rats (Figure 5A), to determine the vagus nerve autonomic electrical discharge which represents changes in the complex enteric nervous system. The frequency and amplitude of vagus nerve autonomic electrical discharge in control rats were

Table 3 Summary of daily general condition

General condition	Control				Braided silk				Half ligation				Wide pipe				Narrow pipe			
	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h
Body weight (E = 10)	--	→	-		-	→	+		-				-	→	+ +		--			
Food intake (B = 5.0, E = 2.5)	±	→	+		±	→	--		+	→	±		+ +	→	±		-			
Feces																				
Dry weight (B = 1.0, E = 0.50)	-	→	+ +		--				-	→	+ +		-	→	--		-	→	+	
Color	Yellow and black → Yellow				Yellow and black → Black				Yellow and black				Yellow and black				Yellow and black → Yellow			
Pill (B =15, E =10)	±	→	+		--	→	-		±	→	+ +		-	→	--		-	→	+	
Score (B = 20, E = 10)	-	→	+		--	→	-		--	→	+ +		--				+	→	+ +	
Death	+	±	±	±	+	±	++	+	++	±	±	+	+	±	±	+	±	±	++	++

B: Base value; E: Extent of increase and reduction; + +: Wider band increase (> E); +: Narrower band increase (< E); ±: No increase or reduction; -: Narrower band reduction (< E); --: Wider band reduction (> E). The values of the score are equal to pill multiplied by color. The color is defined by Yellow = 1, Yellow and black = 1.5, Black = 2.

normal and rhythmic. Those in the wide pipe group were irregular, moderate in the braided silk group, and were normal at each time point in the half ligation and narrow pipe groups (Figure 5B-C). These results demonstrated that the wide pipe can be used successfully to establish a model of incomplete intestinal obstruction in rats.

Considering the possible link between spontaneous mechanical contraction activities and the slow wave of dilated intestine *in vivo* and *in vitro*[17,21], we investigated the waveform and reflected frequency and amplitude of the slow wave in different rats (Figure 6A). We found that the waveform in the wide pipe group resembled a sine curve and showed a notching wave which was irregular at 12 to 72 h. The half ligation group showed no obvious change in the waveform compared with the controls. Peak amplitudes in the braided silk and narrow pipe groups demonstrated different degrees of weakening. In this study, we also found a close relationship between frequency (Figure 6B) and peak amplitudes (Figure 6C) in the vagus nerve electrical discharge at baseline and intestinal motility. This suggested that the wide pipe model may be similar to human incomplete intestinal obstruction.

It is well known that slow waves are relatively regular periodic electrical activities and the basis of intestinal smooth muscle action potentials. In earlier research, we found that the frequency and amplitude of slow wave propagation were reduced in ileac muscles[6]. Other studies[3,22] showed that myoelectrical rhythm is irregular and the slow waves decrease. In this study, the waveform and reflected frequency and amplitude of the slow wave in control rats were normal. Those in the wide pipe group were irregular, were moderate in the braided silk group, and were normal in the half ligation and narrow pipe groups at 12 to 72 h (Figure 6). These results suggest that slow wave abnormalities are responsible for intestinal motility dysfunction.

In our previous intestinal studies, we observed that the enteric nervous system (ENS) - ICC - smooth muscle cell (SMC) network constitute the basic functional unit of gastrointestinal motility and has an important relationship with spontaneous rhythmic activity[12,14,17-18,23]. We supposed that the wide pipe group abolished the propulsive peristaltic waves in the dilated intestine compared with the other obstructed groups, which caused a significant disturbance in spontaneous rhythmic contractions of intestinal smooth muscle. In smooth muscle, there are several factors which increase the frequency of contraction, such as increased pacemaker activity and shortened action potential[24].

It has been previously shown that ICC are regarded as the pacemaker cells of the gastrointestinal (GI) tract[25-28], which suggests that ICC may play a role in GI motility. In order to compare the differences in obstructed rats, and to identify the mutual influence between the ENS-ICC-SMC networks, we investigated the distribution of ICC in intestinal tissue using light microscopy.

Another interesting observation from this study was that, in the wide pipe group the increase in intestinal contractility was closely related to a reduction in the number of ICC and slow wave, which was different in the other obstructed groups. In addition, it was reported that a reduction in the frequency of rhythmic contractions was connected to the disruption of ICC in the dilated intestine of obstructed rats[29]. These findings confirmed those in our study (Figure 7). Therefore, it is likely that ICC lost their original cell phenotype, resulting in an increase in the number of smooth muscle cell-like cells, thereby improving contractility of the ileum. At the present time, we do not know the precise mechanisms underlying such alterations. Further study will be needed to clarify this point.

In conclusion, the results of the present study suggest that there are significant differences *in vivo* and *in vitro* among the four animal models of incomplete intestinal obstruction. The pathogenic mechanism and clinical features in the different models have essential distinctions. Despite being of a similar general condition, the rats in each group showed differences including macroscopic and histological presentation, intestinal transit ratio and contractility, circumference and wet

weight, amplitude and frequency of nerve electrical discharge and slow wave, and ICC numbers. These findings may allow use of the wide pipe to establish a model that is approximate to human incomplete intestinal obstruction. However, a detailed analysis of this is necessary based on the characteristics of the different models used in this study which can be used as a reference.

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COMMENTS

Background

Considering the impairment associated with incomplete intestinal obstruction, the use of animal models is now a focus in this research field, however, to date, no particular model parallels the complex nature of human intestinal obstruction.

Research frontiers

Animal have been used to establish incomplete intestinal obstruction models. However, animal models are not been widely used and research comparing the characteristics in different rat models is rare. In this study, the authors successfully established a reliable animal model and demonstrated that the wide pipe rat model is similar to human incomplete intestinal obstruction.

Innovations and breakthroughs

Recent reports have highlighted the need to establish a reliable animal model of intestinal obstruction. Different types of materials are currently available. This is the first experimental study to compare rat models of incomplete intestinal obstruction. Furthermore, the authors obtained electrophysiologic, morphologic and histologic information which may have some clinical relevance.

Applications

These findings may provide a valuable theoretical basis for selecting incomplete intestinal obstruction models with better feasibility and reproducibility.

Peer review

This is an animal model study which comprehensively demonstrates the results. Overall, it is suitable for publication.

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ORIGINAL ARTICLE

Comparison of modified percutaneous transhepatic variceal embolization and endoscopic cyanoacrylate injection for gastric variceal rebleeding

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Abstract

AIM: To compare the efficacy of modified percutaneous transhepatic variceal embolization (PTVE) with 2-octyl-cyanoacrylate (2-OCA) and endoscopic variceal obturation (EVO) with an injection of 2-OCA for prophylaxis of gastric variceal rebleeding.

METHODS: In this retrospective study, the medical records of liver cirrhosis patients with gastric variceal bleeding who underwent either endoscopic 2-OCA (EVO) or modified PTVE using 2-OCA at Shandong Provincial Hospital from January 2006 to December 2008 were reviewed. Patient demographics, rebleeding rate, survival rate, and complications were compared between the two groups (PTVE and EVO). All results were expressed as mean ± SD, or as a percentage. Quantitative variables were compared by two sample Student *t* tests, and qualitative variables were compared by the Fisher exact test or the χ^2 test (with Yates correction) where appropriate. A *P* value less than 0.05 was considered significant. Statistical computation was performed using SPSS 13.0 software.

RESULTS: A total of 77 patients were included; 45 patients who underwent EVO and 32 patients who received PTVE. During the follow-up (19.78 ± 7.70 mo in the EVO group, *vs* 21.53 ± 8.56 mo in the PTVE group) rebleeding occurred in 17 patients in the EVO group and in 4 patients in the PTVE group (37.78% *vs* 12.5%, *P* = 0.028). The cumulative rebleeding-free rate was 75%, 59%, and 49% in 1, 2, and 3 years respectively for EVO, and 93%, 84%, and 84% for PTVE (*P* = 0.011). Cox analysis was used to identify independent factors that predicted rebleeding after treatment. Variables including age, gender, cause, Child-Pugh classification, size of gastric varices (GV), location of GV, and treatment methods were analyzed. It was revealed that Child-Pugh classification [risk ratio (RR) 2.10, 95%CI: 1.03-4.28, *P* = 0.040], choice of treatment (RR 0.25, 95%CI: 0.08-0.80, *P* = 0.019), and size of GV (RR 2.14, 95%CI: 1.07-4.28, *P* = 0.032) were the independent factors for predicting rebleeding. Follow-up computed tomography revealed that cyanoacrylate was retained in the varices and in the feeding veins of PTVE patients. During the follow-up, eight patients in the EVO group and four patients in the PTVE group died. The cumulative survival rates at 1, 2, and 3 years were 93%, 84%, and 67% respectively in the EVO group, and 97%, 88%, and 74% respectively in the PTVE group. The survival rates were not significantly different between the two groups (*P* = 0.432). Cox analysis showed that the Child-Pugh classification was the most significant prognostic factor of survival (RR 2.77, 95%CI: 1.12-6.80, *P* = 0.027). The incidence of complications was similar in both groups.

CONCLUSION: With extensive and permanent obliteration of gastric varices and its feeding veins, PTVE with 2-OCA is superior to endoscopic 2-OCA injection for preventing gastric variceal rebleeding.

Key words: Gastric varices; Endoscopic variceal obturation; Percutaneous transhepatic variceal embolization; 2-octyl-cyanoacrylate; Bleeding

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INTRODUCTION

Although the incidence of bleeding from gastric varices (GV) is lower than from esophageal varices (EV), once it occurs the outcome is worse, and with higher mortality[1-3]. After an episode of acute variceal bleeding, patients are at high risk for recurrent bleeding and death. Thus, prevention of recurrent bleeding is essential[4,5]. However, rupture from gastric varices, especially varices located in the gastric fundus, poses particular therapeutic challenges because of the location and rapid blood flow. The current treatment methods for gastric varices are far from ideal.

Endoscopic variceal obturation (EVO) with the injection of cyanoacrylate has been widely adopted and proved to be effective in the emergency hemostasis of bleeding from gastric varices since it was proposed in 1986[6]. At present, this method is the first-line treatment for gastric variceal bleeding recommended by Baveno IV consensus and AASLD guidelines[7,8]. However, the long-term rebleeding rate after endoscopic cyanoacrylate injection is still high[9-12]. Additionally, there is also a potential risk of systemic embolism in patients with underlying gastrorenal shunts, and other serious complications such as sepsis, fistula, and pericarditis[13-16].

Balloon-occluded retrograde transvenous obliteration (BRTO) has become the standard treatment for gastric varices in Japan. However, patients without catheterizable gastrorenal shunts cannot be treated by BRTO[17,18]. Therefore for these patients, percutaneous transhepatic variceal embolization (PTVE) with N-butyl-2-cyanoacrylate was introduced and showed satisfactory results[19,20] in a small series of patients.

Based on our previous reports of modified PTVE with 2-octyl cyanoacrylate (2-OCA) for bleeding EV[21,22], we have performed modified PTVE with 2-OCA to prevent gastric variceal rebleeding in recent years.

In this retrospective study, 77 patients with prior bleeding from gastric varices who underwent EVO or modified PTVE for prevention of rebleeding were analyzed. Rebleeding rate, survival, and complications were compared between these two procedures. To our knowledge, at the time of writing, no other report in the literature has compared these two procedures in the prevention of gastric variceal rebleeding.

MATERIALS AND METHODS

Patients

The medical records of liver cirrhosis patients with gastric variceal bleeding who underwent either endoscopic 2-OCA injection or modified PTVE using 2-OCA in our hospital from January 2006 to December 2008 were reviewed. Local ethics committee approval was obtained for the chart review.

The inclusion criteria: (1) diagnosis of liver cirrhosis by biopsy or clinical examination and imaging, including ultrasound, computed tomography (CT), or magnetic resonance imaging; (2) patient suffered bleeding within 6 mo before being admitted or acute bleeding with achieved hemostasis by pharmacological treatment; (3) endoscopically-confirmed bleeding from gastric varices: active spurting or oozing of blood from gastric varices during endoscopy, blood clot coating on gastric varices or the presence of erosive spots on gastric varices, with no other potential source of bleeding; and (4) patient aged between 20-65 years.

Exclusion criteria: (1) hepatocellular carcinoma or other malignancies; (2) a history of transjugular intrahepatic portosystemic shunt (TIPS), surgery, or endoscopic therapy for esophagogastric variceal bleeding; (3) portal vein thrombosis; or (4) infection.

Choice of treatment method was based on the patients' intentions after being given a sufficient explanation of the two treatment methods. Informed written consent was obtained from each patient.

Endoscopic injection procedure

Endoscopic intra variceal injection of 2-octyl cyanoacrylate (Baiyun Medical Adhesive Corporation, Guangzhou, China) was performed using a video endoscope (XQ230, Olympus Optical, Tokyo, Japan) and a 23-gauge disposable injection needle (Medwork Medical Products and Services GmbH). Each injection contained a 0.5-2.0 mL mixture of 2-OCA and Lipiodol (1:1). The injection was aimed at the varices that were either bleeding, possessed red color signs, or were the most prominent. Obliteration was assessed by blunt palpation with a catheter, and presence of hardness indicated a complete obliteration. If the varices remained soft, additional injections were done until all gastric varices became hardened. If necessary, additional injections of cyanoacrylate were performed 2-3 wk after the initial session.

PTVE procedure

PTVE was performed alone, or combined with left renal vein obstruction with a balloon catheter if a large gastrorenal shunt was present (Figure 1). Shortly after a percutaneous transhepatic puncture of the intrahepatic branch of the portal vein, a 5F cobra catheter (Cook,

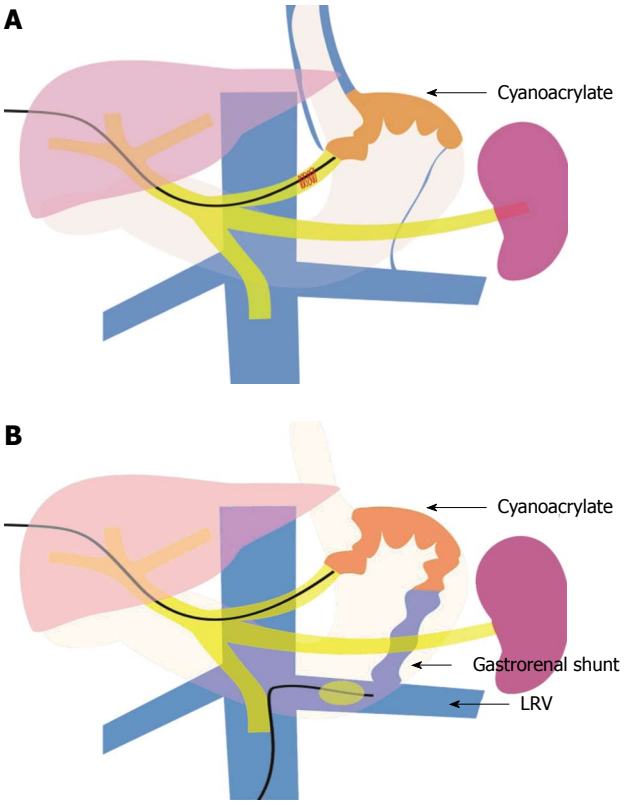

Figure 1 Illustrations of the two types of percutaneous transhepatic variceal embolization. A: Cyanoacrylate was directly injected into the gastric varices by percutaneous transhepatic variceal embolization (PTVE) alone in patients without large gastrorenal shunts; B: Cyanoacrylate injection with PTVE combined with a balloon catheter inserted into the left renal vein (LRV) in patients with large gastrorenal shunt.

Bloomington, IN) was inserted into the splenic vein and a splenoportography was performed to evaluate the gastric varices, the feeding veins, and draining veins. The main gastric feeding vein (*e.g.*, the left gastric vein, short gastric vein, or posterior gastric vein) was then selected with the 5F catheter, and a venography was performed to assess blood flow velocity and the size of varices (the gastrorenal shunt's size being of particular interest).

Based on these data, the embolization of 2-OCA was carried out in the following ways: (1) PTVE alone: In patients without large gastrorenal shunts, the blood flow in gastric varices was slow, and the contrast material could stay in the varices for more than 5 s after injection. In these patients, the cyanoacrylate was directly injected into the gastric varices; and (2) PTVE combined with left renal vein obstruction with a balloon catheter: In patients with large gastrorenal shunts, there was rapid blood flow in the varices and the contrast material disappeared within 3-5 s after injection. For these patients, a 6F balloon catheter (Cook, Bloomington, IN) with a diameter of 15 to 20 mm was inserted into the left renal vein via the right femoral vein to reduce the blood flow of the gastrorenal shunt and varices, and then the cyanoacrylate was injected. In these two ways, the cyanoacrylate could obliterate the entire varices and feeding veins, while cyanoacrylate migration to the systemic circulation could be avoided.

When the cyanoacrylate was flowing into all the gastric varices, the catheter was immediately withdrawn. Splenoportography was again performed to assess the obliteration of the varices. If other feeding veins (such as the short or posterior gastric veins) were present, the procedure would be repeated until the gastric varices and feeding veins were completely filled with cyanoacrylate. Finally, the 5F sheath system was withdrawn, and the puncture tract was embolized with microcoils. Low molecular heparin (100 IU/kg body weight, daily) was subcutaneously administered 24 h after the procedure for 5 to 7 d to prevent portal venous thrombosis.

Follow-up

Follow-up endoscopy was performed for the two groups at intervals of 1, 3, and 6 mo after the procedures, and then every 6-12 mo or when it was considered clinically necessary. In patients with rebleeding, endoscopy was performed to identify the cause of the bleeding. Portal venography was performed at 1 mo after the procedure, and every 6 mo thereafter with 3-dimensional multidetector row CT (GE Medical systems, Milwaukee, WI) to observe the formation of portal vein thrombosis, the location and extent of cyanoacrylate glue, variceal recanalization, and the occurrence of collateral vessels. Rebleeding, survival, and complications were recorded.

Recurrent bleeding was defined as the presence of hematemesis or melena, with the bleeding source being endoscopically proven to originate from gastric or EV, or other resources after the index treatment. Bleeding from gastric varices was distinguished from that of EV on the basis of whether active bleeding or erosive spots were present on the gastric varices themselves.

Complications were defined as any untoward events that required active treatment or prolonged hospitalization.

Statistical analysis

All results were expressed as mean ± SD, or as a percentage. Quantitative variables were compared by two sample Student *t* tests, and qualitative variables were compared by the Fisher exact test or the chi-squared test (with Yates correction) where appropriate. The Kaplan-Meier estimation was used to examine recurrence and rebleeding of gastric varices and rate of survival. Comparisons were performed using the log-rank test. A Cox's analysis was performed to detect possible independent predictors for variceal rebleeding and death. A P value less than 0.05 was considered significant. Statistical computation was performed using SPSS 13.0 software.

RESULTS

Demographic characteristics of patients

From January 2006 to December 2008, EVO or PTVE was performed in a total of 92 cirrhotic patients with a history of gastric variceal bleeding in Shandong Pro-

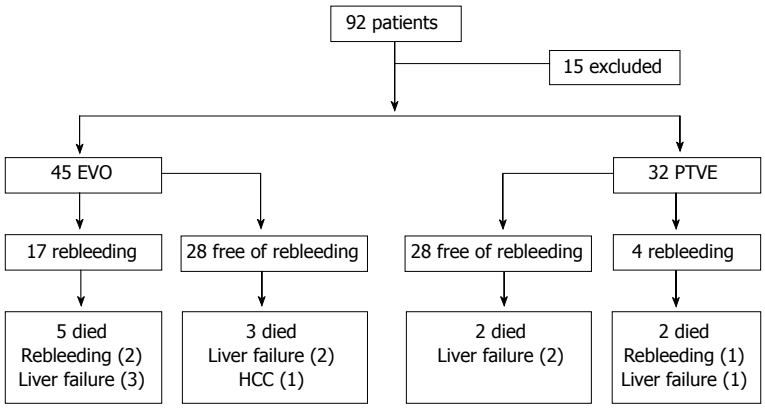

Figure 2 Flow diagram of rebleeding and death after procedures. EVO: Endoscopic variceal obturation; PTVE: Percutaneous transhepatic variceal embolization; HCC: Hepatocellular carcinoma.

Table 1 Clinical characteristics of patients

	EVO (n = 45)	PTVE (n = 32)	P value
Sex (M/F)	33/12	22/10	0.661
Age (mean ± SD, yr)	52.69 ± 8.99	50.65 ± 7.23	0.293
Etiology of cirrhosis			0.999
Hepatitis B	25	18	
Hepatitis C	9	6	
Alcohol	8	6	
Others	3	2	
Child-Pugh class (A/B/C)	14/21/10	9/17/6	0.851
Bleeding onset			1.000
Recent variceal bleeding	40	28	
Acute variceal bleeding	5	4	
Blood transfusion (unit)	4.50±3.32	5.33±3.60	0.542
Form of GV (F1/F2/F3)[1]	12/23/10	7/13/12	0.343
Location of GV[2]			0.467
GOV2	29	18	
IGV1	16	14	
Ascites	26	20	0.857
Duration of follow-up (mo)	19.78 ± 7.70	21.53 ± 8.56	0.350

[1]Forms of gastric varices (GV) were graded by the classification described by Hashizume *et al*[24]: F1, tortuous winding varices; F2, nodular-shaped varices; and F3, tumorous huge varices; [2]Locations of GV were based on the criteria proposed by Sarin *et al*[1]. M/F: Male/female; GOV2: Gastric varices extended from the esophageal varices toward the gastric fundus; IGV1: Isolated gastric varices located in the fundus; PTVE: Percutaneous transhepatic variceal embolization; EVO: Endoscopic variceal obturation.

Table 2 Procedures in endoscopic variceal obturation and percutaneous transhepatic variceal embolization groups

	EVO (n = 45)	PTVE (n = 32)	P value
Status of GV			0.290
Disappeared	17/45	16/32	
Collapsed	16/45	12/32	
Remained	12/45	4/32	
Amount of cyanoacrylate (mL)	3.03 ± 1.04	6.69 ± 2.92	0.000
Rebleeding	17/45	4/32	0.028
Rebleeding from GV	13/45	2/32	0.029
Rebleeding from other sources	4/45	2/32	1.000
EV bleeding	2	1	1.000
PHG	1	1	1.000
Unknown	1	0	1.000
Death during follow-up	8/45	4/32	0.751
Causes of death			
Progressive Liver failure	5/45	3/32	1.000
Rebleeding	2/45	1/32	1.000
HCC	1/45	0/32	1.000

EV: Esophageal varices; GV: Gastric varices; EVO: Endoscopic variceal obturation; PHG: Portal hypertensive gastropathy; HCC: Hepatocellular carcinoma; PTVE: Percutaneous transhepatic variceal embolization.

vincial Hospital. Of the 92 patients, six had hepatocellular carcinoma, and nine had previously received TIPS or shunt surgery; these 15 patients were excluded. Of the remaining 77 patients, EVO was performed in 45, and PTVE was performed in 32 (Figure 2). The clinical characteristics of the 77 patients in the two groups were retrospectively reviewed from a computerized database of our hospital. Gastric varices were subdivided by Sarin classification[4], the form of gastric varices was classified according to Hashizume classification[23], and liver function was estimated based on the Child-Pugh classification[24]. The follow-up records were carefully reviewed. The median follow-up period was 19.78 ± 7.70 mo (range, 3 to 41 mo) in the EVO group and 21.53 ± 8.56 mo (range, 6 to 44 mo) in the PTVE group (P = 0.350). Table 1 shows the patient characteristics in the two groups, for which there was no significant difference.

Technique outcomes

The outcomes of the procedures are shown in Table 2. In the EVO group, 35 patients (77.78%) achieved complete obliteration of gastric varices. Of the 35 patients, one session of EVO was needed to achieve complete obliteration in 23 patients, and two or three sessions were required in 12 patients. The total volume of cyanoacrylate used in the initial session of each EVO group patient was 3.03 ± 1.04 mL (range, 1.5-5.5 mL). In the PTVE group, 18 patients underwent gastric variceal embolization by PTVE alone, while the other 14 patients underwent combined PTVE with left renal vein balloon obstruction. Thirty patients (93.75%) achieved complete obliteration, which was confirmed by a splenoportography after the PTVE procedure. The volume of cyanoacrylate used in

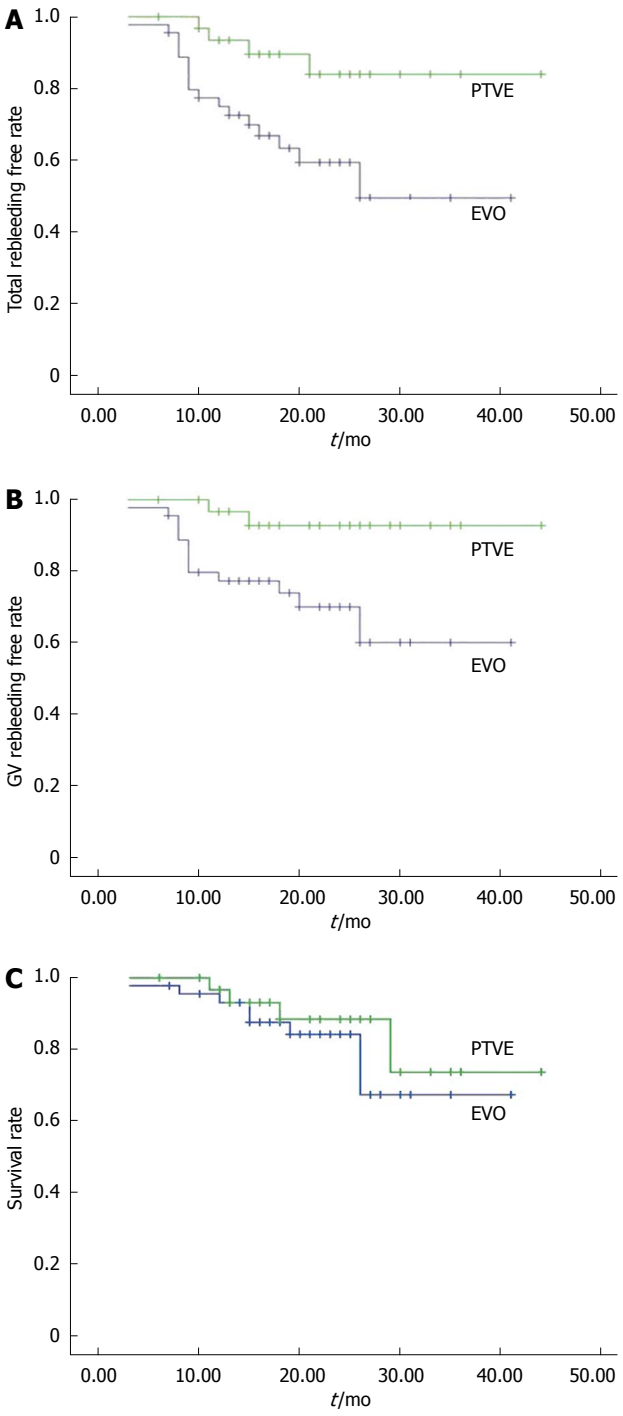

Figure 3 Cumulative survival. A: The cumulative rebleeding free rate was higher in the percutaneous transhepatic variceal embolization (PTVE) group than in the endoscopic variceal obturation (EVO) group, $P = 0.011$, log-rank test; B: The cumulative free gastric varix (GV) rebleeding rate was higher in the PTVE group than in the EVO group, $P = 0.012$, log-rank test; C: The cumulative survival rate was not different between the two groups. $P = 0.432$, Log-rank test.

the PTVE group was 6.69 ± 2.92 mL (range, 3-16 mL); more than in the EVO group ($P = 0.000$).

Endoscopic surveillance

An endoscopic follow-up was performed in all patients in both groups. Generally, 1-3 mo after the injection, the cyanoacrylate plug began to slough off the submucosa in some patients, and the varices disappeared or collapsed after complete or partial expulsion of the glue within one year in the PTVE group. In patients who underwent EVO, the endoscopic findings were similar to that in the PTVE group. During the endoscopic follow-up, the gastric varices disappeared in 16 patients or shrunk in 12 patients in the PTVE group, and disappeared in 17 patients or shrunk in 16 patient in the EVO group ($P = 0.290$).

CT and portal venography follow-up

Twenty patients in the EVO group and fifteen patients in the PTVE group underwent contrast-enhanced CT and portal venography during follow-up. In the PTVE group, it was confirmed that the gastric varices, the perforating veins in the fundus, the perifundus veins, and all the feeding veins were filled with cyanoacrylate 1 mo after the procedure. 3-6 mo after PTVE, the amount of cyanoacrylate in the submucosal varices was reduced compared to previously. Twelve months later, the cyanoacrylate in the submucosal varices almost completely gone, but the cyanoacrylate in the perifundus varices and feeding veins remained the same as at the start. Portal venography showed that there was no blood flow in the eradicated varices with sufficient amount of cyanoacrylate after one year. In the EVO group, CT revealed that the cyanoacrylate was only scattered in the gastric varices and the perifundus varices, which was not as extensive and complete as in the PTVE group.

Rebleeding

During the follow-up, rebleeding occurred in 17 patients (37.78%) in the EVO group and four patients (12.5%) in the PTVE group. The rebleeding rate was significantly lower in the PTVE group compared to the EVO group ($P = 0.028$). The cumulative rebleeding-free rate was 75%, 59%, and 49% in 1, 2, and 3 years respectively for the EVO group, and 93%, 84%, and 84% for the PTVE group ($P = 0.011$, Figure 3A).

The causes of rebleeding included: gastric variceal rebleeding (13 in EVO and 2 in PTVE), aggravated esophageal variceal bleeding (2 in EVO and 1 in PTVE), portal hypertensive gastropathy (1 in EVO and 1 in PTVE), and unknown reasons (1 in EVO and nil in PTVE). The cumulative rate free of GV rebleeding at 1, 2, and 3 years was 77%, 70%, and 60% respectively for the EVO group, and 97%, 93%, and 93% for the PTVE group ($P = 0.012$, Figure 3B). Of the 15 patients with rebleeding from GV, three died of uncontrolled bleeding, four received repeated EVO, three received TIPS, and five received surgery. No patient died of bleeding from EV or other sources.

Cox analysis was used to identify the independent factors that predicted rebleeding after treatment. Variables including age, gender, cause, Child-Pugh classification, size of GV, location of GV, and treatment methods were analyzed. It was revealed that the Child-Pugh classification (RR 2.10, 95%CI: 1.03-4.28, $P = 0.040$), choice of treatment (RR 0.25, 95%CI: 0.08-0.80, $P = 0.019$), and size of GV (RR 2.14, 95%CI: 1.07-4.28, $P = 0.032$) were

Table 3 Procedural complications

	EVO (n = 45)	PTVE (n = 32)	P value
Total complications (%)	29	22	0.881
Bacteremia	4	1	0.395
Fever	19	12	0.857
Abdominal pain	12	15	0.112
Ulcer	3	1	0.637
SBP	2	3	0.644
Ascites	3	6	0.152
Portal vein thrombosis	0	2	0.170
Pulmonary embolism	1	0	1.000

SBP: Spontaneous bacterial peritonitis; EVO: Endoscopic variceal obturation; PTVE: Percutaneous transhepatic variceal embolization.

the independent predicting factors for rebleeding.

Survival

During the follow-up, eight patients in the EVO group and four patients in the PTVE group died. The cumulative survival rates at 1, 2, and 3 years were 93%, 84%, and 67% respectively in the EVO group, and 97%, 88%, and 74% respectively in the PTVE group. The survival rates were not significantly different between the two groups (P = 0.432, Figure 3C). In the EVO group, five patients died of progression of hepatic failure, compared with three patients in the PTVE group. Two patients in the EVO group and one in the PTVE group died of uncontrolled rebleeding. The remaining patient in the EVO group died of hepatocellular carcinoma, which occurred after the procedure.

Seven variables (sex, age, the Child Pugh classification, etiology, choice of modality, form of GV, and location of GV) were taken into consideration in the multivariate analysis using the Cox regression model. Based on the Cox analysis, the Child-Pugh classification was the most significant prognostic factor of survival (RR 2.77, 95%CI: 1.12-6.80, P = 0.027).

Complications

Complications of the procedures are shown in Table 3. Twenty-nine patients experienced complications in the EVO group, compared to twenty-two patients in the PTVE group (P = 0.881). There was no significant difference between the groups. The common complications after treatment were fever and abdominal pain. 19 patients in the EVO group and 12 patients in the PTVE group suffered from fever (P = 0.857). Abdominal pain was encountered in 12 patients in the EVO group and 15 patients in the PTVE group (P = 0.112). The patients were treated with conventional medicinal therapy, with fever and abdominal pain usually being alleviated within 1-2 wk. One patient in the EVO group encountered pulmonary embolism, while no patient encountered systemic embolization in the modified PTVE group (P=1.000). Mild to moderate ascites appeared in 3 patients in the EVO group and 6 in the PTVE group (P = 0.152), and all of them were controlled by medication. Partial portal vein thrombosis appeared in two patients in the PTVE group, but the portal vein was patent under Doppler ultrasound. No patient died of complications in either group.

DISCUSSION

Although gastric varices tend to bleed less than EV, the mortality associated with gastric variceal hemorrhage is substantial[1-3] (Kim, 1997 #5; Sarin, 1992 #4; Ryan, 2004 #6) (Kim, 1997 #5; Sarin, 1992 #4; Ryan, 2004 #6). Furthermore, with the successful management of bleeding EV, as well as successful prophylaxis of first bleeding from EV, "secondary" gastric varices develop in 9% to 15% of patients, which have a higher frequency of bleeding compared with primary gastric varices[1,23]. Unlike EV, gastric varices pose particular therapeutic challenges because of their size and location. Standard endoscopic therapies used for EV, such as sclerotherapy and band ligation, are less effective for gastric varices and have been shown to be associated with high complication rates[6,7]. This leads to the need for more aggressive and costly interventions, such as TIPS and BRTO, however each has its limitations. The current methods for the treatment of gastric varices are far from ideal.

EVO with the injection of agents such as N-buyt1-2-cyanoacrylate for the treatment of GV has been widely adopted, and both the immediate and long-term efficacies have been confirmed in the treatment of GV[25-28]. However, the long-term rebleeding rate after EVO was still high[9-12]. Incomplete obliteration and recurrence of varices are considered to be important causes of rebleeding[29]. However, the unobliterated components of the GV are sometimes small, and a further intraluminal injection might be difficult due to the previously injected polymers. In such circumstances, GV obliteration might not be complete. Over time, the residual GV can become larger, and rebleeding can occur. Incomplete obliteration and recurrence of varices are considered to be important causes of rebleeding[9]. Additionally, although rare, fatal complications do occur[13-16].

PTVE with cyanoacrylate is a modified procedure of the classical percutaneous transhepatic obliteration[30-32]. Though this novel technique was used only in a small number of patients with gastric varices and without gastrorenal shunts who were not candidate for BRTO[19,20], it started a new treatment method for gastric varices. In recent years, based on the good results of modified PTVE with 2-OCA in the treatment of EV reported in our previous study[21,22], we introduced 2-OCA in modified PTVE to treat gastric varices. In this retrospective study, we compared modified PTVE with an endoscopic injection using 2-OCA in the management of gastric varices. Despite the retrospective nature of the study, there were no significant differences in age, sex, cause of disease, or severity of liver disease between the studied patients.

In our study, the rebleeding rate of EVO was 37.8%, while it was only 12.5% in patients who underwent

PTVE with cyanoacrylate. This showed that PTVE with 2-OCA was superior to endoscopic 2-OCA injection with respect to preventing rebleeding. Regarding the risk factors of variceal rebleeding, it was revealed that liver function, size of GV, and treatment methods were independent factors that predicted the rebleeding risk of GV. Although the rebleeding rate of GV was lower in patients who underwent PTVE, this did not yield a decreased mortality compared with patients in the EVO group. Cox analysis showed the liver function in the Child-Pugh classification was the most significant factor.

There are two technical superiorities of PTVE over EVO, which may contribute to the lower variceal rebleeding rate of PTVE. The first is that PTVE can achieve a more extensive obliteration area than EVO. In the present study, with regards to the PTVE procedure, 2-OCA was injected into entire gastric varices, perforating veins in the fundus and perigastric veins, including all the feeding veins in most of the patients (30/32, 93.75%). In the EVO procedure, although we tried to achieve complete obliteration in one session, some patients needed repeated injections, and even then, the obliteration rate was only 77.7%. Contrast CT follow-up showed that the PTVE could obliterate all collaterals in the vicinity of the gastric fundus over a wider area and in deeper layers compared with EVO. Adequate 2-OCA injection is important for complete variceal obliteration. In our study, more cyanoacrylate was used in the PTVE procedure than in EVO, which also indicates a wider obliteration range in the PTVE group. The second technical superiority of PTVE over EVO is that 2-OCA can be permanently embolized in perforating veins in the fundus and perigastric veins, including all its feeding veins. In our study, follow-up endoscopy and CT scans showed that the cyanoacrylate in the submucosa could be released with time, and lead to the eradication of the varices after PTVE. However, CT scans revealed that the 2-OCA still stayed in the perifundus varices and perforating veins in the gastric fundus and all the afferent veins during follow-up. This might explain why PTVE achieved long-term obliteration, and prevented recanalization and rebleeding of gastric varices.

Complications were similar in patients who underwent EVO or modified PTVE. Fever and abdominal pain were the two common complications in both groups. They were given conventional treatment and the fever and abdominal pain were usually alleviated within 1-2 wk. Six patients in the PTVE group and three in the EVO group experienced mild to moderate ascites. They were given diuretics and or albumin transfusion, and no patients experienced refractory ascites. Partial portal vein thrombosis presented in two patients in PTVE group, but the portal vein was patent under Doppler ultrasound without serious consequences. In the present study, one patient in the EVO group encountered pulmonary embolism. However, no patient encountered systemic embolization in the modified PTVE group. The modification of PTVE contributed to this satisfactory result. Firstly, in patients without a large gastrorenal shunt, the blood flow in the gastric varices was slow, so there was enough time for 2-OCA to obliterate the varices, and it was unlikely that the tissue glue would migrate into the systemic circulation. Therefore, the 2-OCA could be injected into the gastric varices with percutaneous transhepatic variceal embolization alone. Secondly, in patients with a large gastrorenal shunt and rapid blood flow in the gastric varices, percutaneous transhepatic embolization combined with left renal vein balloon catheter obstruction was performed to prevent systemic embolization.

Some authors tried to obliterate the afferent vein of the gastric varices using TIPS combined with embolotherapy[33]. However, TIPS for gastric varices is not as effective as that for EV; moreover, stent failure and hepatic encephalopathy also limit its application[34-36]. BRTO embolized the gastric varices through the outflow vein (the gastrorenal shunt), and it is recognized as a safe and effective treatment for gastric varices with a gastrorenal shunt. However, patients without a catheterizable gastrorenal shunt are not suitable for BRTO. In our study, even in patients with a large gastrorenal shunt, the embolization was still performed by PTVE, in which the gastric varices were embolized through the inflow veins, such as the left gastric vein, short gastric vein and/or posterior gastric vein. We suppose that PTVE could achieve a more extensive obliteration range than BRTO. As antegrade transcatheter embolization, PTVE with 2-OCA could embolized the entire gastric varices and all its inflow veins (left gastric vein, short gastric vein and/or posterior gastric vein). But BRTO variceal embolization is performed by gastrorenal shunt (outflow vein), in which the inflow veins perhaps could not be completely embolized.

In conclusion, with extensive and permanent obliteration of both gastric varices and its feeding veins, PTVE with 2-OCA is a prospective modality for the treatment of gastric varices. It is superior to endoscopic 2-OCA injection in terms of preventing rebleeding. However, our study is a retrospective single-center study. A future prospective, randomized, and controlled trial is required.

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COMMENTS

Background

Although gastric varices tend to bleed less than esophageal varices (EV), the mortality associated with gastric variceal hemorrhage is substantial. Unlike EV, gastric varices pose particular therapeutic challenges because of their size and location. The current methods for the treatment of gastric varices are far from ideal.

Research frontiers

At present, endoscopic variceal obturation with the injection of agents such as N-butyl-2-cyanoacrylate is the first-line treatment for gastric variceal bleeding. However, the unobliterated components of the gastric varices (GV) are sometimes small, and a further intraluminal injection might be difficult due to the previously injected polymers. In such circumstances, GV obliteration might

not be complete. Over time, the residual GV can become larger, and rebleeding can occur. Incomplete obliteration and recurrence of varices are considered to be important causes of rebleeding.

Innovations and breakthroughs

Percutaneous transhepatic variceal embolization (PTVE) with cyanoacrylate is a modified procedure of the classical percutaneous transhepatic obliteration. With GV, the perifundus veins and all the feeding veins in the vicinity of the gastric fundus are sufficiently obliterated with cyanoacrylate. PTVE with 2-octyl-cyanoacrylate (2-OCA) can improve long-term efficacy by preventing gastric varices rebleeding. In this retrospective study, the authors compared modified PTVE with endoscopic injection using 2-OCA in the management of gastric varices.

Applications

With extensive and permanent obliteration of both GV and its feeding veins, PTVE with 2-OCA is a prospective modality for the treatment of GV. It is superior to endoscopic 2-OCA injection in terms of preventing rebleeding.

Peer review

The authors report their results of a retrospective control study: modified PTVE with 2-OCA and endoscopic variceal obturation with the injection of 2-OCA for prophylaxis of gastric variceal rebleeding. Important data including the rebleeding rate, survival rate, complications, and prognostic predictors were reported. This study indicates that PTVE with 2-OCA can be a better option for secondary prophylaxis of the gastric variceal hemorrhage. However, given the small sample size and retrospective nature of this study, their results may not be representative of the broader population of patients with gastric variceal and a future prospective, randomized, and controlled trial is required.

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BRIEF ARTICLE

Endoscopic stent therapy in patients with chronic pancreatitis: A 5-year follow-up study

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Abstract

AIM: This study analyzed clinical long-term outcomes after endoscopic therapy, including the incidence and treatment of relapse.

METHODS: This study included 19 consecutive patients (12 male, 7 female, median age 54 years) with obstructive chronic pancreatitis who were admitted to the 2nd Medical Department of the Technical University of Munich. All patients presented severe chronic pancreatitis (stage Ⅲ°) according to the Cambridge classification. The majority of the patients suffered intermittent pain attacks. 6 of 19 patients had strictures of the pancreatic duct; 13 of 19 patients had strictures and stones. The first endoscopic retrograde pancreatography (ERP) included an endoscopic sphincterotomy, dilatation of the pancreatic duct, and stent placement. The first control ERP was performed 4 wk after the initial intervention, and the subsequent control ERP was performed after 3 mo to re-evaluate the clinical and morphological conditions. Clinical follow-up was performed annually to document the course of pain and the management of relapse. The course of pain was assessed by a pain scale from 0 to 10. The date and choice of the therapeutic procedure were documented in case of relapse.

RESULTS: Initial endoscopic intervention was successfully completed in 17 of 19 patients. All 17 patients reported partial or complete pain relief after endoscopic intervention. Endoscopic therapy failed in 2 patients. Both patients were excluded from further analysis. One failed patient underwent surgery, and the other patient was treated conservatively with pain medication. Seventeen of 19 patients were followed after the successful completion of endoscopic stent therapy. Three of 17 patients were lost to follow-up. One patient was not available for interviews after the 1st year of follow-up. Two patients died during the 3rd year of follow-up. In both patients chronic pancreatitis was excluded as the cause of death. One patient died of myocardial infarction, and one patient succumbed to pneumonia. All three patients were excluded from follow-up analysis. Follow-up was successfully completed in 14 of 17 patients. 4 patients at time point 3, 2 patients at time point 4, 3 patients at time point 5 and 2 patients at time point 6 and time point 7 used continuous pain medication after endoscopic therapy. No relapse occurred in 57% (8/14) of patients. All 8 patients exhibited significantly reduced or no pain complaints during the 5-year follow-up. Seven of 8 patients were completely pain free 5 years after endoscopic therapy. Only 1 patient reported continuous moderate pain. In contrast, 7 relapses occurred in 6 of the 14 patients. Two relapses were observed during the 1st year, 2 relapses occurred during the 2nd year, one relapse was observed during the 3rd year, one relapse occurred during the 4th year, and one relapse occurred during the 5th follow-up year. Four of these six patients

received conservative treatment with endoscopic therapy or analgesics. Relapse was conservatively treated using repeated stent therapy in 2 patients. Analgesic treatment was successful in the other 2 patients.

CONCLUSION: 57% of patients exhibited long-term benefits after endoscopic therapy. Therefore, endoscopic therapy should be the treatment of choice in patients being inoperable or refusing surgical treatment.

Key words: Chronic pancreatitis; Pain; Stent therapy; Endoscopic retrograde cholangiopancreatography; Pancreaticolithiasis

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INTRODUCTION

The optimal treatment for chronic pancreatitis is controversial among surgeons and endoscopists. Endoscopic drainage procedures include optional sphincterotomy, stricture dilation, stone removal, and the insertion of a plastic stent. The surgical approaches are classified as drainage and resective procedures. Resective procedures, such as the duodenum-preserving head resections of Beger and Frey, provide good pain control and preserve pancreatic function[1]. Drainage procedures, such as the pancreaticojejunostomy, are comparable to endoscopic treatments because both of these procedures are based on the same pathophysiological idea. The primary goal is to restore pancreatic drainage to lower the pressure within the pancreatic duct and relieve pain[2-4]. Cahen *et al*[5] compared the clinical outcome of patients with endoscopic treatment and patients undergoing pancreaticojejunostomy in a prospective randomized trial. The authors concluded that surgical drainage of the pancreatic duct was more effective than endoscopic treatment. However, some patients are inoperable or refuse surgical treatment. Endoscopic therapy offers as an alternative approach for these patients. We reported previously that endoscopic therapy provided good immediate and short-term benefits to patients with obstructive chronic pancreatitis[6]. The current manuscript provides additional data from a 5-year follow-up to analyze clinical long-term outcomes after the successful completion of endoscopic therapy, including the incidence and treatment of relapse.

MATERIALS AND METHODS

Patients

This section is similar to our previously published 2-year follow-up study[6]. All patients presented severe chronic pancreatitis (stage Ⅲ°) according to the Cambridge classification[7] at the time of sphincterotomy and the majority of the patients (11/19) suffered intermittent pain attacks according to the Amman Score[8]. About 6 of 19 patients had strictures of the pancreatic duct; 13 of 19 patients had strictures and stones.

Methods

The first control endoscopic retrograde pancreatography (ERP) was performed 4 wk after stenting, and the subsequent control ERP was performed after 3 mo to re-evaluate the clinical and morphological conditions. Either a new stent was inserted, or stent therapy was terminated depending on the clinical and morphological conditions.

The long-term effects of stent therapy were analyzed after the successful completion of stent therapy. A short overview of the study protocol is illustrated in Figure 1. Follow-up analyses were conducted from 2004 to 2009. The ethical committee stated that the patient's choice of treatment was protected. Clinical parameters, such as pain and the intake of pain medication, were documented: (1) Prior to endoscopic therapy (time point 1); (2) During endoscopic therapy (time point 2); and (3) Annually during the 5-year follow-up period (time points 3, 4, 5, 6 and 7).

Interviews: The first interview was a two-part session that was conducted during stent therapy. This interview analyzed the technical success of ERP and compared the pain situation prior to and after initial stent therapy (Figure 1). The first session was conducted prior to the first stent implantation (time point 1), and the second session was conducted after the initial stent implantation (time point 2). All 17 patients were interviewed annually during the follow-up period. The interviews primarily evaluated the course of pain. The patients graded their pain on a pain scale from 0 to 10: 0 was no pain, 1-2 slight pain, 3-4 moderate pain, 5-6 strong pain, 7-8 very strong pain, and 9-10 extreme pain. The date and choice of the therapeutic procedure (*e.g.*, conservative pain management, endoscopic or surgical treatment) were documented for instances of relapse.

Statistical analysis

Statistical analysis was not performed. The evaluation of pain was carried out using a pain score in absolute numbers.

RESULTS

Primary outcome, duration and complication rate of stent therapy

This section is similar to our previously published 2-year follow-up study[6].

Long-term follow-up

Nineteen patients with chronic obstructive pancreatitis

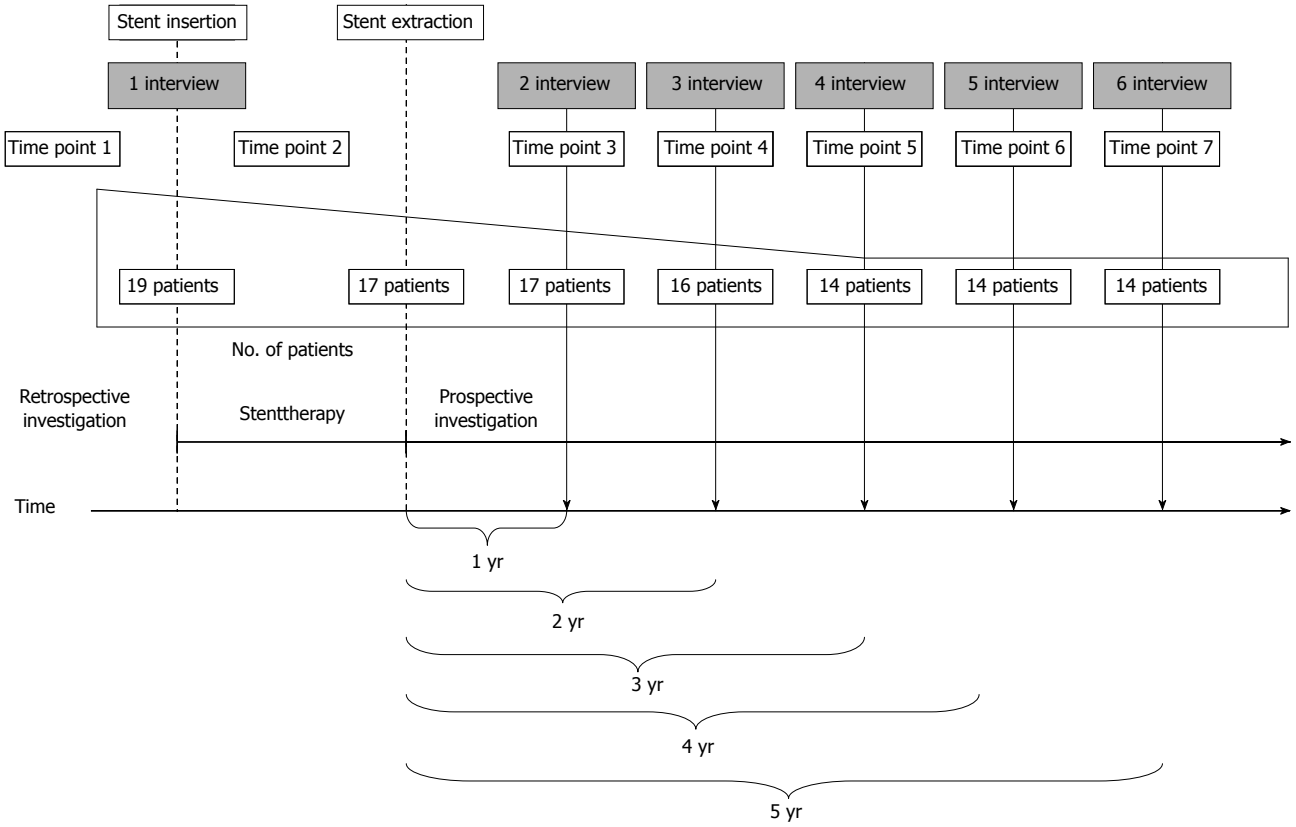

Figure 1 Protocol of the current study, time points of investigation and examination. Nineteen patients with chronic obstructive pancreatitis underwent endoscopic stent therapy. Endoscopic therapy failed in 2 patients. Seventeen of 19 patients showed clinical benefit and were included to follow-up analysis after completion of stent therapy. During the follow-up, patients were interviewed yearly. Three patients were lost to follow-up. One patient was not available any longer and two patients died.

underwent endoscopic stent therapy. Figure 2 is an illustration of a representative ERP procedure. ERP failed in 2 patients in which cannulation or permanent stent therapy was not achieved. One failed patient underwent surgery, and the other patient was treated conservatively with pain medication. We excluded these 2 patients from follow-up analyses. Endoscopic stent therapy was successfully completed in 17 of 19 patients. All 17 patients reported partial or complete pain relief.

The clinical courses of 17 patients were evaluated after the successful completion of stent therapy. Three of the 17 patients were lost during follow-up. One patient was not available for interviews after the 1st year of follow-up (Figure 1). Two patients died during the 3rd year of follow-up, and chronic pancreatitis was excluded as the cause of death. One patient died of myocardial infarction, and one patient succumbed to pneumonia.

Patients consumed the following pain medications during the 5-year follow-up period: paracetamol (500-1000 mg/d), diclofenac (50-100 mg/d), metamizol (500-1000 mg/d) and/or tramadol (100-300 mg/d). 4 patients at time point 3, 2 patients at time point 4, 3 patients at time point 5 and 2 patients at time point 6 and time point 7 used continuous pain medication after endoscopic therapy.

Seven relapses occurred in 6 of the 14 patients (43%). One patient developed 2 relapses during the 1st and 3rd follow-up years. Two relapses were observed during the 1st year, 2 relapses occurred during the 2nd year, one relapse was observed during the 3rd year, one relapse occurred during the 4th year, and one relapse occurred during the 5th follow-up year.

Four of these six patients received conservative treatment with endoscopic therapy or analgesics. Two patients were successfully treated with endoscopic therapy. Two patients achieved pain improvement with analgesic therapy. Only 2 of the 6 patients required surgery.

In contrast, 57% (8/14) of the patients without relapse reported significantly reduced pain or no complaints 5 years after stent extraction. Seven of these patients were completely pain free. Only 1 patient reported continuous moderate pain.

DISCUSSION

Several reviews[9-14] suggest that the majority of physicians consider pancreatic duct stenting a safe and effective therapy for patients with chronic abdominal pain due to chronic pancreatitis. The primary goal of endoscopic therapy is pain relief. Approximately half of the patients in the current study exhibited clinical long-term benefits. Most patients (57%, 8/14) exhibited significantly reduced pain or no pain complaints 5 years after stent extraction. Endoscopic treatment was an effective alternative

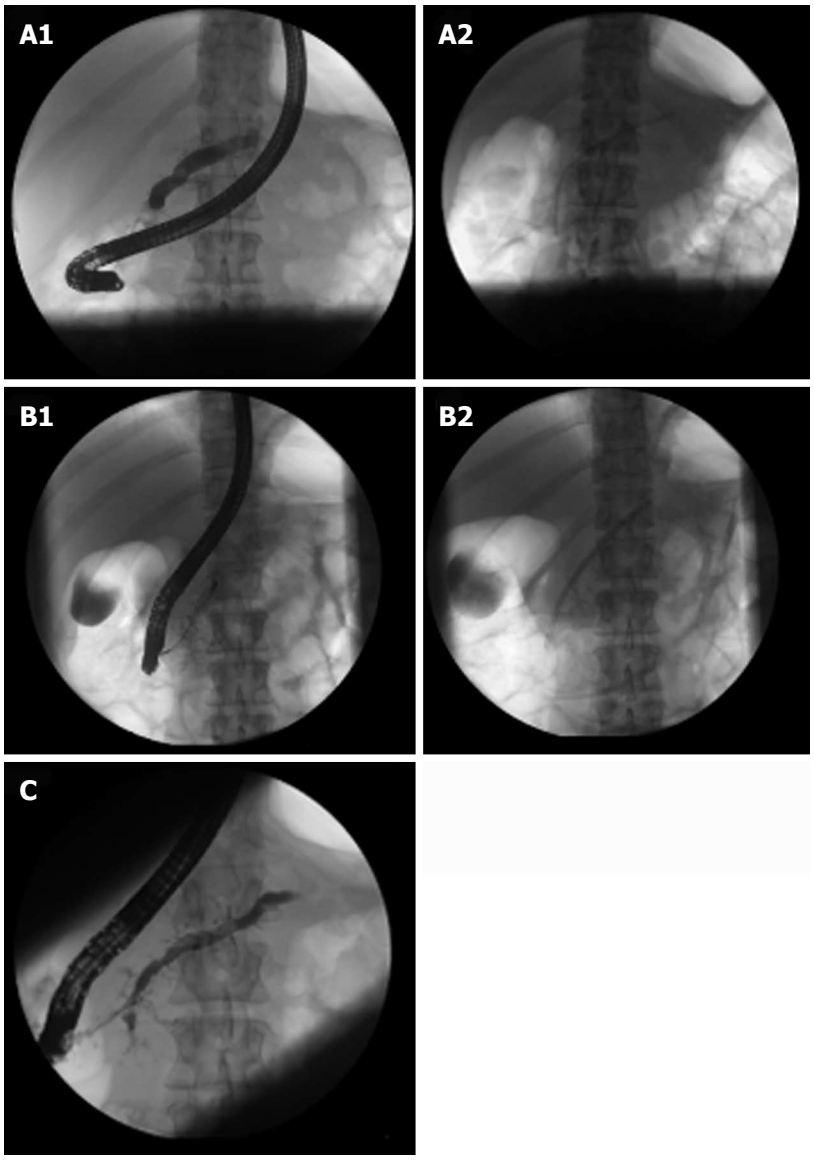

Figure 2 Illustration of a representative endoscopic retrograde pancreatography procedure. A: A dilated pancreatic duct due to a stenosis in the head and several stones in the main duct (A1). The stenosis was passed through with a 5 F catheter followed by a 8.5 F Sohendra Bougie. Subsequently, a 7 F stent was inserted (A2). After initial endoscopic intervention the patient was completely pain free; B: Four weeks later, the patient was readmitted for control endoscopic retrograde pancreatography (ERP). Stones were partially extracted by a dormia basket (B1), and the 7 F stent was replaced by a 10 F stent (B2); C: Three months later, the patient was still free of complaints. ERP revealed a slightly dilated duct with typical stone formations in the corpus and tail of the pancreas. Stones were extracted.

to surgery in instances of relapse. Four of six re-treated patients received conservative treatment with endoscopic or analgesic therapy. Only two of these patients required surgery.

The impact of smoking and alcohol on pain development was not evaluated in this study. However, smoking and alcohol are associated with more a progressive disease course[15-20]. Therefore, the cessation of smoking and alcohol consumption was recommended to all patients in the study. No relationship between the duration of stenting and the occurrence of relapse was observed. However, the small number of patients in this study prohibits valid conclusions. Further studies with a larger number of patients are required.

Surgical procedures seem to be superior to endoscopic therapy. Díte *et al*[21] compared the clinical benefit of endoscopic and surgical therapies in a randomized prospective study. The study included 140 patients with chronic pancreatitis. Endoscopic therapy was performed in 64 patients, and 76 patients underwent surgery. Both therapeutic approaches produced excellent results in initial pain relief (92.1% and 92.2%, respectively). The number of patients with partial or complete pain relief at the 5-year follow-up in the stent group decreased from 92.2% to 65.1%, but 86.2% of patients with surgical procedures reported reduced or no pain. These authors concluded that surgery was superior to endoscopic therapy for long-term pain reduction in patients with painful obstructive chronic pancreatitis. However, 80% of the surgeries were resection procedures. Resection procedures are based on a different pathophysiological concept than endoscopic or surgical drainage. Therefore, these procedures are only

comparable to a limited degree. The prospective randomized study of Cahen *et al*[5] analyzed the clinical benefit of surgical drainage versus endoscopic drainage, which is more suitable for this type of comparison. Nineteen patients underwent endoscopic drainage, and 20 patients received surgical drainage. Two years after intervention, 75% patients with pancreaticojejunostomy were partially or completely pain-free, but complete or partial pain relief was achieved in only 32% of patients with endoscopic therapy. Cahen *et al*[5] concluded that the surgical drainage of pancreatic duct was more effective than endoscopic treatment in patients with obstruction of pancreatic duct due to chronic pancreatitis. In contrast, the data of our current series demonstrated that ERP failed in only 2 of 19 patients. Complete or partial pain relief was achieved in 17 of 19 patients after initial stent insertion. Approximately half of the patients benefited from endoscopic stent therapy 5 years after endoscopic intervention. The less effective outcome of endoscopic stent therapy in the Cahen study may result from the difficulty of managing the endoscopic group. The complication rate of the endoscopic group (58%) was approximately 4 times higher in their study compared to the average results of other studies[22,23].

Many patients present contraindications or refuse to undergo surgery due to the invasiveness of surgical procedures. Consequently, patients who were American Society of Anesthesiologists class Ⅳ, presented with portal vein thrombosis or declined to participate were excluded from the Cahen study[5]. Approximately 12% of patients with chronic pancreatitis exhibit portal hypertension[24]. Izbicki *et al*[25] evaluated the impact of concomitant nonhepatic portal hypertension in chronic pancreatitis on the outcomes after major pancreatic surgery. Patients with portal hypertension required significantly more blood transfusions and longer operative times than their nonhypertensive counterparts. The overall postoperative complication rate was significantly higher in this subgroup. Izbicki concluded that concomitant extrahepatic portal hypertension is a substantial risk in pancreatic surgery for chronic pancreatitis.

The major limitations of this study are the small number of patients, its single-center character and its nonrandomized design. Therefore, randomized long-term follow-up studies with a larger number of patients are required.

In conclusion, endoscopic therapy proved to be a safe and effective alternative to surgery, and endoscopic therapy did not adversely affect the outcome of subsequent surgeries[26]. Our current data demonstrate that endoscopic therapy provided long-term benefits in more than half of the patients (57%) with chronic obstructive pancreatitis. The management of obstructive chronic pancreatitis should be individual. Patients with multiple morbidities profit from low invasive endoscopic therapy. Therefore, endoscopic stent therapy is the treatment of choice in inoperable patients or patients who refuse surgical treatment.

COMMENTS

Background

Obstruction of the pancreatic duct is a common feature of chronic pancreatitis, and it often requires interventional therapy. The optimal treatment for chronic pancreatitis is controversial among surgeons and endoscopists. The primary goal of both surgical and endoscopic drainage procedures is the restoration of pancreatic drainage to lower the pressure within the pancreatic duct and relieve pain.

Research frontiers

This is the first prospective 5-year follow-up assessment of the incidence and treatment of relapse in patients with chronic obstructive pancreatitis after the successful completion of endoscopic stent therapy.

Innovations and breakthroughs

The current data demonstrate that endoscopic therapy provided long-term benefits in more than half of chronic obstructive pancreatitis patients. Most (57 %) of the patients remained free of relapse 5 years after endoscopic treatment. Endoscopic treatment was an effective alternative to surgery in cases of relapse.

Applications

These data suggest that endoscopic therapy is a safe, minimally invasive, and effective procedure in patients who experience pain attacks during chronic pancreatitis. Multimorbid patients benefit from the low invasiveness of endoscopic therapy. Therefore, endoscopic stent therapy should be the treatment of choice in patients who are inoperable or refuse surgical treatment.

Terminology

Chronic pancreatitis is a continuous inflammation of the pancreas that produces morphological changes, such as calcifications, irregularities of the pancreas duct and the formation of pancreatic stones. The pathogenesis of pain in chronic pancreatitis is controversial. Interstitial hypertension, ongoing pancreatic ischemia, neuronal inflammation, and extrapancreatic complications may contribute to the pathogenesis of pain.

Peer review

The current study provides interesting and clinically relevant data. The observed patient group was small, but the differences were obvious. The data support the benefit of long-term endoscopic therapy in a subgroup of patients.

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BRIEF ARTICLE

Australian tertiary care outcomes of entecavir monotherapy in treatment naive patients with chronic hepatitis B

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Abstract

AIM: To evaluate the long-term treatment outcomes of entecavir monotherapy in treatment naive patients in an Australian tertiary care setting.

METHODS: A retrospective analysis of treatment naive patients receiving entecavir monotherapy through Westmead Hospital was performed. Patients were excluded if they had received previous treatment with another nucleoside or nucleotide analogue, were pregnant or less than 18 years old.

RESULTS: Out of 336 patients, 163 patients fulfilled the selection criteria. Range of follow up was 3-46 mo (mean 26 mo). 134 patients (82.2%) had pre-treatment biopsies, with 26 patients (16.0 %) demonstrating F3-4 fibrosis. In total, 153 patients (93.9%) achieved at least Partial Virological Suppression (PVS), with 134 patients (82.2%) achieving complete virological suppression. The cumulative CVS and PVS rates at 36 mo were 92.2% and 97.3%, respectively. 3 patients (1.8%) failed to achieve PVS, while 5 patients (3.0%) developed virological rebound. 128 patients (78.5%) maintained CVS throughout follow up. Predictors of CVS included lower baseline DNA level (P = 0.001), hepatitis B virus e antigen negative status (P = 0.001) and increasing age at treatment (log rank 0.001). No significant adverse effects were reported necessitating cessation of entecavir.

CONCLUSION: Entecavir monotherapy is efficacious and safe in an Australian tertiary care setting. Resistance and rebound rates are very low. This is similar to data from controlled and uncontrolled trials around the world.

Key words: Chronic hepatitis B; Entecavir; Australia; Asia-Pacific; Monotherapy; Hepatitis B virus; Antivirals

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INTRODUCTION

An estimated 350 to 400 million people worldwide have chronic hepatitis B virus (HBV) infection, a leading cause of morbidity and mortality. Australia has an ethnically diverse population, with many migrants from the Asia-Pacific region, where the majority of hepatitis B related deaths occur[1].

The number of people living with chronic hepatitis B in Australia is estimated to be between 153 000 and 175 000, a prevalence of 0.7% to 0.8%[2,3]. In 2010, 228 incident cases of hepatitis B and 6878 notifications of hepatitis B were reported to the Australian government's National Notifiable Diseases Surveillance System[4]. This reflects the major burden of disease in people with childhood acquired chronic hepatitis B.

In chronic HBV infection the goals of antiviral therapy are to suppress HBV DNA and reduce hepatic inflammation [alanine aminotransferase (ALT)], with the aim of preventing progression of liver fibrosis and achieving immune control [hepatitis B e antigen (HBeAg) and/or hepatitis B surface antigen (HBsAg) loss/conversion]. This reduces mortality due to cirrhosis, liver failure and HCC. Antiviral agents available include pegylated interferon, lamivudine, adefovir, entacavir, telbivudine and tenofovir.

Entecavir is an oral deoxyguanosine analog with potent activity against HBV[5]. In multiple clinical trials, entacavir has been found to be highly efficacious in treating nucleoside naive and lamividine refractory patients[6-8]. Emergence of drug resistance during antiviral therapy is well described for HBV, particularly with lamivudine, with resistance appearing in 20% of patients after 1 year and 70% after 5 years[9]. Entecavir has a higher genetic barrier to resistance than lamivudine, with quoted resistance rates of only 1%-2% in treatment naive patients after 5 years[10]. However, cross-resistance with lamivudine is a problem, and in patients who have failed lamivudine, entecavir resistance appears in up to 8% after 5 years[10,11].

The rates of entecavir response and resistance in Australia are unknown. Most published data are from large clinical trials, which do not necessarily reflect the rates seen in "real world" clinical practice. These trials were almost exclusively performed in the Northern Hemisphere, and do not reflect the unique, diverse migrant population seen in Australia. Furthermore, much of this data is based on a 1 mg dose of entecavir, rather than 0.5 mg now recommended for treatment naive patients.

The aim of this study was to evaluate the efficacy, rates of viral resistance and treatment outcomes of entacavir monotherapy in an Australian tertiary referral centre, outside of a clinical trial environment.

MATERIALS AND METHODS

Data collection and clinical setting

Data was entered retrospectively into a database incorporating all patients receiving entecavir through the Westmead Hospital pharmacy (Sydney, Australia) between 1 November 2006 and 31 July 2010.

Selection criteria

All patients receiving entecavir through the Westmead Hospital pharmacy were considered for analysis. Patients were included if they met the following criteria: (1) eligible for entecavir 0.5 mg daily according to AASLD guidelines; (2) HBV infection for greater than 6 mo based on serology; and (3) deranged liver function tests or at least mild fibrosis on liver biopsy (> F2 on Scheuer classification).

Exclusion criteria were: (1) previous treatment with other nucleoside analogues, nucleotide analogues or trial medication (except for interferon); (2) pregnancy; (3) age less than 18 years; (4) hepatitis delta co-infection; and (5) Prophylactic entecavir during immunosuppression (*e.g.*, chemotherapy).

Definitions

Viral load was measured in international units/mL, using the Cobas Taqman assay (12 IU/mL) (Roche Diagnostics, Branchburg, NJ).

Complete and Partial Virologic Suppression were defined as HBV DNA < 12 IU/mL and 12-2000 IU/mL, respectively. Virologic rebound was defined as an increase in viral load > 1 $\log_{10}$ from the previous value, in a patient with initial virologic suppression[12]. Cumulative rates of suppression were calculated by the formula P = 1 - (1 - n1/N1) (1- n2/N2). . .(1 - nx/Nx), where P is the cumulative probability that the event will occur, nx is the number of cases at year x, and Nx is the number of patients still followed up at year x[13].

The Scheuer classification system for grading and staging of chronic hepatitis was used for patients that underwent liver biopsy.

Statistical analysis and ethical consideration

All statistical analyses were performed using SPSS (version 16, Chicago IL). The study was approved by the Westmead Hospital Human Research Ethics Committee.

RESULTS

A total of 336 patients were included in the database, which collected data from 2006 to 2010. 89 patients were excluded due to past or current treatment with another agent. A further 83 patients were excluded as they received treatment for less than 3 mo, were less than 18 years old, received prophylactic entecavir, had hepatitis delta co-infection or were pregnant. Thus, 163 patients were included for analysis. The patient demographics are summarised in Table 1.

Treatment setting

All patients were reviewed at least four-monthly by one of six hepatologists at Westmead Hospital Liver Clinic, with assessment of HBV DNA, viral serology, liver function tests and routine haematological and renal laboratory evaluation. Relevant 6-monthly HCC surveillance was also performed, with serum alpha-fetoprotein and hepatic ultrasound.

The mean and median duration of follow up were 26 and 23 mo, respectively. The range of follow up was 3-46 mo, with 50% of patients followed for greater than 24 mo.

Table 1 Baseline demographics (*n* = 163) *n* (%)

Age (yr)	
Mean age	52.0 (24-86)
Mean age at start	47.4 (20-81)
Gender	
Males	113 (69.3)
Females	50 (30.7)
Pre-treatment Biopsies	134 (82.2)
Duration of therapy (mo)	
Range	3-46
Mean	25.63
Median	23
Patients > 24 mo	79 (48.5)
Patients > 36 mo	49 (30.1)
Co-infection	
Hepatitis C	1 (0.61)
HBV DNA load (IU/mL)	
> 100 000	95 (58.3)
2000-100 000	45 (27.6)
12-2000	23 (14.1)
HBe antigen status	41 (25.2)
e antigen positive	112 (68.7)
e antigen negative	10 (6.1)
Unknown	
ALT (U/L)	
10-40	39 (23.9)
40-400	118 (72.4)
> 400	5 (3.01)
Unknown	1 (0.61)
Fibrosis score	0-10 (6.13)
135 (82.82%)	1-61 (37. 4)
	2-38 (23.3)
	3-17 (10.4)
	4-9 (5.52)
	Unknown-27 (16.6)

HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

Response to treatment

The response to entecavir treatment over time is detailed in Table 2, with a graphical summary of virological suppression in Figure 1. In total, 153 patients (93.9%) achieved partial virological suppression (PVS), with 134 patients (82.2%) achieving complete virological suppression (CVS). The cumulative CVS and PVS rates at 36 mo were 92.2% and 97.3%, respectively. Three patients (1.84%) failed to achieve partial or complete suppression (mean duration of therapy 8 mo). Five patients (3.01%) developed virological rebound (mean duration of therapy 29 mo, 3 females, 4 eAg negative, median fibrosis score 1 (biopsies in 4 patients), mean baseline DNA 9.71×10^6 IU/mL). Four of the 5 patients that developed virological rebound had achieved CVS. Reasons for rebound included non-compliance in 4 patients and suspected entecavir resistance in 1 patient. Specific viral resistance testing was not performed. Four patients (2.45%) were changed to another agent due to failed virologic suppression or virologic rebound. Of these, 3 patients (1.84%) were changed to tenofovir and 1 patient (0.61%) to adefovir, with CVS and PVS achieved in 2 patients each, respectively.

One hundred and twenty eight patients (78.53%) maintained CVS throughout follow up. One hundred and thirty three patients (94.3%) achieved PVS at 12 mo. The annual HBeAg positive to negative seroconversion rate was 14.3%. One patient (HBeAg negative) had loss of surface antigen, resulting in an annual surface antigen loss rate of 0.3% for HBeAg negative patients and 0.2% for all patients, respectively.

Predictors of virological response

The time to achieve CVS was related to baseline DNA levels ($P = 0.001$) and HBeAg status at baseline ($P = 0.001$) (Figure 2).

The mean and median times to CVS were 11.4 (9.77-13.09) and 6 (5.32-6.69) mo, respectively. For HBeAg negative patients the mean time was 6 mo (5.35-6.65), while for HBeAg positive patients it was 12 mo (3.42-20.58) (log rank < 0.001).

There was a statistically significant difference in median time taken to achieve CVS based on age and HBeAg status (Table 3). There was no significant difference in loss of HBeAg based on age (log rank 0.559).

There was no significant effect of duration of therapy, fibrosis score or gender on time to CVS, HBeAg conversion or ALT normalisation. There was a trend towards faster ALT normalisation based on increased age (log rank 0.053).

HCC

Among the 163 patients in the study, 4 patients (2.45%) developed HCC (mean time to development 19.9 mo), while in 6 (3.68%) cases the diagnosis preceded commencement of therapy. Two patients with HCC had a pre-treatment biopsy which did not show cirrhosis (F2 and F3, respectively).

Treatment discontinuation and safety

Twenty one patients (12.88%) failed to follow up in clinic during the study period and thus did not receive ongoing entecavir through the hospital pharmacy. There were no reports of entecavir being ceased due to adverse effects.

DISCUSSION

This study gives an important insight into outcomes of entecavir monotherapy for treatment naive patients with chronic hepatitis B in an Australian tertiary referral setting, with up to 3.5 years follow-up. To our knowledge it is the first study to describe the efficacy, tolerability and resistance rates of entecavir in treatment naive patients in the Australian tertiary care setting. Daily monotherapy with 0.5 mg entecavir was efficacious, with 94% of patients achieving at least partial suppression, and 82% achieving undetectable HBV DNA levels. Only 2% of patients failed to reach any significant reduction in viral load, while 3% developed virologic rebound. Viral resistance testing was not performed. No significant adverse events were reported, and treatment discontinuation was not due to adverse events.

Table 2 Follow up of DNA values and cumulative rates of complete and partial virological suppression *n* (%)

Month	0	6	12	18	24	30	36	42
Patients with DNA	163 (100)	158 (96.9)	141 (86.5)	121 (74.2)	105 (64.4)	76 (46.6)	56 (34.4)	24 (14.7)
Complete virological suppression	0/169 (0)	104/158 (65.8)	117/141 (83.0)	96/121 (74.4)	94/105 (89.5)	65/76 (85.5)	46/56 (82.1)	19/24 (79.2)
Cumulative CVS	0%	66.7%	88.8%	85.1%	94.8%	93.2%	92.2%	91.6%
Partial virological suppression	22/163 (13.5)	141/158 (89.2)	133/141 (94.3)	117/121 (90.7)	100/105 (95.2)	75/76 (98.7)	54/56 (96.4)	23/24 (95.8)
Cumulative PVS	13.5%	89.2%	94.9%	92.1%	96.3%	99.0%	97.3%	96.6%

CVS: Complete virological suppression; PVS: Partial virological suppression.

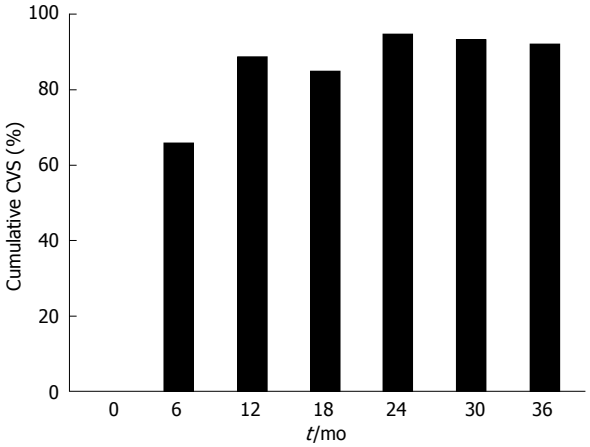

Figure 1 Total cumulative virologic suppression. CVS: Complete virological suppression.

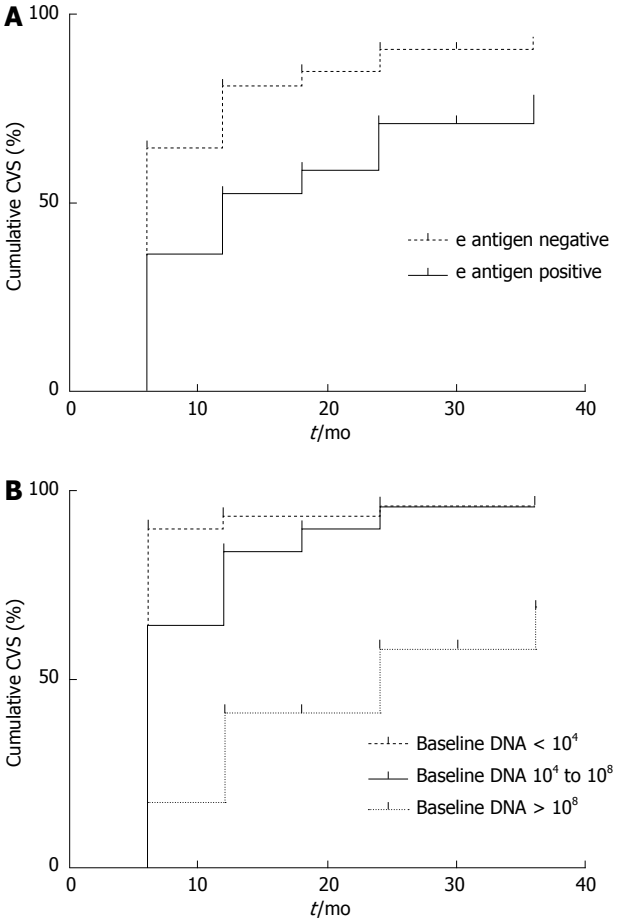

Figure 2 Predictors of complete virological suppression. A: Hepatitis B e antigen status; B: Baseline hepatitis B virus DNA. CVS: Complete virological suppression.

These results are consistent with earlier registration trials, which demonstrated suppression of HBV-DNA to undetectable levels in 67% of HBeAg-positive patients after 12 mo, and in 90% of HBeAg-negative patients[7,8]. Subsequent studies confirmed durable and increasing viral suppression on entecavir, with undetectable HBV DNA achieved by 94% of HBeAg-positive patients over 5 years of treatment, and in 95% of HBeAg-negative patients over 3 years of treatment[14,15]. Clinical trials have also demonstrated minimal emergence of viral resistance (1.2%), after up to 6 years of entecavir treatment[10,16].

Pol *et al*[17] analysed the efficacy and tolerability of entecavir in routine clinical practice settings, by comparing data from 5 international cohorts. In these studies CVS was achieved in 76%-96% of patients, with virologic rebound in 0.6%-4%, and excellent overall tolerability and safety. Therefore the findings from our study are consistent with international data from other "real life" clinical settings, as well as earlier registration trials, which applied more stringent selection criteria.

As has been shown in previous studies, we found that the likelihood of achieving CVS was increased in patients with lower baseline DNA levels and e antigen negative status[18,19]. Amongst patients with baseline HBV DNA $> 10^8$ the CVS was only 67.5% compared to 92% for patients with baseline HBV DNA $< 10^4$. This is most likely explained by the high proportion of eAg positive cases (82%) in the high baseline DNA group compared to the low baseline DNA group (10.5%). The time to achieve CVS was also significantly related to these factors. An interesting and novel finding was that the time taken to achieve CVS was significantly related to age. In patients older than 40 years old the median time to achieve CVS was 6 mo, whereas in patients younger than 40 years old it was 12 mo. This relationship was maintained following HBeAg status stratification, and may reflect better compliance in the older population groups.

A strength of this study is the high proportion of patients in whom pre-treatment liver biopsies were performed (82%). For the duration of this study, liver biopsy

Table 3 Complete virological suppression based on age and HBeAg status

Age-log rank 0.001 (median)		
< 40 yr-	40-55 yr-	> 55 yr-
12 mo (9.25-14.75)	6 mo (5.01-6.99)	6 mo (5.24-6.76)
Hepatitis B e antigen negative status-log rank 0.19 (median)		
< 40 yr-	40-55 yr-	> 55 yr-
6 mo (4.05-7.95)	6 mo (4.99-7.02)	6 mo (5.59-6.41)
Hepatitis B e antigen positive status-log rank 0.19 (median)		
< 40 yr-	40-55 yr-	> 55 yr-
18 mo (7.37-28.63)	18 mo (14.17-26.47)	6 mo (6.5-17.5)
Hepatitis B e antigen seroconversion rate based on age-log rank 0.559 (median)		
< 40 yr-	40-55 yr-	> 55 yr-
36 mo (20.55-51.45)	30 mo (18.46-41.54)	6 mo (9.8-32.2)

was a requirement under the Australian Pharmaceutical Benefit Scheme to access subsidised entacavir treatment. Somewhat surprisingly the degree of fibrosis was not significantly associated with patients achieving CVS, or the time to achieve CVS.

Although there was an association between DNA suppression and ALT normalisation, our study did not take into account other variables that could influence transaminase changes, including anthropomorphic measurements, alcohol intake and intercurrent illness.

The study is limited by its retrospective analysis of medical records and the lack of sufficient follow up data for 13% of patients in the pharmacy registry. Furthermore only a minority of the patients included in the analysis (15%) had completed more than 36 mo of therapy. Finally, viral resistance testing was not performed for patients that failed to achieve PVS or had virologic rebound, as it was not routinely available in our clinic during the study period. The wide range in follow up is mainly due to late commencement in the study period with less time for follow up, drop out of patients and limited treatment duration.

In conclusion, entecavir monotherapy is efficacious and well tolerated for the treatment of patients with chronic hepatitis B infection in Australia. In our cohort baseline HBV DNA levels and HBeAg status influenced time to complete virologic suppression, consistent with previous studies. These factors are not independent, however, as HBeAg negative status most likely correlates with lower baseline HBV DNA levels. There was a very low incidence of failure to suppress DNA, and no apparent emergence of viral resistance on therapy. Our study supports the recommendations that 0.5 mg daily entecavir monotherapy is an appropriate first line agent for patients with treatment naive chronic hepatitis B in Australia.

COMMENTS

Background

An estimated 350 to 400 million people worldwide have chronic hepatitis B virus (HBV) infection, a leading cause of morbidity and mortality. Australia has an ethnically diverse population, with many migrants from the Asia-Pacific region, where the majority of hepatitis B related deaths occur.

Research frontiers

Entecavir is an oral antiviral agent with potent activity against HBV. The rates of entecavir response and resistance in Australia are unknown. Most published data are from large clinical trials, which do not necessarily reflect the rates seen in "real world" clinical practice.

Innovations and breakthroughs

Out of 163 patients, 153 patients (93.9%) achieved at least partial suppression of HBV DNA, with 134 patients (82.2%) achieving complete suppression. Predictors of complete suppression included lower baseline DNA level, HBV e antigen negative status and increasing age at treatment. No significant adverse effects were reported necessitating cessation of entecavir.

Applications

The study results suggest that entecavir monotherapy is efficacious and safe in an Australian tertiary care setting. Resistance and rebound rates are very low. This is similar to data from controlled and uncontrolled trials around the world.

Terminology

Hepatitis B is a viral infection that can lead to persistent inflammation and scarring of the liver. This has significant adverse outcomes including cirrhosis of the liver and liver cancer. Entecavir is a antiviral agent that prevents proliferation of the hepatitis B virus.

Peer review

Well described cohort of entecavir treated patients in clinical practice from Australia; This is an interesting retrospective study from Australia confirms the efficacy and safety of entecavir in clinical practice.

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BRIEF ARTICLE

Can mosapride citrate reduce the volume of lavage solution for colonoscopy preparation?

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Abstract

AIM: To evaluate the possibility of reducing the volume of polyethylene glycol (PEG)-electrolyte solution using adjunctive mosapride citrate for colonoscopy preparation.

METHODS: This was a single-center, prospective, randomized, investigator-blinded, non-inferiority study involving 252 patients of both sexes, aged from 20 to 80 years, scheduled for screening or diagnostic colonoscopy in our department. A total of 126 patients was randomized to receive 1.5 L PEG-electrolyte solution plus 15 mg of mosapride (1.5 L group), and 126 received 2 L PEG-electrolyte solution plus 15 mg of mosapride (2 L group). Patients completed a questionnaire on the acceptability and tolerability of the bowel preparation process. The efficacy of bowel preparation was assessed using a 5-point scale based on the Aronchick scale. The primary end point was adequate bowel preparation rates (score of excellent/good/fair) *vs* (poor/inadequate). Acceptability and tolerability, as well as disease detection, were secondary end points.

RESULTS: A total of 244 patients was included in the analysis. There were no significant differences between the 2 L and 1.5 L groups in age, sex, body mass index, number of previous colonoscopies, and the preparation method used previously. The adequate bowel preparation rates were 88.5% in the 2 L group and 82.8% in the 1.5 L group [95% lower confidence limit (LCL) for the difference = -14.5%, non-inferiority P = 0.019] in the right colon. In the left colon, the adequate bowel preparation rates were 89.3% in the 2 L group and 81.1% in the 1.5 L group (95% LCL = -17.0%, non-inferiority P = 0.066). Compliance, defined as complete (100%) intake of the PEG solution, was significantly higher in the 1.5 L group than in the 2 L group (96.8% *vs* 85.7%, P = 0.002). The proportion of abdominal distension (none/mild/moderate/severe) was significantly lower in the 1.5 L group than in the 2 L group (36/65/22/3 *vs* 58/48/18/2, P = 0.040). Within the subgroup who had undergone colonoscopy previously, a significantly higher number of patients in the 1.5 L group than in the 2 L group felt that the current preparation was easier than the previous one (54.1% *vs* 28.0%, P = 0.001). The disease detection rate was not significantly different between the two groups.

CONCLUSION: Although the 1.5 L group had better acceptability and tolerability, 15 mg of mosapride may be insufficient to compensate for a 0.5-L reduction of PEG solution.

Key words: Mosapride citrate; Bowel preparation; Polyethylene glycol-electrolyte solution; Prokinetics; Colonoscopy

Tajika M, Niwa Y, Bhatia V, Kondo S, Tanaka T, Mizuno N, Hara K, Hijioka S, Imaoka H, Komori K, Yamao K. Can mosapride citrate reduce the volume of lavage solution for colonoscopy preparation? *World J Gastroenterol* 2013; 19(5): 727-735 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i5/727.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i5.727

INTRODUCTION

Polyethylene glycol (PEG)-electrolyte solution is widely used worldwide for bowel cleansing. By consensus of the American Society for Gastrointestinal Endoscopy, the American Society of Colon and Rectal Surgeons, and the Society of American Gastrointestinal and Endoscopic Surgeons, PEG-electrolyte solution is the gold standard for colonoscopic bowel preparation (Grade I A), and aqueous sodium phosphate (NaP) is an alternative regimen to PEG-electrolyte solutions (Grade I A)[1]. Several meta-analyses on the available bowel preparations have favored NaP, concluding that it was effective and better tolerated by patients than PEG-electrolyte solution[2-4]. However, the disadvantages of NaP are its associated side effects. Significant changes in serum electrolyte levels[5], even in patients without renal failure, have prompted recommendations for serum electrolyte evaluation prior to the administration of NaP[6,7].

On the other hand, osmotically balanced electrolyte lavage solutions offer safe and effective cleansing, but volume-related discomfort and adverse experiences have decreased the percentage of patients completing the pre-examination preparation[1,8,9]. This is mainly due to the large volumes of fluid required for bowel preparation, the unpleasant taste, and an increase in the incidence of side effects[10]. Although 3-4 L of this solution is used in Western countries, approximately 2 L of this solution, along with a laxative, is usually considered adequate for bowel preparation in Japan. Despite the lower volume in Japan, the need to drink such large volumes of liquid with an unpalatable taste has a negative impact on patient compliance[11]. Therefore, more effective bowel preparation regimens for colonoscopy are required to improve the acceptability and tolerability of the procedure.

Mosapride citrate (mosapride) is a selective 5-hydroxytryptamine 4 (5-HT4) receptor agonist. Mosapride enhances gastric emptying and motility by facilitating acetylcholine release from the enteric cholinergic neurons, without blocking the dopaminergic D2 receptors[12]. It is known to be effective in gastroesophageal reflux disease[13], functional gastrointestinal disorders, such as functional dyspepsia[14], chronic gastritis with delayed gastric emptying, and diabetic gastroparesis[15]. Since 5-HT4 receptors are also located in the human colon and rectum[16,17], mosapride is also expected to have a prokinetic effect on the colo-rectum. A few clinical studies have reported that mosapride in combination with PEG-electrolyte solution may enhance bowel cleansing and improve patient acceptability and tolerability[18,19]. Furthermore, we previously conducted a randomized, double-blind, placebo-controlled study with mosapride in addition to PEG-electrolyte solution and demonstrated that co-administration of mosapride with PEG-electrolyte solution improved the quality of bowel preparation for colonoscopy, especially in patients without severe constipation[20]. Among the subgroup that had undergone previous colonoscopy, a significantly higher number of mosapride-group patients than placebo-group patients felt that the current preparation was easier. However, mosapride could not improve symptoms such as nausea, abdominal distension, abdominal pain, and willingness to repeat the same regimen compared with placebo. In short, mosapride did not sufficiently improve patient acceptability and tolerability. Therefore, it appears that it is necessary to reduce the volume of PEG-electrolyte solution to improve patient acceptability and tolerability.

The aim of this study was to evaluate the reduction of PEG-electrolyte solution volume when combined with mosapride citrate for colonoscopy preparation.

MATERIALS AND METHODS

This was a prospective, randomized, investigator-blinded study, comparing 1.5 L PEG plus mosapride (1.5 L group), with 2 L PEG-electrolyte solution plus mosapride (2 L group) dosing for patients who underwent colonoscopy. All patients provided written, informed consent prior to entering the study. The study was conducted at Aichi Cancer Center Hospital (ACCH), Nagoya, from January 2010 to June 2010, and was reviewed and approved by the ethics committee of ACCH. This trial was registered in an international clinical trial registry (UMIN000001556).

Study population

All consecutive outpatients of both sexes, aged 20 to 80 years, who were scheduled for screening or diagnostic colonoscopy at ACCH were evaluated for inclusion in the study. Patients with the following clinical features were excluded: significant cardiac, renal, hepatic, or metabolic co-morbidities, ascites, severe constipation (< 2 bowel movements a week), known allergy to PEG-electrolyte solution, history of gastric stapling or bypass procedure, or a history of prior colonic or rectal surgery. Patients were excluded if there was a suspected diagnosis of intestinal obstruction because of advanced colorectal cancer.

Randomization and blinding

Patients were randomly allocated to receive one of two different bowel preparation regimens using a computer-generated random-number list. Patients were randomized in block sizes of two, with serially numbered, sealed, opaque envelopes. Concealed allocation was accom-

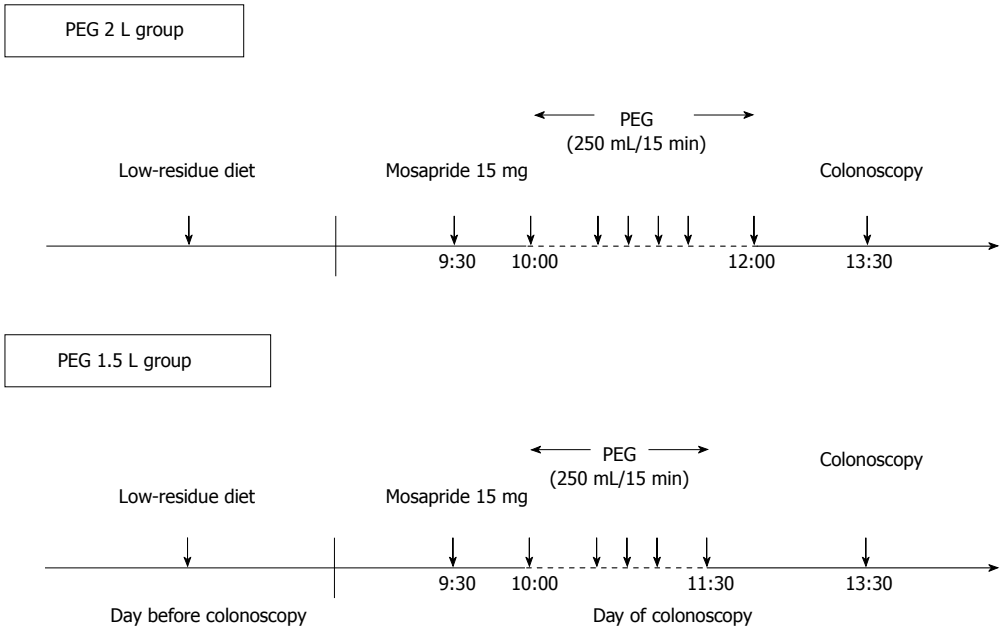

Figure 1 Steps in preparation for colonoscopy. PEG: Polyethylene glycol.

plished through non-research personnel who were not involved in this study. Patients were instructed not to discuss their bowel preparation with anyone other than the unblinded research assistant. With the exceptions of the patient and the unblinded research assistant, all other individuals participating in this study, including the endoscopists and endoscopy nurses, were blinded to the allocated treatment group. Comparisons between the 1.5 L group and the 2 L group were made in an investigator-blind fashion.

Bowel preparation methods

The day before colonoscopy, all patients were instructed to eat a pre-packaged, low residue diet (Enimaclin CS; Horii Pharmaceutical Ind., Ltd., Osaka, Japan) that consisted of a lunch, snack, and dinner, and asked to drink more than 2 liters of clear liquid. On the day of the colonoscopy, all participants reported to the endoscopy room at 9:00 am and received in-hospital bowel preparation. In-hospital preparation was important to ensure uniformity and remove any confounding caused by poor patient adherence. More than 10 toilet facilities were made available in the endoscopy unit for patient comfort. Six mosapride tablets (15 mg) (Gasmotin; Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) were administered orally with water at half past nine. After 30 min, both groups were instructed to drink 0.25 L of PEG-electrolyte solution (Niflec; Ajinomoto Pharmaceuticals Co., Ltd. Tokyo, Japan) every 15 min (Figure 1). Colonoscopies were performed from half past thirteen, and the start times were recorded for each patient.

Evaluation of bowel preparation

The efficacy of bowel preparation was assessed using the Aronchick scale[21]. Participating endoscopists were trained to use the Aronchick scale to achieve a good level of agreement. Investigators performed calibration exercises involving more than 20 colonoscopies prior to study commencement, based on their interpretation of scale anchors, to ensure that their findings agreed. The final assessment of bowel preparation was divided into two categories, adequate and failure. Bowel preparation rated as fair, good, or excellent, based on the Aronchick scale, was considered adequate; poor or inadequate ratings were considered failure. After colonoscopy, two observers, one who was the operator and the other who was a fellow in the procedure room, decided the score by mutual agreement. They scored the quality of the preparation on the right side (proximal to splenic flexure) colon and on the left side (distal to splenic flexure) colon and rectum separately. If the decision was discordant, a third expert reviewer graded and scored the recorded images later, and this evaluation was used in the final analysis. Twelve experienced colonoscopists carried out all colonoscopy procedures, each of whom had performed more than 1000 colonoscopies.

During or immediately following the colonoscopy, the investigator completed a physician questionnaire regarding assessment of bowel preparation, amount of irrigation fluid used, time needed to reach the cecum, and ease of insertion to the cecum and difficulty in observing the lumen of the colo-rectum because of peristalsis.

Patient tolerance and other measurements

The nursing staff recorded the time required to drink the indicated volume of lavage solution. They also recorded the time and number of bowel movements from the start of ingestion to the appearance of clear excretion. Until one hour after finishing the PEG-electrolyte solution plus mosapride, the nursing staff checked patients' excretions.

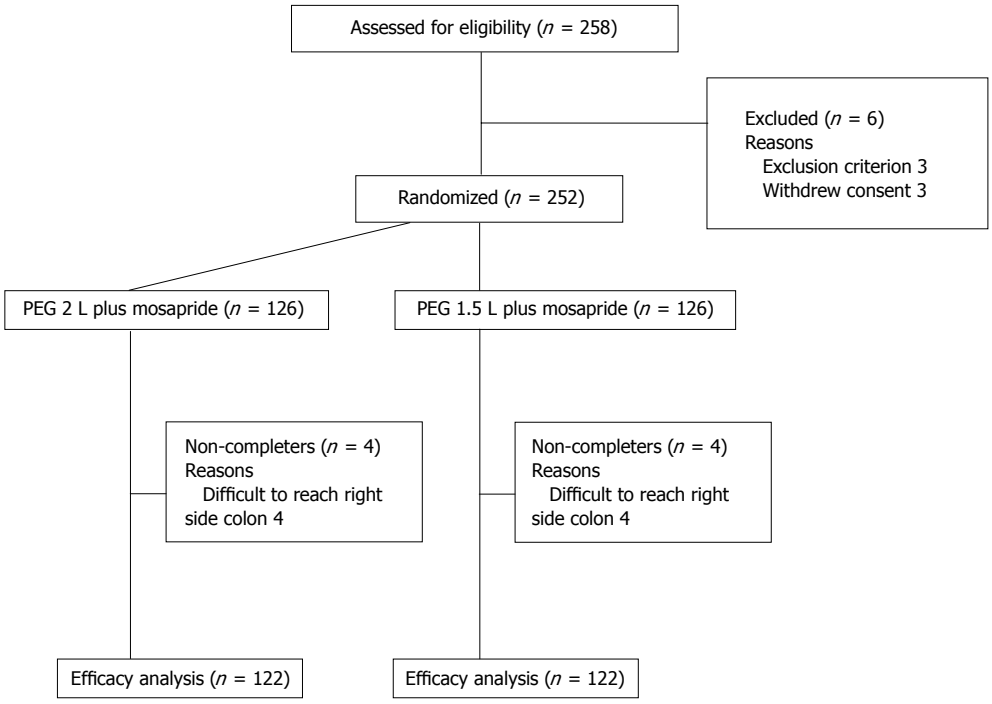

Figure 2 Patient disposition flow chart. PEG: Polyethylene glycol.

If there was a solid stool with muddy excretions or no excretion at that time, the patient was given an additional preparation, such as additional PEG-electrolyte solution or enemas. The patients who received an additional preparation were defined by the Aronchick scale as inadequate. The patient questionnaire consisted of 20 questions[20]. The adverse events were scored using a 4-point scale, where 1 = none, 2 = mild, 3 = moderate, and 4 = severe. The patients completed the questionnaire form before undergoing colonoscopy and submitted it to the nursing staff.

End points

The primary end point was the difference in the rate of adequate colon cleansing between the 1.5 L PEG-electrolyte solution plus 15 mg of mosapride (1.5 L group) and the 2 L PEG-electrolyte solution plus 15 mg of mosapride (2 L group). Secondary end points included differences in patients' acceptability and tolerance of solutions, time to first defecation, frequency of defecation, complete time for colonic preparation, time needed to reach the cecum, amount of irrigation fluid used, subjective difficulty in colonoscopy insertion to the cecum and in observing the lumen of the colo-rectum because of peristalsis, and disease detection rates.

Statistical analysis

Based on a previous study[20], the adequate bowel preparation rate for the PEG-electrolyte solution plus mosapride was expected to be less than 80%. It was expected that about 80% of the 1.5 L group would be judged adequate, and the non-inferiority margin was set at -15%. This study was designed to have 80% power to establish non-inferiority (using a one-sided significance level of 0.025 and a target sample size of 250).

The primary efficacy analysis was based on an intent-to-treat analysis and included patients who were randomized and received any treatment. In patients in this group, the preparation was classified as adequate or inadequate based on the colonoscopists' score of cleansing. Patients who did not undergo colonoscopy because of preparation-related adverse events, or preparation failure, or in whom the right colon could not be reached because of bowel obstruction or technical reasons were excluded. The rates of adequate preparation were compared between the groups by χ^2 test or Fisher's exact test for categorical variables.

For the secondary end points, the Mann-Whitney *U* test was used to compare continuous variables. Categorical variables were tested using the corrected χ^2 test or 2-sided Fisher's exact tests as appropriate. The criterion for significance was $P < 0.05$.

All statistical analyses were performed using Statistical Analysis Software (SAS Ver. 9.2 for the PC, SAS Institute Inc., Cary, NC, United States).

RESULTS

Patients' characteristics

A total of 252 patients was randomized into two groups (Figure 2). Although 252 patients were analyzed, colonoscope insertion to the right colon failed in 4 patients in each group (advanced stenosing cancer in two, and patient refusal in six because of pain). These eight patients were excluded from the efficacy analysis. The baseline characteristics of the patients are shown in Table

Table 1 Baseline characteristics

Variable	2.0 L	1.5 L	*P* value
Patients (*n*)	126	126	
Age (yr, mean ± SD)	65.3 ± 9.9	66.3 ± 9.6	NS
< 60	30	25	
60 - < 70	51	44	NS
⩾ 70	45	53	
Male	77	67	NS
Female	49	59	
Indication (*n*)			
Screening	40	41	
Surveillance	67	63	NS
Diagnostic	19	22	
Body mass index (kg/m^2)	22.5 + 3.2	22.3 + 2.7	NS
Previous colonoscopy			
None (first time)	44	41	NS
⩾ 2	82	85	
Previous preparation for colonoscopy (*n*)			
2L PEG	82	85	NS

PEG: Polyethylene glycol; NS: Not significant.

Table 2 Overall colon-cleansing efficacy *n* (%)

Variable	Right side colon		Left side colon and rectum		*P* value[1]	
	2.0 L	1.5 L	2.0 L	1.5 L	Right	Left
Patients (*n*)	122	122	122	122		
Overall score (*n*)						
Excellent	36	22	48	37		
Good	52	38	49	45		
Fair	20	41	12	17	0.006	NS
Poor	3	3	2	5		
Inadequate	11	18	11	18		
No. adequate	108 (88.5)	101 (82.8)	109 (89.3)	99 (81.1)	NS	NS

[1]*P* value by χ^2 test. PEG: Polyethylene glycol; NS: Not significant.

1. There was no significant difference in age, sex, body mass index, number of previous colonoscopies, and the preparation method used previously between the 2.0 L and 1.5 L groups.

Bowel cleansing efficacy

The efficacy of bowel preparation is shown in Table 2. The adequate bowel preparation rates were 88.5% in the 2 L group and 82.8% in the 1.5 L group (95% lower confidence limit, lower confidence limit (LCL), for the difference = -14.5%, non-inferiority $P = 0.019$) in the right side colon. In the left colon, the adequate bowel preparation rates were 89.3% in the 2 L group and 81.1% in the 1.5 L group (95% LCL = -17.0%, non-inferiority $P = 0.066$). In the right side colon, there were significant differences in the proportion of the overall colon-cleansing score between the two groups ($P = 0.006$). Eleven patients (8.7%) required additional preparation in the 2 L group. On the other hand, 18 patients (14.3%) required additional preparation in the 1.5 L group. However, there was no significant difference in required additional preparation between the two groups.

As shown in Table 3, there were no differences in frequency of defecation, time needed to reach the cecum, elapsed time from last fluid intake to colonoscopy, amount of irrigation fluid used, and subjective difficulties in insertion to the cecum and in observing the lumen of the colo-rectum between the two groups.

Patient acceptability, tolerability, and safety

There was no significant difference in compliance as defined by > 80% intake of the prescribed PEG-electrolyte solution volume. However, complete (100%) intake of the PEG solution was significantly higher in the 1.5 L group than in the 2 L group ($P = 0.002$) (Table 3). The proportion of abdominal distension was significantly less in the 1.5 L group than in the 2 L group ($P = 0.040$), but symptoms such as nausea, vomiting, abdominal pain, and circulatory reactions were similar in both groups. The proportion of patients willing to repeat the same preparation regimen was significantly higher in the 1.5 L group ($P = 0.034$). Furthermore, among the subgroup of patients who had undergone colonoscopy more than twice previously, a significantly higher number of patients in the 1.5 L group than in the 2 L group felt that the current preparation was easier than in the past ($P = 0.001$).

Disease detection rate

In this study, 11 colorectal cancers were detected in 11 patients (4.4%), 4 (3.2%) in the 2 L group and 7 (5.6%) in the 1.5 L group (Table 4). A total of 177 polyps was detected in 74 patients (58.7%) in the 2 L group, and 187 polyps were detected in 73 patients (57.9%) in the 1.5 L group. The proportions of polyps by size and location were similar in the two groups.

DISCUSSION

In this study, 1.5 L PEG-electrolyte solution plus mosapride was found to be non-inferior to 2 L PEG-electrolyte solution plus mosapride with respect to adequate bowel preparation rates only in the right colon, not in the entire colo-rectum. On the other hand, patient tolerability, especially abdominal distension, and acceptability were superior in the 1.5 L group compared to the 2 L group.

This is the first study, to the best of our knowledge, that has evaluated the effect of mosapride when used in conjunction with reduced dose, 1.5 L PEG-electrolyte solution for colonoscopy preparation. We previously conducted a randomized, double-blind, placebo-controlled study with mosapride in addition to PEG-electrolyte solution and demonstrated that co-administration of mosapride with PEG-electrolyte solution improved the quality of bowel preparation for colonoscopy, especially in patients without severe constipation[20]. On the other hand, the beneficial effect of mosapride on gastric emptying[22] was expected to ameliorate nausea, vomiting, and fullness of the abdomen during bowel preparation. Mishima *et al*[19] showed that administration of mosapride prior to PEG-electrolyte solution significantly decreased the incidence of uncomfortable abdominal symptoms. However, there were no significant differences in the

Table 3 Results of preparation, endoscopic findings and patient questionnaire *n* (%)

Variable	2.0 L	1.5 L	*P* value
Patients	126	126	
Time to first defecation (min, mean ± SD)	55.7 ± 27.4	56.4 ± 27.8	NS
Frequency of defecation (times, median, quartile)	7 (4-15)	7 (4-15)	NS
Time to preparation (min, mean ± SD)	157.3 ± 51.9	159.6 ± 57.1	NS
Elapsed time from last fluid intake to colonoscopy (min, mean ± SD)	169.4 ± 56.5	179.7 ± 61.1	NS
Cecal intubation rate	122 (96.8)	122 (96.8)	NS
Insertion time (min, median, quartile)[1]	11.4 (3-76)	10.1 (3-47)	NS
Feel of peristalsis	20 (16.4)	25 (20.5)	NS
Amount of irrigation fluid			
None	38	41	NS
< 50 mL	74	74	
50-100 mL	9	9	
> 100 mL	5	2	
Compliance > 80%	121 (96.0)	125 (99.2)	NS
100% intake	108 (85.7)	122 (96.8)	0.002
Any symptom			
Nausea (none/mild/moderate/severe)	109/13/3/1	117/5/3/1	NS
Vomiting (none/mild/moderate/severe)	0/0/1/0	0/0/0/0	NS
Distension (none/mild/moderate/severe)	36/65/22/3	58/48/18/2	0.04
Abdominal pain (none/mild/moderate/severe)	98/26/2/0	107/18/1/0	NS
Circulatory reactions (none/mild/moderate/severe)	0	0	NS
Willingness to repeat			
The same preparation regimen (much/fair/never)	78/19/29	97/12/17	0.034
How easy/difficult to take preparation compared to previous (easy/no difference/difficult)	23/54/5	46/36/3	0.001

[1]Insertion time was calculated without including the patients whose cecal portion was not examined. NS: Not significant.

Table 4 Characteristics of the endoscopic diagnosis *n* (%)

Variable	2.0 L		1.5 L		*P* value (2.0 L *vs* 1.5 L)	
	Right side colon	Left side colon	Right side colon	Left side colon	Right	Left
Patients	126		126			
Cancer patients	3	1	5	2[1]	NS	NS
Polyp patients	74 (58.7)		73 (57.9)			
Proportion of polyps						
< 5 mm	60	62	65	70		
5-10 mm	15	29	20	19	NS	NS
> 10 mm	6	5	8	5		
Total polyps per study arm	81	96	93	94	NS	NS
Polyps per patient, mean ± SD	0.71 ± 1.19	0.79 ± 1.20	0.78 ± 1.19	0.78 ± 1.11	NS	NS
Diverticulosis	30		37			

[1]Advanced stenosing cancer. NS: Not significant.

frequencies of these symptoms between the mosapride group and the placebo group in the previous study[20]. Therefore, we think that there is a need to reduce the volume of PEG-electrolyte solution to improve patients' acceptability and tolerability. In the present study, it was assumed that 2 L PEG-electrolyte solution plus mosapride was the standard regimen for bowel preparation based on the results of the previous study. Thus, the study was designed to compare a 1.5 L PEG group with a 2 L PEG group.

In the present study, the patients' acceptability and tolerability were superior in the 1.5 L group. The 0.5 L reduction of PEG-electrolyte solution significantly improved patients' acceptability and tolerability; 100% intake of PEG-electrolyte solution was significantly higher in the 1.5 L group than in the 2 L group. For Japanese patients with relatively smaller physiques than Western patients, 2 L PEG-electrolyte solution may be too much to drink. With respect to adverse events, abdominal distension was more common than nausea, vomiting, and abdominal pain. The proportion of abdominal distension was significantly improved in the 1.5 L group compared with the 2 L group. This may be the reason why willingness to repeat the same preparation regimen was significantly higher in the 1.5 L group, and a significantly higher number of patients in the 1.5 L group than in the 2 L group felt that the current preparation was easier.

Although 0.5-L volume reduction improved patient acceptability and tolerability, it would not make sense to decrease the volume of solution if the adequate bowel

preparation rates were worse, and it prolonged the time to preparation. In the present study, 1.5 L PEG was non-inferior to 2 L PEG with respect to adequate bowel preparation rates in the right colon, but the proportion for the overall colon-cleansing score was significantly higher in the 2 L PEG group than in the 1.5 L PEG group. Furthermore, although there was no significant difference between the two groups, 18 patients required additional preparation in the 1.5 L group compared with 11 patients in the 2 L group. One of the reasons why the times to preparation were similar in the two groups is that it took longer for the patients who required additional preparation in the 1.5 L group compared with the 2 L group. From these results, we cannot help but recognize that the dose of 15 mg may be insufficient to compensate for the 0.5-L reduction in PEG solution with respect to cleansing efficacy. In the present study, 15 mg of mosapride was given for colonoscopy preparation. The dose of 15 mg per day is the recommended usual dosage of mosapride citrate for adult patients with chronic gastritis. Since the effects of mosapride are reported to be dose-dependent[23], additional studies that address the optimal dosage are required to clarify the best bowel preparation method for colonoscopy.

Over the years, many researchers have investigated several different combinations and dosages of prokinetic agent or laxatives in search of acceptable, tolerable, and efficacious low-volume bowel preparation that may lead to a better experience for the patient and a more thorough colonoscopic examination[24-26]. Cisapride has been used as a prokinetic agent along with lavage solution for bowel preparation and has been demonstrated to shorten the required time period for precolonoscopic bowel preparation and to decrease the lavage solution volume[27,28], although these results have been difficult to reproduce[29]. However, cisapride was withdrawn from the market because of severe cardiac effects[30]. Other prokinetic agents, including metoclopramide and tegaserod, have been co-administered with oral lavage solution in an attempt to improve the quality of bowel preparation and patient tolerance to lavage solution through increasing the amplitude of gastric contraction and peristalsis of small intestine, and shortening transit time[31,32]. However, the effect of these agents had not yet been clearly established, and the results of studies that have evaluated these agents have thus far been contradictory[33]. The effects of prokinetic agents with the reported timings and doses may not be enough to compensate for the large volume of PEG solution.

Stimulant laxatives such as bisacodyl and magnesium citrate have been used as adjuncts to low-volume PEG-electrolyte solution, achieving results similar to those with full-volume PEG-electrolyte solution[8,34]. Recently, Cohen *et al*[35] compared a reduced-dose 2 L PEG formulation plus ascorbic acid with 2 L PEG formulation plus bisacodyl. The authors found that the use of PEG plus ascorbic acid resulted in better colon cleansing and higher adenoma detection rates than PEG plus bisacodyl. Moreover, Repici *et al*[36] compared a new iso-osmotic sulphate-free formulation (2 L formulation of PEG-citrate-simethicone) in combination with bisacodyl with 2 L formulation PEG plus ascorbic acid. The authors reported that low-volume PEG-citrate-simethicone with bisacodyl provided better bowel cleansing and similar tolerability and acceptance compared with PEG plus ascorbic acid. Unfortunately, these low-volume formulations are currently not available in Japan. In the previous study[20], we selected mosapride from among several prokinetic agents because it is a highly selective agonist for 5-HT4 receptors and does not affect other receptors, including dopamine D2 receptors. However, the results of this study did not demonstrate the efficacy of mosapride in reducing lavage solution volume. A combination with some laxatives may improve the cleansing efficacy of our low-volume 1.5 L PEG formulation plus mosapride.

Few studies designed to assess the quality of bowel preparation for colonoscopy have also examined the disease detection rates, including adenoma detection rates[37-39]. Previous studies demonstrated that a better bowel preparation led to a higher rate of colon lesion detection, enhancing the ability to discern smaller lesions and thus improving the thoroughness of colonoscopy[37,38]. In the present study, there were no differences between the two groups in the polyp detection rate, the proportion of the size of polyps, total polyps per study arm, and polyps per patient. These findings may lead to the conclusion that the efficacy of bowel preparation with 1.5 L PEG is non-inferior to 2 L PEG with respect to bowel cleansing. However, polyp detection rates are indeed affected by several variables, such as patients' background, colonoscopy indication, and endoscopist technique, as well as endoscopy technology, that would introduce uncertainly into the results of this study. Additional studies are necessary to demonstrate the relationship between bowel preparation and the adenoma detection rate.

There are several limitations to consider in interpreting the present results. First, the study was conducted in a single hospital with a small number of patients. Although we hypothesized that the non-inferiority margin was -15%, that margin might be inappropriate. The sample size may have been too small to elucidate the non-inferiority of 1.5 L PEG, which may explain why non-inferiority in only a limited part of colon could be demonstrated. Second, for the evaluation of bowel preparation, the Aronchick scale was used. The merit of the Aronchick scale is that in cases in which additional bowel preparation was needed, such cases could be defined as "inadequate" using the Aronchick scale. However, the Aronchick scale was designed to assess bowel preparation of the entire colon. In the present study, it was scored separately on the right side colon and left side colon; a different scoring system, such as the Ottawa scale[40] and the Boston scale[41], that evaluates different colon segments individually and generates a summary score may have been more appropriate. Finally, biochemical parameters were not evaluated in the two groups. However, co-administration of mosapride at a dose of 40 mg and

PEG-electrolyte solution is already approved in Japan for barium enema examination preparation[42], based on its excellent safety profile.

In conclusion, co-administration of mosapride with 1.5 L PEG-electrolyte solution was non-inferior to mosapride with 2.0 L PEG-electrolyte solution for adequate bowel preparation rates only in the right colon, although better acceptability and tolerability compared to the larger PEG-electrolyte solution volumes were found. A mosapride dose of 15 mg may provide insufficient cleansing efficacy to compensate for a 0.5-L reduction in PEG-electrolyte solution.

COMMENTS

Background

Although polyethylene glycol (PEG) electrolyte solution has been used for colonoscopy preparation since 1980, the need to drink large volumes is a limiting factor that affects patient tolerance. Low-volume bowel preparation regimens for colonoscopy are reported to improve patients' acceptance and compliance.

Research frontiers

Mosapride citrate (mosapride) is a selective 5-hydroxytryptamine 4 (5-HT4) receptor agonist. Mosapride enhances gastric emptying and motility by facilitating acetylcholine release from enteric cholinergic neurons, without blocking the dopaminergic D2 receptors. Since 5-HT4 receptors are also located in the human colon and rectum, mosapride is also expected to have a prokinetic effect on the colo-rectum.

Innovations and breakthroughs

The present randomized trial compared 1.5 L PEG plus mosapride with 2 L PEG plus mosapride dosing for patients who underwent colonoscopy in terms of cleansing effectiveness, patient compliance, tolerability, and disease detection rates.

Applications

The low-volume preparation (1.5 L PEG) represents a valid alternative to high-volume preparations (2 L PEG) with regard to patient compliance and tolerability. However, optimal visualization of colonic wall seems to be one of the primary advantages of high-volume preparations. A mosapride dose of 15 mg may provide insufficient cleansing efficacy to compensate for a 0.5-L reduction in PEG solution.

Terminology

PEG-electrolyte solution: PEG-electrolyte solution is used worldwide for bowel cleansing. Approximately 2 L of this oral solution with some laxatives are usually required for adequate bowel preparation in Japan.

Peer review

This randomized trial compared 1.5 L PEG plus mosapride with 2 L PEG plus mosapride in terms of cleansing effectiveness, patient acceptability, physical tolerability, and endoscopic findings. This is an interesting and well written study. The conclusion sounds good and useful for the general practice.

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BRIEF ARTICLE

Effectiveness of gastric cancer screening programs in South Korea: Organized *vs* opportunistic models

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Abstract

AIM: To investigate the outcome and effectiveness of two screening programs, National Cancer Screening Program (NCSP) and opportunistic screening (OS), for the detection of gastric cancer.

METHODS: A total of 45 654 subjects underwent upper endoscopy as part of the NCSP or OS at the Chung-Ang University Healthcare System in Korea between January 2007 and December 2010. The study population was comprised of subjects over the age of 40 years. More specifically, subjects who took part in the NCSP were Medicaid recipients and beneficiaries of the National Health Insurance Corporation. Still photographs from the endoscopies diagnosed as gastric cancer were reviewed by two experienced endoscopists.

RESULTS: The mean age of the screened subjects was 55 years for men and 54 years for women. A total of 126 cases (0.28%) of gastric cancer were detected from both screening programs; 100 cases (0.3%) from NCSP and in 26 cases (0.2%) from OS. The proportion of early gastric cancer (EGC) detected in NCSP was higher than that in OS (74.0% *vs* 53.8%, $P = 0.046$). Among the 34 416 screenees in NCSP, 6585 (19.1%) underwent upper endoscopy every other year as scheduled. Among the 11 238 screenees in OS, 3050 (27.1%) underwent upper endoscopy at least once every two years during the study period. The detection rate of gastric cancer was found to be significantly higher during irregular follow-up than during regular follow-up in both screening programs (0.3% *vs* 0.2%, $P = 0.036$). A higher incidence of EGC than advanced gastric cancer was observed during regular follow-up compared with irregular follow-up.

CONCLUSION: Compliance to the screening program is more important than the type of screening system used.

Key words: Gastric cancer; National Cancer Screening Program; Opportunistic screening; Early gastric cancer

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INTRODUCTION

Gastric cancer is the second most common cause of cancer death worldwide, and East Asian countries, such

as China and Japan, have high incidence rates of gastric cancer[1,2]. Although the incidence has declined in recent decades, gastric cancer remains the most frequently diagnosed form of cancer in South Korea[3-6]. The symptoms of gastric cancer appear quite late; in fact, a large number of advanced gastric cancer cases, as well as most early gastric cancer (EGC) cases, show no symptoms. Thus, gastric cancer screening is extremely important for the early detection of gastric cancer.

Screening can be defined as the systematic application of a test or inquiry to identify individuals at sufficient risk of a specific disorder who will benefit from further investigation or direct preventive action, among those who have not sought medical attention[7]. Some countries, such as Japan and South Korea, provide gastric cancer screening to populations with an average risk to reduce the disease burden.

Cancer screening may be offered in an organized or opportunistic model[8]. An organized model is primarily distinguished from opportunistic screening (OS) in that invitations to screening are issued from population registers[9]. Organized screening programs have nationally implemented guidelines defining who should be invited, how frequently they should be screened, and how any abnormalities detected on screening should be followed up and treated[8,10]. In South Korea, a nationwide gastric cancer screening program began in 1999 as part of the National Cancer Screening Program (NCSP). NCSP provides screening services free of charge for Medicaid enrollees and people with National Health Insurance with a premium below 50%. The NCSP recommends biennial upper endoscopy or upper gastrointestinal series for men and women aged $\geqslant$ 40 years.

In addition to the organized screening program, OS is also widely available in South Korea. OS programs involve various options in terms of the items screened, intervals between screening, and costs, depending on the preference by the individual, but these services are entirely paid by the users[11]. OS has been highly implemented owing to the low cost and easy accessibility of endoscopy. However, OS involves fewer formal decisions on whether to screen, whom to screen, and at what intervals the screening should be done. Moreover, the evidence and efficiency of this examination tend to be underestimated.

Although screening programs are conducted widely in South Korea, the utilization of such screening programs, including both organized and opportunistic programs, is still unclear[11]. Even though there have been some studies carried out on the characteristics of gastric cancer detected by mass screening, there is neither a study evaluating the efficiency of gastric cancer screening programs or comparison between organized and OS. Therefore, in this study, we investigated the current features of gastric cancer screening programs in South Korea, in terms of the outcome and effectiveness of NCSP and OS conducted at a single center.

MATERIALS AND METHODS

Subjects

The present study was conducted by reviewing medical records. A total of 45 991 subjects underwent upper endoscopy as part of the NCSP or OS at the Chung-Ang University Healthcare System in South Korea between January 2007 and December 2010. The study population was comprised of subjects over the age of 40 years. More specifically, subjects who took part in the NCSP were Medicaid recipients and beneficiaries of the National Health Insurance Corporation. Still photographs from the endoscopies diagnosed as gastric cancer were reviewed by two experienced endoscopists. The gross shape of the lesion was determined as either EGC or advanced gastric cancer (AGC). If there was a discrepancy between the two endoscopists' findings, a final decision was made after further discussion.

Statistical analysis

Statistical analyses in this study were conducted using the SPSS version 12.0 software package (SPSS, Chicago, IL, United States). Categorical data analysis was conducted using the χ^2 test or Fisher's exact test. Continuous data were analyzed using Student's *t*-test. Continuous variables measured in this study are expressed as mean ± SD. All *P* values were 2-tailed. *P* values of less than 0.05 were considered statistically significant. The study was approved by the Institutional Review Board of the Chung-Ang University Medical Center in South Korea.

RESULTS

Demographics of the subjects

A total of 45 991 subjects were screened for gastric cancer during the study period. Of these, 34 481 subjects were included in NCSP and 11 238 subjects in OS. In NCSP, 275 subjects were excluded due to previous gastric surgery (n = 233) and endoscopic resection (n = 42). In OS, 62 subjects were excluded due to previous gastric surgery (n = 45) and endoscopic resection (n = 17). A total of 45 654 subjects (34 416 in NCSP and 11 238 in OS) were subsequently enrolled in this study. The mean age of the screened subjects was 55.1 years for men and 54.4 years for women. The male to female ratio of the screened subjects was 0.7:1. The mean age of the subjects was 56.2 ± 9.2 years in NCSP and 50.0 ± 8.0 years in OS, respectively. In addition, the male to female ratio was 0.5 in NCSP and 1.4 in OS, respectively. The demographics of the subjects are shown in Table 1.

Participation rate of the subjects

In NCSP, a total of 6585 subjects (19.1%) underwent upper endoscopy every other year as scheduled. Their mean age was 58.7 ± 8.3 years, and the male to female ratio was 0.5:1. In contrast, in OS, a total of 3050 subjects (27.1%) underwent upper endoscopy at least once

Table 1 Demographics of participants *n* (%)

	National Cancer Screening Program	Opportunistic screening	*P* value
No. of subjects	34 416 (75.4)	11 238 (24.6)	
Age (yr), mean ± SD	56.2 ± 9.2	50.0 ± 8.0	< 0.001
40-49	8723 (25.4)	6159 (54.8)	
50-59	12 491 (36.3)	3623 (32.2)	
60-69	10 645 (30.9)	1165 (10.4)	
70+	2557 (7.4)	291 (2.6)	
Sex			< 0.001
Male	11 987 (34.8)	6668 (59.3)	
Female	22 429 (65.2)	4570 (40.7)	
No. of regular follow-up	6585 (19.1)	3050 (27.1)	< 0.001
Gastric cancer	100 (0.3)	26 (0.2)	0.299
Endoscopic feature			0.046
EGC	74 (74.0)	14 (53.8)	
AGC	26 (26.0)	12(46.2)	

EGC: Early gastric cancer; AGC: Advanced gastric cancer.

Table 2 Outcomes according to compliance to screening systems *n* (%)

	Regular follow-up	Irregular follow-up	*P* value
No. of subjects	9635 (21.1)	36 019 (78.9)	
Age (yr), mean ± SD	54.4 ± 9.3	55.7 ± 9.1	< 0.001
40-49	2783 (28.9)	12 099 (33.6)	
50-59	3413 (35.4)	12 701 (35.2)	
60-69	2789 (28.9)	9021 (25.1)	
70+	650 (6.8)	2198 (6.1)	
Sex			< 0.001
Male	4170 (43.3)	14 485 (40.2)	
Female	5465 (56.7)	21 534 (59.8)	
Gastric cancer	17 (0.2)	109 (0.3)	0.036
Endoscopic feature			0.942
EGC	12 (70.6)	76 (69.7)	
AGC	5 (29.4)	33 (30.3)	

EGC: Early gastric cancer; AGC: Advanced gastric cancer.

every two years during the study period. Their mean age was 49.4 ± 7.1 years, and the male to female ratio was 1.3:1. Among these subjects, 140 subjects (0.6%) had undertaken upper endoscopy every year during the study period.

Gastric cancer detection rate

As shown in Table 1, gastric cancer was diagnosed in 126 (0.28%) of 45 654 subjects (87 men and 39 women). Their mean age was 60.3 ± 9.5 years. Of these, 88 subjects (0.19%) were endoscopically diagnosed with EGC and 38 subjects (0.08%) with AGC. The proportion of EGCs among the total cases of gastric cancer was 69.8%. Figure 1 shows the annual EGC/AGC ratio in each system.

A total of 100 cases of gastric cancer found in NCSP included 74 cases of EGC (74%) and 26 cases of AGC (26%). The EGC/AGC ratio was 2.9. On the other hand, 14 cases of EGC (53.8%) and 12 cases of AGC (46.2%) were found in OS. The EGC/AGC ratio was 1.2. Hence, the detection rate of EGC in NCSP was significantly higher than that in OS ($P = 0.046$).

Outcomes according to compliance

In this study, we defined regular follow-up as satisfying the biennial checks in NCSP and a check-up at least once every two years during the period in OS. Table 2 shows the outcomes according to compliance to the systems. As a result, their detection rates in regular follow-up were 0.12% for EGC and 0.05% for AGC, respectively. With regard to compliance to the system, EGC was found more than AGC during regular follow-up compared with irregular follow-up in both NCSP and OS.

Among the 100 cases of gastric cancer in NCSP, 10 cases (83.3%) of EGC and 2 cases (16.7%) of AGC were found during regular follow-up. On the other hand, 64 cases (72.7%) of EGC and 24 cases (27.3%) of AGC were found during irregular follow-up. As for OS, 2 cases (40%) of EGC and 3 cases (60%) of AGC were found during regular follow-up, whereas 12 cases (57.1%) of EGC and 9 cases (42.9%) of AGC were found during irregular follow-up. Annual follow-up revealed no cases of gastric cancer among the 140 subjects.

DISCUSSION

In this study, we measured the effectiveness of gastric cancer screening between NCSP and OS. The results showed no significant difference in the gastric cancer detection rate between NCSP and OS. However, the proportion of EGC was higher in NCSP than in OS. In addition, regular check-ups were important regardless of the screening system.

Screening has become an important component in health promotion programs along with the progress of diagnostic technology aimed at early detection of diseases[7]. Screening has the potential to produce benefits in preventing morbidity and mortality as long as the screening program fulfills certain conditions.

The epidemiology and natural history of the disease, including development from the latent to declared disease, should be adequately understood; and there should be a detectable risk factor, disease marker, latent period, or early symptomatic stage. All cost-effective primary prevention interventions should have been implemented as far as practicable. Therefore, many countries have developed a variety of screening programs as part of their preventive health services according to the recommendations of specialized committees[7]. For example, national cancer control programs should be organized to ensure that a large proportion of the target group is screened and that those individuals in whom abnormalities are observed receive appropriate diagnosis and therapy. Agreement should be reached on the guidelines for proper application of national cancer control programs. The purpose of gastric cancer screening is to reduce the cancer-related mortality, and the effect of gastric cancer screening should be evaluated on the basis of this purpose. From the perspective of the South Korean public

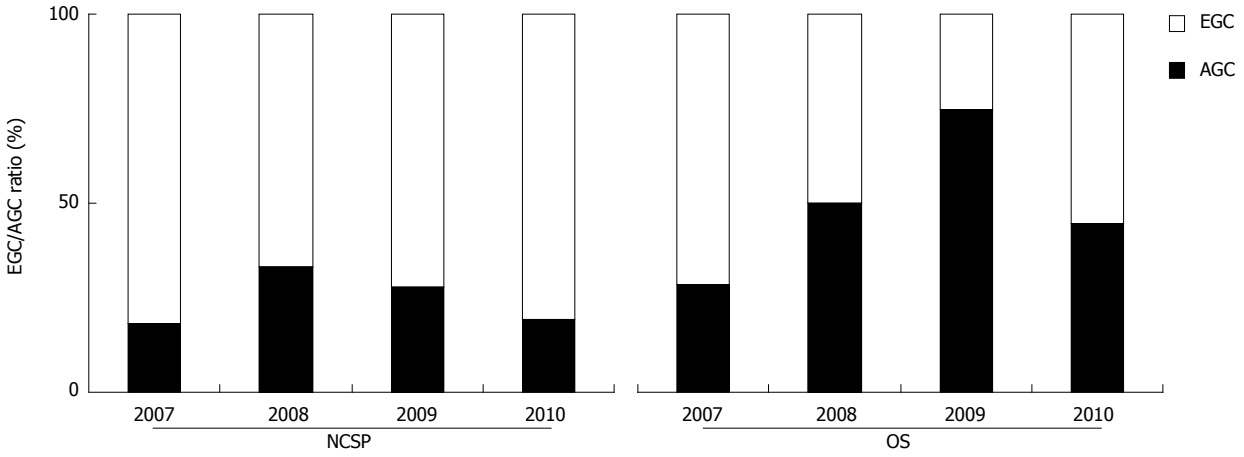

Figure 1 Annual early gastric cancer/advanced gastric cancer ratio in National Cancer Screening Program and opportunistic screening. The proportion of early gastric cancers (EGCs) among a total of 126 gastric cancers was 69.8%. The EGC/advanced gastric cancer (AGC) ratio in National Cancer Screening Program (NCSP) was 2.9, while the EGC/AGC ratio in opportunistic screening (OS) was 1.2. The detection rate of EGC in NCSP was significantly higher than that in OS (P = 0.046).

health care system, reducing the gastric cancer-related mortality rate appears to have the utmost importance[12].

Recently, endoscopy has replaced photofluorography as the initial mass screening method in South Korea. This method has become increasingly useful for gastric cancer screening owing to its high detection rate[13]. Considering the circumstances in South Korea that the cost of endoscopy is as low as that of photofluorography and the incidence of gastric cancer is still high, endoscopy is regarded as the most cost-effective screening method in South Korea. However, gastric cancer screening using endoscopy as a mass screening method has not yet been reported.

NCSP in South Korea has been available for several years, and has played a role in the early detection of gastric cancer. Apart from NCSP, OS has also contributed to screening gastric cancer in South Korea. Although the contents and process of the screening program are similar to those of the NCSP, there are some distinctive features in OS[7]. For this reason, some criticize that this unestablished guideline may drive the population into unnecessary socioeconomic consumption. Our hospital has run both NSCP and OS in the same healthcare system, which is rare in South Korea. A total of 8 well-trained gastroenterologists with at least 5 year of endoscopy experience performed upper endoscopy using a flexible endoscope (Q260 or Q240, Olympus Optical Co., Tokyo, Japan) in our healthcare system (NCSP and OS). They rotated between NCSP and OS as scheduled. Hence, there was little qualitative difference between NCSP and OS. Thus, this study could be regarded as a community-based study conducted for the local population.

So far, numerous studies on gastric cancer screening have focused on either OS or NCSP. Therefore, to our knowledge, this is the first study to compare the efficiency of two gastric cancer screening programs, NCSP and OS, in South Korea.

In this study, a total of 126 cases (0.28%) of gastric cancer were detected from both screening programs; 100 cases (0.3%) from NCSP and in 26 cases (0.2%) from OS during the study period. Screening for gastric cancer revealed that the proportion of EGC detected in NCSP was higher than that in OS (74.0% *vs* 53.8%, P = 0.046).

Kim *et al*[12] reported a significant relationship between the history of gastric examination and severity of gastric cancer. This suggests that gastric cancer screening is effective in detecting early stage gastric cancer. Unfortunately, an appropriate interval for screening gastric cancer has not been determined[14,15]. Despite the lack of evidence, screening for gastric cancer is conducted every other year in South Korea. Therefore, we compared the gastric cancer detection rates between NCSP and OS according to the frequency of check-ups.

We classified the subjects in each system according to the number of tests performed during the study period. This implies compliance to their target screening program. Regular follow-up was defined as satisfying the biennial check-ups in NCSP and a check-up at least once every two years during the period in OS. As a result, only 21.1% of subjects underwent endoscopy.

In this study, compliance to gastric cancer screening programs was determined to be as low as 19.1% in NCSP and 27.1% in OS, respectively. Hence, OS seems to be more effective regarding the examination interval. However, the detection rate of gastric cancer was significantly higher during irregular follow-up than during regular follow-up, regardless of the type of system (0.3% *vs* 0.2%, P = 0.036). While gastric cancer detected during the screening appears to have a good prognosis, several biases should be carefully assessed. Generally, individuals taking part in screening programs are healthier than those who do not. Selection bias has been discussed as a major limitation of case-control studies in which infor-

mation on confounding factors were not obtained[16,17]. Ideally, the effectiveness of a screening program is evaluated by an interventional study in which subjects are randomly allocated to screened and unscreened groups[18]. Due to the wide implementation of gastric cancer screening, however, no such interventional study has been carried out.

The present study had some limitations. First, this study had little information on risk factors. Second, gastric cancer was classified as EGC and AGC based on endoscopic findings, instead of surgical findings. Finally, this study did not cover the clinical course after diagnosis, including stage work-up, treatment, and prognosis, including survival. However, the present study reflects the features of presently available gastric cancer screening programs and may have an influence on future plans for cancer control.

In conclusion, this study demonstrated that NCSP was an effective screening system comparable to OS in the early detection of gastric cancer. The results suggest that compliance to the screening program is more important than the type of screening system itself. However, further studies on the efficiency and analysis of cost-effectiveness will be needed for successful progression of both systems. Furthermore, more comprehensive analyses with extensive nationwide data are warranted.

COMMENTS

Background

Gastric cancer remains one of the leading causes of cancer-related death worldwide. Currently, screening is performed in countries where gastric cancer is highly prevalent such as South Korea and Japan. In South Korea, cancer screening is widely available in two systems; organized cancer screening program, *e.g.*, National Cancer Screening Program (NCSP) and opportunistic screening (OS). However, there are no comparative studies on the effectiveness and outcomes of these two systems.

Research frontiers

Apart from government-led mass screening programs, OS programs by individual needs using an endoscopic examination have become common. However, OS has not been estimated in terms of clinical usefulness as well as cost-effectiveness. This study evaluated the effectiveness of screening systems with regard to early detection of gastric cancer and compliance to the system.

Innovations and breakthroughs

So far, most epidemiologic studies on gastric cancer screening have dealt with screening tools, screening interval and the cost-effectiveness of mass screening. However, little attention has been paid to the differences between two screening systems, *e.g.*, organized and opportunistic models. The authors made a comparison between these two systems and suggest that regular check-up is essential in the early detection of gastric cancer regardless of the screening system used.

Applications

The authors demonstrated that regular check-up is more important than the screening system in the early detection of gastric cancer. This work is interesting and will help health professionals establish a practical program for gastric cancer screening. Furthermore, it will contribute to the exploration of future strategies for gastric cancer prevention and control.

Terminology

NCSP is a cancer screening program which has been conducted by the South Korean government since 1999. OS is a screening program which depends on requests from individuals and has no guidelines.

Peer review

The authors have compared the effectiveness of NCSP and OS for gastric cancer screening. This paper has some valuable information and is worthy of publication.

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BRIEF ARTICLE

Probiotics increase T regulatory cells and reduce severity of experimental colitis in mice

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Abstract

AIM: To investigate the effect of probiotics on regulating T regulatory cells and reducing the severity of experimental colitis in mice.

METHODS: Forty C57/BL mice were randomly divided into four groups. Colitis was induced in the mice using 2,4,6-trinitrobenzene sulfonic acid (TNBS). After 10-d treatment with Bifico capsules (combined bifidobacterium, lactobacillus and enterococcus), body weight, colonic weight, colonic weight index, length of colon, and histological scores were evaluated. $CD4^+CD25^+Foxp3^+T$ cell in mesenteric lymph nodes were measured by flow cytometry, and cytokines in colonic tissue homogenates were analyzed by a cytometric bead array.

RESULTS: The colonic weight index and the colonic weight of colitis mice treated with Bifico were lower than that of TNBS-induced mice without treatment. However, colonic length and percent of body weight amplification were higher than in TNBS-induced mice without treatment. Compared with TNBS-induced mice without treatment, the level of $CD4^+CD25^+Foxp3^+T$ cells in mesenteric lymph nodes, the expression of interleukin (IL)-2, IL-4 and IL-10 in colonic tissues from colitis mice treated with Bifico were upregulated, and tumor necrosis factor-α and interferon-γ were downregulated.

CONCLUSION: Probiotics effectively treat experimental colitis by increasing $CD4^+CD25^+Foxp3^+T$ cell and regulating the balance of Th1 and Th2 cytokines in the colonic mucosa.

Key words: Probiotics; Ulcerative colitis; $CD4^+CD25^+T$ cell; Cytokine

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INTRODUCTION

Ulcerative colitis (UC) belongs to the inflammatory bowel disease (IBD) group, which are chronic, relapsing-remitting gastrointestinal diseases of unknown etiology[1,2]. The primary treatments of UC (san azosulfamide, immunodepressants and surgery) are usually not well tolerated,

have side effects and show a high relapse rate[3,4]. Many reports have indicated that the non-specific inflammation of UC is associated with bacterial overgrowth and dysbiosis[5,6]. As live microbial feed supplements, probiotics can beneficially affect the host by improving the balance of the intestinal microbial flora to effectively and securely treat UC in human and murids[1,7,8]. A pivotal event in the development of UC is that high sensitivity to intestinal symbiotic microbial flora induces serious autoimmunity enteritis, which inhibits the function or lowers the level of $CD4^+CD25^+$T cells. Re-import of $CD4^+CD25^+$T cells may prevent the occurrence or aggravation of IBD[9]. This could be accomplished by secreting suppressor cytokines to recovering immunological tolerance and maintain the stability of internal environment in the intestines[10,11].

To explore the effects of probiotics on regular intestinal mucosal internal environment, we observed the level of $CD4^+CD25^+$T cells in mesenteric lymph nodes and the expression of related cytokines in colonic mucosa from mice with colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS).

MATERIALS AND METHODS

Reagents

TNBS (batch number: 2508-19-2) was purchased from Sigma (St. Louis, MO, United States). Bifico Capsules (batch number: 20101002), which combine bifidobacterium, lactobacillus and enterococcus, was purchased from Bifico Pharma (Shanghai, China). Mesalazine (batch number: 110201) was purchased from Sunflower Pharma (Jiamusi, China). The mouse regulatory T cell staining kit (batch number: 88-8111-40) was purchased from eBioscience CO (San Diego, CA, United States). The BD cytometric bead array (CBA) and mouse Th1/Th2/Th17 Cytokine Kit (batch number: 23-12380-00) was from BD Biosciences (San Jose, CA, United States).

Experimental design

Forty C57/BL mice (half male and half female, weighing 22-26 g, the animal certificate number was SCXK 2009-0004) were provided from Sino-British Sippr/BK Lab Animal Co. Ltd. (Changsha, China), and randomly divided into four groups of ten animals each: the normal group (mice were challenged and administrated with physiological saline), the model group (mice were induced by TNBS without treatment), the Bifico group (mice were induced by TNBS and treated with 345 mg/kg Bifico capsules), the Mesalazine group (mice were induced by TNBS and treated with 300 mg/kg mesalazine). All animals were housed at 20 ± 1 ℃ with a humidity of 50% ± 5% in a 12 h light/dark cycle and fed *ad libitum* with standard mouse feed and water throughout the experiments. The experimental protocols were performed according to the Guidelines of the Jiangxi University of Traditional Chinese Medicine Animal Research Committee.

Experimental colitis in mice was induced with TNBS Solution (100 mg·kg^{-1} body TNBS dissolved in 0.15 mL 30% ethanol) by enema[12]. The mice were maintained in a head-down position for 1 min. After 24 h, mice in the treated groups were administrated Bifico capsules (345 mg/kg) or mesalazine (300 mg/kg) by lavage for 10 d, while mice in the normal and model group received the equivalent volume of physiological saline.

To evaluate the therapeutic effect of probiotics treatment on experimented colitis, body weight, colonic weigh index [colonic weigh index (%) = colonic weigh/body weigh × 100%], colonic length and histological score were analyzed. The histological scoring was performed by Professor Chun Xiao of the pathological department from Jiangxi University of Traditional Chinese Medicine, and was a total score of inflammatory cell infiltration and tissue damage, according to the study of Schmidt and his colleagues[13]. Infiltration scores were as follows: 0, no infiltration; 1, increased number of inflammatory cells in the lamina propia; 2, inflammatory cells extending into the submucosa; 3, transmural inflammatory infiltrates; and for tissue damage: 0, no mucosal damage; 1, discrete epithelial lesions; 2, erosions or focal ulcerations; 3, severe mucosal damage with extensive ulceration extending into the bowel wall[13].

On day 11, mice were euthanized, and mesenteric lymph nodes were separated and triturated with cold physiological saline in a mortar into a cell suspension. The cell suspension was filtered through a 300-micron screen mesh to measure $CD4^+CD25^+Foxp3^+$T cells. The colon from the ileocecal junction to the anus was excised, its length was measured, and then it was cut open longitudinally at the mesenteric attachment, washed with 5 mL of 0.01 mol/L phosphate buffered solution (PBS) (pH 7.4) to remove the fecal remnants and weighted to compute the colonic weigh index. Partial colons were fixed in PBS-buffered 4% paraformaldehyde, and then processed for paraffin embedding and cut into 5-μm thick sections. The sections were stained with hematoxylin and eosin. Other colons were made into tissue homogenates to analyze the expressions of the cytokines.

Assay of $CD4^+CD25^+Foxp3^+$T cells in mesenteric lymph nodes

Cells from mesenteric lymph nodes were resuspended in eFluor® NC Flow Cytometry Staining Buffer at a final cell concentration of 2×10^7/mL. fluorescein isothiocyanate anti-mouse $CD4^+$Ab (RM4-5, 0.125 μg/sample; eBioscience) and APC anti-mouse $CD25^+$Ab (PC61.5, 0.25ug/sample; eBioscience) were added to the cell suspension. Cells were centrifuged at 300 × *g* at 4 ℃ for 5 min, fixed using Fix/Perm Buffer (eBioscience) for at least 12 h at 4 ℃, and then incubated with PE-anti-mouse $Foxp3^+$Ab (FJK-16s, 0.5 μg/sample; eBioscience) for 30 min in the dark at 4 ℃. Cells labeled with PE rat IgG2a were used as the isotype negative control. Fluorescence-activated cell sorting analysis was performed on a FACSCalibur (BD Biosciences)[14].

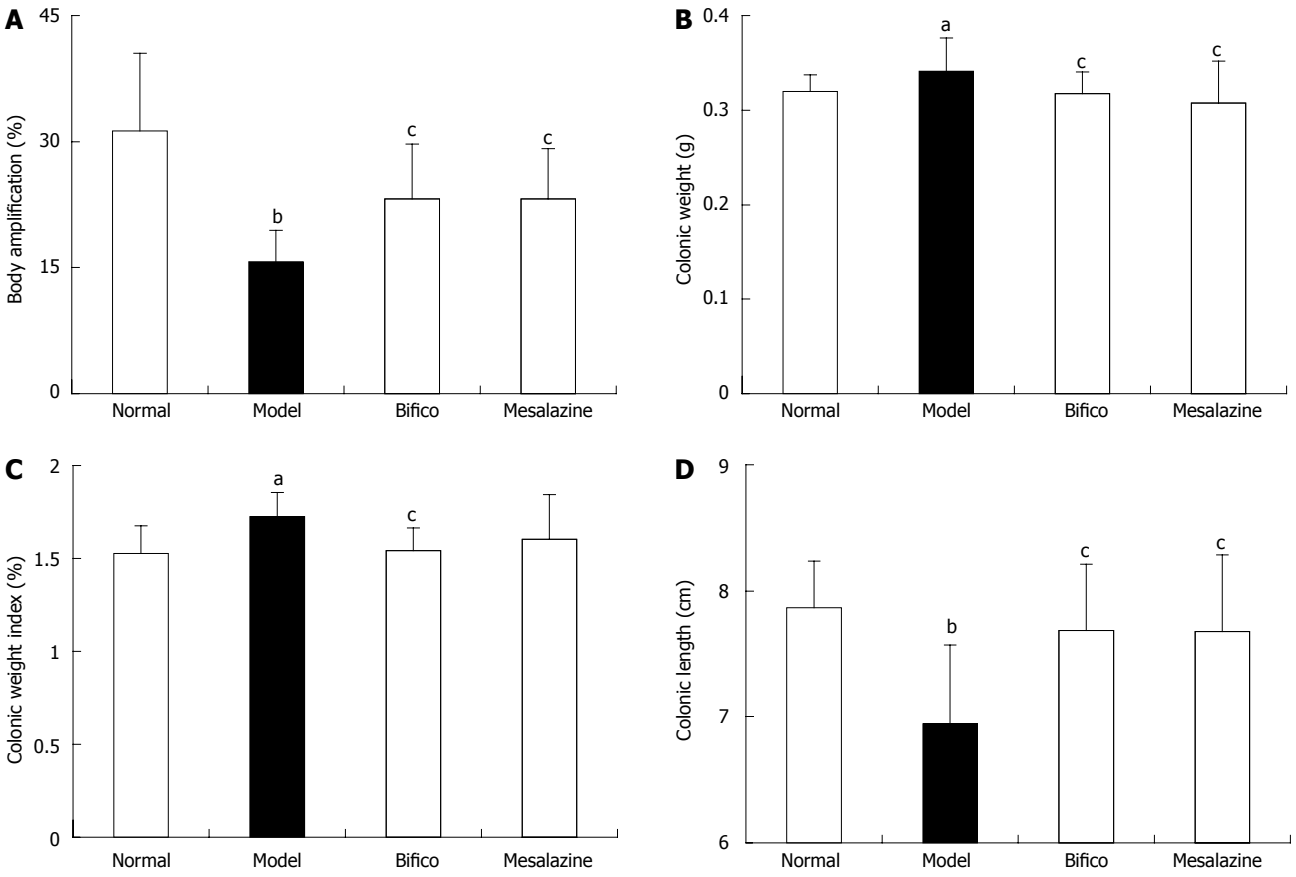

Figure 1 Change of body weight amplification, colonic weight index, colonic weight and colonic length. A: Percent of body weight amplification; B: Colonic weight; C: Colonic weight index; D: Colonic length. Normal: In the normal group, the animals were administrated with physiological saline; Model: In the model group, the animals were challenged with 2,4,6-trinitrobenzene sulfonic acid (TNBS) without treatment; Bifico: In the Bifico group, the animals were challenged by TNBS and treated with Bifico at 345 mg/kg; Mesalazine: In the Mesalazine group, the animals were challenged by TNBS and treated with 300 mg/kg Mesalazine. Data are means ± SE (n = 10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the normal group; $^{c}P < 0.05$ *vs* the model group.

Determination of cytokines in colonic tissue homogenates

The concentrations of cytokines in colonic tissue homogenates were measured using a BD CBA Mouse Th1/Th2/Th17 Cytokine Kit according to the manufacturer's protocol. Fifty microliters of homogenates or standards were incubated with 50 μL of capture beads for 1h at room temperature, and then mixed with 50 μL of phycoerythrobilin (PE)-labeled tumor necrosis factor (TNF)-α, interleukin (IL)-4, interferon (IFN)-γ, IL-10, IL-6, IL-17 and IL-2 detection antibodies and incubated for 2 h at room temperature to form a sandwich complex. Following incubation, 1 mL of washing buffer (BD Biosciences Pharmingen, United States) was added to each tube, and the mean fluorescence intensity was detected using flow cytometry (FACSCalibur, B. D. Co, United States). Data were analyzed using BD Cytometric BeadArray analysis software[15].

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 15.0. Data were presented as means ± SD. Statistical analysis was performed using ANOVA and the F-test. $P < 0.05$ or $P < 0.01$ were considered significant.

RESULTS

Probiotics relieved macroscopic severity of TNBS-induced colitis in mice

Body weight amplifications of mice in the normal, Bifico and mesalazine groups were higher than that of the model group (31.28 ± 9.23, 23.20 ± 6.51, 23.17 ± 6.05, *vs* 15.73 ± 3.73, $P < 0.05$) (Figure 1A); however, the colonic weight index (CWI) and colonic weight (CW) in the Bifico group decreased compared with the model group (CWI: 1.53 ± 0.15, 1.54 ± 0.12 *vs* 1.72 ± 0.13, $P < 0.05$; CW: 0.32 ± 0.02, 0.32 ± 0.02 *vs* 0.36 ± 0.03, $P < 0.05$) (Figure 1B and C). The colonic lengths of mice in the normal, Bifico and mesalazine groups were significantly higher compared with those in the model group (7.86 ± 0.37, 7.68 ± 0.53, 7.68 ± 0.61, *vs* 6.94 ± 0.63, $P < 0.05$) (Figure 1D).

Probiotics attenuated histological severity of TNBS-induced colitis in mice

As shown in Figure 2A, integral colonic epithelium and no inflammatory cell infiltration were observed in the slice from the normal mice. However, ulceration, colonic epithelium loss, incrassate intestinal wall and abundant

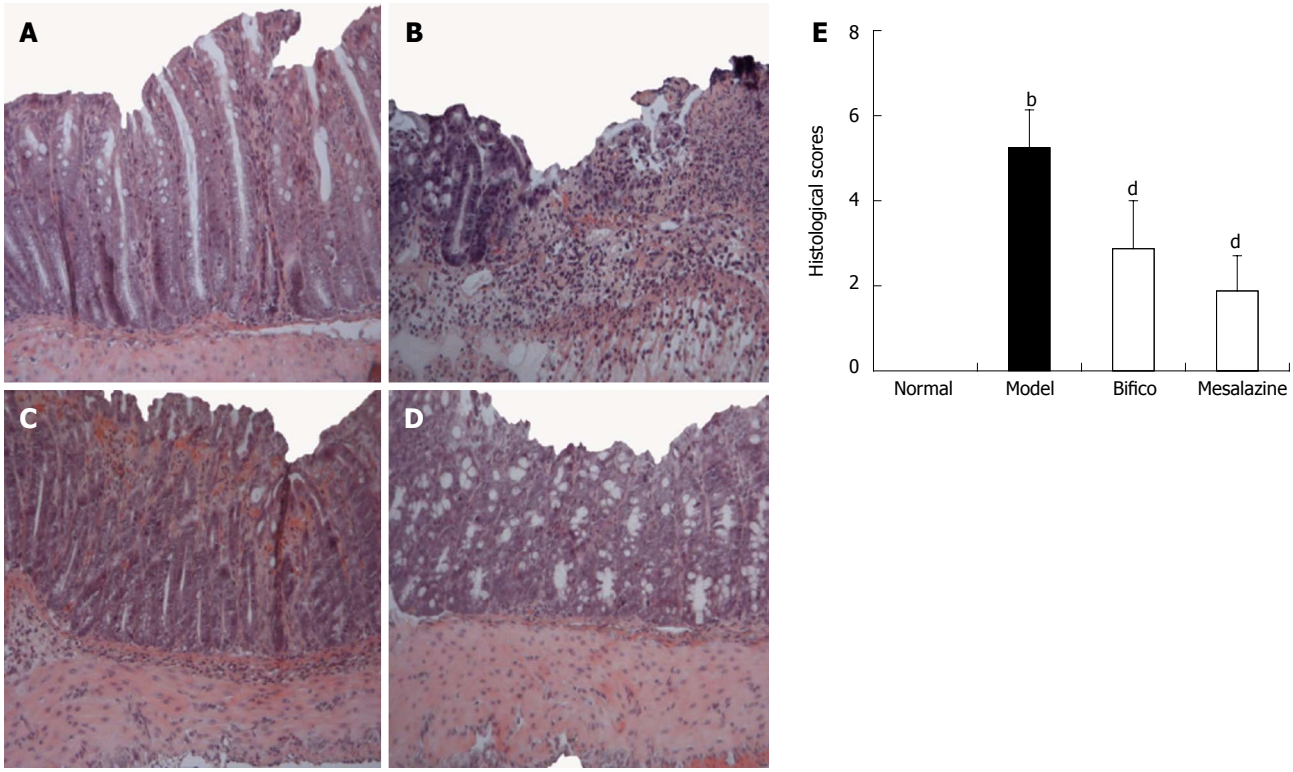

Figure 2 Representative histological images and scores of mice. A: The normal group; B: The model group; C: The bifico group; D: The mesalazine group; E: Histological images and scores (hematoxylin and eosin, light microscope, × 100). Normal: In the normal group, the animals were administrated with physiological saline; Model: In the model group, the animals were challenged with 2,4,6-trinitrobenzene sulfonic acid (TNBS) without treatment; Bifico: In the Bifico group, the animals were challenged by TNBS and treated with Bifico at 345 mg/kg; Mesalazine: In the Mesalazine group, the animals were challenged by TNBS and treated with 300 mg/kg mesalazine. Data are means ± SE (n = 10). $^{b}P < 0.01$ *vs* the normal group; $^{d}P < 0.01$ *vs* the model group.

inflammatory cell infiltration were observed in the colon of mice in the model group (Figure 2B). Meanwhile, the above histological symptoms were remarkably relieved with fewer infiltrated inflammatory cell and restored epithelium in slices from colitis mice treated with Bifico and mesalazine (Figure 2C and D). Compared with the model group, the histological score in the Bifico and mesalazine groups decreased statistically significantly (2.88 ± 1.33, 1.88 ± 0.83 *vs* 5.25 ± 0.89, $P < 0.01$) (Figure 2E). The results indicated that Bifico effectively restrained colonic injury of experimented colitis mice.

Probiotics improved the level of $CD4^+CD25^+Foxp3^+$T cells in mesenteric lymph nodes

To identify the developmental capacity of $CD4^+CD25^+$T regulatory cells, the level of $CD4^+CD25^+Foxp3^+$T cells in mesenteric lymph nodes was analyzed by flow cytometry. As shown in Figure 3, the level of $CD4^+CD25^+Foxp3^+$T cells in the model group was decreased compared with the normal group (29.70 ± 1.87 *vs* 34.73 ± 4.04, $P < 0.05$). But in the Bifico and mesalazine groups, the levels of $CD4^+CD25^+Foxp3^+$T cells were higher than in the model group (34.46 ± 4.11, 32.18 ± 1.44 *vs* 29.70 ± 1.87, $P < 0.05$).

Probiotics regulated the expression of cytokines in colonic tissues

Compared with the normal group, the expressions of TNF-α and IFN-γ in colonic tissues from mice in the model group were significantly increased (TNF-α: 32.67 ± 7.12 *vs* 18.33 ± 4.55, $P < 0.01$; IFN-γ: 28.90 ± 6.13 *vs* 10.24 ± 3.74, $P < 0.01$) (Figure 4A and B), and they were significantly decreased in the Bifico and mesalazine groups compared with the model (TNF-α: 16.87 ± 2.70, 16.96 ± 1.72 *vs* 32.67 ± 7.12, $P < 0.05$; IFN-γ: 10.46 ± 5.44, 5.20 ± 2.10 *vs* 28.90 ± 6.13, $P < 0.01$) (Figure 4A and B). Meanwhile, the expressions of IL-2, IL-4 and IL-10 in the model group were lower than in the normal group (IL-2: 2.00 ± 0.17 *vs* 2.40 ± 0.34, $P < 0.05$; IL-4: 1.58 ± 0.16 *vs* 1.84 ± 0.24, $P < 0.05$; IL-10: 7.50 ± 2.96 *vs* 13.35 ± 2.89, $P < 0.05$) (Figure 4C-E). Compared with the model group, the three cytokines were upregulated in the Bifico and mesalazine groups (IL-2: 2.52 ± 0.40, 2.32 ± 0.11 *vs* 2.00 ± 0.17, $P < 0.05$; IL-4: 1.92 ± 0.34, 1.82 ± 0.20 *vs* 1.58 ± 0.16, $P < 0.05$; IL-10: 14.19 ± 4.14, 12.25 ± 3.41 *vs* 7.50 ± 2.96, $P < 0.05$) (Figure 4C-E). The expressions of IL-6 and IL-17 in colonic tissues from colitis mice without treatment were higher than in the normal group (IL-6: 4.69 ± 1.47 *vs* 2.35 ± 0.19, $P < 0.01$; IL-17: 5.94 ± 1.54 *vs* 3.37 ± 0.96, $P < 0.01$); however, in the two treated groups, they were no different compared with the model.

DISCUSSION

In the present study, the curative effect of probiotics treatment on experimental colitis was demonstrated by

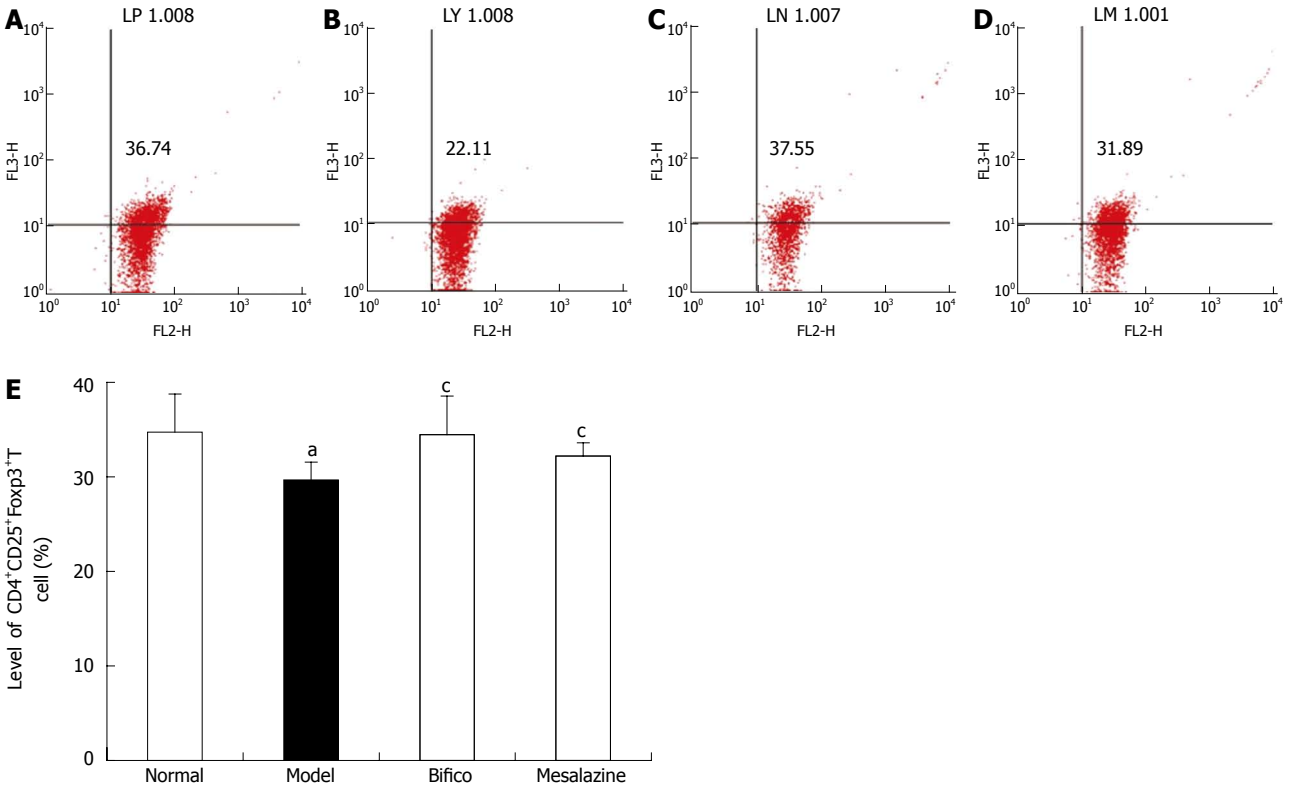

Figure 3 Representative flow plots and level of CD4$^+$CD25$^+$Foxp3$^+$T cell. A: The normal group; B: The model group; C: The bifico group; D: The mesalazine group; E: The level of CD4$^+$CD25$^+$Foxp3$^+$T. Normal: In the normal group, the animals were administrated with physiological saline; Model: In the model group, the animals were challenged with 2,4,6-trinitrobenzene sulfonic acid (TNBS) without treatment; Bifico: In the Bifico group, the animals were challenged by TNBS and treated with Bifico at 345 mg/kg; Mesalazine: In the Mesalazine group, the animals were challenged by TNBS and treated with 300 mg/kg Mesalazine. Data are means ± SE (n = 10). $^aP < 0.05$ *vs* the normal group; $^cP < 0.05$ *vs* the model group.

the increased body weight, the increased length of the colon, and decreasing histological scores followed by restored pathological characters. Probiotics treatment for 10 d inhibited the tendency to decrease CD4$^+$CD25$^+$T cells in mesenteric lymph nodes, downregulated the expression of TNF-α and IFN-γ, and upregulated the products of IL-2, IL-4 and IL-10 in colonic mucosa from TNBS-induced colitis mice.

Many studies have indicated that dysregulation of intestinal microflora plays an important role in initiating and perpetuating colonic inflammation in the development of human UC[16-18]. Findings from animal dextran sulfate sodium (DSS)- or TNBS-colitis models suggest that the intestinal microflora plays a key role in the abnormal immune response leading to mucosal injury in experimental colitis, because germ-free animals do not mount an intestinal inflammatory response[1,19-20]. Recently, probiotic therapy has been recognized as a safe and effective treatment for patients with UC. Lin-Lin Chen and their colleagues demonstrated that every strain of three probiotics in Bifico Capsules (combined bifidobacterium, lactobacillus and enterococcus) had a beneficial effect on DSS-induced experimental colitis in mice[21].

CD4$^+$CD25$^+$T regulatory (Treg) cells are critical for maintaining immune homeostasis and establishing tolerance to foreign, non-pathogenic antigens, including commensal bacteria and food, and are identified by their constitutive expression of Foxp3. CD4$^+$CD25$^+$T cell secretes suppressive cytokines, such as IL-2, IL-10 and TGF-β, to regulate the balance of Th1/Th2 cells and maintain mucosal immunity in the intestine[22-24]. Evidence has shown that non-functional, absent Tregs or genetic mutations in Foxp3 induces hypersensitivity to bacterial antigens[10,11], and destroys the balance of intestinal mucosal immunity to inflammatory injury followed by lymphocytic infiltration of the intestinal mucosa[25,26]. CD4$^+$CD25$^+$Foxp3$^+$ T cells increased in the colon of paracmasis patients with UC[22]. These data indicated that Tregs would be a promising target for treating UC, because increasing the activity of appropriate Tregs in the gut should help to restore inflamed colonic tissues[27].

In the present study, we found that the level of CD4$^+$CD25$^+$Foxp3$^+$ T cells was downregulated in mesocolic lymph nodes from experimented colitis mice without treatment, while IL-2, IL-10 and IL-4 were hypo-expressed in colonic tissues in the model group. The results showed that the suppressed function of CD4$^+$CD25$^+$Foxp3$^+$T cell induced disturbance of Th1/Th2 cytokines in the colonic mucosa, which leads to increased susceptibility to symbiotic bacteria, overexpression of proinflammatory factors (IL-1, TNF-α, IFN-γ), finally resulting in inflammatory injury and ulceration. After 10-d of treatment by probiotics, impaired colonic mucosa was restored with epithelial

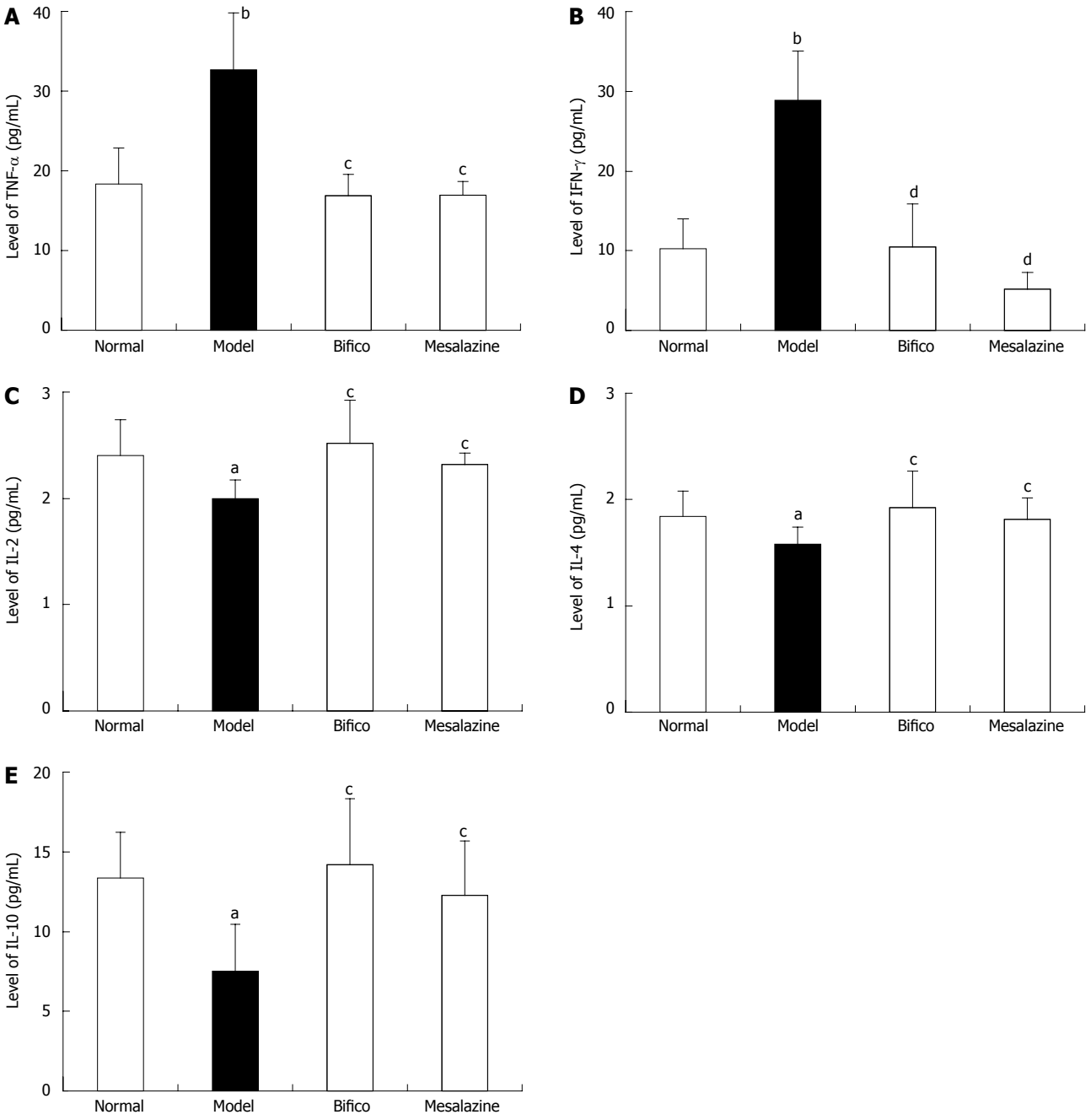

Figure 4 Expression of cytokines in colonic mucosa. A: Level of tumor necrosis factor (TNF)-α; B: Level of interferon (IFN)-γ; C: Level of interleukin (IL)-2; D: Level of IL-4; E: Level of IL-10. Normal: In the normal group, the animals were administrated with physiological saline; Model: In the model group, the animals were challenged with 2,4,6-trinitrobenzene sulfonic acid (TNBS) without treatment; Bifico: In the Bifico group, the animals were challenged by TNBS and treated with Bifico at 345 mg/kg; Mesalazine: In the Mesalazine group, the animals were challenged by TNBS and treated with 300 mg/kg Mesalazine. Data are the means ± SE (n = 10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the normal group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* the model group.

hyperplasia, intact colonic epithelia and fewer infiltrating inflammatory cells infiltration. Probiotics effectively treated mice with colitis. $CD4^{+}CD25^{+}Foxp3^{+}T$ cells were upregulated, with high expression of IL-10, IL-4 and IL-2, and TNF-α and IFN-γ were decreased in colonic mucosa from colitis mice treated by probiotics. In the study, probiotics improved the quantity of $CD4^{+}CD25^{+}Foxp3^{+}T$ cells, and restored the suppressed function of $CD4^{+}CD25^{+}Foxp3^{+}T$ cells. The Treg cells secreted more IL-10 and IL-4, competitively bound with IL-2, and enhanced the immune tolerance of intestinal mucosa to resist bacterial antigens and decreased the activity of proinflammatory factors (as TNF-α and IFN-γ), thereby renewing the immunological barrier of the colonic mucosa. Probiotics alleviated colonic inflammatory injury of colitis mice by alleviating the suppression of or increasing the quantity of $CD4^{+}CD25^{+}Foxp3^{+}T$ cell.

In summary, Probiotics effectively treated experimental colitis by improving $CD4^{+}CD25^{+}Foxp3^{+}T$ cell and regulating the balance of Th1/Th2 cytokines in the co-

lonic mucosa.

COMMENTS

Background

Ulcerative colitis (UC) is a chronic, inflammatory disease of the colonic mucosa, characterized by a relapsing-remitting course; however, the cause is largely unknown. Dysregulation of intestinal microflora and hypofrontality of T regulatory cell play important roles in pathogenicity of UC.

Research frontiers

The barrier of intestinal mucosal immunity may become damaged, which may be primarily caused by decreased inhibition of T regulatory cell by bacterial antigens. This suggested that regulating the function of T regulatory cells and/or the balance of intestinal microflora could potentially be used to treat UC.

Innovations and breakthroughs

Previous studies have highlighted that re-imported T regulatory cells may prevent occurrence or aggravation of UC. In the present study, probiotics (Bifico) inhibited the decreased tendency of $CD4^+CD25^+Foxp3^+$T cell induced by 2,4,6-trinitrobenzene sulfonic acid to effectively treat experimental colitis, followed by regulation of the balance of Th1/Th2 cytokines in the colonic mucosa.

Applications

By understanding the effects of probiotics in the treatment experimental colitis, which was mediated by inhibiting the decreased level of T regulatory cell and regulating the balance of Th1/Th2 cytokines, the study provided some evidence of the effect of probiotics on maintaining stable intestinal mucosal immunity in the treatment of UC.

Peer review

The authors investigated the effects of probiotics on treating experimental colitis and explored its potential mechanism by observing the level of T regulatory cell and expression of Th1/Th2 cytokines in colonic mucosa. It has revealed that probiotics effectively treated experimental colitis, which was accomplished by improving $CD4^+CD25^+Foxp3^+$T cell and regulating balance Th1 and Th2 cytokines in colonic mucosa. The results are valuable for exploring the mechanism of treating UC with probiotics.

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BRIEF ARTICLE

Laparoscopic total mesorectal excision with natural orifice specimen extraction

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Abstract

AIM: To introduce transvaginal or transanal specimen extraction in laparoscopic total mesorectal excision surgery to avoid an abdominal incision.

METHODS: Between January 2009 and December 2011, 21 patients with rectal cancer underwent laparoscopic radical resection and the specimen was retrieved by two different ways: transvaginal or transanal rectal removal. Transvaginal specimen extraction approach was strictly limited to elderly post-menopausal women who need hysterectomy. Patients aged between 30 and 80 years, with a body mass index of less than 30 kg/m^2, underwent elective surgery. The surgical technique and the outcomes related to the specimen extraction, such as duration of surgery, length of hospital stay, and the complications were retrospectively reviewed.

RESULTS: Laparoscopic resection using a natural orifice removal approach was successful in all of the 21 patients. Median operating time was 185 min (range, 122-260 min) and the estimated blood loss was 48 mL. The mean length of hospital stay was 7.5 d (range, 2-11 d). One patient developed postoperative ileus and had an extended hospital stay. The patient complained of minimal pain. There were no postoperative complications or surgery-associated death. The mean size of the lesion was 2.8 cm (range, 1.8-6.0 cm), and the mean number of lymph nodes harvested was 18.7 (range, 8-27). At a mean follow-up of 20.6 mo (range, 10-37 mo), there were no functional disorders associated with the transvaginal and transanal specimen extraction.

CONCLUSION: Transvaginal or transanal extraction in L-TME is a safe and effective procedure. Natural orifice specimen extraction can avoid the abdominal wall incision and its potential complications.

Key words: Laparoscopic total mesorectal excision; Natural orifice specimen extraction; Rectum cancer; Transvaginal; Transanal

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INTRODUCTION

A number of single and multicenter randomized trials and meta-analysis have demonstrated that the laparoscopic total mesorectal excision (L-TME) was superior to the open approach. Laparoscopic surgery resulted in faster postoperative recovery and fewer long-term complications than open surgery without apparently compromising the long-term oncologic outcomes[1-3]. However, this typically requires an abdominal incision for specimen

removal, which is commonly associated with postoperative pain. Surgical wound complications, mainly infection and postoperative hernia, continue to be major problems after both open and laparoscopic colorectal surgery, and the mini-laparotomy is often the most important source of postoperative pain after L-TME. Avoidance of the abdominal wall extraction site may be desirable to improve the short-term outcome of L-TME and limit the high cost of wound complication management[4-6].

The concept of natural orifice transluminal endoscopic surgery (NOTES) has gained interest in the surgical and gastrointestinal community as a technique to potentially reduce the surgical wound complications and trauma associated with conventional surgery[7,8]. However, NOTES has been limited because of lack of suitable instruments and platforms to facilitate a safe performance of such complex procedures. The combination of standard laparoscopy and specimen extraction through a natural orifice has the potential to reduce wound-related complications[9,10]. Natural orifice specimen extraction (NOSE) using either the anus or vagina as a route eliminates the need for abdominal wall incision for specimen extraction and anastomosis construction[11]. The NOSE concept is appealing as it maximizes the benefits of laparoscopic surgery while reducing potential wound complications and provides experience for cautious progression toward less incision surgery[12].

In this report, we adopted the technique of L-TME and transvaginal or transanal extraction in a series of 21 rectal cancer patients who required anterior rectal resection. Technique, results, morbidity, and mortality were described.

MATERIALS AND METHODS

Study participants

All consecutive patients operated on for rectal cancer using laparoscopic TME anterior rectal resection approach and transanal or transvaginal extraction of the specimen from January 2009 to December 2011 at First Hospital of Jilin University were enrolled in the study. Patients were aged between 30 and 80 years, had a body mass index of less than 30 kg/m^2, and underwent elective surgery. Patients operated on in an emergency setting and those who underwent Hartmann or abdominal perineal resection procedures were excluded. Written informed consent was obtained from all subjects and the study protocol was approved by the Ethics Committee of the First Hospital, Jilin University. The procedures were performed by a team consisting of experienced laparoscopic general surgeon and gynecologist. All patients underwent an oral magnesium citrate bowel preparation the day before surgery.

In this study, inclusion criteria for transvaginal specimen extraction were as follows: It is suitable for elderly post-menopausal women who need hysterectomy; female patients who were diagnosed with uterine myoma along with irregular vaginal bleeding, hematochezia, and receive

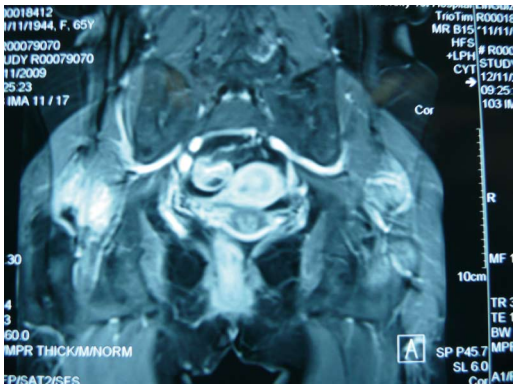

Figure 1 Computed tomography scan showed a rectal tumor combined with uterine myoma in a female patient.

L-TME combined with uterine myomectomy (Figure 1). All patients received preoperative gynecologic examination to rule out vaginal stenosis and congenital abnormalities. Women with a history of endometriosis, narrow vagina, virgins, extensive pelvic adhesions, and lesions > 6 cm, and refusal of the procedure were not considered candidates for the transvaginal removal route. All of the decision was made after consulting with gynecologist.

Transvaginal method

The patients were placed in the supine split leg or modified lithotomy position to allow for vaginal access. Abdominal and vaginal preparations were performed. After vascular control, mesocolic dissection, mobilization of tumor was performed, and the rectum distal to tumor was transected using stapler laparoscopically leaving the proximal rectum in the abdominal cavity. Then, with the assistance of gynecologist, laparoscopic hysterectomy was performed. Next, the specimens were placed in the specimen bag, which is then closed. The proximal rectum with tumor and uterus were delivered transvaginally and the rectum tumor was resected proximally outside body (Figure 2A). The anvil of the circular stapler was then inserted into the proximal colon and closed using purse string suture. The proximal colon was then returned into abdomen with the anvil transvaginally. Bowel anastomosis was completed by inserting the circular stapler via anal canal. Posterior fornix of the vagina was sutured. Final laparoscopic visualization was carried out to ensure hemostasis in the mesentery and inspect the anastomosis site.

Transanal method

The patients were placed in the lithotomy position. Briefly, a three-trocar laparoscopic approach was generally employed for mesenteric dissection and sigmoid colon and rectum mobilization. (1) Laparoscopically TME dissection was done and the upper and lower margins of the tumor determined and rectum was transected by endocutter stapler. Then, the anus was dilated, rectal stump was washed out with a 500 mL povidone-iodine normal saline solution; (2) After transanal lavage was performed,

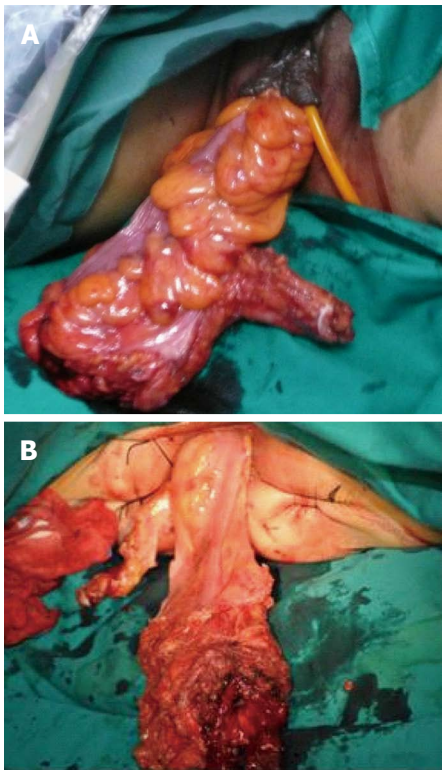

Figure 2 Natural orifice specimen extraction in laparoscopic total mesorectal excision surgery. A: Transvaginal; B: Transana specimen extraction.

the end of distal rectum was opened by ultrasonic scalpel and the specimen was extracted transanally with the specimen bag; (3) The anvil of circular end-to-end anastomosis stapler was introduced to peritoneal cavity transrectally and simultaneously put purse string suture by 2-0 prolene in the proximal colon stump; and (4) Finally, the head of the circular stapler was passed per anal, and a circular anastomosis was created (Figure 2B).

RESULTS

Within a 3-year time period, all 21 laparoscopic resections were performed successfully using a natural orifice removal approach. None of the patients were converted to open operation. The median age was 62 years (range, 50-80 years). Two of 14 patients (14%) had undergone abdominal surgery previously. The mean body mass index was 23.6 kg/m^2 (range, 18-30 kg/m^2). Median operating time was 185 min (range, 122-260 min) and the estimated blood loss was 48 mL. All patients underwent a same postoperative protocol: patient-controlled analgesia for the first 24 to 48 h. The diet was started as tolerated. The mean length of hospital stay was 7.5 d (range, 2-11 d). One patient developed postoperative ileus and had an extended hospital stay. The patient complained of minimal pain. There were no postoperative complications like rupture of the rectus, bleeding or leakage of the anastomosis, anastomosis stenosis or surgery-associated death. All margins in the resected specimens were macroscopically and microscopically free of any tumor. The mean size of the lesion was 2.8 cm (range, 1.8-6.0 cm), and the mean number of lymph nodes harvested was 18.7 (range, 8-27). Patients' demographics and pathologic details are shown in Table 1. At a mean follow-up of 20.6 mo (range, 10-37 mo), no patient experienced pain, drainage from the vaginal extraction site, and dyspareunia, and there was no incisional hernia (Figure 3).

Table 1 Clinical data and outcomes of 21 rectal cancer cases

	Transvaginal	Transanal	*P* value
Patients (*n*)	*n* = 5	*n* = 16	
Sex			> 0.05
Male	0	4	
Female	5	12	
Age, yr (median)	61	62	
Site of tumor [distance to the edge of the anus (cm)]			> 0.05
5-10	4	9	
10-15	1	7	
Operative time (min)	195 ± 35	187 ± 35	> 0.05
Operative bleeding (mL)	36 ± 15	45 ± 20	> 0.05
Post-op flatus (d)	2.0 ± 1.5	1.0 ± 0.8	> 0.05
Post-op hospitalization (d)	7.0 ± 1.2	6.5 ± 1.5	> 0.05
Intra-abdominal infection	0	0	> 0.05
Anastomosis leak	0	0	
Ileus	0	1	
Number of removal lymph node	16 ± 3	18 ± 2	> 0.05
Differentiation			> 0.05
Well	2	4	
Moderate	2	10	
Poor	1	2	
TNM stage			> 0.05
Stage Ⅰ	1	3	
Stage Ⅱ	3	11	
Stage Ⅲ	1	2	
Stage Ⅳ	0	0	

DISCUSSION

At present, despite the advantages of laparoscopic colorectal surgery, during the specimen retrieval through substantial incisions, there is an increased postoperative pain, wound infections, and incisional hernias. Along with the development of the minimally invasive surgery, abdominal wall scarless surgery becomes the new goal for people who pursue laparoscopic surgery[13,14]. In order to leave no scar on the abdominal wall, to achieve fast recovery from surgery using a more minimally invasive technique, and better cosmetic appearance, the idea of the scarless surgery started from NOTES[15,16]. In the era of NOTES, incisionless transrectal or transvaginal approaches for colorectal resections have been investigated with promising results. Transanal retrieval of specimen in laparoscopic TME has been described but not widely adopted[17-21].

Because the NOTES requires advanced technique and equipment, and requests the surgeon to have a higher strain capacity of the anatomy and the techniques. So, at present, it is still at the initial step in many countries. Recently, we have performed fast track rehabilitation in laparoscopic colorectal resection for elderly patients[22].

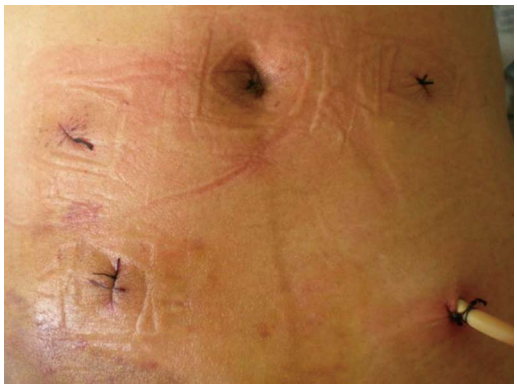

Figure 3 Postoperative scars.

In this article, we described two different ways to retrieve the specimen based on the technique of the traditional laparoscopic radical surgery for rectal cancer, and experienced no additional difficulty during surgery, no needs of specific equipment.

Some additional hints which are helpful during above procedures as follows: First, if the tumor is a medium-lower rectal cancer, large in size or in the fat patient, mesocolon is generally fleshy and contracted, making it shorter and difficult when transanal retrieval of specimen is done. Secondly, before transanal retrieval of specimen, when the upper and lower margin tie off, it is very important to repeat transanal irrigation by povidone iodine solution and saline. This can reduce the chances of infection and tumor cell implantation in the pelvis. Finally, it is important to put the sample in the plastic bag in the abdominal cavity, which will reduce the chances of infection and helps in smooth extraction of specimens[23,24].

In conclusion, the present study showed that NOSE is safe and feasible to perform L-TME for rectal cancer and extract the specimen without scar on abdominal wall. NOSE is an applicable option for patients requiring L-TME and appears to be associated with little incisional pain and rapid recovery. Future randomized controlled trials are necessary to show the superiority of this approach with regard to postoperative pain and morbidity, hospital stay, recovery, function and cosmesis. NOSE constitutes a stepping stone in the transition to future incisionless NOTES colectomy[25,26].

COMMENTS

Background

Despite the advantages of laparoscopic total mesorectal excision rectal surgery, the need for an incision in the abdominal wall to remove the surgical specimen is a morbid factor.

Research frontiers

The development of natural orifice translumenal endoscopic surgery and natural orifice specimen extraction (NOSE) appears to be the next major frontier in minimally invasive surgery.

Innovations and breakthroughs

Present research showed that laparoscopic total mesorectal excision (L-TME) with transvaginal or transanal extraction is a safe and effective procedure. This technique is feasible and simple to perform, avoids the abdominal wall incision and its potential complications.

Applications

Natural orifice specimen extraction may provide both an attractive way to reduce abdominal wall morbidity and a bridge to pure natural orifice transluminal endoscopic surgery for rectal surgery.

Terminology

NOSE in colorectal surgery prevents the need for an enlarged port site or minilaparotomy to extract the surgical specimen. The current trend to develop less invasive laparoscopic techniques by reducing the number and size of abdominal incisions has spurred new interest in practice.

Peer review

The authors performed laparoscopic total mesorectal excision with NOSE, which is associated with rapid recovery. They conclude that NOSE is technically feasible for L-TME. It could be better if the authors could provide data from prospective control study showing that the recovery parameters of this approach is superior to those of L-TME with trans-abdominal incision.

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BRIEF ARTICLE

Laparoscopy-assisted total gastrectomy with trans-orally inserted anvil (OrVil™): A single institution experience

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Abstract

AIM: To investigate the feasibility of laparoscopy-assisted total gastrectomy (LATG) using trans-orally inserted anvil (OrVil™) in terms of operative characteristics and short term outcomes.

RESULTS: Characteristics of 27 patients with gastric cancer who underwent LATG from October 2009 to October 2012 in the Foshan Affiliated Hospital of South Medical University were retrospectively reviewed. Among these patients, six were reconstructed by mini-laparotomy and 21 by OrVil™. The clinicopathological characteristics, total operation time, total blood loss, abdominal incision and complications of anastomosis including stenosis and leakage, were compared between the groups undergoing LATG with OrVil™ and the group undergoing mini-laparotomy.

RESULTS: The operations were successfully performed on all the patients without intraoperative complications or conversion to open surgery. Two (10%) patients received palliative procedure under laparoscope who were prepared for LATG preoperatively. One case had hepatic metastatic carcinoma and 1 case had tumor recurrence near the anastomosis 8 mo after surgery. The mean follow-up duration was 10 mo (range, 2-24 mo). Operation time was significantly reduced by the use of OrVil™ (198.42 ± 30.28 min *vs* 240.83 ± 8.23 min). The postoperative course with regard to occurrence of stenosis and leakage was not different between the two groups. There were no significant differences in estimated blood loss. The upper abdominal incision was smaller in OrVil™ group than in mini-laparotomy group (4.31 ± 0.45 cm *vs* 6.43 ± 0.38 cm).

CONCLUSION: LATG using OrVil™ is a technically feasible surgical procedure with sufficient lymph node dissection, less operation time and acceptable morbidity.

Key words: Total gastrectomy; Esophagojejunostomy; Laparoscopy-assisted total gastrectomy; Reconstruction; OrVil™

Liao GQ, Ou XW, Liu SQ, Zhang SR, Huang W. Laparoscopy-assisted total gastrectomy with trans-orally inserted anvil (OrVil™): A single institution experience. *World J Gastroenterol* 2013; 19(5): 755-760 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i5/755.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i5.755

INTRODUCTION

Laparoscopic gastrectomy (LG) including laparoscopy-assisted gastrectomy (LAG) and totally LG with regional lymph node dissection as an alternative surgical treatment for gastric cancer has become increasingly common worldwide, especially in Asia[1-3]. However, laparoscopy-assisted total gastrectomy (LATG) has not been

accepted as widely as other LGs because of the low incidence of gastric cancer requiring LATG and because it is particularly difficult even for some experienced surgeons to perform[4,5]. Nevertheless, comparing with the conventional open total gastrectomy, laparoscopic surgery as an advanced procedure offers the advantages of less invasiveness and the same curability if surgeons are adroit at performing LATG[6,7].

Since 2009, our institution has adopted laparoscopic modalities for both the early and advanced stage gastric cancer patients, including 27 cases of LATG. Herein, we review our experience with LATG and analyze the results of LATG in terms of operative characteristics and short-term outcomes.

MATERIALS AND METHODS

Patients

We retrospectively reviewed a series of 27 patients who underwent LATG between October 2009 and October 2012. Twenty-one of them were reconstructed by transorally inserted anvil (OrVil™) and six by mini-laparotomy. The mini-laparotomy was performed in relatively early period and set for the comparisons of short-term outcomes.

Preoperative tumor node metastasis (TNM) stage was determined in all the patients according to the International Union Against Cancer (UICC, 7th edition) and based on endoscopic biopsy and abdominal computed tomography. The indication for LATG in gastric cancer was limited to preoperative stage $T_{1-4}N_{0-3}M_0$. Patients whose NRS 2002 score was more than 4 received more than 1-wk nutrition therapy before operation. Patients suitable for endoscopic submucosal dissection or had surgical contraindications were excluded. Written informed consent was signed by each patient who agreed to undergo LATG.

Surgical procedure

All patients were placed in relaxed dorsal lithotomy position. The surgeon usually stood on the left side of the patient, the first assistant surgeon on the right, and the second assistant surgeon holding the camera stood between the patient's legs. At the beginning, five trocars were introduced into the right upper quadrant (5 mm), right middle quadrant (5 mm), subumbilical (10 mm; camera port), left middle quadrant (5 mm), and left upper quadrant (12 mm) regions of the abdomen. The intraperitoneal pressure was maintained as 12 mmHg with carbon dioxide.

Total gastrectomy with complete omentectomy and extended lymphadenectomy (D2) was performed in all the patients. After sufficient mobilization of the duodenum near the pylorus ring and abdominal esophagus, the duodenum and esophagus were transected using EndoGIA™ Universal stapler (60 mm; Covidien). The stomach was bagged in an isolation pocket and pulled out extracorporeally through a 4-6 cm upper midline incision. In the next step, the gastrointestinal continuity was restored in a Roux-en-Y mode extracorporeally through the incision.

The OrVil™ orogastric tube was transorally introduced into the esophagus. The orogastric tube was then used to make a small hole on the middle of the abdominal esophageal stump. The tube was pulled out into the abdominal cavity through the hole until the anvil reached the esophageal stump. The orogastric tube was disconnected from the anvil and taken out of the esophagus. Subsequently, intracorporeal stapling esophagojejunostomy was performed and the jejunal stump was intracorporeally sutured with EndoGIA™ Universal (Figure 1). The intraperitoneal chemohyperthermia was performed and two drains were placed around the esophagojejunal anastomosis and pelvic cavity, respectively.

Postoperative course

The theory of fast track surgery is prevalent in our institution, but we adopt a conservative approach for the LATG patients postoperatively. The preoperatively inserted nasogastric tube for air decompression was removed at the end of surgery. A soft diet commenced orally on postoperative day (POD) 4, and abdominal drain tube was removed after 1 or 2 d when the drainage was less than 30 mL per 24 h. After a meglumine diatrizoate meal examination of esophago-intestinal tract was performed to evaluate anastomotic leakage and stenosis on PODs 8 to 10, patients were discharged on PODs 10 to 13.

Statistical analysis

Data were analyzed by the SPSS statistical software (SPSS 13.0). Quantitative variables were compared using the Student's *t* test and were expressed as means ± SD. *P* values were considered to be statistically significant at 0.05.

RESULTS

Patient characteristics

Patient characteristics including age, gender, body mass index (kg/m^2), history of abdominal surgery, NRS 2002 score and comorbidities are listed in Table 1. The operations were successfully performed in all the patients, without intraoperative complications or conversion to open surgery. Two (10%) cases received palliative procedure under laparoscope who were prepared for LATG preoperatively. One case developed hepatic metastatic carcinoma and 1 case had tumor recurrence near the anastomosis 8 mo after surgery. Mean follow-up duration was 10 mo (range, 2-24 mo).

Surgical procedure

Table 2 shows the surgical outcomes and postoperative complications. All patients underwent LATG with antecolic type Roux-en-Y esophagojejunostomy and D2

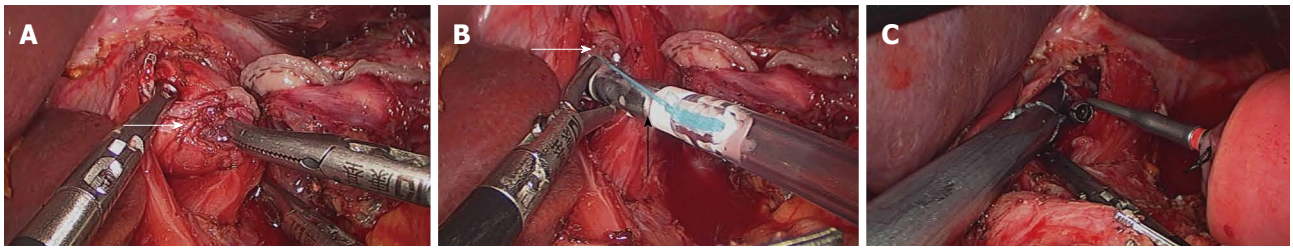

Figure 1 Esophagojejunostomy with trans-orally inserted anvil. A: The trans-orally inserted anvil orogastric tube was transorally introduced into the esophagus (white arrow); B: The tube was pulled out into the abdominal cavity through the hole. The black arrow shows the place to be separated; C: The orogastric tube was disconnected from the anvil and intracorporeal stapling esophagojejunostomy was performed.

Table 1 Characteristics of patients

Case	Gender	Age (yr)	NRS score	BMI	Previous abdominal operation	Comorbidity
1	Female	46	5	27.2	No	Urinary lithiasis
2	Male	77	7	24.1	No	No
3	Male	65	8	21.4	Appendectomy	No
4	Male	61	4	26.4	No	No
5	Male	62	5	22.1	Cholecystotomy	No
6	Female	71	8	20.9	No	Gout; high blood pressure
7	Male	48	5	24.4	No	No
9	Male	62	5	23.7	Cesarean section	Hepatic cyst; high blood pressure; cholecystolithiasis
10	Male	60	2	23.8	No	No
11	Female	55	2	23.2	No	No
12	Female	52	8	17.4	Cesarean section	No
13	Male	70	2	21.5	No	No
14	Male	54	3	20.5	No	Urinary lithiasis; urinary infection
15	Male	42	7	18.8	Gastrectomy	
16	Male	72	3	24.8	No	
17	Male	61	6	22.1	No	High blood pressure
18	Female	63	2	20.8	Appendectomy	
19	Male	66	5	19.2	No	
mean	NA				NA	NA

BMI: Body mass index; NRS: Nutritional risk screening (2002); NA: Not available.

lymph node dissection. One case received combined spleen and pancreatic tail resection. Operation time was significantly reduced by the use of OrVil™ (198.42 ± 30.28 min *vs* 240.83 ± 8.23 min, $P < 0.05$). The postoperative course with regard to stenosis and leakage did not differ between the two groups. There were no significant differences in estimated blood loss (130.57 ± 65.17 mL *vs* 140.83 ± 78.41 mL, $P > 0.05$). The upper abdominal incision was smaller in OrVil™ group than in mini-laparoscopy group (4.31 ± 0.45 cm *vs* 6.43b ± 0.38 cm, $P < 0.05$).

Postoperative course

The mean time to first oral intake and postoperative hospital stay were 3.2 d (range, 2-5 d) and 12.5 d (range, 10-19 d). Anastomotic stenosis and major leakage occurred in one case, respectively. All the patients were evaluated at over stage I and received adjuvant chemotherapy.

Tumor characteristics

Histologically, 13 patients had poorly differentiated carcinoma and 3 patients had signet ring cell carcinoma. The mean tumor size was 4.5 cm (range, 3.2-7 cm). The location of the tumor was the upper body in 7 patients and the mid body in 11 patients. Esophageal invasion was detected in 1 patient and double lesions were detected in 1 patient who had a mid-body cancer. The mean length of proximal resection margin was 4.7 cm (range, 2.2-6.1 cm) and the distal one was 6.2 cm (range, 3.1-9 cm). TNM staging according to the 7th UICC identified stage ⅡA in 2, stage ⅡB in 7, stage ⅢA in 6, stage ⅢB in 5 and stage ⅢC in 1 patient. The mean number of retrieved lymph nodes was 22.4 (range, 16-42). Multiple lymph node metastases were detected, 1-2 lymph nodes in 2 patients, 3-6 in 8 patients and more than 7 in 11 patients.

DISCUSSION

Since the first report of LG in 1992[8], LAG has been carried out not only in distal and proximal gastrectomy, but also in total gastrectomy which was more often used in advanced gastric cancer[9-11]. Although performance of LATG for gastric cancer has been increasing worldwide, especially in Asia, it remains controversial if laparoscopic

Table 2 Comparisons of characteristics between trans-orally inserted anvil group and mini-laparotomy group

	OrVil™ group (n = 21)	Mini-laparotomy group (n = 6)	P value
Total operation time (min)	198 (180-320)	240 (230-290)	0.018
Total blood loss (mL)	130 (100-400)	140 (100-300)	0.211
Abdominal incision (cm)	4.3 (4-6)	6.4 (5.5-7.0)	0.022
Complications of anastomosis	2	0	1.000
Stenosis	1	0	1.000
Leakage	1	0	1.000

In square brackets: Range. OrVil™: Trans-orally inserted anvil.

D2 dissection is equivalent to open surgery for advanced gastric cancer (AGC). In our cases, the dissection of more than 15 lymph nodes was performed and the final cutting edge negative rate was 100%. Some recent studies focus on the outcome of D2 lymph node dissection in LAG and open surgery for gastric cancer[11-13]. Du *et al*[11] evaluated 82 patients with AGC who underwent LATG with D2 dissection compared with 94 patients who received open surgery; a similar number of harvested lymph nodes (HLNs) was obtained in both groups. Cui *et al*[13] retrospectively analyzed 131 cases including a single LATG group, and found that laparoscopic D2 dissection is equivalent to open gastrectomy in the number of HLNs, regardless of tumor location.

The mean operation time for LATG with OrVil™ was 198 min, which was significantly shortened compared with the traditional mini-laparotomy group (240 min), and the mean operation time for LATG was also significantly shorter than for mini-laparotomy (180 min *vs* 406 min) in the previous studies[9,10,13]. It takes a longer time to perform esophagojejunal anastomosis through a narrow mini-laparotomy in LATG, which can be avoided by the use of OrVil™. The same conclusion is confirmed by other operative team and with OrVil™ their mean operation time was 152-243 min which mainly affected by tumor stage[3,5,9,14].

The incidence of postoperative complications in patients who underwent LATG has been reported to be 9.4%-39.4%, and common complications include anastomotic leakage, anastomotic stenosis, and pancreatic fistula[9,2,14,15]. Some studies revealed that the incidence of complications in the LATG group was similar to that in the open total gastrectomy group; however, other studies showed a lower or higher rate of complications in the LATG group[15-17]. In this study, 1 case developed anastomotic leakage and 1 case had anastomotic stenosis. The complication rate was 27%, being slightly lower compared with those from previous studies[15,18,19]. The high frequency of anastomotic complications in patients who underwent LATG might result from the excessive traction of the distal esophagus and the extensive mobilization of the jejunal limb. In our series, the rates of complications associated with anastomosis were not statitistically different between the LATG with OrVil™ and traditional mini-laparoscopy groups. However, it should be mentioned that the number of the mini-laparotomy group was small which may produce statistics bias. The same procedure was performed postoperatively in these two groups, so the comparison of mean time to first oral intake and postoperative hospital stays was meaningless.

There are some reconstructive methods used after LATG, such as Roux-en-Y esophagojejunostomy, and extracorporeal or intracorporeal anastomosis using a hand-sewn, circular stapler, or side-to-side linear stapler[1,9,20,21]. Roux-en-Y esophagojejunostomy by extracorporeal anastomosis through a small skin incision is the most common approach. However, it is difficult to perform through a mini-laparotomy, particularly on obese patients, and too larger laparotomy makes it similar to conventional open surgery[22]. OrVil™ as an intracorporeal circular stapling esophagojejunostomy can simplify the reconstruction procedure after total gastrectomy[23]. This device requires no purse-string sutures and offers wide intracorporeal operating views[24,25]. In this study, compared with control group, the smaller body incision and less operation time were observed. Moreover, two respective studies concluded that this technique was simple, safe, and efficient for performing gastrojejunostomy, and additionally less expensive and accelerated the surgical learning curve[23,26,27]. However, the earlier studies reported postoperative infection and recommended oral gargling with hexamidine solution and abdominal irrigation after anvil insertion[9,28]. No postoperative abdominal infection occurred in our series.

There were some limitations in this study. First, this retrospective analysis might have selection bias as a result of comparison of these nonrandomized groups with a retrospective profile. Second, there was no survival data. Thus, long-term oncological outcomes of LATG with OrVil™ need to be evaluated by future studies. Third, the sample size of the mini-laparotomy group is small and the operation was performed in relatively early period which cause the learning curve effect.

In conclusion, LATG using OrVil™ for gastric cancer may be a technically feasible surgical procedure with advantages of sufficient lymph node dissection, less operation time and acceptable morbidity. However, the number of patients is small in this study. It will be necessary to confirm these results by a large cohort study in the validity of LATG with OrVil™.

COMMENTS

Background

Laparoscopic gastrectomy (LG) including laparoscopy-assisted gastrectomy (LAG) and totally LG with regional lymph node dissection as an alternative surgical treatment for gastric cancer has become increasingly common worldwide, especially in Asia.

Research frontiers

Since the first report of LG in 1992, laparoscopy-assisted gastrectomy has been carried out not only in distal and proximal gastrectomy, but also in total gastrectomy which was more often used in advanced gastric cancer. Although performance of laparoscopy-assisted total gastrectomy for gastric cancer has

been increasing worldwide, especially in Asia, it remains controversial if laparoscopic D2 dissection is equivalent to open surgery for advanced gastric cancer.

Innovations and breakthroughs

LATG using orally inserted anvil (OrVil™) for gastric cancer may be a technically feasible surgical procedure with advantages of sufficient lymph node dissection, less operation time and acceptable morbidity. However, the number of patients is small in this study. It will be necessary to confirm these results by a large cohort study in the validity of LATG with OrVil™.

Peer review

This is an interesting manuscript on LG with trans-orally anastomosis. Since little is known about this technique, many readers would be interested to learn this experience.

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META-ANALYSIS

Preoperative administration of bevacizumab is safe for patients with colorectal liver metastases

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Abstract

AIM: To assess the impact of preoperative neoadjuvant bevacizumab (Bev) on the outcome of patients undergoing resection for colorectal liver metastases (CLM).

METHODS: Eligible trials were identified from Medline, Embase, Ovid, and the Cochrane database. The data were analyzed with fixed-effects or random-effects models using Review Manager version 5.0.

RESULTS: Thirteen nonrandomized studies with a total of 1431 participants were suitable for meta-analysis. There was no difference in overall morbidity and severe complications between the Bev + group and Bev - group (43.3% *vs* 36.8%, $P = 0.06$; 17.1% *vs* 11.4%, $P = 0.07$, respectively). Bev-related complications including wound and thromboembolic/bleeding events were also similar in the Bev + and Bev - groups (14.4% *vs* 8.1%, $P = 0.21$; 4.1% *vs* 3.8%, $P = 0.98$, respectively). The incidence and severity of sinusoidal dilation were lower in patients treated with Bev than in patients treated without Bev (43.3% *vs* 63.7%, $P < 0.001$; 16.8% *vs* 46.5%, $P < 0.00$, respectively).

CONCLUSION: Bev can be safely administered before hepatic resection in patients with CLM, and has a protective effect against hepatic injury in patients treated with oxaliplatin chemotherapy.

Key words: Colorectal cancer; Liver metastases; Bevacizumab; Postoperative complication; Sinusoidal dilatation

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INTRODUCTION

The liver is the most common metastatic site of colorectal cancer (CC). Approximately 50% of CC patients develop colorectal liver metastases (CLM) over the course of the disease[1]. Hepatectomy is the most important modality in the treatment of CLM, offering a 5-year survival rate of approximately 30%-65%[2]. However, the long-term survival of CLM patients remains poor due to the high rate of recurrence and metastases after surgery. It is reported that neoadjuvant chemotherapy before hepa-

tectomy is associated with improved survival of CLM patients[3,4].

Bevacizumab (Bev) is a monoclonal antibody against vascular endothelial growth factor (VEGF) and can inhibit the growth of human tumor xenografts. In addition to inhibiting the antiangiogenic effect, Bev may also improve the delivery of chemotherapy by altering the tumor vasculature and decreasing the elevated interstitial pressure in tumors[5]. Randomized clinical trials[6,7] have demonstrated that the addition of Bev to fluorouracil-based combination chemotherapy can improve overall survival, the response rate and duration of response, and prolong time to disease progression in CLM patients with metastatic colorectal cancer. For this reason, some researchers advocate the use of Bev in the neoadjuvant setting before surgery for resectable CLM[8-11]. However, Bev is known to be associated with bleeding, thrombosis, gastrointestinal perforation, impaired wound healing and liver regeneration[8], thus alerts surgeons to the safe use of Bev at the time of hepatic surgery.

Chemotherapy-specific liver injuries have been more frequently reported as a disadvantage with the use of oxaliplatin in particular, because it is associated with the occurrence of sinusoidal dilation, a distinctive type of hepatic and vascular injury characteristic of hepatic veno-occlusive disease[12]. Nakano *et al*[13] reported that such injury was associated with increased morbidity and mortality after hepatectomy in CLM patients. Although the etiology of sinusoidal dilation remains unclear, it has been documented that VEGF is one of the causative cytokines for the development of sinusoidal dilation in patients undergoing bone marrow transplantation[14]. VEGF blockade by Bev may therefore attenuate sinusoidal injury[15].

Although several recent studies[8-10,15-24] have commented on the impact of preoperative Bev administration on the safety and/or oxaliplatin-associated liver injury after CLM resection, none of these studies were randomized controlled trials. A meta-analysis is therefore required to provide an improved level of evidence on this subject.

MATERIALS AND METHODS

Study selection

A Medline, Embase, Ovid and Cochrane database search was performed to identify all studies published up to March 2012 that assessed the influence of preoperative Bev administration on the outcome after CLM resection. The following Mesh search headings were used: "colorectal cancer", "liver metastases", "bevacizumab", "hepatic resection", "hepatectomy" and "neoadjuvant chemotherapy". A manual search of the reference lists of relevant papers was also carried out to identify additional trials.

Data extraction

Two reviewers (Li B and Wu LP) independently appraised each article and extracted the following parameters: first author, year of publication, study population characteristics, study design, number of patients in each arm, sex, age, inclusion and exclusion criteria, and outcomes of interest. All relevant text, tables and figures were reviewed for data extraction. Discrepancies between the reviewers were resolved through discussion and consensus.

Morbidity and mortality were defined as those events occurring within 30 or 90 d after surgery. Severe complications were defined as events requiring intensive care management or surgical, endoscopic, or radiologic interventions[25].

Criteria for inclusion and exclusion

Only trials that compared postoperative outcomes after hepatectomy between CLM patients treated with neoadjuvant chemotherapy with and without Bev administration before surgery were included. Abstracts, letters, proceedings from scientific meetings, editorials and expert opinions, reviews without original data, case reports, studies lacking control groups, repetitive data, non-English language papers and animal studies were excluded. To avoid drug interactions, studies involving other targeted molecular therapies were also excluded.

Statistical analysis

The meta-analysis was performed using the Review Manager (RevMan) software version 5.0 (The Cochrane Collaboration, Software Update, Oxford, United Kingdom). The Mantel-Haenszel method was used to combine odds ratio with 95%CI for the outcomes of interest, including overall morbidity, major complications, Bev-related complications, general complications, mortality, and incidence and severity of sinusoidal dilation. Heterogeneity between trials was assessed by calculating the Q and I^2 statistic. If $I^2 > 10\%$, a random-effects approach instead of a fixed-effects analysis was undertaken. Publication bias was assessed visually using a funnel plot. Statistical significance was defined as $P < 0.05$.

RESULTS

Eligible studies

Thirteen nonrandomized studies published between 2007 and 2012 met the inclusion criteria and were suitable for meta-analysis[8-10,15-24]. The characteristics of the included studies are summarized in Table 1. Sample size ranged from 31 to 274, with a total of 1431 participants.

Meta-analysis

Results from overall meta-analysis are outlined in Table 2.

Postoperative morbidity

Ten studies reported on overall morbidity, which was found to be comparable in the Bev + group and Bev - group (43.3% *vs* 36.8%, $P = 0.06$) (Figure 1A). Similarly,

Table 1 Study population characteristics of included trials

Ref.	Country	Group	No. of patients	M/F	Age (yr)	No. of lesions	Interval between Bev-treatment and surgery	MR
D'Angelica *et al*[8]	United States	Bev –	32	9/23	51 (34-77)[1]	1 (1-11)[1]	-	17
		Bev +	16	-	-	-	6.9 (3-15) wk[1]	-
Reddy *et al*[9]	United States	Bev –	57	37/20	60 (51-68)[1]	2 (1-4)[1]	-	39
		Bev +	39	25/14	55 (49-64)[1]	2 (1-3)[1]	10 (8-13) wk[1]	27
Mahfud *et al*[10]	Multicenter	Bev –	45	19/26	62 (59-65)[1]	6 (3-8)[1]	-	25
		Bev +	45	31/14	58 (54-61)[1]	4 (3-5)[1]	9 wk[1]	19
Ribero *et al*[15]	United States	Bev –	43	26/17	57 (26-80)[1]	2 (1-8)[1]	-	-
		Bev +	62	36/26	53.5 (34-85)[1]	2 (1-21)[1]	≥ 6 wk	-
Kesmodel *et al*[16]	United States	Bev –	44	30/14	58 (31-80)[1]	2 (1-9)[1]	-	30
		Bev +	81	48/33	57 (29-84)[1]	3 (1-31)[1]	58 (31-117) d[1]	47
Zorzi *et al*[17]	United States	Bev –	13	-	-	-	-	-
		Bev +	19	-	-	-	-	-
Aussilhou *et al*[18]	France	Bev –	20	-	-	-	-	20
		Bev +	11	-	-	-	-	9
Klinger *et al*[19]	Austria	Bev –	50	34/16	62.4[1]	-	-	-
		Bev +	56	32/24	63.0[1]	-	2-5 wk	-
Pessaux *et al*[20]	France	Bev –	21	13/8	63.3 ± 11.7[2]	3 ± 3[2]	-	4
		Bev +	21	10/11	65 ± 8.2[2]	3.8 ± 2.5[2]	11.7 ± 4.7 wk[2]	5
Rubbia-Brandt *et al*[21]	Multicenter	Bev –	204	-	-	-	-	-
		Bev +	70	-	-	-	-	-
Tamandl *et al*[22]	Austria	Bev –	112	35/77	63.6 (28.9-84.2)[1]	2 (1-10)[1]	-	17
		Bev +	102	39/63	63.3 (31.4-81.6)[1]	2 (1-10)[1]	34 (17-99) d[1]	28
Wicherts *et al*[23]	Multicenter	Bev –	97	61/36	62 ± 11[2]	4.5 ± 4.6[2]	-	38
		Bev +	67	42/25	58 ± 11[2]	5.6 ± 4.5[2]	8 (3-19) wk[1]	31
van der Pool *et al*[24]	The Netherlands	Bev –	53	33/20	62 (41-79)[1]	3 (1-7)[1]	-	14
		Bev +	51	29/22	64 (41-77)[1]	2 (1-8)[1]	11 (5-38) wk[1]	9

M: Male; F: Female; MR: Major resections (≥ segments). [1]Median (range); [2]Mean.

Table 2 Results of meta-analysis

Outcome of interest	No. of studies	No. of patients	OR	95%CI	*P*-value	I^2 (%)
Overall morbidity	10[8-10,16-18,20,22-24]	Bev + = 452, Bev - = 494	1.17	1.00, 1.37	0.06	0
Severe complication	8[8-10,16,17,20,22,23]	Bev + = 390, Bev - = 421	1.57	0.97, 2.53	0.07	18
Wound complication	6[8-10,16,22,23]	Bev + = 269, Bev - = 296	1.43	0.82, 2.50	0.21	0
Bleeding/thromboembolic complication	5[8-10,23,24]	Bev + = 218, Bev - = 284	0.99	0.41, 2.38	0.98	0
Cardiovascular complication	2[16,23]	Bev + = 148, Bev - = 141	1.14	0.23, 5.56	0.88	0
Pulmonary complication	6[8,16,18,20,23,24]	Bev + = 247, Bev - = 267	1.10	0.60, 2.02	0.77	0
Renal or urinary complication	6[8,16,18,20,23,24]	Bev + = 247, Bev - = 267	0.73	0.24, 2.23	0.58	0
Hepatic dysfunction	5[16-18,20,23]	Bev + = 223, Bev - = 219	0.48	0.22, 1.05	0.07	0
Mortality	10[8-10,16-18,20,22-24]	Bev + = 452, Bev - = 494	0.63	0.16, 2.56	0.52	0
Overall sinusoidal dilation	6[15,19-21,23,24]	Bev + = 265, Bev - = 395	0.53	0.38, 0.75	< 0.001	0
Moderate or severe sinusoidal dilation	6[15,18-21,24]	Bev + = 267,Bev - = 387	0.34	0.19,0.64	< 0.001	46

OR: Odds ratio; Bev: Bevacizumab.

there was no significant difference in severe complications between the Bev + and Bev - groups (17.1% *vs* 11.4%, $P = 0.07$) (Figure 1B). Nor was there a significant difference in cardiovascular, pulmonary and renal or urinary complications between the Bev + and Bev - groups (2.7% *vs* 2.1%, $P = 0.88$; 10.1% *vs* 9.3%, $P = 0.67$; 1.2% *vs* 2.2%, $P = 0.58$, respectively).

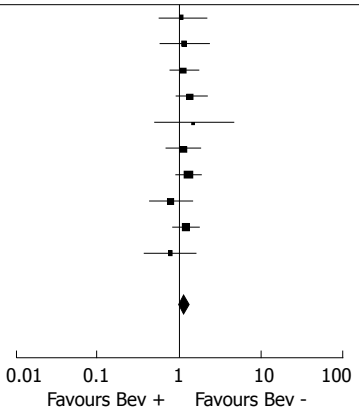
Bev-related complications including wound and thromboembolic/bleeding events were also similar in the Bev + and Bev - groups (14.4% *vs* 8.1%, $P = 0.21$; 4.1% *vs* 3.8%, $P = 0.98$, respectively). Four studies reported other types of Bev-related complications[16,22-24]. Kesmodel *et al*[16] reported hypertension in nine patients and proteinuria in two patients. van der Pool *et al*[24] reported hypertension in one patient. In one study, mild arterial hypertension occurred before surgery in one patient, necessitating dose reduction and treatment with beta-blocker therapy. No bowel perforations occurred in 13 patients with primary colorectal tumor *in situ* who received Bev. Anastomotic leakage with localized peritonitis occurred in one of seven patients who underwent synchronous colorectal and hepatic resections[23]. In another report by Tamandl *et al*[22], one patient developed anastomotic dehiscence after combined hepatic surgery and right colectomy.

Five studies reported on hepatic dysfunction, which was nonsignificantly less frequent in the Bev + group as compared with the Bev - group (5.3% *vs* 9.5%, $P = 0.07$).

A

Study or sub-category	Bev + Events	Bev + Total	Bev - Events	Bev - Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
Aussilhou 2009	6	11	10	20	4.2	1.09 (0.54, 2.19)
D'Angelica 2007	7	16	12	32	4.7	1.17 (0.57, 2.38)
Kesmodel 2008	40	81	19	44	14.5	1.14 (0.76, 1.71)
Mahfud 2010	25	45	18	45	10.6	1.39 (0.89, 2.16)
Pessaux 2010	6	21	4	21	2.4	1.50 (0.49, 4.56)
Reddy 2008	17	39	22	57	10.6	1.13 (0.70, 1.83)
Tamandl 2010	45	102	38	112	21.4	1.30 (0.93, 1.82)
van der Pool 2012	13	51	17	53	9.8	0.79 (0.43, 1.46)
Wicherts 2011	29	67	35	97	16.9	1.20 (0.82, 1.76)
Zorzi 2008	8	19	7	13	4.9	0.78 (0.38, 1.62)
Total (95%CI)		452		494	100.0	1.17 (1.00, 1.37)
Total events	196		182			

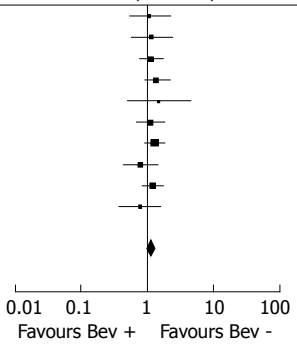
Heterogeneity: χ^2 = 3.94, df = 9 (P = 0.92), I^2 = 0%
Test for overall effect: Z = 1.92 (P = 0.06)



B

Study or sub-category	Bev + Events	Bev + Total	Bev - Events	Bev - Total	Weight (%)	Odds ratio M-H, random, 95%CI
D'Angelica 2007	1	16	3	32	4.0	0.64 (0.06, 6.74)
Kesmodel 2008	16	81	5	44	15.8	1.92 (0.65, 5.65)
Mahfud 2010	14	45	8	45	18.1	2.09 (0.78, 5.63)
Pessaux 2010	0	21	0	21		Not estimable
Reddy 2008	11	39	14	57	20.2	1.21 (0.48, 3.03)
Tamandl 2010	11	102	8	112	19.2	1.57 (0.61, 4.08)
Wicherts 2011	11	67	5	97	15.2	3.61 (1.19, 10.95)
Zorzi 2008	3	19	5	13	7.5	0.30 (0.06, 1.58)
Total (95%CI)		390		421	100.0	1.57 (0.97, 2.53)
Total events	67		48			

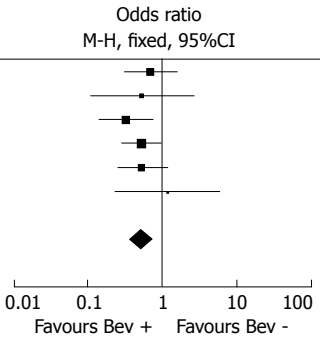
Heterogeneity: Tau^2 = 0.07; χ^2 = 7.29, df = 6 (P = 0.29), I^2 = 18%
Test for overall effect: Z = 1.84 (P = 0.07)



C

Study or sub-category	Bev + Events	Bev + Total	Bev - Events	Bev - Total	Weight (%)	Odds ratio M-H, fixed, 95%CI
Klinger 2009	22	52	24	47	16.7	0.70 (0.32, 1.55)
Pessaux 2010	3	21	5	21	4.9	0.53 (0.11, 2.59)
Ribero 2007	17	62	23	43	22.6	0.33 (0.14, 0.74)
Rubbia-Brandt 2010	45	70	158	204	33.1	0.52 (0.29, 0.94)
van der Pool 2012	25	51	34	53	19.5	0.54 (0.25, 1.18)
Wicherts 2011	3	9	8	27	3.1	1.19 (0.24, 5.96)
Total (95%CI)		265		395	100.0	0.53 (0.38, 0.75)
Total events	115		252			

Heterogeneity: χ^2 = 2.76, df = 5 (P = 0.74), I^2 = 0%
Test for overall effect: Z = 3.58 (P = 0.0003)



D

Study or sub-category	Bev + Events	Bev + Total	Bev - Events	Bev - Total	Weight (%)	Odds ratio M-H, random, 95%CI
Aussilhou 2009	6	11	6	20	11.4	2.80 (0.61, 12.86)
Klinger 2009	5	52	16	46	17.2	0.20 (0.07, 0.60)
Pessaux 2010	2	21	4	21	8.8	0.45 (0.07, 2.76)
Ribero 2007	5	62	12	43	16.7	0.23 (0.07, 0.70)
Rubbia-Brandt 2010	22	70	127	204	28.7	0.28 (0.16, 0.50)
van der Pool 2012	5	51	15	53	17.2	0.28 (0.09, 0.83)
Total (95%CI)		267		387	100.0	0.34 (0.19, 0.64)
Total events	45		180			

Heterogeneity: Tau^2 = 0.26; χ^2 = 9.26, df = 5 (P = 0.10), I^2 = 46%
Test for overall effect: Z = 3.40 (P = 0.0007)

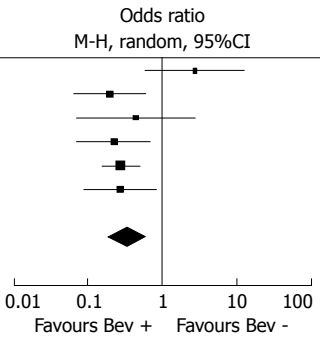


Figure 1 Forest plot. A: Overall morbidity; B: Severe complications; C: Overall sinusoidal dilation; D: Moderate or severe sinusoidal dilation. Bev: Bevacizumab.

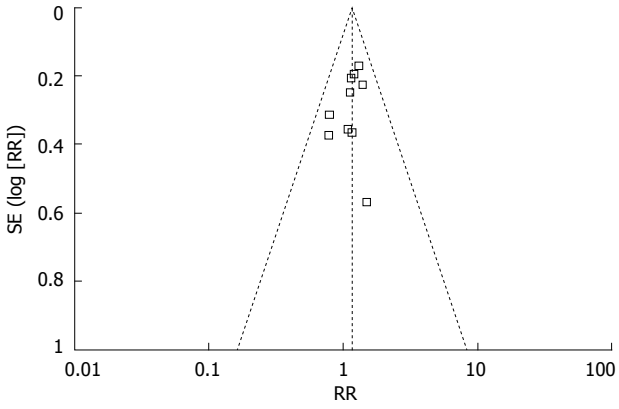


Figure 2 Funnel plot of the results obtained from studies comparing overall morbidity. RR: Risk ratio.

Postoperative mortality

Ten studies reported on postoperative mortality. There were 3 (0.6%) deaths in the Bev + group, which was similar to that in the Bev - group (5 deaths, 1.0%).

Nontumorous liver histology

Seven studies evaluated the effect of Bev for CLM on nontumorous liver histology, and one study reported a significant difference in neoadjuvant treatment regimens between patient groups. To ensure homogeneity within groups, only patients treated with oxaliplatin were included for analysis[23]. Pooled analysis showed that Bev significantly reduced the incidence (Bev + 43.3% *vs* Bev - 63.7%, $P < 0.001$) and severity (Bev + 16.8% *vs* Bev - 46.5%, $P < 0.001$) of sinusoidal dilation (Figure 1C, D).

Publication bias

A funnel plot of the studies included in the meta-analysis reporting on overall morbidity is shown in Figure 2. None of the studies lay outside the limits of the 95%CI, and there was no evidence of publication bias.

DISCUSSION

Liver regeneration is an important component of the recovery process that occurs after various forms of hepatic injury, including partial hepatectomy (PH)[26]. Angiogenesis, the formation of new blood vessels, is a fundamental process in liver regeneration and repair. VEGF is considered a key regulator of normal and pathological angiogenesis. VEGF increases vascular dilatation and permeability, and induces the migration and proliferation of endothelial cells. These activities are mediated *via* two receptors for VEGF: kinase insert domain-containing receptor, and fms-like tyrosine kinase-1 receptor[27,28]. Endogenous expression of VEGF in hepatocytes and its receptors in endothelial cells has been shown to increase after PH[26].VEGF treatment protected the liver against chemically induced cytotoxicity, associated with a marked increase in the proliferation of hepatocytes and sinusoidal endothelial cells (SEC)[28,29]. In addition, exogenous VEGF administration promoted the increase of vessel density, vessel diameter, intrasinusoidal space, liver body weight ratio and hepatocyte proliferation after PH in the rat model. Conversely, these effects were completely suppressed by anti-VEGF treatment[30]. These results suggest that VEGF plays an important role in liver regeneration. Therefore, the safety of VEGF inhibitor administration at the time of hepatic surgery needs to be addressed.

The present meta-analysis shows that both overall and severe complications were not significantly different between the Bev + and Bev - groups. In addition, Bev treatment did not seem to increase the risk of Bev-related complications (wound and bleeding/thromboembolic events), hepatic dysfunction, and postoperative deaths. On the other hand, Wicherts *et al*[23] reported that liver functional recovery parameters including prothrombin time and serum total bilirubin level were equivalent between the Bev + and Bev - groups in the postoperative period, suggesting that Bev administration should be safe before hepatic resection.

To increase the safety of surgery, preoperative portal vein embolization (PVE) has been used for major hepatic resection in CLM patients, because PVE can induce homolateral atrophy and contralateral compensatory hypertrophy of the remnant liver, thus decreasing the risk of postoperative liver failure[31]. In a relatively large cohort study of 100 patients, Covey *et al*[32] found that the mean growth of non-embolized hemiliver was comparable in patients treated with and without neoadjuvant chemotherapy (22% ± 3% *vs* 26% ± 3%) with a similar number of patients with less than 5% growth of liver (4 *vs* 6) after PVE. Similar findings were also reported by other authors[33,34]. Zorzi *et al*[17] found that chemotherapy with Bev did not alter non-embolized liver hypertrophy. In contrast, a study conducted by Aussilhou *et al*[18] demonstrated that the hypertrophy of the future liver remnant after PVE was impaired in patients treated with Bev. These inconsistent results may be attributed to different durations of chemotherapy used in the two studies. It was reported that postoperative morbidity was correlated with the number of cycles of chemotherapy before surgery. Karoui *et al*[35] reported that morbidity in patients who received six or more cycles of chemotherapy was significantly higher that that in patients who received less than six cycles (54% *vs* 19%, $P = 0.047$). Indeed, almost 80% of the patients in the series by Aussilhou *et al*[18] received six or more cycles of chemotherapy compared with 53% in the study by Zorzi *et al*[17].

Although oxaliplatin is often utilized as a chemotherapeutic agent in the treatment of CLM, it can exert adverse effects on the liver. Oxaliplatin-based chemotherapy has been shown to cause hepatic sinusoidal dilation[12]. Our study has shown that Bev can significantly reduce the incidence and severity of sinusoidal dilation,

thus supporting the use of Bev for CLM. An explanation for the above protective effect remains unclear. Increased expression of matrix metalloproteinase (MMP)-9 and MMP-2 by SEC was found to play an important role in sinusoidal dilation development in the monocrotaline-induced rat model[36]. An *in vitro* study[37] suggested that VEGF could up-regulate MMP-9 expression. It is postulated that VEGF blockade by Bev may attenuate sinusoidal injury by down-regulating MMP-9[15].

There is no consensus on the optimal time interval between discontinuation of Bev and hepatic surgery. D' Angelica *et al*[8] found that postoperative complications were more common in patients who received Bev within 8 wk. Similarly, Reddy *et al*[9] noted that patients who underwent hepatectomy within 8 wk of Bev treatment may be at a higher risk of overall, severe and hepatic complications after surgery. Whereas Kesmodel *et al*[16] failed to confirm that the time interval from discontinuation of Bev ($\geq$ 60 *vs* < 60 d) to surgery was associated with an increased likelihood of developing complications. In addition, a subgroup analysis of patients who received Bev also demonstrated that there was no significant difference in complication rates between patients who received Bev 31-45 d, 46-60 d and greater than 60 d before surgery (P = 0.21). In another report, Mahfud *et al*[10] showed that the occurrence of postoperative complications was similar in patients who had received Bev for < 6 wk and in those who had taken Bev $\geq$ 6 wk before liver resection. Based on the evidence that the median half-life of Bev in humans is approximately 21 d (range 11-50 d), some authors recommend waiting at least 6-8 wk from discontinuation of Bev to surgery[8,9,16].

The main limitation of this meta-analysis was that all evidence came from nonrandomized trials which could introduce potential bias in data collection and analysis. However, there is evidence that nonrandomized studies may generally give valid results[38].

In conclusion, our meta-analysis has shown that Bev can be safely administered before hepatic resection in CLM patients, knowing that it has a protective effect against oxaliplatin-related liver injury. The issue of optimal timing of hepatectomy in patients treated with Bev should be addressed in future prospective multicenter trials.

COMMENTS

Background

Bevacizumab (Bev), a monoclonal antibody that targets vascular endothelial growth factor (VEGF) A, has recently been added to standard neoadjuvant chemotherapy in patients with colorectal liver metastases (CLM). However, Bev is reportedly associated with bleeding, thrombosis, gastrointestinal perforation, impaired wound healing and liver regeneration. Therefore, the safety of Bev at the time of hepatic surgery needs to be addressed.

Research frontiers

The study evaluated the impact of preoperative administration of neoadjuvant Bev on the outcome of patients undergoing resection for CLM using a meta-analysis of all relevant studies.

Innovations and breakthroughs

Findings from this meta-analysis suggest that preoperative Bev does not seem to increase postoperative morbidity after hepatic surgery of CLM and has a protective effect against hepatic injury in patients treated with oxaliplatin chemotherapy.

Applications

Bev can be safely administered in CLM patients before hepatic resection.

Terminology

Bev, a monoclonal antibody against VEFG, can inhibit the growth of human tumor xenografts.

Peer review

The topic is interesting and the methodology of the meta-analysis is appropriate.

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CASE REPORT

Degarelix as a new antiangiogenic agent for metastatic colon cancer?

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Abstract

Recently, follicle stimulating hormone receptor was found to be selectively expressed by endothelial cells on tumor-associated blood vessels in a wide range of human cancers. In this context, we hypothesized that degarelix, a new gonadotropin-releasing hormone receptor antagonist developed for patients with prostate cancer, may have antiangiogenic effects *via* its capacity to block follicle stimulating hormone (FSH) production. We report the case of a patient with metastatic colon cancer exhibiting tumor progression after failure of all conventional chemotherapeutic regimens. The addition of degarelix to the last chemotherapeutic regimen was proposed as compassionate treatment. Degarelix induced a rapid decrease in FSH level. This treatment induced radiological stabilization and carcinoembryonic antigen stabilization during 1 year. Contrast-enhanced ultrasonography demonstrated reduction of tumor vasculature. This case represents the first report of an antitumoral effect of degarelix in metastatic colon cancer and suggests an antiangiogenic property of this drug.

Key words: Colon cancer; Degarelix; Chemotherapy; Angiogenesis; Antiangiogenic agents

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INTRODUCTION

In adult humans, the follicle stimulating hormone receptor (FSHR) is expressed only in the testicular sertoli cells and the ovarian granulosa cells. Recently, FSHR was also found to be selectively expressed by endothelial cells on tumor-associated blood vessels in a wide range of human cancers, compared to blood vessels from normal tissue[1]. This leads to the hypothesis that follicle stimulating hormone (FSH) and FSHR could be involved in endothelial cell survival or proliferation, especially during tumor-associated angiogenesis. Thus, the FSH pathway could be a new target for cancer therapy, notably in cancer types in which tumor angiogenesis is critical for cancer progression. Degarelix is a new gonadotropin-releasing hormone (GnRH) receptor antagonist, developed for patients with prostate cancer requiring androgen-deprivation therapy. Degarelix blocks the GnRH receptors in the anterior pituitary gland, resulting in a rapid decrease in the secretion of both luteinising hormone (LH) and FSH[2]. In this context, we hypothesized that degarelix may have antiangiogenic effects *via* its capacity to block FSH production, thereby interrupting the FSH-FSHR pathway on tumor-associated blood vessels.

We report here on a patient with metastatic colon cancer exhibiting tumor progression after failure of all conventional chemotherapeutic regimens, and who ex-

perienced long term disease stabilization associated with tumor devascularization under degarelix treatment.

CASE REPORT

A 69 year-old woman was referred to our center for sideropenic anemia in March 2008. A tumor of the cecum was discovered and the patient underwent right colectomy. The tumor was staged T3 N2, and non-resectable peritoneal carcinomatosis was discovered. The tumor had a K-RAS mutation status. The patient was initially treated starting in July 2008 by FOLFIRI + bevacizumab, and subsequently by LV5FU2 + bevacizumab because of hematological toxicity and grade 3 diarrhea. During treatment, the tumor marker carcinoembryonic antigen (CEA) decreased from 15 ng/mL, to 6 ng/mL in July 2009. Disease progression occurred in December 2009, with a CEA level of 14 ng/mL. The patient received FOLFOX + bevacizumab from December 2009 to September 2010, at which time CEA level increased to 42 ng/mL, and the patient presented an occlusive syndrome with abdominal pain requiring morphine treatment. On compassionate grounds, we proposed the addition of degarelix to LV5FU2 + bevacizumab. Degarelix was initiated subcutaneously at 240 mg for 1 mo, followed by monthly maintenance doses of 80 mg. FSH level was 42.5 IU/L before the first injection. A peritoneal nodule of 135 mm was selected as a target lesion for radiological evaluation by computed tomography (CT) scan and contrast-enhanced ultrasonography. Fifteen days after the first injection, the FSH serum level was < 2 IU/L (Figure 1A), and remained at this level during the entire treatment period. Contrast-enhanced ultrasonography was performed on day 0 and day 15. Radiological evaluation was performed by the same 2 independent radiologists, who were blinded to the patient's clinical, biological and treatment data. A single target tumor was studied and selected on the basis of size (> 2 cm), and site (tumors with a good acoustic window that enabled data acquisition for longer than 3 min). A dynamic study was conducted after a single intravenous bolus injection of 4.8 mL of a contrast agent consisting of sulfur hexafluoride-filled microbubbles (SonoVue; Bracco, Milan, Italy). The investigation recordings and timing were triggered as soon as the contrast agent was injected. A total of 720 images were acquired during each 3-min investigative examination. Three semi-quantitative perfusion parameters were extracted from the time-intensity curves, namely peak intensity, time to peak intensity, total area under the time-intensity curve (AUC)[3]. Before degarelix initiation, AUC of the peritoneal nodule was 1650 mm^2. Fifteen days after treatment initiation, a dramatic reduction of almost 80% in the vascularized surface was observed, at 351 mm^2 (Figure 1B, C) (The area of interest is marked by a dotted white line). Moreover, resolution of the occlusive syndrome was achieved 5 wk after degarelix initiation, and morphine treatment was stopped without residual abdominal pain. Tolerance was excellent, without side effects using NCI-CTCAE v3 grade. Patient was previously treated by amlodipine for bevacizumab related and blood pressure was not modified by degarelix. The patient was menopause at the beginning of the treatment with degarelix so no side affect related to gonad suppression were observed. CT scans performed 2 mo after the beginning of the treatment showed stable disease according to the RECIST criteria with a stable target lesion and a regression of small none target lesion of peritoneal carcinomatosis. Successive CT scans performed between September 2010 and August 2011 confirmed stable disease according to the RECIST criteria (version 1.1) (target lesion evaluated between 137 and 138 mm). CEA levels remained stable during this period (Figure 1A). Unfortunately, occlusive syndrome re-appeared in October 2011. CT scan showed peritoneal progression, and only best supportive care was maintained. The patient died 6 wk later.

DISCUSSION

To the best of our knowledge, this case represents the first report of an antitumoral effect of degarelix in metastatic colon cancer.

The FSH receptor was recently recognized as a specific marker of endothelial cells in blood vessels from different tumor types[1]. Biological data have shown that the binding of FSH to FSH receptor in granulosa cells induces increased production of the hypoxia-inducible factor 1α[4]. This transcriptional factor is commonly induced by hypoxic conditions and drives the up-regulation of vascular endothelial growth factor (VEGF), one of the most important pro-angiogenic factors secreted during cancer growth. Thus, we hypothesized that triggering the FSH-receptor could induce VEGF production and thereby promote tumor angiogenesis. FSH signaling is also known to generate activated Gq protein[5]. Gq protein has been shown to induce VEGFR-2 signaling in human endothelial cells, even in the absence of VEGF[6]. This effect may enhance the proliferation and migration of endothelial cells in cancer, independently of VEGF availability. Thus, blocking FSH-receptor signaling may represent a new antiangiogenic strategy. In this report, we present the first clinical observation that degarelix, a GnRH antagonist used for the treatment of castrate-sensitive prostate cancer, could considerably decrease FSH production in a post-menopausal cancer-bearing woman. Contrast-enhanced ultrasonography seemed to indicate that this period of 12 mo with disease stabilization in a previously chemorefractory patient could be related to an antiangiogenic effect of degarelix, suggesting that this treatment could overcome resistance to anti-VEGF therapies, such as bevacizumab. However, we cannot provide specific immunohistochemical analysis of FSHR expression on tumor-associated blood vessels from the patient's peritoneal tumors. This constitutes a

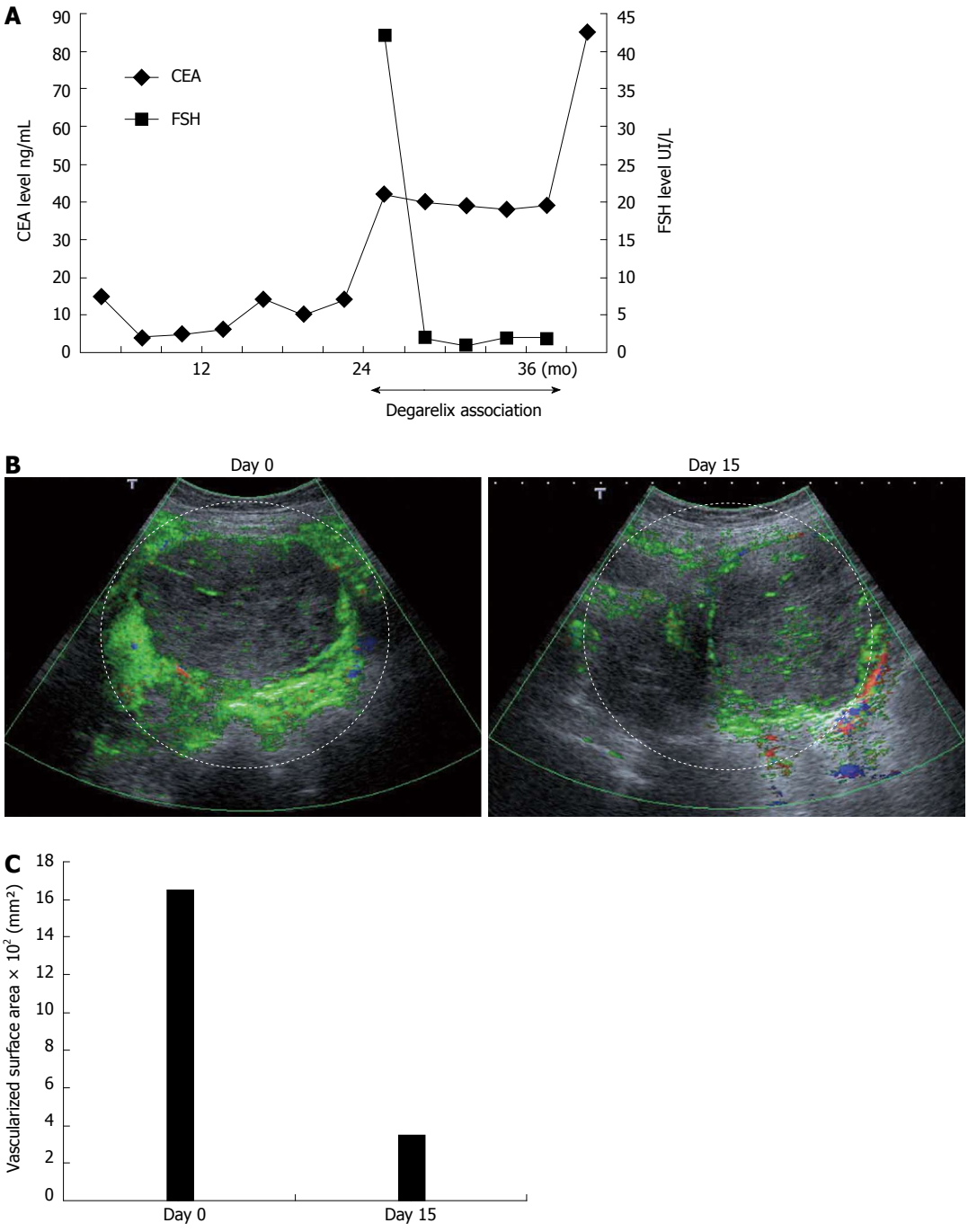

Figure 1 A tumor of the cecum. A: Kinetic of carcinoembryonic antigen (CEA) and follicle stimulating hormone (FSH) serum level; B: Representative images of contrast-enhanced ultrasonography performed before (left panel) and 15 d after (right panel) the beginning of the treatment by degarelix; C: Evaluation of vascularized surface area on contrast-enhanced ultrasonography performed before and 15 d after the beginning of the treatment by degarelix.

limitation of this report, but it should be noted that in the initial report by Radu *et al*[1], consistent expression of FSHR by endothelial cells was detected in all the colorectal cancers analyzed. As we do not test the efficacy of degarelix monotherapy was could not determine if only combination of 5 FU/leucovorin bevacizumab and degarelix or degarelix monotherapy alone could be proposed in further clinical trial. Nonetheless, further clinical trials guided by FSHR expression on tumor vasculature are warranted to investigate the antiangiogenic effect of degarelix in cancer patients.

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CASE REPORT

Internal carotid thrombus in patients with inflammatory bowel disease: Two cases

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Abstract

Increased ischemic stroke risk is observed in patients with inflammatory bowel disease (IBD). Causes and physiopathological aspects of cerebral infarct, in this specific population, are less often described. There is little information to provide guidelines for the best curative and preventive treatment. We report 2 cases of ischemic strokes due to internal carotid thrombus in patients during active phase of IBD. Ulceration of early atherosclerotic plaques activated by a hypercoagulation state may cause a thrombus. A combined therapy with heparin and corticosteroids was used for both our patients. Lysis of the thrombus was obtained after several days without surgical treatment and shown by ultrasonography. These cases highlight an aetiology of stroke in patients with IBD and use of a synergic treatment to respond to hypercoagulability in link with IBD. Benefits and safety of this therapy should be confirmed with clinical studies.

Key words: Inflammatory bowel disease; Ischemic stroke; Carotid thrombus; Hypercoagulable state; Atherosclerosis

Richard S, Mione G, Perrin J, Toussaint-Hacquard M, Lacour JC, Ducrocq X. Internal carotid thrombus in patients with inflammatory bowel disease: Two cases. *World J Gastroenterol* 2013; 19(5): 773-775 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i5/773.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i5.773

INTRODUCTION

Patients with inflammatory bowel diseases (IBD) have a higher risk of arterial cerebral infarction even if they are young with few cardio-vascular risk factors[1]. Causes and physiopathological aspects are less known. We describe 2 cases of IBD patients who presented ischemic strokes due to homolateral internal carotid thrombus.

CASE REPORT

Case report 1

A 32 year-old man, with Crohn's disease (CD) without immunosuppressive treatment for several years, was admitted for sudden right hemiplegia. He was a light smoker without any other vascular risk factor like hypertension, dyslipidemia, diabetes, and had no family history of vascular disease. At admission the National Institute of Health Stroke Scale (NIHSS) was 13, and blood pressure was 110/70 mm Hg. Brain magnetic resonance imaging (MRI) revealed ischemic stroke with thrombosis of the initial part of the left middle cerebral artery (MCA). Intravenous thrombolysis with plasminogen tissue activator was administered. Cervical ultrasonography (US) revealed occlusion of the left internal carotid artery (Figure

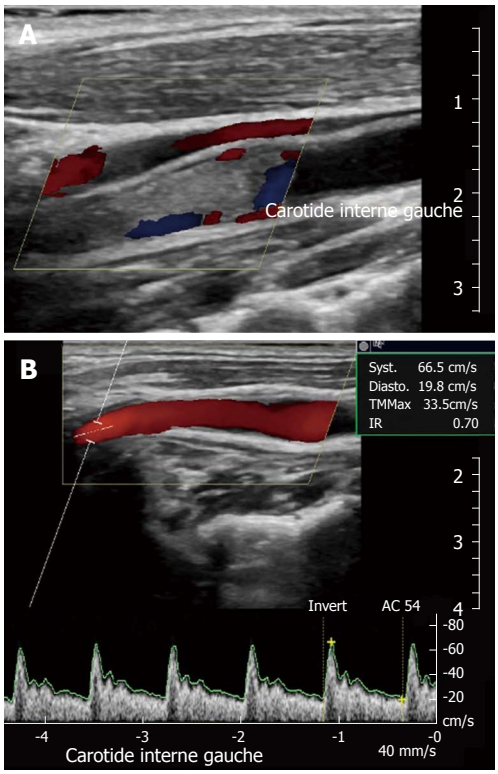

Figure 1 Cervical ultrasonographies in case 1, showing occlusion of the left internal carotid (A), due to a thrombus, and repermeabilization of the vessel with tiny plaque (B) after 15 d of treatment.

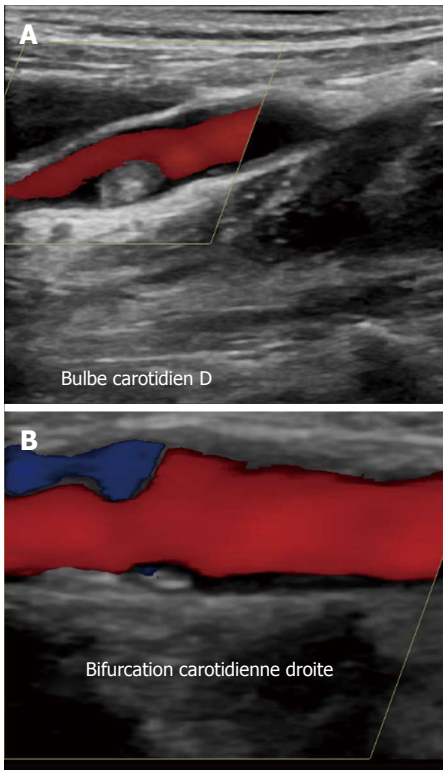

Figure 2 Cervical ultrasonographies in case 2, showing thrombus in the right internal carotid (A) and resorption of this with tiny plaque (B) after 1 wk of treatment.

1A), due to a thrombus, confirmed by computed tomography (CT) angiography. Blood tests were performed: the platelet count was 342 G/L, hyperhomocysteinemia 137 μmol/L; C-reactive protein (CRP) 15.4 mg/L; low-density lipoprotein (LDL)-cholesterol 0.87g/L; preprandial blood glucose level 1.01g/L, and the erythrocyte sedimentation rate (ESR) was elevated at 40 mm. Digestive endoscopy found signs of active CD. Unfractionated heparin and corticosteroid treatment (prednisolone 0.5 mg/kg per day) were initiated and 15 d later, there was total resorption of the thrombus, apart from a tiny plaque as controlled by cervical US (Figure 1B) and CT angiography. One month later, the NIHSS was 6, an overlap treatment of vitamin K antagonist was initiated and the patient was transferred to a rehabilitation center with a daily dose of prednisolone and folic acid. Proctectomy with terminal ileostomy was performed 4 mo later.

Case report 2

A non-smoker 42 year-old woman with ulcerative colitis diagnosed 3 years previously and treated with corticosteroids and mesalazine, was hospitalised for sudden right hemiplegia. She had no personal medical history of diabetes, hypertension and dyslipidemia and no family history of vascular disease. She had already presented several ischemic strokes, revealing intra-cardiac thrombus, but had refused any oral anticoagulant treatment. At admission, the NIHSS was 19 and blood pressure was 120/80 mmHg. A brain MRI showed a recent ischemic stroke with occlusion of the origin of the right MCA. Cerebral angiography with thrombectomy was performed and complicated by hemorrhagic transformation of the cerebral infarct. Severe bloody diarrhoea was controlled by a high dose of corticosteroids (methylprednisolone 1 mg/kg for 10 d before progressive decrease). Cervical US showed a thrombus in the right internal carotid (Figure 2A). There were biological signs of inflammation with an elevated ESR at 64 mm and CRP at 14.4 mg/L, slight hyperhomocysteinemia (17.9 μmol/L), a low level of LDL-cholesterol at 0.80 g/L, a normal preprandial blood glucose level at 0.90 g/L, and a platelet count of 462 G/L. Ten days later, after partial resorption of cerebral haemorrhage, low molecular weight heparin (LWMH), enoxaparine, was introduced at a dose of 100 UI/kg twice a day. After one week of treatment, cervical US revealed regression of the thrombus with only a tiny plaque on the right internal carotid wall (Figure 2B). LWMH was switched for aspirin as the patient rejected any oral anticoagulant. She was finally transferred to a rehabilitation center with a NIHSS of 6.

DISCUSSION

These observations suggest that 2 factors can contribute to carotid thrombus: abnormalities of the coagulation system and disease of arterial wall[2].

Hypercoagulability is almost always observed during the active phase of IBD and can lead to an arterial or

intracardiac thrombus, as described in the second case. Elevated platelets (with hyperactivation), fibrinogen, factor V, Ⅷ, plasminogen activator inhibitor and decreased antithrombin and tissue plasminogen activator have been described. Vitamin K malabsorption causes not only short partial thromboplastin times but also protein C and S deficiency[3]. There may also be a higher prevalence of antiphospholipid antibodies in IBD patients[4]. Common carotid artery intima-media thickness is significantly higher in IBD patients compared with controls with a significant association with homocysteine levels and age[5]. This is due to modifications of the vessel-wall induced by chronic inflammation: cytokines activate endothelium with conversion to a pro-inflammatory thrombogenic phenotype and increased von Willebrand factor[6]. Accelerated atherosclerosis is found in systemic inflammatory disorders[7], and atherosclerotic plaques were found after resorption of carotid thrombus in both the cases described here, as well as in a similar case described by Lafitte *et al*[8]. In IBD patients, an inflammatory state may also lead to endothelial damage and early atherosclerotic lesions. In the cases we describe, the formation of carotid thrombus may have been due to ulceration of plaques which have activated a pro-thrombotic coagulation state. For both our patients, lysis of the thrombus was obtained after several days of heparin treatment with double impact: fibrinolysis and the still debated anti-inflammatory action[9]. Nevertheless, this approach has only been described in a few cases and should be evaluated with clinical studies, especially as it may provoke mucosal[10] and cerebral bleeding in case of extensive stroke. We also administered corticosteroids to both our patients to decrease the inflammation, hypercoagulation state, to promote mucosal regeneration and then, to potentiate the benefits of heparin.

Internal carotid thrombus is a cause of ischemic stroke in IBD patients, and is a consequence of the combination of early atherosclerotic lesions and hypercoagulation state. Combining heparin and anti-inflammatory therapy may be considered as a synergic treatment but, further work is required to establish efficacy and safety.

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CASE REPORT

Strangulated ileal trans-coloanal-anastomotic hernia: A complication of Altemeier's procedure previously never reported

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Abstract

A postoperative complication after Altemeier operation, so far never reported, is described in a 42 years old mentally disabled patient with external full thickness rectal prolapse who usually had prolonged straining at defecation. After 6 d from perineal rectosigmoidectomy, the patient, was discharged free of complications. Four days later he was readmitted in emergency for strangulated perineal trans-anastomotic ileal hernia that occurred at home during efforts to defecate. The clinical feature required an emergency operation for repositioning the ileal loops into the abdomen, resection of the necrotic ileum, and end colostomy. The outcome of the second operation was free of complication and the patient was discharged on the 6th postoperative day. In conclusion, after Altemeier operation prolonged straining at defecation should be carefully avoided

Key words: Rectal prolapsed; Perineal rectosigmoidectomy; Altemeier's procedure; Complication; Hernia

Di Lena M, Angarano E, Giannini I, Guglielmi A, Altomare DF. Strangulated ileal trans-coloanal-anastomotic hernia: A complication of Altemeier's procedure previously never reported. *World J Gastroenterol* 2013; 19(5): 776-777 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i5/776.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i5.776

INTRODUCTION

Full thickness rectal prolapse is an uncommon but very disabling anorectal disease affecting any age and in particular mentally disabled patients. Several surgical approaches have been proposed to treat this disorder including abdominal rectopexy and perineal mucosal (Delorme) or full thickness rectal resection (Altemeier). The Altemeier's procedure is usually preferred (1) in elderly, high-risk, frail patients unsuitable for abdominal operation; (2) for emergency incarcerated external prolapse[1] and for gangrenous rectal prolapse[2]; and (3) in mentally disabled patients because of its short, painless postoperative course and low complication rate.

CASE REPORT

A 42-year-old male patient affected by congenital hypothyroidism with a psychomotor retardation, anorexia and bulimia was admitted to our Colorectal Unit because of irreducible full thickness rectal prolapse. From several years the patient used to have prolonged and forced

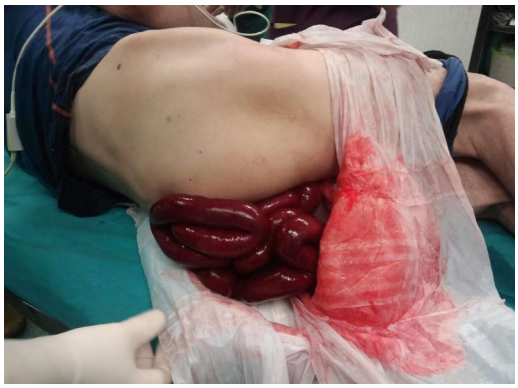

Figure 1 Strangulated ileal trans-coloanal-anastomotic perineal hernia showing ischaemia of the herniated ileum.

straining at defecation (about 30 min) with exteriorization of the prolapse at every defecation. However, in the last 2 mo the rectal prolapse became irreducible. A perineal rectosigmoidectomy with levatorplasty (Altemeier's operation) was performed under general anesthesia with the patient in jack-knife position. Twelve cm of prolapsed rectum was resected and a hand-sewn interrupted coloanal anastomosis was performed. The patient was discharged on the 6th postoperative day free of complication and oral laxatives and antithrombotic prophylaxis were prescribed. Four days later the patient was re-admitted in emergency since a strangulated ileal trans-coloanal-anastomotic hernia occurred after a prolonged and strain defecation (Figure 1). Laparotomy was urgently performed and no signs of intrabdominal infection or ascites were found. However, the distal part of the ileum (about 2 m) was herniated outside the pelvis through a partial posterior dehiscence of the colo-anal anastomosis with torsion of intestinal loops around the vascular stalk. The large portion of small intestine herniated was reduced with difficulty into the abdomen with the help of a second operator from the perineal side. Once the ileum was replaced into the abdomen, the coloanal anastomosis was fully opened and the anus temporarily closed with continue resorbable suture. The vitality of the herniated ileum (up to 5 cm from the ileo-cecal valve) was poor after one hour and, therefore, it was resected. Then, a hand-sewn ileo-cecal anastomosis and a diverting sigmoid end colostomy were performed. The second postoperative course was uneventful and the patient was discharged on the 6th postoperative day. The patient was re-evaluated at follow-up for 4 mo and he is now waiting for a refashioning of the colo-anal anastomosis.

DISCUSSION

Altemeier's procedure is usually recommended in mentally disabled patients with full thickness rectal prolapse because of its short and painless postoperative course. Postoperative complications are uncommon (less than 10%[3,4] of cases), nevertheless they usually occur in the early postoperative days. The complication we herein described, to our knowledge for the first time, was unexpected since it occurred several days after the patient was discharged in good clinical condition and with normal bowel movements.

We suggest that anamnestic data of the behavior of prolonged straining at defecation in mentally disabled patients should have been considered, and adequate advise given to the relatives to assist patients during defecation in order to prevent strain. Finally, the choice of creating a diverting sigmoidostomy instead of trying to repair the coloanal anastomosis seems more prudent, due to the presence of edema in traumatized anastomotic leakage.

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CASE REPORT

Ectopic adrenal cortical adenoma in the gastric wall: Case report

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Abstract

Ectopic adrenal cortical neoplasms are extremely rare. Ectopic adrenocortical tissue can be found in locations such as the celiac axis, the broad ligament, the adnexa of the testes, and the spermatic cord; however, they rarely involve the stomach. We report an unusual case of a patient with an ectopic adrenal cortical adenoma in the gastric wall. The patient was a 72-year old female admitted to our hospital with upper abdominal discomfort. Physical examination revealed tenderness below the xiphoid process. Both computed tomography and fibergastroscopy revealed a mass on the lesser curvature side of the gastric antrum; it was initially diagnosed as a gastric stromal tumor. After adequate preparation, the patient underwent surgery. During the procedure, we found a 30 mm × 30 mm mass with medium density in the lesser curvature near the gastric antrum within the serosa. Following immunohistochemistry examination, we corrected the diagnosis to an ectopic adrenal cortical adenoma; the tumor was nonfunctional.

Key words: Ectopic adrenal cortical neoplasms; Adrenal adenoma; Stomach; Adult; Nonfunctional adenoma

Ren PT, Fu H, He XW. Ectopic adrenal cortical adenoma in the gastric wall: Case report. *World J Gastroenterol* 2013; 19(5): 778-780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i5/778.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i5.778

INTRODUCTION

The adrenal gland arises from primordial mesenchyme in the wall of the dorsal coelom adjacent to the dorsal mesentery and urogenital structures. Therefore, most ectopic adrenocortical tissue is found along the path of embryonic migration within the urogenital tract. Ectopic adrenocortical tissue is found in such locations as the celiac axis, the broad ligament, the adnexa of the testes, and the spermatic cord[1]. However, an ectopic adrenal cortical adenoma rarely involves the stomach. We report an unusual case of a patient with an ectopic adrenal cortical adenoma in the gastric wall and review the literature.

CASE REPORT

The patient was a 72-year old female. She was admitted on 17th November, 2011 with upper abdominal discomfort for 4 d, accompanied by postprandial nausea and vomiting. She denied chills, fever, or diarrhea. Her medical and family history were noncontributory.

Physical examination: temperature 37.5 ℃, pulse 90 bpm, respiration 20 bpm, blood pressure 172/96 mmHg. The heart and lungs were normal. The abdomen was flat and soft, with tenderness below the xiphoid process. No enlarged liver, spleen, or mass was palpable. Murphy's sign was negative. Laboratory and radiology findings: Routine blood work showed white blood cell 12.7 × 10^9/L, neutrophils 85.3%. Liver function tests were in normal range. Fasting blood glucose was 8.88 mmol/L. Adrenocorticotropic hormone was in normal range. On B-ultrasonography, the gallbladder was 64 mm × 37 mm. The gallbladder wall was thickened, with a stone incarcerated

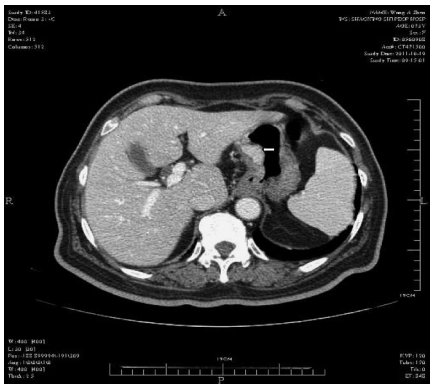

Figure 1 Enhanced image of nodule located in the lesser gastric curvature.

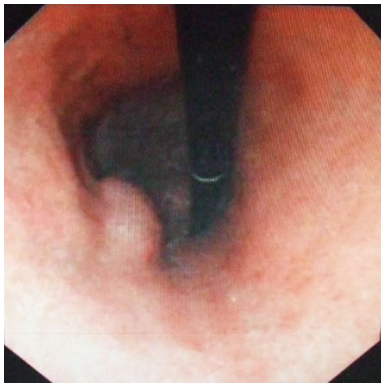

Figure 2 Elevated nodule on the lesser curvature side of the gastric antrum.

in the neck. Computed tomography (CT) scan showed a 15 mm × 25 mm abnormal enhanced nodule located in the submucosa of the lesser curvature of the stomach with a CT value of 150 HU. The size, morphology, and location of the kidneys and adrenal glands were normal bilaterally, and there were multiple cysts in both kidneys. The radiological diagnoses were multiple renal cysts and a gastric stromal tumor (Figure 1). Fibergastroscopy showed no hyperemia in the gastric mucosa. A raised nodule about 30 mm × 25 mm in size was found on the lesser curvature side of the gastric antrum; it was firm with soft mucosa. The endoscopic diagnosis was also a gastric stromal tumor (Figure 2). After adequate preparation, the patient underwent surgery. During the operation, we found a distended gallbladder with a stone inside. There was a 30 mm × 30 mm mass with medium density in the lesser curvature near the gastric antrum and the mass located in the serosa layer. The liver, spleen, pancreas, kidney, and adrenal glands were normal. We resected the gallbladder, ligated the vessels of lesser gastric curvature, and opened the gastric wall 3 cm from the margin of the mass. We found that the gastric mucosa above the mass was integrated, and we performed a simple resection of the mass. On gross examination, the mass was purplish-red in color. It was a 20 mm × 30 mm ellipse with soft margins and a medium texture (Figure 3A). On microscopic exam, the tumor cells were rich in cytoplasm and eosinophilic, and numerous sinusoid capillaries could be seen. The cytoplasm contained melanin, which was confirmed by decolorization. The tumor cells were arranged in cords or gobbets with a low ratio of nucleus to cytoplasm, and rare mitotic figures (Figure 3B, C). Immunohistochemistry: S-100 basement cell negative (Figure 4A), melan-A positive (Figure 4B), P63 basement cell negative, sinusoidal endothelial CD34 positive (Figure 4C). The pathologic diagnosis was of an ectopic adrenal cortical adenoma in the gastric wall.

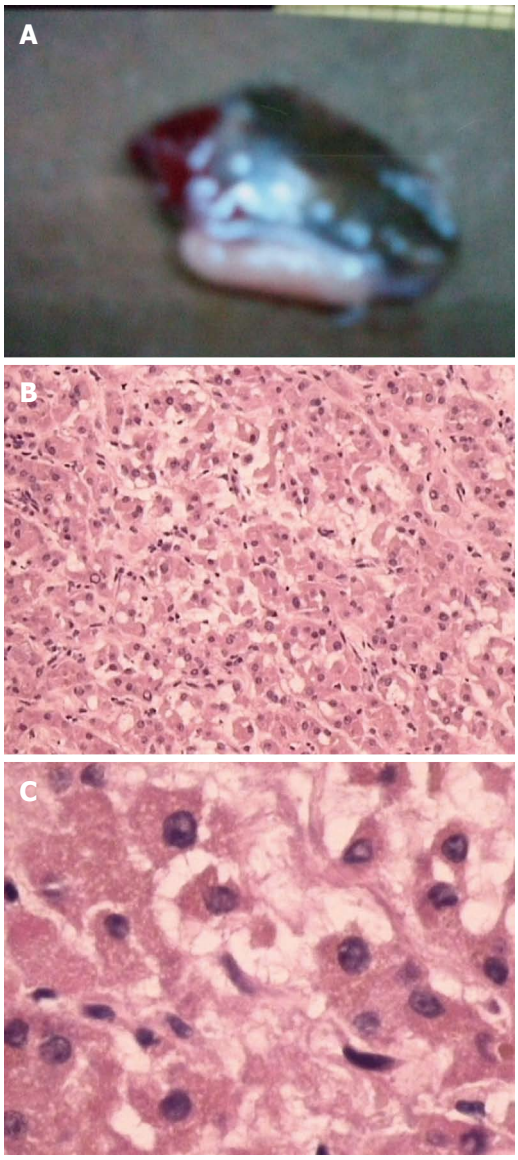

Figure 3 Analysis of tumor cells. A: Gastric wall mass, approximately 20 mm × 30 mm in size; B: Tumor cells under low power magnification; C: Tumor cells under high power magnification.

DISCUSSION

Ectopic adrenal tissue is mostly found in children. According to Anderson *et al*[2], accessory adrenal tissue is found in 50% of post-mortem examinations in neonates

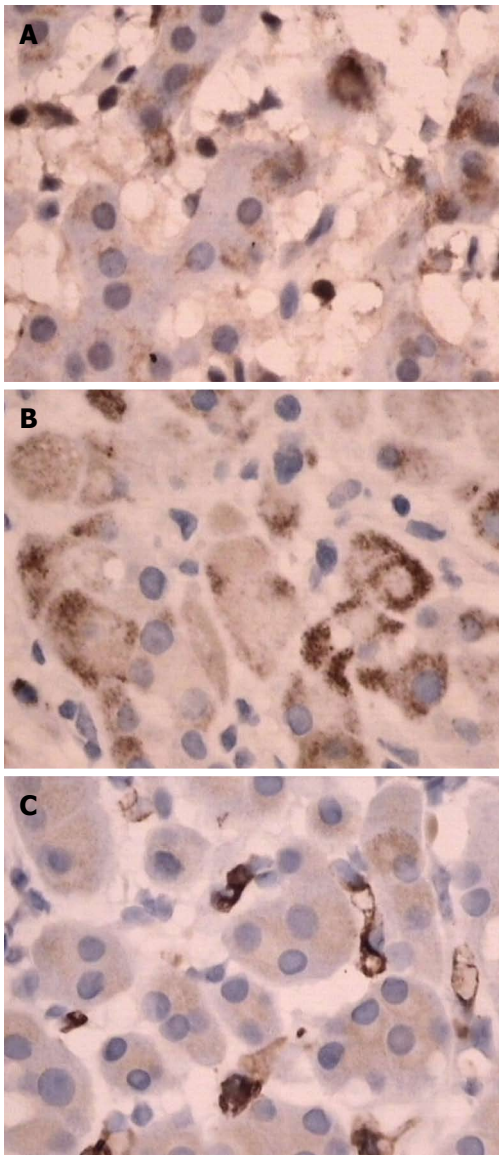

Figure 4 Immunohistochemistry. A: S-100 basement cell negative; B: Melan-A positive; C: CD34 positive.

and children. Usually, maturity leads to atrophy of the ectopic adrenal tissue, so that such tissue is found in only 1% of adults. The adrenal cells have a double embryological origin. The cortex arises from the coelomic mesothelium and the medulla from neural crest ectoderm. At approximately 7 ± 8 wk of pregnancy, the medulla components start moving towards the cortical elements, forming the adrenal gland. During migration of the medulla, fragments of tissue, most frequently the cortex, can be separated, forming accessory adrenal glands. Most ectopic adrenals remain in the vicinity of the adrenal gland, but they are also found to be closely related to the sex organs because of the spatial relationship between the adrenal primordium and the genital ridge in early embryogenesis. Accessory adrenal tissue can also be incorporated into adjacent organs due to incomplete separation of cortical adrenal cells from the coelomic mesothelium. Therefore, most ectopic adrenocortical tissue is found along the path of embryonic migration within the urogenital tract. The most common sites include the fat tissue of the posterior peritoneum near the adrenal glands, the celiac axis, the broad ligament, the adnexa of the testis, and the spermatic cord. There are also rare reported cases of ectopic adrenal cortical adenoma in the lung, spinal region, and brain[3-8].

However, there is no previous report of ectopic adrenal cortical adenoma of the gastric wall in the literature. In the case reported here, the patient presented with abdominal discomfort and the mass was found by CT. It was initially diagnosed as a gastric stromal tumor, which was corrected to ectopic adrenal cortical adenoma by pathology; the tumor was nonfunctional. The patient had no history of operation on adrenal tissue, so we believe the ectopic adrenal cortical adenoma in the gastric wall was due to the malposition or self-differentiation of mesothelial cells during the embryonic period.

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CASE REPORT

Splenic lymphangioma that manifested as a solid-cystic mass: A case report

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Abstract

Lymphangioma, a congenital malformation of the lymphatic system, is usually found in children, and generally occurs in the neck and mediastinum. It is rarely found in the spleen. The clinical features of splenic lymphangioma typically include abdominal pain, nausea, and abdominal distention. Frequently, however, this condition is asymptomatic and is incidentally detected by abdominal ultrasonography or by an abdominal computed tomography (CT) scan. In this paper, we retrospectively describe a case of incidentally detected splenic lymphangioma in a 30-year-old woman with special abdominal contrast material-enhanced CT findings, which was accurately diagnosed by histopathology. The clinical and physical examinations related to the mass were negative. A few cases of splenic lymphangioma have been reported previously; however, the presentation of the mass and the enhancement pattern in the contrast medium-enhanced CT images were quite extraordinary. These findings had misled our abdominal radiologists to consider it as other neoplastic diseases of the spleen.

Key words: Lymphangioma; Benign tumor; Spleen; Splenic neoplasia; Computed tomography

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INTRODUCTION

Splenic lymphangiomas are usually benign tumors that predominantly affect children, whereas only a few cases have been reported in adults[1-3]. Previous case reports revealed no particular findings in the image analysis, which uniformly indicated solitary cysts, or multiple cysts without contrast enhancement or only with slight enhancement of the thin septa[1,3-5], except for one case of a solid mass with diffuse and prolonged enhancement[2]. Here we present a histopathologically proven case in which a solid-cystic mass of the spleen in the computed tomography (CT) images showed no enhancement of the cysts and prominent enhancement of the solid components. To the best of our knowledge, these special contrast-enhanced CT findings are relatively rare.

CASE REPORT

A 30-year year-old woman was admitted to our hospital with a splenic mass detected by abdominal ultrasonography, which was performed for recurrent epigastric pain at another hospital. The epigastric pain was later attributed to gall bladder lithiasis. The patient's past and familial medical histories indicated no abnormalities. There were no positive symptoms related to the splenic mass. Physical examinations detected no mass or splenomegaly in the left upper quadrant of the abdomen. Peripheral blood count, coagulation studies, and liver and kidney function tests were all within normal limits.

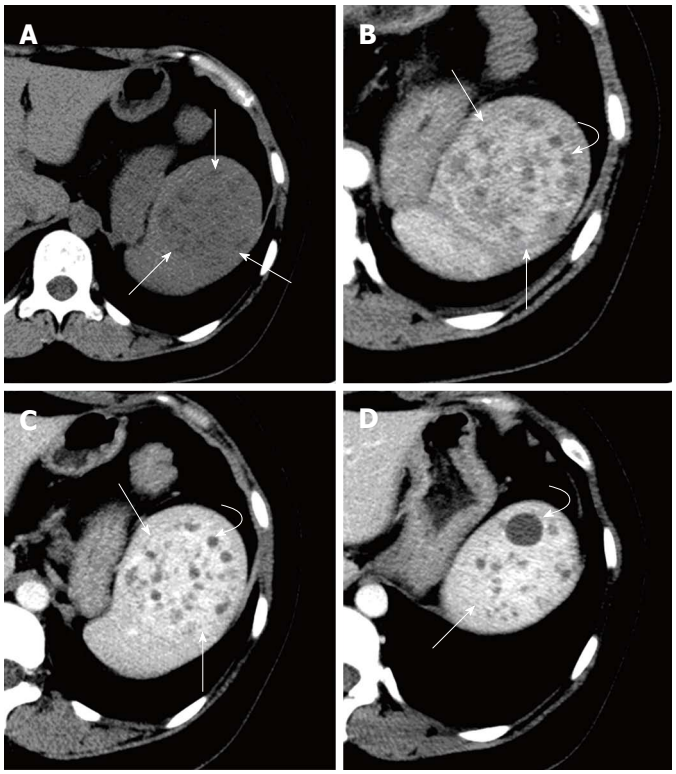

Figure 1 Computed tomography findings of the splenic lymphangioma. A: A heterogeneous solid-cystic mass (straight arrow) with a well-defined margin in the spleen, as shown in the plain image. The solid components show slight hypo-density; B: Arterial phase image shows the mass (straight arrow) with multiple non-enhanced cysts (curved arrow). The solid portions manifest as obvious enhancement; C: Parenchyma phase image shows obviously progressive enhancement of the solid portions; D: Parenchyma phase image of the upper location of the mass showing the same presentation and enhancement pattern.

CT showed a heterogeneous solid-cystic mass with a well-defined margin located in the normal sized spleen in the plain images. Within the mass, there were multiple small cysts of variable size. The cysts showed sharp borders with no enhancement at either the arterial or parenchymal phase, whereas the solid components presented with persistently progressive enhancement (Figure 1). At this point, our radiologists suspected a splenic tumor.

Splenectomy was eventually undertaken for the splenic mass. During the operation, a solid mass was found, which had no clear boundary with the peripheral splenic parenchyma. The final histopathological result was splenic cavernous lymphangioma. The findings revealed multiple lymphatic vesicles of variable size, and the blood sinuses in the red pulp within the mass that were dilated compared with the peripheral red pulp. The vesicles were lined by a single layer of flat endothelial cells, and some vesicles were filled with pink eosinophilic lymphatic fluid (Figure 2).

DISCUSSION

Lymphangiomas are generally considered to be congenital malformations of the lymphatic system, and they occur mostly in the neck, mediastinum and retroperitoneum[4]; they are seldom found in the spleen. Histologically, lymphangiomas are classified into three subtypes according to the congenital dilated lymphatic channels: capillary (super-microcystic), cavernous (microcystic), or cystic (macrocystic)[6]. Our case was ultimately diagnosed as the cavernous type. Cavernous lymphangiomas are composed of numerous irregular cavernous spaces containing amorphous eosinophilic fluid and few lymphocytes, lined by a single layer of flat endothelial cells[7]. Splenic lymphangiomas are mostly asymptomatic; therefore, the final diagnosis should be based on a combination of clinical, radiological, and histopathological findings.

In most cases, splenic lymphangiomas present with thin-walled cystic masses without enhancement or with only slight enhancement of the thin septa in the CT studies. We detailed a histologically confirmed case in which the mass manifested as a solid-cystic lesion. The multiple cysts showed no enhancement, as in previous cases[1,3-5]; however, the solid components showed obvious progressive enhancement. Histopathologically, however, there were no real solid components, and the solid, prominently enhanced portions in the CT images simply represented the dilated blood sinuses in the red pulp within the mass. The presence of reduced blood velocity and collecting blood in the dilated blood sinuses may have resulted in more contrast medium per voxel compared with the peripheral normal sinuses, and may have eventually led to obviously progressive enhancement.

Thus, lymphangioma presenting with this kind of solid-cystic mass in the image studies may be misdiagnosed. The differential diagnoses of splenic lymphangiomas often include other solid and cystic lesions of the spleen, such as hemangioma, chronic infection, lymphoma and metastasis. Hemangiomas may sometimes display solid and cystic portions[8]. Contrast-enhanced CT scans may show delayed enhancement within the solid portion. Punctate calcification can be seen in the central solid portion, and curvilinear calcification may be seen in the cystic areas[8]. Parasitic infection with *Echinococcus granulosus* is the main cause of cystic proliferations of the spleen[9]. The differential factors are the patients' history, calcifica-

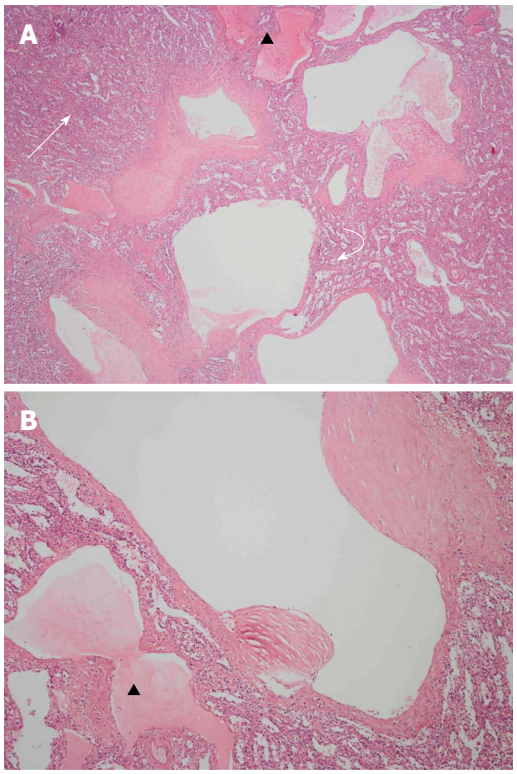

Figure 2 Histopathological findings of the splenic lymphangioma. A: Hematoxylin and eosin (HE) staining of the splenic lymphangioma, × 40, indicates the presence of numerous cystic lesions of variable size, and blood sinuses (curved arrow) in the red pulp within the mass that are dilated as compared with peripheral red pulp (straight arrow); B: HE staining, × 100, indicates that some vesicles are filled with pink eosinophilic lymph sacs (black triangle).

tion in the cystic walls, a cyst with daughter cysts or concomitant cystic lesions in the liver or other organs[10].

In conclusion, dynamic contrast-enhanced CT is a useful method for evaluating splenic lesions. We suggest that lymphangioma should be considered in the differential diagnosis of a splenic mass that manifests as a solid-cystic lesion in the image studies. In our case, the dilated blood sinuses in the red pulp within the mass might have contributed to the indication of solid components with evident enhancement.

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Severe gastrointestinal mucositis following high dose melphalan therapy for multiple myeloma

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Abstract

Mucositis is a known complication following use of chemotherapy, but fatal mucositis is unusual and management of such cases may be challenging. Pathologically there is denudation of mucosa of gastrointestinal tract. Severe cases can develop ileus and even perforation of bowel wall. We report here a case of multiple myeloma who developed World Health Organization grade 4 gut mucositis following the use of high dose melphalan with the expulsion of "intestine-like" material.

Key words: Gastrointestinal mucositis; Chemotherapy

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TO THE EDITOR

Mucositis is the most common complication of chemotherapeutics agents. Melphalan when used in high doses for treatment for multiple myeloma can lead to severe mucositis[1,2]. Patient can develop gastrointestinal ulcers, vomiting and diarrhea with the use of high dose melphalan as a conditioning regimen for autologous transplant for multiple myeloma[3-5]. Pathologically there is denudation of mucosa of gastrointestinal tract. Severe cases can develop ileus and even perforation of bowel wall. We report here a case of multiple myeloma who developed World Health Organization (WHO) grade 4 gut mucositis following the use of high dose melphalan with the expulsion of "intestine-like" material.

A 62-years-old male was admitted for autologous stem cell transplant for multiple myeloma. He was in complete remission following 4 cycles of bortezomib and dexamethasone. Filgrastim (G-CSF) 10 mg/kg was given for 4 d for peripheral stem cell mobilization followed by melphalan 200 mg/m^2 as conditioning regimen. The CD34+ stem cell dose infused was 6×10^6/kg. Cephasol (supersaturated calcium phosphate rinse) was used six times daily to prevent oral mucositis. On day 3 patient developed vomitings and by day 5 he had WHO grade 4 gut mucositis with ileus. Patient also developed fever and was started on empirical antibiotics including meropenem (1 g three times daily, intravenously), metronidazole (500 mg three times daily, intravenously), and teicoplanin (400 mg once daily, intravenously) as per hospital policy, alongwith Ryle's tube aspiration and parenteral feeding. On day 7 he passed 25 cm × 1 cm pinkish, mucosa coated partly digested material mimicking segment of small intestine (Figure 1). Histopathological section of the segment revealed numerous neutrophils surrounding the partly digested material, without any muscle tissue (Figure 2). Blood culture was negative for bacteria and fungus. Viral serology tests for cytomegalovirus immunoglobulin M (IgM) and herpes simplex virus 1 and 2 IgM were negative. Stool examination did not reveal ova or cysts and was negative for Clostridial difficile toxin. Computed tomography of abdomen showed dilated bowel loops, with caecal wall thickness of 10 mm, there was no evidence of perforation or intussusception. Though he had neutrophil engraftment (absolute neutrophil count

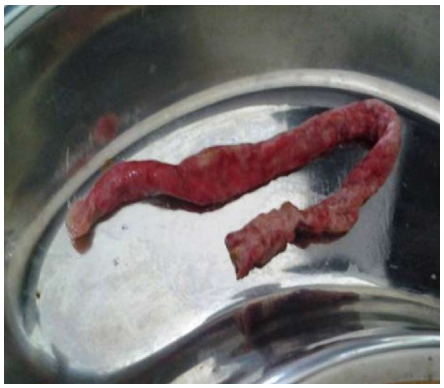

Figure 1 Specimen showing the excreted, blood tinged, elongated material, measuring about 25 cm, resembling part of small intestine.

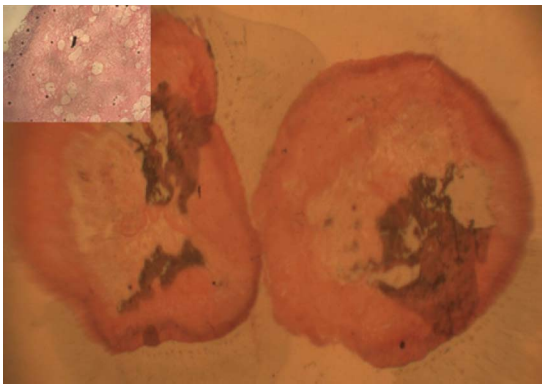

Figure 2 Cross section of the excreted material showing numerous neutrophils surrounding the partly digested material (inset, top left).

more than 0.5×10^9/L) on day 13, platelets remained below 10×10^9/L. Patient succumbed on day 20 because of enterocolitis and sepsis.

Oral and gastrointestinal mucositis due to cancer chemotherapy or radiotherapy continues to be an important clinical problem. Alimentary tract mucositis can involve any part of oral and gastrointestinal mucosa, from the mouth to the anus. The incidence of WHO grade 3 or 4 gastrointestinal mucositis can be as high as 20%-60% in patients undergoing hematopoietic stem cell transplantation, depending on the intensity of the conditioning regimen used[3,4]. In the study by Krishna *et al*[2], of the 47 patients who developed lower alimentary tract mucositis there was no mortality. Our patient developed severe gastrointestinal toxicity following high dose of melphalan, with excretion of a large mucosa coated segment mimicking part of the small intestine, and succumbed to the complications related to mucositis.

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Organization as author

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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