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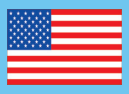
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EDITORIAL

Serum response factor: Look into the gut

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Abstract

Serum response factor (SRF) is a transcription factor that regulates many genes involved in cellular activities such as proliferation, migration, differentiation, angiogenesis, and apoptosis. Although it has only been known for about two decades, SRF has been studied extensively. To date, over a thousand SRF studies have been published, but it still remains a hot topic. Due to its critical role in mesoderm-derived tissues, most of the SRF studies focused on muscle structure/function, cardiovascular development/maintenance, and smooth muscle generation/repair. Recently, SRF has received more attention in the digestive field and several important discoveries have been made. This review will summarize what we have learned about SRF in the gastrointestinal tract and provide insights into possible future directions in this area.

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Key words: Angiogenesis; Cell invasion; Myofibroblast differentiation; Smooth muscle contraction; Serum response factor; Wound healing

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INTRODUCTION

Although serum response factor (SRF) has only 25-year history, its studies have been exponentially grown in several fields including smooth muscle structures, cardiac functions, cellular stress responses and cell motility. SRF is a ubiquitously expressed transcription factor, therefore, its role should be far beyond these areas. When the new millennial dawn broke, we opened a new field for SRF research-digestive system. Several important discoveries have been made in different parts of the system ever since, which foresee a bright and fruitful future for this area. This article is to provide you an update in this line of study and hopefully point you to the right direction.

HISTORY OF SRF

SRF was first identified by Treisman[1] in 1986 based on a previous observation in Greenberg's lab that resting cells responded to serum addition with a rapid activation of *c-fos*[2]. He discovered that it is SRF that initiates the immediate response of c-fos to serum or any other growth factors by binding to a short DNA sequence-serum response element (SRE), which is located about 300 bp upstream of the *c-fos* gene transcription initiation site. Since then, SRE has been identified in as many as 300 human genes, accounting for 1% of our entire genome[3,4]. Although it has only been known for a little over two decades, studies on SRF have been populated exponentially. Last year, more than a hundred SRF studies were documented in PubMed; and ten papers have already been published within the first 3 wk of this

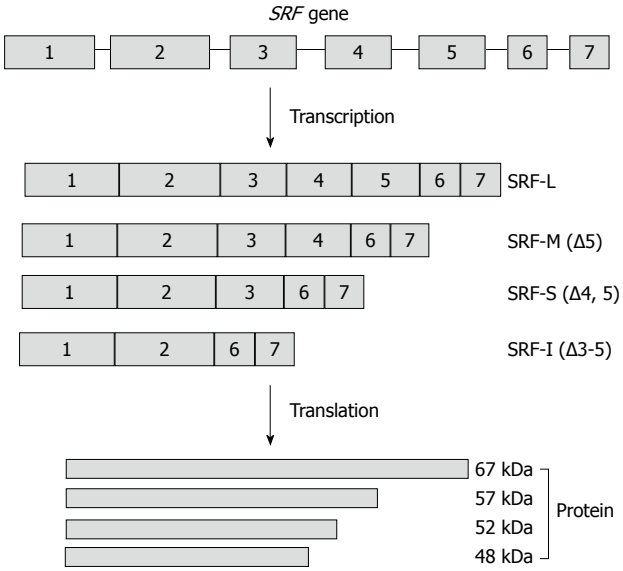


Figure 1 Serum response factor (SRF) splice variants (adapted from Chai and Tarnawski 2002).

year. Most of the published SRF studies deal with its functions in muscle structures or in the regulation of immediate early genes. However, SRF is a ubiquitously expressed protein and thus its role must be far beyond these areas. A few years ago, we started to look for SRF in the gastrointestinal (GI) tract[5,6]. After that, several other groups have expanded our mission. Today, there have been over 20 studies published dealing with SRF in the digestive system.

BIOCHEMISTRY OF SRF

The human *SRF* gene was mapped to chromosome 6p21.1. It is 10607 bp long and contains 7 exons. In both humans and mice, SRF can be expressed in different isoforms due to alternative splicing and some of them appear to display tissue specificity[7]. For instance, SRF-S, which lacks both exon 4 and 5 (Δ4, 5), has only been detected in the aorta, while SRF-I, which is the shortest isoform (missing exon 3, 4 and 5), is specific to embryonic tissues. On the other hand, SRF-M, which lacks only exon 5, has been shown to be a dominant negative mutant (Figure 1).

Full length SRF protein, which is approximately 67 kDa, was shown to contain three distinct domains: a SRE DNA binding domain, a transactivation domain and multiple phosphorylation sites[8]. The DNA binding domain, which also serves for dimerization and interaction with accessory factors, has been highly conserved throughout evolution, showing a 93% homology between fruit flies and humans[9]. Phosphorylation at Serine 103, which is immediately adjacent to the DNA binding domain, was shown to greatly enhance SRF activity[10]. Since its initial discovery in response to serum, SRF has also been shown to be activated by several other agents, including mitogens, cytokines, specific oncogenes and extracellular stimuli, such as antioxidants, UV light and microgravity, to name a few[7].

FUNCTIONS OF SRF

SRF is a master regulator of many cellular activities including cell growth and differentiation, cell migration, and apoptosis. To date, approximately 300 human genes have been estimated to contain an SRE element and be activated by SRF, accounting for 1% of our entire genome[3,4]. Early transgenic data provided important clues to some of the biological functions of SRF, best elucidated through its role in the myocardium, which is of mesodermal origin, and to the different optimal expression requirements during embryogenesis and adulthood[7]. More specifically, mice with complete SRF knockout (*srf* -/-) failed to develop the mesoderm and died in the uterus between E8.5 and E12.5[11], indicating that SRF is required for early embryonic development. For this reason, we generated a mouse model with overexpression of a dominant mutant SRF in cardiac-specific tissue and found that SRF is required for myofiber generation as the transgenic mice died within the first week after birth due to heart dysfunction[12]. For comparison, we also developed a mouse model with overexpression of functional SRF in the heart and demonstrated that too much SRF can cause hypertrophic cardiomyopathy as the mice died of heart failure within 6 mo[13]. From these initial studies, SRF emerged as a key factor in muscle development and maintenance. In addition, modulation of SRF expression levels seems to play an important role in its different functions, where high expression levels of SRF are required for proper embryonic development, while lower levels may be more beneficial in adulthood[7].

IMPLICATIONS IN GI

Even though SRF had been studied extensively in other tissues since its discovery, its role in the GI system was not examined for at least another decade. The earliest record that can be found was in 1997, and this study showed that SRF binding activity is elevated in the liver of Long-Evans Cinnamon rats (animal model of Wilson's disease) compared to Wistar rats[14]. In addition, several other studies used GI-derived cell lines purely as tools to investigate the molecular properties of SRF[15-17]. However, the role of SRF in the GI system was not studied directly until eight years ago, when our group found that SRF is not only expressed in smooth muscle structures, such as muscularis mucosa and muscularis propria, which are of mesoderm origin, but it is also found at intermediate expression levels in the mucosal epithelium, which is of endoderm origin[5]. Since then, work from our group and others has provided important information about the role of SRF in both normal and pathological processes in the digestive system (Table 1).

Esophagus

Esophageal ulcers occur with a great geographical variation, from 5%-10% in the United States to approximately 80% in some Iranian regions. Its causes are also different with locations. While gastroesophageal reflux is its main cause in

Table 1 Identified roles of SRF in the GI tract

GI system	Process involved	Molecules associated	GI disorder associated
Esophagus	Myofibroblast differentiation[19]	TGFβ, ILK	Ulcer
Stomach	Angiogenesis[20]	VEGF, Rho-actin, MEK-ERK	Ulcer
Stomach	Re-epithelialization, muscular structure restoration[6]	Actin, immediate-early genes	Ulcer
Stomach	*H. pylori* activates SRF[21,22]	CagA, villin	Intestinal metaplasia
Intestine	Smooth muscle contraction[23,24]	Smooth muscle actin, smooth muscle myosin, smoothelin, F/G actin	Intestinal obstruction, CIPO
Colon	Alternative splicing, cell survival[26]	SRFΔ5, K-ras	Colon cancer
Liver	Cell cycle; hepatocyte proliferation/survival[29,30]	IGF-1	Liver injury
Liver	Cell proliferation, cell cycle, apoptosis[33]	E2F1	Hepatocellular carcinoma
Liver	Cell invasion[31]	E-cadherin, β-catenin	Liver metastasis
Pancreas	Cell proliferation[32]	Pro-inflammatory cytokines	Pancreatitis

SRF: Serum response factor; GI: Gastrointestinal; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular regulated kinase; *H. pylori*: *Helicobacter pylori*; IGF: Insulin-like growth factors; CIPO: Chronic intestinal pseudo-obstruction.

the United States, in Europe it is alcohol consumption and in the Middle East it is the diet[18]. Healing of esophageal ulcers proceeds *via* a series of overlapping events[19], and among them myofibroblasts make a significant contribution to the wound closure. Our study[20] showed that when the connective tissue has been damaged and denuded of its epithelium during gastrointestinal ulceration, fibroblasts next to the ulcer area are activated to become myofibroblasts and participate in restoration of new epithelial continuity and extracellular matrix. Over-expression of SRF promotes myofibroblast differentiation both *in vitro* and *in vivo*, and knockdown of SRF was sufficient to prevent TGFβ-induced myofibroblast differentiation.

Stomach

While there are nearly 50 thousand publications dealing with stomach ulcers, we are the only researchers to have investigated the role of SRF in this common gastric disorder. SRF is a master regulator of cytoskeleton dynamics and cell motility. We showed that injury-activated SRF is critical to gastric ulcer healing, as local knockdown of SRF severely impairs angiogenesis[21], an essential process for any wound healing. Without SRF, VEGF, the most powerful activator of angiogenesis, loses its power. Since angiogenesis is a key step in tumor progression by providing growing tumors with oxygen and nutrient supplies through generation of new blood vessels, these findings may have potentially important therapeutic implications for blocking cancer progression. We also demonstrated[6] that over-expression of SRF in gastric epithelial cells or in smooth muscle cells (*in vitro*) as well as in gastric tissue (*in vivo*) can promote cell proliferation/migration, and thereby promotes re-epithelialization and restoration of smooth muscle structures damaged by ulcers. These findings show great potential for therapeutic applications of SRF.

While the normal processes of angiogenesis and wound healing have been indirectly associated with promoting cancer when inappropriately activated, therefore making SRF a potential oncogenic factor through its regulation of these processes and a promising target for cancer therapy as elucidated before, more direct evidence that SRF can indeed promote cancer progression has come from different sources. First, two different groups linked SRF to *Helicobacter pylori* (*H. pylori*), a gram-negative bacterium that colonizes the human gastric mucosa, resulting in stomach disorders such as chronic gastritis, peptic ulcers and gastric adenocarcinoma. Of the two *H. pylori* strains, the type one strain contains the cag pathogenicity island (PAI), which confers greater virulence compared to the type two strain lacking PAI. Hirata *et al*[22] showed that transfection of the *CagA* gene into gastric epithelial cells greatly increases *in vitro* binding activity of SRF to SRE. Up to that point, CagA protein had only been linked to cellular cytoskeletal rearrangements, after activation through tyrosine phosphorylation. Therefore, aside from linking SRE and SRF to *H. pylori* pathogenesis, their findings are important for understanding *H. pylori* infection mechanisms by identifying a novel, phospho-tyrosine-independent, mode of action of CagA protein. Rieder and co-workers later built on the story by identifying villin as a new target of SRF and showing that SRF mediates Helicobacter-induced intestinal metaplasia in the stomach through villin[23]. Intestinal metaplasia is a premalignant precursor lesion to several organs of the GI tract, including stomach, gall bladder and pancreas, and it is defined by the presence of intestine-like cells expressing enterocyte-specific markers, such as villin.

Intestine

The importance of SRF in the GI tract was further strengthened by the work from Angstenberger and collaborators on smooth muscle contraction[24], which is a key feature of proper GI function. They developed an inducible mouse model where SRF was conditionally knocked out only in the smooth muscle cells of adult mice. The mutant mice developed symptoms of ileus paralyticus due to impaired contraction of intestinal smooth muscle and died 2 wk after the induction. Through more detailed phenotypic and gene expression analysis of the same model system in collaboration with Feil, Mericskay and co-workers confirmed[25] that SRF plays a central role in maintaining proper smooth muscle function and they provide an inducible mouse model that could have potential implications for studying chronic intestinal pseudo-obstruction

(CIPO). The mutant mice displayed cachexia and autopsy showed severe dilation of the intestinal tract associated with food stasis, indicating impairment of GI motility due to smooth muscle deficiency. Defects in GI contractile function can lead to a variety of different disorders, from common and relatively benign, to more rare but potentially life-threatening. CIPO falls in the latter category, sharing similarities with chronic heart failure in the way that the "intestinal pump" is no longer able to effectively maintain tone and coordinate transit of intestinal contents through the luminal cavity, resulting in intestinal pseudo-obstruction[26]. Several different mechanisms have been associated with CIPO, with both genetic and environmental causes, surely making CIPO a multifactorial condition. Nonetheless, these two studies clearly show that SRF plays a central role in proper smooth muscle contraction and that it could be an important model system to shed new light on CIPO. In their editorial commentary, De Giorgio *et al*[26] propose three different models by which SRF ablation could affect intestinal contractility: "(1) loss of smooth muscle cell (SMC) contractile phenotype due to an impairment of the contractile apparatus; (2) degeneration of SMCs with synthetic phenotype; and (3) derangements of pathways (including neuronal ones) implicated in smooth muscle contraction"[26]. Even though results from Angstenberger and Merickskay made it possible to define these models of potential SRF involvement in CIPO, there is definitely room for additional work, especially in regard to the latter model, which is only briefly touched upon by Merickskay and co-workers.

Colon

As we mentioned earlier, SRF can be expressed in different isoforms in a tissue-specific manner due to alternative splicing of mRNA. Patten and co-workers[27] found that the predominant SRF isoform expressed in colon cancer cell lines derived from poorly differentiated tumors (WiDr, HCT116, LoVo, and SW480) is SRFΔ5, the dominant negative isoform lacking the transactivation domain (Figure 1). SRFΔ5 is normally expressed at high levels in terminally differentiated tissues, such as brain, heart, skeletal muscle, testes and liver. However, aberrant elevated expression of SRFΔ5 in other tissues has been associated with medical conditions. For instance, while normal lungs express very low levels of SRFΔ5, hypoplastic lungs, in which stretching is compromised, express elevated levels of this isoform[28]. Similarly, over-expression of another isoform (SRFΔ4,5), which was shown to inhibit transcription of SRF-dependent cardiac muscle genes, was detected in failing hearts[29]. Patten and co-workers also showed that stable expression of SRFΔ5 in rat intestinal epithelial cells (IEC-6) significantly increased cell survival rates, possibly by preventing apoptosis, as cell proliferation was improved only after day 11 and mRNA levels of pro-apoptotic caspase 3 and Fas were significantly reduced.

Liver

It is known that the liver has a remarkable capacity to regenerate after injury. Latasa *et al*[30] found that SRF and its targeted immediate early genes are rapidly activated after partial hepatectomy in rodents. When they knocked down SRF in the liver, this regeneration capacity was severely damaged. Following up on this idea, Sun and co-workers showed that liver-specific SRF knockout in mice led to a lower survival rate, where surviving animals were generally smaller with smaller and poorly functioning livers[31]. Through gene array analysis of SRF deficient liver fragments, they also showed that loss of SRF prevents activation of a wide array of genes, particularly those involved in IGF-1-mediated cell cycle control, consistent with impaired normal growth, as well as several genes specific to hepatocyte function, suggesting that adequate amounts of SRF are indispensable for proper liver development and function. These findings highlight the different expression requirements for SRF in tissue development and proper function/maintenance, stressing the importance of optimal SRF expression. While cell culture and animal models on the mechanistic action of SRF are quite informative, correlation with cancer progression in patients is often determined based on differential expression between normal and tumor tissue, which implies that up- or down-regulation of a particular gene (or aberrant expression of a different variant of the gene) confers more tumorigenic potential to the cell and is therefore maintained. In this respect, Choi *et al*[32] recently reported that nuclear SRF staining, which was not detected in normal colon tissue, was found in 37% of primary colon cancers and 60% of metastatic liver cancer. A similar trend was observed with loss of E-cadherin expression (14% and 33%, respectively), while nuclear expression of β-catenin was significantly higher in primary tumors (56%) compared to normal tissue but did not change much in metastatic tumors. Loss of E-cadherin expression and translocation of β-catenin from the membrane to the nucleus are fundamental steps in disruption of epithelial cell junctions and acquisition of more migratory potential, which are at the basis of tumor metastasis. Therefore, to follow up on these observations, Choi and co-workers showed that over-expression of SRF in colorectal carcinoma cells enhanced cell motility and invasiveness, paralleled by loss of E-cadherin protein expression and increase in non-phosphorylated (nuclear) β-catenin expression, suggesting that SRF promotes liver metastasis through its action on membrane E-cadherin and β-catenin[32]. The oncogenic potential of SRF overexpression in the liver was further confirmed a few weeks ago by Farra and co-workers. Building on recent advances in the field mentioned above, they decided to test the effectiveness of SRF depletion in highly and poorly differentiated hepatocellular carcinoma (HCC) cell lines[33]. Their studies, which highlight differences in response to SRF depletion among different grades, also support a therapeutic role for SRF depletion against HCC, for which there are currently no effective treatment options[33].

Pancreas

The importance of SRF to the early phase of liver regeneration is well established by the studies above, however, they also noticed that the liver without SRF can still

Table 2 Tools and models available to study SRF functions in GI

Tool	Purpose	Model
SRF in pcDNA3.1, His[6,21]	SRF over-expression	Gene therapy
SRF antisense sequence[21,33]	SRF down-regulation (siRNA)	Gene depletion
Srf Flex1 mice[35]	Conditional *in vivo* SRF deletion	Gene depletion
Srf loxP mice[30]	Conditional *in vivo* SRF deletion	Gene depletion
CreER T2 mice[36]	SMS tissue expression	CIPO
AlfpCre mice[31]	Hepatocyte tissue expression	Liver function
K5-Cre mice[37]	Squamous epithelial expression	Esophagus, foregut
Fabp/Cre mice[38]	Conditional gut tissue expression	Small/large intestine
H. pylori[23]	*H. pylori* infection in human cell lines (*in vitro*) or mice (*in vivo*)	*H. pylori* gastric diseases

develop. A similar situation was also observed in the pancreas. Miralles and co-workers[34] found that mice with conditional inactivation of SRF in the pancreas had normal development of both the exocrine and endocrine pancreas. However, after weaning, these mice developed profound morphological alterations of the exocrine pancreas, which were reminiscent of severe pancreatitis. In these mice, massive acinar injury and pro-inflammatory cytokines release led to complete destruction of the exocrine pancreas and its replacement by adipose tissue.

SRF-related tools and models

Over the last decade, work on SRF in GI tissues has been very productive not only in establishing that SRF plays very important roles in both normal and pathological processes, but also in generating good *in vivo* model systems and validated tools to further study the role of SRF in both normal development and function as well as in related pathologies of the GI tract. Here we provide a detailed list of SRF-related models and tools (Table 2), which will be very useful for further exploration of the role of SRF in the GI tract. These include His-tagged SRF cDNA in the pcDNA3.1 mammalian expression vector under the control of the cytomegalovirus (CMV) promoter[6,21] for SRF over-expression in cell culture and gene therapy *in vivo*; validated antisense SRF oligonucleotide sequences to knock down SRF protein expression[21,33]. Two different conditional SRF knockout mice[30,35], are also available to generate temporally and spatially controlled SRF deletion in any desired tissue through the use of tissue-specific Cre mice. Currently, these have been combined with SMS[36]- and hepatocyte[31]-specific Cre mice to generate the CIPO model[24] and the liver model[30] of SRF, respectively. However, they hold unlimited potential for selectively knocking out SRF in any desired tissue or subpopulation of the GI tract to further study the role of SRF in GI. For instance, crossing either the SRF knockout line to the previously described K5-Cre transgenic mice[37] would allow SRF deletion in the basal cell layers of various squamous epithelial cells, including esophagus and foregut. Moreover, the Gordon group also generated two different Cre model systems (Fabp), which allow for intestinal-specific deletion, which could also be temporally controlled in a doxycycline inducible manner by combining Fabp-rtTA and tetO-Cre with the desired gene knockout[38].

FUTURE DIRECTIONS IN GI

Results summarized here clearly show that SRF is an important factor in mediating both normal and pathological conditions in the GI tract and that different optimal expression levels are associated with its various functions, while deviations from those levels can result in more or less severe pathological conditions. The flip side of that is that SRF could also lend itself to favorable manipulation, if we only know what it is. While providing initial clues and identifying several factors involved in SRF-mediated functions (Table 1), the findings above still leave the door wide open for additional exciting work in these areas of research, with particular focus on the finer details of SRF signaling in the individual processes, which would also help us understand how and when SRF could be a good therapeutic target.

Role of SRF in gastrointestinal ulcers

For instance, we show that SRF is critical for mediating the wound healing process in both gastric[6] and esophageal[21] ulcers, primarily through its role in VEGF-induced angiogenesis in a Rho A/actin- and ERK pathway-dependent manner[21]. However, while both Rho A and ERK have been linked to SRF induction[6], little is known about how they may interact with each other, and more studies along those lines would be extremely helpful in defining the role of SRF in this process and its potential as a therapeutic agent.

Role of SRF in gastrointestinal motility disorders

Similarly, preliminary work by Angstenberger and Merickskay established a very useful model for studying CIPO and trying to find possible treatment options for this very serious condition. Insights for new avenues into this area of research can be found in the three models proposed by de Giorgio[26]. In addition, given the emerging central role of SRF in the contractile function of the intestine, findings in this area of research may also be useful for less severe but more common dysfunctions of the intestine, with wider applications.

Role of SRF in *H. pylori* and related pathologies

H. pylori infection appears to be another major new area of research for SRF function in GI pathogenesis, which definitely deserves more attention. Here too, Hirata[22] and

Rieder[23] outline initial important connections between SRF and *H. pylori* infection and premalignant transformation, however, more work is needed to fill in the gaps. For instance, SRF activation appears to be involved in a new mode of CagA-mediated infection, which, given its more virulent nature, deserves more attention. In addition, work from Rieder and co-workers provide a preliminary molecular framework to further study the signaling components involved in SRF-mediated metaplasia[23]. A better understanding of the signaling cascades downstream and upstream of SRF in this process would be very useful for both diagnosis and more informed development of therapeutic options. For this purpose, the different *H. pylori* strains optimized for either *in vivo* mouse studies or human cell culture studies[23] (Table 2) will be particularly useful.

Role of SRF in gastrointestinal cancers

Overall, several lines of evidence seem to point to a positive role of SRF in various GI cancers, such as its role in driving angiogenesis[21], in mediating *H. pylori* infection[22] and metaplasia[23], which is strongly associated with gastric cancer, and in promoting cell proliferation in HCC[33] and liver metastasis by weakening cell adhesion[32]. Findings by Patten and co-workers that an SRF variant is also over-expressed in colon cancer in response to a very prevalent colon cancer mutation[27] further highlight the potential importance of SRF in cancer. However, the fact that the variant is a known dominant negative form of SRF makes its role in cancer progression not as straight forward. This is particularly true given findings by Choi and co-workers, which clearly correlate SRF over-expression with colon cancer progression[32]. However, since Patten and co-workers never examined actual tumor tissues and, while SRFΔ5 expression was indeed elevated in response to K-ras activation, full-length SRF was also elevated and generally showed much stronger expression than SRFΔ5 itself, more work may be required to better understand the actual prevalence of this variant in colon cancer and, most importantly, its role in cancer progression. More solid evidence to support the conclusions by Patten and co-workers could have important implications for diagnosis, identifying the SRFΔ5 variant as a possible marker for colon cancer. Since it is much easier to routinely collect small biopsies from colon tissue than from some other tissues, this could potentially be an effective way for better risk assessment.

Role of SRF in normal and abnormal liver function

Recent findings reported here suggest an interesting role for SRF in liver function. While Sun and co-workers show that not enough SRF is bad for proper liver function and regeneration after injury[31], studies from Ferra and co-workers raise hopes for effective HCC therapy through SRF depletion[33]. Clearly, this is just the tip of the iceberg and more data is needed in this area of research, where SRF is once again taking a leading role.

CONCLUSION

The last decade has been very prolific in shifting the focus of SRF from its role in the myocardium to a central role in the gastrointestinal tract as well. As summarized here, SRF is critical for proper development and function of most GI tissues in what appears to be a dose-dependent manner, as changes in its expression pattern have been implicated in various GI pathologies from intestinal motility disorders to cancer. In addition, both SRF gene therapy and SRF antisense expression to either elevate or inhibit normal SRF expression have been shown to hold great promise as potential therapeutic agents to either promote ulcer healing or inhibit cancer-related angiogenesis, respectively. Therefore, while great advances have already been made in this field, more in depth studies are warranted to fully understand its various roles and optimal expression requirements in all these processes, with particular attention to the potential therapeutic efficacy of SRF gene therapy and antisense expression where modulation of SRF expression may prove beneficial. For instance, SRF gene therapy could promote wound healing and liver regeneration, while its antisense expression could be more beneficial in slowing down cancer progression, where its effect on VEGF-mediated angiogenesis may play a central role in its dual applications.

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EDITORIAL

Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients

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Abstract

This article reviews the evidence for efficacy and safety of *Saccharomyces boulardii* (*S. boulardii*) for various disease indications in adults based on the peer-reviewed, randomized clinical trials and pre-clinical studies from the published medical literature (Medline, Clinical Trial websites and meeting abstracts) between 1976 and 2009. For meta-analysis, only randomized, blinded controlled trials unrestricted by language were included. Pre-clinical studies, volunteer studies and uncontrolled studies were excluded from the review of efficacy and meta-analysis, but included in the systematic review. Of 31 randomized, placebo-controlled treatment arms in 27 trials (encompassing 5029 study patients), *S. boulardii* was found to be significantly efficacious and safe in 84% of those treatment arms. A meta-analysis found a significant therapeutic efficacy for *S. boulardii* in the prevention of antibiotic-associated diarrhea (AAD) (RR = 0.47, 95% CI: 0.35-0.63, $P < 0.001$). In adults, *S. boulardii* can be strongly recommended for the prevention of AAD and the traveler's diarrhea. Randomized trials also support the use of this yeast probiotic for prevention of enteral nutrition-related diarrhea and reduction of *Heliobacter pylori* treatment-related symptoms. *S. boulardii* shows promise for the prevention of *C. difficile* disease recurrences; treatment of irritable bowel syndrome, acute adult diarrhea, Crohn's disease, giardiasis, human immunodeficiency virus-related diarrhea; but more supporting evidence is recommended for these indications. The use of *S. boulardii* as a therapeutic probiotic is evidence-based for both efficacy and safety for several types of diarrhea.

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Key words: Probiotic; Diarrhea; *Saccharomyces boulardii*; Adult patients; Meta-analysis

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INTRODUCTION

Probiotics have become increasingly popular in the US and are rapidly reaching levels of use in Europe and Asia, which have a longer history of use compared with the US[1-3]. Probiotics are "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host"[4,5]. In one survey, of 435 users of dietary supplements, 50% of women and 33% of men reported use of probiotics[6].

Probiotics are generally recommended to help strengthen host systems and assist in recovery from certain diseases. However, the general public and many health care providers are still confused about how best to utilize probiotics and which ones are effective for specific diseases. There are several challenges in choosing the appro-

priate probiotic; including the wide diversity of probiotic strains, quality control of commercially-available probiotic products and the degree of evidence-based trials for each disease and probiotic. The ability of an organism to be an effective probiotic has been found to be strain-specific and microbial organisms are defined by their genus, species and strain. For example, *Lactobacillus rhamnosus* (*L. rhamnosus*) GG is a specific bacterial strain, which has been shown to be an effective probiotic for several diarrheal diseases, but other strains within the *L. rhamnosus* species may not be an effective probiotic. Similarly, other species under the same genus (*Lactobacillus*) may not be effective probiotics[7]. To add to this confusion, probiotic products are available in diverse forms: capsules of freeze-dried or lyophilized cultures, heat-dried culture supernatants, mixed in diary food (such as yogurts, cheese, milks, or ice cream) or other food (kefir, chocolate, wafers)[8-10]. The variety of probiotic products are also regulated under different guidelines according to the ways of use (food, dietary supplement, over-the-counter use or prescription). Different standards of quality assurance also vary with the type of product, indication for use and country.

Despite these challenges, the awareness of probiotics as a therapeutic modality has increased dramatically and the frequency of peer-reviewed randomized clinical trials have kept abreast with the global interest in this innovative method of therapy. Numerous probiotic strains have been investigated for clinical efficacy, including multiple bacterial strains: *Lactobacilli*, *Bifidobacteria*, *Streptococci*, Clostridia and fungal strains: *Saccharomyces boulardii (S. boulardii), S. cerevisiae*, and *Monascus purpureus*[11-13]. This review examines evidences for Saccharomyces probiotics and focuses on *S. boulardii* for the efficacy and safety of different diseases in adult patients. The two goals of this study were: (1) to provide guidance for clinicians and patients on how to appropriately use *S. boulardii* as a therapeutic probiotic and (2) to review the evidence for efficacy and safety of *S. boulardii* for various disease indications in adults.

SEARCH STRATEGY FOR SYSTEMATIC REVIEW

PubMed, Medline and Google Scholar were searched for articles unrestricted by language from 1976 to 2009. Three on-line clinical trial registers were searched: Cochrane Central Register of Controlled Trials (www.cochrane.org), metaRegister of Controlled Trials (www.controlled-trials.com/mrct) and National Institutes of Health (www.clinicaltrials.gov). Secondary and hand searches of reference lists, other studies cross-indexed by authors, reviews, commentaries, books and meeting abstracts also were performed. Search terms included: *Saccharomyces boulardii*, probiotics, yeast, diarrhea, risk factors, randomized controlled trials, placebo-controlled, and associated author names. Search strategies were broad-based initially, then narrowed to the disease of interest to increase the search network[14].

META-ANALYSIS

If sufficient numbers of clinical trials (> 5) were found on one type of disease indication, a meta-analysis for the efficacy of *S. boulardii* was performed; otherwise a systematic review was done. The procedure for the meta-analysis followed standard meta-analysis methodology with clearly delineated parameters, *a priori* inclusion and exclusion criteria and standardized data extraction[15,16]. Abstracts of all citations and retrieved studies were reviewed and rated for inclusion. In some cases, only published abstracts from meetings were available. Published abstracts from meetings were included to lessen the potential for publication bias due to failure to publish negative findings. Information on study design, methods, interventions, outcomes, adverse effects and treatments was extracted from each article using a standardized extraction table. When necessary, authors were contacted for data not reported in the original article.

The primary objective of the meta-analysis was to determine the overall efficacy of *S. boulardii* within one specific type of disease indications (antibiotic-associated diarrhea, *Clostridium difficile* disease, traveller's diarrhea, irritable bowel syndrome, inflammatory bowel disease or other types of diarrhea). Inclusion criteria were: randomized, controlled, blinded efficacy trials in humans published as full articles or meeting abstracts in peer-reviewed journals. Exclusion criteria included: use of other strains of Saccharomyces (such as *Saccharomyces cerevisiae*) or other strains of probiotics, pre-clinical studies, safety studies, case reports or case series, phase I studies in volunteers, reviews, duplicate reports, trials of unspecified treatments, uncontrolled studies, prebiotic treatments only (no living organisms) or insufficient data in article.

To estimate pooled relative risks across studies, heterogeneity between and within trials was evaluated using the Cochrane Q and I^2 tests[17]. Relative risks (RR) and 95% confidence intervals (CI) were calculated using the Mantel-Haenszel method. The relative risks of responding to *S. boulardii* therapy were pooled using a random-effect model if significant heterogeneity was found or a fixed-effect model if the studies were homogenous. P values less than 0.05 were considered significant. Analyses were performed using Stata software version 9.2 (Stata Corporation, College Station, Texas). A funnel scatterplot and Begg's test were used to assess publication bias[18].

HISTORY

Saccharomyces cerevisiae strains have a long history of use in baking and brewing preparation, but have only infrequently been investigated for probiotic properties[19,20]. Another closely related strain, *S. boulardii*, was discovered by a French microbiologist, Henri Boulard in 1920 when he was in IndoChina searching for new strains of yeast that could be used in fermenting processes. He was a visitor during a cholera outbreak and noticed that some people who did not develop cholera were drinking a special tea. This tea was

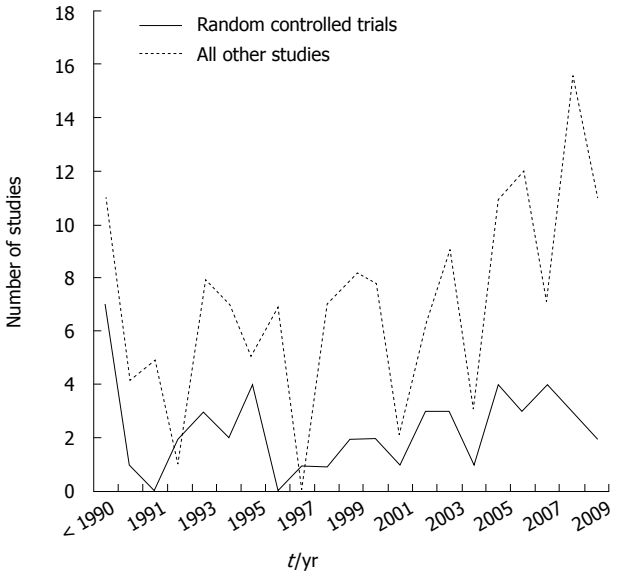


Figure 1 Frequency of peer-reviewed publications on *Saccharomyces boulardii* from 1976 to 2009.

made by taking the outer skin from a tropical fruit (lychee and mangosteens) and cooking them down to make tea. He succeeded in isolating the agent responsible. It was a special strain of yeast he named "*Saccharomyces boulardii*". The patent for this yeast was bought by Laboratories Biocodex in 1947, which began researching and manufacturing protocols. As shown in Figure 1, interest in this strain has increased, as reflected by the increasing number of scientific publications, encompassing both pre-clinical papers in mechanisms of action, animal models of efficacy, pharmacokinetics, early studies in safety and dose-ranging. In addition, there are currently 53 randomized controlled clinical trials, encompassing 8475 subjects, investigating the safety and efficacy of *S. boulardii* in pediatric and adult patients spanning several disease indications, of which 43 (81%) found significant efficacy for *S. boulardii*. As a recent review article on the efficacy of *S. boulardii* was published for pediatric indications[21], this study will focus on *S. boulardii* use in adult patients. There have been 27 randomized controlled trials, with 31 treatment arms testing *S. boulardii* in adult patients for a variety of diseases and most (84%) found a significantly protective efficacy of this probiotic. There have been only a few randomized clinical trials using other strains of Saccharomyces probiotics, which will be discussed later in this paper.

TAXONOMY

Probiotics may be either bacterial or yeast microbes. Yeast probiotics, such as *S. boulardii*, are different from bacterial probiotics (different physiologic structures, large in size, do not acquire antibiotic-resistant genes and are not affected by antibiotics)[12]. Not only is there a confusing array of probiotic products on the global market, but the taxonomy of Saccharomyces strains has been debated[22]. One strain has received considerable discussion about its valid nomenclature[23,24]. It was originally named *S. boulardii* in the 1950's. Advances in typing methods opened a debate as to whether this strain should be reclassified as a strain of *S. cerevisiae* or remain a separate species. Early work using PCR electrophoretic karotyping or rRNA sequencing methods reported that *S. boulardii* was indistinguishable from other strains of *S. cerevisiae*[24,25]. Newer metabolomic tools (microsatellite polymorphism analysis and retrotransposon hybridization analyses) show that *S. boulardii* has a unique clustering different from other strains of *S. cerevisiae*[26-28]. In addition, *S. boulardii* differs from other strains of *S. cerevisiae* by several metabolic and genetic characteristics[29,30]. *S. boulardii* persists longer in gnotobiotic mice models (10 d) compared with rapid clearance of other strains of *S. cerevisiae* (< 1 d)[31]. *S. boulardii* is also different physiologically and metabolically and its optimum growth temperature is 37℃ and it is resistant to low pH and is tolerant to bile acids; whereas other strains of *S. cerevisiae* prefer cooler temperatures (30-33℃) and do not survive well in acid pH ranges[32-34]. *S. boulardii* can be distinguished from other strains of *S. cerevisiae* by advanced typing methods, by differences in metabolism and physiology and by the ability to have anti-pathogen effects (as discussed in the mechanisms of action section).

GUIDELINES FOR CHOOSING APPROPRIATE PROBIOTICS

Challenges in providing guidance to patients regarding the appropriate choice of probiotic include the wide diversity of available products on the market, variances in quality control, stability and formulations in those products, and the requirement to match the type of probiotic with the disease indication. The efficacy of probiotics have been shown to be both strain-specific and disease-specific[11,35,36].

Product to product variation

There are many different Saccharomyces products available commercially. Table 1 lists some examples of products that contain *S. boulardii*, sold as probiotics either as lyophilized or heat-dried powders in capsules, or as one of several strains in a probiotic mixture in capsules or in liquid beverages[12,13,21,37-66]. The quality of these products from different sources have been found to vary. Choosing a probiotic product from a manufacturer with a regulated quality control program is a sound policy. Unfortunately, many of the products available commercially may lack regulated quality control programs. Studies of other probiotics have found a wide diversity in both quality and contamination in products available on the Internet[67]. Marcobal *et al*[68] tested 14 commercial probiotics in the US and found 93% were incorrectly labeled (57% had contaminants and 36% did not list strains on the label). Masco *et al*[69] tested 58 different probiotic products from Europe, UK, Asia, Japan and Canada and found only 38% had the dose stated on the label and 29% did not contain strains listed on the label. Not all products were found to have high standards of manufacture and quality control, as only some conform to Good Manufacturing Practices.

Table 1 Examples of commercially available probiotics containing "*Saccharomyces boulardii*"

Product name	Probiotic strain	Manufacturer	Stable at room temp[1]	Facility certified	Strain confirmed	Colony forming unit (cfu) per mg capsule or mL	Original strain-specific studies	Degree of clinical evidence[2]
Single strain								
Florastor® (US) Perenterol® (Germany) Reflor® (Turkey) Ultra-Levure® (Asia)[3]	*S. boulardii* lyo	Biocodex (France)	Yes	EU GMP	Microsatellite sequency polymorphism [28,29]	5×10^9/ 250 mg	Multiple [37-62]	A
SB+MOS	*S. boulardii*	Jarrow Formulas (Los Angeles, CA) and Gnosis (Italy)	No	EU GMP	Not stated	1.5×10^9	One study[63]	C
Saccharomyces boulardii	*S. boulardii*	Kirkman (Oregon)	No	GMP	DNA fingerprint	3×10^9/ 150 mg	None	F
Saccharomyces boulardii	*S. boulardii*	Allergy Research Group (CA)/NutriCology (CA)	No	GMP	DNA fingerprint	3×10^9/ 150 mg	None	F
Saccharomyces boulardii	*S. boulardii*	Klaire Labs (Reno Nevada)	No	GMP ISO 9001	Ribotyping	3×10^9/ 150 mg	None	F
In mixtures of probiotics								
Protecflor® (Canada) Erce Flora® (Belgium)	*S. cerevisiae boulardii* CNCM I-1077) + *L. rhamnosus* + *L. acidophilus* + Bifido. strain	Institut Rosell-Lallemand (Quebec, Canada)	Not stated	Canadian GMP	Not stated	10^9/2 mL	Two studies [64,65]	D
MitoMix®	*S. boulardii* and *Pediococcus acidilactici*	Imagilin Technology (Maryland)	Yes	Not stated	Not stated	2.3×10^9/ capsule	One study[66]	D
Primal Defense™	*S. boulardii* with 13 other strains[4]	Garden of Life (FL)	Yes	Not stated	No (only certified "organic")	Total 1×10^9 per 410 mg	None	F
Pro-Bio Defense™	*S. boulardii* + 7 other strains[5]	Kirkman (OR)	No	GMP	DNA fingerprint	Total: 2×10^{10}/cap, no data on SB cfu	None	F
ABX Support™	*S. boulardii* + *L. rhamnosus* + *Bifido. bifidum* + *Bifido. breve*	Klaire Labs (NEV)	No	GMP ISO 9001	Ribotyping	5×10^9/ 300 mg	None	F
Kombucha fermented tea	*S. boulardii* + *L. bacterium* + blue-green algae	Millennium Products, Inc.	No	No	Not stated	1×10^9 per 16 oz.	None	F

[1]Products not stable at room temperature require refrigeration, probably heat-dried. Products stable at room temperature are typically packaged in blister packs and are lyophilized; [2]Degree of evidence: A = at least two randomized, controlled clinical trials in patients; B = one randomized controlled clinical trial in patients, C = case reports, uncontrolled randomized trials or open safety/kinetic studies in patients or volunteers, D = *in vivo* animal model or *in vitro* studies only, E = expert opinion only, F = no direct evidence; [3]A few examples, Biocodex has over 40 brand names worldwide; [4]Other strains in Primal Defense include: 11 strains of *Lactobacilli* (*L. acidophilus, L. bulgaricus, L. lactis, L. plantarum, L. casei, L. lactis, L. leichmannii, L. brevis, L. caucasicus, L. fermenti*, *L. helveticus* and 1 strain each: *Bifidobacterium bifidum, Bacillus subtilis, Bacillus lichenformis*); [5]Other strains in Pro-Bio Defense include: 5 strains of *Lactobacilli* (*L. plantarum, L. rhamnosus, L. acidophilus, L. casei, L. bulgaricus*) and *Bifidobacterium lactis* and *Streptococcus thermophilus*. GMP: Good manufacturing practices.

Although most products state they contain at least 1×10^9 *S. boulardii*/mg, independent assays have determined 50% of the products contained a dose less than on the label. In one study comparing six *S. boulardii* products, all had identical PCR typing profiles, but only 50% [Floratil (Merck), Flomicin (NeoChemical), and Florazin (Herald's)], had the same concentration identified on their label. One product [Lactipan (Sigma Pharm)] had 2×10^4 fewer *S. boulardii* than stated on its label. One product [Floratil (Merck)], had the highest concentration (1×10^9/100 mL) and maintained high levels (9.5×10^8) six months later[70]. Even if the label states it contains *S. boulardii*, a variation in efficacy may occur due to lower than stated dose or inaccurate strain composition. Four *S. boulardii* products were tested (along with one *S. cerevisiae* product) in Brazil. Only two (50%) of the *S. boulardii* products were protective in a *Salmonella typhimurum* mouse model (two *S. boulardii* products were ineffective, as well as the one *S. cerevisiae* product)[71]. Without access to specific quality control assays for commercially available probiotic products, the choice of a high quality product can be difficult. One method of selecting a probiotic product is to find a product in which the manufacturing company has sponsored original clinical trials, as this indicates a degree of commitment that may not be present in companies that do not sponsor original research. As shown in Table 1, only a few *S. boulardii* probiotic products are supported by original research. Although at present, most probiotic clinical trials are sponsored by private companies, as more national funding becomes available, this situation may change.

Stability

How the probiotic product is manufactured may significantly affect its potency over time (shelf-life). Probiotics may be available as lyophilized preparations, heat-dried preparations or contained in diary or drink food products.

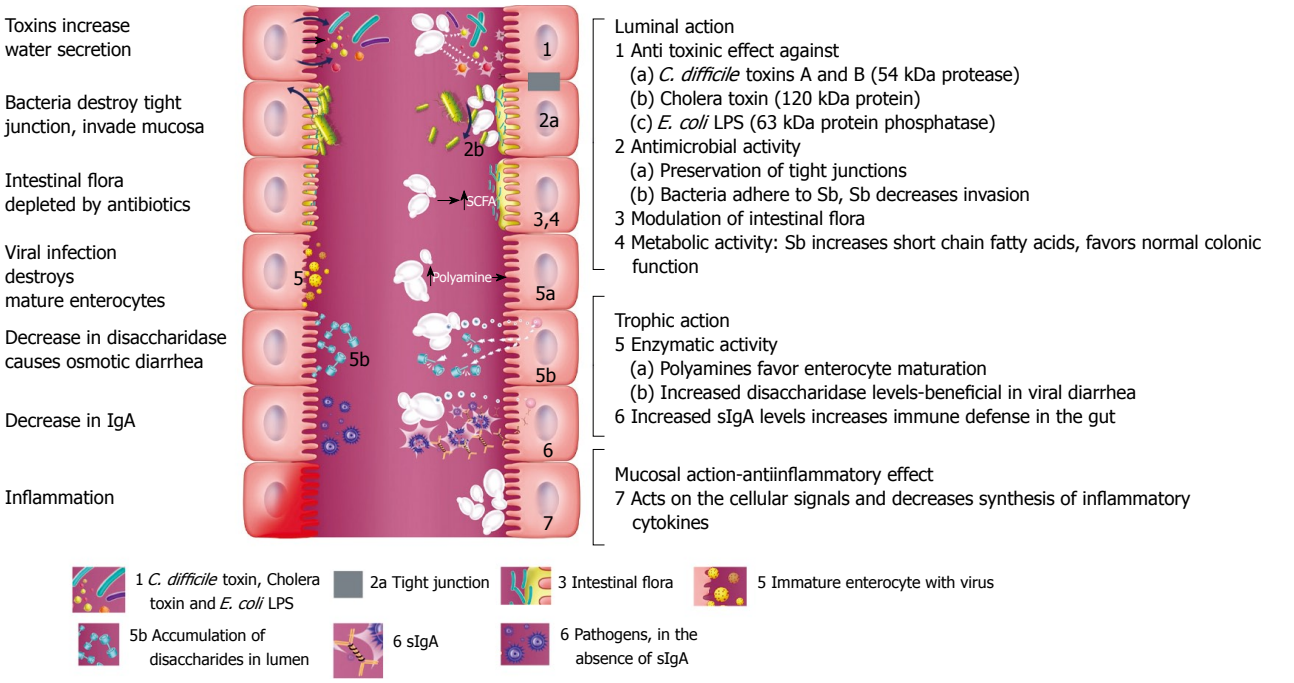


Figure 2 Schematic of intestinal tract, illustrating the different potential mechanisms of action of *Saccharomyces boulardii* (Sb). On the left, effects of different pathogenic microbes are depicted. On the right, seven different protective effects of *S. boulardii* are depicted. Within the lumin of the intestine, *S. boulardii* may degrade toxins of pathogens, interfere with pathogenic adherence, modulate normal microbiota and preserve normal intestinal physiology. *S. boulardii* may also indirectly restore normal short chain fatty acid (SCFA) balance. *S. boulardii* may also increase secretory IgA (sIgA) levels or act as an immune regulator by influencing cytokine levels.

S. boulardii is usually available in capsules of either lyophilized or heat-dried preparations. Heat-dried capsule products may be identified by their labels, which usually state that the products should be refrigerated after opening and they lose their potency rapidly. Lyophilized preparations of *S. boulardii* are stable over one year at room temperature, as long as it is protected from moisture[72]. Lyophilized products are stable at room temperature and have the advantage of portability and convenience and maintain high viability counts over prolonged periods. Heat-dried preparations are not stable at room temperature and must be refrigerated. A study of four *S. boulardii* products in Germany found a lyophilized product [Perenterol Forte (Thiemann)] outperformed three heat-killed *S. boulardii* preparations in terms of higher viable cells and quicker start-up times[73].

Single strain or probiotic "cocktails"

As shown in this review, all the randomized controlled trials using *S. boulardii* probiotics have utilized a single strain preparation. Although mixtures of probiotics, which may contain *S. boulardii*, are available on the market, no randomized controlled trials have been done showing that these mixtures are superior to the single stain preparations. Pre-clinical studies in animal models have found promising results in a probiotic mixture (*L. rhamnosus*, *L. acidophilus, Bifidobacterium* and *S. boulardii*) for reducing *E. coli* diarrhea in rats, but no human clinical trials have been performed with this mixture[65]. Another potential limitation to probiotic mixtures is antagonism between the different probiotic strains and conflicting mechanisms of actions that may tend to attenuate the therapeutic responses of the probiotic strains[74].

Mechanisms of action

An advantage of probiotics is that they are living organisms incorporating a delivery system (most probiotics survive to the target organ) bringing an arsenal of anti-pathogenic strategies into play. *S. boulardii* has several different types of mechanisms of action (Figure 2) which may be classified into three main areas: luminal action, trophic action and mucosal-anti-inflammatory signaling effects[8,75-77]. Within the intestinal lumen, *S. boulardii* may interfere with pathogenic toxins, preserve cellular physiology, interfere with pathogen attachment, interact with normal microbiota or assist in reestablishing short chain fatty acid levels. *S. boulardii* also may act as an immune regulator, both within the lumen and systemically.

Anti-toxin effects: *S. boulardii* may interfere with pathogenesis within the intestinal lumen by several mechanisms: either by blocking pathogen toxin receptor sites[78], or acting as a decoy receptor for the pathogenic toxin[79] or by direct destruction of the pathogenic toxin. Castagliuolo *et al*[80] found a 54 kDa serine protease produced by *S. boulardii* directly degrades *C. difficile* toxin A and B. The efficacy of other strains of Saccharomyces has also been investigated. Only *S. boulardii* produces a protease capable of degrading *Clostridium difficile* toxins and receptors sites on the enterocyte cell surface, unlike other strains of Saccharomyces[78,81]. Buts *et al*[82] found a 63 kDa phosphatase produced by *S. boulardii* destroys the endotoxin of pathogenic *E. coli*. Several investigators showed that *S. boulardii* could reduce the effects of cholera toxin and this may be due to a 120 kDa protein produced by *S. boulardii*[83,84].

Antimicrobial activity: *S. boulardii* is capable of directly or indirectly interfering with intestinal pathogens. *S. boulardii* may directly inhibit the growth of pathogens (such as *Candida albicans*, *Salmonella typhimurum*, *Yersinia enterocolitium*, *Aeromonas hemolysin*[43,85,86]). In animal models testing for the ability to inhibit pathogen growth, several studies using non-*S. boulardii* strains of *S. cerevisiae* did not find any effect unlike the protective effects of *S. boulardii*[31,70]. A few studies have tested other strains of *S. cerevisiae* and found promising results. Brandao *et al*[79] tested *S. cerevisiae* strain W303 in rats and found less histologic damage due to cholera toxin compared with saline controls, but no clinical trials have been done using this strain. *S. boulardii* may also act by enhancing the integrity of the tight junction between enterocytes, thus preserving intestinal integrity and function[87,88]. Wu *et al*[88] found less crypt hyperplasia and cell damage in a *Citrobacter rodentium*-induced mice model of colitis when mice were treated with 1×10^9 *S. boulardii* per day for 7 d. Garcia Vilela *et al*[62] found decreased intestinal permeability when patients with Crohn's disease were given *S. boulardii* (1.6×10^9/d for 4 mo) compared with placebo. *S. boulardii* has also been shown to reduce the translocation of pathogens in rat and pig animal models[64,89,90]. *S. boulardii* can also interfere with pathogenic attachment to intestinal receptor sites[88,91,92]. Gedek *et al*[93] also found that *S. boulardii* acts as a decoy by causing EPEC cells to directly bind to the surface of *S. boulardii* cells rather than enterocytes.

Cross-talk with normal microbiota: Newer techniques, including metagenomics and PCR probes have documented that a typical human may carry over 40 000 bacterial species in the collective intestinal microbiome[94]. The normal intestinal flora has many functions, including digestion of food, but the one that is most germane for this discussion is called "colonization resistance"[77,95,96]. This involves the interaction of many bacterial microflora and results in a barrier effect against colonization of pathogenic organisms. Normal microflora may act by competitive exclusion of nutrients or attachment sites, produce bacteriocins, or produce enzymes detrimental to pathogenic growth. Factors that disrupt this protective barrier, for example antibiotic use or surgery, results in host susceptibility to pathogen colonization until such time as the normal microflora can become re-established. Typically, it takes six to eight weeks for normal microbiota to recover after antibiotic exposure or disease resolution[97]. Probiotics are uniquely qualified to fit into this window of susceptibility and may act as surrogate normal microflora until recovery is achieved. *S. boulardii* has no effect on normal microbiota in healthy human controls[98,99]. In contrast, when *S. boulardii* is given to antibiotic-shocked mice or patients with diarrhea, normal microbiota is re-established rapidly[99,100].

Restoration of metabolic activities: *S. boulardii* has been shown to be able to increase short chain fatty acids (SCFA), which are depressed during disease, indicating altered colonic fermentation[98,101,102].

Trophic effects: *S. boulardii* can reduce mucositis[103], restore fluid transport pathways[84,101,104], stimulate protein and energy production[105], or act through a trophic effect by releasing spermine and spermidine or other brush border enzymes that aid in the maturation of enterocytes[106,107].

Immune response: *S. boulardii* may also regulate immune responses, either acting as an immune stimulant or by reducing pro-inflammatory responses. *S. boulardii* may cause an increase in secretory IgA levels in the intestine[56,108-110]. It has also been found associated with higher levels of serum IgG to *C. difficile* toxins A and B[111]. *S. boulardii* may also interfere with NF-κB-mediated signal transduction pathways, which stimulate pro-inflammatory cytokine production[76,112,113]. Chen *et al*[114] found that *S. boulardii* blocks activation of ERK1/2 and MAP kinases, which typically stimulate IL-8 production and cell necrosis in mice ileal loop models and in *in vitro* models. *S. boulardii* has also been shown to cause the trapping of T helper cells into mesenteric lymph nodes, thereby reducing inflammation[115].

Properties of *S. boulardii*

To be an effective probiotic, it must survive passage to its target organ (most commonly the colon). Organisms need to survive at body temperature (37℃), be resistant to stomach acids and bile acids, and exist in the competitive milieu of the intestinal tract. Probiotic strains of Saccharomyces have been shown to have these abilities. Although the optimal temperature for most strains of Saccharomyces range from 22-30℃, *S. boulardii* survives best at 37℃, giving it a unique advantage of being one of the few yeasts that do best at human body temperatures.

Survival to target sites: Although much of the oral dose is destroyed (usually stool levels are 100-1000 times lower than the oral dose), surviving oral doses have been found to be effective (usually at levels over 10^8 organisms/g stool)[116]. *S. boulardii* is resistant to antibacterial agents and survives gastric acidity[34,117]. In human volunteers, the concentration in the colon was found to be dose-dependent[118]. When *S. boulardii* was given to healthy volunteers at doses typically used therapeutically ($1\text{-}2 \times 10^{10}$/d), colonic levels were 2×10^8/g stool[118]. Few clinical trials using probiotics have documented the level of organisms present in the terminal site (colonic lumen in their study subjects). However, in one trial of patients with recurrent *C. difficile* infection (CDI) given *S. boulardii* (2×10^{10}/d) for 28 d, patients who had a subsequent CDI recurrence were found to have significantly lower numbers of *S. boulardii* (2×10^4/g stool) compared with those without recurrence (1×10^6/g stool)[119].

Pharmocokinetics: *S. boulardii*, when given orally, achieves steady-state concentrations within three days and is cleared within 3-5 d after it is discontinued[34,119,120]. Blehaut *et al*[120] gave eight healthy human volunteers *S. boulardii* (oral dose

of 5×10^9) for six days and followed them for time-to-clearance. They determined *S. boulardii* has half-life of 6 h, fecal steady-state concentrations (2×10^7/g) were reached by day 3 and the yeast was cleared after four days after administration. Klein *et al*[118] confirmed these findings in their studies of human volunteers and also found *S. boulardii* levels were 23 times higher in volunteers with disturbed intestinal microbiota (due to ampicillin exposure) than volunteers who were not given ampicillin. Elmer *et al*[121] found that some types of fiber (psyllium) increased *S. boulardii* levels by 22%, while other types of fiber (pectin) showed no effect.

CLINICAL EFFICACY: ACUTE DISEASES

S. boulardii probiotics have been tested for clinical efficacy in several types of acute diseases including antibiotic-associated diarrhea, *C. difficile* infections, *Helicobacter pylori* (*H. pylori*) disease, acute adult diarrhea, enteral nutrition-related diarrhea, and traveler's diarrhea.

Antibiotic-associated diarrhea

Epidemiology of antibiotic-associated diarrhea: The reported incidence of antibiotic-associated diarrhea (AAD) ranges from 12/100 000 to 34/100[122] depending upon the type of antibiotic, host factors (age, health status, *etc.*), etiology, hospitalization status and presence of a nosocomial outbreak. The highest frequency of AAD is found (26%-60%) during healthcare associated outbreaks, when susceptible patients are clustered by time, exposure and proximity[123]. Healthcare associated (hospital, long-term care facilities, nursing homes) outbreaks of AAD are to be expected because inciting agents (antibiotics), infectious agents and a susceptible patient population are intermixed. Historically, most cases of AAD were reported in hospitalized patients[122]. More recently, although AAD still occurs in hospital settings, rates of 6-33/100 have been reported in outpatient populations[124,125] and lower rates (12/100 000 to 14/100) in non-hospitalized adults[126,127]. Lower rates observed in outpatients may be due to their generally higher health status compared with hospitalized patients and also to the lack of exposure to nosocomial pathogens that commonly contaminate hospital environments. Higher incidences are still found in healthcare associated pediatric and adult patients (ranging from 5-34/100 patients)[128-130].

The clinical presentation of AAD may be mild (uncomplicated diarrhea) or more severe (colitis), or result in toxic megacolon or death[122]. The onset of AAD may occur while the patient is on antibiotics, but delayed-onset AAD is more common[122]. In addition, the onset of AAD may vary according to the type of antibiotic. Elmer *et al*[131] found a variable time of onset using the hamster model of AAD and different types of antibiotics. Early onset of AAD was associated with clindamycin, amoxicillin and ampicillin, while delayed-onset AAD was associated with erythromycin, ciprofloxacin and clarithromycin. Consequences of AAD may result in extended hospital stays, increased medical care costs and increased diagnostic procedures[122,132]. Prevention of AAD has rested on discontinuing the inciting antibiotic or switching to an antibiotic with a narrower spectrum of action, but there are no other current effective preventive measures for AAD.

Efficacy evidence for AAD: There have been ten randomized controlled trials in adults using *S. boulardii* for the prevention of AAD (Table 2)[37,42,44,54,55,58,133-136]. These studies have used a variety of daily doses, durations of treatment, length of follow-up and types of patient population. Of the ten controlled trials, 8 (80%) showed significant efficacy for the prevention of AAD compared with controls. One of the earliest trials by Adam *et al*[37], randomized 388 hospitalized patients to 200 mg of *S. boulardii* [4×10^9 cfu (colony forming units)/d] or placebo for seven days and found a significant reduction in AAD in the *S. boulardii group* (4.5%) compared with the controls (17.5%, $P < 0.05$). The protective effect of *S. boulardii* was confirmed in later studies. Monteiro *et al*[55] enrolled 240 patients receiving oral antibiotics and found significantly fewer patients randomized to *S. boulardii* developed AAD (15.7%) compared with placebo (27.7%, $P < 0.05$). McFarland *et al*[54] enrolled 193 hospitalized patients receiving beta-lactam antibiotics and randomized them to either 1 g of *S. boulardii* or placebo for the duration of the antibiotic treatment plus three additional days. Significantly fewer patients developed AAD while on the beta-lactam antibiotics or in the seven week follow-up period when given *S. boulardii* (7.2%, $P < 0.05$) compared with 14.6% of those given placebo. This study confirmed an earlier study by Surawicz *et al*[58] of 180 hospitalized adults randomized to either *S. boulardii* or placebo for the duration of their antibiotic treatment plus an additional two weeks. Of the 23% patients who were contacted 2-3 wk after the study, only one patient given placebo developed delayed-onset AAD. Only 9.5% of those randomized to *S. boulardii* developed AAD compared with 21.8% of those on placebo ($P < 0.05$). Can *et al*[135] enrolled 151 adult inpatients receiving various types of antibiotics, who were then randomized to *S. boulardii* or placebo for the duration of the antibiotic treatment. Significantly fewer patients (1.4%, $P < 0.05$) of those given *S. boulardii* developed AAD compared with the control group (9.0%). Three trials have been in conducted in patients with *H. pylori* infections receiving triple therapy (usually two antibiotics and an acid suppressor), which is associated with a high rate of AAD development. Duman *et al*[44] enrolled 389 patients with peptic ulcer or non-ulcer dyspepsia in nine hospitals in Turkey to observe if lower rates of side-effects could be achieved. This study compared 204 patients who received 1 g of *S. boulardii* (2×10^{10}/d for 2 wk) and triple therapy with 185 controls, who received only the triple therapy. Of the 389 patients, 376 completed the treatment phase and the four-week follow-up. Significantly fewer patients given *S. boulardii* (6.9%) developed AAD compared with the control group (15.6%, $P = 0.007$). Two other randomized controlled trials in adult patients receiving triple therapy for *H. pylori* infections were conducted and both showed a significant reduction in AAD for those treated with *S. boulardii*. Cremonini *et al*[134] randomized 85 *H. pylori* carriers to placebo, *L. rhamnosus* GG, *S. boulardii* or a mix of

Table 2 Randomized, controlled trials for the prevention of antibiotic associated diarrhea (AAD) using *S. boulardii*

Ref.	Treatment groups	Population	Daily dose: cfu/d (mg/d)	Duration (d)	Follow-up (wk)	AAD in *S. boularii* (%)	AAD in controls (%)
Adam *et al*[37]	*S. boulardii* *vs* placebo	388 hospitalized adults, multisite	4×10^9 (200 mg)	7	0	9/199 (4.5)[a]	33/189 (17.5)
Monteiro *et al*[55]	*S. boulardii* *vs* placebo	240 adults, Portugal	(nr) 4 caps/d	6	0	19/121 (15.7)[a]	33/119 (27.7)
Surawicz *et al*[58]	*S. boulardii* *vs* placebo	180 hospitalized adults at one hospital, Washington	2×10^{10} (1000 mg)	abx + 14 d	2-3	11/116 (9.5)[a]	14/64 (21.8)
McFarland *et al*[54]	*S. boulardii* *vs* placebo	193 hospitalized adults (1 or more beta-lactam antibiotics), 4 hospitals	2×10^{10} (1000 mg)	abx + 3 d	7	7/97 (7.2)[a]	14/96 (14.6)
Lewis *et al*[133]	*S. boulardii* *vs* placebo	72 enrolled, 69 done; elderly patients	4.5×10^9 (226 mg)	14	0	7/33 (21)	5/36 (13.9)
Cremonini *et al*[134]	*S. boulardii* *vs* placebo	43 *H. pylori* + adults on triple therapy	5×10^9 (nr)	14	2	1/22 (5)[a]	6/21 (30)
Duman *et al*[44]	*S. boulardii* *vs* placebo	389 adults in Turkey with *H. pylori* + peptic ulcers, all received triple therapy	2×10^{10} (1000 mg)	14	4	14/204 (6.9)[a]	28/180 (15.6)
Can *et al*[135]	*S. boulardii* *vs* placebo	151 adult inpatients	1×10^{10} (500 mg)	abx	4	1/73 (1.4)[a]	7/78 (9.0)
Cindoruk *et al*[42]	*S. boulardii* *vs* placebo	124 adults with *H. pylori* + dyspepsia	2×10^{10} (1000 mg)	14	6	9/62 (14.5)[a]	19/62 (30.6)
Bravo *et al*[136]	*S. boulardii* *vs* placebo	89 adult outpatients on amoxicillin	1×10^{10} (500 mg)	12	9	3/41 (7.3)	5/45 (11.1)

[a]$P < 0.05$, probiotic *vs* controls. cfu: Colony forming unit; nr: Not reported; abx: Antibiotics.

L. acidophilus and *Bifidobacterium lactis* and found significantly fewer patients developed AAD in only the *S. boulardii* group (5%, $P = 0.05$) compared with placebo (30%). Cindoruk *et al*[42] also found a significant reduction in AAD in adult patients treated with triple therapy for *H. pylori* infections for those randomized to *S. boulardii* (14.5%, $P < 0.05$) compared with placebo (30.6%). None of these trials reported any adverse reactions associated with *S. boulardii*.

It is important to have a sufficiently long follow-up period (usually 4-6 wk after cessation of antibiotics) to capture delayed-onset AAD. Several studies have shown that AAD may occur in patients on antibiotics, but AAD may also be delayed for up to two months (in up to 38% of patients) after antibiotics are discontinued[54,129]. Most studies of AAD followed patients for 4-6 wk after antibiotic treatment to document delayed-onset AAD[42,44,135]. In contrast, Lewis *et al*[133] found no significant reduction in AAD in a study of 69 elderly patients randomized to 226 mg of *S. boulardii* (4.5×10^9 cfu/d) or placebo for 14 d. This failure may have been due to a flawed study design, in that patients were only followed while on antibiotics and no follow-up was done after antibiotics were discontinued. The study by Lewis *et al*[133] therefore, may have missed a significant number of AAD cases due to a too short follow-up period. Short or no follow-up after antibiotic exposure may explain why some studies[133,136] did not find a significant protective effect of *S. boulardii*[137]. No adverse reactions associated with *S. boulardii* were noted in any of these studies. No other strains of Saccharomyces have been found to be effective to prevent AAD.

Meta-analyses for AAD: The literature search yielded 322 unique citations on *S. boulardii*, of which 278 were excluded: non-*S. boulardii* studies ($n = 19$), reviews ($n = 84$), pre-clinical studies including animal models of efficacy, mechanism of action, *in vitro* assays or strain characterization ($n = 130$), case reports or case series ($n = 15$), and phase Ⅱ safety or pharmacokinetic studies ($n = 30$). Of the 44 randomized controlled trials with *S. boulardii* screened, 34 were excluded (pediatric populations, $n = 17$; or non-AAD indications in adults, $n = 17$) and 10 were included (AAD in adult populations). As significant heterogeneity was found among studies, a random effect model was used. The random effect model gave a $\chi^2 = 10.8$, $P = 0.29$, indicating that the heterogeneity was well controlled. A meta-analysis of the ten randomized, controlled trials in adults presented in this review found that *S. boulardii* was significantly protective for AAD (Figure 3), with a pooled relative risk of 0.47 (95% confidence interval 0.35-0.63, $P < 0.001$). Begg's test did not find any significant publication bias ($P = 0.93$), nor did the funnel plot (data not shown). The number needed-to-treat (NTT) to prevent one case of AAD was 10.2.

In the established medical literature, meta-analysis has been used to combine different probiotic strains and studies to obtain a pooled estimate of the efficacy of probiotics for the prevention of AAD. A note of caution, however, while most meta-analyses have concluded that probiotics are effective for preventing AAD[123,138], it is inappropriate to conclude that all probiotic strains are effective. The effectiveness of probiotics is strain-specific and disease-specific, so each probiotic strain must be linked to the disease. Most probiotic meta-analyses have focused on one type of disease indication with a variety of probiotic strains (e.g. antibiotic associated diarrhea)[123,138,139]. When there are sufficient numbers of clinical trials, a meta-analysis limited to one disease indication and one type of probiotic strain may reduce the heterogeneity of studies[140]. Szajewska *et al*[141] limited her meta-analysis to randomized controlled trials of one type of probiotic (*S. boulardii*) in adults and children.

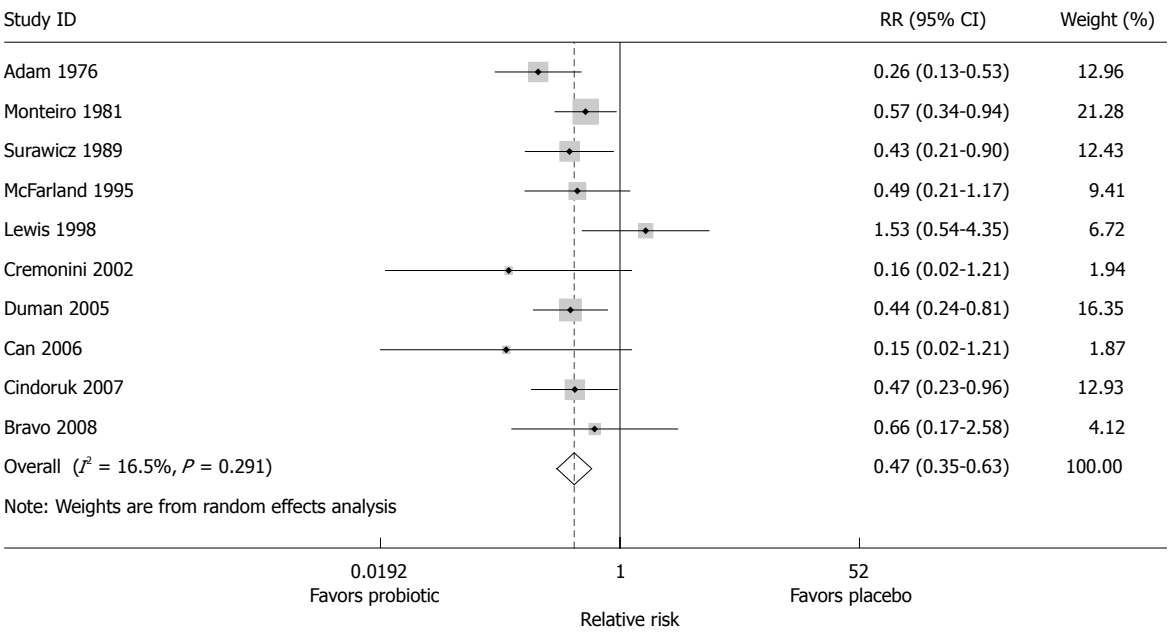


Figure 3 Forest plot of meta-analysis of ten randomized controlled trials measuring protective effect after treatment with *S. boulardii* or placebo for the prevention of antibiotic-associated diarrhea in adult patients. The x-axis depicts effect size, with black dot denoting the relative risk and the line indicating 95% CI, with the size of the grey box proportional to the study size. Relative risks to the left of RR = 1 denote study favored the probiotic, RR to the right denotes the study favors the placebo. Overall, pooled RR was 0.47 (0.35-0.63).

Pooling the results from five trials (involving 1076 subjects), a significantly protective effect of *S. boulardii* was found (pooled RR = 0.43, 95% CI: 0.23-0.78). As an alternative, some meta-analyses have done sensitivity analysis, separating out sub-groups by patient type (e.g. just adults or just children) or by type of probiotic strain[123,142]. One sensitivity analysis found with sufficient evidence that only two probiotic strains had significant efficacy for the prevention of AAD, i.e. *S. boulardii* and *L. rhamnosus* GG. However, *L. rhamnosus* GG probiotics may be contraindicated that this strain is susceptible when the patient is prescribed a type of antibiotic[123]. Although many meta-analyses have been done, pooling studies must be performed with these limitations in mind.

CDI

Epidemiology of *C. difficile* disease: For over two decades, *Clostridium difficile* infections continue to persist as a leading cause of nosocomial gastrointestinal illness[143-146]. The incidence rates of CDI have been increasing globally. In the US, CDI doubled to 301 200 cases from 2001 to 2005[147]. It is estimated that 450 000-750 000 CDI cases will occur per year by 2010 in the US[148,149]. Outbreaks of an emergent strain, BI/NAP1/027, caused large outbreaks of severe CDI with a high mortality rate in Canada between 2003-2005[150]. Studies have documented that CDI extends patients' hospital stays from 4 to 36 d[151-154]. In a study of 1034 CDI cases in Massachusetts in 2000, the average cost ranged from $10 212-$13 675/patient[155], leading to a nation-wide cost of $3.2 billion/year for CDI. There are only two standard antibiotics for CDI (vancomycin and metronidazole) and the response rate of metronidazole has been declining[152]. In addition, 20%-60% of patients may develop recurrent episodes of CDI despite additional antibiotic treatment. Although other investigational antibiotics are under development, no new antibiotics are superior to the two standard antibiotics.

Efficacy evidence for *C. difficile* disease: Probiotics may offer promise as an adjunctive therapy (given along with standard antibiotics vancomycin or metronidazole) for CDI. A meta-analysis of six randomized controlled trials of different probiotics (*S. boulardii*, *Lactobacillus rhamnosus* GG, *L. planatarum* 299v, and a mix of *L. acidophilus* and *Bifidobacterium bifidum* showed probiotics, in general, had a overall significant efficacy to prevent subsequent recurrences of *C. difficile* disease (RR = 0.59, 95% CI: 0.41-0.85, P = 0.005)[123]. However, due to limited number of trials, no meta-analysis was conducted for one probiotic strain.

S. boulardii shows promise for *C. difficile* infection control at several levels: it produces a 54-kDa serine protease that directly degrades *Clostridium difficile* toxins and also directly destroys the colonic receptor site for *C. difficile*[76], *S. boulardii* increases the immune response to *Clostridium difficile* toxins A and B[108], and animal models of *C. difficile* disease respond to this yeast. In addition, case reports or small case series of patients with recurrent *C. difficile* diarrhea treated with *S. boulardii* showed improvement. Toothaker and Elmer found that significantly fewer (51%) hamsters challenged with *C. difficile* died if given *S. boulardii* (5% solution) compared with the saline controls (80%)[156]. Subsequent studies confirmed this efficacy in other animal models: gnotobiotic mice[41,157,158], rats[89,103], and turkeys[159]. Since 1989, case reports or small case series have reported that patients with recurrent CDAD were either cured or improved by *S. boulardii* treatment[40,59, 160,161].

Table 3 Randomized controlled trials for the treatment of *C. difficile* disease using *S. boulardii*

Ref.	Treatment groups	Study population	Daily dose: cfu/d (mg/d)	Duration of treatment (wk)	Follow-up (wk)	*C. difficile* recurrence in probiotic group	*C. difficile* recurrence in placebo group
McFarland *et al*[53]	*S. boulardii vs* placebo	124 adult patients on varied doses of vancomycin or metronidazole; recurrent and initial CDAD cases; 3 referral sites, US	3×10^{10} (1000 mg)	4	4	15/57 (26.3%)[a]	30/67 (44.8%)
Surawicz *et al*[60]	*S. boulardii vs* placebo	168 adult patients recurrent CDAD; on vancomycin (2 g/d, $n = 32$) or V (500 mg/d, $n = 83$) or M (1 g/d, $n = 53$); 4 referral sites, US	2×10^{10} (1000 mg)	4	4	V (2 g/d) 3/18 (17%)[a]; V (500 mg/d) 23/45 (51%); M (1 g/d) 13/27 (48.1%)	V (2 g/d) 7/14 (50%); V (500 mg/d) 17/38 (44.7%); M (1 g/d) 13/26 (50%)

[a]$P < 0.05$, probiotic *vs* controls. V: Vancomycin; M: Metronidazole.

There was a case report[162] and two small case series with 3-4 patients[163,164] with recurrent *C. difficile* disease who seemed to have responded to *S. cerevisiae* administration. However, since no comparison groups or controls were used, there is no foundation to claim the efficacy of other strains of Saccharomyces for *C. difficile* disease.

There have been two randomized, double blinded, controlled trials for the use of *S. boulardii* and antibiotics for patients with recurrent *C. difficile* disease (Table 3)[53,60]. In a multisite, randomized, double-blinded, placebo-controlled trial of 168 patients with recurrent *C. difficile* disease, standard antibiotics were combined with *Saccharomyces boulardii* or placebo[60]. Three antibiotic regimens were used for 10 d: either high-dose vancomycin (2 g/d), low-dose vancomycin (500 mg/d) or metronidazole (1 g/d). Then either *S. boulardii* or placebo (1 g/d for 28 d) was added to the antibiotic treatment. The patients were followed up for two months for subsequent *Clostridium difficile* recurrences. A significant decrease in recurrences was observed only in patients treated with the high-dose vancomycin and *S. boulardii* treatment (16.7%) compared with patients who received high-dose vancomycin and placebo (50%, $P = 0.05$). There were no significant reductions in recurrence rates in either the low-dose vancomycin or metronidazole treatment group, regardless if *S. boulardii* was used. No serious adverse reactions were noted in any of these patients. By the end of the antibiotic treatment, only high-dose vancomycin completely cleared *C. difficile* toxin from the colon, low-dose vancomycin and metronidazole could not clear the toxin. A subsequent investigation of 163 patients with recurrent CDI confirmed this finding[165]. In this study, stool samples were assayed at the end of antibiotic treatment to determine if there was a difference in toxin clearance by the type or dose of antibiotic. Treatment with high-dose (2 g/d) of vancomycin completely cleared *C. difficile* toxin, but 11% of those treated with lower doses of vancomycin and 41% treated with metronidazole were positive for *C. difficile* at the end of antibiotic treatment. *S. boulardii* may be more effective if complete *C. difficile* toxin clearance is achieved before sole reliance upon the yeast probiotic is required.

An earlier randomized, controlled double-blinded study had also found efficacy of the combination treatment using a standard antibiotic (vancomycin or metronidazole) and *S. boulardii* for patients with *C. difficile* disease[53]. This study did not control the dose or duration of either vancomycin or metronidazole (it was under the physician's discretion), but randomized patients to either *S. boulardii* (1 g/d) or placebo for 28 d. All patients were followed up for two months for subsequent recurrences. Approximately half of the enrolled patients had their first episode and half had recurrent *C. difficile* disease. Twenty-six percent of the 57 patients given *S. boulardii* had a recurrence of *C. difficile* disease, which was significantly fewer as compared with 45% of the 67 given placebo ($P < 0.05$). The strongest effect was found in the 60 patients with recurrent *C. difficile* disease; significantly fewer patients (35%) given *S. boulardii* had a recurrence compared with 65% of patients given placebo ($P = 0.04$). Two adverse reactions were associated with *S. boulardii* (thirst and constipation). No randomized controlled trials using stains of *S. cerevisiae* for this disease were found in the literature.

H. pylori

Epidemiology of *H. pylori*: *H. pylori* colonizes the gastric mucosa and usually induces a chronic, asymptomatic carrier state, but some people may develop gastroduodenal ulcers as a result. Carriage of *H. pylori* is a risk factor for developing gastric lymphoma or adenocarcinoma in later life. The prevalence of *H. pylori* carriage is 50%-80% worldwide[166,167]. Standard treatment for *H. pylori* involves a two-week course of triple therapy (usually clarithromycin, amoxicillin and an acid suppressor such as lansoprazole or omeprazole), that also has gastrointestinal side effects.

Efficacy for *H. pylori*: *S. boulardii* induces morphologic changes in *H. pylori* cells consistant with cellular damage[21] and was shown to reduce 12% of *H. pylori* colonization in infected children in one trial[46]. Of four randomized controlled trials testing *S. boulardii* in *H. pylori* infections, two were in children[46,168] and two (Table 4) were in adults[42,134]. Cremonini *et al*[134] first explored the efficacy of probiotics to eradicate *H. pylori* in 85 asymptomatic carriers. They

Table 4 Randomized, controlled trials for acute disease conditions using *S. boulardii*

Ref.	Treatment groups	Study population	Daily dose: cfu/d (mg/d)	Duration of treatment	Follow-up (wk)	Outcome in probiotic	Outcome in controls
	Helicobacter pylori						
Cremonini *et al*[134]	*S. boulardii* (Codex, SmithKine Beecham, Italy) *vs* placebo *vs* LGG *vs* mix (*L acid* and *Bifid lactis*)	85 asymptomatic carriers *H. pylori* 22 SB and 21 placebo; all received triple therapy	5×10^9 (nr)	2 wk	2	Eradicated in: 17/21 (81%) same for LGG and mix Any symptoms: 4/21 (19%)[a]	Placebo, 16/20 (80%) Any symptoms: 12/20 (60%)
Cindoruk *et al*[42]	*S. boulardii* (Reflor, Turkey) *vs* placebo	124 adults with *H. pylori* dyspepsia, all received triple therapy	2×10^{10} (1000 mg)	2 wk	6	Eradication 44/62 (71%) Epigastric distress: 9 (14.5%)[a]	Eradication 37/62 (59.7%) Distress: 27 (43.5%)
	Acute adult diarrheas						
Hochter *et al*[169]	*S. boulardii* *vs* placebo	92 German outpatient adults	8×10^9 (300-600 mg)	8 d	0	Diarrhea score reduction: -17.2 + 6.8[a]	Score: -13.6 + 8.7
Mansour-ghanaei *et al*[51]	*S. boulardii* (UltraLevure, France) *vs* placebo. Both groups got metro and lodoquinol	57 enrolled, 54 done; adults with *E. histolytica* amoebic dysentery	1.5×10^{10} (750 mg)	10 d	4	Cured: 27/27 (100%)[a]	Cured: 5/27 (19%)
	Enteral feeding-related diarrhea						
Tempe *et al*[61]	*S. boulardii* *vs* placebo	40 adults in ICU	1×10^{10} (nr)	11-21 d	0	34/389 days of diarrhea (8.7%)[a]	63/373 days of diarrhea (16.9%)
Schlotterer *et al*[171]	*S. boulardii* *vs* placebo	20 enrolled, 18 done, burnt adults 18-70 yr	4×10^{10} (2000 mg)	8-28 d	0	3/204 days of diarrhea (1.5%)[a]	19/208 days of diarrhea (9.1%)
Bleichner *et al*[39]	*S. boulardii* *vs* placebo	131 enrolled, 128 done, adults in ICU	4×10^{10} (2000 mg)	3 wk	0	50/648 days of diarrhea (7.7%)[a]	87/683 days of diarrhea (12.7%)
	Traveler's diarrhea						
Kollaritsch *et al*[175]	*S. boulardii* *vs* placebo	832 Austrian tourists to hot climates	2×10^9 (250 mg)	5 d before trip and mean 21 d trip	0	143/426 (34%)[a]	173/406 (43%)
Kollaritsch *et al*[175]	*S. boulardii* *vs* placebo	805 Austrian tourists to hot climates	5×10^9 (500 mg)	5 d before trip and mean 21 d trip	0	127/399 (32%)[a]	173/406 (43%)
Kollaritsch *et al*[49]	*S. boulardii* *vs* placebo	713 Austrian tourists to hot climates	2×10^9 (250 mg)	5 d before trip and mean 21 d trip	0	121/352 (34%)[a]	141/361 (39%)
Kollaritsch *et al*[49]	*S. boulardii* *vs* placebo	664 Austrian tourists to hot climates	2×10^{10} (1000 mg)	5 d before trip and mean 21 d trip	0	87/303 (29%)[a]	141/361 (39%)
Bruns *et al*[176]	*S. cerevisiae* Hansen (CBS 5926) *vs* active treatment[1]	60 tourists in Tunisia with traveler's diarrhea, 43 done	1.0×10^{10} (600 mg)	5 d	0	Duration diarrhea 2.1 d	1.4 days of diarrhea[a]

[a]$P < 0.05$, probiotic *vs* controls; [1]Strain currently known as *S. boulardii*.

randomized patients to one of three probiotic treatments (*S. boulardii*, *L. rhamnosus* GG or a mixture of *L. acidophius* and *Bifidobacterium lactis*) or placebo for 14 d. All patients were treated with the triple therapy for the first week. By the end of the second week, eradication rates were similar for all groups (81% for *S. boulardii vs* 80% of placebo). A promising result of this study was a lower rate of antibiotic-associated diarrhea (5%) in all the probiotic groups compared with 30% of the placebo group. A subsequent study by Cindoruk *et al*[42] assessed *S. boulardii* for both eradication of *H. pylori* and the reduction of side-effects of the standard triple treatment. This study enrolled 124 adults with *H. pylori* dyspepsia in Turkey who were receiving the triple therapy and randomized to either 1 g of *S. boulardii* (2×10^{10}/d for 2 wk) or placebo. Patients were followed up for six weeks for side-effects and *H. pylori* clearance. Although there was no significant difference in *H. pylori* eradication (71% in *S. boulardii vs* 60% in placebo), significantly fewer patients randomized to *S. boulardii* reported epigastric distress (14.5%, $P < 0.05$) compared with placebo (43.5%), as well as lower global dyspepsia symptom scores. The frequency of AAD was also significantly reduced in those receiving *S. boulardii* (14.5%) compared with the placebo group (30.6%). These studies indicate that *S. boulardii* may not be effective in eradicating *H. pylori* itself, but it is effective in reducing the side-effects of the standard triple therapy.

Acute adult diarrheas

Epidemiology of acute adult diarrhea: Acute adult diarrhea is a broad classification for diarrhea that includes illnesses that may develop quickly, but are typically short-lived and are sporadic. The etiologies of acute adult diarrhea may include infectious agents (*Entamoeba histolytica*, *E. coli*, or Salmonella) or may be idiopathic. Diarrhea in adults occurring in outbreaks or due to other situations (antibiotic-associated diarrhea, *C. difficile*-associated diarrhea, traveler's diarrhea, inflammatory bowel disease or irritable bowel disease) are not included in this classification.

Clinical efficacy for acute adult diarrhea: Two randomized controlled trials using *S. boulardii* showed that this probiotic may be effective in treating acute diarrhea due to a variety of causes. Hochter *et al*[169] enrolled 92 German

adult outpatients with acute diarrhea and randomized them to *S. boulardii* (8×10^9/d) or placebo for eight days. By the third day, the 43 given *S. boulardii* had a significantly lower diarrhea severity score (5.5 ± 6.8, $P = 0.04$) compared with the 49 given placebo (6.7 ± 8.7). Mansour-Ghanaei *et al*[51] enrolled 57 patients with acute *Entamoeba histolytic* dysentery and treated them with *S. boulardii* (1.5×10^{10}/d for 10 d) or nothing (control). Both groups also received metronidazole and iodoquinol for 10 d. At the end of four weeks, all the patients given *S. boulardii* were cured compared with 19% of those not given the yeast. Unfortunately, the number of trials in this area is small and the etiologies were different in the two trials, so conclusions are limited. No randomized controlled trials using stains of *S. cerevisiae* for this disease were found in the literature.

Enteral nutrition-related diarrhea

Epidemiology of enteral nutrition-related diarrhea: Diarrhea is a common complication associated with enteral tube feeding and may result in a loss of nutrition in an already seriously ill patient. The frequency of diarrhea in enteral tube fed patients has been reported as high as 50%-60%[170] and complications may include life-threatening acidosis, increased morbidity and mortality and increased healthcare costs. Schneider *et al*[102] reported a significant increase in short-chain fatty acids in 10 enteral-fed patients receiving *S. boulardii* (1 g/d for 6 d) compared with 15 healthy controls.

Clinical efficacy for enteral nutrition related diarrhea: Three randomized, controlled trials have assessed the ability of *S. boulardii* to reduce diarrhea in patients receiving this type of nutritional intake (Table 4). Tempe *et al*[61] compared 40 enteral-fed patients randomized to either *S. boulardii* (1×10^{10}/d) or placebo for 11-21 d and found significantly fewer patients (8.7%) given *S. boulardii* developed diarrhea compared with placebo (16.9%). Schlotterer *et al*[171] randomized 18 patients with burns who were receiving enteral nutrition to *S. boulardii* (4×10^{10}/d) or placebo for 8-28 d. Those given *S. boulardii* suffered fewer diarrhea days (3/204 d, 1.5%) than those given placebo (19/208 d, 9.1%, $P < 0.001$). The efficacy of *S. boulardii* was confirmed in a later study of 128 intensive care unit (ICU) patients randomized to either *S. boulardii* (4×10^{10}/d) or placebo for 21 d[39]. Those given *S. boulardii* reported significantly fewer diarrheal days (7.7%) than those given placebo (12.7%). No adverse reactions associated with *S. boulardii* were reported in any of these three trials.

Traveler's diarrhea

Epidemiology of traveler's diarrhea: Traveler's diarrhea (TD) is a common health complaint among travelers; globally 12 million cases are reported every year[172]. Rates of TD range from 5% to 50%, depending upon the destination, with equatorial countries having the highest rates of TD. TD usually occurs as sporadic cases, but outbreaks of TD may occur in large groups traveling or located together (tourists on cruise ships, group tours, military personnel, disaster-relief groups). The etiologies of TD vary widely, but commonly include enterotoxigenic and entroaggregative *E. coli*, *Campylobacter jejuni*, *Salmonella typhimurum*, viruses (Norwalk or Rotavirus) or parasites (*Entamoeba histolytica*, *Giardia lamblia*). Traditional preventive measures including frequent handwashing, avoiding high-risk foods and water and ingestion of bismuth subsalicyclate showed only modest protection[173].

Clinical efficacy for TD: A meta-analysis of 12 randomized, controlled trials of various probiotics (including *S. boulardii*, *Lactobacilli* and mixtures of different probiotic strains) for the prevention of TD found a significant reduction in the risk of TD if probiotics were used (RR = 0.85, 95% confidence interval 0.79-0.91)[174]. Two randomized controlled trials have been done with *S. boulardii* that included a total of four different probiotic treatment arms (Table 4). Kollaritsch *et al*[175] enrolled 1231 Austrian tourists traveling to hot climates and randomized travelers to either of two doses of *S. boulardii* (250 or 500 mg/d) or placebo for 3 wk. The treatment was started 5 d prior to the trip and continued through the duration of the trip. Traveler's diarrhea developed in 43% given placebo and significantly fewer in those given the low-dose *S. boulardii* (34%) and the higher dose *S. boulardii* (32%). A second study by Kollaritsch *et al*[49] was done with 3000 Austrian tourists traveling to northern African, the Middle East and the Far East. Travelers were randomized to either 250 mg/d (5×10^9) *S. boulardii*, 1000 mg/d (2×10^{10}) *S. boulardii* or placebo, starting five days before leaving and throughout the duration of their trip (median of 3 wk). Only 1016 (34%) completed the study, but *S. boulardii* significantly reduced the incidence of traveler's diarrhea in a dose-dependent manner. Of the travelers given placebo, 39% developed diarrhea whereas only 34% of those given the lower dose and 29% of those given the higher dose of *S. boulardii* developed traveler's diarrhea ($P < 0.05$). No adverse reaction was noted by the travelers in either of these studies.

Bruns *et al*[176] tested *S. cerevisiae* Hansen CBS 5926 (Perenterol®, Germany) to treat travelers who had developed TD in Tunisia. Travelers with TD were randomized to 600 mg of *S. cerevisiae* (1×10^{10}/d) for five days or an active control treatment and the duration of their TD tracked. Of 60 enrolled, 43 completed the trial, but the duration of diarrhea was not significantly different between those given *S. cerevisiae* (2.1 d) and those given ethacridine-lactate and tannalbuminate (1.4 d). This strain is known as *S. boulardii* outside of Germany. No other randomized controlled trials have been done for the treatment of TD using *S. boulardii*. These limited numbers of studies indicate that probiotics may be more effective in preventing TD, rather than treating the diarrhea once it becomes symptomatic.

CLINICAL EFFICACY: CHRONIC DISEASES

S. boulardii has been tested for clinical efficacy in several types of chronic diseases including Crohn's disease, irri-

table bowel syndrome, giardiasis and human immunodeficiency virus (HIV)-related diarrhea.

Inflammatory bowel disease

Epidemiology of inflammatory bowel disease: inflammatory bowel diseases (IBDs) are chronic, immune-related inflammatory diarrheas, which include ulcerative colitis, pouchitis and Crohn's disease. The incidence of Crohn's disease varies by countries, with the highest incidences (8-66/100 000) found in Wales, New Zealand, Canada, Scotland, France, Netherlands, intermediate rates (4-7/100 000) in UK, USA, Europe, Australia, and the lowest rates (0.2-3/100 000) in South America, China, Korea, Japan[177]. In Crohn's disease, typically the lower small intestine and colon are the most affected organs. Symptoms include diarrhea, abdominal pain or cramping and loss of appetite. Unlike ulcerative colitis, lesions of Crohn's disease are deep and patchy and often involve thickened areas resulting in intestinal obstruction, which can be life threatening. Disruption of the intestinal wall also allows intestinal bacteria to translocate and incite the immune system. Complications of Crohn's disease are common, in that 40% of Crohn's patients report lower GI bleeding, intestinal obstruction, or perforation[178]. Lifetime risk for surgery in Crohn's patients is extremely high (70%-80%)[179]. Unfortunately, the cause of Crohn's disease is not known, so current therapies are directed at symptom relief. But 10%-60% suffer recurrences of Crohn's disease after treatment is completed[180].

Clinical efficacy for IBD: Guslandi *et al*[48] enrolled 25 adults with mild to moderate ulcerative colitis in a pilot study and treated them with a combination of mesalazine (3 g/d) and *S. boulardii* (1.5×10^{10}/d) for 4 wk. All of these patients had histories of poorly tolerated steroid courses. Most (68%) of the patients responded to the probiotic treatment. This study had a promising result, but the implications were uncertain as patients treated for only a short time, were not followed up for subsequent flare-ups of their disease and there was no control group in this pilot study. Garcia Vilelea *et al*[62] enrolled 31 patients with Crohn's disease in remission (at the time of enrollment). All patients continued their maintenance medications during the trial (mesalamine, azathioprine or others). Patients were randomized to either *S. boulardii* (2×10^{9}/d) for three months or placebo. Those treated with *S. boulardii* were found to have a significant reduction in colonic permeability compared with those given placebo, thus reducing the risk of translocation in these patients.

Two randomized controlled clinical trials have been done testing *S. boulardii* for patients with Crohn's disease (Table 5)[47,57]. Plein *et al*[57] randomized 20 patients with Crohn's disease to either 750 mg of *S. boulardii* (1.5×10^{10}/d) or placebo for seven weeks. All patients continued their maintenance medications during the trial. At the end of seven weeks, patients treated with *S. boulardii* were significantly improved (mean = 3.3 ± 1.2 stools/d, $P < 0.05$) compared with the placebo group (mean = 4.6 ± 1.9 stools/d). Guslandi *et al*[47] studied the effect of *S. boulardii* on the rate of Crohn's disease relapses in a study of 32 patients with Crohn's disease in Italy. Adults (23-49 years old) who were in remission were randomized to either 1 g of *S. boulardii* (2×10^{10}/d) and mesalamine (2 g/d) or mesalamine alone (3 g/d) for 6 mo. Significantly fewer patients treated with *S. boulardii* (6%, $P = 0.04$) relapsed than the control group (38%). No adverse reactions associated with *S. boulardii* were reported in any of these trials.

Crohn's disease is a challenging condition for clinical trials in that it is sporadic, has no defined etiology, has multiple measurable disease outcome and requires lengthy treatment and follow-up period. *S. boulardii* offers promise to this group of patients who are in need of a safe and effective therapy for this chronic condition. The probiotic protection seems to be strain specific. A study tested another strain of *S. cerevisiae* (Baker's yeast) in 19 adults with Crohn's disease, but found the disease was worse in those receiving this strain than in controls[181].

Irritable bowel syndrome

Epidemiology of irritable bowel syndrome: Irritable bowel syndrome (IBS) is a frequent disorder characterized by a triad of symptoms (bloating, abdominal pain, and intestinal transit disturbances). The prevalence of IBS is high (3%-20%) in the general population and IBS may account for 25%-50% of gastroenterologist practice[182]. Consequences of IBS include worse quality of life[183], higher health care costs ($1.7 billion in health care costs)[184], and higher incidences of depression[185].

Clinical efficacy for IBS: Standard treatments target symptom relief, but have not been more effective than placebo. IBS is a recent focus of probiotic clinical trials. A meta-analysis of 20 randomized, controlled trials (with 23 treatment arms) including 1404 subjects found a pooled relative risk (RR) for improvement in global IBS symptoms in 14 probiotic treatment arms (RR = 0.77, 95% CI: 0.62-0.94)[36]. One of those trials (Table 5) randomized 34 patients with IBS to *S. boulardii* (9×10^{9}/d) or placebo for four weeks[52]. A significant decrease in the daily number of stools was found in the *S. boulardii* group and 87.5% of this group reported their IBS had improved compared with 72% of the placebo group ($P < 0.05$). No adverse reactions were noted in this trial. More trials using *S. boulardii* for IBS are required.

Giardiasis

Epidemiology of giardiasis: This condition is characterized by long lasting diarrhea with symptoms ranging from mild to severe diarrhea, weight loss, abdominal pain and weakness. The incidence may be high in developing countries such as Colombia (13%) and lower in developed countries such as Germany (12/100 000)[186,187]. It is also found in people enjoying outdoor hiking and camping who drink contaminated untreated water that appears clean.

Clinical efficacy for giardiasis: Besirbellioglu *et al*[38] randomized 65 adults with giardiasis in Turkey to either *S. boulardii* (1×10^{10}/d) or placebo for 10 d. Both groups

Table 5 Randomized, controlled trials for chronic disease conditions using *S. boulardii*

Ref.	Treatment groups	Study population	Daily dose: cfu/d (mg/d)	Duration of treatment and follow-up	Outcome in probiotic	Outcome in controls
	Crohn's disease					
Plein *et al*[57]	*S. boulardii* (Perenterol®) *vs* placebo	20 enrolled with Crohn's, 17 done, all on maintenance medications	1.5×10^{10} (750 mg)	7 wk	3.3 ± 1.2 stools/d at week 9[a]	4.6 ± 1.9 stools/d at week 9
Guslandi *et al*[47]	*S. boulardii* (Perenterol®) + mesalamine (2 g/d) *vs* mesalamine alone (3 g/d)	32 with Crohn's in remission in Italy (23-49 yr old)	2×10^{10} (1000 mg)	6 mo	1/16 (6%) relapse[a]	6/16 (38%) relapse
	Irritable bowel syndrome					
Maupas *et al*[52]	*S. boulardii vs* placebo	34 with IBS	9×10^{9} (nr)	4 wk	Mean decrease in #stools/d: -2.2/d[a] Improved: 14/16 (87.5%)[a]	Mean decrease -0.5/d Improved: 13/18 (72%)
	Giardiasis					
Besirbellioglu *et al*[38]	*S. boulardii* + metro (2250 mg/d) *vs* placebo + metro	65 adults with giardiasis	1×10^{10} (500 mg)	10 d with 4 wk f/up	100% cured, 0% giardia cysts[a]	100% cured 6/35 (17%) cysts present
	HIV-related diarrhea					
Saint- Marc *et al*[189]	*S. boulardii vs* placebo	35 French AIDS patients with chronic diarrhea	6×10^{10} (3000 mg)	7 d	11/18 (61%) cured[a]	2/17 (12%)

[a]$P < 0.05$, probiotic *vs* controls. IBS: Irritable bowel syndrome; HIV: Human immunodeficiency virus; AIDS: Acquired immune deficiency syndrome; f/up: Follow-up time.

also received metronidazole for the same duration. Two weeks later, both groups reported a resolution of their diarrhea, but none of those on *S. boulardii* had detectable giardia cysts, while significantly more (17%) on placebo still carried giardia cysts.

HIV diarrhea

Epidemiology of HIV-related diarrhea: Patients infected with HIV are susceptible to a variety of diseases and may also develop chronic life-threatening diarrhea. The prevalence of diarrhea in HIV patients ranges from 50%-60% in developed countries and nearly 100% in developing countries[188].

Clinical efficacy for HIV-related diarrhea: A blinded, placebo-controlled dose-ranging study was done in 11 HIV positive patients who had chronic diarrhea[45]. *S. boulardii* was given at 1-3 g/d and six patients reported diarrhea was controlled while taking 3 g/d after one month, while lower doses of *S. boulardii* were not as effective. Patients with HIV-associated diarrhea seem to be one group that requires a higher than typical dose of *S. boulardii*. Saint-Marc *et al*[189] randomized 35 Stage IV AIDS adult patients with chronic diarrhea to either *S. boulardii* (3 g/d or 6×10^{10}/d) or placebo for one week (Table 5). Significantly more of those given *S. boulardii* (61%, $P = 0.002$) had their diarrhea resolved compared with placebo (12%) patients. *S. boulardii* was well tolerated by all HIV patients even treated with high doses of 3 g/d in these two studies.

SAFETY OF *S. BOULARDII*

The use of a living organism as therapy increases the potential risk in four general areas: transfer of antibiotic-resistance genes, translocation of the living organism from the intestine to other areas of the body, persistence in the intestines and the development of adverse reactions. The first three theorical concerns are of minimal impact on *S. boulardii*. Unlike other bacterial strains of probiotics, such as *Enterococcus faecium* and *Lactobacillus rhamnosus*, which have been shown to acquire antibiotic-resistance genes, *S. boulardii* has not developed any antibiotic or antifungal resistance[190,191]. Data from animal models shows reduced translocation with *S. boulardii* treatment[64,89], unlike other strains of *S. cerevisiae*[192]. As pharmacokinetic studies indicate, *S. boulardii* does not persist 3-5 d after oral ingestion is discontinued, so persistence is not a concern for this probiotic[118].

S. boulardii has been used as a probiotic since the 1950s in Europe and has been investigated in clinical trials worldwide. Safety and adverse event data collected during clinical trials, when patients are closely monitored for problems associated with the investigational treatment, has documented a remarkable safety profile of *S. boulardii* in adults. A wide diversity of adult patients have been followed up who were either randomized to *S. boulardii* as part of a clinical trial or were documented in case series or case reports for various disease indications (TD, $n = 1596$; AAD, $n = 958$; adult acute diarrhea, 156; enteral tube feeding, $n = 103$; IBD, $n = 66$; IBS, $n = 16$, HIV-related diarrhea, $n = 18$ and giardia infections, $n = 50$). These studies provide safety data for a total of 2963 adult patients and the only adverse reactions associated with *S. boulardii* was thirst (in 5 patients) and constipation (in 8 patients) in a trial of patients with *C. difficile* infections[53]. No case of *S. boulardii* fungemia has been reported in these diverse patient populations enrolled in clinical trials.

Infrequent cases of fungemia have been reported in case reports or case series in the literature. Most of the adult cases of *S. boulardii* fungemia are with serious co-

Table 6 Summary of recommendations for clinical use of *S. boulardii* in adults

Use for disease	Dose (mg/d)	Duration	Adjunct to	Strength of evidence[1]
Prevention of antibiotic associated diarrhea	500-1000	During antibiotics with additional 3 d to 2 wk after	Nothing	++++
Prevention of Traveler's diarrhea	250-1000	Duration of trip (3 wk)	Nothing	+++
Enteral nutrition-related diarrhea	2000	8-28 d	Nothing	++
H. pylori symptoms	1000	2 wk	Standard triple therapy	++
Treatment of *Clostridium difficile* infections	1000	4 wk	Vancomycin or metronidazole	+
Acute adult diarrhea	500-750	8-10 d	Nothing	+
Inflammatory bowel disease	750-1000	7 wk to 6 mo	Mesalamine	+
Irritable bowel syndrome	500	4 wk	Nothing	+
Giardiasis	500	4 wk	Metronidazole	+
HIV-related diarrhea	3000	7 d	Nothing	+

[1]Strength of evidence, + (weak, needs more randomized controlled trials) to ++++ (strong, efficacy and safety are evidence based from numerous large randomized controlled trials).

morbidities and have central venous catheters. Most cases responded well to treatment with fluconazole or amphotericin B[193]. Some cases developed fungemia, not from the direct ingestion of *S. boulardii* probiotics, but acquired the yeast from contaminated environmental fomites[194]. Fungemia from *S. cerevisiae* (non-boulardii strains) have also been reported and are similar to *S. boulardii* cases, but have poorer prognosis[195,196]. A challenge to determining valid incidence of fungemia is the lack of available advanced yeast identification assays which can distinguish *S. cerevisiae* from *S. boulardii*.

CONTRAINDICATIONS/PRECAUTIONS

Even though fungemia with *S. boulardii* is infrequent, it may be prudent to closely follow up the adult inpatients who are severely ill or in intensive care units and have central catheter for episodes of unexplained fever. Some studies have recommended not to give *S. boulardii* to immunosuppressed patients or those with central catheters to reduce this risk[108].

Unlike many bacterial probiotics, there are few drug or antibiotic interactions with *S. boulardii*. However, if patients are on anti-fungal medications, a staggered dose regime (at least 4 h apart) is suggested[45].

CONCLUSION

The use of *S. boulardii* as a therapeutic probiotic is supported by its mechanisms of action, pharmacokinetics, and efficacy from animal models and clinical trials. The overall safety profile for *S. boulardii* is beneficial. *S. boulardii* can be recommended for several diseases. Other strains of Saccharomyces may have probiotic properties, but clinical efficacy evidence for other strains are still deficient. Guidelines for the most effective use of *S. boulardii* are given in Table 6 and generally, the daily dose is typically $> 10^9$/d, the duration of treatment can vary from 7 d to 6 mo and it may be given alone or as an adjunctive treatment, depending upon the disease indication. *S. boulardii* is significantly effective for the prevention of AAD and prevention of TD. Trials also show evidence for *S. boulardii* in the reduction of side-effects of *H. pylori* treatment and the prevention of enteral nutrition-related diarrhea. More clinical trials are encouraged for the treatment of chronic diseases (Crohn's disease, irritable bowel syndrome and HIV-related diarrhea) and the prevention of *C. difficile* disease recurrences.

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TOPIC HIGHLIGHT

Metin Basaranoglu, PhD, Associate Professor, *Series Editor*

Understanding mechanisms of the pathogenesis of nonalcoholic fatty liver disease

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Abstract

A central issue in the understanding of the pathogenesis of nonalcoholic fatty liver disease is the problem of the underlying mechanisms which are not fully understood. In the setting of excessive central adiposity, insulin resistance is the major underlying cause of fat accumulation in hepatocytes. Because of the difficulties with human trials, several animal models have been developed for this purpose mainly characterized as follows: genetically disturbed or murine fatty liver, methionine-choline deficient diet fed or murine steatohepatitis, and high-fat or sucrose diet fed models. Although these animal models have provided useful information, none of them accurately reflect genetic, metabolic and biochemical characteristics of the human disease.

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Key words: Nonalcoholic fatty liver disease; Pathogenesis; Rat; Rodents

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OBESITY AND NONALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent forms of chronic liver disease[1-4]. The reported prevalence of NAFLD in developed countries is 30% and 13% in adults and children, respectively. It is likely that in type 2 diabetes mellitus, NAFLD is one of the consequences of obesity. The prevalence of NAFLD in the obese population is nearly 95%. Factors contributing to NAFLD include sedentary life style, and increased consumption of foods with high-fat and high fructose corn syrup content. A cafeteria style diet which includes both high fat and fructose corn syrup is the

leading cause of obesity in the population. Furthermore, in the setting of excessive central adiposity, insulin resistance is the major underlying cause of fat accumulation in the liver[5-11]. NAFLD is characterized by hepatic fat accumulation in hepatocytes (> 5% of liver weight).

NONALCOHOLIC STEATOHEPATITIS

Nonalcoholic steatohepatitis (NASH), as a subgroup of NAFLD, is characterized by chronic, progressive liver pathology which has the ability to lead to advanced fibrosis, cirrhosis, hepatocellular carcinoma, and liver-related death[1,2,4,5]. The prevalence of NASH is 3% among adults. The mechanisms underlying NASH pathogenesis are not fully understood. One of the hypotheses has been termed the "two-hit" theory[5,6]. According to this paradigm, NAFLD is a result of inappropriate fat storage or ectopic fat accumulation and the primary abnormality is most likely insulin resistance which leads to the accumulation of triglycerides (TGs) within the hepatocytes. After the first hit of steatosis, the second hit of oxidative stress leads to hepatocyte injury and inflammation.

Animal models

There are several types of animal model used for NAFLD studies, and these are mainly characterized as follows: genetically disturbed or murine fatty liver, methionine-choline deficient (MCD) diet fed mice or murine steatohepatitis model and feeding high-fat and/or sucrose diets with or without high caloric intake model[12-24]. Manipulation of the mouse genome and production of new animal models, such as leptin-deficient ob/ob mice, leptin-resistant db/db rats or knockout mice regarding a special character, have given us great opportunities for research[12-16]. However, it is not possible to extrapolate all the information gained from these animal models into knowledge on the human species as there are some fundamental differences regarding their genetics, mediators, and the mechanisms of the events. For example, although obese patients have higher circulating leptin levels, ob/ob mice exhibit complete leptin deficiency. Additionally, leptin has some immunologic functions, besides its metabolic regulatory capacity. Secondly, although insulin resistance is a universal feature of patients with NASH, the MCD model is not insulin-resistant and not obese[15]. MCD mice have increased insulin hypersensitivity, and their serum has both insulin and glucose levels lower than mice fed a standard diet.

High caloric intake models

With regard to the problems mentioned above, it is reasonable to try to find a suitable model for investigating the mechanism behind human NASH. Actually, there have been several attempts to mimic human species by feeding mice with high fat and/or sucrose diets with or without high caloric intake in order to produce obesity, insulin resistance, and NASH[16-23]. The type and amount of fat, and total daily caloric intake on these diets are very important and there has been no standardized diet reported by study groups. In this regard, Lieber *et al*[25] fed Sprague-Dawley rats with a high-fat, liquid diet (71% of energy from fat which included corn, olive, and safflower oil) for 3 wk and developed a steatohepatitis model in murines which resembled human NASH. These mice displayed obesity and insulin resistance together with increased hepatic tumor necrosis factor (TNF)-α, TNF-α messenger RNA, cytochrome 2E1, cytochrome 2E1 mRNA, increased oxidative stress and lipid peroxidation, fatty liver histopathology, mononuclear cell infiltration, abnormal mitochondria and increased collagen in the liver. Bruce *et al*[26] used a high fat diet (45% kcal from fat, 20% kcal protein, 35% kcal carbohydrate) and Tetri *et al*[27] fed mice with 45% calories in the chow from fat and 30% of the fat in the form of partially hydrogenated vegetable oil (28% saturated, 57% monounsaturated fatty acids, 13% polyunsaturated fatty acids). Both research groups developed mice displaying obesity, insulin resistance and NASH.

There is substantial diversity within and between mouse strains resembling phenotypic variations in human populations. C57 BL6J mice have usually been chosen by NASH researchers because of their predisposition to develop insulin resistance by means of diet and the availability of genetically manipulated mice[14,27-29]. These features of the strain are explained by a strong influence of the genetic background on the susceptibility to diet-induced obesity and insulin resistance[14].

WHAT WE KNOW ABOUT THE MECHANISMS UNDERLYING NASH PATHOGENESIS?

For NASH pathogenesis, it is a prerequisite firstly to develop fatty liver which is then unusually vulnerable to various second hits or injury[5,6]. Although insulin resistance is a universal finding for both simple steatosis and NASH, only a small group of insulin-resistant patients develop NASH, even in patients with metabolic syndrome (MS). MS is the most severe form of insulin resistance and might instigate more advanced NASH. It is believed that oxidative stress and lipid peroxidation might play a central role in the transition of simple steatosis to NASH[30].

Increased production of ROS and lipid peroxidation of hepatocyte membranes and organelles promote necroinflammation, satellite cell activation and fibrosis in the liver. In this context, elevated free fatty acids (FFAs) in both circulation and the liver, mitochondrial abnormalities (dysfunction and structural abnormalities such as mega mitochondria with true crystalline inclusions), gut-derived endotoxins, ethanol secondary to gut and liver interaction, and disturbed production of adipokines should be major concerns in the development of steatohepatitis in an animal model.

Insulin resistance and peripheral lipolysis cause an

increased FFA pool in the circulation[7,21,30]. This pool is one of the major sources of hepatic TGs. FFAs are also the major source of hepatic mitochondrial, peroxisomal and microsomal ROS production. It has been reported that increased hepatic and serum FFA concentrations promote hepatic and systemic insulin resistance by the activation of PKCθ and serine phosphorylation of insulin receptor substrates. Feldstein *et al*[30] reported that FFAs promote lysosomal permeabilization, release cathepsin B which is a lysosomal protease within hepatocytes, and cause hepatocyte apoptosis and injury. In addition to these aspects, increased serum concentrations of FFAs due to obesity were found to be correlated with the severity of fibrosis in patients with NASH[31]. FFAs play a major role in the transition from simple steatosis to NASH.

High-fat diet-induced obesity and insulin resistance have been reported in Wistar rats[32,33], Sprague-Dawley rats[34,35], F344 rats[36], and in Long-Evans rats[37]. Additionally, prolonged feeding periods with high fat (59% fat), such as a 3 mo duration, promoted a greater degree of insulin resistance in Wistar rats[33]. Borst and Conover induced obesity and developed an insulin-resistant animal model by feeding them for 39 d with a high-fat (50%) inclusive diet[38]. In this model, daily caloric intake was not increased, and it was even slightly less than for the normal rat chow (12.4% fat). These mice developed increased visceral and subcutaneous fat mass, insulin resistance, elevated fasting serum insulin, decreased insulin-stimulated glucose transport in skeletal muscle, increased TNF-α expression on visceral adipose tissue, and an undetectable serum TNF-α concentration. Most importantly, serum FFA concentration was not increased significantly in both mice fed high-fat diet and controls, while liver TNF-α expression was not affected. This observation is interesting, as obesity is strongly associated with increased concentration of FFAs in the circulation.

It was previously reported that a five-fold increase of plasma FFAs caused up to 100-fold increase in plasma insulin concentrations[26,30]. Thus, FFAs are more important than TNF-α for inducing insulin resistance. TNF-α expressed in visceral adipose tissue macrophages, and maybe TNF-α in muscle, appears the major cause of systemic insulin resistance in these animal models, since FFA levels were not increased. Oxidative stress is one of the most popular proposed mechanisms of hepatocellular injury in NASH in both animal experiments and human trials. It has been reported that obesity correlates with systemic oxidative stress. Obese adults with MS have a higher plasma concentration of oxidative stress biomarkers than obese adults without MS[2,6,7]. Increased ROS production has been selectively shown in adipose tissue of obese mice. There is also some indirect evidence as to the benefit of antioxidants such as vitamin E, S-adenosylmethionine and betaine, phlebotomy to remove iron, and N-acetylcysteine for treating NAFLD. However, a causal relationship or a pathogenic link between NASH and oxidative stress has so far not been established.

Hepatocyte mitochondria are the main site of β-oxidation of FFAs and adenosine triphosphate (ATP) production is one of the crucial issues in the understanding of NASH pathogenesis. It was previously reported that mitochondrial structural abnormalities, depletion of mitochondrial DNA and ATP, and mitochondrial dysfunction are characteristics of NASH patients as well as of diet-induced and animal models[39-41]. Increased oxidative stress and lipid peroxidation products are integral components of the pathway progressing to NASH from fatty liver. Specifically, rats fed a high-fat diet have been shown to have reduced electron transport chain capacity and increased oxidative stress in liver mitochondria[39,41].

Excessive fatty acids might be used as an alternative pathway to produce mitochondrial injury rather than the mitochondrial β-oxidation pathway. These possible routes of injury include the peroxisomal and microsomal oxidation systems[42]. Alternative fatty acid oxidation systems produce more hydrogen peroxide and thus may contribute to oxidant stress. Increased cytochrome P450 2E1 expression and induction have been well-established previously in both murine SH and human NASH studies.

In a very recent study, researchers developed a novel model which suggested maternal fat intake contributes toward the NAFLD progression in adult offspring; mediated through impaired hepatic mitochondrial metabolism and up-regulated hepatic lipogenesis[26]. Large-scale gene expression study, particularly a whole genome array analysis, is a very powerful technique which was used to measure the differences between the samples in this model[26]. This technique was able to measure the mRNA expressed in the tissue analyzed, assessing parameters such as relative expression of genes involved in inflammation, oxidative stress, lipogenesis, and beta-oxidation.

CONCLUSION

One of the areas for ongoing research is the understanding of how much calorie intake and composition of the diet affect the development of NASH. In conclusion, although these animal NAFLD models are already in use and further improve our knowledge, the underlying mechanisms in NAFLD pathogenesis need further research.

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ORIGINAL ARTICLE

Cost-utility of molecular adsorbent recirculating system treatment in acute liver failure

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Abstract

AIM: To determine the short-term cost-utility of molecular adsorbent recirculating system (MARS) treatment in acute liver failure (ALF).

METHODS: A controlled retrospective study was conducted with 90 ALF patients treated with MARS from 2001 to 2005. Comparisons were made with a historical control group of 17 ALF patients treated from 2000 to 2001 in the same intensive care unit (ICU) specializing in liver diseases. The 3-year outcomes and number of liver transplantations were recorded. All direct liver disease-related medical expenses from 6 mo before to 3 years after ICU treatment were determined for 31 MARS patients and 16 control patients. The health-related quality of life (HRQoL) before MARS treatment was estimated by a panel of ICU doctors and after MARS using a mailed 15D (15-dimensional generic health-related quality of life instrument) questionnaire. The HRQoL, cost, and survival data were combined and the incremental cost/quality-adjusted life years (QALYs) was calculated.

RESULTS: In surviving ALF patients, the health-related quality of life after treatmeant was generally high and comparable to the age- and gender-matched general Finnish population. Compared to the controls, the average cost per QALY was considerably lower in the MARS group (64732€ *vs* 133858€) within a timeframe of 3.5 years. The incremental cost of standard medical treatment alone compared to MARS was 10928€, and the incremental number of QALYs gained by MARS was 0.66.

CONCLUSION: MARS treatment combined with standard medical treatment for ALF in an ICU setting is more cost-effective than standard medical treatment alone.

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Key words: Albumin dialysis; Cost-efficiency; Health-related quality of life; Quality-adjusted life year

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INTRODUCTION

Since its introduction in 1993[1,2], the molecular adsorbent recirculating system (MARS) has been used in the treatment of both acute liver failure (ALF) and acute-on-chronic liver failure (AOCLF). The MARS device is an extracorporeal albumin dialysis apparatus that removes both albumin-bound and water-soluble toxins from the patient's blood, enabling native liver regeneration and allowing time to locate a suitable organ for liver transplantation (Ltx)[3-5].

Numerous studies have documented the favorable effects of MARS treatment on clinical and laboratory parameters[4,6-9] and survival[10-13]. However, only three small non-randomized studies[14-16] have focused on the cost-utility of MARS treatment in AOCLF and the health-related quality of life (HRQoL) of MARS-treated AOCLF patients. Currently, there are no studies on the HRQoL or cost-utility of MARS treatment in ALF patients.

In assessing therapy utility, the subjective feelings of the patient should be taken into account in addition to health benefits (e.g. survival) and cost. To ensure that limited resources are utilized in an ethical manner, the impact of a given treatment on the future HRQoL of the patient must be considered[17]. The effectiveness of a given treatment should be assessed by considering the treatment's impact on both the length and quality of life, which can be combined into the measure quality-adjusted life years (QALYs). In the cost-utility analysis, the QALYs gained by a given treatment are used as the measuring units of efficacy. Thus, the QALYs and cost/QALY-ratios can be used to compare the different treatments in terms of length of life and quality of life. Currently, there is no consensus as to how much a QALY gained can cost, but a 50 000€ threshold has been suggested[17].

The aim of this study was to determine the short-term (3.5 years) cost-utility of MARS treatment in ALF patients.

MATERIALS AND METHODS

Patients

This study included 90 ALF patients treated with MARS from May 2001 to October 2005 and a historical control group of 17 consecutive ALF patients treated from March 2000 to April 2001. ALF was defined as a rapid deterioration of hepatic synthetic function with or without encephalopathy and no previous history of liver disease[18].

All patients were treated in the same intensive care unit (ICU) specializing in liver disease at the Helsinki University Hospital and according to the same main principles of standard medical therapy (SMT). The SMT in our ICU and the operational principles of the MARS device were reported previously[3-5]. Our liver ICU is the only Ltx center in Finland, and all critical ALF patients are referred to our unit for treatment and transplantation evaluation. The indications for MARS treatment and the treatment protocols are summarized in Table 1. The 3-year survival and the need for Ltx were determined in all patients.

Economic evaluation

In the cost-utility analysis, effectiveness was measured as QALYs gained. The costs and outcomes of MARS treatment were compared with those of SMT in the control group over a 3-year time horizon from the perspective of the health care provider. For this comparison, a deterministic decision model was developed using TreeAge Pro HealthCare software (TreeAge, Williamstown, MA, USA). The model was used to combine the data on costs and effectiveness and to incorporate data variability and uncertainty into the analysis (Figure 1). The pathways presented in Figure 1 are mutually exclusive sequences of events, and the expected values are based on the pathway values weighted by the pathway probabilities. The probabilities show the proportion of the patient cohort that is expected to experience the event at any particular point in the tree.

The incremental cost-effectiveness ratio (ICER) was determined by dividing the difference in costs by the difference in QALYs. Both costs and QALYs were discounted 5% as currently recommended in the Finnish guidelines for pharmacoeconomic evaluation. The base-case analysis estimated the cost-effectiveness based on the mean values from the data in the simplified model structure. The effect of model input parameter uncertainty on the results was tested in a one-way and probabilistic sensitivity analysis.

Direct costs

From the year 2000 onwards, the Helsinki University Central Hospital district began to use the clinical patient-administration database Ecomed® for registering all treatment costs. In this study all direct medical costs were obtained from the Ecomed®-database (Datawell Ltd., Espoo, Finland). The total costs included all relevant liver disease-related expenses incurred at the Helsinki University Hospital. All costs incurred between 6 mo before the first MARS treatment (or liver ICU admission in the control group) and 3 years after the treatment were included. Complete cost data were available only for those patients who were living (31 MARS and 16 control patients) and received all hospital care in the catchment area of the Helsinki and Uusimaa Hospital District. Therefore, patients referred from outside the catchment were excluded from the cost analysis. Direct non-medical costs (transportation, domestic help, productivity costs due to absences from work, *etc.*) were not included. The mean total cost within the specified time period was used in the base-case analysis. All costs in Euros were inflated to the 2006 price level.

Table 1 MARS treatment protocols for acute liver failure in Finland

Etiology	MARS treatment initiation criteria	Treatment protocol
Acute liver failure	Rapid deterioration of hepatic synthetic function and clinical condition despite conservative standard medical therapy And one of the following criteria: (1) Ingestion of a lethal dose of a known hepatotoxin (e.g. *Amanita*, paracetamol) (2) The patient fulfills the criteria for highly urgent liver transplantation	22-h sessions daily until: (1) Native liver recovers (2) Suitable transplant organ is found (3) Irreversible multi-organ damage occurs

MARS: Molecular adsorbent recirculating system.

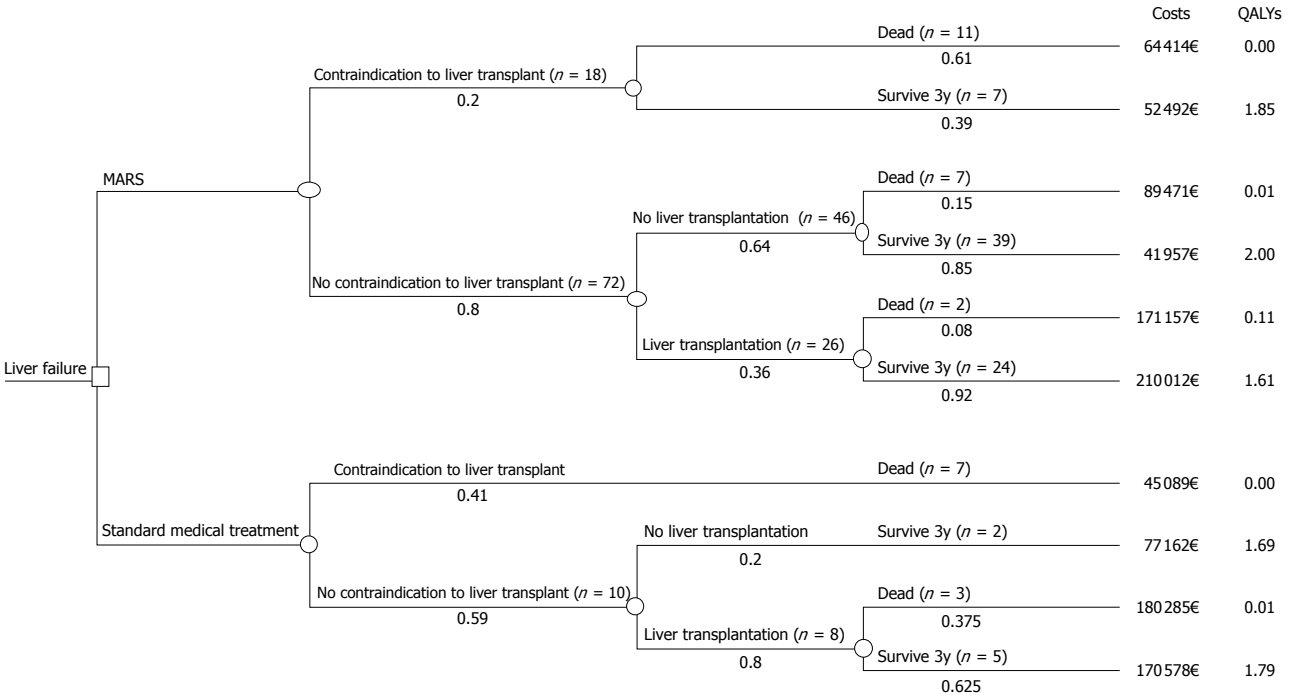


Figure 1 A deterministic decision model. In both treatment arms, the patient cohort was divided based on contraindication *vs* no contraindication to liver transplantation (Ltx) and then on the basis of Ltx *vs* no-Ltx. The "survive 3y" branches include all patients who survived at least three years after the first treatment, and the "death" branches include all patients who died within those three years. The pathway probabilities i.e. the proportion of the cohort that is expected to experience the event, are shown under each branch and were calculated based on real patient data. MARS: Molecular adsorbent recirculating system; QALY: Quality-adjusted life year.

HRQoL

The HRQoL was measured by the 15D (15-dimensional generic health-related quality of life instrument)[19-21], which is a generic, self-administered questionnaire for adults using criteria related to 15 dimensions: moving, seeing, hearing, breathing, sleeping, eating, speech, eliminating, usual activities, mental function, discomfort and symptoms, depression, distress, vitality, and sexual activity. For each dimension, the patient chooses one of five levels that best describes his/her current state of health. A set of utility weights is used to generate a single index number, the 15D score, which ranges from 0 to 1 (1 = full health, 0 = dead)[22]. For most of the important properties (i.e. reliability, content validity, discriminatory power, and responsiveness to change), the 15D is at least equal to other similar HRQoL instruments, such as the EQ-5D, SF-6D, HUI3, and AqoL[19-21,23-25].

Most ALF patients admitted to our liver ICU are seriously ill and encephalopathic, if not unconscious, and thus unable to fill out the HRQoL questionnaires. Therefore, we used expert opinion to assess the pre-treatment HRQoL. Three ICU doctors separately and retrospectively estimated the HRQoL of 30 ALF patients using the 15D instrument and the patients' clinical documents. All patients were divided into five groups according to their pre-treatment encephalopathy grade, which was represented as a number from 0 to 4. The HRQoL was assessed in all patients who were conscious and not intubated. The HRQoL was evaluated for six randomly selected non-intubated patients from each encephalopathy grade 0-3, and their average 15D score was assumed to represent the approximate HRQoL of the entire group (Table 2). Unconscious and intubated patients, which included some encephalopathy grade 3 patients and all encephalopathy grade 4 patients, received a 15D score of 0.0162[22].

In July 2007, the 15D questionnaire was sent to the 68 MARS-treated ALF patients who were still alive (one patient was not found). Two reminder letters were sent to those who did not return the first questionnaire. In total, 79% (54/68) completed the post-treatment 15D questionnaire; 37% of these patients (20/54) had undergone Ltx. At the time of the survey, the time elapsed after the first MARS treatment varied among individual patients

Table 2 Mean pre-treatment 15D scores in different encephalopathy grade groups (mean ± SD)

Pre-treatment encephalopathy grade	15D score
0	0.532 ± 0.151
1	0.461 ± 0.188
2	0.403 ± 0.177
3	0.079 ± 0.077
4	0.016

The average age-matched 15D score of the Finnish population is 0.92, and the standardized 15D score for unconscious/intubated patients is 0.016[22].

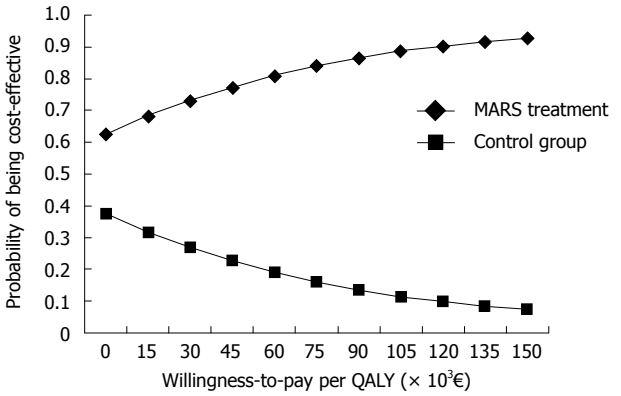


Figure 2 The cost-effectiveness acceptability curve for MARS *vs* standard medical therapy in the historical control group in acute liver failure.

(median 49 mo, range 22-72 mo). Thus, a 15D score 3 years after the first MARS treatment was individually estimated for each patient using linear regression analysis with the patient's age, time since MARS treatment, and the etiology of liver failure as explanatory variables.

The mean 15D scores and resulting QALYs were calculated separately for each pathway in the decision tree (Figure 1). For patients who died within the time horizon, QALYs were calculated based on survival, assuming the 15D score declined linearly from the baseline value to zero at the time of death.

Sensitivity analysis

The effect that model parameter uncertainty had on the results was examined first in a one-way analysis and further in a probabilistic sensitivity analysis. In the one-way sensitivity analysis, the discount rate, the mean cost and QALYs of both the MARS and control groups, the probability of Ltx, and survival rates varied. For the probabilistic sensitivity analysis, probability distributions for tree probabilities, cost, and utility input parameters were estimated. The results of the probabilistic sensitivity analysis are presented as a cost-effectiveness acceptability curve (Figure 2). Probability and utility values are represented by beta distributions (limiting values between 0 and 1 for utility values), the parameters of which were estimated from the patient data. For cost estimates, a gamma distribution was determined. Because the actual patient level cost data were limited, regression analysis was used to estimate costs for all patients. Sex, Ltx and its contraindication, and the number of MARS treatments received were used as explanatory variables in the regression analysis.

Table 3 Baseline data for the MARS and control groups

	MARS group	Historical control group	*P* value
Pre-treatment demographic & clinical data			
Number of patients	90	17	
Age (yr)	45 (14-81)	42 (21-72)	0.714
Sex (male)	37 (41%)	7 (41%)	0.996
Body mass index (kg/m^2)	26 (17-40)	26 (21-37)	0.372
MARS sessions/patient	2 (1-9)	0	
Mechanically ventilated	30 (33%)	6 (35%)	0.875
Vasoactive infusion used	27 (30%)	9 (56%)	0.041
Renal insufficiency	29 (32%)	7 (41%)	0.474
MELD-score	31 (5-50)	30 (19-51)	0.225
Mean encephalopathy grade	1.7 ± 1.6	2.1 ± 1.7	0.335
Contra-indication to Ltx prior to treatment	11 (12%)	4 (24%)	0.253
Became untransplantable during treatment	7 (8%)	3 (18%)	0.195
Number of transplanted patients	26 (29%)	8 (47%)	0.162
Pre-treatment key laboratory values			
Platelets (× 10^9/L)	138 (11-410)	142 (51-448)	0.919
NH_4 ion (μmol/L)	68 (8-512)	90 (14-241)	0.559
Bilirubin (μmol/L)	215 (4-761)	381 (38-880)	0.057
Creatinine (μmol/L)	75 (35-1318)	78 (38-275)	0.946
FV (%)	32 (5-142)	38 (7-71)	0.865

All values are expressed as median (range) or as number of patients (%). Hepatic encephalopathy grade is presented as mean ± SD. MELD score: Mean end-stage liver disease score; Ltx: Liver transplantation; FV: Coagulation factor V.

Statistical analysis

All data were analyzed using SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA). The Wilcoxon signed-rank test, the Mann-Whitney *U*-test, Pearson's χ^2, and Fisher's exact tests were used when appropriate. $P \leqslant 0.05$ was considered significant, and a $\geqslant 0.03$ (absolute value) difference in the HRQoL scores was considered clinically important[26].

RESULTS

Characteristics of the study population

The demographic and clinical characteristics of the MARS-treated and control ALF patients did not differ significantly, with the exception of a need for vasoactive infusion (Table 3). The grade of encephalopathy before treatment was also comparable between the two groups. The etiological distribution of ALF patients differed between the groups ($P = 0.002$). Control patients had mostly ALF of unknown etiology (65%), whereas the majority of MARS-treated patients (57%) had ALF due to known toxicity (e.g. paracetamol or other drugs).

Outcome and 3-year survival

The percentage of transplanted patients was lower (29% *vs* 47%, $P = 0.14$), and the percentage of patients who survived 3 years after treatment was higher (78% *vs* 41%, $P = 0.002$) in the MARS-treated group compared to controls.

Table 4 The survival, cost, and HRQoL data of MARS-treated and control patients

	Subgroups according to outcome	3-yr survival			Cost (€)				HRQoL		
		n	%	Mean (d)	*n*	Mean	Min	Max	*n*	Pre-treatment mean	Post-treatment mean
MARS group	All ALF patients	90	78		31	79745	11961	370573	90	0.30	0.70
	Alive at 3 yr	70			24	78724	11961	370573	70	0.34	0.89
	Dead	20		63	7	83243	32496	171157	20	0.15	0.00
	Contraindication to Ltx	18	39								
	Alive at 3 yr	7			4	52492	30325	112585	7	0.38	0.85
	Dead	11		14	5	64414	32496	95666	11	0.13	0.00
	No contraindication - no Ltx	46	85								
	Alive at 3 yr	39			15	41957	11961	137235	39	0.40	0.93
	Dead	7		110	1	89471			7	0.09	0.00
	Transplanted	26	92								
	Alive at 3 yr	24			5	210012	96984	370573	24	0.22	0.84
	Dead	2		169	1	171157			2	0.47	0.00
Control group	All ALF patients	17	41		16	105820	16862	262481	17	0.27	0.36
	Contraindication to Ltx		0								
	Dead	7		9	7	45089	17591	105917	7	0.27	0.00
	No contraindication - no Ltx		100								
	Alive at 3 yr	2			2	77162	16862	137462	2	0.27	0.85
	Transplanted		63								
	Alive at 3 yr	5			4	170578	117444	262481	5	0.32	0.87
	Dead	3		28	3	180285	129120	250772	3	0.19	0.00

HRQoL: Health-related quality of life; ALF: Acute liver failure.

Table 5 Base case results for MARS

	Cost (€)	Incremental cost (€)	QALYs	Incremental QALYs	Cost per QALY (€)	Incremental cost per QALY
MARS	93214		1.44	0.66	64732	MARS dominates SMT
Control group (SMT only)	104142	10928	0.78		133858	

SMT: Standard medical therapy; QALY: Quality-adjusted life year.

A similar survival benefit favoring MARS treatment compared to controls was noted in both transplanted (92% *vs* 63%, $P = 0.072$) and non-transplanted patients (72% *vs* 22%, $P = 0.006$).

Costs

The total mean direct medical costs related to liver disease were 79745€ for the MARS group and 105820€ for the controls (Table 4). The total mean direct medical costs for transplanted and non-transplanted MARS and control patients are shown in Figure 3 and the expected costs weighted by the pathway probabilities are shown in Table 5.

In all patient groups, most of the costs were incurred within a year of the first MARS or standard medical treatment. The costs during the second and third years were negligible. In all groups, the highest costs were observed in transplanted patients.

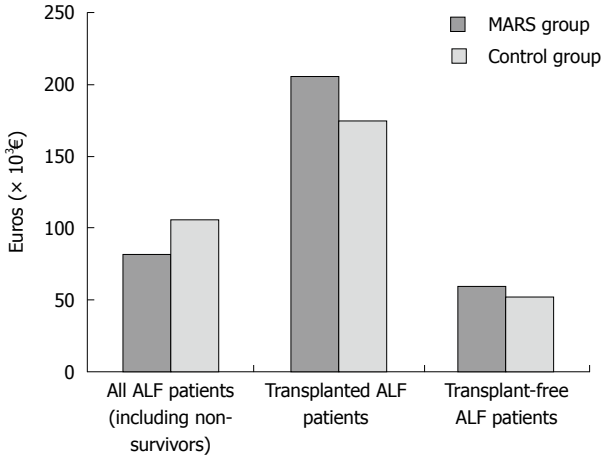


Figure 3 The 3.5-year mean overall direct medical costs per patient in the MARS and control groups. ALF: Acute liver failure.

HRQoL and QALYs

The pre- and post-treatment 15D scores are shown in Figure 4 and Table 4. The estimated HRQoL for all patients prior to treatment was very low compared to an age-standardized reference population in Finland (0.30 *vs* 0.92)[27]. The highest post-treatment 15D scores were observed in MARS-treated patients who did not have a contraindication to Ltx and recovered without transplantation. The same patients also experienced the highest mean number of QALYs (2.00, within the 3-year follow-up). The groups with the smallest mean number of QALYs were patients with a contraindication to Ltx and those who died within 3 years of treatment.

Cost-utility analysis

In the base-case analysis, the MARS group strongly domi-

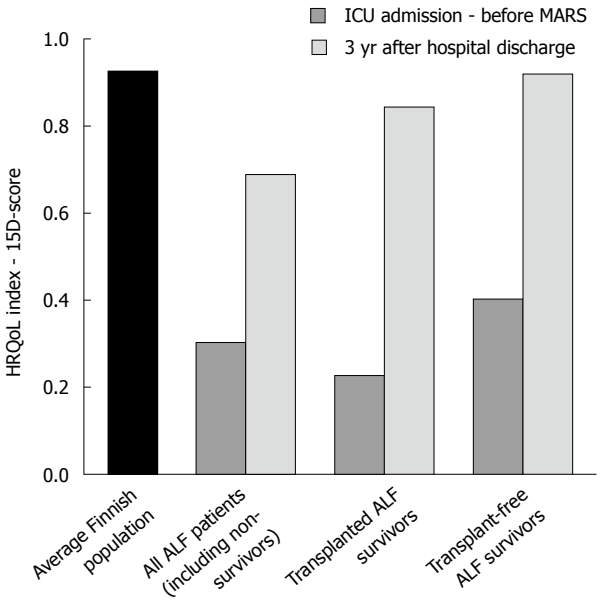


Figure 4 The 15D-score before and 3 years after MARS treatment. HRQoL: Health-related quality of life.

nated the control group receiving only SMT; MARS treatment was both less costly and more effective than SMT in ALF patients (Table 5). Using the 3-year time horizon, the expected outcome of MARS patients was 1.44 QALYs and the expected costs were 93 214€. The corresponding figures for the controls were 0.78 and 104 142€, respectively. Compared to MARS, the incremental cost of SMT in the control group was 10 928€. The incremental number of QALYs gained by MARS was 0.66.

Sensitivity analysis

MARS remained the dominant strategy throughout most of the one-way sensitivity analyses. Neither increasing the proportion of patients with a contraindication to Ltx in the MARS group nor varying the survival rates of patients after Ltx or MARS-treated patients with a contraindication, mitigated the dominance of MARS treatment. MARS also remained the dominant strategy throughout QALY estimate variation in the one-way sensitivity analysis. Using a discount rate of 0%, 3%, or 5% did not eliminate the dominance.

The results were more sensitive to variation in the cost estimates. In the one-way sensitivity analysis, increasing the costs for MARS-treated patients who had no contraindication and survived three years with Ltx removed the dominance of MARS and resulted in an ICER of 63 206€ per QALY gained.

The proportion of liver disease with unknown etiology was significantly higher in the control group (P = 0.002), which can lead to a higher probability of Ltx. We varied the probability of Ltx in the MARS group from 0.89 (observed in controls) to 0.36 (observed in the MARS-treated group); throughout which, MARS remained the more effective alternative. However, when the probability of Ltx in the MARS group was increased to 0.42 or higher, the expected cost of MARS exceeded that of the control group receiving SMT, and its dominance was eliminated. When the probability of Ltx in the MARS group was set to the same level as the control group (0.89), the ICER was 98 686€ per QALY gained.

The cost-effectiveness acceptability curve (Figure 2) of MARS *vs* controls showed the probability that MARS is cost-effective given a range of willingness-to-pay thresholds per QALY gained. If the decision-maker is willing to pay 50 000€ per QALY, the probability of MARS being cost-effective is 78%. Furthermore, the probability of MARS being cost-effective is 95% if the willingness-to-pay threshold is 200 000€ per QALY.

DISCUSSION

To our knowledge, there are no previous studies evaluating the cost-utility of MARS treatment in ALF patients. We found that MARS treatment was both less costly and more effective than SMT in ALF. Compared to the controls, the average cost per QALY was significantly lower in the MARS group (64 732€ *vs* 133 858€), mainly due to the significantly higher 3-year overall survival rate and fewer transplantations in the MARS group.

The average overall 3.5-year costs associated with the treatment of ALF were substantial with or without MARS treatment due to the long ICU stay and, in some cases, Ltx costs. The fewer Ltx in the MARS group resulted in reduced overall average costs compared to conservative treatment. Because the costs associated with the Ltx procedure are high, any intervention that decreases the number of transplantations is bound to have a profound effect on the total expense.

As reported previously[28,29], we found that, even though the HRQoL of surviving transplanted patients was generally very good, it was still somewhat lower than that of a person in the age-standardized general population (0.84 *vs* 0.92)[27]. In ALF patients who recovered without Ltx, the HRQoL after treatment was similar to that of the age-standardized Finnish reference population (0.93 *vs* 0.92)[27].

Only a handful of HRQoL and cost-effectiveness studies have been completed on MARS patients, and all have been limited to AOCLF patients treated in the same center[14-16]. Until now, MARS-treated ALF patients have not been evaluated in terms of total cost and QALYs, possibly owing to the rarity of the condition, which makes it difficult to enroll enough patients for statistical analysis. Furthermore, comparing results from different studies can be difficult because transplant organ availability varies, and centers may have different criteria for MARS treatment. In addition, the heterogeneity of the etiology of ALF in different countries markedly affects survival rates and the percentage of patients who may experience native liver recovery[30,31].

The most recent cost-utility evaluation of MARS treatment included 79 alcohol-related AOCLF patients[14]; their 3-year survival was 52% with mean direct medical costs of 40 032€ and an ICER of 31 448€ per life-year gained. However, transplanted patients and those with serious co-

morbidities were excluded from this analysis, which had a huge impact on the total costs. Another study focused on the impact of MARS treatment on the hospitalization costs and 1-year survival of cirrhotic AOCLF patients[32]; the in-hospital cost for surviving MARS-treated patients was $32 036, $4000 less than in the control group.

The main limitation of this study was the nonrandomized design. Also, due to patient incapacitation, the pretreatment HRQoL was retrospectively assessed by an expert panel rather than by the patients themselves. Studies have shown that the subjective feelings of a patient, such as pain or HRQoL, are usually misjudged or underestimated by the attending doctors, other treatment staff, or even close relatives[33,34]. Another factor affecting the positive outcomes of MARS-treated ALF patients in this study was the uneven distribution of liver failure etiologies between treatment groups. The prognosis and need for Ltx is strongly related to the underlying cause of ALF[30]; the MARS treatment group was at an advantage in that it included many patients with a good prognosis compared to controls. This predisposition must be taken into account when interpreting the results of this study. The sensitivity analysis was used to estimate the effect of this bias, and it seems that even if both the MARS and control groups had a similar distribution of etiologies, MARS treatment would remain the more effective strategy, although more expensive in terms of total cost.

One must bear in mind that this study dealt with a 3.5-year time window, thus severely underestimating the QALYs to be gained over the remaining lifetime. Alternatively, we could have extrapolated the expected lifetime costs and outcomes; however, this would have necessitated further assumptions and resulted in greater uncertainty.

In conclusion, cost-utility analysis found that MARS treatment plus SMT is both less costly and more effective than SMT alone in ALF patients. Although some uncertainty exists in the model input parameters, probabilistic sensitivity analysis showed that the probability of MARS being cost-effective is higher than that of SMT alone.

COMMENTS

Background

The molecular adsorbent recirculating system (MARS) device is an extracorporeal albumin dialysis apparatus which can be used to treat liver failure patients. MARS removes both albumin-bound and water-soluble toxins from the patient's blood, enabling native liver regeneration and allowing time to locate a suitable organ for liver transplantation. Numerous studies have documented the favorable effects of MARS treatment on clinical and laboratory parameters and survival. However, only a few small non-randomized studies have focused on the cost-utility of MARS treatment in patients with acute-on-chronic liver failure.

Research frontiers

The cost-utility of the MARS treatment and its impact on the health-related quality of life in acute liver failure patients has never been investigated previously.

Innovations and breakthroughs

The results from the current study suggest that MARS treatment is both less costly and more effective than standard medical therapy in the treatment of acute liver failure patients. Compared to the controls, the average cost per quality-adjusted life year (QALY) was significantly lower in the MARS group, mainly due to the significantly higher 3-year overall survival rate and fewer transplantations in the MARS group. The authors also found that in MARS-treated acute liver failure patients who recovered without liver transplantation, the health-related quality of life after treatment was similar to that of the age-standardized Finnish reference population.

Applications

The cost-utility analysis suggests that MARS treatment plus standard medical therapy is both less costly and more effective than standard medical therapy alone in acute liver failure patients. Therefore, the additional costs which are associated with the use of the MARS machine seem justifiable in this patient group.

Peer review

This paper deals with the important topic of the cost-utility of molecular adsorbent recirculating system treatment in acute liver failure. The report is concise and informative.

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ORIGINAL ARTICLE

Apoptotic activity of caged xanthones from *Garcinia hanburyi* in cholangiocarcinoma cell lines

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Abstract

AIM: To investigate the growth inhibitory mechanism of four caged xanthones from *Garcinia hanburyi* in cholangiocarcinoma (CCA) KKU-100 and KKU-M156 cells.

METHODS: Four caged xanthones, selected on the basis of their anticancer potency and chemical structure diversities (i.e. isomorellin, isomorellinol, forbesione and gambogic acid) were used in this study. Growth inhibition of these caged xanthones was determined using the sulforhodamine B assay. Induction of apoptosis was assessed by observing cell morphology, ethidium bromide and acridine orange staining and DNA fragmentation assay. Levels of apoptotic-related gene and protein expressions were determined by a real-time reverse transcriptase polymerase chain reaction and Western blotting analysis, respectively.

RESULTS: The compounds were found to inhibit growth of both cell lines in a dose-dependent manner and also showed selective cytotoxicity against the cancer cells when compared with normal peripheral blood mononuclear cells. Growth suppression by these compounds was due to apoptosis, as evidenced by the cell morphological changes, chromatin condensation, nuclear fragmentation, and DNA ladder formation. At the molecular level, these compounds induced down-regulation of Bcl-2 and survivin proteins with up-regulation of Bax and apoptosis-inducing factor proteins, leading to the activation of caspase-9 and -3 and DNA fragmentation. The functional group variations did not appear to affect the anticancer activity with regard to the two CCA cell lines; however, at a mechanistic level, isomorellinol exhibited the highest potency in increasing the Bax/Bcl-2 protein expression ratio (120 and 41.4 for KKU-100 and KKU-M156, respectively) and in decreasing survivin protein expression (0.01 fold as compared to control cells in both cell lines). Other activities at the molecular level indicate that functional groups on the prenyl side chain may be important.

CONCLUSION: Our findings for the first time demonstrate that four caged xanthones induce apoptosis in

CCA cells which is mediated through a mitochondria-dependent signaling pathway.

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Key words: *Garcinia hanburyi*; Caged xanthones; Human cholangiocarcinoma cell lines; Apoptosis

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from bile duct epithelial cells[1], characterized by a poor prognosis and unresponsive to conventional chemotherapeutic agents. The increasing global incidence of this tumor underscores the need for novel and effective therapeutic agents for CCA[2,3].

Much attention has been focused on natural products as potential sources of novel anticancer drugs over the decades[4]. An emerging class of compounds, being potent antiproliferatives as well as having anticancer and anti-tumor activities against mammalian cancer cell lines, is caged xanthones. Recent work by our and other research groups has identified a tropical plant, *Garcinia hanburyi* (*G. hanburyi*) Hook.f. (family Guttiferae), used as a purgative and externally for infected wounds in Thai traditional medicine[5], as a rich source of these compounds[6-11]. In addition, gambogic acid, derived from the gamboges resin of *G. hanburyi,* has been shown to induce apoptosis in human gastric carcinoma cells[9]. These data suggest that these caged xanthones might be effective as chemotherapeutic agents and that they warrant further study of the underlying mechanisms of their anticancer activities.

Our interest in searching for agents for the effective treatment of CCA led us to test a series of caged xanthones from *G. hanburyi*[10] against two human CCA cell lines. Among the compounds tested, isomorellin, isomorellinol, forbesione and gambogic acid (Figure 1) exhibit promising IC_{50} values against both cell lines in comparison with the standard drug doxorubicin. In view of their anticancer potency and chemical structure diversity, these four caged xanthones were selected for a more in-depth study. In order to further delineate the underlying mechanisms, the apoptotic action of these compounds was investigated in detail. The results are the main focus of this communication.

MATERIALS AND METHODS

Reagents and cell culture

Four caged xanthones, i.e. isomorellin, isomorellinol, forbesione and gambogic acid, were isolated from *G. hanburyi* Hook.f. (family Guttiferae) using bioassay-directed fractionation[10]. The KKU-100 and KKU-M156 cells were isolated from Thai CCA patients and the original characterization of these cell lines has been described previously[12,13]. Human peripheral blood mononuclear cells (PBMCs) were freshly isolated using the standard Ficoll-hypaque gradient centrifugation method and used as normal control cells[14]. Cells were grown in RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL streptomycin (GIBCO BRL) at 37℃ in a humidified incubator containing 5% CO_2.

Cell proliferation assay

For the cell proliferation assay, 1.9×10^4 cells/well were seeded in 96-well microtitre plates and incubated for 24 h. After treatment for 72 h with 0-8.8 × 10^4 μmol/L per well for the caged xanthones, 0.04, 0.4, 4, 40 and 400 μmol/L for doxorubicin (Boryung Pharmaceutic Co. LTD, Korea) as a reference compound and DMSO as the solvent-control cells, cell growth was measured using the sulforhodamine B (SRB) assay[15].

Morphological examination

KKU-100 and KKU-M156 cells (1×10^6) were grown in a 25 cm^2 flask at 37℃ for 24 h, and treated with 2 × IC_{50} concentration of each caged xanthone for 48 h. Morphological changes occurring in the cells were observed under bright field inverted Nikon microscope. For nuclear staining, cells (1.9×10^3 cells/well) were grown in 96-well microtitre plates at 37℃ for 24 h, and treated with 2 × IC_{50} concentration of each caged xanthone for 24, 36 and 48 h. The treated cells were stained with 14 μL of 100 μg/mL ethidium bromide/acridine orange (EB/AO) mixture (Sigma Chemical, St. Louis, MO) and observed under a Nikon fluorescent microscope. Apoptotic cells with condensed chromatin or fragmented chromatin were counted and expressed as a percentage from a total of 500 cells each[16].

DNA fragmentation assay

The isolation of fragmented DNA was carried out according to the procedure of Herrmann *et al*[17] with some modifications. Briefly, after culturing for 24 h and starving in medium containing 0.5% FBS for 24 h, cells (1×10^6) were treated with DMSO or 2 × IC_{50} concentrations of the caged xanthones for 24 and 36 h. After extraction, DNA in cell lysate was purified by QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The DNA fragments were precipitated with ethanol, re-suspended in 50 μL of TE buffer, and analyzed by electrophoresis.

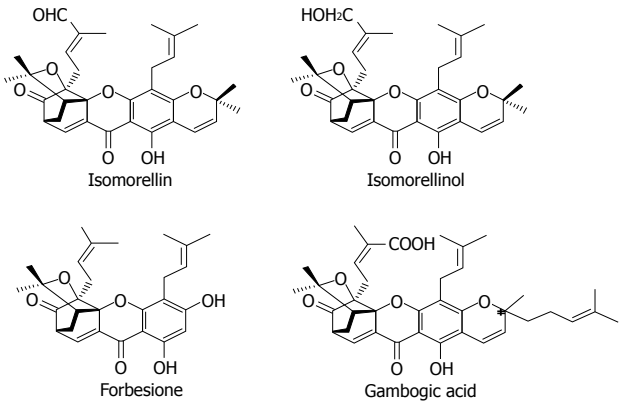


Figure 1 Chemical structure of four caged xanthones.

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction

Cells were treated with 2 × IC_{50} concentrations of the caged xanthones for 0, 6, 12, 24 and 48 h. Total RNA was isolated from the treated and control cells using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The reverse transcription reaction was performed as described[18].

Real-time polymerase chain reaction (PCR) was performed according to the procedure of Namwat *et al*[18]. Real-time PCR of Bcl-2, Bax, survivin and GAPDH were performed in a 20 μL PCR reaction mixture containing first strand cDNA, 5 pmol of each primer, and 10 μL of 2 × SYBR Green PCR Master Mix (Gene Systems Co., Ltd, USA), employing an ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). The PCR primers (Bioservice Unit, Bangkok, Thailand) are shown in Table 1. The sequences of these PCR products were obtained by direct sequencing. Each amplicon was then cloned into the pGEM®-T vector (Promega, Madison, WI, USA) in order to generate standard curves for target cDNA. The mRNA level is reported as the ratios of the copy numbers of target cDNA to GAPDH cDNA. The ratio of fold change in gene expression level between the treated and control cells was determined to identify the up- or down-regulation of gene expression.

Protein extraction and Western blotting analysis

After treatment with DMSO or 2 × IC_{50} concentrations of the caged xanthones for 0, 12, 24 and 48 h, protein extraction and Western blotting were performed as described previously[19]. After blocking, the membranes were incubated at 4℃ overnight with the following antibodies: Bcl-2, Bax, survivin, apoptosis-inducing factor (AIF) (Santa Cruz Biotechnology, CA) or procaspase-9 and -3, activated caspase-9 (Cell Signaling, Beverly, MA) or activated caspase-3, β-actin (Sigma Chemical, St. Louis, MO). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology). Protein bands were detected by an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford). The intensity of the protein bands was quantified using Scion Image software.

Statistical analysis

Data were expressed as mean ± SE. Comparisons between untreated control cells and the caged xanthone-treated cells were made using Student's *t* test. Differences were considered significant at $P < 0.05$. All analyses were performed using SPSS version 10.0 (SPSS Inc., USA).

RESULTS

Anti-proliferative effects of four caged xanthones on CCA cell lines

The treatment of KKU-100 and KKU-M156 cells with four caged xanthones markedly inhibited cell growth in a dose-dependent manner (Figure 2). The IC_{50} values of these caged xanthones and doxorubicin are shown in Table 2. The anticancer potencies of these compounds are comparable to doxorubicin in both cell lines. These caged xanthones induced growth inhibition in the CCA cell lines about 4 to > 8 × 10^6 times greater than that for normal PBMCs (Table 2). These results indicate that these compounds possess considerable potential for further development.

Apoptosis induction by four caged xanthones in CCA cell lines

In both cell lines, treatment with four caged xanthones resulted in cell shrinkage, rounding and membrane blebbing, thus taking on the typical characteristics of apoptotic cells (Figure 3C and D); whereas no effect was observed with the vehicle control cells (Figure 3A and B). Using EB/AO staining, both cell lines showed nuclei with homogeneous chromatin distribution under vehicle-control conditions (Figure 3E and F). In the treated cells, characteristic apoptotic features such as chromatin condensation and nuclear fragmentation were observed (Figure 3G and H). The number of apoptotic cells in both cell lines treated with four caged xanthones was significantly increased in a time-dependent manner (Table 3). Using an agarose gel electrophoresis, a typical ladder pattern of internucleosomal DNA fragmentation, a hallmark of apoptotic cell death[20], was observed in the treated cells (Figure 3I and J).

Effect of four caged xanthones on the expression of apoptosis-related genes and proteins

In both CCA cell lines treated with isomorellinol there was a significant increase in both mRNA (Figure 4A) and protein (Figure 5) of pro-apoptotic Bax, but a marked decrease in the anti-apoptotic Bcl-2 (Figure 4A and Figure 5) in a time-dependent manner leading to an enhanced Bax/Bcl-2 ratio (Figure 4B and Table 4). The same results were obtained when both CCA cell lines were treated with the other three compounds (Figure 4B and Table 4). Using Western blotting, isomorellinol was found to have the highest potency in increasing Bax/Bcl-2 ratio. At 48 h of treatment, isomorellinol increased the Bax/Bcl-2 ratio up to 120 and 41.4 in the KKU-100 and KKU-M156 cells, respectively (Table 4).

Western blotting analysis revealed that treatment of

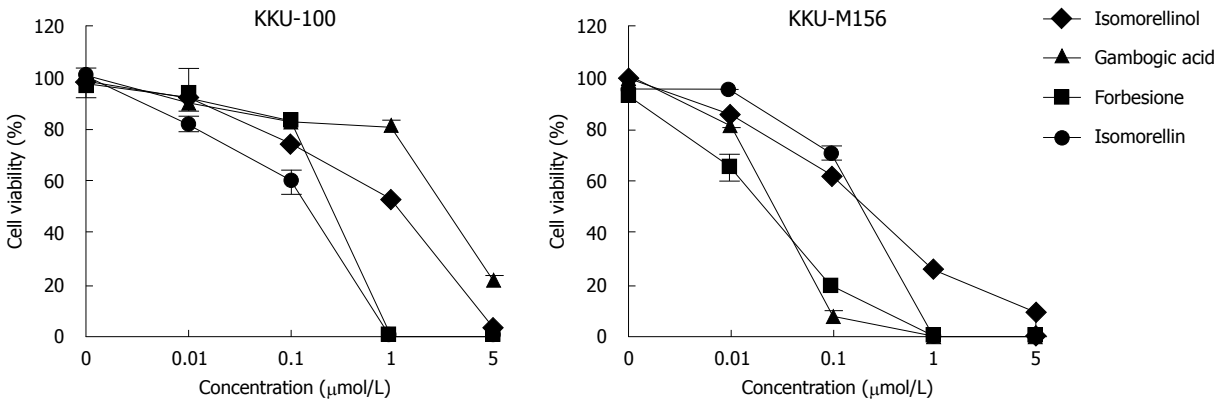


Figure 2 Antiproliferative activities of the four caged xanthones against KKU-100 and KKU-M156 cells. Cells were treated with DMSO or indicated amounts of isomorellinol, forbesione, gambogic acid and isomorellin for 72 h. The percent cell viability was determined by SRB assay. Data are expressed as mean ± SE (n = 3).

Table 1 Primer sequences used for target gene amplification

GenBank accession No.	Gene	Forward primer	Reverse primer
M14745.1	*Bcl-2*	TGGATGACTGAGTACCTGA	TGAGCAGAGTCTTCAGAGA
L22473.1	*Bax*	AACCATCATGGGCTGGA	CGCCACAAAGATGGTCA
AF077350.1	*Survivin*	AAGGCTGGGAGCCAGA	TGGCTCTTTCTCTGTCCA
BC083511	*GAPDH*	TCATCAGCAATGCCTCCTGCA	TGGGTGGCAGTGATGGCA

Table 2 Concentrations of four caged xanthones and doxorubicin causing a 50% inhibitory effect (IC_{50}) in cell proliferation

Cells	IC_{50} values (μmol/L)				
	Isomorellin	Isomorellinol	Forbesione	Gambogic acid	Doxorubicin
KKU-100	0.11 ± 0.004	2.2 ± 0.33	0.15 ± 0.007	2.64 ± 1.29	0.66 ± 0.001
KKU-M156	0.12 ± 0.005	0.43 ± 0.06	0.02 ± 0.002	0.03 ± 0.004	0.36 ± 0.001
PBMC	> 88 × 10^4	59 ± 2.9	59.5 ± 1.0	11 ± 3	ND

Data are mean ± SE of three independent experiments. ND: Not detected.

both cell lines with isomorellinol resulted in a significant reduction of procaspase-3 and -9 while increasing activated caspase-3 and -9 in a time-dependent manner (Figure 5). The treatment of both CCA cell lines with the other three compounds also showed the same results (data not shown). When the relative intensities of activated caspase-3 and -9 to β-actin of treated cells were compared to control cells (0 h), there was a multi-fold increase in the activated caspase-3 and -9 (Table 5). The high degree of multi-fold increase in activated caspase-9 was detected in the isomorellinol-treated KKU-100 and KKU-M156 cells (56 × and 58 ×, respectively), in gambogic acid-treated KKU-100 cells (78 ×) and in isomorellin-treated KKU-M156 cells (34 ×) (Table 5). All four compounds were also shown to increase the activated caspase-3 level in treated KKU-M156 cells, from 10 × to 46 × compared with the control cells (0 h), with a high degree of activated caspase-3 for isomorellinol-treated (33 ×) and gambogic acid-treated KKU-M156 cells (46.7 ×) (Table 5).

The real-time PCR and Western blotting analysis exhibited a significant time-dependent decrease in *survivin* gene (Figure 4A) and protein (Figure 5) expression in the isomorellinol-treated KKU-100 and KKU-M156 cell lines. The same results were obtained when these cell lines were treated with the three other compounds (data not shown). The multi-fold decreases in *survivin* gene and protein expression are shown in Figure 4C and Table 5, respectively. Using Western blotting, isomorellinol showed the highest potency in suppression of survivin protein expression (0.01 ×) (Table 5).

Western blotting analysis revealed that cells treated with isomorellinol resulted in a significant increase of AIF protein (Figure 5). Similar results were obtained for both CCA cell lines treated with the other three compounds (data not shown). Similar multi-fold increases in AIF protein expression were found for the four compound-treated cells as compared to controls (Table 5).

DISCUSSION

The present study demonstrated that the four caged xanthones; isomorellinol, forbesione, gambogic acid and isomorellin, exerted strong growth inhibition with different IC_{50} values in both CCA cell lines in a dose-dependent manner (Table 2 and Figure 2). The results indicate that these two CCA cell lines responded differently to different compounds. However, the potencies of these four compounds were very high, indicating their broad spec-

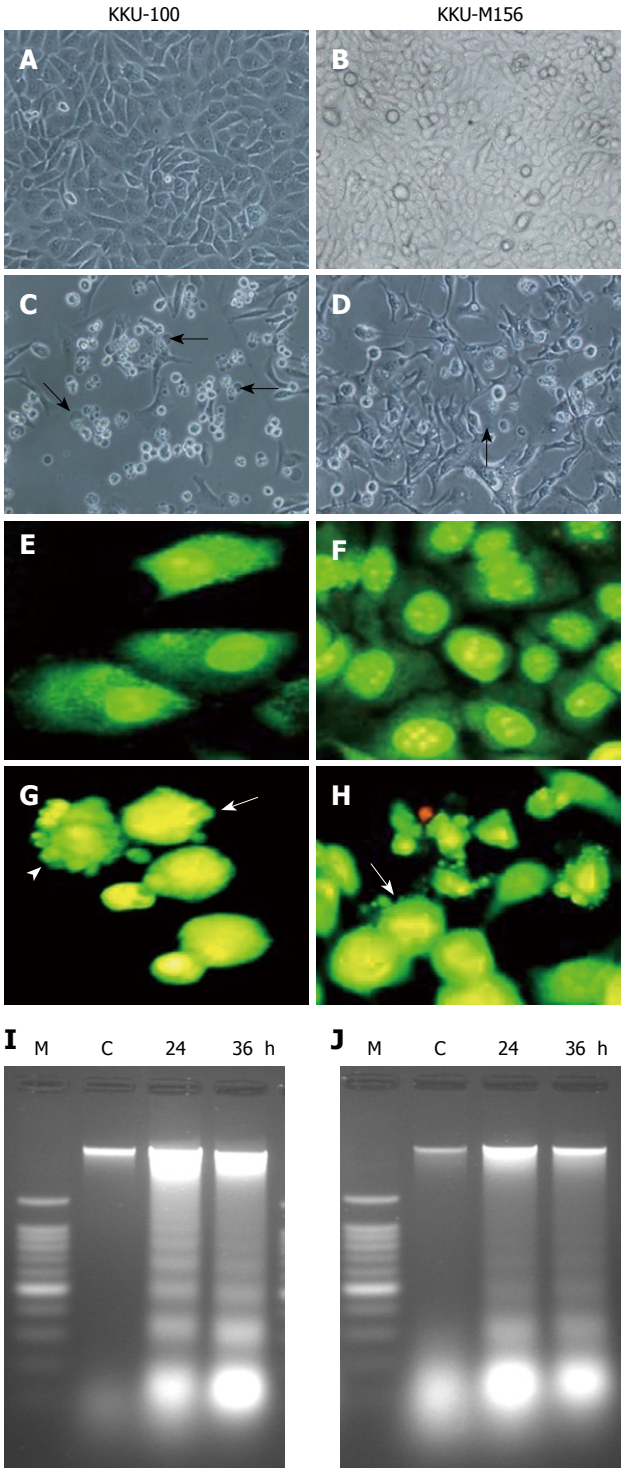


Figure 3 Induction of apoptosis in cholangiocarcinoma (CCA) cell lines by four caged xanthones. Photomicrographs (400 ×) of CCA cell lines KKU-100 (A, C) and KKU-M156 (B, D) treated with DMSO and gambogic acid for 48 h. A, B: Treated with DMSO; C, D: Treated with gambogic acid. Cells with membrane blebbing indicated by arrows; Fluorescence photomicrographs (400 ×) of CCA cell lines KKU-100 (E, G) and KKU-M156 (F, H) treated with DMSO and isomorellinol for 36 h followed by EB/AO staining. E, F: Treated with DMSO; G, H: Treated with isomorellinol. Nuclei of treated cells showing chromatin condensation (arrows) and nuclear fragmentation (arrow heads); DNA fragmentation of CCA cell lines treated with forbesione for the indicated times (24 h and 36 h). Genomic DNA was isolated and separated on 1.6% agarose gels containing 0.1 mg/mL ethidium bromide. I: KKU-100 cells; J: KKU-M156 cells. Lane C: DNA band of CCA cell lines treated with DMSO; Lane M: DNA markers. The figures show representative results of three independent experiments.

Table 3 Percentage of apoptotic cells in the four caged xanthone-treated and non treated CCA cell lines

Compounds	% apoptotic cells			
	KKU-100 (h)		KKU-M156 (h)	
	36	48	24	36
Control	8 ± 1.45	13 ± 0.66	8 ± 0.88	23 ± 0.66
Isomorellinol	44 ± 2.31[b]	79 ± 1.76[b]	34 ± 1.50[b]	89 ± 1.20[b]
Forbesione	34 ± 0.67[b]	78 ± 1.15[b]	38 ± 3.17[b]	83 ± 3.05[b]
Gambogic acid	32 ± 0.87[b]	70 ± 1.45[b]	17 ± 1.50[a]	72 ± 0.66[b]
Isomorellin	25 ± 1.21[b]	69 ± 1.76[b]	31 ± 0.58[b]	77 ± 4.16[b]

Data are mean ± SE of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control. CCA: Cholangiocarcinoma.

Table 4 Bax/Bcl-2 ratios of the four caged xanthone-treated and non treated CCA cell lines

Compounds	Bax/Bcl-2 ratio							
	KKU-100 (h)				KKU-M156 (h)			
	0	12	24	48	0	12	24	48
Isomorellinol	0.98	1.58	1.98	120	0.73	1.04	1.43	41.40
Forbesione	1.02	1.93	2.35	2.91	1.53	2.38	5.12	7.02
Gambogic acid	0.81	1.51	3.42	7.00	0.43	0.72	1.83	3.14
Isomorellin	0.98	1.98	3.12	5.20	0.56	0.70	1.00	1.90

Data are expressed as ratio of mean of protein expression level of Bax to Bcl-2 in control and the caged xanthone-treated CCA cell lines.

trum of anti-CCA effects. In addition, the selectivity of these compounds toward cancer cell lines compared to normal PBMCs was demonstrated (Table 2). Previously, these four caged xanthones have been shown to inhibit the growth of various mammalian cancer cell lines[10]. Gambogic acid was reported to be an effective telomerase inhibitor, displaying potent anticancer activity both *in vitro* and *in vivo*[8-11], and was shown to kill cancer cells selectively with no influence on normal cells[9]. The anticancer potencies of these caged xanthones were comparable to doxorubicin. Due to their potencies, the anticancer mechanisms of these four compounds deserve further investigation.

The growth inhibitory effect of these caged xanthones in both CCA cell lines was modulated through apoptosis which was evidenced by the typical morphological characteristics of apoptosis, such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and DNA ladder formation (Figure 3). Cleavage of DNA at the inter-nucleosomal linker sites yielding DNA fragments has been used as a hallmark of apoptosis[20]. Consistent with our study, the apoptotic effects of gambogic acid in human breast tumor cells T47D[21] and human gastric carcinoma cells MGC-803[9] have been reported.

Apoptosis is regulated by several different genes in response to various stimuli. The Bcl-2 family of proteins plays major roles in apoptotic regulation through the mitochondrial pathway[22]. Our results show that these caged xanthones down-regulated the expression of the Bcl-2

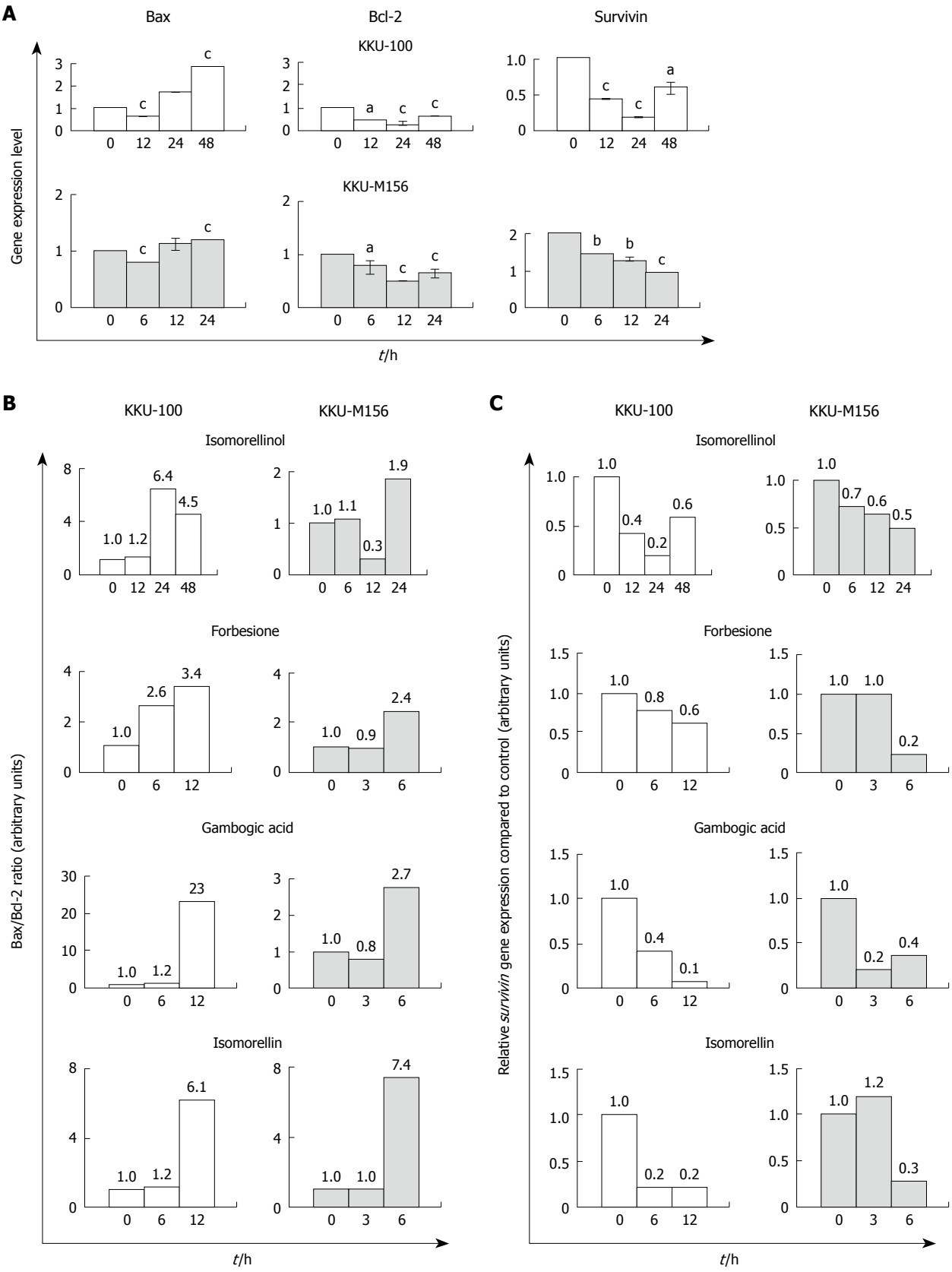


Figure 4 The four caged xanthones induce up-regulation of Bax with down-regulation of *Bcl-2* and *survivin* gene expressions in CCA cell lines. KKU-100 and KKU-M156 cells were treated with DMSO or the four caged xanthones for the indicated times. Real-time polymerase chain reaction for each gene was performed as described in Materials and Methods. Data were expressed as mean ± SE fold increase/decrease in mRNA expression compared to control, normalized with GAPDH (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control. All four compounds showed the same result. A: The histograms of the isomorellinol-treated cells were used as a representative; B: Bax/Bcl-2 ratio; C: Relative *survivin* gene expression compared to control.

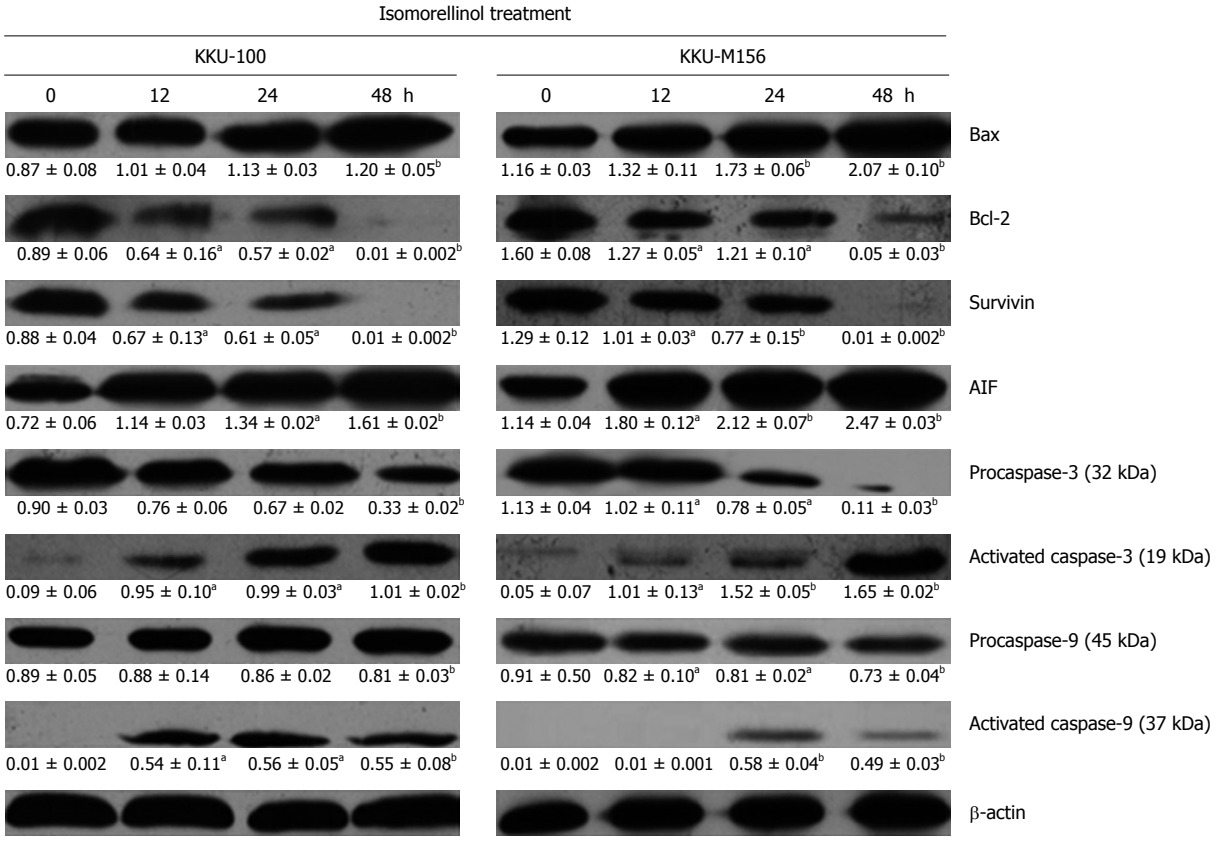


Figure 5 The four caged xanthones induce increase of Bax, AIF, activated caspase-3 and -9 while decreasing Bcl-2, survivin, procaspase-3 and -9 protein levels in CCA cell lines. KKU-100 and KKU-M156 cell lines were treated with DMSO or the four caged xanthones for the indicated times. Western blottings for each protein were performed as described in Materials and Methods. The mean ± SE of density reading of target protein normalized to β-actin was expressed in the respective box (n = 3). $^aP < 0.05$, $^bP < 0.01$ *vs* control. All four compounds showed the same result. The protein bands of the isomorellinol-treated cells were used as a representative.

Table 5 Fold decrease or increase of apoptotic-related proteins of the four caged xanthone-treated compared to non treated CCA cell lines

Apoptotic proteins	Compounds	Fold change in protein of the treated cells compared to control cells							
		KKU-100 (h)				KKU-M156 (h)			
		0	12	24	48	0	12	24	48
Activated caspase-9	Isomorellinol	1	54	56	55	1	1	58	49
	Forbesione	1	9.6	13.1	13	1	5.7	8	5.6
	Gambogic acid	1	30	78	60	1	2.6	4.5	3.9
	Isomorellin	1	6.3	12.3	9.3	1	1	29	34
Activated caspase-3	Isomorellinol	1	10.6	11	11.2	1	20.2	30.4	33
	Forbesione	1	6	9.6	12	1	5.7	9.6	10
	Gambogic acid	1	5.5	7.5	12.2	1	28	39.3	46.7
	Isomorellin	1	8.9	10	13	1	5	5.7	12
Survivin	Isomorellinol	1	0.8	0.7	0.01	1	0.8	0.6	0.01
	Forbesione	1	0.5	0.54	0.3	1	0.7	0.54	0.5
	Gambogic acid	1	0.6	0.4	0.3	1	0.7	0.6	0.4
	Isomorellin	1	0.6	0.5	0.3	1	0.8	0.5	0.3
AIF	Isomorellinol	1	1.6	1.9	2.3	1	1.6	1.9	2.2
	Forbesione	1	1.2	1.6	2.1	1	1.6	2.8	4
	Gambogic acid	1	1.5	1.6	2.7	1	1.6	2.1	3.4
	Isomorellin	1	1.9	3.3	3.5	1	1.3	1.7	2.5

Data are expressed as ratio of mean of protein expression level of the apoptotic-related proteins in the caged xanthone-treated to non treated CCA cell lines.

gene and protein while up-regulating the expression of Bax gene and protein, leading to an increase in the Bax/Bcl-2 ratio (Figure 4B and Table 4). In agreement with our study, gambogic acid-induced apoptosis by regulation of the expression of Bax and Bcl-2 proteins in the human gastric carcinoma cell line MGC-803 has been reported[9]. Bax protein has previously been reported to induce the intrinsic apoptosis pathway by inhibiting the function of

Bcl-2[23], and by increasing the release of death-mediated proteins such as cytochrome C, Smac/DIABLO and AIF from the inter-membrane space to the cytosol[24,25]. Our results show a significant increase in the level of AIF protein in response to the four caged xanthone treatments (Figure 5 and Table 5). This result suggests that the AIF, caspase-independent mitochondrial pathway[25] may be involved in apoptosis induction in the caged xanthone-treated cells.

In the intrinsic apoptotic pathway, the release of cytochrome C from mitochondria triggers caspase-9 and -3 activation through formation of the apoptosome complex. Then the activated caspase-3 is able to cleave multiple cellular substrates leading to DNA fragmentation[24]. Our results show that caspase-9 and -3 were activated by the caged xanthones (Figure 5 and Table 5), suggesting that these caged xanthones induced apoptosis in both CCA cell lines through caspase-9 and -3 activation. Previously, mitochondrial destabilization and caspase-3 activation were reportedly involved in the apoptosis of Jurkat cells induced by gaudichaudione A, a cytotoxic xanthone[26]. Gambogic acid reportedly induced apoptosis and activated caspases in T47D cells[21].

The family of human inhibitor of apoptosis proteins (IAP), including XIAP, c-IAP1, c-IAP2, NAIP and survivin, have been reported to be direct inhibitors of activated caspase-3 and -7[27,28]. Our data show that *survivin* gene and protein expressions were decreased by the four caged xanthones (Figure 4A and C, and Table 5). Since these compounds were found to induce apoptosis in both CCA cell lines by activation of caspase-3, the factor contributing to caspase-3 activation in these treated cells may be a decreased survivin expression. Previously, gambogic acid has been reported to down-regulate IAP, IAP1 and IAP2 in human myeloid leukemia cells[29].

Using Western blotting, isomorellinol was found to have the highest potency in increasing the Bax/Bcl-2 ratio (Table 4) and in decreasing survivin protein expression (Figure 5 and Table 5). This result was also confirmed by the greatly increased amount of activated caspase-3 and -9 in both isomorellinol-treated CCA cell lines (Table 5). Gambogic acid was shown to have lower potency in increasing the Bax/Bcl-2 ratio (Table 4) and decreasing survivin protein expression (Table 5); whereas the increased level of activated caspase-3 was similar to that of isomorellinol (Table 5). These results suggest that the activation of caspase-3 might be stimulated by caspase-12 through endoplasmic reticulum stress-induced apoptosis[30]. Comparing these two CCA cell lines, both isomorellinol and gambogic acid showed higher potency in increasing activated caspase-3 in KKU-M156 cells than KKU-100 cells (Table 5). This may be due to the different histological type of the CCA cell lines. KKU-100 is a poorly differentiated adenocarcinoma which has a higher resistance to chemotherapeutic drugs than KKU-M156, a moderately differentiated adenocarcinoma[13]; whereas forbesione and isomorellin showed the same potency against both CCA cell lines. The latter result may be due to the different functional groups present in these four caged xanthones.

The chemical structure diversity of the four compounds reflects the biological activities. The common feature of all four compounds is the presence of the novel caged structure, which is believed to be a prerequisite for potent anticancer activity in comparison with normal xanthones and chromyl xanthones. The chromene ring is absent in forbesione compared with the other three compounds. Forbesione has only two non-functionalized prenyl side-chains, whereas isomorellin, isomorellinol and gambogic acid are highly prenylated with one of the prenyl methyl groups functionalized as an aldehyde, a primary alcohol and a carboxylic acid functional group, respectively. The functional group variations do not appear to affect the anticancer activity against the two CCA cell lines; however, at a mechanistic level, isomorellinol exhibited the highest potency in increasing the Bax/Bcl-2 ratio and in decreasing survivin protein expression. Other activities at the molecular level indicate that functional groups on the prenyl side chain may be important. It is premature to conclude what the definite structure-activity relationship may be but this preliminary insight provides a basis for further medicinal chemistry studies.

In conclusion, our findings demonstrate for the first time that the four caged xanthones, isolated from *G. hanburyi,* are capable of inhibiting proliferation and inducing apoptosis in CCA cell lines. This is also the first report which shows the molecular mechanism of the growth inhibitory effects of isomorellinol, forbesione and isomorellin. These compounds induce down-regulation of Bcl-2 protein while up-regulating the Bax and AIF proteins, leading to the activation of caspase-9 and -3 and DNA fragmentation. Moreover, the down-regulation of survivin protein thus maintains caspase-3 in an active state and stimulates the molecular cascade of apoptosis. Based on these results, we conclude that these four caged xanthones stimulate both caspase- and non-caspase mitochondrial-dependent apoptosis in KKU-100 and KKU-M156 cells. Furthermore, it appears the mitochondrial apoptosis pathway is induced by the upstream receptor-ligand mediated apoptosis pathway. The precise target of action of these four caged xanthones therefore remains to be elucidated. There is a potential role for these caged xanthones in cancer therapy.

ACKNOWLEDGMENTS

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COMMENTS

Background

Cholangiocarcinoma (CCA) is a malignant tumor, characterized by a poor prognosis and unresponsive to conventional chemotherapeutic agents. Therefore, searching for novel and effective therapeutic agents for CCA is necessary. Several caged xanthones from *Garcinia hanburyi* (*G. hanburyi*) have been reported to be potent antiproliferatives, as well as having anticancer and anti-tumor activities, and induce apoptosis in various cancer cell lines.

Research frontiers

The four caged xanthones from *G. hanburyi*; isomorellin, isomorellinol, forbesione and gambogic acid, exerted strong growth inhibition in both KKU-100 and KKU-M156 cells, indicating their broad spectrum of anti-CCA effects. These compounds were shown to kill cancer cells selectively with no influence on normal peripheral blood mononuclear cells (PBMCs). Growth suppression by these compounds was due to apoptosis which correlated with an increase in Bax/Bcl-2 ratio and apoptosis-inducing factor, activation of caspase-9 and -3, and a decrease in survivin expression. At mechanistic level, isomorellinol exhibited the highest potency.

Innovations and breakthroughs

This is the first report which shows the growth inhibitory effect and molecular mechanisms of the four caged xanthones isolated from *G. hanburyi* in CCA cell lines. The results suggest that these compounds stimulate both a caspase- and non-caspase mitochondrial-dependent signaling pathway in CCA cell lines.

Applications

The chemical structure diversity of the four compounds reflects the biological activities. At the molecular level, isomorellinol exhibited the highest potency, indicating that functional groups on the prenyl side chain may be important. It is premature to conclude what the definite structure-activity relationship may be, but this preliminary insight provides a basis for further medicinal chemistry studies. Based on these results, The authors suggest that these four caged xanthones are compounds with great promise and may serve as a potential source and lead-structure for the development of a drug for the treatment of CCA.

Peer review

The manuscript by Hahnvajanawong *et al* evaluates the mechanism by which various caged xanthones exert their antiproliferative activity on cholangiocarcinoma cell lines. The data presented are convincing and the manuscript well written.

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ORIGINAL ARTICLE

Roux-en-Y gastric bypass promotes expression of PDX-1 and regeneration of β-cells in Goto-Kakizaki rats

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Abstract

AIM: To study the effects of Roux-en-Y gastric bypass (RYGB) on the expression of pancreatic duodenal homeobox-1 (PDX-1) and pancreatic β-cell regeneration/neogenesis, and their possible mechanisms in diabetics.

METHODS: Three groups of randomly selected non-obese diabetic Goto-Kakizaki (GK) rats were subjected to RYGB, sham-RYGB and sham-operation (sham-op) surgery, respectively. The rats were euthanized at post-operative 1, 2, 4 and 12 wk. Their pancreases were resected and analyzed using reverse transcription polymerase chain reaction to detect the mRNA of PDX-1. Anti-PDX-1 immunohistochemical (IHC) staining and Western blotting were used to detect the protein of PDX-1. Double IHC staining of anti-Brdu and -insulin was performed to detect regenerated β-cells. The index of double Brdu and insulin positive cells was calculated.

RESULTS: In comparison with sham-RYGB and sham-op groups, a significant increase in the expressions of PDX-1 mRNA in RYGB group was observed at all experimental time points (1 wk: 0.378 ± 0.013 *vs* 0.120 ± 0.010, 0.100 ± 0.010, $F = 727.717$, $P < 0.001$; 2 wk: 0.318 ± 0.013 *vs* 0.110 ± 0.010, 0.143 ± 0.015, $F = 301.509$, $P < 0.001$; 4 wk: 0.172 ± 0.011 *vs* 0.107 ± 0.012, 0.090 ± 0.010, $F = 64.297$, $P < 0.001$; 12 wk: 0.140 ± 0.007 *vs* 0.120 ± 0.010, 0.097 ± 0.015, $F = 16.392$, $P < 0.001$); PDX-1 protein in RYGB group was also increased significantly (1 wk: 0.61 ± 0.01 *vs* 0.21 ± 0.01, 0.15 ± 0.01, $F = 3031.127$, $P < 0.001$; 2 wk: 0.55 ± 0.00 *vs* 0.15 ± 0.01, 0.17 ± 0.01, $F = 3426.455$, $P < 0.001$; 4 wk: 0.39 ± 0.01 *vs* 0.18 ± 0.01, 0.22 ± 0.01, $F = 882.909$, $P < 0.001$; 12 wk: 0.41 ± 0.01 *vs* 0.20 ± 0.01, 0.18 ± 0.01, $F = 515.833$, $P < 0.001$). PDX-1 mRNA and PDX-1 protein production showed no statistical significance between the two sham groups. Many PDX-1 positive cells could be found in the pancreatic islets of the rats in RYGB group at all time points. In addition, the percentage of Brdu-insulin double staining positive cells was higher in RYGB group than in the other two groups (1 wk: 0.22 ± 0.13 *vs* 0.03 ± 0.06, 0.03 ± 0.06, $P < 0.05$; 2 wk: 0.28 ± 0.08 *vs* 0.00 ± 0.00, 0.03 ± 0.06, $P < 0.05$; 4 wk: 0.24 ± 0.11 *vs* 0.07 ± 0.06, 0.00 ± 0.00, $P < 0.001$; 12 wk: 0.20 ± 0.07 *vs* 0.03 ± 0.06, 0.00 ± 0.00, $P < 0.05$).

CONCLUSION: RYGB can increase the expression of pancreatic PDX-1 and induce the regeneration of β-cells in GK rats. The associated regeneration of islet cells may be a possible mechanism that how RYGB could improve type 2 diabetes mellitus.

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Key words: Gastric bypass; Diabetes mellitus; Regene-

ration; β-Cells; Animals; Pancreatic duodenal homeobox-1

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by insulin resistance and progressive deterioration of islet functions[1]. The apoptosis of β-cells may be a major contributor to the failure of these cells at the late stages of T2DM[2]. An effective treatment for diabetes can improve the functions of β-cells and increase their numbers. Roux-en-Y gastric bypass (RYGB) is a commonly used surgical treatment for patients with morbid obesity. It could not only significantly and persistently reduce patients' body weight, but also lower the serum levels of glucose, and glycosylated hemoglobin in 80%-100% of T2DM patients[2,3]. Furthermore, this treatment could also prevent impaired glucose intolerance from developing into diabetes among these patients[3]. Animal experiments have shown that RYGB treatment can improve the functions of pancreatic islets and the metabolism of glucose in both obese and non-obese diabetic rats[4]. These results suggest that RYGB surgery is effective in the treatment of diabetes.

Efforts have been made to understand the mechanism of RYGB treatment on T2DM patients, however, the exact mechanism still remains elusive[5,6]. RYGB treatment may be correlated to the changes of gastrointestinal hormones, which then influence the enteroinsular axis to regulate the endocrine function of the pancreas[7]. One of the most important intestinal hormones involved in this mechanism is glucagon-like peptide 1 (GLP-1). It has been found that RYGB surgery can increase GLP-1 levels in the sera of T2DM patients and in the experimental animals. This effect may last 1 year and in some cases, even up to 20 years after RYGB surgery[5,8]. This may be one of the reasons why the post-operative diabetes is well controlled in both human beings and experimental rodents.

Recently, it has also been reported that after RYGB surgery, some patients developed nesidioblastosis, a serious life-threatening postprandial hyperinsulinism with hypoglycemia[9-12]. These symptoms may be alleviated, after part of the pancreas is removed. Hypertrophy and hyperplasia pancreatic islets are common features seen in the resected specimens, and even multiple islet tumors detected in some specimens. Since the hyperplasia of islet cells are rarely found in the normal population[9,12,13], the above data suggest that RYGB may be associated with the regeneration and neogenesis of the pancreatic islets. Thus, RYGB surgery may occasionally cause the pathological overgrowth and hyperplasia of β-cells in the islets[14]. However, this hypothesis has not been supported by any animal experiments.

Pancreatic duodenal homeobox-1 (PDX-1) is known as the first and most important transcription factor expressed during the embryonic development of the pancreas in experimental animals. All pancreatic cells are derived from the precursor cells with expression of PDX-1[15]. The embryonic pancreas failed to develop in the PDX-1 knockout mice[16]. The mice with heterozygous mutations of *PDX-1* gene could develop a normal pancreas, but may show abnormal glucose tolerance and diabetes in adulthood[17]. If PDX-1 is removed from mature β-cells by Cre-recombinase-mediated deletion, the expression of these genes is impaired and the loss of β-cells is accelerated[18]. The expression level of PDX-1 in the pancreas reach the peak in the first 10.5 d during the development of mouse embryos, and decline gradually afterwards. After the birth and in the adulthood, the expression of PDX-1 becomes very weak, which is specifically found only in 90% of β-cells and 10% of δ-cells in the islets[19].

PDX-1 is significantly re-expressed in proliferating cells in the ducts[20,21] and islets[22] during pancreas regeneration[23] and may serve as a marker of cells that regain their pluripotency to differentiate into all types of pancreatic cells. Subtotal pancreatectomy promoted the PDX-1 expression of duct epithelial cells in Sprague-Dawley rats[21], and the PDX-1 positive cells were also obvious in mouse model with pancreas injury[24]. PDX-1 has significant effect on cell proliferation and could induce a dramatic cell proliferation, which is similar to the formation of the pancreas during embryonic development[25]. Studies of animal models suggested that the down-regulation of PDX-1 expression in the β-cells may underlie the pathogenesis of β-cell failure and the onset of T2DM[26]. Therefore, the regeneration of islet β-cells requires the activation and expression of PDX-1. PDX-1 expression is the basis of regeneration of the pancreas.

Goto-Kakizaki (GK) rats are used in a genetically non-overweight type 2 diabetes model, which has the features of slightly elevated blood glucose, impaired secretion of glucose-stimulated insulin and reduced mass of pancreatic β-cells[1]. It is similar to those in T2DM patients. Therefore, GK rats have been widely used in an experimental animal model for T2DM studies. The aim of this study was to investigate the effects of RYGB surgery on the expression of PDX-1 as well as on the regeneration of pancreatic β-cells in non-obese diabetic GK rats, and the possible mechanisms.

MATERIALS AND METHODS

Animals

Sixty male GK rats weighing 200-230 g, at age of 10-12 wk, were provided by Shanghai Laboratory Animal Center,

Chinese Academy of Sciences, and kept in the animal facility at the Henan Key Lab of Biological Psychiatry, China. The rats were randomly divided into 3 groups with 20 rats in each group: RYGB group, sham-RYGB group and sham-operation (sham-op) group.

Abdominal surgeries

For experiments, GK rats in the RYGB group were fasted overnight and anesthetized with 4% chloral hydrate (Wako Co., Japan). Upper abdomen was cut open with midline incision and then the stomach was sutured bisecting it to produce two separate gastric cavities, distal one and a proximal one. The jejunum was cut off about 8 cm from the Tretiz ligaments. The proximal part of the stomach was opened and anastomosed with the distal jejunum. The proximal jejunum was anastomosed with the side of distal jejunum at the site about 10 cm away from the above stomach-jejunum anastomosis. The abdominal incisions were closed with absorbable sutures. For controls, the stomachs of rats were transected across the pylorus followed by discontinuously suturing back the two gastric stumps in the sham-RYGB group, and in the sham-op group there was only an anterior abdominal wall incision which was immediately sutured without disturbing the internal organs. After surgery, all rats were fasted and only fed with water on the same day of the operation (day 0). They were fed with food starting from day one. Animals were administered with the antibiotics, cefradine powder (Bright Future Pharmaceuticals Factory, Hong Kong), at a dose of 100-150 mg/kg body weight, once every 24 h, for a total of 3 d to prevent infections. Animals were euthanized on the 1st, 2nd, 4th and 12th wk after operation, with five rats from each group at each time-point, separately.

Reverse transcription polymerase chain reaction

On the sacrifice day, each rat was first intraperitoneally injected with 5-bromo-2-deoxyuridine (Brdu, from Sigma, USA) at 50 mg/kg and two hours later, the rats were euthanized with overdose anesthesia. The pancreatic tissues were isolated and rinsed with 0.01 mol/L phosphate buffered saline (PBS). Eighty mg of pancreatic tissues from each rat was homogenized using Trizol reagent (Sigma, USA), and the total RNA was extracted according to the manufacturers' protocol. The optical density of RNA was read with the spectrophotometry at the wavelength of 260 nm (A_{260}). The cDNAs were synthesized by adding 1-2 μg DNA-free RNA to the RT mixture. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. PCR was performed with the following primers: GAPDH (forward: 5'-ACCACAGTCCATGCCATCAC-3'; reverse: 5'-TCCACCACCCTGTTGCTGTA-3'), PDX-1 (forward: 5'-CCAAAACCGTCGCATGAAGTG-3', reverse: 5'-TCTGGGTCCCAGACCCG-3'). The combination of the DNA primers produced single PCR products of 550 and 208 bp in length, respectively. PCR was carried out in a DNA thermal cycler (Biometra, Goettingen, Germany) after denaturation at 95℃ for 5 min. The number of total PCR cycles was 35 for PDX-1 and 30 for GAPDH, and each cycle consisted of denaturation at 94℃ for 15 s, annealing at 65℃ (GAPDH at 62℃) for 30 s, and extension at 72℃ for 90 s. For semiquantitative analysis, 25 μL of each PCR product was examined by electrophoresis in 1.8% agarose gel containing 0.5 μg/mL of ethidium bromide. DM 1000 (LEICA, Germany) was used as a molecular marker. OD readings of PDX-1 mRNA were normalized to those of GAPDH mRNA, and the ratios were analyzed.

Paraffin sectioning

The pancreatic tissues obtained from the above rats were also immediately fixed with 4% paraformaldehyde at 4℃ overnight. The tissues were then dehydrated in graded ethanol, cleared in xylene and finally embedded in paraffin. The tissues were sectioned to produce 4 μm-thick sections, which were mounted onto slides for further staining.

Immunohistochemical staining

In brief, the pancreatic sections were deparaffined in xylene and rehydrated in graded ethanol. The sections were blocked in H_2O_2 and sequentially incubated with goat anti-PDX-1 polyclonal primary antibody (1:50 dilution; Santa Cruz, USA) overnight and anti-goat secondary antibody (1:100 dilution; Santa Cruz, USA) for 2 h. For negative control, the primary antibody was replaced with 0.01 mol/L PBS.

Western blotting

Total protein was obtained from the pancreatic tissues, that were lysed in the buffer containing 50 mmol/L Tris, pH 7.5, 0.3 mol/L NaCl, 0.5% Triton X-100 and 0.1% sodium azide, and protease inhibitor cocktail CLAP (chymostatin, leupeptin, aprotinin and pepstatin, 100 μmol of each, Sigma Aldrich, USA). Then protein samples (50 μg) were separated in 10% sodium dodecyl sulphate polyamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham, UK). The membranes were then blocked in 0.01 mol/L PBS containing 5% dry milk and 0.3% Tween 20 for 1 h. The membranes were then incubated with goat anti-PDX-1 antibody (1:10000 dilution) at room temperature for 2 h and horseradish peroxidase-labelled secondary antibody (1:5000 dilution; Amersham, Japan) at room temperature for 1 h, respectively. Finally, the proteins were visualized on enhanced chemiluminescence hyperfilm (Amersham, Japan) by application of Amershem's Enhanced Chemiluminescence Western blotting Detection System. The band intensity was quantitated with GelAnalysis software (GreyStone-Iconix).

Double immunohistochemical staining

Briefly, the pancreatic sections were sequentially incubated with guinea pig anti-insulin polyclonal antibody (1:50 dilution; Santa Cruz, USA), biotinylated rabbit anti-guinea pig antibody (1:50 dilution; Santa Cruz, USA), and streptavidin-alkaline phosphatase complex (Santa Cruz, USA), for a period of 45 min each. The alkaline phosphatase activity was identified using new fuchsin under light microscope. The sections were counterstained with Harris hematoxylin.

To detect the second antigen Brdu, the sections were placed in 2 N hydrochloric acid and kept at 70℃ for 15 min for antigen retrieval. The reaction was stopped by 0.1 mol/L sodium borate and left in 0.01 mol/L PBS for 20 min. Tissue sections were incubated with hydrogen peroxide for 45 min, mouse anti-Brdu monoclonal antibody for 3 h, biotinylated goat anti-mouse antibody for 45 min, and streptavidin-peroxidase complex for 45 min, respectively. Finally, the peroxidase activity was visualized with diaminobenzidine under light microscope, and the reaction was stopped by rinsing with PBS. Avidin and biotin were then applied to the sections for 45 min each.

Cell count

The images of the above double-stained sections were captured at 100 × magnifications. In each rat, a total of 1000 nuclei from five sections were counted, and the sections were randomly selected from tail (3 sections), body (1 section) and head (1 section). The index of Brdu-labeling was calculated as follows: The index of Brdu labeling = (The number of double-stained cells/1000) × 100%.

Statistical analysis

All data were presented as mean ± SD (n = 5). The data were analyzed using SPSS13.0 statistical package. The differences were analyzed by factorial design of variance. The comparison of variance between two groups was analyzed by LSD method. Missing variance was analyzed using Dunnett T3. Non-normal data were analyzed non-parametrically using a number of independent samples and tested by Kruskal-Wallis method. $P < 0.05$ was considered to indicate statistical significance of the test results.

RESULTS

Reverse transcription polymerase chain reaction

Compared with internal control, GAPDH, the data of relative mRNA expression of PDX-1 at weeks 1, 2, 4 and 12 after surgery were 0.378 ± 0.013, 0.318 ± 0.013, 0.172 ± 0.011 and 0.140 ± 0.007 in the rat pancreas of RYGB group, 0.120 ± 0.010, 0.110 ± 0.010, 0.107 ± 0.012 and 0.120 ± 0.010 in sham-RYGB group, and 0.100 ± 0.010, 0.110 ± 0.010, 0.090 ± 0010 and 0.097 ± 0.015 in sham-op group, respectively. The above data were plotted as shown in Figure 1B. The F values of the relative expression level of PDX-1 mRNA in the rat pancreas at 1, 2, 4 and 12 wk after surgery in the three groups were 727.717, 301.509, 64.297 and 16.392, respectively, all $P < 0.05$. There was statistically significance at each time point. In addition, the relative mRNA expression level of PDX-1 in the RYGB group showed statistical significance (F = 512.105, $P < 0.05$), being higher at 1 and 2 wk after surgery. In contrast, there was no significant difference at different time points between sham-RYGB and sham-op groups (F = 0.750, both $P > 0.05$).

Immunohistochemical staining

Anti-PDX-1 immunohistochemical (IHC) staining showed that there were many PDX-1 positive cells with brown nuclei in the rat pancreas in the RYGB group. These cells were mainly located in the islets. A few PDX-1 positive cells could be observed in the ducts and acini (Figure 2A and B). In contrast, few PDX-1 positive cells were found in the islets of rat pancreas at each time point in both sham-RYGB and sham-op groups (Figure 2C).

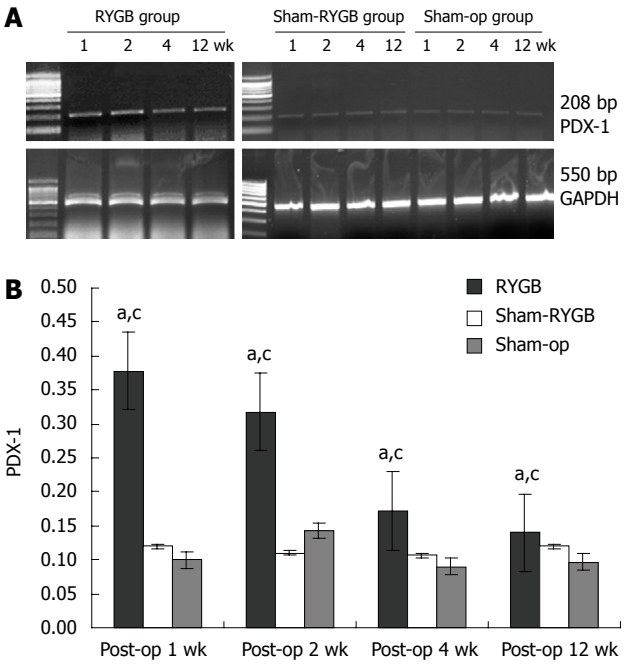


Figure 1 The expression of PDX-1 mRNA in the pancreas of GK rats. A: Representative gel from RT-PCR with primers to PDX-1 (208 bp) and GAPDH (550 bp); B: The intensities of PDX-1 mRNA bands relative to those of GAPDH bands. $^{a}P < 0.05$ *vs* sham-RYGB; $^{c}P < 0.05$ *vs* sham-op group.

Western blotting

The data of the expressions of PDX-1 protein in the rat pancreas are plotted in Figure 3A. Compared with the internal control, GAPDH, the relative expressions of PDX-1 protein in the rat pancreas are shown in Figure 3B. The F values of the relative expression of PDX-1 protein in the rat pancreas at 1, 2, 4 and 12 wk after surgery in the three groups were 3031.127, 3426.455, 882.909 and 515.833, respectively (all $P < 0.05$). In addition, there was statistical significance at each time point among the three groups. The relative expression levels of PDX-1 protein in the rat pancreas of RYGB group showed statistical significance (F = 676.157, $P < 0.05$), being highest at 1 and 2 wk after surgery.

Double IHC staining

Anti-Brdu and anti-insulin double stained cells could be found in the rat pancreas in the RYGB group at each time point (Figure 4A-C). The Brdu and insulin double positive cells were found primarily in the islets and occasionally in the ducts (Figure 4C). Single insulin positive cells could be observed in the acini (Figure 4B) and there were many single Brdu positive cells in the acini of the pancreas (Figure 4A-D). However, few Brdu and insulin double positive cells could be found in the rat pancreas in both sham-RYGB

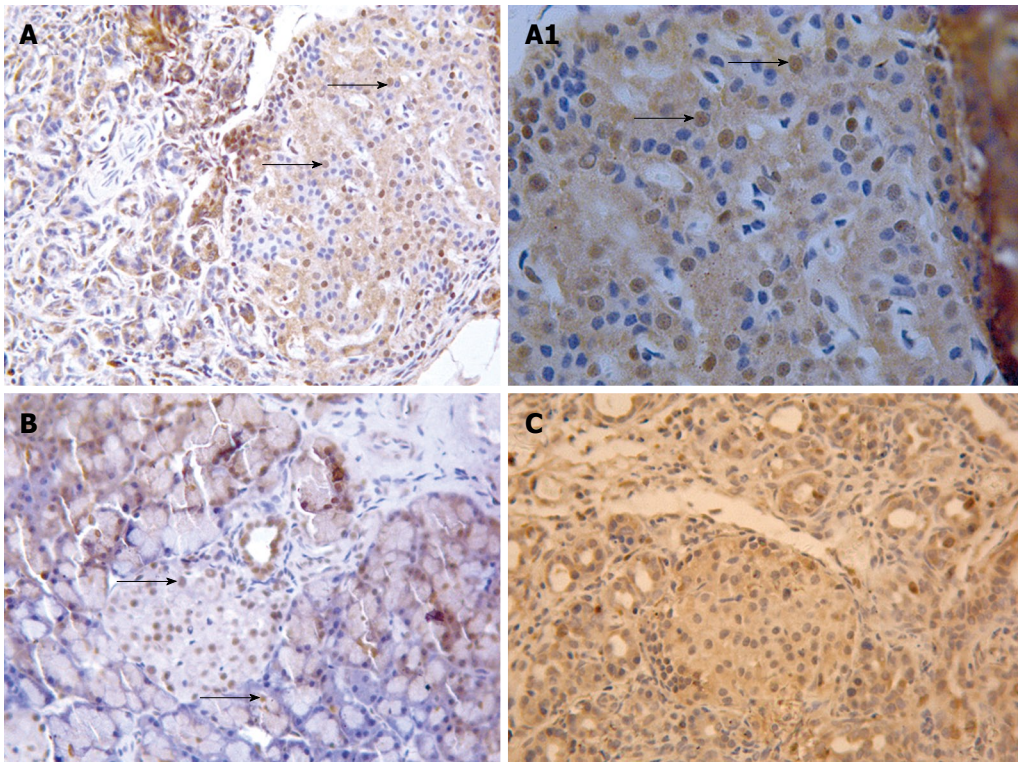


Figure 2 The expression of PDX-1 protein in the pancreas of GK rats. PDX-1 positive signals were stained in dark brown color (arrows), and mainly located the islets. Occasionally, they were found in the duct and acinar cells. A, B and A1 in RYGB group; C in sham-op group. (Hematoxylin & anti-PDX-1 IHC stain. A, B and C at 200 × magnifications; A1, at 400 × magnifications).

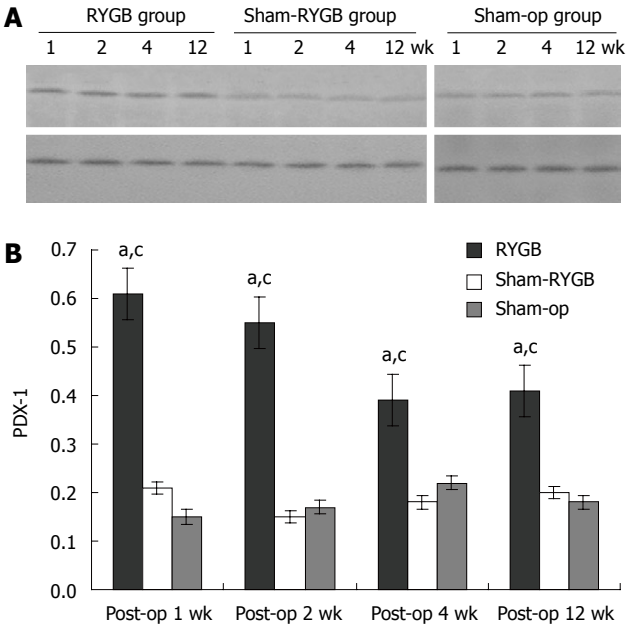


Figure 3 Western blotting analysis of PDX-1. A: Representative Western blotting analysis of PDX-1 protein; B: The relative expression level of PDX-1 protein. $^{a}P < 0.05$ *vs* sham-RYGB; $^{c}P < 0.05$ *vs* sham-op group.

and sham-op groups at each time point after surgery. The percentage of both Brdu and insulin positive cells showed a significant increase at 1, 2, 4 and 12 wk after surgery as compared with the other two groups (Figure 4E). The χ^2 values were 6.197, 8.485, 7.808 and 8.166, respectively; the corresponding *P* values were 0.045, 0.014, 0.020 and 0.017. The rank of RYGB group was the highest at each time point. In addition, there were more single Brdu positive cells in RYGB group than in the other two groups.

DISCUSSION

In this study, the expression levels of PDX-1 mRNA in the RYGB group were significantly higher than those in the sham-RYGB and sham-op groups at all time points of 1, 2, 4 and 12 wk after surgery. It suggests that RYGB surgery may result in an increase in the expression of pancreatic PDX-1 mRNA in GK rats. In other words, RYGB surgery may promote the expression of PDX-1 mRNA, which may last at least 12 wk. The expression of PDX-1 mRNA in RYGB group peaked at the first two weeks and decreased afterwards, which may suggest that RYGB surgery played a role in promoting the expression of PDX-1 mRNA primarily at the early stage after the surgery.

Similarly, the protein expressions of PDX-1 in RYGB group were also significantly higher than those in the other two groups at all time points. The protein expression level of pancreatic PDX-1 in RYGB group also peaked at the first two weeks after surgery, and decreased afterward. Furthermore, the results of anti-PDX-1 IHC staining also showed that PDX-1 positive cells in the pancreas of GK rats increased significantly in RYGB group when compared with those in both sham-RYGB and sham-op groups. The PDX-1 positive cells were mainly located in the islets, but not in the acinus and ducts.

It is important to increase the number of β-cells in the pancreatic islets in the treatment of diabetes. The regenerated β-cells in the islets may be double labeled by anti-Brdu and anti-insulin double IHC staining. In this experiment, Brdu and insulin double positive cells were visible in the pancreas in all GK rats at all time points. In addition, the percentages of Brdu and insulin double positive cells in RYGB group were significantly higher than those in both sham-RYGB and sham-op groups. The results also suggest

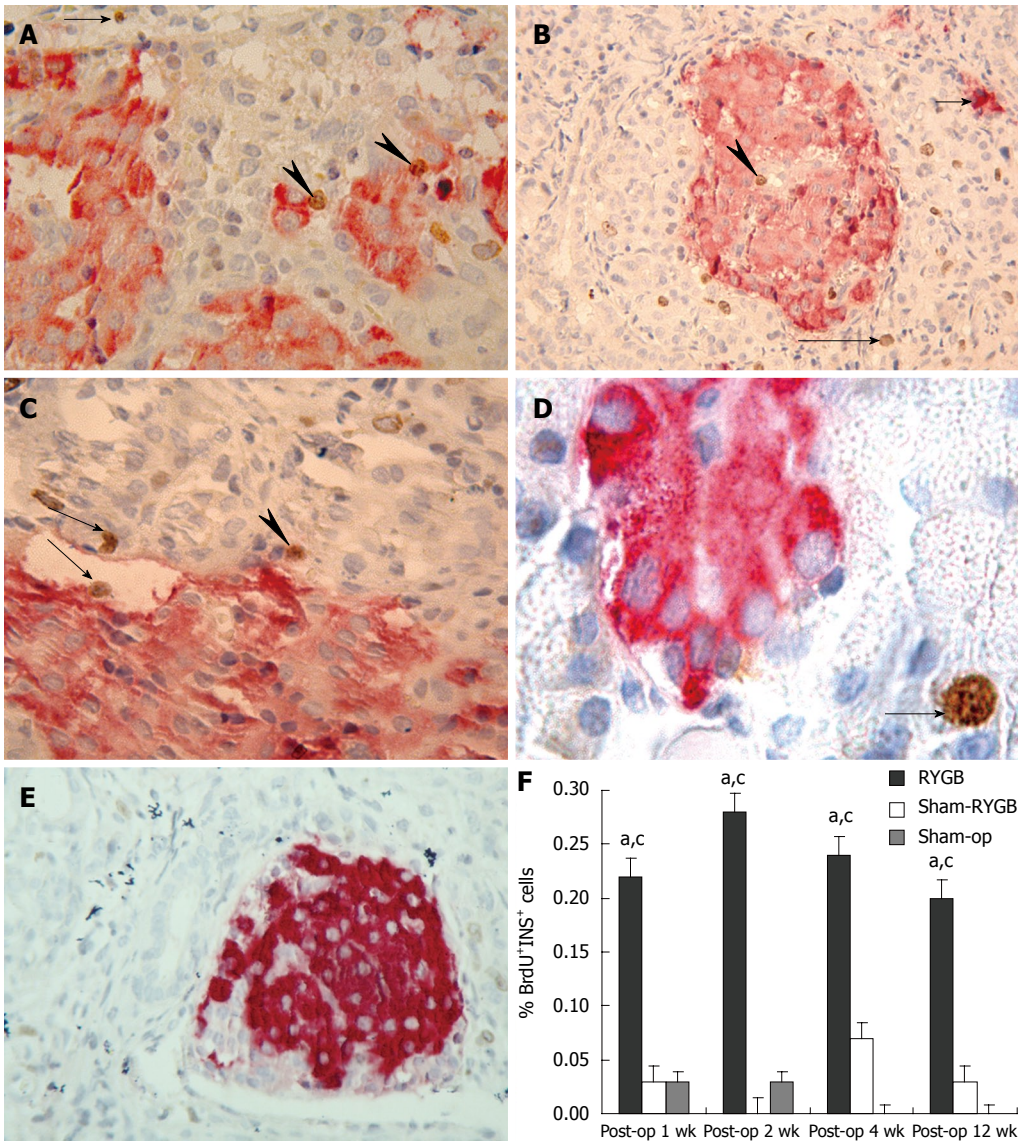


Figure 4 Anti-Brdu and -insulin double IHC staining. A: Brdu and insulin double positive cells in the islets or acini (arrowheads), single Brdu positive cells in the acini (thin-tail arrow); B: Brdu and insulin double positive cells in the islets (arrowhead) and single Brdu positive cells in the acini (thin-tail arrow), insulin positive cells in the acini (short-tail arrow); C: Brdu and insulin double positive cells in the ducts (thin-tail arrows) and islets (arrowhead); D: Single Brdu positive cells in the acini (thin-tail arrow); E: No Brdu and insulin double labeling cells in the pancreas of sham-RYGB rats; F: The index of Brdu and insulin double positive cells. (A and C at 400 × magnifications; B and E at 200 × magnifications; D at 1000 × magnifications). $^{a}P < 0.05$ *vs* sham-RYGB; $^{c}P < 0.05$ *vs* sham-op group.

that RYGB surgery may promote the regeneration of islet β-cells in GK rats, which is likely another mechanism why RYGB surgery may decrease the blood glucose in diabetes patients.

The results in this study have shown that RYGB surgery may result in an increase in expressions of PDX-1 mRNA and PDX-1 protein, the number of PDX-1 positive cells and the percentage of Brdu and insulin double positive cells in GK rats. All these suggest a regeneration of islet β-cells, which may be related to persistent elevations of serum GLP-1 after RYGB surgery. It is reported that GLP-1 and its long-acting analog exendin-4 (Ex-4) enhance the expression of pancreatic PDX-1 and regeneration of islet β-cells, and inhibit the apoptosis of β-cells[27,28]. GLP-1 can stimulate the differentiation and proliferation of β cells in GK rats[29]. GLP-1 increases the number of β cells by inducing the differentiation and neogenesis of ductal progenitor cells into islet endocrine cells. Koizumi *et al*[27], have found that Ex-4 can significantly stimulate β-cell proliferation in PDX-$1^{+/+}$ mice, but not obviously influence the apoptosis of β-cells. However, in PDX-$1^{-/-}$ mice, the proliferation of β-cells showed no significant change, whereas the apoptosis of β-cells increased significantly in number. Therefore, functions of GLP-1 may rely on the *PDX-1* gene activation and expression[24,30]. Ex-4 has been approved by the Food and Drug Administration for the clinical treatment of diabetes[31]. GLP-1 is decomposed *in vivo* by enzyme dipeptidyl peptidase-Ⅳ (DPP-Ⅳ)[31], whose inhibitor, vildagliptin, has been applied in the treatment of diabetes and is at the stage of advanced clinical trials[30]. This compound may increase the mass of β-cells, and slow or even reverse the progressive islet-cell deterioration in diabetes. It is possible that the effect of GLP-1 on increasing β-cells is partly mediated by an increase in the circulating insulin, which may act as a

growth factor. But, long-term over-stimulation of GLP-1 signaling may cause pathologic hypertrophy and hyperactivity of β-cells, resulting in hypoglycemia.

New pancreatic β-cells are formed in two ways: proliferation of preexisting β-cells, which is called regeneration, and transdifferentiation of non-β-cells from either duct or acinar cells to new β-cells, which is called neogenesis. Dor *et al*[32], have found that the removal of most of the pancreas in mice led to hyperplasia of the pancreas, and suggested that the new β-cells came from the proliferation of pre-existing β cells in the adult pancreas. Injury in the mouse model caused by pancreatic duct ligation has shown that the progenitors of β-cells exist in adult mouse pancreas, which could be activated and then produced new functional β-cells by differentiation and proliferation[33]. It has been reported that new β-cells in adult pancreas were transdifferentiated from epithelial cells in pancreatic ducts whose characteristics as duct cells were lost either in normal or injured pancreas[27,33-37]. Stem cells also existed in adult human pancreatic ducts, which not only expressed nestin and PDX-1, but also exhibited the markers of mesenchymal stem cells[38]. These data suggest that the new β-cells in pancreas may be formed through a variety of channels and derived from different cells.

Similarly, *in vitro* cultured stem cells from adult bone marrows[35] and adipose-derived mesenchymal stem cells[36] can be induced to differentiate into insulin-like cells which produce insulin. This indicates that the stem cells from the mesoderm may cross over the embryonic layer and differentiate into insulin-like cells in the endoderm. In other words, the mesenchymal stem or progenitor cells have a strong reserving function in the body. Yuan *et al*[39] and Kodama *et al*[40] have also proposed similar hypothesis, in which progenitor cells exist in the adult pancreas and may transform to endocrine cells under pathological conditions. In Streptozotocin-treated mice, regeneration of β-cells seemed to occur mainly from intra-islet stem/progenitor cells[40].

In this study, the regeneration of β-cells was mainly located within the islets, which is consistent with the experimental results of Kodama *et al*[40]. In addition, in this experiment, Brdu and insulin double positive cells were mainly found in the pancreatic duct and single insulin positive cells were mainly in the acini. These results suggest that the duct epithelial cells and acinar cells can be transdifferentiated into insulin-like cells, which are consistent with the experimental results of De Haro-Hernández *et al*[41]. Single Brdu positive cells were found within the pancreatic acini in RYGB rats, which suggests that RYGB promotes not only regeneration/neogenesis of pancreatic β-cells, but also regeneration/neogenesis of other cells. However, the emergence of these new cells depends on the PDX-1 activation and expression.

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COMMENTS

Background

Roux-en-Y gastric bypass (RYGB) is a commonly used surgical treatment for patients with morbid obesity. It significantly and persistently decreases the levels of blood glucose and glycosylated hemoglobin in 80%-100% of type 2 diabetes mellitus (T2DM) patients. Efforts have been made to understand the mechanism of RYGB treatment in T2DM patients, however, the exact mechanism is still elusive.

Research frontiers

Some patients who underwent RYGB surgery developed nesidioblastosis, a serious life-threatening postprandial hyperinsulinism with hypoglycemia, which is rarely found in the normal population. The article suggests that RYGB may be associated with the regeneration and neogenesis of the pancreatic islets. However, this hypothesis has not been supported by any animal experiment.

Innovations and breakthroughs

This study showed that RYGB could increase the expression of pancreatic PDX-1, which is the first and most important transcription factor found during the embryonic development of the pancreas and pancreas regeneration, and induce the regeneration of β-cells in GK rats used in a model of genetic non-overweight type 2 diabetes. The regeneration of islet cells is a possible mechanism how RYGB can treat T2DM.

Applications

T2DM is one of the most common chronic metabolic diseases that is characterized by insulin resistance and progressive deterioration of islet cell functions. Findings of this study explain the possible mechanism of treating T2DM with RYGB surgery. This study has provided a new basis for surgery to treat T2DM and explained why the post-surgical nesidioblastosis occurs in RYGB patients.

Peer review

This is a very important paper since it is dealing with an issue of high relevance, nevertheless not properly addressed in most of the publications at present. Moreover, an extensive literature review is reported and examined.

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ORIGINAL ARTICLE

Expression of interleukin 6 in brain and colon of rats with TNBS-induced colitis

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Abstract

AIM: To characterise expression of interleukin 6 (IL-6), a potent proinflammatory cytokine, in the occurrence and development of inflammatory bowel disease (IBD) and investigate its effect on neuroimmunomodulation and immune homeostasis regulation.

METHODS: In this study, rats with colitis induced by trinitrobenzene sulfonic acid (TNBS) were sacrificed on days 3, 7, 14, 21 and 28 after induction. In the controls, the TNBS was just replaced by equivalent amount of phosphate buffered solution (PBS, 0.01 mol/L). IL-6 mRNA expression in brain and colon tissues in each phase was evaluated by real-time reverse transcription-polymerase chain reaction, and cellular localisation and protein level of IL-6 was determined by immunohistochemistry.

RESULTS: At day 7, mRNA expression of IL-6 was significantly higher in the colon and brain of IBD rats than that of the controls. The protein level was also significantly higher in colon, hypothalamus and cerebral cortex of IBD rats compared with the controls. So there are similar temporal trends in IL-6 mRNA expression and protein levels in all positions with a persistent increase to a peak at day 7, followed by a decline and gradual return to normal levels.

CONCLUSION: These results revealed that changes in IL-6 expression in brain and colon tissues occur in different phases of IBD. Therefore, we propose that the nerve centre regulates and controls the occurrence and development of IBD *via* IL-6.

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Key words: Inflammatory bowel disease; Interleukin 6; Neuroimmunomodulation; Real-time transcription-polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Inflammatory bowel disease (IBD) includes two chronic pathologies characterised by alternating phases of active inflammation and quiescence: ulcerative colitis (UC) and Crohn's disease (CD)[1]. Although the pathogenesis of IBD has not been well established, it has been suggested that some frequently observed clinical manifestations such as abdominal pain, nausea, vomiting, ileus, or diarrhoea, can be attributed to deranged gastrointestinal motility associated with inflammation[2]. The hypothesis that immune abnormalities induced by Th1/Th2 imbalances play a core role in the pathogenesis of IBD has been widely accepted[3,4]. Th1 cells predominantly secrete interferon-γ (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor α (TNF-α), while Th2 cells secrete interleukins 4, 5 and 10 (IL-4, IL-5, IL-10). Cytokines can be divided into pro-inflammatory and anti-inflammatory cytokines and growth factors. Pro-inflammatory cytokines such as IL-1, IL-2, IL-8, IL-12, TNF-α, TNF-β and IFN-γ are produced by monocytes and macrophages and are involved in cell-mediated immune responses. Anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 are produced mainly by T cells and are involved in the humoral immune response.

Communication between the periphery and the brain takes place *via* neural and humoral pathways. Cytokines released by activated immune cells are mediators of inter- and intra-cellular communication[5-11] and are part of a chemical signalling system that regulates development, tissue repair, haemopoiesis, inflammation and specific and nonspecific immune responses. Potent cytokine polypeptides (such as interleukins 1, 6, 8 and TNF-α) have pleiotropic activities and functional redundancy and exist in a complex, intermingled network in which one cytokine can influence the production of and response to many other cytokines[12]. Of note, modulation of cytokines and their antagonists also exhibits therapeutic potential in several chronic and acute diseases[10,11]. The immune system is regulated in part by the central nervous system (CNS), acting principally *via* the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS)[13-15]. IL-6 is a pleiotropic cytokine whose expression in physiological conditions is important for host responses to a number of infections; in addition, it exerts antigen-specific immune responses and has both pro- and anti-inflammatory effects[16,17]. In CD, IL-6 is present at high levels in both serum and intestinal tissues[18]. Increased levels of IL-6 receptor (IL-6R) and gp130 expression have been also demonstrated in peripheral lymphocytes of Crohn's patients, along with increased serum soluble IL-6R[19].

To explore the role of IL-6 in the initiation and development of IBD with respect to the neuroimmunomodulation and regulation of immunological homeostasis at the organismic level, we used real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) to evaluate the mRNA expression and cellular localisation of IL-6 in brain and colon tissues in the inflammatory and quiescent phases of IBD in an animal model with trinitrobenzene sulfonic acid (TNBS)-induced IBD.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma Chemical, unless otherwise stated.

Animals

Eighty female Wistar rats (Laboratory Animal Service of Jilin University, Changchun, China), 8-10 wk old and weighing 180-200 g, were maintained under specific pathogen-free conditions according to our institutional guidelines for animal care. This study was carried out in accordance with guidelines established by a consensus statement entitled the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Experimental model

After a seven-day acclimation period, 80 rats were divided randomly into 8 cages (one cage for 10) and fed in the same condition. Among them, 4 cages were experimental group and the others were controls. Rats were fasted overnight, and colitis was induced in the TNBS group by the method originally described by Morris *et al*[20], with some modifications made by our group. Briefly, rats were anaesthetised with ethylether and given a 5% TNBS (Sigma, R&D, USA) and ethanol mixture (2:1) at a dose of 0.3 mL/100 g inserted through the anus to a distance of 8 cm. Rats from the non-colitis group were given an intracolonic dose of 0.25 mL phosphate buffered saline (PBS) instead of TNBS. Animals were maintained in a vertical position for 30 s and returned to their cages.

Assessment of experimental model

Macroscopic scoring: At 3, 7, 14, 21, and 28 d after induction of colitis, rats were exsanguinated under ether anaesthesia, and the small and large intestines and the brain were excised. Samples of the descending colon were obtained for paraffin section and measurement of myeloperoxidase activity (see below). The remaining descending colon, sigmoid colon, and rectum were opened longitudinally and fixed onto a board with pins, and the macroscopic appearance was scored by two blinded investigators on a 0-4 scale similar to the original scoring system of Morris and colleagues[20]; briefly, 0 = no evidence of inflammation, 1 = erythema only, 2 = erythema with slight oedema and small erosions, 3 = two or more bleeding ulcers and/or inflammation and/or moderate adhesions, and 4 = severe ulceration and/or stenosis with prestenotic dilations with or without severe adhesions.

Determination of myeloperoxidase activity: Colonic myeloperoxidase activity was measured as previously described by Grisham *et al*[21] and Suzuki *et al*[22]. Briefly, tissue was homogenised in approximately 10 mL 50 mmol/L

KPO_4 at pH 7.4 and centrifuged for 20 min at 20 000 *g*. The pellet was homogenised in 10 mL 50 mmol/L KPO_4, and 10 mmol/L EDTA at pH 6.0 with 0.5% hexadecyltrimethylammonium bromide (wt/vol). After one freeze-thaw cycle, the homogenate was sonicated, and myeloperoxidase activity was determined by measuring H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine and expressed as units per gram of tissue.

Microscopic scoring: Specimens were fixed in PBS containing 4% formaldehyde and embedded in paraffin. Multiple sections (5 μm thick) were stained with haematoxylin and eosin using standard techniques. The degree of inflammation was assessed by two blinded investigators using the 0-4 scoring system described by Neurath *et al*[23] (0 = no signs of inflammation, 1 = low levels of leucocyte infiltration, 2 = moderate levels of leucocyte infiltration, 3 = high levels of leucocyte infiltration, high vascular density, thickening of bowel wall, and 4 = transmural infiltrations, loss of goblet cells, high vascular density, significant bowel wall thickening).

Isolation of mRNA and analysis of mRNA expression by real-time RT-PCR

A total of 80 rats (normal and IBD model) were used for real-time RT-PCR analysis. The proximal part of the colon (the area with macroscopically visible mucosal folds) and the brain were stored in RNAlater (Ambion, Austin, TX) at -80℃ for use in RNA extraction. RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. The integrity and quality of the isolated RNA was examined by quantifying the A260/280 ratio (BioPhotometer, Eppendorf, Hamburg, Germany) and resolving the RNA on a 1% agarose gel. The cDNA was prepared from 1.0 μg RNA (OD = 1.9-2.0) in the presence of 2.5 μmol/L oligo (dT) primer and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV; Promega, Madison, WI) in a total volume of 20 μL. The reaction mixture was incubated for 1 h at 42℃ and stopped by heating at 90℃ for 5 min. The primers and FAM-labelled probes were designed with Primer Express v1.5 software (Applied Biosystems). For IL-6, the forward primer was 5'-GCCCTTCAGGAACAGCTATGA-3', the reverse was 5'-TGTCAACAACATCAGTCCCAAGA-3', and the probe was 5'-CTCTCCGCAAGAGACTTCCAGCCAGTT-3'. For the housekeeping gene beta-actin, the forward primer was 5'-GTCAGGTCATCACTATCGGCAAT-3', the reverse was 5'- AGAGGTCTTTACGGATGTCAACGT-3', and the probe was 5'-CCTTCCTTCCTGGGTATGGAATCCTGTG-3'. All the primers and probes were obtained from Applied Biosystems. PCR was performed in the ABI PRISM 7000 Sequence Detection operated by Sequence Detector v1.3 software (Applied Biosystems), using TaqMan Real-time PCR Master Mix (Toyobo, Japan). A threshold was set at the linear part of the amplification curve, and the number of cycles needed to reach it were calculated. Relative mRNA levels were determined by using a standard curve and by further normalisation to the housekeeping gene beta-actin. The annealing temperatures were both set to 60℃.

Immunohistochemistry

IHC for detection of IL-6 was performed on paraffin sections of rat brain and colon samples; female rats were perfused under chloral hydrate anaesthesia *via* the abdominal aorta. After a 30 s rinse with physiological saline, a fixative containing 4% freshly prepared paraformaldehyde in 0.01 mol/L PBS buffer (pH 6.0) was perfused for 5 min. The brains and colons were cut into 1-2 mm frontal or sagittal slices and were embedded in paraffin using an automated vacuum tissue processor (Leica ASP300, Germany). For experiments, 4 μm sections were cut and mounted on specific slides. For antigen retrieval, the protocol recently described by Pizarro *et al*[24] and Strober *et al*[25] was used. Briefly, the deparaffinised and rehydrated sections were subjected to a 5 min digestion with 0.01% trypsin (Sigma) in PBS and an additional microwave treatment in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 720 W, followed by a 20 min cooling period in the same buffer. After pretreatment, sections were treated for 15 min with 3% hydrogen peroxidase to block endogenous peroxidase activity and were blocked with 0.5% blocking reagent for 30 min to reduce non-specific reactions. The sections were incubated for 36 h at 4℃ with rabbit anti-rat-IL-6 IgG (Biozol, Eching, Germany; diluted 1:400) in blocking buffer. Sections were washed in PBS, and biotinylated linking antibody solution (Biozol, Eching, Germany) was applied (100 μL/section) for 20 min at 37℃. Sections were then washed in PBS before application of HRP conjugated streptavidin (100 μL/section, 15 min) at 37℃. Slides were stained with DAB (IBL, Germany) after being washed in PBS for 5 min, washed in PBS and DAB (BIOS) for 5 min and counterstained with hematoxylin. For negative controls, the primary antibody was replaced with the corresponding affinity-purified preimmune IgG.

Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was performed using SPSS 17.0. Differences were considered to be statistically significant when $P < 0.05$ by analysis of variance (ANOVA).

RESULTS

Effects of TNBS-induced colitis on clinical and macroscopic appearance

After administration of TNBS, we found that Wistar rats regularly developed colitis with severe diarrhoea, losing 15% of their body weight and suffering rectal prolapse and extensive wasting. Gross blood adhesion to the anus was noted in some rats. Macroscopically, colitis was characterised by bowel wall thickening, adhesions and necrotic foci (Figure 1). As these severe inflammatory changes covered 2-4 cm of the distal colonic mucosa, this resulted in a higher median macroscopic damage score in TNBS-treated rats compared to controls (Figure 2A, $P < 0.01$), especially by day 7.

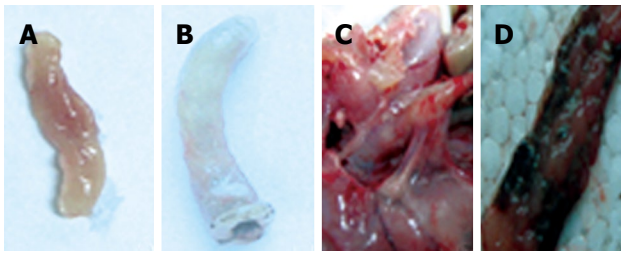


Figure 1 The macroscopic appearance of colon tissue in trinitrobenzene sulfonic acid (TNBS)-treated *vs* control rats. A: The colon of control rats, with macroscopic damage score = 0; B-D depict colonic tissue of TNBS-treated rats: B: Significant swelling and distension, with macroscopic damage score = 5; C: Extensive adhesions around the colon, with macroscopic damage score = 7; D: Necrotic foci in colon, with macroscopic damage score = 8.5.

Effects of TNBS-induced colitis on colonic myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined by using accumulation of polymorphonuclear leucocytes as an indicator. Compared with the control rats, MPO activity increased gradually, peaked by day 7, and decreased to nearly the baseline value (Figure 2B).

Effects of TNBS-induced colitis on colonic histology

The degree of inflammation was assessed using the scoring system at 3, 7, 14, 21, and 28 d after TNBS induction. The score reached a peak at day 7, and then decreased gradually to nearly the normal value (Figure 2C). Microscopic evaluation of colons from control rats showed normal colonic histological features (Figure 3A). Histological evaluation of the colon at each period after TNBS induction is shown in Figure 3B-F. TNBS caused the inflammatory features typical of colitis in the rats' colonic architecture: severe oedema and haemorrhage at the muscular layer of the mucosa, ulceration, goblet cell depletion, and a mixed cell infiltration that was mainly composed of mononuclear cells such as macrophages, lymphocytes and plasmocytes.

Analysis of IL-6 mRNA expression

We used real-time RT-PCR to measure expression of IL-6 mRNA in the brain and colon at various time points after induction of colitis. Our results demonstrated a remarkable difference ($P < 0.01$) in colon and brain tissues in the experimental *vs* the control group. IL-6 mRNA expression levels increased by day 3 and returned to near baseline levels by day 28, reaching their highest point at day 7. Thus, there is similarity on the increasing trend (Figure 4A).

IHC

IHC was performed to determine IL-6 localisation in the colon, cerebral cortex and hypothalamus. The distribution, cellular localisation, and intensity of staining were assessed using light microscopy. The distribution and relative abundance of IL-6 in the parts of the colon, cerebral cortex and hypothalamus examined in this study are presented in Figure 4B; representative sections are shown in Figure 5. Control sections immunolabeled with the preimmune serum were devoid of immunolabeling for IL-6. We found that

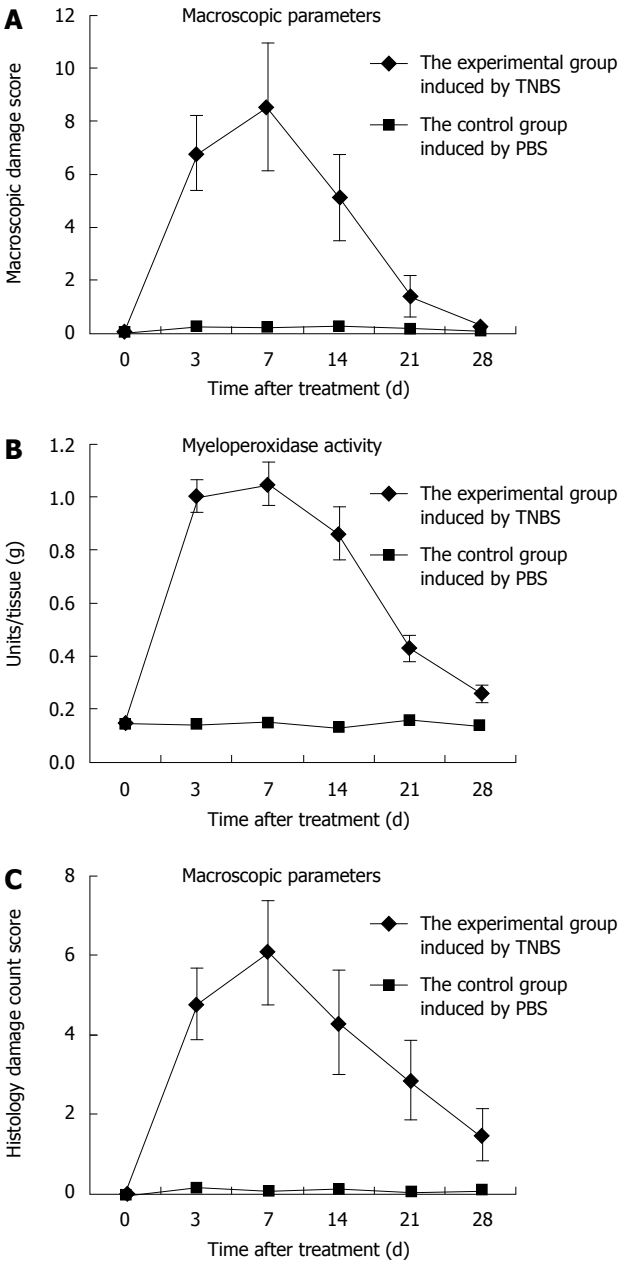


Figure 2 Assessment of TNBS-induced colitis rats by time points. As these severe inflammatory changes covered 2-4 cm of the distal colonic mucosa, this resulted in a higher median macroscopic damage score in TNBS-treated rats compared to controls (A, $P < 0.01$), especially by day 7; compared with the control rats, MPO activity increased gradually, peaked by day 7, and decreased to nearly the baseline value (B); The score reached a peak at day 7, and decreased gradually to nearly the normal value (C).

immunostaining was clearly increased in the colon, cerebral cortex and hypothalamus after induction of colitis, reaching a peak at day 7 and decreasing thereafter. Morphologically, analyses showed that the number of IL-6 immunopositive cells was significantly increased ($P < 0.01$) in the colon, cerebral cortex and hypothalamus after induction compared to control animals.

DISCUSSION

Over the past several years, a variety of IBD animal mod-

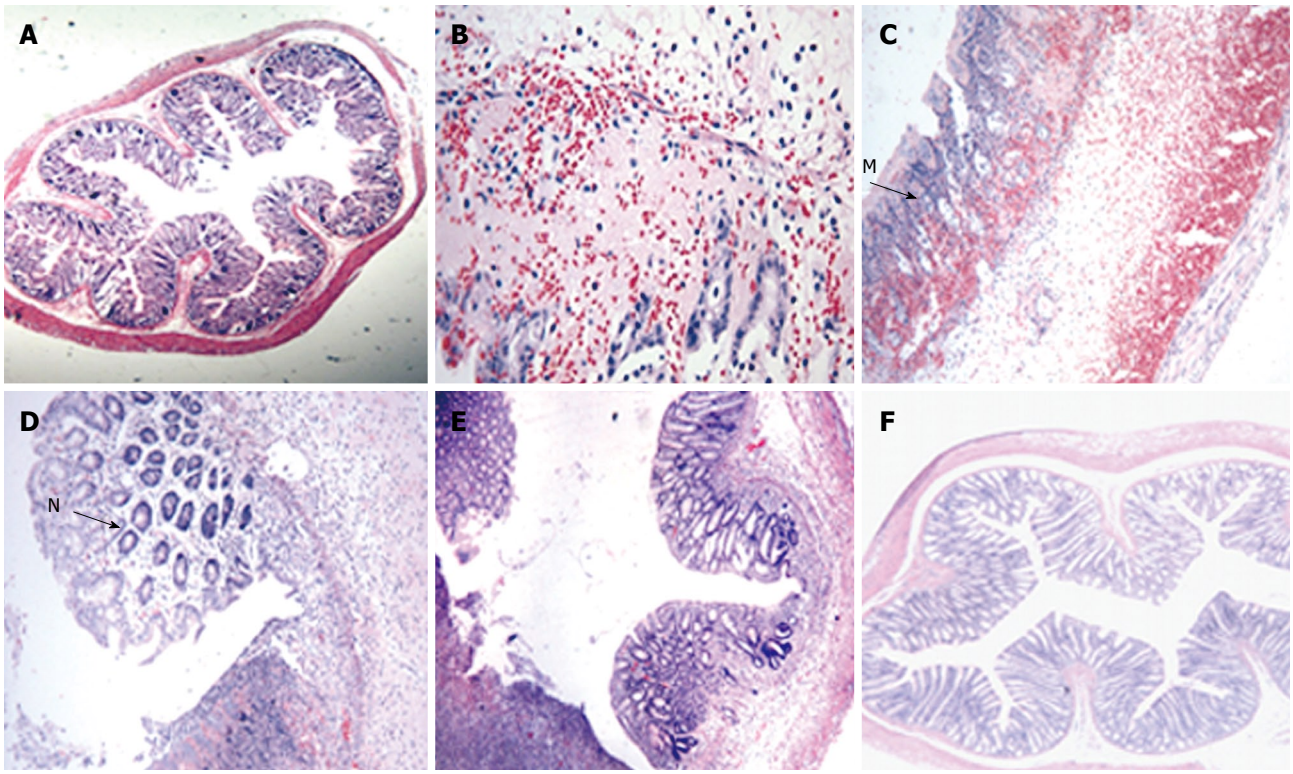


Figure 3 Light photomicrograph of colon tissues from TNBS-induced colitis rats. In control rats, normal histological colon morphology is evident (A, × 4). At day 3, oedema and haemorrhage at the mucosa with goblet cell depletion were seen (B, × 20). At day 7, severe transmural infiltration of the colon wall developed, with submucosal oedema and haemorrhage, lymphocytic and neutrophilic infiltration (M) and coagulative necrosis (C, × 10). At day 14, goblet cell regeneration (N), oedema and haemorrhage regressed by degrees (D, × 20). At day 21, abundant goblet cells and crypts developed (E, × 10). At day 28, oedema and haemorrhage had almost entirely disappeared, the structure of the muscular layer became compact and the intestinal glands developed highly (F, × 4).

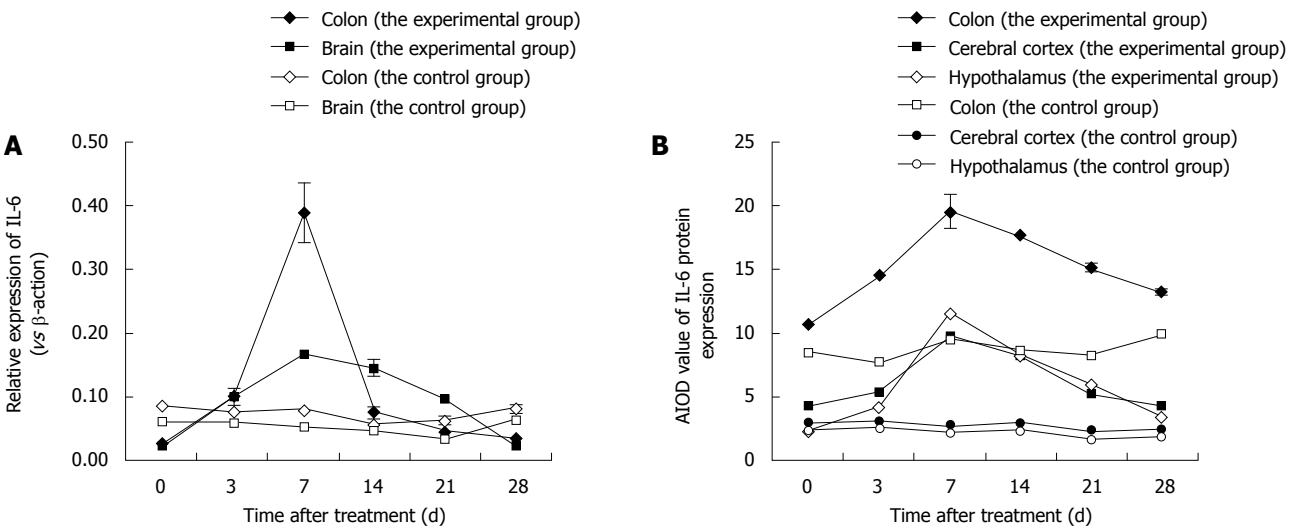


Figure 4 Variation in expression of interleukin-6 (IL-6) in the colon and brain of TNBS-induced colitis rats by time points (at days 3, 7, 14, 21 and 28) *vs* control rats. A: The expression of IL-6 mRNA after real-time RT-PCR; Data shown as mean ± SD, n = 4-8; B: The average integrated optical density (AIOD) values for IL-6 protein expression after immunohistochemical staining. The results were determined by ANOVA.

els have been developed and investigated, with the ultimate goal of understanding the causes of CD and UC[24,25]. To study the inflammatory pathways and pathogenesis of IBD, we have established models of colitis after chemical induction (TNBS) of hapten-induced gut inflammation; with this model, we have detected mRNA expression and cellular localisation of IL-6 using RT-PCR and IHC in brain and colonic tissues.

Our IHC and RT-PCR analysis of the colon demonstrate an important role for IL-6 in the development of IBD. The induction-related changes in IL-6 described here are not limited to our animal models and are seen in human IBD as well[26,27]. Although the pathogenesis of IBD is unclear, many earlier experiments have proved that IL-6

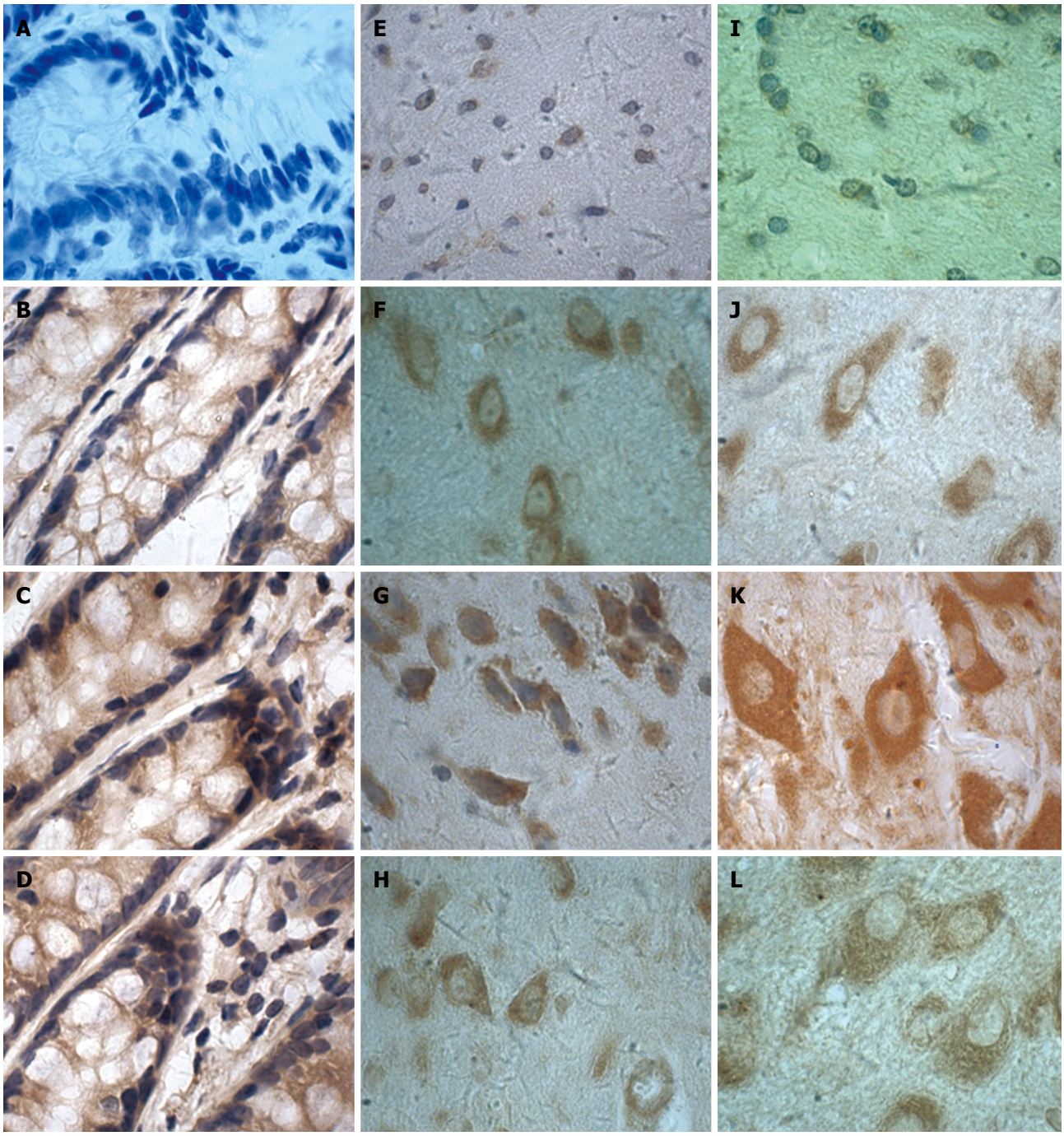


Figure 5 Immunohistochemical localisation of IL-6 proteins in colon (A-D), cerebral cortex (E-H) and hypothalamus (I-L) from TNBS-induced colitis (× 100). C, G, K: Day 7; D, H, L: Day 28; B, F, J: Day 0; A, E, I: Negative control (the primary antibody was replaced with the corresponding affinity-purified preimmune IgG).

plays an important role in the course of colitis[28,29]. IL-6 is one of several inflammatory mediators that induce lesions in the intestinal mucosa; however, if a small amount is generated, the organism's immune function may be enhanced (particularly anti-viral, anti-infectious and anti-tumour activities), while excessive production of IL-6 may induce septic shock and pyosepticaemia. IL-6 expression in CD and UC has been found to be increased[30]. Using RT-PCR, Ishiguro *et al*[31] found enhanced mRNA expression of IL-1β, IL-6, IL-8 and TNF-α in CD and UC, as well as a positive correlation of IL-6 expression levels with severity of inflammation. These results showed that when pathological changes in intestinal canal were increased, IL-6 expression in colonic tissues was gradually enhanced; with the alleviation of pathological changes in the intestinal canal, colonic IL-6 expression gradually declined and tended towards normal. It should be noted that inflammatory cells secreting IL-6 were present in intestinal inflammation sites, and their number was closely related to the severity of inflammation.

There is increased evidence to support the hypothesis of bidirectional circuits between the immune system, CNS and endocrine system[12]. Neuroimmunomodulation is one of the fastest-growing fields of study in current biomedi-

cal science. There is also abundant evidence suggesting that the CNS is not an immune-privileged area and that T lymphocytes are present in the CNS. Microglial cells and astrocytes in the CNS that are equivalent to macrophages in other tissues are the primary cells responsible for an inflammatory reaction. In normal cerebral tissues, these glial cells are quiescent, but they are highly reactive and can secrete cytokines rapidly; this is followed by changes in internal cellular environments and in cell-surface antigens. Cytokines produced by the CNS, including interleukins 1-24, TNF and TGF, can reduce the production of proinflammatory cytokines, lowering the occurrence of some pathological behaviours regulated by the CNS[32,33]. Thus, cytokines may not only play an important role in the immune system, but may also show wide-ranging central regulatory effects. Together with neuromediators and endocrine hormones, cytokines act as the intercellular signalling molecules in organisms and are involved in immune activation and information transmission using neurotransmitters and hormones; thus they are closely related to psychological reactions and mental disorders. In-depth studies on cytokines will contribute to our understanding of IBD pathogenesis and potential therapeutic options. Because neural cells produce IL-6 and have IL-6 receptors, several studies have suggested that IL-6 plays a role in regulating their response to stress. Moreover, IL-6 may be an essential neurotrophic factor in the midbrain after long-term stress[34]. Loss of IL-6 in the CNS does not lead to an improvement in memory function, but instead to memory impairment[35]. Moreover, IL-6 trans-signalling may be of paramount importance in the pathogenesis of several immune-mediated disorders, as well as diseases of the CNS[36,37]. IL-6 is an important CNS-related cytokine and is involved in disease occurrence and course, by direct or indirect regulation of neuronal excitability and neurotransmitter release after receptor binding. IL-6 also plays a significant role in glial cell differentiation and proliferation and reduces antigen presentation by glial cells in the CNS[38]. It is currently recognised that neurons and glial cells can also secrete and express cytokines, and cytokines are active during nerve growth and in the adult nervous system under normal and pathological conditions. This evidence speaks for a potential and crucial role for IL-6 in regulating the interaction of the immune system, CNS and endocrine system. We have confirmed this conclusion through the detection of IL-6 expression in the brains of IBD rats, using IHC and RT-PCR.

Over the course of IBD, we found that expression of IL-6 in cerebral cortex and hypothalamus increased when IBD was exacerbated and gradually declined with disease alleviation. Neither cytokines in cerebral tissues nor those in peripheral blood can cross the blood-brain barrier[39], and there are no reports of an association between cerebral IL-6 expression and colonic cytokines or of what their relationship might be in IBD. The CNS is a central link in the nerve-endocrine-immune regulation network and plays a leading role in the maintenance of the nerve-endocrine-immune network homeostasis. Therefore, by exploring the role of IL-6 in IBD rats from the perspective of the nerve-endocrine-immune network and homeostatic regulation, we have explored the general role of the nerve-endocrine-immune network over the course of IBD. Our results show that pathological changes were most obvious and severe at day 7 after induction of colitis, with necrosis and exfoliation of the intestinal epithelial cells and severe oedema and haemorrhage in the mucosa and muscularis layers, as well as increased expression of IL-6 in the cerebral tissues. Conversely, with the resolution of enteropathy and alleviation of symptoms, IL-6 expression in cerebral tissues began to decline and gradually trended towards levels in normal rats. Therefore, there may be a correlation between IL-6 expression in cerebral and colonic tissues. We propose that cerebral tissues may regulate and control the occurrence and development of IBD *via* IL-6.

This experiment provides new evidence concerning IBD pathogenesis, as well as an experimental model for further enriching and improving the nerve-endocrine-immune network theory. Future studies that explore signal transduction of IL-6, e.g. in IBD rats, may help to further elucidate its role in neuroimmunomodulation.

COMMENTS

Background

Since inflammatory bowel disease (IBD) appeared in Northern Europe and North America, the incidence has been rising in these areas. Currently the incidence of IBD is increasing rapidly in Asian countries especially in the last two decades. Although considerable progress has been made, a major gap in knowledge of the pathogenesis of IBD remains and has precluded the discovery of lasting, effective forms of therapy.

Research frontiers

It has become increasingly evident that interactions between the nervous system and the immune system play an important role in the pathophysiology of IBD. However, how neuroimmunomodulation plays its role in the occurrence and development of IBD is unknown. In this study, the authors demonstrate that interleukin-6 (IL-6) could play a potential role in the course of colitis.

Innovations and breakthroughs

Recent reports have highlighted the importance of cytokines, including pro- and anti-inflammatory cytokines, in the development of IBD. In particular, the imbalance of the cytokines can lead to improper immune responses and initiation of the inflammatory process. So the authors demonstrated similar trends of IL-6 in the brain and in the colon of the experimental IBD model.

Applications

By understanding the role of IL-6 in the development of IBD, this study may open a new strategy for research into the pathogenesis, prevention and treatment of the autoimmune colitis.

Peer review

This is simple and solid work with some new and interesting results.

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BRIEF ARTICLE

Covered nitinol stents for the treatment of esophageal strictures and leaks

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Abstract

AIM: To compare 2 different types of covered esophageal nitinol stents (Ultraflex and Choostent) in terms of efficacy, complications, and long-term outcome.

METHODS: A retrospective review of a consecutive series of 65 patients who underwent endoscopic placement of an Ultraflex stent (n = 33) or a Choostent (n = 32) from June 2001 to October 2009 was conducted.

RESULTS: Stent placement was successful in all patients without hospital mortality. No significant differences in patient discomfort and complications were observed between the Ultraflex stent and Choostent groups. The median follow-up time was 6 mo (interquartile range 3-16 mo). Endoscopic reintervention was required in 9 patients (14%) because of stent migration or food obstruction. No significant difference in the rate of reintervention between the 2 groups was observed (P = 0.8). The mean dysphagia score 1 mo after stent placement was 1.9 ± 0.3 for the Ultraflex stent and 2.1 ± 0.4 for the Choostent (P = 0.6). At 1-mo follow-up endoscopy, the cover membrane of the stent appeared to be damaged more frequently in the Choostent group (P = 0.34). Removal of the Choostent was possible up to 8 wk without difficulty.

CONCLUSION: Ultraflex and Choostent proved to be equally reliable for palliation of dysphagia and leaks. Removal of the Choostent was easy and safe under mild sedation.

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Key words: Dysphagia; Esophageal neoplasms; Endoscopy; Palliative care; Surgical anastomosis; Stricture; Neoadjuvant therapy; Self-expanding metal stents

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INTRODUCTION

Less than 50% of patients with esophageal carcinoma are suitable for surgery at the time of diagnosis. Most of these patients present with locally advanced or metastatic disease and/or significant comorbidities. In such circumstances, the only therapeutic option is palliative care to treat dysphagia and prevent respiratory complications secondary to aspiration.

Over the past 20 years, the use of covered self-expanding metal stents (SEMS) has proven effective in

patients with unresectable esophageal carcinoma and in those with a tracheo-esophageal fistula[1-3]. In addition, covered SEMS have been successfully used in the management of patients with anastomotic leaks or fistulas[4,5]. Despite the large number of different covered metal stents available on the market, the superiority of one type over another has not yet been proven[6,7]. The aim of this study was to review our experience with 2 types of covered nitinol stents in the management of patients with esophageal strictures and anastomotic complications.

MATERIALS AND METHODS

From July 2001 to October 2009, 422 patients with esophageal stricture due to cancer were seen at our institution. A total of 263 (62.3%) patients had surgical resection with or without neoadjuvant chemotherapy or chemoradiotherapy. We retrospectively reviewed the charts of 65 consecutive patients who underwent endoscopic placement of a covered nitinol stent. Two types of stents were used: Ultraflex (Boston Scientific, MA, USA), and, more recently, Choostent (M.I. Tech, Seoul, Korea). Ultraflex stents had a diameter of 18 or 23 mm and a length of 10 or 12 cm; Choostent stents had a diameter of 18 mm and lengths of 8, 11, 12 or 14 cm.

The indications for placement of the stent are shown in Table 1. The grade of dysphagia at presentation was defined using a 0 to 4 grading system as shown in Table 2. Demographic and clinical data are presented in Table 3.

Assessment of the extent of disease included a videoesophagram, upper endoscopy with biopsy, chest and abdominal computed tomography scan. In a subset of patients, total body positron emission tomography was performed to rule out the presence of distant metastases. If the tumor was in close proximity with the carina, a fiber-optic bronchoscopy was performed to evaluate the presence of infiltration and/or compression that could affect ventilation after placement of the stent. In some patients, Savary esophageal dilators were used to simulate the presence of the stent during the bronchoscopic examination. Written consent for the treatment was obtained from all patients.

Prior to the procedure, an antibiotic (ceftazidime 1 g iv) was administered to the patient. Endoscopic stent placement was performed with fluoroscopic guidance in the operating room, with the patient in left lateral decubitus. In 95% of the cases, conscious sedation with midazolam was used; in 3 patients propofol was required to achieve an adequate level of sedation. If the diameter of the lumen was too small to allow placement of the stent, dilatation up to 9 mm in diameter was performed with Savary bougies.

In patients with tumors of the thoracic esophagus, the proximal and distal margins of the lesion were marked with water-soluble ink injected into the submucosa. In patients with tumors located at the gastroesophageal junction, only the proximal margin was marked. In a few cases the proximal and distal limits of the tumor were marked using metallic endoclips[8]. Complete opening of the stent was expected within 48-72 h after implantation. In most patients, no attempt was made to pass the stent with the endoscope after its deployment in order to prevent migration.

Table 1 Indication for use of the stent in 65 patients

	n (%)
Esophageal carcinoma	51 (78.5)
Adenocarcinoma	31
Squamous-cell carcinoma	20
Complications of esophagogastric anastomosis	8 (12.3)
Fistula	3
Stricture	3
Tumor recurrence	2
Extrinsic compression	4 (6.1)
Lung cancer	2
Pleural mesothelioma	1
Mediastinal lymphoma	1
Post-radiotherapy stricture	2 (3.1)

Table 2 Classification of dysphagia

Grade	Definition
0	Normal swallowing
1	Able to swallow some solid food
2	Able to swallow semi-liquid food
3	Able to swallow liquids only
4	Absolute dysphagia

After the procedure, a non-steroidal antiinflammatory (ketorolac) and proton pump inhibitors (e.g. pantoprazole) were administered intravenously. Chest X-ray and a gastrografin swallow study were performed the day after stent placement. Patients were then allowed to eat a semi-liquid diet until discharge.

RESULTS

Stent placement was technically successful in all patients. Nine patients (13.8%) presenting with severe stricture required preliminary dilation before deployment of the Ultraflex (n = 5) or the Choostent (n = 4). The procedure took a mean of 16 min (range, 12-35 min) with the Ultraflex, and 17 min (range, 13-27 min) with the Choostent (P = 0.8). There were no deaths related to the procedure. Periprocedural complications occurred in 4 patients (6.1%): 2 had fever probably related to aspiration pneumonia, 1 had an episode of atrial fibrillation managed with amiodarone iv, and 1 had acute urinary retention requiring catheterization. The 2 types of stent showed equal palliative efficacy against dysphagia. Most patients were discharged within 48 h. The results of the treatment are summarized in Table 4.

Early and late post-procedural complications are shown in Table 5. Severe chest pain immediately after stent insertion was present in 3 patients who had an Ultraflex implanted. The pain disappeared within 36 h of iv infusion of morphine. Overall, 9 patients (14%) needed a second endoscopic intervention. In 1 patient of the

Table 3 Demographic and clinical data of the patient population

	Total	Ultraflex	Choostent	*P*-value
Patients	65	33	32	-
Male/female	43/22	23/10	20/12	0.5
Mean age (range)	67.5 (34-86)	66.8 (34-83)	68.3 (42-86)	0.6
Grade of dysphagia (mean ± SD)	3.4 ± 0.4	3.4 ± 0.3	3.5 ± 0.4	0.9
Diagnosis				
Adenocarcinoma	31	16	15	0.9
Squamous cell carcinoma	20	11	9	0.8
Extrinsic compression	4	2	2	0.9
Anastomotic fistula	3	2	1	0.6
Anastomotic stricture	3	1	2	0.5
Anastomotic recurrence	2	1	1	0.9
Post-radiotherapy stricture	2	0	2	0.1
Site of lesion				
Cervical	3	2	1	0.6
Upper thoracic	9	6	3	0.3
Middle thoracic	21	11	10	0.9
Lower thoracic	32	14	18	0.4
Tumor stage (n = 51)				
Ⅲ	19	12	7	0.6
ⅣA	17	8	9	0.7
ⅣB	15	7	8	0.5

Table 4 In-hospital characteristics and long-term outcome after stent placement

	Ultraflex (n = 33)	Choostent (n = 32)	*P*-value
Duration of the procedure (min)	16 (12-35)	17 (13-27)	0.8
Median hospital stay (d)	2 (2-7)	2 (2-4)	0.7
Hospital mortality (%)	0	0	1.0
Hospital morbidity (%)	6.1 (2/33)	6.2 (2/32)	0.8
Pain score (scale 0-10)	6.3	4.8	0.2
Residual dysphagia (grade)	1.9 ± 0.3	2.1 ± 0.4	0.6
Mean survival (mo)	6.5 (1-19)	6 (3-26)	0.7

Table 5 Early and late complications after stent placement

	n (%)	Ultraflex (n = 33)	Choostent (n = 32)	*P*-value
Abrasion of soft palate	1 (1.5)	1	0	0.3
Odynophagia	1 (1.5)	0	1	0.3
Malposition	1 (1.5)	0	1	0.3
Late distal migration	3 (4.6)	2	1	0.6
Persistent chest pain	3 (4.6)	2	1	0.6
Persistent hiccups	3 (4.6)	1	2	0.6
Gastroesophageal reflux	14 (22)	7	7	0.9
Food obstruction	5 (7.6)	2	3	0.6

Choostent group, the radiographic control showed malpositioning of the stent (too distal release) thus requiring the insertion of a second device overlapping the first one. No stent migration was observed within 72 h after starting oral intake. Interestingly, symptomatic gastroesophageal reflux occurred in 14 (43.7%) of the 32 patients with a stent placed in the lower esophagus.

Upper gastrointestinal endoscopy was performed 1 mo after the procedure in 21 patients with the Ultraflex and in 19 patients with the Choostent. None of these individuals were complaining of dysphagia. The cover membrane of the Choostent appeared to be damaged more frequently compared to the Ultraflex (26% *vs* 14%, $P = 0.34$).

Satisfactory palliation of dysphagia was achieved also in patients with stricture of the esophagogastric anastomosis, and post-radiotherapy stricture. In the majority of these individuals the Choostent was easily removed 3 to 4 wk after the insertion under mild intravenous sedation. One of the 2 patients with an Ultraflex stent required general anesthesia for removal because of a marked tissue reaction and embedding of the proximal edge of the stent.

The worst clinical outcome was recorded in patients suffering from extrinsic malignant compression. One of these patients with dysphagia caused by a bronchial carcinoma died because of massive bleeding 21 d after stent placement. The other 3 patients did not achieve complete palliation of dysphagia and died within 2 mo because of progression of the underlying disease.

The stenting procedure was effective in 2 of the 3 patients with fistula of the esophagogastric anastomosis. The stent was successfully removed in all patients after a mean of 4 wk. Radiological evaluation showed persistent leakage in 1 patient who required insertion of another stent.

Twenty-six of the 65 patients (40%) received chemotherapy or chemoradiotherapy after stent implantation. In 7 patients, a Choostent was uneventfully removed under mild sedation within 8 wk from the beginning of chemotherapy and oral intake was well tolerated. Three of these patients showed significant down-staging of the disease that eventually allowed esophagectomy to be performed without complications.

The incidence of mechanical complications requiring further endoscopic intervention after stent implantation was similar in patients treated or not with chemotherapy or chemoradiotherapy. In treated patients (n = 26) there was an 11.5% incidence of stent dislocation, whereas in

patients who did not receive additive treatment (n = 31) there was a 12.9% incidence of food obstruction.

DISCUSSION

Self-expanding metal stents came onto the market at the beginning of the 1990s and gradually replaced the old Celestin tube. The endoscopic placement of SEMS has proven to be technically easier, requiring minimal dilatation, and resulting in less morbidity and better palliation of dysphagia. In addition, SEMS provide a better quality of life[9], particularly for patients with a Karnofsky index greater then 50[10]. The efficacy of these stents, the ease of insertion, and the large spectrum of diameters and lengths available has resulted in their widespread use also in patients with anastomotic leaks[11].

The standardization of the endoscopic technique and the precise placement mechanism have reduced, but not eliminated, the rate of intraoperative complications. Late complications range from 26% to 52%, especially in patients with adenocarcinoma, and complications requiring additional intervention are frequent[12]. The choice of the correct stent diameter in each patient may represent an important factor for the success of the procedure. The use of an Ultraflex stent with a large diameter significantly reduced the chances of recurrent dysphagia, formation of granulation tissue, and the risk of food obstruction compared to other SEMS[13]. Despite the reported high incidence of covered stent migration at the gastroesophageal junction[14], we believe this is still the best available palliative option in these patients. However, the overall incidence of symptomatic gastroesophageal reflux was 22% in our series, and was almost double (43.7%) among the 32 patients with a stent placed in the lower esophagus. Theoretically, the use of stents provided with an antireflux valve can prevent gastroesophageal reflux, thereby avoiding the risk of aspiration pneumonia[15].

To date, no significant differences in outcomes or complication rates have been reported with the available covered SEMS. The present study shows that there are no statistically significant differences between the Ultraflex and the Choostent, although insertion of the Choostent in the oro-pharynx was less traumatic and post-procedural pain was reduced in the Choostent group. In general, we found it more convenient to use the Ultraflex in patients with strictures of the proximal esophagus because of the ease of stent application under visual control (proximal release). In contrast, the distal release mechanism of the Choostent still allows the operator to modify the site of delivery before the stent has reached 50% of the maximum diameter. Interestingly, a greater frequency of degeneration of the covering film was observed at the 1-mo follow-up endoscopy in the Choostent group compared to the Ultraflex group.

Another finding of this study is that temporary stent placement has indeed a role in patients undergoing neo-adjuvant therapy and in those with anastomotic complications or post-radiotherapy strictures. In such circumstances, the complete cover membrane of the Choostent may cause less granulation tissue and allows easier removability a few weeks later. The benefit of temporary stent insertion has been suggested as a "bridge" to surgery in patients undergoing neo-adjuvant therapy[16]. This minimally invasive and reversible treatment can represent an alternative to trans-nasal feeding tube placement, endoscopic percutaneous gastrostomy, or jejunostomy. Stent placement is usually better accepted by patients, but no conclusive scientific evidence exists on this issue[16,17]. In our series, the use of a Choostent device allowed optimal nutrition and tolerance of neo-adjuvant therapy until tumor downstaging was documented. The stent was easily removed under mild sedation within 2 mo in 7 patients, 3 of whom underwent surgical resection without complications.

In conclusion, covered nitinol stents are safe and effective devices for palliation of dysphagia in patients with esophageal strictures. The Ultraflex and the Choostent proved to be equally reliable in the achievement of this goal. Close patient monitoring is required to avoid late complications. When temporary stent insertion is required, as in patients undergoing neo-adjuvant therapy and in those with anastomotic complications or post-radiotherapy strictures, the Choostent is preferable because of its easy and safe removal.

COMMENTS

Background

Less than 50% of patients with esophageal carcinoma are suitable for surgery at the time of diagnosis. In such circumstances, the self-expanding metal stents (SEMS) represent an excellent palliative option. Covered SEMS have also been used in the management of patients with anastomotic complications, malignant extrinsic compression of the esophagus, and post-radiotherapy stricture.

Research frontiers

Despite the large number of covered metal stents available on the market, the superiority of one type over another has not been proven yet in the management of esophageal strictures and leaks.

Innovations and breakthroughs

This study compared two different types of nitinol stents that were proven to be equally reliable for palliation of dysphagia and leaks. Both stents also proved to be easily removable up to 2 mo after insertion.

Applications

Temporary stent placement has a role in patients undergoing neo-adjuvant therapy and in those with anastomotic complications or post-radiotherapy strictures. This minimally invasive and reversible treatment can represent an alternative to trans-nasal feeding tube placement, endoscopic percutaneous gastrostomy, or jejunostomy in selected patients.

Peer review

This is an interesting and worthwhile report on the use of expandable covered metal stents for the treatment of esophageal problems.

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BRIEF ARTICLE

Insulin resistance is associated with hepatocellular carcinoma in chronic hepatitis C infection

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Abstract

AIM: To elucidate the role of insulin resistance (IR) and serum adiponectin level in hepatocellular carcinoma (HCC) associated with chronic hepatitis C.

METHODS: Clinical and biochemical characteristics were collected from 165 consecutive patients with newly diagnosed HCC. Homeostasis model assessment of IR (HOMA-IR) and serum adiponectin level were investigated in 188 patients with different stages of hepatitis C virus (HCV) infection.

RESULTS: Among HCC patients, type 2 diabetics (DM) was more prevalent in HCV subjects (35.6%, n = 59) compared to hepatitis B virus (HBV; 12.7%, n = 63) or non-HBV, non-HCV cases (7.1%, n = 28). In patients with chronic hepatitis C, HCC subjects had higher blood sugar (P < 0.001), insulin level (P = 0.003) and HOMA-IR (P = 0.018) than those with chronic hepatitis and advanced fibrosis. Age, male sex and body mass index were significantly associated with serum adiponectin level, whereas HOMA-IR was not. Based on stepwise logistic regression analysis, age (OR: 1.124, P < 0.001), serum insulin level (OR: 1.585, P < 0.001), HOMA-IR (OR: 0.495, P = 0.001), DM (OR: 11.601, P = 0.002) and male sex (OR: 3.877, P = 0.016) were independently associated with HCC. This result was similar even if the diabetic subjects were excluded for analysis.

CONCLUSION: Insulin resistance measured by HOMA-IR, regardless of the presence of diabetes, is significantly associated with HCC development in patients with chronic HCV infection.

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Key words: Hepatitis C virus; Hepatocellular carcinoma; Insulin resistance; Diabetes; Adiponectin

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INTRODUCTION

Hepatitis C virus (HCV) infects hundreds of millions of

people persistently, and induces a spectrum of chronic liver disease worldwide[1]. Chronic HCV infection causes progressive hepatic fibrosis and cirrhosis in up to 20% of patients, and approximately 10%-20% of cirrhotic patients may develop hepatocellular carcinoma (HCC) within 5 years[2-4]. Recent cohort studies have indicated that HCC is the most frequent cause of death in patients infected with HCV, and epidemiological trends suggest that the mortality rate is rising[5]. Thus, understanding the risk factors for HCC development in patients infected with HCV is of great importance.

HCV may contribute to carcinogenesis by causing advanced fibrosis or cirrhosis, which represents a precancerous state accompanied by increased DNA synthesis[6,7]. Nevertheless, several factors associated with HCC development in chronic hepatitis C have been reported, such as male sex, older age at infection, excessive alcohol consumption, coinfection with hepatitis B virus (HBV) and some viral variability in HCV itself[8-11]. Recently, epidemiological studies have demonstrated that diabetes mellitus (DM) is associated with a 2-4-fold increase in the risk of HCC, regardless of the presence of other major HCC risk factors (HBV, HCV, and alcoholic liver disease)[12-15]. In particular, two large cohort studies have shown that DM is associated with a higher risk of HCC development in patients with chronic hepatitis C compared to HBV-infected subjects or those without HBV and HCV infections[12,13].

The mechanisms that may link DM with carcinogenesis in chronic HCV infection remain unclear. Insulin resistance (IR), which correlates inversely with circulating adiponectin concentration, is a consistent finding in patients with type 2 DM[16,17]. Previous studies have shown that patients infected with HCV have significantly higher IR than healthy controls matched for age, sex and body mass index (BMI)[18,19]. Recent studies have suggested that IR plays a crucial role in fibrosis progression, and has been demonstrated to have a negative impact on treatment responses to antiviral therapy in patients with chronic hepatitis C[18,20,21]. IR has emerged as a risk factor for a wide variety of cancers, including endometrial and breast (especially after menopause), colon and rectal, esophageal, kidney, pancreatic, biliary, ovarian and cervical cancer[22-24]. To the best of our knowledge, the role of IR and serum adiponectin level in the development of HCC associated with chronic HCV infection has not been established. In this present study, we prospectively investigated the IR assessed by the homeostasis model (HOMA-IR) and serum adiponectin level in patents with HCC and those with other clinical stages of chronic HCV infection.

MATERIALS AND METHODS

Patients

From January 2007 to August 2008, a total of 165 newly diagnosed patients with HCC (122 men and 43 women; median age: 60.1 ± 12.4 years) who fulfilled all criteria outlined below were consecutively collected in a single center. The diagnosis of HCC was based on either the histopathological findings in tumor tissue, one typical HCC feature on a dynamic image or alpha-fetoprotein (AFP) > 200 ng/mL if the nodule was > 2 cm in cirrhotic liver, or two typical HCC features of dynamic images if the nodule was between 1 and 2 cm in a cirrhotic liver[25]. Patients with concurrent human immunodeficiency virus infection, significant change of body weight (≥ 3 kg within 3 mo), previous history of interferon-based antiviral therapy, and current treatment with any dosage of insulin therapy were excluded.

Of these patients, 63 were positive for hepatitis B surface antigen (HBsAg) (Abbott Laboratories, North Chicago, IL, USA), 59 were positive for anti-HCV antibody (third-generation ELISA; AxSYM HCV 3.0; Abbott Laboratories), 15 were positive for both HBsAg and anti-HCV, and 28 were negative for HBsAg and anti-HCV, in whom alcoholic liver disease (n = 11, 39%) was the major cause of HCC.

During the same period, 129 consecutive patients (61 men and 68 women, 23-77 years old; median age: 53.0 ± 11.5 years) with chronic HCV infection who consulted our clinics were studied, including 86 with chronic hepatitis (F0-2) and 43 with advanced fibrosis (F3-4). All patients had positive anti-HCV antibody and detectable HCV RNA (Amplicor™; Roche Diagnostics, Branchburg, NJ, USA). Pathological diagnosis of chronic hepatitis or advanced fibrosis was made by percutaneous liver biopsies according to the modified Knodell histological activity index[26], which were analyzed by pathologists who were blind to the patients' characteristics. The Human Research and Ethics Committee (Institutional Review Board) approved the study, and informed consent was obtained from each patient involved in the study.

Clinical and laboratory assessments

Patients with a BMI of 18.5-24.9 kg/m^2 were classified as normal, 25-29.9 as overweight, and ≥ 30 as obese. The diagnosis of type 2 DM was based on the American Diabetes Association revised criteria, using a value of fasting blood glucose of ≥ 126 mg/dL on at least two occasions[27], or ongoing treatment with hypoglycemic agents.

Blood glucose, serum insulin level and stored serum samples for adiponectin were collected after 12 h of overnight fasting from each individual. For HCC patients, serum samples were collected before any treatment for tumor. Serum insulin was measured by radioimmunoassay (Coat-A-Count insulin kit; Diagnostic Products Corp., Los Angeles, CA, USA). IR was calculated by the HOMA-IR using the following formula: HOMA-IR = fasting insulin (μU/mL) × plasma glucose (mmol/L)/22.5. Circulating level of adiponectin was measured in duplicate by sandwich ELISA using commercial kits according to the manufacturer's instructions (Quantikine ELISA kits; R&D Systems, Inc., Minneapolis, MN, USA). The differences between duplicate wells were consistently less than 10% of the mean values. The mean values of duplicate measurements were used in the analyses.

Table 1 Comparison of baseline features among HBV, HCV, dual HBV and HCV, and non-HBV, non-HCV related hepatocellular carcinoma patients

	HBV *n* = 63	HCV *n* = 59	HBV + HCV *n* = 15	Non-HBV, non-HCV *n* = 28	*P* value
Age (yr)[1]	55 (46-66)[a,c]	66 (57-75)[a,d]	60 (50-68)[d]	61 (55-67)[c]	< 0.001
Sex (M/F)	45/18	41/18	10/5	26/2	0.093
DM (%)	8 (12.7)[a]	21 (35.6)[a,e]	4 (26.7)	2 (7.1)[e]	0.003
BMI (kg/m^2)[1]	24.3 (22.2-26.8)	23.8 (21.8-26.6)	23.6 (20.9-25.5)	24.5 (23.3-26.7)	0.439
Overweight (%)	24 (38)	22 (37)	4 (27)	10 (36)	0.797
Obesity (%)	4 (6)	5 (8)	1 (7)	2 (7)	0.965
AST (U/L)[1]	48 (38-71)[a]	72 (39-123)[a,e]	72 (47-111)	38 (29-52)[e]	0.002
ALT (U/L)[1]	45 (34-63)[a]	63 (29-100)[a,e]	82 (54-117)	27 (21-39)[e]	0.011
Child–Pugh class (A/B, C)	47/16	46/13	11/4	23/5	0.857
Platelet (10^4/μL)[1]	16.5 (12.0-25.0)[a]	13.2 (8.7-17.1)[a,e]	12.7 (7.6-18.1)[f]	21.1 (15.3-24.1)[e,f]	0.001
AFP (ng/mL)[1]	15 (6-525)	28 (7-256)	38 (11-4388)	15 (6-8027)	0.468
Tumor size (≤ 3 cm) (%)	14 (22.2)[a]	29 (49.2)[a,e]	7 (46.7)	5 (17.9)[e]	< 0.001
BCLC (≤ early stage) (%)	20 (31.7)[a,b]	31 (52.5)[a,e,f]	9 (60.0)[b]	4 (14.3)[e,f]	0.001
HOMA-IR	3.5 (2.0-8.6)[a]	4.4 (2.9-6.6)[a]	3.2 (1.7-10.7)	3.4 (2.0-4.6)	0.108
Insulin (μU/mL)[1]	12.8 (8.4-25.0)	14.8 (9.9-21.30)	11.9 (8.1-23.5)	9.7 (7.6-14.8)	0.256
Adiponectin (μg/mL)	4.7 (2.9-8.1)[a,c]	7.9 (5.2-11.0)[a,e]	8.0 (3.8-10.9)[f]	3.7 (2.0-6.0)[c,e,f]	0.002

[1]Median (interquartile range); *P* value by one-way ANOVA test or χ^2 test; [a]$P < 0.05$ between HBV and HCV; [b]$P < 0.05$ between HBV and HBV + HCV; [c]$P < 0.05$ between HBV and non-HBV, non-HCV; [d]$P < 0.05$ between HCV and HBV + HCV; [e]$P < 0.05$ between HCV and non-HBV, non-HCV; [f]$P < 0.05$ between HBV + HCV and non-HBV, non-HCV HCC with LSD post-hoc correction. HBV: Hepatitis B virus; HCV: Hepatitis C virus; DM: Diabetes mellitus; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; BCLC: Barcelona clinic liver cancer; HOMA-IR: Homeostasis model assessment of insulin resistance.

Statistical analysis

Continuous data are expressed as the median (interquartile range), and the categorical data are expressed as a number (percentage). Comparisons of differences in the categorical date between groups were performed using the χ^2 test. Distributions of continuous variables were analyzed by the Mann-Whitney *U* test or one-way ANOVA test with least significant difference (LSD) post-hoc correction between groups where appropriate. Spearman's correlation coefficient analysis was used to evaluate the factors associated with HOMA-IR and adiponectin level. Multiple linear regression analysis with stepwise variable selection was performed to assess the independent factors. Stepwise logistic regression analysis was performed to assess the influence of each factor on the risk of developing HCC. All analyses were carried out using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA). All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of baseline features among HBV, HCV, dual HBV/HCV, and non-HBV, non-HCV-related HCC

Table 1 shows the comparison of baseline features among the 165 patients with HCC related to different etiology. The median age of HCV-related HCC patients (66 years) was significantly higher than that in HCC patients infected with HBV (55 years) or dual HBV/HCV (60 years) ($P < 0.001$). There was a male predominance among all four groups. The prevalence of DM was higher (35.6%) in patients with HCV-related HCC compared to those infected with HBV (12.7%) ($P < 0.005$) or non-HBV, non-HCV subjects (7.1%) ($P < 0.005$). HOMA-IR was higher in HCC patients with HCV (median 4.4) than in those with HBV (median 3.5) ($P < 0.05$). However, there was no significant difference in BMI and prevalence of overweight and obesity among the four groups. Patients with HCV-related HCC had significantly higher aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and lower platelet counts than those infected with HBV or non-HBV, non-HCV subjects. In addition, patients with HCV-related HCC had smaller tumors and earlier Barcelona clinic liver cancer stage (BCLC) than HBV or non-HBV, non-HCV subjects.

Comparison of baseline features, serum insulin, HOMA-IR and adiponectin level among chronic hepatitis, advanced fibrosis and HCC patients

The comparison of baseline features, serum insulin, HOMA-IR and adiponectin level in different clinical stages of chronic HCV infection is shown in Table 2. The median age for HCC patients (66 years) was significantly higher than those with advanced fibrosis (56 years) and chronic hepatitis (53 years) ($P < 0.001$). Patients with HCC had a higher male-to-female ratio and higher prevalence of DM than those with advanced fibrosis or chronic hepatitis. There was no significant difference in BMI among these three groups. The HCC subjects had lower AST and ALT levels compared to those with advanced fibrosis; however, the platelet count was comparable between these two groups. Patients with HCC had higher blood sugar ($P < 0.001$), insulin level ($P = 0.003$) and HOMA-IR ($P = 0.018$) than those with chronic hepatitis and advanced fibrosis. As shown in Figure 1, patients with HCC had a higher ratio of HOMA-IR > 4 (61.8%, 95% CI: 48.6%-75.1%) than those with chronic hepatitis (39.5%, 95% CI: 29.0%-50.1%) and advanced fibrosis (48.8%, 95% CI: 33.3%-64.4%) ($P = 0.036$). There was no significant difference in serum adiponectin among the three groups.

Table 2 Comparison of baseline features, HOMA-IR and adiponectin level among chronic hepatitis, advanced fibrosis and HCC patients with chronic HCV infection

	Chronic hepatitis (F0-2) *n* = 86	Advanced fibrosis (F3-4) *n* = 43	Hepatocellular carcinoma *n* = 59	*P* value[2]
Age (yr)[1]	53 (45-58)[a,b]	56 (49-63)[a,c]	66 (57-75)[b,c]	< 0.001
Male sex (%)	44 (51)[b]	17 (40)[c]	41 (69)[b,c]	0.008
DM (%)	13 (15)[b]	12 (28)	21 (36)[b]	0.016
BMI (kg/m^2)[1]	24.3 (21.8-26.3)	25.0 (22.0-29.4)	23.8 (21.8-26.6)	0.227
AST (U/L)[1]	90 (64-122)	114 (87-178)[c]	72 (39-123)[c]	0.085
ALT (U/L)[1]	127 (92-187)[b]	140 (102-199)[c]	63 (29-100)[b,c]	0.001
Platelet (10^4/μL)[1]	20.2 (15.8-22.4)[a,b]	13.7 (10.6-17.9)[a]	13.2 (8.7-17.1)[b]	< 0.001
Sugar (mg/dL)[1]	91 (87-101)[b]	98 (88-122)[c]	114 (94-172)[b,c]	< 0.001
Insulin (μU/mL)[1]	10.3 (7.7-14.4)[b]	11.7 (6.9-15.9)[c]	14.8 (9.9-21.3)[b,c]	0.003
HOMA-IR[1]	3.5 (2.6-4.7)[b]	4.1 (2.6-5.9)[c]	4.4 (2.9-6.6)[b,c]	0.018
Adiponectin (μg/mL)[1]	5.0 (3.4-8.4)	5.8 (4.1-9.7)	7.9 (5.2-11.0)	0.222

[1]Median (interquartile range); [2]*P* value by one-way ANOVA test or χ^2 test; [a]*P* < 0.05 between chronic hepatitis and advanced fibrosis; [b]*P* < 0.05 between chronic hepatitis and hepatocellular carcinoma; [c]*P* < 0.05 between advanced fibrosis and hepatocellular carcinoma with LSD post-hoc correction.

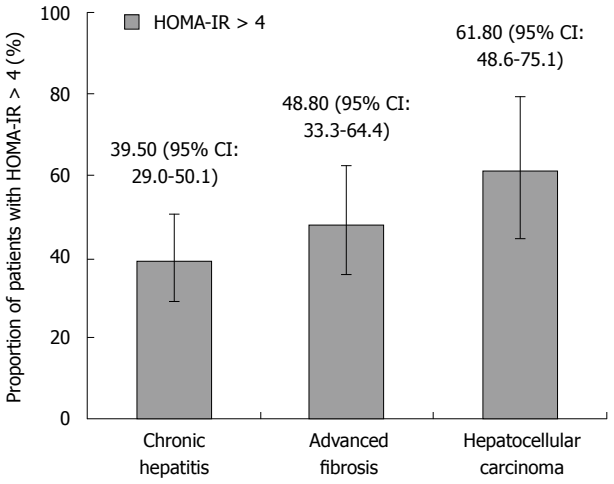


Figure 1 Comparison of high homeostasis model assessment of insulin resistance (HOMA-IR) (> 4) among different stages of chronic hepatitis C virus (HCV) infection (*P* = 0.036).

Factors associated with serum adiponectin level in patients with chronic hepatitis C

Table 3 shows the factors associated with serum adiponectin level in 188 patients with chronic HCV infection. By univariate analysis, age (r = 0.388, P < 0.001), male sex (P < 0.001), BMI (r = -0.281, P < 0.001), AST level (r = 0.159, P = 0.030), platelet count (r = -0.198, P = 0.009), insulin level (r = -0.179, P = 0.014) and HOMA-IR (r = -0.290, P < 0.001) were significant factors associated with serum adiponectin level. Multiple linear regression analysis showed that age (regression coefficient = 0.140, P < 0.001), male sex (regression coefficient = -2.925, P < 0.001) and BMI (regression coefficient = -0.495, P < 0.001) were independent variables.

Stepwise logistic regression analysis for factors associated with development of HCC

Based on stepwise logistic regression analysis, significant factors associated with development of HCC in patients with chronic HCV infection were age (OR: 1.124, 95% CI: 1.067-1.183, P < 0.001), serum insulin level (OR: 1.585, 95% CI: 1.269-1.980, P < 0.001), HOMA-IR (OR: 0.495, 95% CI: 0.330-0.743, P = 0.001), DM (OR: 11.601, 95% CI: 2.50-53.8, P = 0.002) and male sex (OR: 3.877, 95% CI: 1.282-11.729, P = 0.016) (Table 4).

When excluding DM cases, factors independently associated with HCC development in 142 non-DM patients were age (OR: 1.170, 95% CI: 1.075-1.272, P < 0.001), serum insulin level (OR: 2.434, 95% CI: 1.555-3.811, P < 0.001), HOMA-IR (OR: 0.158, 95% CI: 0.055-0.452, P = 0.001) and male sex (OR: 6.111, 95% CI: 1.310-28.49, P = 0.021).

Table 3 Univariate and multivariate analysis of factors associated serum adiponectin level in 188 patients with chronic HCV infection

	Univariate		Multivariate		
	Coefficient	*P* value[1]	Regression coefficient	SE	*P* value[3]
Age	0.388	< 0.001	0.140	0.028	< 0.001
Male sex[2]	NA	< 0.001	-2.925	0.744	< 0.001
BMI	-0.281	< 0.001	-0.495	0.101	< 0.001
HCC[2]	NA	0.136	-	-	-
DM[2]	NA	0.629	-	-	-
Child-Pugh classification	0.107	0.145	-	-	-
AST (U/L)	0.159	0.030	-	-	-
ALT (U/L)	-0.096	0.195	-	-	-
Platelet (10^4/μL)	-0.198	0.009	-	-	-
Insulin (μU/mL)	-0.179	0.014	-	-	-
HOMA-IR	-0.290	< 0.001	-	-	-

[1]*P* value by Spearman's test or [2]*P* value by Mann-Whitney *U* test; [3]*P* value by stepwise linear regression analysis. NA: Not applicable.

DISCUSSION

There is increasing evidence linking chronic HCV infection and type 2 DM. Large community-based studies have shown that the prevalence of DM in HCV-infected patients is much higher than that observed in the general population, and in patients with other chronic liver diseases such as HBV and alcoholic liver disease[28,29].

Table 4 Stepwise logistic regression analysis of factors associated with HCC

	Comparison	OR	95% CI	*P* value
All patients				
Age	Per 1 year increase	1.124	1.067-1.183	< 0.001
Insulin	Per 1 μU/mL increase	1.585	1.269-1.980	< 0.001
HOMA-IR	Per 1 increase	0.495	0.330-0.743	0.001
DM	Yes *vs* no	11.601	2.500-53.800	0.002
Sex	Male *vs* female	3.877	1.282-11.729	0.016
Non-DM patients				
Age	Per 1 year increase	1.170	1.075-1.272	< 0.001
Insulin	Per 1 μU/mL increase	2.434	1.555-3.811	< 0.001
HOMA-IR	Per 1 increase	0.158	0.055-0.452	0.001
Sex	Male *vs* female	6.111	1.310-28.499	0.021

In this study, we found that among HCC patients, type 2 DM was more prevalent in those infected with HCV compared to those with HBV or non-HBV, non-HCV infection. This observation in accordance with previous studies suggests a strong synergistic effect of metabolic factors and viral hepatitis in HCC development among HCV-infected patients[12,13]. Although there was no significant difference in BMI among HCC patients with different etiology, this could be explained by the low prevalence of obesity in our study population.

Chronic HCV infection is associated with the development of hepatic steatosis and unique, virus-specific alterations in host metabolism leading to the development of IR[19,30,31]. In this present study, we provide the first evidence that IR could potentially increase the risk of developing HCC in patients with chronic HCV infection. In a cross-sectional, hospital-based setting, we prospectively assessed the HOMA-IR value in different clinical stages of chronic HCV infection. Our data showed that patients with HCC had a higher ratio of HOMA-IR > 4 than those with chronic hepatitis and advanced fibrosis. Furthermore, after adjusting for age and sex, HOMA-IR was an independent factor associated with the development of HCC. A novel finding of our work, not specifically evaluated in other studies, was the association of IR, regardless of diabetes, with the development of HCC. An alternative explanation for the observed association between HOMA-IR and HCC is that advanced hepatic fibrosis and disease severity results in IR and impairs insulin clearance. This possibility could be excluded by the similar prevalence of DM and platelet count that has been considered as a fibrosis marker in chronic HCV infection between patients with HCC and those with advanced fibrosis. Also, HOMA-IR did not correlate with Child-Pugh classification, which suggests that disease severity was not associated with IR in patients with HCC or advanced fibrosis.

Although our work was not designed to clarify the pathogenic interaction between IR and the development of HCC, a few hypotheses can be put forward. IR is defined as an increased requirement for insulin to maintain normal metabolic function, which results in the compensatory development of hyperinsulinemia[32]. Recent evidence has suggested that hyperinsulinemia can promote the synthesis and biological activity of insulin-like growth factor 1 (IGF-1), which is a peptide hormone that regulates energy-dependent growth processes[33]. IGF-I stimulates cell proliferation and inhibits apoptosis and has been shown to have strong mitogenic effects on a wide variety of cancer cell lines. Changes in the expression pattern of IGF-system components have been observed in patients with HCC, in human HCC cell lines and in their conditioned culture medium, as well as in rodent models of hepatocarcinogenesis[34].

To study the role of adiponectin in HCC may be more complex because of its underlying chronic hepatitis infection[19,35,36]. Previous studies have demonstrated that circulating adiponectin levels are inversely associated with the risk of malignancies associated with IR, including endometrial, breast, colon and gastric cancer[37-40]. Moreover, serum adiponectin level has been reported to be significantly elevated in chronic liver disease, and correlated with stage of liver cirrhosis, liver cell injury, e.g. aminotransferase activity, and inflammatory markers[35,36]. Thus, serum adiponectin level is modified according to the two opposing factors, IR and underlying liver condition. In this study, we found no difference in serum adiponectin level among different clinical stages of chronic HCV infection. Although HOMA-IR score was inversely associated with serum adiponectin level by univariate analysis, multiple linear regression analysis did not support this correlation.

There are some limitations to our study. First, the analysis was carried out in a cross-sectional setting with a relatively small number of HCC patients, and it would be interesting to determine whether this association holds true in longitudinal follow-up studies of larger groups of patients. Second, the cohort of patients, at low prevalence of obesity, was enrolled in a tertiary referral center for liver disease, which limits the broader application of the results. A further methodological issue resides in the inability to dissect the temporal relation between IR and HCC. Another limitation lies in the fact that there is some concern on the use of HOMA-IR in the presence of long-standing DM. However, a diagnosis of DM is *per se* expression of IR, and HOMA-IR is a less invasive, inexpensive, and less labor-intensive method to measure IR as compared with the glucose clamp test.

In conclusion, we demonstrated the independent association between IR and HCC development in chronic HCV infection. These findings may have important prognostic and therapeutic implications in the management of chronic HCV-infected patients. Since IR is a potentially modifiable factor, therapeutic intervention aimed at decreasing IR may be warranted in these patients.

COMMENTS

Background

Recent studies have demonstrated that diabetes mellitus (DM) is associated with high risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C. Insulin resistance (IR), which correlates inversely with circulating adiponectin concentration, is a consistent finding in patients with type 2 DM. Chronic hepatitis C virus (HCV) infection has been reported to be associated with increased IR. Recent studies have suggested that IR plays a crucial role in

fibrosis progression, and has been demonstrated to have a negative impact on treatment responses to antiviral therapy in patients with chronic hepatitis C.

Research frontiers

IR has emerged as a risk factor for a wide variety of cancers. In a cross-sectional, hospital-based setting, the authors assessed the role of IR assessed by the homeostasis model (HOMA-IR) and serum adiponectin level in the development of HCC associated with chronic HCV infection.

Innovations and breakthroughs

The authors have provided the first evidence that IR can potentially increase the risk of developing HCC in patients with chronic HCV infection. A novel finding, not specifically evaluated in other studies, is the association of IR, regardless of diabetes, with the development of HCC.

Applications

These findings may have important prognostic and therapeutic implications in the management of chronic HCV-infected patients. Since IR is a potentially modifiable factor, therapeutic intervention aimed at decreasing IR may be warranted in these patients.

Peer review

The authors present a clinical investigation of the correlation between IR and HCC. The title accurately reflects the major contents of the article, and the abstract delineates briefly and concisely the research background, objectives, materials and methods, results and conclusions.

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BRIEF ARTICLE

Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains

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Abstract

AIM: To investigate the resistance of *Helicobacter pylori* (*H. pylori*) to ciprofloxacin (CIP), levofloxacin (LVX) and moxifloxacin (MOX) in the Beijing area and to elucidate the resistance mechanisms.

METHODS: Seventy-nine *H. pylori* clinical strains, isolated from patients who had undergone upper gastrointestinal endoscopy in Peking University First Hospital from 2007 to 2009, were tested for their susceptibility to CIP, LVX and MOX using the *E*-test method. *H. pylori* strain 26695 was included in the susceptibility testing as a control strain. According to the minimal inhibitory concentration (MIC) values, a strain was classified as resistant to CIP, LVX or MOX when the MIC was > 1 μg/mL. We amplified by polymerase chain reaction (PCR) and sequenced the quinolone resistance-determining regions of the *gyrA* and *gyrB* genes from 29 quinolone-resistant and 16 quinolone-susceptible *H. pylori* strains selected at random.

RESULTS: In this study, the resistance rates of *H. pylori* to CIP, LVX or MOX were 55.7% (44/79), and the primary resistance rates were 26.6% (21/79). Patients with secondary resistance had received LVX in previous eradication treatments, but not MOX or CIP. Forty-five strains, including 29 CIP, LVX or MOX-resistant strains (MIC: 1.5-32 μg/mL) and 16 susceptible strains, were selected randomly from the 79 strains and used in PCR analysis. Among these 45 strains, 27 resistant strains had mutations in the *gyrA* gene, including 11 strains with mutations corresponding to Asp-91 (MIC: 2-32 μg/mL), one of which also had a mutation corresponding to Val-150, and 16 strains had mutations at Asn-87 (MIC: 4-32 μg/mL), three of which also had mutations corresponding to Arg-140 or Val-150. In addition, Arg-140, Val-150 or Ala-97 mutations were separately detected in three susceptible strains. Analysis of the *gyrB* gene showed that one strain of low resistance had a mutation corresponding to Ser-457 that coexisted with an Asp-91 mutation. There was a significant difference in the occurrence of mutations in the *gyrA* gene between CIP, LVX and MOX-resistant and -susceptible strains ($P < 0.05$), but 2 resistant strains were found to possess no quinolone resistance-determining region mutations.

CONCLUSION: Resistance is primarily mediated through point mutations in gyrA. Whether other mechanisms are responsible for resistance in strains without mutations in the QRDR should be detected.

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Key words: *Helicobacter pylori*; Antibiotic resistance; Quinolones; Mutation; *gyrA*

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Wang LH, Cheng H, Hu FL, Li J. Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains. *World J Gastroenterol* 2010; 16(18): 2272-2277 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i18/2272.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i18.2272

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection, which affects half of the world's population, is responsible for gastritis[1], peptic ulcers[2,3] and gastric mucosa-associated lymphoid tissue lymphoma[4], and is a major risk factor for the development of gastric adenocarcinoma[5]. Eradication therapy plays an important role in the treatment of *H. pylori* infection. According to the Maastricht Ⅲ and Chinese Consensus Report, triple therapy using a proton-pump inhibitor (PPI) combined with clarithromycin and amoxicillin or metronidazole, is recommended as the first choice treatment[6,7]. With the increasing frequency of clarithromycin-resistance among *H. pylori* strains, there is rising concern about the potential decline in the eradication rate of this infection[8,9]. There is therefore an urgent need to introduce other treatment options. Fluoroquinolones, such as levofloxacin (LVX) and moxifloxacin (MOX), have been evaluated as an alternative to standard antibiotics against *H. pylori*[6,7].

Some studies have shown good results when using fluoroquinolone-based triple therapies for *H. pylori* eradication. In a German study, a 7-d course including LVX in patients with persistent *H. pylori* infection, resulted in eradication rates of greater than 85%[10]. In an Italian study, *H. pylori* eradication was achieved in 90% of patients treated with MOX, clarithromycin and lansoprazole[11]. However, the widespread use of fluoroquinolones for the treatment of *H. pylori* infection has led to an increase in its resistance rate in some areas, leading to unacceptably low eradication rates[12]. Several studies have shown that LVX-based therapies are not superior to traditional quadruple therapy or Maastricht triple therapy in the treatment of *H. pylori* infection, especially in the case of resistant *H. pylori* strains[13,14]. A Turkish study speculated that the low eradication rate with MOX-containing treatment regimens may be due to the development of resistance to this quinolone[15]. The findings from all of these studies indicate that a regimen that is effective in one area may not be effective in another area, as antibiotic-resistant rates for *H. pylori* may be different in different areas.

The fluoroquinolone resistance rates of *H. pylori* have been reported for several regions, including China (Mainland), Hong Kong, Taiwan, France, Japan and Korea[12,16-20], and range from 3% to about 20%. It was reported that LVX resistance was associated with prior fluoroquinolone use over the previous 10 years, and with the total number of fluoroquinolone courses prescribed[21]. A study from Korea speculated that resistance to MOX might be acquired through prior use of fluoroquinolones due to the development of cross-resistance to other fluoroquinolones like ciprofloxacin (CIP) and LVX[12]. Bogaerts *et al*[22] reported that mutations in the *gyrA* gene elevated the minimal inhibitory concentrations (MICs) to LVX, CIP and MOX.

The mechanism of action of fluoroquinolones is *via* inhibition of DNA gyrase and topoisomerase, which control and modify the topological state of DNA in cells. Fluoroquinolone then interferes with bacterial DNA replication. Both DNA gyrase and topoisomerase are composed of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes for DNA gyrase and the *parC* and *parE* genes for topoisomerase Ⅳ. The mechanism of fluoroquinolone resistance in *H. pylori* has been found to be linked to mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene. Mutations in the *gyrB* gene have also been reported in LVX-resistant strains isolated in Japan, but these often occurred along with *gyrA* mutations[19]. The resistance of *H. pylori* strains to fluoroquinolones in the Beijing area has not yet been reported. The aim of this study was to assess the prevalence of fluoroquinolone resistance against CIP, LVX and MOX in *H. pylori* strains isolated from patients over the past 3 years in Beijing and to compare the susceptibility of these strains with CIP and two newer fluoroquinolones *in vitro*. Mutations in the QRDRs of the *gyrA* and *gyrB* genes were also evaluated in these strains.

MATERIALS AND METHODS

Patients and bacterial strains

Seventy-nine clinical *H. pylori* strains were collected from adult patients, who were randomly selected and had undergone a gastroduodenoscopy at Peking University First Hospital between January 2007 and July 2009. Some patients had received *H. pylori* eradication therapy before, but none of the patients had received MOX or CIP-based therapy. The biopsy specimens were cultured on Colombia blood agar (BBL Microbiology Systems, Cockeysville, MD, USA), supplemented with 8% defibrinated sheep blood, and incubated for 5-7 d under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Clinical isolates were identified as *H. pylori* based on positive tests for urease, oxidase and catalase. All cultures were stored at -80℃ in brain-heart infusion broth (BHI, Difco Laboratory, Detroit, MI, USA) supplemented with 30% glycerol.

MIC determination

The MICs of LVX, CIP and MOX were determined by *E*-test strips (AB Biodisk, Solna, Sweden) according to the recommendations of the Clinical and Laboratory Standards Institute (Pennsylvania, USA) and the manufacturer's instructions. *H. pylori* 26695 was used as the quality control strain. The resistance breakpoints of fluoroquinolones were defined as > 1 μg/mL, as previously suggested[19,20].

Polymerase chain reaction amplification and nucleotide sequence analysis

H. pylori genomic DNA was extracted by the High Pure

polymerase chain reaction (PCR) Template Preparation kit (Tiangen, Beijing China). Oligonucleotide primers gyr APF (5'-AGCTTATTCCATGAGCGTGA-3') and gyr APR (5'-TCAGGCCCTTTGACAAATTC-3'), gyrBPF (5'-CCCTAACGAAGCCAAAATCA-3') and gyr BPR (5'-GGGCGCAAATAACGATAGAA-3') were designed to amplify a 582-bp and 465-bp region of the *H. pylori gyrA* and *gyrB* genes respectively. Primers were synthesized by Shenggong (Shanghai, China). PCR was performed in a 25 μL reaction volume containing 2 pmol of the oligonucleotide primer, 200 μmol/L (each) of dATP, dCTP, dGTP and dTTP (GE Healthcare, Little Chalfont, UK), 1.5 mmol/L of reaction buffer (GE Healthcare), 1 μL of template DNA, and 2.5 U of *Taq* polymerase (GE Healthcare). Thermocycling conditions were 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 53℃ for 30 s and 72℃ for 30 s, with a final extension step of 72℃ for 10 min. The reaction products were visualized by running 5 μL of the reaction mixture on 1.5% agarose gels. Sequencing of the amplified DNA was performed on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were then compared with the published sequence of the *H. pylori gyrA* and *gyrB* gene (GenBank accession number NC_000915.1).

Statistical analysis

The association between MIC levels and the occurrence of *gyrA/B* mutations relating to quinolone susceptibility was determined using Fisher's exact probability test, a P-value < 0.05 was considered significant.

RESULTS

Antimicrobial susceptibility

The MICs of CIP, LVX and MOX were determined for the 79 *H. pylori* strains isolated between 2007 and 2009 from gastric biopsies. The resistance rate to either CIP, LVX or MOX was 55.7% (44/79). Primary CIP, LVX and MOX resistance was detected in 21 (26.6%) isolates. Based on the breakpoints, we found that the strains susceptible to CIP (MIC ⩽ 1.0 μg/mL) were also susceptible to LVX and MOX, whereas the isolates resistant to CIP (MIC > 1.0 μg/mL) were also resistant to LVX and MOX (Tables 1 and 2).

Mutation analyses of the gyrA and gyrB genes and the association with LVX, MOX and CIP resistance

Of the 45 strains selected randomly, 29 were resistant and 16 were susceptible to LVX, MOX and CIP. Various substitutions in the QRDR of the *gyrA* and *gyrB* genes were observed in 31 of the strains. Mutations in the *gyrA* gene were identified in 27 resistant strains, 11 of which had mutations corresponding to Asp-91 and 16 of which had mutations corresponding to Asn-87. As for the *gyrB* gene, one low-level resistant strain had a mutation corresponding to Ser-457 which coexisted with the Asp-91 mutation. One susceptible strain exhibited a mutation corresponding to Val-451 of the GyrB protein. Amino acid substitutions in the GyrA protein associated with quinolone resistance fell into the following categories: (1) substitution of the amino acid at position 91 in 10 of the 27 isolates; (2) substitution of the amino acid at position 87 in 13 isolates; and (3) two mutations leading to substitution of amino acids in four isolates (Table 1). We also found 2 resistant strains with no QRDR mutations; in these strains the MICs to fluoroquinolones were > 12 μg/mL (Table 2).

Table 1 MICs and amino acid changes in quinolone-resistant and susceptible strains of *H. pylori*

Strains	MIC (μg/mL)			Mutations of *GyrA*	Mutations of *GyrB*
	LVX	MOX	CIP		
1R[1]	1.5	2	32	Asp91Gly and Val150Ala	0
2R[1]	2	2	32	Asp91Asn	0
3R[1]	2	2	32	Asp91Gly	0
4R[1]	3	1.5	1.5	Asp91Asn	Ser457Ala
5R[1]	4	3	4	Asp91Tyr	0
6R	4	3	32	Asp91Asn	0
7R[1]	6	4	32	Asp91Asn	0
8R	6	6	12	Asn87Lys and Arg140Lys	0
9R	8	4	12	Asn87Tyr	0
10R	12	8	32	Asp91Gly	0
11R	12	12	12	Asn87Ile and Arg140Lys	0
12R[1]	12	12	32	Asp91Asn	0
13R	32	12	32	Asn87Tyr	0
14R[1]	32	16	32	Asp91Asn	0
15R[1]	32	32	32	Asp91Asn	0
16R[1]	32	32	32	Asn87Ile	0
17R	32	32	32	Asn87Ile and Val150Ala	0
18R[1]	32	32	32	Asn87Lys	0
19R[1]	32	32	32	Asn87Lys	0
20R	32	32	32	Asn87Lys	0
21R	32	32	32	Asn87Lys	0
22R	32	32	32	Asn87Lys	0
23R	32	16	8	Asn87Lys	0
24R	24	32	32	Asn87Lys	0
25R	12	12	32	Asn87Lys	0
26R	6	6	32	Asn87Lys	0
27R	3	6	12	Asn87Lys	0
1S	0.02	0.02	0.016	Val150Ala	0
2S	0.016	0.016	0.032	Ala97Thr	0
3S	0.064	0.094	0.064	Arg140Lys	0
4S	0.064	0.02	0.023	0	Val451Gly

[1]Primary resistance. R: Resistant strains; S: Susceptible strains; 0: No mutation; MIC: Minimal inhibitory concentration; LVX: Levofloxacin; MOX: Moxifloxacin; CIP: Ciprofloxacin; *H. pylori*: *Helicobacter pylori*.

Table 2 MICs and amino acid changes in quinolone strains of *H. pylori* with no QRDR mutations

Strains	MIC (μg/mL)			Mutations of *GyrA*	Mutations of *GyrB*
	LVX	MOX	CIP		
28R[1]	24	16	32	0	0
29R[1]	16	32	32	0	0

[1]Primary resistance. QRDR: Quinolone resistance-determining region.

DISCUSSION

In this study, we investigated the resistance of *H. pylori* strains isolated from patients at Peking University First Hospital during 2007-2009 to three quinolone antibiotics. Of the 79 *H. pylori* strains isolated, 44 (55.7%) strains

were found to be resistant to quinolones and 21 (26.6%) showed primary resistance to quinolones. This is the first report of primary *H. pylori* resistance to quinolones in the Beijing area. In a previous study carried out in Hong Kong, the prevalence of LVX resistance in *H. pylori* was reported to be 11.5%[16]. An increase in fluoroquinolone resistance has also been reported in other areas, for example, the resistance rate to either CIP or LVX increased from 2.8% (1998-2003) to 11.8% (2004-2007) in southern Taiwan[17]. In other parts of the world, geographical differences and differences in treatment regimens result in a range of resistance rates, for example, rates of more than 20% in Korea[12], 15% in Japan[19] and 14% in Italy[23] have been reported.

According to research from Japan[19], caution is needed when interpreting these results because no criteria have been published for establishing the breakpoint of fluoroquinolones against *H. pylori*. According to the Maastricht Ⅲ Consensus Report[6], the threshold of clarithromycin resistance at which the empirical use of this antibiotic should be abandoned, or pretreatment clarithromycin susceptibility testing performed, is 15%-20%. Although some studies have reported good eradication rates using fluoroquinolones in some areas of China[24,25], quinolone treatment regimens in Beijing should be based on the findings of local antimicrobial susceptibility tests as the resistance rate is higher than 20% in this area.

In China, quinolone antibiotics have been widely used in hospitals to treat various infections and in the poultry industry as a supplement in feed. This has resulted in drug resistance becoming a serious problem[26-28]. In our study and a previous study from Korea[12], cross-resistance of the *H. pylori* strains to MOX, CIP and LVX was observed in patients who had not received MOX before the other eradication regimens.

Fluoroquinolone resistance is attributed to specific mutations in the genes encoding DNA gyrase and/or topoisomerase Ⅳ. In *Neisseria gonorrhoeae*[29], for example, mutations in the QRDRs of the *gyrA* and *parC* genes may be responsible for fluoroquinolone resistance. In *H. pylori*, only mutations in the DNA gyrase gene have been considered responsible for fluoroquinolone resistance because neither *parC* nor *parE* have been detected in the genome sequence and the drug efflux system is not considered important in this organism[30,31]. Consequently, DNA gyrase is the unique target for quinolones in *H. pylori*. Fluoroquinolone resistance of *H. pylori* is considered to depend on point mutations in the QRDR of the *gyrA/B* gene. GyrA mutations at Asn-87 and Asp-91 have been reported previously[19,22]. In our study, 11 resistant strains (37.93%) possessed mutations at Asp-91, 16 resistant strains (55.17%) possessed mutations at Asn-87, but mutations at both Asp-91 and Asn-87 were not found to coexist in any strain. Mutations at position Asn-87 were more frequent than at Asp-91 in LVX-resistant strains in Japan, according to a previous report [19]. We also identified more mutations at Asn-87 than at Asp-91 in this study. In *Escherichia coli*, Nakamura *et al*[32] found that mutations in the *gyrB* gene also lead to low-level quinolone resistance. Yoshida *et al*[33] identified two possible mutations in the GyrB protein of *Escherichia coli*: Asp426→Asn and Lys447→Glu. We also identified mutations in the *gyrB* gene in this study, but there was no statistically significant difference between the genotype of this gene and quinolone resistance. The role of GyrB in fluoroquinolone resistance therefore remains to be determined. In *H. pylori*, one mutation within the QRDR of the *gyrA* gene was associated with low level fluoroquinolone resistance, and two or more mutations may relate to high level of fluoroquinolone resistance[19]. One previous study reported that in *Campylobacter spp.*[34], a single mutation in the *gyrA* gene can lead to a high level of resistance not only to nalidixic acid but also to CIP, but it requires a second mutation to become MOX-resistant. In our study, two strains with two mutations in the QRDR of the *gyrA* gene had different levels of resistance to quinolones. Consistent with the findings of the Japanese study, we were also unable to identify any significant association between *gyrA* mutation patterns and the MICs of three fluoroquinolones[19]. In our study, we found that two resistant strains had no mutations in the QRDR of the *gyrA* and *gyrB* gene, and in these strains the MICs of three fluoroquinolone antibiotics reached 32 μg/mL. We also found that the same mutation can exist in resistant and susceptible strains. We therefore speculate that low-level resistance is most likely mediated through a point mutation in the *gyrA* gene. There may be other mutations in non-QRDRs of the *gyrA* and *gyrB* genes or other mechanisms, such as efflux systems, that lead to high level resistance.

A previous Japanese study reported that differences in amino acid substitutions associated with fluoroquinolone resistance correlate with geographical differences[19], with the occurrence of the Asn-87 GyrA mutation being more frequent than the Asp-91 GyrA mutation in LVX-resistant strains. Different from the findings of our study, it has previously been reported that in Hong Kong the most frequent mutation site in the GyrA protein was position 91[16].

In conclusion, the prevalence of resistance of *H. pylori* to fluoroquinolones is of concern in the Beijing area. Our results suggest that the susceptibility of *H. pylori* to fluoroquinolones should be tested before administration of a therapy, especially in the Beijing area. Resistance is most likely mediated through point mutations in the *gyrA* gene. Future studies should investigate whether other mechanisms are responsible for resistance in strains in which no mutations in the QRDR were detected.

COMMENTS

Background

Resistance to antibiotics is one of the most important reasons behind *Helicobacter pylori* (*H. pylori*) eradication failure. *H. pylori* resistance to metronidazole and clarithromycin is becoming an increasingly serious problem and as a result first-line treatment programs based on these drugs are declining. As an alternative to these standard regimens, quinolones can be recommended for the first-line treatment of *H. pylori* infection.

Research frontiers

Fluoroquinolone-based regimens for *H. pylori* eradication are widely employed

in some areas of China, but studies investigating fluoroquinolone resistance are limited.

Innovations and breakthroughs

Traditional *E*-tests and modern genetic mutation analysis of 45 *H. pylori* isolates showed a high rate of resistance to fluoroquinolones in Beijing. Resistance was found to be most likely mediated through point mutations in the *gyrA* gene, but two resistant strains were found with no quinolone resistance-determining region mutations. The possibility that other mechanisms are responsible for fluoroquinolone resistance in these strains remains to be determined.

Applications

The results of this study show a high prevalence of *H. pylori* resistance to fluoroquinolones in Beijing. The authors recommend that the susceptibility of *H. pylori* to fluoroquinolones is tested before the administration of a therapy.

Terminology

gyrA: The gene encoding DNA gyrase (type Ⅱ topoisomerase), subunit A; *gyrB*: The gene encoding DNA gyrase (type Ⅱ topoisomerase), subunit B.

Peer review

The manuscript investigates the prevalence and mechanisms of resistance of *H. pylori* to fluoroquinolones in Beijing. The methodology of resistance determination consists of a traditional *E*-test and modern genetic testing of *gyrA* mutation. The results show a primary resistance of 26% and secondary resistance of 55%, which is higher than in European countries. The article is good, using a sound methodology and correct conclusions.

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BRIEF ARTICLE

Most common *SLC25A13* mutation in 400 Chinese infants with intrahepatic cholestasis

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Abstract

AIM: To establish the real time fluorescence polymerase chain reaction (RT-PCR) with dual labeled probes for fast detection of *SLC25A13* gene mutation 851del4.

METHODS: Four hundred infants (< 1 year of age) with unexplained intrahepatic cholestasis from 18 provinces or municipalities in China were enrolled in this study for detecting their *SLC25A13* gene mutation 851del4. Suitable primers and fluorescence-labeled probes for detecting *SLC25A13* gene mutation 841del4 were designed. Normal and mutant sequences were detected by PCR with two fluorescence-labeled probes. After a single RT-PCR, results were obtained by analyzing the take-off curves. Twenty-four positive and 14 negative samples were retested by direct sequencing.

RESULTS: Eight homozygous and 30 heterozygous mutations were detected in 46 mutant alleles with a 851del4 mutation rate of 5.8% (46/800). Twenty-six and 20 mutant alleles were observed respectively, in 474 and 242 alleles from the intermediate and southern areas of China. No mutant allele was detected in 84 alleles from northern China. Twenty-four positive samples including 4 homozygous and 20 heterozygous mutations, and 14 negative samples were retested by direct sequencing, which confirmed that the accuracy of RT-PCR was 100%.

CONCLUSION: RT-PCR can detect the mutation 851del4 in infants with intrahepatic cholestasis with an accuracy of 100%.

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Key words: 851del4 mutation; Neonatal intrahepatic cholestasis; Real-time fluorescent polymerase chain reaction; *SLC25A13* gene

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INTRODUCTION

The *SLC25A13* gene, localized in chromosome 7q21.3, encodes a 3.4kb transcript that is expressed most abundantly in liver[1,2]. *SLC25A13* gene mutations can lead to citrin deficiency, which causes not only onset of type II citrullinemia (CTLN2) in adults[2], but also neonatal intrahepatic cholestasis (NICCD)[3,4]. Clinical manifestations of NICCD

include intrahepatic cholestasis, fatty liver, failure to thrive, hyperaminoacidemia, liver dysfunction, hypoglycaemia, hypoproteinemia, and coagulation disorders. Symptoms of most NICCD patients disappear by 12 mo of age. Specific treatment, except for nutritional program including supplementation of fat soluble vitamins and formulas containing medium-chain triglycerides[3,5-7], is usually not required. However, NICCD is not always benign and a few NICCD patients may have a less favorable clinical course with relatively early liver dysfunction that necessitates management with liver transplantation under the age of 1 year[8]. Some infants with homozygous mutations may suffer from CTLN2 one or more decades later[9,10].

Since the full genomic sequence of the *SLC25A13* gene was published in 1999, over 50 mutations have been identified[11]. It has been reported that more than 100 000 East Asians have two homozygous mutated *SLC25A13* alleles, and the carrier rate in Chinese population is 1/63[11,12]. Mutation 851del4, is a 4-bp (GTAT) deletion from nt 851-854 in exon 9, leading to premature truncation of a protein. Population analysis has shown that mutation 851del4 accounts for 70% of all detected *SLC25A13* gene mutations in general Chinese population[13]. However, its prevalence in infants with intrahepatic cholestasis is unclear.

Traditionally, mutation 851del4 is tested by direct agarose gel electrophoresis or GeneScan[2,12-14]. Since the differences in length between the mutant fragment and wild sequence are quiet small, direct agarose gel electrophoresis depends greatly on experience of the investigator, while GeneScan requiring expensive equipments is time consuming. In this study, real-time fluorescence polymerase chain reaction (RT-PCR) with dual labeled probes was used to detect the most common *SLC25A13* gene mutation 851del4 in 400 Chinese infants with intrahepatic cholestasis.

MATERIALS AND METHODS

Study design

A gene test for mutation 851del4 in 14 infants with intrahepatic cholestasis was carried out by direct sequencing. Four cases were detected to be with mutant alleles, including one homozygote and three heterozygotes. These patients were used as samples to develop the method of RT-PCR. The other ten cases with wild sequence served as the control.

Then, RT-PCR was performed to detect the prevalence of 851del4 mutation in 386 infants with unexplained intrahepatic cholestasis. If a heterozygous mutation 851del4 was found in an infant, the second mutation was identified in all the 18 exons and their flanking sequences by direct sequencing.

The accuracy of RT-PCR was retested by direct sequencing in 24 infants with mutant sequences and 14 infants with normal sequence.

Subjects

From June 2003 to May 2009, 400 infants with idiopathic intrahepatic cholestasis were enrolled in this study for testing their genes. The inclusion criteria were conjugated hyperbilirubinaemia with its serum total bilirubin (TBil) exceeding 5 mg/dL and conjugated bilirubin level > 20% of TBil or > 1 mg/dL if the total serum bilirubin < 5 mg/dL, and development of conjugated hyperbilirubinaemia under the age of 1 year. Those were excluded from the study if they had diseases affecting their extrahepatic biliary system (such as biliary atresia, choledochal cyst or tumor, inspissated bile, or haemangioma), low γ-glutamyl transpeptidase (GGT) level (no more than 50 U/L) that may indicate progressive familial intrahepatic cholestasis[15], and obvious extrahepatic abnormalities or positive serology other than cytomegalovirus (CMV) that may also indicate infection. CMV infection occurs frequently in infants with neonatal hepatitis in Mainland China[16,17], and the outcome is similar in those infected with or without CMV[18,19].

The infants included in this study came from 18 provinces or municipalities in China. Because the most likely boundary between northern and southern China was drawn at an altitude of 30°N, the Yangtze River was considered a historically significant border between northern and southern China as previously described[13,20]. According to such criteria, 237 infants came from a border area (Shanghai City, and Jiangsu, Anhui, Hubei, Sichuan Provinces), 121 from the southern area (Hunan, Jiangxi, Zhejiang, Fujian and Guangdong Provinces), and 42 from the northern area (Jilin, Liaoning, Hebei, Shandong, Ningxia and Henan Provinces) in our study (Figure 1).

RT-PCR assay for mutation 851del4

This study was approved by the Ethics Committee on Human Research of the Children's Hospital of Fudan University. Informed consent was obtained from the parents or the guardians of each participant.

The sequence of upstream primer and downstream primer is 5'-TTGGTATATTTGTTGCTTGTGTTT-3' and 5'-AGAGGGAACTCTGCCCTTTAA-3', respectively. Their normal and mutated sequences were detected with the probe FAM-CCTACAGACGTATGACCTTAGCA-TAMRA and the probe HEX-CCTACAGACGACCTTAGCAGA-TAMRA, respectively. Twenty-five microlitre reaction mixture used in this study contained 2 μL of genomic DNA, 10 μL of 2.5* real Master Mix [Tiangen Biotech (Beijing) Co. Ltd.], 1.25 μL of 20*probe enhancer, 0.25 μL of each FAM- and HEX-labeled probe, 0.5 μL of each upstream primer and downstream primer (diluted to 10 μmol/L), and 10.25 μL of ddH_2O. After overlaying 25 μL liquid, RT-PCR was performed under the following conditions: denaturation at 95℃ for 2 min, followed by 40 thermal cycles, each at 95℃ for 15 s, at 56℃ for 30 s, and at 68℃ for 40 s. The results were obtained by analyzing the curves.

Conventional PCR assay for mutation 851del4

To ensure the specificity and sensitivity of amplification, nested PCR was performed to amplify exons 8 and 9. Thirty cycles of PCR were performed in 30 μL reaction mixture at

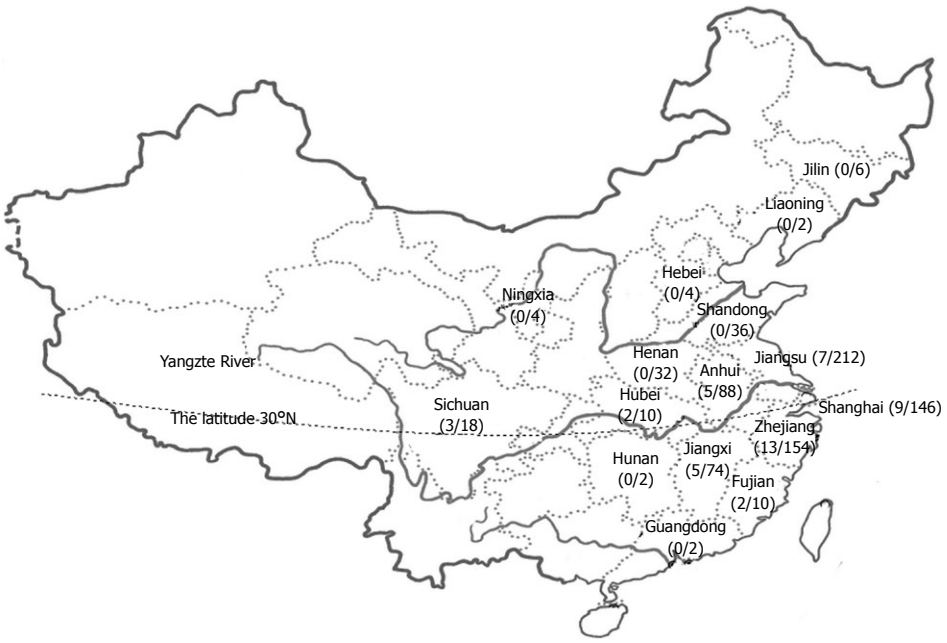


Figure 1 Distribution of idiopathic intrahepatic cholestasis infants enrolled in our study. As shown in the map, the provinces were separated into three parts by the Yangtze River and the northern latitude circle of 30°. The numbers in parentheses are the number of alleles of 851del4 mutation over the tested alleles.

94℃ for 3 min followed by 25 cycles, each at 94℃ for 30 s, at 50℃ for 30 s, at 72℃ for 1 min, and a final extension at 72℃ for 4 min, using primers Ex8/9F(5'-GCCTG-TAATCCCAGCACTT-3') and Ex8/9R(5'-GTTGAGA-GATGTAGCGAAAGAA-3'). The outer PCR reaction mixture consisted of 2*Taq- plus 15 μL PCR Master MIX, 1 μL in each primer (10 μmol/L), 2 μL DNA template, and 11 μL ddH_2O. After the initial outer PCR, inner PCR was conducted at 94℃ for 3 min followed by 30 cycles, each 94℃ for 30 s, at 50℃ for 30 s, at 72℃ for 1 min, and a final extension at 72℃ for 4 min using 1 μL of primers IVS7F (5'-TCACTCATTCCAGTGCCTTG-3') and IVS9B (5'-GCAGTTGCCTTTGCGGCATTG-3') as previously described[2], 2 μL outer PCR product as template, 2*Taq-plus 15 μL PCR Master MIX, and 11 μL ddH_2O.

The PCR products were analyzed by direct sequencing, in which IVS7B and IVS9B were taken as sequence primers for forward and backward sequencing identification. Mutation 851del4 was identified using software (Bioedit, North Carolina State University, NC, USA) and checked by two of the investigators.

RESULTS

Accuracy of RT-PCR

The curves were analyzed in accordance with the direct sequencing in 14 infants with known sequences. In infants with homozygous 851del4 mutation, positive fluorescence was observed only on the HEX-530 curve but not on FAM-490 curve. In those with heterozygous 851del4 mutation, positive fluorescence was found on both FAM-490 and HEX-530 curves, which was very close to each other, with the difference in *Ct* value always less than 2 (Figure 2). In those with the wild sequence, positive fluorescence was detected only on the FAM-490 curve. For any given sample, the experiment was considered a failure if negative fluorescence appeared on both HEX-530 and FAM-490 curves.

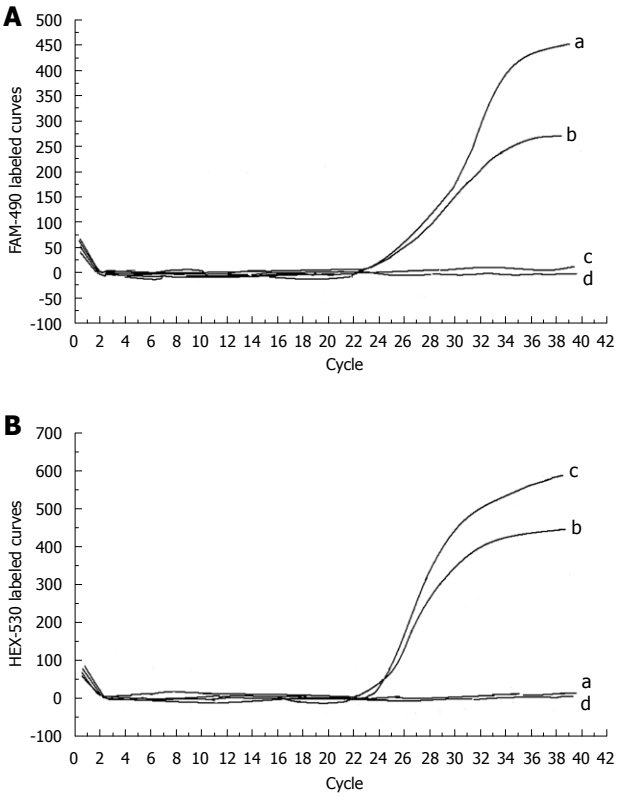


Figure 2 Positive fluorescences on FAM-labeled (A) and HEX-labeled (B) curves for different samples. As shown in the figure, positive fluorescence was observed on both FAM-labeled and HEX-labeled curves in sample b, indicating that b is a heterozygote. In sample c, positive fluorescence was only observed on the HEX-labeled curve, indicating that c is a homozygote. Positive fluorescence was observed on the FAM-labeled curve, otherwise HEX-labeled curve is a horizontal line in sample a, indicating that sample a has a wild sequence. d is a blank control with negative fluorescence on both FAM-labeled curve and HEX-labeled curve.

Positive mutation 851del4 was detected in 24 samples including 4 homozygous and 20 heterozygous mutations, and negative mutation 851del4 was retested in 14 infants

by nested PCR and direct sequencing, which was consistent with that detected by RT-PCR.

Prevalence of mutation 851del4

Eight homozygous and 30 heterozygous mutations were detected in the 400 infants with intrahepatic cholestasis, with a 851del4 mutation rate of 5.8% (46 mutant alleles with 851del4 were detected in 800 tested alleles). Subsequent testing of 18 exons and their flanking sequences in 30 heterozygous mutations showed another 9 mutant alleles including 1638ins23 in 5, R184X in 1 and novel mutations (data will be reported separately, manuscript in preparation) in 3, respectively.

Geographical variation of mutation 851del4 in China

Twenty-six and 20 mutant alleles were detected, respectively in 474 and 242 alleles of the intermediate and southern areas,with a 851del4 mutation rate of 5.5% and 8.3%, respectively. No mutant allele was detected in alleles of northern China (Figure 1).

DISCUSSION

To our knowledge, this is the first study to detect the most common mutation 851del4 of the *SLC25A13* gene in Chinese infants with intrahepatic cholestasis in a relatively large sample size. In this study, we developed a simple, fast, and accurate RT-PCR for detecting the mutation 851del4, the most common mutation of the *SLC25A13* gene, and detected 46 mutant alleles from 800 alleles with it. The prevalence of mutation 851del4 in Chinese infants with intrahepatic cholestasis was 1/17, which is much higher than that in general Chinese population (1/93)[13].

Compared with conventional nested PCR and agarose gel electrophoresis or GeneScan, RT-PCR could detect the mutation alleles and identify patients with homozygous or heterozygous 851del4 mutation, indicating that it can be used in diagnosis of intrahepatic cholestasis in infants.

In the present study, the mutation rate of 851del4 was about 5.8%, which was significantly higher than that in the general population, indicating that the *SLC25A13* gene mutation plays an important role in the pathogenesis of intrahepatic cholestasis of Chinese infants and should be detected in such infants, especially in those with symptoms of NICCD.

Screening of mutation 851del4 by RT-PCR may contribute to the diagnosis of intrahepatic cholestasis in patients with homozygous mutation because testing 18 exons of the *SLC25A13* gene can be omitted and diagnosis of citrin deficiency can be made. Early diagnosis and intervention of NICCD are extremely important[20] for the prevention of its serious consequences.

In this study, 30 heterozygous mutations of 851del4 were detected in 400 intrahepatic cholestasis infants with 9 compound heterozygous mutations detected by examining 18 exons of the *SLC25A13* gene. It is noteworthy that if suspicious patients were found to be heterozygous or when no mutation was found, all the 18 exons and their flanking sequence of the *SLC25A13* gene should be examined.

The *SLC25A13* gene mutation 851del4 has geographical variations in China and can be frequently found in southern China (1/70) but rarely in northern China (1/940)[13]. In this study, mutant alleles were detected in most infants from the intermediate and southern areas but not in those from northern China, showing that the distribution of the *SLC25A13* gene mutation 851del4 is different in south and north areas of China. Our finding is consistent with the reported data[13]. Thus, different methods should be used in dignosis of intrahepatic cholestasis in infants according to their geographical origin.

RT-PCR was established and the *SLC25A13* gene mutation 851del4 was detected in 400 infants using it in this study. However, it was used in detecting only one specific *SLC25A13* gene mutation, its prevalence might be underestimated. Further study is needed to highlight the importance of the *SLC25A13* gene mutations in infants with intrahepatic cholestasis and the application of RT-PCR in clinical practice.

In conclusion, RT-PCR can detect the most common *SLC25A13* gene mutation 851del4 in patients with intrahepatic cholestasis, thus providing a useful and effective tool for the diagnosis of NICCD.

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COMMENTS

Background

SLC25A13 gene mutations lead to citrin deficiency, which causes onset of type Ⅱ citrullinemia (CTLN2) in adults and neonatal intrahepatic cholestasis (NICCD). Up to date, over 50 mutations of *SLC25A13* have been identified, in which 851del4 is the most common mutation type. Traditionally, mutation 851del4 is screened by direct agarose gel electrophoresis or GeneScan. Since these methods are highly dependent on personal experience or need special equipments, a simple and accurate method for screening the mutation 851del4 is needed.

Research frontiers

Population analysis has shown that mutation 851del4 accounts for 70% of all mutations of the *SLC25A13* gene in general Chinese population. Mutation 851del4 is a 4-bp (GTAT) deletion from nt 851-854 in exon 9, leading to premature truncation of a protein. Early diagnosis of citrin deficiency is extremely important.

Innovations and breakthroughs

Real-time fluorescent polymerase chain reaction (RT-PCR) with dual-labeled probes was established, which could detect the mutation 851del4. The homozygosity, heterozygosity and wild sequence can be differentiated clearly. Compared with conventional nested PCR and agarose gel electrophoresis or GeneScan, RT-PCR does not require expertise in experimental operation.

Applications

The prevalence of mutation 851del4 in Chinese infants with intrahepatic cholestasis is 1/17, which is much higher than that in general Chinese population (1/93). RT-PCR may be used in diagnosis of intrahepatic cholestasis in infants.

Peer review

The authors obtained infants with intrahepatic cholestasis from various parts of China. Using RT-PCR with dual-labeled probes, they identified patients with homozygous or heterozygous 851del4 mutations and found that the mutation is more prevalent in southern than in northern part of China, indicating that RT-PCR can also be used in diagnosis of other genetic disorders in pediatric patients.

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BRIEF ARTICLE

Adhesion and immunomodulatory effects of *Bifidobacterium lactis* HN019 on intestinal epithelial cells INT-407

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Author contributions: Liu C and Zhang ZY performed the majority of experiments; Dong K and Guo XK provided vital reagents and analytical tools and were also involved in editing the manuscript; Guo XK provided financial support for this work; Liu C and Guo XK designed the study and wrote the manuscript.
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Abstract

AIM: To elucidate the adherence and immunomodulatory properties of a probiotic strain *Bifidobacterium lactis* (*B. lactis*) HN019.

METHODS: Adhesion assays of *B. lactis* HN019 and *Salmonella typhimurium* (*S. typhimurium*) ATCC 14028 to INT-407 cells were carried out by detecting copies of species-specific genes with real-time polymerase chain reaction. Morphological study was further conducted by transmission electron microscopy. *Interleukin-1β* (*IL-1β*), *interleukin-8*, and *tumor necrosis factor-α* (*TNF-α*) gene expression were assessed while enzyme linked immunosorbent assay was used to detect IL-8 protein secretion.

RESULTS: The attachment of *S. typhimurium* ATCC 14028 to INT407 intestinal epithelial cells was inhibited significantly by *B. lactis* HN019. *B. lactis* HN019 could be internalized into the INT-407 cells and attenuated IL-8 mRNA level at both baseline and *S. typhimurium*-induced pro-inflammatory responses. IL-8 secretion was reduced while IL-1β and TNF-α mRNA expression level remained unchanged at baseline after treated with *B. lactis* HN019.

CONCLUSION: *B. lactis* HN019 does not up-regulate the intestinal epithelium expressed pro-inflammatory cytokine, it showed the potential to protect enterocytes from an acute inflammatory response induced by enteropathogen.

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Key words: Probiotics; Intestinal epithelium; Enteropathogen; Immunomodulation; Adhesion

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INTRODUCTION

The intestinal tract acts as a reservoir for the intestinal microbiota that exert both harmful and beneficial effects on human health; intestinal microbiota contains an extraordinarily complex variety of metabolically active bacteria and fungi which interact with the host's epithelial cells and provide constant antigenic stimulation to the mucosal immune system[1]. The intestinal epithelium presents the

first line of defense against invading or attaching bacteria. In addition to serving as a physical barrier to microbial penetration, the intestinal mucosa is the main interface between the immune system and the luminal environment[2]. Intestinal epithelial cells (IECs) appear as an essential link in communicating with the immune cells in the underlying mucosa and the microflora in the lumen *via* the expression of many mediators. These mediators included antibacterial peptides such as defensins[3], mucins (MUC3), and chemokines and cytokines such as interleukin (IL)-8.

Under certain pathological circumstances, such as enteropathogens infection, the intestinal epithelium releases the chemokine IL-8 and other pro-inflammatory molecules that provoke an acute inflammatory response[4]. Therefore, a prolonged infection can result in a high level and protracted IL-8 release by IECs. The final outcome is a considerable infiltration of neutrophils that may perpetuate inflammation and eventually lead to cell damage, epithelial barrier dysfunction and pathophysiologic change of diarrhea[5].

For the protection against enteropathogen infections, the possibility of using food supplements containing probiotic bacteria has been recently considered. Probiotics are a group of live micro-organisms administered in adequate amounts which confer a beneficial effect on the health of the host[6]. Most probiotics found are resident members of the intestinal flora and are of major economic importance to the food industry. Several health-related effects associated with the intake of probiotics have been reported in different animal models as well as in human studies[7]. This bacterial community plays a pivotal role in human nutrition and health by promoting the supply of nutrients, preventing pathogen colonization and shaping and maintaining normal mucosal immunity[8]. While the precise mechanistic basis of the beneficial effects of probiotics is still obscure and will most likely vary depending on the strain and species used, a number of mechanisms have been suggested[9]. Protecting the host from enteropathogen colonization (barrier effects) and immunomodulatory effects toward host immune response have been demonstrated in humans and laboratory animals[10,11].

The interaction between probiotic strains and the intestinal epithelium is a key determinant for cytokine production by enterocytes, and probably the initiating event in probiotic immunomodulatory activity, as it occurs prior to the encounter with the immune system cells. It has been reported that several strains of probiotics belonging to *Bifidobacterium* and *Lactobacillus* are highly relevant to the prevention of the invasion of tissues by enteropathogens[12]. Moreover, by inhibiting the production of IL-8 in enterocytes, these strains are also found to be effective in modulating the pro-inflammatory response in IECs challenged by enteropathogens such as *Salmonella typhimurium* (*S. typhimurium*); such induction is species and strain specific[13,14].

Since the immunomodulatory properties are strain-specific[15-17], for each probiotic strain, profiles of the cytokines secreted by lymphocytes, enterocytes and/or DCs that come into contact with the strain should be established. Strains belonging to the *Bifidobacterium* are the most widely used probiotic bacteria, and are included in many functional foods and dietary supplements.

We focused our study mainly on how IEC respond to widely used probiotic bacteria. The aim of this work was to study the cytokine pattern induced by the interaction of probiotics with intestinal epithelium cells. Furthermore, whether probiotic bacteria still function after inactivation was evaluated. This study may contribute to verify the pro- and anti-inflammatory properties of the strain in question and define specific clinical applications.

MATERIALS AND METHODS

Bacterial strains and related preparations

A globally consumed commercial probiotic strain *Bifidobacterium lactis* (*B. lactis*) HN019 was studied in the experiment. The *B. lactis* HN019 powder was a gift from Danisco. Live strain was inoculated in De Man, ROGOSA and SHARPE (MRS) broth (Difco) supplemented with 0.05% (w/v) cysteine hydrochloride at 37℃ under anaerobic conditions for 24 h. The stationary-phase bacteria were harvested and Gram-stained to confirm purity. Heat-killed *B. lactis* HN019 were prepared by heating the bacteria at 80℃ for 20 min. Effectiveness of the heat-killing method was confirmed by plating the treated bacteria on MRS agar at 37℃ for 48 h. *S. typhimurium* ATCC 14028 was used as an enteropathogenic reference strain. *S. typhimurium* was cultured in Tryptone Soy broth (TSB) at 37℃ with shaking overnight.

Bacteria number was estimated by measuring the absorbance at 600 nm, and correlating the absorbance value to a standard curve of colony-forming units (CFU) on MRS agar (Merck) estimated in preliminary experiments.

For storage, liquid cultures of *B. lactis* HN019 grown for 24 h were frozen in 30% glycerol at -80℃; vacuum freeze-drying method was also applied.

Cell lines and media

INT-407 cells which derived from human intestinal epithelium, were purchased from American Type Culture Collection (ATCC strain CCL-6). Cells were grown in a humidified incubator at 37℃ under 5% CO_2. INT-407 cells were cultured in Dulbcco's Modifed Eagle Medium (DMEM) (GIBCO) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells were routinely propagated in 25 cm^2 tissue culture flasks until they approached 80%-90% confluences. Subsequently, the cells were subcultured and the medium was replaced every two to three days in the culturing process.

Adhesion assays

Each adhesion assay was conducted in triplicate over three to five successive passages of INT-407 cells. Briefly, INT-407 cells were seeded on sterile six-well plates and grown to 80% confluence. The monolayers of INT-407 cells were rinsed three times with modified Hank's Balanced Salt Solution (mHBSS) without calcium and magnesium. Late exponential cultures of *B. lactis* HN019 or/

and *S. typhimurium* ATCC 14028 were adjusted at an optical density at 600 nm corresponding to 1×10^8 cells/mL. After rinsing, the bacterial cells were resuspended in DMEM and used for the adhesion assay. After incubation for 2 h and 6 h at 37℃ in the atmosphere of 5% CO_2 and 95% air, unattached bacteria were removed by washing the monolayers four times with sterile PBS. After detachment of the INT-407 cells from the plastic surface by incubation with 200 μL trypsin/EDTA per well (10 min, 37℃), the INT-407 cells and the adhesive bacteria were transferred into a 1.5 mL reaction tube. The wells were rinsed with 200 μL sterile PBS which was also transferred into the 1.5 mL-reaction tube.

Bacterial adhesion to INT-407 cells was evaluated by quantification of INT-407-bound bacteria with genus-specific primers *via* real-time polymerase chain reaction (PCR) as reported by Candela *et al*[18]. To quantify the bacterial cells by real-time PCR, the cell suspensions obtained from the adhesion assays were thawed at room temperature and, after mixing, an aliquot of 20 μL was transferred into a 0.2 mL-reaction tube and incubated for 10 min at room temperature with 3.8 μL of Trypsin inhibitor solution. Then the bacterial cells (Bifidobacterium and Salmonella) were specifically quantified by real-time PCR performed with the genus-specific primers listed in Table 1. pMD19 T-vector which carried genus-specific genes was constructed as internal standards.

Real-time PCR was performed in ABI 7500 instrument and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescent signal. Amplification was carried out in a 20 μL final volume containing 2 μL of cell suspension, 10 μL SYBR Premix Ex Taq (TAKARA), 0.2 μmol/L of each primer and 0.4 μL ROX Reference Dye Ⅱ. The experimental protocol consisted of the following programs: starting preincubation at 95℃ for 10s; amplification including 40 cycles of 95℃ for 5 s, and 60℃ for 34 s. Since the copy numbers of standard plasmids could be quantified, we amplified serial dilutions of the plasmids that represent bacteria number ranging from 1×10^7 to 1×10^3 CFU/μL.

The data reported represent mean values obtained in 3-5 independent experiments. Each experiment was performed in duplicate.

Preparation of samples for electron microscopy

INT-407 cells treated with *B. lactis* HN019 for 2 h and 6 h as previously described were washed three times with PBS buffer solution. Formaldehyde (40%) and glutaraldehyde (10%) were added for fixation. Ultrastructure of the cells was examined under transmission electron microscopy. The micrographs were produced at 5800 × and 13500 × magnification. Cells from the control group which had not been treated with bacteria were processed in the same way. Two samples from each group were analyzed.

INT-407 cells treatment

Monolayer of INT-407 cells were washed in PBS and supplemented with DMEM plus 2% FBS without antibiotics overnight before infection. *B. lactis* HN019 and *S. typhimurium* ATCC 14028 was harvested in stationary-phase, centrifuged and resuspended in fresh DMEM medium. After heat-killing treatment, viable *B. lactis* HN019, *S. typhimurium* ATCC 14028 and inactivated *B. lactis* HN019 were added into six-well plates for co-culture with INT-407 cells at a bacterial to epithelial cell ratio of 10:1. Untreated cells with normal medium were used as negative control. The plates were kept in humidified atmosphere containing 5% CO_2 for 2 h.

Table 1 Primer sequences of genus-specific genes used in adhesion assays

Primer name	Primer sequences (5′-3′)	Specificity	Ref.
BIF 164	GGGTGGTAATGCCGGATG	*Bifidobacterium*	[37]
BIF 662	CCACCGTTACACCGGGAA		
MINf	ACGGTAACAGGAAGCAG	*Salmonella*	[38]
MINr	TATTAACCACAACACCT		

Bacterial interference assays

The ability of *B. lactis* to inhibit binding of *S. typhimurium* to INT-407 cells was also assessed. INT-407 cells (1×10^6) seeded in six-well plates in antibiotic-free medium were incubated for 24 h. After replenishing the medium, the following assays were performed. (1) Coincubation of *B. lactis* HN019 and *S. typhimurium* ATCC 14028 with INT-407 cells for 2 h; (2) INT-407 cells were pretreated with 10:1 *B. lactis* HN019 for 2 h prior to infection with 10:1 *S. typhimurium* ATCC 14028 for further 2 h; (3) INT-407 cells were pre-stimulated with 10:1 *S. typhimurium* ATCC 14028 for 2 h prior to retreatment with 10:1 *B. lactis* HN019 for further 2 h; and (4) Untreated INT-407 cells were used as baseline control.

RNA extraction

Following bacterial treatment, cell culture supernatants were removed and washed three times with sterile D-Hank's. INT-407 cells collected for RNA preparation were immediately lysed in TRIZOL Reagent (Invitrogen). Total RNA was isolated from the lysed cells according to the manufacturer's instructions. Purification of RNA was performed by RNase-free DNase and standard phenol chloroform extraction method. The quantity of RNA was evaluated by spectrophotometric analysis and the quality of RNA samples was determined based on the A260:A280 ratio. All RNA samples were further analyzed by agarose gel electrophoresis to check for the integrity of 5S, 18S and 28S rRNA.

Real-time RT-PCR analysis

Total RNA (2 μg) was reverse-transcribed to yield cDNA using SuperScript Ⅲ First-Strand Synthesis System (Invitrogen) as the manufacture's protocol for the following experiments. Primers targeting four genes of interest were designed for detecting mRNA expression. Real-time RT-PCR was performed in an ABI 7500 system (Applied Biosystems) using SYBR Premix Ex Taq as mentioned

Table 2 Primer sequences used for quantitative real-time PCR analysis

Gene	Primer sequences (5′-3′)	Product size (bp)
IL-1β	ACTCACTTAAAGCCCGCCTG TCAGAATGTGGGAGCGAATG	80
IL-8	CAAGAGCCAGGAAGAAACCAC TGCAGAAATCAGGAAGGCTG	101
TNF-α	TCTTCTCGAACCCCGAGTGA CCTCTGATGGCACCACCAG	150
β-actin	TGTTACAGGAAGTCCCTTGCC AATGCTATCACCTCCCCTGTG	101

PCR: Polymerase chain reaction; IL: Interleukin; TNF: Tumor necrosis factor.

above. Calculations were performed using β-actin as an internal standard. Primer sequences are shown in Table 2. All reactions were performed in triplicate. Relative gene expression data was determined by the $2^{-\Delta\Delta CT}$ method as previously described[19]. Fold changes > 2 or < -0.5 was defined as significantly changed between treated and untreated cells.

IL-8 ELISA

Subconfluent or confluent INT-407 cells were incubated with varying groups of bacteria: (1) Cells were incubated with *B. lactis* HN019 or heat-killed *S. typhimurium* ATCC 14028 at a bacterial to epithelial cell ratio of 10:1 for 12 h; (2) Confluent monolayers of INT-407 cells were stimulated with 10 ng/mL LPS (derived from *S. typhimurium,* Sigma) for 12 h; (3) INT-407 cells were co-incubated with *B. lactis* HN019 (multiplicity of infection = 10) and LPS (10 ng/mL) simultaneously for 12 h; (4) Pretreated with 10:1 *B. lactis* HN019 for 2 h prior to stimulation with 10 ng/mL LPS for further 12 h; (5) Pre-stimulated with 10 ng/mL LPS for 2 h, and then treated with 10:1 *B. lactis* HN019 for further 12 h; and (6) Untreated cells were used as negative control.

Cells from all treatments were washed three times with PBS. Immunoreactive IL-8 protein levels in cell-culture supernatants were quantified using an ELISA Duo-Set kit (R & D Systems) according to the manufacturer's protocol.

Statistical analysis

Values were given as mean ± SD of triplicate measurements. Statistical analyses were performed using unpaired two-tailed Student's *t* test. *P* value < 0.05 was considered to be statistically significant.

RESULTS

Inhibitory effects of B. lactis HN019 on S. typhimurium ATCC 14028 attachment

The *B. lactis* HN019 strains in this study were evaluated with regard to their ability to inhibit the attachment of pathogenic *S. typhimurium* ATCC 14028 to INT-407 cells. Adherence activity of *B. lactis* HN019 and/or *S. typhimurium* ATCC 14028 was evaluated by a real-time PCR as shown in Figure 1.

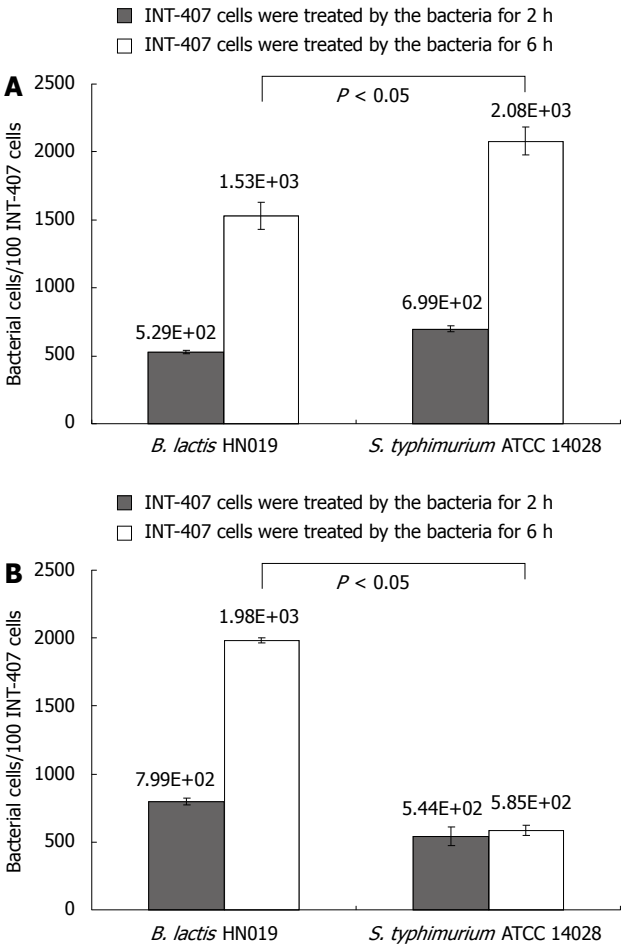


Figure 1 Adherence of *Bifidobacterium lactis* (*B. lactis*) HN019 and *Samonella typhimurium* (*S. typhimurium*) ATCC 14028 to INT-407 cells. A: Number of bacteria bound to 100 INT-407 cells obtained in assays contain each single strain; B: Number of bacteria bound to 100 INT-407 cells obtained in assays contain *S. typhimurium* ATCC 14028 plus *B. lactis* HN019. The error bars represent ± SD of the mean values.

Candela *et al*[18] defined bacterial strains as non-adhesive with less than 5 bacterial cells adhered to one cell. Following this adhesion score, both *B. lactis* HN019 and *S. typhimurium* ATCC 14028 could effectively adhere to INT-407 cells. Moreover, the adhesive capacity of *B. lactis* HN019 was not higher than *S. typhimurium* ATCC 14028 when treated with either strain alone, showing the adhesion values of 5.29×10^2 and 6.99×10^2 bacterial cells/100 INT-407 cells after 2 h while 1.53×10^3 and 2.08×10^3 bacterial cells/100 INT-407 cells after 6 h.

Nevertheless, the adhesion values obtained in co-treated assays showed that the *B. lactis* HN019 could significantly decrease the attached number of *S. typhimurium* ATCC 14028 from 2.08×10^3 to 5.85×10^2 bacterial cells/100 INT-407 cells after 6 h treatment, and the number of attached *B. lactis* HN019 was not affected by the existence of *S. typhimurium* ATCC 14028.

Transmission electron microscopy studies

In these studies we observed that *B. lactis* HN019 was able to interact with the epithelial cells of small intestine (Figure 2), and it was interesting that the *B. lactis* HN019

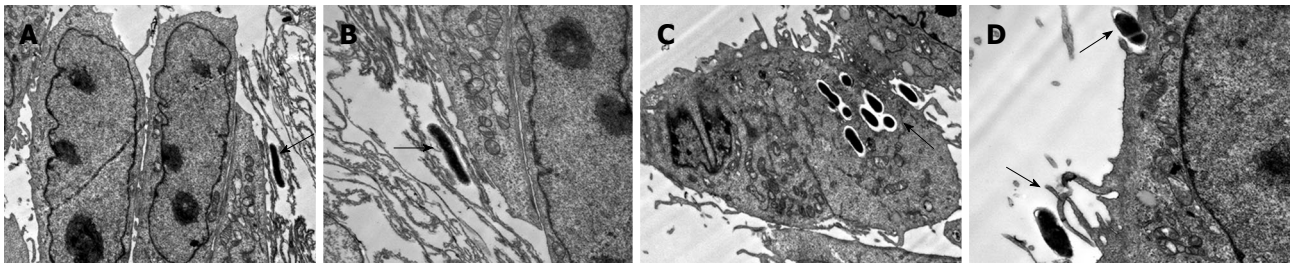


Figure 2 Transmission electron micrograph of INT-407 cells after stimulation with *B. lactis* HN019 (arrows). A: Stimulation for 2 h (× 5800); B: Stimulation for 2 h (× 13500); C: Stimulation for 6 h (× 5800); D: Stimulation for 6 h (× 13500).

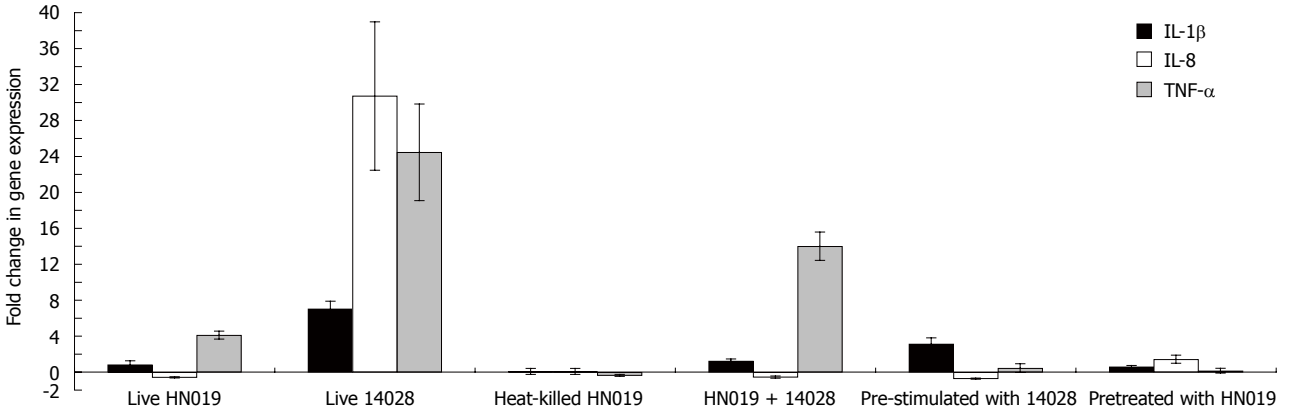


Figure 3 Effects of different bacterial treatment on expression of three genes assessed by real-time polymerase chain reaction. IL: Interleukin; TNF: Tumor necrosis factor.

could be internalized into the INT-407 cells after 6 h treatment.

Inflammatory gene expression following bacteria incubation

Inflammatory gene expression in bacteria-treated INT-407 cells was analyzed with real-time PCR. The results showed that live *B. lactis* HN019 had no effect on gene expression of IL-1β, but could up-regulate TNF-α expression and down-regulate IL-8 expression. Heat-killed *B. lactis* HN019 had no effect on gene expression of IL-1β, IL-8 and TNF-α. Pathogenic *S. typhimurium* ATCC 14028 could significantly up-regulate the mRNA level of pro-inflammatory cytokines. Although co-incubation with *B. lactis* HN019 and *S. typhimurium* ATCC 14028 could induce the gene expression of TNF-α and IL-1β, the extent is significantly lower than in those stimulated with *S. typhimurium* ATCC 14028 alone (Figure 3).

In bacterial interference assays, IL-1β, IL-8 and TNF-α mRNA expression was not changed at baseline after pre-treated with *B. lactis* HN019, and addition with *S. typhimurium* ATCC 14028 also did not activate these pro-inflammatory cytokines expression. For groups pre-stimulated with *S. typhimurium* ATCC 14028, *B. lactis* HN019 lowered the *S. typhimurium* ATCC 14028 induced IL-1β, IL-8 and TNF-α levels from 8.73, 47.97 and 9.3 folds to 2.6, 0.89 and 0.37 folds as compared with the untreated group. The results are summarized in Figure 3.

Through either incubating the INT-407 cells with *B. lactis* HN019 and *S. typhimurium* ATCC 14028 simultaneously or pre-treating with each strain respectively, the pro-inflammatory cytokines induced by *S. typhimurium* ATCC 14028 could be suppressed by *B. lactis* HN019. We therefore compared the inhibitory effects of these three groups and found that for IL-1β, the inhibition rate of *B. lactis* HN019 pretreated group was much lower than the other two groups. For IL-8, there was no significant difference among the three groups. For TNF-α, the inhibition rate of co-treated group was much lower than the other two groups (Figure 4).

IL-8 secretion by INT-407 cells following bacteria incubation

To assess the effects of probiotic strain on IL-8 secretion, INT-407 cells were incubated with live or heat-killed *B. lactis* and *S. typhimurium* and/or lipopolysaccharide. The experiments were performed 2-5 times in replicate; the results are demonstrated in Figure 5.

Firstly, we tested the effects of live *B. lactis* HN019, heat-killed *B. lactis* HN019 and *S. typhimurium* ATCC 14028, LPS derived from *S. typhimurium* on IL-8 secretion. The results showed that both live and dead *B. lactis* HN019 decreased IL-8 levels (844 ± 64 pg/mL and 897 ± 80 pg/mL) compared with the untreated group (1963 ± 77 pg/mL). Co-culturing with heat-killed *S. typhimurium* ATCC 14028 induced no change in IL-8 concentration. However, stimulation of INT-407 cells with LPS resulted in a significant increase of IL-8 production (3104 ± 80 pg/mL).

Secondly, in order to assess the anti-inflammatory

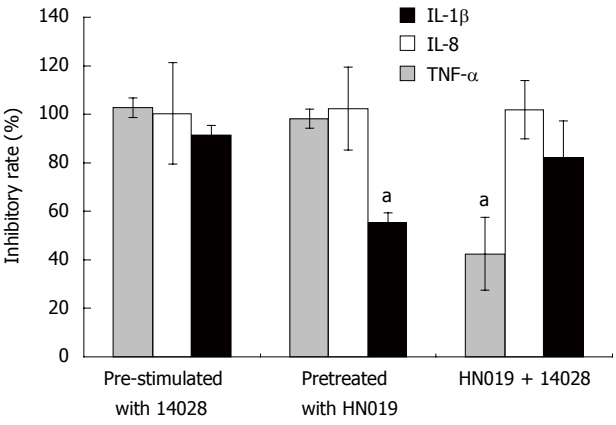


Figure 4 Inhibitory effects of *B. lactis* HN019 on gene expression of pro-inflammatory cytokines. $^{a}P < 0.05$ *vs* other treatment methods.

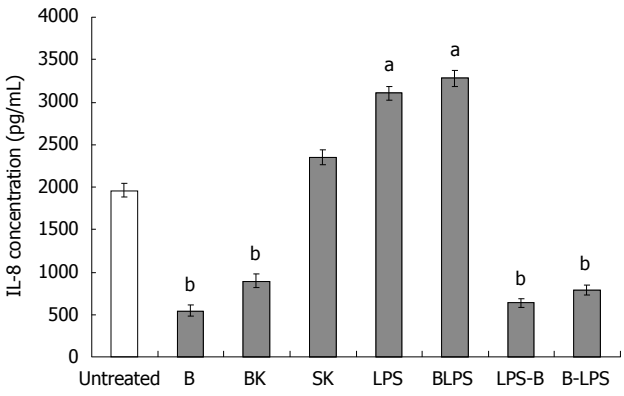


Figure 5 Bacteria modulate IL-8 secretion at baseline. B: *Bifidobacterium lactis* HN019; BK: Heat-killed *B. lactis* HN019; SK: Heat-killed *Salmonella typhimurium* ATCC 14028; LPS: Lipopolysaccharide (10 ng/mL); BLPS: Co-incubation with *B. lactis* HN019 and LPS; LPS-B: Treated with *B. lactis* HN019 after pretreatment with LPS for 2 h; B-LPS: Treated with LPS after pretreatment with *B. lactis* HN019 for 2 h. The data are expressed as pg/mL and represent the mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* untreated monolayers.

properties of the probiotic strain, the IL-8 production was evaluated by incubating INT-407 cell monolayers with *B. lactis* HN019 in the presence of LPS. As presented in Figure 5, IL-8 protein level secreted by the cells noticeably increased after co-incubated with LPS and probiotic strain compared with untreated group. However, the probiotic strain could decrease the LPS-induced IL-8 expression, and the cells pretreated with probiotic strain could also remain at a low level IL-8 secretion even after re-stimulated with LPS as compared with untreated group. This suggests that this probiotic strain *B. lactis* HN019 exerted anti-inflammatory effects on the epithelium by down-regulating the secretion of IL-8.

DISCUSSION

The role of probiotic bacteria derived from normal intestinal microbiota in the physiology of the gastrointestinal tract is not completely understood. In particular, the mechanisms underlying the benefits from biotherapy with probiotics have not been sufficiently elucidated. *B. lactis* HN019 is a world-wide consumed probiotic strain which is associated with several health-related immunomodulatory effects: such as reducing the severity of pathogen infection and enhancing immunity in the elderly[20-22]. Nevertheless, the mechanism is yet to be known. This is the first study on the differential pro-inflammatory cytokine responses of intestinal epithelium under conditions employing live and heat-killed *B. lactis* HN019. Furthermore, the enteropathogen exclusion effect of *B. lactis* HN019 was not evaluated.

In the present study, we used a rapid, accurate and sensitive method for studying bacteria adhesion. Provided specific primers for bifidobacteria are known, our method allows the quantification of this organism's cell number adhering to INT-407 cells which is used as a model in our investigation. A further important advantage of the real-time PCR based method is its efficacy in detecting and quantifying different bacterial genera and species simultaneously adhering to an epithelial cell monolayer. This method can be useful for probiotic strain selection. By this method, we found that *B. lactis* HN019 exerted a strong adhesive activity to human intestinal epithelial cells INT-407. The ability to adhere to the intestinal epithelium represents a significant prerequisite for the transient intestinal colonization of probiotic bacteria[23]. Bifidobacteria are normal gastrointestinal flora of many animals and have been shown to be adherent to a variety of epithelial cells *in vitro*[18,24,25]. However, adherence to INT-407 cells is seldom reported. This adherence to INT-407 cells may be mediated *via* fibronectin binding[26]. It is known that probiotic strains have the ability to counteract the pathogenic bacteria invasion[27,28]. In particular, the bifidobacteria strain tested is effective in inhibiting the adhesion of pathogenic *S. typhimurium* ATCC 14028 to INT-407 cells. This behavior is a fundamental prerequisite for probiotic bacteria to exert antagonistic activities against enteropathogens[29]. Such inhibitory effects of bifidobacteria can be explained by the mechanisms of competition for common adhesive sites or producing antibacterial lipophilic factor(s)[30]. In addition, it is reported that *bifidobacteria* could produce a proteinaceous factor that inhibits *in vitro* adherence of an enterotoxigenic *E. coli* strain to gangliotetraosylceramide molecules, which are physiological constituents of the mammalian intestinal epithelial surface[31].

Interactions of *B. lactis* HN019 and INT-407 cells observed under transmission electron microscope (TEM) indicated that the bacteria are able to contact with the epithelial cells of the small intestine. Moreover, the *B. lactis* HN019 could be internalized into INT-407 cells. It has been described that pathogens (bacteria, virus and parasites) can cross the mucosal barrier through different routes: transcellular route, paracellular route across cells for the tight junctions and *via* M cells[32]. The *B. lactis* used in this study was originally isolated from a human host. Therefore, it may be that this strain can internalize into epithelial cells because of its host specificity. We considered that such internalization may be of potential risks if the safety status of the internalized bacteria were not carefully assessed, especially the translocation capacity which correlate with its ability to induce infections[33].

The colonization of the intestinal tract by enteropathogenic bacteria induces the activation of the inflammatory cascade, resulting in the epithelial cell secretion of IL-8 and other pro-inflammatory molecules and in the consequent recruitment of neutrophils and other inflammatory cells. IL-8 amplifies an ongoing acute immune response and provides a set of signals for the activation of mucosal inflammatory responses in the earliest phases of microbial invasion. In some cases, a massive and prolonged infiltration of neutrophils may perpetuate inflammation and ultimately lead to cell damage, epithelial barrier dysfunction and pathophysiologic change of diarrhea. In the present study, INT-407 cells behaved like human enterocytes and could secrete IL-8 either constitutively or after stimulation with physiological agonists. Probiotic strains presented differences in their capacity to augment IL-8 expression, however, some strains seemed to rather decrease epithelial-cell production of IL-8[15-17,34-36]. Our results showed a robust IL-8 expression of INT-407 cells induced when exposed to *S. typhimurium*. However, live and heat-killed *B. lactis* HN019 not only functionally modulate the epithelium by inhibiting the constitutive mRNA level of IL-8 and attenuating *S. typhimurium*-induced *IL-8* gene expression, but also protect the INT-407 cells from IL-8 protein production activated by LPS. The same results were discovered on *TNF-α* and *IL-1β* gene expression except for up-regulation of TNF-α expression by live *B. lactis* HN019, but the *S. typhimurium* could induce much higher TNF-α expression than live *B. lactis* HN019. High concentrations of IL-1β and TNF-α cause cachexia, tissue injury, disseminated intravascular coagulation and shock. Therefore, the control of secretion of these pro-inflammatory cytokines *in vivo* is very important. *B. lactis* HN019 has a beneficial effect in converting the infection-induced excessive cytokine expression and helping maintain an immunological balance. These data indicated that INT-407 displayed immunological quiescence when exposed to both live and dead *B. lactis* HN019. *B. lactis* HN019 can modulate epithelial responses to limit inflammatory signals and markedly inhibit the inflammatory response induced by pathogen.

We used three approaches to detect the influence of probiotic strain on the pathogenic strain, and found that although the *B. lactis* HN019 could reduce the expression of pro-inflammatory cytokines, the inhibitory effect was different depending on the treatment methods. Pre-stimulated INT-407 cells with *S. typhimurium* ATCC 14028 could strongly induce a high-level of pro-inflammatory cytokine expression and such up-regulation could be reversed to a normal level after re-treatment with probiotic *B. lactis* HN019. This treatment achieved the maximum inhibitory rate. These results indicated that *B. lactis* HN019 may be more effective as adjunctive therapeutic agent than as preventive agent in inflammatory diseases.

In conclusion, our studies revealed that as a probiotic strain, *B. lactis* HN019 could modulate immune system towards anti-inflammatory action and exclude enteropathogenic adhesion, thus contributing to the homeostasis of the intestinal epithelium. This finding will offer, in the near future, new therapeutic means to counteract the inflammatory disorders observed in human inflammatory bowel disease.

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COMMENTS

Background

Bifidobacterium lactis (*B. lactis*) HN019 is a world-wide consumed probiotic strain and several health-related effects of the strain have been reported, especially the immunomodulatory effect. The immunomodulatory properties are strain-specific, and the interaction between probiotics and the intestinal epithelium is the initiating event in immunomodulatary activity. The authors focused their studies on how intestinal epithelial cells (IEC) respond to widely used probiotic bacteria to explore the mechanism of the immunomodulatory effects of *B. lactis* HN019.

Research frontiers

Studies in probiotics are one of the hottest research fields at present. The interactions between bacteria and the host may reveal the mechanisms of bacteria on the host. This study may contribute to better applications of probiotic bacteria.

Innovations and breakthroughs

This is the first study on different pro-inflammatory cytokine responses of intestinal epithelium under conditions employing *B. lactis* HN019. The authors used a rapid, accurate and sensitive method for studying bacteria adhesion and found that *B. lactis* HN019 exerted a strong adhesive activity to human intestinal epithelium cells and the bifidobacteria strain tested was effective in inhibiting the adhesion of pathogen. The authors also indicated that *B. lactis* HN019 could modulate epithelial responses to limit inflammatory signals and markedly inhibit the inflammatory response induced by pathogen.

Applications

The study revealed that as a world-wide consumed probiotic strain, *B. lactis* HN019 could modulate immune system towards anti-inflammatory action and exclude enteropathogenic adhesion. These findings may help with better applications of the *B. lactis* HN019 and will contribute to offering new therapeutic means to counteract the inflammatory disorders observed in human inflammatory disease.

Peer review

The manuscript by Liu *et al* describes the adherence and immuno-modulatory properties of *B. lactis* HN019 against INT-407 intestinal epithelial cells, and additionally reports the anti-*S. typhimurium* properties of this probiotic strain. This is an interesting manuscript which has been well executed.

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BRIEF ARTICLE

Establishment of a human hepatoma multidrug resistant cell line *in vitro*

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Abstract

AIM: To establish a multidrug-resistant hepatoma cell line (SK-Hep-1), and to investigate its biological characteristics.

METHODS: A highly invasive SK-Hep-1 cell line of human hepatocellular carcinoma, also known as malignant hepatoma was incubated with a high concentration of cisplatin (CDDP) to establish a CDDP-resistant cell subline (SK-Hep-1/CDDP). The 50% inhibitory dose (IC_{50}) values and the resistance indexes [(IC_{50} SK-Hep-1/CDDP)/(IC_{50} SK-Hep-1)] for other chemotherapeutic agents and the growth curve of cells were all evaluated using cell counting kit-8 assays. The distribution of the cell cycles were detected by flow cytometry. Expression of acquired multidrug resistance P-glycoprotein (MDR1, ABCB1) and multidrug resistance-associated protein 1 (MRP1, ABCC1) was compared with that in parent cells by Western blotting and immunofluorescence combined with laser scanning confocal microscopy.

RESULTS: The SK-Hep-1/CDDP cells (IC_{50} = 70.61 ± 1.06 μg/mL) was 13.76 times more resistant to CDDP than the SK-Hep-1 cells (IC_{50} = 5.13 ± 0.09 μg/mL), and CDDP-resistant cells also demonstrated cross-resistance to many anti-tumor agents such as doxorubicin, 5-fluorouracil and vincristine. Similar morphologies were determined in both SK-Hep-1 and SK-Hep-1/CDDP groups. The cell cycle distribution of the SK-Hep-1/CDDP cell line exhibited a significantly increased percentage of cells in S (42.2% ± 2.65% *vs* 27.91% ± 2.16%, $P < 0.01$) and G2/M (20.67% ± 5.69% *vs* 12.14% ± 3.36%, $P < 0.01$) phases in comparison with SK-Hep-1 cells, while the percentage of cells in the G0/G1 phase decreased (37.5% ± 5.05% *vs* 59.83% ± 3.28%, $P < 0.01$). The levels of MDR1 and MRP1 were overexpressed in the SK-Hep-1/CDDP cells exhibiting the MDR phenotype.

CONCLUSION: Multiple drug resistance of multiple drugs in the human hepatoma cell line SK-Hep-1/CDDP was closely related to the overexpression of MDR1 and MRP1.

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Key words: Hepatoma; Cell line; Multidrug resistance; *In vitro*; Cisplatin

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INTRODUCTION

Multidrug resistance (MDR)[1] is a major obstacle in the chemotherapy of cancer patients and is also one of the reasons for tumor relapse and metastasis. Hepatocellular

carcinoma (HCC) is one of the most common gastrointestinal tumors worldwide, accounting for 70%-90% of primary liver cancers[2], with a high degree of malignancy and poor prognosis. A majority of patients are surgically unresectable at the time of diagnosis, and even for those where surgery is possible, the risk of recurrence is extremely high. Consequently, chemotherapy is an important treatment for most HCC patients. However, HCC responds poorly to chemotherapy owing to acquired MDR[3,4]. The ability to overcome this MDR is a major concern in clinical oncology in successfully treating HCC[5].

Cisplatin (CDDP) is a key drug that is widely used for cancer chemotherapy, but the effectiveness of CDDP for HCC is unsatisfactory because of MDR. To elucidate the CDDP-resistant mechanism in HCC *in vitro*, we established a CDDP-resistant SK-Hep-1 cell line and analyzed its biological behavior.

MATERIALS AND METHODS

Drugs and chemicals

CDDP, doxorubicin (DOX), penicillin, streptomycin, propidium iodide (PI) and RNase A were obtained from the Sigma-Aldrich Chemical Co (St.Louis, MO, USA). Vincristine (VCR) was obtained from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). 5-fluorouracil (5-FU) was obtained from Shanghai Xudong Haipu Pharmaceutical Co. Ltd (Shanghai, China). Dulbecco's modified Eagle's medium/high glucose (DMEM/H), fetal bovine serum (FBS) and Trypsin were purchased from Invitrogen (Carlsbad, CA, USA). The phospho-glycoprotein (P-gp, MDR1) mouse monoclonal antibody, multidrug resistance-associated protein 1 (MRP1)-specific mouse anti-human antibody and FITC-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MitoTracker Red CMXRos dye purchased from Invitrogen (Molecular Probes, Invitrogen Corp.). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Dojindo, Japan). The cell culture supplies were purchased from Corning Life Sciences (Lowell, MA, USA). Anti-tumor agents were prepared extemporaneously in complete culture medium immediately prior to use *in vitro*.

Cells and cell culture

The human hepatoma cell line, SK-Hep-1, was obtained from the Cell Culture Center, Institute of Basic Medical Sciences within the Chinese Academy of Medical Sciences. SK-Hep-1 cells were cultured in DMEM/H containing 10% (v/v) FBS, penicillin (200 U/mL), streptomycin (100 μg/mL), and were incubated at 37℃ in a humidified incubator with an atmosphere of 50 mL/L CO_2.

Establishment of a CDDP-resistant SK-Hep-1 subline *in vitro*

SK-Hep-1/CDDP was produced by exposing SK-Hep-1 cells to CDDP repeatedly at a single high concentration over a period of 24 h. Briefly, SK-Hep-1/CDDP was selected by a procedure consisting of 6 pulse drug treatments with 5 μg/mL CDDP, with SK-Hep-1 cells (1×10^6) plated onto a 25 cm^2 culture flask in each pulse treatment. When cells were growing exponentially, they were exposed to CDDP for 24 h. The majority of the cells were dead following 24 h exposure to CDDP. The treated cells were then washed with 0.01 mol/L phosphate-buffered saline (PBS) and cultured in CDDP-free growth medium. After 1-2 d, the dead cells were washed out with PBS and fresh medium again added. The resistant subclones were isolated by limiting dilution. After 4 weeks' incubation at 37℃ in a humidified incubator containing 50 mL/L CO_2, the cells recovered at an exponential rate and were then subcultured at a density of 1×10^6 cells/25 cm^2 flasks. Once cells reached 60%-70% confluence, the cells were preserved for further study as described above. The CDDP-resistant subclone was established 6 mo after the treatment was initiated, then the resistant phenotype developed. For maintenance of CDDP-resistant cells, the SK-Hep-1/CDDP cells were grown in the presence of 0.01 μg/mL CDDP. Before experimentation, SK-Hep-1/CDDP cells were maintained in a CDDP-free culture medium and subcultured at least 3 times. The MDR characteristics of these SK-Hep-1/CDDP cells were tested using various concentrations of anticancer drugs including CDDP, DOX, VCR and 5-FU.

CCK-8 cell proliferation and cytotoxicity assay

Cell proliferation assays were performed with CCK-8 according to the manufacturer's instructions. Cells (2×10^3) were seeded into each well of a 96-well plate and cultured in 100 μL of DMEM/H supplemented with 10% FBS. At the indicated time points, medium was exchanged for 110 μL of DMEM/H with CCK-8 reagent (10 μL CCK-8 and 100 μL DMEM/H), and the cells were incubated for 2 h. Absorbance was measured for each well at a wavelength of 450 nm, with the reference wavelength set at 600 nm. An increase or decrease in absorbance values at 450 nm in the experimental wells relative to the initial value indicated cell growth or death, respectively. Cell growth was monitored every 24 h over 5 d, and was repeated in 6 wells. All assays were carried out independently in triplicate.

The effects of chemotherapeutic agents on the growth of SK-Hep-1 and SK-Hep-1/CDDP cells were also evaluated with CCK-8. Cells (2×10^3/well) were seeded into 96-well plates in 100 μL of DMEM/H with 10% FBS incubated at 37℃ in a humidified atmosphere containing 50 mL/L CO_2. After 24 h the medium was aspirated, and exchanged with media containing a test chemotherapeutic agent at various concentrations. After incubation for 24 h at 37℃, the drug-containing growth medium was replaced with 110 μL medium containing CCK-8 reagent. After 2 h, the absorbance was read at 450 nm with a reference wavelength at 600 nm. Six wells were used for each drug concentration and the experiment was replicated 3 times. The IC_{50} was calculated by SPSS13.0 (SPSS Inc., Chicago, IL, USA). The lower the IC_{50} value, the higher the potency against cell proliferation.

Cell cycle analysis

Control and CDDP-resistant cells were harvested, washed twice with ice-cold PBS (pH 7.2) and fixed in ethanol: PBS (9:1) at -20℃ for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 mg/mL PI in the presence of 25 mg/mL RNase A. Cell cycle phase distribution was analyzed in 3 different experiments using a COULTER® EPICS™ XL™ flow cytometer (Beckman Coulter Inc., Brea, CA, USA). Data from 50 000 events/sample were collected and analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Immunolabeling and quantification

For immunofluorescence, cells were grown on glass slides in 6-well culture plates. Two days later, MitoTracker Red in DMEM/H (200 nmol/L) was added to live cells for 30 min at 37℃, and washed twice with ice-cold PBS (0.01 mol/L, pH 7.2). The cells were fixed for 30 min in 4% ice-cold paraformaldehyde followed by permeabilization with 0.3 mg/mL Triton X-100 for 30 min at room temperature. Fixed, permeabilized cells were washed twice with 0.01 mol/L PBS and incubated with blocking buffer containing goat serum for 30 min at room temperature. Cells were washed twice with 0.01 mol/L PBS again and the expression of MDR1 was determined using the mouse monoclonal anti-P-gp antibody (1:100), Expression of MRP1 was determined using the mouse monoclonal anti-MRP1 antibody (1:100). Negative controls for immunofluorescence involved replacing the primary antibodies with an IgG isotype control. The primary antibody incubations were carried out in blocking buffer overnight at 4℃. After incubation with primary antibodies, cells were washed 3 times with 0.01 mol/L PBS, and incubated with secondary FITC-conjugated goat anti-mouse antibody (1:50) in the dark for 1.5 h at 37℃. Cell nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) solution (1 μg/mL in PBS) for 5 min at room temperature. After washing with 0.01 mol/L PBS, cells were mounted on glass slides using Antifade Mounting Medium (Beyotime Institute of Biotechnology, Jiangsu, China). Confocal laser scanning microscopic analysis of immunolabeling was performed on a Leica TCS SP2 confocal laser imaging system (Leica Microsystems, Wetzlar, Germany). To ascertain localization of the mitochondria, cells were stained with MitoTracker Red CMXRos dye. A merged double image of green MDR1 or MRP1 and MitoTracker Red was obtained. To allow quantitative comparisons of the relative fluorescence intensity of cells between groups, cells were chosen on a random basis and scanned at more than 3 points for analysis. Software Image-Pro Plus Version 6.0 (MediaCybernetics, Bethesda, MD, USA) was used to count the mean value of the fluorescence intensity. Average green fluorescence intensity per cell was determined as the quantity of P-gp or MRP1 protein expression.

Detection of P-gp and MRP1 protein by Western blotting

Total protein was collected from cultured SK-Hep-1 and SK-Hep-1/CDDP cells and the concentration was measured using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The protein was denatured in lithium dodecyl sulfate (LDS) sample buffer (106 mmol/L Tris-HCl, 141 mmol/L Tris base pH 8.5, 0.51 mmol/L EDTA, 10% glycerol, 2% LDS, 0.22 mmol/L SERVA blue G250, 0.175 mmol/L phenol red, 0.1 mmol/L 2-mercaptoethanol) for 5 min at 95℃, electrophoresed on a 7.5% SDS-PAGE and blotted onto 0.2 μm PVDF membranes (Roche, Indianapolis, IN, USA). Membranes were blocked with 5% (w/v) dry milk in TBS-T (Tris-buffered saline containing 0.05% Tween 20) for 1 h at room temperature and incubated overnight at 4℃ with antibodies against P-gp (1:500) or MRP1 (1:100). After incubation with the respective primary antibodies, the membranes were washed 3 times for 5 min in TBS containing 0.05% Tween-20, and then the membranes were exposed to species-specific horseradish peroxidase-labeled secondary antibodies (ZhongShan Goldenbridge Biotechnology, Beijing, China) at 37℃ for 1 h, and developed using the ECL plus Western blotting reagent with visualization on X-ray films. The expression of β-actin was detected as an internal control. The band intensity on X-ray films was quantified using Gel-Pro Analyzer software. The ratio of the band intensity for P-gp/β-actin and MRP1/β-actin was used as the relative expression level for P-gp and MRP1 protein, respectively.

Statistical analysis

All experiments were run in triplicate, and the results are given as mean ± SD. Statistical analyses were performed using either an analysis of variance (ANOVA) or Student *t* test. The difference was considered statistically significant when the *P* value was less than 0.05. All statistical analyses were carried out with SPSS 13.0 software.

RESULTS

Establishment of a cisplatin-resistant SK-Hep-1/CDDP cell line

We established CDDP-resistant SK-Hep-1 (SK-Hep-1/CDDP) cells by pulse exposure of SK-Hep-1 cells to high concentrations of CDDP for short periods over 24 h. This caused a marked change in cellular morphology with many elongated cell dendrites and deposits of intracytoplasmic vacuoles observed, and cells membrane became less clear, or cells died. These effects were clearly observed after 24 h treatment with CDDP at 5 μg/mL. The surviving cells recovered exponentially and then were further selected by a procedure consisting of 6 pulse drug treatments with 5 μg/mL CDDP. The SK-Hep-1-resistant subclone was established 6 mo after the treatment was initiated. Microscopic observation revealed that the SK-Hep-1/CDDP cells (Figure 1B) adopted a spindle shape, similar to that of the parent cells (Figure 1A). The sensitivity of SK-Hep-1 and SK-Hep-1/CDDP cells to various concentrations of CDDP was determined by CCK-8 assay. As shown in Table 1, IC_{50} values for CDDP on SK-Hep-1 and SK-Hep-1/CDDP cells were 5.13 ±

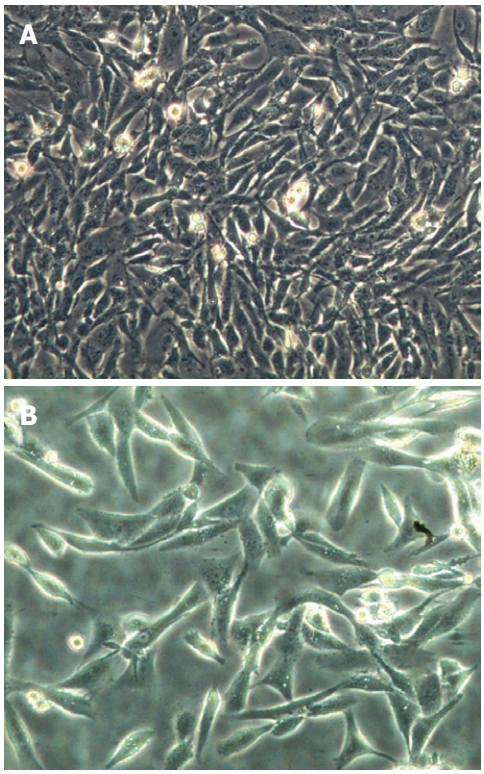


Figure 1 The cell shape of SK-Hep-1 (A) cell line and SK-Hep-1/CDDP (B) cell line (× 100). SK-Hep-1: A highly invasive human hepatoma cell line with a epithelial-like morphology; SK-Hep-1/CDDP: A cisplatin-resistant cell line from the parental cell line.

Table 1 IC_{50} and RI values of SK-Hep-1 and SK-Hep-1/CDDP cells

Drug (μg/mL)	IC_{50} (mean ± SD)		RI
	SK-Hep-1	SK-Hep-1/CDDP	
CDDP	5.13 ± 0.09	70.61 ± 1.06[b]	13.76
DOX	0.74 ± 0.04	4.13 ± 0.23[b]	5.58
VCR	0.76 ± 0.02	2.28 ± 1.06[b]	3.12
5-FU	12.49 ± 0.27	52.79 ± 3.85[b]	4.23

[b]Significant difference in comparison with the parental cells ($P < 0.01$). SK-Hep-1 and SK-Hep-1/CDDP cells were exposed to indicated concentrations of CDDP, DOX, VCR and 5-FU for 24 h and determined using the CCK-8 assay. The IC_{50} values were calculated. Values of 3 independent experiments are represented as mean ± SD. Resistance index (RI) = (IC_{50} SK-Hep-1/CDDP cells)/(IC_{50} SK-Hep-1 cells). CDDP: Cisplatin; IC_{50}: 50% inhibitory dose; DOX: Doxorubicin; VCR: Vincristine; 5-FU: 5-fluorouracil.

0.09 μg/mL and 70.61 ± 1.06 μg/mL, respectively. SK-Hep-1/CDDP cells were 13.76-fold more resistant to CDDP than the parent cells. We also compared the cross-resistance to other anticancer drugs (DOX, VCR, 5-FU) between the parent and CDDP-resistant cells, with our results indicating that the SK-Hep-1/CDDP cells also had cross-resistance to DOX, VCR and 5-FU.

Cell growth and cell cycle

The growth curves for SK-Hep-1 cells and the CDDP-resistant SK-Hep-1 subline are shown in Figure 2A, and the cell cycle distribution of each cell line is shown in Figure 2B and C, and Table 2. The resistant cells grew

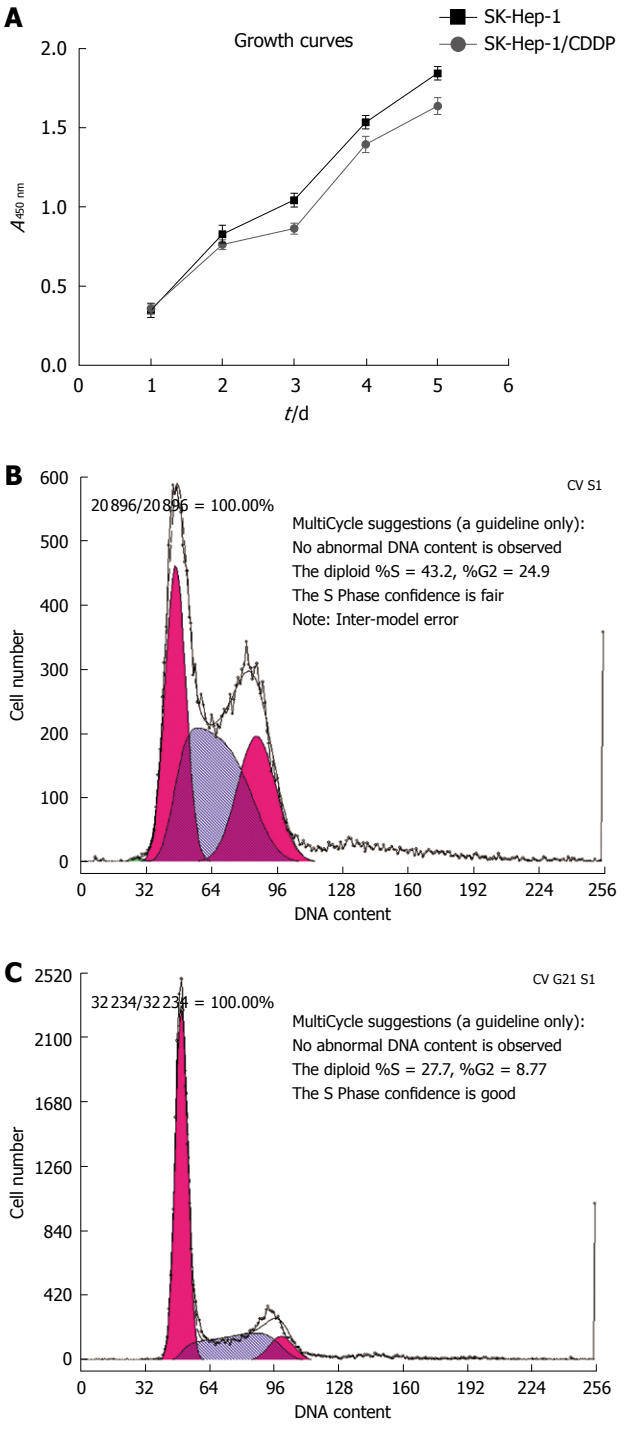


Figure 2 Growth curves of 2 cell lines, SK-Hep-1 and SK-Hep-1/CDDP (A), flow cytometric analysis of cell cycle distribution of SK-Hep-1/CDDP (B) and SK-Hep-1 (C).

more slowly than the the parent cells ($P < 0.05$). Cell cycle analysis revealed that the number of SK-Hep-1/CDDP cells in the G0/G1 phase decreased, accompanied by an increased proportion of cells in the S phase and G2/M phases ($P < 0.05$, Table 2).

Evaluation of MDR1 and MRP1 by laser scanning confocal microscope

We examined the expression of the ATP-binding cassette (ABC) transporters[6], MDR1 and MRP1, by immunofluo-

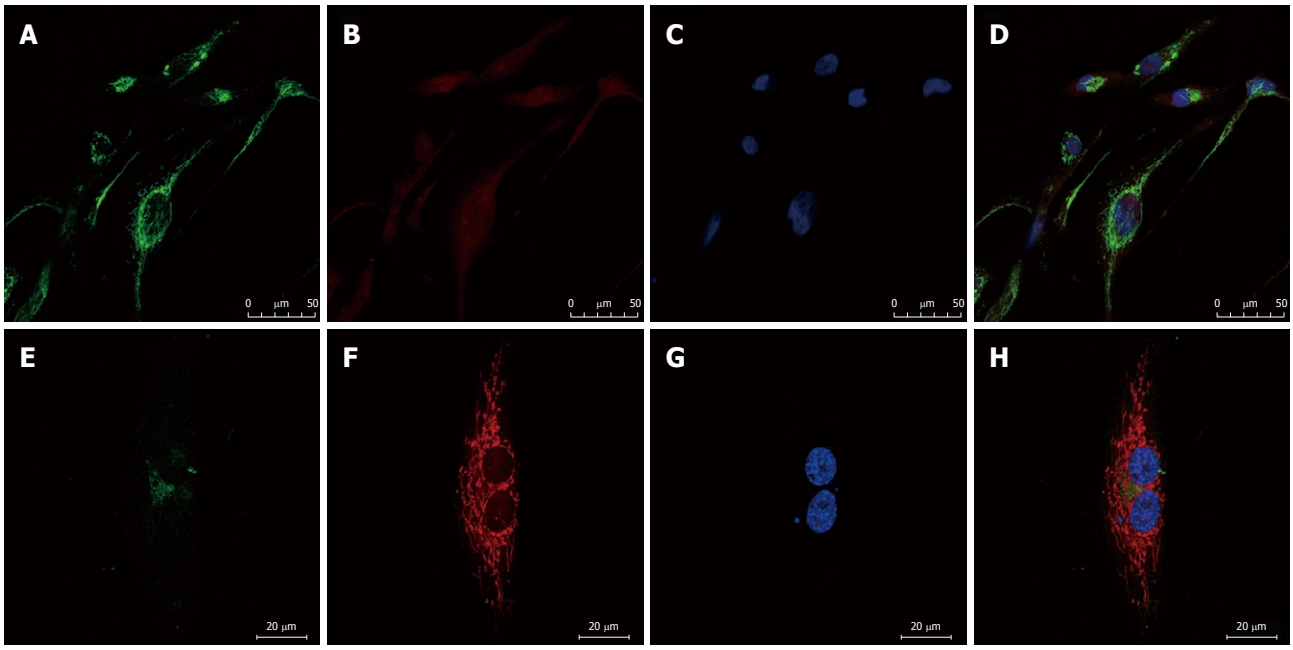


Figure 3 Laser scanning confocal microscopy for MDR1/P-gp in resistance SK-Hep-1/CDDP (A-D) and parent SK-Hep-1 (E-H) cell lines. SK-Hep-1/CDDP (A) and SK-Hep-1 (E) cells were simultaneously stained for the anti-P-gp antibody-FITC (green), (B) and (F) were stained with mitochondria (red), (C) and (G) immunofluorescence images for the nuclei (blue), (D) and (H) the expression of the merged image. MDR1: Multidrug resistant protein 1; P-gp: Phospho-glycoprotein.

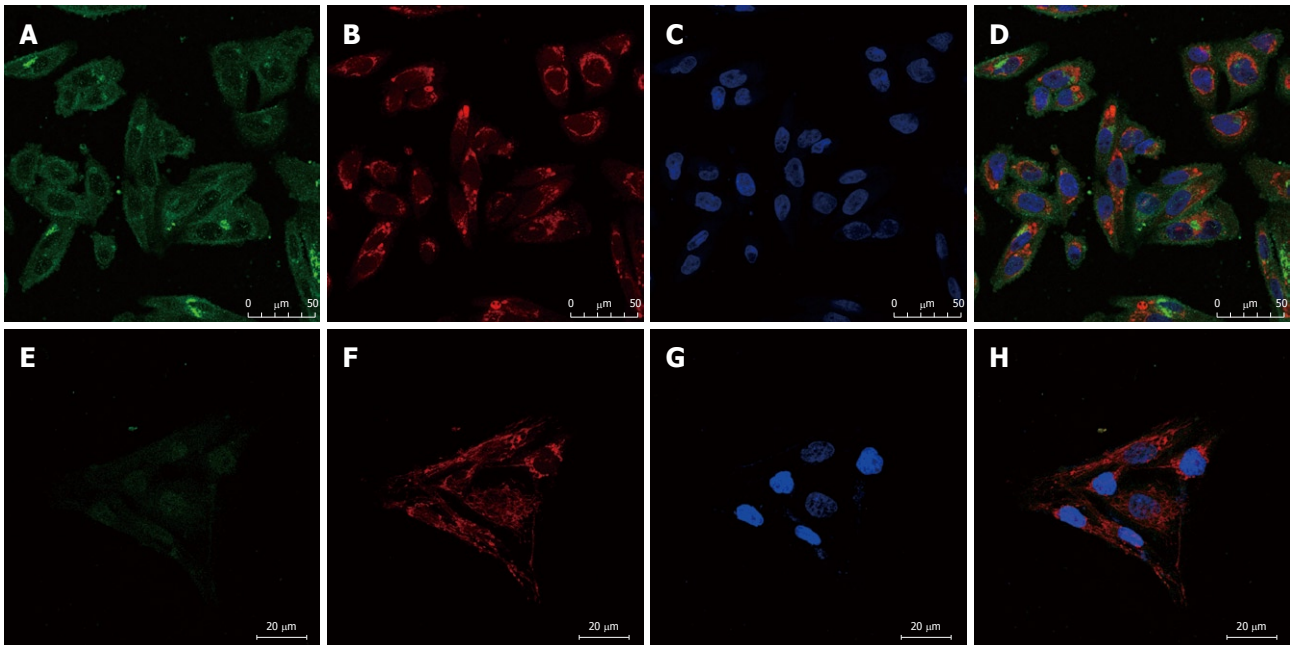


Figure 4 Overexpression of MRP1 in resistant SK-Hep-1/CDDP (A-D) and SK-Hep-1 (E-H) cell lines. Immunofluorescence images for MRP1 (A and E, green), mitochondria (B and F, red), and nuclei (C and G) on the same section were merged to illustrate areas of overlap (D and H). MRP1: Multidrug resistance-associated protein 1.

rescence. Confocal microscopic analysis confirmed that expression of both MDR1 and MRP1 was elevated in SK-Hep-1/CDDP and SK-Hep-1 cells (Figures 3 and 4), MDR1 or MRP1 appeared to localize around the nuclear membranes. To further assess the localization of MDR1 and MRP1, we performed a double stain with MitoTracker Red and DAPI. The green fluorescein fluorescence image was ubiquitous (MDR1 or MRP1). Figure 3 shows the relative level of MDR1/P-gp (green fluorescence) in SK-Hep-1/CDDP (Figure 3A-D) and parent SK-Hep-1 cell (Figure 3E-H) as viewed by confocal microscopy. Figure 4 shows the relative level of MRP1 (green fluorescence) in SK-Hep-1/CDDP (Figure 4A-D) and parent SK-Hep-1 cell (Figure 4E-H).

The results of the quantitative assessment are shown in Table 3. CDDP-resistant cells demonstrate pronounced high levels of green fluorescence and high mean gray density when compared with SK-Hep-1 cells ($P < 0.05$).

Table 2 Cell cycle distribution of SK-Hep-1 and SK-Hep-1/CDDP cells (%)

Cell	G0/G1	S	G2/M
SK-Hep-1	59.83 ± 3.28	27.91 ± 2.16	12.14 ± 3.36
SK-Hep-1/CDDP	37.50 ± 5.05[a]	42.20 ± 2.65[a]	20.67 ± 5.69[a]

[a]$P < 0.05$, resistant cells *vs* SK-Hep-1. SK-Hep-1/CDDP in G0/G1 phase decreased accompanied by an increased proportion of cells in the S phase and G2/M phase.

Table 3 Semi-quantification of P-gp and MRP1 immunofluorescence (mean ± SD)

Cell	P-gp (n = 3)	MRP1 (n = 3)
SK-Hep-1	20.57 ± 6.59	3.75 ± 0.02
SK-Hep-1/CDDP	64.79 ± 12.4[a]	35.78 ± 6.17[a]

[a]$P < 0.05$, resistant cells *vs* SK-Hep-1.

P-gp and MRP1 expression in cells by Western blotting

The expression of P-gp/MDR1 and MRP1 in SK-Hep-1/CDDP cells was evaluated by Western blotting analyses. These proteins were detected in the parental SK-Hep-1 and SK-Hep-1/CDDP cells. The levels of P-gp and, MRP1 were significantly higher in the SK-Hep-1/CDDP resistant cells than in the SK-Hep-1 cells ($P < 0.01$, Figure 5). We considered it important to confirm the maintenance of MDR1 and MRP1 expression and its functionality for our culture conditions. The gray intensity of P-gp/β-actin was 0.58 ± 0.02 for SK-Hep-1 and 1.32 ± 0.04 for SK-Hep-1/CDDP ($P < 0.01$, $n = 3$). The gray intensities of MRP1/β-actin were 0.24 ± 0.01 and 0.59 ± 0.03 for SK-Hep-1 and SK-Hep-1/CDDP cells, respectively ($P < 0.01$, $n = 3$).

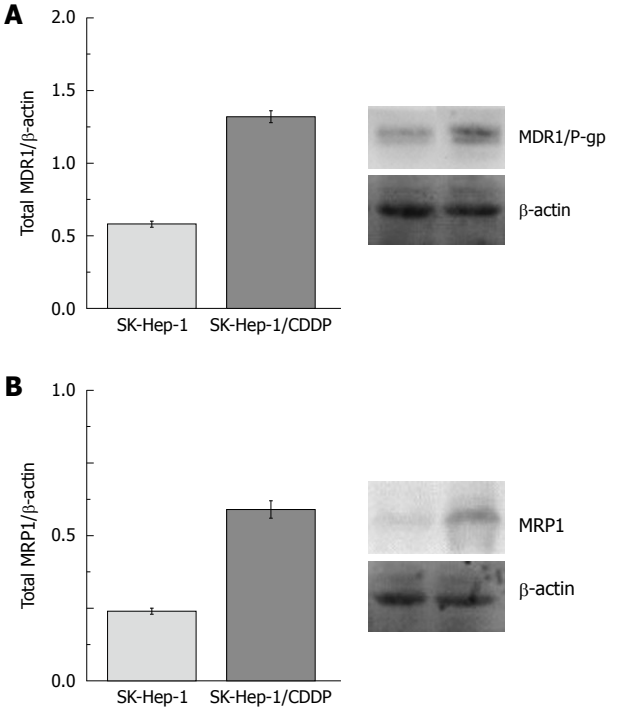


Figure 5 Representative Western blotting of total protein extracts from SK-Hep-1 and SK-Hep-1/CDDP cells. A: Left: The relative level of MDR1/β-actin in each cell line in the bar graph ($P < 0.05$, $n = 3$). Right: Western blotting analysis of MDR1/P-gp; B: Left: The relative level of MRP1/β-actin in each cell line in the bar graph ($P < 0.05$, $n = 3$). Right: Expression level of total MRP1 protein in SK-Hep-1 and SK-Hep-1/CDDP cells. The data are representative of those obtained in 3 other experiments.

DISCUSSION

Intrinsic and acquired resistance to multiple chemotherapeutic drugs is a major obstacle in the clinical treatment of cancer, and the mechanisms responsible for MDR remain unclear. Tumor-derived cell lines in which MDR is induced by the intermittent exposure to drugs[7,8] are frequently used models to study these mechanisms. In order to elucidate the molecular mechanisms of multiple chemotherapeutic drugs resistance in HCC, we established a CDDP-resistant hepatoma cell line as model for investigating chemotherapy resistance.

In this study, the CDDP-resistant subline SK-Hep-1/CDDP was induced by high-concentration, short-duration drug treatment[9]. SK-Hep-1/CDDP cells exhibited strong resistance to CDDP, 13.76 times greater than in SK-Hep-1 cells. The SK-Hep-1/CDDP cells showed significant changes in cell growth compared with SK-Hep-1, growing at a slower rate and exhibiting changes in the cell cycle following the development of drug resistance. The proportion of SK-Hep-1/CDDP cells in the G2/M and S phases increased significantly. Meanwhile SK-Hep-1/CDDP cells also demonstrated cross-resistance to multiple, structurally diverse chemotherapeutic agents, such as DOX, VCR and 5-FU. These resistant phenotypes were stable and the IC_{50} values and resistance index demonstrated no significant change over a 3-mo period in drug-free medium.

HCC resistance to chemotherapeutic treatment is possibly related to the overexpression of MDR proteins belonging to the ABC family. Recent studies showed that HCC expressed *MRP1*, *MDR1*, *MRP3* and breast cancer resistance protein (BCRP/ABCG2), and these might confer tumor cells with a MDR phenotype[10,11]. Some reports stated that MRPs were more likely to be candidates to mediate chemo-resistance in HCC than MDR1[12,13]. *MDR1*, *MRP1* and *MRP2* genes are well known as ABC transporters associated with CDDP-resistance, as these proteins cause the efflux of many anticancer agents that have different mechanisms. Our study revealed that both a CDDP-resistant subline and its parental cells expressed MDR1, but the subline cells overexpressed MDR1 and MRP1.

In summary, although chemotherapy-resistant tumor cell lines have some disadvantages, they do provide model systems where all biological parameters can be controlled and assessed. We established a CDDP-resistant human hepatoma cell line, which provided the basis for further study of resistant mechanisms and reversal of clinical HCC drug resistance.

COMMENTS

Background

Drug resistance, in particular multidrug-resistance (MDR), is still the major cause of anticancer chemotherapy failure. The most important factor of medicating a MDR phenotype is the increased activity of the membrane-embedded drug extrusion pump, MDR1/P-glycoprotein (MDR1/P-gp or ABCB1). The mechanism of the reverse MDR phenotype is thought to involve direct binding to P-gp and displacement of the anticancer drugs, which enhances intracellular toxic efficacy of these agents. Analysis of novel chemotherapy-resistant cell lines may duplicate as far as possible the treatment conditions used *in vivo*.

Research frontiers

Hepatocellular carcinoma (HCC) responds poorly to chemotherapy owing to MDR. Recent studies have shown the tumors derived from the colon, kidney, or adrenal cortex, and HCC exhibited overexpression of MDR1/P-gp. This overexpression results in a primary MDR phenotype of these cancers. Tumor-derived cell lines are one of the most important tools for investigation of the biological mechanisms directly leading to drug resistance in patients. Today, the experimental search for drug resistant mechanisms that are clinically relevant targets whose circumvention can improve cancer therapy is still ongoing.

Innovations and breakthroughs

The model SK-Hep-1/CDDP cell line can be used as an *in vivo* model to investigate the molecular mechanisms involved in MDR-related genes of hepatocarcinoma and to explore the targeted approaches for overcoming MDR in tumor cells.

Applications

The SK-Hep-1/CDDP cells can be employed as a model system as its biological parameters can be controlled and assessed, and novel biomarkers of chemotherapy resistance can be identified. The methods described in this study and the results presented might provide the basis for advanced studies in this field.

Peer review

The manuscript is interesting and could be useful. The establishment of a human hepatoma multidrug-resistance cell line *in vitro* presents interesting results. The data show that a cisplatin-resistant hepatoma cell line increased MDR1 comparing with control cells, and expressed MRP1. They concluded that this fact conferred cross-resistance to the cell line.

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CASE REPORT

Diagnosis of ruptured superior mesenteric artery aneurysm mimicking a pancreatic mass

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Abstract

Aneurysms and pseudoaneurysms of the superior mesenteric artery are potentially lethal and should be treated as urgently as possible. In a 52-year-old man with occasional epigastric pain, we accidentally discovered a superior mesenteric artery aneurysm that was ruptured with spontaneous tamponade in the uncinate process and in the head of the pancreas. The ruptured aneurysm had a heterogeneous appearance due to its thrombotic and hemorrhagic content, and it simulated a voluminous mass in the head and uncinate process of the pancreas, associated with mild dilatation of the main pancreatic duct. Recent advances in multidetector computed tomography and magnetic resonance imaging have enabled radiologists to develop a correct diagnosis of mesenteric aneurysms and pseudoaneurysms of the visceral branches of the abdominal aorta, and to differentiate this diagnosis from that of pancreatic or peripancreatic masses; angiography is currently used to confirm a diagnosis and to develop therapeutic treatments.

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Key words: Superior mesenteric artery; Magnetic resonance imaging; Computed tomography; Ruptured aneurysm

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INTRODUCTION

Aneurysms of the visceral branches of the abdominal aorta are rare and potentially life-threatening, with a documented prevalence of 0.1%-2%[1]. The superior mesenteric artery is the third most common site of splanchnic aneurysm[2]. Recent advances in computed tomography (CT) vascular imaging and magnetic resonance angiography have provided a highly accurate representation of abdominal splanchnic vessels[3]. Multi-detector computed tomography (MDCT) with 3D post-processing allows for an accurate representation of vascular course and caliber. Thanks to its high-contrast resolution, magnetic resonance imaging (MRI) is used to determine the composition of aneurysmatic masses.

The aim of this case report is to describe the imaging features of a ruptured superior mesenteric artery aneurysm, which created a giant hematoma and mimicked a pancreatic mass, with mild dilatation of main pancreatic duct.

CASE REPORT

A 52-year-old man with intermittent epigastric pain was admitted to our hospital. He had undergone cholecystectomy and left colonic resection for cancer of the large intestine 4 and 2 years earlier, respectively. Preoperative liver ultrasonography and chest X-rays performed during previous hospitalization were normal.

During his last hospital admission, the patient underwent laboratory tests that revealed a mild increase in serum pancreatic amylase and lipase activity (129 and 116 UI/L, respectively). Coagulation tests were normal, and clinical examination did not reveal significant results. The patient was scheduled for abdominal CT. MDCT showed an 8.2 cm × 6.6 cm mass in the area of the uncinate process of the pancreas (Figure 1A). A post-contrast infusion MDCT scan showed enlargement of the proximal portion of the superior mesenteric artery, which had a transverse diameter of 2.2 cm. Multiplanar reconstructions of MDCT images indicated communication of the mass with the enlarged superior mesenteric artery (Figure 1B). The artery was englobed into the mass, which resulted in its complete obstruction. Liver, spleen and kidneys were normal, as was the small bowel, as shown by CT. CT did not show any relapse or residual sign in the area where surgical anastomosis of the colon had been performed.

Enhanced and unenhanced MRI was performed to investigate the mass composition. T1-weighted MR images showed heterogeneous high signal content (Figure 1C); a low peripheral "rim sign" on T1 and T2 images was revealed. Post-gadolinium images confirmed the enlarged superior mesenteric artery and its communication with the mass; there was no significant contrast enhancement in the mass itself. The initial diagnostic hypothesis was of a giant mass of the pancreas, which involved the superior mesenteric artery. The radiologist who performed the examinations described "an expansive lesion in the pancreatic area involving the proximal tract of the superior mesenteric artery", whereas the mass was caused by development and fissuration of the aneurysm into the uncinate process of the pancreas.

Angiography confirmed dilatation of the superior mesenteric artery, without any sign of bleeding. The patient underwent surgical intervention with resection of the thrombotic clot-filled aneurysm (Figure 2), which ruptured with spontaneous tamponade in the pancreatic region and formed a giant hematoma; bowel vitality was good during the provocative occlusive test, and subsequent ligation of the superior mesenteric artery was performed. Distal pancreatectomy was also required for the retropancreatic extension of the hematoma. MDCT performed 6 mo later did not show any complications.

DISCUSSION

Early diagnosis and treatment of aneurysms and pseudoaneurysms of the superior mesenteric artery are essential to prevent them from rupturing and threatening patients' lives[4]. Etiology includes arteriosclerosis, fibromuscular dysplasia, connective tissue disease, infection, and arterial dissection[1,5]. Mycotic aneurysms affect the superior mesenteric artery in 33%-66% of cases[6]. Superior mesenteric artery pseudoaneurysms are often associated with chronic pancreatitis, or they can be a consequence of external or iatrogenic trauma[5,7].

We describe a case of superior mesenteric artery aneurysm which ruptured with spontaneous tamponade in the uncinate process and head of the pancreas, which mimicked a pancreatic mass on MDCT and MRI. The etiology of the superior mesenteric aneurysm in our patient has still not been established; a previous history of pancreatitis, vascular hypertension, connective disease or traumatic abdominal injury was not revealed.

It is known that, on unenhanced MDCT, a superior mesenteric aneurysm can simulate a pancreatic mass[8]; thrombotic or hemorrhagic material in the aneurysm sac provides heterogeneous signal intensity. In our case, the sac filled with blood clots showed a heterogeneous elevated signal on T1 images, which suggested the possibility of a mass with hemorrhagic content or a hematoma.

Without contrast enhancement during the arterial phase, it is very difficult to establish the vascular nature of aneurysms, which is why the radiologist made an incorrect diagnosis[9]. The radiologist who first described MDCT and MRI signs did not explore the vascular structures with multiplanar reconstructions and hypothesized aneurysm not as a primary diagnosis, but as a consequence of a giant mass in the pancreatic tissue. Initially, he did not consider the possibility of a fissurated aneurysm. Pre-angiographic evaluation of multiplanar reconstruction, performed with other colleagues, finally oriented him towards diagnosing a giant aneurysm ruptured into the uncinate process. Axial images and CT reconstructions showed the continuity of the mass and the superior mesenteric artery, and enabled radiologists to diagnose a vascular aneurysm.

There is a large group of peripancreatic structures that can simulate pancreatic cystic lesions, such as aneurysms of the superior mesenteric artery, superior mesenteric vein, hepatic artery and portal vein[10]. Large aneurysms compress the duodenum and biliary duct, thus causing obstructive jaundice[7]. In our case, magnetic resonance cholangiopancreatography (MRCP) showed the dislocation of the pancreatic tract of the main biliary duct, which was caused by the large ruptured aneurysmatic sac (Figure 1D). The choledochus had a caliber of approximately 1 cm, but this was not considered a significant feature since the patient had been cholecystectomized; a diameter of up to 1 cm is frequent in such patients. Dilatation of the main pancreatic duct has not been reported very frequently in the literature. MRCP showed moderate dilatation of the main pancreatic duct and mild ectasia of the side branches

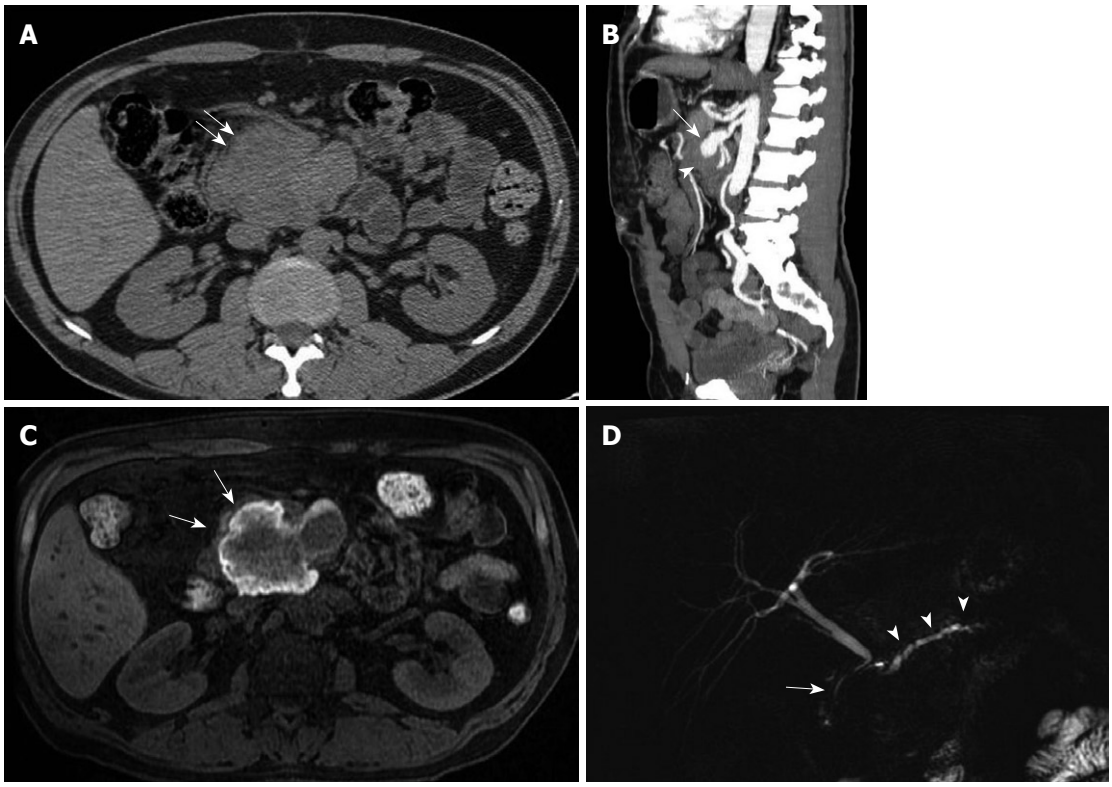


Figure 1 Images of the patient. A: Axial unenhanced multi-detector computed tomography (MDCT) shows a soft-tissue mass (arrows) in the area of the uncinate process of the pancreas. There is no sign of calcification or fluid level; B: Sagittal multiplanar reconstruction clearly shows the superior mesenteric artery (arrow) involved in a giant soft-tissue attenuated mass - the hematoma (arrowhead) - at the pancreatic level. After contrast administration, there was no significant enhancement in the hematoma, due to its thrombotic content; C: Magnetic resonance, axial unenhanced gradient echo T1-weighted imaging with fat suppression. The T1 hyperintense perilesional signal (arrows) was caused by the blood content; D: Magnetic resonance cholangiopancreatography (MRCP) shows dilatation of the main pancreatic duct (arrowheads); the choledochus (arrow) was compressed and dislocated, with mild narrowing of the lumen in the distal tract.

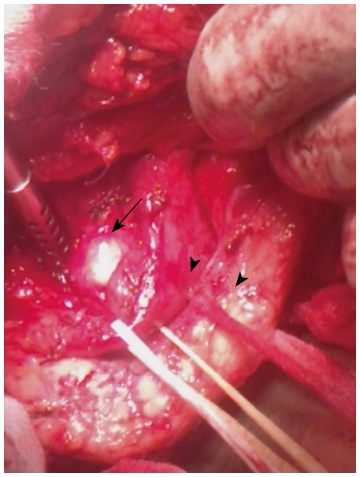


Figure 2 Surgical intervention. Intervention confirmed an aneurysm of the superior mesenteric artery, which ruptured with hematoma in the head of the pancreas. The figure shows the aneurysm (arrow) and superior mesenteric artery and vein (arrowheads).

of the main pancreatic duct (Figure 1D). The dilatation of the pancreatic duct was caused by the hemorrhagic sac, with evident dislocation of pancreatic parenchyma. Mild compression of the main pancreatic duct by the aneurysm sac can also explain minimally increased values of amylase and lipase activity discovered through laboratory tests, which initially physicians interpreted as symptoms of pancreatic disease.

Contrast administration is necessary to demonstrate a patent lumen, and suggests the correct diagnosis. Recent advances in CT technology have allowed for a more accurate multiplanar and 3D reconstruction, which shows the communication between the aneurysm sac and the blood vessels. High table-speed and rapid image acquisition during contrast injection clearly show the arterial anatomy. On CT, large aneurysms and pseudoaneurysms can simulate pancreatic pseudocysts with fluid content[11]. Blood clots and thrombotic deposits in aneurysms and pseudoaneurysms have a density similar to that of soft tissues or of high-density fluid collections. In some cases, pseudocysts have a hemorrhagic content, and on unenhanced CT scans, their density is the same as that of aneurysms.

MRI is very useful in terms of diagnosis because it provides radiologists with accurate information on mass composition: on T1-weighted images the presence of thrombotic and hemorrhagic material is easily demonstrated by the high-signal content of the mass. However, these features are not specific. Since hemorrhagic content on unenhanced T1 images can also be found in mucinous cystic neoplasms, a differential diagnosis is called for. Mucinous cystic lesions have variable signal intensity and sometimes proteinaceous material provides hyperintensity on T1-weighted images. Post-gadolinium images did not reveal internal septa and were very helpful in differentiating the diagnosis from that of cystic mucinous lesions.

In conclusion, aneurysms and pseudoaneurysms of the superior mesenteric artery at the pancreas level can be very insidious for radiologists because they can mim-

ick pancreatic masses. Significant advancements in 3D and multiplanar imaging software have made it possible to obtain high-resolution images of the abdominal aorta and its branches: radiologists need to keep in mind the diagnostic value of multiplanar reconstructions.

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CASE REPORT

Stroke and dilated cardiomyopathy associated with celiac disease

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Abstract

Celiac disease (CD) is manifested by a variety of clinical signs and symptoms that may begin either in childhood or adult life. Neurological symptoms without signs of malabsorption have been observed for a long time in CD. In this report, an 8-year-old girl with CD presented with rarely seen dilated cardiomyopathy and stroke. The girl was admitted with left side weakness. Her medical history indicated abdominal distention, chronic diarrhea, failure to thrive, and geophagia. On physical examination, short stature, pale skin and a grade 2 of 6 systolic murmur were detected. Muscle strength was 0/5 on the left side, and 5/5 on the right side. Coagulation examinations were normal. Tests for collagen tissue diseases were negative. Factor V Leiden and prothrombin GA20210 mutations were negative. Tandem mass spectrophotometry and blood carnitine profiles were normal. Brain magnetic resonance imaging and cerebral angiography showed an infarction area at the basal ganglia level. Examinations of serologic markers and intestinal biopsy revealed CD. We emphasize that in differential diagnosis of ischemic stroke, CD should be kept in mind.

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Key words: Celiac disease; Stroke; Cardiomyopathy; Children

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INTRODUCTION

Celiac disease (CD) is a disease of the small intestine caused by an immune response to ingested gluten. This response results in characteristic damage to the villi, leading to malabsorption[1]. CD is manifested by a variety of clinical signs and symptoms that may begin in either childhood or adult life. Neurological symptoms without malabsorption signs have been observed for a long time in CD. Epilepsy, bilateral occipital calcification, cerebellar ataxia, degenerative central nervous system disease, peripheric neuropathy, myopathy and, rarely, stroke were defined as neurologic manifestations[2]. Tissue transglu-

taminase enzyme is an auto-antigen, which is related to gluten-associated immune events[3]. In this case report, an 8-year-old girl with CD presented with rarely seen stroke.

CASE REPORT

An 8-year-old girl was admitted to our emergency department with weakness of the left lower and upper extremities. It was learnt that the patient had geophagia, abdominal distention and chronic diarrhea for 2 years. On physical examination, her body weight and height were 16 kg (-2 SD) and 110 cm (-2.6 SD), respectively. Blood pressure was 110/70 mmHg. She had pale skin and mucosa, and a grade 2 of 6 systolic murmur at the inferior left side of the sternum. Muscle strength was determined as 0/5 on the left upper and lower extremities and 5/5 on the right side. The laboratory analysis, hemogram, serum electrolytes, glucose, cholesterol, triglyceride levels, liver and renal function tests were within normal limits. Thyroid hormone values were also found to be within the normal range [free T_4: 1.4 ng/dL (normal range: 0.8-2.2 ng/dL), total T_4: 7.4 mg/mL (normal range: 5.5-12.8 mg/L), thyroid stimulating hormone: 1.2 mU/mL (normal range: 0.6-5.5 mU/mL), total T_3: 170 ng/dL (normal range: 119-218 ng/dL), free T_3: 3.2 pg/mL (normal range: 2.0-4.0 pg/mL)]. C-reactive protein was negative and erythrocyte sedimentation rate was 19 mm/h. Vitamin B12 and folate levels were normal. Prothrombin time and activated partial thromboplastin time were 13.6 s (normal range: 11-14 s) and 29 s (normal range: 31-40 s), respectively. Fibrinogen, protein C, protein S, factors V, Ⅶ, Ⅷ, Ⅺ, Ⅻ and antithrombin Ⅲ levels were within normal limits. Serologic tests for human immunodeficiency virus, lupus anticoagulants, antinuclear antibody, anti-double-stranded DNA, anticardiolipin antibody immunoglobulin (Ig) G and M serologies were negative. Factor V Leiden and prothrombin GA20210 mutations were not detected. The tandem mass metabolic disease screening panel and detailed blood carnitine profile were normal.

On brain magnetic resonance imaging, an infarction measuring 31 mm × 14 mm at the right basal ganglia level was seen (Figure 1). On cerebral angiography examination, a 1 cm segment occlusion was detected at the M2 branch of the right middle cerebral artery (Figure 2). Although dilated cardiomyopathy was identified on transesophagial and transthoracic echocardiographic examination, thrombus or vegetation was not seen. Serologic markers of CD were examined because of chronic diarrhea, abdominal distention, short stature, cerebral infarction and dilated cardiomyopathy, and anti-tissue transglutaminase IgA and IgG, and anti-endomysium IgA were found at a highly positive rate. Additionally, the examination of intestinal biopsy revealed CD. Duodenal biopsy showed villous atrophy with hyperplasia of the crypts and an increased intraepithelial lymphocyte count (above 40%).

A gluten-free diet and nadroparin calcium treatment were initiated and physiotherapy was performed. At the 18th day of hospitalization, the patient, whose symptoms had regressed, was discharged with a gluten-free diet, nadroparin calcium, co-enzyme Q and salicylate treatment. Symptoms resolved by the following 7th wk. Muscle strength at the left upper and lower extremities was 5/5, and other neurologic examinations were normal.

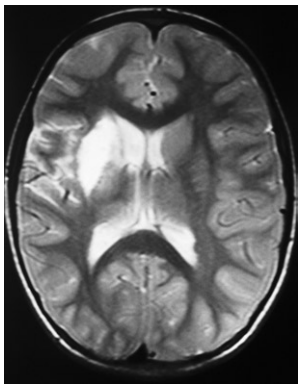


Figure 1 Brain magnetic resonance imaging shows an infarction area measuring 31 mm × 14 mm at the right basal ganglia level.



Figure 2 Cerebral angiography examination shows a 1 cm segment occlusion at the M2 branch of the right middle cerebral artery.

DISCUSSION

Classical findings of CD usually begin at 1-3 years of life. Toddlers and young children classically present with chronic diarrhea, vomiting, poor appetite, abdominal distension, abdominal pain, irritability, and failure to thrive some time after the introduction of gluten in the diet[1]. In adults, a variety of neuropsychiatric conditions, such as depression and anxiety, have been reported in individuals with CD[1]. In our case, abdominal distention, chronic diarrhea, geophagia, and short stature were observed. In addition, infarction and dilated cardiomyopathy were present.

The diagnosis of CD is established by positive results of serological testing and evidence of characteristic histopathology on intestinal biopsy[4]. If results of serologic testing are negative but clinical suspicion is high, intestinal biopsy should be performed. Characteristic histologic features of CD include varying degrees of villous atrophy, with hyperplasia of the crypts and an increased intraepithelial lymphocyte count[5]. Consistent with the literature, our case was positive for anti-tissue transglutaminase IgA and IgG, and anti-endomysium IgA, and duodenal biopsy examination showed villous atrophy

with hyperplasia of the crypts and increased intraepithelial lymphocyte count (above 40%).

Development of autoimmune diseases is one of the complications of the CD. Tissue transglutaminase enzyme is an auto-antigen that is related to gluten-associated immune events. Evidence of a central nervous system vasculitis was reported by Ozge *et al*[6] in a patient with recurrent stroke and CD. Pratesi *et al*[7] found that sera from patients with active CD contain IgA antibodies that reacted with human brain vessel structures, giving intense fluorescence. These antibodies were not present in sera from celiac patients on a gluten-free diet or non-celiac controls. They emphasized that this finding might be involved in the abnormal nervous system manifestations frequently described in association with CD. Tissue transglutaminase is the major auto-antigen in CD and is thought to maintain vascular endothelial integrity. Anti-endomysial IgA antibodies, demonstrated to be the same autoantibody as anti-transglutaminase, react with the cerebral vasculature, suggesting an autoimmune mechanism for CD-associated vasculopathy. Because CD is a potentially treatable cause of cerebral vasculopathy, serology (specifically for anti-tissue transglutaminase antibodies) should be included in the evaluation of cryptogenic stroke in childhood, even in the absence of typical gut symptoms.

Dilated cardiomyopathy is the most commonly seen type of cardiomyopathy. Regarding its etiology, genetic causes, endocrine disorders, collagen vascular diseases, drugs, congenital metabolism diseases, muscular dystrophies, structural heart diseases, acute and chronic myocarditis and toxins can be present. However, 50% of cases remain idiopathic. An increased incidence of CD in patients with idiopathic dilated cardiomyopathy as well as in patients with secondary cardiomyopathy has been reported recently[8]. In our case, dilated cardiomyopathy was diagnosed with echocardiography. However, dilated cardiomyopathy was not thought to be the primary factor causing the stroke, because there was no thrombus or vegetation at echocardiography, and regression of symptoms occurred with a gluten-free diet. In addition, in patients with a cryptogenic stroke, the presence of a patent foramen ovale should be evaluated. Transthoracic 2-dimensional echocardiography can generally show the atrial septum and the flap of the foramen ovale in infants and small children. Color Doppler flow across the atrial septum proves the presence of the foramen ovale. In older children and adults, transthoracic echocardiography does not visualize the atrial septum as well. Transesophageal echocardiography is preferred in patients where the atrial septum is inadequately visualized by transthoracic echocardiography. Older children and adults fall into this category. In addition to a patent foramen ovale, redundancy of the septum primum can also be seen. When the redundancy of the septum moves more than 1 cm, it is called an atrial septal aneurysm. In the presence of a patent foramen ovale in patients who have had a prior stroke, an atrial septal aneurysm confers an increased risk for a subsequent neurologic event[9,10]. In our patient, a patent foremen ovale was not found in both transthoracic and transesophageal echocardiographic examination. Therefore, a patent foremen ovale was not thought to be a cause of the stroke in our patient.

In conclusion, the cause of ischemic stroke in our case is thought to be multifactorial. We suggest that CD was a primary factor in its etiology, secondary to a contribution from dilated cardiomyopathy. In conclusion, we emphasize that in the differential diagnosis of ischemic stroke, CD should be kept in mind.

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CASE REPORT

Primary endoscopic approximation suture under cap-assisted endoscopy of an ERCP-induced duodenal perforation

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Abstract

Duodenal perforation during endoscopic retrograde cholangiopancreatography (ERCP) is a rare complication, but it has a relatively high mortality risk. Early diagnosis and prompt management are key factors for the successful treatment of ERCP-related perforation. The management of perforation can initially be conservative in cases resulting from sphincterotomy or guide wire trauma. However, the current standard treatment for duodenal free wall perforation is surgical repair. Recently, several case reports of endoscopic closure techniques using endoclips, endoloops, or fully covered metal stents have been described. We describe four cases of iatrogenic duodenal bulb or lateral wall perforation caused by the scope tip that occurred during ERCP in tertiary referral centers. All the cases were simply managed by endoclips under transparent cap-assisted endoscopy. Based on the available evidence and our experience, endoscopic closure was a safe and feasible method even for duodenoscope-induced perforations. Our results suggest that endoscopists may be more willing to use this treatment.

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Key words: Duodenal perforation; Endoscopic retrograde cholangiopancreatography; Endoscopic therapy; Endoclip

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INTRODUCTION

Although MR cholangiopancreatography has almost completely replaced endoscopic retrograde cholangiopancreatography (ERCP) in the diagnosis of pancreato-biliary diseases, the risk of ERCP complications has increased as therapeutic endoscopists have taken on increasingly more complex cases, particularly at tertiary referral centers[1]. The frequency of duodenal perforation is 0.5%-2% of patients[2]. However, because of a relatively high mortality rate of 16%-18%, all duodenal perforations should be treated immediately after diagnosis[2-5].

Traditionally, the standard treatment for traumatic or iatrogenic duodenal perforation is surgical closure. Recently, endoscopic trials of perforation management have increased and successful primary repair of duodenal perforation using the endoscope itself has been reported. However, there is no clear consensus for primary repair due to the limited number of cases seen[6-8].

We report four cases in which ERCP done at tertiary referral centers induced a large duodenal perforation, which was then successfully managed with endoscopic approximation suture using multiple endoclips under transparent cap-assisted endoscopy.

CASE REPORT

Case 1

An 80-year-old woman with Klatskin tumor underwent ERCP. Three days later after endoscopic biliary drainage and biopsy, ERCP was attempted for the placement of the metallic stent. During the procedure, the duodenoscope slipped into the duodenum and caused an approximately 13 mm-sized perforation in the duodenal bulb. After early recognition of perforation, an immediate attempt to seal the perforation with 8 endoclips (Endoclip HX-600-090L; Olympus Optical Co., Ltd., Tokyo, Japan) was made successfully under transparent cap-assisted endoscopy (Figure 1A and E). Following the endotherapy, placement of percutaneous transhepatic biliary drainage (PTBD), peripheral parenteral nutrition, intravenous high-dose proton pump inhibitor (PPI), and broad-spectrum antibiotics (3rd generation cephalosporin + metronidazole) was initiated. A nasoduodenal tube was not placed. An abdominal X-ray showed a pneumoperitoneum in the subphrenic area (Figure 2A). However, she remained symptom-free without additional complications. Six days later, a contrast passage *via* endoscope showed no evidence of leakiness and the pneumoperitoneum was completely resolved (Figure 2B). She resumed a scheduled diet 6 d after the clipping and was discharged on day 12 (Tables 1 and 2).

Case 2

A 72-year-old woman with a history of left hepatic lobectomy and cholecystectomy 11 years prior to the procedure underwent ERCP for the segmental stricture of right intrahepatic duct and stones after PTBD. During the insertion of the duodenoscope into the stomach, severe rigidity was felt and the duodenoscope suddenly slipped into the duodenum. This caused an approximately 13 mm-sized perforation in the lateral wall of the second portion of the duodenum. Subsequently, an attempt to seal the perforation was successfully made with 5 endoclips under transparent cap-assisted endoscopy (Figure 1B and F). Peripheral parenteral nutrition, intravenous high-dose PPI, and broad-spectrum antibiotics were initiated. An abdominal computed tomography (CT) showed a pneumoretroperitoneum (Figure 3A). She remained symptom-free 2 d later, and did not develop any complications. A repeat abdominal CT performed 6 d later showed markedly decreased pneumoretroperitoneum and fluid collection, and there was no contrast leak on upper gastrointestinal investigations (UGIs) (Figure 3B). She resumed a normal scheduled diet 7 d after clipping and was discharged on day 10.

Case 3

A 69-year-old woman was referred due to pancreatic cancer. An abdominal CT scan revealed about a 5 cm-sized pancreatic head cancer with multiple hepatic metastases. ERCP was considered for jaundice and evaluation of the biliary system. At first, a trainee who had less than 6 mo experience inserted the duodenoscope. He tried to shorten the scope loop in the duodenum several times, but failed to shorten the scope loop due to duodenal rigidity resulting from the duodenal invasion of pancreatic cancer. The trainee then notified his supervisor, who was observing the procedure. He found about a 10 mm-sized duodenal perforation, which might have been caused by excessive shortening of the scope in the restricted lumen. Successful closure was immediately performed using 4 endoclips under transparent cap-assisted endoscopy (Figure 1C and G). The next day, the patient complained of abdominal pain, but there was no interval change except mild leukocytosis. Fasting and hydration were ordered and abdominal CT scans were taken twice, 24 h apart, but the pneumoperitoneum was not found to be progressive (Figure 4A). On the 8th day after duodenal perforation, UGIs showed no contrast leakage (Figure 4B), and she was put on a diet. After discharge from the hospital 10 d later, the patient received gemcitabine chemotherapy.

Case 4

A 63-year-old man with a history of cholecystectomy 10 years before the procedure underwent ERCP for choledocholithiasis. During insertion of the duodenoscope, an approximately 12 mm-sized perforation occurred in second portion of the duodenum secondary to trauma from difficult passage of the duodenoscope due to bulb deformity caused by recurrent duodenal ulcers. A successful attempt to seal the perforation with 5 endoclips was made under long transparent cap-assisted endoscopy (Figure 1D and H), followed by PTBD. Conservative managements were initiated as mentioned above. Simple X-ray and abdominal CT showed a pneumomediastinum, pneumoperitoneum, and subcutaneous emphysema. The patient remained symptom-free 3 d later and did not develop any complications. An abdominal CT repeated 4 d later showed markedly decreased pneumoretroperitoneum (Figure 5) and a scheduled diet was started. The remaining CBD stones were removed by ERCP 25 d later.

DISCUSSION

Duodenal perforation is an infrequent complication of ERCP. It extends beyond the intramural portion of the bile duct and is usually associated with sphincterotomy in about 1% of patients[9]. Retroperitoneal duodenal perforations represent the majority of cases and usually are

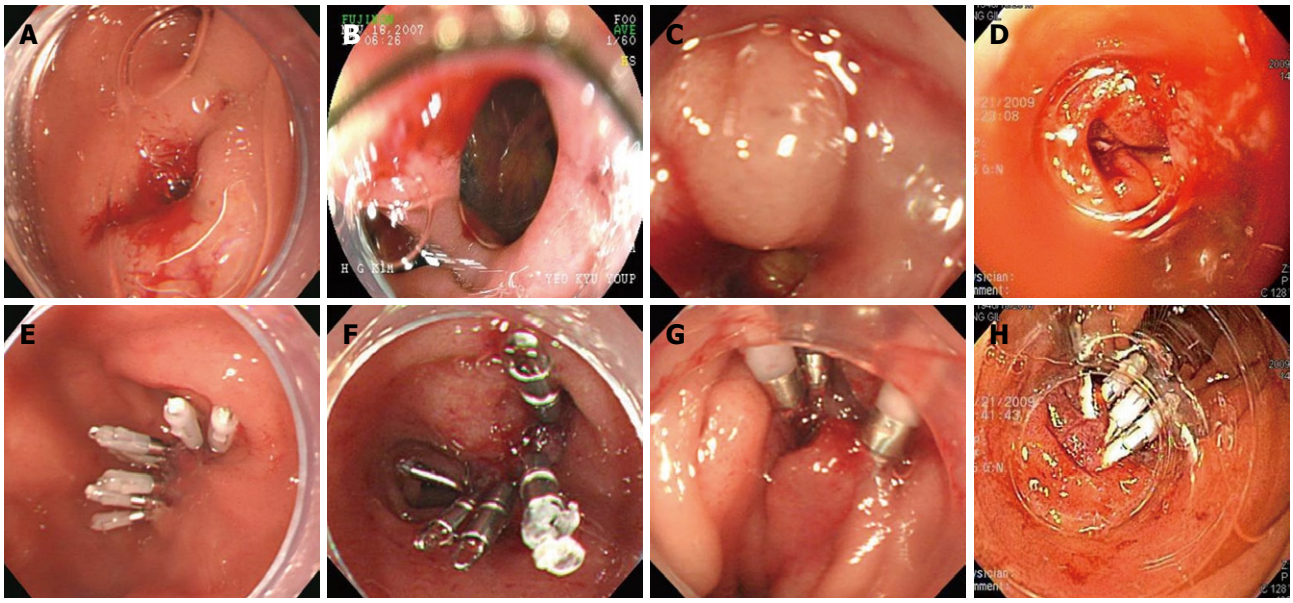


Figure 1 Endoscopic images of four cases demonstrating a large perforation on bulb and lateral wall of the second portion of duodenum, and successful primary endoscopic closure using multiple endoclips.

Table 1 Patient characteristics

Case No.	Age (yr)/sex	ERCP indication	Perforation site	Perforation size (mm)	Diagnosis	Experience of ERCPs (yr)
Case 1	80/F	Klatskin tumor	Bulb	> 10	During ERCP	< 2
Case 2	72/F	IHD stricture	2nd portion, lateral wall	> 10	During ERCP	< 2
Case 3	69/F	Jaundice	2nd portion, lateral wall	> 10	During ERCP	< 1
Case 4	63/M	Choledocholithiasis	2nd portion, lateral wall	> 10	During ERCP	< 1

ERCP: Endoscopic retrograde cholangiopancreatography; IHD: Intrahepatic duct.

Table 2 Therapeutic outcomes

Case No.	No. of endoclip	Antibiotics (d)	PPI	Gastric drainage	Biliary drainage	Start diet/ hospital stay (d)	Mortality or Cx.
Case 1	8	3rd generation cephalosporin/metronidazole (7/5)	Pantoprazole 40 mg IV	-	PTBD	6/12	-
Case 2	5	3rd generation cephalosporin/metronidazole (7/3)	Pantoprazole 40 mg IV	-	PTBD, PTCS	7/10	-
Case 3	4	3rd generation cephalosporin/metronidazole (8/5)	Pantoprazole 40 mg IV	-	PTBD	8/10	-
Case 4	5	3rd generation cephalosporin/metronidazole (14/7)	Pantoprazole 40 mg IV	-	PTBD	4/27	-

PPI: Proton pump inhibitor; PTBD: Percutaneous transhepatic biliary drainage; PTCS: Percutaneous transhepatic cholangioscopy.

due to papillotomy, whereas intraperitoneal perforations are much less common and caused by the endoscope itself[10]. Direct duodenoscope-induced perforation is much less common, accounting for 0.1% of patients who undergo ERCP, but tends to be large and further away from the ampulla[4,5,11,12]. Known risk factors of an ERCP-related perforation might include old age, suspected sphincter of Oddi dysfunction, dilated bile duct, papillary stenosis, Billroth-Ⅱ reconstruction, pre-cut sphincterotomy, and long procedure duration[5,13-15].

Immediate surgery after diagnosis is the current standard treatment for duodenal perforations caused by an endoscope. Stapfer *et al*[4] and Howard *et al*[3] have proposed a classification scheme for duodenal injury by dividing it into four and three types, respectively, according to anatomical location, mechanism of injury, and severity. Type I (lateral or medial wall duodenal perforation; Stapfer *et al*) or Group Ⅲ (duodenal perforation remote from the papilla; Howard *et al*) injuries are usually large and require immediate surgery for repair. In a study by Stapfer *et al*, surgery was recommended for patients with the following criteria: large contrast extravasation on ERCP/UGIs, contrast-enhanced CT scans showing intra- or retroperitoneal fluid collection, massive subcutaneous emphysema or suspected perforation in association with retained material (i.e. stones, ERCP wire/basket)[4]. In cases of perivaterian injuries, they suggested conservative management with serial radiographic examination. Howard *et al*[3] also suggested the use of endoscopic drainage to divert the bile, pancreatic, and/or duodenal

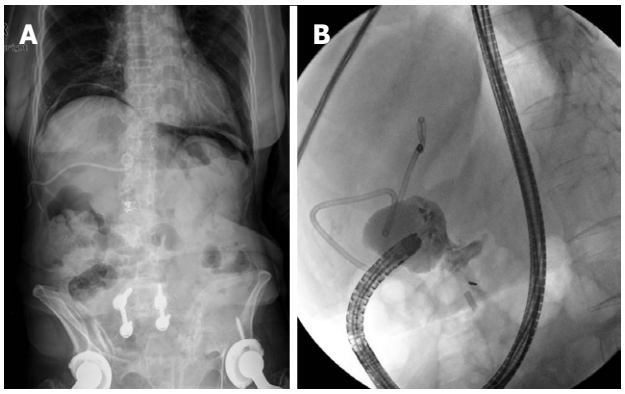


Figure 2 A simple abdomen X-ray shows both subphrenic pneumoperitoneum (A) and 6 d later, the follow-up upper gastrointestinal investigation (UGI) reveals no contrast leaks (B).

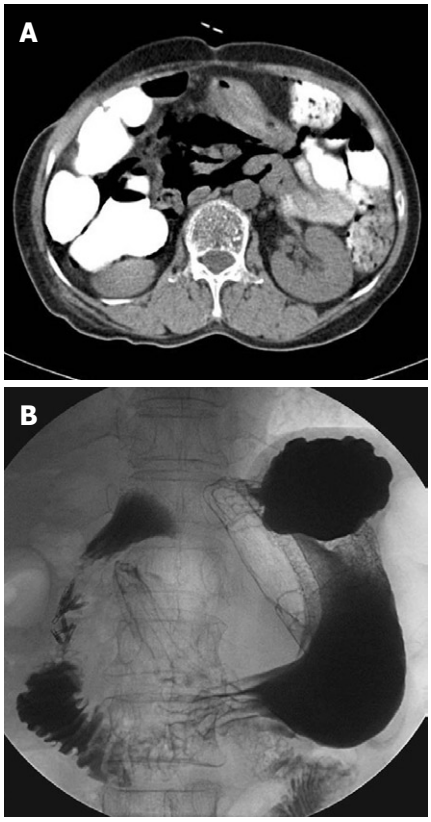


Figure 3 An abdominal computed tomography (CT) shows a severe pneumoretroperitoneum (A), and follow-up UGIs done 6 d later reveal no contrast leaks (B).

fluids away from the perforation, and showed that the endoscopic approach reduced the rates of surgery, mortality, and length of hospital stay.

However, unlike more common spontaneous perforation resulting from peptic ulcer disease, endoscopic therapy-related iatrogenic perforations have a relatively lower chance of bacterial contamination in a fasting state, and there is therefore sometimes an opportunity to manage these patients using nonsurgical means. A small amount of bacterial contamination may be controlled by the administration of antibiotics[16]. Recently, trials of endoscopic

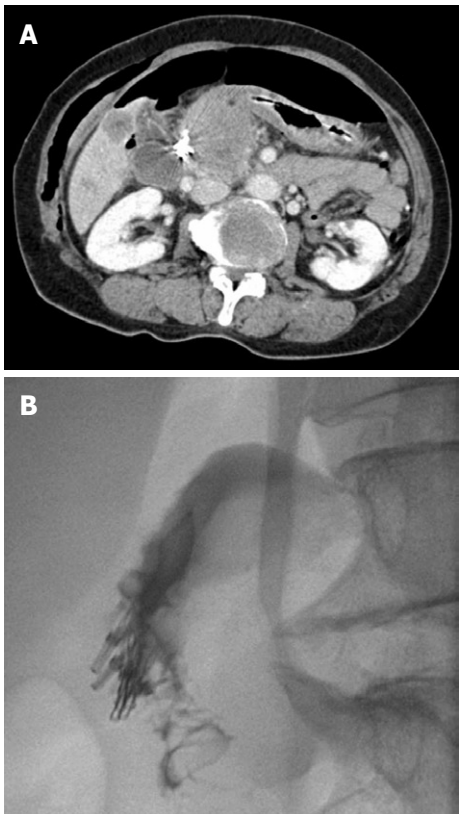


Figure 4 Initial abdominal CT following perforation shows pneumoperitoneum and subcutaneous emphysema (A), and follow up UGIs performed 8 d later reveal no contrast leaks (B).

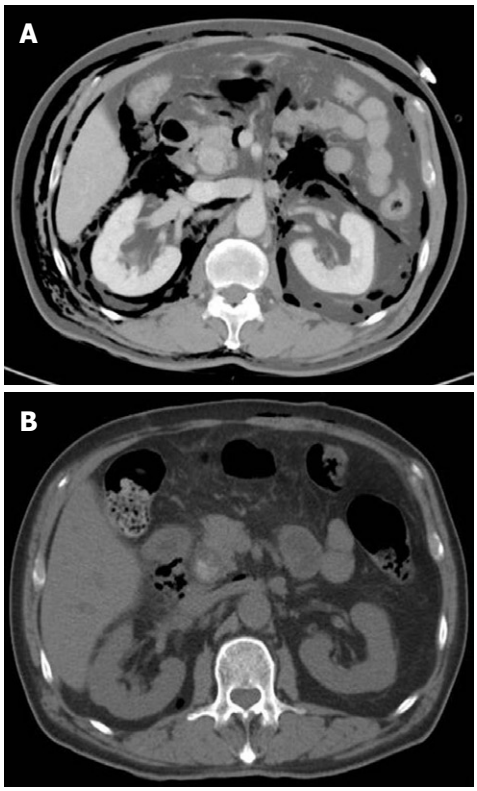


Figure 5 Abdominal CT images showing a pneumoretroperitoneum, and subcutaneous emphysema following perforation (A) and a marked improvement 4 d after conservative management (B).

management have been performed. There have been sporadic reports about the use of an endoscopic clipping device for the closure of iatrogenic perforations during endoscopic mucosal resection (EMR) or sphincterotomy in the esophagus, stomach, and duodenum[17-19]. Though surgery remains the standard treatment for duodenal perforations caused by the endoscope itself, the outcomes from case reports support the beneficial role of endoclips in the closure of these defects[6-8,20]. In particular, some reports described that nonsurgical treatment is possible for the perforation of the upper gastrointestinal tract when peritonitis remains localized. The clinician's familiarity with endoclips and their immediate availability and proper use may avoid surgery for a selected group of patients with a high surgical risk.

Kaneko *et al*[16] suggested some conditions for endoscopic repair using a clipping device in EMR-related perforation. Their suggestions included prior preparation of the patients, quick recognition of perforation, the diameter of perforation being less than the width of the clip's nail, the shape of the opening must be smooth and suitable for drawing the edges together, and an excellent visual field. In endoscopic management, quick recognition and rapid endoscopic closure were the keys to success in limiting the degree of peritoneal contamination and pneumoperitoneum, as delayed diagnosis and surgery are associated with a high mortality rate[4,21]. However, nonsurgical suturing therapy using endoclips is not yet widely accepted as the primary management of ERCP-related duodenal perforation.

In the four cases presented here, the experience of endoscopist and patient old age may be risk factors. All the perforations were done by inexperienced endoscopists who only had one or two years of therapeutic ERCP experiences. However, routine surgery was not required in all patients. The endoscopists could detect the injury early, the visual field was relatively clear, and the endoscopic manipulation was performed in minimal time in all cases. These were the reasons why the primary closure was successful despite a large perforation of more than 10 mm. The perforation was detected very early during ERCP because it occurred in the course of duodenoscope insertion. Cap-assisted endoscopy method under direct vision through a transparent hood was also helpful in reducing the manipulation time of the procedure by allowing a good visual field and ensuring the safety margin during clipping. The cap can facilitate the displacement of any mucosal folds that obscure the lumen and is very useful for overcoming the sharp angulations[22].

Conservative treatment includes giving the patients nothing by mouth, broad-spectrum antibiotics, PPI, and diversion of the bile and pancreatic secretion, or nasogastric or nasoduodenal decompression. However, there were differences in the follow-up method and interval, duration of conservative management methods (fasting, PPI, and antibiotics), and the time when a normal diet was started. Following immediate closure, the use and duration of broad-spectrum antibiotics or PPI was not clear. In our cases, routine nasogastric or duodenal drainage was not used because of early successful closure and biliary diversion by PTBD. Therefore, we think that these procedures are not always required in such cases. Normal diet should be resumed after confirming the complete closure of the wound by UGIs. If patients don't have any clinical symptoms and contrast leakage, earlier resumption of normal diet may be possible.

In summary, primary approximation closure using endoclips under cap-assisted endoscopy of iatrogenic duodenal perforation during ERCP was a safe and feasible technique for even a large free wall perforation. Although the surgical operation remains the standard treatment for duodenal perforation, these cases support the use of endoscopic closure of the perforation with conservative treatments for selected cases of the injury caused by the endoscope itself.

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CASE REPORT

Living donor liver transplantation for an adult patient with situs inversus totalis

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Abstract

This recipient with situs inversus totalis (SIT) was a 60-year-old female who had hepatitis B-related end-stage liver disease. Preoperative donor evaluation showed that the right posterior section satisfied graft volume and was space-fitting in the recipient hepatic fossa when it was rotated 180 degrees. The operation and postoperative course progressed satisfactorily. Three weeks after living donor liver transplantation (LDLT), the graft function was disturbed by compression of bottom-placed right hepatic vein. This was treated with a vascular stent and subsequently the graft function was normalized. The present case shows that LDLT for patients with SIT using a right posterior section graft is feasible.

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Key words: Living donor liver transplantation; Situs inversus totalis; Right posterior section graft

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INTRODUCTION

Situs inversus totalis (SIT) is a rare congenital anomaly with complete reversal of all visceral organs. It occurs once in about 15 000 to 20 000 births and has unknown cause. Sometimes SIT is associated with other anomalies such as biliary atresia, visceral vascular anomaly, and polysplenia. In general, most people with SIT have no complications and perform normal physical activities. However, this condition could be a serious problem when he/she needs liver transplantation because the liver has asymmetrical anatomy that causes technical difficulties in graft positioning and vascular anastomoses in the SIT recipient. For this reason, it has been regarded as a contraindication to liver transplantation.

There are some technical reports of whole graft liver transplantation for adult SIT recipients[1-3]. Also, cases of living donor liver transplantation (LDLT) for the adult patient with SIT have been reported using the left or right lobe with midline positioning of the graft[4,5]. However, midline positioning in the SIT recipient may present two major concerns. Firstly, graft displacement due to redundant space of recipient's hepatic fossa could result in vascular torsion and kinking; and secondly, the recipient's inferior vena cava could be compressed by the graft. To overcome these problems, we performed reversed orthotopic implantation using right posterior section, Couinaud segment VI + VII, grafted into the recipient's hepatic fossa[6].

CASE REPORT

Our recipient was a 60-year-old female with advanced liver cirrhosis caused by hepatitis B virus. Her body weight was 60 kg and the Model for End-stage Liver Disease (MELD) score was 19. The donor was her nephew, a 45-year-old male with a suitable volume of right posterior sector on preoperative computed tomography (CT) scan. Preoperative CT scan showed hilar trifurcation of portal vein and measurement of total liver volume (TLV) was 1100 mL. The right liver volume of the donor was 780 mL (71% of TLV), the left lobe volume was 320 mL, and the right posterior section volume was 510 mL (46.4% of TLV). A right posterior section graft was the only suitable type of donor hepatectomy to ensure donor safety and appropriate volume of graft to the recipient. Preoperative measurement of the right posterior section graft to recipient weight ratio (GRWR) was 0.85%. Preoperative simulation showed that the donor's right posterior section fit well into the recipient's left subphrenic space with 180-degree flip-over (facing backward) implantation method.

The donor operation was performed uneventfully according to the usual procedure of right posterior sectionectomy including right hepatic vein (RHV). There was no significant inferior right hepatic vein in the graft. The actual graft weight was 470 g. On the back table, protruded liver tissue behind the right posterior portal vein and RHV was cut out with an ultrasonic dissector to expose the portal and hepatic veins in reversed graft position. Under laparotomy of the recipient with inverted T-incision, complete rotation of all internal organs along the midline was observed without any vascular anomaly. Recipient hepatectomy was not complicated. The anhepatic time was 45 min. The reversed right posterior section graft was located in the recipient's left subphrenic space. To gain sufficient graft outflow, a piece of cryopreserved vein patch was applied on the ventral portion of the graft's RHV and a modified piggyback anastomosis was done. Vascular anastomoses of the portal vein and hepatic artery were performed as usual. The cold ischemic time was 2 h and the warm ischemic time was 45 min. In the reversed position of the right posterior section graft, the bile duct was located behind the portal vein. Thus, temporary downward traction of the portal vein was necessary for biliary anastomosis (Figure 1). Biliary anastomosis between the recipient's common hepatic duct and the graft's duct was performed successfully with 7-0 prolene continuous suture. Splenectomy was performed according to our indication of thrombocytopenia and splenomegaly. Total operation time was 11 h and 35 min.

The patient's postoperative course was excellent for 3 wk after transplantation, but the serum levels of liver enzymes subsequently started to elevate. However, there was no evidence of acute rejection on needle biopsy. Abdominal CT scan and hepatic Doppler ultrasound showed disturbance of blood flow in segment 6 RHV (V6 RHV) due to compression by the graft itself. A long endovascular stent was inserted into V6 RHV and the liver recovered quickly (Figure 2). Consequently, the patient was discharged at postoperative day 45 with good liver function. She has maintained good general condition and liver function for 8 mo postoperatively, up to the present time.

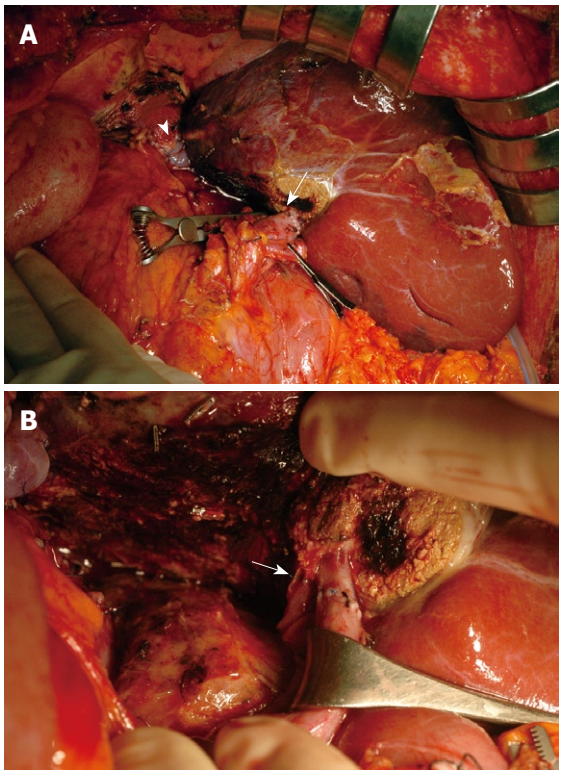


Figure 1 Reversed implantation of the right posterior section graft. A: Modified piggyback anastomosis of right hepatic vein (arrow head) and portal vein anastomosis (arrow); B: Duct opening of the graft (arrow).

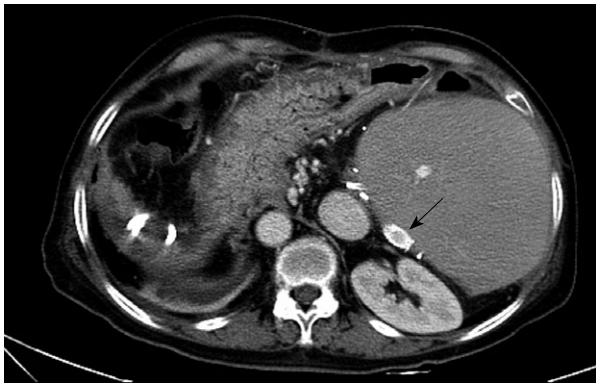


Figure 2 Postoperative 6 mo computed tomography scan showing good graft positioning and patent endovascular stent in the right hepatic vein (arrow).

DISCUSSION

The major technical problem of liver transplantation for adult SIT is the spatial mismatch between the graft's and recipient's left upper quadrant space. Liver transplantation for the SIT patient has been performed at several centers, mainly using techniques of midline positioning or left shifting with/without clockwise rotation of the graft.

However, these techniques have possible complications, such as graft displacement due to unfavorable position and vena cava compression by large right lobe of the graft.

Recently, Rayhill *et al*[7] reported a novel technique of 180-degree flip-over (facing backward) implantation of the whole liver graft into the recipient's left subphrenic space. They reported excellent outcome without complication. Their technique provided not only orthotopic implantation of the whole graft but also good arrangement of portal vein, hepatic artery, and bile duct. We agree that orthotopic reversed graft implantation could overcome the possible complications of midline graft positioning for the SIT recipient.

Regarding LDLT, Chun *et al*[8] reported orthotopic implantation of a right lobe graft with 180-degree flip-over method from a living SIT donor to a recipient with normal anatomy. This technique is similar to ours in reversing the graft, but when using a reversed right lobe graft, the hilar plate is located more ventrally due to voluminous anterior sector behind the hilum. This could result in shortage of portal vein and bile duct, which leads to unavoidable tension in the anastomoses, and consequent vascular stretching or kinking. To avoid such complications, Chun *et al* used a special technique to achieve sufficient length of recipient's portal vein and bile duct.

In our case, the location of the hilar plate of the right posterior sector was almost at the same level within the SIT recipient's hepatic fossa in the reversed position. This allowed us to perform easy vascular and duct-to-duct biliary anastomoses without any of the complications mentioned above. To our knowledge, this is the first report of liver transplantation using a right posterior section graft for the adult patient with SIT. According to our experience, the only disadvantage of this technique using right posterior section graft is the possible compression of the V6 RHV, located at the bottom, by the graft itself during rapid liver regeneration after LDLT. The compression of the bottom-located RHV in reverse positioning of the right posterior section graft would be a common phenomenon, because there is little space to expand behind the RHV during rapid liver regeneration. Thus, we suggest that the surgeon should be alert to detect compression of the RHV during the early postoperative period. However, this complication could easily treated by inserting an endovascular stent.

In conclusion, reversed implantation of the right posterior section graft is one good option for liver transplantation in the adult recipient with SIT. This procedure provides not only orthotopic transplantation to the SIT recipient but also convenient vascular and duct-to-duct biliary anastomoses. In using this technique, however, we have to be alert during the postoperative course because the bottom-located RHV could be compressed by the graft.

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CASE REPORT

Radiological diagnosis of duodenocaval fistula: A case report and literature review

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Abstract

Duodenocaval fistula (DCF) is an uncommon but lethal clinical entity. The high mortality has been attributed to the difficulty of diagnosis before attempts at definitive therapy. In this case report, we describe a patient with a series of computed tomography (CT) examinations over a 2-mo period in hospital. A low-density air bubble appeared in the inferior vena cava (IVC) on the second day in hospital and became clear on day 19, and gradually enlarged. Magnetic resonance imaging (MRI) also clearly demonstrated a high-signal enteric contrast medium or thrombus and signal-void air bubbles in the IVC. However, cavography did not show the filling defect. We suggest that noninvasive CT and MRI should be chosen as a first-line investigation, and IVC, including the surrounding structures, should be carefully reviewed on images if DCF is clinically considered.

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Key words: Duodenocaval fistula; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Duodenocaval fistula (DCF) is an uncommon but lethal clinical entity that has previously been reported in only 39 patients in the English-language literature[1-9]. The high mortality of 41% (16/39) has been attributed to the difficulty of diagnosis before attempts at definitive therapy. We present a case of DCF that underwent a series of endoscopy, computed tomography (CT), magnetic resonance imaging (MRI), and cavography examinations, and a review of the literature with respect to radiological manifestation of DCF.

CASE REPORT

A 78-year-old man was hospitalized because of right-sided abdominal pain for 1 mo, associated with fever, rigors, and chills for 1 d. His medical history was significant for right nephrectomy 5 years ago because of renal cell carcinoma. A mass was found in the right adrenal gland and gamma knife radiotherapy was performed 10 mo ago. On physical examination, his initial vital signs were temperature 39.5℃, heart rate 120 beats/min, blood pressure 110/70 mmHg, respiration 25 breaths/min, and SaO_2 97%. Initial laboratory studies showed a leukocyte count of 22 × 10^9/L, hemoglobin 80 g/L, platelet count 33 × 10^9/L, and normal blood chemistry, clotting parameters, and liver function tests. Blood cultures revealed *Enterococcus faecium* and *Enterobacter cloacae* bacteremia and *Candidia albicans* fungemia. Treatment with intravenous broad-spectrum antibiotics and antifungal

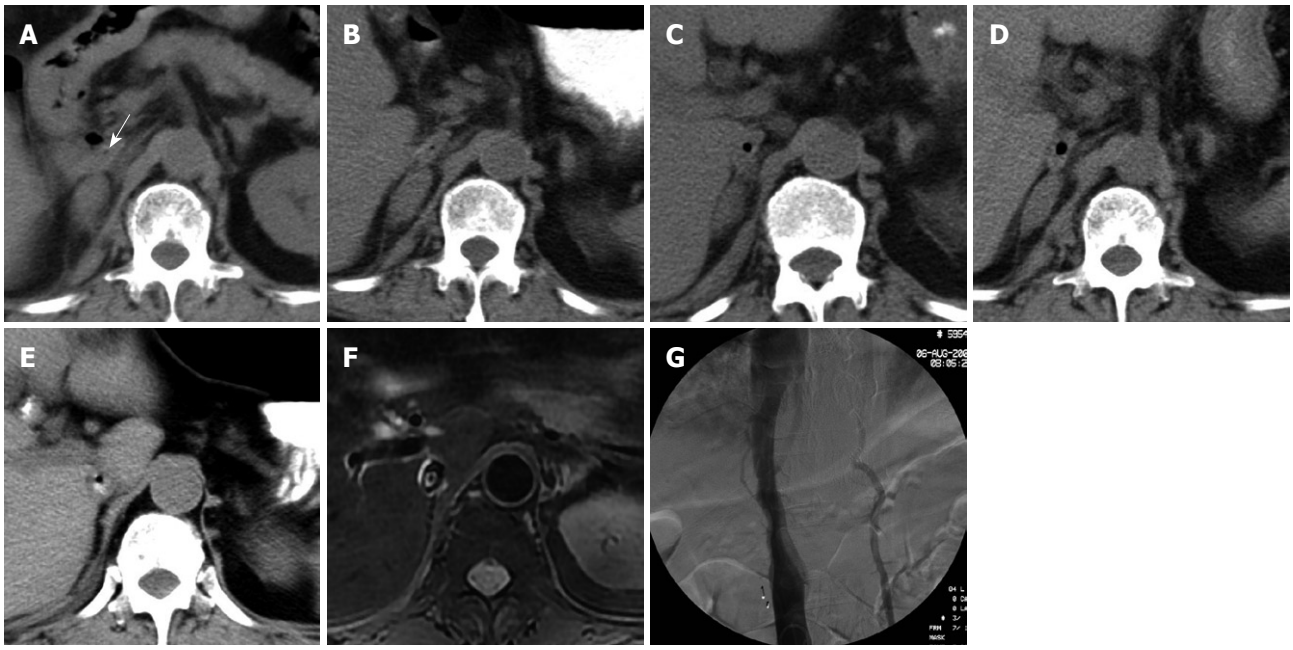


Figure 1 During the 2-mo hospital stay, a small, low-density air bubble appeared in the inferior vena cava (IVC) and gradually enlarged on follow-up computed tomography (CT) scans. Enteric contrast medium leaked into the IVC. Magnetic resonance imaging (MRI) clearly demonstrated the high-signal enteric contrast medium or thrombus and signal-void air bubbles. Cavography did not reveal the thrombus in the IVC. A: CT scan on the second hospital day. A low-density dot loomed up in the IVC (arrow). A mass in the right side adrenal gland was also seen; B: CT scan on the 19th hospital day. The low-density dot became more evident; C, D: CT scan on the 59th and 67th hospital day. The low-density dot (air bubble) was gradually enlarged; E: CT scan on the 73rd hospital day with water-soluble enteric contrast demonstrated air bubble surrounded by contrast medium in the IVC; F: MRI T2WI on the 74th hospital day demonstrated the high signal enteric contrast medium or thrombus and signal void air bubble in the IVC; G: Inferior vena cavogram on the 75th hospital day showed no evident thrombus in the IVC.

therapy (meropenem and caspofungin) resulted in some improvement. However, during the 76 d in hospital, the patient developed intermittent fever, high leukocyte count, sepsis and fungemia. About 1 mo after admission, the patient had watery stools, which were positive for occult blood. A series of upper gastrointestinal (GI) endoscopy examinations, abdominal ultrasound, CT, MRI and inferior vena cavography were performed during the hospital stay. Upper GI endoscopy (on day 3 in hospital) revealed a giant ulcer in the second portion of the duodenum, but no active bleeding. Abdominal ultrasound found nothing except a mass in the right adrenal gland. A series of abdominal CT scans were performed on days 2, 19, 59, 67 and 73 in hospital (Figure 1). Low-density air bubbles were incidentally found in the inferior vena cava (IVC) (at the level of the first lumbar vertebra, adjacent to the duodenum) by CT on day 67. A review of the previous CT scans showed a low-density dot in the IVC on the second day in hospital, which became clear on day 19, and gradually enlarged. A repeat abdominal CT scan (day 73) with water-soluble enteric contrast demonstrated low-density air bubbles surrounded by high-density contrast medium or thrombus in the IVC, which suggested DCF. T2-weighted MRI (day 74th day of hospitalization, Figure 1) revealed high-signal enteric contrast medium or thrombus and signal-void air bubbles in the IVC. However, inferior vena cavography (day 75 of hospitalization) showed no evidence of IVC thrombus (Figure 1). Surgical consultation was scheduled but before it could be performed, the patient had an episode of hematemesis. Massive hemorrhage ensued and the patient died despite aggressive resuscitation efforts. Postmortem examination revealed a fistula between the second portion of the duodenum and the IVC.

DISCUSSION

DCF is a rare occurrence that typically arises as a complication from migrating IVC filters, peptic ulcer disease related to retroperitoneal tumor resection in association with radiation therapy, or transmural migration of ingested foreign bodies. Diagnosis of this entity is challenging because of the nonspecific nature of the symptoms and is rarely made before laparotomy or autopsy. The most common presentations of DCF are sepsis and GI hemorrhage. Sepsis is generally polymicrobial in origin, and caused by Gram-positive and Gram-negative enteric bacteria in the systemic circulation. Fungemia may also occur, as in the present case[1-9]. Endoscopy typically discloses a duodenal ulcer that may show visible bleeding, but the extent of penetration of the ulcer is often underappreciated[1]. CT provides noninvasive evaluation of the IVC and the adjacent structures. It is capable of detecting thrombus and air bubbles in the IVC, infectious fluid collection or abscess around the IVC and duodenum, incarcerated foreign bodies, and migrated caval filters[1-4]. CT correctly can identify DCF in approximately 50% of patients[2]. In this case, a low-density dot appeared in the IVC on the second day in hospital, became clear on day 19, and gradually enlarged over the 2-mo hospital stay. Air bubbles in the IVC may have been produced by bacteria or pushed in by gut peristalsis. Repeated CT

examinations are necessary when air bubbles are too small to make a diagnosis. For detecting thrombus in the IVC, enhanced CT is essential. MRI was not used for the diagnosis of DCF in previous studies. With conventional or flow-sensitive sequences, MRI can clearly delineate thrombus in the IVC, even without intravenous contrast medium[10,11]. Thrombus, intestinal contents and enteric contrast show abnormal signals against signal-void blood flow in the IVC. In the present case, MRI clearly demonstrated high-signal enteric contrast medium or thrombus and signal-void air bubbles in the IVC. Cavography has revealed thrombus or filling defects in the IVC, but was diagnostic of DCF in only two out of six cases[1,4,12]. In the present case, cavography did not show enteric contrast medium or thrombus in the IVC. The most likely reason was that the thrombus was flushed out by the high-pressure injection of intravenous contrast medium. Fatal pulmonary embolization composed of intestinal contents through a DCF has been reported[13]. Our patient died of hematemesis 24 h after cavography. Therefore, care should be taken to perform invasive cavography to detect unstable thrombus of DCF in the IVC.

In summary, we report this case to remind clinicians that noninvasive CT and MRI should be chosen as a first-line investigation for diagnosis of DCF. If DCF is clinically considered, IVC and the surrounding structures should be carefully reviewed by imaging.

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SCIENTOMETRICS

Role of *Orvosi Hetilap* in the development of Hungarian gastroenterology

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Abstract

AIM: To analyze the contribution of *Orvosi Hetilap* (Hungarian Medical Journal) to the field of gastroenterology.

METHODS: All issues of the journal between 1857 and 2008 and identified original articles and reviews dealing with gastroenterology were reviewed. The rate of publications, the thematic distribution and foreign sources of knowledge were assessed. The dates that major achievements in gastroenterology were introduced in Hungary were compared to those dates in Western medicine.

RESULTS: A total of 4799 original/research articles on gastroenterology were published, which represents 11.1% of the total publications. Thematic rankings showed that liver and biliary diseases represented 20.36% of the total, followed by gastric diseases (9.35%) and surgery (8.77%). A total of 268 foreign journals were reviewed: 50.9% were German, 30.4% English, 12.1% French and only 6.6% were in other languages. The major achievements of gastroenterology were introduced with varying delays compared to Western countries.

CONCLUSION: *Orvosi Hetilap* has made a large contribution to the development of Hungarian gastroenterology. The high proportion of gastroenterology studies underlines the importance of digestive diseases in public health.

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Key words: Content analysis; Gastroenterology; Hepatology; *Orvosi Hetilap*; Scientometrics

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INTRODUCTION

Nineteenth century Europe witnessed the publication of national medical journals. In Hungary, *Orvosi Hetilap* (*OH*; Hungarian Medical Journal) was first published in 1857, thus making it one of the first medical journals worldwide (Table 1). The importance of the journal in the development of Hungarian medical science and medical language is unrivalled, a fact that has been recognized many times. Much less is known, however, about the role of *OH* in the development of special fields, such as Hungarian gastroenterology. Medical historians divide the past 150 years into six periods according to the editors-in-chief of *OH* (Table 2)[1]. Most of the editors were eminent figures in Hungarian medicine, and were quoted in foreign textbooks of medical history. To the best of my knowledge, this is the first complete content analysis of a medical journal in the field of gastroenterology.

The aim of the study was to track the timeline and make a thematic analysis of the papers dealing with diseases of the gastrointestinal tract, as published in *OH* between 1857 and 2008.

Table 1 Publishing timeline of the national medical journals

1798: *Journal de Medécine*	Paris
1809: *Journal of the Royal Society of Medicine*	London
1812: *New England Journal of Medicine*	Boston
1823: *The Lancet*	London
1840: *British Medical Journal*	London
1850: *Wiener Medizinische Wochenschrift*	Vienna
1854: *Nederlander Tijdschrift der Geeneskunde*	Amsterdam-Utrecht
1857: *Orvosi Hetilap*	Budapest
1875: *Deutsche Medizinische Wochenschrift*	Stuttgart
1883: *Journal of American Medical Association*	Chicago
1893: *La Presse Médicale*	Paris

Table 2 Editorial periods of *OH*

Period	Editor-in-chief	Specialty	Duration (yr)
1857-1888	L. Markusovszky (1815-1893)	Public health	32
1889-1905	E. Hőgyes (1847-1906)	Bacteriologist, pharmacologist	16
1905-1922	M. Lenhossék (1863-1937)	Anatomist	17
1923-1944	Z. Vámossy (1868-1953)	Pharmacologist	21
1948-1989	T. Trencséni (1907-1996)	Internist	41
1989-2008	J. Fehér	Gastroenterologist, hepatologist	20

OH: *Orvosi Hetilap*.

Table 3 Rate of gastroenterology publications in *OH* between 1857 and 2008 *n* (%)

Editorial period	Total original articles	Gastroenterology articles	Total journal reviews	Gastroenterology article reviews
1857-1888	3416	312 (9.1)	1386	126 (9.0)
1888-1904	2102	143 (6.8)	435	39 (8.9)
1905-1922	2906	406 (13.9)	7332	658 (8.9)
1923-1944	7757	696 (8.9)	11586	937 (8.0)
1948-1989	19583	2175 (11.1)	31489	3965 (12.5)
1989-2008	7682	1067 (13.8)	19575	1698 (8.6)
Total No.	43446	4799 (11.1)	71803	7423 (10.3)

MATERIALS AND METHODS

I manually reviewed the journal volumes between 1857 and 2008. The full papers (both original research and reviews) as well as foreign journal article reviews were identified and classified according to their subjects and origin. All gastrointestinal, liver, biliary tract and pancreatic diseases were included. Articles on epidemics (cholera and typhus) that involved the digestive tract were excluded, being more the subject of epidemiology and microbiology, as considered by medical historians[2]. The thematic analysis of the articles and journal reviews was performed using specific key words that occurred in the titles. The data were entered into an Excel database. The differences between the editorial periods were assessed using analysis of variance and the Kruskal-Wallis test, with a significance level of $P = 0.05$. The statistical work was performed using Statsoft Inc. version 9.0 software (Tulsa, OK, USA). The dates when some major achievements in gastroenterology were introduced in Hungary were compared to those for Western medicine.

Table 4 Thematic breakdown of full papers published in *OH* between 1857 and 2008[1] *n* (%)

Subject	Articles
Anatomy	18 (0.30)
Pathology	121 (2.02)
Physiology	132 (2.20)
Esophagus	194 (3.24)
Stomach	559 (9.65)
Small bowel	298 (4.98)
Appendix	93 (1.55)
Large bowel	268 (4.63)
Liver	871 (14.57)
Biliary tract	346 (5.79)
Pancreas	354 (5.92)
Peritoneum	102 (1.70)
Tumors	195 (3.26)
Surgery	524 (8.92)
Radiology	165 (2.76)
Endoscopy	246 (4.11)
Ultrasound	69 (1.25)
Pediatrics	185 (3.09)
Laboratory medicine	145 (2.42)
Peptic ulcer	189 (3.16)
Inflammatory bowel diseases	102 (1.70)
Viral hepatitis	219 (3.96)
Infectious diseases (non-epidemic)	200 (3.34)
Laparoscopic surgery	27 (0.45)
Transplantation	39 (0.67)
Genetics	61 (1.02)
General topics	252 (4.28)
Total	5974 (100)

[1]Some articles are included more than once, based on keywords in the title.

RESULTS

General data

The volume of *OH* gradually increased from about 400 pages a year at the outset to 800 pages at the turn of the century, before increasing further. During World Wars I and II, both the extent and the number of publications decreased. Between 1944 and 1948, the publication of *OH* was interrupted because of the post-war economic depression (mainly due to lack of paper and printing facilities). After 1948, the volume averaged out at 2469 pages/year, which reflected the increasing number of original articles, journals and book reviews.

Full papers (original research and reviews)

A total of 43446 papers were published during the period studied (Table 3). While the editorial periods were unequal, the number of papers/year was calculated and it increased from 36 (1857-1904) to 64 (1905-1922) ($P = 0.0002$), before changing to 60 (1923-1944) ($P = 0.46$), 89 (1948-1989) ($P = 0.0001$) and 53 (1989-2008) ($P = 0.48$). For the whole period, gastroenterology articles represented 11.1% (6.8-13.9) of the total number of papers published in *OH*. The thematic breakdown of full papers on gastroenterology is shown in Table 4.

Table 5 Introduction of some major gastroenterology achievements in Western countries and Hungary[2-6]

Achievement	International application (year, author, location)	Hungarian application (year, author, location)
Rigid gastroscopy	1868 (A. Kussmaul, Freiburg)	1902 (J. Zimmermann, Budapest)
Gastric resection	1880 (L. Rydiger, Vienna)	1900 (M. Herczel, Budapest)
Radiography	1895 (K. Roentgen, Würzburg)	1896 (J. Klupathy, Budapest)
Cholecystectomy	1881 (C. Langenbuch, Berlin)	1889 (D. Velits, Budapest)
Liver biopsy	1938 (I. Silverman, New York)	1948 (L. Friedrich, Budapest)
Vagotomy	1943 (L. Dragstedt, Chicago)	1948 (E. Hedri, Budapest)
B-mode abdominal ultrasound	1957 (I. McDonald, London)	1973 (Á. Szebeni, Budapest)
Fiberoptic gastroscopy	1958 (B. Hirschowitz, Birmingham, USA)	1965 (T. Jávor, Debrecen)
Fiberoptic colonoscopy	1970 (F. Matsunaga, Tokyo)	1972 (L. Simon, Jászberény)
Endoscopic retrograde cholangio-pancreatography	1970 (I. Oi, Tokyo)	1971 (L. Sáfrány, Budapest)
Endoscopic sphincterotomy	1973 (M. Classen, Munich)	1976 (J. Papp, Budapest)
Liver transplantation	1967 (T. Starzl, Denver)	1995 F. Perner, Budapest)
Laparoscopic cholecystectomy	1985 (T. Mühe, Bobelein)	1990 (I. Kiss, Pécs)
Capsule endoscopy	2000 (P. Swain, London)	2002 (I. Rácz, Győr)

Table 6 Profile of foreign journal reviews in *OH* between 1857 and 2008

Editorial period	No. of articles reviewed	No. of journals	English (%)	German (%)	French (%)	Other Languages (%)
1857-1888	211	86	17.9	52.5	15.9	13.2
1888-1904	381	113	12.6	64.5	17.7	5.2
1905-1922	658	112	15.6	62.2	14.6	7.6
1922-1944	936	121	19.6	57.1	16.9	6.4
1948-1989	3965	178	52.4	38.4	2.9	6.3
1989-2008	1698	113	64.4	31.0	2.9	0.9
Total	7849	268	30.4	50.9	12.1	6.6

Table 7 Ranking of core journals reviewed in *OH* between 1857 and 2008 *n* (%)

Journal	Articles reviewed
Deutsche Medizinische Wochenschrift	1147 (31.2)
Lancet	584 (15.9)
British Medical Journal	410 (11.2)
New England Journal of Medicine	313 (8.5)
American Journal of Roentgenology	263 (7.1)
Zentralblatt für Chirurgie	227 (6.1)
Radiology	204 (5.5)
Schweizerische Medizinische Wochenschrift	194 (5.2)
Journal of American Medical Association	167 (4.5)
Münchener Medizinische Wochenschrift	157 (4.2)
Total	3666 (100)

The dates when some major achievements in gastroenterology were first introduced in Hungary were compared with their first application in Western countries (Table 5)[2-7].

Journal article reviews

Foreign medical journal reviews appeared in the pages of *OH* from the very beginning. Two hundred and sixty-eight different journals were reviewed. Between 1948 and 1959, however, their publication was interrupted for political reasons. The number and language breakdown of the journals in the editorial periods are given in Table 6.

The 10 most reviewed core journals are listed in Table 7. Overall, 3666 articles were reviewed in these journals, which represented 9.7% of the total reviews. The six English journals (60%) had 1941 articles reviewed (52.5%), while the four German journals had 1725 articles reviewed (47.5%).

DISCUSSION

During the 150 years of its publication, *OH* has covered all aspects of developing gastroenterology, from basic sciences (anatomy, physiology and pathology) to the latest achievements (abdominal imaging, laparoscopic surgery, transplantation and genetics) (Table 5). Published every week, it became the main source of professional knowledge for Hungarian physicians. The 11.7% ratio of papers dealing with diseases of the gastrointestinal tract underlines their importance in public health. Diseases of the liver and biliary tract (20.36%) and the stomach (9.35%) (especially peptic ulcers, 3.16%) were studied in most detail, although there were considerable fluctuations in the publication rates. Content analysis revealed that milestone developments of gastroenterology, as quoted, mentioned or even canonized by leading historians[3-8], were introduced in Hungary with varying degrees of delay.

A large variety of foreign journals were reviewed. An analysis of journal reviews reveals that until the 1960s, German literature was the main source of information. The reasons for this are rooted in history, given that the country was part of the Austro-Hungarian monarchy. From the 1960s onwards, British and American journals gradually became the dominant source of information until the dawn of the internet. Even so, the German weekly *Deutsche Medizinische Wochenschrift* was and remains the most frequently reviewed journal. For a closer look at the foreign sources, it would have been better to conduct a citation analysis of the full articles. However, accurate reference lists only appeared after the 1920s. Until then, references were mentioned only as footnotes, indi-

cating the name of the authors; therefore, the impact of major advances and seminal articles on the Hungarian literature cannot be calculated.

In spite of its longevity and indexing in Medline/PubMed, Index Medicus, Embase, Index Copernicus and Google Scholar, *OH* has not been assigned an impact factor. To overcome this, the current Editor-in-Chief, J. Fehér, introduced a 2-monthly English version of *OH* called the *Hungarian Medical Journal*, which has published selected articles of the parent journal since 2007. Another alternative would be the bilingual (Hungarian-English) publication of the high-quality articles. *OH* has been accessible on the internet (http://www.akademiaikiado.hu) since 2008.

The complete archive of *OH* is available in just a few university and hospital libraries. These collections have not benefited from the passing of time. In the future, the digitalization of the journal is vital to save its valuable scientific content for future generations.

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COMMENTS

Background

The development of a medical specialty can be followed by the analysis of articles, journals and book reviews published over time in a given field.

Innovations and breakthroughs

The paper is believed to be the first content analysis of the complete edition of a time-honored medical journal, *Orvosi Hetilap* (Hungarian Medical Journal; 1857-2008).

Applications

The author overviews the development of gastroenterology in Hungary over 150 years by analyzing and classifying the original articles, journal and book reviews in the field of gastroenterology, covering both basic (anatomy, pathology and physiology) and applied sciences (esophageal, gastric, intestinal, liver and biliary diseases, and diagnostic procedures), with an emphasis on the main achievements as they have occurred in the literature.

Peer review

This is an interesting historical review of 150 years of gastroenterology articles published in a weekly Hungarian medical journal.

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Comments on the article about the treatment of peripancreatic infection

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Abstract

We read with great interest the article by Tang *et al* published in issue 4 of *World Journal of Gastroenterology* 2010. The results of their study indicate that percutaneous catheter drainage in combination with choledochoscope-guided debridement is a simple, safe and reliable treatment procedure for peripancreatic infections secondary to severe acute pancreatitis. However, there are some points that need to be addressed, including data about the patients in the study and their clinical characteristics, data about infection and superinfection during the treatment and type of treatment of patients with acute necrotizing pancreatitis.

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Key words: Acute pancreatitis; Pancreatic necrosis; Infection; Non-surgical management

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TO THE EDITOR

We read with great interest the article by Tang *et al*[1] published in issue 4 of *World Journal of Gastroenterology* 2010. The article provides important data because it evaluated the new method used in treatment of pancreatic and peripancreatic infections secondary to severe acute pancreatitis (SAP). The results of their study indicate that percutaneous catheter drainage in combination with choledochoscope-guided debridement is a simple, safe and reliable treatment for peripancreatic infections secondary to SAP. The authors suggested that the procedure performed in their study could be used as an alternative to the conventional open-abdominal surgical or laparoscopic debridement in treatment of peripancreatic infection of early SAP patients.

However, there are some points that need to be addressed. In the section of RESULTS, the authors specified "the time from the onset of pancreatitis to drainage was 4-11 d (mean 5.3 d). Before the sinus tract was expanded, the external drainage was maintained for 3-5 d (mean 3.6 d)". Hence, practically all interventions were performed during the first two weeks from the onset of pancreatitis. However, it is known that SAP runs a biphasic course[2]. During the first 1-2 wk, there is a pro-inflammatory response, resulting in a systemic inflammatory response syndrome. It is a sterile response in which sepsis or infection hardly ever occurs. However, after the first 1-2 wk, there is a transition from a pro-inflammatory to an anti-inflammatory response. It is during this anti-inflammatory response that the patient is at risk for the translocation of intestinal flora and the development of infection of necrotic tissue and fluid collections. It is quite uncommon that all patients in the study had pancreatic infection in the first two weeks of the disease. In the article, there are no data confirming that peripancreatic necroses and collections were infected. Also, it is well known that continuous percutaneous drainage often leads to colonization of the cavity with microorganisms and results in frequent superinfection[3,4]. However the authors did not report any data about it.

The authors had impressive results comparing to other similar studies[3-5], but it is disputable if comparable patients were treated in this study since the authors did not present data about the clinical scoring and multiple organ failure of the included patients.

Finally, on the basis of our long-term experience, we believe that the disease catheter drainage of infected necrotic tissue is very poor in the beginning, irrespective of the catheter size we used. However, during the course of SAP, a transition from solid necrotic tissue to more liquid contents leads to a higher success rate of the evacuation of necrotic tissue from the cavities, regardless of the catheter size. Therefore, we consider that only conservative treatment with proper intravenous hydration and administration of proper antibiotics should be performed in beginning of the disease. Percutaneous drainage with vigorous irrigation should be considered when truly conservative treatment fails to resolve infected pancreatic necrosis. We consider that necrosectomy, including choledochoscope-guided debridement, may represent an overtreatment in beginning of the disease in these patients with usually poor general condition. However, it is difficult to discriminate between necrotic tissue and normal tissue, and a very high risk of bleeding from vessels may occur in necrotized tissue during or immediately after the intervention.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
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Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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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Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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/eid.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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Write as mean ± SD or mean ± SE.

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