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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007
[3]Passed away on June 14, 2008

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INTRODUCTION

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EDITORS FOR THIS ISSUE

Surgical treatment of anal stenosis

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Abstract

Anal stenosis is a rare but serious complication of anorectal surgery, most commonly seen after hemorrhoidectomy. Anal stenosis represents a technical challenge in terms of surgical management. A Medline search of studies relevant to the management of anal stenosis was undertaken. The etiology, pathophysiology and classification of anal stenosis were reviewed. An overview of surgical and non-surgical therapeutic options was developed. Ninety percent of anal stenosis is caused by overzealous hemorrhoidectomy. Treatment, both medical and surgical, should be modulated based on stenosis severity. Mild stenosis can be managed conservatively with stool softeners or fiber supplements. Sphincterotomy may be quite adequate for a patient with a mild degree of narrowing. For more severe stenosis, a formal anoplasty should be performed to treat the loss of anal canal tissue. Anal stenosis may be anatomic or functional. Anal stricture is most often a preventable complication. Many techniques have been used for the treatment of anal stenosis with variable healing rates. It is extremely difficult to interpret the results of the various anaplastic procedures described in the literature as prospective trials have not been performed. However, almost any approach will at least improve patient symptoms.

Key words: Anal canal surgery; Anal stenosis; Anoplasty; Hemorrhoidectomy; Complications; Lateral internal sphincterotomy; Surgical flap

INTRODUCTION

Anal stenosis is an uncommon disabling condition[1-5]. It is a narrowing of the anal canal. This narrowing may result from a true anatomic stricture or a muscular and functional stenosis. In anatomic anal stenosis, the normal pliable anoderm, to a varying extent, is replaced with restrictive cicatrized tissue. Stenosis produces a morphologic alteration of the anal canal and a consequent reduction of the region's functionality, leading to difficult or painful bowel movements[6-8].

Anal stenosis is a serious complication of anorectal surgery. Stenosis can complicate a radical amputative hemorrhoidectomy in 5%-10% of cases[9-14], particularly those in which large areas of anoderm and hemorrhoidal rectal mucosa from the lining of the anal canal is denuded, but can also occur after other anorectal surgical procedures.

Treatment, both medical and surgical, should be modulated based on stenosis severity[4,15]. Mild stenosis can be managed conservatively with stool softeners or fiber supplements. Daily digital or mechanical anal dilatations may be used. Sphincterotomy may be quite adequate for a patient with a mild degree of narrowing. For more severe anal stenosis, a formal anoplasty should be performed to treat the loss of anal canal tissue. Several techniques have been described for the treatment of moderate to severe stenosis refractory to non-operative management. In the literature, several studies have been conducted on anal stenosis treatment, but there is not yet universal consent on the anaplastic procedure to use. This review examines some of the evidence concerning the surgical treatment of anal stenosis.

ETIOLOGY AND PATHOPHYSIOLOGY

Stenosis may be caused by an intrinsic or extrinsic pathologic process of the anorectum. Anal stenosis may follow almost any condition that causes scarring of the anoderm. The causes of anal stenosis include surgery of the anal canal, trauma, inflammatory bowel disease, radiation therapy, venereal disease, tubercolosis, and chronic laxative abuse. We focus on the treatment of postsurgical anal stenosis.

Ninety percent of anal stenosis is caused by overzealous hemorrhoidectomy[16]. Removal of large areas of anoderm and hemorrhoidal rectal mucosa, without sparing of adequate muco-cutaneous bridges, leads to scarring and a progressive chronic stricture. The surgical procedure influences the incidence of anal stenosis, particularly after "Whitehead hemorrhoidectomy" because, later, surgeons misinterpreted Whitehead's description and anchored the mucosa to the anal verge (Whitehead deformity)[14,17-19]. After Milligan-Morgan and stapled rectal mucosectomy (SRM), stenosis is less frequent. In a study of 1107 patients treated with stapled hemorrhoidectomy, 164 of 1107 patients registered a complication: anal stenosis was observed in only 0.8% of cases[19]. Stenoses caused by SRM are presumably rectal stenoses, since the causing event was a resection of rectal mucosa. The stenosis rate following stapled mucosectomy generally ranges from 0.8%-5.0%. The calculated actuarial one-year stenosis rate is 6%, which is higher than the above-mentioned published stenosis rates.

In addition, anal fissure surgery can lead to anal stenosis, if an internal sphincterotomy is not performed. Stenosis may follow anterior resection of the rectum, if complicated by anastomotic dehiscence. Inflammatory bowel diseases may cause anal stenosis, particularly Crohn's disease. These stenoses are characterized by a transmural scarred inflammatory process. Patients with anal fissure or who abuse paraffin laxatives may develop a disuse stenosis. Radiotherapy treatment for pelvic tumors (i.e. uterine carcinoma, prostatic carcinoma, *etc.*) promotes anal stenosis formation. Also sepsis, ischemia from occlusion of lower mesenteric artery or upper rectal artery, AIDS, venereal lymphogranuloma, gonorrhea, amoebiasis and anorectal congenital disease may lead to anal stenosis. Finally, chronic abuse of ergotamine tartrate for the treatment of migraine headache attack may lead to anorectal stricture[20].

In the natural anatomic configuration, the anal canal is an upside down funnel, where its diameter is lower than the diameter of the anal verge. Physiologically, during evacuation, the internal sphincter relaxes and dilates to the cutaneous side, where the diameter is greater, to allow the regular passage of stool. On this subject, it is important to distinguish acute from chronic anal stenosis. Acute anal stenosis is determined by a severe and sudden spasm of persistent pain (i.e. in the anal fissure). These spasms are dynamic and reversible. In this case, the ano-rectal passage is cylindrical. Chronic anal stenosis, occurs secondary to surgical procedures, infections and fibrosis, and spasms are adynamic and irreversible[3,4].

Thus, the anal canal progressively reduces its diameter. In patients who use laxatives improperly, physiologic regular dilatation is abolished. Gradual and irreversible fibrosis occurs in the sub-cutaneous space of the anal canal with a pathologic funnel-shaped configuration in which the diameter of the anal canal is greater than the diameter of the anal verge.

DIAGNOSIS

Diagnosis of this condition is straightforward. The patient usually reports difficult or painful bowel movements. The patient may also have rectal bleeding and narrow stools. The fear of fecal impaction or pain usually causes the patient to rely on daily laxatives or enemas. Suspicion of anal stenosis is heightened by a history of hemorrhoidectomy, Crohn's disease, or excessive laxative use.

Physical examination confirms the diagnosis. Visual examination of the anal canal and perianal skin, along with a digital rectal examination, is usually suffice to establish the presence of anal stenosis. Occasionally the patient is too anxious or the anal canal too painful to allow an adequate examination. In this situation, anesthesia is needed to perform a proper examination of the anal canal. The anesthetic abolishes the spasm associated with an acute fissure but will not produce an increased luminal diameter in a patient with a true stenosis. Anorectal manometry is an objective method for assessing anal musculature tone, rectal compliance, anorectal sensation, and verifying the integrity of the rectoanal inhibitory reflex. Several methods are available for obtaining this information. No single method is universally accepted and manometric data from different institutions are difficult to compare. Manometry has been widely used to document sphincter function prior to procedures, such as lateral internal sphincterotomy, which may affect continence.

It is important to ascertain the cause of the stricture in order to determine proper therapy; a malignant disease must be treated by excision or resection, and anal Crohn's disease is an absolute contraindication to anoplasty[4].

CLASSIFICATION AND TOPOGRAPHY

When planning treatment of anal stenosis, it is useful to categorize the severity of the stenosis. Anatomic anal stenosis may be classified on the grounds of stricture severity, its structure and the level of involvement in the anal canal. On the basis of severity, Milsom and Mazier[6] distinguished mild (tight anal canal can be examined by a well-lubricated index finger or a medium Hill-Ferguson retractor), moderate (forceful dilatation is required to insert either the index finger or a medium Hill-Ferguson retractor), and severe anal stenosis (neither the little finger nor a small Hill-Ferguson retractor can be inserted unless a forceful dilatation is employed). Furthermore, stenosis may be diaphragmatic (after inflammatory bowel disease, characterized by a thin strip of constric-

tor tissue), ring-like or anular (after surgical or traumatic lesions, of length less than 2 cm), and tubular (length more than 2 cm). On the basis of the anal canal levels, stenosis may also be distinguished as low stenosis (distal anal canal at least 0.5 cm below the dentate line, 65% of patients), middle (0.5 cm proximal to 0.5 cm distal to the dentate line, 18.5%), high (proximal to 0.5 cm above the dentate line, 8.5%), and diffuse (all anal canal, 6.5% of cases)[6].

TREATMENT

The best treatment of postsurgical anal stenosis is prevention. Adequate anorectal surgery reduces the incidence of anal stenosis[3,16]. It is essential to treat tissues delicately and not to draw them. Also it is important to use absorbable sutures and minimal resection of tissues. Khubchandani[3] condemned the use of manual dilatation under anesthesia for the non-operative treatment of mild to moderate stenosis because the resultant hematoma in the sphincter apparatus may cause fibrosis and progressive stenosis. In Milligan-Morgan hemorrhoidectomy, internal sphincterotomy, if necessary, associated with preservation of adequate muco-cutaneous bridges, prevents anal stenosis. However, if anal stenosis is present, treatment should be modulated based on severity, cause and localization[6].

Non-operative treatment is recommended for mild stenosis and for initial care of moderate stenosis. Also, with severe stenosis, conservative treatment can lead to good results, however, surgery is always necessary. The use of stool softeners and fiber supplements with adequate gain of fluids is the basis of non-operative treatment. This gradual and natural dilation is very effective in most patients. Anal dilatation is another important part of this treatment. Anal dilation can be performed daily both digitally or with any of a number of graduated mechanical dilators. Patients are instructed to sit down on the toilet, bear down, and gradually insert the smallest dilator with ample lubrication. If the patient can persist with the dilations on a regular basis, the result is usually excellent. Many patients do not tolerate this procedure. On the other hand, a dilator may tear the canal. In fact, a complication from the use of dilators may itself precipitate the need for surgical intervention. However, it would be a rare circumstance when mild stenosis would require surgery[4].

Moreover, if the patient remained symptomatic with the usual measures, it is important to be certain that anal stenosis is indeed the cause of the patient's complaints; particularly in the postoperative patient, anal fissure must be ruled out as a possible source of the problem. If stenosis is refractory to non-operative management, surgery represents the last solution. However, a long course of conservative, medical management is indicated in the treatment of mild anal stenosis before resorting to a surgical approach.

Many different surgical techniques have been described for the management of moderate to severe anal stenosis. Moderate stenosis is generally treated initially in the same fashion as mild stenosis. Fiber supplementation is initiated and dilations are carried out if necessary. Furthermore, partial lateral internal sphincterotomy may be quite adequate for a patient with a mild degree of narrowing. This technique is simple and safe and use is limited to functional stenosis. It is important that the sphincterotomy is done in the open fashion so that the associated scarred anoderm is divided at the same time to allow full release of the scar. The resulting wound is then left open and allowed to heal by secondary intent. This provides relief of the partial obstruction and pain caused by the stenosis, but the relief will be short-lived without appropriate medical management. The importance of a high-fiber diet and fiber supplements must be emphasized to the patient and instituted immediately after surgery. Although the results have been reported as excellent[21,22], it is difficult to interpret whether the patients had significant narrowing or spasm associated with the anal fissure.

For more severe anal stenosis, a formal anoplasty should be performed to treat the loss of anal canal tissue. Various types of flaps have been described for anal stenosis which allow delivery of the more pliable anoderm into the anal canal to replace the scarred lining at that level. A lateral internal sphincterotomy is also usually necessary at the time of anoplasty.

Lateral mucosal advancement flap
This is a modification of Martin's anoplasty (Figure 1A)[1,23,24]. A midlevel stenosis is corrected by excision of the scar tissue. An undermining of the proximal rectal and anal mucosa through a transverse incision at the dentate line is performed. An internal sphincterotomy is performed if a functional component is present. The resulting flap is advanced to the distal edge of the internal sphincter near the anal verge. The vascular supply is maintained through the submucosal plexuses. The external part of the wound is left open to minimize ectropion formation.

Y-V advancement flap
This procedure is performed in the gynaecological prone position. It is important to administer adequate antibiotic therapy (cephazoline and metronidazole) at the time of surgery. A mechanical bowel preparation is usually done the day before surgery. After anal dilatation with a medium Hill-Ferguson retractor, the initial incision overlying the area of stricture is the vertical limb of the Y (Figure 1B). This incision is extended on the perianal skin in two directions for creating a V flap. Incisions are carried proximally for 5 to 8 cm. The V flap is incised with fatty subdermal tissue, providing an adequate blood supply. The resulting V advancement flap is sutured into the vertical limb of the Y incision in the anal canal with the internal apex of the triangular flap sutured to the internal sphincter and to rectal mucosa (dentate line) with interrupted long-term absorbable sutures[5,20,23,25-30]. This flap can be done in the posterior midline or in either lateral position. It can also be done bilaterally if needed to relieve the stenosis. Postsurgical management consists of fiber supplements and pain control. Sitz baths can also

Figure 1 Operative procedure for the surgical treatment of anal stenosis. A: Martin's anoplasty; B: Y-V advancement flap; C: V-Y advancement flap; D: Diamond-shaped flap; E: House-shaped flap; F: U-shaped flap; G: Rotational S-flap.

be instituted to assist with local hygiene. In the post-operative period, a constipating regimen is recommended for 2 d. Antibiotic therapy is usually continued for 7 d. This technique is simple and quite useful for stenosis associated with an anal fissure. However, if more than 25% of the circumference of the anal canal needs to be covered, another anaplastic procedure is indicated[4].

V-Y advancement flap

This procedure is an alternative to Y-V anoplasty. The base of the triangular V flap is sutured to the dentate line (Figure 1C). In addition, the underlying vascular pedicle is contained in the subcutaneous fat. Thus, it is necessary to preserve fatty subcutaneous tissue with wide mobilization to maintain flap viability. The skin is then closed behind the V at the external portion of the perineum to push the V into the anal canal and widen the stenotic area[31].

Diamond-shaped flap

After adequate mechanical bowel preparation and antibiotic therapy in the pre-operative period, this procedure is performed in the gynaecological prone positition. To avoid bleeding, epinephrine can be used. On the basis of stenosis severity, one or two flaps can be created. The scar tissue is incised leaving a diamond-shaped defect (Figure 1D). A diamond-shaped flap is designed so that it will cover the intra-anal portion of the defect[5,23,29]. The preparation of the flap is a crucial step in the procedure: the flap should be well mobilized to reduce tension and to provide enough blood supply to preserve the underlying vascular pedicle.

House flap

After the use of stool softeners in the pre-operative period, enema is performed on the day of surgery. This technique is performed in the gynaecological prone position. If stenosis is extended from the dentate line to perianal skin, a house flap is recommended (Figure 1E). With the use of a Hill-Ferguson retractor, a longitudinal incision is made toward the perianal skin, from the dentate line to the end of the stenosis. The length of incision corresponds to the length of the flap wall. Proximal and distal transverse incisions are centered on the longitudinal incision. The flap is then designed in the shape of a house with the base oriented proximally. The width of the base of the house is designed to match the transverse incisions and hence the width of the mucosal defect to be replaced. It is necessary to preserve the subcutaneous vascular pedicle[2,7,26,31,32]. The flap is then easily advanced into the anal canal and sutured. This procedure offers two advantages: (1) the creation of a wide flap increases the anal canal diameter along its length, (2) the technique allows primary closure of the donor site.

U flap

This procedure is used for the treatment of anal stenosis associated with mucosal ectropion. A U-shaped incision is made in the adjacent perianal skin (Figure 1F). Mobilization and suture of the flap are the same as for diamond-shaped anoplasty. The donor site is left open, and covered with fatty gauzes.

C flap

This procedure is performed in the lithotomy position.

Table 1 Anoplasty for anal stenosis

Procedures	Indications	Advantages/Disadvantages
Partial lateral internal sphincterotomy	Functional stenosis; mild and low stricture in the anal canal	This technique is simple and safe. Use is limited to functional stenosis
Mucosal advancement flap	Middle or high localized stricture	Ectropion formation if the flap is sutured at the anal verge
Y-V advancement flap	Low and localized stricture below the dentate line	Proximal part of the flap is very narrow and will not allow for a significant widening of the stricture above the dentate line. Also, the tip of the V within the anal canal is subject to ischemic necrosis from lack of mobilization, tension of the flap or loss of vascularization
V-Y advancement flap	Mild to severe stricture at the dentate line. Middle or high localized strictures, associated with mucosal ectropion	The tip of the V is subject to ischemic necrosis
Diamond flap	Moderate to severe long stricture, localized or circumferential stricture above the dentate line, associated with mucosal ectropion	A diamond-shaped flap is designed so that it will cover the intra-anal portion of the defect. The flap is mobilized with minimal undermining to preserve the integrity of the subcutaneous vascular pedicle
House flap	Moderate to severe long stricture, localized or circumferential or diffuse, and stricture above the dentate line, associated with mucosal ectropion	It allows primary closure of the donor site and increases anal canal diameter along its length. Because of the wide base, it avoids the pitfall of having a narrow apex present inside the anal canal that may become ischemic
U flap	Moderate to severe stricture, localized or circumferential, associated with mucosal ectropion	This technique is particularly useful when there is need to excise a significant area of ectropion. The donor site is left open
C flap	Moderate to severe stricture, localized or circumferential, associated with mucosal ectropion	The donor site is left open
Rotational S flap	High severe stricture, circumferential or diffuse, associated with mucosal ectropion	It provides for adequate blood supply, avoids tension, and can be performed bilaterally if necessary for coverage of large areas of skin. Complex technique: high morbidity and longer hospital stay

With the use of a small Hill-Ferguson retractor, a radial lateral incision is made from the dentate line to the anal verge. Then a C-shaped incision is made in the perianal skin starting from the distal point of radial incision. Preparation of a C flap should guarantee an adequate blood supply.

Rotational S-flap
The S-plasty is best used for the treatment of Bowen's disease or Paget's disease, where a large amount of skin has to be excised and new skin rotated into the area[29,33]. The S-plasty does not open a stricture as well as the advancement flap. In the gynaecological prone position, after scar tissue has been excised, a full-thickness S-shaped flap is made in the perianal skin, with the size of the base as great as its length, starting from the dentate line for approximately 8 cm to 10 cm. The flap is then rotated and sutured to the normal mucosa (Figure 1G).

Internal pudendal flap anoplasty
A solitary case report has been reported where extensive coverage was required concomitant with excision of Paget's disease of the anal canal[34].

Foreskin anoplasty
This interesting operation has been described for the treatment of mucosal ectropion. The procedure uses the foreskin (suitable prepuce) to provide a full-thickness skin graft to the anal canal. Since the initial report by Freeman with six children in 1984[35], no further publications have been noted.

Choise of procedure
The choice of an adequate procedure is related to the extent and severity of the stenosis (Table 1) as it may involve the skin, transitional zone to the dentate line, anal canal, or all of these. Y-V anoplasty is not used in the treatment of stricture above the dentate line. V-Y anoplasty has been used in the treatment of severe low anal stenosis with good results.

Various types of anoplasties with adjacent tissue transfer flaps have been devised to relieve anal stenosis. All of these flaps share the concept of an island of anoderm that is incised completely around its circumference. A significant advantage of these flaps over the Y-V anoplasty is that there is significantly greater mobility of the flap, so it can be advanced well into the anal canal. The diamond flap, House flap, and island flap have all been reported to yield excellent results[3-8,10,15,19,20,22-32,34-44]. The type of flap to be used is based on the surgeon's familiarity and choice as well as the patient's anatomy and the availability of adequate perianal skin for use in the various flaps. For any of these flaps, the preoperative preparation is the same as for the Y-V flap. A partial lateral internal sphincterotomy is often required as well. Once the flap is fully mobilized, it can be advanced into the anal canal and sutured in place with interrupted long-term absorbable sutures. Similar to the Y-V flap, these flaps can be done in any location and can be done bilaterally if needed.

The House flap is recommended if stenosis extends from the dentate line to perianal skin, allowing primary closure of the donor site and an increase in anal canal diameter along its length. U flap anoplasty is used for the treatment of anal stenosis associated with mucosal ectropion. If less than 50% of the anal circumference is involved, an advancement flap should suffice; however, if 50% or more of the anal canal needs to be recon-

structed, a rotational flap of skin should be considered, as it is necessary to cover a large area providing adequate blood supply and avoiding tension.

Postoperative care

Single and limited flaps may be performed in the outpatient setting. For the simplest procedures, patients are started on a high-fiber diet, bulk laxatives and mineral oil for a short time in the postoperative period. Sitz baths and showers are recommended for comfort and hygiene. Flaps with multiple and extensive dissection or reconstruction will require hospital admission. These extensive procedures may require bowel confinement for 3 d to 5 d after which a high-fiber diet is initiated.

Complications

In the literature, various complications have been reported after anoplasty. These include flap necrosis from loss of vascular supply, infection or local sepsis, suture dehiscence from excessive suture line tension, failure to correct the stenosis, donor site problems, sloughing of the flap, ischemic contracture of the edge of the flap, pruritus, urinary tract infection subsequent to *Clostridium difficile* enterocolitis only in a few cases, fecal incontinence, constipation without stenosis, urinary retention, restenosis and ectropion if the flap is advanced too far and sutured at the anal verge[4-7,10,15,19,20,22,23,25,26,31,32,34,36-39,41,42,44].

COMMENTS

Anal stenosis, although rare, is one of the most feared and disabling complications of anorectal surgery. It has been documented that hemorrhoidectomy is the most frequent cause, but stenosis may be a consequence of other causes. Several operative techniques to treat hemorrhoids have been described. Milligan-Morgan's open hemorrhoidectomy is most commonly used; other procedures, such as Ferguson's closed hemorrhoidectomy and Parks' submucosal hemorrhoidectomy, are technically more complex. We feel that the surgeon's choice of technique is primarily based on personal experience and technical training, and only a competently performed technique produces satisfying results: hemorrhoidectomy needs skilled operators. If technical guidelines are rigorously followed, the feared complications associated with surgical procedures, such as anal stricture and sphincteric injuries, are largely reduced.

Furthermore, anal stenosis became a focus of interest after the introduction of SRM. Anorectal stenosis is not a specific problem of SRM but is a considerable problem after all anal interventions. In a direct comparison in prospective randomized trials there was no significant difference in stenosis rate between conventional hemorrhoidectomy and SRM. Nevertheless, a substantial rate of stenoses was observed following conventional hemorrhoidectomy, and probably the highest stenosis rate was described after Whitehead hemorrhoidectomy. One potential mechanism that might cause stenosis following SRM is ring dehiscence followed by submucous inflammation. Another theoretical cause is that the

stapled ring is placed too deep in the anal canal and that the squamous skin cells react by scarring and shrinking. One major aspect of the potential risk of developing a stenosis is the distance to the anal verge. A full thickness excision of the rectal wall is another potential cause for stenosis after SRM.

A number of corrective surgical procedures have been designed aiming to bring a healthy lining to the narrowed portion of the anal canal. Since more complex techniques, such as S-plasty, have now been abandoned due to high morbidity and longer hospital stay, easier techniques are still being performed with good results (Table 2). The ideal procedure should be simple, should lead to no or minimal early and late morbidity, and should restore anal function with a good long-term outcome.

Each of the surgical techniques described can be performed safely and have been used with variable healing rates. It is extremely difficult to interpret the results of the various anaplastic procedures in the literature for the obvious reason that prospective trials have not been performed. There are no controlled studies on the advantages and disadvantages of the various anaplastic maneuvers; however, almost any approach will at least improve symptoms in the patient. Oh and Zinberg[41] used C anoplasty in 12 patients with anal stenosis (10 by previous hemorrhoidectomy, 1 by fistulectomy and 1 by fissurectomy), and 11 patients obtained satisfactory results with a total healing rate of 91%. Khubchandani[3] published a study in which 53 patients underwent mucosal advancement flap anoplasty with a healing rate of 94%. Similar results have been reported in a total of 33 patients treated with Y-V anoplasty in two studies[23,28]. A total healing rate of 100% was obtained using diamond flap anoplasty in a total of 23 patients affected by anal stricture and mucosal ectropion. The healing rate was 91.5% in 53 patients who suffered from anal stenosis and ectropion treated with island flap anoplasty[29,39].

Aitola and coworkers[25] conducted a retrospective study in 10 patients who had undergone Y-V anoplasty combined with internal sphincterotomy between 1991 and 1995. After a median follow up period of 12 mo, all but one patient improved. Six patients had good results, three had fair results and in one the result was poor. This patient later developed a restenosis. Total healing rate was 60% with improvement rate of 30%. In a recent study[5], a Y-V anoplasty was perfomed in 29 cases and a diamond flap anoplasty in the remaining 13 cases. At 2 years follow-up, all patients who underwent diamond flap anoplasty had complete resolution of the stenosis (healing rate 100%). Among 29 patients who underwent Y-V anoplasty, 26 (90%) judged their clinical results as excellent while 3 patients (10%) required periodical use of anal dilators. Those three patients had post-operative complications (two suture dehiscence and one ischemic contracture of the edge of the flap).

Rakhmanine and colleagues[24] published a study in which 95 patients underwent lateral mucosal advancement anoplasty. Mean follow up was 50 mo. Only 63% of patients had undergone previous surgery: 35 patients

Table 2 Experiences in literature

Authors	No. of cases	Procedure	Results			Healing rate (%)
			Good	Fair	Poor	
Sarner *et al*[45]	21	Sarner's flap	-	-	-	100
Nickell *et al*[46]	4	Advancement flap anoplasty	4	-	-	100
Oh *et al*[41]	12	C anoplasty	11	-	1	90
Khubchandani[40]	53	Advancement flap anoplasty	Nr	Nr	Nr	94
Milsom *et al*[6]	24	V-Y anosplasty and Sarner's anoplasty	-	-	-	90
[1]Gingold *et al*[28]	14	Y-V anoplasty	9	5	-	64
Caplin *et al*[11]	23	Diamond flap anoplasty	23	-	-	100
Ramanujam *et al*[30]	21	Y-V anoplasty	18	2	1	95
Pearl *et al*[29]	25	Island flap anoplasty	16	7	2	92
[2]Angelchik *et al*[23]	19	Y-V anoplasty (12 cases)	8	4	0	100
		Diamond flap anoplasty (7 cases)	7	-	-	100
Pidala *et al*[42]	28	Island flap anoplasty	25		3	91
Eu *et al*[38]	9	Lateral internal sphincterotomy (5 patients) and anoplasty (4 cases)	9	-	-	100
Gonzalez *et al*[39]	17	S anoplasty (6 cases) and advancement flap anoplasty (11 cases)	16	-	1	92
Sentovich *et al*[32]	29	House advancement flap	26	-	3	90
Saldana *et al*[34]	1	Internal pudendal flap anoplasty	1	-	-	100
Aitola *et al*[25]	10	Y-V anoplasty with internal sphincterotomy	6	3	1	60
de Medeiros[47]	30	Sarner's flap or Musiani's flap	-	-	-	100
Maria *et al*[5]	42	Y-V anoplasty (29 cases)	26	-	3	90
		Diamond flap anoplasty (13 cases)	13	-	-	100
Stratmann *et al*[44]	3	-	-	-	-	100
Ettorre *et al*[26]	1	House advancement flap	1	-	-	100
Saylan[20]	3	Y-V anoplasty	-	-	-	100
[3]Rakhmanine *et al*[24]	95	Lateral mucosal advancement anoplasty	74	-	8	90
Carditello *et al*[36]	149	Internal sphincterotomy and mucosal flap anoplasty	Nr	Nr	Nr	97
Habr-Gama *et al*[15]	77	Sarner's flap (58 patients) and Musiani's flap (19 patients)	-	-	-	87
Filingeri *et al*[27]	7	Y-V anoplasty	-	-	-	100
Casadesus *et al*[1]	19	Y-V anoplasty (7 patients) and lateral mucosal advancement flap anoplasty (12 patients)	-	-	-	100
Alver *et al*[31]	8	House advancement flap (14 flaps: 1 flap for 2 patients, and 2 flaps for 6 patients)	6	-	-	100

Nr: Not reported. [1]No sphincterotomies were done; [2]Not all patients underwent sphincterotomy, and some of the procedures were for anal ectropion; [3]Depending on the degree of stenosis, patients initially underwent either unilateral (62%) or bilateral (38%) anoplasty. Thirteen patients with a follow up of less than 6 mo were excluded from the analysis for restenosis.

had had hemorrhoidectomy, 10 operations for anal fissure, 4 for fistula, 1 transversal excision of a neoplasm and 10 other operations. The overall complication rate was 3% (one abscess and two seepage of liquid stool).

CONCLUSION

Anal stenosis is most often a preventable complication. A well-performed hemorrhoidectomy is the best preventative measure. Anoplasty should be part of the armamentarium of colorectal surgeons for treating severe anal stenosis. The anatomic configuration of the anorectum and perianal region is very complex and knowledge of this area is essential before performing any surgical procedure. Most post-anoplasty complications can be avoided by respecting the rectal wall anatomy in the execution of surgical procedures. The preparation of flaps is important for treatment success. In all cases, in fact, it is necessary to preserve as much sub-cutaneous fat as possible with wide mobilization, and to maintain viability and to avoid excessive suture line tension. In addition, it is important to treat tissues delicately and not to draw them, to use absorbable sutures and perform minimal tissue resection.

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EDITORIAL

Measurement of serum paraoxonase-1 activity in the evaluation of liver function

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Abstract

Paraoxonase-1 (PON1) is an esterase and lactonase synthesized by the liver and found in the circulation associated with high-density lipoproteins. The physiological function of PON1 seems to be to degrade specific oxidized cholesteryl esters and oxidized phospholipids in lipoproteins and cell membranes. PON1 is, therefore, an antioxidant enzyme. Alterations in circulating PON1 levels have been reported in a variety of diseases involving oxidative stress including chronic liver diseases. Measurement of serum PON1 activity has been proposed as a potential test for the evaluation of liver function. However, this measurement is still restricted to research and has not been extensively applied in routine clinical chemistry laboratories. The reason for this restriction is due to the problem that the substrate commonly used for PON1 measurement, paraoxon, is toxic and unstable. The recent development of new assays with non-toxic substrates makes this proposal closer to a practical development. The present editorial summarizes PON1 biochemistry and function, its involvement with chronic liver impairment, and some aspects related to the measurement of PON1 activity in circulation.

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INTRODUCTION

The paraoxonase (PON) enzyme family comprises 3 members, PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7q21-22[1]. In humans, PON1 and PON3 are mainly found in the circulation bound to high-density lipoproteins (HDL)[2]. Conversely, PON2 is an intracellular enzyme[3]. Their physiological roles have not been completely ascertained. PON1 has esterase and lactonase activities[4] and is involved in protection against xenobiotic toxicity[5]. PON2 and PON3 have only lactonase activity[6]. All the PONs are able to reduce low density lipoprotein (LDL) oxidation[7], while PON2 reduces cellular oxidative stress and prevents apoptosis in vascular endothelial cells[8]. PON1 is the best known among these enzymes. Alterations in circulating PON1 levels have been reported in a variety of diseases involving oxidative stress. These include cardiovascular disease, Alzheimer's disease, chronic renal failure, HIV-infection, metabolic syndrome, and chronic liver impairment[9]. As such, increased knowledge of the physiological significance of PON1 and its involvement in human pathology would be of critical importance in the years to come. In the present article we review fundamental concepts regarding PON1 biochemistry and function, and the relationships with chronic liver diseases. We also discuss the possible application of its measurement in serum for an improved evaluation of hepatic function.

PON1 IS AN ANTIOXIDANT ENZYME

The first approximation to the physiological role of PON1 was suggested by Mackness *et al*[10]. The authors investigated the protection against copper-induced LDL oxidation *in vitro* provided by purified PON1. They observed that this enzyme prevents the generation of

lipoperoxides during the process of LDL oxidation. Further studies from this and other groups reached the conclusion that PON1 protects LDL and HDL from lipid peroxidation by degrading specific oxidized cholesteryl esters and specific oxidized phospholipids contained in oxidized lipoproteins[11-13]. PON1 is, in turn, inactivated by oxidized lipids. This was shown by Aviram et al[14], who demonstrated that the incubation of PON1 in vitro with oxidized palmitoyl arachidonoyl phosphatidylcholine, lysophosphatidylcholine, and oxidized cholesteryl arachidonate, inactivated PON1 activity, as well as did oxidized LDL. Cysteine-284 was required for this effect of oxidized lipids on PON1 because, in recombinant PON1 in which this aminoacid had an induced mutation, no inactivation was observed. A further article from the same group showed that, under oxidative stress, PON1 may be inactivated by S-glutathionylation, a redox regulatory mechanism characterized by the formation of a mixed disulfide between a protein thiol (i.e. cysteine-284) and oxidized glutathione[15].

Identifying the native function of PON1 has, for a long time, been hampered by confusion with respect to the structure and mechanism-of-action of this enzyme. Several studies established the primordial function of PON1 as that of a lipolactonase[16-18] which subsequently evolved new substrate specificities. These studies also established that the preferred substrates of PON1 are 5- and 6-membered ring lactones, typically with aliphatic side-chains[19]. A model has been proposed to link lactonase activity and the degradation of lipid peroxides[20] by which oxidized lipids containing hydroxyl groups at the 5'-position could be lactonized by PON1 to yield lysophosphatidylcholine and δ-valerolactone products. As such, according to this hypothesis, the PON1 ability to degrade lipid peroxides is secondary to its lipolactonase activity.

PON1 IS ESSENTIALLY SYNTHESIZED BY THE LIVER

PON1 is found mainly in serum and in the liver. Northern blot analysis performed in human and rabbit tissues detected PON1 mRNA only in the liver, although reverse transcriptase PCR (RT-PCR) studies in mice identified PON1 mRNA in liver, kidney, heart, brain, intestine, and lung[1]. It seems highly likely that the liver is the main source of serum PON1 since it is the organ with the highest PON1 gene expression, and where an important percentage of HDL is synthesized and secreted into the circulation. Over the last 15 years, there have been several attempts to purify hepatic PON1 to homogeneity with the aim of comparing its properties with those of the serum enzyme. This task is complicated by the hepatic PON1 being an enzyme associated with membrane vesicles derived from the endoplasmic reticulum[21].

In 1993, the first method for the partial purification of rat liver PON1 was published[22]. Essentially, the process consisted of the preparation of microsomes, solubilization with Triton X-100, adsorption on to hydroxyapatite, and chromatography with DEAE-52

cellulose to yield a 77-fold purified product. Later, Huang et al[23] isolated PON1 from mouse hepatic microsomes, and Rodrigo et al[24] in 1997, purified rat liver PON1 to homogeneity. They achieved a 415-fold purified product by using hydroxyapatite adsorption followed by three chromatography steps including DEAE-Sepharose, affinity chromatography, and Mono Q HR fast-performance liquid chromatography. The N-terminal sequence and two internal sequences of the purified protein were similar to those of rabbit and human PON1 of serum and mouse liver PON1. Subsequent studies with rat and human liver PON1 demonstrated many biochemical characteristics in common with those of serum PON1. These included optimum pH, substrate affinity (K_M), kinetic constants, heat inactivation, and calcium requirement[25]; all of which strongly suggested a high degree of identity between both enzymes.

What is the true role of hepatic PON1? If HDL-bound serum PON1 is an antioxidant enzyme, it may not seem illogical to infer that a similar function could apply to intracellular PON1. Indeed, liver microsomes are the major sites for the catabolism of xenobiotic compounds, in the course of which process an increased production of free radical species is observed. Rodrigo et al[26] observed PON1 protein expression mainly in the hepatocytes from the centrolobular region, thus supporting the hypothesis of intrahepatic PON1 participation in oxidative by-product inactivation.

OXIDATIVE STRESS AND CHRONIC LIVER IMPAIRMENT

Increased oxidative stress and inflammation play a fundamental role in the onset and development of liver diseases. The most important causes of chronic liver disease are alcohol abuse, obesity, and hepatitis C virus infection.

Alcoholic liver disease (ALD) comprises a broad spectrum of hepatic alterations ranging from steatosis and minimal injury to advanced fibrosis and cirrhosis[27]. The involvement of oxidative injury in ethanol toxicity has emerged from reports showing that alcohol-fed animals and patients with ALD present with a high content of lipid peroxidation products in their livers and in the circulation[28]. Oxidative stress associated with ethanol intake comes mainly from reactive oxygen species (ROS) generated by the mitochondrial respiratory chain and cytochrome P4502E1 from hepatocytes, and the NADPH oxidase from Kupffer cells and recruited macrophages[29]. The impairment of mitochondrial lipid oxidation is one of the mechanisms responsible for hepatic fat accumulation[30]. Pan et al[31] reported that lipid peroxidation reduces hepatic lipoprotein secretion by enhancing the degradation of newly synthesized apolipoproteins and this effect, together with alterations in lipoprotein glycosylation in the Golgi apparatus[28], might contribute to microvesicular steatosis. Further evidence suggests that alcohol-induced oxidative stress interferes with the regulation of lipid synthesis by the peroxisome proliferator-activated receptor-α and the sterol

regulatory element binding protein 1[32]. The possible role of oxidative stress in promoting an inflammatory reaction in ALD has emerged from the observation that ethanol-induced lipid peroxidation increases the hepatic production of cytokines, growth factors, and collagen[33-35].

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are hepatic lesions which appear frequently in obese and diabetic individuals despite the fact that they may not have a history of alcohol abuse. These lesions resemble those of ALD, and are characterized by steatosis, hepatocyte hydropic degeneration, and inflammatory infiltrates. In addition, alterations in mitochondrial shape and function, and varying degrees of fibrosis are usually found[36]. NAFLD is an emerging lesion in modern societies, and will become more prevalent in the future, as it is associated with insulin resistance, metabolic syndrome, diabetes, and obesity. Oxidative stress plays a pivotal role in the evolution of "benign" steatosis to the more severe NASH. Several studies have shown that mitochondria in patients with NASH are abnormal from both the morphological and the functional points of view and, as in ALD, alterations in the fatty acid β-oxidation promote increased free radical production and lipid peroxidation[36]. The consequences of oxidative stress in NASH would be similar to those of ALD, with altered lipoprotein synthesis and secretion, an inflammatory reaction, and fibrosis.

Hepatitis C virus (HCV) is a major cause of viral hepatitis. In the USA, about 4 million people are infected, and 35 000 new HCV cases are estimated to occur every year[37]. The infection by this virus frequently does not resolve, and about 80% of the infected individuals become chronic carriers who may then progress to the most severe forms of liver impairment, as cirrhosis or hepatocellular carcinoma. Lipid peroxidation products, aldehydes as 4-hydroxynonenal, and 8-hydroxyguanosine (a marker of oxidative DNA damage) are elevated in HCV infection[38]. The increased oxidative stress may be explained by chronic inflammation and the generation of free radicals by Kupffer cells and recruited macrophages. The NS3 protein of HCV has been found to activate Nox 2 protein from macrophages, leading to increased generation of ROS that can exert oxidative stress to the nearby cells[39]. Furthermore, studies have indicated that HCV can directly induce oxidative stress in hepatocytes. HCV core gene expression has been associated with increased ROS, decreased reduced glutathione content, and increased thioredoxin in parenchymal cells. Recent studies showed that HCV core proteins bind to the outer mitochondrial membrane resulting in mitochondrial dysfunction by Ca^{2+} accumulation. These alterations would inhibit electron transport and promote ROS production[37]. Another HCV protein, NS5A, has also been reported to increase free radical production by Huh7 cells[40]. As in ALD and NASH, increased oxidative stress would produce a multifactorial reaction involving the synthesis of pro-inflammatory and pro-fibrogenetic cytokines and chemokines.

Therefore, it seems evident that chronic liver diseases share common biochemical alterations irrespective of their etiology. They are all accompanied by an increased oxidative stress secondary to mitochondrial abnormalities, promoting changes in lipid and lipoprotein metabolism, fat accumulation, an exacerbation of the inflammatory reaction due to increased cytokine synthesis, and extracellular matrix deposition.

THE MEASUREMENT OF SERUM PON1 ACTIVITY

There are no standardized methods for measuring PON1 esterase activity. The most widely used method is the hydrolysis of paraoxon. However, this method is not free of drawbacks, because paraoxon is very unstable and extremely toxic. The solution to the former problem is to prepare the reagent immediately before use. The solution to the latter problem requires that the stock solutions be handled in an air-extraction cupboard and the operator to take appropriate safety precautions such as wearing masks and gloves to protect against accidental contact or inhalation of the toxic fumes. Recent significant advances in the search for reliable PON1 lactonase activity assays may facilitate the measurement in a routine clinical chemistry laboratory setting. A new serum test based on this capacity of PON1, and employing 5-thiobutyl butyrolactone (TBBL) as a substrate, was recently proposed[41,42]. TBBL is a synthetic chromogenic lactone that resembles the natural lipolactone substrate of PON1. The method enables PON1 activity to be measured using a more 'physiological-like' substrate.

SERUM PON1 ACTIVITY IN CHRONIC LIVER IMPAIRMENT

In chronic liver diseases, oxidative stress influences the pathophysiological changes leading to liver cirrhosis and to hepatocellular carcinoma. Since PON1 exerts a protective effect against oxidative stress, it is logical to find an association between this enzyme and liver impairment. Ferre *et al*[43] observed, in rats with carbon tetrachloride-induced fibrosis, that an inhibition of hepatic PON1 activity was an early biochemical change related to increased lipid peroxidation and liver damage. They investigated the relationships between hepatic microsomal PON1 activity, lipid peroxidation and the progress of the disease in this experimental model. They found that PON1 activity decreased while lipid peroxidation increased in carbon tetrachloride-administered rats while the addition of zinc, which possesses antioxidant and anti-fibrogenetic properties, was associated with enhanced PON1 activity and a normalization of lipid peroxidation. This study suggested that PON1 activity may be involved in the defence against free radical production in liver organelles.

Pioneer studies in the 1970's observed for the first time a significant decrease in serum PON1 activity in small groups of patients with liver cirrhosis[44,45]. This results were confirmed by Ferre *et al*[46,47] in a wider series of patients with various degrees of chronic liver damage. These latter studies noted a significant decrease of serum

PON1 activity in patients with chronic hepatitis, and an even greater decrease in cirrhotic patients, compared to a control group. In alcoholic patients, the effects of alcohol intake on serum PON1 levels depend on the degree of liver dysfunction. In a study conducted in chronic alcohol abusers, subjects were classified into several sub-groups according to their degree of liver disease. The results demonstrated that serum PON1 activity was decreased in alcoholic patients, and that the magnitude of the alteration was related to the degree of liver damage[48]. These findings differ from those described in normal volunteers reporting moderate alcohol consumption, and in whom serum PON1 activity and HDL cholesterol were found to be slightly increased[49]. Changes in serum PON1 activity has also been studied in relation to outcomes of liver transplantation in patients with severe liver disease[50]. The serum PON1 activity was low, but tended to increase, in liver transplanted patients when the hepatic arteries had become blocked. Since PON1 activity is closely related to the recovery of liver function, its measurement could provide more accurate information on the success, or otherwise, of the liver transplant.

Serum PON1 measurement has been proposed as an useful test for the evaluation of the degree of liver impairment. Clinical diagnosis of chronic liver impairment and/or liver fibrosis is currently conducted *via* the invasive procedure of needle biopsy followed by histological evaluation. This procedure has important drawbacks, including a significant mortality rate (1/10 000-1/1000), sampling error, and subjectivity. Therefore, the development of non-invasive tests for the diagnosis of liver disease and the extent of the disease is an important goal of current research. Unfortunately, most of the individual laboratory tests to assess liver impairment have low specificity and sensitivity and, hence, the standard approach is to perform a battery of several tests followed by an algorithmic evaluation of the results. It is for this reason that several years ago, Ferre *et al*[46] proposed the addition of serum PON1 paraoxonase activity measurement as a biomarker of liver impairment. Serum PON1 measurement has an important feature in that the measured value is inversely related to the degree of liver derangement i.e. it decreases while most of the standard laboratory test values increase with the extent of the disease. Thus, PON1 measurement makes an additional contribution in improving current algorithms, and the ratios between tests. These authors estimated, by multiple logistic regression analysis, that the addition of paraoxonase measurement to a battery of standard liver function tests increased the overall sensitivity up to ≥ 90%, while keeping the specificity close to 100%. However, the measurement of this enzyme is, to-date, restricted to research laboratories and has not been extensively applied as yet in routine clinical chemistry laboratories, due to the problems associated with the use of paraoxon as a substrate. These drawbacks preclude full automation of PON1 measurement and, as such, can rarely be justified for inclusion in panels of standard biochemical tests. The recent development of new assays, such as the TBBL lactonase assay, makes this proposal closer to practical development. The TBBL assay has been shown to be equivalent to the paraoxon assay in terms of diagnostic accuracy[42], but with better safety of the TBBL substrate for the laboratory worker, and makes the lactonase measurement a strong candidate for inclusion into routine clinical laboratory testing of liver impairment, or for the study of other diseases involving oxidative stress.

CONCLUSION

Research into paraoxonases has flourished over the last 10 years. It seems now evident that PON1 is a lactonase with the ability to degrade lipid peroxides in lipoproteins and in cells, and that plays a protective role against oxidative stress and inflammation, which are key processes involved in the pathophysiology of chronic liver diseases. In the years to come, more reliable, practical, and accurate methods to measure PON activity and concentration will become available and these will facilitate more research in this field, and also enable the addition of PON measurement to the battery of routine analyses in clinical chemistry laboratories.

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Five years' experience with capsule endoscopy in a single center

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Abstract

Capsule endoscopy (CE) is a novel technology that facilitates highly effective and noninvasive imaging of the small bowel. Although its efficacy in the evaluation of obscure gastrointestinal bleeding (OGIB) has been proven in several trials, data on uses of CE in different small bowel diseases are rapidly accumulating in the literature, and it has been found to be superior to alternative diagnostic tools in a range of such diseases. Based on literature evidence, CE is recommended as a first-line investigation for OGIB after negative bi-directional endoscopy. CE has gained an important role in the diagnosis and follow-up of Crohn's disease and celiac disease and in the surveillance of small bowel tumors and polyps in selected patients. Capsule retention is the major complication, with a frequency of 1%-2%. The purpose of this review was to discuss the procedure, indications, contraindications and adverse effects associated with CE. We also review and share our five-year experience with CE in various small bowel diseases. The recently developed balloon-assisted enteroscopies have both diagnostic and therapeutic capability. At the present time, CE and balloon-assisted enteroscopies are complementary techniques in the diagnosis and management of small bowel diseases.

INTRODUCTION

Examination of the small bowel (SB) has been considered a challenge for several anatomical (i.e. distance from external orifices, length) and physiological (i.e. active peristalsis) reasons. Conventional techniques of endoscopy are limited by length while radiologic examinations, such as barium studies, are insensitive for the evaluation of pathology in the SB. An ingestible miniature camera device capable of obtaining images of the whole small intestine was developed due to a need for the exploration of this "final frontier". Video capsule endoscopy (CE) is a breakthrough in medical history for noninvasive imaging of the entire small intestine[1-3]. It was first introduced in 2000, and since then more than 700 studies have been published, which is indicative of its ease and the widespread acceptance of this new diagnostic tool[4]. According to reports by Given Imaging, more than 650 000 CEs have been performed, representing an increase in the utilization of this technology of approximately 15% over the previous year[5]. Problems with reimbursement, physician training, time requirements for interpretation and lack of therapeutic capability limit the further widespread use of this technology.

A wide range of uses for CE has been reported in the literature, but the majority of the studies have aimed to evaluate the cause of obscure gastrointestinal bleeding (OGIB). Recent studies showed the superiority of CE over conventional methods, but passive features such as inability to insufflate the bowel and to biopsy and lack of therapeutic capability have generated a debate on its advantages[6-14]. Newly developed balloon assisted enteroscopes are also available and have the potential to outscore CE in terms of diagnostic indications and therapeutic applications.

The purpose of this article was to review and share our institution's results using small bowel CE, with special reference to the existing literature.

PROCEDURE

Technical features of the capsule

The Given M2A (Given Imaging; Yoqneam, Israel) video CE is a pill-shaped wireless device with a slippery coating for easy ingestion and measures 11 mm × 26 mm. It is composed of a white light-emitting diode as light source, lens, imaging chip, batteries and a radio transmitter with internal antenna. The image field is 140 degrees and magnification is × 8[4]. Once swallowed, the capsule moves thorough the intestine *via* peristalsis and is excreted in the stool. The camera takes two images per second as it sweeps the intestine and transmits these to eight lead sensor arrays, arranged in a specific manner and taped to the anterior abdominal wall, connected to a recording device in the belt for the duration of the battery life, which is 6-8 h. Once the study is completed, the recording device and sensor arrays are removed and the images (50 000-60 000 images total) are downloaded to a computer with reporting and processing of images and data (Rapid, Given Imaging) software that displays the video images on a computer monitor. This software includes a localizing system, blood detector and some features to assist the interpreter. The suspected blood indicator is quite good at detecting active bleeding, but is not so useful at detecting other lesions and does not replace careful examination of the CE. It is recommended that patients avoid magnetic fields such as magnetic resonance imaging (MRI), and metal detectors until the capsule is excreted in the stool, which usually occurs in 24-48 h.

Bowel preparation

Pre-procedure bowel preparation is a controversial issue. Some favor the bowel preps and prokinetics. Incomplete SB transit during the examination occurs in about 20% of patients[6]; however, according to data from the international conference on capsule endoscopy, it was suggested that there was no need for routine use of bowel preparations[11]. We performed CE in an ambulatory outpatient setting, but there were some inpatients. All of the patients undergoing CE examination had bowel preparations before the procedure. Each patient was administered 3 L of polyethylene-glycol solution for bowel cleansing. Patients fasted overnight for at least 12 h before taking the capsule. After ingestion of the capsule, patients were allowed to drink clear liquids after 2 h and eat a light meal after 4 h and were observed for 8 h at the study site.

INDICATIONS

Capsule endoscopy is mainly indicated for the evaluation of SB diseases, particularly for the diagnosis of OGIB. CE can be used in a variety of conditions including Crohn's disease (CD), malabsorption, chronic diarrhea, evaluation

Table 1 Indications and contraindications of capsule endoscopy	
Indications	**Contraindications**
Small bowel	Absolute
Obscure gastrointestinal bleeding	Bowel obstruction
Overt GI bleeding	Extensive and active Crohn's
Occult (positive FOBT)	Disease ± strictures
Evaluation of iron deficiency anemia	Intestinal pseudo-obstruction
Crohn's disease	Young children (< 10 years)
Suspected Crohn's disease	Relative
Indeterminate colitis	Cardiac pacemakers
Assessment of mucosal healing	Implanted electromedical
Determine post-operative recurrence	Devices
Abdominal pain	Dysphagia
Graft-versus-host disease	Previous abdominal surgery
Surveillance of polyposis syndromes	Pregnancy
Celiac disease	Diverticulosis
Suspected small bowel tumors	
Follow-up of small intestine transplantation	
Evaluation of abnormal small bowel imaging	
Evaluation of drug induced injury	
Esophagus	
Barrett esophagus	
Esophagitis	
Variceal evaluation	

of refractory iron deficiency anemia, abdominal pain, polyposis syndromes, celiac disease, and detection of SB tumors. Graft versus host disease (GVHD) and follow-up of small intestine transplantation are rare indications, but our experience thus far did not include such patients. CE with high frame rate (PillCam Eso, Given Imaging) can be used for esophageal disorders, such as noninvasive evaluation of esophageal varices, esophagitis and Barrett's esophagus[11]. Table 1 shows the indications and contraindications for Capsule Endoscopy.

We reviewed our database in a retrospective evaluation of the characteristics and findings of patients who underwent CE examination between 2003 and 2008. All patients had upper and lower GI endoscopies before the CE study. There was no clinical sign of intestinal obstruction, but patients with suspected CD had radiologic examinations to exclude obstruction. A total of 120 CE examinations were performed from 2003 to 2008 for various indications. The average patient age was 47.7 ± 18.2 (min: 13 - max: 97), 45 were female (37.5%) and 75 male (62.5%). The CE completely evaluated the entire SB in 89 patients (74.2%). Indications for CE were OGIB (57.5% of cases), diarrhea (15%), abdominal pain (5.8%), other indications such as known CD, and surveilance for polyposis syndromes. CE study was normal without any finding in 22.5% of patients. We did not use CE for esophageal disorders and there were no findings suggestive of esophageal diseases.

OGIB

Gastrointestinal bleeding is a common problem encountered by gastroenterologists during clinical practice. Proximal and distal bleeding sites are mostly identified by means of endoscopy and colonoscopy. The bleeding

Figure 1 VCE images of lesions found in patients with obscure-overt GI bleeding. A: Multiple angiodysplasias in the jejunum; B: A jejunal mass with active bleeding; C: An ileal ulcer in a patient with newly diagnosed Crohn's disease. VCE images of small bowel polyps; D: Benign lymphoid hyperplasia located diffusely through the GI tract in a patient with CVID; E: A jejunal polyp in a patient with peutz-jeghers disease; F: Multiple small polyps in the ileum in the same patient depicted in Figure 1 E.

source is not identified in 3%-5% of cases despite the utilization of multiple studies[15,16]. OGIB is defined as bleeding from an unidentified source that persists and recurs after a negative endoscopy examination[16,17].

Obscure GI bleeding is the most common indication for CE examination. CE has a high diagnostic yield in OGIB, which may lead to early diagnosis and revision of the management strategy. CE facilitates effective decision-making regarding subsequent investigations and treatments[4].

Diagnostic yield of CE for OGIB varied between 31% and 91%[9,17-31]. Lema and Ruano-Ravina[32] reviewed the published studies of CE for OGIB and reported that sensitivity ranged from 79% to 95% and specificity from 75% to 100%. The positive predictive value (PPV) varied from 94% to 100% and the negative predictive value (NPV) from 80% to 100%. CE led to a change in therapeutic management in 9%-77% of patients. A recent study by Albert et al[33] reported that CE detected the bleeding source in 76.8% of patients.

The diagnostic yield of CE in OGIB depends on the type of bleeding. Pennazio et al[17] found that the highest yield of CE was in patients with active bleeding (92.3%) compared to those with obscure occult bleeding (44.2%). Researchers observed a reverse relationship between findings and time after last bleeding episode. The longer the time from last bleed, the lower the diagnostic yield. Do the lesions discovered by CE have any bleeding potential or clinical importance in terms

of management change? Saurin et al[18] showed that CE detects more lesions, but only half of them have true bleeding potential.

Several studies examined the diagnostic role of CE in OGIB and mostly compared the diagnostic yield of CE to other diagnostic modalities. CE is superior to other techniques in diagnosing the source of bleeding. The yield for CE is 63% and 67% compared with 28% for push enteroscopy (PE) and 8% for barium study[34].

Obscure GI bleeding was the most common (57.5% of cases) indication for CE study in our cohort. SB ulcerations were found in 25.8% of patients. Angiodysplasias were present in 12.5% of cases (Figure 1A). Active bleeding was observed in 8.3% of patients. Figure 1B shows a jejunal mass, which was found to be adenocarcinoma, with active bleeding. Diagnostic yield of CE for OGIB was 72.5% in our series. We have been performing single balloon enteroscopy (SBE) (Olympus; Tokyo, Japan) and a few patients underwent both CE and SBE. CE revealed angiodysplasias in two patients with OGIB who were treated with argon plasma coagulation during SBE examination. Balloon assisted enteroscopy and CE should be used as complementary studies. It is advisable to use CE to detect lesions and direct enteroscopy for the therapeutic interventions.

Crohn's disease
Crohn's disease is a chronic inflammatory disease that can involve any part of the GI system, and disease is

confined to the SB in about one-third of the patients. There is no single test to diagnose CD completely, so CD diagnosis can be established with a combination of clinical, endoscopic and histological findings. Most imaging studies lack sensitivity to identify early changes, and endoscopy does not allow total examination of the bowel. CE is able to identify mucosal changes before other technologies. It has a valuable role in the evaluation of the SB in patients with suspected or known CD. The use of CE in the diagnosis of small bowel CD has been examined in several studies. Triester *et al*[35] compared the yield of CE with other modalities in patients with suspected small bowel CD. Diagnostic yield of CE was 63% compared with 23% for barium radiography. When compared with ileocolonoscopy, CE had a higher yield (61% *vs* 46%). Compared with PE, CE had a 38% higher yield, and when compared with CT enterography, the yield of CE was 69% to 30%. Due to its high diagnostic yield, CE will have a very important place in the diagnostic workup of patients with CD, but more studies are needed to make such suggestions. Triester *et al*[35] reported in their meta analysis that there was no statistical significance in the incremental yield between CE and other diagnostic modalities in patients suspected of having CD. However, there was a significant difference in yield of CE over alternative methods in patients with known CD who were being evaluated for SB recurrence[35]. Yield of CE is low when performed in patients with abdominal pain alone; when other criteria are added, this yield is increased[34].

Capsule endoscopy can be used for the assessment of mucosal healing after treatment. The only limitation of CE is its inability to offer biopsy for histological examination. A scoring system has been proposed to evaluate CD on the basis of CE findings of villous structure, ulceration and stenosis. Each variable is assessed by size and extent of the change[36]; however, further studies are needed to clarify the helpfulness of this system. The score provides a common language to quantify mucosal changes associated with any inflammatory process. The index does not diagnose or measure a disease, it measures mucosal change. In addition, this scoring index does not have the discriminatory ability to differentiate between illnesses. This index could be helpful in determining mucosal healing after therapy in CD[34]. Mucosal breaks and aphthous ulcers or erosions are also seen in asymptomatic healthy volunteers. Since non-steroidal antiinflammatory drugs (NSAIDs) may cause ulcerations resembling those of CD, patients should be advised to stop such drugs at least one month before the CE examination[10]. It is difficult to differentiate these findings with the presence of CD.

Mucosal ulcerations were the most common finding in our patient series, determined in almost one out of four patients. CD was the third most common indication for CE study (6.7% of patients). Patients with CD had severe ulcerations and two patients had strictures

that resulted in regional transit abnormality. However, no capsule retention occurred in this group. Moreover, CE changed the management strategy in 10% of patients with a new diagnosis of CD. Another interesting finding was that 37.5% of the patients diagnosed as suspected CD did not have complete examination. Nonspecific jejunoileitis and NSAID-induced erosions were observed in 6.7% of patients. Figure 1C shows a mucosal ulceration.

Celiac disease

Celiac disease is an immune-mediated disease characterized by chronic SB inflammation that may result in mucosal atrophy, malabsorption and related clinical manifestations. Diagnosis is based on the combination of serologic, endoscopic and typical histological changes of the SB biopsy in clinically suspected patients. Its prevalence is around 1% in the United States. There are four endoscopic changes suggestive of villous atrophy: loss of mucosal folds, mosaic mucosal pattern, scalloping of the duodenal folds and nodularity of the mucosa[37]. It is no surprise that CE provides high resolution images that contain such changes. Rondonotti[38] evaluated 43 patients with signs or symptoms suggestive of celiac disease and positive serological markers. Patients underwent both CE and upper GI endoscopy. Characteristic histological changes were observed in 32 patients. Using this as a gold standard, 87.5% of patients were diagnosed by CE. Mucosal changes beyond the duodenum were detected in 18 (66.6%) patients and in 3 (11.1%) patients the whole SB was affected.

Another newly published study, searching for celiac disease in older adults, also showed that duodenal mucosa was normal in appearance on CE in 71% of patients, but classic abnormalities of celiac disease were present distally[39].

Overall, CE can detect endoscopic markers of celiac disease. In addition, CE seems to be able to recognize the extent of disease and may be a tool for follow-up. CE has a high sensitivity (range, 70%-95.2%), specificity (range, 63.6%-100%) and high PPV and NPV (96.5%-100% and 71.4%-88.9%, respectively)[38,40-43]. When an atrophic pattern is detected by CE, the patient has a high probability of having celiac disease[37]. CE has also been reported to be able to demonstrate diseases such as adenocarcinoma, lymphoma or ulcerative jejunoileitis, which may complicate the course of celiac disease. A limitation is that CE is able to detect Marsh III lesions, which are associated with clear mucosal abnormalities, but may not distinguish between Marsh I and II lesions[37]. At present, CE is an alternative to endoscopy with biopsy in patients with suspected celiac disease who do not consent to the conventional methods.

Chronic diarrhea was the second most common indication for CE study in our series. Half of these patients did not have any condition that may cause diarrhea. Lymphoid hyperplasia and nodularity were observed in 6.7% of patients. Lymphoid hyperplasia due to common variable immune deficiency was detected in three pa-

tients. Celiac disease was investigated in only one patient but CE examination was completely normal. One patient with iron deficiency anemia had mucosal atrophy on CE examination and was diagnosed as having celiac disease. Figure 1D shows benign lymphoid nodular hyperplasia in a CVID patient.

Small bowel tumors and polyps

Capsule endoscopy is a major advance in the diagnosis of SB tumors. Before the introduction of CE, malignant neoplasms of the SB were often diagnosed at a later stage of the disease, mostly during the work-up of obstructive symptoms. Diagnosis is delayed because conventional imaging techniques fail to detect small neoplasms in almost half of the patients. SB tumors are a rare disease, accounting for 1%-3% of all primary GI tumors. SB mass lesions are responsible for OGIB in up to 10% of patients[44-48]. Early clinical studies of CE have reported a frequency of SB tumors ranging between 6% and 9%[49-54]. This has led to an idea that CE doubled the rate of diagnosing SB tumors. However, a recent multicenter European study showed that the frequency of SB tumors was 2.4% and the most common indication for CE was OGIB[55,56]. SB tumors appear as masses or polyps in most patients and ulcer or stenoses in a minority of patients. It is not possible to distinguish the type of tumor based only on CE pictures. Most of the tumors reside in the mid SB[56].

Capsule endoscopy is also useful for the surveillance of polyps in patients with inherited GI polyposis syndromes (familial adenomatous polyposis and Peutz-Jeghers syndrome), who are at increased risk of developing polyps in the SB. Several studies comparing the yield of CE to other imaging modalities in patients with polyposis syndromes have shown that CE is accurate in the detection of polyps. The same studies also emphasized that CE is not reliable for sizing and determining localization of polyps[57-60]. The duodenum is a potential blind point of CE because the capsule passes quickly with tumble and results in inadequate examination. Wong et al[61] reported that CE underestimated the total number of polyps and did not reliably detect larger polyps in that portion.

In our series, SB masses were diagnosed in 4.2% of patients who had tumor resection, and two patients had benign tumors. CE examination was done in only one patient with Peutz-Jeghers disease. CE revealed a few proximal jejunal polyps measuring < 2 cm (Figure 1E and F). Subsequent enteroscopy showed multiple jejunal polyps with diameters up to 8 cm. CE definitely has a potential for use in patients with polyposis syndromes, but more studies are needed.

Other indications

Abdominal pain is one of the most common symptoms of patients referred to the gastroenterologist. Use of CE for the evaluation of abdominal pain is debated. Although some serious causes are identified in such patients, CE is mostly unyielding. If patients with other signs and symptoms of inflammation were selected, than

the diagnostic yield was considerably higher[62].

Capsule endoscopy may be helpful in the diagnosis of the following diseases: surveillance for NSAID side effects, Henoch-Schönlein purpura, indeterminate colitis, protein losing enteropathy, intestinal lymphangiectasia, Meckel's diverticulum, follow-up of SB transplantation, GVHD, and bowel changes in refractory pouchitis[1-10,62].

COMPLICATIONS, LIMITATIONS AND SAFETY ISSUES OF CAPSULE ENDOS-COPY

Capsule endoscopy is a safe and well-tolerated procedure for patients, with very low complication rates. Contraindications to CE include the presence of intestinal obstruction, fistulas and strictures. Swallowing abnormalities and esophageal stricture are other contraindications for the procedure. Capsule retention is the major complication of CE. Retention is defined as the indefinite presence of a capsule in the SB. This is different from slow transit, incomplete transit or regional transit abnormalities. In these cases, the capsule stays in the ileum but ultimately passes via peristalsis. Retention can cause symptoms of SB obstruction that in turn lead to need for endoscopic or surgical removal of the capsule[63,64].

Retention risk is high in patients with known CD, NSAID stricture, radiation enteritis and SB tumors. The capsule retention rate ranges from 0% to 13%. The rate of retention in patients with OGIB is 5% and in suspected CD 1.4%, and it can be as high as 8% in patients with known CD. Interestingly, no capsule retention was reported in healthy volunteers. The overall frequency of capsule retention is usually 1%-2%[10,63,64]. A negative SB series does not prevent capsule impaction[17]. It is advisable to perform abdominal radiographs within two weeks to identify capsule retention if the capsule did not enter the colon. Therapeutic intervention can be instituted anytime unless the patient becomes symptomatic[4].

The patency capsule (Agile Patency System, Given Imaging Ltd; Yoqneam, Israel) has been developed for the detection of high-risk patients before the procedure. This capsule is identical to the video capsule, with the same dimensions, and is made of lactulose and 5% barium, which make the capsule radiopaque and it dissolves spontaneously after 40 h. The capsule has a radiofrequency identification tag that enables easy detection by a special handheld device. In a recent study that included patients with known strictures, no CE retention occurred if the patency capsule passed safely[65]. Although there are promising data on patency capsule use before CE, it is still not definitive to predict capsule retention based on results of barium studies or patency capsule.

Another theoretic risk is electromagnetic interference with implantable medical devices, pacemakers, etc. In a small series of patients, no adverse cardiac effect or

image distortion due to interference was noted. Large sample sized studies are needed to confirm the safety of capsule in this context[66].

Reading the procedure is a time-consuming process and reading time is another limitation of this procedure. The optimal review rate is 15 images/s and it takes over 1 h to read a full 8-h procedure[62]. The reliable interpretation of the CE procedure requires experienced readers (experience of reading at least 20 studies).

Another clinical problem is sizing and locating SB lesions, since location and size are important findings for subsequent management. CE underestimates the number of SB polyps and does not reliably detect large polyps[61]. Technical problems related to the battery and failure of image downloading are also reported. The overall rate of technical failure is around 9%[10]. Incomplete study occurs due to delayed gastric emptying, previous SB surgery, hospitalization and poor bowel cleansing. A gastric transit time longer than 45 min was identified as a risk factor[67]. Reported incompletion rates vary between 0% and 50%, approximately 20% to 30% in most studies[67]. Effect of prokinetic drugs on completion rates is uncertain. Real time viewers of CE may help to identify prolonged gastric stay and in such case, endoscopy can be done to push the CE into the SB.

The overall miss rate of CE is about 11%, ranging between 0.5% for ulcerative disease and 18.9% for neoplastic disease. Of course, this rate is much lower than conventional examinations[47]. Inability to take biopsy or perform any therapeutic procedure is also a limitation of the CE, which makes balloon assisted enteroscopies a good choice for a number of indications.

In our patient cohort, the most common cause for an incomplete examination was premature battery failure in 20 patients (16.7%), followed by technical problems, of the capsule itself, in seven patients (5.8%). No complication related to the CE procedure was observed. There was no capsule retention event. Two patients' studies showed regional transit abnormality. One was due to severe CD with stricture, and the other patient had an ileal adenocarcinoma that was diagnosed after operation for ileal perforation. Although there was a temporal relation of perforation to CE study (2 d after the study), no capsule was detected in the preoperative radiograms and CE was not the likely cause of perforation. There was no patient with an implantable cardio defibrillator or pacemaker among our cohort, but it seems safe to use the capsule in these patients. Based on our data, we can say CE is a safe procedure. Placing the capsule directly in the duodenum by means of dedicated devices or endoscopy may lower the incomplete examination rate. However, by doing so, we can miss esophageal and gastric disorders in which CE is also informative. Therefore, if selective placement of the capsule is preferred, the proximal GI tract should be carefully re-examined. Higher capture rate and longer battery life could resolve these obstacles.

OTHER TYPES OF CAPSULE ENDOSCOPE

The Olympus Endo Capsule (Olympus; Tokyo, Japan) has been in the Turkish market for a while, but there is not yet sufficient experience with its use. It differs from the PillCam by having a high resolution image chip and an external real time viewer. There are additional SB capsule systems that are not currently available in Turkey. One is from China, the OMOM pill (Jinshan Science and Technology; Chongqing, China) and there is also a Korean model (MicroCam, Intromedic; Seoul, Korea)[68,69]. Both the capsule endoscopes are similar to the PillCam in terms of battery life, dimensions, field of view and picture intervals. The first trials of the MiRo capsule and OMOM capsule were published in 2008 but they were without FDA approval. The MiRo capsule uses a novel telemetry technology known as "electric-field propagation", which uses the human body as a conductive medium for data transmission. A pair of gold plates coated on the surface of the capsule acts as a transmitter. This is claimed to be superior in terms of battery life since the CE has few power-consuming components. Bang *et al*[68] used this new capsule in 45 healthy adults and it produced good image quality and capture rates. This capsule may also be used for the colon due to the long battery life. The first trial of the OMOM CE revealed comparable results to the PillCam. The authors express the cost advantage over other CEs, which could affect the choice of CE systems because of reimbursement problems[69]. PillCam SB2 and EndoCapsule have real time viewer capability that may shorten the examination once the cecum is seen. PillCam ESO was specially designed for investigation of esophageal disorders. It may be an accurate noninvasive method for detection of esophageal varices and portal hypertensive gastropathy, but it may not be suitable as a screening tool for Barrett's esophagus[12]. PillCam COLON is bigger than the standard PillCam SB capsule (11 mm × 31 mm). It was developed for detection of colonic neoplasia. It is a promising tool but further studies and improvements are needed before its regular use[70].

In summary, capsule endoscopy is a new diagnostic modality for the diagnosis and management of GI disorders. It is a simple and well-tolerated procedure. Capsule retention is the major complication. Care must be taken in patients with symptoms suggesting partial obstruction and CD. SB series and computerized tomography enteroclysis before CE may reveal stenosis. The newly developed patency capsule may be an alternative for detection of stenoses.

The value of CE in patients with OGIB appears to be high and is supported by high yields in the literature. CD and celiac disease appear to be areas where use of CE would be helpful. There may also be an indication for CE in CD surveillance and follow-up. The diagnostic role of CE extends beyond the SB. PillCam ESO and COLON showed promising outcomes in diagnosing esophageal and colonic diseases. More

research is needed to explore the feasibility of CE in these contexts.

Blind spots of CE such as the duodenum should be examined by a second look endoscopy before the CE procedure, especially in patients with OGIB. After negative endoscopic examinations, CE should be recommended as a first-line investigation over balloon assisted enteroscopies in view of its noninvasiveness, higher probability of visualizing the entire small intestine and the similar diagnostic yield of both investigations. Such an approach may decrease the time between diagnosis and intervention. A second look CE may reveal more findings in up to 35% of patients who had prior nondiagnostic CE.

CONCLUSION

The newly announced CEs would fire up the competition for new innovations and possible cost reductions, making possible the widespread use of this technology. Improvement in capsule design for better luminal visualization by coupling with a second backward camera, higher frame rates for viewing and longer battery life will definitely overcome the blind spots resulting in complete and detailed examination of the whole GI tract from the mouth to anus with just one capsule, as the capsule named M2A has denoted.

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ORIGINAL ARTICLES

Effects and mechanisms of silibinin on human hepatocellular carcinoma xenografts in nude mice

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Abstract

AIM: To investigate the *in vivo* effects and mechanisms of silibinin on the growth of hepatocellular carcinoma (HCC) xenografts in nude mice.

METHODS: Nude mice bearing HuH7 xenografts were used to assess the anti-HCC effects and mechanisms of silibinin.

RESULTS: Silibinin resulted in a potent dose-dependent reduction of HuH7 xenografts in association with a significant decrease in Ki-67 and α-fetoprotein production, nuclear NF-κB content, polo-like kinase 1, Rb phosphorylation, and E2F1/DP1 complex, but increased p27/CDK4 complex and checkpoint kinase 1 expression, suggesting that the *in vivo* effects of silibinin are mediated by inhibiting G1-S transition of the cell cycle. Silibinin-induced apoptosis of HuH7 xenografts was associated with inhibited survivin phosphorylation. Silibinin-reduced growth of HuH7 xenografts was associated with decreased p-ERK, increased PTEN expression and the activity of silibinin was correlated with decreased p-Akt production, indicating involvement of PTEN/PI₃K/Akt and ERK pathways in its *in vivo* anti-HCC effects. Silibinin-reduced growth of HuH7 xenografts was also associated with a significant increase in AC-H3 and AC-H4 expression and the production of superoxide dismutase (SOD)-1.

CONCLUSION: Silibinin reduces HCC xenograft growth through the inhibition of cell proliferation, cell cycle progression and PTEN/P-Akt and ERK signaling, inducing cell apoptosis, and increasing histone acetylation and SOD-1 expression.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents approximately 6% of all human cancers[1,2]. The global incidence of HCC has risen significantly in the past 2 decades[2], and prognosis of HCC is usually poor[3]. Limited treatment options and the poor prognosis of HCC emphasize the importance of developing an effective chemoprevention for this disease.

Milk thistle (*Silybum marianum*) is a popular dietary supplement that has been reported to be safe, well-tolerated, and protects the liver from drug or alcohol-related injury[4]. Silibinin, the major biologically active compound of milk thistle is a polyphenolic flavonoid, and a strong antioxidant and radical scavenger[5-7]. Studies have demonstrated the inhibitory effects of silibinin on multiple cancer cell lines, including prostate, lung, colon, skin, and bladder cancers[8-16]. Recently, we and Varghese *et al*[17] reported the *in vitro* anti-HCC effects of silibinin[18], however, additional studies are needed to further determine its *in vivo* inhibitory effects and mechanisms on the growth of human HCC. Clearly, nude mice bearing human hepatoma xenografts represent a suitable model for such a study[19,20].

Plasma α-fetoprotein (AFP) has been used as a

clinical marker in the diagnosis and monitoring of HCC[21-23]. We demonstrated that silibinin reduces AFP production and secretion from human hepatoma cells, but the AFP value in monitoring the *in vivo* anti-HCC effects of silibinin has not yet been tested.

Hepatocarcinogenesis is a complicated process that alters cell cycle progression and apoptosis. This may be mediated by altering signal transduction through cell cycle modulators, phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphatidylinositol 3'-kinase (PI3K) and Akt (PTEN/PI3K/Akt) pathways[24-30], and histone acetylation[31-33]. p-Rb, p21, and p27 are molecules that are involved in cell cycle regulation[17]. Nuclear factor (NF)-κB activation stimulates G1 to S phase progression and transcription of a wide variety of genes that are involved in cell proliferation[34,35]. Checkpoint kinase 1 (Chk1) and polo-like kinase 1 (Plk1) are the up-stream molecules. Chk1 controls cell cycle progression and inhibits mitosis[36]. Plk1 has long been recognized as a potential target for cancer therapy. Inhibition of Plk1 function may increase anti-tumor activity *in vivo*[37]. It is unclear whether these pathways are involved in silibinin-mediated anti-HCC effects.

Studies have also indicated that signals related to reactive oxygen species (ROS) may play important roles in the development of HCC[38]. The cellular levels of ROS are regulated by the antioxidant defense systems, that is, the enzymatic activities of superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase[39]. Altered expression of SOD has been associated with the development and differentiation of HCC[40,41]. Silymarin significantly increased suppressed SOD activity in patients with chronic alcoholic liver disease[42]. However, it is unclear whether silibinin-reduced growth of HCC cells is mediated by enhanced expression of SOD.

In the present study, we demonstrated that silibinin can effectively inhibit growth of HuH7 xenografts, a human HCC cell line, in nude mice and examined the related mechanisms.

MATERIALS AND METHODS

Reagents
The cell culture media were the same, as previously reported[19,20]. Anti-activated caspase-3 antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibodies against human Ki-67, AFP, p-Rb, E2F1, DP1, CD1, CDK4, p21 and p27, active caspase-9, phosphorylated-AktThr308, PTEN, AC-histone3 and AC-histone4, survivin phosphorylation (p-survivin), Plk1, Chk1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The NF-κB assay kit was from Panomics, Inc. (Redwood City, CA, USA).

Cell culture
HuH7 cells, a human HCC cell line, were cultured in DMEM with 10% FBS, as previously reported[17,19,20], and used to establish HCC xenografts in nude mice as described below.

Development and treatment of nude mice bearing HuH7 xenografts
After subcutaneous inoculation of $5 \times 10^6/0.25$ mL of HuH7 cells[19,20], the mice were randomized to 3 groups, 10 mice in each group, respectively. The control group received daily gavage of a vehicle solution. The other 2 groups received silibinin at a dose of 80 mg/kg per day and 160 mg/kg per day, respectively, started 24 h after inoculation. The silibinin dose was adjusted weekly based on changes in body weight. Tumor volumes were recorded weekly and the experiment lasted for 5 wk. At the end of the experiments, xenograft tumors were measured, isolated, and weighted after euthanasia. Blood specimens were collected from the tail vein and plasma was used to quantify AFP. Three HCC xenograft specimens which were closest to the mean volume were taken from each group. Three hundred milligram of tumor tissue from each xenograft was homogenized with lysing buffer. After centrifuging, the clarified supernatants were stored in -80℃ and used for the experiments described below.

Quantification of plasma and tissue AFP levels
The plasma AFP level was quantified using an enzyme immunoassay (EIA) kit as previously reported[20]. A standard curve was obtained using the manufacturer's internal control and was used to calculate plasma AFP levels.

Analysis of apoptosis
Apoptosis was quantified using an EIA kit, as previously reported[19,20]. The degree of apoptosis was expressed based on the ratios of absorbance of the treated *vs* control xenograft tissue specimens.

Immunoprecipitation and Western blotting analysis
The supernatants of xenograft lysates were used to detect Ki-67, p21, p27, E2F1, CDK4, p-Rb, activated caspase-3 and caspase-9, PTEN, AC-H3, AC-H4, p-Akt, p-survivin and p-ERK, Plk1, Chk1, and SOD1. To determine whether silibinin could affect binding of p21 and p27 with CDK4, and binding of DP1with E2F1, an immunoprecipitation technique was used. β-actin was used as an internal control. The relative amount of each protein was quantified by digitally scanning its hybridizing bands, and the optical density of the scanned Western blotting results, as previously reported[19,20].

PTEN activity assay
PTEN protein was immunoprecipitated with 10 μL of rabbit anti-human antibodies at 4℃ overnight, followed by the addition of 25 μL of anti-rabbit IgG-conjugated agarose beads at 4℃ for 2 h. The phosphatase reaction was performed using the PTEN activity assay kit in accordance with the manufacturer's instructions[19,20].

NF-κB assay
NF-κB was quantified using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Briefly, after incubation

Table 1 Silibinin reduced the frequency and volume of HuH7 xenografts in nude mice

Groups	Treatment	Tumor frequency (%)	Tumor volume (cm³)
Group 1	Placebo	100	4.0 ± 0.9
Group 2	80 mg/kg per day	50[a]	2.1 ± 0.3[a]
Group 3	160 mg/kg per day	30[a]	0.6 ± 0.2[a]

[a]$P < 0.05$ *vs* Group 1, $n = 10$/group.

for 1 h with 10 μL of the sample solution at room temperature the sample was washed 3 times, NF-κB p50 antibody (1:1000) was added and incubated for another hour at room temperature, followed by anti-rabbit HRP antibody (1:1000) and substrate reaction. The *A* absorbance at 450 nm was recorded.

Statistical analysis

The descriptive statistics are provided with mean ± SD. *t*-test was used to assess the effect (i.e. mean differences) of silibinin treatment on AFP production, apoptosis, as well as the scanning data of Western blots. $P < 0.05$ was considered statistically significant.

RESULTS

Silibinin reduced the frequency and volume of HuH7 xenograft growth

As shown in Table 1, silibinin treatment significantly reduced the frequency and volume of HuH7 xenografts in a dose-dependent fashion. The frequency of HuH7 xenografts was reduced by 50% in the group treated with silibinin 80 mg/kg per day and by 70% in the group treated with 160 mg/kg per day. The mean reduction in HuH7 xenograft volume was 48% in the group treated with silibinin 80 mg/kg per day and was 85% in the group treated with 160 mg/kg per day. The silibinin-reduced frequency and volume of HuH7 xenografts was associated with a significant decrease in Ki-67 expression (Figure 1A). These findings demonstrated that silibinin produced a significant *in vivo* inhibition of HCC growth through a reduction in HCC cell proliferation.

Silibinin-reduced growth of HCC xenografts was associated with decreased AFP production and secretion

Consistent with our previous *in vitro* report[17], we found that silibinin treatment significantly reduced AFP levels in both xenograft tissue and plasma obtained from the mice (Figure 2A and B). This indicated that silibinin-reduced HuH7 xenograft growth was associated with decreased production of AFP in xenograft tissue and secretion of AFP into blood circulation.

Effects of silibinin on cell cycle progression

Uncontrolled progression of the cell cycle promotes multiplication of cancer cells. We have reported the inhibitory effects of silibinin on p-Rb formation *in vitro*[17]. In the present study, we demonstrated that silibinin

Figure 1 Effects of silibinin on proliferation and cell cycle progression in HuH7 xenograft tissue specimens. A: Silibinin inhibited Ki-67 expression; B: Silibinin inhibited Rb phosphorylation; C: Silibinin inhibited E2F1/DP1 complex formation; D: Silibinin increased p27/CDK4 complex formation; E: Silibinin inhibited Plk1 expression; F: Silibinin increased Chk1 expression; G: β-actin as internal control; H: Silibinin inhibited nuclear NF-κB content. [a]$P < 0.05$; [b]$P < 0.01$ *vs* control.

resulted in a significant and dose-dependent inhibition of p-Rb production (Figure 1B), which was associated with decreased E2F1/DP1 complex formation in HuH7 xenograft tissue (Figure 1C).

By binding to the cyclin/CDK complexes, cyclin dependent kinase inhibitors, such as p21 and p27, halt uncontrolled cell proliferation. P21/CDK4 and p27/CDK4 complexes are involved in the transition from G1 into S phase. Consistent with our *in vitro* report[17], silibinin treatment significantly and dose-dependently increased p27/CDK4 complex (Figure 1D), but did not affect p21/CDK4 complex formation (data not shown) in HuH7 xenograft tissue. To further determine whether silibinin could also alter the levels of up-stream molecules that control cell cycle progression, the changes in Plk1, Chk1 and nuclear NF-κB were determined. As shown in Figure 1E-H, silibinin increased Chk1 expression, but inhibited Plk1 expression and nuclear NF-κB level.

Figure 2 Effects of silibinin on AFP production and secretion. A: Silibinin reduced AFP production; B: Silibinin reduced AFP secretion. [a]$P < 0.05$; [b]$P < 0.01$ vs control.

Figure 3 Effects of silibinin on apoptosis in HuH7 xenograft tissue. A: Silibinin induced apoptosis; B: Silibinin inhibited survivin phosphorylation. [a]$P < 0.05$ vs control.

Figure 4 Effects of silibinin on P-Akt and P-ERK pathways in HuH7 xenograft tissue. A: Silibinin inhibited p-Akt expression; B: Silibinin increased PTEN expression; C: Silibinin inhibited P-ERK expression; D: β-actin as internal control; E: Silibinin increased PTEN activity. [a]$P < 0.05$; [b]$P < 0.01$ vs control.

Silibinin-reduced HuH7 xenograft growth was associated with increased apoptosis and reduced survivin phosphorylation

Apoptosis is another important mechanism that controls cancer cell growth. We previously reported that silibinin promotes HuH7 cell apoptosis in vitro[17]. In the present study, we examined apoptosis in HuH7 xenograft tissue specimens. As showed in Figure 3A, we observed that silibinin significantly increased apoptosis in HuH7 xenograft tissue. To further define the mechanisms involved in the apoptosis pathway, activated caspase-3 and 9, Bcl-2, and p-survivin expression were assessed. We demonstrated that silibinin treatment significantly inhibited p-survivin (Figure 3B), as previously reported in the in vitro system[17]. However, inconsistent with our previous in vitro findings, silibinin did not affect production of activated caspase 3 and 9, or Bcl-2 (data not shown).

In vivo effects of silibinin on p-Akt and P-ERK pathways

P-Akt and p-ERK pathways are involved in modulating cancer development and growth[24-30]. Our previous study indicated that silibinin increased PTEN activity and reduced p-Akt expression in vitro[17]. In the present study, we found that significantly reduced p-Akt production was only seen in HuH7 xenograft tissue treated with silibinin at a dose of 160 mg/kg per day, but not 80 mg/kg per day (Figure 4A). However, silibinin-reduced HuH7 xenograft growth was associated with a silibinin dose-dependent increase in PTEN production (Figure 4B) and its activity (Figure 4E). In addition, silibinin-reduced HuH7 xenograft growth was also dose-dependently associated with a decrease in p-ERK (Figure 4C).

Figure 5 Effects of silibinin on AC-H3, AC-H4, and SOD1 in HuH7 xenograft tissue. A: Silibinin increased AC-H3 expression; B: Silibinin increased AC-H4 expression; C: Silibinin increased SOD1 expression in HuH7 xenograft tissue; D: β-actin as internal control. [a]*P* < 0.05 *vs* control.

In vivo effects of silibinin on histone acetylation

Histone acetylation plays an important role in controlling cell proliferation and cell cycle progression[31-33]. Our previous *in vitro* results indicated silibinin increases AC-H3 and AC-H4[17]. In the present study, we found that silibinin-reduced HuH7 xenograft growth was associated with significantly increased AC-H3 and AC-H4 production (Figure 5A and B). These results further confirmed the *in vivo* effects of silibinin on AC-H3 and AC-H4 production, indicating their potential role in HCC growth.

In vivo effects of silibinin on SOD1 expression

SOD1 is one of the most important enzymes in reducing ROS levels. It was reported that SOD1 may play a role in the effects of silymarin on alcoholic-induced liver injury[42]. We demonstrated that silibinin-reduced growth of HuH7 xenografts was associated with a significant and dose-dependent increase in SOD1 production in the xenograft tissue (Figure 5C). Our results indicate a possible mechanistic role of SOD1 in silibinin-reduced growth of HuH7 xenografts.

DISCUSSION

HCC is one of the most common malignancies globally. A rise in incidence, limited treatment options, and poor prognosis of this disease emphasize the importance of developing effective chemoprevention for this disease. Silibinin is the major biologically active compound of milk thistle which has been reported to be safe and well-tolerated, and protects the liver from drug or alcohol-related injury[5-7]. Recently, the potent *in vitro* anti-HCC effects of silibinin have been demonstrated[5,17], which have provided us with a rationale to further define the *in vivo* effects and mechanisms of silibinin on HCC growth.

In the present study, we examined the *in vivo* effects and mechanisms of silibinin on HCC growth using the nude mouse model bearing human HCC xenografts following inoculation of HuH7 cells[19,20]. We demonstrated that silibinin treatment resulted in a

significant dose dependent decrease in both frequency and mean volume of HuH7 xenograft growth. Our previous data recently reported the *in vivo* anti-HCC effects of silymarin[43]. The silibinin dose used in our study was lower than that of silymarin[43]. The fact that silibinin is a purified bioactive component from silymarin may explain why silibinin at the lower dose can achieve a potent anti-HCC effect. The anti-HCC effects of silibinin were associated with a significant reduction in Ki-67 expression, a biomarker of cell proliferation. These findings were consistent with our previous *in vitro* results and those of Varghese *et al*[17], and were further supported by the recent reported effects of silibinin on colorectal cancer[14,17]. Thus, our data suggest that silibinin-reduced *in vivo* growth of human HCC xenografts is associated with down regulation of cell proliferation.

Plasma AFP has been widely used as a noninvasive biomarker for HCC[21-23]. As we previously reported in the cell culture system, it was demonstrated that silibinin treatment resulted in a significant decrease in xenograft production and plasma levels of AFP which was correlated with growth inhibition of HCC xenografts. Since AFP overexpression has been associated with uncontrolled growth of HCC, our data provided additional *in vivo* evidence that silibinin-reduced growth of human HCC is associated with down regulation of cell proliferation. These findings also indicate the potential value of using plasma AFP as a non-invasive biomarker to determine the *in vivo* anti-HCC effects of silibinin.

Uncontrolled G1-S progression results in continued proliferation with potential malignant transformation and carcinogenesis. Increased E2F1/DP1 complex promotes cell cycle progression. Our results indicated that silibinin could significantly inhibit E2F1/DP1 complex formation in association with inhibition of HCC xenograft growth. Consistent with these findings, we also demonstrated that silibinin significantly decreased p-Rb expression, an important modulator that induces E2F1/DP1 formation.

P21 and p27 inhibit cell cycle progression by forming p21/CDK4 or p27/CDK4 complexes. Consistent with our previous *in vitro* report[17], we demonstrated that silibinin significantly increased p27/CDK4 complexes in HuH7 xenograft tissue. Similar effects of silibinin were previously reported in a skin carcinogenesis model. In contrast to the *in vitro* data[17], silibinin did not enhance p21/CDK4 complex formation in HuH7 xenograft tissue.

Chk1 is a critical enzyme in DNA damage-induced G2/M arrest, and blocks mitosis by phosphorylating Cdc25C and has been proposed as a novel tumor suppressor[36]. Both Plk1 and NF-κB promote cell cycle progression. NF-κB mediates activation of cyclin D1 gene transcription, induces cell cycle progression and inhibits cell apoptosis[34]. Inhibition of NF-κB activation induced an early G1 cell cycle arrest in primary rat hepatocytes[35]. In human cells, Plk1 has

been implicated in the regulation of different processes, including mitotic entry, spindle formation, and plays a role at multiple points during the restart of the cell cycle following DNA damage[37]. Our results demonstrated that silibinin at 80 mg/kg and 160 mg/kg significantly reduced Plk1 expression and the level of nuclear NF-κB. The higher dose of silibinin (160 mg/kg) also increased Chk1 expression. Taken together, our data indicate that silibinin reduced *in vivo* HCC xenograft growth by decreasing HCC cell proliferation and cell cycle progression which was mediated by inhibiting translocation of NF-κB to the nucleus, Plk1, p-Rb expression, E2F1/DP1, and increasing Chk1 expression and formation of the p27/CDK4 complex.

Increasing cell apoptosis is another important step that inhibits tumor growth[44]. We demonstrated that silibinin promotes *in vivo* apoptosis in HuH7 xenografts, which reconfirmed the previous *in vitro* findings[17,18]. Survivin is an apoptosis inhibitor that is overexpressed in most cancers in a cell cycle-dependent manner. P-survivin is necessary for cancer cell viability[45]. Our results demonstrated that silibinin inhibited p-survivin in association with increased apoptosis in HuH7 xenograft tissue. These results reconfirmed our previous *in vitro* findings[17] and indicated the important role of the survivin-mediated decrease in apoptosis in HCC growth.

We reported that silibinin-enhanced apoptosis of cultured HuH7 cells was associated with increased production of activated caspase 3 and 9, however, these changes were not reproducible in HuH7 xenograft tissue. Additionally, silibinin seemed not to alter Bcl-2 expression, another modulator of apoptosis, in HuH7 xenograft tissue. These data indicated that a discrepancy of silibinin-mediated apoptosis signaling may occur in these two systems.

Studies have indicated the important roles of PTEN/PI₃K/Akt and ERK signaling in carcinogenesis and cancer progression[24-30,46]. Phosphorylation of Akt results in its activation, which promotes cell cycle progression by phosphorylating several other key proteins[47-51]. PTEN is an up-stream molecule that inhibits p-Akt. We found that silibinin significantly increased PTEN expression and activity that was further associated with reduced p-Akt production in HCC xenograft tissue. These results indicate a possible pathogenic role of the PTEN/PI₃K/p-Akt pathway in HCC growth that may also serve as an important silibinin target. Increased p-ERK activates transcription of the mitogenic and cell regulatory genes and promotes oncogenesis[46]. P-ERK was reportedly increased in HCC[52], suggesting its involvement in HCC development. A previous *in vitro* study reported that silibinin can inhibit ERK phosphorylation in human osteosarcoma[53]. In the present study, we found silibinin-reduced HuH7 xenograft growth was also associated with a significant inhibition of p-ERK production. These results are also in agreement with an effect on colorectal cancer reported by Singh *et al*[14]. The results revealed that the p-ERK pathway is likely involved in silibinin-reduced HCC growth, another possible novel

target of HCC chemoprevention and therapy in future research.

Histone acetylation has been reported to be involved in cell proliferation, differentiation, and cell cycle regulation. A decrease in acetylation status in the cells is associated with carcinogenesis[31-33]. Our results demonstrated that silibinin significantly increased AC-H3 and AC-H4 expression, suggesting that increased histone acetylation may mediate silibinin-reduced HCC growth.

ROS stress has been associated with the development of HCC. SOD is one of the important enzymes in reducing ROS levels. Altered expression of SOD has been associated with the development and differentiation of HCC. Although the effects of silibinin on SOD were reported in patients with alcoholic liver disease[42], it is unknown whether the same mechanism has any role in the anti-HCC effects of silibinin. We demonstrated that silibinin-reduced growth of HuH7 xenografts was associated with a significant increase in the production of SOD1 in the xenograft tissue of nude mice. This was particularly evident when a higher dose of silibinin was used. Thus, our results indicate a possible mechanistic role of SOD1 in silibinin-reduced growth of HuH7 xenografts.

COMMENTS

Background
Hepatocellular carcinoma (HCC) is one of the most common malignancies. Silibinin has been reported to be safe, well-tolerated, and protects the liver from drug or alcohol-related injury. A recent demonstration of the potent *in vitro* anti-HCC effects of silibinin has provided us with a rationale to further assess the *in vivo* effects of silibinin on HCC growth. The present study examined the *in vivo* effects and mechanisms of silibinin on HCC growth using a nude mouse model bearing HuH7 (a human HCC cell line) xenografts.

Research frontiers
The search for safe and well-tolerated chemopreventive agents is one of the significant research frontiers in HCC chemoprevention. Many studies have demonstrated that silibinin can effectively inhibit the growth of various types of tumor cells, however, little is known about the *in vivo* effects and mechanisms of silibinin on HCC growth.

Innovations and breakthroughs
Previous study demonstrated that silibinin can inhibit HCC cell growth *in vitro*. In the present study, we confirmed that silibinin can effectively inhibit growth of human HCC xenografts in mice by affecting cell cycle progression, apoptosis, and several other pathways.

Applications
These results provide a rationale to further pre-clinical investigations which may result in clinical trials assessing the application of silibinin in HCC chemoprevention.

Terminology
Xenografts: Tissue or organs from an individual of one species inoculated, transplanted into or grafted onto an organism of another species, genus, or family. Chemoprevention: The use of chemical compounds to intervene in the early stage of carcinogenesis and thereby reverse tumor formation.

Peer review
This is a well-designed and very interesting study, methods are appropriated and results are consistent with the conclusions.

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ORIGINAL ARTICLES

Histological and biochemical alterations in early-stage lobar ischemia-reperfusion in rat liver

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Abstract

AIM: To investigate the structural and biochemical changes in the early stage of reperfusion in the rat livers exposed to lobar ischemia-reperfusion (IR).

METHODS: The median and left lobes of the liver were subjected to 60 min ischemia followed by 5, 10, 30, 45, 60 and 120 min reperfusion. Blood samples were taken at different time intervals to test enzyme activities and biochemical alterations induced by reperfusion. At the end of each reperfusion period, the animals were killed by euthanasia and tissue samples were taken for histological examination and immunohistochemistry.

RESULTS: Cell vacuolation, bleb formation and focal hepatitis were the most important changes occur during ischemia. While some changes including bleb formation were removed during reperfusion, other alterations including portal hepatitis, inflammation and the induction of apoptosis were seen during this stage. The occurrence of apoptosis, as demonstrated by apoptotic cells and bodies, was the most important histological change during reperfusion. The severity of apoptosis was dependent on the time of reperfusion, and by increasing the time of reperfusion, the numbers of apoptotic bodies was significantly enhanced. The amounts of lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, creatinine and urea were significantly increased in serum obtained from animals exposed to hepatic IR.

CONCLUSION: Inflammation and subsequent apoptotic cell death were the most important changes in early-stage hepatic reperfusion injury, and the number of apoptotic bodies increased with time of reperfusion.

INTRODUCTION

Reperfusion of a previously ischemic tissue is associated with additional injury that leads to structural and functional alterations in many organs including the liver. The hepatic injury that occurs during reperfusion has been shown to be the major problem associated with stroke, shock, cirrhosis, liver surgery and transplantation[1-4]. The mechanisms of reperfusion-induced pathological and functional alterations are under intensive investigation, but the results of different studies are controversial. Some studies suggest that the reintroduction of oxygen to the ischemic (hypoxic) tissues stimulates the production of reactive oxygen species (ROS), which contribute to cell damage. Others have argued that the liver tissues are basically resistant to the oxidative stress followed by ischemia-reperfusion (IR)[5-7]. However, many studies have shown that the ischemic livers undergo moderate[8,9] to severe structural and functional alterations by IR[10,11].

It has been shown that the injury induced during

reperfusion has a biphasic pattern that consists of an early stage that starts upon reoxygenation and a delayed phase. The early stage is associated with hepatocellular damage during 2-6 h after reperfusion (reoxygenation), and the delayed phase occurs 18-24 h after reperfusion and is accompanied with a massive neutrophil infiltration[12-15]. The injury in early stage (acute phase) is mediated by ROS, but the damage in the delayed stage (subacute phase) is associated with the inflammatory responses mediated by neutrophil activity. It is thought that ROS formation during reperfusion induces a cascade of cellular events that eventually leads to hepatocellular injury, including inflammation, necrosis, and/or apoptosis[16-19]. However, the detailed mechanisms of cell death and the structural alterations induced during different stages of reperfusion injury are not yet completely determined. Some studies have reported that the morphological changes induced by reperfusion are predominantly limited to non-parenchymal cells[20-23], whereas others have shown that some changes are seen in parenchymal cells[8,24].

While necrosis has been shown to be the cause of hepatic IR injury, many studies have shown that programmed cell death or apoptosis is the cause of cell death during liver reperfusion after long-term ischemia[20,25,26]. However, the role of apoptosis as the main cause of the injury and the level of morphological changes induced by this type of cell death have not been determined in detail.

The present study was designed to characterize the features of the injury induced in the early stage of reperfusion in rat liver. The ischemia was established by a lobar model and the ischemic liver was exposed to different reperfusion times. The hepatic alterations were assessed by both histological and biochemical observations.

MATERIALS AND METHODS

Animals and experimental groups

Female Sprague-Dawley rats weighing 230-280 g were used in all experiments. The animals were group-housed with a 12-h light-dark cycle and fed a standard laboratory diet. All experiments were performed according to the standard procedures outlined by our institutional guidelines. Rats were fasted overnight for at least 16 h prior to the experiments, but access to water was uninterrupted.

A group of four animals were subjected to 60 min lobar ischemia only. They were sacrificed at the end of this period and then liver tissue samples were taken for histology and immunohistochemistry (IHC). Seven groups of animals underwent 60 min ischemia followed by 5, 10, 15, 30, 54, 60 or 120 min reperfusion. A sham-operated group was selected for each test groups as a control.

In vivo (lobar) models of IR

This term refers to the model in which the ischemia is induced in the anesthetized animals through application of a vascular clamp simultaneously to branches of the hepatic portal vein, hepatic artery and bile duct. The reperfusion is commenced by removal of the clamp, thus restoring normal blood flow. Anesthesia was induced by a single intraperitoneal (i.p.) injection of ketamine (80 mg/kg) plus xylazine (10 mg/kg). After injection of 300 U of heparin via the femoral vein, the right jugular veins were catheterized by polyethylene tubing for blood sampling and infusion of normal saline solution to replace the removed blood. After laparotomy, the median and left lobes of the liver were removed from the abdominal cavity. Then, in vivo lobar ischemia was induced by clamping the left branches of the hepatic portal vein, hepatic artery and bile duct with a microvascular occlusion clip for a period of 60 min. This, caused occlusion of all blood vessels supplying the median and left lobes of the liver, which is reported to produce approximately 70% (partial) liver ischemia[27]. Upon release of the clamp, reperfusion was commenced and the blood flow was continued for different times as described above. Control (sham-operated) groups underwent the same surgical procedure, except that the blood supply to the liver lobes was not interrupted.

Biochemical assays

Blood samples were taken at different times before ischemia, during ischemia and after reperfusion. The plasma was separated by centrifuging the blood, which was kept in the freezer until analysis. The release of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes, as well as the level of glucose, urea and creatinine were measured by a Hitachi 747 analyzer (Boehringer Mannheim).

Histological examination

Small pieces of liver were taken from both left and median hepatic lobes. Parts of samples were fixed in 10% formalin for light microscopy. Paraffin embedded sections of 5-μm thickness were stained with hematoxylin and eosin and/or periodic acid-Schiff. The remaining samples were fixed with 3% glutaraldehyde/ 4% paraformaldehyde in 0.1 mmol/L sodium cacodylate buffer for electron microscopy. They were transferred into sodium cacodylate buffer on the following day and then stored at 4℃ until processing.

IHC

IHC was carried out to detect the presence of apoptosis protease-activating factor 1 (APAF-1) as a marker of apoptosis induction in tissue samples. Serial embedded sections were prepared from formalin-fixed samples, which were cut at a thickness of 3 μm and dried at 37℃ overnight. IHC was performed by the avidin-biotin complex (ABC) procedure, including heat-induced epitope-retrieval and enzymatic antigen-retrieval procedures. Incubation with the primary antibody (NCL-APAF-1; Novocastra; 1:100 dilutions) was carried out in a moist chamber at 37℃ for 1 h. Negative controls were treated identically, with the primary antibody omitted; positive controls consisted of normal hepatic tissue.

Table 1 Data analysis of different biochemical tests obtained from livers subjected to 60 min ischemia followed by 30-120 min reperfusion

Time of sampling	Glucose (μg/dL)	Urea (μg/dL)	Creatinine (μg/dL)	AST (IU/L)	ALT (IU/L)	LDH (IU/L)
Before ischemia	261.33 ± 36.16	17.66 ± 2.88	0.55 ± 0.058	54.82 ± 19.82	78.2 ± 24.3	153.75 ± 0.11
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 16$
During ischemia	189.66 ± 18.8	18.33 ± 1.2	0.57 ± 0.075	12.74 ± 9.51	18.19 ± 4.39	39.69 ± 8.58
	$n = 5$	$n = 5$	$n = 7$	$n = 5$	$n = 5$	$n = 16$
After reperfusion	160.83 ± 10.63	26.50 ± 1.66	2.62 ± 1.29	61.58 ± 9.12	142.28 ± 30.94	200.52 ± 60.52
	$n = 14$	$n = 14$	$n = 13$	$n = 10$	$n = 7$	$n = 23$

The values are expressed as the mean ± SE taken from at least 12 samples in test.

Table 2 Comparison of data obtained from biochemical analysis of blood samples taken from the animals exposed to 60 min ischemia followed by different times of reperfusion

| Sampling phase | *P*-value | | | | | |
	Glucose	Urea	Creatinine	AST	ALT	LDH
Before ischemia/ during ischemia	0.38	0.72	0.79	0.043	0.049	0.034
Before ischemia/ after reperfusion	0.31	0.05[a]	0.006[a]	0.08	0.043[a]	0.008[a]
During ischemia/ after reperfusion	0.17	0.05[a]	0.05[a]	0.08	0.005[a]	0.014[a]

[a]$P < 0.05$.

Statistical analysis

Data from biochemical assays are expressed as the mean ± SD obtained from at least four experiments in each group. They were analyzed by ANOVA using the SPSS program and the significance of the differences between groups were tested by Tukey's post-hoc test, with $P < 0.05$ considered statistically significant. Pathological changes in both untreated and treated groups were scored semi-quantitatively from + (mild) to ++++ (severe).

RESULTS

Biochemical changes induced by reperfusion injury

There were significant changes in enzyme release and blood urea and creatinine level in the animals exposed to hepatic IR, compared to the controls. The data obtained from analysis of biochemical assays are summarized in Table 1, and comparison of the results at different times of reperfusion is shown in Table 2. As seen in the tables, while enzyme release was significantly reduced during ischemia ($P < 0.05$), the level of glucose, creatinine and urea did not change in the blood of animals exposed to lobar hepatic ischemia. However, plasma level of LDH, AST, creatinine and urea was significantly increased during reperfusion (Table 2).

Morphological findings

The histological changes induced by IR were examined, based on portal inflammation, focal inflammation, sinusoidal congestion, cytoplasmic vacuolation, bleb formation, apoptotic cells and apoptotic bodies production. The liver samples from the sham-operated group did not show significant histological alterations,

Figure 1 Zone 3 cytoplasmic vacuolation and hepatocyte bleb formation in the liver subjected to 60 min ischemia only. A: The white arrow shows cytoplasmic vacuolation and the black arrows show the cytoplasmic blebs formation with light microscopy; B: The arrows shows the cytoplasmic blebs with electron microscopy.

similar to those of the non-operated control group. Changes observed in the liver exposed to lobar ischemia alone were limited to mild to moderate focal hepatitis, sinusoidal congestion, vacuolation and bleb formation (Figure 1).

During reperfusion, whilst some changes including blebbing of hepatocytes were improved, induction of portal hepatitis and mild apoptosis were added at 5 min of reperfusion. By increasing the time of reperfusion, the induction of portal and local hepatitis were reduced, but the amount of apoptosis was moderately increased, so that at 30 min reperfusion, the presence of apoptotic cells but not apoptotic bodies was the most important change in the majority of tissue samples (Figure 2A). Sixty minutes ischemia followed by 45 or 60 min reperfusion caused an increased amount of apoptosis

Figure 2 Histological changes in the livers exposed to 60 min lobar ischemia followed by different times of reperfusion. A: Nuclear pyknosis (P), nuclear fragmentation (F) and cytoplasmic vacuolation in the liver exposed to 60 min ischemia followed by 30 min reperfusion; B: Apoptotic bodies (A.B) and nuclear fragmentation (F) in the liver exposed to 60 min ischemia followed by 60 min reperfusion; C: Nuclear fragmentation (F), apoptotic cell (A.C) and apoptotic bodies (A.B) in the liver exposed to 60 min ischemia followed by120 min reperfusion.

Figure 3 Apoptotic bodies of an endothelial origin apoptotic cell phagocyted by a hepatocyte in the liver exposed to 60 min ischemia followed by 60 min reperfusion.

IHC, in which the expression of APAF-1 was positive in the stained sections of livers exposed to IR, and the presence of apoptosis and/or apoptotic bodies was seen by light and electron microscopy (Figure 5A). Representative staining patterns for APAF-1 expression showed that the occurrence of apoptosis was limited to the pericentral area (Figure 5B). The presence of cells labeled with brown color indicated that the expression of APAF-1 was increased during 5-15 min of reperfusion. This showed that the occurrence of apoptosis occurred upon the initiation of reperfusion. However, during 30-60 min of reperfusion, the presence of cells with a brown-colored cytoplasm was accompanied with apoptotic bodies in which, when reperfusion time was increased (120 min), the number of apoptotic bodies also increased (Figure 5B).

DISCUSSION

In the present study, an *in vivo* lobar (partial) model of rat liver IR injury was established by clamping the vessels to the left lateral and median hepatic lobes, which account for 70% of the rat liver mass. This hepatic insult is similar to the clinical situation when the liver is rendered ischemic during total vascular exclusion for liver resection[28]. We tried to illustrate the pathological profile of the liver exposed to 60 min ischemia followed by different periods of 5, 10, 30, 45, 60 and 120 min reperfusion. It was found that cell vacuolation, bleb formation and focal hepatitis were the most important changes induced by *in vivo* lobar ischemia in rat liver. However, during reperfusion, not only some changes including bleb formation was reduced, but some other alterations including portal hepatitis, inflammation and the induction of apoptosis, occurred. It appears that the occurrence of apoptosis, as demonstrated by the formation of apoptotic cells and bodies, is the most important histological change during the early stage of reperfusion. The severity of apoptosis was dependent on the time of reperfusion, so that by increasing the time of reperfusion, the number of apoptotic bodies was significantly enhanced. To accompany these changes,

with phagocytic apoptotic bodies and sinusoidal congestion (Figure 2B). However, in the livers that underwent ischemia and 60 or 120 min reperfusion, phagocytic apoptotic bodies were seen in most tissue samples (Figures 2C and 3). To organize different groups exposed to different reperfusion times, they were classified into three durations: short time, 5 and 10 min; middle time, 30, 45 and 60 min; and long time (120 min). Statistical analysis of the histological alterations and the severity of hepatic changes in the liver subjected to 60 min ischemia followed by short, middle and long times of reperfusion in comparison with the changes induced by 60 min ischemia only are summarized in Figure 4.

The occurrence of apoptosis was confirmed by

Figure 4 A summary of statistical analysis of histological alterations in the livers exposed to ischemia or ischemia-reperfusion. A: Hepatic changes in the liver exposed to 60 min lobar ischemia only; B: Changes in the liver subjected to 60 min ischemia followed by short, middle and long times of reperfusion in which: 0= Normal or no changes, +: Mild injury, ++: Moderate injury, +++: Severe injury; 0 < A.C/A.B ≤ 2 = +, 2 < A.C/A.B ≤ 5 = ++, 5 < A.C, A.B = +++.

Figure 5 Confirmation of apoptosis by IHC assay in the staining sections of livers exposed to ischemia-reperfusion and the presence of apoptosis cells and/or apoptotic bodies. A: Representative staining patterns for *APAF-1* positive, illustrating the occurrence of apoptosis in the sections of the liver exposed to 60 min ischemia followed by long time of reperfusion; B: *APAF-1* positive staining that shows the high level of apoptosis incidence in the pericentral area of the liver exposed to 60 min ischemia followed by long time of reperfusion.

the serum level of LDH, ALT, creatinine and urea was significantly increased in rats exposed to hepatic IR, which indicates the induction of cell injury in the liver and other organs, including the kidneys.

Hepatic IR injury is a common pathological phenomenon that may be induced after severe liver trauma, extensive hepatic lobe excision, liver transplantation, and shock. By initiation of reperfusion injury, a series of functional, humoral and structural alterations occur in the liver tissues that directly influence the prognosis of patients[3-5,9]. The mechanisms by which the reperfusion injury induces pathological and functional alterations and the methods of intervention have been under intensive investigation. However, the detailed pathological mechanisms of liver injury induced by this phenomenon are complex and not yet fully understood. Different *in vivo* and *in vitro* models have been used to establish the pathological process of hepatic IR injury, such as the liver transplantation model, the partial warm or cold IR model, and the total hepatic IR model[6,28-30].

There is evidence that the pathogenesis of reperfusion injury involves a series of events, including Kupffer cell activation, cytokine release, neutrophil activation, increased expression of adhesion molecules, sinusoidal endothelial cell death, and hepatocyte injury[15,19,31-33]. Among these, the inflammatory process and activation of Kupffer cells, which result in the release of excessive quantities of cytokines and ROS formation, play a major role[4,17]. The inflammatory aspect of the injury includes cellular and humoral components. A growing body of evidence, primarily from animal models of IR and preliminary human studies, has revealed that the inflammatory mechanisms may play a major role in the pathogenesis of the injury induced by reperfusion. It has been shown that hepatocyte injury followed by reperfusion is partly dependent on Kupffer cell activation and production of inflammatory mediators. This occurs in a biphasic pattern that consists of acute-phase (ROS-mediated) and subacute-phase (neutrophil-mediated) damage[8,19,20,24].

Our findings strongly suggest the occurrence of inflammation and the subsequent cell death by apoptosis as an important morphological change observed in the early stage (acute phase) of reperfusion. It is proposed that in the early stage of hepatic reperfusion injury, these inflammatory reactions and the different stress processes that follow may result in the activation of the apoptotic pathway mediated by mitochondria. This may lead to an increased number of apoptotic cells and apoptotic body formation, which is associated with a reduction in the total number of parenchymal cells, thus damaging the hepatic tissues and resulting in liver dysfunction[20,27].

The role of apoptosis as a cell death mechanism in reperfusion injury has been shown by some studies previously[20,34,35]. Although other alterations including hepatocyte blebbing, sinusoidal congestion, and portal and focal hepatitis were shown have been seen in liver histology, the integrity and organizational arrangement of the hepatic acinus remains intact. This suggests that the liver parenchyma is able to resist against these types of insults[9]. This has been confirmed with further studies that have demonstrated that parenchymal cells are not susceptible to damage under some conditions of ischemia-reperfusionIR[21-23].

In the present study, the occurrence of apoptosis was confirmed by IHC of the liver for APAF-1 expression. Positive APAF-1 staining in most sections of liver exposed to IR confirmed the role of apoptosis as the main cause of cell death in early-stage hepatic reperfusion injury. APAF-1 has been identified as a key protein that plays an essential role in the induction of apoptosis in different mammalian cells. In response to apoptotic stimuli, such as ROS, Ca^{2+} and cytokines released by reperfusion, APAF-1 binds to cytochrome c and procaspase 9, to yield a complex entitled the apoptosome. Activation of procaspase 9 through an autocatalytic process initiates a cascade of downstream effector caspases, which finally leads to mitochondrial apoptosis. The mitochondrial/cytochrome c apoptotic pathway and the expression of APAF-1 have attracted close attention of researchers to determine the induction of apoptosis[36-39]. Using IHC, we found that the occurrence of apoptosis was started in the initial phase of reperfusion, and it was completed as the reperfusion time increased. This was shown by the abundance of apoptotic bodies phagocytosed by macrophages or neighboring hepatocytes during long periods of reperfusion. Increased expression of APAF-1 in zone 3 of the liver indicated the greater susceptibility of this area of the liver to reperfusion injury.

In conclusion, we showed that inflammation and apoptosis were the major histological alterations induced by early-stage reperfusion injury in the liver exposed to lobar ischemia. It appears that apoptosis is the most important histological change induced in the early stage of hepatic reperfusion injury, during which the number of apoptotic bodies was increased with the time of reperfusion.

ACKNOWLEDGMENTS

COMMENTS

Background

Reperfusion of a previously ischemic tissue is associated with additional injury that leads to structural and functional alterations in many organs, including the liver. The mechanisms of reperfusion-induced pathological and functional alterations are under intensive investigation, but the results of different studies are controversial.

Research frontiers

The injury induced during reperfusion evolves in a biphasic pattern that consists of an early stage that starts with reoxygenation and a delayed phase. The early stage is associated with hepatocellular damage at 2-6 h after reperfusion, and the delayed phase occurs at 18-24 h after reperfusion, and is accompanied by massive neutrophil infiltration. The injury in the early stage is mediated by reactive oxygen species (ROS), but the damage in the delayed stage is associated with the inflammatory responses mediated by neutrophil activity. It is thought that ROS formation during reperfusion induces a cascade of cellular events that eventually leads to hepatocellular injury. However, the detailed mechanisms of cell death and the structural alterations induced during different stages of reperfusion injury are not yet completely understood.

Innovations and breakthroughs

In the present study, the authors demonstrated that the occurrence of inflammation and the subsequent cell death by apoptosis were the most important changes in the early stage of hepatic reperfusion injury.

Applications

By characterizing the feature of the injury induced in the early stage of reperfusion, this study may represent a future strategy for therapeutic intervention of reperfusion injury induced under different conditions, such as stroke, shock, cirrhosis, liver surgery and transplantation.

Terminology

Lobar ischemia refers to the model in which the ischemia is induced in the anesthetized animals through application of a vascular clamp simultaneously to branches of the hepatic portal vein, hepatic artery and bile duct.

Peer review

The authors examined the effects of 60 min lobar ischemia followed by different periods of 5, 10, 30, 45, 60 and 120 min reperfusion. It was found that the occurrence of apoptosis, as demonstrated by apoptotic cells and bodies, was the most important histological change during reperfusion. The severity of apoptosis was dependent on the time of reperfusion, such that by increasing the time of reperfusion, the number of apoptotic bodies was significantly enhanced.

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ORIGINAL ARTICLES

Rocket "*Eruca sativa*": A salad herb with potential gastric anti-ulcer activity

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Abstract

AIM: To validate gastric anti-ulcer properties of Rocket "*Eruca sativa*" on experimentally-induced gastric secretion and ulceration in albino rats.

METHODS: Gastric acid secretion studies were undertaken using pylorus-ligated rats. Gastric lesions in the rats were induced by noxious chemicals including ethanol, strong alkalis, indomethacin and hypothermic restraint stress. The levels of gastric wall mucus (GWM), nonprotein sulfhydryls (NP-SH) and malondialdehyde (MDA) were also measured in the glandular stomach of rats following ethanol administration. The gastric tissue was also examined histologically. The extract was used in two doses (250 and 500 mg/kg body weight) in all experiments.

RESULTS: In pylorus-ligated Shay rats, the ethanolic extract of Rocket "*Eruca sativa* L." (EER) significantly and dose-dependently reduced the basal gastric acid secretion, titratable acidity and ruminal ulceration. Rocket extract significantly attenuated gastric ulceration induced by necrotizing agents (80% ethanol, 0.2 mol/L NaOH, 25% NaCl), indomethacin and hypothermic restraint stress. The anti-ulcer effect was further confirmed histologically. On the other hand, the extract significantly replenished GWM and NP-SH levels, as well as the MDA level significantly reduced by extract pretreatment.

CONCLUSION: Rocket extract possesses anti-secretory, cytoprotective, and anti-ulcer activities against experimentally-induced gastric lesions. The anti-ulcer effect is possibly through prostaglandin-mediated activity and/or through its anti-secretory and antioxidant properties.

INTRODUCTION

Gastric ulcer is an illness that affects a considerable number of people worldwide. The etiological factors of this disorder include: stress, smoking, nutritional deficiencies, infections, frequent and indiscriminate use of nonsteroidal anti-inflammatory drugs (NSAIDs)[1]. The pathogenesis of gastroduodenal ulcers are influenced by various aggressive and defensive factors, such as mucus secretion, mucosal barrier, acid-pepsin secretion, blood flow, cellular regeneration and endogenous protective agents (prostaglandins and epidermal growth factor)[2]. Although the introduction of proton-pump inhibitors to the classic anti-ulcer therapy had revolutionized treatment of peptic ulcers and other gastrointestinal disorders, but there is still no complete cure for this disease. It has been shown that long term use of these drugs leads to various adverse

and side effects. Relapses of the malady, ineffectiveness of different drug regimens and even resistance to drugs are emerging[3]. Thus, there is an urgent requirement to identify more effective and safe anti-ulcer agents. During the past few decades, a widespread search has been launched to identify new anti-ulcer therapies from natural sources. Herbs, medicinal plants, spices, vegetables and crude drug substances are considered to be a potential source to combat various diseases including gastric ulcer. In the scientific literature, a large number of medicinal plants with gastric anti-ulcer potential have been reported[4-8]. In recent years, Rocket "*Eruca sativa* L." (EER), a member of the Brassicacae family, has gained greater importance as a salad vegetable and spice, especially among Middle Eastern populations and Europeans[9]. It is believed that plants belonging to the Brassicacae family possess diversified medicinal and therapeutic properties including inhibition of tumorigenesis[10], anti-ulcer[11], and hepatoprotective[12] activities. Rocket, locally known as Jarjeer, is used in salads, by local herbal practitioners and in Unani medicine, and is used as a diuretic, stimulant, and in the treatment of stomach disorders and scurvy[13]. The seeds and tender leaves are known in Arabian countries to increase sexual desire and are considered to be an aphrodisiac. It is also used as a carminative and to alleviate abdominal discomfort and improve digestion. It has been reported that the rocket seed ethanolic extract possesses potent antioxidant and renal protective and diuretic activities[14-16]. Phytochemical studies of rocket leaves and seeds have revealed the presence of glucosinolates[17,18]. Weckerle *et al*[19] isolated and identified three new quercetins from *Eruca sativa* leaves. In view of the acclaimed medicinal value of rocket in Unani, Ayurvedic and Arab traditional medicine as well as its diversified therapeutic uses, we have undertaken the present study to evaluate the anti-ulcerogenic property of EER in different ulcer models in rats.

MATERIALS AND METHODS

Plant material and preparation of extract

Fresh *Eruca sativa* leaves were purchased from a local vegetable market in Riyadh, and the identity of these leaves was confirmed by an expert taxonomist of the Department of Pharmacognosy, where a voucher specimen (No. 8208) of the plant has been kept in the Herbarium of the College of Pharmacy, KSU, Riyadh. Shade-dried, coarsely pulverized rocket leaves were placed in a glass percolator with ethanol and were allowed to stand at room temperature for about 72 h. The percolate was collected and dried under reduced pressure in vacuo. The extract obtained was later used and dissolved in distilled water for evaluation of anti-ulcer activity.

Animals and dosing

Albino Wistar rats of either sex, approximately the same age, weighing 150 to 200 g and fed on a diet of standard chow were used in this study. They were randomly

divided into experimental groups of 6 rats each. Aqueous solutions of ulcerogens and EER were freshly prepared before administration. EER at doses of 250 and 500 mg/kg were given orally in the anti-ulcer studies and intraperitoneally for gastric secretion evaluation. The rats were sacrificed, and the stomachs removed and opened along the greater curvature. After washing with saline, the gastric lesions were quantified by a person unaware of the treatments. The animal study protocol was approved by the Research and Ethics Committee of the Experimental Animal Care Society, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Pylorus-ligated rats

Rats were fasted for 36 h with access to water *ad libitum* before pylorus ligation under ether anesthesia was carried out. Care was taken not to cause bleeding or to occlude blood vessels[20]. EER was administered intraperitoneally immediately after pylorus ligation (Shay). The rats were sacrificed at 6 h after pylorus ligation. The stomachs were removed, the contents were collected, volumes measured, centrifuged and analyzed for titratable acidity against 0.01 mol/L NaOH at pH 7.

Gastric lesions induced by necrotizing agents (cytoprotection)

Each rat was administered 1 mL of a necrotizing agent (80% ethanol, 0.2 mol/L NaOH or 25% NaCl). Rocket extract was given 30 min before the administration of necrotizing agents. One hour after the administration of ethanol and the alkalis, the rats were sacrificed and examined for stomach lesions. The scoring of stomach lesions was as follows: Patchy lesions of the stomach induced by ethanol were scored according to the method described by Robert *et al*[21] using the following scale: 0 = normal mucosa; 1 = hyperemic mucosa or up to 3 small patches; 2 = from 4 to 10 small patches; 3 = more than 10 small or up to 3 medium-sized patches; 4 = from 4 to 6 medium-sized patches; 5 = more than 6 medium-sized or up to 3 large patches; 6 = from 4 to 6 large patches; 7 = from 7 to 10 large patches; 8 = more than 10 large patches or extensive necrotic zones. "small" was defined as up to 2 mm across (max. diameter), "medium-sized" between 2 and 4 mm across and "large" more than 4 mm across.

Gastric lesions induced by indomethacin

Indomethacin was suspended in 1.0% carboxy-methylcellulose (CMC) in water (6 mg/mL) and administered orally to the 36 h fasted rats at a dose of 30 mg/kg body weight. Control rats were treated similarly with an equivalent amount of vehicle[22]. The rocket extract was given 30 min prior to indomethacin administration at a dose of 250 and 500 mg/kg. The animals were sacrificed 6 h after treatment. The stomachs were excised, rinsed with normal saline and examined for ulceration.

Hypothermic restraint stress-induced ulcers

The method described by Senay *et al*[23] was adopted

with slight modifications. Animals were fasted for 36 h but had access to water *ad libitum*. Thirty minutes after the oral administration of EER (250 and 500 mg/kg), the rats were immobilized in restraint cages and placed inside a ventilated refrigerator maintained at 3 ± 1℃ for 3 h. The animals were then sacrificed and the stomachs were excised. They were examined for ulceration and the severity of intraluminal bleeding according to the following arbitrary scale described by Chiu *et al*[24]. 0 = no blood detectable; 1 = thin blood follows the rugae; 2 = thick blood follows the rugae; 3 = thick blood follows the rugae with blood clots in certain areas and 4 = extensive covering of the whole gastric mucosal surface with thick blood.

Determination of gastric wall mucus (GWM)

Gastric wall mucus was determined according to the modified procedure of Crone *et al*[25]. The glandular segment of the stomach was separated from the rumen of the stomach, weighed, and transferred immediately to 10 mL of 0.1% w/v Alcian blue solution (in 0.16 mmol/L sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for 2 h in Alcian blue, and excess dye was removed by two successive rinses with 10 mL of 0.25 mmol/L sucrose, firstly after 15 min and then after 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mmol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract were then vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 r/min for 10 min and the absorbance of the aqueous layer was recorded at 580 nm. The quantity of Alcian blue extracted per gram of wet glandular tissue was then calculated.

Estimation of non-protein sulfhydryls (NP-SH)

Gastric mucosal non-protein sulfhydryls were measured according to the method of Sedlak and Lindsay[26]. The glandular part of the stomach was homogenized in ice-cold 0.02 mmol/L ethylenediaminetetraacetic acid (EDTA). Aliquots of 5 mL of the homogenates were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10 min and centrifuged at 3000 r/min. Two milliliters of supernatant were mixed with 4 mL of 0.4 mol/L Tris buffer at pH 8.9. 0.1 mL of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was measured within 5 min of DTNB addition at 412 nm against a reagent blank.

Determination of malondialdehyde (MDA)

The method reported by Utley *et al*[27] was followed. The animals were killed 1 h after ethanol administration. The stomachs were removed and each was homogenized in 0.15 mol/L KCl (at 4℃) in a Potter-Elvehjem type C homogenizer to give a 10% w/v homogenate. Aliquots

of homogenate 1 mL in volume were incubated at 37℃ for 3 h in a metabolic shaker. Then 1 mL of 10% aqueous TCA was added and mixed. The mixture was then centrifuged at 800 *g* for 10 min. One milliliter of the supernatant was removed and mixed with 1 mL of 0.67% w-thiobarbituric acid in water and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 mL distilled water. The absorbance of the solution was then read at 535 nm. The content of malondialdehyde (nmol/g wet tissue) (index of the magnitude of lipid peroxidation) was then calculated, by reference to a standard curve of malondialdehyde solution.

Histopathological evaluation

Gastric tissue samples were fixed in neutral buffered formalin for 24 h. Sections of gastric tissue were histopathologically examined to study the ulcerogenic and/or anti-ulcerogenic activity of EER. The tissues were fixed in 10% buffered formalin and processed using a VIP tissue processor. The processed tissues were embedded in paraffin blocks and sections about 5 μm thick were cut using an American optical rotary microtome. These sections were stained with haematoxylin and eosin using routine procedures[28]. The slides were examined microscopically for pathomorphological changes such as congestion, hemorrhage, edema, and erosions using an arbitrary scale for severity assessment of these changes.

Statistical analysis

Values in tables and figures are given as mean ± SE. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Student's *t*-test.

RESULTS

Effect of EER on gastric secretions in 6 h pylorus-ligated rats

When the rats were subjected to pylorus ligation for 6 h, a considerable amount of basal gastric acid secretion was noted (10.83 ± 1.16 mL) in the control group. In the same control group, the titratable acidity was found to be 196.57 ± 15.50 mEq/L and the ulcer index was recorded as 2.33 ± 1.5. EER at both doses (250 and 500 mg/kg) significantly reduced gastric acid secretion, titratable acidity and ulcer formation (6.33 ± 1.63 mL, 4.16 ± 1.16 mL $P < 0.05$, $P < 0.01$; 132.77 ± 17.43, 55.55 ± 10.46 mEq/L $P < 0.05$, $P < 0.001$, 0.66 ± 0.51, 0.50 ± 0.54, $P < 0.05$, $P < 0.05$), respectively (Table 1).

Effect of EER on necrotizing agents-induced gastric lesions

The treatment of rats with 80% ethanol, 0.2 mol/L NaOH and 25% NaCl produced extensive gastric lesions in the glandular mucosa of the stomach in all the control rats. The ulcer index was 6.00 ± 0.89, 6.66 ± 1.36 and 6.66 ± 0.51, respectively in control rats 1 h after administration of the necrotizing agents. Pretreatment of rats with EER at doses of 250 mg/kg (ulcer index in 80% ethanol, 0.2 mol/L

Table 1 Effects of EER on gastric secretion, acidity and gastric lesion index in pylorus-ligated shay rats (mean ± SE)

Group serial	Treatment	Dose (mg/kg, i.g.)	Volume of gastric content (mL)	Titratable acidity (mEq/L)	Ulcer index
1	Control (distilled water)	-	10.83 ± 1.16	196.57 ± 15.50	2.33 ± 1.50
2	EER	250	6.33 ± 1.63[a]	132.77 ± 17.43[a]	0.66 ± 0.51[a]
3	EER	500	4.16 ± 1.16[b]	55.55 ± 10.46[d]	0.50 ± 0.54[a]

Six rats were used in each group. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control (distilled water) group, Student's *t*-test.

Table 2 Effect of EER on gastric lesions induced by necrotizing agents (mean ± SE)

Group serial	Treatment	Dose (mg/kg, i.g.)	Ulcer index		
			80% EtOH	0.2 mol/L NaOH	25% NaCl
1	Control (distilled water)	-	6.00 ± 0.89	6.66 ± 1.36	6.66 ± 0.51
2	EER	250	2.00 ± 0.89[b]	2.66 ± 1.21[a]	2.83 ± 0.98[b]
3	EER	500	1.66 ± 1.03[b]	1.50 ± 0.54[b]	2.16 ± 0.75[d]

Six rats were used in each group. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control (distilled water) group, Student's *t*-test.

Table 3 Effect of EER on indomethacin-induced gastric mucosal lesions (mean ± SE)

Group serial	Treatment	Animals (*n*)	Dose (mg/kg, i.g.)	Ulcer Index
1	Control (indomethacin only)	6	-	44.50 ± 5.82
2	EER	6	250	25.50 ± 6.88
3	EER	6	500	13.50 ± 4.84[b]

Six rats were used in each group. [b]$P < 0.01$ *vs* control (indomethacin only) group, Student's *t*-test.

Table 4 Effect of EER on hypothermic restraint stress-induced intraluminal bleeding and gastric lesions in rats (mean ± SE)

Group serial	Treatment	Dose (mg/kg, i.g.)	Intraluminal bleeding score	Gastric lesion ulcer index
1	Control (distilled water)	-	2.60 ± 0.50	24.00 ± 3.24
2	EER	250	1.20 ± 0.83	15.20 ± 3.96
3	EER	500	0.83 ± 0.40[a]	7.66 ± 6.12[a]

Six rats were used in each group. [a]$P < 0.05$ *vs* control (distilled water) group, Student's *t*-test.

NaOH and 25% NaCl = 2.00 ± 0.89, $P < 0.01$, 2.66 ± 1.21, $P < 0.05$ and 2.83 ± 0.98, $P < 0.001$); 500 mg/kg (ulcer index = 1.66 ± 1.03, $P < 0.01$, 1.50 ± 0.54, $P < 0.01$ and 2.16 ± 0.75, $P < 0.001$), respectively, significantly inhibited the formation of gastric lesions as shown in Table 2.

Effect of EER on gastric lesions induced by indomethacin

The oral administration of indomethacin induced marked damage in the rat glandular stomach. EER at the 500 mg/kg dose significantly prevented the development of gastric lesions in the rat stomach ($P < 0.01$), however, no significant preventive effect of EER at the 250 mg/kg dose, in indomethacin-treated rats was observed (Table 3).

Effect of EER on hypothermic restraint stress-induced gastric mucosal lesions

Table 4 shows that EER at a dose of 500 mg/kg body weight significantly inhibited intraluminal bleeding and ulcer formation induced by hypothermic restraint stress ($P < 0.05$). Although the intraluminal bleeding and ulcer index was reduced at a dose of 250 mg/kg body weight, this reduction was not found to be statistically significant.

Effect of EER on ethanol-induced changes in gastric wall mucus (GWM)

Rats treated with ethanol showed a significant decrease in the Alcian blue binding capacity of gastric wall mucus (148.41 ± 18.81 μg/g of tissue, $P < 0.001$) as compared to control (normal) rats (426.73 ± 39.15 μg/g). Pretreatment of rats with EER at doses of 250 mg/kg (263.87 ± 32.65 μg/g, $P < 0.05$) and 500 mg/kg (313.90 ± 24.30 μg/g, $P < 0.001$) significantly enhanced the Alcian blue binding capacity of gastric mucosa (Figure 1).

Effect of EER on ethanol-induced depletion of gastric mucosal NP-SH

The level of NP-SH in the gastric mucosa of control rats was 5.26 ± 0.63 mmol/g of tissue, which was significantly decreased to 2.38 ± 0.33 mmol/g ($P < 0.001$) following the administration of 80% ethanol. Pretreatment of rats with EER at both doses (250 and 500 mg/kg) significantly replenished the ethanol-induced depletion of NP-SH (3.63 ± 0.22, $P < 0.001$; 4.53 ± 0.56, $P < 0.01$), respectively (Figure 2).

Effect of EER on ethanol-induced increase in MDA

As depicted in Figure 3, MDA levels in the gastric mucosa used as an index of lipid peroxidation were significantly higher in the ethanol only treated group than in the untreated control group (7.09 ± 0.60 μmol/g of tissue; 2.77 ± 0.19 μmol/g of tissue), respectively. EER at both doses (250 and 500 mg/kg) significantly

Figure 1 Effect of EER on the changes in gastric wall mucus induced by 80% ethanol.

Figure 2 Effect of EER on NP-SH concentration in gastric ulcer induced by 80% ethanol.

Figure 3 Effect of EER on MDA concentration in gastric ulcer induced by 80% ethanol.

Figure 4 Light micrographs showing the effect of EER on ethanol-induced gastric lesions of rats. A: Normal mucosa; B: Ethanol-induced gastric mucosal congestion and necrosis; C: Pretreatment of rats with EER 250 mg/kg; D: Pretreatment of rats with EER 500 mg/kg.

decreased the MDA content (4.55 ± 0.66 μmol/g and 3.58 ± 0.49 μmol/g), respectively.

Effect of EER on histopathological evaluation

Histopathological studies (Figure 4) further confirmed that pretreatment with rocket extract prevented ethanol-induced necrosis in the superficial layers of the gastric mucosa with congestion.

DISCUSSION

The results of this study show that the ethanolic extract

of Rocket possesses significant anti-secretory, anti-ulcer and cytoprotective properties in rats. Pretreatment with EER produced a dose-dependent decrease in the volume of basal gastric secretion, titratable acidity and lesions in pylorus-ligated Shay rats. It has been reported that anti-secretory agents such as histamine H_2-receptor antagonists ameliorate the decrease in gastric mucosal blood flow caused by factors that disturb the gastric mucosa such as NSAIDs and ethanol[29]. Gastric acid is an important factor in the genesis of ulceration in pylorus-ligated rats[20]. The activation of the vagus-vagal reflux by stimulation of pressure receptors in the antral gastric mucosa in the hypersecretion model of pylorus ligature is believed to increase gastric acid secretion[30]. Since EER markedly inhibited gastric acid secretion and ruminal ulcers in pylorus-ligated rats, this observed effect could be related, at least in part, to the ability of EER to reduce gastric acid secretion. It is now accepted that gastric acid secretion plays an important role in the progression from an erosive mucus layer to a gastric lesion. On the other hand, substances which have the ability to suppress gastric acid secretion, such as proton pump inhibitors and histamine H_2-receptor antagonists are believed to accelerate the healing process of the gastric lesions or inhibit the formation of mucosal injury[31]. Rocket extract was found to offer the gastric mucosa, a statistically significant and dose-dependent protection against ulceration caused by various necrotizing agents including ethanol and strong alkalis. Ethanol-induced gastric ulcers have been widely used in the evaluation of gastroprotective activity. Ethanol is metabolized in the body and releases superoxide anion and hydroperoxy free radicals. It has been found that oxygen-derived free radicals are implicated in the mechanism of acute and chronic ulceration in the stomach[32]. The genesis of ethanol-induced gastric lesions is of multifactorial origin with a decrease in gastric mucus, and is associated with the significant production of free radicals leading to increased lipid peroxidation which in turn causes damage to cells and cell membranes[33]. The cytoprotective effect of EER may be related to its ability to prevent gastric acid secretion and/or enhance the mucosal defensive factors such as prostaglandins and decrease lipid peroxidation[34]. Treatment of rats with indomethacin, a non-selective cyclooxygenase inhibitor is known to induce gastric damage through multiple mechanisms which include suppression of prostaglandin generation, overproduction of leukotrienes, acting as a topical irritant and by reducing the local blood-flow[35]. Rats pretreated with EER produced significant protection in this model. It is possible that an enhanced level of gastric mucus, generating prostaglandins and inhibiting leukotriene may contribute to the gastroprotective effect of rocket extract.

Hypothermic-restraint stress ulcers have been used as an experimental model in the evaluation of anti-ulcer activity in rats due to data reproducibility[36]. Disturbances of gastric mucosal microcirculation, enhancement of acid secretion and reduction in mucus production are mediated by histamine release and abnormal gastric motility[37]. It is also reported that

free radicals may play a major role in stress-induced gastric injury[38]. Stress is reported to inactivate mucosal prostaglandin syntheses by accumulating hydrogen peroxide, a prostaglandin biosynthesis inhibitor, which also causes reactive oxygen species (ROS) generation[39]. In addition, a positive correlation has been reported between the level of gastric mucosal lipid peroxidation products, a marker of oxidative stress, and stomach damage in cold restraint-stressed rats[40]. The protective efficacy against cold restraint-stress may be due to the antioxidant activities of EER, as an antioxidant activity was reported earlier in *Eruca sativa*[14], this together with its antisecretagogue potential, thereby strengthens the animals physiological capabilities to decrease stress ulcers.

Our results revealed that the rocket ethanol extract significantly protected gastric mucosa against the depletion of gastric wall mucus. The mucus gel adhering to the gastric mucosal surface protects the underlying epithelium against acid[41,42], pepsin[43] and necrotizing agents such as ethanol and indomethacin[11]. Gastric wall mucus however, plays a more important role in the defense of the gastric mucosa against chemical or mechanical aggression than the soluble mucus in the lumen of the stomach[44]. The gastric mucus coat is thought to be important in facilitating the repair of the damaged gastric epithelium[45]. It seems likely that the cytoprotective activity of EER could result, at least in part, from interaction with the adhering gastric mucus layer.

Sulfhydryl compounds in living organisms plays a central role in the maintenance of gastric integrity, particularly when ROS are involved in the pathogenesis of tissue damage[46]. A significant decrease in gastric NP-SH following ethanol administration indicated massive generation of oxygen derived free radicals (ODFR). Our findings are in agreement with earlier reports showing depletion of sulfhydryls in ethanol-induced gastric lesions[3,47]. Treatment of rats with glutathione depletors has been shown to significantly potentiate ulcerogen-induced gastric mucosal injury[48], whereas an increase in mucosal NP-SH exerts a gastroprotective effect[49]. Our observations clearly point towards the mediation of sulfhydryls in EER gastric mucosal protection.

Furthermore, the extract also showed significant inhibition of lipid peroxidation. The generation of MDA from lipids that react with thiobarbituric acid was found to be inhibited by the EER. Thus, it appears that the antioxidant property of the rocket extract may possibly counteract oxidative damage caused by alcohol toxicity. The observed anti-ulcerogenic activity may be due to its antioxidant effects and appears to strengthen the mucosal barrier, which is the first line of defense against endogenous and exogenous ulcerogenic agents.

The preliminary phytochemical screening of rocket revealed the presence of flavonoids, sterols and/or triterpenes. Moreover, quercetin and its derivatives were also reported in rocket leaves. Previous studies have shown that flavonoids may be related to the anti-ulcer activity[50], and play a major role in the mechanism of

gastroprotection[51,52]. In addition to flavonoids, other constituents in rocket such as sterol and/or triterpenes are known for their antioxidant activities, which may contribute to some of the anti-ulcer mechanisms[53].

In conclusion, the data obtained confirm the traditional indications for this salad herb and present a new therapeutic option for the treatment of gastric ailments. The exact mechanism(s) underlying this anti-ulcerogenic effect remain unknown, but the extract contains substances, which might increase endogenous prostaglandins and mucus synthesis through its potent antioxidant activity. Furthermore, the anti-secretory mechanism can not, however, be dismissed.

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COMMENTS

Background

Gastric ulcer is an illness that affects a considerable number of people worldwide. The etiological factors of this disorder include: stress, smoking, nutritional deficiencies, infections, frequent and indiscriminate use of nonsteroidal anti-inflammatory drugs (NSAIDs). Herbs, medicinal plants, spices, vegetables and crude drug substances are considered to be a potential source to combat various diseases including gastric ulcer. In the scientific literature, a large number of medicinal plants with gastric anti-ulcer potential have been reported.

Research frontiers

Although the introduction of proton-pump inhibitors to the classic anti-ulcer therapy has revolutionized treatment of peptic ulcers and other gastrointestinal disorders, there is still no complete cure for this disease. It has been shown that long term use of these drugs leads to various adverse and side effects. Relapses of the malady, ineffectiveness of different drug regimens and even resistance to drugs are emerging. Thus, there is an urgent requirement to identify more effective and safe anti-ulcer agents. During the past few decades, a widespread search has been launched to identify new anti-ulcer therapies from natural sources.

Terminology

The anti-secretory, cytoprotective and antioxidant properties of the rocket extract caused an inhibition of chemically and stress-induced gastric ulceration in rats.

Peer review

The authors examined an ethanolic extract of rocket "Eruca sativa L." for its claimed beneficial effects on gastrointestinal disorders. The results are interesting and may provide a better understanding and give a clue for further investigations and innovations for an effective and safe phytotherapy for peptic ulcer disease.

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ORIGINAL ARTICLES

In vitro and *in vivo* suppression of hepatocellular carcinoma growth by midkine-antisense oligonucleotide-loaded nanoparticles

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Abstract

AIM: To synthesize antisense oligonucleotides (ASODNs) of midkine (MK), package the ASODNs with nanoparticles, and to inhibit hepatocellular carcinoma (HCC) growth using these nanoparticles.

METHODS: HepG2 cell proliferation was analyzed *in vitro* using the 3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium, inner salt assay. The *in vivo* activity of nanoparticles delivering the MK-ASODNs was analyzed by histopathological and immunohistochemical staining and quantitative real time polymerase chain reaction (PCR).

RESULTS: The *in vitro* proliferation of HepG2 cells was significantly inhibited by the nanoparticles packaged with MK-ASODNs (NANO-ASODNs). Furthermore, the NANO-ASODNs significantly inhibited the growth of HCC in the mouse model.

CONCLUSION: NANO-ASODNs can significantly suppress the growth of HCC *in vitro* and *in vivo*.

Dai LC, Yao X, Wang X, Niu SQ, Zhou LF, Fu FF, Yang SX, Ping JL. *In vitro* and *in vivo* suppression of hepatocellular carcinoma growth by midkine-antisense oligonucleotide-loaded nanoparticles. *World J Gastroenterol* 2009; 15(16): 1966-1972 Available from: URL: http://www.wjgnet.com/1007-9327/15/1966.asp DOI: http://dx.doi.org/10.3748/wjg.15.1966

INTRODUCTION

Hepatocellular carcinoma (HCC), a primary malignancy of the liver, is one of the most common tumors worldwide. The mortality rate from HCC is the third highest worldwide for any cancer-related diseases, and since the 1990s, HCC has been the cause of the second highest mortality rate due to cancer in China[1]. Globally, the 5-year survival rate of HCC is less than 5% and 598000 HCC patients die each year[2], mainly because no satisfactory treatment is available and chemotherapy has been extremely ineffective. Recent insights into the biology of HCC suggest that certain pathways and molecular alterations are likely to play essential roles in HCC development by promoting cell growth and survival. Growth factors and the downstream signaling factors are often overexpressed in tumors and will become the targets of diagnosis or treatment.

Midkine (MK), a heparin-binding growth factor or cytokine, has been reported to be generally overexpressed in malignant tumors[3-5], whereas in normal adult tissues, MK levels are low or undetectable[6-10]. MK exhibits several cancer-related activities, including fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic and chemotactic functions. The antisense oligonucleotide (ASODN) that targets MK has been reported to suppress the growth of tumors in nude mice[11,12]. Additionally,

siRNA or an ASODN that targets MK inhibits neointima formation[13] and renal injury after ischemia[14]. MK has been suggested to play an important role in carcinogenesis, thus MK can serve as a novel tumor marker or therapeutic target.

At present, the delivery tools of siRNAs or ASODNs are ineffective and toxic. Although lentiviral technology is a proven tool in the laboratory setting, it has several adverse effects, such as toxic immune responses and genetic alterations. Therefore, in the present study, we have used the systemic delivery of nanoparticles incorporated with MK-ASODNs (NANO-ASODNs). This delivery approach is a much safer and more effective alternative to viral therapy for the treatment of tumors, such as HCC. NANO-ASODNs have been found to play an important role in the suppression of HCC growth *in vitro* and *in vivo*, and provid insights into their future clinical application to tumor therapy.

MATERIALS AND METHODS

ASODN synthesis

Antisense phosphorothioate oligonucleotide MK-ASODN (5'-CCCCGGGCCGCCCTTCTTCA-3') targeting 108-127 base positions of MK mRNA was synthesized by an Applied Biosystems Model 391 DNA synthesizer (Amersham, Piscataway, NJ, USA) and purified by HPLC (Waters Delta Prep 4000, Milford, MA, USA) using a SOURCE 15Q column (Amersham). In our previous study[15], this antisense sequence has been identified to be the most effective sequence for the down-regulation of MK. Consequently, this sequence was synthesized and applied in this study.

Nanoparticles liposome packageing

Acyl-chloride-cholesterol 2.25 g was dissolved in 5 mL anhydrous chloroform and transferred to a 25-mL three-necked flask. Two milliliters of N', N'-dimethyl-ethanediamine was dissolved into another 5 mL of anhydrous chloroform. The solution was added to the acyl-chloride-cholesterol solution at a constant temperature of 0℃. After dropwise addition, the chloroform was removed by reduction vaporization and the product was purified three times by recrystallization with dehydrated alcohol, and finally eluted with dehydrated alcohol. Following recrystallization, the product was dehydrated by vacuum dehydration for 12 h and the white DC-Chol was obtained. This target product was confirmed by thin-layer chromatography and ^{1}H-NMR exosyndrome analysis. Ten milligrams of DC-Chol and 8 mg of dioleophosphatidyl-ethanolamine were dissolved into chloroform and transferred into a pear shape. The shape was filled with nitrogen gas on a rotary evaporation instrument. Organic solvent was removed by reduction vaporization at a constant temperature of 40℃, until an even lipid film formed on the shape wall. Fifteen milliliter chloroform and 6 mL PBS (pH 7.4) were added into the shape to produce a water-in-oil emulsion adjuvant, using water-bath ultrasound. Chloroform was removed by reduction vaporization on a rotary evaporation instrument to produce a proteoliposome suspension. The suspension

was filled with nitrogen gas, made up to a volume of 10 mL with PBS, shattered using a transducer-ultrasound (1 s ultrasound with 2 s breaks for 150 times, with a work rate of 200 W) and filtered using a 0.1-μm polycarbonate membrane five times using a miniextruder. Nanometer liposomes were finally produced. One milliliter of the stock solution (5% manicol) was stored at -70℃ for more than 3 h before vacuum dehydration. The shape and size of the nanometer liposomes were detected by transmission electron microscopy (TEM) and dynamic light scattering.

Cell culture and transduction assay

Human liver HCC cell line HepG2 (HepG2 cells were purchased from the Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ and 5% CO₂. Cells (3×10^3) were seeded in each well of a 96-well microtiter plate and allowed to attach overnight. ASODNs and NANO-ASODNs with different concentrations were added to the cells at different time points. Furthermore, the transduction rate of NANO-ASODNs was analyzed using a confocal microscope (Leica, Heidelberg, Germany).

Cell proliferation assay

NANO-ASODNs with concentrations of 0.1, 0.2, 0.4 and 0.6 μmol/L were added into the HepG2 cell cultures. ASODNs (0.6 μmol/L) transfected into the cells with Lipofectin (Invitrogen), following the manufacturer's instructions, acted as a positive control. Free nanoparticles were added as a negative control. The effects of ASODNs on cellular viability were measured by an MTS [3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. After 48 h of incubation following transfection, 20 μL MTS (Sigma, St Louis, MO, USA) was added to each well and incubated at 37℃ for 2 h. The absorbance values were determined at 490 nm using a MR600 microplate reader (Wallac 1420 Multilable counter; Wallac, Turku, Finland).

In vivo tumor studies

Athymic nude mice (BALB/c-nu/nu females, 6-8 wk old) were purchased from the Academy of Military Medical Science (Beijing, China) and housed in a controlled environment at 22℃ on a 12 h light/12 h dark cycle. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The *in situ* HCC models were established as described previously[16]. Three days after the *in situ* HCC models were established, mice were randomly divided into six groups and each experimental group consisted of eight mice. The mice were intravenously injected with PBS (negative control group), 5-fluoro-2, 4 (^{1}H, ^{3}H) pyrimidinedione (5-FU, positive control group) at 10 mg/kg per day and free nanoparticles as a second positive control group. 25, 50 and 100 mg/kg per day of NANO-ASODNs or

free ASODNs were injected into the mice for 20 d. The distribution of NANO-ASODNs was detected with the *in vivo* imaging systems (ICE-FM-1024B, LumazoneFM). Briefly, mice were anesthetized and FAM-NANO-ASODNs or Free ASODNs were intravenously injected through the tail vein. Images were obtained using the YAP-(S) PET scanner (ISE) at 0, 30, 60 and 90 min after injection. The data were acquired in list mode from 256 views over an angle range of 360 degrees. Images were reconstructed using an iterative reconstruction algorithm that provided transaxial, coronal and sagittal slices. At the end of the second *in vivo* imaging study, animals were sacrificed and tumors were removed for radioactivity counting. The body weights of the animals were recorded weekly. Two days after the intravenous injections were completed, mice were sacrificed and the tumors were removed and weighed. Tumor sizes were monitored with calipers; the tumor volume (V, mm^3) was calculated as: V = length × width × depth/2. The percentage of tumor growth inhibition was calculated as: Inhibitory rate = ($W_{control}$-W_{treat})/$W_{control}$ × 100%. The blood of the mice was taken for routine blood tests and the α fetoprotein (AFP) test. The tissues of livers and tumors were taken for hematoxylin and eosin (HE) staining and histological examination.

Animal HCC model

The virgin female BALB/c mice used in this experiment were obtained from the Academy of Military Medical Science (Beijing, China). All animal experiments were carried out according to the standards of animal care as outlined in the NIH guide for the Care and Use of Laboratory Animals. The human HCC tumor model was described previously[17]. Briefly, the HCM-Y89 tumor derived from a surgical specimen of HCC was cut into 1 mm × 1 mm × 1 mm pieces, and implanted into the liver of mice. Twenty days later, the mice treated with or without drugs were killed. The tumors were removed and fixed in neutral buffered 10% formalin, processed by standard methods, embedded in paraffin and sectioned and stained with HE. The HCC model maintains various important features similar to clinical liver cancer patients, including local growth, regional invasion, lymph nodes and pulmonary metastasis, peritoneal seeding with bloody ascites, and secretion of AFP in the recipient animals[17].

Histopathological and immunohistochemical analysis

The liver and tumor specimens were fixed and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC, USA). Five-micrometer sections were cut and stained with HE for histopathological analysis. The immunohistochemical demonstration of anti-MK protein binding was achieved with a rabbit polyclonal anti-MK antibody and an LSAB 2 kit, with visualization of the binding using 3,3'-diaminobenzidine tetrahydrochloride. Staining intensities were classified according to the proportions of positive cells as: negative, none; slightly positive, 50%; positive, 50%-90%; and strongly positive, > 90%. The specificity of the binding was confirmed by negative control staining using a rabbit non-immune serum rather than the primary antibody.

RNA isolation and real-time polymerase chain reaction (PCR)

Total cellular RNA from cell cultures and tissues isolated from the livers and the tumors of mice were extracted using the RNeasy kit according to the manufacturer's protocol. cDNA of the tissue was synthesized from 5 μg of total RNA using a reverse transcription kit. Subsequently, the first strand of cDNA was used as a PCR template. Aliquots of 1 μL of 10-fold diluted cDNA solutions were subjected to PCR in a 20-μL reaction mixture (2 μL PCR buffer; 2 μL dNTP mix; 0.1 μL Taq DNA polymerase; 0.2 μL primers; 14.7 μL autoclaved, distilled water). The primers were as follows: MK, sense primer: 5'-CTCCGCGGTCGCCAAAAAGAAAGA-3'; anti-sense primer: 5'-CCCCCATCACACGCACCCCAGTT-3'. GAPDH, sense primer: 5'-GGAGCCAAAAGGGTCATCATCT-3'; anti-sense primer: 5'-AGGGGCCATCCACAGTCTTCT-3'. PCR was conducted using a SYBR Premix ExTaqTM kit (TaKaRa, Dalian, China) with the following conditions: pre-heating at 95℃ for 2 min, 40 cycles of 30 s denaturation at 94℃, a 20 s annealing at 56℃, and a 40-s extension at 72℃.

Statistical analysis

All parameters were analyzed by analysis of variance. Analysis of variance and Student's *t* test were used to compare each post operative result of each level among all groups. A minimum level of 0.05 was chosen.

RESULTS

Generation of nanoparticles

The produced nanometer liposomes were used to package the MK-ASODNs using a ratio of 1.8 μL of the nanometer liposomes to 1 μg MK-ASODNs (Figure 1A). The nanoparticles packaged with MK-ASODNs were stained with 1% uranyl acetate and examined with an electron microscope. The size of the micelles was determined with a Zetasizer 5000 (Malvern Instruments, Malvern, Worcestershire, UK). The morphology of the nanoparticles packaged with MK-ASODNs was examined using dynamic light scattering and TEM (Figure 1B).

Inhibition of growth of HCC *in vitro*

In order to determine the transduction of NANO-ASODNs, the ASODNs were conjugated with FAM and the rate of transduction under the confocal microscope at different time points was observed. Figure 2A shows that the NANO-ASODNs were effectively transduced into the HepG2 cells at the indicated times (6, 12 and 18 h). Since the NANO-ASODNs readily enter into the cells, we wanted to know whether they could decrease expression of MK in HepG2 cells. Figure 2B shows that the NANO-ASODNs (0.1, 0.2, 0.4 and 0.8 μg/mL) could significantly down-regulate the MK mRNA levels (Figure 2B). The MTS assay was used to analyze the effect of NANO-ASODNs on HCC cell proliferation. Figure 2C shows that the inhibition rates ranged from 20% to 80%, which correlated with the ASODNs concentrations.

Figure 1 Nano-assembly of MK-ASODNs and nanoliposomes and characterization of NANO-ASODNs. A: Schematic illustration of the self-assembly of MK-ASODN and nanoliposomes; B: TEM image of the empty nanoliposomes stained with 1% uranyl acetate; C, D: TEM image of the NANO-ASODNs.

Table 1 NANO-ASODNs inhibit growth of HCC *in vivo*

Group (mg/kg)	Tumor volume (mm³)	Tumor weight (g)	Tumor inhibition (%)	AFP (ng/mL)
Control (PBS)	1633.38 ± 525.93	1.92 ± 0.45	-	457.63 ± 141.47
ASODN-100	321.56 ± 85.55[b]	0.57 ± 0.16[b]	70.31	97.63 ± 23.79[b]
ASODN-50	509.29 ± 300.85[b]	0.73 ± 0.22[b]	61.98	179.86 ± 210.27[b]
ASODN-25	835.25 ± 263.33[a]	1.14 ± 0.12[b]	40.63	428.63 ± 141.47
5-FU10	717.19 ± 281.25[b]	0.98 ± 0.16[b]	48.96	315.25 ± 195.77
Nano-ASODN-100	225.81 ± 128.75[b]	0.35 ± 0.17[b]	81.77	58.25 ± 30.83[b]
Nano-ASODN-50	457.88 ± 249.29[b]	0.52 ± 0.21[b]	72.92	89.38 ± 61.75[b]
Nano-ASODN-25	584.00 ± 261.92[b]	0.83 ± 0.20[b]	56.77	205.38 ± 125.16[b]
Nano control	1319.25 ± 340.70	1.59 ± 0.18	17.18	419.25 ± 148.46

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control (PBS).

Inhibition of growth of HCC *in vivo*

NANO-ASODNs mainly target the liver. In the present study, we used an *in situ* mouse HCC model to evaluate the antitumor activity of NANO-ASODNs. Figure 3 shows that NANO-ASODNs mainly targeted the liver after injection through the tail vein. We also found that the concentration of the NANO-ASODNs reached a peak 90 min after the injection, and then slowly decreased.

Effects of NANO-ASODNs treatment on *in situ* HCC xenograft growth: After establishing the mouse HCC model for 2 d, PBS, free nanoparticles, 10 mg/kg per day 5-FU, various doses of NANO-ASODNs or ASODNs (25, 50 and 100 mg/kg per day) were administered through the tail vein for 20 d. The tumors were removed after sacrificing the mice. The tumors were measured and weighed. Table 1 and Figure 4 show the final tumor volumes and weights after 20 d of treatment. The results showed that the tumor volumes decreased in both free ASODNs and NANO-ASODNs treated groups compared with the PBS control group ($P < 0.01$).

Additionally, the effect of NANO-ASODNs on tumor growth inhibition was superior to the free ASODNs ($P < 0.05$). Moreover, the effect of NANO-ASODNs on inhibiting tumor proliferation was dose-dependent (Table 1). In addition, the NANO-ASODNs treatment also resulted in a significant inhibition of tumor weight compared with the PBS- and free-nanoparticle-treated mice. In contrast to the PBS group, it had the highest inhibitory efficacy for the tumor weight which was 81.77% and 10 mg/kg 5-FU had an inhibitory efficacy of 48.96%; however, for the free nanoparticles treatment, the inhibitory rate was only 17.18% ($P < 0.01$) (Table 1).

Histopathological analysis: The morphology of the tumors treated by NANO-ASODNs, ASODNs, 5-FU and PBS were evaluated by HE staining. The tumors were excised at the endpoint of the treatment of each protocol. Figure 5 shows the representative sections of the tumors from each experimental group. The tumors from the mice treated with NANO-ASODNs, ASODNs and 5-FU showed a marked increase in the necrotic

Figure 2 Transduction and function of NANO-ASODNs *in vitro*. A: 0.2 μmol/L FAM-conjugated NANO-ASODNs transduced into HepG2 cells. The results were observed under a confocal microscope at indicted times of 6, 12 and 18 h; B: NANO-ASODNs down-regulated expression of MK mRNA; C: The proliferation of HepG2 cells was significantly inhibited by NANO-ASODNs.

Figure 3 NANO-ASODNs target the liver. The kinetic results of the NANO-ASODNs were observed through *in vivo* imaging systems at indicated times after NANO-ASODNs were injected through the tail vein. A: Free ASODNs did not concentrate within the liver and these ASODNs disappeared quickly; B: NANO-ASODNs were found to mainly target the liver (the arrow represents the NANO-ASODNs).

Figure 4 Morphological changes of HCC following treatment with NANO-ASODNs. The volume of HCC decreased significantly following treatment with 100, 50 and 25 mg/kg per day of NANO-ASODNs for 20 d. MK-ASODNs were the positive control. The PBS or free nanoparticles represent the negative controls.

area compared with the PBS-treated animals. This result suggests that the NANO-ASODNs, ASODNs and 5-FU treatment induced HCC necrosis *in vivo*.

Inhibition of plasma AFP secretion: AFP is often expressed in high levels in fetal liver, the gastrointestinal tract and the yolk sack, but AFP is transcriptionally down-regulated after birth and frequently re-expressed in HCC. Therefore, it is often used as an indicator of HCC[18]. In this experiment, we used radioimmunoassay to detect serum AFP concentrations at the endpoint of the treatment. Table 1 shows that NANO-ASODNs at the dose of 100, 50 and 25 mg/kg per day significantly

decreased AFP secretion compared with the ASODNs or control groups. This result suggests that there were fewer liver tumor cells in the NANO-ASODNs-treated mice and this treatment reduced circulating AFP.

Systemic toxicity of NANO-ASODNs: Drug treatment of cancer is usually associated with terrible side effects, which result in severe reduction of white blood cell counts or weight loss. In order to evaluate the toxicity of the NANO-ASODNs, we compared the systemic toxicity

Figure 5 Histopathological analysis. A: Tissue sections of the tumors from *in situ* xenograft HCC; B: Tissue sections of the tumors from nanoparticles; C: Tissue sections of the tumors from 5-FU (10 mg/kg per day); D: Tissue sections of the tumors treated with ASODNs 50 mg/kg per day; E-G: Tissue sections of tumors treated with NANO-ASODNs 100, 50 and 25 mg/kg per day of NANO-ASODN treated tumors, respectively; H: Tissue sections of the tumors treated with 5-FU (10 mg/kg per day) and 50 mg/kg per day of NANO-ASODNs. Regions showing an increase of necrosis and fibrosis were observed in the 5-FU or ASODN treatment groups (C-H, × 200) compared with the free nanoparticles and untreated groups (A and B, × 200).

of free ASODNs, 5-FU and NANO-ASODNs. Mice without tumors were administered a dose of 10 mg/kg per day ASODNs, 5-FU and NANO-ASODNs, and PBS as the control. The body weight was subsequently monitored. The results showed that the mean body weight decrease of the NANO-ASODNs group was not significantly different compared with the ASODNs or PBS groups ($P > 0.05$). However, the decrease in the weight of ASODN- and 5-FU-treated mice was more significant (data not shown). In addition, no inflammatory infiltrate was observed surrounding the solid tumor after treatment with different concentrations of the NANO-ASODNs (data not shown).

All the above studies suggest that nanoparticles packaged with ASODNs were safer than free ASODNs or chemical drugs.

DISCUSSION

MK is a heparin-binding growth factor identified as a product of a retinoic acid response gene[19,20]. MK is overexpressed in a wide range of human carcinomas and believed to contribute to tumorigenesis and tumor progression. HCC is the most common primary liver malignancy, with a rising incidence worldwide. At present, although surgery and chemotherapy are effective in patients with localized tumors, the prognosis of patients with advanced or metastatic tumors is not ideal. Therefore, novel treatment approaches for the cancer are urgently needed. Recently, HCC tumor cells were found to overexpress MK. In our previous studies, ASODNs that target MK were demonstrated to play an important role in anti-tumor functions[15,16]. However, the anti-tumor effect was not satisfactory and found to be toxic because of the absence of smart and safer delivery tools. At present, the strategies that have been adopted to improve the uptake of various nucleic-acid-based therapeutic agents are microinjection, passive diffusion, endocytosis (i.e. receptor

mediated endocytosis, fluid phase pinocytosis, adsorptive endocytosis) and artificially enhanced uptake (i.e. using delivery vectors like liposomes, micro- or nanoparticles or dendrimers)[21,22].

In the present study, we used more effective and less toxic nanoparticle liposomes that have previously been used effectively to deliver siRNA for the treatment of lymphoma and ovarian cancer, as well as colorectal carcinoma. Liposomes, the first nanotechnology to benefit cancer patients, are continuing to evolve as tools for delivering potentially useful therapies for the treatment against tumors. In this study, MK-ASODNs incorporated into nanoliposomes have been used effectively *in vivo*. Evidence from these experiments showed that nanoliposomes packaged with MK-ASODNs could suppress HCC growth both *in vitro* and *in vivo*. In addition, the data also indicated that nanoliposomes could effectively deliver MK-ASODNs and showed less systematic toxicity. Consequently, nanoliposomes incorporated with MK-ASODNs should represent an effective and less toxic approach for treatment of HCC, and potentially, other tumor types.

In summary, our results suggest that nanoliposomes packaged with MK-ASODNs can increase the therapeutic effect of MK-ASODNs, both *in vivo* and *in vitro*, for the treatment of HCC. The combination of nanoliposomes and MK-ASODNs showed a more effective and less toxic tool for therapy against HCC and should provide a novel strategy for cancer treatment.

COMMENTS

Background
Midkine (MK) is a 13-kDa protein with a heparin-binding growth factor function. MK has been found to play important roles in carcinogenesis, including mitogenic, anti-apoptotic, transforming, fibrinolytic, chemotactic and angiogenic cancer-related activities.

Research frontiers
MK plays an important role in tumor development and progression.

Nanoliposomes packaged with MK-antisense oligonucleotides (ASODNs) can inhibit growth of hepatocellular carcinoma (HCC), both *in vitro* and *in vivo*, and potentially represents a significant clinical benefit.

Innovations and breakthroughs

In this study, nanoliposomes effectively delivered MK-ASODNs *in vitro* and *in vivo* with low toxicity. Additionally nanoliposomes packaged with MK-ASODNs inhibited proliferation of HepG2 cells and the growth of HCC xenografts.

Applications

In this study, the authors addressed the potential therapeutic effect of nanoliposomes packaged with MK-ASODNs on the suppression of HCC growth. Significant inhibition of HCC growth was achieved using the nanoliposomes packaged with MK-ASODN. This observation indicates that the nanoliposomes packaged with MK-ASODN are an effective anti-tumor agent.

Terminology

MK is a growth protein, which is overexpressed in HCC and promotes the growth of tumors. Nanoparticles represent powerful delivery tools, which can effectively deliver drugs or molecules into cells.

Peer review

The authors studied the growth inhibition of HCC *in vitro* and *in vivo* with nanoparticles delivering MK-ASODNs. MK-ASODNs have been shown to inhibit the growth of HCC. They found that the proliferation of HepG2 cells was significantly inhibited in the presence of different concentrations of nanoparticles packaged with MK-ASODNs (NANO-ASODNs). Furthermore, in the HCC mouse model, the NANO-ASODNs mainly accumulated in the liver and significantly inhibited the growth of the HCC tumors.

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Colonoscopic polypectomy in anticoagulated patients

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Abstract

AIM: To review our experience performing polypectomy in anticoagulated patients without interruption of anticoagulation.

METHODS: Retrospective chart review at the Veterans Affairs Palo Alto Health Care System. Two hundred and twenty five polypectomies were performed in 123 patients. Patients followed a standardized protocol that included stopping warfarin for 36 h to avoid supratherapeutic anticoagulation from the bowel preparation. Patients with lesions larger than 1 cm were generally rescheduled for polypectomy off warfarin. Endoscopic clips were routinely applied prophylactically.

RESULTS: One patient (0.8%, 95% CI: 0.1%-4.5%) developed major post-polypectomy bleeding that required transfusion. Two others (1.6%, 95% CI: 0.5%-5.7%) had self-limited hematochezia at home and did not seek medical attention. The average polyp size was 5.1 ± 2.2 mm.

CONCLUSION: Polypectomy can be performed in therapeutically anticoagulated patients with lesions up to 1 cm in size with an acceptable bleeding rate.

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INTRODUCTION

Current guidelines for the management of anticoagulants during colonoscopic polypectomy recommend that clotting parameters should be normalized at the time of the procedure[1,2]. These guidelines are based largely on expert opinion: polypectomy is considered to be a high risk procedure, and the risk of temporary discontinuation of anticoagulants was previously considered low[3-5]. However, recent data suggest that the risk of thromboembolic events is significant when anticoagulants are discontinued for endoscopic and other procedures: the risk of stroke was 1% in a study of 987 patients with atrial fibrillation undergoing 1137 endoscopic procedures, and the risk of thromboembolic events was 0.7% in a study of 1293 warfarin interruptions in 1024 patients[6,7].

One strategy that may decrease this risk by shortening the period of subtherapeutic anticoagulation, is to use intravenous heparin or subcutaneous low-molecular-weight heparin in the perioperative period, rather than simply holding warfarin for several days before and resuming warfarin after the procedure[8,9]. An alternative strategy to decrease the thromboembolic risk is to perform polypectomy on lesions up to 1 cm in size while patients remain anticoagulated. In 2007, we reported a series of 41 polypectomies performed in 21 patients with an average international normalized ratio (INR) of 2.3 (range 1.4-4.9)[10]. The patients were maintained on warfarin until 36 h before the procedure; the medication was held for 36 h in order to avoid supratherapeutic anticoagulation as a result of dietary restriction during colonoscopy preparation. Endoscopic clipping was performed prophylactically immediately after polypectomy. There were no episodes of post-polypectomy bleeding in that small case series. In this study we report our experience of performing polypectomy in a significantly larger number of patients who were anticoagulated.

MATERIALS AND METHODS

We reviewed available data from all anticoagulated patients from July 2004 to May 2008 who underwent colonoscopy at the Veterans Affairs Palo Alto Health Care System. Informed consent for the procedure was obtained from all patients, including discussion of the potentially high risk of bleeding due to anticoagulation. Institutional review board approval was obtained for retrospective data analysis. Our clinical protocol, which was followed by all patients, was to continue warfarin until 36 h before the procedure, when a clear liquid diet was initiated in preparation for the procedure. Warfarin was not taken while patients were on a clear liquid diet in order to avoid supratherapeutic anticoagulation during this period of potentially low vitamin K intake. In the first 21 patients, as reported in our original case series, INR was measured on the day of the procedure[10]. Subsequently, INR was no longer routinely measured. Colonoscopic polypectomy was generally only performed on polyps up to 1 cm in size (the size was estimated by comparison to a fully opened 1 cm snare), and patients with larger lesions were rescheduled at a later date with cessation of anticoagulation. On five occasions, lesions larger than 10 mm were removed and these patients were also included in the series. Immediately after polypectomy, one or more endoclips were placed prophylactically to close the polypectomy defect. Following the procedure, warfarin anticoagulation was continued on the patient's standard schedule. Follow-up was available on all patients *via* telephone and/or clinic visits.

RESULTS

Two hundred and twenty five polypectomies were performed in 123 patients (Table 1). The most common indications for colonoscopy were screening, history of polyps, iron-deficiency anemia, hematochezia and occult bleeding. The most common indications for warfarin therapy were atrial fibrillation, history of thromboembolism and mechanical heart valves. Characteristics of resected polyps are described in Table 2. The average diameter of resected polyps was 5.1 ± 2.2 mm, with a range of 2-15 mm. Most of the polyps were removed by cold snare (snare removal without cautery or submucosal saline injection) or by snare with cautery following submucosal saline injection. Seventy percent of the resected polyps were neoplastic, consisting mainly of tubular adenomas. Twenty percent of the resected polyps were non-neoplastic. Ten percent of the specimens were lost.

One patient (0.8%, 95% CI: 0.1%-4.5%) developed major post-polypectomy bleeding. He was a 79-year-old man with atrial fibrillation, dilated cardiomyopathy, emphysema and a history of alcohol abuse who had 6 mm, 8 mm and 12 mm tubular adenomas removed. The smallest polyp was removed by cold snare and the larger two were removed by snare with cautery after saline injection. A subsequent upper endoscopy on the same day as the colonoscopy demonstrated portal hypertensive gastropathy. On post-procedure day 4, he developed hematochezia. He was admitted to a local hospital and received 2 units of packed red blood cells. Repeat colonoscopy was not performed, and the bleeding resolved without further treatment. Two patients (1.6%, 95% CI: 0.5%-5.7%) had self-limited hematochezia at home and did not seek medical attention; these were classified as minor bleeding complications. No thromboembolic events were observed.

DISCUSSION

Current practice guidelines for the management of anticoagulation during colonoscopy are largely based on expert opinion. According to current guidelines, colonoscopy with or without biopsy can be performed in anticoagulated patients, but polypectomy is considered a high risk procedure for which anticoagulation must be temporarily discontinued in order to achieve normalization of coagulation function at the time of the polypectomy. The risk of withholding warfarin for several days has generally been estimated based on extrapolation from the annual incidence of thromboembolic events in patients with various clinical conditions who do not receive anticoagulants. However, more recent data suggest that actual observed thromboembolic complication rates in patients who have interruption of anticoagulation for endoscopic

Table 1 Patient characteristics

Patient characteristics	Number or percentage
Number of patients	123
Average age (range)	68.4 ± 9 (49-90) yr
Male, female	122, 1
Indication for procedure	Screening (48%)
	History of polyps (24%)
	Iron def anemia (9%)
	Hematochezia (7%)
	Occult-blood positive stool (7%)
	Other (5%)
Indication for warfarin	Atrial fibrillation (65%)
	Thromboembolism (16%)
	Mechanical valve (9%)
	Other indications (13%)

Table 2 Polypectomy characteristics

Polyp characteristics	Number or percentage
Number of polyps	225
Polyps per patient	1.8
Average polyp size (mm)	5.1 ± 2.2
Range of polyp size (mm)	2-15
Polypectomy method	Cold snare (48%)
	Snare/cautery after saline injection (30%)
	Snare/cautery, no saline injection (16%)
	Cold biopsy (4%)
	Cold snare after saline injection (1%)
Polyp histology	Neoplastic (70%)
	Non-neoplastic (20%)
	Lost specimen (10%)

procedures are higher than the theoretical predictions; this may be due to a rebound increase in clotting factors in this setting[6-7,11].

Post-polypectomy bleeding is a relatively common complication of colonoscopic polypectomy, with a reported incidence of approximately 0.3%-2% that depends on multiple factors including lesion size[12,13]. A multivariate regression analysis suggested that anticoagulation increases this risk[14]. Post-polypectomy bleeding is generally divided into two types: immediate bleeding following the polypectomy, and delayed bleeding that can occur up to 2-3 wk following the procedure. Immediate bleeding is familiar to therapeutic endoscopists, as it is particularly common following endoscopic mucosal resection of large lesions[15]. In this situation, it is generally treated very effectively by methods such as clip application[15-17]. In contrast, delayed bleeding typically occurs when the patient is already at home and is therefore a significant concern when polypectomy is undertaken in patients who require anticoagulation. Even when current guidelines are followed and warfarin is held in anticipation of polypectomy, it is quite possible that the patient will be therapeutically anticoagulated at the time that delayed bleeding occurs several days after the procedure. The major issues with performing polypectomy while patients are anticoagulated are therefore as follows: will there be difficulty in controlling any immediate bleeding, will there be a significantly increased risk of delayed bleeding, and are the bleeding risks outweighed by the risk of thromboembolic events that could occur if anticoagulation was interrupted? This study systematically evaluated the risks associated with performing polypectomy while patients were anticoagulated.

Our study demonstrated that polypectomy of lesions up to approximately 1 cm in size can be performed with relative safety in anticoagulated patients. Because we anticipated some degree of immediate bleeding, our uniform practice was to immediately apply endoscopic clips to the polypectomy sites rather than observing the site and waiting to see if bleeding developed. With this strategy, we did not observe any extraordinary episodes of immediate bleeding that could not be controlled with clip application. There was only 1 episode of major delayed bleeding out of 123 patients and 225 polypectomies, a major bleeding rate of 0.8% (CI: 0.1%-4.5%). There were no thromboembolic events, although follow-up was often performed by telephone and typically did not include a neurological exam. The complication profile observed in this study suggests that the strategy of removing small lesions up to 1 cm in size may compare favorably with the standard practice of discontinuation of warfarin for elective procedures, where two major recent studies found thromboembolic rates of 0.7% and 1%[6,7].

It must be emphasized that in our patients warfarin was withheld for approximately 36 h in order to avoid supratherapeutic anticoagulation due to dietary restriction and possibly other factors relating to bowel preparation. We previously published our experience with the first 21 patients in our series, in whom INR was routinely measured before the procedure[10]. In those patients the average INR at the time of colonoscopy was 2.3. Following this experience, we stopped routine measurement of INR before colonoscopy as this involved significant logistical difficulty. The polypectomy techniques utilized in our series varied, with cold snare, standard snare with cautery and inject & cut mucosectomy comprising the majority of polypectomies. Although our data suggest that all three techniques may be safe, our current preference is to perform cold snare in lesions smaller than 5 mm and to perform submucosal injection for larger lesions in which cautery is used in order to minimize potential injury to the bowel wall. In addition, prophylactic clipping was performed immediately at all polypectomy sites. While there is no data to demonstrate the efficacy of clipping in this circumstance, and a randomized study of clipping in average-risk patients demonstrated no benefit, we felt compelled to perform clipping because of the absence of data in anticoagulated patients[18]. The drawbacks of clipping include additional endoscopy time, clip cost, and in our case also the practice of immediate clipping before specimen retrieval may have contributed to a relatively large fraction, 10%, of lost specimens.

Limitations of our study include the retrospective, single center design. It is possible that the bleeding rate could be higher in different patient populations, with alternative polypectomy techniques, and/or with less experience in clip placement. Anecdotally, we have observed that endoscopists in our unit apply significantly less cautery during polypectomy than many of our colleagues and it is possible that this will influence delayed bleeding rates due to cautery ulcers. An additional limitation is the absence of INR measurements on patients after the first 21 in the series. However, there were no changes in our colonoscopy preparation during the study period so we expect that the INR levels in subsequent patients would have been similar.

Our study suggests that a reasonable strategy for screening and surveillance colonoscopy in anticoagulated patients may be to perform the procedure while patients are anticoagulated. Small polyps can be removed with a relatively low risk of bleeding. The proportion of patients with lesions larger than 1 cm is relatively low in most clinical settings, so only a relatively small number of patients would undergo a repeat colonoscopy with normalization of coagulation parameters to resect these larger lesions[19,20]. A more refined strategy, which would be ideal, would be to develop a highly predictive algorithm to determine which patients are likely to harbor a lesion larger than 1 cm and proceed directly to colonoscopy with interruption of anticoagulation only in these select patients. This strategy could potentially resolve a dilemma facing endoscopists and patients who follow current American Society for Gastrointestinal Endoscopy guidelines: whether to perform screening/surveillance colonoscopy while patients are anticoagulated and repeat

the procedure off anticoagulation in the large number of patients who have small polyps, or to risk thromboembolic complications by normalizing coagulation parameters even in screening/surveillance colonoscopies where potentially no polyps may be found.

A recent survey of endoscopists in the United Kingdom demonstrated a wide variation in the management of anticoagulants for colonoscopy, with 49% of responding physicians routinely stopping anticoagulants and 37% routinely continuing anticoagulants[21]. This wide variation in practice suggests that the management of anticoagulants is a significant clinical dilemma. A prospective randomized trial would be the ideal method to resolve this dilemma, and by demonstrating that polypectomy of small lesions can be performed with relative safety, our series demonstrated that it would be reasonable to prospectively compare a strategy of anticoagulant interruption to one of colonoscopy in anticoagulated patients where small polyps can be removed at the discretion of the endoscopist.

COMMENTS

Background
Current guidelines recommend discontinuation of anticoagulation prior to colonoscopic polypectomy. However, small colon polyps are exceedingly common and the risk of significant complications from anticoagulant interruption may outweigh the benefit in these patients.

Research frontiers
In this study, the authors report their experience with resection of small colon polyps up to 1 cm in diameter without interruption of anticoagulation.

Innovations and breakthroughs
The authors report that the bleeding risk is very low (0.8%) when small polyps are removed in this setting. This suggests that it is reasonable to perform polypectomy without interruption of anticoagulation.

Applications
Patients who are on chronic anticoagulation and are undergoing screening colonoscopy can be considered for colonoscopy without interruption of anticoagulation, and if small polyps are found then polypectomy can be considered.

Terminology
Polyps are growths in the colon that are often precancerous. During colonoscopy, the colon is examined for polyps using an endoscope. Polyps are usually removed using a variety of instruments during colonoscopy. Bleeding is a relatively common complication of polyp removal, so there is widespread concern about performing polyp removal in patients who are taking anticoagulants.

Peer review
The authors performed a retrospective review that demonstrated that the risk of post-polypectomy bleeding is low in patients with small polyps who are anticoagulated. A comparison to published risk estimates for anticoagulant interruption suggests that it may be favorable to perform polypectomy in this setting rather than interrupting anticoagulation.

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BRIEF ARTICLES

Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model

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Abstract

AIM: To test the effect of the dephytinization of three different commercial infant cereals on iron, calcium, and zinc bioavailability by estimating the uptake, retention, and transport by Caco-2 cells.

METHODS: Both dephytinized (by adding an exogenous phytase) and non-dephytinized infant cereals were digested using an *in vitro* digestion protocol adapted to the gastrointestinal conditions of infants younger than 6 mo. Mineral cell retention, transport, and uptake from infant cereals were measured using the soluble fraction of the simulated digestion and the Caco-2 cells.

RESULTS: Dephytinization of infant cereals significantly increased ($P < 0.05$) the cell uptake efficiency (from 0.66%-6.05% to 3.93%-13%), retention (from 6.04%-16.68% to 14.75%-20.14%) and transport efficiency (from 0.14%-2.21% to 1.47%-6.02%), of iron, and the uptake efficiency (from 5.0%-35.4% to 7.3%-41.6%) and retention (from 4.05%-20.53% to 14.45%-61.3%) of zinc, whereas calcium only cell uptake showed a significant increase ($P < 0.05$) after removing phytate from most of the samples analyzed. A positive relationship ($P < 0.05$) between mineral solubility and the cell uptake and transport efficiencies was observed.

CONCLUSION: Removing phytate from infant cereals had a beneficial effect on iron and zinc bioavailability when infant cereals were reconstituted with water. Since in developing countries cereal-based complementary foods for infants are usually consumed mixed with water, exogenous phytase additions could improve the nutritional value of this weaning food.

Frontela C, Scarino ML, Ferruzza S, Ros G, Martínez C. Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model. *World J Gastroenterol* 2009; 15(16): 1977-1984 Available from: URL: http://www.wjgnet.com/1007-9327/15/1977.asp DOI: http://dx.doi.org/10.3748/wjg.15.1977

INTRODUCTION

Insufficient mineral intake during infancy is responsible for many diseases which can not only influence immediate health, but may also have an adverse impact on adult health. Anaemia, rickets, osteoporosis, and immune diseases are caused by a deficiency of iron, calcium and/or zinc[1]. An adequate intake of these minerals is important for meeting infant nutritional needs[2]. Cereals are considered a rich plant source of carbohydrate, proteins, vitamins, and minerals, and are therefore usually introduced to an infant's diet between the ages of 4 and 6 mo. However, cereals are also rich in phytate, which can decrease the bioavailability of critical nutrients such as iron, calcium, and zinc because of its high ability to chelate and precipitate minerals[3,4]. Dephytinization by adding an

exogenous phytase or by activating the naturally occurring plant phytases has been proposed as a sustainable strategy for reducing mineral deficiency by increasing mineral bioavailability in infant complementary cereal-based foods[5]. An estimate of mineral bioavailability from infant cereals is important because not only must the absolute amounts of minerals be increased in the edible portions of foods, but they must also be in forms bioavailable to infants. Bioavailability should preferably be determined by *in vivo* testing, however, these studies could also be based on preliminary *in vitro* methods[6]. Caco-2 cells are human intestinal adenocarcinoma cells exhibiting biochemical and morphological characteristics of small intestinal absorptive enterocytes, and together with a simulation of gastrointestinal digestion, have been used widely for mineral bioavailability studies[7-9]. Caco-2 cells grown on microporous supports, allow the measurement of mineral uptake and transport across cell monolayers, improving the estimation of bioavailability by *in vitro* methods used until now (solubility and dialysis)[10,11]. In western countries, infants are usually fed with infant cereals reconstituted with follow-on formula; nevertheless in developing countries, where mineral deficiencies are particularly frequent, cereal-based complementary foods destined to infants are usually consumed mixed with water[12]. Given the importance of an adequate intake of minerals during infancy, the purpose of the current investigation was to study the effect of dephytinization on iron, calcium and zinc solubility, retention, transport, and uptake by Caco-2 cells from infant cereals when reconstituted with water, with the aim of obtaining data on mineral bioavailability from infant cereals with this kind of reconstitution.

MATERIALS AND METHODS

Chemicals

Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO): pepsin (porcine, catalogue no. P-7000), pancreatin (porcine, catalogue no. P-1750), and bile extract (porcine, catalogue no. B-8756). Pepsin solution was prepared by dissolving 1.6 g of pepsin in 10 mL of 0.1 mol/L HCl. Pancreatin-bile extract solution was prepared by dissolving 0.2 g of pancreatin and 1.25 g of bile extract in 50 mL of 0.1 mol/L NaHCO3. Millipore Milli-Q distilled-deionized water (Millipore Ibérica S.A., Barcelona, Spain) was used throughout the experiments. Cell culture media, antibiotics (penicillin and streptomycin), glucose, 4-(2-hydroxyethyl)-1-piperazineethanosulfonic acid (HEPES), 2-(N-morpholino) ethanesulfonic acid (MES), and Hank´s Balanced Salt Solution (HBSS) were obtained from Gibco BRL Life Technologies (Paisley, Scotland).

Inositol phosphate content

Inositol phosphates were determined by HPLC using a Merck Hitachi chromatograph [pump L-7100, refraction index (RI)-detector L-7490, and L-7350 column oven] according to the method of Lehrfeld[13]. Inositol phosphates were extracted from the different samples with 0.5 mol/L HCl at room temperature for 2 h. Because

of the high binding capacity of inositol pentaphosphate (IP5) and inositol hexaphosphate (IP6) to minerals, we consider the sum of IP5 and IP6 to determine phytate content[4,13]. The molar ratios of phytate to iron, calcium, and zinc were calculated as the millimoles of phytate present in the sample divided by the millimoles of iron, calcium, and zinc present in the sample, respectively. To find the phytate × (Ca/Zn) molar ratio, the total amount of Ca (mmol) in 100 g of infant cereal was multiplied by the phytate/Zn molar ratio.

Samples

Both commercial and dephytinized infant cereals were dried in an oven at 120℃ overnight to obtain the dry weight, and were then milled. Infant cereals were reconstituted according to the recommendations of the manufacturer: 200 mL of water was mixed with 35 g of infant cereal. The infant cereals were dephytinized using an exogenous phytase from *Aspergillus oryzae* (EC 3.1.3.26 from Stern-Enzym GmbH & Co. KG, Ahrensburg, Germany, 2500 PU/g). The phytase was added to the aqueous slurry at a concentration of 3.2 U/g of sample and incubated at pH 5.5 with stirring at 55℃ for 20 min. The dephytinized samples were dried in an oven at 120℃ overnight to obtain the dry weight, and then ground in an electrical mill to a fine powder similar to that of commercial infant cereals. Dephytinization was checked by HPLC[13].

Caco-2 cells

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK) and used in assays at passages 28-55. For iron, calcium, and zinc uptake assays, cells were seeded onto polycarbonate membrane chamber inserts (24 mm diameter, 0.4 μm pore size; Transwell, Costar Corp.) at a density of 50000 cells/cm^2 and allowed to differentiate on filters for 21 d. During this period, cells were maintained in minimum essential medium (MEM) with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% v/v nonessential amino acids, 1% v/v L-glutamine and 1% antibiotic solution (penicillin-streptomycin) at 37℃ in an incubator with 5% CO2, 95% air atmosphere and 95% relative humidity. The medium was changed every 2 d. During the cell differentiation period, monolayer formation and tight junction maturation and sealing were assessed by measuring the passage of phenol red across the monolayer according to Ferruzza et al[14]. Briefly, following three washes of cell monolayers with phosphate-buffered saline (PBS), 0.5 mL of 1 mmol/L phenol red was added in the apical compartment, whereas 1 mL of PBS was added in the basolateral compartment. After 1 h of incubation at 37℃, 0.9 mL of basolateral medium was collected, treated with 0.1 mL of 0.1 mol/L NaOH, and read at 560 nm using a molecular absorption spectrophotometer (UV-Vis, U-200, Hitachi Ltd. Tokyo, Japan) to determine the phenol red concentration. The passage of phenol red was expressed as apparent permeability (Papp) and obtained from the following formula: Papp = Ct × V$_{BL}$/Δt × C$_0$ × A, where V$_{BL}$ is the volume of the basolateral compartment

(cm^3), A is the filter area (cm^2), Δt is the time interval (s), Ct is the phenol red concentration in the basolateral compartment at the end of time interval, and C_0 is the phenol red concentration in the apical compartment at time zero. Apparent permeability of monolayers at the end of differentiation period was 1.71×10^{-5} cm/s, indicating that tight junctions were functionally mature[14]. The experiments were conducted on day 21 from seeding. Microscopic examination of the cultures revealed that confluence was reached after 3-4 d of growth. Cell viability 3 h after the addition of the soluble fraction was assessed by trypan blue exclusion and was typically 85%-95%.

In vitro gastrointestinal digestion

Gastrointestinal digestion was applied to infant cereals, whether or not dephytinized, and reconstituted with deionized distilled water using the *in vitro* method described by Miller *et al*[15] with modifications aimed at reducing the amounts of the enzymes used because the gastrointestinal tract in the early stages of life is not yet fully developed[16,17]. The method consisted of two phases: gastric and intestinal. Prior to the gastric stage, the pH of 17.5 g of each infant cereal homogenized with 100 mL of deionized-distilled water was lowered to pH 4 with 6 mol/L HCl. Then, 3 g of pepsin solution was added, and the sample was incubated in a shaking water bath at 37℃ and 120 strokes/min for 2 h to allow pepsin digestion. The digest was then maintained in ice for 10 min to stop pepsin digestion. For intestinal digestion, the pH of the gastric digests was raised to 5.0 by dropwise addition of $NaHCO_3$ 1 mol/L. Then a freshly prepared pancreatin-bile solution sufficient to provide 0.005 g of pancreatin and 0.03 g of bile salts/g of sample was added, and incubation was continued for 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. Then the pH was adjusted to 7.2 by dropwise addition of 0.5 mol/L NaOH. The intestinal digest was heated for 4 min at 100℃ to inhibit the sample proteases and then cooled by an ice bath. The gastrointestinal digest were centrifuged at $9187 \times g$ for 30 min at 4℃. The supernatant fraction was filtered through a centrifugal filter devices with a 30 000 MW cut-off (Millipore Corporation Bedford, MA 01730, USA) and then centrifuged at $4000 \times g$ for 90 min at 4℃ using a Sorvall centrifuge (Model RC5C, with SS-34 rotor; Sorvall instruments, DuPont, Mississauga, ON, Canada). Prior to addition of the soluble fraction to the cells, glucose (5 mmol/L final concentration), HEPES (50 mmol/L final concentration), and MES (30 mmol/L final concentration) (pH 6.5-6.9) were added to make the soluble fraction similar to the culture media; and finally, water was added to adjust the osmolarity to 310 $\pm$ 10 mOsm/kg (Freezing point osmometer 030, Berlin, Germany) according to Ekmekcioglu[18]. Then the supernatants (soluble fraction) were analyzed for mineral content and used in cell uptake assays.

Uptake, retention and transport experiments

The soluble fraction obtained from gastrointestinal digestion was used to carry out uptake, retention, and

Figure 1 TEER values of Caco-2 monolayers incubated in the presence of digested infant cereals.

transport experiments with Caco-2 cells because it is more similar to the *in vivo* digests[19]. Before each experiment growth medium was removed and apical and basolateral cell surfaces of the monolayers were washed three times with phosphate-buffered saline (PBS) at 37℃. One millilitre of soluble fractions was added to the apical chamber and 1.5 mL of HBSS (pH 7.4) was added to the basal chamber of each cell monolayer. After incubation, the apical samples were collected, and the monolayers were carefully washed three times with 1 mL ice-cold HBSS to remove any nonspecific-bound mineral and residual soluble fractions. Cells on filters were lysed by the addition of 0.5 mL of deionized water to each well, and then harvested; HBSS in basal chamber also was removed. Total mineral content was measured in the apical solutions, cell monolayer, and basal solutions.

Assessment of cell monolayer integrity during experiments

To investigate possible adverse effects of infant cereal components or digestive enzymes from the supernatants on Caco-2 cells, the integrity of the monolayer was assessed by measuring the transepithelial electrical resistance (TEER) according to the method of Okada *et al*[20]. At the end of differentiation period control, monolayers used for experiments had a resistance higher than 900 $\Omega \cdot cm^2$ (Figure 1). During the uptake, retention, and transport experiments measurements of TEER were taken every 30 min. Background resistance was determined by measuring across a filter without cells in Hank's Balanced Salt Solution (HBSS). Monolayers with resistances < 500 $\Omega \cdot cm^2$ were discarded.

Iron, calcium, and zinc determination

To estimate mineral bioavailability, iron, calcium, and zinc contents in the sample (before digestion), soluble fraction (apical solution), blank (HBSS), basal solution and in cell homogenates were determined by flame atomic absorption spectrophotometry (AAS; Perkin-Elmer, mod. 3100 Norwalk, U.S.A) according Perales *et al*[10] with slight modifications; dry organic matter destruction (450℃) was applied prior to analysis. An amount of lanthanum chloride sufficient to obtain a final content of 0.1% was added to eliminate phosphate

Table 1 Mineral content (per 100 g), phytate (IP₅ + IP₆) content (per 100 g) and molar ratios of phytate to iron, calcium and zinc, and phytate × calcium/zinc of commercial infant cereals

Infant cereal	Fe (mg)	Ca (mg)	Zn (mg)	Phytate (mg)	Phytate/Fe	Phytate/Ca	Phytate/Zn	Phytate × Ca/Zn
Eight cereals-honey	8.3 ± 0.4	137.3 ± 5.6	0.6 ± 0.3	319.6 ± 3.1	3.8	0.16	53.1	182.3
Rice cream	8.8 ± 0.1	283.1 ± 27.7	1.2 ± 0.2	167.1 ± 27.5	1.6	0.11	14.4	101.9
Multicereals	8.7 ± 0.2	174.4 ± 21.0	1.5 ± 0.4	143.5 ± 10.6	1.4	0.07	9.8	42.7
Gluten-free cereals	7.5 ± 1.0	154.4 ± 38.9	1.0 ± 0.3	299.8 ± 16.9	3.5	0.18	31.8	122.7

interferences in the calcium determination. To dissolve the ashes, 2 mL of concentrated HCl (sp gr = 1.19) was added, and the vessel was covered with a watch glass and gently warmed (70-75℃) for 4 h, leaving about 1 mL of liquid at the end of heating. The solution was then transferred to a 10 mL volumetric flask, and the volume was completed with water. Solubility percentages were determined as follows: solubility % = 100 × S/C, where S = soluble mineral content (μg of mineral/g of sample), and C = total mineral content of the sample (μg of mineral/g of sample). Differences between the mineral content of the monolayer incubated with soluble mineral fraction and the content of monolayer not exposed (retention blank) yielded an estimation of the cellular retention (micrograms) of minerals. Transport (T) was evaluated by the difference between the mineral amount in basal chamber solutions of treated samples and HBSS. The following calculation was used for retention percentages: retention % = 100 × R/C, where R = mineral retention (μg of mineral/well), and C = mineral soluble added (μg). Transport percentages were calculated as follows: transport % = 100 × T/C, where T = cellular transport (μg of mineral/well), and C = mineral soluble added (μg)/well. The differences between the mineral content of cells cultures incubated with samples or HBSS (blank) gave an estimation of the cellular uptake (cell retention plus transport) of these mineral elements. Uptake percentage values were calculated as the percentage of the mineral applied to the Caco-2 cell monolayer which was taken up by the cells and results were used as a measure of mineral availability. Due to the differences among samples in terms of the solubility of minerals after *in vitro* digestion, mineral transport and uptake were normalized for solubility as follows: % transport efficiency = (% solubility × % transport)/100, % uptake efficiency = (% solubility × % uptake)/100.

Quality control of the iron, calcium, and zinc analyses

The absence of matrix interferences in AAS determination of Fe and Ca in the samples was checked by the addition's method. Community Bureau of reference material CRM-189 (wholemeal flour) (Brussels, Belgium) was used as a control to test the method for accuracy. For Fe, Ca and Zn, the measured mean values were 66.9 μg/g, 519 μg/g and 54.9 μg/g, respectively, which were in accordance with the certified range of 68.3 ± 1.9 μg/g for Fe, 520 μg/g (standard deviation non-certified) for Ca and 56.5 ± 1.7 μg/g for Zn. The detection limit was determined to be 0.6 mg/L. The method was shown to

be linear ($r \geqslant 0.998$) over the range 1-5 mg/L for both Fe ($y = 2.86 \times 10^{-3} + 4.76 \times 10^{-2} X$) and Ca ($y = 2.67 \times 10^{-3} + 5.24 \times 10^{-2} X$).

Statistical analysis

Results are reported as mean ± SD of five experiments. After testing for normality and equal variances, the mean solubility, retention, transport efficiency, and uptake efficiency percentages of Fe, Ca, and Zn from infant cereals, whether or not dephytinized, were compared by one-way analysis of variance (ANOVA) including the Tukey post-test in the data treatment to determine significant differences among means ($P < 0.05$). A Pearson correlation analysis was performed to investigate the possible correlation between phytate content; Fe, Ca, and Zn contents; mineral solubility (%); retention (%); uptake (%); and transport (%) by Caco-2 cells. Values of $P < 0.05$ were considered significant. All statistical analyses were performed with the Statistical Package for the Social Sciences (version 14.0; SPSS).

RESULTS

Inositol phosphate content

Molar ratios of phytate (IP₅ + IP₆) to iron, phytate to calcium and phytate to zinc, as well as the phytate × (Ca/Zn) molar ratio, are shown in Table 1. For iron, values ranged from 1.4 to 3.8; for calcium, from 0.07 to 0.18; and for zinc, 42.7 to 182.3.

Uptake, retention and transport experiments

The results obtained in the iron retention, transport, and uptake assays by Caco-2 from infant cereals are summarized in Table 2. The iron retention percentage and transport and uptake efficiencies of eight cereals-honey, Multicereals and Gluten-free cereals after dephytinization were significantly higher ($P < 0.05$) than that of commercial infant cereals. The iron solubility percentage was higher from eight cereals-honey and Multicereals after dephytinization; conversely the solubility percentage of commercial rice cream was higher than that of the same sample after phytase treatment. Total iron content of each infant cereal before and after phytase treatment was equal.

Table 3 shows the results obtained for calcium cell retention, transport, and uptake assays by Caco-2 cells. Calcium uptake efficiency percentage was higher from infant cereals analyzed after phytase treatment with the exception of eight cerals honey; however, results were significant ($P < 0.05$) only for Gluten-free cereals.

Table 2 Iron retention, transport and uptake from infant cereals by Caco-2 cells

	Infant cereal	Iron added (μg)	Solubility (%)	Retention (μg)	Retention (%)	Transport (μg)	Transport efficiency (%)	Uptake (μg)	Uptake efficiency (%)
- phytase	A	12.51	17.42 ± 6.1[a]	1 ± 0.1	7.99 ± 2.2[b]	1.59 ± 0.6	2.21 ± 1[a]	2.59 ± 0.8	3.6 ± 0.9[b]
	B	13.08	8.72 ± 2.7[b]	0.79 ± 0.4	6.04 ± 0.6[b]	0.21 ± 0	0.14 ± 0.03[c]	1 ± 0.2	0.66 ± 0.2[d]
	C	13.13	24.21 ± 1.9[a]	2.19 ± 0.1	16.68 ± 3.8[a]	1.09 ± 0.7	2.01 ± 0.8[a]	3.28 ± 1.1	6.05 ± 2.2[a]
	D	11.18	20.93 ± 4.8[a]	0.78 ± 0.2	6.98 ± 0.8[b]	0.18 ± 0.04	0.33 ± 0.1[b]	0.96 ± 0.2	1.8 ± 0.3[c]
+ phytase	A	12.51	34.53 ± 8.6[1]	2.52 ± 0.3	20.14 ± 3.9[1]	2.18 ± 0.9	6.02 ± 1.3[1]	4.7 ± 0.9	13 ± 2.5[1]
	B	13.08	21.1 ± 1.8[1]	2.1 ± 0.2	16.28 ± 2.1[1]	2.49 ± 0.9	4.01 ± 0.8[1]	4.62 ± 1.6	7.45 ± 3.9[1]
	C	13.13	19.19 ± 3[1]	2.41 ± 0.3	18.35 ± 3.4	1.66 ± 0.6	2.43 ± 0.6	4.0 ± 1.2	5.95 ± 1.1
	D	11.18	16.64 ± 6.2	1.65 ± 0.2	14.75 ± 1.3[1]	0.99 ± 0.2	1.47 ± 0.3[1]	2.64 ± 0.8	3.93 ± 0.7[1]

A: Eight cereals honey; B: Multicereals; C: Rice cream; D: Gluten-free cereals. mean ± SD, *n* = 5. Different letter (a-d) denotes significant differences (*P* < 0.05) between commercial infant cereals (without phytase treatment) to assess the effect of different phytate content. [1]*P vs* the same infant cereal dephytinized or not.

Table 3 Calcium retention, transport and uptake from infant cereals by Caco-2 cells

	Infant cereal	Calcium added (μg)	Solubility (%)	Retention (μg)	Retention (%)	Transport (μg)	Transport efficiency (%)	Uptake (μg)	Uptake efficiency (%)
- phytase	A	206	38.9 ± 11.1[a,1]	5.72 ± 0.3	2.78 ± 1[a,1]	25.99 ± 8	4.9 ± 1.2[a]	31.71 ± 4.9	5.99 ± 2[a]
	B	261.6	15.2 ± 1.8b[1]	7.33 ± 0.2	2.8 ± 0.6[a]	16.1 ± 3.9	0.94 ± 0.1[b,1]	23.43 ± 3.6	0.66 ± 0.2[b]
	C	424.7	2.8 ± 1[c]	2.18 ± 0.3	0.51 ± 0.2[b]	33.1 ± 6.1	0.22 ± 0.02[d]	35.28 ± 7.2	0.23 ± 0.2[c]
	D	231.6	4.53 ± 1.3[c]	4.45 ± 0.2	1.92 ± 0.3[a]	15.64 ± 4.4	0.31 ± 0.08[c]	20.09 ± 1.8	0.39 ± 0.1[c]
+ phytase	A	206	22.5 ± 2.3	0.7 ± 0.2	0.34 ± 0.09	29.59 ± 7.2	3.24 ± 1	30.29 ± 6.8	3.31 ± 0.9
	B	261.6	8.03 ± 2.7	7.33 ± 1.4	2.8 ± 0.6	23.08 ± 2.1	0.71 ± 0.04	30.41 ± 2.2	0.93 ± 0.2
	C	424.7	3.72 ± 1.9	5.5 ± 0.1	1.3 ± 0.7	28.1 ± 4.4	0.25 ± 0.07	33.6 ± 7.1	0.43 ± 0.09
	D	231.6	8.31 ± 1.2[1]	2.98 ± 0.7	1.29 ± 0.9	66.16 ± 10.8	2.38 ± 0.1[1]	69.14 ± 8.2	2.48 ± 0.3[1]

Table 4 Zinc retention, transport and uptake from infant cereals by Caco-2 cells

	Infant cereal	Zinc added (μg)	Solubility (%)	Retention (μg)	Retention (%)	Transport (μg)	Transport efficiency (%)	Uptake (μg)	Uptake efficiency (%)
- phytase	A	2.12	36.4 ± 7.1[a]	0.18 ± 0.08	8.5 ± 2[b]	1.88 ± 0.6	32.3 ± 3[a,1]	2.06 ± 0.8	35.4 ± 4.1[a]
	B	2.22	18.9 ± 3.8[b]	0.09 ± 0.01	4.05 ± 2[c]	0.5 ± 0.08	4.25 ± 1.1[c]	0.59 ± 0.6	5 ± 0.9[d]
	C	2.63	17 ± 1.9[b]	0.54 ± 0.1	20.53 ± 3.8[a]	0.62 ± 0.1	4 ± 0.8[c]	1.16 ± 0.4	7.5 ± 0.2[c]
	D	1.63	37.8 ± 6.2[a,1]	0.09 ± 0.01	5.5 ± 1.6[c]	0.71 ± 0.2	16.5 ± 0.3[b]	0.8 ± 0.4	18.6 ± 1.2[b]
+ phytase	A	2.12	46.9 ± 6.6	0.84 ± 0.2	22.2 ± 2.7[1]	1.04 ± 0.5	23 ± 3.6	1.88 ± 0.9	41.6 ± 6.5
	B	2.22	18.92 ± 2.7	0.42 ± 0.1	18.92 ± 2.8[1]	0.44 ± 0.2	3.74 ± 0.3	0.86 ± 0.2	7.3 ± 0.2[1]
	C	2.63	27.3 ± 6[1]	0.38 ± 0.1	14.45 ± 3.4	1.59 ± 0.6	16.5 ± 2.6[1]	1.97 ± 0.8	20.4 ± 2.1[1]
	D	1.63	21 ± 4.3	0.18 ± 0.03	61.3 ± 9.9[1]	1.43 ± 0.4	18.4 ± 4.1	1.61 ± 0.2	20.7 ± 0.6[1]

After phytase treatment, this infant cereal showed that solubility and transport efficiency percentages of calcium increased significantly as well. From samples not dephytinized, significant highest solubility and retention percentages of calcium were observed for eight cereals-honey, and highest solubility and transport efficiency percentages for multicereals. Total calcium content of each infant cereal before and after phytase treatment was equal.

As shown in Table 4, the effect of dephytinization caused an increase (*P* < 0.05) in retention and uptake efficiency percentages of zinc in most of samples analyzed. Rice cream showed an increase in solubility, transport, and uptake efficiencies percentages after phytase treatment. Significant differences on the solubility percentage of zinc were observed for Gluten-free cereals and rice cream between samples, whether or not dephytinized. Total zinc content of each infant cereal before and after phytase treatment was equal.

With significant values at *P* < 0.05, a negative correlation between phytate content and retention percentages of iron and zinc (r = -0.730 and r = -0.538) and iron transport (r = -0.507), and uptake efficiency (r = -0.519) percentages were found. Calcium content showed a negative correlation with transport efficiency percentage of iron (r = -0.426) and with uptake efficiency percentage of zinc (r = -0.855). Mineral solubility percentage showed for each mineral analyzed (Fe, Ca, and Zn) a positive correlation with transport efficiency percentage (r = 0.735, r = 0.912, r = 0.732, respectively) and with uptake efficiency percentage (r = 0.794, r = 0.923, r = 0.838, respectively).

DISCUSSION

Fe, Ca, and Zn contents of infant cereals analyzed were

in accordance with recommendations of the European Economic Community (Directive 2006/125)[21]. In the present study, the values found for the phytate (IP$_5$ + IP$_6$) to iron molar ratios were higher than 1.4. Although the critical ratio (capable of compromising bioavailability) has not yet been well established, according to Hurrell[5,12], values obtained in infant cereals of this study have the potential to compromise iron bioavailability. It is interesting to note the very low phytate to calcium molar ratio for all infant cereals analyzed; apparently phytate could not compromise Ca availability since the critical value for which the absorption of calcium is compromised has been reported to be > 0.24[4]. Three of the four infant cereals analyzed showed a phytate to zinc molar ratio above 12, a value implicated in interference with zinc bioavailability in humans[22,23]. The infant cereal named eight cereals-honey showed phytate × calcium/zinc molar ratios above the critical value within the range of 150-200, which have been associated with a decrease in zinc bioavailability.

It has been reported that removing phytate increases iron bioavailability in the Caco-2 cell *in vitro* model[24,25]. Since iron solubility, iron retention, transport efficiency, and uptake efficiency percentages can be used as bioavailability predictors[8,26,27], our results showed that three of the four infant cereals analyzed after phytase treatment increased iron bioavailability. The source of iron used for the enrichment of infant cereals (elemental iron) plays an important role in iron solubility[28]; in this regard, values found in our study were lower than those reported by other authors[29,30] for the same element, probably due to the phytate content or fiber components from cereals, the different pH conditions applied for the assays[29], or the previously reported the inhibitory effect of calcium on iron availability[10] since infant cereals used in this study were calcium-enriched. Differences in iron bioavailability parameters between dephytinized infant cereals could indicate that other components of infant cereals can also decrease iron bioavailability; in this regard, it has been reported that some dietary fiber components can bind mineral ions decreasing their bioavailability[30,31]. Commercial infant cereal (Multicereals) showed the highest values of iron bioavailability parameter measures which can be justified because of its lower molar ratio of phytate to iron, compared to eight cereals-honey, rice cream and Gluten-free cereals.

It should be noted that the positive correlation observed between iron solubility with cell transport and uptake efficiencies percentages found in our study is in agreement with Bergqvist et al[26], studying iron absorption from carrot juice, and with Proulx and Reddy[32] studying iron bioavailability of maize, both using Caco-2 cells. Meanwhile, it has been reported by other authors[27,33] that solubility and bioavailability by Caco-2 cells did not show parallel trends.

In our study, a lack of significant effect of dephytinization on calcium bioavailability parameters by Caco-2 cells was found in most of the infant cereals analyzed, although a great variability was observed. The inhibitory effect of phytate on calcium bioavailability has been reported[34-36] but only for high ratios. Our observation could be explained by the binding of calcium phytate to the membrane of Caco-2 cells. In this regard, Phyllippy[36] reported that Caco-2 cells may not be useful for studying the effects of inositol phosphates on the calcium uptake by cells. Calcium solubility from eight cereals-honey and Multicereals decreased after phytase treatment whereas Gluten-free cereals showed a higher calcium solubility after phytase treatment. Since it has been reported that calcium solubility depends on the phytate to calcium molar ratio[34] the low ratio Ca: phytate ratio (≤ 0.18) of all infant cereals analyzed could explain the lack of effect of phytase on calcium solubility. In fact, only Gluten-free cereals (the sample with the highest phytate/Ca molar ratio) showed a significant increase in transport and uptake efficiencies after dephytinization.

Percentages of soluble zinc did not show significant differences before and after dephytinization for most of the samples analyzed. Zn solubility percentages obtained (< 37.8% for commercial infant cereals; < 46.9% for dephytinized infant cereals) were lower than values obtained by Cámara et al[27] in school meals, and by Lyon[37] in cereal products. The same trend was observed in a previous analysis in our laboratory studying infant cereals[38], since a lack of phytase effect on zinc solubility was found for most of the samples studied; moreover, Kayode et al[39] observed similar results studying opaque sorghum beer. Probably, as reported Perales et al[10], the calcium added as enrichment to infant cereals that are not Zn-fortified exerted a negative effect on Zn solubility.

However, when bioavailability was evaluated in Caco-2 cells, all infant cereals analyzed showed an increase in the Zn uptake efficiency percentage after phytase treatment. Values obtained for Multicereals, rice cream and Gluten-free cereals were significant ($P < 0.05$). Eight cereals-honey, Multicereals and Gluten-free cereals presented a higher percentage of zinc retention after phytase treatment with respect to the same infant cereal not dephytinized. The retention and uptake efficiencies of zinc data obtained in our study clearly demonstrate that phytate impaired bioavailability, since significant differences were found between samples dephytinized and not dephytinized. The inhibitory effect of phytate on Zn bioavailability by Caco-2 cells has been previously reported[40,41].

In conclusion, dephytinization of infant cereals by an exogenous phytase resulted in increasing bioavailability parameters of iron and zinc measured in a Caco-2 cell line. However, for calcium, a lack of effect of dephytinization of infant cereals on bioavailability by Caco-2 cells was found in our study.

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COMMENTS

Background

An adequate intake of minerals is particularly important for infants in the first year of life. Although breast-feeding is considered the natural and preferred method for infant feeding, after the 6th mo of age cereals are introduced to supplement breast milk. Given the importance of an adequate nutrition during infancy, a better knowledge of the gastrointestinal conditions of infants and its effects on food components affecting intestinal absorption of minerals are essential.

Research frontiers

The research hotspot is the study and improvement of mineral bioavailability from cereal foods with a different matrix under gastrointestinal conditions of infants and to obtain major knowledge about intestinal absorption of iron, calcium and zinc using the Caco-2 cell line.

Innovations and Breakthroughs

Low absorption of minerals from infant foods is considered to be a factor in the aetiology of mineral deficiencies in infants. Gastrointestinal conditions (pH and digestive enzymes) are keys in mineral absorption. Data indicate that the Caco-2 cell line is a useful tool to study iron and zinc absorption and simultaneously to characterize the effect of some food components on mineral intestinal absorption.

Applications

Better knowledge of intestinal mineral absorption process and interactions with dietary factors at a gastrointestinal level would be helpful to develop infant foods with improved mineral availability.

Terminology

Phytic acid: (my-inositol hexaphosphoric acid), a dietary factor found principally in cereals and legumes which is a potent inhibitor of mineral absorption owing to its strong ability to bind multivalent metal ions. Mineral bioavailability: the proportion of minerals that can be absorbed and used for physiological purposes.

Peer review

This is a descriptive study that shows the effect of dephytinization on the bioavailability of iron, calcium and zinc in different commercial infant cereals using the *in vitro* Caco-2 cell model. The manuscript is easy to understand and in general terms well written. The work described is well done.

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BRIEF ARTICLES

Intussusception in adults: Clinical characteristics, diagnosis and operative strategies

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Abstract

AIM: To evaluate 20 adults with intussusception and to clarify the cause, clinical features, diagnosis, and management of this uncommon entity.

METHODS: A retrospective review of patients aged > 18 years with a diagnosis of intestinal intussusception between 2000 and 2008. Patients with rectal prolapse, prolapse of or around an ostomy and gastroenterostomy intussusception were excluded.

RESULTS: There were 20 cases of adult intussusception. Mean age was 47.7 years. Abdominal pain, nausea, and vomiting were the most common symptoms. The majority of intussusceptions were in the small intestine (85%). There were three (15%) cases of colonic intussusception. Enteric intussusception consisted of five jejunojejunal cases, nine ileoileal, and four cases of ileocecal invagination. Among enteric intussusceptions, 14 were secondary to a benign process, and in one of these, the malignant cause was secondary to metastatic lung adenocarcinoma. All colonic lesions were malignant. All cases were treated surgically.

CONCLUSION: Adult intussusception is an unusual and challenging condition and is a preoperative diagnostic problem. Treatment usually requires resection of the involved bowel segment. Reduction can be attempted in small-bowel intussusception if the segment involved is viable or malignancy is not suspected; however, a more careful approach is recommended in colonic intussusception because of a significantly higher coexistence of malignancy.

INTRODUCTION

Intestinal invagination or intussusception is the leading cause of intestinal obstruction in children, but in adults it accounts for only 5% of all intussusceptions, and 0.003%-0.02% of all adult hospital admissions. In contrast to childhood intussusception, which is idiopathic in 90% of cases, adult intussusception has a demonstrable lead point, which is a well-definable pathological abnormality in 70%-90% of cases[1-3].

The presentation of pediatric intussusception often is acute with sudden onset of intermittent colicky pain, vomiting, and bloody mucoid stools, and the presence of a palpable mass. In contrast, the adult entity may present with acute, subacute, or chronic non-specific symptoms[4]. Therefore, the initial diagnosis often is missed or delayed and may only be established when the patient is on the operating table. Most surgeons accept that adult intussusception requires surgical resection because the majority of patients have intraluminal lesions. However, the extent of resection and whether the intussusception should be reduced remains controversial[5].

Therefore in this paper, we report our experience in an attempt to clarify the cause, clinical features, diagnosis, and management of this uncommon entity.

MATERIALS AND METHODS

The clinical, operative, and pathological records of 20 adult patients (> 18 years of age) with a diagnosis of intussusception, surgically treated between 2000 and 2008 were reviewed retrospectively. Patients with rectal prolapse, prolapse of or around an ostomy and gastroenterostomy intussusception were excluded.

Intussusception was classified as enteric or colonic. When the pathologic lead point was located in the small bowel, including jejunojejunal, ileoileal and ileocolic intussuceptions, it was classified as enteric. Colonic intussusception included ileocecal-colic, colocolonic, sigmoidorectal, and appendicocecal intussusception. Ileocolic and ileocecal-colic intussusception was distinguished by the site of the pathological lead point. When the lead point was at the ileum, intussusception was classified as ileocolic, whereas when the lead point was at the ileocecal valve, it was classified as ileocecal-colic.

RESULTS

Demographics

A total of 20 patients were identified who had a diagnosis of intussusception and were older than 18 years of age. The average age of the patients was 47.7 years, with a range of 21 to 75 years. Nine (45%) were male and 11 (55%) were female.

Clinical manifestations

Pain was the most common presenting complaint and was present in 17 patients (85%). Nausea, vomiting, constipation, rectal bleeding, and diarrhea were other symptoms. Table 1 shows the symtoms and signs. A palpable mass was found in only one patient (5%). The mean duration of symptoms was 7.9 d (range, 1 d to 3 mo). Six patients (30%) had acute symptoms (< 4 d), five (25%) had subacute symptoms (4-14 d), and nine (45%) had chronic symptoms (> 14 d).

Preoperative diagnostic studies

Plain abdominal X-rays were first obtained in patients with acute symptoms, which revealed air-fluid levels that suggested intestinal obstruction in five patients (25%). It was normal in the other 15 patients (75%).

Intussusception was a preoperative diagnosis in 14 patients (70%). Six patients (30%) who were diagnosed with intussusception in the operating room showed serious signs of bowel strangulation and were not diagnosed preoperatively because they were transferred to the operating room without further radiological evaluation. Abdominal computed tomography (CT) scan was performed in 12 patients, of whom 10 (83.3%) were diagnosed with intussusception. The finding on CT was an in-homogeneous soft-tissue mass that was target- or sausage-shaped (Figure 1). Three patients underwent colonoscopy, and intussusception was confirmed in two. A small-bowel series were performed in three patients. Two patients in diagnostic studies had findings

Table 1 Symptoms and signs of intussusception

Symptoms and signs	n (%)
Pain	17 (85)
Nausea	15 (75)
Vomiting	14 (70)
Constipation	3 (15)
Rectal bleeding	1 (5)
Diarrhea	1 (5)
Abdominal mass	1 (5)
Fever	1 (5)

Figure 1 Abdominal CT scan showing an in-homogeneous soft tissue mass that is target or sausage-shaped in an jejunojejunal intussusception (white arrow).

suspicious of intussusception caused by obstruction with polyps or tumors.

Location

The majority of intussusceptions were enteric (17/20 or 85%) (Table 2). There were three (15%) cases of colonic intussusception. Five cases of enteric intussusception were jejunojejunal (Figure 2A), nine were ileoileal, and four had ileocecal invagination detected.

Pathology

The pathological cause of intussusception was identified in 18 (90%) cases (Table 2). Benign pathology was seen in 14 cases (77.8%) and malignancy in four (22.2%). Among enteric intussusception, 14 cases were secondary to a benign process, including submucosal lipoma, Peutz Jeghers polyps, inflammatory fibroid polyp, intussuscepting Meckel diverticulum, fibroid polyp (Figure 2B), and congenital band adhesions. One malignant case was secondary to metastatic lung adenocarcinoma. All colonic intussusceptions resulted from a malignant lesion. The causes of colonic intussusception were secondary to primary adenocarcinoma in two cases and primary colonic lymphoma in one. No colorectal or rectorectal intussusception was identified in this study.

Treatment and consequences

All patients underwent operative treatment (Table 2). No hydrostatic reduction was attempted in any case. The choice of procedure was determined by the location, size, and cause of the intussusception and the viability

Table 2 Location, treatment and pathology

No. of patients	Age	Gender	Location of the lesion	Preoperative diagnosis	Surgical treatment	Pathology
1	33	M	Enteric (jejunojejunal)	+ (small bowel series)	Reduction + enterotomy + polypectomy	Peutz-Jeghers (hamartomatous polyp)
2	49	F	Enteric (ileocecal)	+ (CT)	Right hemicolectomy	Ileal lipoma
3	60	F	Enteric (ileoileal)	- (Urgent)	Reduction + segmental ileal resection	Inflammatory fibroid polyp
4	30	F	Enteric (ileocecal)	+ (CT)	Reduction + segmental ileal resection	Fibrous polyp
5	63	M	Colonic (colocolic)	+ (colonoscopy)	Near totalcolectomy + ileorectal anastomosis	Lenfoma
6	56	F	Enteric (ileocecal)	+ (CT)	Right hemicolectomy	Inflammatory fibroid polyp
7	21	F	Enteric (jejunojejunal)	+ (CT)	Reduction + segmental jejunal resection + enterotomy + polypectomy	Peutz-Jeghers (hamartomatous polyp)
8	36	M	Enteric (ileoileal)	- (Urgent)	Reduction	Idiopathic
9	75	M	Colonic (colocolic)	+ (colonoscopy)	Left hemicolectomy	Adeno CA
10	25	M	Enteric (ileoileal)	- (Urgent)	Reduction	Congenital band
11	60	M	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Ileal lipoma
12	28	F	Enteric (jejunojejunal)	- (Urgent)	Reduction + segmental jejunal resection	Inflammatory fibroid polyp
13	48	M	Enteric (jejunojejunal)	+ (CT)	Reduction + segmental jejunal resection	Idiopathic
14	55	F	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Ileal lipoma
15	62	F	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Inflammatory fibroid polyp
16	26	M	Enteric (jejunojejunal)	+ (small bowel series)	Reduction + enterotomy + polypectomy	Peutz-Jeghers (hamartomatous polyp)
17	48	F	Enteric (ileoileal)	- (Urgent)	Reduction + diverticulectomy	Meckel's diverticulum
18	72	M	Colonic (colocolic)	+ (CT)	Left hemicolectomy	Adeno CA
19	56	F	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Metastatic Adeno CA
20	51	F	Enteric (ileoileal)	- (Urgent)	Reduction + diverticulectomy	Meckel's diverticulum

Figure 2 Operative picture. A: Jejunojejunal intussusception; B: The fibroid lesion that acted as a lead point for ileocecal intussusception.

of the bowel. All 20 patients underwent laparotomy. En bloc resection (without reduction) was performed in five patients (25%), four of whom underwent right or left hemicolectomy, and one, subtotal colectomy and ileorectal anastomosis because of suspicion of malignancy. Reduction of the intussuscepted bowel was performed on the remaining 15 patients (75%). Among these, segmental resection was performed in nine, two of whom underwent diverticulectomy, two, enterotomy and polypectomy, and one, congenital band exision.

Postoperative complications occurred in four of 20 patients (20%): superficial wound infection in two (10%), pneumonia in one (5%), and severe sepsis in one (5%). There were no anastomotic leaks or intra-abdominal abscesses. There was one perioperative death (5%), which was secondary to severe sepsis complicated by multiple organ failure 6 d after the operation.

DISCUSSION

Adult intussusception is an uncommon clinical entity encountered by surgeons. The exact mechanism is unknown, and it is believed that any lesion in the bowel wall or irritant within the lumen that alters normal peristaltic activity is able to initiate invagination[2,6]. Ingested food and the subsequent peristaltic activity of the bowel produce an area of constriction above the stimulus and relaxation below, thus telescoping the lead point (intussusceptum) through the distal bowel lumen (intussuscipiens)[1,2]. The most common locations are at the junctions between freely moving segments and retroperitoneally or adhesionally fixed segments[6,7].

About 90% of occurrences in adults have a lead point, a well-definable pathological abnormality. In general, the majority of lead points in the small intestine

consist of benign lesions, such as benign neoplasms, inflammatory lesions, Meckel's diverticuli, appendix, adhesions, and intestinal tubes. Malignant lesions (either primary or metastatic) account for up to 30% of cases of intussusception in the small intestine[2,5]. On the other hand, intussusception occurring in the large bowel is more likely to have a malignant etiology and represents up to 66% of the cases[2,5,6,8]. In our study malignant etiology was detected in all cases of colonic intussusception.

The clinical presentation in adult intussusception is often chronic, and most patients present with non-specific symptoms that are suggestive of intestinal obstruction. Abdominal pain is the most common symptom followed by vomiting and nausea[1,2]. Abdominal masses are palpable in 24%-42% of patients, and identification of a shifting mass or one that is palpable only when symptoms are present is suggestive of intussusception or volvulus[1,2,5]. In our series, an abdominal mass was only palpable in one patient (5%).

The symptoms in cases of adult intussusception are so non-specific that a clinical diagnosis beyond bowel obstruction is rarely made before surgery.

Several imaging techniques may help to precisely identify the causative lesion preoperatively. Plain abdominal X-rays are typically the first diagnostic tool and show signs of intestinal obstruction, and may provide information regarding the site of obstruction[8,9]. Contrast studies can help to identify the site and cause of the intussusception, particularly in more chronic cases. Upper gastrointestinal series may show a "stacked coins" or "coiled spring" appearance[8]. Barium enema examination may be useful in patients with colonic or ileocolic intussusception in which a "cup-shaped" filling defect is a characteristic finding[8]. Barium studies are obviously contraindicated if there is the possibility of bowel perforation or ischemia.

Colonoscopy is also a useful tool for evaluating intussusception, especially when the presenting symptoms indicate a large bowel obstruction[2,10,11]. It may not be advisable to perform endoscopic biopsy or polypectomy in those individuals with long-term symptoms because of the high risk of perforation, which is more likely to happen in the phase of chronic tissue ischemia, and perhaps necrosis because of vascular compromise in intussusception[12].

In our series, three patients underwent colonoscopy, and intussusception was confirmed in two (66.6%). A small-bowel series were performed in three patients. Two (66.6%) patients in diagnostic studies had findings suspicious of intussusception because of obstruction with polyps or tumors.

Ultrasonography has been used to evaluate suspected intussusception. The classic features include the "target and doughnut sign" on transverse view and the "pseudokidney sign" in longitudinal view. The major disadvantage of ultrasound is masking by gas-filled loops of bowel, and operator dependency[10,11,13,14].

In recent years, CT has become the first imaging method performed, after plain abdominal X-rays, in the evaluation of patients with non-specific abdominal complaints. The characteristics of intussusception on CT are an early "target mass" with enveloped, excentrically located areas of low density. Later, a layering effect occurs as a result of longitudinal compression and venous congestion in the intussusceptum[15]. Abdominal CT has been reported to be the most useful tool for diagnosis of intestinal intussusception and is superior to other contrast studies, ultrasonography, or endoscopy[15-18]. The reported diagnostic accuracy of CT scans was 58%-100%, especially in recent series[1,4,6,16,19]. The diagnosis of intussusception was based on CT findings in the majority of our cases (10/12); two were based on colonoscopy and two on a small-bowel series. The accuracy was 83.3% for CT, 66.6% for colonoscopy, and 66.6% for small-bowel series. The preoperative diagnostic accuracy was 70% in our series.

Although few reports have described magnetic resonance imaging (MRI) of adult intussusception, the general imaging characteristics of intussusception on MRI are similar to those on CT[18,20], but fast MR examination, unlike CT, is not technically limited by the presence of previously administered barium for small bowel series[21].

The optimal management of adult intussusception remains controversial. Most of the debate focuses on the issue of primary en bloc resection versus initial reduction, followed by a more limited resection[1,2,19,22]. Proponents of primary resection cite the high incidence of underlying malignancy, especially in colonic lesions, which mandates en bloc resection. Furthermore, the inability to differentiate malignant from benign etiology preoperatively or intraoperatively also dictates that small bowel intussusception be resected without reduction. The reduction of an intussusception secondary to a malignant lead point is potentially detrimental, as there is the theoretic risk of intraluminal seeding and venous embolization in regions of ulcerated mucosa. Other drawbacks include the increased risk of anastomotic complications (the bowel wall may be weakened during manipulation) and the potential for bowel perforation[1,5,6,23,24].

On the other hand, some authors have recommended a selective approach to resection, taking into consideration the site of intussusception, which influences the type of pathology[2,25]. They advocate resection of all colonic lesions but a more selective approach for small bowel pathology, as the lower malignancy rate for small bowel intussusception makes the argument for initial resection less convincing.

Recently, minimally invasive techniques have been applied to the treatment of small or large bowel obstructions, specifically to the diagnosis and treatment of adult intussusception. There are several case reports about laparoscopic small bowel resection because of intussusception[26,27]. The choice of using a laparoscopic or open approach depends on the clinical condition of the patient, the location and extent of intussusception, the possibility of underlying disease, and the availability of surgeons with sufficient laparoscopic expertise[28,29]. In the present study, we did not use laparoscopy for diagnosis or treatment.

In conclusion, intussusception in adults is a rare entity and diagnosis may be challenging because of non-specific symptoms. Surgeons should be familiar with the various treatment options, because the real cause of the intussusception often is accurately diagnosed by laparotomy. CT is the most useful imaging modality in the diagnosis of intussusception. Treatment usually requires resection of the involved bowel segment. Reduction can be attempted in small-bowel intussusception if the segment involved is viable or malignancy is not suspected; however, a more careful approach is recommended in colonic intussusception because of a significantly higher chance of malignancy.

COMMENTS

Background
Intestinal intussusception in adults is a rare entity and there is an ongoing controversy regarding the optimal management of this problem. Most surgeons accept that adult intussusception requires surgical resection because the majority of patients have intraluminal lesions. However, the extent of resection and whether the intussusception should be reduced remains controversial.

Research frontiers
Authors aimed to evaluate their experience with 20 adult intussusception cases and to clarify the cause, clinical features, diagnosis, and management of this uncommon entity.

Applications
The present study was retrospective; however, it highlights the clinical features in adult intussusception and may guide surgeons who encounter this problem.

Terminology
Intussusception occurs when a segment of bowel and its mesentery (intussusceptum) invaginates the downstream lumen of the same loop of bowel (intussuscipiens). Sliding within the bowel is propelled by intestinal peristalsis and may lead to intestinal obstruction and ischemia.

Peer review
This is an interesting retrospective study with excellent figures. Although the authors do not show any case with laparoscopic approach, the general statement should be more positive and oriented towards the practice in 2009.

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Computer simulation of flow and mixing at the duodenal stump after gastric resection

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Abstract

AIM: To investigate the flow and mixing at the duodenal stump after gastric resection, a computer simulation was implemented.

METHODS: Using the finite element method, two different Billroth Ⅱ procedure cases (A and B) were modeled. Case A was defined with a shorter and almost straight duodenal section, while case B has a much longer and curved duodenal section. Velocity, pressure and food concentration distribution were determined and the numerical results were compared with experimental observations.

RESULTS: The pressure distribution obtained by numerical simulation was in the range of the recorded experimental results. Case A had a more favorable pressure distribution in comparison with case B. However, case B had better performance in terms of food transport because of more continual food distribution, as well as better emptying of the duodenal section.

CONCLUSION: This study offers insight into the transport process within the duodenal stump section after surgical intervention, which can be useful for future patient-specific predictions of a surgical outcome.

INTRODUCTION

The duodenum is a short, complex and functionally highly specialized part of the small intestine. It has many motor, sensitive and secretory functions. Transit of chyme through the duodenum is a very complicated process (which includes intestinal peristalsis, gastric emptying, and pyloric sphincter tone) and is regulated by many neurological and hormone-dependent feedback mechanisms[1-6].

Two layers of smooth muscle cells (inner-longitudinal and internal-circumferential), as well as two neurological intramural networks (*Auerbach's* and *Meisner's* complexes), are responsible for gastroduodenal peristalsis. Electrical activity of the smooth muscle cell syncytium of the duodenum and in other parts of the gastrointestinal tract is represented by two basic types of electrical waves: slow waves and the spikes. Slow waves represent basic electrical activity and they are caused by activity of the Na-K pump. When the resting membrane potential become less negative than -40 mV, the spikes appear on the top of slow waves with a frequency of 1-10 Hz,

Figure 1 Normal physiological gastric outlet, pylorus, and superior duodenum.

and cause smooth muscle contraction. The number of duodenal contractions is about 12/min[7]. Several new investigations suggest that slow waves and spikes are regulated and spread through different cell networks[8].

The pylorus shown in Figure 1 comprises a collection of tissue structures that connect the antrum to the duodenum. Its luminal diameter is controlled by a sphincter muscle complex that sets resistance to the bulk gastric effluent, through regulation of the pyloric orifice tone. During gastric digestion, the proximal and distal pyloric muscle loops occlude the pyloric lumen, preventing premature discharge of unprocessed material into the duodenum. Once the stomach has completed its task of breaking down large solid agglomerates into smaller particles, the pylorus relaxes and peristaltic contractions in the antrum begin to force chyme distally. At this point, antral contraction waves approach the pyloric orifice and, along with the sphincter complex and mucosal folds, cause steady constriction of the pyloric lumen. Chyme continues to be forcibly transported through this lumen until it is fully occluded, a process thought to induce an effluent jet into the superior duodenum[9]. Dysfunction of the duodenum can occur as a result of many disorders of gastric emptying and dyspeptic complaints, which demonstrates the vital role of the duodenum[10]. This role becomes especially apparent in surgical interventions on the gastroduodenum[11].

A large number of studies have investigated the intact gastrointestinal tract anatomically[12-14], but there have been relatively few studies on the consequences of cutting muscles, nerves and other important anatomical structures of the gastroduodenum. These are unavoidable during surgical interventions, with disturbance of many fine, highly sophisticated feedback systems. These undesirable conditions lead to negative feedback mechanisms[15,16] that cause changes in physiological processes with respect to the preoperative state[17,18].

To date, it seems that insufficient attention has been paid to how the geometry and flow conditions affect the gastroduodenal system after distal gastric resection. There are various types of reconstruction

Figure 2 Schematic representation of reconstruction of gastrointestinal continuity after gastric resection. A: Normal anatomy of gastroduodenal region with resection lines; B: Billroth II antiperistaltic anastomosis.

of gastrointestinal continuity after gastric resection. In studying the treatment of gastric cancer, Devin reported in 1968 about 300 types of reconstruction after surgery of the gastroduodenal region[19]. During recent years, a few types have become more frequent. The first and most physiological variant is a state in which continuity of the gastrointestinal tract is reconstructed with anastomosis between the gastric stump and the duodenum; this procedure is called Billroth I gastric resection (gastroduodenal anastomosis). The other type of reconstruction is the Billroth II operation, which is shown in Figure 2, in which the anastomosis is located between the gastric stump and a loop of jejunum (gastrojejunal anastomosis, gut to side), and this type of intervention is the subject of our study. Anastomosis may include the entire circumference of the gastric stump (today it is a rarely used technique because of many unwanted consequences) or just a part of the circumference when a smaller diameter anastomosis is created. This is a better method of adaptation of the gastric stump and jejunal loop-the Hoffmeister-Finsterer modification. The length of the proximal jejunal loop is variable and depends on anatomical variations (e.g. length of the mesenterium of the small intestine, and adhesions), as well as surgeons' ability. The jejunal loop conducts duodenal juice toward the gastric stump and the rest of the intestine. There is a hypothesis that increased intraluminal pressure in the afferent loop is the dominant cause of duodenal suture

dehiscence (caused by the length of afferent jejunal loop, narrow gastrojejunoanastomosis, *etc*[20-22]). The distal or efferent loop is the part of the duodenum that is downstream of the anastomosis, and it conducts duodenal and gastric stump content distally to the small intestine. The anastomosis is antecolic when the jejeunal loop is positioned in front of the transverse colon, or the jejunal loop may be brought up posteriorly through an opening made in the transverse mesocolon (retrocolic anastomosis). Adequate position, shape and diameter of the anastomosis facilitate gastric emptying. The orientation of the jejunal loop may be two-fold, isoperistaltic, when the stomach and jejunum have the same peristalsis direction, and antiperistaltic, when the stomach and afferent loop of the jejunum have the opposite direction of peristalsis.

There are other methods of gastrointestinal tract reconstruction after gastric resection, and one of these is called Roux en Y gastrojejunoanastomosis, in which the small bowel is cut distal to the ligament of Treitz, and the anastomosis is created between the distal limb of the jejunum and remaining gastric tissue (or esophagus in cases of total gastrectomy). The proximal limb of the jejunum is positioned downstream to the jejunum at a distance of about 45 cm, where a termino-lateral end-to-side anastomosis is created[23,24].

In gastrointestinal tract reconstruction, the small bowel is not prepared to receive acidic content from the stomach, especially when duodenal juice is not present to neutralize it[25]. Suture dehiscence with postoperative peritonitis can occur as a complication after surgical intervention. Duodenal stump blowout is considered to be a serious postoperative complication, because of its high mortality rate[26,27]. Suture dehiscence after surgical intervention in the gastroduodenal region has been the subject of many investigations, but still many pathological mechanisms involved in this surgical problem remain unclear[28].

This study offers a new approach to this problem. Using finite element analysis with computer modeling and clinical experiments, we attempted to determine physiological constants relevant to the above-mentioned surgical complication. In order to achieve this goal, we computed flow and mixing in the duodenal junction after distal gastric surgical resection and a Billroth II procedure.

MATERIALS AND METHODS

A prospective, randomized, placebo-controlled, multicenter study was performed on patients treated with Billroth II Hofmeister-Finsterer subtotal gastrectomy. Our task was to investigate and quantify the effects of increased intraluminal pressure in the duodenal stump on disruption of the duodenal suture.

Subjects

Measurements were performed on five patients after Billroth II Hofmeister-Finsterer surgical intervention. The indication for surgery was a malignant gastric

Figure 3 Schematic representation of the manometry equipment positioned in a patient during pressure measurement.

process in three cases and ulcer disease in two. Patients with no significant co-morbidity were chosen for the study. Each patient gave written consent to our investigation. No complications were observed, and no complaints were made by these patients in relation to our actions for the purpose of this study.

Manometry equipment

We created hardware and software equipment for measurement of pressure in the duodenal stump after distal gastric resection. The manometry assembly consisted of a Miller-Abbott tube, connecting catheters with control valves, transducers, analog-to-digital (AD) converter, and a computer with appropriate software. A Miller-Abbott tube 3.7 m in length, with an outer diameter of 5 mm, was placed in the duodenum after the operation (Figure 3). The volume of the balloon on top of the Miller-Abbott tube was 30 mL and the balloon expanded under pressure. The length of the balloon was 7 cm. The proximal end of the two-lumen tube was divided in two channels: one that connected the balloon with the measurement assembly of catheters, transducers, AD converter and personal computer; and the other used for aspiration of the duodenal stump. Distilled water was injected manually through the first channel into the balloon positioned in the duodenal stump. Data were recorded by an in-house developed software and stored to disk for later analysis. The measuring system was mobile and could be used outside the laboratory.

Protocol

The balloon of the Miller-Abbott tube was positioned at the duodenal bulb with minimum air insufflation, followed by direct visual control. The duodenum and anterior wall of the gastrojejunal anastomosis were sutured after appropriate placing of the balloon. The entire procedure lasted 3-5 min and did not have any influence on the operative procedure itself. The final location of the catheter was documented by checking X-rays before starting measurements and again before removal. Catheter migration from the duodenum was not observed. However, duodenal motility activity was noticed in two cases.

A

Figure 4 Computer simulation of pressure measurement inside duodenum bulb. A: Schematic representation of the computational model of balloon pressure measurement inside the duodenum. Geometry of the model (a); 3D model view (b); B: Distribution of solid deformation (wall displacement) at the maximum pressure measurement (a); Fluid pressure distribution inside the duodenum (b).

B

Figure 5 Geometry and finite element mesh of two selected cases. Case A showed a straight duodenal part with shorter length. Case B had a much longer and curved duodenal section.

After the catheter was positioned and each subject was at rest for at least 24 h, all measurements were performed on patients in the supine position. Data files were recorded for each patient during seven postoperative days, starting 24 h after intervention. Data files consisted of pressure measurements over time. Prostigmine was used to stimulate bowel peristaltic movements. Every measurement lasted about 30 min. All external impacts that could cause some changes in duodenal intraluminal pressure were recorded in diagram form, as contractions of the anterior abdominal wall, cough, patient movements and administration of prostigmine.

Patients were discharged from the clinic after 10 d.

They continue to be under observation and come to follow-up clinical examinations.

Details about the calculation of pressure inside the duodenal bulb using finite element fluid-solid analysis are as follows.

Computer simulation of pressure measurement inside duodenum bulb: We modeled balloon measurement of duodenal intraluminal pressure by using a finite element method[29,30]. The balloon and the duodenal walls were discretized by 2D solid finite elements, while 2D fluid elements were used for the fluid domain (Figure 4A). A simplified axi-symmetric model was adopted. The thickness of the balloon wall was 0.05 cm and the outer wall was 0.2 cm. The length of the balloon was 7.0 cm, with a distance from the outer wall of 2.0 cm. Fluid density was taken as 1 g/cm^3, dynamic viscosity μ was 1000 cP, Young's elasticity modulus E was 1 MPa. Fluid and solid domains had common boundaries at the interfaces on the balloon wall and duodenal wall. Air pressure was applied to the balloon wall, which caused flow of the fluid surrounding the balloon. The air pressure p_1 inside the balloon was measured, and the fluid pressure p_2 between the balloon and duodenal wall was calculated. The balloon deformed as a result of the difference between the air and fluid pressures. The deformed walls and fluid pressure distribution inside the duodenum are shown in Figure 4B. They corresponded to a maximum inlet balloon pressure of 2000 Pa (15 mmHg). The maximum balloon displacement was 0.4 cm, and the maximum

fluid pressure within the duodenum was around 338 Pa (2.54 mmHg), which was in the physiological range.

Computational analysis

Computational analysis aimed to examine two different Billroth Ⅱ antiperistaltic anastomosis cases. Case A represents gastric resection with a shorter and straight part of the duodenum, while case B, has a much longer and curved duodenal section. The geometry with finite element mesh for both cases is shown in Figure 5.

A viscous incompressible fluid flow was considered here as the model for the transit of chyme. This flow is governed by the Navier-Stokes equations and continuity equation that can be written as[30]:

$$\rho \left(\frac{\partial v_i}{\partial t} + v_j \frac{\partial v_i}{\partial x_j} \right) = -\frac{\partial p}{\partial x_i} + \mu \left(\frac{\partial^2 v_i}{\partial x_j \partial x_j} + \frac{\partial^2 v_j}{\partial x_j \partial x_i} \right) \quad (1)$$

$$\frac{\partial v_i}{\partial x_i} = 0 \quad (2)$$

where v_i is the chyme velocity in direction x_i (the coordinate axes are $x_1 \equiv x$ and $x_2 \equiv y$), ρ is the fluid density, p is pressure, μ is the dynamic viscosity; and summation is implied on the repeated (dummy) indices, $i,j = 1.2$. The first equation represents balance of linear momentum, while equation (2) expresses incompressibility condition.

To take into account mass transport of food, we used the additional transport equation. The assumption is that the concentration of food does not affect the gastric fluid flow (i.e. a diluted mixture is considered). The food mass transport process is governed by the convection-diffusion equation,

$$\frac{\partial c}{\partial t} + v_x \frac{\partial c}{\partial x} + v_y \frac{\partial c}{\partial y} = D \left(\frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} \right) \quad (3)$$

where c denotes the food concentration; v_x, v_y are the gastric velocity components in the coordinate system x, y; and D is the diffusion coefficient, assumed constant, of the transported food.

The code was validated using the analytical solution for shear stress and velocities through a straight tube[29,30]. The finite element equations were solved with respect to velocities after the pressure was eliminated by a so-called penalty procedure. The solution was obtained by use of an incremental-iterative approach and implicit integration scheme over time. The system of algebraic equations of balance for a finite element, and for a time step and equilibrium iteration "m", can be written as:

$$\begin{bmatrix} \frac{1}{\Delta t}\mathbf{M}_v + {}^{t+\Delta t}\mathbf{K}_{vv}^{(m-1)} + {}^{t+\Delta t}\mathbf{K}_{mv}^{(m-1)} + & \\ {}^{t+\Delta t}\hat{\mathbf{K}}_{mv}^{(m-1)} + {}^{t+\Delta t}\mathbf{J}_{vv}^{(m-1)} + \mathbf{K}_{1v} & \mathbf{0} \\ {}^{t+\Delta t}\mathbf{K}_{cv}^{(m-1)} & \frac{1}{\Delta t}\mathbf{M}_c + {}^{t+\Delta t}\mathbf{K}_{cc}^{(m-1)} + {}^{t+\Delta t}\mathbf{J}_{cc}^{(m-1)} \end{bmatrix} \begin{Bmatrix} \Delta \mathbf{v}^{(m)} \\ \Delta c^{(m)} \end{Bmatrix} = \begin{Bmatrix} {}^{t+\Delta t}\hat{\mathbf{F}}_v^{(m-1)} \\ {}^{t+\Delta t}\mathbf{F}_c^{(m-1)} \end{Bmatrix}$$

$$(4)$$

where the detailed expressions for the matrices and vectors can be found elsewhere (e.g.[29-31]).

Figure 6 Geometry and boundary conditions for a Billroth Ⅱ Hoffmeister-Finsterer operation.

RESULTS

We here present typical results obtained by computer modeling and by our experimental investigation.

To examine different flow conditions, a few cases were considered within a simple 2D model. The steady state inflow conditions were imposed by specifying inlet fluxes at two different locations: gastric inlet with Q_1 = 2.2 (l/d) and duodenum inlet with Q_2 = 2.0 (l/d), as shown in Figure 6. These two flow conditions corresponded to a postoperative period of 3-4 d after distal gastric resection. Fluid density was taken as 1 g/cm³ and dynamic viscosity μ was 1000 cP. For the convective-diffusion equation (3), boundary conditions were prescribed by the inlet food concentration c_0 = 1.0 (g/cm³).

For the prescribed flow conditions at the inlets, steady state velocity distributions for both cases are shown in Figure 7. It can be seen that maximum velocity occurred at the inlet duodenal section, which was dominant during steady state flow conditions, such that fluid was moving distal to the remainder of the small intestine. Also, a maximum pressure (shown in Figure 8) was found near the pyloric section in the duodenal zone where the gastric resection was performed. Case B showed a higher pressure in comparison to case A, which was expected because of the longer section of duodenum in case B. This directly implies possible complications for case B because the risk of undesirable duodenal stump blowout was higher.

Mixing effect during postoperative period was examined by using mass transport analysis coupled with Navier-stokes equations. The influence of the diffusivity coefficient on the mass transport was investigated. Some authors[8] have suggested a negligible diffusivity which means that mass species simply advect along the fluid streamlines. Mass concentration distribution for the diffusion coefficient D = 2.0e-3 cm²/s for both cases A and B is presented in Figure 9. It can be seen from Figure 9 that maximal food concentration was located in the duodenal zone for both cases, which means that the food stays in this zone if the diffusion coefficient D is 2.0e-3 cm²/s. If the coefficient of diffusion is four times larger, at 8.0e-3 cm²/s, mass concentration distribution is

Case A

Case B

5.28e-02
4.95e-02
4.62e-02
4.29e-02
3.96e-02
3.63e-02
3.30e-02
2.97e-02
2.64e-02
2.31e-02
1.98e-02
1.65e-02
1.32e-02
9.90e-03
6.60e-03
3.30e-03
0.00e+00

5.07e-02
4.75e-02
4.43e-02
4.12e-02
3.80e-02
3.48e-02
3.17e-02
2.85e-02
2.53e-02
2.22e-02
1.90e-02
1.58e-02
1.27e-02
9.50e-03
6.33e-03
3.17e-03
0.00e+00

Figure 7 Velocity distribution for the steady state flow conditions [in (cm/s)].

Case A

Case B

2.01e+00
1.89e+00
1.76e+00
1.64e+00
1.51e+00
1.39e+00
1.26e+00
1.13e+00
1.01e+00
8.83e-01
7.58e-01
6.33e-01
5.07e-01
3.82e-01
2.56e-01
1.31e-01
5.29e-03

2.54e+00
2.38e+00
2.22e+00
2.06e+00
1.91e+00
1.75e+00
1.59e+00
1.43e+00
1.27e+00
1.11e+00
9.55e-01
7.97e-01
6.39e-01
4.80e-01
3.22e-01
1.64e-01
5.41e-03

Figure 8 Pressure distribution for the steady state flow conditions [in (mmHg)].

Case A

Case B

3.55e+00
3.39e+00
3.23e+00
3.07e+00
2.91e+00
2.75e+00
2.59e+00
2.43e+00
2.27e+00
2.12e+00
1.96e+00
1.80e+00
1.64e+00
1.48e+00
1.32e+00
1.16e+00
1.00e+00

2.34e+00
2.26e+00
2.17e+00
2.09e+00
2.00e+00
1.92e+00
1.84e+00
1.75e+00
1.67e+00
1.59e+00
1.50e+00
1.42e+00
1.34e+00
1.25e+00
1.17e+00
1.08e+00
1.00e+00

Figure 9 Mass concentration [in (g/cm^3)] distribution for the diffusion coefficient D = 2.0e-3 cm^2/s.

different (Figure 10). Obviously, for the higher diffusivity coefficient (according to our analysis, D = 8.e-3 cm^2/s) mass transport of food will go smoothly to the rest of gastrointestinal system, which is a desirable outcome. This effect of diffusivity is expected since diffusion enhances mass transport. There is also a difference in these two cases A and B, when the diffusivity is higher. It can be seen from Figure 9 that in case B, the duodenum section looked empty, while in case A there was approximately uniform mass distribution. This was probably the effect of the duodenal section. Therefore, the conditions in case B were better for the patient. A

lower diffusivity coefficient D caused a more dominant convective term in equation (3), which resulted in food accumulation in the duodenal section. This is clearly shown in Figure 9, in which cases A and B had maximum food concentration in the duodenal zones. This means that a patient after this surgical procedure may have food accumulation in the duodenal zone, which is not desirable.

We tested cases A and B for D > 8.e-3 cm^2/s (data not shown), for which computational analysis indicated a slightly better performance in the gastroduodenal connection for case A, for which more food passed

Figure 10 Mass concentration [in (g/cm^3)] distribution for the diffusion coefficient D = 8.0e-3 cm^2/s.

Figure 11 Pressure measurement of Miller-Abbott (MA) tube and connecting catheter in the duodenum at 3 d after gastric resection.

through the duodenal zone.

A measurement technique described in the Methods section and Appendix was implemented in order to measure pressure inside the duodenum a few days after surgical intervention. Patients agreed that this measurement technique could be used at 3-4 d after gastric resection. Among five patients, there was one with duodenal stump leakage, and one died from a non-surgical complication (pulmonary embolism). The contractile events associated with pressure changes were detected and recorded with our in-house software. We recorded pressure changes every 0.1 s. The pressure range shown in Figure 11 for the air inside the balloon was around 15 mmHg, while the fluid pressure inside the duodenum was between 3.5 and 4.5 mmHg (which was in the physiological range[32]). Also, numerical simulations (Figure 8) for cases A and B showed similar pressure distributions (around 2.5 mmHg).

DISCUSSION

The rate of postoperative gastric resection complication seems to be primarily influenced by increased duodenal pressure. Localization and extent of the malignant process, suture material, surgical technique, presence of potentially pathogenic microorganisms colonizing the digestive tract, and tissue quality, also may play a role in

the pathogenesis of this complication[33,34]. In this study, we focused on numerical simulation of two different Billroth Ⅱ antiperistaltic anastomosis cases, with a slightly different geometrical connection of the duodenal and gastric parts during gastric resection. Numerical solutions of velocity and pressure distribution for both cases gave similar results. As expected, the maximum pressure was localized in the duodenal suture area and it was in the range of experimental pressure measurements obtained by using a Miller-Abbott tube and connecting catheter. Case B showed higher pressures, which was the result of the longer duodenal section.

Additional transport analysis showed that the food distribution in the gastric and duodenal connection zone varied depending on the diffusivity coefficient. The results of this analysis implied that case B was more favorable with respect to food transport because of continual food distribution and an empty duodenal section. Therefore, the longer duodenal section in case B provides conditions for food not to stay within the duodenal zone.

Experimental results have shown that intraluminal duodenal stump pressure after gastric resection was higher by an order of magnitude with respect to the pre-operative state. Comparison of models with different lengths of afferent loops showed that the afferent loop syndrome was an important factor in postoperative complications.

By changing input parameters of our interactive computational model (fluid parameters, model geometry), we can improve prediction of the potential *in vivo* events. Also, it is possible to obtain insight into possible outcomes of different types of surgical reconstruction of the digestive tract after operations on the gastroduodenal region. This may include variations of the same surgical intervention, related to surgeons' ability or the specific anatomy of a single patient.

The practical aim of this study was to provide an insight into the physical conditions within the duodenal stump after gastric resection, in relation to the pathogenesis of duodenal stump blowout, and the implications for the surgical technique itself.

New, more comprehensive computational models may first include duodenal pressure quantification,

which causes disruption of the duodenal stump closure. Such models will require determination of shear stress and pressure distribution at the inner surface of the duodenal stump, as well as the stress-strain state within the duodenal wall, especially in the suture area. The stress-strain state and the critical state at the point of disruption depend on tissue quality and tissue material characteristics. Furthermore, these sophisticated models need to establish a correlation between duodenal flow and the wall dynamics of contractions, in order to quantify critical levels of increased intraluminal duodenal pressure.

COMMENTS

Background
A malignant process and ulcer disease in the gastric distal section are the main causes for surgical intervention, which has become a standard procedure over recent years. Duodenal stump dehiscence after gastric resection occurs relatively rarely, but is of particular importance because it is associated with a high rate of mortality. A computer simulation can provide better understanding of this process.

Innovations and breakthroughs
Computer simulation offers better insight into fluid flow, mass transport and pressure distribution in the duodenal section before and after surgical intervention.

Applications
This computer simulation study can be applied to new follow-up studies that will help clinicians with diagnosis and treatment.

Terminology
Computer simulation and a finite element method solve a large number of equations in which physical laws such as fluid flow and mass transport are solved with differential partial equations. This methodology has been well known in industrial applications over several decades, and recently, it has been used for biological systems. Many questions regarding parameters in this computational study are still unknown and future computer simulation will try to answer these.

Peer review
The title describes well the manuscript. The introduction is clear. The description of the materials and methods is clear. The results are reported well. The discussion is well organized. The references are well reported. the tables and figures are clear.

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Transjugular intrahepatic portosystemic shunt in liver transplant recipients

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Abstract

AIM: To evaluate the efficacy of transjugular intrahepatic portosystemic shunts (TIPSs) after liver transplantation (LT).

METHODS: Between November 1996 and December 2005, 10 patients with severe recurrent hepatitis C virus infection (n = 4), ductopenic rejection (n = 5) or portal vein thrombosis (n = 1) were included in this analysis. Eleven TIPSs (one patient underwent two TIPS procedures) were placed for management of therapy-refractory ascites (n = 7), hydrothorax (n = 2) or bleeding from colonic varices (n = 1). The median time interval between LT and TIPS placement was 15 (4-158) mo.

RESULTS: TIPS placement was successful in all patients. The mean portosystemic pressure gradient was reduced from 12.5 to 8.7 mmHg. Complete and partial remission could be achieved in 43% and 29% of patients with ascites. Both patients with hydrothorax did not respond to TIPS. No recurrent bleeding was

seen in the patient with colonic varices. Nine of 10 patients died during the study period. Only one of two patients, who underwent retransplantation after the TIPS procedure, survived. The median survival period after TIPS placement was 3.3 (range 0.4-20) mo. The majority of patients died from sepsis with multiorgan failure.

CONCLUSION: Indications for TIPS and technical performance in LT patients correspond to those in non-transplanted patients. At least partial control of therapy-refractory ascites and variceal bleeding could be achieved in most patients. Nevertheless, survival rates were disappointing, most probably because of the advanced stages of liver disease at the time of TIPS placement and the high risk of sepsis as a consequence of immunosuppression.

INTRODUCTION

Since the first attempts were made over 30 years ago, placement of transjugular intrahepatic portosystemic shunts (TIPSs) has become an established procedure in patients with complications of portal hypertension[1]. The two main indications for TIPS are therapy-refractory ascites and variceal bleeding unresponsive to endoscopic treatment[2-7]. In many patients with these complications,

TIPS is used as a bridge to liver transplantation (LT). In contrast, there is rather limited experience with the use of TIPS following LT. One reason is the rare occurrence of portal hypertension after LT. The causes of portal hypertension in such patients can be impaired venous outflow, recurrence of the underlying liver disease, size mismatch between donor and recipient organs and/or vessels[8,9], or increased vascular resistance as a consequence of repeated rejection episodes[10]. This might result in the development of ascites, hepatic hydrothorax or variceal bleeding comparable to the non-grafted population. Therapeutic options for these conditions are basically the same, including TIPS. The placement of a TIPS can be rendered more difficult by the altered anatomy after transplantation. Furthermore, patients undergoing chronic immunosuppression are at higher risk of infection.

Only few data have been published on TIPS after LT and its role in LT recipients is largely undefined. The aim of our retrospective analysis was to critically scrutinize the indications, efficacy and safety of TIPS placement in liver recipients at our center.

MATERIALS AND METHODS

Between November 1996 and December 2005, a total of 11 TIPSs were placed in 10 liver recipients at Innsbruck Medical University Hospital, which represents 5% of all 217 TIPS procedures carried out during this time period. One of the patients received a TIPS before and after retransplantation. The mean age of the six male and four female patients was 56.8 (37-71) years. The underlying liver diseases were hepatitis C virus (HCV) cirrhosis ($n = 4$), alcoholic liver disease ($n = 2$), primary biliary cirrhosis ($n = 2$), hemochromatosis ($n = 1$) and autoimmune hepatitis ($n = 1$).

Patients underwent full-sized deceased donor LT, three of them using a piggyback technique, and the remaining patients by replacement of the retrohepatic vena cava. The mean time interval between LT and TIPS was 29.7 mo (range: 3.8-158 mo). In four patients, recurrent HCV cirrhosis was present at the time of TIPS implantation, five had ductopenic rejection, and one had portal vein thrombosis.

Therapy-refractory ascites was the indication for TIPS in seven patients, resistant hydrothorax in two, and bleeding from colonic varices in one. Ascites and hydrothorax were assessed by ultrasound and chest X-ray, respectively.

All four patients with recurrent HCV presented with decompensated cirrhosis at the time of TIPS implantation. One patient was in Child-Turcotte-Pugh class B and three were in class C. The median model for end-stage liver disease (MELD) score for all patients was 20 (12-35).

The TIPS procedure used for LT recipients did not differ from that for non-transplanted patients, as described previously[6,7].

The immunosuppressive regimen at the time of the TIPS procedure consisted of calcineurin inhibitors,

alone ($n = 1$) or in combination with steroids ($n = 2$), mycophenolate mofetil (MMF; $n = 3$), or an mTOR-inhibitor ($n = 1$). An mTOR-inhibitor was used with MMF in one patient or with low-dose steroids in three patients.

Variables were compared using Student's t test, and $P < 0.05$ was considered statistically significant. Kaplan-Meier plots were calculated using SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

TIPS were placed in all patients without any procedural complications. One patient with pre-existing atrial fibrillation developed cardiac failure during the procedure but responded to specific treatment.

The mean portosystemic pressure gradient was reduced from 12.5 (8-22) mmHg to 8.7 (5-14) mmHg after the procedure. Although pressure gradients below 12 mmHg were found in three patients with refractory ascites and one with hydrothorax, the TIPS procedure was continued, with the aim of further decreasing the final pressure gradients (around 5 mmHg), in order to improve the clinical condition.

Regarding patients with refractory ascites, complete resolution of ascites was achieved in three and a partial response in two patients, whereas no response was seen in two others. TIPS failed to improve the condition in both patients with hydrothorax. After TIPS implantation, no more bleeding was seen in the patient who suffered from colonic variceal hemorrhage.

Seven out of 10 patients developed TIPS-related hepatic encephalopathy, which necessitated TIPS reduction in two patients with a later closure in another. In the other patients, encepaholpathy was successfully treated with standard medical therapy. One patient developed TIPS dysfunction, which was corrected by dilatation.

Only one patient in our cohort, who underwent retransplantation, survived long-term. All other patients died, mainly from sepsis associated with multiorgan failure. The median survival time of all patients was 3.3 mo (range 0.4-20 mo; Figure 1).

The course of all 10 patients is summarized in Table 1. Although TIPS was able to reduce ascites in patients 1 and 2, both died at 1 and 3 mo after TIPS placement because of HCV recurrence, with sepsis and multiorgan failure. Both patients presented with a high MELD score of 22 and 26, respectively.

The third patient with therapy-refractory ascites first responded well to TIPS. Four months later, however, she developed massive bleeding in the upper gastrointestinal tract and lungs because of severe coagulopathy, secondary to graft failure associated with ductopenic rejection, and died.

No improvement in ascites was seen in the fourth patient. Eight months after TIPS implantation, the patient underwent retransplantation for recurrent HCV cirrhosis. A few months after retransplantation, he again developed a rapidly progressive HCV recurrence, with

Table 1 Summary of clinical data and outcomes

Patient no.	Age, sex	Cause of liver disease	Transplant pathology before TIPS	Indication for TIPS	MELD score at TIPS	CPC at TIPS	Time from transplantation to TIPS (mo)	Encepha-lopathy-post-TIPS	Follow-up after TIPS
1	71, F	Hepatitis C	HCV recurrence	Ascites	22	C	15	Yes	Died 1 mo
2	59, M	Hepatitis C	HCV recurrence	Ascites	26	C	16	Yes	Died 3 mo
3	37, F	Primary biliary cirrhosis	Vanishing bile duct syndrome	Ascites	18	B	4	Yes	Died 4 mo
4	56, M	Hepatitis C	HCV recurrence	Ascites	15	C	158	Yes	ReLT 8 mo, died after ReLT, see below
	59, M	Hepatitis C	HCV recurrence	Ascites	13	C	18	Yes	TIPS reduction day 11 and 16; died at 2 mo
5	52, M	Fatty liver cirrhosis	Vanishing bile duct syndrome	Ascites	35	C	10	No	ReLT 3 mo; alive 32 mo
6	51, F	Hepatitis C	HCV recurrence	Ascites	19	B	61	No	Died 3 mo
7	71, M	Primary biliary cirrhosis	Thrombosis of the portal vein at the anastomosis	Ascites	12		4	No	TIPS revision 3 mo; died 12 mo
8	62, M	Hemochromatosis	Vanishing bile duct syndrome	Hydrothorax	21	C	17	Yes	Died 1.5 mo
9	68, M	Fatty liver cirrhosis	Vanishing bile duct syndrome	Hydrotho-rax/Ascites	30	B	11	Yes	Died 11 d
10	38, F	Autoimmune hepatitis	Vanishing bile duct syndrome + thrombosis of the portal vein	Bleeding	1	B	8	Yes	TIPS reduction 2.5 mo; died 19 mo

[1]MELD score calculation not possible due to incomplete laboratory data. ReLT: Liver retransplantation.

Figure 1 Kaplan-Meier plot of patients' overall survival after TIPS implantation.

massive ascites that was resistant to diuretic therapy. Therefore, 18 mo after retransplantation, a TIPS was placed again. Eleven days after placement, a reduction in the TIPS, with subsequent complete closure was necessary because of severe hepatic encephalopathy. The patient died 2 mo after the second shunt placement from sepsis with multiorgan failure.

In the fifth patient, TIPS implantation resulted in complete resolution of ascites. Three months after TIPS placement, the patient developed ductopenic rejection in association with renal failure, and underwent combined liver and kidney transplantation. Thirty-two months later, the patient is doing well with stable graft function.

The sixth patient with TIPS placement for ascites did not respond to the procedure and died 3 mo later from HCV recurrence.

Ascites further deteriorated in patient seven after TIPS placement. Shunt occlusion was suspected and the patient was given another TIPS 3 mo later, which

led to a reduction of portosystemic pressure from 19 to 12 mmHg, and disappearance of ascites. The patient, however, died 1 year after TIPS insertion from cholangitis secondary to multiple ischemic-type intrahepatic biliary strictures.

TIPS placement did not result in any improvement in the two patients with hydrothorax (patients 8 and 9). One of them died at 6 wk and the other at 11 d after TIPS, as a result of sepsis.

The patient with colonic bleeding as indication for TIPS placement developed severe encephalopathy at 2.5 mo after the procedure, which required a reduction in TIPS. The portal pressure increased from 5 to 9 mmHg, and encephalopathy improved thereafter. Although there was no recurrence of bleeding, the patient died 19 mo after TIPS because of chronic rejection.

DISCUSSION

TIPS is an established therapeutic modality for the management of complications of portal hypertension, in particular, therapy-refractory ascites or pleural effusion, as well as variceal bleeding resistant to endoscopic treatment. However, there is much less experience with TIPS after LT[11-15]. Although the indications for TIPS should be essentially the same as in patients without LT, certain specific points need to be considered.

At our center, the indications for TIPS placement did not differ between the transplant and non-transplant patients, with therapy-refractory ascites being the main indication also in LT recipients. Therapy-refractory hydrothorax and variceal bleeding, not manageable by endoscopic means, were less frequent indications. Our analysis showed that, in principle, TIPS was technically feasible in patients with portal hypertensive

complications after LT, and was efficacious in the majority of patients. Complete resolution was achieved in three out of eight patients, and a partial response in two further patients with therapy-refractory ascites. In addition, severe variceal hemorrhage in one patient did not recur after TIPS. No improvement, however, was seen in both patients with severe hepatic hydrothorax. Similar response rates have been reported in four previously published series, including a small study of 6-12 patients[11-14]. In these studies, improvement or complete resolution of ascites was achieved in 50%-90% of patients, and only two out of 10 patients with variceal hemorrhage experience recurrent bleeding.

The success rate of TIPS placement for managing ascites and variceal bleeding in LT was comparable with that in non-transplanted patients at our department. In the present cohort, complete resolution or significant reduction in the amount of ascites was achieved in about 70% of patients, and recurrence of bleeding was prevented in about 77% of patients (data not shown).

With an average survival of 3.3 mo, the survival in our series was extremely poor. This might mainly have been the result of the advanced stage of liver disease and the already poor prognosis of our patients at the time of TIPS placement, and not by the intervention itself. TIPS should serve as a bridge to a possible liver retransplantation. As we had only little experience with TIPS in LT recipients, this intervention was indicated with great caution. As a consequence, TIPS was placed in LT patients as the last therapeutic option, after all conservative modalities had failed. Several studies have shown that survival rates of TIPS patients with advanced disease are markedly poorer than those in earlier stages of liver disease[4,16,17]. In fact, almost all of our patients presented with an advanced stage of graft dysfunction and high MELD scores (median score of 20). The MELD score was originally developed for patients undergoing TIPS[18], and then slightly modified to predict survival of patients with liver cirrhosis in general[19]. In our analysis, there was a trend towards a higher MELD score being associated with poor survival. The correlation was not statistically significant, most probably as a result of the small number of patients.

It is well known that the natural course of recurrent HCV infection is more aggressive and leads more rapidly to cirrhosis of the allograft and graft failure than HCV infections in non-transplanted patients. Subsequently, the long-term outcome of HCV-positive patients after LT is worse compared to those with other indications for transplantation[20-23]. It has been shown that the prognosis of HCV patients is poor after decompensation, with a median survival of less than 1 year[20]. All of our four patients with recurrent hepatitis C infection presented with decompensated cirrhosis (Child-Pugh stage B or C and MELD scores between 13 and 26).

Chronic rejection with progressive loss of bile ducts inevitably leads to irreversible loss of the allograft[24], with liver retransplantation rates of 50%-90%[25,26]. Prognosis is especially poor in patients with bilirubin values > 10 mg/dL[27]. In our study, all three patients with chronic rejection presented with a bilirubin above this level.

Therefore, an advanced stage of graft dysfunction caused by recurrent HCV cirrhosis and chronic ductopenic rejection might have been responsible for the poor survival rate in our patients. In addition, the number of liver retransplantations, the only potentially curative therapy for these patients, was lower in our series compared to other studies. Only two of our patients underwent liver retransplantation, whereas the retransplantation rate was 50% in the largest series of Amesur and co-workers[13]. Three of our patients died while being on the waiting list for a second LT, which suggests that retransplantation should be considered as early as possible when graft decompensation occurs.

The most frequent cause of death ($n = 5$) in our cohort was sepsis associated with multiorgan failure. Thus, two interacting factors were responsible for the frequency of sepsis in our patients. Patients with impaired liver function or recurrent cirrhosis frequently develop bacterial infections, which lead to death in 30%-50% of cases[28,29]. The risk of infection is further increased by chronic immunosuppression. Therefore, we recommend prophylactic antimicrobial therapy following TIPS placement.

The altered anatomy of the hepatic vessels that results from LT should be kept in mind before the TIPS procedure. The two most frequently used techniques are the replacement of the retrohepatic vena cava and the piggyback-type of transplantation. Previous studies have shown that there are no difficulties in TIPS placement with either of these procedures. In contrast, in patients with cava-cava liver transplantation, probing for the hepatic and portal veins in the recipient's organ might be difficult[11-14]. No technical problems were encountered in our series, in which, three had undergone the piggyback-type of transplantation and the remaining patients had replacement of the retrohepatic vena cava. This indicates that the anatomical situation in these patients creates no problems for the TIPS procedure.

Only one patient (10%) developed dysfunction of the TIPS, which was managed successfully by TIPS dilatation. In contrast, the rate of TIPS revision in our non-transplanted patients was markedly higher at 35% ($P = 0.059$). This low rate of TIPS dysfunction in the LT group might be attributed to the fact that immunosuppressive therapy can lead to reduced intima proliferation[11], but can also be ascribed to the very short survival of these patients.

Noticeable in our LT recipients was a low pre-interventional mean portosystemic pressure gradient of 12.5 mmHg, which suggests that ascites and hydrothorax post-transplant may not be as well-correlated with portal pressure as in the pre-transplant phase. Other factors beside the portosystemic pressure gradient, such as renal function, may play a major role in the efficacy of TIPS in LT transplant recipients. Chronic renal dysfunction is a common complication in transplant recipients, especially if calcineurin inhibitors are used[30]. In our cohort, both patients with hepatic hydrothorax presented with markedly impaired renal function (glomerular filtration

rate < 20 and 45 mL/min per 1.73 m², respectively), which may explain their non response to TIPS treatment, although the postinterventional pressure gradients could be successfully lowered to 10 and 5 mmHg, respectively.

In the ascites group, only three of seven patients showed normal renal function, and in two of these, complete resolution of ascites was achieved. No statistically significant correlation was found between the reduction of the portal pressure gradient and response to TIPS.

Hepatic encephalopathy developed in 70% of our patients. A similar rate of encephalopathy was also described in a previous study, and is markedly higher than the incidence reported for non-transplant TIPS recipients[4,11]. We could not find a relation between final pressure gradients and the development of encephalopathy. Mild pre-existing hepatic encephalopathy was found in two patients, and in both, a deterioration of encephalopathy was noticed after TIPS creation. Therefore, we conclude that the main reasons for the high occurrence of hepatic encephalopathy in this cohort might be the advanced stage of disease in these patients, as well as the potentially neurotoxic effects of immunosuppressive drugs, in particular the calcineurin inhibitors.

In summary, our study showed that the TIPS procedure in LT recipients was feasible without technical difficulties. Indications for TIPS seemed not to differ from those of the non-transplanted TIPS group. The TIPS procedure was efficacious in the management of therapy-refractory ascites and severe variceal bleeding unresponsive to endoscopy, in the majority of patients. TIPS did not appear to be useful in patients with hepatic hydrothorax. However, the outcome was very disappointing. The low survival rate shows that, in LT patients with an already advanced stage of graft dysfunction, TIPS does not improve prognosis. Similar to the recent work of Kim and co-workers[15], we conclude that TIPS may not be useful in most transplant patients with an advanced graft disease. It may have its place in treating some vascular problems after LT. Otherwise retransplantation remains the only possibility to improve the survival of patients with portal hypertensive complications after LT.

ACKNOWLEDGMENTS

COMMENTS

Background
Transjugular intrahepatic portosystemic shunt (TIPS) is a well-established therapeutic procedure in patients suffering from complications of portal hypertension, such as ascites or variceal bleeding. In rare cases, portal hypertension develops in liver transplant (LT) recipients. The use of TIPS in this special patient cohort has been discussed in the literature, but only few cases have been reported so far.

Research frontiers
In this study, the authors report a series of TIPS placements in 10 LT recipients

because of ascites, hydrothorax and variceal bleeding. In the majority of patients, at least a partial response could be achieved, however, the outcome of these patients was poor with a median survival of 3.3 mo.

Innovations and breakthroughs
Portal hypertension is a rare but severe complication after LT and leads to graft loss in the majority of patients. The pathophysiology is not well understood and the best therapeutic modality for these patients remains to be defined. TIPS placement has been discussed. This paper shows that TIPS placement is not efficacious in this cohort.

Applications
Results from this study will help to refine the post-transplant care of patients and should encourage physicians to consider retransplantation as the only effective treatment in LT patients with portal hypertensive complications.

Terminology
TIPS is an interventional technique for the creation of an intrahepatic decompressive shunt between a branch of the portal vein and the main hepatic vein, using expandable metallic stents. This leads to a decrease in portosystemic pressure gradient and has become an established therapy for patients with therapy-refractory ascites and unresponsive variceal bleeding.

Peer review
This is a well written report on a single institution's experience on the use of TIPS after LT for the treatment of portal hypertension recurrence related complications. Although the series is small, the paper gives a clear message to the readers about a selected topic in liver transplantation.

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BRIEF ARTICLES

Efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy: A prospective, randomized study

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Abstract

AIM: To determine the efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy.

METHODS: A prospective, double-blind, randomized, placebo-controlled study was conducted on 70 patients who underwent elective laparoscopic cholecystectomy under general anesthesia at Siriraj Hospital, Bangkok, from January 2006 to December 2007. Patients were randomized to receive either 20 mg parecoxib infusion 30 min before induction of anesthesia and at 12 h after the first dose (treatment group), or normal saline infusion, in the same schedule, as a placebo (control group). The degree of the postoperative pain was assessed every 3 h in the first 24 h after surgery, and then every 12 h the following day, using a visual analog scale. The consumption of analgesics was also recorded.

RESULTS: There were 40 patients in the treatment group, and 30 patients in the control group. The pain scores at each time point, and analgesic consumption did not differ between the two groups. However, there were fewer patients in the treatment group than placebo group who required opioid infusion within the first 24 h (60% *vs* 37%, *P* = 0.053).

CONCLUSION: Perioperative administration of parecoxib provided no significant effect on postoperative pain relief after laparoscopic cholecystectomy. However, preoperative infusion 20 mg parecoxib could significantly reduce the postoperative opioid consumption.

INTRODUCTION

Preemptive analgesia has become a popular adjunct to conventional postoperative pain control. The concept of preemptive analgesia is based on the hypothesis that the most effective way to eliminate or reduce postoperative pain is to prevent nociceptive input from afferent stimuli to the central nervous system so that central nervous system hyperexcitability does not occur[1]. Clinically, this strategy predicts not only less pain during the initial postoperative period but also a reduced intensity of pain during the days after the procedure[2]. A variety of preoperative or preemptive analgesic regimens have been used such as intravenous administration of opioids or non-steroidal anti-inflammatory drugs (NSAIDs), local anesthetic infiltration, nerve block, and epidural block[3].

Recent research indicates that cyclooxygenase-2 (COX-2) inhibitors, a selective class of NSAIDs, could

play an important role in perioperative pain management by reducing the inflammatory response in the periphery, modulating nociceptors, and attenuating central sensitization[4]. The COX-2 inhibitors provide effective pain control, in addition to a lesser degree of platelet dysfunction and gastrointestinal toxicity compared to nonselective NSAIDs. Reuben et al[5] reported that patients receiving perioperative oral COX-2 inhibitors experienced less postoperative pain, required fewer analgesic drugs, and had a greater range of motion after total knee arthroplasty than those receiving placebo.

The injectable COX-2 inhibitor, parecoxib, could play an important role in pain control in the perioperative and immediate postoperative period, especially in those who cannot take an oral analgesic such as a patient about to undergo intraabdominal surgery under general anesthesia. Laparoscopic cholecystectomy is the most common laparoscopic procedure performed in a general surgical unit worldwide, and accounts for about two-thirds of total laparoscopic operations in the authors' unit. However, the efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy remains controversial[6-11].

The aim of this study was to determine the efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy in a university hospital.

MATERIALS AND METHODS

After obtaining approval from our Institutional Ethics Committee, a prospective double-blind, randomized, controlled study was conducted at the Department of Surgery, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand, involving 70 consecutive ASA class I - III patients who underwent elective laparoscopic cholecystectomy from January 2006 to December 2007. All patients gave written informed consent.

Patients were excluded from the study for one of the following reasons: age under 18, hypersensitivity to NSAIDs, or conversion to open cholecystectomy. Patients were randomized into one of two groups by opening a sealed envelope in the operating theater: the treatment group received 20 mg parecoxib infusion 30 min before induction of anesthesia and at 12 h after the first dose; the control group received normal saline infusion as a placebo in a similar time schedule. All patients underwent laparoscopic cholecystectomy under a balanced general anesthetic technique, using fentanyl for premedication and during the intraoperative period. The procedures were performed by an experienced laparoscopist.

The degree of the postoperative pain was assessed every 3 h in the first 24 h after surgery and then every 12 h the following day, using a visual analog scale (0 = no pain, 10 = worst possible pain), by nursing staff who were unaware of the perioperative intervention. A standard postoperative analgesic regimen was administered to all patients. This consisted of intravenous pethidine 1 mg/kg prn for patients with a pain score = 5, every 3 h during the first 24 h after the operation, or until oral analgesics could be taken. The consumption of analgesics was also recorded.

Table 1 Comparison of patients' characteristics, indication for surgery and operative details between treatment group and control group (mean ± SD) n (%)

	Treatment group (n = 40)	Control group (n = 30)	P-value
Age (yr)	57.6 ± 12.7	56.2 ± 15.1	0.67
Female	25 (63)	16 (53)	0.44
Co-morbid diseases	24 (60)	15 (50)	0.41
Indication for surgery			0.26
Symptomatic gallstone	28 (70)	23 (77)	
Common bile duct stone	6 (15)	6 (20)	
Gallstone induced pancreatitis	6 (15)	1 (3)	
Operative time (min)	63.7 ± 36.9	58.1 ± 31.5	0.51

Figure 1 Average visual pain score (0 = no pain, 10 = worst possible pain) at 3, 6, 9, 12, 15, 18, 21 and 24 h after surgery in the treatment group and control group.

All data were prepared and compiled using SPSS software. Mean and SD were assessed. The Kolmogorov-Smirnov test was used to test for the pattern of data distribution. The student's unpaired t-test was used to compare data between the two groups when the data were in a normal distribution pattern. The Mann-Whitney U test was used to compare data between the two groups when the data were in a non-normal distribution. $P < 0.05$ was considered statistically significant.

RESULTS

There were 40 patients in the treatment group, and 30 patients in the control group. The patient's characteristics, indication for surgery and operative details between the treatment group and control group were well matched (Table 1). The pain scores at each time point did not significantly differ between the two groups (Figure 1). However, there were fewer patients in the treatment group than in the placebo group who required opioid infusion within the first 24 h (60% vs 37%, $P = 0.053$).

DISCUSSION

Parecoxib, the first injectable COX-2 inhibitor, was

introduced into clinical practice in 2001. It was found that preoperative administration of parecoxib was more effective than postoperative administration for postoperative pain relief in patients undergoing elective general surgical procedures such as appendectomy, open cholecystectomy and hernioplasty[12]. Parecoxib can be injected intravenously or intramuscularly with good patient tolerance. The lack of platelet inhibition allows COX-2 inhibitors such as parecoxib to be administered preoperatively. Parecoxib is now increasingly used in ambulatory or day-case surgery because it reduces opioid consumption, improves pain scores, and results in earlier hospital discharge and return to normal function[13].

In the present study, perioperative administration of parecoxib provided no significant effect on postoperative pain relief after laparoscopic cholecystectomy. One possible explanation for our observation is that administration of a total of 40 mg parecoxib is not an optimal dose for perioperative pain control. Puolakka *et al*[8] found that a single dose of 80 mg parecoxib resulted in the least pain intensity after laparoscopic cholecystectomy compared with 40 mg parecoxib or placebo. However, preoperative infusion of 40 mg parecoxib could significantly reduce the postoperative opioid requirement, and the incidence of opioid-related adverse effects if oral COX-2 inhibitors have been taken in the early postoperative period[9]. There is evidence that the administration of preoperative intravenous parecoxib followed by oral COX-2 inhibitors after laparoscopic cholecystectomy resulted in a shorter length of stay in the postoperative anesthesia care unit, a better quality of postoperative recovery, and a faster return to normal activity, with greater patient satisfaction[10].

It is also possible that pain after laparoscopic cholecystectomy has several components such as incisional and visceral pain; the latter type of pain seems to be more resistant to the analgesic effect of NSAIDs[8]. Therefore, systemic administration of COX-2 inhibitors alone is relatively ineffective. Several investigators have suggested that intraperitoneal administration of local anesthetics could improve postoperative pain control by means of attenuation of the visceral pain. Jabbour-Khoury *et al*[14] reported that intraperitoneal spray of an aliquot of bupivacaine and NSAIDs, or intraperitoneal spray of local anesthetics following by intravenous NSAIDs resulted in significantly lower abdominal pain scores and incidence of vomiting after laparoscopic cholecystectomy, compared to the non-treatment group. Meanwhile, Elhakim *et al*[15] revealed that a combination of intraperitoneal lidocaine and tenoxicam provided better analgesia on movement, and faster return of bowel function compared with intraperitoneal lidocaine and intravenous tenoxicam after laparoscopic cholecystectomy[15].

In conclusion, perioperative administration of intravenous parecoxib provided no significant effect on postoperative pain relief after laparoscopic chole-cystectomy. However, preoperative infusion of 20 mg parecoxib could significantly reduce the postoperative opioid consumption.

COMMENTS

Background
Preemptive analgesia has become a popular adjunct to conventional postoperative pain control. The concept is based on the hypothesis that the most effective way to reduce postoperative pain is to prevent nociceptive input from afferent stimuli to the central nervous system so that central nervous system hyperexcitability does not occur.

Research frontiers
The injectable Cyclooxygenase-2 (COX-2) inhibitor, parecoxib, could play an important role in perioperative pain control and is increasingly used in day-case surgery because it reduces opioid consumption, improves pain scores, and results in earlier discharge and return to normal function.

Innovations and breakthroughs
This study determined the efficacy of perioperative 20 mg parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy.

Applications
Perioperative administration of intravenous 20 mg parecoxib provided no significant effect on postoperative pain relief, but could significantly reduce the postoperative opioid consumption after laparoscopic cholecystectomy.

Terminology
COX-2 inhibitors, a selective class of non-steroidal anti-inflammatory drugs (NSAIDs), could play an important role in perioperative pain management by reducing the inflammatory response in the periphery, modulating nociceptors, and attenuating central sensitization. The COX-2 inhibitors provide effective pain control, in addition to a lesser degree of platelet dysfunction and gastrointestinal toxicity compared to nonselective NSAIDs.

Peer review
This is a well written manuscript with concise data. The concept of perioperative COX-2 infusion can make GI surgeons pay much attention.

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Effect of Lianshu preparation on lipopolysaccharide-induced diarrhea in rats

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Abstract

AIM: To investigate the effect of Lianshu preparation on lipopolysaccharide (LPS)-induced diarrhea in rats.

METHODS: A diarrhea model was established in Sprague Dawley rats *via* injection of 1 mL of 30 mg/kg LPS. A total of 40 rats were randomly divided into normal group, LPS group, LPS + Lianshu group, LPS + berberine group ($n = 10$ in each group). Their intestinal mucosal barrier and frequency of diarrhea were observed. Levels of glucose, serum Na^+, K^+, Cl^- and hematocrit, plasma nitrogen monoxide (NO), diamine oxidase (DAO), and D (-)-lactate were measured. The number of IgA+ plasma cells in small intestine was detected and SIgA levels in the intestinal fluid were measured. The antipyretic activity of Lianshu preparation in rats was evaluated using Brewer's yeast-induced pyrexia (10 mL/kg of 20% aqueous suspension). Acetaminophen (250 mg/kg, intragastric administration, *bid*) was used as a standard drug for comparison. Temperature was recorded 1 h before and 6 h after Brewer's yeast injection. Finally, small intestinal transmission in mice treated with Lianshu was detected after intraperitoneal injection of methyl prostigmin (2 mg/kg). Atropine (10 g/kg) was used as a control. The ink content in intestine was determined and the total length of intestine was measured.

RESULTS: The frequency of diarrhea was higher in LPS group than in LPS + Lianshu group and LPS + berberine group (36.70 ± 5.23 *vs* 28.50 ± 4.06 and 32.70 ± 9.30 respectively, $P < 0.01$), and lower in LPS + Lianshu group than in LPS + berberine group ($P = 0.03$). The levels of Na^+, glucose, Cl^-, K^+ were significantly lower in LPS + Lianshu group than in LPS + berberine group (140.35 ± 3.19 mmol/L *vs* 131.99 ± 4.86 mmol/L, 8.49 ± 1.84 mmol/L *vs* 6.54 ± 2.30 mmol/L, 106.29 ± 4.41 mmol/L *vs* 102.5 ± 1.39 mmol/L, 5.08 ± 0.66 mmol/L *vs* 4.32 ± 0.62 mmol/L respectively, $P < 0.05$). The level of hematocrit was lower in LPS + Lianshu group than in LPS + berberine group (0.50% ± 0.07% *vs* 0.59% ± 0.10% respectively, $P < 0.05$). The plasma levels of NO, DAO and D (-)-lactate were higher in LPS group than in normal group (79.74 ± 7.39 μmol/L *vs* 24.94 ± 3.38 μmol/L, 2.48 ± 0.42 μ/mL *vs* 0.82 ± 0.33 μ/mL, 5.63 ± 0.85 μg/mL *vs* 2.01 ± 0.32 μg/mL respectively, $P < 0.01$), and lower in LPS + Lianshu group than in LPS + berberine group (48.59 ± 4.70 μmol/L *vs* 51.56 ± 8.38 μmol/L, 1.43 ± 0.53 μmol/mL *vs* 1.81 ± 0.42 μmol/mL, 4.00 ± 0.54 μg/mL *vs* 4.88 ± 0.77 μg/mL respectively, $P < 0.05$). The morphology of the intestinal mucosa showed destroyed villi in LPS group and atrophied intestinal mucosa in other groups. The pathological intestinal mucosal changes were less in LPS + Lianshu group than in LPS group. The number of IgA+ plasma cells and amount of SIgA were higher in LPS + Lianshu group than in LPS group (1.16 ± 0.19/μm² *vs* 1.09 ± 0.28/μm², $P = 0.026$; 0.59 ± 0.12 mg/L *vs* 0.15 ± 0.19 mg/L respectively, $P = 0.000$). Lianshu had counteractive effects on yeast-induced pyrexia and enterokinesia in rats.

CONCLUSION: Lianshu preparation has therapeutic effects on LPS-induced diarrhea and enterokinesia in rats.

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Key words: Lianshu preparation; Lipopolysaccharide; Diarrhea; Nitrogen monoxide; D-lactate

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INTRODUCTION

Infectious diarrhea is often caused by Gram-negative bacteria such as *Escherichia coli*. These organisms contain lipopolysaccharide (LPS). Antibiotics developed in the 1940s have saved millions of people's life. However, because of the widespread and inappropriate use of antibiotics, some bacterial strains become antibiotic-resistant. Antibiotic-resistant bacteria and side effects of antibiotics severely threaten the general welfare and health of people. Folk herbal drugs have been extensively studied in recent years for the treatment of acute diarrhea. This study was to investigate the effect of Lianshu preparation on LPS-induced diarrhea in rats.

MATERIALS AND METHODS

Animals
Eighty adult female and male Sprague-Dawley rats, weighing 230-280 g (Experimental Animal Breeding Center, Fudan University), and thirty 5-wk-old female and male Kunming mice, weighing 18-20 g, were used in this study following the guidelines of the Animal Care Committee of Institute of Chinese Medicine, Tongji University. The animals were housed at 22 ± 2℃ with a humidity of 55% ± 5% in a 12-h light/dark cycle for at least 1 wk prior to use, with free access to laboratory chow and tap water.

Lianshu preparation
Coptis chinensis, atractylodis, pulsatillae and portulacae, provided by Leiyunshang Company, were treated twice at 100℃ for 2 h, divided into 1:5:5:5 proportions, then treated at 100℃ for 1 h, filtered and concentrated to a relative density of 1.25 (60℃), and finally, desiccated. The effective constituents of Lianshu preparation were determined by Ley's Company (Shanghai, China). Lipopolysaccharide from *Escherichia coli* serotype 0128: B12 was purchased from Sigma Aldrich (St Louis, MO, USA). Nitrogen monoxide (NO) assay kit was provided by Jiancheng Bionic Research Center (Nanjing, China). Diamine oxidase (DAO) standard preparation, O-dianisidine, horseradish peroxidase, 1,5-pentanediamine-di-hydrochloride, D-lactate standard preparation, NAD- aminoacetic acid, and D-lactic dehydrogenase were purchased from Sigma Aldrich (St Louis, MO, USA). Anti-rat IgA mAb was purchased from Santa Crus Company (USA) and SIgA assay kit was purchased from Bethyl Labs Inc. (USA).

Detection of inhibitory effect of Lianshu preparation on LPS-induced diarrhea in rats
Rats were randomly divided into normal group, LPS group, LPS + Lianshu group, LPS + berberine group (n = 10 in each group). Before exposure to LPS, rats in normal and LPS groups were treated with distilled water, rats in LPS + Lianshu group were given Lianshu preparation (1.8 g/kg), rats in LPS + berberine group received berberine (0.2 g/kg) twice a day for 3 d. The rats were allowed to have water alone for 12 h. One hour after the last administration of drugs, rats in all groups apart from those in the normal group were treated with LPS (30 mg/kg).

Detection of diarrhea frequency before specimen collection
After treated with LPS, rats in each cage had a filter paper couch. Filter paper was changed once an hour for 4 h. The frequency of diarrhea was determined by counting the number of feces deposits on the filter paper.

Measurement of erythrocrit, blood glucose, and serum Na+, K+, Cl- levels
Four hours later, 8 mL of blood was drawn from the abdominal aorta of each rat after anesthetized with 10 mg/kg intraperitoneal pentobarbital. Of the 8 mL blood, 2 mL was treated with heparin for erythrocrit analysis, 2 mL was centrifuged at $3000 \times g$ for 15 min at 4℃. Blood glucose and serum Na^+, K^+, Cl^- levels were measured.

Measurement of plasma NO, DAO and D (-)-lactate levels
Four milliliters of blood was centrifuged at $3000 \times g$ for 15 min at 4℃. Plasma was collected to measure NO at 550 nm as previously described[1]. DAO and D (-)-lactate levels were measured by spectrophotometry at 436 nm[2] and enzyme-linked ultraviolet spectrophotometry[3], respectively. The absorbance at 340 nm was recorded.

Observation of morphologic changes in intestinal mucosa
After humane killing, a 5-cm long section of ileum 10 cm below the Treitz ligament was removed, dissected longitudinally, fixed in 100 mL/L formalin, embedded in paraffin, stained with hematoxylin and eosin, and observed under a light microscope.

Detection of IgA+ plasma cells in rat small intestine
ABC immunohistochemistry staining of tissue paraffin sections was performed. The brown stained IgA + plasma cells were detected and analyzed using medical image analysis software (Taimeng Technology Co. Chengdu, China).

Determination of SIgA in small intestine
A small intestine segment about 5 cm long, 15 cm

Table 1 Serum electrolyte levels in different groups (mean ± SD)

Group	Sodium (mmol/L)	Glucose (mmol/L)	Chlorine (mmol/L)	Kalium (mmol/L)
Normal	143.46 ± 3.34	8.37 ± 1.06	102.03 ± 2.82[c]	5.37 ± 0.96
LPS	102.48 ± 3.67[b]	4.99 ± 1.23[b]	98.40 ± 1.42[a]	3.38 ± 0.29[b]
LPS + Lianshu	140.35 ± 3.19[d]	8.49 ± 1.84[d]	106.29 ± 4.41[b,d]	5.08 ± 0.66[d]
LPS + berberine	131.99 ± 4.86[b,d,f]	6.54 ± 2.30[a,e]	102.5 ± 1.39[d,e]	4.32 ± 0.62[b,d,e]

[a]$P < 0.05$, [b]$P < 0.01$ vs normal group; [c]$P < 0.05$, [d]$P < 0.01$ vs LPS group; [e]$P < 0.05$, [f]$P < 0.01$ vs LPS + Lianshu group.

Figure 1 Frequency of diarrhea in different groups. [a]$P < 0.01$ vs normal group; [b]$P < 0.01$ vs LPS group; [c]$P < 0.05$ vs LPS + Lianshu group.

below the Treitz ligament, was removed. Intestinal fluid was collected by passing 5 mL PBS and 0.02% sodium into the small intestine. The first intestinal washing removed 85%-90% of total IgA, and the variability of samples was less than 5%. The washed out material was centrifuged at 3000 × g for 10 min at 4℃. The supernatant was harvested and stored at -70℃. Total IgA was determined by ELISA.

Assay of antipyretic activity of Lianshu preparation

Antipyretic activity of Lianshu preparation in rats was evaluated using Brewer's yeast-induced pyrexia. Forty rats were divided into 4 groups. Rats in control group were given sodium chloride (2 mL) and rats in experimental groups were given Lianshu preparation (1.8 g/kg), twice a day before yeast was injected. Acetaminophen (250 mg/kg, *bid*) was used as a standard drug to compare the antipyretic action of Lianshu preparation. One hour after the last administration of drugs, fever was induced by hypodermic injection of a 20% suspension of 10 mL/kg Brewer's yeast and rectal temperature was recorded 1 h before and 6 h after injection of Brewer's yeast. The antipyretic activity of Lianshu preparation in rats was assayed.

Detection of effect of Lianshu preparation on small intestine transmission in mice

Thirty Kunming mice, after 12 h fasting, were divided into sodium chloride group, Lianshu preparation group and atropine group ($n = 10$). Mice in the sodium chloride group received 0.5 mL sodium chloride by intragastric administration, twice a day for 1 d. Mice in the Lianshu preparation group received 1.8 g/kg Lianshu preparation,

twice a day for 1 d. Mice in the atropine treatment group received 10 g/kg intraperitoneal atropine, twice a day for 1 d. All mice were given 2 mg/kg methyl prostigmin followed by 0.1 mL ink after 10 min, and killed by cervical vertebral dislocation with their belly cut open to collect intestines. The effect of Lianshu preparation on small intestinal transmission in mice was detected.

Statistical analysis

Data were expressed as mean ± SE and analyzed by analysis of variance and Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

General health of rats

No diarrhea and loose stools occurred in rats of the normal group. The fur of rats was smooth and the rats were active. Rats in the other groups that received LPS drank more water and had loose stools. The fur of rats was fluffy, and the rats were affected by lassitude. The respiration of rats was rapid.

Frequency of diarrhea after injection of LPS

The frequency of diarrhea in LPS group, LPS + Lianshu group and LPS + berberine group was increased compared to normal group (36.70 ± 5.23, 28.50 ± 4.06, 32.70 ± 9.30 vs 12.40 ± 3.20 respectively, $P < 0.01$), significantly increased in LPS + Lianshu group and LPS + berberine group compared with normal group ($P < 0.01$), and decreased in LPS + Lianshu group compared with LPS + berberine group ($P = 0.03$) (Figure 1).

Glucose, serum electrolyte and hematocrit levels in rats

The levels of glucose, Na^+, Cl^-, and K^+ were significantly lower in LPS + Lianshu group than in LPS + berberine group (140.35 ± 3.19 mmol/L vs 131.99 ± 4.86 mmol/L, 8.49 ± 1.84 mmol/L vs 6.54 ± 2.30 mmol/L, 106.29 ± 4.41 mmol/L vs 102.5 ± 1.39 mmol/L, 5.08 ± 0.66 mmol/L vs 4.32 ± 0.62 mmol/L respectively, $P < 0.05$), while the hematocrit level was lower in LPS + Lianshu group than in LPS + berberine group (0.50% ± 0.07% vs 0.59% ± 0.10%, $P < 0.05$, Table 1, Figure 2).

Plasma NO and DAO levels in rats

The plasma NO and DAO levels were higher in LPS group than in normal group ($P < 0.01$) and lower in LPS + Lianshu group than in LPS + berberine group ($P < 0.05$, Table 2).

Figure 2 Hematocrit in different groups. [b]$P < 0.01$ vs normal group; [d]$P < 0.01$ vs LPS group; [e]$P < 0.05$ vs LPS + Lianshu group.

Figure 3 Comparison of plasma D (-)-lactate in different groups. [a]$P < 0.01$ vs normal group; [b]$P < 0.01$ vs LPS group; [c]$P < 0.05$ vs LPS + Lianshu group.

Plasma D (-)-lactate levels in rats

Plasma D (-)-lactate level was higher in LPS group, LPS + Lianshu group and LPS + berberine group than in normal group (5.36 ± 0.85, 4.00 ± 0.54, 4.88 ± 0.77 vs 2.01 ± 0.32 respectively, $P < 0.01$), and lower in LPS + Lianshu group, LPS + berberine group than in LPS group and LPS + berberine group ($P < 0.01$, Figure 3).

Morphological changes in intestinal mucosa

The villi of intestinal mucosa maintained their integrity and the shape of epithelial cells remained unchanged in the normal group. The villi were destroyed in the LPS group. Intestinal mucosal atrophy and inflammatory reaction were detectable in the other groups (Figure 4).

Staining and number of IgA+ plasma cells in intestinal mucosa

IgA+ cells, round or elliptical in shape, were distributed in the intestinal villi, lamina propria and intestinal glands. The endochylema was stained brown (Figure 5).

Number of IgA+ cells and SIgA in intestinal fluid

The number of IgA+ cells and amount of SIgA in intestinal fluid were less in LPS group than in normal group ($P < 0.01$) and higher in LPS + Lianshu

Table 2 Plasma NO and DAO levels in different groups (mean ± SD)

Group	No (μmol/L)	DAO (μmol/mL)
Normal	24.94 ± 3.38	0.82 ± 0.33
LPS	79.74 ± 7.39[b]	2.48 ± 0.42[b]
LPS + Lianshu	48.59 ± 4.70[b,d]	1.43 ± 0.53[b,d]
LPS + berberine	51.56 ± 8.38[b,d,f]	1.81 ± 0.42[b,d,e]

[b]$P < 0.01$ vs normal group; [d]$P < 0.01$ vs LPS group; [e]$P < 0.05$, [f]$P < 0.01$ vs LPS + Lianshu group.

Table 3 Number of IgA + cells and SIgA in intestinal fluid of different groups (mean ± SD)

Group	IgA+ cells (/μm²)	SIgA (mg/L)
Normal	0.94 ± 0.21	0.50 ± 0.09
LPS	0.73 ± 0.22[a]	0.22 ± 0.66[b]
LPS + Lianshu	1.16 ± 0.19[b,d]	0.59 ± 0.12[d]
LPS + berberine	1.09 ± 0.28[a,d,e]	0.15 ± 0.19[b,c,e]

[a]$P < 0.05$, [b]$P < 0.01$ vs normal group; [c]$P < 0.05$, [d]$P < 0.01$ vs LPS group; [e]$P < 0.05$ vs LPS + Lianshu group.

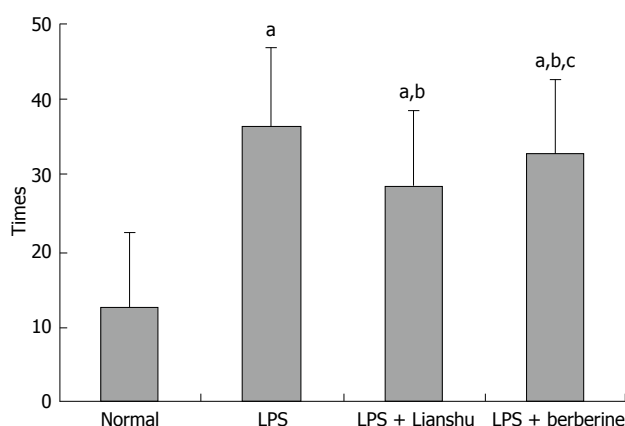group than in LPS + berberine group ($P = 0.05$, Table 3).

Counteractive effect of Lianshu preparation on yeast-induced pyrexia in rats

The temperature of rats in yeast group and sodium chloride group was similar 1 h after treatment with Lianshu preparation and higher in yeast group than in sodium chloride group 2-5 h after treatment with Lianshu preparation ($P < 0.05$). The temperature was not significantly changed in yeast + Lianshu group compared to yeast group 1 h or 2 h after treatment with Lianshu preparation. The temperature in yeast + Lianshu group decreased 3 h after treatment with Lianshu preparation ($P = 0.025$), and was almost identical to that in the normal group 4 h after treatment with Lianshu preparation. The temperature was lower in yeast + acetaminophen group than in the sodium chloride group 2 h after treatment with Lianshu preparation. Yeast + acetaminophen exhibited their effect 1 h earlier than yeast + Lianshu preparation (Table 4).

Counteractive effect of neostigmine on enterokinesia in mice

The rate of small intestinal peristalsis for mice after treatment with Lianshu preparation and atropine was different from that in mice after treatment with sodium chloride ($55.20\% \pm 10.16\%$ and $39.96\% \pm 8.57\%$ vs $65.10\% \pm 10.93\%$ respectively, $P < 0.05$, Figure 6).

DISCUSSION

A group of Japanese workers reported that parenteral LPS can lead to proliferation of gastrointestinal luminal bacteria in mice[4]. Subsequent work established that this enhancement of bacterial growth is secondary to

Figure 4 Optical microscopic observation of ileum mucosa of rats (HE, × 100). A:Normal group; B: LPS group; C: LPS + Lianshu group; D: LPS + berberine group.

Figure 5 Staining and number of IgA+ plasma cells in intestinal mucosa (× 200). A: Normal group; B: LPS group; C: LPS + LianShu group; D: LPS + berberine group.

fluid exudation, since the growth of bacteria could be suppressed by enteral unabsorbable antibiotics like neomycin[5]. Subcutaneous LPS from Gram-negative bacteria induces intestinal diarrhea characterized by destruction of the intestinal mucosa barricade[6-9].

In the present study, the frequency of diarrhea and gastrointestinal transit (GIT) were detected, and the levels of NO, DAO and D (-)-lactate were measured in mice after treatment with intraperitoneal LPS. Lianshu

preparation could effectively prevent diarrhea by inhibiting enterokinesia. Generally, NO can protect and repair gastrointestinal mucosa. Endogenous NO has therapeutic effects on hypoxia, inflammation and damage. However, excess NO is probably one of the most important mediators that induce blood poisoning, septic shock and multi-organ dysfunction[10]. When stimulated by LPS, NO is excessively expressed and induces multi-organ functional lesions in stomach and intestine[11-13].

Table 4 Counteractive effects of yeast on pyrexia in different groups (Δ℃) (mean ± SD)

Groups	1 h	2 h	3 h	4 h	5 h	6 h
Normal	0.58 ± 0.28	0.24 ± 0.71	0.20 ± 0.83	0.47 ± 0.30	0.17 ± 0.51	0.27 ± 0.65
Yeast	0.14 ± 0.94	0.75 ± 0.28[b]	1.06 ± 0.28[b]	1.16 ± 0.39[b]	1.69 ± 0.43[b]	1.46 ± 0.59[b]
Yeast + Lianshu	0.30 ± 0.38	0.58 ± 0.38	0.44 ± 0.56[c]	0.42 ± 0.29[d]	1.01 ± 0.43[c]	0.50 ± 0.44[d]
Yeast + acetaminophen	0.11 ± 0.73	0.21 ± 0.39[c]	0.37 ± 0.53[c]	0.66 ± 0.50[d]	0.74 ± 0.97[a,d]	0.53 ± 0.98[d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* yeast group.

Figure 6 Counteractive effects of neostigmine on enterokinesia of mice. [a]$P < 0.05$, [b]$P < 0.01$ *vs* sodium chloride group; [d]$P < 0.01$ *vs* Lianshu group.

In this study, Lianshu preparation prevented enteritis necroticans by inhibiting the production of excess NO. DAO is an ideal index of intestinal mucosal structure and function. The activity of DAO is closely related with villi, nucleic acid and protein synthesis in mucosal cells. When intestinal mucosal epidermis is damaged, DAO is released into blood, indicating that DAO in blood reflects the destruction of the intestinal mucosal epithelial cell layer and intestinal mucosa barricade[14,15]. In our study, DAO was localized mostly in the epithelial cell layer of mucous membrane as previously described[16], and Lianshu preparation could effectively decrease DAO induced by LPS. However, its mechanism of action remains unclear. In addition, the function of the intestinal mucosa barricade could also be evaluated by measuring D (-)-lactate in plasma[17-19]. D (-)-lactate is a product of bacterial metabolism and schizolysis. However, mammals cannot produce D (-)-lactate[20]. D (-)-lactate cumulation in plasma reflects membrane permeability and barrier function of the intestinal mucosa[21,22]. In our study, Lianshu preparation could reduce D (-)-lactate in plasma ($P < 0.01$) by protecting the barrier function of the intestinal mucosal and suppressing the overgrowth of harmful bacteria in the intestinal tract.

IgA is the main immunoglobulin in the mucosal immune system[23]. Polymeric IgA antibodies produced by plasma cells in the lamina propria of the intestinal tract bind to polymeric immunoglobulin receptors at the base of the epithelium. The complex of IgA and polymeric immunoglobulin receptors undergoes endocytosis and vesicular transport to the apical surface of enterocytes, and is secreted into the lumen. Generally, plasma cells are derived from B cells. It was reported that B cells are able to switch from IgM expression to IgA expression by Th2 cytokine activity in Peyer's patches of the lamina propria and mesenteric lymph nodes, and then return to lamina propria of the intestinal tract *via* systemic circulation[24-26]. LPS, a mouse B-cell mitogen, induces B cell multiplication[26] and differentiation into plasma cells. LPS also activates macrophages to promote the production of cytokines such as interleukin (IL)-1, IL-6 and nitric oxide, which might regulate immunoglobulin production. Therefore IgA inhibits diarrhea caused by viruses[27-29]. Moreover, LPS might activate IgA production in the intestine[30]. However, in our study, the number of IgA+ cells and SIgA in the intestinal fluid was less in the LPS group than in normal group, and greater in the LPS + Lianshu group than in the LPS group, suggesting that high dose LPS leads to severe destruction of intestinal mucosa.

ACKNOWLEDGMENTS

COMMENTS

Background

Infectious diarrhea is often caused by Gram-negative bacteria such as *Escherichia coli*. These organisms contain lipopolysaccharide (LPS). It is the important task of modern medicines to control infectious diarrhea. Antibiotics developed in the 1940s have saved millions of people's lives. However, because of the widespread and inappropriate use of antibiotics, some bacterial strains become antibiotic-resistant. Folk herbal drugs have been studied for the treatment of acute diarrhea.

Research frontiers

The method to treat diarrhea is mainly to kill bacteria or viruses. Lianshu preparation could destroy bacteria, prevent diarrhea, and protect the intestinal mucosa.

Innovations and breakthroughs

Lianshu preparation, comprised of non-antibiotic botanic components, has been used in the treatment of infectious diarrhea with a satisfactory effect. Lianshu preparation could effectively prevent diarrhea, relieve electrolyte disturbances and protect immune barriers. Its effect is better than berberine. In addition, Lianshu preparation has counteractive effects on pyrexia.

Applications

Lianshu preparation can be used as a natural medicine in treatment of infectious diarrhea with pyrexia. Chinese herbal drugs are effective against both chronic and acute diseases.

Peer review

The authors confirmed, in their study, that Lianshu preparation was effective against LPS-induced diarrhea, its effect was better than berberine, suggesting that it can be used as a natural medicine in treatment of infectious diarrhea.

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BRIEF ARTICLES

High level of ezrin expression in colorectal cancer tissues is closely related to tumor malignancy

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Abstract

AIM: To investigate the ezrin expression in normal colorectal mucosa and colorectal cancer tissues, and study the correlation between ezrin expression in colorectal cancer tissues and tumor invasion and metastasis.

METHODS: Eighty paraffin-embedded cancer tissue samples were selected from primary colorectal adenocarcinoma. Twenty-eight patients had well-differentiated, 22 had moderately differentiated and 30 had poorly differentiated adenocarcinoma. Forty-five patients and 35 patients had lymph node metastasis. Forty-five patients were of Dukes A to B stage, and 35 were of C to D stage. Another 22 paraffin-embedded tissue blocks of normal colorectal epithelium (> 5 cm away from the edge of the tumor) were selected as the control group. All patients with colorectal cancer were treated surgically and diagnosed histologically, without preoperative chemotherapy or radiotherapy. The immunohistochemistry was used to detect the ezrin expression in paraffin-embedded normal colorectal mucosa tissues and colorectal cancer tissue samples.

RESULTS: Ezrin expression in colorectal cancer was significantly higher than in normal colorectal mucosa (75.00% *vs* 9.09%, $P < 0.01$), and there was a close relationship between ezrin expression and the degree of tumor differentiation, lymph node metastasis and

Dukes stage (88.46% *vs* 50.00%, $P < 0.01$; 94.28% *vs* 51.11%, $P < 0.01$; 94.28% *vs* 51.11%, $P < 0.01$).

CONCLUSION: Ezrin expression is obviously higher in colorectal cancer tissues than in normal colorectal mucosa tissues, and the high level of ezrin expression is closely related to the colorectal cancer invasion and metastasis process.

INTRODUCTION

Ezrin belongs to the ezrin/radixin/moesin (ERM) protein family, which act as membrane organizers and linkers between the plasma membrane and cytoskeleton[1,2]. Ezrin is mainly expressed on the cell surface to maintain the polarity of endothelial cells[3]. Recent studies have found that, through regulating adhesion molecules and signal transduction pathways, ezrin is involved in cell-cell and cell-matrix interactions, and might play an important role in the process of tumor cell invasion and metastasis[4]. Overexpression of ezrin protein is correlated with the metastatic potential of several cancers[5-8], and a high level of ezrin protein expression can induce conversion of a variety of cell lines, as well as abnormal hyperplasia[9]. Tumor cell lines with stronger metastatic abilities are usually accompanied by overexpression of ezrin[10]. Through testing the expression of ezrin protein in normal colonic mucosa

and colorectal cancer tissues, we aimed to establish the relationship between ezrin expression and clinical parameters, evaluate its molecular action mechanisms in the process of colorectal cancer carcinogenesis, invasion and metastasis, and provide the evidence for clinical prognosis and suitable adjuvant therapy.

MATERIALS AND METHODS

Patients and their pathological samples

The immunohistochemistry was performed in paraffin-embedded tissue samples. Eighty colorectal adenocarcinoma patients diagnosed by postoperative pathology were investigated. There were 44 male and 36 female patients, whose ages ranged from 31 to 80 years, with an average age of 55.5 years. Histologically, 28 patients had well-differentiated, 22 had moderately differentiated, and 30 had poorly differentiated adenocarcinoma. Forty-five patients were without and 35 patients had lymph node metastasis. Forty-five patients were of Dukes A to B stage, and 35 were of C to D stage. Another 22 paraffin-embedded tissue blocks of normal colorectal epithelium (> 5 cm away from the edge of the tumor) was the control group.

Drugs and reagents

Mouse anti-human ezrin mAb was purchased from Fujian Maixin Biotechnology Development Co. Ltd, and SP kit DAB from Beijing Zhong Shan Jinqiao Biotechnology Development Co. Ltd. Experiments were performed following the instructions of the manufacturers. PBS (0.01 mmol/L) was used to replace the first antibody as a negative control, while the normal colorectal mucosa was a positive control.

Result judgment

Each stained slide was assessed and given a score according to the classification standard of Mathew *et al*[11]: score 0, no expression; score 1, < 50% of cells staining positive expression or less; score 2, ≥ 50% of cells staining positive expression. Score 0-1 was recorded as negative, and score 2 recorded as positive.

Statistical analyses

SPSS for Windows version 11.0 was used for statistical analyses. The χ^2 test was used in the analysis of the relationship between ezrin and colorectal cancer clinicopathological parameters. $P \leqslant 0.05$ was considered as a significant difference.

RESULTS

The positive expression of ezrin in colorectal cancer was significantly higher than that in normal colorectal mucosa (Figure 1A-E). The positive rate of ezrin protein in normal colorectal mucosa was 9.09% (2/22) and 75.00% (60/80) in colorectal cancer tissues. There were significant differences between the two groups (75.00% *vs* 9.09%, $P < 0.01$), as shown in Table 1.

Table 1 Ezrin expression in different colorectal tissues *n* (%)

Group	*n*	Positive expression (%)
Normal colorectal mucosa	22	2 (9.09)[b]
Colorectal cancer tissues	80	60 (75.00)

[b]$P < 0.01$ *vs* colorectal cancer tissues.

Table 2 Relationship between ezrin expression in colorectal cancer tissues and clinicopathological parameters *n* (%)

Clinicopathological parameters	*n*	Ezrin positive expression (%)
Well-differentiated	28	14 (50.00)[b]
Moderately and poorly differentiated	52	46 (88.46)
Lymph node metastasis	35	33 (94.28)[d]
Without lymph node metastasis	45	27 (51.11)
Dukes A to B stage	45	27 (51.11)[e]
Dukes C to D stage	35	33 (94.28)

There was a close relationship between ezrin expression and the degree of tumor differentiation, lymph node metastasis and Dukes stage. There were significant differences between the well-differentiated and the moderately and poorly differentiated groups ([b]$P < 0.01$); lymph node metastasis group *vs* group without lymph node metastasis ([d]$P < 0.01$); Dukes A to B stage *vs* Dukes C to D stage ([e]$P < 0.01$).

Relationship between ezrin expression in colorectal cancer tissues and clinicopathological parameters

There was a close relationship between ezrin expression and the degree of tumor differentiation, lymph node metastasis and Dukes stage (88.46% *vs* 50.00%, $P < 0.01$; 94.28% *vs* 51.11%, $P < 0.01$; 94.28% *vs* 51.11%, $P < 0.01$), as shown in Table 2.

DISCUSSION

Ezrin protein expression in specific cell membrane regions is mainly involved in the connection between the epithelial cell cytoskeleton and the cell membrane, through membrane surface signaling molecules and some transmembrane signal transduction pathway. It participates in the regulation of cell survival, adhesion, proliferation and migration processes. Recent studies have found that ezrin protein may play an important role in the tumorigenesis, development, invasion and metastasis process, probably through regulating adhesion molecules and participating in cell signal transduction, and other channels in the tumor[12-17]. Ezrin protein is an indispensable factor for tumor cell metastasis of osteosarcoma[18], breast cancer[19], nasopharyngeal carcinoma[20], and prostate cancer[21]. In addition, in malignant tumor tissues, there are also changes in subcellular localization of ezrin expression. Moilanen *et al*[22] found that ezrin expression in normal ovarian epithelial cells is a kind of cell polarity expression, and that ezrin expression in malignant ovarian tumor cells is more diffusive, with a different degree of tumor cell differentiation, and the location and intensity of ezrin expression in cells is quite different. Therefore, we speculate that ezrin subcellular localization in normal

Figure 1 Ezrin expression (HE, × 400). A: Normal colorectal mucosa; B: Well-differentiated adenocarcinoma; C: Poorly differentiated adenocarcinoma; D: Adenocarcinoma without lymph node metastasis; E: Adenocarcinoma with lymph node metastasis.

cells forms the foundation of various physiological functions and cell structure. Abnormal ezrin expression or distribution will also lead to abnormal cell structure and physiological function, and accordingly, these abnormal changes participate in the occurrence, development, invasion and metastasis of malignant tumors.

The role of ezrin in tumor progression is very important and deserves much attention. Recent studies have found that ezrin is a key factor in Fas-mediated apoptosis[23], in the P-gp1-mediated multidrug resistance of cancers, and in cannibalism of metastatic tumors[24]. The active ezrin C-terminal is connected with the actin cytoskeleton, and the N-terminal is connected with cell adhesion molecules such as E-cadherin, and CD44[25,26], *etc*. Ezrin participates in regulating cell-cell and cell-extracellular matrix adhesion, thus influencing tumor cell invasion and other biological behavior[27-30]. CD44 is a cellular membrane receptor which can specially recognize hyaluronic acid and collagen, and regulate cell-cell and cell-extracellular matrix adhesion. Some studies have found that ezrin, CD44 and CD44 variants could make up a compound that is co-expressed in the tumor cells[1]. Pujuguet *et al*[31] have found that ezrin can regulate E-cadherin expression in the cell membrane through Rho protein, thereby regulating cell adhesion. At the same time, ezrin also has regulating function in the E-cadherin membrane localization, and activated ezrin can make the E-cadherin protein aggregate in the cell, thereby undermining the cell-to-cell contact and intercellular adhesive ability, and the overexpression of ezrin in the tissues also has the same function of weakening the intercellular adhesion[32]. Through activation of RhoA and the MAPK pathway, ezrin can promote the cell adhesion plaque formation, thereby promoting the adhesive function between the tumor cells and other cells, as well as stoma cells[33]. Therefore, we believe, through participation in the formation of the cell adhesion plaque, cytoskeletal connections and cell surface compounds assembly, and other biological functions, ezrin protein mediates and regulates cell-cell and cell-extracellular matrix adhesion, and is also involved in the malignant tumor invasion and metastasis process. This study showed that, the overexpression of ezrin in colorectal cancer tissues may

be involved in cancer invasion and metastasis. The studies on the correlation between ezrin protein and cancer might help us further reveal the tumor invasion and metastasis mechanism, and find the targets for inhibiting tumor metastasis, or indicators that forecasts the prognosis of patients with tumors.

COMMENTS

Background

Ezrin belongs to the ezrin/radixin/moesin (ERM) protein family, which act as membrane organizers and linkers between the plasma membrane and cytoskeleton. Ample evidence has indicated that ezrin is regarded as a metastatic determinant and a key component in tumor progression and metastasis; however, its role in the process of colorectal cancer growth and metastasis is not clearly understood.

Research frontiers

Recent studies have found that a high level of ezrin protein expression can induce a variety of cell line conversions, as well as abnormal hyperplasia. Ezrin is a key factor in Fas-mediated apoptosis, the P-gp1-mediated multidrug resistance of cancers, and cannibalism of metastatic tumors.

Applications

This preliminary study about ezrin in colorectal cancer growth and metastasis may pave the way for further clinical studies on colorectal cancer dissemination and metastasis.

Terminology

Ezrin belongs to the ERM protein family, which are expressed in specific cell membrane regions, and acts as membrane organizers and linkers between the plasma membrane and epithelial cell cytoskeleton.

Peer review

This study is interesting, and discusses the ezrin expression in normal colorectal mucosa and colorectal cancer tissues, and the clinical relevance of ezrin expression to tumor invasion and metastasis. The study was carefully performed and the data and conclusions drawn are sound.

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BRIEF ARTICLES

Overexpression of DNA methyltransferase 1 and its biological significance in primary hepatocellular carcinoma

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Abstract

AIM: To explore the relationship between DNA methyltransferase 1 (DNMT1) and hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) and its biological significance in primary HCC.

METHODS: We carried out an immunohistochemical examination of DNMT1 in both HCC and paired non-neoplastic liver tissues from Chinese subjects. DNMT1 mRNA was further examined in HCC cell lines by real-time PCR. We inhibited DNMT1 using siRNA and detected the effect of depletion of DNMT1 on cell proliferation ability and cell apoptosis in the HCC cell line SMMC-7721.

RESULTS: DNMT1 protein expression was increased in HCCs compared to histologically normal non-neoplastic liver tissues and the incidence of DNMT1 immunoreactivity in HCCs correlated significantly with poor tumor differentiation ($P = 0.014$). There were more cases with DNMT1 overexpression in HCC with HBV (42.85%) than in HCC without HBV (28.57%). However, no significant difference in DNMT1 expression was found in HBV-positive and HBV-negative cases in the Chinese HCC group. There was a trend that DNMT1 RNA expression increased more in HCC cell lines than in pericarcinoma cell lines and normal liver cell lines. In addition, we inhibited DNMT1 using siRNA in the SMMC-7721 HCC cell line and found depletion of DNMT1 suppressed cells growth independent of expression of proliferating cell nuclear antigen (PCNA), even in HCC cell lines where DNMT1 was stably decreased.

CONCLUSION: The findings implied that DNMT1 plays a key role in HBV-related hepatocellular tumorigenesis. Depletion of DNMT1 mediates growth suppression in SMMC-7721 cells.

INTRODUCTION

DNA methylation plays an important role in transcriptional regulation, chromatin remodeling and genomic stability[1-3]. It is catalyzed in mammalian cells by a family of highly related DNA methyltransferases (DNMTs) that use S-adenosylmethionine as the methyl donor[4]. Alteration of DNA methylation is one of the most consistent epigenetic changes in human cancers and is involved even in the early and precancerous

stages of human carcinogenesis[5,6]. Overexpression of DNMT1 has been detected in several human cancers[7-11] and showed a relatively significant correlation with tumorigenesis, but not in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). HCC is a devastating disease with a very poor prognosis, and is the fourth or fifth largest cause of cancer-related death worldwide[12,13]. More than 85% of Chinese HCC patients have HBV infection history. Data on hepatocarcinogenesis in other countries showed that DNMT1 mRNA and protein expression were significantly higher in HCCs[14,15]. Therefore, DNMT1 may play an important role during hepatocarcinogenesis. However, the relationship between HBV infection and DNMT1 in HCC has still to be elucidated, especially in Chinese subjects. In the present study, we examined the expression of DNMT1 both in HBV-related HCC cases and cell lines from Chinese subjects. DNMT1 expression was significantly increased both in tumor tissues and HCC cell lines compared with corresponding controls. To further investigate and evaluate the possibility of DNMT1 knockdown strategies for cancer therapy, we inhibited DNMT1 using siRNA and examined the cell growth and cell proliferation ability in the HCC cell line SMMC-7721.

MATERIALS AND METHODS

Tissue specimens
We selected 42 cases of surgically resected livers from the surgical pathology files of the Department of Pathology at The Qidong County Hospital, China. The patients' clinicopathologic features are shown in Table 1. All the HCC samples were diagnosed by a single pathologist and appropriate consent was obtained. Paired samples of primary HCC and matched non-cancerous normal tissues were obtained from each patient. The pathological classification of HCC tissues was carried out and the stage of each HCC was determined according to criteria. Histological examination of noncancerous liver tissues from HCC patients revealed no remarkable findings. Thirty-five cases were associated with HBV infection, and 7 cases were negative for HBV.

Cell lines
Nine human cell lines were cultured for the study. Human hepatocellular carcinoma cell lines (BEL-7402, BEL-7404, BEL-7405, QGY-7703, QGY-7701, SMMC-7721), an immortalized human hepatocellular normal cell line (HL-7702) or a pericarcinoma (QSG-7701) cell line were cultured in RPMI-1640 (Life Technologies, Inc., Rockville, MD) containing 10% new born bovine serum in 5% CO_2 incubation at 37℃.

Preparing a vector-based siRNA construct for DMMT1
siRNAs targeting DNMT1 were designed and prepared as described previously[16]. The siRNA sequences against DNMT1 were designed as sense and antisense oligonucleotides corresponding to nucleotide position 2620-2638 of human DNMT1 (GenBank accession No. NM001379.1). The siRNA sequence and scrabble

Table 1　Clinicopathological features of hepatocellular carcinoma patients

Patients and tissue specimens	*n*
Sex	
Male	36
Female	6
Viral status	
HBs-Ag positive	35
HBs-Ag negative	7
Histology of noncancerous liver tissues	
Histologically normal	18
Liver cirrhosis	24
Tumor differentiation	
Well differentiated	0
Moderately differentiated	22
Poorly differentiated	20
Total	42

sequence were sub-cloned into the pSUPER-EGFP vector (gift from Dr. Dianqing Wu), which was identified by *Hin*dⅢ (TAKARA) and *Bgl*Ⅱ (TAKARA), to be the DNMT1 siRNA construct named pMT1, with sMT1 as a control.

Transfection of DNMT1 RNAi construct to hepatocellular carcinoma cell line SMMC-7721
The human HCC cell line SMMC-7721 (No. TCHu13 Cell Bank Shanghai, China) was cultured in RPMI-1640 containing 10% new born bovine serum in 5% CO_2 incubation at 37℃. Cells were transfected with 1.5 μg of DNMT1 siRNA (pMT1) construct or sMT1 using transfectamine™ 2000 reagent (Invitrogen), and selected with 0.4 mg/mL genetincin (Life Technologies). SMMC-7721 cells were transfected with pMT1 and named 7721-MT1 cell lines or transfected with sMT1 and named 7721-sMT1 as a control.

Immunohistochemistry
Four micrometre thick sections of formalin-fixed, paraffin embedded tissue specimens from all 42 patients were deparaffinized and dehydrated. For antigen retrieval, the sections were heated for 10 min at 120℃ in an autoclave, and nonspecific reactions were blocked with 5% normal horse serum. All sections were incubated with specific primary antibodies that recognized DNMT1 (goat polyclonal antibody, dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). We previously confirmed the specificity of the goat anti-human DNMT1 polyclonal antibody by Western blotting analysis: an immunoreactive band of 193.5 kDa, corresponding to the molecular mass of DNMT1, was detected in human cancer cells, but no nonspecific bands were detected with this antibody[16]. All primary antibody incubations were conducted at 4℃ overnight and were followed by incubation with biotinylated secondary antibodies (anti-goat IgG, anti-mouse IgG, dilution 1:200; QIAGEN Laboratories) at room temperature for 30 min. The sections were then treated with Vectastain Elite ABC reagent (Vector Laboratories). All sections were counterstained with hematoxylin. For negative

control preparations, the primary antibody was omitted from the reaction sequence.

Real time PCR to detect mRNAs of DNMT1

Total RNA was purified from a normal liver cell line, a cell line established from pericarcinoma tissue and HCC cell lines with TRIzol (Invitrogen). The first-strand cDNA was synthesized from 2 μg total RNA using Oligo (dT) 18 primer and SuperScript II reverse transcription kit (Life Technologies). A PCR reaction was performed in a 50 μL volume with 5U polymerase (TAKARA) and cDNA samples equivalent to 1 ng of RNA. SYBR green with 20 000 dilutions was included in each reaction for relative quantification in the ABI 7300 sequence detection system (Applied Biosystems). To normalize the input load of cDNA among samples, β-actin was quantified and used as an endogenous standard. The relative level of expression of each DNMT1 among different cell lines was then calculated accordingly (ABI PRISM 7300 Sequence Detection System, USA). The primers used for PCR were as follows: DNMT1: sense primer, 5'-CCGAGTTGGTGATGGTGTGATC-3'; antisense primer, 5'-AGGTTGATGTCTGCGTGGTAG C-3'. β-actin: 5'-AAAGACCTGTACGCCAACAC-3'; antisense primer, 5'-GTCATACTCCTGCTTGCT GAT-3'. PDCD4: sense primer 5'-TGGATGAAAGGG CATTTGAGA-3'; antisense primer, 5'-AGCCTTCCC CTCCAATGCTA-3'.

Western blotting analysis

Cells were grown and harvested at 80%-85% confluency, and cellular proteins were extracted with lysis buffer containing 0.5% NP-40, 150 mmol/L NaCl, and 1 mmol/L EDTA in 50 mmol/L Tris-HCl at pH 7.5, supplemented with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). The protein concentration of each extract was quantified by BCA assay (Pierce, Rockford, IL). Two to forty microliter of total protein was electrophoresed on 7%-15% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (PVDF, Amershan) electrophoretically. After blocking with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, membranes were incubated with goat anti-DNMT1 (Santa Cruz Biotechnology), proliferating cell nuclear antigen (PCNA) (mouse monoclonal antibody, dilution 1:200, Lexington, KY) or mouse monoclonal anti-actin (Sigma) antibodies. The membranes were then developed with peroxidase-labeled antibodies (Amersham Pharmacia, Piscataway, NJ) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL). Actin protein levels were used as a control for equal protein loading.

In vitro analysis of cell growth

Cell proliferation of transiently transfected SMMC-7721 cells was measured by trypan blue dye cell count assay. Cells were cultured in triplicate in 12-well plates at a concentration of 3×10^4 cells per well. Cells were collected at 1, 3, 5 and 7 d and exposed to trypan blue (Sigma), and nonviable cells took up the dye. Both viable (unstained) and nonviable (stained) cells were counted, and the relative survival rate (%) of each siRNA treatment was then calculated.

Flow cytometric (FCM) analysis

Stably transfected SMMC 7721 cells were washed, resuspended in staining buffer, and examined by ApoAlert Annexin V Apoptosis kit (BD Biosciences) and PI according to the manufacturer's instructions. Stained cells were analyzed by FACS (FACScalibur, BD Biosciences).

RESULTS

Immunohistochemical analysis of DNMT1 in pericancerous liver tissues and HCCs

Immunoreactivity for DNMT1 was detected in the nuclei and cytoplasm, but not in the cell membranes, of cancer cells (Figure 1). To discriminate definitely positive cases from cases with a leaky background level signal, if more than 30% of the cells in a tissue sample exhibited nuclear and cytoplasm staining the sample was considered to show positive immunoreactivity. Nuclear and cytoplasmic DNMT1 immunoreactivity was detected and overexpressed in 40.48% of patients. The incidence of DNMT1 immunoreactivity in carcinoma correlated significantly with poor tumor differentiation (Table 2, $P = 0.014$, χ^2 test). The incidence of DNMT1 immunoreactivity was significantly higher in HCCs than in pericancerous liver tissues. DNMT1 protein overexpression was not significantly associated with other parameters relating to cancer aggressiveness, such as the depth of invasion, vascular involvement, or lymph node metastasis. In HBV-related HCC cases, 70% had different degrees of hepatocirrhosis. To investigate whether there was an HBV-induced increase in DNMT1 expression in HCC, we evaluated the relationship between HBV infection and up-regulated DNMT1. There was higher expression of DNMT1 in HCC with HBV than in HCC without HBV. However, there was no significant correlation between the incidence of DNMT1 immunoreactivity in HCCs and the patients' viral status (HBs-Ag-positive, HBs-Ag-negative) by statistical analysis.

Expression of DNMT1 in nine cell lines

In order to determine whether the expression of DNMT1 was different between HCC cell lines and normal hepatocellular cell lines which were cultured under the same conditions, real-time RT-PCR was carried out in ABI 7300. DNMT1 was increased more in most HCC cell lines, especially in BEL-7405, BEL-7402 and SMMC-7721, than in a normal cell line and a pericarcinoma cell line. There were a trend that DNMT1 increased more in HCC cell lines than in a normal liver cell line and a pericarcinoma cell line. In contrast to the low expression level of DNMT1 in the pericarcinoma cell line, the levels of DNMT1 in most HCC cell lines were increased more than 2-fold (Figure 2).

Figure 1 DNMT1 expression was analyzed in pericancerous liver tissues and HCCs by immunohistochemistry (x 100). A:The expression of DNMT1 was detected in cirrhotic noncancerous liver tissue obtained from HCC patients; B: The expression of DNMT1 was detected in well differentiated HCC; C: The expression of DNMT1 was detected in poorly differentiated HCC. The arrow is to show DNMT1 antibody staining in cell nuclei.

Figure 2 DNMT1 mRNA expression levels analysis in HCC cell lines by quantitative real-time PCR. DNMT1 mRNA expression levels were normalized according to the β-actin mRNA level of the same cell line. DNMT1 was increased more in most hepatocellular carcinoma cell lines, especially in BEL-7402, BBEL-7405 and SMMC-7721, than in the normal cell line HL-7702 and pericarcinoma cell line QSG-7701.

Figure 3 Inhibition of DNMT1 affects cell growth in HCC cell lines. Expression of endogenous DNMT1 protein in 7721-MT1 cells transfected with DNMT1 RNAi construct were decreased more than 80%. A: The SMMC-7721 cells were transfected transiently with DNMT1 siRNA and analyzed for their survival by trypan blue staining assay. The assays were performed in triplicates and the data are the mean ± SE; B: Western blotting analysis of DNMT1 expression in cells transfected transiently with DNMT1 siRNA for 1, 3, 5, 7 d; C: The SMMC-7721 cells were transfected stably with DNMT1 siRNA for 2 mo and the inhibition of DNMT1 and expression of PCNA were evaluated.

Decrease in DNMT1 suppresses growth of human hepatocellular carcinoma cells in vitro

We analyzed the proliferation ability of SMMC-7721 cells transfected with DNMT1 siRNA constructs and its control by FCM and trypan blue assay. The resulting RNA interference had a negative effect on SMMC-7721 growth (Figure 3). This growth suppression was noticeable two days after transfection, and the effect was more prominent in DNMT1 knockdown cells than at seven days after transfection (Figure 3A). For the determination of DNMT1 protein expression in construct pMT1 transiently transfected SMMC 7721 cells, β-actin was employed for adjustment of DNMT1 expression data to protein content. PCNA, an established cell proliferation marker and the DNA replication factor to which DNMT1 binds, was employed to evaluate cell proliferation. Comparison with PCNA expression levels

revealed that the changes in DNMT1 expression were independent of the cell proliferation status (Figure 3B). The data showed that DNMT1 levels started to decrease on day 3 and was maintained until seven days after transfection with cell growth inhibition. However, there were no significant changes in PCNA expression, even in a stably decreased DNMT1 HCC cell line (Figure 3C).

DNMT1 knockdown induced cell apoptosis in SMMC-7721 cell line

Cell apoptosis detection was performed by flow cytometric analysis as described above. The data from

Table 2 Relationship between DNMT1 and pathological change in patients with HCC n (%), χ^2 test

| | Subtotal | DNMT1 expression (P/OR: up-regulated/normal + down-regulated) | | | | |
		Up-regulated	Normal	Down-regulated	P	OR (95% CI)
Total or subtotal	42 (100.00)	17 (40.48)	13 (30.95)	12 (28.57)		
Age (yr)					0.54	1.49 (0.41-5.35)
> 50	15 (35.71)	7 (46.67)	4 (26.67)	4 (26.67)		
< 50	27 (64.29)	10 (37.04)	9 (33.33)	8 (29.63)		
Size of tumor (cm)					0.23	0.45 (0.12-1.67)
> 5	17 (40.48)	5 (29.41)	6 (35.29)	6 (35.29)		
< 5	25 (59.52)	12 (48.00)	7 (28.00)	6 (24.00)		
Histological differentiation					0.014	0.20 (0.05-0.75)
Moderate	22 (52.38)	5 (22.72)	9 (40.91)	8 (36.37)		
Poor	20 (47.62)	12 (60.00)	4 (20.00)	4 (20.00)		
HBsAg					0.169	4.5 (0.829-24.4)
Positive	35 (83.33)	15 (42.86)	12 (34.28)	8 (22.86)		
Negative	7 (16.67)	2 (28.57)	1 (14.29)	4 (57.14)		
Hepatocirrhosis					0.083	3.57 (0.81-15.71)
With	24 (57.14)	10 (41.67)	10 (41.67)	4 (16.67)		
Without	18 (42.86)	3 (16.67)	7 (38.89)	8 (44.44)		

Figure 4 Knock-down DNMT1 induces cell apoptosis in HCC cell lines. A: The SMMC-7721 cells were transfected stably with DNMT1 siRNA for 2 mo and apoptosis was detected by FACS; B: Knock-down DNMT1 induced apoptosis gene PDCD4 expression in 7721-MT1 cell line compared with control cell line by quantitative real-time PCR. [a]Show the significant difference between 7721-pMT1 and control.

the present study indicated that DNMT1 knockdown induced cell apoptosis. The apoptotic rate increased from 8.78% ± 0.44% to 14.24% ± 0.12%. These results indicated that DNMT1 knockdown may induce HCC cell apoptosis (Figure 4). The apoptosis gene *PDCD4* was induced by DNMT1 siRNA in SMMC-7721.

DISCUSSION

HCC is well known to develop through the stages of dysplasia and early HCC in a background of chronic liver diseases, including chronic hepatitis and liver cirrhosis. Increased DNMT1 mRNA expression has been reported in a number of human cancers[17-19]. DNMT1 may play an important role during hepatocarcinogenesis even at the precancerous stage[9,20]. Although DNMT1 expression and its role were evaluated in different populations, information from Chinese subjects was still unclear. HCC is one of the most lethal and prevalent cancers in China, where HBV is one of the main attributable risk factors[4,21]. To study the significance of aberrant DNMT1 expression during hepatocarcinogenesis in a Chinese population, we used an immunohistochemical technique to directly evaluate DNMT1 protein expression in noncancerous liver tissues and HCCs, and real-time PCR to evaluate DNMT1 expression in HCC cell lines and a normal liver cell line.

After all tumors were analyzed, HBV infection was present in 83.3% of the samples, indicating that our study cohort is representative of the subset of HCC arising from HBV infection, which is the most considerable risk factor for HCC in studied cases. In the present study, more than 80% of nonneoplastic liver tissues with either chronic hepatitis or liver cirrhosis showed variable nuclear and cytoplasm immunopositivity for DNMT1. DNMT1 immunoreactivity was certainly detected in HCCs, as it was in other cancers[22-24]. DNMT1 overexpression was detected in 40.48% of the patients and the incidence of DNMT1 immunoreactivity was significantly higher in HCC than in pericancerous liver tissues. Specifically, the incidence of DNMT1 immunoreactivity in HCCs correlated significantly with poor tumor differentiation. The above evidence indicates that increased DNMT1 protein expression may play a role in the malignant progression of HCCs in Chinese subjects. Alternatively, in virus-associated cancers, viral proteins have been shown to disturb the host DNA methylation system by up-regulating DNMT activities, thereby increasing tumor susceptibility[25].

Park *et al*[26] showed that HBx promoted specific regional hypermethylation of tumor suppressor genes and genome-wide hypomethylation by transcriptional regulation of DNMTs. HBx expression elevates overall intracellular DNMT activity by inducing DNMT1 and DNMT3A. HBx promotes epigenetic abnormalities by modulating the expression of DNMTs immediately after HBV infection, thus epigenetically accelerating hepatocarcinogenesis. In this study, we evaluated DNMT1 expression in HBV-positive cases and HBV-negative cases. No significant relationship was found between HBV infection and DNMT1 up-regulation. These data suggested there is a different mechanism of HBV affected by DNMTs expression in diverse ethnic populations although additional studies with larger sample sizes are required to confirm our findings.

According to the results of both immuno-histochemistry and real time RT-PCR, progressive increases in DNMT1 expression may be accompanied by hepatocarcinogenesis from the precancerous stage to the malignant progression of HCC. Immunohistochemical analysis of DNMT1 in biopsy specimens obtained for diagnostic purposes and/or surgically resected materials may show that DNMT1 is a biologic predictor of both HCC recurrence and poor prognosis in HCC patients. To address whether DNMT1 overexpression plays an important role in hepatocellular carcinogenesis in Chinese subjects and to evaluate DNMT1 knockdown strategies for cancer therapy, it is necessary to deplete DNMT1 in the HCC. To avoid the problem of DNMT1 siRNA not being sufficient to inhibit cell growth due to the short-term inhibition of DNMT1 expression, we employed the RNAi technique to knockdown DNMT1 expression in an HCC cell line and assessed tumor cell growth in transiently transfected and stably expressed DNMT1 siRNA cell lines, respectively. Fortunately, we observed that depletion of DNA methyltransferase 1 mediates growth suppression in the HCC cell line SMMC-7721. In order to explore whether this inhibition of DNA replication reflects a distinct alteration in cell cycle kinetics, similar to the DNA damage checkpoints that trigger arrest at distinct phases of the cell cycle[27], we detected PCNA in the treated cells, and found there were no significant changes in PCNA expression in treated cells and controls. However, DNMT1 knockdown inhibited cell growth and induced cell apoptosis in the SMMC-7721 cell line. With a view to the results of cDNA microarray[16], PDCD4, an over-expression apoptosis gene, may contribute to the apoptosis rate in SMMC-7721 cells treated by siRNA. PDCD4 is a proapoptotic molecule involved in TGF-beta1-induced apoptosis in human HCC cells, and a possible tumor suppressor in hepatocarcinogenesis[28-30]. These results provide a rationale for the development of a DNMT1-targeted strategy as an effective epigenetic cancer therapy.

COMMENTS

Background
Hepatocellular carcinoma (HCC) is the fourth or fifth largest cause of cancer-related death worldwide. The aberration of DNA methylation (DNMT), which is catalyzed by DNA methyltransferases, is common in HCC. DNMT1 is the main and maintenance methyltransferase in mammals. DNMT1 may play an important role during hepatocarcinogenesis. However, the relationship between abnormal DNMT1 expression and hepatocellular carcinogenesis is still to be elucidated, especially in a Chinese population.

Research frontiers
Overexpression of DNMTs is a common event in nearly all malignancies, and DNMT1 may play an important role during hepatocarcinogenesis. In the present study, the authors demonstrated that the overexpression of DNMT1 is correlated significantly with poor tumor differentiation in HCC. Inhibition of DNMT1 through siRNA affected the proliferation ability of an HCC cell line.

Innovations and breakthroughs
Recently, most reports focus on the overexpression of DNMT1 and DNA methylation in tumors. However, abnormal expression of DNMT1 may be involved in tumorigenesis, especially in HBV-related HCC. RNA interference is a direct and efficient way to suppress the target gene in a gene function study. This is the first study to report that inhibition of DNMT1 *via* RNAi suppressed cell proliferation and induced cell apoptosis in an HCC cell line.

Applications
This study implied that DNMT1 maybe considered as a target for HCC therapy. The study also gives a general and better understanding of tumor cell biology and the potential epigenetic mechanism of hepatocellular carcinogenesis.

Terminology
DNMTs are crucial components of DNA methylation. DNMT1 is the major and best-known DNMT in somatic cells. DNMT1 overexpression correlated significantly with poorer tumor differentiation, but not with the phenotype of the cancer cells, in several tumors. DNMT1 protein might have a more direct and immediate effect on the state of cellular growth and transformation.

Peer review
The manuscript by Hong *et al* shows an increased expression of DNMT1 in hepatocellular carcinoma tissue samples and cell lines compared to controls. The increase was correlated with the degree of differentiation. Knockdown of DNMT1 expression in HCC cell lines decreased the number of cells at the end of the experiment. This did not correlate with decreased proliferating cell nuclear antigen (PCNA) expression but was associated with an increase in apoptosis and the expression of apoptosis-related gene PDCD4. For the most part the data is convincing and the experiments are well carried out.

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Synchronous incidental gastrointestinal stromal and epithelial malignant tumors

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Abstract

AIM: To investigate the incidence of incidental gastrointestinal stromal tumor (GIST) and its etiopathogenesis.

METHODS: From January 1, 2000 to December 31, 2007, 13 804 cases of gastrointestinal epithelial malignant tumor (EMT) and 521 cases of pancreatic adenocarcinoma (PAC) were successfully treated with surgery at the Department of General Surgery and the Department of Thoracic Surgery, West China Hospital, Sichuan University, China. The clinical and pathologic data of 311 cases of primary GIST, including 257 cases with clinical GIST and 54 cases of incidental GIST were analyzed.

RESULTS: Of the 311 patients, 54 had incidental GIST, accounting for 17.4%. Of these tumors, 27 were found in 1.13% patients with esophageal squamous cell carcinoma (ESCC), 22 in 0.53% patients with gastric adenocarcinoma (GAC), 2 in 0.38% patients with PAC, 2 in 0.03% patients with colorectal adenocarcinoma, and 1 in one patient with GAC accompanying ESCC, respectively. Patients with incidental GIST presented symptoms indistinguishable from those with EMT. All incidental GIST lesions were small in size, and the majority had a low mitotic activity while only 1.9% (5/257) of clinical GIST lesions had a high risk.

CONCLUSION: Incidental GIST may occur synchronously with other tumors and has a high prevalence in males. Surgery is its best treatment modality.

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of gastrointestinal (GI) tract, probably arising from precursor interstitial cells of Cajal. Significant advances have been made in symptomatic GIST in the last two decades[1,2]. However, little is known about the incidental GIST detected during examinations or surgery for other reasons. Its clinicopathologic characteristics are unclear. Many cases of synchronous or asynchronous GIST with other tumors have been reported as single cases[3-6]. We discovered 54 cases of incidental GIST during surgery for epithelial malignant tumor (EMT). This study was to investigate the incidence of incidental GIST and its etiopathogenesis.

MATERIALS AND METHODS

Patients

From January 1, 2000 to December 31, 2007, 13 804 cases of gastrointestinal EMT and 521 cases of pancreatic adenocarcinoma (PAC) were successfully treated with surgery at the Department of General

Table 1 Location of 54 incidental GIST lesions and their corresponding EMT

EMT	Patients (n)	Median age	Gender (M/F)	Incidental GIST site (No. of patients)							
				Gastric cardia	Gastric fundus	Gastric body	Gastric antrum	Esophagus	Terminal ileum	Colon	Omentum
GAC	22	64.5 (45-79)	19/3	1	7	13	1	-	-	-	-
ESCC	27	63 (44-77)	24/3	1	3	19	1	2	-	-	1
GAC + ESCC	1	79	1/0	-	-	1	-	-	-	-	-
CRA	2	57.5 (54-61)	2/0	-	-	-	-	-	1	1	-
PAC	2	67.5 (65-70)	2/0	-	1	1	-	-	-	-	-
Total	54	63 (44-79)	48/6	2	11	34	2	2	1	1	1

GIST: Gastrointestinal stromal tumor; EMT: Epithelial malignant tumor; GAC: Gastric adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; CRA: Colorectal adenocarcinoma; PAC: Pancreatic adenocarcinoma.

Surgery and the Department of Thoracic Surgery, West China Hospital, Sichuan University, China. Gastrointestinal EMT cases included 2382 cases of esophageal squamous cell carcinoma (ESCC), 35 cases of esophageal adenocarcinoma (EAC), 4168 cases of gastric adenocarcinoma (GAC), 329 cases of small intestinal adenocarcinoma (SAC), and 6890 cases of colorectal adenocarcinoma (CRA). During this period, 311 cases of primary GIST (121 females, 190 males) were identified in our center, including 257 cases of clinical GIST and 54 cases of incidental GIST.

Methods

Hospital records of patients with incidental GIST were reviewed. Each patient was followed up by telephone or mail. Histopathologic features of primary GIST were evaluated by two experienced pathologists, blinded to their respective findings and patient outcomes, at the Department of Pathology, West China Hospital. The largest diameter of tumor was recorded. In patients with multiple GIST lesions, only the largest GIST lesion was included in pathological analysis. The risk category for GIST was defined by assessing the tumor size and mitotic count following the consensus guidelines of the National Institutes of Health-(NIH-NCI) workshop[7]. In addition to the assessment of CD117 in tumor cells, reactions with CD34, SMA, and S-100 proteins were also studied. Immunohistochemical examination of these proteins was performed on tumor tissues embedded in paraffin with DAKO (Glostrup, Denmark) antibodies according to the manufacturer's instructions.

Statistical analysis

Categorical variables were compared by χ^2 test or by Fisher's exact test where applicable. Survival analysis was performed using the Kaplan-Meier method. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of the 311 patients, 54 had incidental GIST, accounting for 17.4%. Among these tumors, 27 were found in 1.13% patients with ESCC, 22 in 0.53% patients with GAC, 2 in 0.38% patients with PAC, 2 in 0.03% patients with CAC, and 1 in one patient with GAC accompanying ESCC,

respectively.

The median age of the 54 cases of incidental GIST was 63 years (range, 44-79 years). Interestingly, 48 of them (88.9%) were males, and 6 (11.1%) were females ($P < 0.001$). The patients presented symptoms of EMT without specific clinical manifestations indicative of GIST. Among the 54 patients, only a submucous lesion in gastric fundus, 2.5 cm in diameter, was preoperatively detected in 1 patient with GAC by gastroscopy, and a single-lesion was postoperatively detected in 4 patients by specimen examination. A total of 58 incidental GIST lesions were discovered in the 54 patients, including 51 single-lesions, 2 double-lesions, and 1 triple-lesion. A total of 90.7% incidental GIST lesions occurred in stomach, 3.6% in esophagus, 1.9% in terminal ileum, 1.9% in colon and 1.9% in omentum, respectively. The most common sites were the gastric fundus and body. In our series, 4 cases with a unique coexistence style (esophageal GIST + ESCC: 2, gastric GIST + ESCC + GAC: 1, colonic GIST + CRA: 1) have not been reported previously. The location of 54 incidental GIST lesions and their corresponding EMT lesions are shown in Table 1.

Of the incidental GIST lesions, 37 (68.5%) were of spindle-cell morphology, 9 (16.7%) epithelioid morphology, and 8 (14.8%) a mixed histological type. Immunohistochemical staining showed that 50 cases (92.6%) and 52 cases (96.3%) of incidental GIST were positive for CD117 and CD34, respectively. None of them was proven to have a metastasis of GIST, while 29 cases were confirmed with metastasis derived from EMT. Incidental GIST was small in size. The majority (90.7%) had a low mitotic activity and a very low risk, while only 1.9% cases of clinical GIST had a very low risk ($P < 0.001$), and 38.5% had a high risk with a marked mitotic activity (Table 2).

All the GAC patients received radical excision (distal gastrectomy for 3, proximal gastrectomy for 2, total gastrectomy for 12, esophagogastrectomy for 5). All the ESCC patients including the patient with triple tumors underwent esophagogastrectomy. The two PAC patients underwent duodenopancreatectomy and distal pancreatectomy, respectively, with local gastrectomy. Right and left hemicolectomy was performed for the two CRA patients, respectively. Thirty-four out of the 54 patients received either adjuvant chemotherapy and/or radiotherapy after operation. None of them received oral Imatinib mesylate (Glivec) treatment. On September

Table 2 Distribution of gender, age, tumor site, tumor size, and risk in 311 patients with GIST

GIST	Patients (*n*)	Gender (M/F)	Median age in yr (range)	Tumor site (No. of patients)	Tumor size (cm)			Risk patients, *n* (%)
					Median	Mean	Range	
Incidental GISTs	54	48/6	63 (44-79)	Gastric (49), esophagus(2), ileum(1), colon (1), omentum (1)	0.8	0.9	0.2-2.5	VL: 49 (90.7); L: 5 (9.3)
Clinical GISTs	257	142/115	57 (22-87)	Gastric (147), duodenum (10), jejunum-ileum (57), colon (25), rectum (3), anal canal (3), mesenterium (6), omentum (4), pancreatic (2)	7.5	6.2	1.5-30.0	VL: 5 (1.9); L: 86 (33.5); Int: 67 (26.1); H: 99 (38.5)
Total	311	190/121	61 (22-87)	Gastric (196), esophagus(2), duodenum (10), jejunum-ileum (58), colon (26), rectum (3) anal canal (3), mesenterium (6), omentum (5), pancreas (2)	6.3	5.5	0.2-30.0	VL: 54 (17.4); L: 91 (29.3); Int: 67 (21.5); H: 99 (31.8)

Risk was determined as previously described[7]. VL: Very low risk; L: Low risk; Int: Intermediate risk; H: High risk.

Figure 1 Kaplan-Meier survival curves. A: 22 patients with GIST accompanying GAC; B: 27 patients with GIST accompanying ESCC.

1, 2008, four of the patients were alive while 50 died of recurrence or distal metastasis of other malignancies. The remaining two patients died of other causes. Recurrent GIST was not found during the survival period of all dead patients, and the follow-up time of the remaining four. The overall 5-year survival rate of the 22 patients with GAC and incidental GIST was 31.8%, with a median survival time of 36 mo (Figure 1A). The 5-year survival rate of the 27 patients with ESCC and incidental GIST was 22.2%, with a median survival time of 32 mo (Figure 1B). The average survival time of the

two PAC and two CRA patients was 26 mo and 52 mo, respectively, and the survival time of the patients with triple tumors was 47 mo.

DISCUSSION

In our series, incidental GIST occurred simultaneously with EMT in 17.4% (54/311) of the GIST patients, which is higher than the reported incidence (14%)[8]. However, assessment of the actual incidence of incidental GIST with EMT is difficult, because the data are only based on patients who have been surgically treated, whereas EMT patients managed with non-surgical measures are unaccounted for. Moreover, during examination or surgery, identification of GIST is incidental rather than intentional, and many lesions are missed as a result.

Notably, in addition to those with EMT, many synchronous and asynchronous cases of GIST with non-epithelial tumors have been reported, such as osteosarcoma, Burkitt's lymphoma, plasmocytoma, neuroblastoma, somatostatinoma, chronic lymphatic leukemia, lipoma and ectopic pancreas[4,9-13]. Synchronous incidental GIST and non-tumorous diseases have been reported, such as ulcerative colitis, Meckel's diverticulum, rapidly progressive glomerulonephritis, HIV carriers, and Crohn's disease[5,14-17]. Sanchez *et al*[18] reported that incidental gastric GIST is found in 0.8% of patients undergoing laparoscopic Roux-en-Y gastric bypass surgery for obesity. Kawanowa *et al*[19] showed that microscopic GIST can be found in 35% of stomach-resected patients with gastric cancer. It has been shown that microscopic GIST can be found in 10% of patients undergoing surgery for esophageal carcinoma[20]. Especially, incidental GIST has also been detected in 0.2% of all autopsies, accounting for 10% of all patients with primary GIST[21]. These findings suggest that incidental GIST may occur synchronously with other diseases more frequently than expected, and the incidence of incidental GIST might be much higher than that of clinical GIST.

Particular attention has been paid to clinical GIST because of its striking symptoms such as gastrointestinal bleeding, pain, dyspepsia, abdominal mass and obstruction[22,23]. On the contrary, incidental GIST may emerge asymptomatically, and even if symptomatically, the symptoms may often be vague and nonspecific[18]. In our study, all the 54 patients presented symptoms

indistinguishable from those of EMT, which might have been overlooked because of the progressing symptoms of EMT such as severe dysphagia, weight loss, abdominal pain and anemia. The size of incidental GIST was small, and the majority (90.7%) of them had a very low risk. Also, only a few reports are available on incidental GIST with a high risk[21,24,25]. In this study, only 1.9% of clinical GIST lesions had a very low risk, and 38.5% had a high risk, indicating that GIST is malignant. Perhaps, incidental GIST might have emerged later than EMT, or their development may have been depressed by EMT through mechanisms which are yet to be studied.

Generally, the preoperative detection rate of incidental tumors is very low. In this study, except for two patients with PAC, the other patients received endoscopic examinations preoperatively, yet only one GIST lesion was found. Difficulty in detecting the lesion might be attributed to its small size and intramural location. Incidental GIST, if detected at CT or MRI, is often mistaken for metastatic lymph nodes derived from EMT. Therefore, radiological examination is minimally helpful for its diagnosis. As a result, the endoscopist and surgeon should take the major responsibility of detecting incidental GIST. Incidental GIST occurs most commonly in stomach, esophagus, small bowel, colon and omentum. Consistent with the reported findings[19], incidental GIST was observed in gastric fundus and body in the present study. Careful assessment of the hotspot (i.e. the upper portion of stomach) is important for both endoscopist and surgeon.

Interestingly, we found that there was a significant difference of the incidence of incidental GIST in male and female patients. Because of the unclear pathogenesis of incidental GIST, we cannot explain this finding. Further studies are needed on the gene expression in primary tumor cells from male and female patients and signal transduction may also provide us with some clues to this question.

In the absence of prospective control studies, whether resection of incidental GIST lesions helps to improve the quality of life and/or the survival rate of EMT patients remains unclear. There are two major concerns for incidental GIST if missed during operation for other tumors. First, residual GIST lesions may progress to invasive disease and cause intestinal obstruction and/or life-threatening gastrointestinal hemorrhage because the malignant potential is unpredictable based on gross appearance alone. Second, a residual incidental GIST may be mistaken for the relapse or metastasis of a previously removed neoplasm, which may result in inappropriate treatment of patients in follow-up after operation. Therefore, an en bloc resection with other tumors or an additional local resection with adequate margins has been recommended by surgeons[6,18,25]. Making surgeons aware of this will help to correct surgical procedures, and ultimately improve the quality of life and avoid inappropriate treatment of patients during follow-up after operation.

Common carcinogenic agents, which result in a simultaneous proliferation of different cell lines (epithelial and stromal cells), may be involved in the development of incidental GIST as a mere coincidence. In this study, males with primary GIST were more likely to have a synchronous tumor than females ($P < 0.001$). Synchronous tumors may have a high prevalence in males. Simultaneous neoplastic proliferation of epithelial and stromal cells might be stimulated by the same carcinogenic factors, such as *Helicobacter pylori* infections, germline mutations, and exposure to ionizing radiation[6,24,26,27]. To clarify possible common carcinogenic agents against synchronous tumors, further studies are needed.

In conclusion, incidental GIST coexists with EMT at a higher incidence than expected. Surgeons are advised to be alert against possible primary GIST accompanying other tumors.

ACKNOWLEDGMENTS

COMMENTS

Background

Gastrointestinal stromal tumor (GIST) is one of the most common tumors in gastrointestinal (GI) tract, probably arising from precursor cells that serve as a pacemaker to trigger gut contraction. It may exist alone with clinical manifestations or coexist with other diseases. The former is usually diagnosed by its clinical presentations and called clinical GIST, while the latter is usually found during examination or surgery for other diseases and called incidental GIST.

Research frontiers

Clinical GIST has been extensively studied in the past twenty years. Many cases of GIST existing alone or coexisting with other diseases have been reported, but GIST coexisting with other GI tumors has only been reported as single cases. It is necessary to conduct a comprehensive study with a large sample size to determine its incidence and features.

Innovations and breakthroughs

For the first time, the authors report an extensive study on incidental GIST coexisting with other GI tumors. This study revealed some important and interesting information regarding incidental GIST coexisting with other GI tumors. Firstly, they found that incidental GIST coexisted most frequently with esophageal and gastric tumor (1.13% and 0.53% respectively), and least with colorectal tumor (0.03%). Secondly, the majority of clinical GISTs had a moderate or a high risk. In contrast, the majority of incidental GISTs had a very low risk. Thirdly, the incidence of incidental GIST was significantly higher in male than in female patients (88.9% *vs* 11.1%). Finally, this study also provided the statistics for age, survival time and prognosis of studied patients and outlined the other features of incidental GIST, such as the number of lesions, lesion location and cellular morphology, *etc*.

Applications

The incidence of incidental GIST coexisting with other GI tumors is much higher than expected. However, without specific manifestations, preoperative detection of incidental GIST is difficult. Residual GIST lesions may progress to invasive diseases, cause intestinal obstruction and/or life-threatening gastrointestinal hemorrhage. In addition, residual incidental GIST may be mistaken for the relapse or metastasis of previously removed tumors, resulting in inappropriate treatment of patients during follow-up after operation. A careful inspection for GIST is highly recommended during surgery for GI tumors.

Terminology

GIST is one of the tumors in the GI tract, probably arising from precursor cells that serve as a pacemaker to trigger gut contraction. GI epithelial malignant tumor (EMT) refers to a tumor arising from the surface cells of the GI tract.

Peer review

This article is the first report to present the incidence of incidental GIST accompanying gastrointestinal EMT. In this study, the authors evaluated the

incidental GIST and its clinical significances. The title of the paper reflects the major contents of the article. The abstract gives a clear delineation of the research background. Results and discussion are well organized. The conclusion is reliable and valuable.

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BRIEF ARTICLES

Effect of 5-FU on modulation of disarrangement of immune-associated cytokines in experimental acute pancreatitis

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Abstract

AIM: To investigate the effects of 5-Fluorouracil (5-FU) on modulation of pro-inflammatory and anti-inflammatory cytokines in acute pancreatitis and the mechanism of it in the treatment of acute pancreatitis.

METHODS: Male Sprague Dawley rats were assigned to 3 Groups: Group A, sham operated rats as controls ($n = 7$); Group B, acute pancreatitis induced by ductal injection with 5% sodium cholate at a volume of 1.0 mL/kg without any other treatment; Group C, after the pancreatitis was induced as in Group B, the rats were injected intravenously with 5-FU 40 mg/kg. The animals in Groups B and C were killed at 2, 6 and 24 h after operation ($n = 7$), and blood samples were taken for measurement of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) (by bioassay), and interleukin-10 (IL-10), transforming growth factor-β (TGF-β) (by ELISA). The wet weight of pancreatic tissue, serum amylase levels and white blood cells were also measured.

RESULTS: Four rats in Group B and one in Group C died after pancreatitis was induced. Both pro-inflammatory cytokines (TNF-α, IL-1, IL-6) at the 2 and 6 h period and the anti-inflammatory cytokines (IL-10, TGF-β) at 24 h increased significantly ($P < 0.05$) in rats of Group B. After treatment with 5-FU, TNF-α, IL-1, and

IL-6 in serum of rats of Group C were inhibited at 2 and 6 h after operation ($P < 0.05$), and IL-10, TGF-β were inhibited at 24 h compared to Group B ($P < 0.05$). Obvious improvements in the severity of the acute pancreatitis, including the amylase levels, wet weight of pancreatic tissue and neutrophil counts, were also observed after treatment with 5-FU.

CONCLUSION: 5-FU is an anti-metabolic and immunosuppressive agent which can minimize the abnormal immune cytokine response and relieve the pathophysiological disorders associated with experimental acute pancreatitis.

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INTRODUCTION

5-Fluorouracil (5-FU) has been used in the treatment of acute pancreatitis both experimentally and clinically since 1970[1-3]. Several animal experiments of pancreatitis treated with 5-FU have shown very promising results, especially for a decrease in amylase and trypsin levels and improvement of survival rates[1,2]. It has been reported that prolongation of pancreatic allograft survival and protection from pancreatitis in dog pancreas allografts occur after pretreatment with 5-FU[4]. A prospective controlled clinical study was carried out in 1983, which showed that treatment with 5-FU was of some benefit in the modulation of clinical pancreatitis[5]. Clinical studies conducted in Russia documented that both the mortality and the length of hospital stay were reduced after treatment with 5-FU[6-8]. In China, administration of 5-FU

has been considered as an adjuvant therapy of acute pancreatitis. More than one thousand patients with acute pancreatitis have received the treatment of 5-FU each year in China, with many reports showing some beneficial results[9-12]. While there are many studies focusing on clinical observation of 5-FU treatment, research involving the mechanisms is sparse, but many investigators felt that the effect of 5-FU treatment for pancreatitis was derived from inhibiting the activities of pancreatic enzymes[1-3,5,9,10]. Recently, it has been increasingly clear that disarrangement of the immune system during acute pancreatitis is the determining factor in the pathophysiological process[13-19]. Considering that abnormal inflammation-associated cytokines (pro- and anti-inflammatory cytokines) present a primary index of disarrangement of immune function during acute pancreatitis[20-24], we designed this animal experiment to investigate the inhibiting effect of 5-FU on the inflammatory cytokines (TNF-α, IL-1, IL-6) and anti-inflammatory cytokines (IL-10, TGF-β) in acute pancreatitis and the relationship between the level of cytokines in serum and degree of acute pancreatitis.

MATERIALS AND METHODS

Materials

Sodium cholate was purchased from Sigma. Rat TGF-β and IL-10 EIA kits were purchased from R & D Co., USA. Reagents and instruments for measurement of TNF-α, IL-1, IL-6 were supplied by the Immunology Department, Medical Center, Sichuan University. Sprague Dawley (SD) rats were purchased from the Experimental Animal Center, Sichuan University, China.

Animals and pancreatitis model

SD rats (male, 10-12 wk-old, weighing 200-250 g) were fasted but allowed to drink water freely for 16 h before the experiment. They were allocated randomly into three Groups: Group A ($n = 7$), sham operation, with the same laparotomy under general anesthesia as Group B and sham intubation of the cholo-pancreatic duct but without any drug injection. These rats were killed 2 h later. In Group B, the acute pancreatitis Group, SD rats were injected with 5% sodium cholate into the cholo-pancreatic duct at a volume of 1.0 mL/kg using a midline laparotomy under general anesthesia and strict aseptic conditions to establish acute pancreatitis; Group C, acute pancreatitis with treatment of 5-FU. After pancreatitis was induced as in Group B, the rats were injected intravenously 40 min later with 5-FU 40 mg/kg. (This dosage is equal to 10-15 mg/kg in humans based on body surface). All the animals in Groups B and C were resuscitated post-operatively with 0.9% sodium chloride, subcutaneously at 6 mL/kg per hour. The surviving animals in Groups B and C were killed at 2, 6 and 24 h after operation ($n = 7$). Blood samples were taken for measurement of TNF-α, IL-1, IL-6, IL-10, and TGF-β. The wet weight of pancreatic tissue (the index of pancreatic edema), serum amylase levels and

the total and differential count of leukocytes, were also measured and recorded.

All the measurements of cytokines were done in the Department of Immunology, Medical Center, Sichuan University. IL-1, IL-6 and TNF-α were measured by bioassay according to Lederer, Kimura and Heo's methods[25-27]. IL-10 and TGF-β1 were measured by EIA according to the manufacturer's instructions. Amylase and white blood cells levels were tested by the Clinical Laboratory, Medical Center, Sichuan University.

Statistical analysis

We used the analysis of variance for continuous variables to detect variation among Groups with the same time (version 9.0 SAS Institute, Inc, Cary, NC). Statistical significance was regarded as $P < 0.05$. All reported P values are 2 sided. Continuous variables were described as mean ± SD unless stated.

RESULTS

There were 4 deaths in Group B at the 4, 6, 8, 15 h time points after pancreatitis was induced, and one rat died in Group C at 12 h.

In Group A, TNF-α, IL-1, IL-6, IL-10, and TGF-β in the serum of rats were detected as basic concentrations because of tissue injury resulting from sham operation. After acute pancreatitis was induced in Group B, the concentrations of pre-inflammatory cytokines such as TNF-α, IL-1 and IL-6 in the serum of rats increased rapidly. At the 2, 6, and 24 h periods, TNF-α, IL-1, IL-6 in Group B and Group C were significantly higher than that of Group A ($P < 0.05$). After pancreatitis was treated with 5-FU, the concentrations of IL-1 and IL-6 in serum of rats in Group C were significantly lower than those of Group B at 2, 6 h periods after operation ($P < 0.05$). At 6 h after operation the concentration of TNF-α in serum of Group C was also lower than that of Group B ($P < 0.05$). But at the time point 24 h, TNF-α, IL-1, IL-6 in Groups B and C still maintained a higher level and there was no significant difference between these two Group (Table 1). We presume that this is due to 5-FU being quickly catabolised in the body so that its regulating action disappeared swiftly and was not maintained up to 24 h. Serum IL-10 and TGF-β were significant higher in Group B and Group C than in Group A at the 24 h period ($P < 0.05$). At 24 h after operation, compared to Group B, the concentrations of IL-10 and TGF-β in serum in rats of Group C were decreased significantly ($P < 0.05$) (Table 2). When we collected the samples of pancreas after rats were sacrificed, we found that samples of pancreas in Group C were more obviously swollen and congested than those of Groups A and C. Since some doctors have investigated histopathological change of pancreas in detail in similar animal experiments with 5-FU, here we only chose the wet weight of pancreas (index of pancreatic edema) and serum amylase as indexes of severity of acute pancreatitis. In the control Group, the weight of pancreatic tissue was 0.5 ± 0.09 g. At 2, 6, 24 h

Table 1 Change of level of pro-inflammatory cytokines in serum

Group	IL-1 (ng/mL)	IL-6 (IU/mL)	TNF (IU/mL)
A	0.28 ± 0.06	34.5 ± 6.40	11.82 ± 1.87
B (2 h)	1.02 ± 0.12[1]	98.83 ± 12.43[1]	43.67 ± 5.72[1]
(6 h)	1.13 ± 0.17[1]	101.0 ± 15.07[1]	48.67 ± 5.32[1]
(24 h)	1.15 ± 0.13[1]	127.17 ± 13.91[1]	55.33 ± 12.79[1]
C (2 h)	0.80 ± 0.07[1,2]	76.33 ± 7.42[1,2]	35.33 ± 4.50[1]
(6 h)	0.70 ± 0.06[1,2]	74.33 ± 11.02[1,2]	31.17 ± 4.54[1,2]
(24 h)	1.02 ± 0.18[1]	112.67 ± 20.06[1]	42.33 ± 11.64[1]

A: Sham operation Group without acute pancreatitis and drug injection; B: Acute pancreatitis Group; C: Acute pancreatitis with 5-FU group. 2 h, 6 h, 24 h: 2 h, 6 h, 24 h after operation. [1]Compared to sham operation Group (Group A), $P < 0.05$; [2]Compared to pancreatitis Group (Group B), $P < 0.05$.

Table 2 Change of level of anti-inflammatory cytokines in serum

Group	IL-10 (pg/mL)	TGFβ (pg/mL)
A	22.05 ± 14.87	66.40 ± 13.20
B (2 h)	36.52 ± 9.76	64.58 ± 10.56
(6 h)	37.75 ± 6.54	72.87 ± 18.34
(24 h)	68.13 ± 19.90[1]	103.77 ± 28.95[1]
C (2 h)	28.82 ± 6.63	61.15 ± 30.31
(6 h)	45.5 ± 4.72[1]	80.27 ± 19.83
(24 h)	24.0 ± 7.86[2]	68.52 ± 11.51[2]

[1]Compared to sham operation Group (Group A), $P < 0.05$; [2]Compared to pancreatitis Group (Group B), $P < 0.05$.

Table 3 Change of index of severity of acute pancreatitis

Group	Amylase (U/L)	W.Weight of P (g)	W.B.C (× 10⁹/L)[2]	Neutrophils (%)
A	374.20 ± 92.84	0.51 ± 0.90	6.69 ± 2.59	0.35 ± 0.09
B (2 h)	371.25 ± 86.28	1.63 ± 0.54[1]	7.59 ± 2.25	0.74 ± 0.38[1]
(6 h)	508.83 ± 344.82	1.85 ± 0.25[1]	9.46 ± 2.16	0.75 ± 0.08[1]
(24 h)	1817.25 ± 459.35[1]	1.30 ± 0.13[1]	6.93 ± 0.67	0.62 ± 0.11[1]
C (2 h)	352.2 ± 118.15	0.87 ± 0.22[2]	7.90 ± 2.33	0.48 ± 0.09[2]
(6 h)	434.4 ± 138.35	0.68 ± 0.24[2]	10.72 ± 1.45	0.69 ± 0.06
(24 h)	794.40 ± 225.99[1,2]	0.88 ± 0.13[1,2]	6.10 ± 1.44	0.53 ± 0.10

W.Weight of P: wet weight of pancreatic tissue. [1]Compared to sham operation Group (Group A), $P < 0.05$; [2]Compared to pancreatitis Group (Group B), $P < 0.05$.

after acute pancreatitis being induced, the wet weights of pancreatic tissue in Group B were 1.63 ± 0.54 g, 1.85 ± 0.25 g and 1.53 ± 0.13 g, respectively; but at 2, 6, 24 h after treatment with 5-FU in Group C, the wet weights of pancreatic tissue were 0.87 ± 0.22 g, 0.58 ± 0.24 g and 0.88 ± 0.13 g, respectively. There was a significant difference between the acute pancreatitis Group (Group B) and the treatment with 5-FU Group (Group C) at all time periods ($P < 0.05$). In the control Group, the concentration of serum amylase was 374.2 ± 92.84 U/L. At 24 h after operation, the concentration of serum amylase was 1817.25 ± 459.35 U/L, but after treatment with 5-FU the concentration was 797.4 ± 225.9 U/L at the same time period. The concentration of serum amylase in the acute pancreatitis Group (Group B) was higher than that in the treatment with 5-FU Group (Group C), and it reached statistical significance ($P < 0.05$). In the control Group, leukocyte count in the peripheral blood of the rats was $(6.59 ± 2.59) × 10^9$/L. At 24 h after experiment, the leukocyte counts in the Groups A and B were $(6.93 ± 0.67) × 10^9$/L and $(6.1 ± 1.44) × 10^9$/L respectively and there was no significant difference between the two Groups. Percentage of neutrophils in rats in Group A was 0.35% ± 0.09%. In Group B, the percentage of neutrophils was increased significantly at each time period. After treatment of 5-FU, the percentage of neutrophils in Group C decreased compared to Group B, but it did not reach statistical significance until after the 2 h period (Table 3).

DISCUSSION

More and more studies have been reported to support the theory that the severity of acute pancreatitis largely depends on the degree of secondary disarrangement of inflammatory mediators[13-16]. Damage caused by trypsin at the initial stage of acute pancreatitis is an event that triggers the systemic inflammatory response syndrome (SIRS)[28]. The pathological process of SIRS results in the clinical manifestation and damage to other distant organs in acute pancreatitis[29,30]. If SIRS persists and anti-inflammatory cytokines are not adequate to suppress this response, SIRS may lead to clinical sepsis and the multiple organ dysfunction syndrome, which could account for one of the main causes of death in severe pancreatitis[31,32]. Along with the production and release of large amounts of pro-inflammatory cytokines in acute pancreatitis, anti-inflammatory cytokines (IL-10, TGF-β, IL-4, IL-13) and other immunosuppressive factors (PGE2, glucocorticosteroids) start to be synthesized and released. This process could be helpful to restrain SIRS and restore the balance between the inflammatory and anti-inflammatory responses. However, when the anti-inflammatory cytokines and other immunosuppressive factors became predominant in severe acute pancreatitis, these mediators will inhibit the immunity against pathogens, especially inhibiting the cellular immune function, and this will result in the so-called compensatory anti-inflammatory response syndrome (CARS), a secondary immunological deficiency syndrome[33-36]. CARS seems to be related to the systemic infection and pancreatic abscess which develop during severe acute pancreatitis[37-39]. In the present experiment, after pancreatitis was induced in animals in Group B, pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, increased promptly, and 24 h later, the anti-inflammatory cytokines IL-10 and TGF-β increased sequentially. These results indicate that there is a pro-inflammatory process (SIRS) followed by an anti-inflammatory process in acute pancreatitis. It is thus suggested that the strategy for acute pancreatitis should not only include modulation of SIRS, but also prevention of CARS.

With this knowledge, the mechanism of treatment of

acute pancreatitis with 5-FU should be evaluated further. Previously, it was thought that inhibition of exocrine secretion of the pancreas was a fundamental mechanism of treatment of acute pancreatitis with 5-FU[1,2,9]. 5-FU traditionally is classified as an anti-metabolic agent. 5-FU is a derivant of pyrimidine, and interferes with the synthesis of DNA and RNA both in normal cells and tumor cells. 5-FU also inhibits the synthesis of protein. Essentially, 5-FU can serve as a proteinase inhibitor and exert general action throughout the whole process of acute pancreatitis. 5-FU decreases the synthesis and secretion of pancreatic enzymes. Thus, it can alleviate the damage to pancreatic tissues by auto-digestion at the initial stage. This function has been confirmed previously[1-4]. Results from the present experiment provide evidence that 5-FU can reduce inflammation-associated cytokines. We also presume that 5-FU inhibits proteinases produced by leukocytes, which is thought a powerful factor in development of MODS. In this study, after the treatment of acute pancreatitis with 5-FU, the activity of inflammation-related cytokines was inhibited. Meanwhile, the level of serum amylase and the weight of pancreatic tissue, factors that reflect the severity of pancreatic injury and pathological lesions, were improved significantly. This indicated that 5-FU could improve the severity of acute pancreatitis by means of modulation of disarrangement of inflammation.

We cannot recommend that 5-FU could be a definite therapy for acute pancreatitis based only on the results of this experiment. We know that there is much difference between animal experiments and clinical practice with regard to results of medical research. Deterioration of acute pancreatitis is never due to simple inflammatory processes, many factors may be involved including secondary infection, derangement of blood circulation, even genetic predisposition so that clinical effects of 5-FU on acute pancreatitis need to be validated by large scale, prospective controlled studies. But we do think that 5-FU may be a candidate for treatment of SIRS based on results of this experiment. After the immunopathogenesis of sepsis following surgical disease was elucidated, many biological products were introduced for use against the pre-inflammatory cytokines[40-43] and the prevention of SIRS, such as anti-endotoxin antibodies[44], anti-TNF-α antibodies[45,46], IL-1 receptor antagonists[47-49] and monoclonal anti-interleukin 8 antibody[50]. None of these interventions have been shown to improve the prognosis of sepsis, possibly because many patients were already in a state in which anti-inflammatory responses dominated[51,52]. Because inflammation plays an important role in the defense against pathogenic microbes and reparation of injured tissue, there is a possibility of infection becoming lethal by excessive anti-inflammatory therapy. In our study, elevated TGF-β and IL-10 levels in an animal model of acute pancreatitis predicted the potential tendency of immunodepression. We think that the decrease of both pro-inflammatory cytokines in addition to the decrease of anti-inflammatory cytokines after treatment with 5-FU may offer a rational strategy for treatment of SIRS. As observed above, 5-FU has multiple

actions and biphasic regulation for the disarrangement of immunity in acute pancreatitis. Compared with the effect of single inflammatory cytokine blockers, treatment with 5-FU for SIRS and CARS in surgical disease may be the more effective method. Moreover, immunoregulation with 5-FU displayed in this experiment opens a new possible pathway towards the search for therapy of surgical systemic inflammatory response syndrome.

COMMENTS

Background
5-Fluorouracil (5-FU) has been used in the treatment of acute pancreatitis both experimentally and clinically since 1970, but the mechanisms of the therapeutical effect of 5-FU are not clear, and it has been considered an adjuvant therapy of acute pancreatitis. Recently, it has been increasingly clear that disarrangement of the immune system during acute pancreatitis is the determining factor in the pathophysiologic process. Abnormal inflammation-associated cytokines (pro- and anti-inflammatory cytokines) present a primary index of disarrangement of immune function during acute pancreatitis and lead to sepsis. Sepsis revealed as self-destructive inflammatory reaction remains a puzzle worldwide with respect to its pathological mechanism and corresponding preventive and therapeutic strategies for the clinicians.

Research frontiers
The hotspots of sepsis therapy research have focused on the modification of the inflammatory factors existing in sepsis, ever since the basis of sepsis injuries were revealed as self-destructive inflammatory reactions. Although with some frustrations, research is still focused on the immune regulation.

Innovations and breakthroughs
The authors designed an acute pancreatitis animal model to investigate the inhibiting effect of 5-FU on the inflammatory cytokines (TNF-α, IL-1, IL-6) and anti-inflammatory cytokines (IL-10, TGF-β) in acute pancreatitis and the relationship between the level of cytokines in serum and degree of acute pancreatitis. The experiments obtained encouraging results that the 5-FU, as an immunosuppressive agent, could be effective because of its regulation of immunity. Previously it was thought that inhibition of exocrine secretion of pancreas was a fundamental mechanism of treatment of acute pancreatitis with 5-FU. This trial reveals the immunoregulatory effect of 5-FU in the therapy of acute pancreatitis. The majority of research in the last 20 years on sepsis focused on the blocking agents of inflammatory factors, which failed in clinical trials. According to their trial, 5-FU has multiple actions and biphasic regulation for disarrangement of immunity in acute pancreatitis. Compared with the effects of single inflammatory cytokine blockers, treatment with 5-FU for SIRS and CARS in surgical disease may be the more effective method.

Applications
This trial reveals the potential of 5-FU treatment against acute pancreatitis and sepsis in the clinic. 5-FU is cheaper and safer as a typical immunosuppressive agent, and has been a familiar therapy compared to new medicines.

Terminology
5-FU is one of the first pyrimidine antagonists to be synthesized as an antimetabolite; 5-FU *in vivo* is transformed enzymatically into 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), which covalently binds and inhibits thymidylate synthase (TS) and interferes with the synthesis of nucleic acids and prevents the cell from making DNA. Another bio-transformed form of 5-FU, 5-fluorouridine-5'-triphosphate (FUTP), also incorporates itself into RNA and disrupts biological activity of RNA and protein. Systemic inflammatory response syndrome (SIRS) is the self-destructive, severe systemically inflammatory reaction of the body responding to invasion of pathogen, trauma or ischemia.

Peer review
This is an innovative study in which authors analyze the regulative effect of 5-FU on inflammatory reaction in rats. The results are encouraging and suggest that 5-FU is a potential therapeutic substance that could be used in acute pancreatitis, and sepsis caused by other etiological factor.

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CASE REPORT

Hepatic failure caused by plasma cell infiltration in multiple myeloma

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Abstract

Although plasma cell infiltration is not rare in autopsy of patients with multiple myeloma (MM), it is very rarely detected in living patients. This is because MM rarely causes significant liver dysfunction that requires further evaluation. A 49-year-old man presented with acute renal failure and was diagnosed with kappa light chain MM stage ⅡB. Thalidomide and dexamethasone were initiated. The patient developed a continuous increase in bilirubin that led to severe cholestasis. A liver biopsy revealed plasma cell infiltration. He then rapidly progressed to liver failure and died. Treatment options are limited in MM with significant liver dysfunction. Despite new drug therapies in MM, those patients with rapidly progressive liver failure appear to have a dismal outcome.

Key words: Hepatic failure; Multiple myeloma; Cell infiltration

Rahhal FE, Schade RR, Nayak A, Coleman TA. Hepatic failure caused by plasma cell infiltration in multiple myeloma. *World J Gastroenterol* 2009; 15(16): 2038-2040 Available from: URL: http://www.wjgnet.com/1007-9327/15/2038.asp DOI: http://dx.doi.org/10.3748/wjg.15.2038

INTRODUCTION

Plasma cell infiltration of the liver can be detected in up to 45% of patients with multiple myeloma (MM) at autopsy[1,2]. However, only rare cases have been reported of massive plasma cell infiltration of the liver that leads to non-obstructive cholestasis with progression to liver failure[3-10]. Here, we report a patient with MM with biopsy-proven plasma cell involvement of the liver. The patient died of rapidly progressive liver failure despite treatment for MM.

CASE REPORT

A 49-year-old black man presented with acute renal failure with a serum creatinine of 13 mg/dL. He was found to have circulating plasma cells in the blood and was diagnosed with kappa light chain MM by a bone marrow biopsy, which revealed 90% plasma cells with kappa light chain restriction. Free kappa light chains were noted in the serum. Cytogenetics of the bone marrow plasma cells revealed an abnormal hypodiploid clone with deletions of chromosomes 4, 13, 16, 17 and 21. A skeletal survey was negative for lytic bone lesions. The serum hemoglobin was 10 g/dL and the serum calcium was normal. Liver enzymes were normal at presentation. The physical examination was remarkable only for cachexia. Therapy with thalidomide (200 mg/d) and high dose dexamethasone (40 mg on days 1-4, 7-11, 14-17 and 21-24) was initiated. After receiving three cycles over a 3-mo period, the patient had clearance of circulating plasma cells on the peripheral blood smear but no response on repeat bone marrow biopsy. His therapy was switched to bortezomib.

One month after the initiation of thalidomide and dexamethasone, he started developing a gradual increase in total and direct bilirubin from a normal baseline. Right-upper-quadrant ultrasound revealed an enlarged liver with a largest dimension of 23 cm; however, no mass lesions were noted. The parenchymal echogenecity was within normal limits. Abdominal computed tomography performed with oral and Ⅳ contrast agents revealed a diffusely enlarged liver with no mass lesions and no evidence of intra- or extrahepatic ductal dilatation. After three cycles of thalidomide and dexamethasone therapy, the total bilirubin had increased to 3.9 mg/dL.

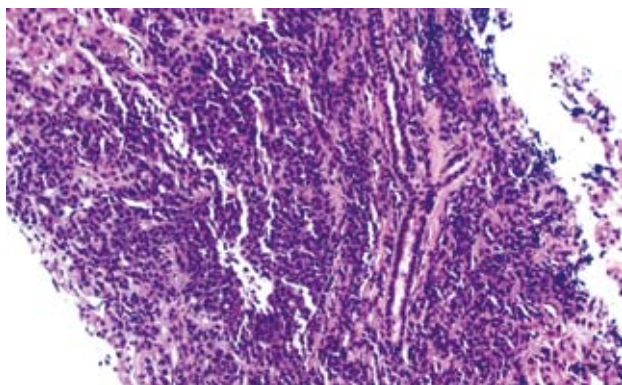One week after switching to bortezomib, the patient

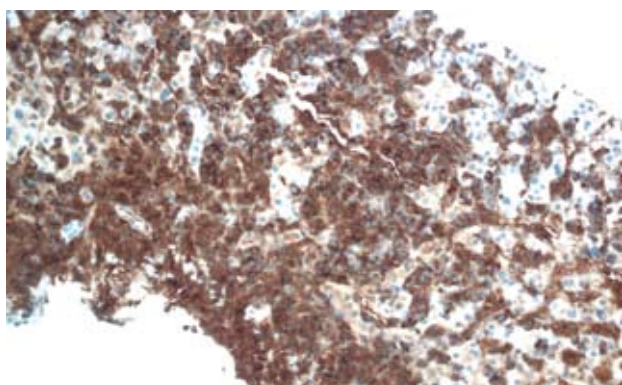**Figure 1 HE liver biopsy showing massive plasma cell infiltration (HE).**

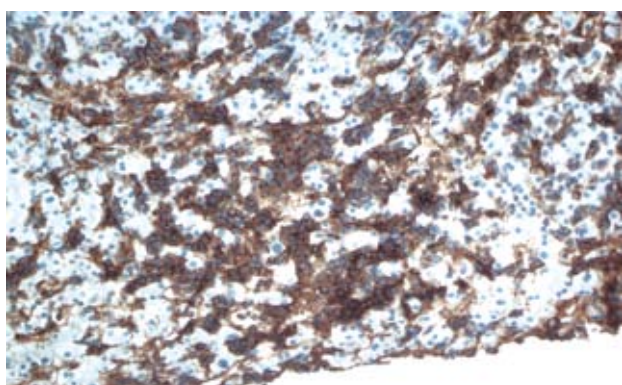**Figure 2 Positive kappa light chain stain on liver biopsy.**

Figure 3 Positive CD138 (syndecan-1), a plasma cell marker. CD138 is expressed on plasma cells, including the malignant plasma cells of MM and some lymphomas.

was admitted to the hospital for further evaluation after the total bilirubin acutely increased to 9.4 mg/dL. The remainder of the liver function tests revealed aspartate aminotransferase of 41 U/L, alanine aminotransferase of 38 IU/L, lactate dehydrogenase of 306 IU/L, and gamma-glutamyltransferase of 286 IU/L. The direct bilirubin was 5.8 mg/dL. The physical examination was remarkable for frank jaundice and hepatomegaly. A repeat ultrasound of the abdomen showed significant liver enlargement up to 26 cm over a period of 2 mo with no evidence of biliary obstruction. A hepatobiliary imino-diacetic acid scan revealed delayed hepatic uptake consistent with severe

hepatocellular dysfunction. Hepatitis serology for hepatitis A/B/C, and antinuclear and antineutrophil cytoplasmic antibodies was negative. Cytomegalovirus serology was also negative. A transjugular liver biopsy was performed to rule out drug toxicity. It demonstrated massive infiltration of the liver parenchyma with plasma cells (Figure 1). The immunological profile of the liver biopsy revealed diffuse kappa light chain restriction that was similar to that in the bone marrow biopsy, which supported the diagnosis of MM with hepatic infiltration (Figures 2 and 3). Flow cytometry of the liver biopsy revealed plasma cells with CD38/CD138 co-expression and kappa light chain restriction. No evidence of amyloidosis was evident on immunostaining. During his 2 wk of hospitalization, the patient rapidly developed hepatic failure, characterized by coagulopathy, hyperbilirubinemia, encephalopathy and ascites. He was discharged to hospice care and expired 1 wk later.

DISCUSSION

Pathologic liver involvement in patients with MM has been reported in up to 45% of patients[1,2]. An autopsy series of 128 cases with MM done by Perez-Soler *et al*[3] showed diffuse infiltration of the liver by plasma cells in 10 of 21 patients with liver tissue involvement. Thomas *et al*[4] reviewed 64 cases of MM, including autopsy reports and prior medical records; 58% of patients had hepatomegaly, defined as percussible dullness of more than 12 cm or a liver edge palpable 4 cm below the right costal margin, and 25% had splenomegaly. Jaundice was reported in nine patients (14%) and serum bilirubin values ranged from 3.2 to 17.3 mg/dL. Only six patients (9%) had a completely normal liver on pathological examination, while 40% had plasma cell involvement of the liver in the form of plasmacytoma or diffuse sinusoidal infiltration. Both these two autopsy series excluded cases with plasma cell leukemia[3,4].

The histological pattern of liver involvement in MM can be in the form of light chain deposition disease, extramedullary plasmacytoma, amyloidosis, or a diffuse infiltrative pattern. Massive liver involvement can be either from tumor-forming plasmacytomas or diffuse sinusoidal flooding[4]. The latter can consist of sinusoidal flooding by plasma cells of varying degrees of differentiation with little or no propensity to destroy liver parenchyma[5]. Some of these patients may present with non-obstructive jaundice and show elevations in alkaline phosphatase from plasma cell infiltration, as did our patient[3]. Only two prior cases of severe cholestasis associated with hepatic failure have been reported[6,7]. Radiographic imaging is unable to detect this diffuse infiltrative pattern and a liver biopsy is essential to make a diagnosis.

Plasmacytomas (nodular forming pathology) are less common. This entity can be detected radiographically as space-occupying lesions in the liver or in the head of the pancreas, and may be associated with biliary obstruction[8-10].

Amyloidosis manifested as tissue deposition of clonal light-chain fibrils is seen in 15% of the general MM patient population. Liver involvement has been reported

in MM patients as well as those with primary systemic amyloidosis[11]. Elevation in alkaline phosphatase is more common and liver enzymes are often normal or mildly elevated. Obstructive jaundice occurs rarely and only a few cases have been reported, with some associated with hepatic failure[12-18].

The clinical significance of liver involvement in MM is uncertain. Treatment of MM with hepatic involvement requires systemic therapy. Successful treatment with combination chemotherapy or steroids alone has been reported[6,19,20]. Talamo et al[21] have reviewed the medical records of 24 patients with MM involvement of the gastrointestinal (GI) system as documented by tissue biopsy, 11 of whom had hepatic involvement. GI involvement at the time of initial diagnosis of MM was less common than later in the disease course.

The treatment of MM patients with significant liver dysfunction is challenging because of the inability to deliver most chemotherapeutic agents in the setting of liver failure. Substantial dose reductions are required to administer anthracyclines and vincristine in the setting of liver failure. Steroids, and the newer agents thalidomide and bortezomib have been administered in these settings, although thalidomide has been cited in a single case report as causing fulminant hepatic dysfunction, and liver toxicity has been reported rarely with bortezomib[22,23].

Stem cell transplantation (SCT) is now the standard first-line therapy for myeloma patients who respond to chemotherapy. Patients are analyzed to evaluate the role of treatment with high-dose chemotherapy with autologous or allogeneic SCT, and its impact on survival[21]. It has been found that GI involvement is common during relapse after SCT. Plasmablastic morphology was common (Bartl grade Ⅲ) in 29% of cases with GI involvement. Monosomy 13, which is one of the most powerful negative prognostic factors, was observed in 46% of the cases, while it is present in 15% of the general MM patient population. SCT is effective in inducing remissions but relapse is common.

In conclusion, there are no classical clinical manifestations of liver infiltration in MM. The initial presentation can be subtle, but then very rapidly progressive, as in our case. The number of clinically reported cases of liver involvement with MM is small, therefore, it is difficult to ascertain the prognosis of this clinical presentation of the disease or its response to therapy. We suspect that the prognosis is poor because of the limited number of chemotherapeutic agents that can be administered to patients with severe liver dysfunction. The optimal approach in managing these cases can only be standardized after studying a larger number of patients.

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CASE REPORT

Chemical ablation of the gallbladder using alcohol in cholecystitis after palliative biliary stenting

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Abstract

Chemical ablation of the gallbladder is effective in patients at high risk of complications after surgery. Percutaneous gallbladder drainage is an effective treatment for cholecystitis; however, when the drain tube cannot be removed because of recurrent symptoms, retaining it can cause problems. An 82-year-old woman presented with cholecystitis and cholangitis caused by biliary stent occlusion and suspected tumor invasion of the cystic duct. We present successful chemical ablation of the gallbladder using pure alcohol, through a percutaneous gallbladder drainage tube, in a patient who developed intractable cholecystitis with obstruction of the cystic duct after receiving a biliary stent. Our results suggest that chemical ablation therapy is an effective alternative to surgical therapy for intractable cholecystitis.

Key words: Percutaneous cholecystostomy; Cholecystitis; Biliary stenting; Alcohol; Chemical therapy

INTRODUCTION

Tumor obstruction of the cystic duct is a known risk factor for the development of cholecystitis following biliary stent placement. Percutaneous cholecystostomy is an effective treatment for such cholecystitis[1,2]. However, recurrent cholecystitis or retractable symptoms may be troublesome. Recently, chemical ablation of the gallbladder has been shown to be effective in patients at high risk for complications after surgery[3]. Absolute alcohol or 95% ethanol causes necrosis and fibrosis in the gallbladder epithelium, which reduces the gallbladder to a shrunken fibrous remnant[4].

However, until now there have been few human studies of which sclerosants are safe and feasible, and for how long the sclerosant has to be in contact with the mucosa.

In this report, we describe the successful chemical ablation of the gallbladder in a patient who developed intractable cholecystitis with obstruction of the cystic duct, after undergoing palliative stenting for the management of a malignant biliary obstruction.

CASE REPORT

An 82-year-old woman presented with gradually aggravated right upper-abdominal pain after undergoing biliary stent implantation for the palliative management of a cholangiocarcinoma 2 wk previously. Upon presentation, clinical examination revealed severe tenderness of the right upper abdomen without rebound tenderness. Laboratory tests revealed the following: white blood cell count, 14.380×10^9/L (normal 4.0-10.8 $\times 10^9$/L); total bilirubin, 5.4 g/dL (normal 0.1-1.0 g/dL); aspartate aminotransferase, 118 IU/L (normal < 40 IU/L); alanine aminotransferase, 118 IU/L (normal < 40 IU/L); alkaline phosphatase, 118 IU/L (normal 39-117 IU/L); and carbohydrate antigen 19-9, 98 U/mL (normal <

Figure 1 Abdominal CT scan revealing a markedly enlarged and distended gallbladder with a thickened wall.
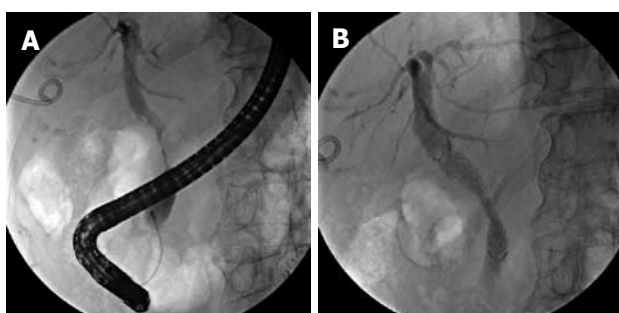

Figure 2 Cholangiographic findings. A: Endoscopic retrograde cholangio-pancreatography showed a severe irregular segmental stricture at the mid-CBD, without visualization of the cystic duct; B: A covered metal stent, 60 mm in length, was implanted in the narrowed CBD.

Figure 3 Chemical ablation therapy. A: Before chemical ablation of the gall-bladder, the gallbladder volume was measured by infusing contrast medium through the drain tube; the contrast medium did not pass into the CBD; B: Three weeks later (after a total of three chemical ablation sessions), cholecystography showed a marked collapse in the lumen of the gallbladder.

34 U/mL). Abdominal computed tomography (CT) revealed a markedly enlarged and distended gallbladder with a thickened wall (Figure 1). Clinically, both cholecystitis and cholangitis were suspected, based on CT and laboratory data. Following decompression of the gallbladder via percutaneous cholecystostomy, endoscopy using a duodenoscope (TJF 240; Olympus, Tokyo, Japan) was performed, which showed a completely occluded plastic biliary stent. The occluded stent was subsequently removed. Cholangiography showed a severe irregular segmental stricture at the mid common bile duct (CBD), without visualization of the cystic duct, a finding that indicated cystic duct occlusion caused by tumor invasion (Figure 2A).

For the management of cholecystitis and malignant stricture, the percutaneous drainage tube was left in place and a covered metal biliary stent (Niti-S; Taewoong Medical Co., Ltd., Seoul, Korea), 60 mm in length, was implanted through the peroral route (Figure 2B). Seven days later, the percutaneous cholecystostomy was draining less than 50 mL/d; therefore, removal of the drain tube was attempted. However, the patient complained of recurrent abdominal pain and discomfort whenever the drain tube was closed, and the amount of fluid draining continued at a rate of > 40 mL/d. Consequently, removal of the drain tube failed. As a result of the patient's advanced age and her refusal of palliative cholecystectomy, medical ablation of the gallbladder was considered to be a good option for treating the symptoms and to allow the removal of the percutaneous drain tube.

After informed consent from the patient and approval by the ethics committee of our hospital, 99% absolute ethanol was used as a sclerosant for chemical ablation of the gallbladder. The volume of the gallbladder was measured by filling it with contrast medium, followed by aspiration (Figure 3A). Absolute ethanol, 1-2 mL less than the volume of the gallbladder, was infused into the gallbladder through the drain tube. Initially, a total of 55 mL of ethanol was infused and the drainage tube was closed. Then, the patient changed positions every 10 min for a total of 30 min, and the sclerosant was drained. Cholecystography was performed 1 wk after the first chemical ablation, and it showed a decrease in the size of the gallbladder. The same method was repeated twice more in weekly sessions with 40 and 25 mL of ethanol, respectively. During the final week, a small amount of bright yellow fluid (< 10 mL/d) was draining, and cholecystography showed a marked collapse in the lumen of the gallbladder (Figure 3B). During each procedure, the patient's vital signs were closely monitored. The patient had no abdominal pain or other complications related to the procedure. The cholecystostomy drain tube was removed, and there were no complications in the following 8 wk, during which she remained under outpatient observation.

DISCUSSION

Endoscopic insertion of biliary stents is a well-established palliative treatment for obstructive jaundice caused by unresectable malignant disease[5-7]. As a result of increased use, complications such as cholangitis, cholecystitis, pancreatitis, stent migration, and stent occlusion are being reported increasingly[8]. In particular, cholecystitis has been reported in 1.9%-12% of stent insertion cases[9,10]. For several reasons, obstruction of the cystic duct by a tumor is a risk factor for the development of cholecystitis following biliary stent placement[2].

Although cholecystectomy is a safe and effective treatment in patients with cholecystitis, the morbidity

and mortality of this operation increases considerably in the elderly and unfit patients who often have concomitant diseases. Percutaneous gallbladder drainage or aspiration, transpapillary gallbladder drainage, and endoscopic-ultrasound-guided gallbladder drainage have been reported for the management of cholecystitis after stent placement or for cystic duct invasion by a tumor[1,2,11]. However, in cases such as those reported here, when the drain tube cannot be removed because of recurrent symptoms, retaining it causes problems for the patient, and its experimental removal may cause other complications.

Chemical ablation of the gallbladder may be a useful alternative to cholecystectomy in high-risk patients or in those who refuse surgery. Recently, experimental studies on chemical ablation of the gallbladder *in vitro* and *in vivo* have demonstrated that many sclerosants, including 95% ethanol, 3% sodium tetradecyl sulfate, 5% tetracycline, and 5% trifluoroacetic acid, ablate gallbladder mucosa[3,12-14]. Oh *et al*[15] used 99.9% ethanol for the chemical ablation of cystic tumors of the pancreas. Xu *et al*[3] reported that minicholecystostomy followed by chemical ablation of the gallbladder was safe and effective. In that study, 95% ethanol was in contact with the gallbladder mucosa for 30 min every 4 h, for a total of eight times after occlusion of the cystic duct. A suitable chemical for gallbladder mucosal ablation must be safe, effective, and require brief contact time with the mucosa. However, there have been few human studies to determine which sclerosants are feasible and the duration for which the sclerosant must be in contact with the mucosa. Some studies have reported complications, including mucocele, gallbladder hydrops, abscess formation, and perforation; however, there have been no serious, life-threatening complications[3,4,12-14].

In our case, we used absolute ethanol as a chemical sclerosant. In animal and human studies, alcohol has been found to be safe and has resulted in few complications; however, it requires more treatments of longer duration than other sclerosants. More studies are needed to determine which sclerosants are suitable, how often they need to be applied and at what interval, and the contact duration required for chemical ablation. Absolute ethanol is a safe sclerosant and procedure-related complications did not develop during the procedure or follow-up period. Especially in the case of cystic duct obstruction by tumors, this method is easy and feasible, and is not affected by the presence of additional biliary stents. However, if the cystic duct is patent, the use of chemical agents is restricted and another approach is necessary.

In summary, following development of cholecystitis after stent placement, with tumor obstruction of the cystic duct, or in patients with recurring symptoms,

chemical ablation using absolute ethanol may be an alternative to percutaneous cholecystostomy or surgical cholecystectomy. Further investigation of this technique and the identification of suitable sclerosants are necessary, and long-term follow-up should be conducted.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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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
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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]

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5   **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 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]

Volume with supplement

7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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)

11   **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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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

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15   Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/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 $\pm$ SD or mean $\pm$ SE.

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Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

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Italics

Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
Biology: *H pylori*, *E coli*, *etc*.

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