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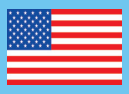

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BRIEF ARTICLE

Endoclips *vs* large or small-volume epinephrine in peptic ulcer recurrent bleeding

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Abstract

AIM: To compare the recurrent bleeding after endoscopic injection of different epinephrine volumes with hemoclips in patients with bleeding peptic ulcer.

METHODS: Between January 2005 and December 2009, 150 patients with gastric or duodenal bleeding ulcer with major stigmata of hemorrhage and nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa) were included in the study. Patients were randomized to receive a small-volume epinephrine group (15 to 25 mL injection group; Group 1, n = 50), a large-volume epinephrine group (30 to 40 mL injection group; Group 2, n = 50) and a hemoclip group (Group 3, n = 50). The rate of recurrent bleeding, as the primary outcome, was compared between the groups of patients included in the study. Secondary outcomes compared between the groups were primary hemostasis rate, permanent hemostasis, need for emergency surgery, 30 d mortality, bleeding-related deaths, length of hospital stay and transfusion requirements.

RESULTS: Initial hemostasis was obtained in all patients. The rate of early recurrent bleeding was 30% (15/50) in the small-volume epinephrine group (Group 1) and 16% (8/50) in the large-volume epinephrine group (Group 2) (P = 0.09). The rate of recurrent bleeding was 4% (2/50) in the hemoclip group (Group 3); the difference was statistically significant with regard to patients treated with either small-volume or large-volume epinephrine solution (P = 0.0005 and P = 0.045, respectively). Duration of hospital stay was significantly shorter among patients treated with hemoclips than among patients treated with epinephrine whereas there were no differences in transfusion requirement or even 30 d mortality between the groups.

CONCLUSION: Endoclip is superior to both small and large volume injection of epinephrine in the prevention of recurrent bleeding in patients with peptic ulcer.

Key words: Peptic ulcer; Hemorrhage; Hemoclip; Epinephrine; Nonvariceal upper gastrointestinal bleeding

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INTRODUCTION

Peptic ulcer disease accounts for 50% to 70% of cases

of acute nonvariceal upper gastrointestinal bleeding (UGIB)[1,2]. Initial haemostatic rates of 80% to almost 100% can be achieved with various endoscopic techniques. However, after initial control, bleeding recurs in 10% to 30% of patients[3].

Among various endoscopic techniques a recent International consensus on nonvariceal UGIB recommends combination therapy with clear statement that epinephrine injection alone provides suboptimal efficacy and should be used in combination with another methods[4]. However, several recent studies found injection of a large volume of epinephrine to be superior to injection of a small epinephrine volume with respect to recurrent bleeding from peptic ulcer[5,6]. Since epinephrine injection is effective (initial hemostasis obtained with epinephrine injection range from 85% to 100%), safe, inexpensive and technically easy, the concept of a beneficial effect of large volumes of epinephrine in preventing recurrent ulcer bleeding seems to be very challenging. Therefore, the aim of this prospective study was to compare the rates of recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution (15-25 mL *vs* 30-40 mL) with endoscopic placement of hemoclips in patients with peptic ulcer bleeding. Since it has been suggested that the useful baseline factor for stratification in UGIB trials may be stigmata of hemorrhage in an ulcer, we decided to include in the study only patients presenting with acute UGIB and endoscopically proven gastric or duodenal ulcer with visible vessel in an ulcer bed (Forrest Ⅱa)[7].

MATERIALS AND METHODS

Between January 2005 and December 2009, consecutive patients presenting with acute UGIB were considered for this study. These patients were referred to the Emergency Unit of the Department of Internal Medicine at the "Sestre milosrdnice" University Hospital, Zagreb, Croatia and then if necessary hospitalized at the Interventional Gastroenterology Unit at the same hospital.

UGIB was suspected if hematemesis, melena or hematochezia were seen and/or bloody nasogastric aspirate was observed. In all those patients upper gastrointestinal endoscopy was performed within 6 h of hospital admission. Patients were included only if emergency endoscopy disclosed a gastric or duodenal bleeding ulcer with major stigmata of hemorrhage ("coffee ground" material or blood in the stomach and/or duodenum) and nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa)[8]. Exclusion criteria were as follows: major comorbid or terminal illness that made endoscopy hazardous; inability or unwillingness to consent to endoscopy and endoscopic therapy; gastric malignancy; minor stigmata of hemorrhage at endoscopy such as oozing from ulcer borders without a visible vessel, flat-pigmented spots, or clean ulcer base. Patients with gastric and duodenal ulcer with either an actively bleeding vessel (spurting or oozing; Forrest Ⅰ), or adherent clot (Forrest Ⅱb) were also excluded.

Endoscopy was performed with standard upper endoscopes (GIF Q140 and GIF Q160, Olympus Optical Co., Japan). Endotherapy was carried out by the well-trained endoscopists, each with at least five years experience in the treatment of patients with GI bleeding. Endoscopic characteristics, including ulcer localization, ulcer size, and type of stigmata, were recorded (Endobase, Olympus, Japan).

Possible complications of endoscopic treatment and complete study protocol were discussed with patients and their relatives, and written informed consent was obtained before endoscopy and entry into the trial. The ethics committee of our hospital approved the treatment protocol. Randomization of eligible patients was carried out at the time of endoscopy by an individual uninvolved with the procedure who opened sealed numbered envelopes containing treatment assignments generated with a computer randomization program. The treatment group allocation was then communicated to the endoscopist in the endoscopy suite. Patients were randomized to a small-volume epinephrine group (15 to 25 mL injection group; Group 1), a large-volume epinephrine group (30 to 40 mL injection group; Group 2) and a hemoclip group (Group 3). In the small-volume epinephrine group (Group 1) 15 to 25 mL of a 1:10 000 solution of epinephrine was injected around the visible vessel (2-4 mL/injection at 2-3 mm from the visible vessel). In the large-volume epinephrine group (Group 2), 30-40 mL of a 1:10 000 solution of epinephrine was injected around the visible vessel at the ulcer bed as in the small-volume epinephrine group. Mechanical hemostasis was performed with stainless steel hemoclips (Olympus, Japan) as has been previously described[9,10]. During endoscopy and endotherapy, electrocardiographic monitoring was used to detect arrhythmias.

Once hemostasis was achieved the bleeding site was observed for at least 10 min and it was assessed by water irrigation at maximal pressure. Failure of the initial hemostasis has been defined if any hemorrhage occurred immediately (within 10 min) after initial endoscopic hemostasis. In these patients crossing over to the other treatment group was not allowed. In all patients two biopsy specimens were taken from the gastric antrum and body, and the presence of *Helicobacter pylori* (*H. pylori*) infection was assessed by histopathological examination of the specimens. In patients with gastric ulcer in whom recurrent bleeding was not observed, control endoscopy was performed 4 d to 5 d after initial hemostasis and biopsy specimens were obtained from the margins and base of gastric ulcers to exclude malignancy.

After initial endoscopic hemostasis, patients were hospitalized and cared for by a physician who was blinded to the endoscopic treatment that had been delivered. Vital signs were monitored hourly whereas blood counts were observed every 6 h for the first 48 h and every 12 h to 24 h thereafter. All patients were given acid suppressive therapy: pantoprazole 80 mg iv, (bolus) and then 40 mg iv, every 8 h for at least 48 h, followed by 40 mg daily by mouth, or esomeprazole 80mg iv, (bolus) and then 40 mg iv, every 8 h for at least 48 h, followed by 20 mg once a day by mouth. Shock was defined as a systolic blood

pressure of less than 90mmHg with symptoms or signs of organ hypoperfusion.

Recurrent bleeding was defined as one or more signs of ongoing bleeding, including fresh hematemesis or melena, hematochezia, aspiration of fresh blood *via* nasogastric tube, instability of vital signs, and a reduction of Hb by more than 2 g/dL over a 24 h period (early recurrence) or over a 7 d period (late recurrence) after initial stabilization of puls, blood pressure and Hb concentration. If recurrent bleeding was suspected, endoscopy was performed immediately. If "coffee ground" material or blood in the stomach and/or duodenum has been found together with active bleeding or a fresh blood clot in the ulcer base were found, recurrent bleeding was considered confirmed. For ethical reasons, additional endoscopic methods for treatment of recurrent bleeding were discussed with patients and their relatives and therapeutic option in all patients with recurrent bleeding was hemoclip application. Patients in whom endoscopic treatment or retreatment was unsuccessful underwent emergency surgery.

The rate of recurrent bleeding, as the primary outcome, was compared between the groups of patients included in the study. Secondary outcomes compared between the groups were primary hemostasis rate (defined as the absence of hemorrhage occurred immediately after initial endoscopic hemostasis), permanent hemostasis (defined as the absence of recurrent bleeding within the 30 d period after initial or secondary endoscopic hemostasis), need for emergency surgery, 30 d mortality and bleeding-related deaths, length of hospital stay, and transfusion requirements.

Statistical analysis

Base on assumption that injection of a large-volume epinephrine decreased the expected rate of recurrent bleeding from 17.1% after injection of small-volume epinephrine solution to zero, 39 patients would have been needed in each group for a power of 80% and a significance level of 0.05[6].

Continuous data were summarized as mean [95% confidence interval (CI)]. The Student *t* test was used to compare the mean values of continuous variables. The Pearson chi-square test and the Fisher exact test were used when appropriate for the comparison of categorical variables. All analyses were performed with a statistical package (SPSS for Windows, United States). A *P* values less than 0.05 were regarded as statistically significant.

RESULTS

From January 2005 to December 2009, 150 patients were included in this study; they were randomly assigned to receive small-volume (15 to 25 mL) injection of epinephrine (Group 1, $n = 50$), large-volume (30 to 40 mL) injection of epinephrine (Group 2, $n = 50$), and hemoclip (Group 3, $n = 50$). During the same period a total of 1516 patients with UGIB were encountered; of these 47.8% had gastric or duodenal bleeding ulcer, 41.2% had non-ulcer lesions (acute erosive gastropathy, portal hypertensive gastropathy, malignancy, Mallory-Weiss tear, angiomata, Dieulafoy's lesion), 9.1% had esophageal or gastric variceal bleeding, and 1.9% had no source of bleeding (Figure 1). Among the 161 patients with UGIB and endoscopically proven peptic ulcer with visible vessel (Forrest Ⅱa), randomly assigned to receive small-volume or large-volume of epinephrine, or hemoclip, 11 patients were excluded because they refused to participate in the study.

Clinical and endoscopic data obtained for patients included in the study are outlined in Table 1. There were no significant differences between the groups with respect to age, gender, ulcer size and location, positive *H. pylori* status, NSAID or alcohol consumption, shock, bleeding stigmata, history of previous peptic ulcer or peptic ulcer

Table 1 Clinical and endoscopic characteristics of the patients at study entry *n* (%)

	Group 1 ($n = 50$)	Group 2 ($n = 50$)	Group 3 ($n = 50$)
Age (yr)	68 (40-96)	61 (30-92)	67 (40-94)
Gender (M/F)	31/19	33/17	34/16
Location of ulcer			
Stomach	26 (52)	23 (46)	28 (56)
Duodenum	24 (48)	27 (54)	22 (44)
Ulcer size (cm)			
< 2	36 (72)	44 (88)	37 (74)
⩾ 2	14 (28)	6 (12)	13 (26)
Gastric content			
Blood	19 (38)	22 (44)	21 (42)
Coffee ground	31 (62)	28 (56)	29 (58)
Shock	4 (8)	2 (4)	1 (2)
Hb level (g/dL)	9.3 (3.9-14.7)	9.0 (3.6-14.2)	9.4 (5.6-14.3)
Comorbid disease	36 (72)	35 (70)	33 (60)
NSAIDs	15 (30)	23 (46)	29 (58)
Alcohol consumption	20 (40)	23 (46)	30 (60)
Smoker's	12 (24)	16 (32)	10 (20)
Previous ulcer disease	14 (28)	10 (20)	11 (22)
Previous ulcer bleeding	12 (24)	10 (20)	8 (16)

Continuous data are expressed as mean (95% CI). NSAIDs: Non-steroidal anti-inflammatory drugs.

Table 2 Clinical outcomes of endoscopic therapy *n* (%)

	Group 1	Group 2	Group 3
Primary outcome			
Early recurrent bleeding	15 (30)	8 (16)	2 (4)[c,b]
Stigmata			
Spurting	4 (26.7)	1 (12.5)	1 (50)
Oozing	5 (33.3)	2 (25)	1 (50)
Visible vessel	6 (40)	5 (62.5)	0
Secondary outcomes			
Initial hemostasis	50 (100)	50 (100)	50 (100)
Permanent hemostasis	44 (88)	46 (92)	48 (96)
Emergency surgery	6 (12)	4 (8)	2 (4)
30-d mortality	3 (6)	0 (0)	4 (8)
Blood transfusion (mL)	1041 (120-1997)	912 (0-2039)	840 (0-1893)
Hospital stays (d)	7.5 (1-14)	7.6 (1-15)	5.7(1-15)[b,d]

Continuous data are expressed as mean (95% CI). [b]$P < 0.01$ *vs* group 1; [c]$P < 0.05$, [d]$P < 0.01$ *vs* group 2. Pearson χ^2 test.

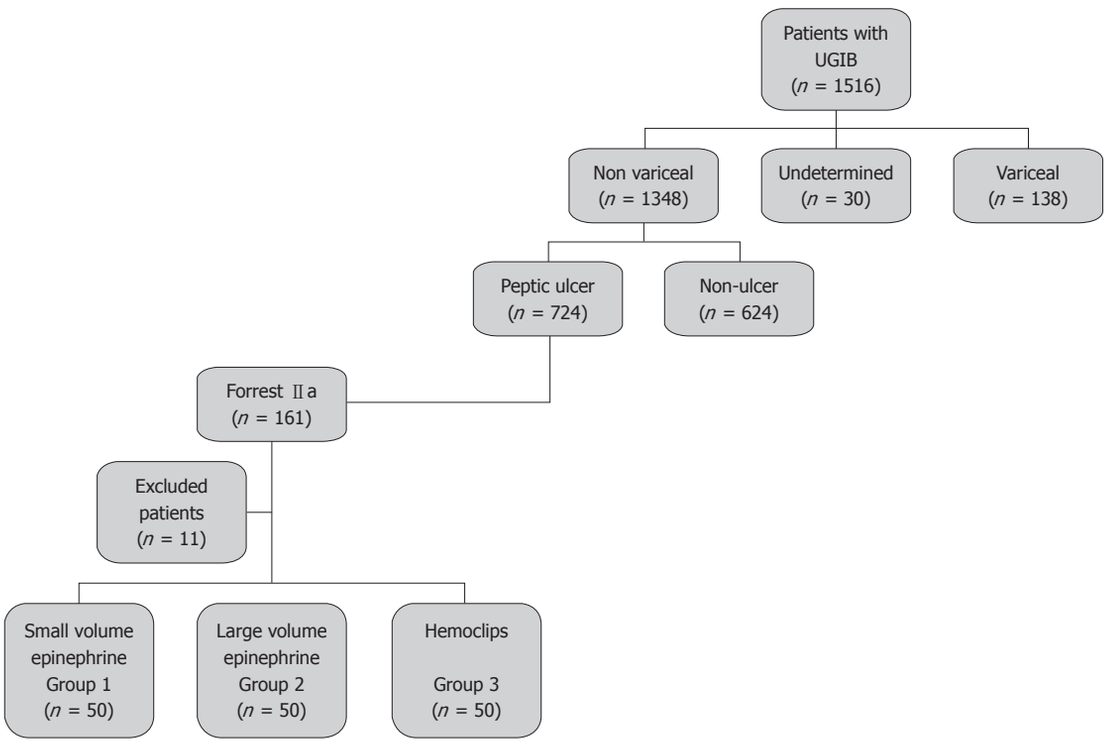

Figure 1 Patients with upper gastrointestinal bleeding. UGIB: Upper gastrointestinal bleeding.

bleeding, comorbid diseases or hemoglobin and hematocrit levels at admission.

Clinical outcome data are summarized in Table 2. Initial hemostasis was obtained in all patients. In the small-volume epinephrine group (Group 1) the mean volume of epinephrine injected was 19.1 mL (range, 16 to 25 mL) whereas in the large-volume epinephrine group (Group 2) the mean volume of epinephrine injected was 37.9 mL (range, 30 to 40 mL). Among patients endoscopically treated with hemoclips (Group 3), multiple clips (up to three) were needed in majority of cases with a median of 1.6 clips per patient.

The rate of early recurrent bleeding was 30% (15/50) in the small-volume epinephrine group (Group 1) and 16% (8/50) in the large-volume epinephrine group (Group 2); the difference did not reach statistical significance ($P = 0.09$). The rate of recurrent bleeding was 4% (2/50) in the hemoclip group (Group 3); the difference was statistically significant with regard to patients treated with either small-volume or large-volume epinephrine solution ($P = 0.0005$ and $P = 0.045$, respectively). Late recurrent hemorrhage was not observed in our patients. With regard to ulcer location and ulcer size as well, there were no significant differences in the rate of early recurrent bleeding between the groups. Also, there were no differences in transfusion requirement or even 30 d mortality between the groups. However, duration of hospital stay was significantly shorter among patients treated with hemoclips than among patients treated with epinephrine (Table 2). There was no bleeding-related death or procedure-related death. Three patients from Group 1 (causes of death were colon malignancy in one patient, cardiac failure in one patient, and obstructive pulmonary disease with pneumonia in one patient) and four patients from Group 3 died (pulmonary embolism in two patients and myocardial infarction in one patient). Among patients from Group 2 no one died. One patient in whom large-volume injection of the epinephrine solution was administered (35 mL) required emergent surgery because of a perforation.

Of the 15 patients in the small-volume epinephrine group (Group 1), eight patients in the large-volume epinephrine group (Group 2), and two patients in the hemoclip group (Group 3) who had recurrent bleeding, all were treated with hemoclips. Emergency surgery was performed in all patients in whom re-treatment with hemoclips did not produce hemostasis: six patients from group 1, three patients from Group 2, and two patients from Group 3. Majority of patients in whom emergency surgery has been performed had duodenal ulcer located on the duodenal bulb posterior wall (Group 1, 5/6; Group 2, 2/3; Group 3, 3/3, respectively). Successful permanent hemostasis was not statistically different among groups of patients (Table 2).

Therapeutic efficacy of the small-volume epinephrine *vs* large-volume epinephrine and hemoclips is given in Table 3. Small-volume *vs* large-volume epinephrine was not significant in NNT benefit prediction, although small-volume epinephrine (NNT = 4) and large-volume epinephrine (NNT = 9) showed different significant benefits concerning hemoclip treatment.

There were no procedure-related cardiovascular complications in the three groups. Electrocardiographic monitoring did not record any serious cardiac arrhythmia except of occasional sinus tachycardia and isolated supraventricular extrasystoles observed among all patients

Table 3 Therapeutic efficacy of small-volume and large-volume epinephrine, and hemoclips in reducing recurrent bleeding (95% CI)

Recurrent bleeding rate (%)	RRR (%)	ARR (%)	NNT
Small-volume *vs* large-volume epinephrine	46.6 (-11.2-75.0)	14.0 (-2.6-30.3)	8.0 (37.7-3.3)
Small-volume epinephrine *vs* hemoclips	86.7 (51.7-96.5)	26.0 (12.3-40.4)	4.0 (2.5-8.1)
Large-volume epinephrine *vs* hemoclips	75.0 (9.0-23.0)	12.0 (0.2-25.0)	9.0 (4.0-476.0)

RRR: Relative risk reduction; ARR: Absolute risk reduction; NNT: Number needs to treat.

treated with large-volume epinephrine injection. The number of patients who complained of epigastric pain during and/or immediately after the procedure of endotherapy was significantly higher in the large-volume epinephrine group (34/50) than in the small-volume epinephrine (3/50) or hemoclips (2/50) groups ($P < 0.001$).

DISCUSSION

Emergency endoscopy is accepted as the method of choice in the early identification and treatment of a bleeding peptic ulcer[1,10]. A variety of endoscopic hemostatic methods have been developed and all were found to be similarly effective[3,11]. Epinephrine as the most commonly used agent for endoscopic injection therapy has been demonstrated to be effective for initial hemostasis but appears less effective in preventing further bleeding than other monotherapies, and definitely is less effective than epinephrine followed by a second modality such as sclerosant or a thermal contact device[4,12]. However, when the analysis was restricted to studies that used routine second-look endoscopy with re-treatment of high-risk stigmata, epinephrine injection was not found to be less effective than other monotherapies or epinephrine followed by second modality[12]. On the other hand, limited data indicate that injection of a large volume epinephrine seems to be superior to injection of a small epinephrine volume with respect to recurrent bleeding[5,6,13]. These studies suggested local tamponade is the major effect in sustained hemostasis and that injection of larger volumes of epinephrine may be beneficial in preventing recurrent bleeding by prolonging the hemostatic effect of mechanical compression. Lin *et al*[5] demonstrated that injection of a large volume (13-20 mL) of epinephrine can reduce the rate of recurrent bleeding in patients with high-risk peptic ulcer and is superior to injection of lesser volumes (5-10 mL) of epinephrine (15.4% *vs* 30.8%). Park *et al*[6] reported that injection of 35 to 45 mL of a epinephrine solution was more effective in preventing recurrent bleeding than an injection of 15 to 25 mL of the same solution (0% *vs* 17.1%). Similar results have been found by Liou *et al*[13,14] demonstrated that injection of a large volume (30 to 40 mL) of epinephrine significantly reducing the rebleeding rate in patients with active bleeding ulcer.

The current study clarifies the low value of endoscopic injection therapy with epinephrine alone in patients with peptic ulcer bleeding showing major stigmata of hemorrhage (patients with endoscopically proven peptic ulcer with a visible vessel in an ulcer bed; Forrest Ⅱa). Disparities in inclusion criteria using Forrest classification, across the majority of studies that demonstrated higher effectiveness of large volume diluted epinephrine injection significantly limit the interpretation of those results. Unlike many mentioned studies, this trial was carried out on adequate patient's sample with clearly predefined groups of patients.

Our results have clearly shown that endoscopic therapy with hemoclip represents safe and effective method, superior to both, small-volume (15 to 25 mL) and large-volume (30 to 40 mL) injection of diluted epinephrine in the prevention of early recurrent bleeding from peptic ulcer. Reduction in recurrent hemorrhage rates observed among our peptic ulcer patients treated with hemoclip method positively affected length of hospital stay, reflecting the possibility of significant cost savings.

It has been postulated that possible mechanisms that underlie hemostasis in response to endoscopic injection of diluted epinephrine are vasoconstriction, vessel compression, and platelet aggregation[15,16]. Among these, mechanical compression of the bleeding vessel is the most important factor with respect to initial hemostasis[6,16]. Therefore, it has been assumed that injection of larger volumes of diluted epinephrine may be beneficial in preventing recurrent peptic ulcer bleeding by prolonging the hemostatic effect of mechanical effect and compression[5,6,14]. Despite the fact that previously mentioned assumption has been indirectly confirmed by several studies demonstrating a significantly lower rate of recurrent peptic ulcer bleeding following large volume epinephrine injection, we strongly believe that even sustained mechanical compression achieved by a larger volumes of diluted epinephrine injection is not sufficiently sustained to produce vessel compression that will last enough to provoke platelet aggregation in a greater extent, that would finally result vessel thrombosis. The results observed in this study indicate that local tamponade observed even after larger volumes of diluted epinephrine injection was not effective as hemoclip for the preventing of recurrent bleeding. This observation strongly suggested that vessel compression, produced by a hemoclip has an important role in the mechanisms involved in vessel occlusion, thus preventing the recurrent bleeding.

To our knowledge, this is the first prospective randomized study comparing the rates of the recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution and mechanical endoscopic method in patients with UGIB and endoscopically proven peptic ulcer with nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa). Our results have confirmed that

endoclip is safe and effective method, pointing out to its superiority to both, small volume and large volume injection of diluted epinephrine in the prevention of early recurrent bleeding from peptic ulcer.

COMMENTS

Background

Peptic ulcer disease accounts for 50% to 70% of cases of acute nonvariceal upper gastrointestinal bleeding (UGIB). Initial haemostatic rates of 80% to almost 100% can be achieved with various endoscopic techniques, but, after initial control, bleeding recurs in 10% to 30% of patients. Several recent studies found injection of a large volume of epinephrine to be superior to injection of a small epinephrine volume with respect to recurrent bleeding from peptic ulcer. Since epinephrine injection is effective (initial hemostasis obtained with epinephrine injection range from 85% to 100%), safe, inexpensive and technically easy, the concept of a beneficial effect of large volumes of epinephrine in preventing recurrent ulcer bleeding seems to be very challenging.

Research frontiers

To compare the rates of recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution (15-25 mL *vs* 30-40 mL) with endoscopic placement of hemoclips in patients with acute peptic ulcer bleeding and endoscopically proven gastric or duodenal ulcers with visible vessel in an ulcer bed (Forrest Ⅱa).

Innovations and breakthroughs

This is the first prospective randomized study comparing the rates of the recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution and mechanical endoscopic method in patients with UGIB and endoscopically proven peptic ulcer with nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa). Unlike many studies, this trial was carried out on adequate patient's sample with clearly predefined groups of patients, in a unique center. A detailed description is provided to allow other investigators to reproduce or validate.

Applications

The results provide sufficient experimental evidence to draw firm scientific conclusions. The results have clearly shown that endoscopic therapy with hemoclip represents safe and effective method, superior to both, small-volume (15 to 25 mL) and large-volume (30 to 40 mL) injection of diluted epinephrine in the prevention of early recurrent bleeding from peptic ulcer. Reduction in recurrent hemorrhage rates observed among our peptic ulcer patients treated with hemoclip method positively affected length of hospital stay, reflecting the possibility of significant cost savings.

Peer review

This is the paper in which authors compere two most commonly used hemostatic methods in patients with bleeding peptic ulcer. The sample size is adequate, in a unique center. A detailed description is provided to allow other investigators to reproduce or validate. The statistical methods used are appropriate. The results provide sufficient experimental evidence or data to draw firm scientific conclusions. The discussion is well organized and provide systematic theoretical analyses and valuable conclusions.

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BRIEF ARTICLE

Beneficial effects of fucoidan in patients with chronic hepatitis C virus infection

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Abstract

AIM: To evaluate the effects of fucoidan, a complex sulfated polysaccharide extract from marine seaweed, on hepatitis C virus (HCV) RNA load both *in vitro* and *in vivo*.

METHODS: HCV-1b replicon-expressing cells were cultured in the presence of fucoidan obtained from *Cladosiphon okamuranus Tokida* cultivated in Okinawa, Japan, and quantified the level of HCV replication. In an open-label uncontrolled study, 15 patients with chronic hepatitis C, and HCV-related cirrhosis and hepatocellular carcinoma were treated with fucoidan (0.83 g/d) for 12 mo. The clinical symptoms, biochemical tests, and HCV RNA levels were assessed before, during, and after treatment.

RESULTS: Fucoidan dose-dependently inhibited the expression of HCV replicon. At 8-10 mo of treatment with fucoidan, HCV RNA levels were significantly lower relative to the baseline. The same treatment also tended to lower serum alanine aminotransferase levels, and the latter correlated with HCV RNA levels. However, the improved laboratory tests did not translate into significant clinical improvement. Fucoidan had no serious adverse effects.

CONCLUSION: Our findings suggest that fucoidan is safe and useful in the treatment of patients with HCV-related chronic liver diseases. Further controlled clinical trials are needed to confirm the present findings.

Key words: Fucoidan; Hepatitis C virus; Replicon

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INTRODUCTION

Hepatitis C virus (HCV) infection often advances to chronic hepatitis due to the low viral clearance rate, leading to liver cirrhosis (LC) and subsequent development of hepatocellular carcinoma (HCC)[1,2]. The estimated global number of people infected with HCV is 170 million and more than 3.5 million new sufferers are diagnosed annually[3]. Currently, there is no vaccine available

for prevention of HCV infection due to the extreme sequence variability within the HCV genome. The first-line treatment for chronic hepatitis C (CHC) includes the combination of pegylated α-interferon (IFN) and ribavirin, a broad spectrum antiviral drug[4,5]. Although the reported HCV eradication rate by this combination therapy is 75%-90% for genotypes Ⅱ and Ⅲ and 45%-52% for genotypes Ⅰ and Ⅳ[6], these rates are still far from ideal. Because of the high rate of nonresponders among those infected with genotype Ⅰ, the predominant strain in Japan, and because antiviral treatment causes frequent, unpleasant and sometimes serious adverse effects[7], the establishment of a new treatment modality without serious adverse effects is desirable[8].

Considering the prolonged period (20-30 years) required for development of LC and HCC in individuals infected with HCV, progression of the disease might be influenced by nutritional status and diet. Although herbal supplements, including silymarin (an extract of milk thistle), are frequently used by patients with chronic liver diseases[9,10], the available scientific evidence for the beneficial effects of these supplements is limited[11]. However, administration of EH0202, a mixture of four herbal extracts, is reported to induce IFN activity and reduce HCV RNA levels in patients with high viral titers[12]. Furthermore, a recent study reported the hepatoprotective effect of birch bark extract in patients with CHC[13].

Fucoidan is a sulfated polysaccharide extracted from marine brown seaweeds that possess some biological activities including anti-inflammatory properties[14,15]. Sulfated polysaccharides, including fucoidan, are also reported to inhibit the replication of viruses such as herpes simplex virus, Sindbis virus, human immunodeficiency virus, parainfluenza virus type Ⅱ, and dengue virus[16-18]. We have also reported recently that oral administration of fucoidan for 12 mo resulted in 42.4% decrease in the human T-cell leukemia virus type I proviral load in patients with human T-cell leukemia virus type Ⅰ -associated neurological disease[19]. Since fucoidan shows no toxicity or irritation in humans, it may be useful also as an anti-HCV agent.

To our knowledge, there are no data on the anti-HCV effect of fucoidan. In the present study, we examined the anti-HCV activity of fucoidan extracted from the marine alga, *Cladosiphon okamuranus Tokida* (*C. okamuranus Tokida*) cultivated in Okinawa, Japan. Our pilot study is the first clinical trial that investigated the effect of fucoidan in patients with HCV-related chronic liver diseases.

MATERIALS AND METHODS

Preparation of fucoidan from seaweed

The unsalted brown seaweed *C. okamuranus Tokida* cultivated in Okinawa, Japan, was suspended in water, 0.57% (w/v) citric acid was added to the solution, and then heated at 90 ℃ for 40 min. The suspension was neutralized with NaOH and cooled to 40 ℃. It was centrifuged at 3500 *g* by decantation centrifugal separator. The supernatant was collected, filtered using Cohlo filter, and concentrated by ultrafiltration (molecular weight cutoff 6000). The extracts were dried by spraydrier. They were composed of carbohydrates (72%), uronic acids (24%), and sulfate (8%). Total carbohydrates were determined by the phenol-H_2SO_4 method using fucose as the standard. Uronic acids were determined by the carbazole-H_2SO_4 method using D-glucuronic acid as the standard. The sulfate contents were measured by ion chromatography. The main carbohydrates were fucose. Fucoidan content determined by high-performance liquid chromatography was 83% and the molecular weight was 21-kDa. Fucoidan was dissolved in phosphate-buffered saline at a concentration of 30 mg/mL.

Inhibition assay of HCV replicon cells by fucoidan

Fucoidan was added to Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum of HCV subgenomic replicon cells FLR3-1 (genotype I b, Con-1;)[20] at a final concentration of 62.5, 125, 250, 500, 1000, 2000, and 3000 μg/mL. FLR3-1 cells were established from human hepatoma HuH-7 cells[21] by stable transfection with subgenomic selectable RNA in which the encoding HCV structural proteins were replaced by the firefly luciferase gene, the internal ribosome entry site of the *Encephalomyocarditis* virus, and the neomycin phosphotransferase gene[22]. After 72 h-incubation, the cells were washed in phosphate buffered saline and lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for luciferase activity with the luciferase assay system (Promega) using the instructions provided by the manufacturer. With this HCV subgenome, the efficiency of subgenomic HCV expression could be estimated by measuring luciferase activity in the replicon cells.

Measurement of cell viability

Cell viability was measured using the cell proliferation reagent, WST-8 (Wako Pure Chemicals, Osaka, Japan). This method relies on mitochondrial dehydrogenase cleavage of WST-8 to formazan dye to estimate the level of cell viability. Briefly, FLR3-1 cells were incubated in a 96-well microculture plate. After 24 h incubation, fucoidan was added to the cells at various concentrations. After 72 h culture, WST-8 (5 μL) was added for the last 4 h of incubation and absorbance at 450 nm was measured using an automated microplate reader. WST-8 solution was added to the media-only wells to correct for background.

Patients

Table 1 lists the characteristics of the patients. The subjects included in the study were 15 patients with chronic liver diseases (7 men and 8 women; age, 66.1 ± 11.1 years; mean ± SD, range, 42-86), who visited the Nakasonekazu Medical Clinic. This study was carried out as an open-label study. All patients were infected with HCV genotype I b, with a serum viral load in excess of 10^5 copies/mL. Nine patients had been diagnosed with CHC, 4 with HCV-related LC, and 2 with HCV-related cirrhosis and HCC.

Table 1 Characteristics of patients

Patient No.	Age (yr)	Gender	Diagnosis	Previous IFN therapy	Other medications
1	73	F	LC	Not eligible	Glycyrrhizin
2	78	F	LC	Not eligible	Glycyrrhizin
3	49	M	LC	Not eligible	Glycyrrhizin
4	72	M	LC + HCC	Not eligible	Glycyrrhizin
5	66	M	LC + HCC	Not eligible	Glycyrrhizin
6	70	M	LC	Not eligible	None
7	70	F	CHC	Not eligible	Glycyrrhizin
8	86	F	CHC	Not eligible	Glycyrrhizin
9	55	M	CHC	Intolerant	None
10	69	M	CHC	Non-responder	Glycyrrhizin
11	71	M	CHC	Non-responder	Glycyrrhizin
12	68	F	CHC	Non-responder	Glycyrrhizin
13	61	F	CHC	Non-responder	Glycyrrhizin
14	62	F	CHC	Non-responder	None
15	42	F	CHC	Non-responder	None

M: Male; F: Female; LC: Liver cirrhosis; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; IFN: Interferon.

Eight patients were not eligible for IFN treatment because of LC, complication (depression), or advanced age. Seven patients had received IFN therapy in the past. Six patients were non-responders to IFN and 1 discontinued therapy because of side effect (depression). All patients assessed the tolerability as excellent.

During fucoidan treatment, 11 patients received a glycyrrhizin preparation. Fucoidan (provided by Kanehide Bio Co., Okinawa, Japan) was given orally as capsules containing 166 mg of dry extract from *C. okamuranus Tokida* per capsule in a dose of five capsules daily for 12 mo. Informed consent was obtained from all patients enrolled in the study, after a thorough explanation of the aims, risks, and benefits of this therapy.

Test parameters

The outcome parameters included the course of alanine aminotransferase (ALT), aspartate aminotransferase (AST), quantitative HCV RNA levels, subjective symptoms associated with CHC, LC, and HCC (such as fatigue, abdominal discomfort, depression, and dyspepsia), safety, and compliance. Data on all clinical parameters were documented at each visit. HCV RNA levels were determined using the AMPLICOR GT HCV Monitor test (Roche Diagnostics, Basel, Switzerland), which has a lower limit of quantitation of 0.5 kIU/mL at a linear range up to 850 kIU/mL.

Statistical analysis

Data are expressed as mean ± SD. The results of biochemical tests and HCV RNA levels were compared by the Student's *t* test. A $P < 0.05$ was considered significant.

RESULTS

Fucoidan suppresses HCV replication

To assess the effects of fucoidan on intracellular replication of the HCV genome, HCV subgenomic replicon cells FLR3-1 were cultured in the presence of various concentrations of fucoidan in the medium. The luciferase activities of the FLR3-1 cells showed a concentration-dependent suppression of replication of HCV replicon by fucoidan. The WST-8 assay showed that fucoidan had negligible effect on cell viability (Figure 1). These results suggest that fucoidan inhibits HCV replication but does not have cytotoxic effects.

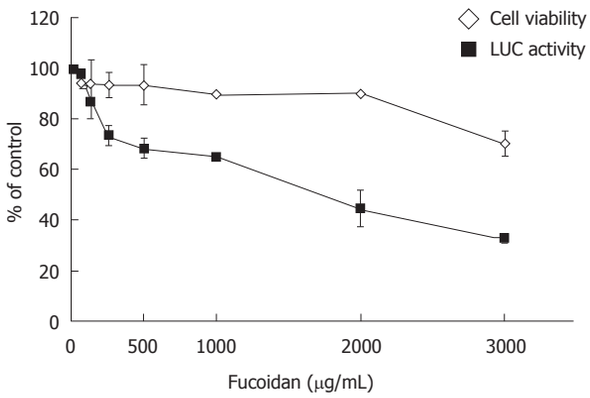

Figure 1 Anti-hepatitis C virus effects of fucoidan in hepatitis C virus replicon cells. Luciferase (LUC) activity (a marker of replication level) and cell viability of FLR3-1 cells, which constitutively express hepatitis C virus replicon, were measured in the presence of various concentrations of fucoidan. LUC and WST-8 assays were performed in triplicate. Data are mean ± SD.

Effect of fucoidan therapy on HCV RNA and alanine aminotransferase levels

Changes in HCV RNA and serum ALT levels in patients treated with fucoidan are shown in Figure 2. The mean HCV RNA for the 15 patients was 736 ± 118 kIU/mL (range, 100-850 kIU/mL) before fucoidan therapy. As shown in Figure 2A, fucoidan tended to reduce the mean HCV RNA level with time relative to the baseline, with significant falls registered at 8-10 mo of treatment. However, HCV RNA increased after 11 and 12 mo. Biochemical tests showed that the mean serum ALT level, but not AST, correlated with the mean HCV RNA level, although the decrease in ALT level was not significant relative to the baseline (Figure 2B). Whereas the above changes were not associated with improvement in clinical symptoms in every patient, none of the patients showed progression of LC or adverse events.

DISCUSSION

It is estimated that 170 million people worldwide are infected with HCV[3], and some 2 million (1%) of these reside in Japan[23]. Of the HCC cases in Japan, around 80% are caused by HCV infection. The increase in the number of HCC patients contributes to the increase in total deaths in Japan from HCC. This trend is expected to continue until 2015[23]. The general strategies followed in the treatment of CHC include eradication of HCV and suppression of hepatitis.

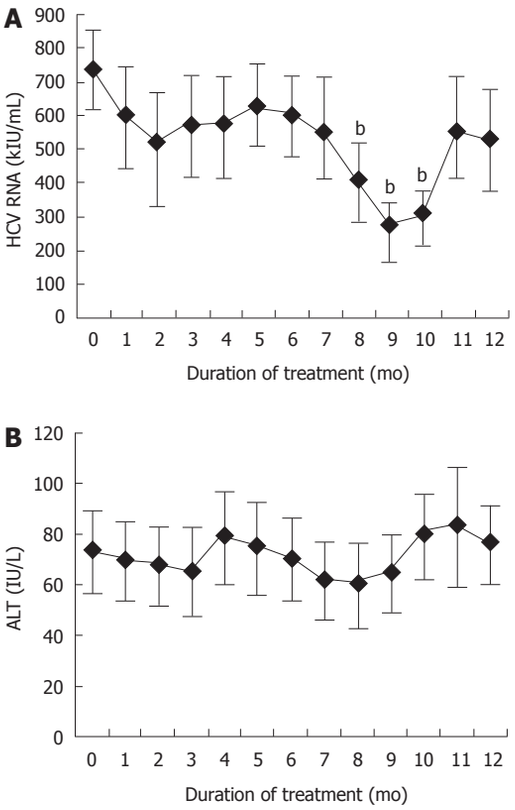

Figure 2 Effects of treatment with fucoidan on hepatitis C virus RNA and alanine aminotransferase levels in patients with liver diseases. A: Hepatitis C virus (HCV) RNA levels; B: Serum alanine aminotransferase (ALT) levels. Values are mean ± SD. $^{b}P < 0.01$ *vs* pretreatment value.

Sulfated polysaccharides including fucoidan are reported to inhibit the growth of various enveloped viruses[16-18]. Fucoidan is thought to inhibit virus adsorption to the cell surface by binding to the cell surface, with subsequent prevention of cell infection[18]. In addition, fucoidan interacts directly with the envelope glycoprotein on dengue virus type Ⅱ[17]. In the present study, we demonstrated a novel mechanism of action for fucoidan. Using the HCV replicon system, we demonstrated here that fucoidan inhibits intracellular replication of the HCV genome *in vitro*.

To our knowledge, this is the first clinical study to investigate the effects of fucoidan in patients with liver diseases. The rationale for this study was stimulated by experimental data showing the efficiency of fucoidan in cell cultures. Patients with chronic HCV infection, who were not eligible for, did not respond to, or were intolerant of IFN treatment, were treated for 12 mo with fucoidan at 830 mg/d to investigate the effect of this treatment on HCV RNA level. In Case 6 (baseline HCV RNA 380 kIU/mL), fucoidan treatment successfully eradicated HCV at 9 mo, although HCV RNA was 5 kIU/mL at 10 mo. Thus, fucoidan was effective in lowering HCV RNA level in this study, although its effect was temporary. There was a significant decrease in HCV RNA at month 8, 9 and 10 of fucoidan commencement ($P < 0.01$). However, the level increased later at month 12 to become equivalent to the baseline.

We also measured serum IFNα levels to determine the indirect effect of fucoidan on IFNs, especially whether it increases the antiviral activity of IFNs. However, IFNα could not be detected in the serum of patients treated with fucoidan. Furthermore, fucoidan did not enhance IFNs expression in FLR3-1 replicon cells (data not shown). It has been reported that the protective effect of fucoidan is based on direct inhibition of viral replication and stimulation of both innate and adaptive immune defense functions[24]. We are currently investigating the effect of fucoidan on the host immune system including natural killer cell cytotoxic activity.

Our study has certain limitations. First, the study comprised only a small number of patients, including 6 patients who were known non-responders to IFN therapy. Second, all patients harbored HCV virus genotype I b and 6 had cirrhosis. Thus, at least some patients in this cohort could be classified as likely non-responders to IFN therapy[25,26]. Thus, the selection criteria employed in the present study may have favored a poor response to fucoidan.

The abnormally high levels of ALT tended to decrease temporarily during fucoidan treatment, suggesting a correlation between viral load and indices of hepatic dysfunction. Thus, fucoidan may be effective in the management of HCV-related chronic liver diseases, although long-term clinical improvement was not observed in the present study. Importantly, no adverse events were observed in all patients, similar to the results reported in a previously study on fucoidan[19], suggesting that daily oral administration of fucoidan for 12 mo is safe and tolerable.

There is no doubt that patients who fail to respond to conventional treatments often seek alternative therapies. In conclusion, our study demonstrated that fucoidan from *C. okamuranus Tokida* has HCV replication suppressive effects in a replicon cell system. Furthermore, our relatively small uncontrolled pilot study showed that fucoidan has temporary but beneficial effects on HCV RNA levels in HCV infected patients. The preliminary findings suggest that fucoidan may be a useful health-food additive with antiviral activity to be used in the treatment of chronic liver diseases. To suppress the viral titer as much and for as long as possible, we need to define the daily effective dosage. Further studies on the mechanism of fucoidan-induced HCV inhibition may provide alternative strategies for the design of novel anti-HCV drugs.

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COMMENTS

Background

Hepatitis C virus (HCV) is a major cause of chronic liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The standard care

for chronic hepatitis C involves the administration of pegylated α-interferon in combination with the nucleotide analog ribavirin. However, this regimen has limited success rate for genotype Ⅰ and Ⅳ, and unfavorable side effects. Thus, it is important to discover more effective and safer agents to improve the clinical treatment on HCV carriers. Fucoidan, a sulfated polysaccharide, has significant biological activities, such as antiviral and anti-inflammatory effects. Nevertheless, there has been no investigation on the efficacy of fucoidan against HCV infection.

Research frontiers

Natural products have been used for the treatment of various diseases as an alternative to conventional chemical agents. So far, several natural products have been screened for their antiviral effect against various viral infections. Screening of natural potent inhibitors for HCV has also become a research hotspot.

Innovations and breakthroughs

Previous studies have shown the efficacy of natural products against HCV replication in a cell-based HCV replicon system. However, there have been few clinical studies that evaluated the safety and efficacy of these natural products. In the present study, the authors investigated the anti-HCV activity of fucoidan obtained from the *Cladosiphon okamuranus Tokida* cultivated in Okinawa, Japan, both *in vitro* and *in vivo*. This pilot study is the first clinical trial that investigated the effect of fucoidan in patients with HCV-related chronic liver diseases.

Applications

Fucoidan inhibited HCV RNA replication in the HCV replicon assay system. The experimental data on fucoidan efficiency in cell culture stimulated the rationale for clinical study. Oral fucoidan administration resulted in temporary reduction of viral loads of genotype Ⅰb in patients with chronic HCV infection, who were not eligible for, did not respond to, or were intolerant of interferon therapy. Fucoidan is well tolerated and no serious adverse events were observed in any of the patients. Fucoidan exhibited antiviral properties against HCV both *in vitro* and *in vivo*, and would be expected to become a new strategy for HCV infection. Further controlled clinical trials will be required to confirm the present findings.

Terminology

Fucoidan is a complex sulfated polysaccharide found in the cell walls of several edible brown algae, including *Fucus vesiculosus*. The HCV replicon system replicates a modified HCV genome containing luciferase gene to high levels in human hepatoma cells. The efficacy of subgenomic HCV expression was estimated by measuring luciferase activity in the replicon cells. This system provides a powerful tool for studying virus replication and for screening anti-HCV drugs.

Peer review

The paper studied the effects of fucoidan, a complex sulfated polysaccharide extracted from marine seaweed, on HCV RNA load *in vitro* and *in vivo*. The research is of significance because of the high rate of nonresponders in HCV genotype Ⅰ, which is the predominant strain in Japan. Moreover, antiviral treatment causes frequent, unpleasant and sometimes serious adverse effects, Thus the search for a new treatment modality without serious adverse effects is desirable.

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BRIEF ARTICLE

Plasma levels of acylated ghrelin in patients with functional dyspepsia

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Abstract

AIM: To investigate the relationship between plasma acylated ghrelin levels and the pathophysiology of functional dyspepsia.

METHODS: Twenty-two female patients with functional dyspepsia and twelve healthy volunteers were recruited for the study. The functional dyspepsia patients were each diagnosed based on the Rome Ⅲ criteria. Eligible patients completed a questionnaire concerning the severity of 10 symptoms. Plasma acylated ghrelin levels before and after a meal were determined in the study participants using a commercial human acylated enzyme immunoassay kit; electrogastrograms were performed for 50 min before and after a standardized 10-min meal containing 265 kcal.

RESULTS: There were no significant differences in plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia. However, in patients with functional dyspepsia, there was a negative correlation between fasting plasma acylated ghrelin levels and the sum score of epigastric pain (r = -0.427, P = 0.047) and a positive correlation between the postprandial/fasting plasma acylated ghrelin ratio and the sum score of early satiety (r = 0.428, P =0.047). Additionally, there was a negative correlation between fasting acylated ghrelin plasma levels and fasting normogastria (%) (r = -0.522, P = 0.013). Interestingly, two functional dyspepsia patients showed paradoxically elevated plasma acylated ghrelin levels after the meal.

CONCLUSION: Abnormal plasma acylated ghrelin levels before or after a meal may be related to several of the dyspeptic symptoms seen in patients with functional dyspepsia.

Key words: Functional dyspepsia; Acylated ghrelin; Electrogastrogram; Rome Ⅲ criteria; Dyspeptic symptoms

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INTRODUCTION

Functional dyspepsia is defined as the presence of dyspeptic symptoms thought to originate in the gastroduodenal region that occur in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms[1,2]. To date, functional dyspepsia has been associated with various physiological abnormalities, including delayed gastric emptying[3], altered antro-duodenal motility[4], impaired gastric accommodation[5], visceral hypersensitivity[6], gastric dysrhythmia[7-10]; functional dyspepsia has also been associated with multiple psychiatric and personal factors, such as somatization[11], depression, anxiety[12], and changes in coping skills[13]. However, a number of studies have failed to find associations between dyspeptic symptoms and the putative pathophysiology of functional dyspepsia in patients. The underlying etiology of functional dyspepsia remains unclear.

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor, and it has potent growth hormone-releasing activity. Ghrelin is predominately produced by endocrine cells in the oxyntic mucosa of the stomach[14,15]. Ghrelin has two subtypes: a deacylated form and an acylated form[16]. The physiologic functions of ghrelin are pleiotropic as follows. First, ghrelin stimulates food intake. An appetite stimulatory effect is associated with both central regulation and peripheral signals[17,18]. Second, ghrelin regulates gastric acid secretion. Ghrelin acts in the central nervous system to stimulate gastric acid secretion[19]. Third, ghrelin induces the migrating motor complex and promotes gastric emptying. Lastly, ghrelin has also been reported to have a gastroprotective effect in the context of the generation of nitric oxide and prostaglandins[20,21].

Given these diverse functions, ghrelin has been hypothesized to play a role in the pathophysiology of functional dyspepsia. The aim of this study was to investigate the role of acylated ghrelin in the pathophysiology of functional dyspepsia. This study measured plasma acylated ghrelin levels of female subjects, before and after a meal; these data were then compared between females with functional dyspepsia and healthy female volunteers. Also, in patients with functional dyspepsia, we determined the correlation between plasma acylated ghrelin levels, symptom scores, and electrogastrogram (EGG) parameters. In addition, patients with functional dyspepsia were divided into two subgroups according to the Rome Ⅲ criteria: patients with postprandial distress syndrome (PDS) and patients with epigastric pain syndrome (EPS). Differences in plasma acylated ghrelin levels between these two subgroups were also evaluated.

MATERIALS AND METHODS

Study subjects

Female subjects between 18 and 60 years of age were recruited from Sep. 2006 to Jan. 2007 at Soonchunhyang University Hospital, Seoul, South Korea. We recruited healthy volunteers by advertisement. Consecutive patients who were diagnosed with functional dyspepsia were invited to participate in the study. To diagnose functional dyspepsia, patients with dyspeptic symptoms thought to originate in the gastroduodenal region were asked to answer a questionnaire based on the Rome Ⅲ functional dyspepsia criteria after the exclusion of organic disease using endoscopic examination. In addition, eligible patients were asked to complete a questionnaire regarding the severity of 10 symptoms; severity was determined using a self-devised scale of absent (0), mild (1), relevant (2), moderate (3), and severe (4). The 10 symptoms were epigastric pain, epigastric burning, upper abdominal discomfort, nausea, upper abdominal fullness, gastric retention, upper abdominal distention, early satiation, vomiting, and belching. According to the predominant symptom, patients were classified as either PDS or EPS. Patients were examined for *Helicobacter pylori (H. pylori)* infection with a rapid urease test (Pronto Dry, Gastrex Corp., Warsaw, Poland) or the ^{13}C-urea breath test (UBiT-IR 300, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan). The study protocol was approved by the Soonchunhyang University Hospital Institutional Review Board. Written informed consent was obtained from all participants at the time of enrollment.

Study design

This prospective case-control study was designed to elucidate the role of plasma acylated ghrelin levels in the pathophysiology of functional dyspepsia. The primary endpoint was to investigate the difference in the plasma levels of acylated ghrelin between the two investigated groups. The second endpoint was to evaluate correlations between fasting and postprandial plasma acylated ghrelin levels, symptom severity, the EGG parameters in patients with functional dyspepsia. The tertiary endpoint was to examine whether there were any differences in plasma acylated ghrelin levels between patients with PDS and EPS.

Inclusion and exclusion criteria

To be included in the study, patients had to meet the following inclusion criteria: (1) one or more bothersome dyspeptic symptoms, such as postprandial fullness, early satiation, epigastric pain, and epigastric burning, for the last 3 mo, with symptom onset at least 6 mo prior to diagnosis; (2) the ability to cease all medical treatment that could influence the gastrointestinal motility at least 1 wk prior to the test; and (3) informed written consent.

The following exclusion criteria were utilized: (1) subjects who suffered from structural diseases, such as esophagitis, erosive gastroduodenal lesions or ulcers that could explain symptoms; (2) subjects who suffered from systemic diseases, such as diabetes mellitus or thyroid disease; (3) subjects with a history of peptic ulcers or major abdominal surgery; and (4) subjects who were obese, as defined by a body mass index of over 30.

Plasma acylated ghrelin measurement

Fasting blood samples were taken and analyzed for plasma levels of acylated ghrelin, glucose, insulin and growth

Table 1 Baseline subject characteristics

	Healthy volunteers (n = 12)	Patients with functional dyspepsia (n = 22)	P value
Age (yr)	24 (23-34)	46 (23-59)	0.000[1]
Height (cm)	162 (154-167)	160 (150-175)	0.435[1]
Weight (kg)	52.8 (44-58)	55 (45-70)	0.170[1]
Body mass index (kg/m^2)	20.2 (18.1-22.9)	21.2 (17.6-27.3)	0.135[1]
Growth hormone (ng/mL)	0.8 (0.1-3.3)	0.4 (0.01-14.7)	0.339[1]
Insulin (μIU/mL)	8.3 (2.3-14.6)	8.3 (4.0-14.4)	0.639[1]
Fasting blood sugar (mg/dL)	82 (72-97)	88.5 (70-107)	0.026[1]
Helicobacter pylori infection rate (%)	66.7 (8/12)	36.4 (8/22)	0.151[2]

[1]Analysis by Mann-Whitney *U* test; [2]Analysis by Fisher's exact test.

hormone. Postprandial blood samples were obtained fifty minutes after a 10-min meal and analyzed for postprandial plasma levels of acylated ghrelin. For each sample, whole blood was directly drawn into a centrifuge tube that contained 500 U of aprotinin and 1.25 mg of EDTA - 2Na per 1 mL of whole blood. Blood samples in the tubes were immediately centrifuged at 1500 × *g* for 15 min at 4 ℃. Plasma samples were stored at -80 ℃ for later use after immediately adding 100 μL of 1 mol/L HCl per 1 mL of collected plasma. The commercially available human acylated ghrelin enzyme immunoassay kit (Cayman Chemical Co., Michigan, United States) was used to measure the acylated ghrelin levels.

Electrogastrogram

Study subjects visited the office in the morning, after an overnight fast. Three EGG electrodes were connected to the subject's abdomen according to standard method[9,10]. The EGG electrodes were then attached to the Digitrapper EGG recorder (Medtronic Co. WA, United States). A fasting EGG signal was obtained for fifty minutes. After a 10-min break for a standardized meal, the postprandial EGG was also recorded for fifty minutes. Subjects were given a standardized soft diet that contained a total of 265 kcal, composed of 72% carbohydrate, 16% protein, and 12% fat. Nine parameters were measured, namely, the proportions of bradygastria (%), normogastria (%), and tachygastria (%) during the fasting and postprandial periods, the instability coefficient for both periods and the power ratio. The instability coefficient represents the stability of the slow wave and the power ratio represents the fasting electrical power of the slow wave divided by the postprandial electrical power.

Statistics analysis

SPSS software version 17.0 was used for statistical analyses. The Mann-Whitney *U* test was utilized to compare plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia. Fisher's exact test was used to compare the prevalence of *H. pylori* infection between the two groups. In patients with functional dyspepsia, correlation analysis by the Spearman's rho correlation coefficient was performed to assess the correlations between plasma acylated ghrelin levels, symptom scores and EGG parameters.

RESULTS

Subject characteristics

In total, thirty-four female subjects were enrolled. Twelve women were healthy volunteers and twenty-two were patients suffering from functional dyspepsia. Patient baseline characteristics are summarized in Table 1.

The healthy volunteers were significantly younger compared to the patients with functional dyspepsia (P = 0.00). The median fasting blood glucose level was significantly higher in patients with functional dyspepsia compared to healthy volunteers (P = 0.03), because two functional dyspepsia patients exhibited glucose intolerance. However, no significant differences were observed between the two groups for median growth hormone and insulin levels and body mass index values.

For the assessment of *H. pylori* infection status, thirty participants were tested by upper endoscopy with the rapid urease test, while the remaining four subjects underwent urea breath tests because they had undergone an upper endoscopy within 6 mo preceding study enrollment. *H. pylori* infection rates were 66.7% (8/12) in healthy volunteers, and 36.4% (8/22) in patients with functional dyspepsia. However, the prevalence of *H. pylori* between the two groups was not statistically significant.

The difference in plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia

In healthy volunteers, the median level of plasma acylated ghrelin was 52.2 pg/mL (1.6-228) during fasting and 32.1 pg/mL (1.1-104.2) postprandially. In patients with functional dyspepsia, the median levels of plasma acylated ghrelin during fasting and postprandially were 56.4 pg/mL (25.6-139.1) and 34.2 pg/mL (12.4-141.2), respectively. Interestingly, two patients with functional dyspepsia exhibited a paradoxical increase in postprandial plasma acylated ghrelin levels (Figure 1).

The association between plasma acylated ghelin levels, symptom scores, and electrogastrogram parameters in twenty patients with functional dyspepsia

Correlations between plasma acylated ghrelin levels and the symptom scores of the 10-investigated symptoms: There was negative correlation between fasting plasma levels of acylated ghrelin and total epigastric pain

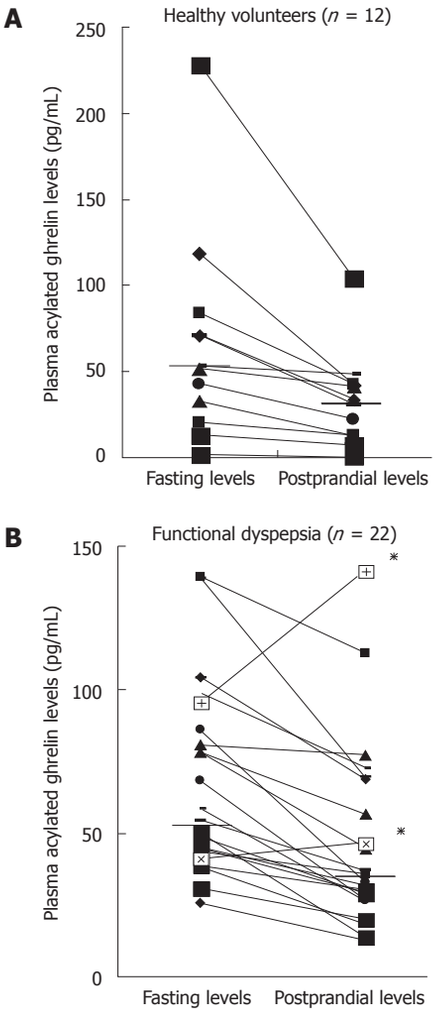

Figure 1 Plasma acylated ghrelin levels before and after a meal in healthy volunteers (A) and in patients with functional dyspepsia (B). Bars represent the median values. Note that two patients with functional dyspepsia (*) showed a paradoxical increase in postprandial plasma acylated ghrelin levels.

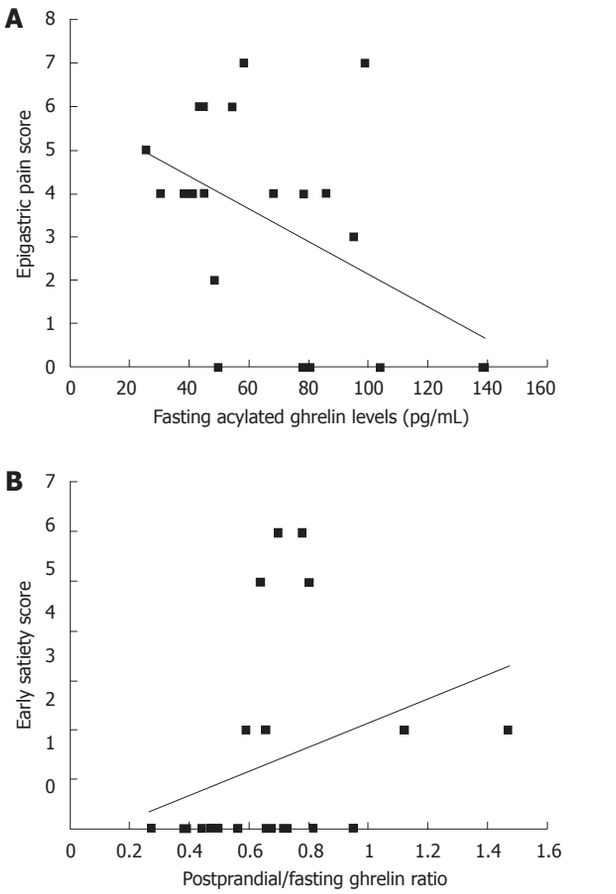

Figure 2 Relationship between plasma acylated ghrelin levels and dyspeptic symptoms in patients with functional dyspepsia. A: There was a negative correlation between fasting acylated ghrelin levels and epigastric pain scores (r = -0.427, P = 0.047); B: There was a positive correlation between the postprandial/fasting acylated ghrelin ratio and early satiety scores (r = 0.428, P = 0.047).

scores (r = -0.427, P = 0.047) (Figure 2A). In contrast, we found a positive correlation between the postprandial/fasting acylated ghrelin ratio and the total early satiety scores (r = 0.428, P = 0.047) (Figure 2B).

Correlations between plasma acylated ghrelin levels and the nine electrogastrogram parameters: There was a negative correlation between fasting plasma levels of acylated ghrelin and fasting normogastria (%) (r = -0.522, P = 0.013) (Figure 3).

The difference in plasma acylated ghrelin levels between postprandial distress syndrome and epigastric pain syndrome patients

In the thirteen PDS patients, the median plasma level of acylated ghrelin was 58.31 pg/mL (30.53-139.12) during fasting and 35.66 pg/mL (19.61-112.25) postprandially. In the 9 EPS patients, the median plasma level of acylated ghrelin was 49.79 pg/mL (25.56-95.14) during fasting and 32.78 pg/mL (12.35-141.16) postprandially. The differences in plasma acylated ghrelin levels between PDS and EPS patients were not statistically significant (Table 2).

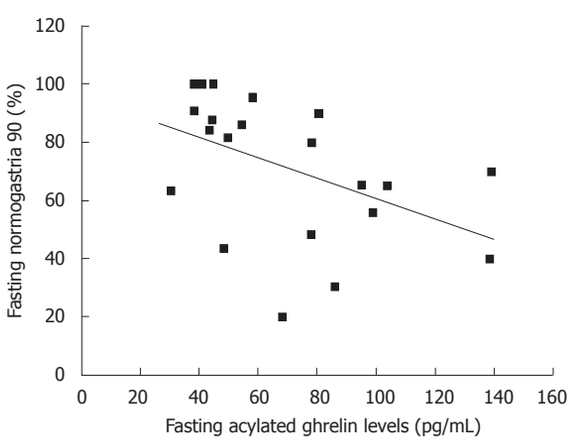

Figure 3 Relationship between fasting plasma acylated ghrelin levels and electrogastrographic parameters. There was a negative correlation between fasting plasma acylated ghrelin levels and fasting normogastria (%) (r = -0.522, P = 0.013).

Table 2 Postprandial distress syndrome and epigastric pain syndrome patient characteristics

	Postprandial distress syndrome (n = 13)	Epigastric pain syndrome (n = 9)	P value
Age (yr)	46 (23-59)	44 (37-58)	0.713[1]
Height (cm)	160 (154-175)	161 (150-168)	0.946[1]
Weight (kg)	54 (45 -70)	57 (47-62)	0.841[1]
Body mass index (kg/m^2)	20.7 (17.63-27.34)	22.27 (18.44-26.22)	0.640[1]
Growth hormone (ng/mL)	0.41 (0.03 -9.16)	0.46 (0.01-14.72)	0.404[1]
Insulin (μIU/mL)	8.99 (4.12-14.38)	7.88 (4.00-13.80)	0.664[1]
Fasting blood sugar (mg/dL)	92 (81-107)	86 (70-95)	0.114[1]
Helicobacter pylori infection rate (%)	53.8 (7/13)	11.1 (1/9)	0.074[2]
Fasting plasma levels of acylated ghrelin (pg/mL)	58.31 (30.53-139.12)	49.79 (25.56-95.14)	0.443[1]
Postprandial plasma levels of acylated ghrelin (pg/mL)	35.66 (19.61-112.25)	32.78 (12.35-141.16)	0.616[1]

[1]Analysis by Mann-Whitney *U* test; [2]Analysis by Fisher's exact test.

DISCUSSION

Ghrelin has two subtypes: deacylated ghrelin, which lacks an acyl group at third serine residue, and acylated ghrelin, which has the acyl modification necessary for noctanoic acid hormonal activity[16]. Although deacylated ghrelin circulates in far greater amounts compared to the acylated form and is involved in cell proliferation and adipogenesis, only acylated ghrelin exhibits physiologic activity and can stimulate growth hormone release and food intake[22-25]. Therefore, we focused our investigation on acylated ghrelin.

In a study by Takamori *et al*[26] that focused on two age-matched groups, the authors found that fasting levels of deacylated ghrelin were significantly lower in patients with functional dyspepsia compared to controls; however, they reported that both fasting and postprandial levels of acylated ghrelin and postprandial levels of deacylated ghrelin were similar in the two groups. Consistent with these findings, our study did not show any differences in either fasting or postprandial plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia. These results suggest that plasma acylated ghrelin levels may not be directly associated with the pathophysiology of functional dyspepsia.

Plasma acylated ghrelin levels differ significantly in males and females. A study by Akamizu *et al*[16] showed that fasting levels of acylated, but not deacylated ghrelin, in female subjects were higher compared to males after adjustment for body mass index. For this reason, we limited our study to female participants.

H. pylori infection was more prevalent in the healthy volunteers compared to the patients with functional dyspepsia in our study; however, the difference in prevalence was not statistically significant. Although the prevalence of *H. pylori* infection in Korea has been decreasing from 66.9% in 1998 to 59.6% in 2005[27,28], it is still high even in asymptomatic adult subjects. This could be the reason why *H. pylori* was present more in the healthy volunteers in our study.

In our study, fasting plasma acylated ghrelin levels ranged from 1.61 to 227.98 pg/mL and postprandial plasma acylated ghrelin levels ranged from 1.09 to 141.16 pg/mL. Interestingly, plasma acylated ghrelin levels vary more than 100-fold between the extremes.

Most studies have reported the plasma levels of total ghrelin between 300 and 800 pg/mL[19]. Low total plasma ghrelin levels during fasting are associated with insulin resistance, hypertension, and type 2 diabetes[29]. Plasma ghrelin levels rise before the meal and sharply decline as soon as eating commences[30]. The surge of ghrelin levels before the meal is similar to the increase of gastric acid that occurs in the cephalic phase. Plasma ghrelin levels do not begin to recover until thirty minutes after a meal. This delayed recovery suggests that the mechanism for immediate intragastric inhibition of ghrelin release is not present in the stomach, but is instead associated with feedback inhibition, either *via* the release of an intestinal hormone or by insulin release in conjunction with food intake[31]. Interestingly, in our study, postprandial plasma acylated ghrelin levels were paradoxically higher compared to fasting levels in two individuals. Test repetition confirmed these results. One individual had a fasting ghrelin level of 95.1 pg/mL with a postprandial ghrelin level of 141.2 pg/mL. In the other individual, the fasting and postprandial plasma ghrelin levels were 41.1 pg/mL and 46.4 pg/mL, respectively. Both individuals listed epigastric pain and burning sensations as their main symptoms. These results suggest that an abnormal acylated ghrelin response after a meal may be one of the mechanisms involved in the pathophysiology of functional dyspepsia. Further study including more subjects will be needed to confirm this hypothesis.

A study by Shinomiya *et al*[32] revealed that the fasting levels of plasma acylated ghrelin correlated with subjective symptoms of functional dyspepsia in female patients. Similarly, we observed that plasma acylated ghrelin levels were associated with symptom scores and several EGG parameters. First, there was a negative correlation between fasting plasma acylated ghrelin levels and total epigastric pain scores (r = -0.427, P = 0.047). Therefore, patients with higher plasma levels of acylated ghrelin before the meal suffered from less epigastric pain. Taking into consideration a previous report showed that basal gastric acid secretion was normal in patients with functional dyspepsia[33],the relationship between higher fasting

plasma levels of acylated ghrelin and decreased epigastric pain appear to result from a gastroprotective effect exerted by ghrelin on the gastric mucosa[19-21]. Second, we found a positive correlation between the ratio of acylated ghrelin level (i.e., the postprandial plasma acylated ghrelin level divided by the fasting plasma acylated ghrelin level) and the total early satiety scores (r = 0.428). Thus, blunted ghrelin decreases after the meal were associated with higher early satiety scores. Our results suggest that abnormal responses following a meal might play a role in the impairment of gastric accommodation. Third, we report a negative correlation between fasting plasma acylated ghrelin levels and fasting normogastria (3 cpm) (%). Accordingly, in patients with functional dyspepsia, higher fasting plasma acylated ghrelin levels were associated with fasting gastric dysrhythmia. Gastric dysrhythmia is reported in 40%-50% of patients with dysmotility-like dyspepsia[7,8]; also,abnormal myoelectrical activity of the stomach is associated with dyspeptic symptoms, especially nausea and vomiting[9,10]. Further study will be required to evaluate the relationship between fasting plasma acylated ghrelin levels and fasting gastric dysrhythmia.

A study by Shindo *et al*[34] showed that fasting plasma levels of acylated ghrelin in PDS patients were significantly lower compared to healthy volunteers; these levels also tended to be lower compared to EPS patients. However, in our study, no significant differences were observed in either the fasting or postprandial plasma levels of acylated ghrelin between PDS and EPS patients. Interestingly, the two patients who exhibited paradoxical increases in postprandial plasma acylated ghrelin levels had EPS.

The limitations of our study should be noted. These limitations include the small sample, which permitted only the use of non-parametric tests in the statistical analysis. Also, the study group and the control group were not age-matched. Although plasma ghrelin levels are not known to change with age, age may be important factor for this kind of functional study. Additionally, the high level of *H. pylori* infection in our participants was not ideal. However, *H. pylori* infection has no direct relationship to the diagnostic criteria for functional dyspepsia[2]. Further, although a study by Isomoto *et al*[35] showed that the fasting levels of total ghrelin in *H. pylori*-positive patients were significantly lower compared to *H. pylori*-negative patients, the relationship of the plasma acylated ghrelin levels to *H. pylori* infection status has not been evaluated to date. Another factor that was not taken into account was the phase of the participants' menstrual cycles. This is potentially an important variable to control for because a study from De Souza *et al*[36] found that fasting ghrelin plasma concentrations were at least 85% greater in subjects with exercise-associated amenorrhea. Finally, as approximately one third of ghrelin is produced in extra-intestinal organs such as the pancreas and the hypothalamus[14,15], further studies measuring exclusively gastric ghrelin levels are needed.

In conclusion, although no significant differences in plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia were observed, we found that abnormal plasma acylated ghrelin levels before and after the meal may be related to several dyspeptic symptoms in patients with functional dyspepsia. We also observed a paradoxical increase in postprandial plasma ghrelin levels in two patients with EPS, suggesting that abnormal plasma acylated ghrelin levels might be involved in the pathophysiology of functional dyspepsia. Further study in a larger sample size is needed to elucidate the complicated pathophysiology of functional dyspepsia.

COMMENTS

Background

Dyspepsia occurs in approximately 25 percent of the population each year. The most common cause of dyspepsia is functional dyspepsia. However, the pathophysiology of functional dyspepsia is unclear. As ghrelin, an acylated peptide produced predominantly by the stomach, has a well-established role in increasing appetite and food intake and in stimulating gastric emptying and acid secretion, it may play a role in the pathophysiology of functional dyspepsia.

Research frontiers

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor, which is present on pituitary cells that secrete growth hormone. However, ghrelin exerts many endocrine and extraendocrine biological activities beyond the control of growth hormone secretion. In this study, the authors demonstrate that abnormal plasma acylated ghrelin levels before and after a meal may be related to several dyspeptic symptoms in patients with functional dyspepsia.

Innovations and breakthroughs

The authors found a negative correlation between fasting plasma levels of acylated ghrelin and epigastric pain scores, and a positive correlation between the postprandial/fasting acylated ghrelin ratio and early satiety scores. Further, they also found a negative correlation between fasting plasma acylated ghrelin levels and fasting normogastria. The results regarding the relationship between plasma ghrelin levels and epigastric pain and early satiety scores are interesting.

Applications

By revealing which symptoms in patients with functional dyspepsia is associated with plasma ghrelin levels, this study may represent a future strategy for the research on the relationship between plasma ghrelin levels and the pathophysiology of functional dyspespsia.

Peer review

The authors examined whether fasting and postprandial plasma acylated ghrelin levels exhibited differences in patients with functional dyspepsia compared to healthy volunteers. In addition, they attempted to demonstrate which dyspeptic symptoms and electrogastrogram parameters in patients with functional dyspepsia correlated with fasting and postprandial levels of plasma acylated ghrelin. Overall, this paper is unique despite several weak points.

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BRIEF ARTICLE

Knowledge levels and attitudes of health care professionals toward patients with hepatitis C infection

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Abstract

AIM: To study knowledge levels and attitudes of health care providers toward patients with hepatitis C virus infection in Guilan, a northern province of Iran.

METHODS: This cross-sectional study was performed on 239 health care professionals from the Razi Hospital, including doctors, nurses, and operating room technicians. The questionnaires consisted of questions on demographic characteristics, knowledge levels, and attitudes toward hepatitis C patients. The questionnaire was tested in a pilot study and validated by Cronbach's alpha coefficient. Data were analyzed using SPSS16 software.

RESULTS: The mean ± SD knowledge score was 17.43 ± 2.65 (from a total of 22). 51.9% of the participants achieved scores higher than the mean. There was a significant relationship between knowledge score and age (P = 0.001), gender (P = 0.0001), occupational history (P = 0.0001), and educational history (P = 0.027). There was also a significant relationship between attitude level and age (P = 0.002), gender (P = 0.0001), occupational history (P = 0.0001), and educational history (P = 0.035). Physicians were significantly more knowledgeable and showed more positive attitudes. There was a positive correlation between knowledge and attitude scores (P = 0.02).

CONCLUSION: Discriminatory attitudes are common among health care providers toward hepatitis C patients. It is therefore necessary to improve their knowledge level and attitude toward this disease.

Key words: Knowledge; Attitude; Hepatitis C; Health professional; Patient care

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INTRODUCTION

Hepatitis C is a hepatotropic viral infection caused by hepatitis C virus (HCV), which is a major cause of acute hepatitis and chronic liver disease. It is estimated that 170 million people worldwide (3% of the world population) are chronically infected with HCV and are under the risk of cirrhosis and liver cancer. Chronic HCV infec-

tion is usually slowly progressive. HCV infection leads to chronic hepatitis in 50% to 80% of individuals[1-5]. The increasing global prevalence of this disease puts extra demands on health care services and increases the likelihood that health care workers (HCWs) will care for, or have personal contact with, people with hepatitis C[6]. Occupational exposure from percutaneous injuries is a substantial source of infection by blood borne pathogens among HCWs. However, studies of HCWs exposed to HCV by a needle stick injury (NSI), or any other percutaneous injury, have found that the incidence of anti HCV seroconversion is 1.8% (0%-7%) on average[7]. Physicians, laboratory technicians, nurses, and dialysis unit personnel are the main HCWs at risk. Nurses are the most at risk group because they have close contact with patients and are more likely to be exposed to a NSI[8].

NSIs are common in our country. In a study in Qazvin, 52.9% of nurses had a history of NSI[9]. Patients with hepatitis C have met with discrimination and stigmatization in the work place, by family members and by members of their communities. In addition, they may face discrimination from HCWs[10,11]. The discriminatory practices of HCWs may result from a lack of knowledge and negative attitudes toward these kinds of diseases, which could interfere with their willingness to treat these patients because of a fear of contracting hepatitis C. In a study by Richmond *et al*[6], most health care workers had sufficient knowledge about hepatitis C; however, some of them showed discriminatory attitudes toward patients with hepatitis C. Until now, little research has been conducted on HCW knowledge levels and attitudes toward people with hepatitis C in Iran. We therefore studied HCW knowledge levels and attitudes toward treating people with hepatitis C as a blood borne disease, to investigate how attitudes can be influenced by education and how this can affect their willingness to treat these patients.

MATERIALS AND METHODS

A cross-sectional study was conducted using a questionnaire that was created and standardized by Richmond *et al*[6] and translated into Persian. The questionnaire was considered by a panel of consulting experts and its validity was documented by a pilot study using a random sample (n = 20) drawn from the subgroups to be surveyed in the main study. The sample was calculated as 345 health care workers, based on the positive attitude proportion among the random subgroup (32%) and considering the precision of 0.06 and the type one error of 0.05. The questionnaire's validity and reliability were also confirmed by Cronbach's alpha coefficient (alpha = 0.7). We used this specifically designed questionnaire for the study because no other instrument was identified that explored HCW attitudes toward treating people with hepatitis C in the detail that was required for the current research. The questionnaire consisted of four parts and 49 questions. Seven questions obtained demographic characteristics, 22 questions were used to explore knowledge levels, 10 questions determined attitudes, and 10 questions were designed to determine compassion levels toward hepatitis C patients, willingness to treat hepatitis C patients, their self reported fear of contracting hepatitis C, and their self reported behavior toward injecting drug users. The mean of the total score from a possible score of 22, based on 22 questions, was used as the discriminant level. Scores higher than the mean indicated a good knowledge level and lower than the mean indicated a poor knowledge level[12].

Regarding attitude, participants were asked to what extent they agreed or disagreed (using a five-point Likert scale ranging from "strongly agree" to "strongly disagree") with each of the statements. For the 10 statements, total scores that could be achieved ranged from 10 to 50. Scores between 10 and 30 were considered as negative attitudes and scores higher than 30 were considered as positive attitudes[13,14]. The knowledge level, attitude questions, and self-behavior statements are shown in Table 1. Demographic data containing participants' age, sex, working history (years which health care workers had worked for health care service), occupation (doctor, nurse), needle stick injury (NSI) history, education (participation in education classes on NSI), were registered for each participant. Questionnaires were filled by direct interviews, which were performed by a trained general practitioner from the research team.

Statistical analysis

Data were entered into SPSS 16 software and analyzed by descriptive statistics (i.e., mean, SD, frequency) and comparison means (i.e., one way ANOVA, χ^2 test). A P value less than 0.05 was considered statistically significant.

RESULTS

Response rate and demographic data

The mean age of the participants was 33.06 ± 7.72 years and the mean working history was 7.51 ± 6.49 years. The overall response rate was 69% (239 of 345 HCWs). There was no significant difference between the age, sex, occupation, and working history of responders and non-responders. Table 2 presents the demographic data. Overall, 52.3% of the participants reported having a history of NSI with hepatitis C patients.

Nurses (54%) were most likely to have a history of NSI. Most of the HCWs (79.1%) reported that they had received information on hepatitis C patients and NSI (although they did not refer to the type of education they had received). Nurses (85.3%) were significantly more likely to have received training on hepatitis C, while 72.2% of the physicians had received training and operating room technicians (40%) were the least likely to have received training (P = 0.001).

Hepatitis C knowledge level and education

Overall, HCW level of knowledge was satisfactory and

Table 1 Hepatitis C knowledge level, attitude and self-reported statement questions in the questionnaire

Hepatitis C knowledge questions (response options: true, false and uncertain)
Hepatitis C is caused by a virus
Hepatitis C is caused by bacteria
Hepatitis C can be spread through close personal contact such as kissing
Hepatitis C can be spread through sharing injecting equipment, such as needles, tourniquets, spoons, filters and swabs
Hepatitis C can be spread by mosquitoes
Hepatitis C is spread through blood-to-blood contact
Having a medical and/or dental procedure performed in the Middle East, South East Asia or the Mediterranean increases a person's chances of contracting hepatitis C
Hepatitis C is spread through the air in an enclosed environment (e.g., crowded buses and elevators)
Sexual transmission is a common way hepatitis C is spread
Some people with hepatitis C were infected through unsterile tattooing
Some people with hepatitis C were infected through blood transfusions
People with hepatitis C should be restricted from working in the food industry
Hepatitis C can lead to cirrhosis
Hepatitis C is associated with an increased risk of liver cancer
Hepatitis C is a mutation of hepatitis B
A person can be infected with hepatitis C and not have any symptoms of the disease
There is a pharmaceutical treatment available for hepatitis C
There is a vaccine for hepatitis C
HIV is easier to catch than hepatitis C
An individual can have hepatitis C antibodies without being currently infected with the virus
People with hepatitis C should restrict their alcohol intake
Once you have had hepatitis C, you cannot catch it again because you are immune
Attitudes and self-reported behavior statements according to theme (response options: strongly agree, agree, uncertain, disagree and strongly disagree)
Attitudes and self-reported behavior toward the implementation of infection control guidelines
When receiving health care, patients with hepatitis C (HCV) should be identified for safety reasons
Patients with HCV should be given the last appointment for the day (ICG)
Health professionals who are HCV positive should be discouraged from having contact with patients
All patients should be tested for HCV before they receive health care
I deliver the same standard of care to patients with HCV as I do for other patients
I feel that I do not have the skills needed to effectively and safely treat patients with HCV
Following infection control guidelines will protect me from being infected with HCV at work
I often use additional infection control precautions when treating patients with HCV
I would prefer to wear two pairs of gloves when treating a bleeding person with HCV
The infection control guidelines necessary to treat patients with hepatitis C would be a financial burden on my practice/ward
Attitudes and self-reported compassion toward people with hepatitis C
I feel sorry for people who contracted HCV through a blood transfusion
I feel sorry for people who contracted HCV through HIV drug use
Attitudes and self-reported willingness to treat people with hepatitis C
I do not like treating people with HCV
I am willing to treat people with HCV
I believe my profession should have central role in the treatment of HCV

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; ICG: International crisis group.

Table 2 Health care workers' demographic data

Variable		*n* (%)
Age (yr)	≤ 30	108 (45.2)
	31-40	88 (36.8)
	≥ 41	43 (18.0)
Sex	Male	47 (19.7)
	Female	192 (80.3)
Working history (yr)	≤ 5	117 (49)
	6-10	60 (25.1)
	≥ 11	62 (25.9)
Health care group	Physicians	79 (33.1)
	Nurses	150 (62.8)
	Technicians	10 (4.2)
NSI history	Yes	125 (52.3)
	No	114 (47.7)
Education	Yes	189 (79.1)
	No	50 (20.9)

NSI: Needle stick injury.

the mean knowledge score was 17.43 ± 2.65 from a total score of 22. Scores higher than 17.43 indicated a good knowledge level and lower than 17.43 indicated a poor knowledge level. For example, 95.4% of the participants answered the questions about cirrhosis correctly, 97.5% knew that hepatitis C is contracted through blood contact, 95% knew that non-sterile tattoos are also a method of transmission, and 98.3% of HCWs knew that HCV can be spread through sharing injection equipment, such as needles, tourniquets, spoons, filters, and swabs. However, some deficits were identified in their knowledge level. For example, only 37.7% of HCWs answered correctly to the statement: Sexual contact is a common mode of transmission.

Only 54.8% knew that there are effective treatments for hepatitis C, 35.1% did not know, and 10% were not sure about it.

The mean knowledge score of males was 18.78 ±

Table 3 Health care workers' hepatitis C knowledge scores in association with different variables (mean ± SD)

Variables		*n*	Mean knowledge score (SD)	*P* value
Sex	Male	47	18.78 (2.12)	0.0001
	Female	192	17.1 (2.66)	
Age	≤ 30	108	16.75 (2.9)	0.001
	31-40	88	17.93 (2.22)	
	≥ 41	43	18.13 (2.41)	
Working history	≤ 5	117	17.05 (2.97)	0.073
	6-10	60	17.6 (2.91)	
	≥ 11	62	17.98 (2.54)	
Professional group	Physicians	79	19.26 (1.97)	0.0001
	Nurses	150	16.61 (2.51)	
	Technicians	10	15.3 (1.25)	
NSI history	Yes	125	17.28 (2.81)	NS
	No	114	17.59 (2.45)	

NSI: Needle stick injury; NS: Not significant.

Table 4 Association of health care workers' attitudes with different variables

Variables	*P* value
Sex	0.0001
Age	0.002
Working history	0.002
Professional group	0.0001
NSI history	NS
Education	0.035
Knowledge score	0.0001

NSI: Needle stick injury; NS: Not significant.

2.12 and the mean knowledge score of physicians was 19.26 ± 1.97. Physicians were the most knowledgeable, while technicians were the least knowledgeable group (P = 0.0001). Those who were more than 30 years old were the least knowledgeable group among different professional groups (P = 0.0001) (Table 3). In this study, 55.6% of the HCWs who had received training had a good knowledge score, but only 38% of those who had not received training showed good scores (P = 0.027).

Attitudes and self-reported behaviors

In this sample, 159 HCWs (66.5%) showed negative attitudes (score = 10-30) and 80 HCWs (33.5%) showed positive attitudes (score = 31-50). Males who were over 40 years old and had a working history of more than 10 years were more likely to show positive attitudes toward patients with hepatitis C (P = 0.002 for both) (Table 4). In the physicians group, 55.7% showed positive attitudes and, in the nurses group, 24% showed positive attitudes. Physicians were significantly more positive (P = 0.0001). All of the technicians showed negative attitudes. Those who received training also showed significantly more positive attitudes (P = 0.035).

HCWs who were weak in knowledge were more likely to show negative attitudes and those who were knowledgeable were more likely to show positive attitudes (P = 0.02. There was a positive correlation between knowledge score and attitude (r = 0.227, Figure 1).

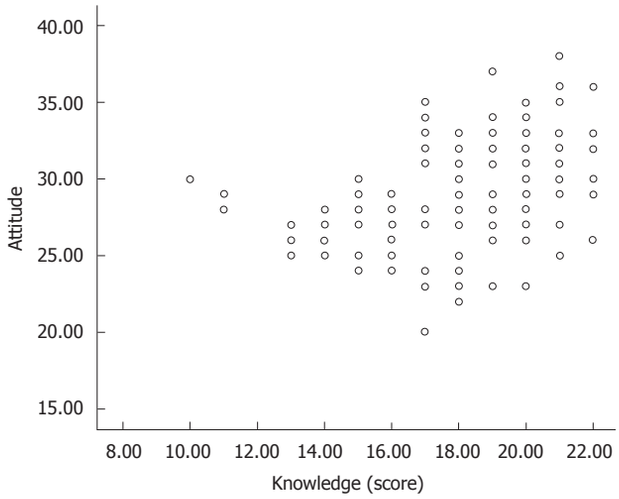

Figure 1 Pearson correlation between health care workers knowledge levels and attitudes.

Regarding attitude statements, 95.8% of the HCWs believed that hepatitis C patients should be identified for infection control purposes, 82.8% of the participants indicated that they used additional infection control precautions when they knew patients had hepatitis C; 74.4% were double-gloved when they treated a bleeding person with hepatitis C. 48.5% of the participants indicated that patients with hepatitis C should be given the last appointment of the day.

Compassion toward people with hepatitis C was measured in two statements (Table 1). Among the HCWs, 92.1% felt compassion when hepatitis C was acquired through a blood transfusion, compared with 55.6% when it was contracted through injection drug use. Participants' willingness to care for people with hepatitis C was identified by responses to three questions (Table 1). Among HCWs, 82.8% believed that they liked to treat these patients and 15.5% said that they did not like treating these patients. Regarding attitudes toward intravenous (IV)-drug users, 78.7% showed fear toward IV-drug users, while 77% said that they were worried they might contract a disease from them. 35.6% believed that IV-drug users deserved the disease and 40.2% indicated that they did not want to treat IV-drug users. 26.8% believed that opiates should not be used for pain relief in patients with a history of injection drug use. There was an association between HCW knowledge level and fear of contracting a disease (P = 0.0001) and attitudes toward IV-drug users (P = 0.0001); however, there was no association between knowledge level and willingness to treat these patients. In addition, HCW attitudes toward IV-drug users emerged as a significant issue that affected willingness to treat people with hepatitis C (P = 0.035).

DISCUSSION

The total response rate in the present study was rather

high compared to other studies on this issue[6,11,15]. In the present study, the reasons for not responding included not having enough time and not having enough interest. Some part of not having enough interest may have been because of incomplete knowledge on the issue.

In the current study, 49.4% of physicians and 54% of nurses had a history of NSI and the difference, unlike in Zafar's study[16], was not significant. Vitale *et al*[17] and Wicker *et al*[18] reported a lower rate of NSI history. Perhaps the reason for the high rate of NSI among HCWs was not receiving enough practical education, HCW stress, or carelessness because of an increased workload.

HCWs are currently receiving training about blood borne diseases. In the present study, however, we did not refer to the contents and efficacy of training. Nurses in the current study were most likely to have received training, possibly because of their close contact with these patients and their greater interest in having a correct approach toward them.

In the current study, the mean knowledge level score was acceptable. Physicians were more knowledgeable than other groups. Richmond showed a significant relationship between medical groups and mean knowledge scores: doctors were the most knowledgeable group[6]. In the study by Shehab *et al*[19], the knowledge level of internal medicine residents on hepatitis was suboptimal. The higher knowledge level of physicians was likely because of more advanced and professional education on gastrointestinal and liver diseases.

In the present study, males and HCWs who were more than 40 years old were more knowledgeable. In Richmond's study[6], HCWs who were 30-49 years old were the most knowledgeable and those above 40 were the least knowledgeable. This finding shows that older age and greater experience can be associated with greater knowledge. However, in elders, the efficacy of initial education decreases.

In the present survey, HCWs were insufficiently knowledgeable about the complications of hepatitis C. Sood *et al*[20] showed that more than half of the participants answered correctly to the questions about hepatitis C complications. In a study by Nicklin *et al*[21], half of the personnel indicated cirrhosis was caused by hepatitis C and 37% thought it caused liver cancer. In the present study, the knowledge about modes of transmission was also acceptable. In the studies of D'Souza *et al*[11] and van de Mortel *et al*[22], most of the participants indicated that blood transfusion is a major mode of transmission. However, some deficits were seen in HCW knowledge on sexual contact as a mode of transmission for hepatitis C.

In some prospective studies, in which the effect of HCW training on HCV knowledge was investigated, participants who received training showed significant advances in their knowledge levels[23-25]. Zdanuk *et al*[25] showed the same advances after receiving training. However, D'Souza *et al*[11] and Shehab *et al*[19] indicated that education did not produce any advance in knowledge levels. In the current study, education was introduced as an effective agent for developing HCW knowledge. However, we should not ignore the role of clinical practice as a form of education. For example, in Richmond's survey, while complementary therapists were the group most likely to have been educated about hepatitis C, they were not the most knowledgeable[6]. In addition, in the current study, nurses were the most likely group to have been educated, but were not the most knowledgeable. Therefore, other factors must influence the knowledge level of HCWs. However, we should not deny the fact that the information presented cannot be effective if it has not been repeated and recorded in the mind. In some studies, it has been suggested that one method for getting better results is active and problem-based learning[6,20].

In the present study, attitude scores were significantly different among different groups ($P = 0.0001$). Physicians were the most positive group towards people with hepatitis C and technicians showed negative attitudes. This may be related to the fact that physicians were the most knowledgeable group and the better attitudes of males and elders reflect this, as these groups are mainly composed of physicians. Perhaps the positive attitudes are not related to age or experience.

Education had a significant influence on developing positive attitudes, which was also noted by van de Mortel[22] and Richmond *et al*[6]. However, it should be considered that HCW attitudes on hepatitis C patients might be influenced by the attitudes of colleagues. A problem associated with consulting colleagues is that the information provided could be inaccurate, outdated, or reflect just subjective clinical experiences about people with hepatitis C.

In the current study, most of the participants indicated willingness to treat patients with hepatitis C, as in the studies by Hu *et al*[26] in dentistry students and a study by van de Mortel[22].

The HCWs' approach toward giving opiates to the IV-drug users for pain relief reflects deficits in their understanding of pain management and drug dependence. This also demonstrates the powerful influence of attitude on their clinical behavior. In the present study, some HCWs said that IV-drug users deserved to contract hepatitis C and this attitude was affected by their knowledge level and influenced their willingness to treat patients with hepatitis C. In addition, Richmond *et al*[6] showed that HCW willingness to treat patients with hepatitis C was significantly under the influence of their belief on injection drug users, rather than their knowledge of hepatitis C. This shows the role of social prejudice on self-reported behavior. Access to health services could be difficult for people with hepatitis C because HCWs believe that they are injection drug users[27].

Finally, as we expected, there was a significant correlation between HCW knowledge levels and attitudes ($P = 0.0001$). This finding was also reported in the studies of Richmond[6], Vitale *et al*[17] and van de Mortel[22]. We suggest that occupational experience and fear of contracting hepatitis C can also influence the willingness to treat people with hepatitis C.

This study had the advantage of direct interviews

with the responders, and not just distributing the questionnaires among them. The response rate in the present study (69%) was higher than some other similar surveys[6,11,15]. However, some limitations should be noted. For example, like other similar studies, self-reported behavior was not validated against actual clinical behavior. Self-reported responses may not reflect responders' actual attitudes[6].

In conclusion, we showed that discriminatory behaviors are common among HCWs towards patients with hepatitis C. Attitudes are directly under the influence of knowledge levels; therefore, it is necessary to increase the level and quality of training among HCWs to prevent discrimination and prejudice towards patients with hepatitis C.

ACKNOWLEDGMENTS

We would like to thank all the hospital staff that assisted us in this study.

COMMENTS

Background

Hepatitis C is a chronic prevalent liver infection worldwide which is caused by a blood borne pathogen (hepatitis C virus, HCV). Occupational exposure due to percutaneous injuries is a source of infection with this pathogen among health care workers. Because this group are in close contact with the patients with hepatitis C their knowledge and attitude toward the disease and the patients is so critical and can influence on their own health and their behavior toward the patients. This issue has not evaluated enough until now.

Research frontiers

In contacting with hepatitis C patients, prejudice and discrimination is so prevalent. The research hotspot is to evaluate health care workers' knowledge toward hepatitis C and its effect on their attitude toward the patients and this way lowering discrimination against the patients through improving the knowledge level among this critical group.

Innovations and breakthroughs

Until now no survey has investigated the association of knowledge and attitude of health care workers toward patients with hepatitis C in Iran, a country with high prevalence of the disease. In most of the previous surveys, the questionnaires were passively distributed among the participants. But the present study has the advantage of direct interviewing with the responders. Also the response rate in this study was much higher than some other similar investigations.

Applications

The present study showed that health care professionals' attitude and behavior toward hepatitis C patients is directly under the influence of their knowledge and education on hepatitis C. So by increasing education on this issue maybe we can do something on the care given to the patients with hepatitis C.

Terminology

Hepatitis C: A viral liver infection caused by hepatitis C virus which can be transmitted by blood. This kind of hepatitis will usually end to chronic liver disease and cause cirrhosis; Knowledge: Mental perception and clearly realizing the information and learning; Attitude: Mental backgrounds which are achieved by experiences and can influence person's reactions and behaviors toward others and have close association with the personality.

Peer review

This is a good study which gives important information which could be used in improvement of the treatment of the hepatitis C patients. The survey reports the knowledge and attitudes of health care worker (HCW) impacting on the care given to patients with HCV. Their findings reflect on the significant relationship between attitude level and knowledge score, and various demographic factors of HCW. The study is well reported and holds significant value (on a regional level) towards the care of HCV patients.

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BRIEF ARTICLE

Antibiotic resistance and *cagA* gene correlation: A looming crisis of *Helicobacter pylori*

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Abstract

AIM: To determine antibiotic resistance of *Helicobacter pylori* (*H. pylori*) in Pakistan and its correlation with host and pathogen associated factors.

METHODS: A total of 178 strains of *H. pylori* were isolated from gastric biopsies of dyspeptic patients. Susceptibility patterns against first and second-line antibiotics were determined and trends of resistance were analyzed in relation to the sampling period, gastric conditions and *cagA* gene carriage. The effect of *cagA* gene on the acquisition of resistance was investigated by mutant selection assay.

RESULTS: The observations showed that monoresistant strains were prevalent with rates of 89% for metronidazole, 36% for clarithromycin, 37% for amoxicillin, 18.5% for ofloxacin and 12% for tetracycline. Furthermore, clarithromycin resistance was on the rise from 2005 to 2008 (32% *vs* 38%, $P = 0.004$) and it is significantly observed in non ulcerative dyspeptic patients compared to gastritis, gastric ulcer and duodenal ulcer cases (53% *vs* 20%, 18% and 19%, $P = 0.000$). On the contrary, metronidazole and ofloxacin resistance were more common in gastritis and gastric ulcer cases. Distribution analysis and frequencies of resistant mutants *in vitro* correlated with the absence of *cagA* gene with metronidazole and ofloxacin resistance.

CONCLUSION: The study confirms the alarming levels of antibiotic resistance associated with the degree of gastric inflammation and *cagA* gene carriage in *H. pylori* strains.

Key words: *Helicobacter pylori*; Antibiotic resistance; *cagA*; Pakistan; Clarithromycin; Metronidazole; Fluoroquinolones

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is among the most widespread infectious agents because of its high colonization rate and persistent nature in its host's stomach. Mostly the colonization is silent but overt damage of gastric mucosa occurs in certain cases leading to the development of gastritis, duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC) and mucosa associated lymphoid tissue lymphoma. It is believed that CagA toxin is responsible for the underlying virulence mechanism because of its ability to translocate into gastric epithelial cells. In host cells, it binds with cellular SRC homology 2 domain-containing tyrosine phosphatase (SHP-2) protein and subsequently damage of gastric mucosa.

Triple therapy constituting the combination of a proton pump inhibitor or bismuth citrate and two antibiotics such as amoxicillin (AML), clarithromycin (CLR) or metronidazole (MTZ) is the internationally recommended first-line regime to eradicate *H. pylori* in symptomatic patients[1,2]. However, the combination might be delivered concomitantly, sequentially or in the form of a traditional straight course for 10-15 d to attain the highest cure rate[3]. In case of treatment failure, several other antibiotics such as fluoroquinolones and tetracycline (TE) are used as secondary options[3,4]. The increased rates of CLR and MTZ resistance have further compounded the already challenging treatment strategy within the harsh acidic environment of the stomach. Patient compliance has declined to less than 80% due to resistance against one of the antibiotics used in the first-line regime[5,6].

Pakistan is among the countries with high prevalence of *H. pylori* infection. The organism is not only associated with severe clinical outcomes[7] but is also carried by the healthy population. Empirical treatment has always been in practice without examining whether it matches *in vitro* antibiotics susceptibility testing (AST) or not. As a result, patient compliance has diminished up to 70%-75% in the last decade[8,9]. As most of studies from Pakistan are based on the outcome of therapy, information is scanty on the status of AST profiles of local isolates. Previous attempts, some of which are based on alternate AST methods, provide limited information[10,11], while no data is available on AST profiles for second-line options.

The paucity of information on the issue and the key role it plays in controlling *H. pylori* infection led us to conduct the present investigation that not only provides a detailed AST profile of local *H. pylori* isolates against first- and second-line regimes but also analyzes their distribution in various groups of patients. The study also contributes toward the better understanding of the role of *cagA* gene in the evolution of resistance.

MATERIALS AND METHODS

Patients and sampling

A total of 178 *H. pylori* strains isolated from gastroduodenal biopsies were included in this study. Biopsy samples were taken from symptomatic patients (n = 450) who underwent gastroduodenal endoscopy at Medical Unit Ⅱ, Civil Hospital, Dow University of Health Sciences, Karachi, from March 2005 to November 2008. They were grouped as non ulcerative dyspepsia (NUD), gastritis, GU and DU on the basis of endoscopic findings. Patients with previous treatment history for *H. pylori* infection and/ or GC were excluded. Samples were collected in 20% sterile glucose solution, transported in ice, and processed within two hours of collection. The study was conducted upon approval from the ethical review board of the University of Karachi, Pakistan.

Molecular diagnosis

Genomic DNAs were extracted from crushed tissue samples by SDS-PK method[12] (Khan, 2006 No. 354). Molecular diagnosis for the presence of *H. pylori* was conducted by polymerase chain reaction (PCR) targeting the *16SrRNA* gene as described previously[13]. Samples that were found positive with the *16SrRNA* gene of *H. pylori* were further examined for the presence of the *cagA* gene by PCR using primers designed for the entire 3' repeat conserved region. Amplification was performed at 35 cycles of 95 ℃ for 1 min, 52 ℃ for 1 min, and 72 ℃ for 1 min with a final extension of 7 min at 72 ℃[14]. A segment of human β-globulin gene was amplified as the internal control.

Isolation and identification of Helicobacter pylori strains

For the isolation of *H. pylori,* biopsy samples were crushed with the help of a sterile disposable tissue homogenizer and inoculated on Columbia Blood Agar (Oxoid, United Kingdom) containing 7% laked horse blood (Oxoid, United Kingdom) and *H. pylori* selective supplement Dent (Oxoid, United Kingdom). Plates were incubated at 37 ℃ for 5 d under microaerophilic conditions using a Campygen gas generating kit (Oxoid, United Kingdom). Suspicious small dew drop colonies were subjected for morphological and biochemical identification.

For further confirmation, genomic DNA was extracted and subjected to PCR analysis for the *16SrRNA* gene of *H. pylori* per the above-mentioned protocol.

Determination of minimum inhibitory concentrations of antibiotics

Susceptibility patterns of *H. pylori* isolates was determined against a battery of antibiotics including MTZ, AML, CLR, DA, TE, ofloxacin (OFX) and erythromycin (E). Various concentrations of antibiotics were added to Mueller-Hinton agar containing 5% old sheep blood (SB-MHA). Bacterial suspensions were prepared in sterile phosphate buffered saline (PBS) with density equivalent to 3 McFarland's turbidity standard. Ten microliters of each strain was spotted on the plates and incubated for 72 h under microaerophilic conditions. The lowest concentration of antibiotic able to inhibit visible bacterial growth was considered as minimum inhibitory concentrations (MIC). Results were interpreted according to standard criteria[15].

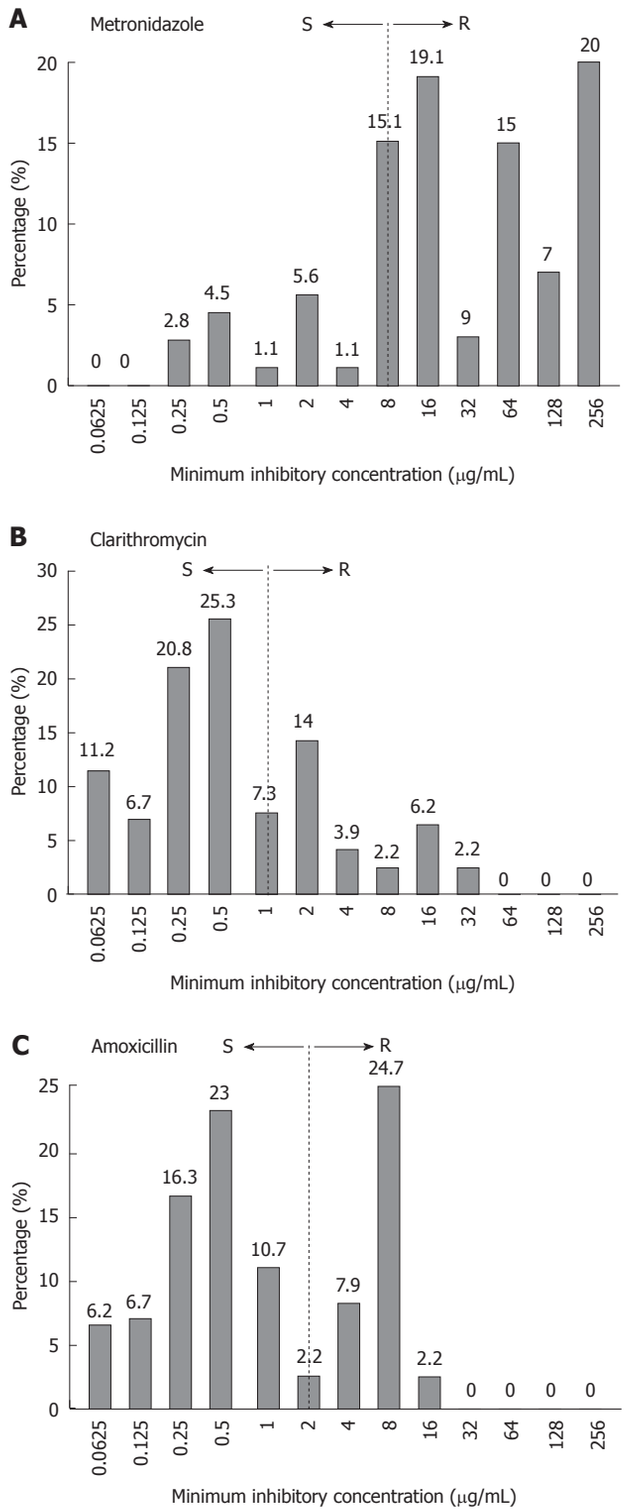

Figure 1 Prevalence of *Helicobacter pylori* resistance to first-line drugs. Dotted box represents the distribution of resistant strains at different minimum inhibitory concentrations (MICs). MIC breakpoints were ⩾ 8 μg/mL (A), ⩾ 1 μg/mL (B) and ⩾ 2 μg/mL (C). S: Sensitive; R: Resistant.

Mutant selection assay

To determine the role of *cagA* gene in the emergence of resistance, two *cagA*$^+$ and two *cagA*$^-$ strains were subjected to mutant selection assay. A total of 1.2 × 10^6-1.5 × 10^6 colony forming units (CFU) of bacterial strains suspended in PBS was spread over SB-MHA plates containing varying concentrations of antibiotics and incubated for 72 h at 37 ℃ under microaerophilic conditions. Frequency of resistant mutants was determined as the CFU of each strain grown on antibiotic supplemented plates divided by the starting inocula[16].

Statistical analysis

Statistical analyses were performed by PASW statistics 18 (SPSS Inc., Chicago, IL, United States). Pearson's χ^2 test was applied to compare categorical data. Linear regression was applied to correlate the frequency of resistance with *cagA* gene. A P value of < 0.05 was considered statistically significant.

RESULTS

Antibiotic susceptibility profile

Out of 450 dyspeptic patients, 201 (45%) were found positive for *H. pylori* by PCR and 178 (40%) by culture. AST profile of 178 *H. pylori* strains revealed high levels of resistance against the first-line regime. A total of 149 (84%) were found to be resistant (MIC ⩾ 8 μg/mL) to MTZ. As shown in Figure 1A, 34 (19%) strains had MIC 16 μg/mL, 16 (9%) had 32 μg/mL, 27 (15%) had 64 μg/mL, 12 (7%) had 128 μg/mL and 35 (20%) had MIC 256 μg/mL whereas 27 (15%) isolates were inhibited at boarder-line concentration 8 μg/mL. In case of CLR, 64 (36%) strains showed resistance with MICs ⩾ 1 μg/mL. The highest MIC 32 μg/mL was observed only in 4 (2.2%) isolates while 25 (14%) had MIC 2 μg/mL (Figure 1B). Sixty six (37%) strains showed resistance to AML with MICs ⩾ 2 μg/mL. Surprisingly, 44 (24.7%) strains exhibited MIC 8 μg/mL (Figure 1C).

Emergence of resistance against first-line therapy led us to investigate the susceptibility patterns of antibiotics usually given as second-line treatment options, such as TE, fluoroquinolones and clindamycin (DA). Data analysis shows an evenly distributed pattern of DA activity with MICs ranging from 0.25 to 32 μg/mL (Figure 2A). Resistance to TE was found in 21 (12%) strains. MIC 4 μg/mL was observed in 15 (8.4%) strains and 8 μg/mL in 7(3.9%) strains (Figure 2B). As shown in Figure 2C, a total of 33 (18.5%) strains were resistant to OFX. MIC of OFX was 2 μg/mL and 4 μg/mL for 21 (11.8%) and 12 (6.7%) strains respectively. We further analyzed the rate of multidrug resistance (MDR) in our studied population. A total of 46 (26%) of the isolates were resistant to two antibiotics i.e. MTZ and AML whereas 62 (35%) were resistant to MTZ and CLR. MDR isolates who were resistant to all first-line antibiotics (R-phenotype; MTZ^{r}CLR^{r}AML^r) were 20 (11%). Of these 7 (4%) were also resistant to tetracycline (R-phenotype; MTZ^{r}CLR^{r}AML^{r}TE^r) as shown in Figure 2D.

Correlation of resistance with demographic and disease factors

We next examined whether the proportion of strains showing antibiotic resistance were increased over time or not. For the purpose, the strains were divided into four groups; 2005 (n = 24), 2006 (n = 55), 2007 (n = 73) and

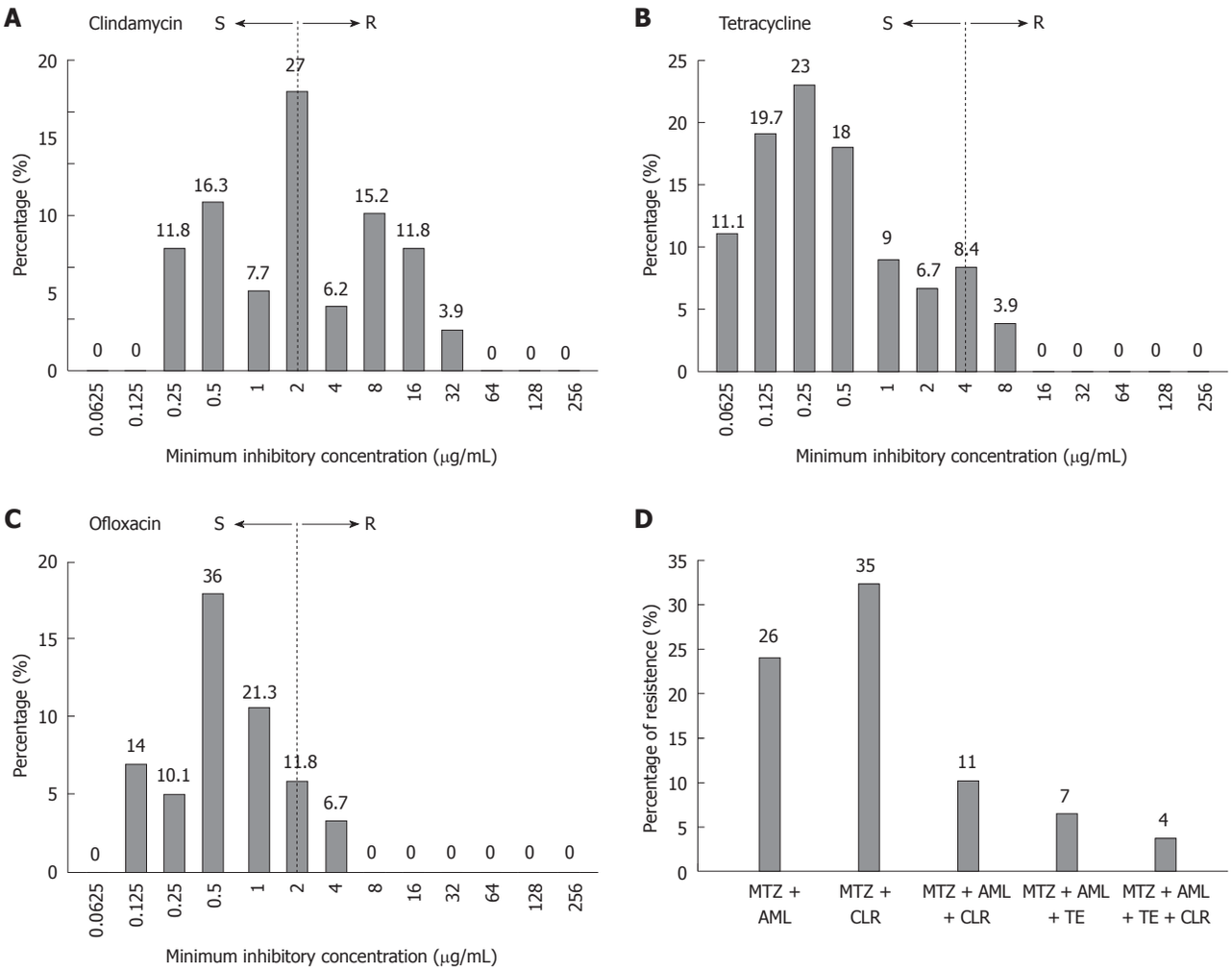

Figure 2 The prevalence of *Helicobacter pylori* resistance to second-line drugs. Dotted box represents the distribution of resistant strains at different minimum inhibitory concentrations (MICs). MIC breakpoints were ⩾ 1 μg/mL (A), ⩾ 4 μg/mL (B) and ⩾ 2 μg/mL (C); D: Distribution of multidrug resistant strains. AML: Amoxicillin; CLR: Clarithromycin; MTZ: Metronidazole; TE: Tetracycline. S: Sensitive; R: Resistant.

2008 (n =26) according to the year of sample collection. In general, a significantly progressive trend was only observed in CLR resistance from 32% (n = 8) in 2005 to 35% (n = 19) in 2006, 37.5% (n = 27) and 38% (n = 19) in 2008 (P = 0.004). On the contrary, MTZ resistant strains went down by year from 72% (n = 17) in 2005 to 69% (n = 38) in 2006, 68% (n = 50) in 2007 and 65% (n = 17) in 2008. However AML and OFX resistance remained steady with the rates of approximately 37% and 19% throughout the study period whereas overall resistance rates of TE were 10% (n = 2) in 2005, 13% (n = 7) in 2006, 12% (n = 9) in 2007 and 11% (n = 3) in 2008 (Figure 3A).

Comparative analysis was also performed according to the endoscopic findings of each patient from which *H. pylori* strains were isolated. Out of 178, a total of 25 strains were isolated from NUD cases, 89 gastritis cases, 26 GU cases and 38 from DU cases. Our findings indicate that AML and OFX resistance rates were more common in gastritis patients at 48% (n = 43) (P = 0.005) and 22% (n = 20) of cases (P = 0.08) respectively. In contrast, respective resistance rates of AML and OFX were 31% (n = 8) and 19% (n = 5) in GU and 16% (n = 6) and 21% (n = 8) in DU patients while no OFX resistant strain was found in NUD group. In case of MTZ, resistance rate was significantly higher among the patients with damaged mucosa such as gastritis (90%), GU (100%) and DU (92%) compared with NUD cases (70%) (P = 0.001). CLR resistance was observed in 53% (n = 13) of NUD, 20% (n = 18) gastritis, 18% (n = 4) GU and 19% (n = 5) of DU cases (P = 0.000) as shown in Figure 3B.

Correlation of drug resistance with cagA gene

The *cagA* genotypes of *H. pylori* usually correlate with the severity of disease; therefore, we determined the relationship of *cagA* gene with the susceptibility profile in local isolates. In this study 83 out of 178 (47%) *H. pylori* strains carried the *cagA* gene. The percentage of $cagA^+$ strains was 49% (n = 44) among patients with gastritis, 69% (n =18) GU and 60% (n = 23) DU whereas only 8% (n = 2) of the strains isolated from NUD cases carried this gene. Due to the low frequency of $CagA^+$ strains in NUD cases, further analysis was only based only on a total of 153 strains which were isolated from gastritis, GU and DU

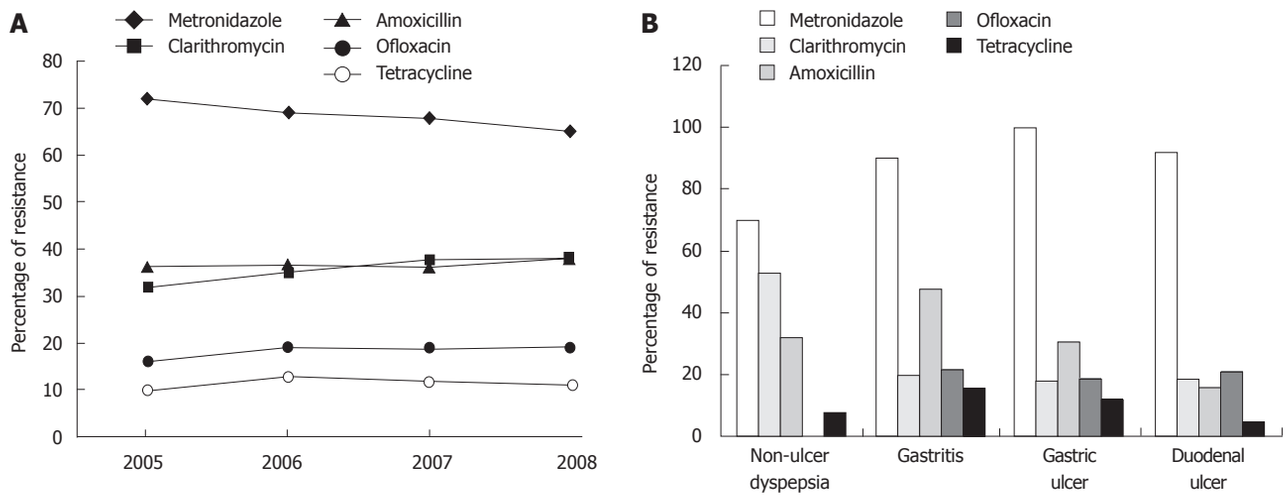

Figure 3 The trend analysis antimicrobial drug resistance in *Helicobacter pylori* strains (*n* = 178). A: From 2005 to 2008, significant increase in clarithromycin resistance was observed by linear regression ($P = 0.004$); B: Correlation of drug resistant strains in various groups of patients. Significant distribution of metronidazole ($P = 0.001$), clarithromycin ($P < 0.000$) and amoxicillin ($P = 0.005$) resistance was observed by Pearson's χ^2 test. NUD: Non ulcerative dyspepsia; GU: Gastric ulcer; DU: Duodenal ulcer.

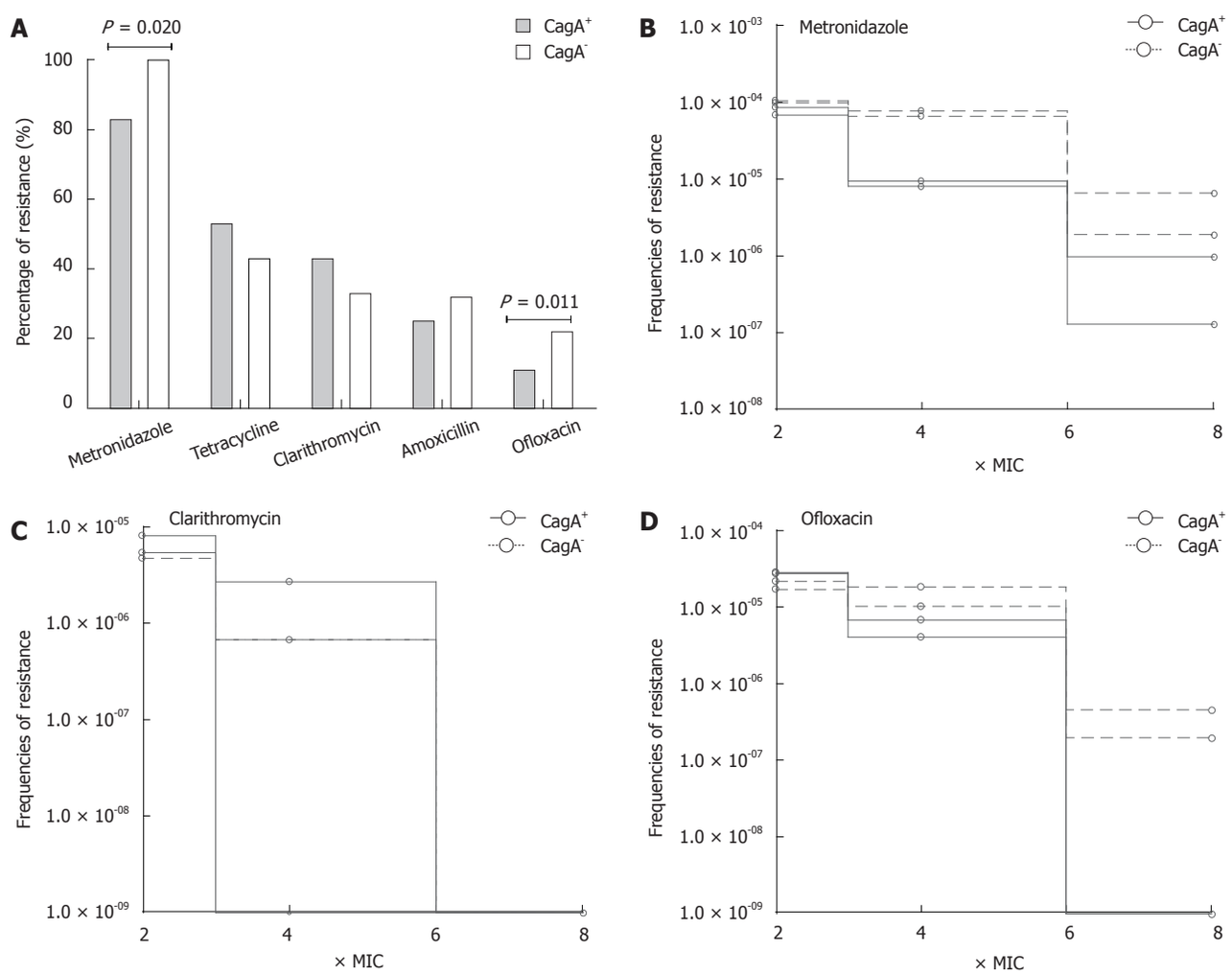

Figure 4 The correlation of antimicrobial drug resistance with *cagA* gene. A: Rate of drug resistance in *Helicobacter pylori* (*H. pylori*) strains carrying (*n* = 81) and devoid of (*n* = 72) of *cagA* gene. Statistical differences were observed by Pearson's χ^2 test. To determine the effect of cagA carriage on the development of resistance, cagA$^+$ (*n* = 2) and cagA$^-$ (*n* = 2) *H. pylori* strains were exposed to the increasing concentrations of metronidazole (B) clarithromycin (C) and ofloxacin (D). Bacterial growth was monitored at different concentration of antibiotic and frequencies of resistant mutants were determined as the colony forming units of *H. pylori* strain divided by the starting inocula.

cases. Of these, 81 (53%) were *cagA*$^+$ and 72 (47%) were *cagA*$^-$. Analysis of the drug resistance indicates the lower prevalence of OFX (11%, n = 9), MTZ (83%, n = 67) and AML (25%, n = 20) resistance in *cagA*$^+$ strains compared with 22% (n = 16), 100% (n = 72) and 32% (n = 23) in *cagA*$^-$ strains respectively (Figure 4A). In contrast, CLR resistance was more prevalent in *cagA*$^+$ strains (43%, n = 35) than in *cagA*$^-$ (33%, n = 24). The analysis indicates a possible link between *cagA* gene and the development of drug resistance.

To determine whether the rate of acquisition of antibiotic resistance varies between *cagA*$^+$ and *cagA*$^-$ strains, we exposed selected strains to increasing concentrations of MTZ, OFX and CLR. Bacterial growth was monitored at each concentration of antibiotics and frequency of resistant mutants was determined as the CFU of *H. pylori* strain divided by the starting inocula. We observed that *cagA*$^-$ strains were able to mutate more frequently under the selective pressure of MTZ since they were able maintain their frequencies even after the exposure of 6 × MIC of MTZ (r^2 0.9966, P = 0.0374). In contrast, more than 1 $\log_{10}$ decrease in bacterial growth was observed in *cagA*$^+$ strains with increasing concentrations of MTZ (Figure 4B). Similarly *cagA*$^-$ strains maintained their frequency in the increasing concentration of OFX (r^2 = 0.9966, P = 0.0374) whereas a sharp decline was observed in the development of resistant mutants of *cagA*$^+$ strains (Figure 4D). However, no significant difference was observed in the case of clarithromycin (Figure 4C).

DISCUSSION

H. pylori is often neglected for antimicrobial susceptibility testing because of its complex growth requirement and low recovery rate by bacterial culture. Increasing reports of treatment failure necessitate surveillance studies to analyze the trend of drug resistance especially in developing countries where MDR is quite common in other bacterial species. To determine the trend of antibiotic resistance in Pakistan, we conducted a 4-year longitudinal study comprised of 178 *H. pylori* strains. AST profile revealed high levels of resistance against the first-line regime including MTZ (84%), CLR (36%) and AML (37%). Our results and those from recently published papers from other countries show comparable prevalence rates; for example 33% resistance to AML was observed in the United States[17] whereas MTZ and CLR resistance rates were 31% and 33% in Ireland[18], 61% and 26% in France[19], 48% and 28% in Saudi Arabia[20], 80% and 45% in India[21], and 77% and 15% in Bangladesh respectively[22]. Moreover the trends of CLR and MTZ resistance were also in agreement with previously published reports[6,23]. In contrast with our observations, available data indicate a low occurrence of such strains in southeast Asian countries such as Malaysia and Taiwan where *H. pylori* is endemic[6,24]. However, the distinct genotypic nature of Southeast Asian strains provides a possible explanation for the differences in resistance profiles compared with rest of the world, including Pakistan.

The global analysis of clinical data clearly indicates that drug resistance to AML, MTZ and CLR has a central role in poor patient compliance to "gold standard" triple therapy to *H. pylori* infection, especially in the case of CLR if the point mutations in peptidyltransferase of 23S *rRNA* gene are responsible to phenotypic behavior[25]. Therefore, Maastricht Ⅲ consensus guidelines proposed not to provide CLR based empirical therapy if primary resistance rates are more than 15%-20% in the respective territory[4]. The present study clearly indicates the upward trend in the primary resistance to CLR in our population with an average of 36% in mono-resistance and 22% in MDR (*R-phenotype*; MTZ^{r}CLR^{r}AML^r) strains which provides an possible reason for the poor patient compliance (up to 70%-75%) with CLR based therapies in Pakistan as reported earlier.[8,9] Although MDR strains were equally present in our studied population when compared with the rates in other countries[26,27], they were less prevalent than mono-resistant strains.

Fluroquinolones such as ofloxacin or ciprofloxacin and tetracycline are usually considered as second-line therapy for *H. pylori* infection. In this study, the prevalence of TE resistance was comparable to that of other countries, however resistance to OFX was at a higher level than that seen other countries[18,28]. Mutations in the *gyrA* gene that are responsible for fluoroquinolone resistance have been directly linked with the failure of *H. pylori* eradication[29] therefore the higher rate of OFX resistance is alarming. These antibiotics are generally used to treat gastrointestinal infections in Pakistan; consequently, the resistance occurs in other Gram negative bacteria such as *Salmonella, Shigella* and *Escherichia coli*[30] and therefore the transmission of resistance in *H. pylori* can be anticipated. To combat the situation, broad-spectrum fluoroquinolones such as levofloxacin have been introduced; however, the development of resistance and intense side effects hamper its wide use despite its better compliance rate[31].

Genotypic differences of *H. pylori* directly influence the pathogenesis of infection. Such effects have been widely evidenced with the *cagA* gene carriage however the exact mechanism remains elusive. In this study, the differential prevalence of MTZ and OFX resistance in *cagA*$^+$ and *cagA*$^-$ strains clearly indicate the absence of *cagA* gene contributes in the acquisition of resistance which was further evidenced by the differential frequencies of resistant mutants developed with the increasing amount of each antibiotic as previously observed by Taneike *et al*[16] previously. The underlying phenomenon is usually explained by the ability of *cagA*$^+$ strains to cause intense inflammation which might increase the availability of antibiotics at the site of infection and eventually lead to better eradication of infection. In other words, it describes no direct role of *cagA* gene in antibiotic resistance. However, undermining the hypothesis, we observed that drug sensitive strains were more prevalent in NUD cases, despite of the absence of *cagA* gene, compared to those patients with damaged gastric mucosa. Taken together, the present study suggests that *cagA* gene and the degree

of tissue damage might be two independent factors that affect the drug susceptibility of *H. pylori*.

In summary, we observed that the magnitude of drug resistance in *H. pylori* strains is alarming in Pakistan. The degrees of gastric inflammation and bacterial genotypes are independently implicated in the development of resistance. The study reaffirms the need for both the continuous surveillance for drug resistance and the development of effective prevention and treatment strategies at national and regional levels.

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COMMENTS

Background

Eradication of *Helicobacter pylori* (*H. pylori*) is directly associated with symptomatic relief in the patients of gastroduodenal diseases. However, the failure of combination therapy containing two antibiotics and a proton pump inhibitor often results because of antibiotic resistance.

Research frontiers

Pattern of antibiotic resistance in *H. pylori* varies in different settings. However it is yet to determine that how host and pathogenic factors affect the prevalence of resistance.

Innovations and breakthroughs

This study indicates the alarming level of antibiotic resistance among Pakistani strains of *H. pylori* especially the magnitude of clarithromycin resistance is on rise and more commonly observed in non ulcerative dyspeptic patients. We further describe that *cagA* gene carriage and the degree of gastric inflammation are two independent factors affecting the metronizadole and ofloxacin resistance in *H. pylori*.

Applications

It is important to conduct continuous surveillance of antibiotic resistance in *H. pylori*. This study helps to comprehend antibiotic resistance pattern in *H. pylori* that facilitate to developing effective treatment strategy in different groups of patients.

Terminology

Non ulcerative dyspepsia (NUD) is defined as presence of upper gastrointestinal tract symptoms such as stomachache, indigestion and vomiting in patients who did not have damaged gastric mucosa.

Peer review

Overall, the study was well carried out and generally well written. However there are a few areas that needs further clarification mainly in the results section.

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BRIEF ARTICLE

CD74 and macrophage migration inhibitory factor as therapeutic targets in gastric cancer

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Abstract

AIM: To investigate the relationship and molecular features of CD74/macrophage migration inhibitory factor (MIF)/Toll-like receptor 4 (TLR4) in gastric cancer.

METHODS: CD74, MIF and TLR4 expression in the paraffin-embedded sections of gastric cancer from 120 patients were detected by immunohistochemical staining. Knock down of CD74 expression in gastric cancer cell line MKN-45 was performed by lentivirus transduction and detected by Western blotting. MKN-45 cell proliferation assay under the stimulants was measured by the cell counting kit 8 (CCK8) assay and MIF concentration in the culture medium was detected by enzyme-linked immunosorbent assay. Surface staining of CD74 in the MKN-45 cell line under the stimulation of lipopolysaccharide (LPS) was measured by flow cytometry. MIF, CD74 and TLR4 co-localization in the MKN-45 cell line was performed by the immunoprecipitation.

RESULTS: CD74, MIF and TLR4 were found to be expressed in gastric cancer and increased significantly in the advanced stage, and were also associated with lymph node metastasis. Correlation analysis revealed that CD74 was positively correlated with MIF (r = 0.2367, P < 0.01) and both proteins were also associated with TLR4 (r = 0.4414, r = 0.5001, respectively, P < 0.01). LPS can significantly promote MKN-45 cell proliferation (3.027 ± 0.388 *vs* 4.201 ± 0.092, P < 0.05), induce MIF production (54.333 ± 2.906 pg/mL *vs* 29.667 ± 3.180 pg/mL, P < 0.01) and cell surface expression of CD74 (75.6% ± 4.046% *vs* 9.4% ± 0.964%, P < 0.01) at LPS concentration of 1 μg/mL compared to medium control. Knockdown of CD74 or using anti-CD74 and MIF antagonist ISO-1 significantly reduced LPS-induced MKN-45 cell proliferation (4.201 ± 0.092 *vs* 3.337 ± 0.087, 4.534 ± 0.222 *vs* 3.368 ± 0.290, 4.058 ± 0.292 *vs* 2.934 ± 0.197, respectively, P < 0.01). MIF, CD74 and TLR4 could co-localize in the MKN-45 cell line.

CONCLUSION: Upregulation of MIF, CD74 and TLR4 are associated with increasing clinical stage and provide an opportunity as novel gastric cancer chemoprevention and/or treatment strategy.

Key words: Gastric cancer; CD74; Migration inhibitory factor; Toll-like receptors; Gastric epithelial cells

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INTRODUCTION

CD74 is a transmembrane glycoprotein that associates with MHC Ⅱ, and is an important chaperone that regulates antigen presentation for the immune response. CD74 is expressed at high levels by antigen-presenting cells (APCs), including B cells, monocytes, macrophages and dendritic cells in normal tissues[1,2]. Although cell surface expression of CD74 is low in many cell types, rapid internalization with concomitant re-expression at the cell surface provides a steady state level of CD74-MHC Ⅱ complex at the cell surface that is sufficient for biological function[3]. More recently, CD74 expression has been examined in cell types other than APCs, such as epithelial cells, and is particularly important in the complex immunological mechanisms and in the link between chronic inflammation and carcinogenesis in the gastrointestinal tract[4]. Substantial evidence has demonstrated that CD74 protein is upregulated in cancer cells, indicating its role in tumorigenesis and angiogenesis[5]. The contribution of CD74 to carcinogenesis is multifaceted. High levels of CD74 expression associated with class Ⅱ MHC expression might prevent tumor antigen presentation by blocking the peptide binding cleft and preventing antigenic peptide binding for presentation to T cells, rendering tumors less immunogenic[6]. In addition, CD74 is the receptor for macrophage migration inhibitory factor (MIF), which, when bound to CD74, initiates survival pathways and cell proliferation[7,8] and facilitates adhesion of *Helicobacter pylori* to gastric epithelial cells (GECs)[9,10].

MIF is an upstream activator of innate immunity that regulates subsequent adaptive responses. In addition to its roles in inflammation and immunity, recent studies have shown that MIF contributes to tumorigenesis. MIF is overexpressed in several tumors including breast cancer, gastric cancer, lung cancer, hepatocellular carcinoma, and cervical cancer[11-15]. MIF binding to CD74 might contribute to carcinogenesis in chronic conditions through the upregulation of proinflammatory cytokines, including interleukin (IL)-8, which upregulates CD74 and has its own mechanisms leading to increased proliferation, tumor growth, and angiogenesis[16]. MIF binding to CD74 affects proliferation and cell cycle events, including antagonism of p53, inhibition of retinoblastoma function, and activation of Akt[17]. This combination of properties suggests that MIF may play a pivotal role in tumor biology.

Pattern-recognition receptors such as Toll-like receptors (TLRs) act as sensors that detect microbial infections and induce a proinflammatory response[18]. TLRs are a family of mammalian homologs of the *Drosophila* Toll proteins and they recognize pathogen-associated molecular patterns that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. In mammalian systems, TLR4 confers responsiveness to Gram-negative lipopolysaccharide (LPS), induces cyclo-oxygenase (COX)2, and is important for proliferation and apoptosis in response to gastrointestinal injury[19].

In previous studies, it has been reported that CD74 and MIF are upregulated in gastric cancer[12,20]. However, how CD74 and MIF are elevated in gastric cancer remains unclear. The relationship between MIF/CD74/TLR4 expression by the tumor and clinicopathological factors in gastric carcinoma needs to be further demonstrated. In this study, we examined CD74, MIF and TLR4 expression in gastric cancer and analyzed their correlations with clinicopathological factors. Also, we used the gastric cancer epithelial cell line MKN-45 to confirm that, under LPS stimulation, MIF production and surface CD74 expression increased, thus promoting cell proliferation. These results suggest that the MIF/CD74 pathway may greatly induce gastric tumorigenesis in infection.

MATERIALS AND METHODS

Patients, specimens and immunohistochemistry

One hundred and twenty patients with gastric cancer, who underwent surgery at Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine, China, were included in this study. Prior to sample collection, appropriate permission was granted from the research ethical committee of Xinhua Hospital. The surgical specimens were fixed in formalin and embedded in paraffin before they were archived. For immunohistochemical staining, paraffin-embedded sections were deparaffinized in xylene and hydrated in 95%, 85%, 75% and 50% ethanol sequentially. Antigens were retrieved by heating for 15 min with 10 mmol citrate buffer (pH 6.0) in a microwave oven. The sections were incubated with 3% hydrogen peroxide to quench endogenous tissue peroxidase activity, and normal goat serum was used as the blocking agent (DakoCytomation, Glostrup, Denmark). The sections were then incubated with CD74 monoclonal antibody (mAb) (1/200 dilution; clone LN2; BD Pharmingen) or MIF Ab (1/100 dilution; clone 2A10-4D3; Sigma-Aldrich) or TLR4 antibody (1/100 dilution; clone 76B357.1; Abcam) at 4 ℃ overnight. Affinity-purified goat anti-mouse IgG conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as secondary antibody. The sections were developed using the liquid diaminobenzidine-substrate chromogen system (DakoCytomation). CD74, MIF and TLR4 expression was separately assessed by two observers who were blinded to the clinical data. CD74 expression was evaluated based on Ishigami's classification[12], by which, according to the percentage of positive cells, cases were divided into two groups: negative, CD74-positive cells < 10%, and positive, CD74-positive cells ⩾ 10%. MIF and TLR4 staining was evaluated as follows: -: undetectable;

+: weakly positive; ++: moderately positive; and +++: strongly positive.

Cell culture

The gastric epithelial cell line MKN-45 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) in 5% CO_2 at 37 ℃.

CD74 shRNA and cell sorting

Four different shRNA sequences for CD74 (NM_00 1025158.1) were purchased (GeneChem, Shanghai, China). These sequences were inserted into the pGCSIL-green fluorescent protein (GFP) plasmid (Takara Bio Inc, Otsu, Shiga, Japan) and transformed in *Escherichia coli* for propagation. Purified shRNA plasmids were then used to generate lentiviral particles by expressing them together with a gag-pol-env-encoding plasmid in a HEK293T packaging cell line. Centrifuged cell culture supernatants that contained lentivirus particles were used to infect MKN-45 cells. On day 5, GFP^+ cells were sorted by FACSAria (BD Biosciences, NJ, USA) to > 98% purity. Cells that had been infected with the lentiviral shRNA that gave rise to the strongest CD74 knockdown (target sequence: GCATGAAGCTTCCCAAGCCTC) were cultured and used for further experiments.

Flow cytometry

For surface staining of CD74 in the MKN-45 cell line, cells were harvested and washed with PBS supplemented with 2% FBS. Mouse anti-human FITC-conjugated CD74 and isotype control (BD Biosciences) were used and cultured at 4 ℃ for 30 min, after two washes and detected by flow cytometry (BD Biosciences).

Immunoprecipitation

Two nanograms of recombinant MIF (rMIF) (R&D Systems, Mineapolis, MN, USA) was added to MKN-45 cell lysates, which were rotated for 2 h at 4 ℃. Lysate mixtures were precleared with protein A/G beads (GE Healthcare, Pittsburgh, PA, USA) for 2 h at 4 ℃. MIF was immunoprecipitated using protein A/G beads that were preincubated with anti-MIF mAb (R&D Systems) for 2 h at room temperature. After washing, beads were incubated with the lysate mixture of MIF and cell lysates. Beads were then washed four times and the bound material was eluted for immunoblotting.

Immunoblot analysis

GFP^+ MKN-45 cell lysates or eluted antigens were subjected to 10% SDS-PAGE. Immunoblot analysis was performed by transfer of proteins onto nitrocellulose membranes (Schleicher and Schuell Microscience, Dassel, Germany) using a mini Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). After 2 h blocking, the membranes were incubated overnight at 4 ℃ with anti-human CD74 (clone EPR4064; Origene, Rockville, MD, USA), anti-human TLR4 (clone 76B357.1; Abcam) specific antibody, and β-actin antibody (Sigma-Aldrich, St Louis, MO, USA). After washing, subsequent incubation with appropriate horseradish-peroxidase-conjugated secondary Antibodies for 1 h at room temperature, and extensive washing, signals were visualized by ECL substrate (Pierce Chemical, Rockford, IL, USA).

LPS and MIF stimulation and proliferation assay

Approximately 10^4 cells/well were grown in 96-well microtiter plates and incubated overnight in 200 μL culture medium. Cells were starved without FCS overnight at 80%-90% confluence and then treated with recombinant human MIF (R&D Systems) and LPS (Sigma-Aldrich) at different concentrations, with or without 2 h pretreatment with ISO-1 [(S,R)-3-(4-hydroxyphenyl)-4,5- dihydro-5-isoxazole acetic acid methyl ester] at 100 nmol (Cal-Biochem, Darmstadt, Germany), or anti-CD74 5 μg/mL (C-16; Santa Cruz Biotechnology) and isotype control (BD Biosciences). Cells without any treatment were used as controls. After 24 h culture, OD was measured using the microplate computer software (Bio-Rad Laboratories) according to the protocol of the CCK8 assay kit (Dojindo, Kumamoto, Japan).

MIF enzyme-linked immunosorbent assay

MKN-45 cells were cultured in 96-well plates and stimulated with the LPS at different concentrations for 24 h. Supernatants from wells were used to quantitate the production of MIF by enzyme-linked immunosorbent assay. The MIF enzyme-linked immunosorbent assay kit was obtained from R&D Systems, and assays were performed according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± SD. Comparison of any two groups was performed by Student's *t* test, and one-way ANOVA was performed for multiple comparisons. CD74, MIF and TLR4 protein expression related to clinicopathological parameters was tested using the Mann-Whitney *U* test and Kruskal-Wallis ANOVA. The relationship between immunohistochemistry scores for CD74, MIF and TLR4 was explored using Spearman's correlation coefficient. Statistical significance was assumed if the *P* value was < 0.05. All analyses were performed using SPSS v14.0.

RESULTS

Overexpression of CD74, MIF and TLR4 in gastric cancer

We routinely collected tissue specimens from patients undergoing surgical operation of known gastric cancer and cut 4-μm-thick sections to stain for the presence of CD74, MIF and TLR4 in the adjacent sections. We stained a total of 120 specimens. CD74, MIF and TLR4 immunoreactivity was identified on the surface of the tumor cells. Some populations of tumor-infiltrating lymphocytes were

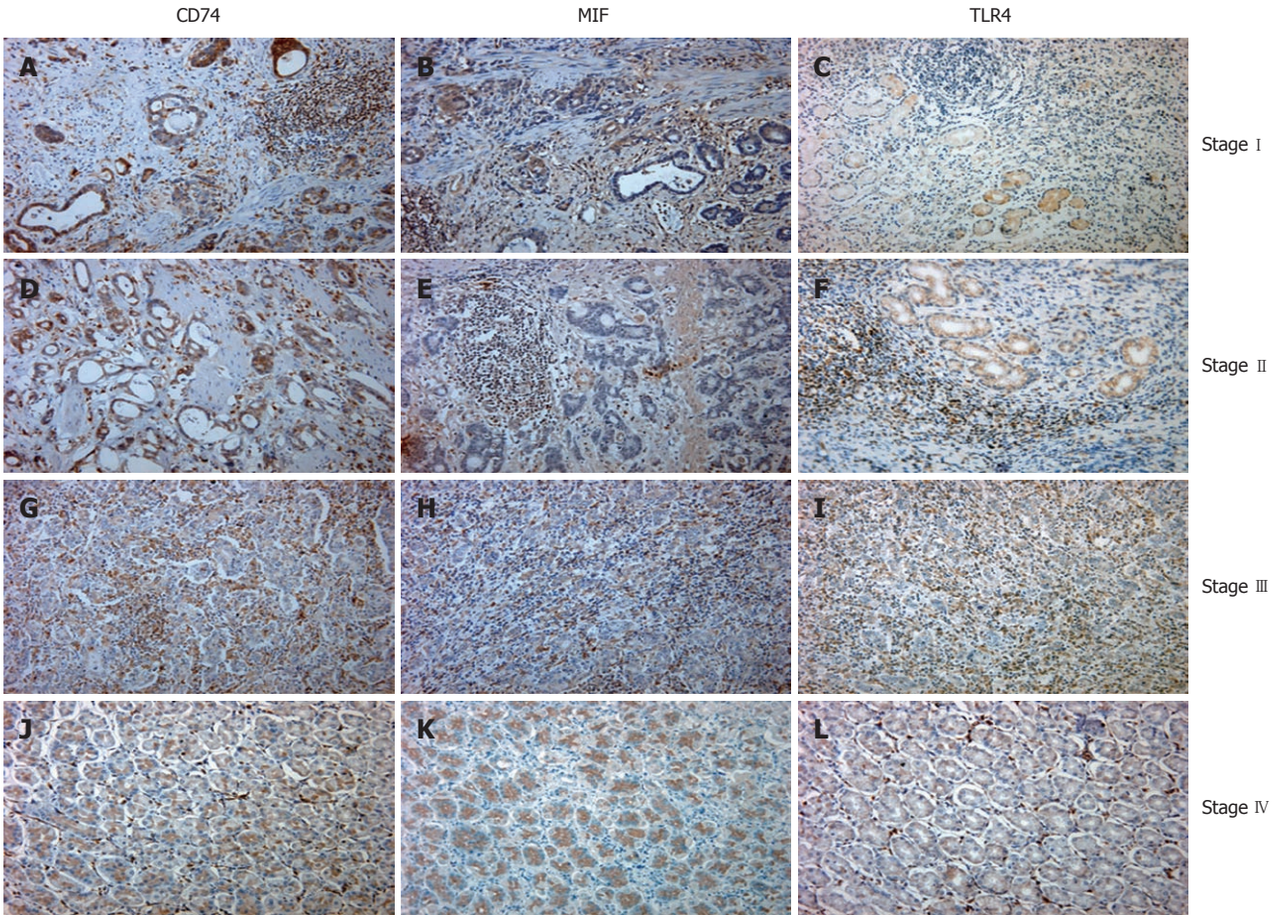

Figure 1 Representative sections show CD74, migration inhibitory factor and toll-like receptor 4 staining pattern in gastric cancer in each clinical stage. Gastric tumor sections stained for CD74 (A, D, G and J), MIF (B, E,H and K), toll-like receptor 4 (TLR4) (C, F, I and L) in each clinical stage, and CD74, Migration inhibitory factor (MIF) and TLR4 staining for the same stage are from the same patient, demonstrating that MIF and its receptor CD74 and TLR4 are expressed in close proximity in the tumor microenvironment. Original magnifications: 200 ×.

also immunopositive for those markers (Figure 1). Positive antigen expression of CD74 was observed in 100 of 120 specimens (81%), with the overwhelming majority of CD74-positive specimens with localization of the marker to the apical and perinuclear region of the cytoplasm (Figure 1A, D, G and J and Table 1). There was no difference in CD74 scores between adenocarcinoma from patients aged above or below 60 years (Table 1, $P = 0.5969$). Also, there was no difference between male and female patients (Table 1, $P = 0.9910$) and cell differentiation ($P = 0.3565$). However, a significant difference in CD74 scores between adenocarcinoma with different clinical stage was observed (Table 1, $P = 0.0141$) and lymph node metastasis ($P = 0.0158$).

Immunohistochemical staining also showed that MIF and TLR4 were primarily localized in the cytoplasm and occasionally on the membrane or nuclei of GECs (Figure 1). The positive staining of MIF and TLR4 was observed in 97 (81%) and 99 (83%) respectively of 120 gastric cancer, and the representative example of positive staining in each stage was shown in Figure 1. Like the CD74 staining, there was no difference in age, sex, and cell differentiation, but there were significant in clinical stage and lymph node metastasis (Table 1).

TLR4 and its correlation with CD74 or MIF in gastric cancer

The function of cell surface CD74 as a receptor for MIF provided the rationale for dual analysis of CD74 and MIF immunoreactivity in gastric cancer. A combined MIF and CD74 epithelial score might have a higher predictive value than either parameter alone. Table 2 shows the distribution of CD74 and MIF epithelial staining. There was a significant correlation between MIF and CD74 epithelial scores in individual adenocarcinomas ($r = 0.2367$, $P < 0.01$). TLR4 engagement by ligands such as bacterial LPS leads to proinflammatory cytokine production. Furthermore, from the correlation analysis, we observed that TLR4 had a significant correlation with CD74 ($r = 0.4414$, $P < 0.01$) and MIF ($r = 0.5501$, $P < 0.01$) (Table 2), which suggests that chronic inflammation might have an important association with gastric carcinogenesis.

LPS induces MIF production and surface CD74 expression in gastric cancer cell line

As with immunohistochemical staining, TLR4, CD74 and MIF were highly correlated with the tumor stage and lymph node metastasis, thus, we sought to determine MIF production or CD74 expression by GECs in response to LPS stimulation. Gastric epithelial cell line

Table 1 Correlation of migration inhibitory factor, CD74 and toll-like receptor 4 expression with clinicopathological variables in gastric cancer *n* (%)

Variables	No.	CD74 expression		*P*	MIF expression		*P*	TLR4 expression		*P*
	Cases	Positive	Negative		Positive	Negative		Positive	Negative	
Age (yr)				0.5959			0.8612			0.6421
< 60	46	40 (87)	6 (13)		38 (83)	8 (17)		38 (83)	8 (17)	
> 60	74	60 (81)	14 (19)		59 (80)	15 (20)		61 (82)	13 (18)	
Sex				0.9910			0.5817			0.6358
Male	75	61 (81)	14 (19)		60 (80)	15 (20)		60 (80)	15 (20)	
Female	45	39 (87)	6 (13)		37 (82)	8 (18)		39 (87)	6 (13)	
Histological type				0.3565			0.8440			0.2172
Well	20	16 (80)	4 (20)		16 (80)	4 (20)		17 (85)	3 (15)	
Moderate	40	30 (75)	10 (25)		33 (83)	7 (17)		30 (75)	10 (25)	
Poor	60	54 (90)	6 (10)		48 (80)	12 (20)		52 (87)	18 (13)	
TNM stage				0.0141			0.0281			0.0153
Ⅰ	26	16 (62)	10 (38)		17 (65)	9 (35)		17 (50)	9 (50)	
Ⅱ	28	20 (71)	8 (29)		22 (79)	6 (21)		20 (57)	8 (43)	
Ⅲ	33	29 (88)	4 (12)		28 (85)	5 (15)		30 (67)	3 (33)	
Ⅳ	33	31 (94)	2 (6)		30 (91)	3 (19)		32 (85)	1 (15)	
Lymph node metastasis				0.0158			0.0251			0.0152
Negative	50	37 (74)	13 (26)		36 (72)	14 (28)		33 (66)	17 (34)	
Positive	70	63 (90)	7 (10)		61 (87)	9 (13)		66 (94)	4 (6)	

MIF: Migration inhibitory factor; TLR4: Toll-like receptor 4.

Table 2 Correlation analysis of CD74, migration inhibitory factor and toll-like receptor 4 epithelial staining in 120 human gastric cancer patients

	MIF expression			*r*	*P*		TLR4 expression			*r*	*P*		TLR4 expression			*r*	*P*
CD74	(+)	(-)	Total	0.2367	< 0.01	CD74	(+)	(-)	Total	0.4414	< 0.01	MIF	(+)	(-)	Total	0.5501	< 0.01
(+)	85	15	100			(+)	90	10	100			(+)	89	8	97		
(-)	12	8	20			(-)	9	11	20			(-)	10	13	23		
Total	97	23	120			Total	99	21	120			Total	99	21	120		

MIF: Migration inhibitory factor; TLR4: Toll-like receptor 4.

MKN-45 was cultured in 96-well plates and stimulated with LPS (0.1, 0.5, 1 and 5 μg/mL) for 24 h. LPS significantly induced MIF production (54.333 ± 2.906 pg/mL *vs* 29.667 ± 3.180 pg/mL, $P < 0.01$) at a concentration of 1 μg/mL (Figure 2A), suggesting that under conditions of inflammation, such as Gram-negative infection, MIF can be induced. MKN-45 cell line expressed high amounts of TLR4, but LPS stimulation did not significantly induce TLR4 expression (Figure 2B). Although immunohistochemistry confirmed the presence of MIF receptor in gastric tumors, for extracellular MIF signaling to be mediated by CD74 *in vivo*, it must be present on the cell surface. Therefore, we analyzed the MKN-45 cell line with LPS stimulation, to determine whether surface expression of CD74 was present. We detected CD74 by flow cytometry and revealed a detectable but low level of surface CD74 expression. Stimulation with 1 μg/mL LPS for 24 h increased surface expression of CD74 from the basal level of 9.4% ± 0.964% to 75.6 ± 4.046% ($P < 0.01$) (Figure 2C), suggesting that surface CD74 expression by GECs is dependent on LPS stimulation. LPS stimulation can greatly induce MIF and surface CD74 expression and enhance the MIF/CD74 pathway.

LPS induces MIF and CD74 expression increases GEC proliferation

Various reports have shown that MIF or LPS increases proliferation of some cell types[21,22]. We investigated the ability of MIF or LPS to induce proliferation of GECs. rMIF or LPS were incubated with MKN-45 cells for 24 h. Proliferation was measured by nonradioactive cell proliferation colorimetric assay, as used in several recent studies[23]. Standard curves of known numbers of cells were run with each assay to extrapolate cell number from treated samples. As seen in Figure 3B and C, MKN-45 cell proliferation was significantly increased when stimulated with LPS (3.027 ± 0.388 *vs* 4.201 ± 0.092, $P < 0.01$) or MIF (3.160 ± 0.054 *vs* 4.856 ± 0.068, $P < 0.05$) at 1 μg/mL compared with medium control.

To investigate the role of CD74 in the observed proliferation, we used lentivirus shRNA that targeted CD74. Figure 3A shows that the transduction efficiency of MKN-45 cells between the control and CD74 shRNAs was equal, and after sorting, the GFP^+ cells reached 98%. Western blotting showed that CD74 expression was strongly knocked down (Figure 3B). When CD74 expression was knocked down, the proliferation of MKN-45 cells

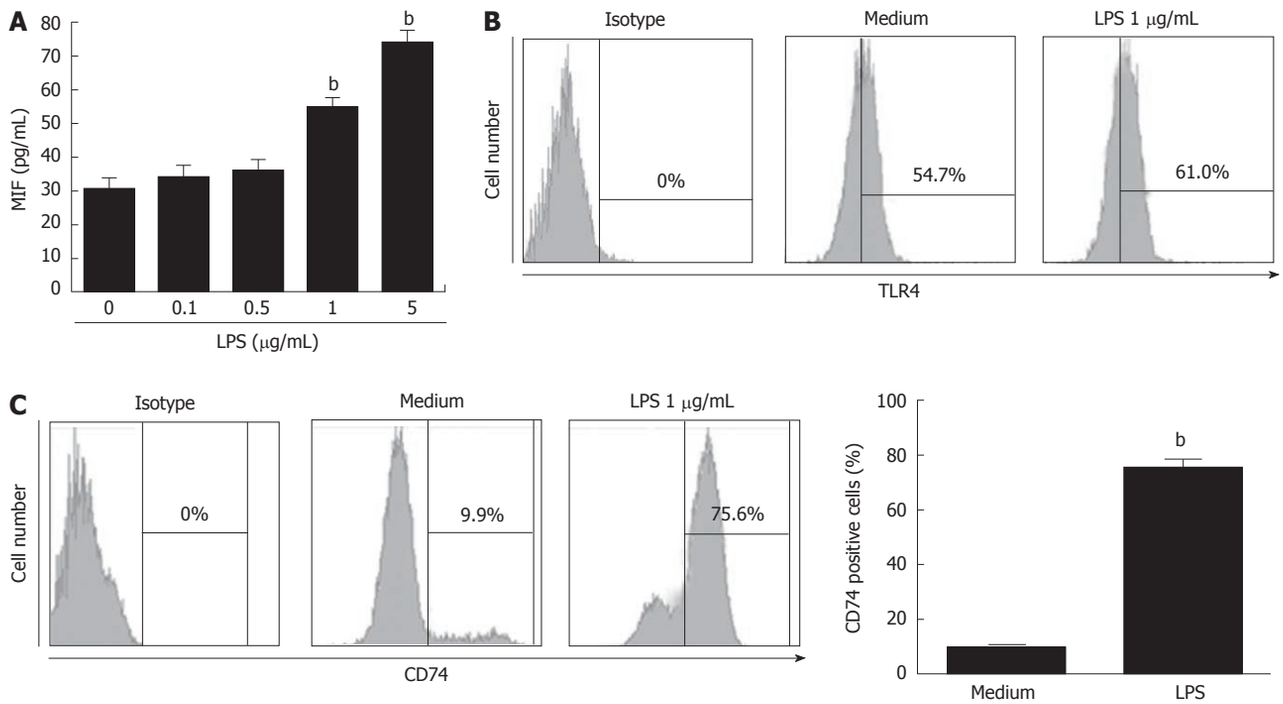

Figure 2 Lipopolysaccharide stimulation induced migration inhibitory factor and surface CD74 expression in gastric cancer cell line MKN-45. A: MKN-45 cell line was stimulated with lipopolysaccharide (LPS) at the indicated concentration respectively for 24 h, the supernatants were collected and migration inhibitory factor (MIF) concentration was measured by enzyme-linked immunosorbent assay; B and C: MKN-45 cell line was stimulated with or without LPS (1 μg/mL) for 24 h, toll-like receptor 4 (TLR4) (B) and CD74 surface expression was detected by flow cytometry (C, left panel), the mean values of CD74-positive cells were compared between the medium and condition group (right panel). $^{b}P < 0.01$ *vs* medium group.

after stimulation by LPS or MIF was greatly inhibited (4.201 ± 0.092 *vs* 3.337 ± 0.087, 4.856 ± 0.068 *vs* 3.160 ± 0.054, respectively, $P < 0.01$) (Figure 3C and D). The same effect was observed when anti-CD74 blocking antibodies were incubated with cells at 2 h before addition of LPS (4.534 ± 0.222 *vs* 3.368 ± 0.290, $P < 0.01$), (Figure 3E). Notably, after anti-CD74 treatment, proliferation levels were decreased to levels similar to those of untreated cells. To investigate further the role of MIF in LPS-induced GEC proliferation, using the MIF specific inhibitor ISO-1, MKN-45 cell proliferation was greatly inhibited (4.058 ± 0.292 *vs* 2.934 ± 0.197, $P < 0.01$) (Figure 3F). These data suggest that LPS stimulated GEC proliferation through the MIF/CD74 pathway.

TLR4 and MIF/CD74 co-localization

CD74 has been suggested to act as a receptor for MIF in several studies. We have shown that GECs express large amounts of CD74, which is upregulated under inflammatory conditions. Consequently, we examined the role of CD74 as a receptor for MIF on GEC by immunoprecipitation and western blotting. rMIF was incubated with MKN-45 cell lysates. MIF was immunoprecipitated by the MIF antibody along with GEC proteins bound to it. Western blotting using anti-CD74 mAb revealed that CD74 was co-precipitated with MIF, and TLR4 was co-precipitated with MIF (Figure 4). These results suggest that TLR4/CD74/MIF can form a complex to promote cell proliferation.

DISCUSSION

Recent data have expanded the concept that inflammation is a critical component of tumor progression. Many cancers arise from chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration[24,25]. TLRs are evolutionarily conserved transmembrane molecules that help the immune system to recognize pathogen-associated molecular patterns, and TLR4 sensitizes immune cells to bacterial LPS. When stimulated by LPS, many intracellular signaling pathways are activated, and lead to the generation of nuclear factor-κB, which in turn promotes proinflammatory cytokine production and release[26]. The unique biological activities of MIF have the potential to contribute to an *in vivo* microenvironment favoring tumor growth and invasiveness. These functional activities include: tumor suppressor downregulation, COX-2 and prostaglandin E2 upregulation, and potent induction of angiogenesis[27,28]. Recent evidence has suggested another important role for the CD74 molecule in the activation of cell survival pathways. CD74 is a cell receptor for the proinflammatory cytokine, MIF. Although CD74 itself is able to bind MIF, when bound to surface expressed CD44, the CD74-CD44 complex is able to initiate several survival pathways, including the extracellular signal-regulated kinase-1/2-mitogen-activated protein kinase

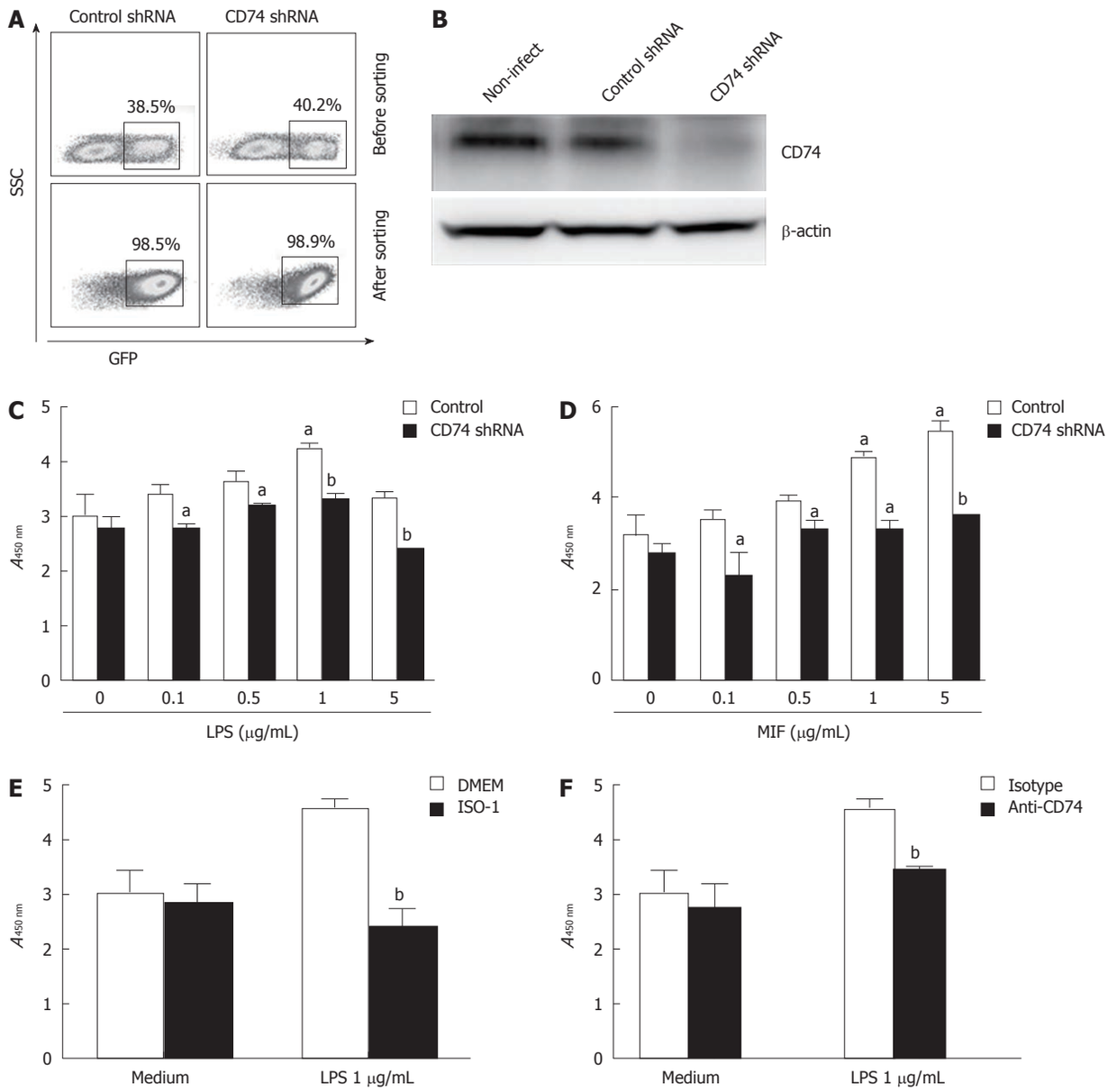

Figure 3 Lipopolysaccharide stimulation induced migration inhibitory factor and surface CD74 expression in gastric cancer cell line MKN45. A: MKN-45 cells were transduced with control or CD74-specific shRNA, and the percentage of GFP$^+$ cells is shown before or after flow cytometry sorting; B: CD74 expression was measured by western blotting when the MKN-45 cells were infected by control or CD74-specific shRNA; C and D: MKN-45 cells were knocked down for CD74 and stimulated with lipopolysaccharide (LPS) (C) or migration inhibitory factor (MIF) (D) for 24 h; cell proliferation was measured by CCK8; E and F: MKN-45 cells were stimulated with LPS at 1 μg/mL, and blocked with MIF antagonist ISO-1 (E) or CD74 antibody (F) for 24 h, and cell proliferation was measured by CCK8. GFP: Green fluorescent protein. $^aP < 0.05$, $^bP < 0.01$.

signaling cascade, and to stimulate cell proliferation by enhanced expression of cyclins and other regulatory factors[29].

It has been reported that CD74 surface expression is increased under inflammatory conditions and during *H. pylori* infection, and the bacterium can also use CD74 as a point of attachment to GECs[30]. The dramatic increase in CD74 expression during infection and the high turnover rate of CD74 suggests that both MIF and *H. pylori* can use CD74 as a receptor. Our data demonstrate that in gastric cancer, TLR4 expression is increased and has a strong association with disease stage and lymph node metastasis. GEC proliferation was significantly increased by LPS stimulation, suggesting that gastric cancer is strongly correlated with inflammation. Similarly, rMIF induced proliferation of GECs in a dose-dependent manner. Proliferation was decreased when CD74 was blocked by knockdown of CD74 gene or with antibodies or MIF was blocked by the antagonist ISO-1.

Immunohistochemical staining showed that CD74, MIF and TLR4 has a strong association with cancer stage, suggesting that CD74, MIF and TLR4 have a role in tumor progression. Ishigami *et al*[12] have reported that CD74 expression in gastric cancer is a useful prognostic marker and is correlated with surgical outcome. McClelland *et al*[13] have observed coexpression of CD74 in close proxim-

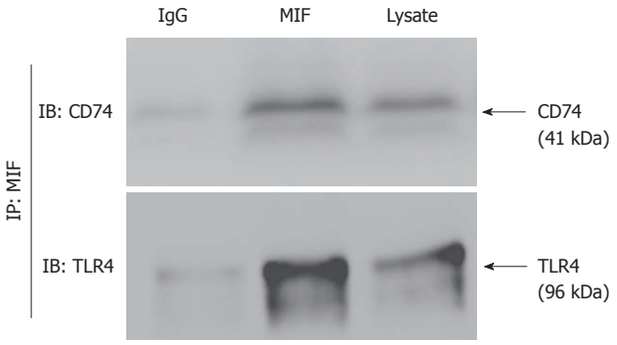

Figure 4 Migration inhibitory factor binds to CD74 and toll-like receptor 4 on gastric epithelial cells. r-Migration inhibitory factor (MIF) was mixed with MKN-45 cell lysates and immunoprecipitated with anti-MIF with bound cell proteins. Western blotting analysis with anti-CD74 and anti-toll-like receptor 4 (TLR4). MKN-45 lysates were run as a control in the right lane, and MKN-45 cell lysates immunoprecipitated with isotype control antibody were run in the left lane.

ity to the ligand MIF in non-small cell lung cancer, and have found that coexpression is associated with higher levels of CXC chemokines. In the current study, we also found positive correlation between MIF and CD74 and TLR4 in gastric cancer through correlation analysis. We further showed that CD74, MIF and TLR4 could form a complex, and under LPS stimulation, greatly induced cell proliferation. These findings suggest that TLR4, MIF and CD74 overexpression may be related to the pathogenesis of gastric cancer, and they could become promising therapeutic targets.

In summary, our study demonstrated the positive correlation of CD74/MIF/TLR4 in gastric cancer, suggesting that inflammation, as induced by LPS stimulation, can enhance the CD74/MIF pathway, promoting GEC proliferation and gastric carcinogenesis. Blocking of CD74 or MIF may provide a novel strategy for gastric cancer chemoprevention and/or treatment.

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COMMENTS

Background

CD74 is an important chaperone of MHC Ⅱ that regulates antigen presentation for the immune response. It is also expressed on epithelial cells, and is particularly important in the complex immunological mechanisms and in the link between chronic inflammation and carcinogenesis in the gastrointestinal tract. Macrophage migration inhibitory factor (MIF) binding to CD74 might contribute to carcinogenesis in chronic conditions, leading to increased proliferation, tumor growth, and angiogenesis. Toll-like receptor 4 (TLR4) confers responsiveness to Gram-negative lipopolysaccharide (LPS), and is important for proliferation and apoptosis in response to gastrointestinal injury.

Research frontiers

Recent data have expanded the concept that inflammation is a critical component of tumor progression. In a previous study, it has been reported that CD74 and MIF are upregulated in the gastric cancer. However, how CD74 and MIF are elevated in gastric cancer remains unclear. The relationship between MIF/CD74/TLR4 expression by the tumor and clinicopathological factors in gastric carcinoma needs to be further investigated.

Innovations and breakthroughs

In this study, CD74, MIF and TLR4 were found to be expressed in gastric cancer and increased significantly in the advanced stage; they were also associated with lymph node metastasis. Correlation analysis revealed that CD74 was positively correlated with MIF and both proteins were also associated with TLR4. LPS can significantly promote MKN-45 gastric cancer cell proliferation, and induce MIF production and cell surface expression of CD74. Knockdown of CD74 or using anti-CD74 and MIF antagonist ISO-1 significantly reduces LPS-induced MKN-45 cell proliferation. MIF, CD74 and TLR4 can co-localize in MKN-45 cells.

Applications

The study demonstrates the positive correlation of CD74/MIF/TLR4 in gastric cancer, suggesting that inflammation, as caused by LPS stimulation, can enhance the CD74/MIF pathway, promoting gastric epithelial cell proliferation and gastric carcinogenesis. Blocking of CD74 or MIF may provide a novel strategy for gastric cancer chemoprevention and/or treatment.

Terminology

CD74, also known as the invariant chain, participates in several key processes of the immune system, including antigen presentation, B-cell differentiation and inflammatory signaling. Recently, studies have revealed that CD74 is a receptor for macrophage MIF and is upregulated in inflammation, which has the potential to contribute to an *in vivo* microenvironment favoring tumor growth and invasiveness. As a participant in several immunological processes and an indicator of disease in some conditions, CD74 has potential as a therapeutic target.

Peer review

In this study, the authors demonstrated the positive correlation between CD74, MIF and TLR4 in gastric cancer and their association with clinicopathological factors. They revealed that LPS stimulation induced gastric cancer cell proliferation through enhanced MIF production and CD74 expression, and that knockdown of CD74 or using anti-CD74 antibody and MIF antagonist could reduce LPS-induced MKN-45 cell proliferation. This study certainly provides a novel mechanism of gastric carcinogenesis associated with the CD74/MIF pathway. The results suggest that CD74 and MIF could be novel therapeutic and chemopreventive targets in gastric cancer treatment.

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BRIEF ARTICLE

Preventive effects of geranylgeranylacetone on rat ethanol-induced gastritis

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Abstract

AIM: To establish a rat ethanol gastritis model, we evaluated the effects of ethanol on gastric mucosa and studied the preventive effects of geranylgeranylacetone on ethanol-induced chronic gastritis.

METHODS: One hundred male Sprague-Dawley rats were randomly divided into 4 equal groups: normal control group, undergoing gastric perfusion of normal saline (NS) by gastrogavage; model control group and 2 model therapy groups that underwent gastric perfusion with ethanol (distillate spirits with 56% ethanol content) by gastrogavage for 4 wk. Low or high doses of geranylgeranylacetone were added 1 h before ethanol perfusion in the 2 model therapy groups, while the same amount of NS, instead of geranylgeranylacetone was used in that model control group. The rats were then sacrificed and stomachs were removed. The injury level of the gastric mucosa was observed by light and electron microscopy, and the levels of prostaglandin 2 (PGE_2), endothelin-1 (ET-1) and nitric oxide (NO) were measured by radioimmunoassay and the Griess method.

RESULTS: The gastric mucosal epidermal damage score (EDS; 4.5) and ulcer index (UI; 12.0) of the model control group were significantly higher than that of the normal control group (0 and 0 respectively, all P = 0.000). The gastric mucosal EDS and UI of the 2 model therapy groups (EDS: 2.5 and 2.0; UI: 3.5 and 3.0) were significantly lower than that of the model control group (all $P < 0.01$). There was no statistically significant difference between the low-dose and high-dose model therapy groups. The expression value of plasma ET-1 of the model control group was higher than that of the normal control group ($P < 0.01$) and the 2 model therapy groups (all $P < 0.01$). The expression values of gastric mucosal PGE_2 and serum NO of the model control group were lower than those of the normal control group (all $P < 0.05$) and the 2 model therapy groups (all $P < 0.05$). The thickness of the gastric mucous layerand the hexosamine content in the model control group were significantly lower than that in the normal control group (all $P < 0.01$) and the 2 model therapy groups (all $P < 0.05$). Scanning and transmission electron microscopy observation showed that in the model control group, the epithelial junctions were vague, the intercellular joints disappeared and damage of the intracellular organelles were significantly worse than those in the normal control group. However, in the 2 model therapy groups, damage to the intercellular joints and organelles was ameliorate relative to the model control group.

CONCLUSION: Administration of geranylgeranylacetone was correlated with a more favorable pattern of gastric mucosa damage after ethanol perfusion. The mechanism could be related to regulation of ET-1, NO and PGE_2.

Key words: Geranylgeranylacetone; Gastritis; Ethanol; Endothelin-1; Nitric oxide; Prostaglandin 2

Peer reviewer: Dr. Pantelis Antonodimitrakis, Division of Endocrine Oncology, University Hospital of Uppsala, 76031 Uppsala, Sweden

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INTRODUCTION

The liver is a main organ for ethanol metabolism. The gastrointestinal tract is also involved in the ethanol metabolic process. Research[1] has reported that binge drinking or long-term drinking can cause acute or chronic gastric mucosal injury. Ethanol can be converted into acetaldehyde in first-pass metabolism in the gastrointestinal tract, which may have a carcinogenic affect on the gastrointestinal tract through local toxic effects. Geranylgeranylacetone is a derivative of terpenes. Studies have shown that geranylgeranylacetone has a therapeutic effect on chronic gastritis, digestive ulcers and portal hypertensive gastropathy[2-5]. There have been few studies on the repair mechanisms of gastric mucosal damage caused by ethanol. By using a rat ethanol gastritis model, we aimed to study the effect and mechanisms of geranylgeranylacetone on repairing gastric mucosa by observing the histological and ultrastructure changes and detecting the expression levels of plasma endothelin-1 (ET-1), serum nitric oxide (NO) and gastric mucosal prostaglandin 2 (PGE_2) in this research.

MATERIALS AND METHODS

Materials

Adult male Sprague-Dawley (SD) rats, 8 wk old, weighing 200 ± 50 g, were purchased from the Animal Center of Zhejiang University of Traditional Chinese Medicine. They were fed in a specific pathogen free environment with 12 h of light a day with unlimited drinking water. Geranylgeranylacetone was manufactured by Japan Eisai Co., Ltd., and packed by the Suzhou Eisai Pharmaceutical Co. A kind of liquor (Trade name: Red Star Erguotou, with 56% ethanol, manufactured by a general Beijing brewing factory) was used for gastrogavage to establish an ethanol-induced gastritis model in rats. The ET-1 radioimmunoassay determination kit was purchased from Beijing North Institute of Biotechnology Technology. The PGE_2 radioimmunoassay kit was purchased from the Beijing Huaying Biotechnology Research Institute.

Animal treatments

Groups: One hundred adult male SD rats were randomly divided into four groups, including the normal control group, the model control group, the low-dose model therapy group (50 mg/kg) and the high-dose model therapy group (200 mg/kg). The ethanol gastritis model was built by following an established procedure[6]. Red Star Erguotou liquor with 56% ethanol content was used for feeding the laboratory rats by gastrogavage. On every Tuesday and Friday, the rats were fed with ethanol after fasting for 12 h (food was removed on every Monday and Thursday at 9 pm, and gastrogavage was performed on the next day at 9 am). The dose of ethanol was 8 g/kg body weight. The conversion formula was: weight of ethanol (A) = liquor volume (mL) × ethanol content (vol/vol) × ethanol density (8 g/kg). The normal control group received the same amount of normal saline instead of ethanol. All laboratory rats were administered treatment for 4 wk. In the model therapy group, geranylgeranylacetone was dissolved in pure water and was administered by gastrogavage 1 h before ethanol feeding each time. In the normal control and model control groups, only normal saline, instead of geranylgeranylacetone was administered.

Specimen collection: Observations were made of the reactivity, activity and death of rats in each group during the experiment. The animals were killed by cervical dislocation after administration of an overdose of sodium pentobarbital on the 4th weekend of the experiment. The abdomen was opeded immediately. The whole stomach was cut and removed 1.5 cm away from the cardia and the pylorus. Dissection was done along the greater curvature for general specimen observation. The obviously damaged gastric mucosa specimen was rinsed in cold saline solution. Then, the specimen was placed in formaldehyde and glutaraldehyde, and stored in liquid nitrogen solution for later observation. All operations of all specimens were assigned to the same experienced professional laboratory personnel. All animal studies were approved by the Animal Care and Use Committee of Zhejiang University in accordance with the Chinese guidelines for the care and use of laboratory animals.

Determination of gastric mucosal injury index: The length and the width of the injured gastric mucosa region were measured with a vernier caliper. The gastric mucosa ulcer index (UI) was determined according to the Guth standard[7]: spot erosion was recorded as 1 point, erosion length < 1 mm was recorded as 2 points, 1-2 mm was recorded as 3 points, 2-3 mm was recorded as 4 points, and > 3 mm was recorded as 5 points, the score doubled if the erosion width was > 1 mm.

Determination of the thickness of the gastric mucous layer and the mucus glycoprotein content: The thickness of the gastric mucous layer in each group was measured by converted fluorescence microscopy with a thick smear method (using an ink staining method to enhance the contrast). The thickness of the gastric mucous gel layer was detected by measuring the thickness of the

centric bright area with a micrometer eyepiece. Detection of the levels of hexosamine (the main component of mucus glycoprotein) was performed by colorimetric assay using a spectrophotometer.

Histopathology: Four percent formalin-fixed gastric mucosa tissues were embedded by paraffin after gradient dehydration, 4 μm serial sections were obtained and HE staining was performed. We used the epithelial damage score (EDS) to rate morphological changes in the gastric mucosa under light microscopy: normal gastric mucosa was is recorded as 1 point, mucosal epithelial cell damage was recorded as 2 points, damage involving the glandular cells was recorded as 3 points, and mucosal erosion, bleeding or ulceration was recorded as 4 points. We observed a 1 cm length in each slice under light microscopy, and then calculated the cumulative score for each slice. Light microscopy was used to evaluate the degree of gastric damage, which was performed by two pathologists who were unaware of the treatment.

Observation of the ultrastructure of the gastric mucosa: We took 5 mm × 5 mm specimens close to the gastric antrum. According to the electron microscopy procedure, specimens were double fixed by 2.5% glutaraldehyde and 2% osmium tetroxide and subjected to conventional ethanol dehydration, and iso-amyl acetate transition. Critical point drying was carried out on a HCP-2 type critical point drying apparatus. The sample was stuck and a gilded target alloy was placed on the specimens using an IB-5 ion sputter coater. Specimens were observed on the sample stage of the electron microscope. We observed the cell morphology of the gastric mucosa, the junctions of the gastric mucosa epithelial cells, the shape of the gastric pit, and the ultrastructural changes of the intracellular mitochondria as well as the Golgi apparatus and other organelles in all of the laboratory rats by scanning electron microscopy and transmission electron microscopy.

Detection of the levels of serum nitric oxide, plasma endothelin-1, and gastric mucosal prostaglandin 2: We drawed 2 mL of blood from the abdominal aortic vein and injected it into the anticoagulant tube containing 30 μL 10% ethylenediaminetetraacetic acid disodium and 800 U aprotinin and mixed it well. We carried out centrifugal separation of the plasma at 4 ℃ at 3000 r/min for 30 min. Detection of plasma ET-1 levels and gastric mucosal PGE_2 content was carried out by a radioimmunoassay method with strict attention to the instructions. Detection of serum NO levels was carried out by a chemiluminescence method (according to the Griess method).

Statistical analysis

All values are expressed as mean ± SE. The Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

RESULTS

Observation of the general situation of rats

In the model control group, 3 of 25 rats died and most of the others were listless, had a bad appetite, and were unresponsive to external stimuli. In the normal control group and the model therapy groups, most of the rats had alert consciousness, normal appetites, agile responses to outside stimuli, and no deaths. The differences between the model control group and the other groups were significant.

The gastric mucosa ulcer index and the gastric epithelial damage score

In the normal control group, there were only 2 rats (2/25, 8%) that had punctate erosion on the gastric body mucosa. The mucosa of the others was not damaged and the UI was 0. In the model control group, the erosion and ulcers on the gastric mucosa were obvious and the median ulcer index was 12.0, which was significantly different ($P < 0.01$) from the normal control group. The gastric mucosal damage of the low-dose and high-dose model therapy groups was significantly reduced and the UIs were decreased (3.5 and 3.0, respectively). The results were statistically significant difference ($P < 0.01$) compared with the model control group. But there was no statistically significant difference between the low-dose and high-dose model therapy groups($P > 0.05$). In the model control group, the EDS values (median 4.5) were higher than that in the normal control group (median 0, $P < 0.01$). However, in the low-dose and high-dose therapy groups, the EDS values (median 2.5 and 2.0 respectively) were lower than the model control group (median 4.5, $P < 0.05$). The EDS values of the low-dose and the high-dose model therapy groups were not statistically significantly different ($P > 0.05$) (Figure 1 and Table 1).

Histological changes

In the control group, the gastric mucosa was smooth, the layers of the gastric mucosa had clear boundaries under high-power microscopy, and there was no significant inflammatory cell infiltration and edema. In the model control groups, the gastric mucosal surface was uneven with erosion, ulcers and bleeding, and under high-power microscopy the gastric mucosa were congested and had edema. With telangiectasia, the surface mucus layers were damaged and the submucosal gastric glands were incomplete. In the model therapy groups, the gastric mucosal injuries were obviously slighter than the model control group. In the model control group, there was only scattered mucosal damage and local congestion. Under high-power microscopy, the gastric surface mucus layer in the model therapy groups was basically intact and the submucosal layers were slight congested and had less inflammatory cell infiltration, especially in the high-dose model therapy groups (Figure 2).

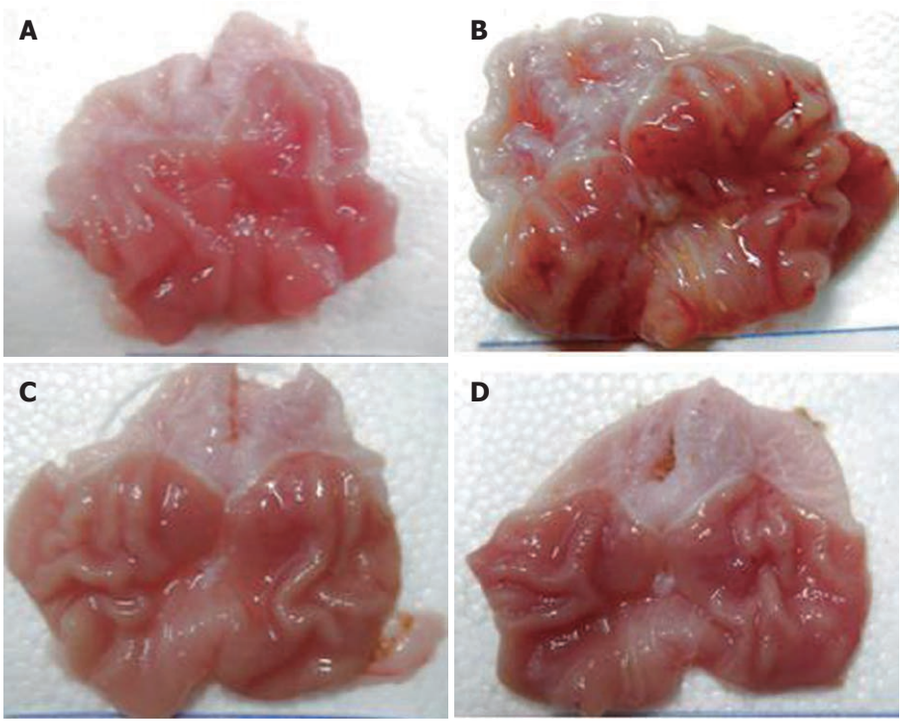

Figure 1 The gross anatomy of gastric mucosa in different groups. A: Gastric mucosa of normal control group was non-destructive; B Gastric mucosa of model control group showed erosion and ulcer; C: The gastric mucosa damage of low-dose model therapy group was relatively reduced according to model control group; D: The gastric mucosa damage of high-dose model therapy group was significantly reduced according to model control group.

Ultrastructural changes

Ultrastructural changes under scanning electron microscopy: In the normal control group, the gastric mucosa epithelial cells were closely joined and were ring-wise arranged around the gastric gland openings. The gastric pits were clear with ordered cells (according to the arrow). The model control group showed extensive gastric epithelial cell loss, disappearance of gastric pits, and revealed the glandular epithelium (according to the arrow). In the low-dose and high-dose model therapy groups, the gastric epithelial cells showed a basically complete structure and a small amount of ruptured epithelial cells (according to the arrow) (Figure 3).

Ultrastructural changes under transmission electron microscopy: In the normal control group, the gastric mucosal organelles had integrated structures with no degeneration, and the microvillis were arranged in neat rows with no loss. The model control group showed widened cell gaps, vague intercellular junctions, sparse and deciduous microvillis, and swollen mitochondria and endoplasmic reticulum. In the model therapy groups, the cells were arranged in neat rows and the intercellular junctions were clear. The structures of the mitochondria and endoplasmic reticulum were clear with mild swelling (Figure 4).

The thickness of the gastric mucous layer and hexosamine assay results

The thickness of the gastric mucous layer and the contents of the hexosamine in the model control group were significantly lower than the normal control group ($P < 0.01$). While the thickness of the gastric mucous layer and the contents of the hexosamine in the model therapy groups were lower than the normal control group, they obviously were higher than the model control group, and the differences was statistically significant ($P < 0.05$) (Table 2).

Detection of plasma endothelin-1, serum nitric oxide and gastric prostaglandin 2 levels

Plasma endothelin-1 levels: Compared with the normal control group, the levels of plasma ET-1 were significantly higher in the model control group ($P < 0.01$) and were significantly lower in the high-dose model therapy group ($P < 0.05$). While the levels of plasma ET-1 in the model therapy groups (including both the high-dose and low-dose groups) were significantly decreased compared to the model control group ($P < 0.01$) (Table 2).

Serum nitric oxide levels and gastric mucosal prostaglandin 2 levels: Compared with the normal control group, the levels of serum NO and gastric mucosal PGE_2 were significantly decreased in the model control group ($P < 0.05$). In both model therapy groups, the content was significantly higher than in the model control group ($P < 0.05$), and this was especially the case in the high-dose model therapy group (Table 2).

DISCUSSION

The integrity of the gastric mucosa depends on the protec-

Table 1 A comparison of the injury of gastric mucosa in different groups

Group	The gastric mucosa ulcer index		The epithelium damage scores	
	Median	Interquartile range	Median	Interquartile range
Normal control group	0.00	0.5	0.00	1
Model control group	12.00[d]	4.5	4.5[d]	2
Low-dose model therapy group	3.50[b,d]	1.5	2.5[a,d]	1
High-dose model therapy group	3.00[b,d]	1.5	2.0[a,d]	1

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the model control groups; [d]$P < 0.01$ *vs* normal control groups.

Table 2 Comparison of the thickness of gastric mucous layer, the content of hexosamine, the content of plasma endothelin-1, serum nitric oxide and gastric mucosal prostaglandin 2 in different groups

Group	The thickness of gastric mucous layer (μm)	The content of hexosamine (mg/g protein)	Plasma endothelin-1 (pg/mL)	Serum NO (μmol/L)	Gastric mucosal PGE_2 (pg/mg)
Normal control group	86.25 ± 3.21	65.57 ± 3.85	52.19 ± 2.82	30.20 ± 2.39	298.7 ± 9.28
Model control group	66.18 ± 5.11[b]	21.51 ± 4.54[b]	74.65 ± 8.84[b]	17.6 ± 3.37[a]	163.2 ± 8.84[a]
Low-dose geranylgeranylacetone	79.43 ± 6.67[a,c]	31.78 ± 5.78[a,c]	35.98 ± 4.78[a,d]	50.60 ± 10.68[c]	205.7 ± 10.39[c]
High-dose geranylgeranylacetone	81.34 ± 5.98[a,c]	37.78 ± 4.98[a,c]	26.87 ± 4.87[a,d]	69.10 ± 9.56[c]	265.5 ± 13.39[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* model control group. NO: Nitric oxide; PGE_2: Prostaglandin 2.

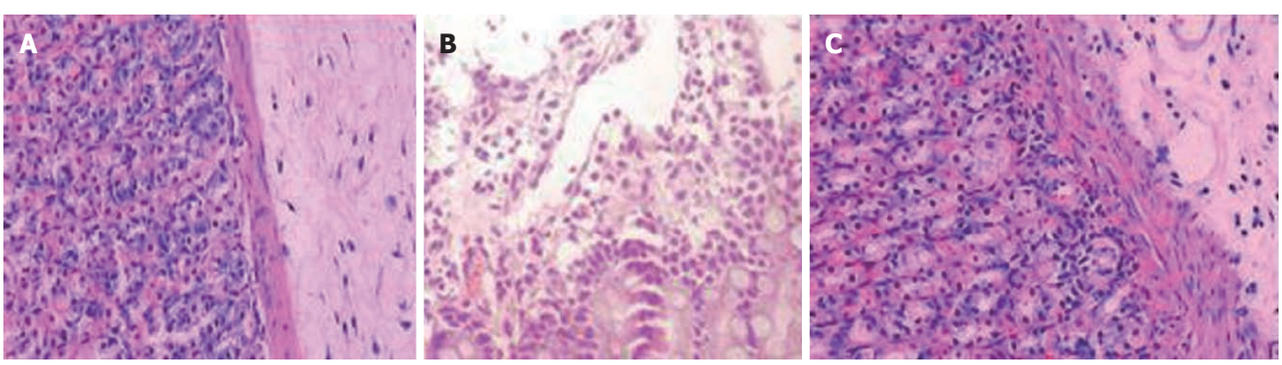

Figure 2 Histological changes of different groups. A: Normal control group: gastric mucosa was smooth, with clear boundaries, and no significant inflammatory cell infiltration and edema (HE staining × 200); B: Model control group: the gastric mucosa surface was uneven with erosion, ulcer bleeding, edema and telangiectasia, the submucosal gastric glands was incompleted (HE staining × 200); C: Geranylgeranylacetone treated group: the gastric surface mucus layer was basic intact, with scattered mucosal damage, local congestion and edema, fewer inflammatory cell infiltration (HE staining × 200).

tion of the gastric mucosal barrier (including, for example, the mucus-bicarbonate barrier and mucosal microcirculation) and can be damaged by a variety of factors (internal or external) with the production of a number of inflammatory mediators and cytokines, resulting in secondary mucosal damage[8-10]. Of the damaging factors, ethanol is an important external factor. With both hydrophobic alkyl and hydrophilic hydroxyl in its molecular stucture, ethanol can damage the gastric mucosal barrier defense system, diminish the capacity of the gastric mucosa to defend the invasion of gastric acid, bile and many digestive enzymes, causing mucosal edema, erosion, hemorrhage and necrosis. In this study, excessive ethanol intake can cause laboratory rats to become apathetic, lose appetites, have slow responses, and have increased mortality. In our study, damage of the gastric mucosa in the model control group, including the general view, microscopic structures and ultrastructure changes, was significantly serious compared to the normal control group.

The gastric mucous layer is the first defensive line of the gastric mucosa to against external stimuli. Studies[11-13] have shown that changes in thickness and content; i.e., mucous glycoprotein (hexosamine) can reflect the anti-invasive ability of the gastric mucous. The results of this study showed that excessive ethanol intake can significantly reduce the thickness of the gastric mucous layer, reduce the content of hexosamine in the mucus gel layer, and finally result in the decline of the anti-invasive ability of the gastric mucosa to ethanol and other external attacks.

Studies[14,15] in recent years have shown that the impaired microcirculation is one of the pathological reasons for gastric mucosal barrier damage, which is accompanied by elevated levels of ET-1, and declining levels NO and PGE_2 in blood and the gastric mucosa. NO and PGE_2 are recognized vasodilator factors *in vivo*, which can inhibit platelet aggregation and thrombosis, accelerate the flow of the gastric mucosal microcirculation, promote the

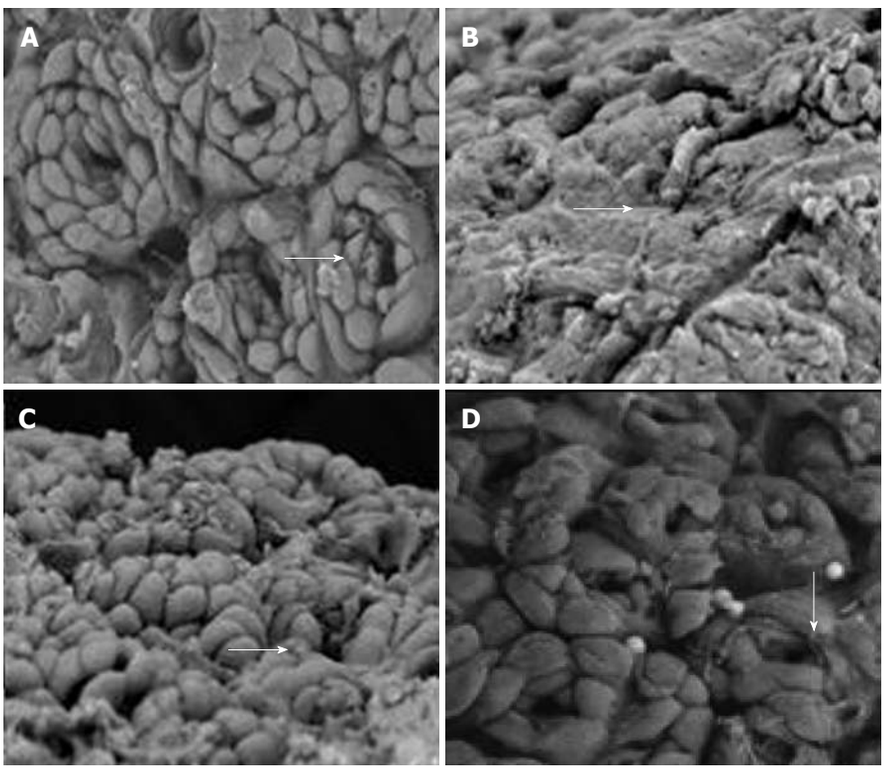

Figure 3 Ultrastructural changes under scanning electron microscopy. A: Normal control group: Epithelial cells were closely joined and ringwise arranged around the gastric gland openings (arrow), the gastric pits were clear with ordered cells (× 2500); B: Model control group: extensive gastric epithelial cell loss, disappearance of gastric pits (arrow), and revealed glandular epithelium (× 2500); C: Low-dose geranylgeranylacetone treated group: the gastric epithelial cells showed basically complete structure and fewer ruptured epithelial cells (arrow, × 2500); D: High-dose geranylgeranylacetone treated group: the gastric epithelial cells showed relatively perfect structure and fewer ruptured epithelial cells (arrow, × 2500).

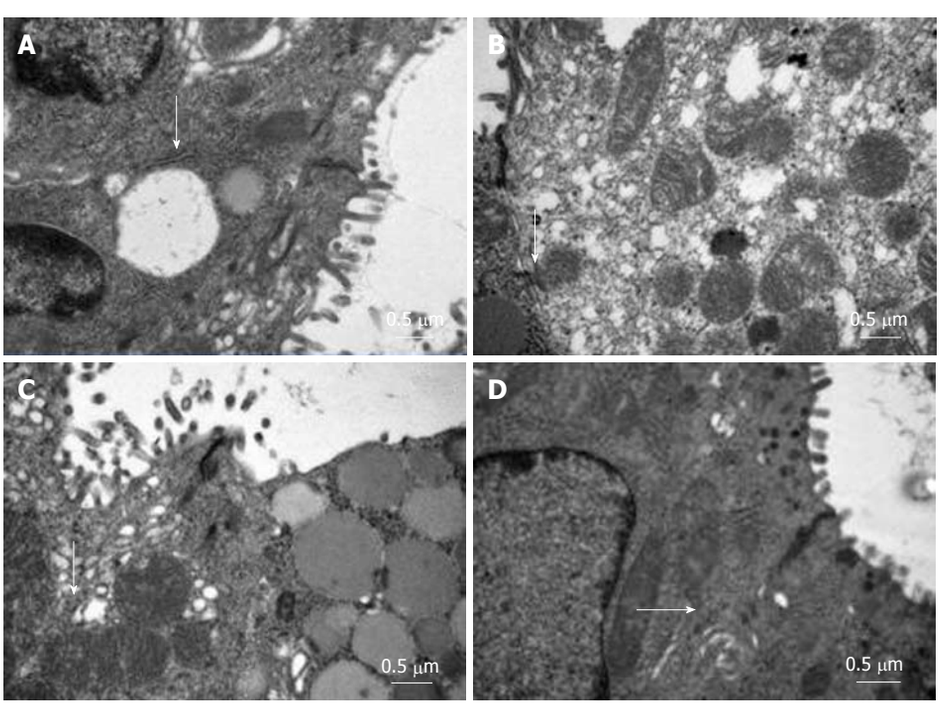

Figure 4 Ultrastructural changes under transmission electron microscope. A: Normal control group: the microvillis were arranged in neat rows and with no loss, organelles had integrated structure. intercellular junction were distinct (arrow, x 30 000); B: Model control group: widened cell gaps, vague intercellular junction(according to arrow), sparse and deciduous microvillis, and swelling mitochondria and endoplasmic reticulum (× 30 000); C: Low-dose geranylgeranylacetone treated group: the cells were arranged in neat rows and the intercellular junction were relative clear (arrow × 30 000), the structure of mitochondria and endoplasmic reticulum were mild swelling; D: High-dose geranylgeranylacetone treated group: the cells were arranged in neat rows and the intercellular junction were obvious clear (arrow × 30 000), the structure of mitochondria and endoplasmic reticulum were mild swelling.

secretion of bicarbonate, mediate the adaptive immune protective function, increase protein synthesis and cell renewal, and finally enhance the repair ability of the damaged gastric mucosa. ET-1 is the strongest vasoconstrictor *in vivo*. Lazaratos *et al*[16] reported that after injection of exogenous ET-1 in the rat gastric artery, the gastric mucosa was obviously damaged, while injection of an endothelin receptor antagonist in advance can significantly reduce the gastric mucosal damage. Under physiological conditions, these factors work together to regulate the gastric mucosal microcirculation and maintain homeostasis. However, imbalanced regulation caused by various factors will disorder the gastric mucosal microcirculation, affect the integrity of the gastric mucosa, and thus lead to a variety of gastrointestinal diseases. This study[16] showed that the content of gastric mucosal PGE_2 and serum NO were decreased in the model control group compared with the normal control group, and were negatively correlated with the gastric mucosal UI and EDS. The study also found that the levels of plasma ET-1 in excessive ethanol intake rats were higher than that in the normal control group, and were positively correlated with the UI and EDS. All these results suggested that the role of ethanol in damage to the gastric mucosa and weakening of its ability to repair may be caused by it stimulating the secretion of ET-1, and inhibiting the synthesis and secretion of endogenous NO and PGE_2.

Geranylgeranylacetone

Geranylgeranylacetone (a derivatives of terpenes with a molecular formula of $C_{23}H_{38}O$) has been a widely used gastrointestinal mucosa protective agent in recent years. It can stimulate the synthesis and secretion of macromolecule glycoprotein and phospholipids in the gastric mucus layer and maintain the normal structure and function of the gastric mucus layer. It therefore has a strong role in renovation of various experimental and clinical gastric mucosal lesions. Studies[17,18] have shown that geranylgeranylacetone can stimulate the regenerated gastric mucosal cells to secrete hexosamine and carry out the biosynthesis of gastric mucosal PGE_2. The mechanism may be related to the fact that geranylgeranylacetone changes the fluidity of membrane phospholipids, and increases the production of phospholipase A2, which is an important intermediate product for the PGE_2 and hexosamine. Hexosamine is an essential component of polymer glycoprotein in the gastric mucosa gel layer, and PGE_2 is a local hormone in the gastric mucosa. Studies have reported that PGE_2 is involved in the improvement of gastric mucosal microcirculation, and the continuous secretion of PGE_2 under the external stimulation helps to renovate gastric mucosa lesions[19-21]. Studies[22-24] have also shown that NO takes part in the process of geranylgeranylacetone inducing gastric mucus synthesis, in which NO synthase plays an important role, Meanwhile the synthesis of ET-1 is inhibited. Geranylgeranylacetone was used to pre-treat ethanol gastritis in the rat model in this study. We also showed that geranylgeranylacetone can elevate the serum NO and gastric mucosal PGE_2 content and decrease the plasma ET-1 content to varying degrees with different dosages. Meanwhile, the gastric anti-invasion ability of ethanol showed a corresponding increase. In conclusion, Administration of GGA was correlated with a more favourable pattern of gastric mucosa damage after alcohol perfusion. The mechanism could be related to regulation of ET-1, NO and PGE_2. The molecular pathways and mechanisms, however, need to be studied further.

COMMENTS

Background

Binge drinking or long-term drinking can cause acute or chronic gastric mucosal injury. Ethanol can be converted into acetaldehyde in the first-pass metabolism in the gastrointestinal tract, which may have a carcinogenic affect on the gastrointestinal tract through local toxic effects. Studies have shown that geranylgeranylacetone has a therapeutic effect on chronic gastritis, digestive ulcers and portal hypertensive gastropathy.

Research frontiers

By establishing rat ethanol gastritis model, the authors evaluated the effects of ethanol on the gastric mucosa and studied the preventive effects of geranylgeranylacetone on ethanol-induced chronic gastritis.

Innovations and breakthroughs

There have been few studies on repair mechanisms for gastric mucosal damage caused by ethanol. Moreover, the effect and mechanisms of geranylgeranylacetone on repairing ethanol-induced gastritis have seldom been evaluated. This study demonstrated the administration of geranylgeranylacetone was correlated with a more favorable pattern of gastric mucosa damage after ethanol perfusion. The mechanism could be related to regulation of prostaglandin 2 (PGE_2), endothelin-1 (ET-1) and nitric oxide (NO).

Applications

The study results suggest that geranylgeranylacetone can protect the rat gastric mucosa from ethanol-induced injury by changing the mobility of the cell membrane phospholipid bilayer, which further promotes the synthesis of endogenous PGE_2 and NO, and inhibits the secretion of ET-1.

Terminology

Geranylgeranylacetone is a derivative of terpenes, which has a therapeutic effect on chronic gastritis, digestive ulcers and portal hypertensive gastropathy. NO and PGE_2 are recognized vasodilator factors *in vivo*, which can inhibit platelet aggregation and thrombosis, accelerate the flow of the gastric mucosal microcirculation, promote the secretion of bicarbonate, mediate the adaptive immune protective function and increase protein synthesis and cell renewal. ET-1 is the strongest vasoconstrictor *in vivo*.

Peer review

It is an interesting study that confirms alcohol can damage the gastric mucosa. The study demonstrated the protective effect of geranylgeranylacetone on alcohol damage to the gastric mucosa and it elucidated the underlying mechanisms of this protective action.

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BRIEF ARTICLE

Role of bone marrow-derived mesenchymal stem cells in a rat model of severe acute pancreatitis

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Abstract

AIM: To investigate the role and potential mechanisms of bone marrow mesenchymal stem cells (MSCs) in severe acute peritonitis (SAP).

METHODS: Pancreatic acinar cells from Sprague Dawley rats were randomly divided into three groups: non-sodium deoxycholate (SDOC) group (non-SODC group), SDOC group, and a MSCs intervention group (i.e., a co-culture system of MSCs and pancreatic acinar cells + SDOC). The cell survival rate, the concentration of malonaldehyde (MDA), the density of superoxide dismutase (SOD), serum amylase (AMS) secretion rate and lactate dehydrogenase (LDH) leakage rate were detected at various time points. In a separate study, Sprague Dawley rats were randomly divided into either an SAP group or an SAP + MSCs group. Serum AMS, MDA and SOD, interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α levels, intestinal mucosa injury scores and proliferating cells of small intestinal mucosa were measured at various time points after injecting either MSCs or saline into rats. In both studies, the protective effect of MSCs was evaluated.

RESULTS: *In vitro*, The cell survival rate of pancreatic acinar cells and the density of SOD were significantly reduced, and the concentration of MDA, AMS secretion rate and LDH leakage rate were significantly increased in the SDOC group compared with the MSCs intervention group and the Non-SDOC group at each time point. *In vivo*, Serum AMS, IL-6, TNF-α and MAD level in the SAP + MSCs group were lower than the SAP group; however serum IL-10 level was higher than the SAP group. Serum SOD level was higher than the SAP group at each time point, whereas a significant between-group difference in SOD level was only noted after 24 h. Intestinal mucosa injury scores was significantly reduced and the proliferating cells of small intestinal mucosa became obvious after injecting MSCs.

CONCLUSION: MSCs can effectively relieve injury to pancreatic acinar cells and small intestinal epithelium, promote the proliferation of enteric epithelium and repair of the mucosa, attenuate systemic inflammation in rats with SAP.

Key words: Bone marrow mesenchymal stem cells; Severe acute pancreatitis; Intestinal barricade function; Pancreatic acinar cells

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INTRODUCTION

Acute pancreatitis (AP) is characterized by a rapid onset and disease progression, with high fatality. Severe acute pancreatitis (SAP) is extremely challenging to treat and the mortality rate is approximately 20%-40%[1]. Several studies currently suggest that the pathogenesis of AP involves complicated cascade reactions that start from the activation of pancreatin in pancreatic acinar cells. Pancreatin causes injury to the acinar cells and induces both local and systemic inflammation[2]. Inflammatory factors such as C-reactive protein, tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, nitric oxide (NO) and endothelin (among others) are thought to be involved in both the genesis and progression of AP and play a critical role in the progression from slight acute pancreatitis to severe acute pancreatitis[3]. Intestinal barricade function is significantly injured in SAP permitting bacteria to invade the enteric cavity and allowing endotoxin to enter the circulatory system thereby inducing a systemic inflammatory factor cascade reaction that aggravates the condition[4].

Mesenchymal stem cells (MSCs) are multipotent stem cells. One previous study demonstrated that MSCs had strong immunoregulatory effects and multidirectional differentiation potency[5]. Other recent studies found that MSCs also played a special role in inhibiting inflammatory reactions and promoting tissue repair[6]. For example, Hagiwara *et al*[7] found that rats with renal injury caused by ischemia-reperfusion induced a significant reduction in renal cell apoptosis after the injection of thymidine kinase-expressing MSCs (TK-MSCs). In addition, nitric oxide synthase (NOS) and NO levels were significantly reduced, which significantly inhibited: the infiltration of neutrophils and mononuclear macrophages; reduced the activity of peroxidase; delayed the production of peroxide, phosphorylation of p38 extracellular signal regulated kinase, the expression of TNF-α, and monocyte chemoattractant protein-1 cell adhesion. TK-MSCs also inhibited H_2O_2-induced cell apoptosis and increased Akt phosphorylation and cell activity in the periphery of the renal tubular cells. Tögel *et al*[8] administered MSCs to mice with acute renal failure for 24 h. The proinflammatory cytokines IL-1β, TNF-α, IFN-γ and NOS were all significantly reduced, whereas the anti-inflammatory factors IL-10, β fibroblast growth factor, TGF-α and B cell lymphoma-2 appeared highly expressed. In a pulmonary injured animal model, Iyer *et al*[9] found that MSCs attenuated a self-inflammatory reaction and enhanced the anti-inflammatory reaction by regulating the proliferation, differentiation, and delomorphous nature of immunocytes.

MSCs have also been demonstrated to have therapeutic effects in inflammatory diseases. For example, Imberti *et al*[10] injected MSCs in a cisplatin-induced acute renal injury model in mice and found that the MSCs enhanced mitosis. In addition, the production of insulin-like growth-factor-1 promoted the repair of renal tubules. In the treatment of chronic ischemic cardiomyopathy, MSCs were injected into ligate ramus descendens anterior arteril coronariae sinistrae. They were also injected into acute myocardial infarction regions. MSCs in both cases enhanced the contractile force of the cardiac muscle cells, regulated the contents and composition of collagen fibers in the tissue, and prevented the reconstruction of cardiac ventricles, thereby protecting the basic structure of cardiac muscle[11]. After intravenous injection of MSCs in experimental rats with spinal injury, MSCs assembled and survived in the host injury spinal cord and promoted the neural repair and recovery of nerve function[12]. In yet another study, rats with a radioactive intestinal injury were injected with labeled MSCs and the intestinal chorioepithelium regeneration occurred in the injured intestinal mucosa for 3 d and the radial related regions (e.g., kidney, spleen, stomach) also had MSCs[13]. Finally, after injecting MSCs into rats with an intestinal injury (ischemia/reperfusion), the permeability of the intestine was reduced and the injury to the intestinal villi was attenuated[14]. Together, these data indicate that MSCs can reduce the expression of various inflammatory factors and promote the repair of various tissues and organ injury.

Because the treatment of AP with stem cells has not been studied to date, and based on the ability of MSCs to inhibit inflammatory reactions and promote tissue repair, the purpose of this study was to explore the role, and the possible mechanisms, of MSCs in rats with SAP.

MATERIALS AND METHODS

Animals

Healthy Sprague Dawley rats weighing 200-300 g were provided by Shanghai SLK experimental animal Company [Batch No. SCXK (Shanghai) 2007-0005, China]. The study was approval by the Institutional Animal Care and Use Committee Fujian Medical University. The care and handling of all animals were in accordance with guidelines for animal ethics.

Drugs, reagents and instruments

The following reagents were used in the experiments: sodium deoxycholate (SDOC), 3-(4,5-dimethylthiazol-2-yl)-2-,5-diphenyltetrazolium bromide (MTT; Sigma), fetal bovine serum (Purpleflower holly leaf, Hangzhou), Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher, United States), ethylenediaminetetraaceticacid (EDTA)-trypsin (Amersco Co., United States), 4,6-diamidino-2-phenylindole (DAPI; Roche, Switzerland), malonaldehyde (MDA), superoxide dismutase (SOD), amylase (AMS) secretion rate and lactate dehydrogenase (LDH) kits (Nanjing Jianchen Scientific Co. Ltd), transwell double layer culture dish (Corning Costar, United States), IL-10 enzyme-linked immunosorbent assay (ELISA) kit for rats, IL-10 ELISA for rats, and TNF-α ELISA kit for rats (all ELISAs from Wuhan Youer Bio-scientific Co., Ltd), and antibody against proliferating cell nuclear antigen (Shanghai Zhuokang Bio-scientific Co., Ltd).

Culture, identification, and labeling of mesenchymal stem cells

Male rats weighting about 200 g were humanely sacrificed by cervical dislocation. The bone marrow was aseptically collected and subsequently cultured using whole marrow differential adherence methods. MSCs were obtained by multiple digestions and passages. The cellular identification of the expression of MSCs surface markers (i.e., CD29, CD34, CD45 and CD90) were detected using flow cytometry. The cells were labeled with DAPI and observed under fluorescence microscope. Third generation MSCs was acquired for subsequent experimentation.

Cell experiments

Pancreatic acinar cells from the rats were separated using the collagenase method[15]. The cells were seeded in Hanks buffer solution containing 10% fetal calf serum at a density of 1×10^6 cells/mL. The purity of the pancreatic acinar cells was > 80% and the survival rate was > 90%). Cells were seeded in 35 mm × 35 mm culture dishes and incubated at 37 ℃ and 55% CO_2 for 2 h. Cell morphology was examined using phase contrast microscopy. The cultured acinar cells were seeded in the underlayer of transwell double-deck culture dishes.

Pancreatic acinar cells were randomly divided into three groups: non-sodium deoxycholate group (non-SODC group), SDOC group, and a MSCs intervention group. In the SDOC group, the pancreatic acinar cells were seeded in the bottom of the transwell double-layer culture dishes and had a final concentration of 50 μmol/L SDOC. In the Non-SDOC group, the pancreatic acinar cells were seeded in the bottom of the transwell double-layer culture dishes and were not cultured with SDOC. In MSCs intervention group, the insert of the transwell plates was inserted into the poles and the third generation MSCs were seeded at a density of 1×10^6 cell/mL. The culture medium in the insert and the six-pole plate were fused, thereby establishing the co-culture system of MSCs and acinar cells. The co-culture medium was LG-DMEM with SDOC at a final concentration of 50 μmol/L. Subsequently, the cells in each group were incubated for 0.5 h, 1 h, 4 h and 10 h. Alterations in cell morphology were examined and cell survival was quantitatively detected by the MTT assay. The cell survival rate was expressed by the percentage in each group using the following equation: 100% × absorbance at 490 in each group/absorbance at 490 in the fresh separated pancreatic acinar culture medium.

The supernatants were also collected and the concentration of MDA was determined using the thiobarbituric acid method. The density of culture serum SOD was also determined using the xanthine oxidase method. The AMS secretion rate and LDH leakage rate of acinar cells were measured by enzyme kinetics methods. The AMS secretion rate was cell supernatant AMS/cell total AMS × 100% and the LDH leakage rate was cell supernatant LDH/cell total LDH × 100%.

Animal experiment

Thirty-six male rats were randomly divided into either the SAP group or the SAP + MSCs group. The SAP model was established by injecting deoxy-STC under the pancreatic capsule[16]. Specifically, following a peritoneal injection of 2.5% thiopental sodium, the pancreas of each rat was sufficiently exposed after entering into the abdomen *via* a median abdominal incision. Next, 1 mL of 3.8% STC was slowly injected into the inferior aspect of capsule using a No. 4 needle from the tail of pancreas, which made the entire pancreas swell. The pancreas was replaced 2 min later and the abdominal cavity was sutured closed routinely. In the SAP + MSCs group, 2 mL of the MSCs cell suspension (containing approximately 1×10^6 cells/mL determined *via* DAPI fluorescence immunity labeling) were injected into the caudal vein. In the SAP group, 2 mL of normal saline was injected. Six mice were randomly collected from both groups 6 h, 24 h and 72 h postinjection. Blood was collected from the apex of the heart and 5 cm of the small intestine (the section from the terminal ileum and extending distally) was obtained. Serum AMS was detected and the concentrations of serum IL-6, IL-10 and TNF-α were determined using ELISAs. Serum MDA concentration was determined using the thiobarbituric acid method, and the concentration of serum SOD was measured *via* the xanthine oxidase method.

The small intestinal tissue was flash frozen, and the number of DAPI positive cells was measured under fluorescence microscopy. Conventional hematoxylin and eosin staining was performed on sections of small intestine and injury to the intestinal mucosa was assessed in six different, randomly selected, high-power fields (original magnification × 400). According to the injury scoring criteria of Chiu's intestinal tissue[17], injury to intestinal mucosa, infiltration of inflammatory cells, and degree of hemorrhage and hyperemia were scored. The proliferating cell nuclear antigen Ki-67 immunohistochemistry staining was performed to note any proliferation of intestinal mucosa cells. Again, six different high-power fields (× 400) were randomly selected and the number positive cells were counted.

Statistical analysis

All data were expressed by mean ± SD. The mono-factor variance analysis was applied for comparisons between groups. A $P < 0.05$ was considered statistically significant, and all analyses were performed using SPSS 13.0.

RESULTS

General morphology of mesenchymal stem cells and the expression of surface markers

Third generation of MSCs were examined under an inverted microscope. The cells assumed a fusiform and swirling colony (Figure 1). As shown in Figure 2, the positive rate of CD29 was 98.6% and the positive rate of CD90 was 99.6%. In contrast, CD34 and CD45 were

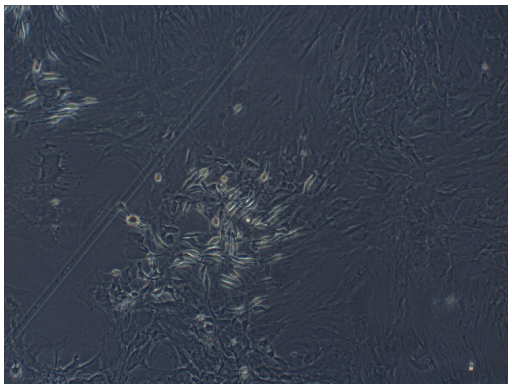

Figure 1 Third generation mesenchymal stem cells were spindle-shaped and formed spiral-like colonies (original magnification × 100).

negative (0.56% and 0.89%, respectively), demonstrating that the purity of the MSCs was > 95%.

Morphology of pancreatic acinar cells

After the pancreatic acinar cells were cultured for 2 h, no adherence was noted under the inverted microscope. Instead, the cells assumed a cluster formation and assembled with lumping. The boundary of the cells was clear and the refraction was strong. High density particles containing proenzymes could be seen in the cells (Figure 3).

The cell survival rate of fresh separated pancreatic acinar cell was comparatively high and that in each group was reduced. This reduction was most evident in the SDOC group. The cell survival rate at each time point in the MSCs intervention group was significantly increased compared with the SDOC group (Table 1).

Amylase secretion and lactate dehydrogenase leakage rates

The AMS secretion rate and LDH leakage rate of pancreatic acinar cell in the SDOC group at each time point was significantly higher than the other two groups. The AMS secretion rate and LDH leakage rate in the MSCs intervention group at each time point was significantly reduced compared with the SDOC group (Table 2).

Oxidative stress

MDA and SOD were measured in the supernatants collected from each group (Table 3). In SDOC group, SOD activity significantly reduced and this difference was significant compared with the non-SDOC group ($P < 0.05$). With the extension of SDOC reaction time, the SOD activity in the cell culture supernatants was further reduced, which was also significantly lower than the non-SDOC group at the same time points ($P < 0.05$). However, MDA content in cell culture supernatants was significantly higher in the SDOC group than the non- SDOC group at the corresponding time points ($P < 0.05$). The SOD activity in the MSCs intervention group at each time point was significantly increased compared with the SDOC group, whereas MDA content was significantly lower than the SDOC group ($P < 0.05$).

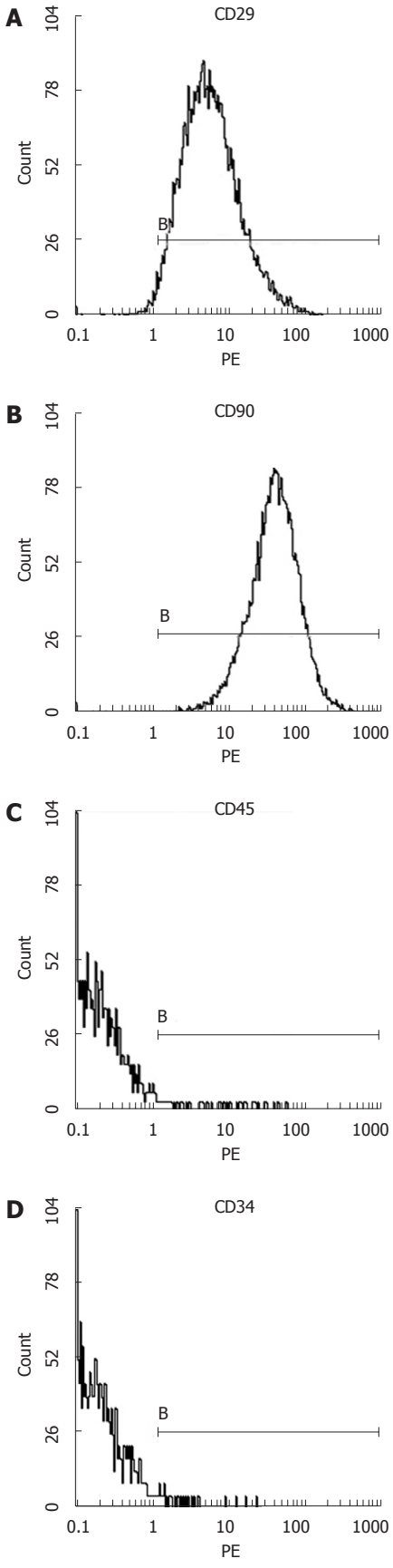

Figure 2 The expression of mesenchymal stem cells surface markers detected by flow cytometry. The proportion of CD29+ (A) cells was 98.6%, the proportion of CD90+ (B) cells was 99.6%, the proportion of CD45+ (C) cells was 0.89% and CD34+ (D) cells was 0.56%. B: The boundary of the cells.

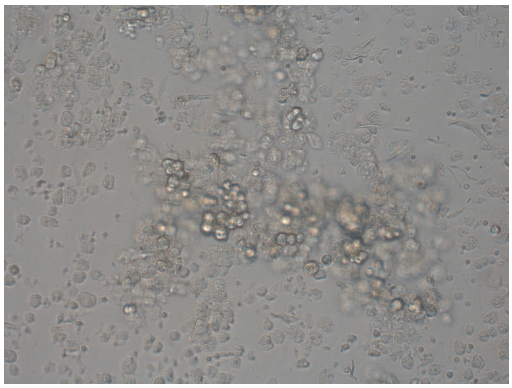

Figure 3 Separated pancreatic acinar cells (original magnification × 100).

Permanent planting of mesenchymal stem cells in small intestine

Blue fluorescing cells (DAPI positive cells) were observed in sections of small intestinal tissue of rats that were flash frozen in the rats included in the SAP + MSCs group (Figure 4).

Effect of mesenchymal stem cells transplantation on serum amylase levels and oxidative stress

Serum AMS levels of rats in the SAP group at each time point was significantly enhanced compared with those measured in the SAP + MSCs group ($P < 0.05$, Table 4). Serum MDA and SOD levels have been summarized in Table 5. Serum MDA levels tended to initially increase, but were then reduced following the injection of MSCs. Serum MDA levels at each time point in the SAP + MSCs group were significantly lower than in the SAP group ($P < 0.05$). Serum SOD levels in the SAP + MSCs group was higher than in the SAP group, whereas a significant between-group difference in SOD level was only noted after 24 h ($P < 0.01$).

Regulation of mesenchymal stem cells transplantation on inflammatory factors

Serum IL-6, IL-10 and TNF-α levels in the two groups have been summarized in Table 5. Serum IL-10 and TNF-α after MSCs transplantation tended to increase then decrease. IL-6 was persistently elevated and was obvious in the SAP + MSCs group. After MSCs transplantation, serum IL-6 and TNF-α levels were significantly lower than in the SAP group ($P < 0.05$), Further, serum IL-10 was significantly higher in the SAP + MSCs group than the SAP group ($P < 0.05$). After 72 h, each cytokine was not significantly different between the two groups.

Assessment and scoring of intestinal tissues at different time points after mesenchymal stem cells transplantation

Using a conventional light microscope, the intestinal mucosa was clearly damaged in the SAP group. Specifically, the lamina propria was destroyed, the blood capillary network was exposed, there was bulk infiltration of neutrophils, local regions of hemorrhage, there was a depopulation of intestinal villi, and the glands of the lamina propria showed a variable degree of destruction. In contrast, these changes were rarely noted in the SAP + MSCs group. The main changes noted were neutrophil infiltration of the proper layer and engorgement of the capillaries. The Chiu intestinal tissue damage score in the SAP + MSCs group was significantly lower than that in the SAP group after 6 h (36.33 ± 5.72, $P = 0.045$), 24 h (46.33 ± 2.80, $P < 0.05$), and 72 h (26.67 ± 3.08, $P < 0.05$) as described in Table 6.

Cell proliferation in the small intestinal mucosa at different time points following mesenchymal stem cells transplantation

Cellular regeneration in the small intestinal mucosa in the SAP + MSCs group was more obvious than that in the SAP group, which was in accordance with conventional pathology (Figure 5). For 6 h after transplantation, neither of the two groups had any evidence of proliferation. Then the cell proliferation of small intestinal mucosa in the SAP + MSCs group became significant different than the SAP group ($P < 0.05$) as shown in Table 6 and Figure 6.

DISCUSSION

The goal of this study was to explore the role of bone marrow MSCs in a model of SAP to provide a new, practical basis for the intervention of this often fatal disease. The results of this study are supported by a recently published study on the inhibition of inflammation and reduction of acute pancreatitis in rats by human bone marrow-derived clonal mesenchymal stem cells[18]. Other studies have also demonstrated that SAP induces functional disturbances of the intestinal barrier, resulting in the displacement of bacteria in enteric cavity. Endotoxin subsequently enters the blood and induces a systemic inflammatory factors cascade reaction that aggravates the pathogenic condition. In this course, impairment of free radicals is thought to be one of the most important links between endotoxin and inflammatory reaction. Specifically, oxygen-derived free radicals can induce lipid peroxidation of biological membranes, change the activity of proteins and enzymes, and directly assault DNA and injure the mitochondria, *etc.*, thereby elevating oxidative stress levels in cells[19,20].

Previous studies have also demonstrated that MSCs reduce oxygen-derived free radical levels in the body *via* multiple pathways and maintain the stability of membranes. Exogenous MSCs protected vascular endothelial cells to avoid the damage of oxidative stress[21], and relieve the oxidative damage of neuroblastoma[22]. In one study[23], Kallikrein-modified MSCs were transplanted into the renal tissues of rats with ischemic/reperfusion injury. Those MSCs inhibited the infiltration of neutrophils and mononuclear macrophages, reduced the activity of myeloperoxidases, diminished the formation of superoxides, and relieved H_2O_2-induced apoptosis. Another study reported that MSCs reduced amylase and lipase levels in the serum of rats with injury to the pancreas and

Table 1 Measurement of cell survival rate by the 3-(4,5-dimethylthiazol-2-yl)-2-,5-diphenyltetrazolium bromide assay of pancreatic acinar cells at various time points (mean ± SD)

Group	0.5 h	1 h	4 h	10 h
Non-SODC group	93.83% ± 3.13%	89.00% ± 2.83%	81.83% ± 3.06%	75.00% ± 6.54%
SODC group	87.83% ± 6.59%[a]	77.50% ± 9.35%[a]	65.83% ± 8.23%[a]	39.17% ± 8.26%[a]
MSCs intervention group	90.00% ± 3.41%	82.17% ± 7.47%	75.17% ± 5.85%[c]	51.83% ± 6.79%[c]

[a]$P < 0.05$ *vs* the non-sodium deoxycholate (SDOC) group, [c]$P < 0.05$ *vs* the SDOC group. MSCs: Mesenchymal stem cells.

Table 2 Changes in amylase secretion rate and lactate dehydrogenase leakage rate of pancreatic acinar cells at various time points (mean ± SD)

Index	Group	0.5 h	1 h	4 h	10 h
AMS	Non-SDOC group	7.47 ± 0.67	8.97 ± 0.69	20.32 ± 2.00	24.28 ± 2.47
	SDOC group	11.75 ± 2.40[a]	17.23 ± 2.43[a]	40.88 ± 3.61[a]	60.38 ± 4.01[a]
	MSCs intervention group	10.18 ± 1.53	14.48 ± 1.74[c]	29.33 ± 2.16[c]	40.33 ± 4.27[c]
LDH	Non-SDOC group	3.00 ± 0.63	3.47 ± 0.59	13.17 ± 2.86	23.40 ± 2.55
	SDOC group	7.65 ± 1.75[a]	12.00 ± 3.17[a]	39.02 ± 2.38[a]	53.70 ± 6.73[a]
	MSCs intervention group	5.35 ± 1.01[c]	8.33 ± 3.08[c]	27.67 ± 3.39[c]	38.33 ± 3.20[c]

[a]$P < 0.05$ *vs* the non-sodium deoxycholate (SDOC) group; [c]$P < 0.05$ *vs* the SDOC group. MSCs: Mesenchymal stem cells; AMS:Amylase; LDH: Lactate dehydrogenase.

Table 3 Comparison of superoxide dismutase and malonaldehyde levels in pancreatic acinar cell culture supernatants at various time points (mean ± SD)

Index	Group	0.5 h	1 h	4 h	10 h
Superoxide dismutase (U/mL)	Non-SDOC group	194.83 ± 26.48	185.83 ± 37.79	170.00 ± 25.42	165.00 ± 31.72
	SDOC group	116.17 ± 28.85[a]	108.00 ± 41.52[a]	102.00 ± 33.45[a]	90.67 ± 33.55[a]
	MSCs intervention group	125.50 ± 39.20	138.50 ± 42.03	147.67 ± 37.25[c]	139.00 ± 46.22[c]
Malonaldehyde (μmol/L)	Non-SDOC group	3.50 ± 5.84	4.17 ± 0.75	4.33 ± 1.27	4.67 ± 1.21
	SDOC group	4.40 ± 1.33	6.33 ± 1.63[a]	7.33 ± 1.21[a]	8.00 ± 1.10[a]
	MSCs intervention group	3.97 ± 0.89	5.00 ± 1.41	5.33 ± 1.63[c]	5.83 ± 2.04[c]

[a]$P < 0.05$ *vs* the non-sodium deoxycholate (SDOC) group; [c]$P < 0.05$ *vs* the SDOC group.

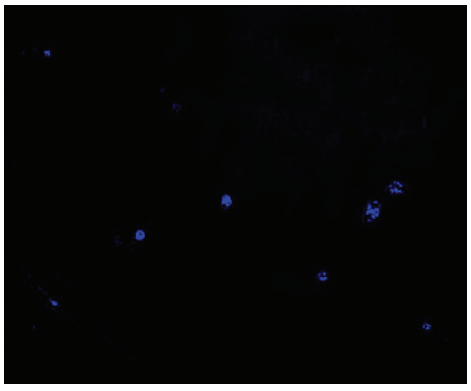

Figure 4 Transplanted mesenchymal stem cells were stained with 4,6-diamidino-2-phenylindole in advance, flash-frozen then observed under fluorescence microscope. The blue fluorescent 4,6-diamidino-2-phenylindole-positive cells (mesenchymal stem cells) were noted in the small intestinal tissue.

Table 4 Comparisons of serum amylase levels (U/L) in rats that were or were not treated with mesenchymal stem cells following establishment of an severe acute pancreatitis model (n = 36, mean ± SD)

Group	Post-MSCs or saline injection (h)		
	6	24	72
SAP	3753.83 ± 791.65	5344.67 ± 649.63	7762.50 ± 977.30
SAP + MSCs	2671.33 ± 547.57[a]	4235.83 ± 554.57[a]	5615.17 ± 809.30[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the severe acute pancreatitis (SAP) group. MSCs: Mesenchymal stem cells.

repaired the necrotic pancreatic tissue. MSCs might also inhibit inflammation and involve in the reaction by producing some soluble materials[24].

The production of MDA and lipid peroxidation are parallel; therefore, detecting MDA is thought to represent lipid peroxidation. In addition, SOD is a critical free radical scavenger in mammals and its concentration reflects the ability of the body to scavenge oxygen-derived free radicals[25,26]. In this study, serum MDA of rats in the SAP + MSCs group was reduced while SOD level was heighten, indicating that MSCs transplantation could reduce the oxidative stress level of SAP rats, relieve lipid peroxidation, protect the stability of the membranes, improve the

Table 5 Comparison of serum malonaldehyde, superoxide dismutase levels, interleukin-6, interleukin-10 and tumor necrosis factor-α measured at various time points after injecting either mesenchymal stem cells or saline into rats after establishing an severe acute pancreatitis model (n = 36, mean ± SD)

Index	Group	Post-mesenchymal stem cells or saline injection (h)		
		6	24	72
Malonaldehyde (nmol/mL)	Severe acute pancreatitis group	4.89 ± 0.97	5.20 ± 1.21	4.43 ± 0.42
	Severe acute pancreatitis + mesenchymal stem cells group	3.68 ± 0.38[a]	3.89 ± 0.59[a]	3.36 ± 0.98[a]
Superoxide dismutase (U/mL)	Severe acute pancreatitis group	43.16 ± 6.94	48.13 ± 3.93	45.83 ± 4.72
	Severe acute pancreatitis + mesenchymal stem cells group	48.05 ± 3.83	61.29 ± 7.81[b]	50.75 ± 7.59
Interleukin-6 (pg/mL)	Severe acute pancreatitis group	107.70 ± 13.08	128.52 ± 8.52	134.06 ± 13.12
	Severe acute pancreatitis + mesenchymal stem cells group	90.16 ± 9.55[a]	107.33 ± 12.13[b]	143.24 ± 12.11
Interleukin-10 (pg/mL)	Severe acute pancreatitis group	31.08 ± 6.64	45.02 ± 4.28	40.11 ± 8.39
	Severe acute pancreatitis + mesenchymal stem cells group	40.84 ± 7.05[a]	52.08 ± 5.79[a]	41.76 ± 3.37
TNF-α (pg/mL)	Severe acute pancreatitis group	106.15 ± 9.01	132.62 ± 8.64	122.42 ± 13.44
	Severe acute pancreatitis + mesenchymal stem cells group	91.47 ± 10.00[a]	119.47 ± 10.83[a]	110.91 ± 9.92

[a]P < 0.05, [b]P < 0.01 *vs* the severe acute pancreatitis group. TNF-α: Tumor necrosis factor-α.

Table 6 Comparison of intestinal mucosa injury scores (each slice/score) and proliferating cells of small intestinal mucosa (each slice/number) determined at various time points after injecting either mesenchymal stem cells or saline after establishment of an severe acute pancreatitis model (n = 36, mean ± SD)

Group	Post-MSCs or saline injection (h)					
	6		24		72	
	Intestinal mucosa injury scores	proliferating cells number	Intestinal mucosa injury scores	proliferating cells number	Intestinal mucosa injury scores	proliferating cells number
SAP group	43.33 ± 4.84	39.50 ± 5.09	52.83 ± 5.27	59.67 ± 6.80	32.17 ± 4.17	81.50 ± 7.89
SAP + MSCs group	36.33 ± 5.72[a]	40.83 ± 5.12	46.33 ± 2.80[a]	68.00 ± 3.22[a]	26.67 ± 3.08[a]	101.00 ± 11.58[b]

[a]P < 0.05, [b]P < 0.01 *vs* the severe acute pancreatitis (SAP) group. MSCs: Mesenchymal stem cells.

scavenging ability of oxygen-derived free radicals, relieve oxygen-derived free radical-induced multiple injury, and protect SAP-induced intestinal tissue damage.

After functional damage of the intestinal barrier, the displacement of endotoxin has multiple pathologic and physiologic consequences. For instance, endotoxin induces pyrogenic reactions, activates the complement system, affects mononuclear macrophages and endothelial cells, induces the genesis of endogenous mediators including TNF, IL, oxygen-free radicals, interferons, *etc*.[27], resulting in the aggravation of pathogenic conditions or even death. The displacement of endotoxin impairs intestinal epithelial cells and increases intestinal permeability[25]. MSCs also inhibit multiple immunocytes, such as T lymphocytes[28], secrete inhibitory mediators of inflammation such as IL-4 and IL-10, parasecrete IL-10, HGF, VEGF, reduce apoptosis signals[29], and relieve endotoxin-induced inflammatory reactions[30]. In the current study, mediators of inflammation, including serum IL-6 and TNF-α of rats in the SAP + MSCs group was higher than the SAP group, whereas IL-10 levels (an anti-inflammatory mediator) were lower in the SAP + MSCs group than the SAP group. This result is similar to several previous studies[13,31] indicating that MSCs might have a role in immunosuppression by reducing the expression of inflammatory factors and promoting the expression of anti-inflammatory mediators. The study reported herein also found that the Chiu intestinal tissue injury scores at 6 h, 24 h and 72 h after transplantation were significantly lower in the SAP + MSCs group than the SAP group, and cellular regeneration in the small intestinal mucosa in the SAP + MSCs group was more evident than in the SAP group. Therefore, MSCs appear to relieve the degree of injury to the small intestinal epithelium, promote the repair of enteric epithelium of rats, and maintain the integrity of the barrier of the intestinal mucosa.

Pancreatic acinar cells are the functional unit for the external secretion of the pancreas, which accounts for 80% of pancreatic tissue. SAP is caused by a functional disorder and impairment of pancreatic acinar cells[32]. During the process of SAP, inflammatory mediators, metabolic products of arachidonic acid, and oxygen-derived free radicals might reduce the antioxidative ability of pancreatic cells[33], enhance vascular permeability, and cause tissue thrombosis and hemorrhage, thereby inducing necrosis of the pancreas[34]. Thus, maintaining the function of pancreatic cells has a critical significance in relieving the severity of SAP. In this study, MDA levels in the MSCs intervention group were lower than in the SDOC group; however, SOD levels in the MSCs intervention group were higher than the SDOC group indicating that MSCs could impact the oxidative stress level of pancreatic acinar cells of injury rats inducted by SDOC, abrogate lipid peroxidation, protect the stability of membranes, improve the scavenging ability of free radicals, relieve free radical-induced injury to protect pancreatic acinar cells.

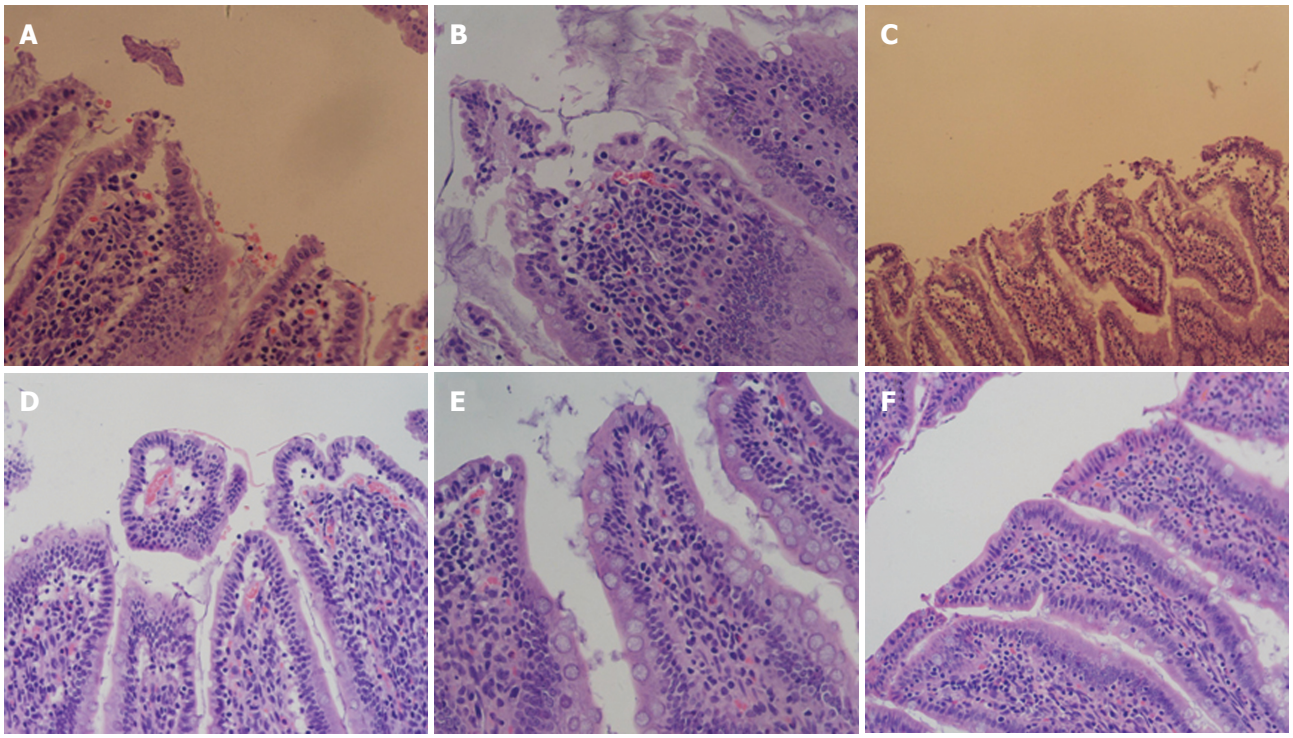

Figure 5 Description of the intestinal pathologic manifestations 6 h after mesenchymal stem cells transplantation. A: Extensive injury of the intestinal mucosa was obvious in the severe acute pancreatitis (SAP) group; B: The dissection of the upper cortex of the intestinal mucosa was noted in the SAP + marrow mesenchymal stem cells (MSCs) group; C, D: Injury to the intestinal mucosa in the SAP (C) and SAP + MSCs groups (D) 24 h after MSCs transplantation were more severe than at 6 h; E, F: Repair of the intestinal mucosa was seen in the SAP (E) and SAP + MSCs groups (F) (HE staining, original magnification × 200).

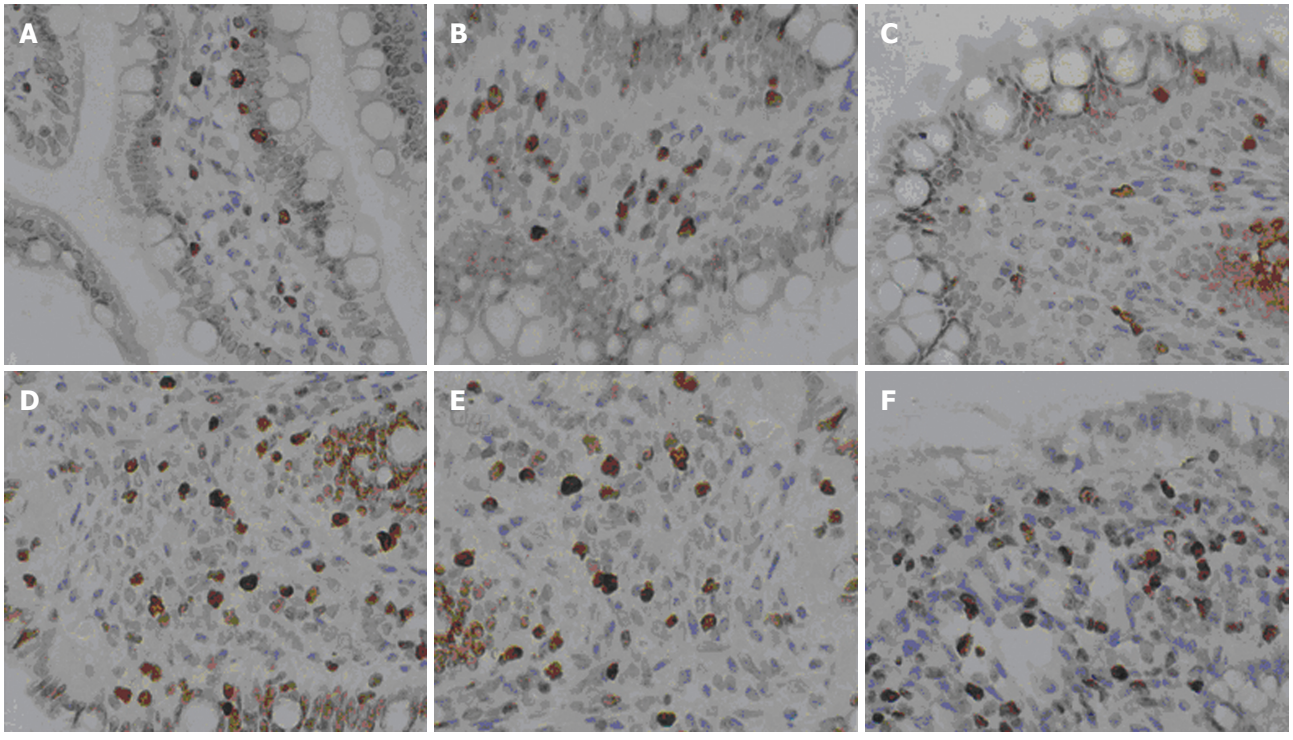

Figure 6 The immunohistochemical staining of proliferating cell nuclear antigen Ki-067 after mesenchymal stem cells transplantation at 6 h (A, B), 24 h (C, **D) and 72 h (E, F).** Cell proliferation (brown cells) was obvious. The number of stained (brown) cells in the severe acute pancreatitis (SAP) + mesenchymal stem cells group (B, D, F) were significantly higher than the SAP group. Cell numbers gradually increased with time (original magnification × 400).

In conclusion, this study found that MSCs could relieve injury to pancreatic acinar cells in rats with SAP, attenuate inflammation and injury in the small intestinal epithelium, promote the proliferation of enteric epithe-

lium and repair of the mucosa, and maintain the integrity of the intestinal barrier function. Potential mechanisms might involve regulating the oxidative stress levels of rats with SAP, inhibiting the extensive release of mediators of inflammation and cytokines, promoting the secretion of mediators of inflammation, and scavenging oxygen-derived free radicals. The specific mechanisms remain worthy of further study.

COMMENTS

Background

Acute pancreatitis (AP) is characterized by a rapid onset and disease progression with high fatality. Severe acute pancreatitis (SAP) is extremely challenging to treat. Several studies currently suggest that the pathogenesis of AP involves complicated cascade reactions of inflammation. Mesenchymal stem cells (MSCs) are multipotent stem cells which had strong immunoregulatory effects and multidirectional differentiation potency. Recent studies found that MSCs also played a special role in inhibiting inflammatory reactions and promoting tissue repair in various inflammation-based diseases such as kidney disease in ischemia/reperfusion injury, collagen-induced arthritis, and acute renal failure. However, very few studies to date have investigated the potential role of cell therapy for pancreatitis.

Research frontiers

Inflammation plays an important role in the pathology of AP. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 as proinflammatory cytokines are produced mainly during AP. The research hotspot is to explore whether MSCs could reduce the level of inflammatory factors and promote the repair of various tissues and organ injury in AP.

Innovations and breakthroughs

Recent reports have indicated that MSCs can reduce the expression of various inflammatory factors and promote the repair of tissues and organ injury. In the present study the authors found that MSCs can also effectively relieve injury to pancreatic acinar cells and small intestinal epithelium, promote the proliferation of enteric epithelium and repair of the mucosa, attenuate systemic inflammation in rats with SAP.

Applications

The study results suggest that MSCs have a special role in inhibiting inflammatory reactions and promoting tissue repair in rats with SAP that might be developed as a cell therapy for pancreatitis.

Terminology

Severe acute pancreatitis (SAP): SAP is a serous gastrointestinal disorder which caused by a functional disorder and impairment of pancreatic acinar cells. The disease is characterized by a rapid onset and disease progression which has a high fatality. Mesenchymal stem cells (MSCs): MSCs are multipotent stem cells which derived form bone marrow. They have strong immunoregulatory effects and multidirectional differentiation potency.

Peer review

The authous detected the cell survival rate, the concentration of malonaldehyde (MDA), the density of superoxide dismutase (SOD), serum amylase (AMS) secretion rate and lactate dehydrogenase leakage rate in pancreatic acinar cell experiments, and measured serum AMS, MDA and SOD, IL-6, IL-10, and TNF-α levels, intestinal mucosa injury scores and proliferating cells of small intestinal mucosa at various time points in animal experiments. The results are interesting and suggest that MSCs could relieve injury to pancreatic acinar cells in rats with SAP, attenuate inflammation and injury in the small intestinal epithelium, promote the proliferation of enteric epithelium and repair of the mucosa. It is believable that MSCs infusion might be a promising treatment method for AP or SAP.

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BRIEF ARTICLE

Clinical significance of connective tissue growth factor in hepatitis B virus-induced hepatic fibrosis

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Abstract

AIM: To determine the utility of connective tissue growth factor (CCN2/CTGF) for assessing hepatic fibrosis in hepatitis B virus (HBV)-induced chronic liver diseases (CLD-B).

METHODS: Enzyme-linked immunosorbent assay was used to measure CCN2 in sera from 107 patients with chronic hepatitis B (CHB) and 39 patients with HBV-induced active liver cirrhosis and 30 healthy individuals. Liver samples from 31 patients with CHB, 8 patients with HBV-induced liver cirrhosis and 8 HBV carriers with normal liver histology were examined for transforming growth factor β-1 (TGF-β1) or CCN2 mRNA levels by *in situ* hybridization, and computer image analysis was performed to measure integrated optimal density (IOD) of CCN2 mRNA-positive cells in liver tissues. Histological inflammation grading and fibrosis staging were evaluated by H and E staining and Van Gieson's method.

RESULTS: Serum CCN2 concentrations were, respectively, 4.0- or 4.9-fold higher in patients with CHB or active liver cirrhosis as compared to healthy individuals ($P < 0.01$). There was good consistency between the levels of CCN2 in sera and CCN2 mRNA expression in liver tissues ($r = 0.87$, $P < 0.01$). The levels of CCN2 in sera were increased with the enhancement of histological fibrosis staging in patients with CLD-B ($r = 0.85$, $P < 0.01$). Serum CCN2 was a reliable marker for the assessment of liver fibrosis, with areas under the receiver operating characteristic (ROC) curves (AUC) of 0.94 or 0.85 for, respectively, distinguishing normal liver controls from patients with F1 stage liver fibrosis or discriminating between mild and significant fibrosis.

CONCLUSION: Detection of serum CCN2 in patients with CLD-B may have clinical significance for assessment of severity of hepatic fibrosis.

Key words: Connective tissue growth factor; Liver fibrosis; Chronic hepatitis B; Chronic liver disease; Chronic hepatitis C

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INTRODUCTION

Hepatic fibrosis, characterized by an excessive accumu-

lation of extracellular matrix (ECM) components, is a common feature of many chronic liver diseases (CLD-B) and can ultimately lead to liver cirrhosis[1,2]. Hepatitis B virus (HBV) is a predominant cause of chronic liver disease and presents a high risk of fibrosis progression[3]. While the pathobiology of HBV-induced hepatic fibrosis has not been fully clarified, HBV presents a huge medical challenge because one third of the world's population has been infected and 350 million people are carriers of the virus. Hepatitis B is endemic in China (> 8% prevalence) and has caused epidemics in other parts of Asia and Africa.

Over last two decades, hepatic stellate cells (HSCs) have dominated studies exploring mechanisms of hepatic fibrosis[4]. In response to chronic liver injury, quiescent HSCs become activated myofibroblast-like cells that express α-smooth muscle actin (α-SMA) and produce components of the ECM, including fibrillar collagens[1,2]. This process is driven by a variety of growth factors, cytokines and matricellular proteins. Connective tissue growth factor (CCN2, also known as CTGF) is a secreted matricellular protein that is recognized increasingly as a central player in hepatic fibrosis[5]. Previously, we have shown that CCN2 production and secretion is enhanced by transforming growth factor-β1 (TGF-β1) in rat HSC and exposure of HSCs to CCN2 induces cell adhesion, migration, and proliferation[6,7]. We and others have shown that CCN2 induces expression of α-SMA or type I collagen in HSCs consistent with a role in activation and fibrogenesis[8,9]. In addition, CCN2 also stimulates survival pathways in activated HSCs thereby prolonging their fibrogenic potential[10]. In human or experimental liver fibrosis, CCN2 expression is higher than in normal liver, with strong correlation between hepatic CCN2 production and the degree of liver fibrosis[11].

Given the central role of CCN2 in hepatic fibrosis, we investigated the levels of serum CCN2 of patients with HBV-induced CLD-B, and determined the potential clinical value of hepatic or serum CCN2 levels in diagnosing the severity of HBV-induced hepatic fibrosis.

MATERIALS AND METHODS

Ethics

This work was approved by First Hospital of Jilin University and was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Informed consent was obtained from all patients prior to sample collection.

Clinical data

Serum was collected from 146 patients with CLD-B who were either outpatients or inpatients at First Hospital (Jilin University, Changchun, China) from June 2008 to September 2011. Of the 146 patients, there were 107 cases with chronic hepatitis B (CHB) (69 men and 38 women; mean age: 43.9 years, range: 18-76 years), 39 cases with active liver cirrhosis (27 men and 12 women; mean age: 41.9 years, range: 26-68 years). As determined using the Child-Pugh score to assess the severity of liver cirrhosis, 25 cases were class A and 14 cases were class B.

Serum was collected from 30 healthy individuals (18 men and 12 women; mean age: 33.6 years, range: 19-56 years) for controls. Serum and liver samples were collected from 8 HBV carriers who had histological normal livers (6 men and 2 women; mean age: 28.6 years, range: 19-38 years), and were studied as normal controls for the assessment of liver fibrosis of CLD-B patients. Patients and HBV carriers meet the diagnostic criteria for chronic HBV infection[12].

Liver tissue samples from 39 CLD-B patients and 8 HBV carriers were obtained using a percutaneous needle. The length of each sample was more than 1.5 cm. There were 31 cases with CHB (23 men and 8 women; mean age: 29.8 years, range: 18-50 years) and 8 cases with HBV-induced active liver cirrhosis (6 men and 2 women; mean age: 36.6 years, range: 26-54 years). Importantly, initial studies showed that levels of serum CTGF, Collagen Ⅰ, Collagen Ⅲ or aminotransferases in CLD-B patients receiving a liver biopsy were not significantly different as compared to CLD-B patients without a biopsy.

CCN2 enzyme linked immunosorbent assay

Sera were stored at -70 ℃ for 1 to 6 mo before analysis. The level of CCN2 in sera from 146 patients with CLD-B, 8 HBV carriers and 30 healthy individuals were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer's protocols (USCN Life Science and Technology Co, TX, United States). Briefly, microtiter wells were pre-coated for 2 h at 37 ℃, with 100 μL of each standard or 1:20 dilutions of sera. The plates were then developed by sequential addition of biotinylated anti-CCN2 antibody, avidin-conjugated horseradish peroxidase and tetramethylbenzidine substrate solution, and the color reaction was measured at 450 nm.

Grading and staging of liver biopsies

Liver tissue samples from 31 CHB patients, 8 patients with HBV-induced liver cirrhosis and 8 HBV carriers were individually fixed, paraffin-embedded and subjected to H and E staining or Van Gieson's method to determining histological inflammation and fibrosis which were scored using the Metavir system. Fibrosis was staged on a 4-point scale (F0: No fibrosis; F1: Minimal fibrosis; F2: Fibrosis with a few septa; F3: Numerous bridging fibrosis without cirrhosis; F4: Cirrhosis or advanced severe fibrosis). F1-F2 was defined as mild fibrosis and F3-F4 as significant fibrosis. Inflammation was graded on a four-point scale from A0, which indicated no inflammatory activity, up to A3, which indicated severe activity.

In situ hybridization

In situ hybridization (ISH) was performed using digoxigenin-labled sense or anti-sense probes for CCN2 or TGF-β1 (*Boster Biotechnology* Co. Ltd. Wuhan, China). In

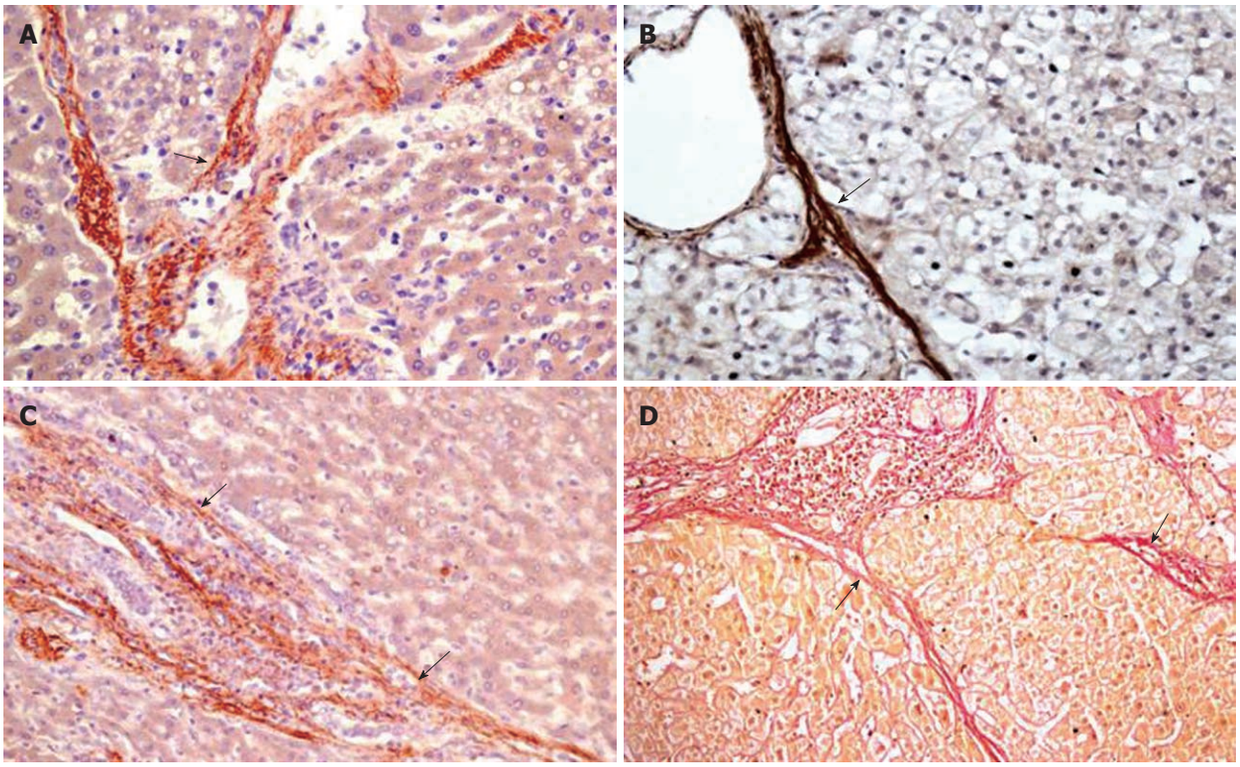

Figure 1 Production of connective tissue growth factor or transforming growth factor β-1 in fibrous septa of hepatitis B virus-infected livers. Connective tissue growth factor mRNA (A) or protein (B) were detected by *in situ* hybridization (ISH) or immunohistochemistry respectively. transforming growth factor β-1 mRNA (C) was detected by ISH while collagen bundles (D) were stained red using Van Gieson's method. Original magnification, × 200 in A, B, C and D. Examples of positively stained cells or structures in each panel are arrowed.

brief, liver tissue samples from CLD-B patients were formaldehyde-fixed and parafin-embeded. The tissue sections (5 μm) were deparaffinized, rehydrated with PBS, digested with pepsin (30 μg/mL) for 10 min at 37 ℃, fixed in 4% paraformaldehyde in PBS and washed in 3 ×SSC. The samples were pre-hybridized at 40 ℃ for 2 h, and hybridization was performed overnight at 40 ℃ with sense or anti-sense probes. After hybridization, excess probes were removed by sequential washing in twice concentrated (2×) saline-solution citrate buffer (SSC), 0.5×SSC and then 0.2×SSC at 37 ℃ for 2 h. The tissue sections were incubated at 37 ℃ for 1 h with biotinylated mouse anti-digoxigenin, followed by addition of the streptavidin-biotin-peroxidase complex for 20 min. The slides were then developed with 3-amino-9-ethylcarbazole (Boster Biotechnology). Ten random images (original magnification ×400) of each slide underwent computer image analysis using Image-Pro Plus 6.0 software to assess the integrated optimal density (IOD) of CCN2-positive cells in liver tissues.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5 μm) were de-waxed and re-hydrated. Sections were incubated overnight at 4 ℃ with mouse anti-human α-SMA monoclonal antibody (Zhongshan Goldbridge Biotechnology, Beijing, China) or rabbit anti-human CCN2 polyclonal antibody (Santa Cruz, Heidelberg, Germany) or rabbit anti-human F4/80 polyclonal antibody (Spring Bioscience, United States). Sections were washed in PBS and incubated at room temperature for 10 min with biotinylated *goat anti-mouse* and *rabbit* IgG (Maixin Bio, Fuzhou, China). After washing with PBS, sections were incubated with streptavidin-peroxidase (Maixin Bio, Fuzhou, China) for 10 min and then developed with diaminobenzidine or 3-amino-9-ethylcarbazole.

Statistical analysis

The values reported represent the median [95% confidence interval (CI)] of the measurements. Statistical analysis of the data was performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, United States). The nonparametric Wilcoxon signed ranks test was used for pair-wise comparison of groups and Spearman's rank correlation analysis was used to determine the relationship between two variables. Areas under the receiver operating characteristic (ROC) curves (AUC) were calculated for comparing the accuracy of the CCN2 in sera in different subgroups.

RESULTS

Localization of CCN2 mRNA or protein in HBV-induced chronic liver disease

In normal livers, only mild CCN2 mRNA staining was detected in portal tracts and there was no staining in the

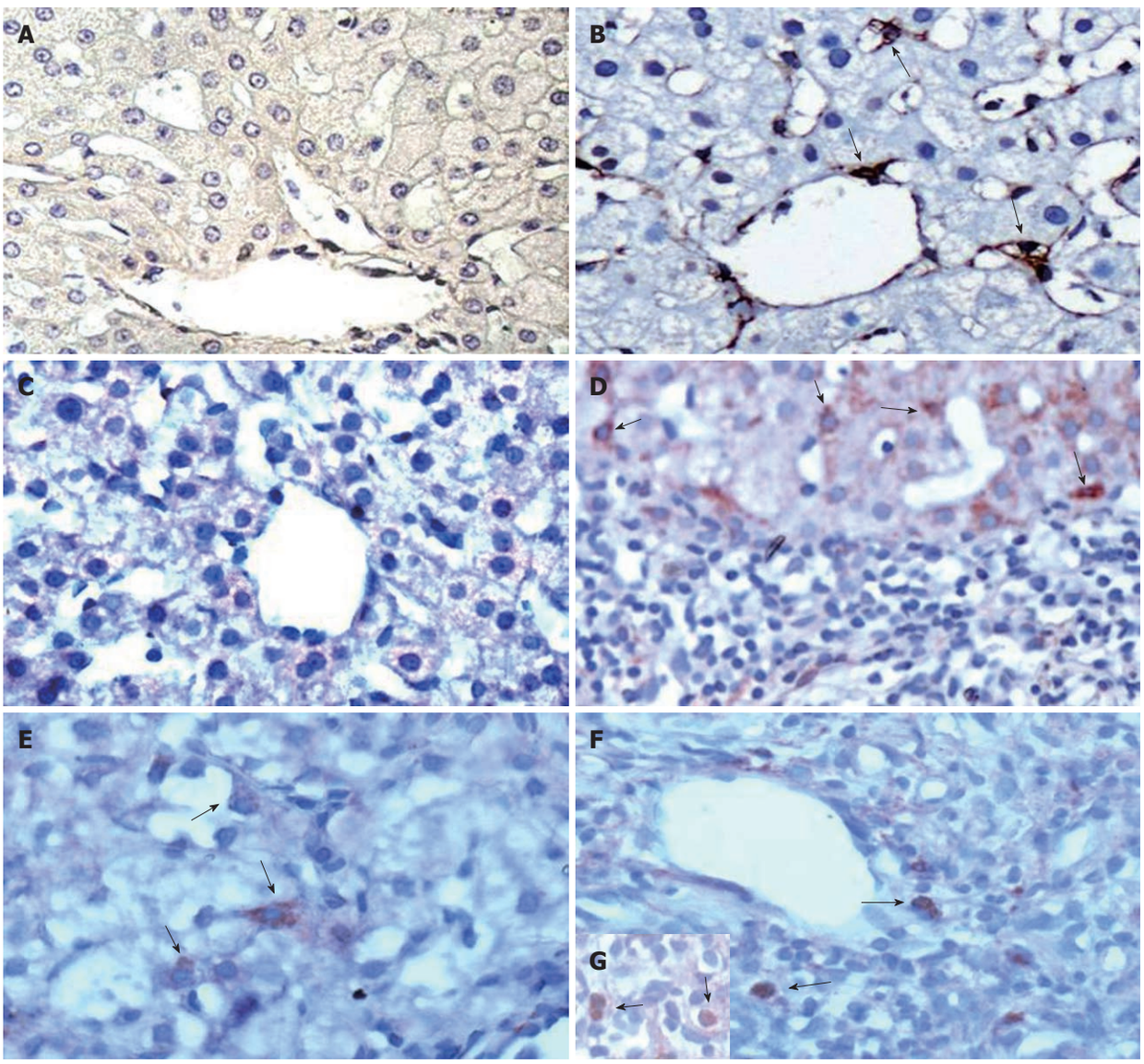

Figure 2 Cellular localization of key fibrotic markers in hepatitis B virus -induced liver fibrosis. α-SMA-positive hepatic stellate cells (HSCs) were not detectable in hepatitis B virus carriers who had normal liver histology (A) but were present in chronic hepatitis B (CHB) liver (B). In CHB liver samples, there was no staining when the *in situ* hybridization probes were omitted (C) but their inclusion demonstrated the presence of either connective tissue growth factor mRNA in activated HSCs (D) or transforming growth factor β-1 mRNA in activated HSCs (E) or Kupffer cells (F). F4/80 antigen-positive Kupffer cells (G). Original magnification, × 400 in A-G. Examples of postively stained cells in each panel are arrowed.

central vein or lobule (data not shown). However, CCN2 mRNA and protein were localized to fibrotic septa in CLD-B patients with hepatic fibrosis (Figure 1A and B) or cirrhosis (data not shown), and the pattern of CCN2 staining was well correlated with the distribution of collagen fibers (Figure 1D). By comparison to normal livers, CHB patients demonstrated strong α-SMA-positive staining in presumptive activated HSC (Figure 2A and B) and these cells also stained strongly for CCN2 mRNA (Figure 2C and D).

Since CCN2 acts downstream of TGF-β1 to drive fibrosis[13], we examined the expression and distribution of TGF-β1 mRNA in the liver of CLD-B patients. TGF-β1 mRNA was detected in the fibrotic septa of patients with hepatic fibrosis (Figure 1C) or cirrhosis (data not shown) and was localized to activated HSCs in the lobule (Figure 2E) and Kupffer cells (Figure 2F and G) within inflammatory area in the livers of CHB patients.

Serum levels of CCN2 in HBV-induced chronic liver diseases

Since CCN2 is synthesized with a signal peptide and can exist extracellularly in soluble or matrix-associated forms, we examined serum from CLD-B patients for the presence of CCN2 protein by ELISA. As shown in Table 1, serum CCN2 concentrations were, respectively, 4.0- or 4.9-fold higher in patients with CHB or active liver cirrhosis as compared to healthy individuals ($P < 0.01$). There was no difference in the serum CCN2 levels between the HBV carriers and healthy individuals.

Table 1 Serum connective tissue growth factor concentrations in patients with chronic liver diseases (μg/L)

Group	*n*	Median (95% CI)
Healthy control	30	2.2 (1.6-2.8)
HBV carrier	8	2.2 (1.5-2.9)
Chronic hepatitis B	107	8.8 (6.0-12.3)
Active liver cirrhosis	39	10.9 (7.0-14.6)

HBV: Hepatitis B virus.

Table 2 Relationship between fibrosis stage and hepatic or serum connective tissue growth factor content

Fibrosis stage	*n*	Serum CCN2 (μg/L) median (95% CI)	Hepatic CCN2 mRNA (IOD) median (95% CI)
Normal control	8	2.2 (1.5-2.9)	6.0 (3.9-8.8)
F1	11	6.8 (5.0-8.9)	19.4 (12.3-26.4)
F2	9	8.9 (7.1-10.7)	25.6 (13.9-34.8)
F3	11	9.4 (7.3-12.0)	31.9 (19.7-44.6)
F4	8	10.1 (8.2-12.1)	39.6 (25.5-52.8)

Normal Control samples were from hepatitis B virus carriers and had normal liver histology; F1-F2: Mild fibrosis; F3-F4: Significant fibrosis. CCN2: Connective tissue growth factor; IOD: integrated optimal density.

CCN2 production as a function of severity of fibrosis or inflammation

Having shown that hepatic and serum CCN2 concentrations were higher in CLD-B patients than in healthy individuals, we next investigated if there was a correlation between CCN2 and fibrosis stage. As shown in Table 2, serum concentrations and hepatic content of CCN2 increased in proportion to the severity of fibrosis; Spearman's rank correlation analysis showed that correlation coefficients were 0.85 and 0.89 (both $P < 0.01$), respectively. However, the levels of CCN2 in sera were not correlated with the degree of inflammation in CHB patients.

Diagnostic performance of serum CCN2

We further analyzed the diagnostic performance of serum CCN2 for assessing liver fibrosis using the ROC curves. Calculation of the areas under ROC curves (AUC) showed that serum CCN2 could be used to distinguish either normal liver controls from patients with F1 stage liver fibrosis (AUC = 0.94) or mild fibrosis (F1/F2) from significant fibrosis (F3/F4) (AUC = 0. 85) (Figure 3).

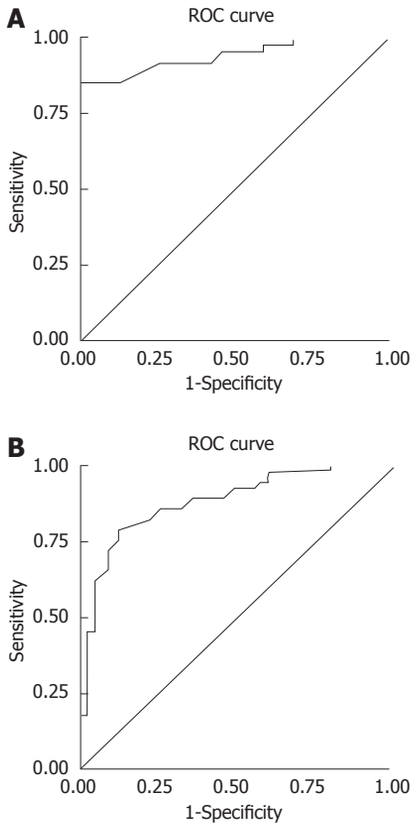

Figure 3 Receiver operating characteristic curve of connective tissue growth factor. Receiver operating characteristic (ROC) curves of connective tissue growth factor distinguishing normal liver controls from patients with F1 stage liver fibrosis (A) or discriminating between mild and significant fibrosis (B) with areas under the ROC curves of 0.94 or 0.85, respectively.

DISCUSSION

Chronic HBV infection can cause hepatic fibrosis and eventually cirrhosis. Over the last few years, HBV infection has been studied extensively *in vitro* with the finding that expression of the HBV X protein (HBx) in hepatocytes results in paracrine activation and proliferation of human or rat HSC resulting in their increased expression of collagen I , CCN2, α-SMA, matrix metalloproteinase-2, or TGF-β[14,15]. Although hepatocytes serve as a suitable host for HBV and permit viral replication and antigen production, HBV can also transiently infect and replicate in human HSCs which directly increases the production of collagen type in the cells[5,16]. Collectively, these findings have shown that activation of fibrogenic pathways in HSC following HBV infection of either hepatocytes or HSC is a key event in HBV-mediated hepatic fibrosis. In this regard, CCN2 has emerged as a potential key fibrogenic mediator in response to HBV in as much as CCN2 supports HSC activation, promotes HSC proliferation and survival, and acts downstream of TGF-β to drive HSC collagen production[6,7,10,13,17]. In this study, we showed that CCN2 mRNA and protein were expressed at high levels by myofibroblasts (including presumptive activated HSC) in fibrotic septa in CLD-B patients with hepatic fibrosis or cirrhosis, and that increased levels of hepatic or circulating CCN2 were associated with severe fibrosis. This result is consistent with previous observations by others[11].

Activation of Kupffer cells, the resident macrophage population of the liver, serves as a central determinant of the liver's response to injury and repair, and the resulting inflammatory reaction is an important prerequisite for HSC activation and progression to hepatic fibrosis[18,19]. Macrophage-derived TGF-β1 has been identified as a potential paracrine stimulator of HSC activation[20] and addition of TGF-β antibodies to Kupffer cell conditioned medium inhibits its ability to induce expression of

CCN2, collagen I and TIMP-1 when added to cultured HSC[21]. In the present study, both TGF-β1 and CCN2 mRNA were detected in presumptive activated HSCs, while TGF-β1 mRNA alone was detected in Kupffer cells within inflamed areas of livers from CHB patients. These data support the notion that TGF-β1 upregulates CCN2 production in HSCs *via* paracrine and autocrine pathways, and further enhance the effects of CCN2 during fibrogenesis. This is supported by *in vitro* studies showing that CCN2 is a downstream mediator of TGF-β1-induced collagen I production in human HSCs[16].

CCN2 is a secreted protein that has been detected in several human body fluids including serum, cerebrospinal fluid, follicular fluid, uterine fluid, or urine[22]. This has led to examination of the potential utility of evaluating CCN2 concentrations in highly accessible fluids such as serum or plasma for non-invasive diagnostic evaluation of the extent or severity of various fibrotic pathologies. Thus, serum levels of CCN2 have been shown to be correlated with the extent of systemic skin sclerosis and severity of pulmonary fibrosis in human subjects[23] and to serve as a biomarker of progressive kidney fibrosis in chronic allograft nephropathy in a clinical and experimental study[24]. Studies on circulating CCN2 levels in assessment of hepatic fibrosis have just begun to gain momentum and are founded on the well documented over-expression of CCN2 in fibrotic livers due to its production by multiple cell types (including HSC, hepatocytes, biliary epithelial cells). An early study reported as association between elevated CCN2 serum levels and progression of hepatic fibrosis in biliary atresia[25] while a more recent investigation demonstrated significantly elevated serum levels of CCN2 in patients with chronic hepatitis and cirrhosis that were well correlated with the progression of hepatic fibrosis[26-28]. In the present research, we found that increased CCN2 concentrations were present in the serum of patients with CHB and HBV-induced cirrhosis. Serum CCN2 levels were consistent with those in liver tissue and were strongly correlated with the stage of hepatic fibrosis. Taken together, our data indicate that CCN2 is a potential valuable biomarker of HBV-induced hepatic fibrosis, and further support the classification of CCN2 as class I fibrosis biomarker, defined as one that is derived from changes of the fibrogenic cell types and which reflects the activity of the fibrogenic and/or fibrolytic process[29].

Finding the best method to evaluate and diagnose the stage of liver fibrosis continues to be a challenge[30]. Although liver biopsy is a gold-standard procedure for determining the grade of liver inflammation and stage of fibrosis[30-33], there are well recognized difficulties including complications, high hospital expenses[30,34], false sample recording[35], contra-indications during the procedure, and dependence on the pathologists' skills in examining samples. Serum fibrosis tests with AUCs ranging from 0.85 to 0.90 have been proposed as good biochemical markers with high diagnostic value[36,37]. In our research, serum CCN2 was valuable not only in distinguishing normal liver controls from patients with F1 stage liver fibrosis but also in distinguishing between mild and significant liver fibrosis. We therefore propose that further studies are warranted to further evaluate the potential utility of serum CCN2 as a biomarker of liver fibrosis in HBV-induced CLD-B.

COMMENTS

Background

Millions of individuals around the world are infected with hepatitis B virus (HBV), resulting in chronic liver disease. In many cases, affected individuals suffer from hepatic fibrosis, a highly debilitating pathology in which the normal cellular architecture and function in the liver are severely compromised through the deposition of collagen and other insoluble extracellular matrix molecules. This process is driven by connective tissue growth factor (CCN2) which is known to be produced at high levels in fibrotic livers and which acts to drive fibrogenic pathways in hepatic stellate cells (HSCs), a principal fibrotic cell type in the liver.

Research frontiers

Currently, parameters used to assess liver fibrosis are inaccurate. There is optimism that measurement of CCN2 levels in either the livers or serum of affected patients will have useful diagnostic or prognostic value.

Innovations and breakthroughs

To date, there have been a limited number of studies regarding the value of serum CCN2 for assessment of hepatic fibrosis. In this study, the authors employed more systemic detection techniques to evaluate the relationship among serum CCN2 levels, hepatic CCN2 content and liver fibrosis severity in patients with chronic liver diseases. Furthermore, the authors described the expression characteristics of CCN2 in liver tissues and its role and mechanism in HBV-induced hepatic fibrosis.

Applications

These studies suggest that serum CCN2 concentrations are a reliable diagnostic indicator of HBV-induced liver fibrosis and that CCN2 can be used a part of the platform for evaluation of the severity of liver fibrosis.

Terminology

CCN2: a pro-fibrogenic molecule that is over-expressed in many fibrotic diseases and which stimulates collagen synthesis in HSC.

Peer review

This is an interesting and important issue in the utility of CCN2 for assessing hepatic fibrosis. Correlations of the serum levels of CCN2 in HBV infected patients with hepatic fibrosis have been well documented in literature.

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CASE REPORT

Mycophenolate mofetil for maintenance of remission in steroid-dependent autoimmune pancreatitis

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Abstract

Systemic corticosteroids represent the standard treatment for autoimmune pancreatitis with IgG4-associated cholangitis. For steroid-dependent disease, azathioprine has been used for maintenance of remission. Mycophenolate mofetil has been used for transplant immunosuppression and more recently for autoimmune hepatitis; however, there are no case reports to date on the use of mycophenolate mofetil in adult patients with autoimmune pancreatitis. A patient with IgG4-mediated autoimmune pancreatitis and IgG4-associated cholangitis refractory to steroids and intolerant of azathioprine was treated with mycophenolate mofetil, which inhibits de novo guanosine synthesis and blockade of both B and T lymphocyte production. Introduction of mycophenolate mofetil and uptitration to 1000 mg by mouth twice daily over a treatment period of 4 mo was associated with improvement in the patient's energy level and blood glucose control and was not associated with any adverse events. The patient was managed without a biliary stent. However, there was a return of symptoms, jaundice, increase in transaminases, and hyperbilirubinemia when the prednisone dose reached 11 mg per day. In the first report of mycophenolate mofetil use in an adult patient with IgG4-associated autoimmune pancreatitis and IgG4-associated cholangitis, the introduction of mycophenolate mofetil was safe and well-tolerated without adverse events, but it did not enable discontinuation of the steroids. Mycophenolate mofetil and other immunomodulatory therapies should continue to be studied for maintenance of remission in the large subset of patients with refractory or recurrent autoimmune pancreatitis.

Key words: Autoimmune diseases; Pancreatitis; Mycophenolate mofetil; Recurrence

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INTRODUCTION

Autoimmune pancreatitis (AIP) represents a chronic dis-

ease of the pancreas with a presumed autoimmune etiology. There are characteristic serologic, morphologic, clinical and pathologic features, specifically, lymphoplasmacytic infiltration with parenchymal fibrosis. Some patients develop comorbid involvement of the biliary tract characterized by steroid responsive stricturing, a condition that has been termed IgG4-associated cholangitis (IAC). Steroid therapy is the first-line and mainstay treatment for AIP but relapse is frequent on steroid withdrawal. Azathioprine has been used for patients who fail steroid tapering, but is not tolerated by some patients and may be contraindicated in patients with deficient thiopurine methyltransferase (TPMT) activity. The use of mycophenolate mofetil as a possible treatment alternative for autoimmune pancreatitis with IAC has been described once in the pediatric population[1] and has been mentioned in discussions of AIP[2,3]. However, there have been no case reports to date in an adult population. We describe a 65-year-old man with a history of AIP and IAC who failed multiple attempts at steroid tapering and was intolerant of azathioprine. This is the first case report of the use of mycophenolate mofetil in an adult patient with IgG4-mediated AIP and IAC.

CASE REPORT

A 65-year-old Caucasian male presented to another medical center for a routine colonoscopy. A chest radiograph obtained prior to the procedure revealed multiple nodules in the lower lung fields with pleural scarring and a small pleural effusion. A subsequent computed tomography (CT) scan of the chest revealed multiple pulmonary nodules in the right lower lobe, right lateral middle lobe, and left middle lobe, as well as intrathoracic lymphadenopathy. Because of concerns of malignancy, bronchoalveolar lavage was performed, which revealed infection with *Aspergillosis fumigatus*. The patient began treatment with micofungin.

At the time of bronchoscopy, the patient was also noted to be jaundiced. Laboratory findings revealed an elevated serum total bilirubin level of 4.8 mg/dL and an elevated conjugated bilirubin level of 4 mg/dL. Serum lipase was 16 U/L and serum amylase was 40 U/L, both within normal limits. CT scan of the abdomen revealed diffuse enlargement of the pancreas, evidence of cholelithiasis, as well as biliary ductal dilatation. An upper endoscopic ultrasound with fine-needle aspiration of the pancreas revealed ductal epithelium with fibrosis, inflammatory cells, and debris and no evidence of malignancy, suggestive of autoimmune pancreatitis. Endoscopic retrograde cholangiopancreatography (ERCP) revealed a stricture in the intra-pancreatic portion of the main bile duct, for which the patient underwent stent placement. Biliary ductal brushings revealed no malignant cells. The serum IgG4 level was elevated at 213 mg/dL. Treatment with a tapering course of prednisone for 6 weeks was initiated, with significant improvement in symptoms, reduction in the size of the pancreas on cross sectional imaging, and normalization of the liver enzymes.

After 3 mo, the patient presented to the same hospital emergency department with a relapse of jaundice, steatorrhea, and chills. The total bilirubin was 6 mg/dL, direct bilirubin was 5.2 mg/dL, aspartate aminotransferase (AST) was 122 U/L, alkaline phosphatase (ALP) was 557 U/L, and lactate dehydrogenase was 369 mg/dL. Ultrasound of the gallbladder showed development of mild intrahepatic biliary dilatation, possibly representing stent malfunction. CT of the abdomen revealed significant interval increase in the size of the pancreas. ERCP was performed with exchange of the common bile duct stent. Cholangiography revealed diffuse irregular stricturing of the common bile duct to the hilum of the liver. The patient was restarted on prednisone 40 mg daily. There was symptomatic and clinical improvement.

The patient was referred to our medical center to receive care closer to his home. He was taking 30 mg prednisone daily on presentation and reported feeling well. The biliary stent was extracted. His serum IgG4 level was elevated at 120 mg/dL. The other autoimmune work-up was negative: Anti-nuclear antibody IgG was not detected, Anti-neutrophil cytoplasmic antibody IgG was less than 1:20, f-actin antibody IgG was 15 units (negative), anti-mitochondrial M2 antibody IgG was 0.9 units (negative), and liver kidney microsomal antibody IgG was less than 1:20 (negative). Two months later, the patient presented once again with recurrent jaundice, fatigue, and discomfort. By this time, he had been tapered to a dose of 15 mg prednisone daily and had completed micafungin treatment for his pulmonary aspergillosis. Magnetic resonance imaging (MRI) of the abdomen with and without gadolinium revealed a diminished T1 signal and abnormal delayed enhancement in the pancreas. There was no pancreatic ductal dilatation, and the overall morphology was normal to mildly enlarged. The T2 signal was mildly elevated in the pancreatic parenchyma. The impression was of changes consistent with autoimmune pancreatitis. The MRI also revealed diffuse intrahepatic biliary ductal dilatation and intrahepatic ductal stenosis as well as periportal arterial blush enhancement and delayed uptake of contrast, consistent with active cholangitis. ERCP showed a single severe 18 mm stricture in the distal common bile duct with irregular stricturing of the bilateral intrahepatic ducts (Figure 1). A stent was placed in the bile duct. Biopsy of the pancreatic ampulla demonstrated chronic active inflammation with approximately 20-25 IgG4-containing plasma cells/high power field, consistent with the clinical impression of autoimmune pancreatitis (Figure 2). The patient was maintained on 15 mg prednisone and was started on 50 mg azathioprine daily in an attempt to transition to a steroid-sparing immunosuppressive regimen. However, he was unable to tolerate azathioprine due to debilitating nausea, fatigue, lethargy and diarrhea despite dose reduction, evening administration, and concurrent antiemetic therapy.

Over the next 6 mo, the patient underwent 2 additional biliary stent exchanges at 3-mo intervals. Brush specimens obtained from the common bile duct stricture were

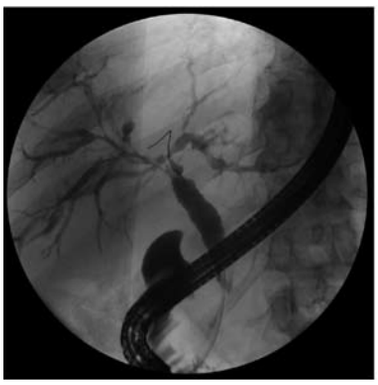

Figure 1 Cholangiogram obtained during endoscopic retrograde cholangiopancreatography demonstrating a marked 18 mm stricture in the distal common bile duct with diffuse irregular stricturing of the intrahepatic ducts bilaterally.

negative for malignant cells by cytology and fluorescence *in situ* hybridization analysis. The patient was maintained on 15 mg prednisone daily. Mycophenolate mofetil was discussed as an alternative immunomodulatory treatment and an alternative to long-term prednisone. The patient was initiated on mycophenolate mofetil at a dose of 750 mg twice daily.

The patient tolerated the mycophenolate mofetil without side effects. At this point, he was being maintained on mycophenolate mofetil and prednisone. After 3 mo on mycophenolate mofetil and prednisone, he had no jaundice or steatorrhea. His hyperglycemia was mild and his diabetes medications were being tapered, likely a result of the lower dose of prednisone required. He required 3 half-tablets of glipizide 5 mg per month to maintain normal serum glucose values. The serum AST level was 30 U/L, ALP level was 101 U/L, total bilirubin level was 0.7 mg/dL, albumin was 3.4 mg/dL, amylase was 43 U/L and lipase was 16 U/L. At the time of his last scheduled stent exchange, the stent had passed spontaneously and no procedure was performed. However, over this 3-mo period, he had several self-limited episodes of fatigue, malaise, and pruritus lasting 24 to 48 h, associated with transient elevations in his liver enzymes. He remained without a biliary stent and felt well overall. Given this course, the mycophenolate mofetil dosage was increased to 1000 mg twice daily and a steroid taper was again resumed, decreasing the dose by 1 mg per week from an initial dose of 15 mg prednisone daily. When the patient reached 10 mg prednisone daily, he experienced a recurrence of nausea and abdominal pain, as well as darkened urine. His total bilirubin was elevated to 9.5 mg/dL, AST 146 IU/L, and ALT 266 IU/L. His prednisone dosage was increased back to 15 mg per day and his symptoms again resolved. He is presently maintained on 15 mg prednisone per day and the mycophenolate mofetil is being tapered due to an inability to stop systemic corticosteroids. He is able to continue an active lifestyle working 12-14 h days on his farm. He is being considered for alternative immunomodulatory therapy.

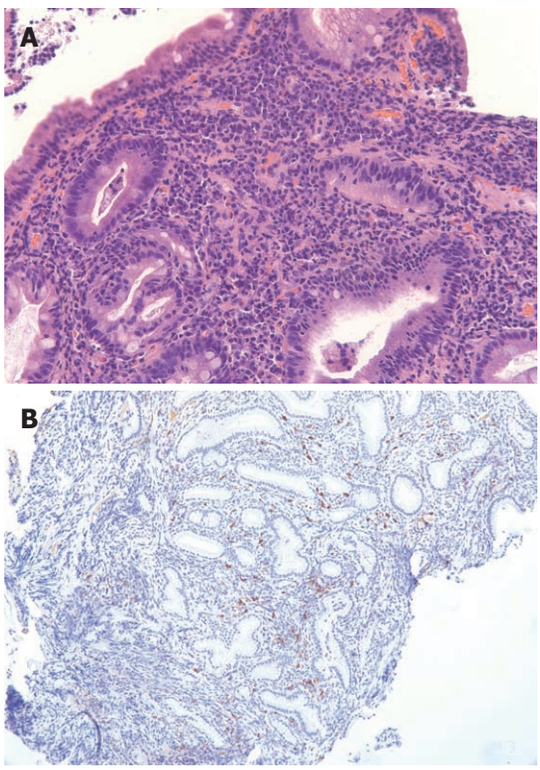

Figure 2 Histopathologic images demonstrating the findings from the patient's ampullary biopsy. A: A hematoxylin eosin stained biopsy at 20 x magnification revealed chronic active inflammation; B: An IgG4 stain revealed approximately 20-25 IgG4-containing plasma cells/high power field, consistent with autoimmune pancreatitis.

DISCUSSION

Systemic corticosteroid therapy is the standard treatment for AIP with IAC[3]. AIP and IAC likely represent organ-specific manifestations of a broader systemic disease described as IgG4-related sclerosing disease. The disease process involves deposition of IgG4 antibodies into various tissues, causing fibrosis and organ dysfunction. Other proposed manifestations of IgG4-associated disease include Sjögren's syndrome, primary sclerosing cholangitis, and retroperitoneal fibrosis[4]. There is a strong association between AIP and IAC, as was seen in our patient[5].

Although the response to corticosteroids is a defining feature of AIP, representing one of the 5 diagnostic criteria (histology, imaging, serology, other organ involvement and response to therapy) for the disease[6], relapse of biliary strictures after steroid withdrawal in cases of IAC is not uncommon. In one recent study of 53 patients with IAC, 54% of patients experienced relapse after steroid withdrawal[3]. In such cases, alternative treatment with immunomodulating medications such as azathioprine and 6-mercaptopurine has been proposed to avoid long-term steroid use and its associated consequences[3]. However, azathioprine and 6-mercaptopurine are not tolerated by all patients. In addition, reduced TPMT activity may be found in approximately 11% of the population, and undetectable enzyme activity in 0.3% of the population[7]. Patients with insufficient TMPT activity may develop

bone marrow toxicity and myelosuppression[8]. In addition, while azathioprine and 6-mercaptopurine may be tolerated, not all patients started on these medications will be able to taper their steroid therapy. For these reasons, alternatives are being examined. For example, a patient refractory to steroid taper and 6-mercaptopurine was successfully managed with rituximab, experiencing cholangiographic improvement, normalization of liver enzymes, and resolution of extrapancreatic manifestations of IgG4 disease[2]. However, rituximab requires infusional therapy, is not indicated for AIP, and has a high cost, which limits its use in this setting.

There have been 2 references to mycophenolate mofetil as a potential alternative treatment in refractory cases of autoimmune pancreatitis[2,3]; however, there are no case reports in the adult populations to date. Mycophenolate mofetil acts as an inhibitor of inosine monophosphate dehydrogenase, which inhibits *de novo* guanosine nucleotide synthesis. Through this mechanism, mycophenolate mofetil exhibits a cytostatic effect on T and B lymphocytes by blocking proliferation[9]. Mycophenolate mofetil has been used in transplantation medicine, inflammatory bowel disease, and rheumatoid arthritis, often as a second-line immunosuppressant. More recently, mycophenolate mofetil use has been described in cases of refractory autoimmune hepatitis[10].

In the case described herein, the steroid taper was not successful with the use of mycophenolate mofetil and the patient had recurrence of symptoms, jaundice and elevated liver enzymes; however, the medication may be efficacious in a subset of patients with refractory disease, and we believe that this merits further investigation in larger studies.

In summary, this is the first case report of mycophenolate mofetil for AIP and IAC. The addition of mycophenolate mofetil to the patient's therapy was well-tolerated but did not permit steroid tapering below 11 mg prednisone per day. The patient may have experienced an improvement in energy and in remission of mild diabetes while on mycophenolate mofetil, possibly because the treatment allowed for a lower dose of prednisone. However, the decision was made to taper and ultimately discontinue the mycophenolate mofetil due to failure to enable cessation of systemic corticosteroids. We believe that mycophenolate mofetil should continue to be examined for use in patients with steroid-dependent AIP and/or IAC, especially in the subset for whom azathioprine and 6-mercaptopurine are ineffective or contraindicated. Additional immunomodulatory therapies are needed for maintenance of remission in the large cohort of AIP and IAC patients who fail steroid tapering.

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CASE REPORT

Hepatic artery pseudoaneurysm caused by acute idiopathic pancreatitis

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Abstract

Hepatic artery pseudoaneurysm (HAP) is a very rare disease but in cases of complication, there is a very high mortality. The most common cause of HAP is iatrogenic trauma such as liver biopsy, transhepatic biliary drainage, cholecystectomy and hepatectomy. HAP may also occur with complications such as infections or inflammation associated with septic emboli. HAP has been reported rarely in patients with acute pancreatitis. As far as we are aware, there is no report of a case caused by acute idiopathic pancreatitis, particularly. We report a case of HAP caused by acute idiopathic pancreatitis which developed in a 61-year-old woman. The woman initially presented with acute pancreatitis due to unknown cause. After conservative management, her symptoms seemed to have improved. But eight days after admission, abdominal pain abruptly became worse again. Abdominal computed tomography (CT) was rechecked and it detected a new HAP that was not seen in a previous abdominal CT. Endoscopic retrograde cholangiopancreatography (ERCP) was performed because of a suspicion of hemobilia as a cause of aggravated abdominal pain. ERCP confirmed hemobilia by observing fresh blood clots at the opening of the ampulla and several filling defects in the distal common bile duct on cholangiogram. Without any particular treatment such as embolization or surgical ligation, HAP thrombosed spontaneously. Three months after discharge, abdominal CT demonstrated that HAP in the left lateral segment had disappeared.

Key words: Hepatic artery; Pseudoaneurysm; Pancreatitis; Acute; Hemobilia

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INTRODUCTION

Hepatic artery pseudoaneurysm (HAP) is rare[1,2]. But rupture is common and occurs in 76% of patients with HAP. There are many causes of HAP, but it usually results from procedures such as liver biopsy, transhepatic biliary drainage, cholecystectomy, and hepatectomy[3]. HAP may also occur with complications such as infections or inflammation associated with septic emboli[4-7]. HAP has rarely been reported in patients with acute pancreatitis[8,9]. As far as we are aware, there is no report of a case caused by acute idiopathic pancreatitis. We report a case of HAP thought to be caused by acute pancreatitis due to unknown cause, in a 61-year-old Korean woman.

HAP spontaneously thrombosed without any particular treatment and finally disappeared several months later. We also review the literature concerning HAP.

CASE REPORT

A 61-year-old Korean woman was admitted to the emergency room with right upper quadrant pain and vomiting starting 8 h before admission. She was previously well-nourished, and had no past medical history. The patient denied a history of percutaneous intervention, abdominal surgery, trauma, viral hepatitis, bleeding disorders, jaundice or blood transfusion. On admission, her blood pressure was 120/80 mmHg, pulse rate was 78/min, and body temperature was 36.8 ℃. On examination, her appearance was that of acute illness and her tongue was mildly dehydrated. But she was clinically non-anemic and non-icteric. Abdominal examination revealed right upper quadrant tenderness without rebound and hepatomegaly. Rectal examination showed no melena.

Laboratory data on admission were as follows: white blood cell count 14 900/μL with 90.5% increase in neutrophils; hemoglobin 13.4 g/dL; hematocrit 39.0%; platelet count 226 000/μL; prothrombin time 15.0 [international normalized ratio (INR) 1.27]; serum total protein 7.0 g/dL; albumin 4.4 g/dL; alkaline phosphatase 103 U/L; total bilirubin 1.0 g/dL; Alanine aminotransferase (ALT) 87 U/L; aspartate aminotransferase (AST) 56 U/L; gamma-glutamyl transferase 46 U/L. Amylase (1167 U/L) and lipase (2630 U/L) were increased. Abdominal computed tomography (CT) demonstrated infiltration with swelling in an uncinate process of the pancreas head and infiltration and mural enhancement in adjacent duodenum, described as grade C according to Balthazar classification (Figure 1).

After conservative management, her symptoms were improved but eight days after admission, her abdominal pain abruptly deteriorated again. Laboratory data were as follows: hemoglobin 12.2 g/dL; hematocrit 35.7%; white blood cell count 9200/μL with 69.2% neutrophils; platelet count 118 000/μL; prothrombin time 14.1 s (INR 1.20); alkaline phosphatase 83 U/L; total bilirubin 0.6 g/dL; ALT 13 U/L; AST 22 U/L; gamma-glutamyl transferase 33 U/L. Amylase (69 U/L) and lipase (153 U/L) were within normal ranges. On a second check-up with abdominal CT, the infiltration and swelling of the pancreas head was improved and there was no stone in the biliary tract. But it showed a newly developed hepatic artery pseudoaneurysm in the left lateral segment and no evidence of acute bleeding related to it as a complication (Figure 2). We performed endoscopic retrograde cholangiopancreatography (ERCP) in order to find the cause of aggravated pain with suspicion of hemobilia. Several filling defects with labile and indeterminate shapes were observed on cholangiogram and, after sphincterotomy, these findings were confirmed to be caused by fresh blood clots without a stone, and blood clots were removed with a balloon catheter and basket (Figure 3). Emergent

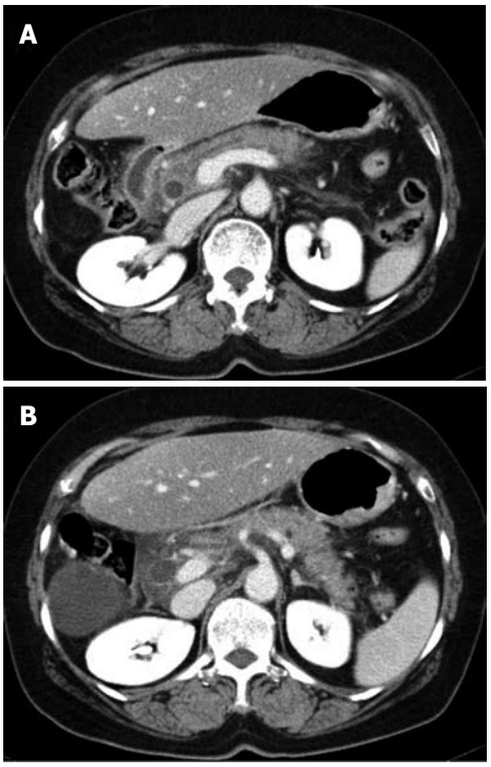

Figure 1 Abdominal computed tomography scans showed diffuse swelling and infiltration in head, body (A) and tail (B) of pancreas without obvious stone, indicating acute pancreatitis.

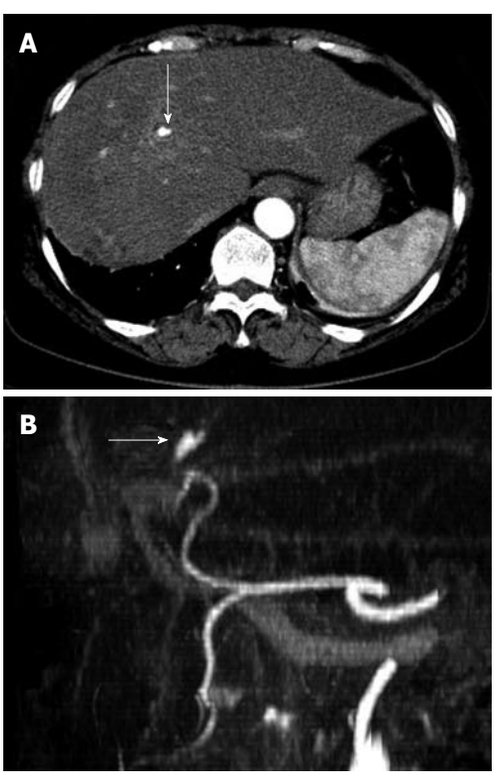

Figure 2 Follow up of abdominal computed tomography scan (A) due to abruptly aggravated abdominal pain and reconstructed computed tomography angiogram (B) showed newly developed hepatic artery pseudoaneurysm in the left lateral segment of liver (white arrows).

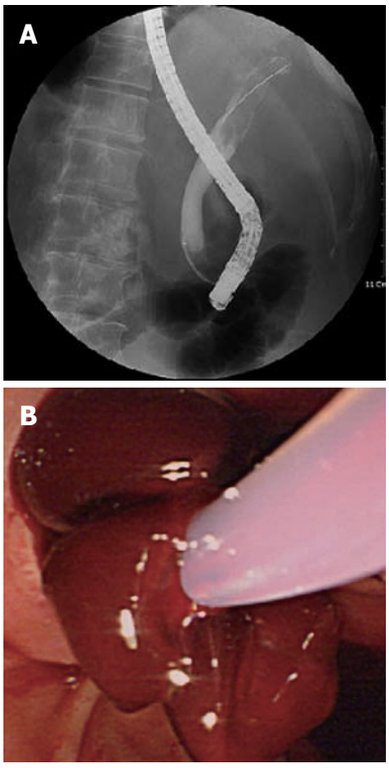

Figure 3 Cholangiogram by endoscopic retrograde cholangiopancreatography showed several amorphous filling defects in the common bile duct (A) and lots of blood clots were seen and removed by basket after endoscopic sphincterotomy (B).

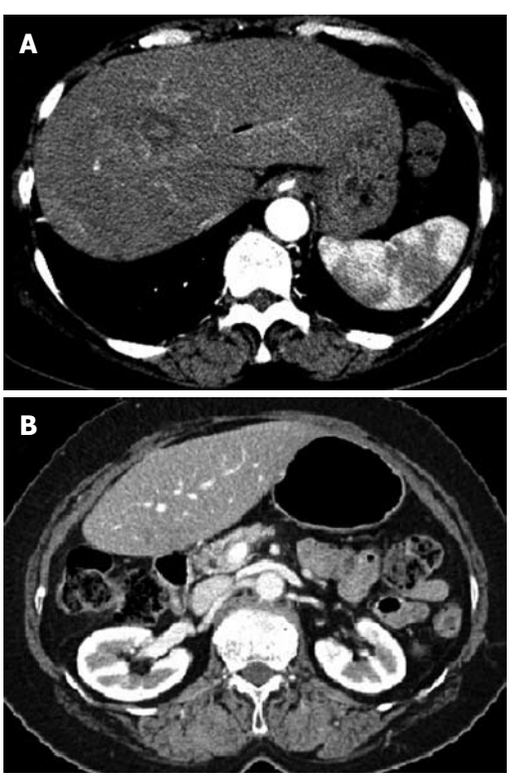

Figure 5 Follow-up of abdominal computed tomography scans 3 mo later showed that both previously noted hepatic artery pseudoaneurysm in the left lateral segment (A) and diffuse swelling and infiltration of pancreas (B) had disappeared.

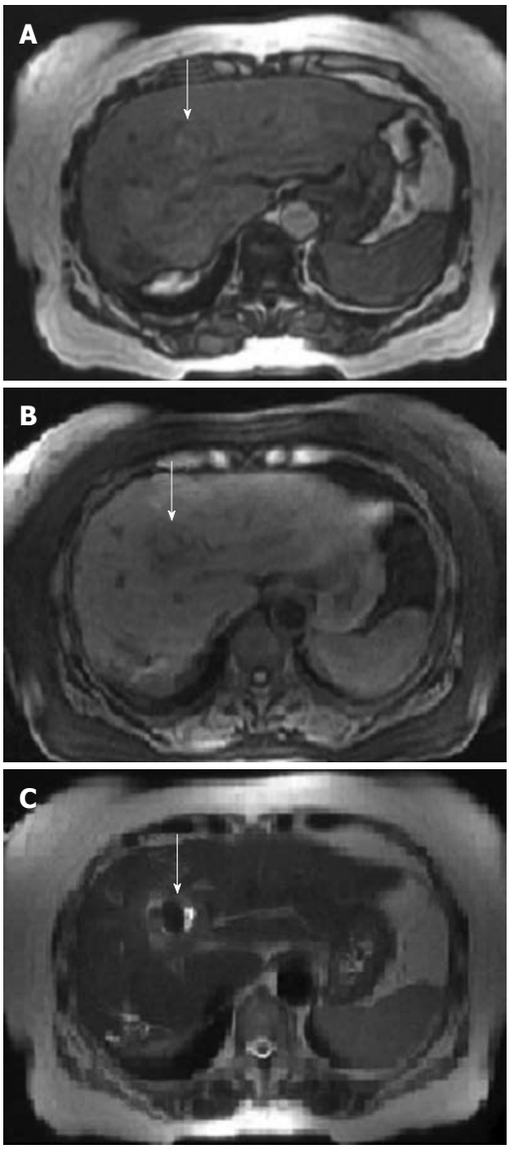

Figure 4 Magnetic resonance imaging scans of the abdomen revealed that hepatic artery pseudoaneurysm was replaced by thrombus formation (white arrows) in the left lateral segment in-phase (A) and out-of-phase (B) T1-weighted images and T2-weighted image (C).

angiography was not performed due to no further acute bleeding evidence.

After ERCP, the patient's pain improved and filling defects caused by blood clots of the extrahepatic duct were not observed on endoscopic nasobiliary drainage cholangiogram. Magnetic resonance imaging showed that dilatation or filling defects of the bile duct disappeared and bleeding of proximal HAP was replaced by thrombus formation (Figure 4). Two days later, angiography revealed no visible contrast leakage and definite HAP. Her symptoms resolved rapidly, and she was discharged on the 16th day after clot removal.

Three months after discharge, abdominal CT demonstrated that minimal bile duct dilatation was noted but HAP in the left lateral segment had disappeared and pancreatitis improved (Figure 5).

DISCUSSION

HAP is mainly caused by acute or chronic artery injuries such as blunt or penetrating injuries and interventional radiological procedures[10]. A minority of their formation may occur as a result of bile duct damage usually associated with stone impaction or procedure-related infection[4-6]. Unlike these reports, this case suggests that HAP occurred due to no invasive procedure and infection but acute pancreatitis. In this case, acute pancreatitis due to unknown cause may have eroded the arterial wall and led to pseudoaneurysm formation and then HAP's rupture causing hemobilia. The mechanism of pseudo-aneurysm formation in pancreatitis is thought to be due to autodigestion of pancreatic enzymes. Among HAP caused by pancreatitis, most cases have been reported to be due to chronic pancreatitis, but HAP caused by acute pancreatitis has only rarely been reported[11]. Furthermore, among reports of HAP due to acute pancreatitis, acute idiopathic pancreatitis causing HAP has not been reported so far.

In this case, it can be presumed that a rupture of preexisting HAP was not detected on initial abdominal CT imaging, resulting in hemobilia and eventually, acute pancreatitis was complicated by hemobilia. However, considering the fact the patient had normal levels of hemoglobin and no bile duct dilatation or filling defects when visiting hospital and the clinical course worsened (e.g., pain) despite the improvement of acute pancreatitis during the treatment, this possibility is very low. She was hospitalized for 8 d without any evidence of this procedure, and hemobilia-induced abdominal pain developed without aggravation of acute pancreatitis. This makes the scenario of pancreatitis-induced HAP more reasonable.

HAP is a rare and potentially fatal disease if it ruptures. In most cases, conservative management is not recommended due to the high rupture rate. Treatment comprises reconstructive surgery, or ligation depending on the size of the lesion and its location in the past. Today, the treatment of choice is selective transcatheter embolization[10,12,13]. However, in very limited cases, which means, unless HAP is not at risk of immediate rupture because of progressively enlarging size or instability, it can be managed by closed medical observation and imaging follow-up with appropriate treatment of the associated infection[14]. In this case, there was only conservative management of acute pancreatitis which is thought to be a cause of HAP. But we could confirm that HAP spontaneously thrombosed without any particular treatment at the image study. Three months after discharge, abdominal CT demonstrated that HAP in the left lateral segment had disappeared.

In summary, we report a rare case of a 61-year-old Korean woman who had HAP caused by acute idiopathic pancreatitis. In this case, HAP spontaneously thrombosed and then disappeared after recovery of acute pancreatitis. This case alerts clinicians that acute pancreatitis can be considered as a potential cause of HAP.

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FRONTIER

Pathogenesis of NSAID-induced gastric damage: Importance of cyclooxygenase inhibition and gastric hypermotility

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Abstract

This article reviews the pathogenic mechanism of non-steroidal anti-inflammatory drug (NSAID)-induced gastric damage, focusing on the relation between cyclooxygenase (COX) inhibition and various functional events. NSAIDs, such as indomethacin, at a dose that inhibits prostaglandin (PG) production, enhance gastric motility, resulting in an increase in mucosal permeability, neutrophil infiltration and oxyradical production, and eventually producing gastric lesions. These lesions are prevented by pretreatment with PGE_2 and antisecretory drugs, and also *via* an atropine-sensitive mechanism, not related to antisecretory action. Although neither rofecoxib (a selective COX-2 inhibitor) nor SC-560 (a selective COX-1 inhibitor) alone damages the stomach, the combined administration of these drugs provokes gastric lesions. SC-560, but not rofecoxib, decreases prostaglandin E_2 (PGE_2) production and causes gastric hypermotility and an increase in mucosal permeability. COX-2 mRNA is expressed in the stomach after administration of indomethacin and SC-560 but not rofecoxib. The up-regulation of indomethacin-induced COX-2 expression is prevented by atropine at a dose that inhibits gastric hypermotility. In addition, selective COX-2 inhibitors have deleterious influences on the stomach when COX-2 is overexpressed under various conditions, including adrenalectomy, arthritis, and *Helicobacter pylori*-infection. In summary, gastric hypermotility plays a primary role in the pathogenesis of NSAID-induced gastric damage, and the response, causally related with PG deficiency due to COX-1 inhibition, occurs prior to other pathogenic events such as increased mucosal permeability; and the ulcerogenic properties of NSAIDs require the inhibition of both COX-1 and COX-2, the inhibition of COX-1 upregulates COX-2 expression in association with gastric hypermotility, and PGs produced by COX-2 counteract the deleterious effect of COX-1 inhibition.

Key words: Non-steroidal anti-inflammatory drug; Gastric damage; Pathogenesis; Gastric motility; Neutrophil

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to treat inflammatory pain. A major limitation to their use, however, is the adverse reaction they cause to the gastrointestinal (GI) tract, including the formation of gastric lesions, the potentiation of ulcerogenic responses to stress, and the impairment of gastric ulcer healing[1-4]. Concerning the mechanism of NSAID-induced gastric damage, prostaglandin (PG) deficiency is

of prime importance to the gastric ulcerogenic response to NSAIDs, yet it has proven to be more complicated than expected and involves multiple, closely interacting elements, including hypermotility, neutrophils, free radicals, and so on[1,5-13].

The PG deficiency caused by NSAIDs is due to the inhibition of cyclooxygenase (COX). COX exists in two isozymes, COX-1 and COX-2; the former is constitutively expressed in various tissues, including the stomach, while the latter appears to be expressed in most tissues in response to growth factors and cytokines[14,15]. This tissue specificity of the COX isozymes has led to the idea that COX-1 is critical for housekeeping actions in the GI mucosa, whereas COX-2 functions under pathological conditions such as inflammation. Indeed, it has been reported that the gastric ulcerogenic properties of NSAIDs are due to the inhibition of COX-1, but not COX-2[16]. However, studies using selective COX-1 and COX-2 inhibitors demonstrated that the GI ulcerogenic effects of NSAIDs are not accounted for solely by inhibition of COX-1, but require inhibition of COX-2 as well[17-21]. It has also been shown that inhibition of COX-1 upregulated COX-2 expression in the GI mucosa, and PGs produced by COX-2 may help maintain the mucosal integrity when there is a deficiency of PGs due to COX-1 inhibition[18,20,21]. This idea was supported by the finding that the selective COX-2 inhibitor by itself damaged the gastric mucosa when the expression of COX-2 was upregulated in the stomach of rats subjected to adrenalectomy (glucocorticoid deficiency) or induction of adjuvant arthritis or *Helicobacter pylori* (*H. pylori*) infection[22-24].

In this article, we reviewed the pathogenesis of NSAID-induced gastric damage, mainly based on our own publications, including the roles of functional events, particularly, gastric hypermotility, as well as the influences of arthritis and *H. pylori* infection, and discussed the relation between COX-1 or COX-2 inhibition and pathogenic elements such as gastric motility and neutrophil infiltration.

GENERAL ASPECTS OF NSAID-INDUCED GASTRIC DAMAGE

Relation to PG deficiency

There is no doubt that a deficiency of endogenous PG is a background factor in NSAID-induced gastric ulceration. Indeed, when various NSAIDs, such as indomethacin (30 mg/kg), flurbiprofen (20 mg/kg), naproxen (40 mg/kg), dicrofenac (40 mg/kg) and aspirin (200 mg/kg), were administered to rats subcutaneously, all of these agents, except aspirin, produced damage in the stomach at doses that significantly decreased the mucosal prostaglandin E2 (**PGE2**) concentration[18,19] (Figure 1). Characteristically, the damage was observed along the long axis of the stomach and consisted mostly of hemorrhagic lesions, with a few non-hemorrhagic lesions. Interestingly, aspirin given subcutaneously did not produce any damage, despite inhibiting PG production as effectively as other NSAIDs[18,25]. Notwithstanding, it is assumed that PG deficiency is causally related to the gastric ulcerogenic

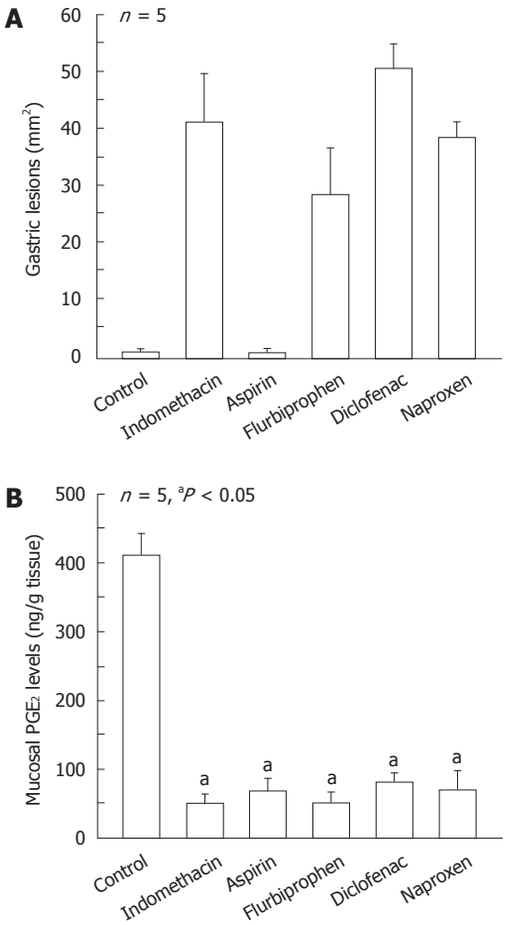

Figure 1 Gastric ulcerogenic responses (A) and changes in mucosal prostaglandin E2 content (B) induced by various non-steroidal anti-inflammatory drugs in rat stomach. The animals were given indomethacin (30 mg/kg), aspirin (200 mg/kg), naproxen (40 mg/kg), flurbiprofen (20 mg/kg) and diclofenac (40 mg/kg) s.c., and killed 4 h later. Data are presented as the mean ± SE in 5 rats. $^{a}P < 0.05$ *vs* control (data from ref. 18 after modification).

action of NSAIDs, but this factor alone is not sufficient for the development of gastric lesions. The reason why parenterally administered aspirin does not cause gastric damage will be discussed in another section of this article.

Effect of various drugs

The development of gastric lesions in response to indomethacin was inhibited by prior administration of PGE2. These lesions were also prevented by antisecretory drugs such as cimetidine, omeprazole and atropine[6,26,27], confirming the importance of luminal acid in the pathogenesis of these lesions. Of interest, since atropine was effective even when 150 mmol of HCl was applied to the lumen, it is assumed that this protective action is not associated with the antisecretory effect and initiated by factors other than inhibition of acid secretion (Figure 2). Neither cimetidine nor omeprazole was effective against indomethacin-induced gastric damage in the presence of exogenous acid. In addition, anti-neutrophil antiserum also reduced the severity of these lesions, but much less effectively than other agents[28]. Pretreatment with both

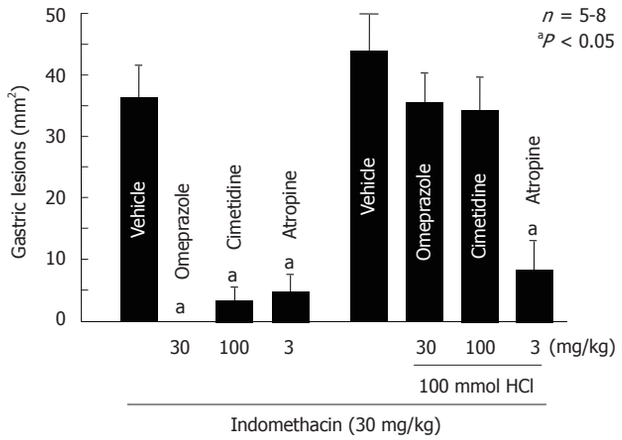

Figure 2 Effects of atropine, cimetidine and omeprazole on gastric lesions produced by indomethacin in rats. The animals were given indomethacin (30 mg/kg, s.c.) and killed 4 h later. Omeprazole (30 mg/kg), cimetidine (100 mg/kg) and atropine (3 mg/kg, s.c.) were given 1 h before indomethacin. In some cases, the animals were given 1 mL of 100 mmol HCl p.o. immediately after the administration of indomethacin. Data are presented as the mean ± SE for 5-8 rats. $^aP < 0.05$ *vs* vehicle (data from refs. 6, 26 and 27 after modification).

atropine and dmPGE2 significantly inhibited the development of gastric lesions at all time points during a 4 h-test period following administration of indomethacin. By contrast, the anti-neutrophil antiserum did not affect the onset but significantly reduced the severity of lesions at 4 h after indomethacin treatment. It is assumed that the gastric ulcerogenic response to indomethacin is prevented by supplementation with PGE2 and inhibition of acid secretion as well as an atropine-sensitive mechanism, not related to the antisecretory action. Neutrophils do not play a role in the onset of these lesions but may be involved in the later extension of the damage. Suematsu *et al*[29] recently reported that the severity of gastric lesions produced by indomethacin was worse in mice lacking heat shock factor 1 (HSF1), a transcription factor for *HSP* genes, than in control mice, while these lesions were ameliorated in transgenic mice expressing HSP70. They suggested that expression of HSP70 ameliorates indomethacin-induced gastric damage by affecting mucosal apoptosis, probably *via* the activation of Bax.

Functional alterations involved in pathogenesis

Gastric hypermotility: Mersereau *et al*[7] first emphasized the importance of stomach hypermotility and mucosal foldings in the genesis of gastric lesions in response to phenylbutazone. As expected, all NSAIDs, except aspirin, increased gastric motility at ulcerogenic doses, leading to the development of gastric lesions[19] (Figure 3). Gastric hypermotility causes microvascular disturbances, especially at specific sites on mucosal foldings, leading to various events including neutrophil-endothelial interaction. Garrick *et al*[30] reported that high-amplitude contractions during cold-restraint stress resulted in a temporal restriction of mucosal blood flow and lowered the mucosal resistance to injury. The gastric damage induced by indomethacin occurred linearly along the long axis of the stomach, and microscopically, was seen at the top or the bottom of mucosal foldings, the sites most influenced by mucosal compression due to contraction of the stomach, where mucosal blood flow is restricted, leading to microvascular disturbances (Figure 4A and B). The inhibition of gastric motility may lead to a flattening of the mucosal foldings and a decrease in microvascular disturbances, resulting in prevention of the fold-related band-like lesions, as observed after the administration of indomethacin[6,10,26,31]. A role for muscle elements in the pathogenic mechanism of gastric ulceration has been demonstrated[6,10,31-33]. Yamaguchi *et al*[32]monitored gastric mucosal hemodynamics and motility simultaneously and found oscillatory changes in the hemodynamics during gastric hypermotility induced by water-immersion stress. We also found that indomethacin caused oscillatory changes in mucosal blood flow associated with hypermotility of the stomach, and such blood flow changes were prevented when the hypermotility was inhibited by atropine[10] (Figure 4C). It is assumed that indomethacin induces the sequential events in the early stage of lesion formation in the stomach during hypermotility; the microcirculatory disturbances due to abnormal compression of the gastric wall, followed by increased vascular permeability, leading to cellular damage[10,33]. Anyway, the indomethacin-induced gastric hypermotility was inhibited by both atropine and PGE2 but not by either omeprazole or the anti-neutrophil antiserum[6,10]. Since atropine prevented indomethacin-induced gastric damage, even in the presence of exogenous acid[26], the inhibitory effect on gastric hypermotility may account for the protective action of this agent. In addition, indomethacin caused oxyradical production and lipid peroxidation in the gastric mucosa, probably resulting from the ischemic-reperfusion changes due to rhythmic hypercontraction of the stomach[10]. Certainly, these changes were prevented by atropine, again confirming an importance of gastric hypermotility. At present, the exact mechanism by which NSAIDs cause gastric hypermotility remains unknown. However, it is assumed that indomethacin-induced gastric hypermotility is mediated by a vagal-cholinergic mechanism, involving a glycoprivic response[6,31].

Neutrophils: Neutrophils have been implicated in the damage associated with NSAIDs[35]. These cells are recruited to a site of injury by chemotaxins and participate in amplifying the inflammatory response. Many studies including ours have shown that indomethacin-induced gastric damage could be prevented by an anti-neutrophil antiserum or monoclonal antibody against the CD18 adhesion molecule[13,34,35]. However, there have been few studies showing the less importance of neutrophils in NSAID-induced gastric damage[36,37]. Trevethick *et al*[36] reported that neutrophil infiltration does not contribute to the ulcerogenic effects of indomethacin in the rat gastric mucosa. Similarly, Melange *et al*[37] showed that neutropenia does not prevent indomethacin-induced gastrointestinal damage in rats. Santucci *et al*[38] even showed that

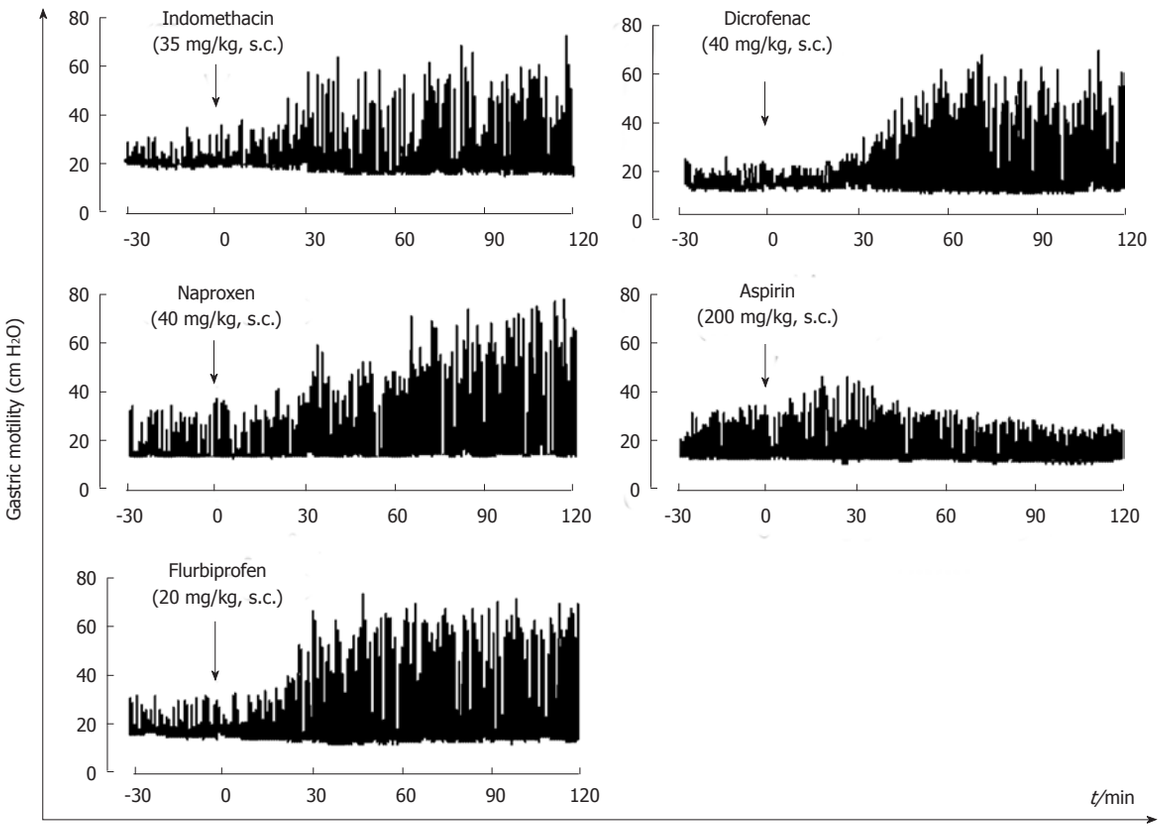

Figure 3 Representative recordings showing the effects of various non-steroidal anti-inflammatory drug on gastric motility in rats. Indomethacin (35 g/kg), aspirin (200 mg/kg), naproxen (40 mg/kg), flurbiprofen (20 mg/kg) or diclofenac (40 mg/kg) was given s.c. after basal motility had stabilized (data from ref.18 after modification).

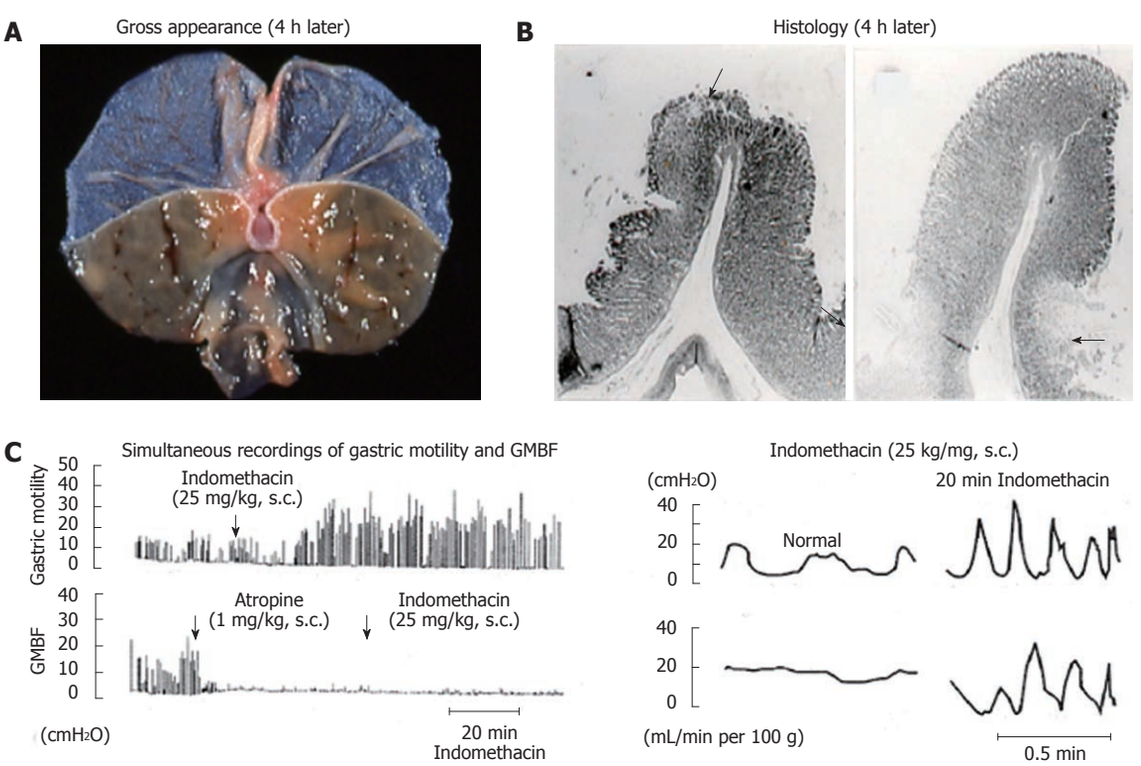

Figure 4 Macro- and microscopical observations of gastric lesions induced by indomethacin in rats (A and B) and simultaneous recordings of gastric motility and mucosal blood flow in the rat before and after administration of indomethacin (C). A, B: The animals were given indomethacin (25 mg/kg, s.c.), and the stomachs were excised 4 h later. Note that the lesions were located, in most cases, on the upper part of the mucosal folds (arrow) and in some cases at the base of the folds (arrows); C: Indomethacin (25 mg/kg, s.c.) was given, while atropine (1 mg/kg, s.c.) was given 1 h after indomethacin treatment. Note that during hypermotility states the mucosal blood flow repeated a decrease and an increase, respectively, corresponding to contraction and relaxation of the stomach wall (data from refs. 8 and 33 after modification). GMBF: Gastric mucosal blood flow.

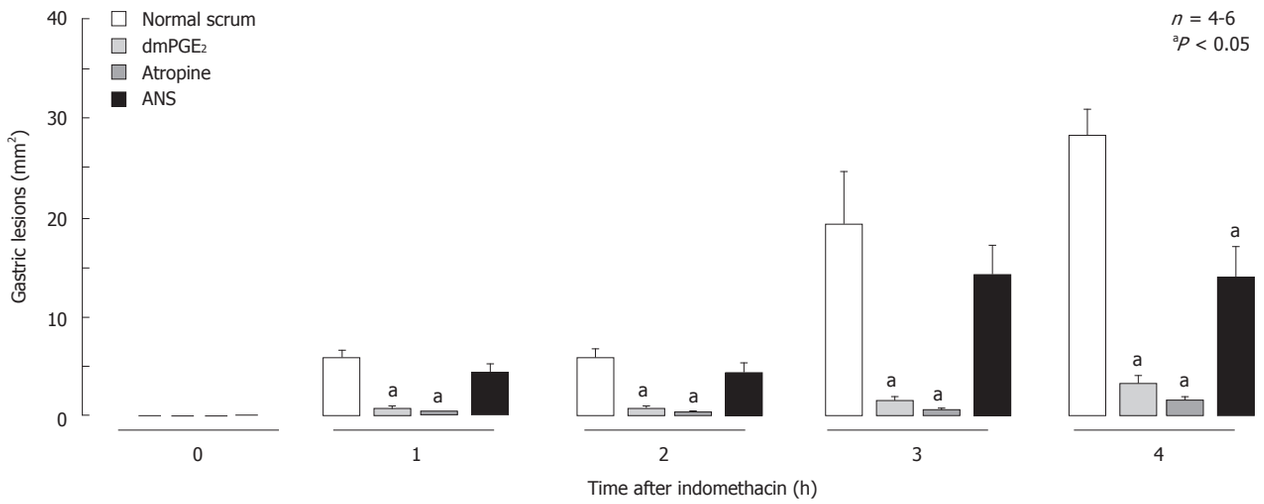

Figure 5 Time-course of changes in gastric lesions following administration of indomethacin (30 mg/kg, s.c.) in rats, with or without pretreatment. Atropine (1 mg/kg) was given s.c. 30 min before indomethacin, while dmPGE$_2$ (10 μg/kg) or anti-neutrophil antiserum (ANS, 0.2 mL/rat) was given i.v. 10 min and 1 h, respectively, before indomethacin. Data are presented as the mean ± SE in 4-6 rats. $^aP < 0.05$ *vs* control group given normal serum (data from ref. 28 after modification).

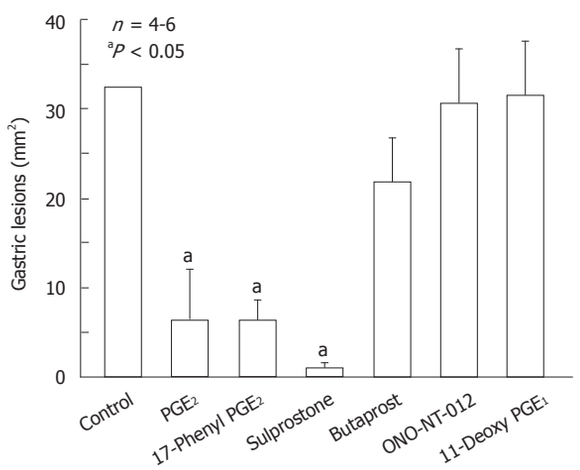

Figure 6 Effects of various prostaglandin E agonists on gastric lesions generated by indomethacin in rats. The animals were given indomethacin (30 mg/kg) s.c. and killed 4 h later. Prostaglandin E$_2$ (PGE$_2$, 0.3 mg/kg), 17-phenyl PGE$_2$ (0.3 mg/kg; EP1 agonist), sulprostone (0.3 mg/kg; EP1/EP3 agonist), butaprost (10 mg/kg; EP2 agonist), ONO-NT-012 (10 mg/kg; EP3 agonist) and 11-deoxy PGE$_1$ (3 mg/kg; EP3/EP4 agonist) were given i.v. 10 min before indomethacin. Data are presented as the mean ± SE in 4-6 rats. $^aP < 0.05$ *vs* control (data from ref. 39 after modification).

granulocyte colony stimulating factor, though it markedly increased myeloperoxidase (MPO) activity, significantly prevented gastric lesions from forming, suggesting no relationship between MPO activity and the ulcerogenic response to indomethacin. A study by Morise *et al*[34] also showed that indomethacin provoked the development of gastric lesions even in CD18, intercellular adhesion molecule 1, or P-selectin-deficient mice, the degree of severity being about 70% of that in wild-type mice. We reported that the anti-neutrophil antiserum caused a significant inhibition of indomethacin- induced gastric damage, yet the degree of inhibition was much less than that shown by atropine or dmPGE$_2$[28] (Figure 5). Furthermore, it was shown that the anti-neutrophil antiserum did not prevent the onset of damage until 3 h after indomethacin treatment and significantly reduced the severity of damage 4 h later. These results suggest that the neutralization of neutrophils itself is not sufficient to prevent the onset of damage but reduces the overall expression of gastric lesions in response to indomethacin. Anthony *et al*[32] examined the sequence of histological changes in the rat stomach after indomethacin treatment and identified an early phase of injury that involves mucosal contraction and vascular fibrin deposition but does not involve neutrophil infiltration. Thus, the neutrophil infiltration may be secondary to the events associated with gastric hypermotility, and not a primary event preceding the onset of gastric damage. Indeed, the increase in MPO activity as well as formation of lesions induced by indomethacin was prevented when the enhanced gastric motility was inhibited by atropine[28].

PROSTAGLANDIN E RECEPTOR SUBTYPE INVOLVED IN PGE$_2$-INDUCED PROTECTION

Although exogenous PGs, especially PGE$_2$, prevent NSAID-induced gastric damage, how they do so remains unknown. We examined the effect of various prostanoids, subtype-specific prostaglandin E (EP) agonists, on the development of gastric lesions in response to indomethacin and determined which functional alteration is most closely associated with this action[39]. Such an approach would be helpful to understanding of which event(s) may be critically important to the pathogenic mechanism of NSAID-induced gastric damage.

Gastric ulcerogenic response

PGE$_2$ exhibited a potent inhibitory effect on indomethacin-induced gastric damage. This effect was mimicked by other prostanoids such as 17-phenyl PGE$_2$ (EP1 agonist) and sulprostone (EP1/EP3 agonist)[39] (Figure 6). Neither

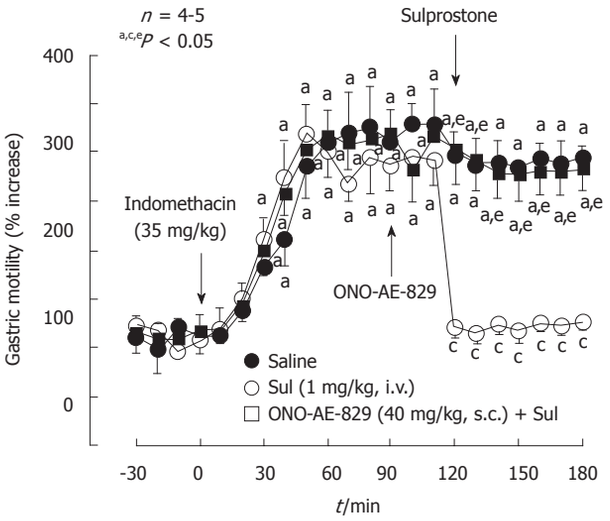

Figure 7 Effect of sulprostone on the increased gastric motility caused by indomethacin in rats. Indomethacin (35 mg/kg) was given s.c.. Sulprostone (Sul, 1 mg/kg) was given i.v. as a single injection 2 h after indomethacin, while ONO-AE-829 (40 mg/kg) was given s.c. 30 min before the administration of sulprostone. Data are presented as the mean ± SE of values determined every 10 min in 4-5 rats. Significant difference at $P < 0.05$; [a]from basal values in the corresponding group; [c]from saline group; [e]from indomethacin plus sulprostone (data from ref. 39 after modification).

butaprost (EP2 agonist), ONO-NT-012 (EP3 agonist), nor 11-deoxy PGE_1 (EP3/EP4 agonist), was effective in reducing the severity of these lesions, indicating that the activation of the EP2, EP3, and EP4 receptors does not provide gastric protection against indomethacin[39,40]. These results strongly suggest that the protective effect of PGE_2 against indomethacin- induced gastric damage is brought about by activation of the EP1 receptor. This idea is supported by the finding that the protective action of PGE_2 against indomethacin was totally mitigated by prior administration of ONO-AE-829, a selective EP1 receptor antagonist. In addition, indomethacin caused gastric damage similarly in both wild-type and knockout mice lacking EP1 or EP3 receptors, yet the protective action of PGE_2 was observed in wild-type and EP3-receptor knockout mice but not in mice lacking EP1 receptors. Given the above findings, it is assumed that PGE_2 prevents indomethacin-induced gastric ulceration through the activation of EP1 receptors.

Gastric functional alterations

The prostanoids exhibiting a preference for the EP1 receptors inhibited gastric hypermotility and damage in response to indomethacin (Figure 7). These effects were antagonized by ONO-AE-829, an EP1 antagonist, strongly suggesting that the antigastric motility effect of PGE is paralleled by a reduction in gross mucosal injury of the stomach with the use of indomethacin. Both butaprost and ONO-NT-012 reportedly increased gastric mucosal blood flow[41], yet these drugs did not provide any protection against indomethacin-induced gastric damage, suggesting that the protective action is not functionally associated with the increased mucosal blood flow. Certainly, since inhibition of gastric motility may lead to an attenuation of microvascular disturbances due to contraction of the stomach, prostanoids acting through EP1 receptors may help maintain mucosal blood flow after the administration of indomethacin. It is assumed that the actions of PGE_2 to prevent indomethacin-induced gastric damage are functionally associated with the inhibition of gastric hypermotility. The mechanism by which PGE_2 inhibits gastric motility through EP1 receptors remains unknown. Milenov *et al*[42] reported that PGE_2 relaxed the circular muscle but contracted the longitudinal muscle of the canine stomach. Narumiya and his group reported the distribution of mRNA of the EP receptors along the gastrointestinal tract[43,44]. They found that strong signals for EP1 transcripts occurred in the smooth muscle cells in the muscularis mucosa throughout the tract. Since EP1 receptors are coupled to phosphatidyl inositol turnover[45], it is assumed that contraction of longitudinal smooth muscle by PGE_2 is associated with an increase of cytosolic calcium. Contraction of circular smooth muscle leads to the appearance of mucosal folds, which have been implicated in the pathogenesis of ulcers including indomethacin-generated gastric lesions[6-8,10,13]. At present, the mechanism by which PGE_2 relaxes circular smooth muscle through activation of EP1 receptors still remains unclear.

It is known that PGE_2 has an inhibitory effect on neutrophil functions, including chemotaxis[46]. We confirmed that PGE_2 exhibited an inhibitory effect on the migration of neutrophils caused by formyl-methionyl-leucyl-phenylalanine *in vitro*[39]. The same inhibitory action was shown by both butaprost and 11-deoxy PGE_1, but not by 17-phenyl PGE_2, sulprostone, or ONO-NT-012, clearly indicating that the anti-neutrophil chemotaxis action of PGE_2 is mediated by activation of EP2 and EP4 receptors (Figure 8). Thus, it is assumed that the inhibition of neutrophil migration by itself is not sufficient to reduce the overall expression of gastric lesions in response to indomethacin. Since the increase in MPO activity as well as ulceration induced by indomethacin was prevented when the enhanced gastric motility was inhibited by atropine[13,28,47], it is likely that the neutrophil infiltration is secondary to the event associated with gastric hypermotility following indomethacin treatment. As mentioned before, Melange *et al*[37] even showed that NSAID-induced gastric injury is neutrophil-independent in the neutropenic rats. These results strongly suggest that the protective effect of PGE_2 is functionally associated with the inhibition of gastric motility, but not neutrophil infiltration.

ROLE OF COX INHIBITION IN NSAID-INDUCED GASTRIC DAMAGE

Ulcerogenic properties of various COX inhibitors

COX, the enzyme responsible for PG production, exists in two isozymes, the constitutively expressed COX-1 and the inducible COX-2[14,15]. NSAIDs inhibit the activity of both COX-1 and COX-2, yet it is believed that the inhibi-

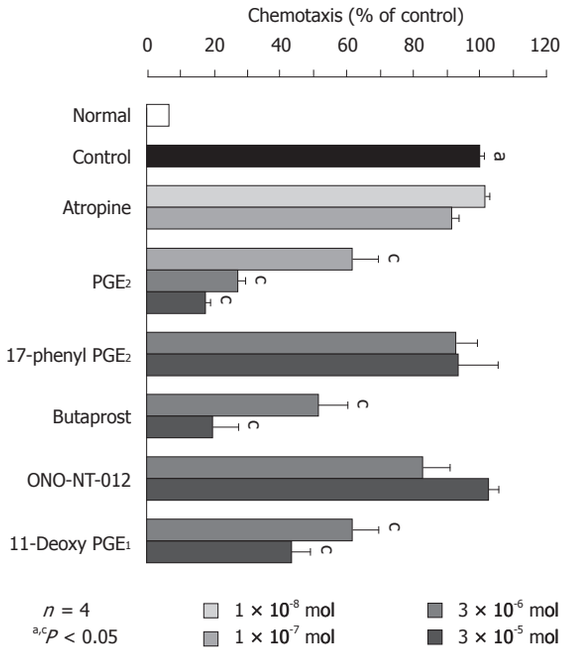

Figure 8 Effects of atropine and various prostaglandin E agonists on the neutrophil chemotaxis stimulated by formyl-methionyl-leucyl-phenylalanine. Neutrophils were pretreated for 45 min with atropine and various prostaglandin E agonists such as PGE_2, 17-phenyl PGE_2, butaprost, ONO-NT-012 and 11-deoxy PGE_1 at the indicated concentrations, and then the cells were stimulated by incubation with formyl-methionyl-leucyl-phenylalanine (fMLP, $1x10^{-7}$ mol) for another 45 min. Data are expressed as a percentage of the stimulated values (control) observed in the presence of fMLP and represent the mean ± SE from 4 experiments. Significant difference at $P < 0.05$; [a]from normal; [c]from control (data from ref. 39 after modification).

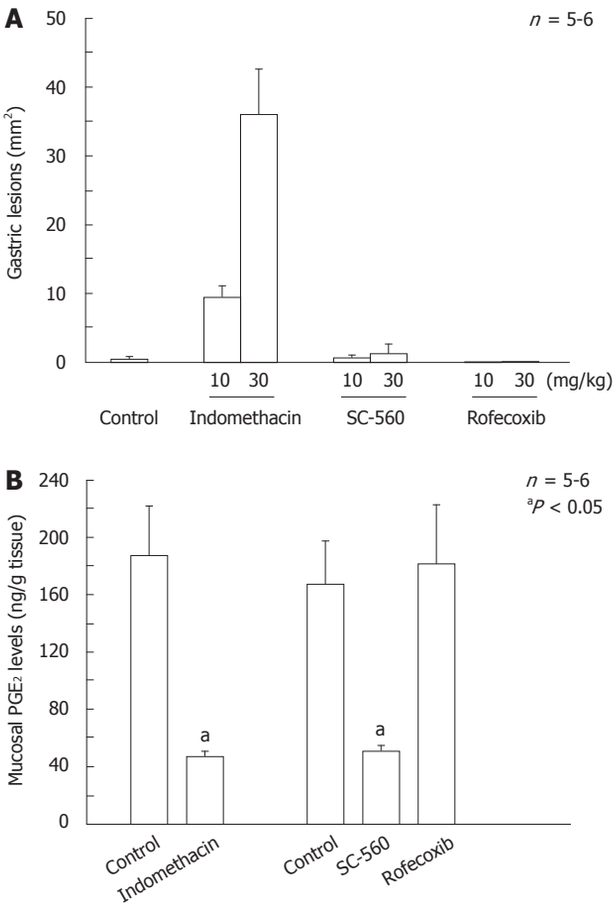

Figure 9 Gastric ulcerogenic responses to various cyclooxygenase inhibitors and their effects on prostaglandin E_2 content in rat stomachs. A: Gastric ulcerogenic responses induced by various cyclooxygenase (COX) inhibitors in rat stomach. The animals were given indomethacin (nonselective COX inhibitor; 10 and 30 mg/kg), SC-560 (selective COX-1 inhibitor; 10 and 30 mg/kg), or rofecoxib (selective COX-2 inhibitor; 10 and 30 mg/kg) p.o. and killed 8 h later; B: Effects of various COX inhibitors on gastric mucosal prostaglandin E_2 (PGE_2) content in rats. The animals were given indomethacin (10 mg/kg), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg) p.o. and killed 2 h later. Data are presented as the mean ± SE in 5-6 rats. [a]$P < 0.05$ *vs* control (data from refs. 18 and 19 after modification).

tion of COX-1 is critical for their ulcerogenic properties in the stomach. However, Wallace *et al*[17] reported that inhibition of both COX-1 and COX-2 is required for the induction of gastric lesions. This finding was confirmed in our experiment using the selective COX-1 inhibitor SC-560 and the COX-2 inhibitor rofecoxib[19,20,40]. As shown in Figure 9, indomethacin at 30 mg/kg produced gastric lesions with a marked decrease in mucosal PGE_2 content. As expected, the selective COX-2 inhibitor rofecoxib did not induce any damage at 30 mg/kg, with no effect on mucosal PGE_2 content. Likewise, the COX-1 inhibitor SC-560 did not cause gastric damage even at 30 mg/kg, despite inhibiting PGE_2 production, as effectively as indomethacin. However, these agents given together provoked damage in the stomach. In this case, when SC-560 at 10 mg/kg was given together with various doses of rofecoxib, the severity of the damage increased depending on the dose of the selective COX-2 inhibitor (Figure 10). Similarly, when rofecoxib at 10 mg/kg was given together with SC-560, the damage increased in a manner dependent on the dose of SC-560. These results do not support the paradigm that COX-1 but not COX-2 plays a "housekeeping" role in the stomach, and strongly suggest that inhibition of both COX-1 and COX-2 is required for the occurrence of NSAID-induced gastric injury. Langenbach *et al*[47] reported that the indomethacin-induced gastric lesions were inhibited in animals lacking the COX-1 enzyme, casting a doubt on the role of PG/COX-1 in the pathogenesis. However, since the inhibition of COX-1 induces the expression of COX-2[19,48], it is possible that the PGs produced by COX-2 compensate for the PG deficiency in COX-1 knockout animals.

COX inhibition and various pathogenic events

The pathogenic mechanism of NSAID-induced gastric damage involves multiple functional alterations, including gastric hypermotility, microcirculatory disturbance, neutrophil activation, and microvascular permeability[5-13]. A marked increase in gastric motility was observed after the administration of SC-560 but not rofecoxib, although the duration of the hypermotility was short as compared with that induced by nonselective COX inhibitors, suggesting that gastric hypermotility induced by NSAIDs is associated with a PG deficiency caused by COX-1 inhibition[18]. Likewise, SC-560 but not rofecoxib increased

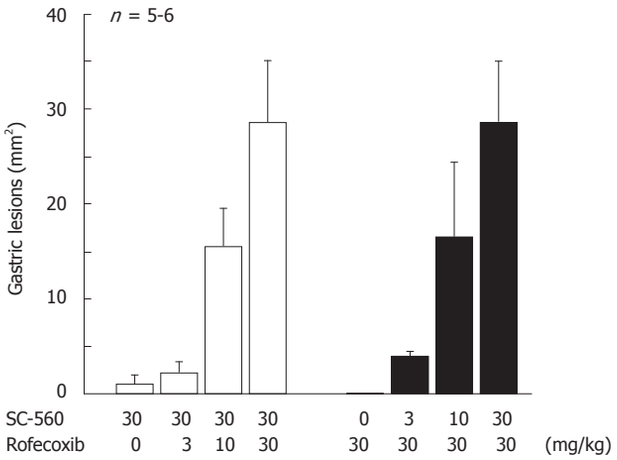

Figure 10 Gastric ulcerogenic response induced by combined administration of SC-560 and rofecoxib in rats. The animals were administered SC-560 (3-30 mg/kg) and rofecoxib (3-30 mg/kg) p.o. either alone or in combination, and killed 8 h later. Data are presented as the means ± SE in 5-6 rats (data from ref. 18 after modification).

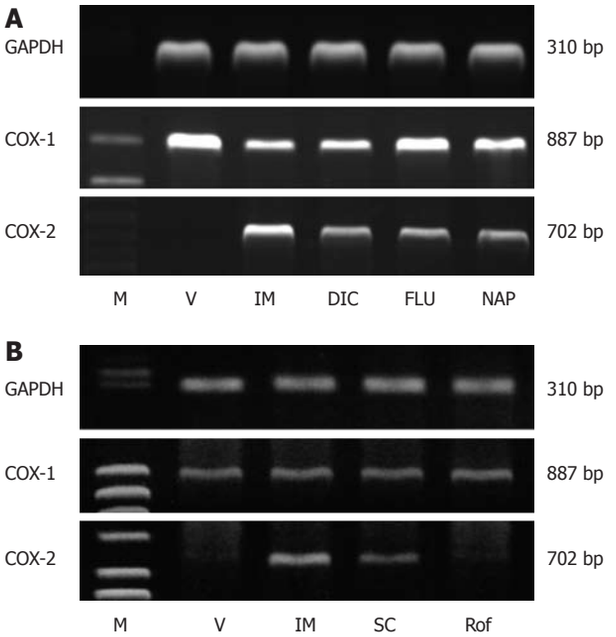

Figure 11 Gene expression of cyclooxygenase-1 and cyclooxygenase-2 in rat gastric mucosa after administration of various non-steroidal anti-inflammatory drugs (A) or various cyclooxygenase inhibitors (B). The animals were given indomethacin (IM, 30 mg/kg), naproxen (NAP, 40 mg/kg), flurbiprofen (FLU, 20 mg/kg), dicrofenac (DIC, 40 mg/kg), SC-560 (SC, 30 mg/kg), or rofecoxib (Rof, 30 mg/kg) p.o., and the expression of cyclooxygenase (COX)-1 and COX-2 mRNA was examined by reverse transcription polymerase chain reaction 4 h later. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; M: Marker, V: Vehicle (data from refs. 18 and 19 after modification).

microvascular permeability in the stomach, similar to indomethacin. These results for SC-560 are reasonable, because indomethacin at an ulcerogenic dose is known to cause microcirculatory disturbances resulting from abnormal mucosal compression of the stomach wall due to gastric hypermotility[10]. On the other hand, Wallace *et al*[17] reported that SC-560, but not celecoxib, decreased the gastric mucosal blood flow, suggesting a role for PGs derived from COX-1 in the maintenance of mucosal blood flow. They also showed that the selective COX-2 inhibitor celecoxib elicited neutrophil adherence in mesenteric venules, as potently as indomethacin, whereas the selective COX-1 inhibitor SC-560 did not. However, we observed that neither SC-560 nor rofecoxib alone affected MPO activity in the gastric mucosa, yet these two agents together apparently increased MPO activity to the levels comparable to those induced by indomethacin[19]. This event might be hampered by PGs derived from COX-2, probably at later stages following gastric hypermotility, since microcirculatory disturbances are known to enhance the adhesion of neutrophils to endothelial cells[13,34]. These results strongly suggest that the inhibition of both COX-1 and COX-2 is required for enhancement of neutrophil migration in the gastric mucosa and that neutrophils may be involved in the damage process later on, but do not play a role in the onset of gastric damage induced by NSAIDs.

Upregulation of COX-2 expression

The most important event is that the expression of COX-2 mRNA was induced in the gastric mucosa after administration of NSAIDs[18,19] (Figure 11A). The upregulation of COX-2 expression was similarly observed in the rat stomach after administration of SC-560 but not rofecoxib, suggesting a causal relationship between COX-1 inhibition and COX-2 expression (Figure 11B). We also reported the upregulation of COX-2 expression in the small intestine following administration of both indomethacin and SC-560[20,48,49]. It is assumed that inhibition of COX-1 induces a PG deficiency but upregulates the expression of COX-2, which contributes to a restoration of PG production. Indeed, the mucosal PGE_2 content of the stomach was markedly decreased by SC-560, yet values recovered significantly 8 h after the administration in a rofecoxib-sensitive manner[18] (Figure 12A). Thus, the upregulation of COX-2 expression following inhibition of COX-1 may represent a compensatory response to inhibition of PG biosynthesis and contribute to maintenance of the mucosal integrity of the stomach. This speculation is supported by the fact that combined treatment with SC-560 and rofecoxib did provoke gross damage in the stomach, and that such damage was prevented by administration of PGE_2 4 h after the use of COX inhibitors[19]. The exact mechanism by which the expression of COX-2 is induced by inhibition of COX-1 remains unknown. Since the expression of COX-2 induced by indomethacin was attenuated by atropine at the dose that inhibited the gastric hypermotility[6,21,48], it is possible that the upregulation of COX-2 expression is due to vascular injury caused by abnormal mucosal compression of the stomach wall during gastric hypermotility (Figure 12B). Indeed, atropine significantly inhibited the recovery of PGE_2 levels following administration of SC-560, similar to rofecoxib[48]. Alternatively, because NSAIDs release tumor necrosis factor α (TNF-α)[38,50],

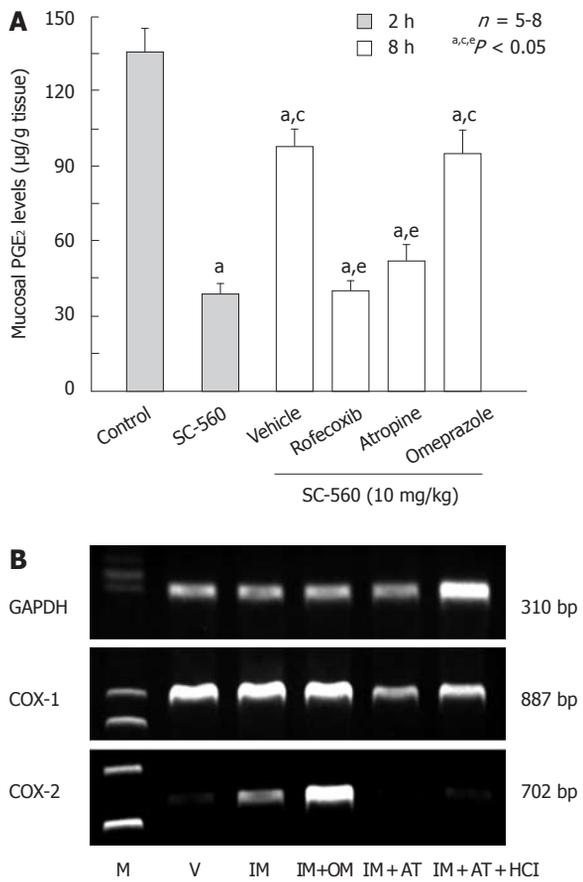

Figure 12 Effects of various drugs on prostaglandin E_2 production and cyclooxygenase-2 expression in rat gastric mucosa. A: Effects of various drugs on prostaglandin E_2 (PGE_2) levels in the rat gastric mucosa at 8 h after administration of SC-560. The animals were administered SC-560 (10 mg/kg) p.o., and killed 2 or 8 h later. Rofecoxib (30 mg/kg) was given p.o. together with SC-560 while omeprazole (OM, 30 mg/kg) or atropine (AT, 3 mg/kg) was given s.c. 1 h before the administration of SC-560. Data are presented as the mean ± SE in 6 rats. Significant difference at $P < 0.05$; [a]from control; [c]from SC-560 (2 h); [e]from vehicle; B: Effect of OM and AT on cyclooxygenase (COX)-2 expression after administration of indomethacin (IM) in rat stomach. The animals were administered IM (30 mg/kg) p.o., and killed 4 h later. OM (30 mg/kg) or AT (3 mg/kg) was given s.c. 1 h before indomethacin. Some animals were given 1 mL of 100 mmol HCl immediately after administration of IM. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; M: Marker, V: Vehicle (data from ref. 48 after modification).

the upregulation of COX-2 expression observed under COX-1 inhibition is mediated by TNF-α. Omeprazole had no effect on the expression of COX-2 induced by indomethacin, suggesting no role for luminal acid in this phenomenon[48].

POTENTIATION OF NSAID-INDUCED GASTRIC DAMAGE

Adrenalectomy

Takeuchi *et al*[9] demonstrated that indomethacin-induced gastric damage was markedly aggravated in adrenalectomized rats and the dose required to produce lesions was decreased in these rats. Filaretova *et al*[22] confirmed the aggravation of NSAID-induced gastric ulceration in adrenalectomized rats (glucocorticoid-deficient conditions) and further investigated the influence of adrenalectomy on the expression of COX-2 in the stomach as well as the ulcerogenic effect of celecoxib (a selective COX-2 inhibitor) in these rat stomachs. It was found that adrenalectomy decreased plasma corticosterone levels and markedly aggravated indomethacin-induced gastric lesions. This aggravation was significantly prevented by corticosterone replacement, suggesting that glucocorticoid deficiency is the reason for the aggravation of indomethacin-induced gastric injury in adrenalectomized rats. Moreover, in adrenalectomized rats, celecoxib provoked gross damage that was prevented by corticosterone pretreatment. Mucosal PGE_2 content was increased 3-fold after adrenalectomy, and this response was prevented by both celecoxib and corticosterone. COX-2 mRNA expression was up-regulated in the stomach of adrenalectomized rats, but suppressed by corticosterone replacement. It is assumed that adrenalectomy, probably *via* a glucocorticoid deficiency, increases PGE_2 production in the stomach due to COX-2 expression, and the selective COX-2 inhibitor produces gastric lesions by suppressing this additional PG production in adrenalectomized rats. These findings also support the idea that COX-2 as well as COX-1 play a role in maintaining gastric mucosal integrity under glucocorticoid-deficient conditions.

Adjuvant arthritis

Patients with rheumatoid arthritis (RA) are reportedly more susceptible to NSAID-induced gastropathy than other NSAID users[51,52]. This observation has been validated in arthritic rat models induced by injecting Freund's complete adjuvant into the planter region of a hindfoot, where the gastric ulcerogenic response to indomethacin was markedly aggravated in comparison with normal animals[23,53,54]. Since the aggravation of these lesions in arthritic rats was dependent on the degree of arthritic change, it is assumed that there is a cause-effect relationship between the systemic inflammation and the increased gastric mucosal susceptibility to indomethacin. As several studies including ours showed increased serum gastrin levels and acid secretion in arthritic rats[53,55], it is speculated that the increased gastric ulcerogenic response is partly attributable to hyperacidity in the stomach. However, because the aggravation of these lesions was similarly observed in arthritic rats, even in the presence of exogenous acid to mask endogenous hyperacidic conditions[53], it is unlikely that the increased mucosal susceptibility to indomethacin in arthritic rats is associated with the increase of acid secretion. Interestingly, the aggravation of indomethacin-induced gastric damage in arthritic rats was prevented by prior administration of N^G-nitro-L-arginine methyl ester, a nonselective nitric oxide synthase (NOS) inhibitor, and aminoguanidine, a selective inducible NOS (iNOS) inhibitor, as well as dexamethasone, an inhibitor of iNOS mRNA transcription, although they did not affect the severity of the lesions observed in normal rats[53]. Moreover, the distinct expression of iNOS mRNA was observed in the stomach of arthritic rats, accompanied with an increase in NO production. These

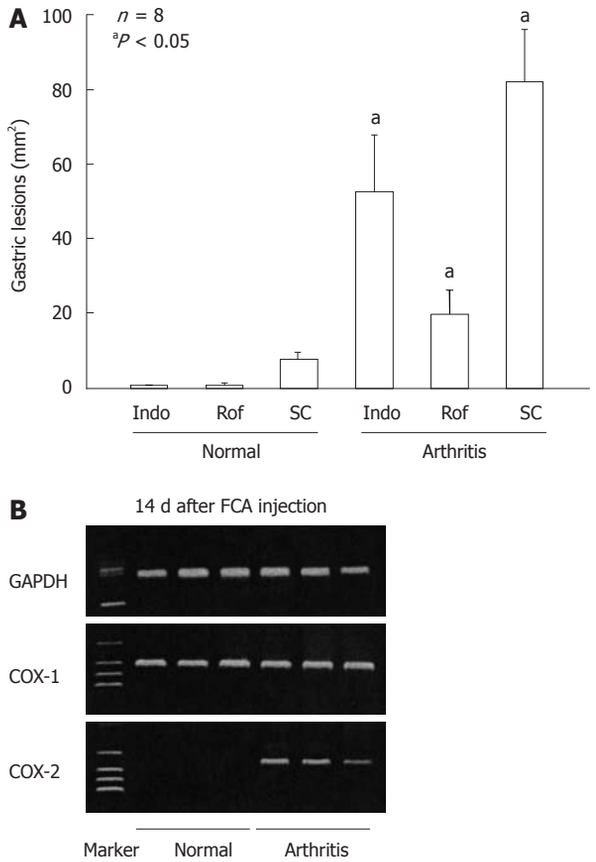

Figure 13 Gastric ulcerogenic effect of indomethacin, rofecoxib and SC-560 and the expression of cyclooxygenase-1 and cyclooxygenase-2 mRNA in the gastric mucosa of normal and arthritic rats. A: Arthritis was induced by injecting Freund's complete adjuvant (FCA) into the plantar region of the right hindfoot, and the experiments were performed 14 d after the injection. Indomethacin (Indo) (3 mg/kg), rofecoxib (Rof) (30 mg/kg), or SC-560 (SC) (30 mg/kg) were administered p.o., and the animals were killed 4 h later. Data are presented as the mean ± SE in 4-8 animals, $^aP < 0.05$ *vs* the corresponding group in normal rats; B: COX-2 mRNA was not detected in the normal rats, but clearly observed in the arthritic rats on day 14 after the FCA injection, whereas COX-1 mRNA was observed in the stomach of both normal and arthritic rats. Lane 1, marker; lanes 2-4, normal rats; lanes 5-7, arthritic rats (data from ref. 23 after modification). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

findings suggest that the increased ulcerogenic response to NSAIDs in arthritic rats is associated at least partly with endogenous NO, mainly produced by iNOS. It is possible that the increased susceptibility of arthritic rat stomachs to NSAIDs might be explained by production of peroxynitrite, resulting from the interaction of NO/iNOS with superoxide radicals[56].

As mentioned, selective COX-2 inhibitors such as rofecoxib and celecoxib, even at a higher dose (100 mg/kg), did not damage the normal rat stomach[18]. However, they produced gross lesions in the stomach of arthritic rats[23] (Figure 13A). Moreover, PG generation in the arthritic rat stomach was significantly enhanced with a concomitant increase of COX-2 expression (Figure 13B). Certainly, the mucosal PG content was reduced by indomethacin in both normal and arthritic rat stomachs. In contrast, the COX-2 inhibitor rofecoxib did not affect PG generation in normal rats but significantly decreased PG content in the stomach of arthritic rats, suggesting that COX-2 activity caused the increase in PG production in arthritic rat stomachs. These findings suggest that COX-2 plays an important role in maintaining the integrity of the gastric mucosa in arthritic rats. It is possible that the increased COX-2 expression level in the stomach occur in association with inflammation or stress caused by pain. Since SC-560, a selective COX-1 inhibitor, worsened stress-induced gastric lesions[57], SC-560 may produce hemorrhagic lesions in the stomach by potentiating the ulcerogenic response to arthritis-related stress. Further study is certainly required to verify this point.

H. pylori infection

Takahashi *et al*[24] examined the expression of COX proteins and production of PGE_2 in the gastric mucosa during *H. pylori* infection. The level of COX-1 remained nearly constant during the infection. In contrast, the COX-2 protein was not found in normal mucosa or in *H. pylori* -infected mucosa at 2 wk, but was markedly elevated 4 wk after the infection, with a significant rise in PGE_2 production. To investigate the role of COX-2 in *H. pylori*-induced gastritis, they also examined the effects of NSAIDs on PGE_2 production and gastric pathology caused by *H. pylori*. NS-398 (a COX-2-selective inhibitor) at 10 mg/kg or indomethacin at 2 mg/kg was administered for 4 wk to normal and *H. pylori*-infected animals. NS-398 failed to inhibit PGE_2 production in normal mucosa but significantly reduced the *H. pylori*-increased PGE_2 production. In contrast, indomethacin potently inhibited PGE_2 production in both normal and *H. pylori*-infected mucosa. Hemorrhagic erosions, neutrophil infiltration, lymphoid follicles, and epithelium damage were induced by *H. pylori* infection. NS-398 and indomethacin aggravated these pathological changes, but did not increase viable *H. pylori* numbers. Overall, these results indicate that both COX-2 and COX-1 might play anti-inflammatory roles in *H. pylori*-induced gastritis. Similar findings were also obtained by Tanigawa *et al*[58] who showed that PGE_2 derived from either COX-1 or COX-2 is involved in the regulation of gastric mucosal inflammation and contributes to the maintenance of mucosal integrity during *H. pylori* infection *via* inhibition of TNF-α expression.

BIPHASIC EFFECT OF ASPIRIN

Conventional NSAIDs cause gastric damage with a concomitant decrease in mucosal PGE_2 production, irrespective of the route of administration[18,19]. However, since aspirin is not ulcerogenic in the stomach, despite that it reduces mucosal PGE_2 production as effectively as other NSAIDs, it is likely that a depletion of endogenous PGs by itself is not sufficient for gastric lesions to form and other factors are required for the onset of gastric damage. However, when administered orally, aspirin damages the stomach, similar to other NSAIDs. Several studies have proposed a role for neutrophils or TNF-α in the

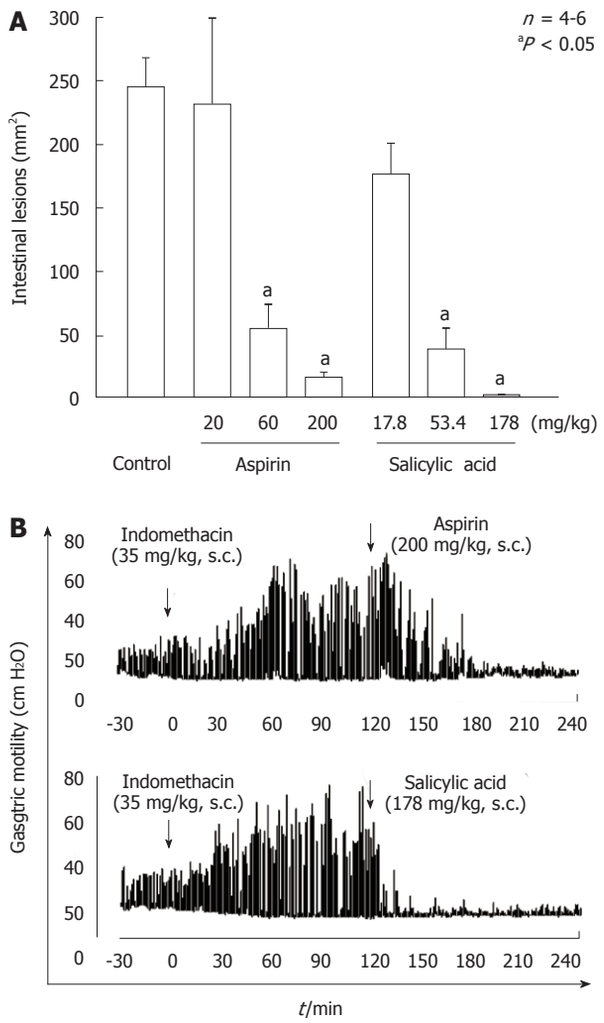

Figure 14 Effects of aspirin and salicylic acid on gastric lesions (A) and gastric hypermotility (B) caused by indomethacin in rats. A: The animals were given indomethacin (35 mg/kg) s.c., and killed 24 h later. Aspirin (20-200 mg/kg) or salicylic acid (17.8-178 mg/kg) was given s.c. 30 min before indomethacin. Data are presented as the mean ± SE in 4-6 rats. $^aP < 0.05$ *vs* control; B: Animals were given indomethacin (30 mg/kg) s.c. and subsequently aspirin (200 mg/kg) or salicylic acid (178 mg/kg) s.c. 2 h later. Note that both aspirin and salicylic acid markedly inhibited the intestinal hypermotility induced by indomethacin, with the effect of salicylic acid appearing much earlier than that of aspirin (data from ref. 25 after modification).

pathogenesis of NSAID-induced gastric damage[34,35,50]. These events are considered to occur in relation to a decrease in PG biosynthesis in the gastric mucosa due to suppression of COX activity. However, aspirin given parenterally inhibited PGE_2 production in the stomach, yet did not cause any damage in the mucosa[19]. Furthermore, salicylate reportedly inhibited TNF-α production by suppressing nuclear factor kappa B[59]. Considering all these points, we assumed that the topical irritant action of oral aspirin is most crucial in causing gastric mucosal damage.

As mentioned earlier, aspirin does not damage the stomach but shows a dose-dependent inhibition of indomethacin-induced gastric injury[24] (Figure 14A). This result is consistent with the finding by Robert *et al*[60], who showed for the first time that aspirin provided protection against gastric damage in response to various noxious agents including indomethacin. Following the subcutaneous administration of aspirin (200 mg/kg) in rats, plasma levels of salicylate increased with time, reaching almost a plateau within 30 min, and remained elevated for more than 4 h. A small amount of aspirin was detected in the blood for the first 15 min, but it had disappeared almost totally 30 min later. As expected, since salicylate, the major metabolite of aspirin, also prevented indomethacin-induced gastric damage, it is possible that the protective action of aspirin is mediated by salicylate. Interestingly, aspirin and salicylate did not increase basal gastric motility but suppressed the enhanced gastric motility following indomethacin treatment, suggesting again a relationship between the inhibition of gastric hypermotility and prevention of gastric damage (Figure 14B)[25]. At present, the exact mechanism by which salicylate (aspirin) suppresses the gastric hypermotility induced by indomethacin remains unknown.

Unlike other NSAIDs, COX-2's acetylation by aspirin switches eicosanoid biosynthesis from PGE_2 to lipoxin A4, which exerts protective effects in the stomach. Co-administration of aspirin and a selective COX-2 inhibitor, such as celecoxib or rofecoxib, resulted in substantially more severe gastric injury than that produced with either agent alone[61,62]. We also observed that the gastric ulcerogenic response to aspirin was significantly worsened by co-administration of rofecoxib but not SC-560[63]. These results confirmed the importance of COX-2's inhibition in this phenomenon related to the suppression of lipoxin A4's production.

SUMMARY AND FUTURE PROSPECTS

The gastric ulcerogenic properties of NSAIDs are not accounted for solely by the inhibition of COX-1 and require the inhibition of both COX-1 and COX-2[17-19]. This idea is supported by the finding that neither the selective COX-1 nor COX-2 inhibitor alone caused gross damage in the stomach, but the combined administration of these two inhibitors provoked the development of gastric lesions. Indomethacin caused an increase of gastric motility, microvascular permeability and MPO activity following administration of indomethacin[6,10,26,28,34,64,65] and showed that the former two events were due to COX-1 inhibition, but the increase of MPO activity occurred only when both COX-1 and COX-2 were inhibited[19]. On the other hand, NSAIDs up-regulate the expression of COX-2, and the PGs produced by COX-2 may suppress the neutrophil-endothelial interaction caused by the vascular disturbances due to COX-1 inhibition. These sequential events related to COX-1 and/or COX-2 inhibition explain why gastric damage occurs only when both COX-1 and COX-2 are inhibited (Figure 15). It should also be noted that selective COX-2 inhibitors by themselves damage the gastric mucosa when an overexpression of COX-2 occurs in the stomach under conditions of adrenalectomy, arthritis, or *H. pylori* infection[22-24]. Independent of the type of NSAIDs, the users of NSAIDs should be aware of these side effects if they are

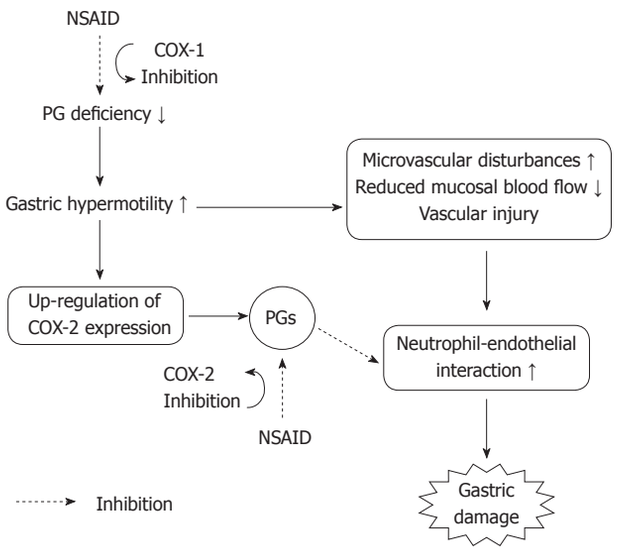

Figure 15 Working hypothesis on the roles of COX-1 and COX-2 in the pathogenic mechanism of non-steroidal anti-inflammatory drug-induced gastric damage. Non-steroidal anti-inflammatory drugs (NSAIDs) cause gastric hypermotility, followed by microvascular disturbances and neutrophil activation, leading to gastric damage. Gastric hypermotility and subsequent vascular disturbances are associated with a prostaglandin (PG) deficiency caused by COX-1 inhibition. The inhibition of COX-1 up-regulates COX-2 expression, and PGs produced by COX-2 may suppress the neutrophil-endothelial interaction caused by microvascular disturbances due to COX-1 inhibition.

infected with *H. pylori* or have a glucocorticoid deficiency or arthritic condition. Interestingly, aspirin acts to protect against indomethacin-induced gastric damage, although this agent given p.o. damages the stomach due to its direct irritative action. The failure of aspirin to induce gastric injury may be explained, at least partly, by a protective action of salicylic acid, the metabolite of aspirin, and this action is also functionally associated with inhibition of gastric hypermotility in response to indomethacin.

There is no doubt that gastric hypermotility plays a primary role in the pathogenesis of NSAID-induced damage in the stomach[3-7]. This response, causally related with PG depletion due to COX-1 inhibition, occurs prior to other pathogenic events involved in NSAID-induced gastric damage, such as microvascular disturbances and neutrophil infiltration as well as COX-2 expression[6,10,19,28,63]. However, the mechanism underlying NSAID-induced gastric hypermotility remains unknown. Since the gastric hypermotility induced by indomethacin was inhibited by atropine and vagotomy as well as intravenous glucose infusion[6,7,66], it is assumed that the response occurs in association with PG deficiency caused by COX-1 inhibition and is mediated by the vagal-cholinergic pathway through central glucose receptors. The upregulation of NSAID-induced COX-2 expression is functionally associated with gastric hypermotility[47]. Because atropine prevented both gastric hypermotility and COX-2 expression in response to indomethacin[21,47] and because gastric microvascular permeability increased in association with gastric hypermotility[8,63], the upregulation of COX-2 expression may result from mild mucosal injury and/or vascular injury caused by gastric hypermotility. However, the cells responsible for COX-2 expression induced by COX-1 inhibition also remain to be identified. In addition, other possible actions, such as inhibition of phosphorylative oxidation, injury of mitochondrial membrane and cell apoptotic change, have been demonstrated as the cellular mechanisms of NSAID-induced gastropathy[66-70], although these effects are shared by NSAIDs, including aspirin that does not cause gastric damage through parenteral administration. Further study is certainly needed to clarify these points, and these approaches should contribute to the development of gastric-sparing NSAIDs that are devoid of ulcerogenic properties.

BIOGRAPHY

Professor Koji Takeuchi received his PhD degree from the University of Tokyo, Tokyo, Japan. He had an extensive 4-year postdoctoral training at Department of Physiology and Cell Biology, University of Texas, Houston and Department of Surgery, Harvard Medical School, Boston, United States. He is presently Professor and Chairman of the Department of Pharmacology and Experimental Therapeutics, Dean of the Graduate School and Vice President of the Kyoto Pharmaceutical University, Kyoto, Japan. His research interest covers numerous areas of GI pharmacology and physiology, and one of his most notable contributions is the understanding of mucosal defense, focusing on the regulation of acid/bicarbonate secretion, the influences of non-steroidal anti-inflammatory drugs and prostaglandins; their mode of action, cyclooxygenase isoforms, receptors that drive physiological responses, and their role in mucosal injury, protection and healing. He has had 448 papers published on peer-reviewed journals, including 45 book chapters, and gave numerous presentations at national and international meetings. Professor Koji Takeuchi has enjoyed quite a few prestigious academic awards and honors.

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TOPIC HIGHLIGHT

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Clopidogrel and proton pump inhibitors - where do we stand in 2012?

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Abstract

Clopidogrel in association with aspirine is considered state of the art of medical treatment for acute coronary syndrome by reducing the risk of new ischemic events. Concomitant treatment with proton pump inhibitors in order to prevent gastrointestinal side effects is recommended by clinical guidelines. Clopidogrel needs metabolic activation predominantly by the hepatic cytochrome P450 isoenzyme Cytochrome 2C19 (CYP2C19) and proton pump inhibitors (PPIs) are extensively metabolized by the CYP2C19 isoenzyme as well. Several pharmacodynamic studies investigating a potential clopidogrel-PPI interaction found a significant decrease of the clopidogrel platelet antiaggregation effect for omeprazole, but not for pantoprazole. Initial clinical cohort studies in 2009 reported an increased risk for adverse cardiovascular events, when under clopidogrel and PPI treatment at the same time. These observations led the United States Food and Drug Administration and the European Medecines Agency to discourage the combination of clopidogrel and PPI (especially omeprazole) in the same year. In contrast, more recent retrospective cohort studies including propensity score matching and the only existing randomized trial have not shown any difference concerning adverse cardiovascular events when concomitantly on clopidogrel and PPI or only on clopidogrel. Three meta-analyses report an inverse correlation between clopidogrel-PPI interaction and study quality, with high and moderate quality studies not reporting any association, rising concern about unmeasured confounders biasing the low quality studies. Thus, no definite evidence exists for an effect on mortality. Because PPI induced risk reduction clearly overweighs the possible adverse cardiovascular risk in patients with high risk of gastrointestinal bleeding, combination of clopidogrel with the less CYP2C19 inhibiting pantoprazole should be recommended.

Key words: Clopidogrel; Thienopyridine; Proton pump inhibitors; Drug interaction; Platelet reactivity; Antiplatelet therapy; Cytochromes; Acute coronary syndrome; Gastrointestinal bleeding

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CLOPIDOGREL AND PROTON PUMP INHIBITORS - WHERE DO WE STAND IN 2011?

Clopidogrel in association with Aspirine has become

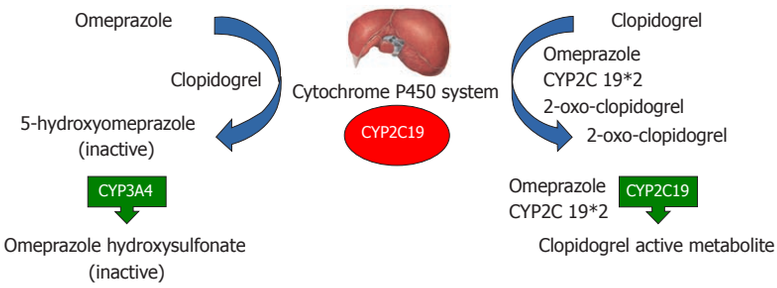

Figure 1 Potential intrahepatic mechanism of Proton pump inhibitor-clopidogrel interaction by the example of omeprazole (adapted from Tantry *et al*[50]). CYP2C19: Cytochrome 2C19; CYP2C19*2: Poor metabolizing cytochrome 2C19 isoenzyme.

the basis of pharmaceutical treatment in patients treated either medically or with percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS), by significantly reducing the risk of new ischemic cardiovascular events[1].

To prevent gastrointestinal bleeding as a drug-induced side effect, proton pump inhibitors (PPI) are often associated with clopidogrel use. This strategy is recommended by consensus guidelines[2] and endorsed by a recent meta-analysis, especially for patients taking dual antiplatelet therapy but in a lesser extent for those on clopidogrel alone due to sparse data[3-5]. Gilard *et al*[6,7] first reported in 2006 and 2008 a significant decrease of the clopidogrel effect in association with omeprazole *in vitro*. In opposite to that, no decrease was found in further pharmacodynamic studies for pantoprazole or esomeprazole[8-13]. Several retrospective observational studies showed an increased risk of new cardiovascular events in patients on clopidogrel-PPI association[14-22], thus leading the United States Food and Drug Administration and the European Medicines Agency to recommend to avoid the clopidogrel-PPI combination, especially with omeprazole[23,24]. More recently, one randomized double-blind trial[25], one post-hoc analysis of a randomized double-blind trial comparing prasugrel with clopidogrel[26] and several predominantly propensity matched cohort studies[27-35] have not shown clinically relevant adverse cardiovascular interaction between clopidogrel and PPI. Moreover, three recent meta-analyses, one by Kwok *et al*[36] reviewing 23 studies with the majority in abstract form, one by Siller-Matula *et al*[37] including 25 studies and the most recent by Lima *et al*[38] reviewing 18 studies pointed out that an elevated risk of bias was present in these studies indicating a possible interaction between clopidogrel and PPI. Furthermore, there was no significance for a drug interaction by analysing propensity matched and randomized trials.

The aim of this review is to focus on these recent studies, in order to reevaluate the present recommendations.

CLOPIDOGREL

Clopidogrel is a thienopyridine, inhibiting adenosine diphosphate (ADP) induced platelet activation by blocking the $P2Y_{12}$ receptor on the platelet surface. It is a prodrug that needs to be metabolized in an intrahepatic two-step oxidative process. First, the cytochrome P450 isoenzymes CYP1A2, CYP2B6 and CYP2C19 form 2-oxo-clopidogrel, which is then oxidized by CYP2B6, CYP2C19 and CYP3A4 to the clopidogrel active metabolite. The further formation of a disulfide bond with the $P2Y_{12}$ receptor unables the binding of ADP and finally platelet activation[12,39]. This is associated with dephosphorylation of intraplatelet vasodilator-stimulated phosphoprotein (VASP), providing an index of platelet reactivity to clopidogrel: the higher the platelet reactivity index (PRI), the less important the antithrombotic effect of clopidogrel[7]. Cytochrome P450 CYP2C19 seems to be of major importance in the metabolisation and activation of clopidogrel (Figure 1). Recent studies investigating the genetic polymorphism of the CYP2C19 allele have found a decreased platelet inhibition and increased cardiovascular risk in patients treated by clopidogrel, when carriers of even one reduced function CYP2C19 allele[40-42]. The CYP2C19*2 mutation was the most frequent variant found in the poor metabolizer (decreased platelet inhibition) group[43-45]. The prevalence of reduced function alleles differs among various populations, while an increase effect is observed from West to East: In the Caucasian population, 30%-40% of the normal function *1/*2 genotype and 2%-5% of the reduced function *2/*2 genotype are reported, whereas in East Asian and Chinese populations up to 24% of the poor metabolizing genotypes *2/*2, *2/*3 and *3/*3 are present[46-48].

PROTON PUMP INHIBITORS

PPI are benzimidazole derivates consisting of two heterocyclic moieties linked *via* a methylsulfinyl group. Being weak bases, they reach the parietal cell membrane as prodrugs and can thereby cross cell membrane to accumulate in the canalicular space, where the environment is highly acid. After a two step pronation, the drug reacts with cysteine sulfhydryls on the gastric H+/K+-ATPase by forming covalent disulfide bonds and inhibiting its activity[49-53]. So far, we dispose of five different PPIs on the market: omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole. Each among them is mainly metabolized by the intrahepatic P450 cytochrome system, especially CYP2C19 and CYP3A4, inhibiting them competitively. Interestingly, *in vitro* studies showed important differences in the inhibition of CYP2C19, with lansoprazole

and omeprazole being the most powerful inhibitors while pantoprazole and rabeprazole are the less potent inhibitors[49,54,55]. Of note, only pantoprazole showed significant acid inhibition after a single dose in the fast metabolizing genotype CYP2C19*1[46].

PHARMACODYNAMIC STUDIES ON CLOPIDOGREL- PPI INTERACTION

Gilard *et al*[6] demonstrated in 2006 an *in vitro* reduction of the antiaggregatory activity of clopidogrel in patients after coronary revascularisation under PPI treatment. The same group ran out the randomized double-blind OCLA (Omeprazole Clopidogrel Aspirine) trial in 2008: 124 patients undergoing elective coronary artery stent implantation receiving 75 mg of aspirine and clopidogrel daily, were randomized to receive either omeprazole 20 mg/d or placebo. The clopidogrel effect was assessed by measuring the phosphorylated VASP expressed in the PRI on day 1 and 7. On day 7, the mean PRI was significantly higher in the omeprazole-associated group (51.4% *vs* 39.8%, $P > 0.0001$), indicating less effective platelet antiaggregation. To investigate whether this potential interaction was due to a class effect, Cuisset *et al*[11] compared in the PACA (PPI And Clopidogrel Association) study 104 patients undergoing coronary stent implantation for non-ST-elevation ACS by randomizing them to a 20 mg omeprazole or pantoprazole treatment in association with 75 mg of aspirine and 150 mg of clopidogrel. After 1 mo, the VASP PRI was significantly lower in the pantoprazole group (36% ± 20% *vs* 48% ± 17%, $P = 0.007$), suggesting that pantoprazole, being a less potent CYP2C19 inhibitor, leads to a lower decrease of the clopidogrel antithrombotic effect. These results were confirmed in a prospective observational study, including a multivariable logistic regression analysis on 300 patients with coronary artery disease undergoing PCI and being already under aspirine 100 mg/d and clopidogrel 75 mg/d for at least 5 d. No difference was found for the VASP-PRI and the ADP induced platelet aggregation (ADP Ag) either between the PPI and no-PPI-group (51% *vs* 49%, $P = 0.724$) or between the different PPIs (pantoprazole and esomeprazole)[8]. In the same line, a prospective observational study including 336 patients undergoing coronary stent implantation showed no difference in ADP induced platelet aggregation between patients treated concomitantly by clopidogrel (600 mg loading and 75 mg maintenance dose) and pantoprazole *vs* clopidogrel only (OR 0.59, 95% CI: 0.31-1.13). For omeprazole and esomeprazole, a non significant increase in platelet aggregation persisted even after multiple adjustment (OR 1.84, 95% CI: 0.64-5.31), but due to the relatively small number of patients (26 *vs* 122 pantoprazole users), definite conclusions couldn't be drawn[13]. The authors of the post-hoc analysis of the PRINCIPLE (Prasugrel In Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation)-TIMI 44 study evaluated the impact of concomitant PPI use in 201 patients undergoing planned PCI and randomly assigned to either prasugrel (a new third generation thienopyridine) or high dose clopidogrel (600 mg loading dose and 150 mg/d maintenance dose) treatment. Fifty-six patients (26.4 %) were recorded to take a PPI at the time of randomization and the mean inhibition of platelet aggregation measured by ADP induced platelet aggregation was significantly lower at 2, 6 and 24 h after the loading dose, with a non-significant trend still persisting after 15 d. For prasugrel, no significant lowering of the mean inhibition of platelet aggregation was observed in the first 24 h, becoming only significant after 15 d in patient treated by PPI[26]. Recently, Angiolillo *et al*[12] conducted four randomized placebo-controlled crossover comparison studies among 282 healthy subjects, addressing the questions whether the PPI-clopidogrel interaction should be considered as a class effect or is rather due to more or less potent CYP2C19 inhibition and if a time interval between clopidogrel and PPI administration might diminish the inhibitory effect as evoked by the rapid metabolization of clopidogrel and omeprazole. After randomization in either interventional or placebo groups, the interventional arm entered a two period (clopidogrel only and clopidogrel with PPI) crossover study with four interventions during the clopidogrel-PPI period: The first study investigated an interaction between clopidogrel (300 mg loading and 75 mg maintenance dose) and omeprazole 80 mg/d when administered simultaneously. Study 2 investigated the administration of clopidogrel and omeprazole staggered by 12 h and study 3 an increased clopidogrel dose (600 mg loading and 150 mg maintenance dose) with omeprazole 80 mg/d. Finally, study 4 used a standard clopidogrel dose with pantoprazole 80 mg/d. Dosages of the active metabolite of clopidogrel (clopi H4) were significantly decreased in study 1, 2 and 3 while ADP induced platelet aggregation as well as VASP-PRI were significantly increased, indicating a less effective platelet antiaggregation in patients treated concomitantly with clopidogrel and omeprazole. Of note, these results were irrespective of the administration time or the clopidogrel dose. In contrast, the decrease of clopi H4 (40%, $P < 0.001$ for omeprazole and 14%, $P < 0.002$ for pantoprazole) was smaller in study 4 as well as the increase of ADP induced platelet aggregation, both differences remaining statistically significant. The increase of VASP-PRI was not significant when treated with pantoprazole, leading the authors to conclude that the clopidogrel-PPI interaction was not a class effect, whereas the combination with pantoprazole was a more optimal treatment option[12]. However, omeprazole was given at 80 mg per day, which represents 2 to 4 times the dose commonly prescribed, leaving unclear the hypothesis of a possible interaction when using standard doses. Furthermore, other molecules like rabeprazole, which does not inhibit the CYP2C19 isoenzyme, haven't been tested. In the same line, Ferreiro *et al*[56] conducted two supplementary randomized crossover studies in healthy subjects: In the first study, 20 volunteers received a 600 mg loading dose followed by 75 mg of clopidogrel combined with 40 mg of omeprazole concomitantly or staggered by 8-12 h with

Table 1 Overview of important pharmacodynamic studies on the clopidogrel-proton pump inhibitor interaction

Study	PPIs used	Population	Primary outcome	Author's conclusions
Gilard *et al*[7] OCLA study (double-blind, placebo-controled, randomized)	Omeprazole	124 patients undergoing elective coronary stent implantation	VASP-PRI on 7 d	Omeprazole significantly decreases clopidogrel inhibitory effect
Cuisset *et al*[11] PACA study (prospective, randomized)	Omeprazole *vs* Pantoprazole	104 NSTE-ACS patients undergoing coronary stenting	VASP-PRI/ADP-Ag after 1 mo	Significantly better platelet response under pantoprazole (VASP-PRI), no difference for ADP-Ag
O'Donoghue *et al*[26] PRINCIPLE-TIMI 44 (post hoc analysis of a RCT)	Not specified	201 patients undergoing planned PCI	ADP Ag	Mean inhibition of platelet aggregation significantly lower for patients on PPI
Siller-Matula *et al*[8] (prospective observational)	Pantoprazole esomeprazole	300 patients with CAD undergoing PCI	VASP-PRI/ADP-Ag in the catheter laboratory	No association of PPIs with impaired response to clopidogrel
Neubauer *et al*[13] (prospective observational)	Pantoprazole omeprazole esomeprazole	336 patients undergoing coronary stent implantation	ADP Ag	Pantoprazole does not diminish the antiplatelet effectiveness of clopidogrel
Angiolillo *et al*[12] (placebo controlled, randomized, cross-over)	Omeprazole pantoprazole	282 healthy subjects	Clopi H4 ADP Ag VASP-PRI after 5 d	Presence of a metabolic drug-drug interaction between clopidogrel and omeprazole but not for pantoprazole

VASP-PRI: Vasodilator-stimulated phosphoprotein platelet reactivity index; NSTE-ACS: Non-ST-elevation acute coronary syndrome; ADP Ag: Adenosine DiPhospate induced platelet aggregation; CAD: Coronary artery disease; PCI: Percutaneous coronary intervention; PPI: Proton pump inhibitor; OCLA: Omeprazole Clopidogrel Aspirine; PACA: PPI and clopidogrel association; **PRINCIPLE-TIMI: Prasugrel in comparison to clopidogrel for inhibition of** platelet activation and aggregation-TIMI.

a crossover washout period after a 2-4 wk followed by 1 wk of clopidogrel alone after a new washout period: No difference was observed in VASP-PRI after 1 wk between the concomitant and the staggered omeprazole administration, but PRI was significantly lower in the clopidogrel alone period compared with the omeprazole period, irrespective of the timing of administration (comcomitant omeprazole: $P = 0.02$; staggered omeprazole: $P = 0.001$). In the second study, 80 mg of pantoprazole were administered with the same regimen, but no differences in VASP-PRI were found between a concomitant or staggered administration of pantoprazole. Moreover, no difference was noted between clopidogrel alone and clopidogrel plus pantoprazole after 1 wk of treatment. The authors concluded that a time interval between the administration of clopidogrel and PPI doesn't afford any benefit and that pantoprazole seems to be a safer choice when combined with clopidogrel[57] (Table 1).

CLINICAL TRIALS ON CLOPIDOGREL- PPI INTERACTION

In 2009, Ho *et al*[15] published a retrospective cohort study including 8205 patients hospitalized for ACS in Veterans Affairs Hospitals. Analysis of prescription records identified 63.9% of patients being concomitantly under clopidogrel and PPI with a mean follow-up of 521 d. Concomitant use of clopidogrel and PPI (predominantly omeprazole and rabeprazole) was associated with an elevated risk of death or rehospitalisation for ACS after multivariable analysis (OR 1.25, 95% CI: 1.11-1.41). Of note, 98% were men and no information on the patient's race was available. In the same line, a Canadian population-based nested case-control study based on discharge abstracts and prescription records of 13636 patients being hospitalized for ACS, found an increased risk of re-infarction when under concomitant clopidogrel and PPI use (OR 1.27, 95% CI: 1.03-1.57). An analysis according to the PPI molecule used found no association with increased myocardial reinfarction for pantoprazole users in contrast to a 40% risk increase when using other PPIs (OR 1.40, 95% CI: 1.10-1.77). This result should be interpreted carefully, due to the small number of pantoprazole users (46 of 734 reinfarction patients)[16]. Moreover, only patients aged 66 years or older were included, introducing potential age bias. Another retrospective observational study based on diagnosis and prescription records of two Dutch health insurances, included 18139 new clopidogrel users, of whom 5734 (32%) were on concomitant PPI treatment. In this particular study, patients under PPI cotherapy had a significantly higher risk for the composite endpoint of myocardial infarction, unstable angina, stroke and all-cause mortality (HR 1.75, 95% CI: 1.58-1.94). In the subanalysis of secondary endpoints, PPI use was associated with a higher risk of myocardial infarction (ST-elevation and non-ST-elevation), unstable angina and all-cause mortality, but not with stroke[14]. Selection bias may be present in these two insurance databases, covering only 25% of the Dutch population. All three studies evidence significant differences in the baseline characteristics between the clopidogrel and the clopidogrel-PPI groups with significantly older patients with several comorbidities (e.g., heart failure, diabetes mellitus and renal failure) in the latter group, raising concern about unmeasured confounders in patients whith cardiovascular risk treated by PPI. In addition to that, no data about the efficacy of antihypertensive and statin treatment as well as on smok-

ing status were available. Finally as the medication exposure was based on prescription records, drug compliance data were not available.

More recently, several studies have been designed to include propensity scores in their analysis to improve confounding adjustment. Especially confounding by indication, an important bias in pharmacoepidemiologic studies, is diminished by using propensity score matching by calculating the probability to be exposed to a treatment or not. Moreover, adjustment for unmeasured or mismeasured covariates is improved by including hundreds of items in the propensity score calibration[58]. However, by the fact that many unexposed subjects of the initial study population aren't matched to exposed subjects and unmatched exposed subjects are excluded from the propensity matched analysis, precision of the estimated drug interaction could be decreased[59-62]. Rassen *et al*[31] analysed 18 565 patients aged over 65 years having been hospitalized for ACS and consecutive PCI in a retrospective cohort study based on Canadian and United states insurance records. Patients under clopidogrel and PPI had a slightly increased risk for rehospitalization for myocardial infarction or death of any cause (RR 1.26, 95% CI: 0.97- 1.63) leading the authors to conclude to no evidence of a substantial interaction. Major efforts for bias reduction have been made in this study by including only clopidogrel naïve patients, using a 7 d run-in period and a high-dimensional propensity score, permitting further adjustment for 400 additional variables empirically identified in their databases[63]. However, Aspirine use was unfortunately not measured in this coronary disease population[31]. A similar analysis was conducted on 20 596 patients of the Tennessee Medicaid program after hospitalization for ACS and PCI. Concomitant clopidogrel and PPI use was not associated with serious cardiovascular disease (HR 0.99, 95% CI: 0.82-1.19). Subanalysis concerning the different types of PPI has not found any increased risk of serious cardiovascular disease either, but confidence bounds were wide except for pantoprazole[27]. Another retrospective cohort study using the national Danish patient and prescription registry, included 56 406 patients older than 30 years and hospitalized for acute myocardial infarction. Concomitant clopidogrel and PPI users had a significant increased risk for cardiovascular death or rehospitalization for myocardial infarction and stroke compared to non-PPI users (HR 1.35, 95% CI: 1.22-1.50). In the same time, PPI users not receiving clopidogrel presented a similar increased risk (HR 1.43, 95% CI: 1.34-1.53), indicating no interaction between clopidogrel and PPI. The authors suspected that the increased cardiovascular risk in PPI users might be due to imperfectly measured differences in the baseline characteristics (lack of data on smoking status, lipid levels and body mass index)[28]. The strength of this study lies in the unselected nationwide population (patients older than 30 years hopitalized for myocardial infarction allover in Denmark) and the probably high concordance between the measured drug dispension (from data of the Danish national prescription registry) and real drug consumption due to only partial reimboursement of drug expenses and the fact that PPIs weren't available over the counter during the study period. However, the study is based on data from 2000 to 2006 and the low antiplatelet drug exposure (only 50%-70% of patients were under aspirine and 27% under clopidogrel on follow-up) dramatically contrasts with the current practice and questions the validity of the final conclusions.

In 2011, two analyses of PCI registries, including large data on cardiovascular risk factors and comorbities, were not able to show any difference on cardiovascular events: The American Guthrie Health Off-Lable Stent (GHOST) investigators studied 2651 patients discharged after coronary stenting and found no increase of Major Adverse Cardiovascular Events (MACE: death, myocardial infarction, target vessel revascularisation or stent thrombosis) for PPI users after propensity adjusted analysis (HR 0.89, 95% CI: 0.63-1.27) and in the propensity matched subgroup including 685 pairs of patients [42 (6.1%) without PPI against 40 (5.8%) with PPI; adjusted $P = 0.60$], the latter indicating even a trend to a protective effect of PPI treatment when under clopidogrel, perhaps due to less discontinuation of the antiaggregation as shown at the 6 mo follow-up (78% under clopidogrel in the PPI group against 70% without PPI, $P = 0.0085$). Furthermore, no difference according to the PPI used (omeprazole and esomeprazole) was observed[29]. Similar results came from the French Registry of Acute ST-Elevation and Non-ST Elevation Myocardial Infarction (FAST-MI), including 3670 post myocardial infarction patients. No increase in death, reinfarction or stroke was observed for concomitant PPI and clopidogrel use after one year (HR 0.98, 95% CI: 0.90-1.08). Furthermore, no difference existed regarding the PPI used (predominantly omeprazole and esomeprazole) and the presence of no or 1 to 2 CYP2C19 loss-of-function alleles. Of note, only a low number of 2 CYP2C19 loss-of-function alleles patients has been integrated (44 of 1579), leaving a higher risk of adverse cardiovascular outcome in this group still possible[30]. Both studies are based on PCI registries with detailed data assessment on baseline until hospital discharge. In contrast, follow-up was restricted on recording the patient's hospital readmission or death, without reliable information on medication exposure after hospitalization. The analysis restricted to the clopidogrel naïve population (2651 of 4421 respectively 2744 of 3670 patients) in order to avoid bias du to the occurrence of the index episode, limited the number of patients included, and may have underpowered the individual subgroups to detect a significant difference. Pointing out that the majority of the previous observational studies relied on discharge prescription records, Banerjee *et al*[32]. conducted a study on 23 200 post-PCI patients, including postdischarge drug exposure patterns using data from the Veteran Affairs Pharmacy Benefits Management database to assess drug exposure during the follow-up throughout a 6 years period. After propensity score adjustment, no difference in MACE (composite of all-cause death, nonfatal myocardial infarction or repeated revasularisation)

was observed between PPI and no PPI use in the group of continuous clopidogrel users (HR 0.97, 95% CI: 0.65-1.44). A rigorous control according to the consistency and duration of the clopidogrel and PPI exposure has been done, by revising daily exposure derived from prescription release dates and days of supply-a method considered superior to patient self-reported medication use[64]. In a subanalysis, rescue nitroglycerin and/or PPI use in patients < 30 d before MACE was significantly greater in patients taking clopidogrel and PPI ($P < 0.001$), suggesting a potential indication bias for PPI use due to misdiagnosed angina, a fact that may have contributed to a confounding bias in previous observational studies[32].

Conducting a post hoc analysis of the randomized Clopidogrel for Reduction of Events During Observation (CREDO) trial, Dunn *et al*[65] reported an increased risk of death, myocardial reinfarction or urgent target vessel revascularization at 28 d for patients using PPIs, independent on the underlying treatment [clopidogrel (OR 1.63, 95% CI: 1.02-2.63) or placebo (OR 1.55, 95% CI: 1.03-2.34)]. Baseline characteristics of the PPI group are not available, but as already discussed by Charlot *et al*[28], patients under PPI might be sicker than those who are not, explaining the higher rate of adverse cardiovascular events. Another post hoc analysis of a double-blind randomized trial, the Trial to Assess Improvement in Therapeutic Outcomes by Optomizing Platelet Inhibition with Prasugrel (TRITON)-TIMI 38 trial included 13 608 patients with an ACS undergoing PCI and being randomly assigned to prasugrel or clopidogrel. Thirty three percent (4529 patients) were on PPIs at randomisation and exposure during the follow-up was identified by landmark analyses at 3 d, 3 and 6 mo and at the end of follow-up. Baseline characteristics showed that patients treated with PPIs were once again significantly older and had more often pre-existing cardiovascular disease. After multivariable adjustment and propensity score matching, PPI use was not associated with the composite endpoint of cardiovascular death, myocardial infarction or stroke when prescribed with either clopidogrel (HR 0.94, 95% CI: 0.80-1.11) or prasugrel (HR 1.00, 95% CI: 8.84-1.20). Sensitivity analysis of patients being on PPI during the whole follow-up and patients never taking PPIs has not found any increase in adverse cardiovascular events either. Finally, no difference regarding the PPI subtype prescribed was found[26]. Both analyses have the advantage, that each end-point was strictly defined and controlled according to the initial randomized design. However, the analyses weren't designed to assess PPI use and therefore didn't randomize PPI treatment, leaving a potential risk of residual confounding even after multivariable adjustment and propensity score analysis. Furthermore, PPI compliance has not been memorized during follow-up- a fact attempt to be adjusted by landmark analyses in the second study.

So far, the only existing randomized controlled double-blind multicenter trial is the Clopidogrel and the Optimization of Gastrointestinal Events (COGENT) trial, including 3878 patients presenting with an ACS or undergoing PCI. Patients were randomized to receive CGT-2168, a fixed combination of 75 mg of clopidogrel and 20 mg of omeprazole *vs* 75 mg of clopidogrel alone. After a median follow-up of 106 d, a significant reduction in the primary endpoint, a composite of upper gastrointestinal bleeding, was observed in the CGT-2168 group (1.1% *vs* 2.9%, HR 0.34, 95% CI: 0.18-0.63, $P < 0.001$). Moreover, analysis of the primary cardiovascular safety end-point, (a composite of death from cardiovascular causes, myocardial infarction, coronary revascularisation and ischemic stroke) have not shown any difference between the placebo and omeprazole group (HR with omeprazole 0.99, 95% CI: 0.68-1.44, $P = 0.96$). Unfortunately, the study was interrupted prematurely due to the bankruptcy of the sponsor, after having included only 3873 of the 5000 initially planned patients. Moreover, wide confidence intervals around the hazard ratio of cardiovascular events and the fact that 94% of the study population was white, do not permit to rule out any significant clinical interaction between clopidogrel and omeprazole[25].

The first of three recent meta-analyses was conducted by Kwok *et al*[36] selecting 23 studies with 93 278 patients. Of note, more than half of the included studies have been available only as abstracts (12 of 23). Studies have been divided into three groups: nonrandomized studies with unadjusted risk ratios, nonrandomized studies with adjusted RR and randomized trials or studies including propensity score matching. Overall analysis of 19 studies reporting the incidence of MACE showed a significantly increased risk in the PPI group (RR 1.43, 95% CI: 1.15-1.77), but data were substantially heterogeneous (I^2 = 77%), partially due to considerable variation of the definition of MACE within the studies. Of interest, subanalysis of the propensity matched and randomized trails didn't show any increased risk (RR 1.15, 95% CI: 0.89-1.48) and data were much less heterogeneous (I^2 = 53%). Identical results were found when analysing the risk of myocardial infarction or ACS, leading the authors to conclude that unmeasured confounders may contribute to the results of the lower quality studies[36]. Siller-Matula *et al*[37] re-analysed 25 studies with 159 138 patients, finding a 29% increase of MACE (RR 1.29, 95% CI: 1.15-1.44) and myocardial infarction (RR 1.31, 95% CI: 1.12-1.53) for concomitant PPI and clopidogrel use. Again heterogeneity in the overall analysis was very important (I^2 = 72% *vs* 77%, respectively) and sensitivity analysis assessing the study quality showed a decreased risk of MACE in high quality studies (RR 1.23, 95% CI: 1.09-1.39) *vs* low quality studies (RR 1.65, 95% CI: 1.43-1.90), rising again the question of unmeasured confounders and differences in baseline characteristics[37]. Lima *et al*[38] reviewed 18 studies according to the PRISMA guidelines[66] by classifying them into high (well-performed randomized clinical trials), moderate (post hoc analysis of RCTs and propensity matched studies) and low (observational studies without propensity matching) quality studies. Due to important study heterogeneity, data pooling was a priori not effected. A stratified analysis comparing studies of low (13) with those of moderate quality (5) demonstrated an inverse correlation

Table 2 Overview of important clinical studies on the proton pump inhibitor-clopidogrel interaction

Study	PPIs used	Procedures to minimize bias	Population	Primary outcome	Results
Bhatt *et al*[25] (randomized, controlled, double-blind trial)	Omeprazole		3873 patients with ACS or undergoing PCI	Mean 133 d- composite safety endpoint of cardiovascular death, MI, coronary revascularisation	No difference between PPI and placebo group (HR with omeprazole 0.99, 95% CI: 0.68-1.44)
O'Donaghue *et al*[26] (post-hoc analysis of a RCT)	Pantoprazole omeprazole esomeprazole lansoprazole rabeprazole	Propensity score matching; multivariable and sensitivity analysis	13 608 patients undergoing planned PCI for ACS	Composite endpoint of cardiovascular death, MI or stroke after 6-15 mo	No difference between PPI and clopidogrel alone group (HR 0.94, 95% CI: 0.80-1.11)
Dunn *et al*[65] (post-hoc analysis of a RCT)	Not specified	Multivariable analysis	2116 patients undergoing PCI	28 d death, MI, urgent target vessel revascularisation 1 yr death, MI or stroke	Increased risk for adverse cardiovascular outcome regardless of clopidogrel use (clopidogrel/PPI: OR 1.63, 95% CI: 1.02-2.63 *vs* placebo/PPI: OR 1.55, 95% CI: 1.03-2.34)
Charlot *et al*[28] (retrospective cohort study)	Esomeprazole pantoprazole lansoprazole omeprazole rabeprazole	Propensity score matching; multivariable and sensitivity analysis	56 406 patients discharged with first-time myocardial infarction	1 yr composite end point of MI, stroke or cardiovascular death	Increased risk for adverse cardiovascular outcomes in PPI users regardless of clopidogrel use (HR for PPI/clopidogrel: 1.35, 95% CI: 1.22-1.50 *vs* HR for PPI alone: 1.43, 95% CI: 1.34-1.53)
Banerjee *et al*[32] (retrospective cohort study)	Predominantly omeprazole (88,9%)	Propensity score matching; multivariable and sensitivity analysis	23 200 post PCI patients	6-yr MACE	No increased risk for MACE in PPI users (HR 0,97, 95% CI: 0.65-1.44)
Ray *et al*[27] (retrospective cohort study)	Pantoprazole lansoprazole esomeprazole omeprazole rabeprazole	Propensity score matching; multivariable and sensitivity analysis	20 596 patients discharged after PCI or ACS	1 yr composite end point of ACS, stroke or cardiovascular death	No increased risk for serious cardiovascular disease in PPI users (HR 0.99, 95% CI: 0.82-1.19)
Rassen *et al*[31] (retrospective cohort study)	Pantoprazole omeprazole rabeprazole lansoprazole esomeprazole	Propensity score matching;	18 565 patients discharged after PCI or ACS (age > 65 yr)	180 d composite end point of hospitalization for MI and PCI or death of any cause	Trend towards a higher risk of composite end point in PPI users (RR 1.26, 95% CI: 0.97-1.63)
Simon *et al*[30] (retrospective cohort study)	Omeprazole esomeprazole pantoprazole lansoprazole	Propensity score matching; multivariable and sensitivity analysis	2744 clopidogrel and PPI-naive patients with definite MI	In hospital and 1-yr death, reinfarction or stroke	No increased risk of cardiovascular events and mortality in PPI users (HR 0.98, 95% CI: 0.90-1.08)
Harjai *et al*[29] (retrospective cohort study)	Omeprazole esomeprazole	Propensity score matching; multivariable and sensitivity analysis	2651 patients discharged after PCI for stable and unstable CAD	6-mo MACE	No increased risk for MACE in PPI users (HR 0.89, 95% CI: 0.63-1.27)
van Boxel *et al*[14] (retrospective cohort study)	Pantoprazole omeprazole rabeprazole lansoprazole	Multivariable analysis	18 139 clopidogrel users	2 yr composite endpoint of ACS, stroke and any cause death	Increased risk of composite endpoint (HR 1.75, 95% CI: 1.58-1.94), myocardial infarction (HR 1.93, 95% CI: 1.40-2.65) and unstable angina pectoris (HR 1.79, 95% CI: 1.60-2.03)
Juurlink *et al*[16] (population-based nested case-control study)	Omeprazole rabeprazole lansoprazole pantoprazole	Nested case –control; multivariable and sensitivity analysis	13 636 patients discharged after ACS (age > 65 yr)	90-d readmission for acute MI	Increased risk of reinfarction (OR 1.27, 95% CI: 1.03-1.57) in PPI users except pantoprazole
Ho *et al*[15] (retrospective cohort study)	Omeprazole rabeprazole lansoprazole pantoprazole	Multivariable and sensitivity analysis	8205 patients discharged after ACS	3 yr death or rehospitalization for ACS	Increased risk for death or rehospitalization in PPI users (OR 1.25, 95% CI: 1.11-1.41)

ACS: Acute coronary syndrome; PPI: Proton pump inhibitor; OR: Odds ratio; CI: Confidence interval; MI: Myocardial infaction; PCI: Percutaneous coronary intervention; RR: Relative risk; HR: Hazard ratio; MACE: Major adverse cardiovascular event; RCT: Randomized controlled trial.

between clopidogrel-PPI interaction and study quality (P = 0.007), as none of the moderate quality studies reported an association *vs* 10 in the low quality group[38]. The authors pointed out that according to the large CURE (Clopidogrel in Unstable Angina to Prevent Recurent Events) trial[67] no or very little advantage in reduction of

Table 3 Summary of studies reporting on adverse bleeding events

Study	Observed adverse event	Ascertainment	Results
Bhatt *et al*[25]	Composite of upper gastrointestinal bleeding (of known and unknown origin): overt bleeding, ulcers, symptomatic erosions, obstruction, perforation or decrease in hemoglobin of 2 g/dL	Endoscopic and radiologic confirmation (in known origin subgroup)	Significative reduction of upper gastrointestinal bleeding in the omeprazole treated group (1.1% against 2.9% under placebo; HR 0.34, 95% CI: 0.18-0.63)
Ray *et al*[27]	Hospitalization for bleeding at a gastroduodenal site (excluding angiodysplasia) or other gastrointestinal and non-gastrointestinal sites	Validated diagnostic codes with PPV of 91%	Adjusted 50% reduction of hospitalization in the PPI treated group (HR 0.50, 95% CI: 0.39-0.65), no significant difference concerning bleeding at other sites
van Boxel *et al*[14]	Occurence of complicated or non complicated peptic ulcer disease	ICD-9 diagnostic codes	Low incidence (0.7% with PPI against 0.2%) but significant increase of peptic ulcer disease in the PPI treated group even after multivariable adjusting (HR 4.76, 95% CI: 1.18-19.17)
Charlot *et al*[28]	Hospitalization for gastrointestinal bleeding	ICD-9 diagnostic codes	No reduction between the clopidogrel with PPI and clopidogrel alone group
Harjai *et al*[29]	TIMI major bleeding: intracranial hemorrage or a $\geqslant$ 5 g/dL decrease in hemoglobine TIMI minor bleeding: observed blood loss with decrease $\geqslant$ 3 g/dL in hemoglobine	Guthrie Health System database	No significant difference between the clopidogrel with PPI and clopidogrel alone group
Simon *et al*[30]	In-hospital major bleeding (not specified) or need for blood transfusion	FAST-MI registry	No significant difference between the clopidogrel with PPI and clopidogrel alone group

HR: Hazard ratio; CI: Confidence interval; PPV: Positive predictive value; PPI: Proton pump inhibitor; ICD-9: International classification of disease-9th revision; FAST-MI: French registry of acute-ST-elevation and non-ST-elevation myocardial infarction.

adverse cardiovascular events when treated with clopidogrel was observed later than 3 mo after an ACS. In contrast to that, in the study of Ho *et al*[15] the increased risk of adverse cardiovascular events for concomitant PPI and clopidogrel use appears in the long term (not before 180 d), a period when clopidogrel has not been shown to be therapeutically useful any more[38]. Theses results might be explained by unmeasured residual confounders rather than by the existence of a clopidogrel-PPI interaction, a hypothesis endorsed by the three studies having found an elevated risk for adverse cardiovascular events in PPI-users, regardless whether on clopidogrel or not[28,65,68]. Characteristics and results of the cited studies are overviewed in Table 2 by classifying them according to their scientific weight, while Table 3 summarizes the studies reporting on adverse bleeding events.

To summarize, pharmacodynamic studies suggest an existing interaction between clopidogrel and omeprazole but not with pantoprazole, a phenomenon that may be explained by the higher inhibitory potency of omeprazole for the cytochrome P450 CYP2C19[54], a key enzyme in the metabolic activation of clopidogrel[39].

Nevertheless, the clinical impact of this biochemical interaction still remains unclear, as several cohort studies report an interaction and consecutive increase in adverse cardiovascular events for omeprazole[14-22]. In contrast to that, recent retrospective studies including propensity score matching in order to minimise underlying bias have not show any clopidogrel-PPI interaction[27-35] (except one recent study using a larger endpoint including overall death, myocardial infarction, stroke and critical limb ischemia[69]). In addition to that, post-hoc analyses of randomized trials[26,65] and the only randomized double blinded trial available so far have not found any increase in adverse cardiovascular events for the PPI treated group[25]. Finally, several meta-analyses pointed out that there was an inverse correlation between study quality and a reported statistically positive interaction[36-38]. Despite of that, the United states Food and Drug Administration and the European Medecines Agency still discourage the use of PPI (especially omeprazole) concomitantly with clopidogrel[23,24].

Three recommendations to health care providers could therefore be made for the moment: (1) A gastrointestinal risk evaluation (e.g., history of gastrointestinal bleeding, dyspepsia, therapeutic anticoagulation, concomitant NSAIDS use especially in elderly persons and in the presence of helicobacter pylori[70-73]) has to be performed in each patient, as clopidogrel treatment and dual antiplatelet therapy rise the risk of adverse gastrointestinal events and mortality[3,4,25,74,75]. Patients at high risk of gastrointestinal bleeding should have prescribed concomitant PPIs when under clopidogrel, due to the high mortality rate in case of bleeding[2]; (2) Favoring pantoprazole over omeprazole pharmacologically leads to less inhibition of the CYP2C19 isoenzyme, but the clinical impact of this pharmacologic difference has not been proved so far. Nevertheless, to the best of our knowledge, no clinical trial (regardless of its quality) has ever demonstrated a clear interaction for pantoprazole, making it a rather safe choice, especially regarding recent moderate and high quality publications. Furthermore the standard daily dose of 40 mg doesn't seem to induce any significant pharmacodynamic interaction with clopidogrel, as none was found for a 80 mg/d dose[12,57]; and (3) Widening the delay between clopidogrel and PPI intake by a minimum of 12 h (a concept based on the rapid metabolization of clopidogrel[49]), doesn't seem to avoid the possible drug interaction between clopidogrel and PPIs[12,56,57].

In conclusion, rising evidence accumulates to infirm an interaction between PPIs and clopidogrel. This point

suggests that the bleeding reduction benefit overweighs the possible adverse cardiovascular risk in patients with an indication for PPI treatment taking dual antiplatelet treatment. Of course, adequate powered randomized controlled trials with pharmacodynamic assessment are still needed to infirm the persisting doubt upon the PPI-clopidogrel interaction.

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REVIEW

Enhanced apoptosis in post-liver transplant hepatitis C: Effects of virus and immunosuppressants

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Abstract

Hepatitis C (HCV)-infected patients have a poorer survival post-liver transplantation compared to patients transplanted for other indications, since HCV recurrence post-transplant is universal and commonly follows an aggressive course. There is increasing evidence that in the non-transplant setting, induction of hepatocyte apoptosis is one of the main mechanisms by which HCV drives liver inflammation and fibrosis, and that HCV proteins directly promote apoptosis. Recent studies have shown that post-liver transplant, there is a link between high levels of HCV replication, enhanced hepatocyte apoptosis and the subsequent development of rapidly progressive liver fibrosis. Although the responsible mechanisms remain unclear, it is likely that immunosuppressive drugs play an important role. It is well known that immunosuppressants impair immune control of HCV, thereby allowing increased viral replication. However there is also evidence that immunosuppressants may directly induce apoptosis and this may be facilitated by the presence of high levels of HCV replication. Thus HCV and immunosuppressants may synergistically interact to further enhance apoptosis and drive more rapid fibrosis. These findings suggest that modulation of apoptosis within the liver either by changing immunosuppressive therapy or the use of apoptosis inhibitors may help prevent fibrosis progression in patients with post-transplant HCV disease.

Key words: Hepatitis C; Liver transplantation; Apoptosis; Immunosuppressive agents; transforming growth factor-β

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INTRODUCTION

Hepatitis C (HCV)-related liver failure is now the commonest indication for liver transplantation in the United States, Australia and Europe[1]. HCV-infected patients have a poorer survival post-transplantation compared to patients transplanted for other indications[2]. This is because HCV recurrence occurs in virtually all patients and commonly follows an aggressive course, with 20%

or more of patients developing cirrhosis within 5 years of transplantation[3]. The cause of this accelerated disease has not been fully elucidated, but risk factors include advanced donor age, early high HCV viral load post-transplant[4], acute graft rejection and treatment thereof, and the degree of immunosuppression[5].

In the non-transplant setting, induction of hepatocyte apoptosis is one of the main mechanisms via which HCV drives liver inflammation and fibrosis[6]. Recent evidence suggests a link between high levels of HCV replication, high rates of apoptosis and the subsequent development of rapidly progressive graft injury and fibrosis after liver transplantation[7]. The mechanisms responsible for this high levels of apoptosis found in aggressive post-liver transplant HCV disease remain unclear. It is well known that immunosuppressants impair immune control of HCV, thereby allowing increased viral replication. There is also recent evidence that some commonly used immunosuppressants may directly induce apoptosis and this may be facilitated by the presence of high levels of HCV replication. This suggests that HCV and immunosuppressants may synergistically interact to enhance apoptosis and drive rapid fibrosis.

OVERVIEW OF APOPTOSIS

Apoptosis is a highly regulated physiological process that plays an important role in organogenesis and the maintenance of tissue homeostasis[8]. Cells posing a threat to the integrity of an organ, such as virus-infected cells, may be eliminated by apoptosis, which occurs by two major pathways - extrinsic and intrinsic. The extrinsic pathway is activated when death ligands [tumor necrosis factor (TNF), FasL/CD95L and TRAIL] secreted by cells of the immune system in response to foreign (for example, viral) antigens bind to their respective cell surface receptors, to trigger signaling pathways that result in the activation of caspases[9]. The caspases are a class of enzymes responsible for the execution of apoptosis within the cell. In the intrinsic pathway, intracellular apoptotic stimuli, such as viral antigens, cause disruption of mitochondrial membrane integrity, releasing cytochrome c that activates the caspase pathway[10]. The integrity of the outer mitochondrial membrane is predominantly maintained by anti-apoptotic members of the Bcl-2 family (e.g., Bcl-2 and Bcl-xL), which antagonize pro-apoptotic members (for example, Bax and Bak).

LINK BETWEEN HEPATOCYTE APOPTOSIS AND LIVER FIBROSIS

There are increasing amounts of experimental data implicating apoptosis as a driving force for fibrogenesis in a range of different liver diseases, including alcohol-related and cholestatic liver diseases and viral hepatitis[11]. Apoptotic hepatocytes are engulfed and cleared by both Kupffer cells and hepatic stellate cells (HSCs). Activated HSCs are the primary cell type responsible for promoting fibrogenesis within the damaged liver, and the uptake of apoptotic bodies by HSCs result in their activation and secretion of the key pro-fibrogenic cytokine transforming growth factor-β (TGF-β)[12]. In activated HSCs, TGF-β induces a marked upregulation of genes encoding fibrillar collagens and other extracellular matrix components, resulting in the abnormal deposition of collagen within the liver[13]. Kupffer cells, which are the resident liver macrophages, upon ingestion of apoptotic hepatocytes, also secrete TGF-β, thereby promoting a pro-fibrogenic response in activated HSCs[14]. Furthermore, TGF-β itself induces hepatocyte apoptosis via two independent pathways, SMAD and DAXX[15], thus providing a positive feedback loop that could further potentiate apoptosis-induced fibrosis. In support of these *in vitro* observations, inhibition of apoptosis reduces hepatic inflammation and fibrosis in experimental models of fibrotic liver disease[16].

HEPATITIS C AND HEPATOCYTE APOPTOSIS

In HCV infection, hepatocyte apoptosis is an important part of the host anti-viral defense mechanism since it interrupts viral replication and assists in the elimination of virus-infected cells. However, in keeping with the observed effects of apoptosis in laboratory studies, there is now evidence to suggest that the severity of liver damage in chronic HCV is associated with the degree of hepatocyte apoptosis[6]. Furthermore, the degree of apoptosis correlates with the level of viraemia[17]. Bantel and colleagues have studied a serum apoptosis biomarker, the proteolytic neoepitope of the caspase substrate cytokeratin-18, as a means of determining caspase activity to monitor liver injury and predict the progression of hepatic fibrosis in HCV-infected patients[18]. This biomarker was markedly elevated in the sera of HCV-infected patients compared to healthy controls, and in patients with normal transaminase levels, raised serum caspase activity was associated with advanced fibrosis on liver biopsy.

Hepatocyte apoptotic rates on liver biopsy are significantly greater in HCV-positive patients post-liver transplant compared to the non-transplant setting, with the severity of liver inflammation correlating with the level of hepatocyte apoptosis[7], and HCV viral load is known to be higher post-liver transplantation[19]. Thus one potential explanation for accelerated fibrosis post-transplantation is that the high levels of HCV replication that occurs due to impaired immune control of HCV replication may drive increased hepatocyte apoptosis.

How then does HCV affect apoptosis? One likely mechanism is that virus-specific cytotoxic T-cells may induce apoptosis of HCV-infected hepatocytes by upregulating death receptor ligands (TNF, FasL/CD95L and TRAIL), by producing antiviral cytokines (for example, interferon-γ), and by direct cell killing with perforins and granzymes[20]. HCV infection is also associated with an upregulation of death receptors on hepatocytes, and the levels of Fas/CD95 and FasL/CD95L have been shown

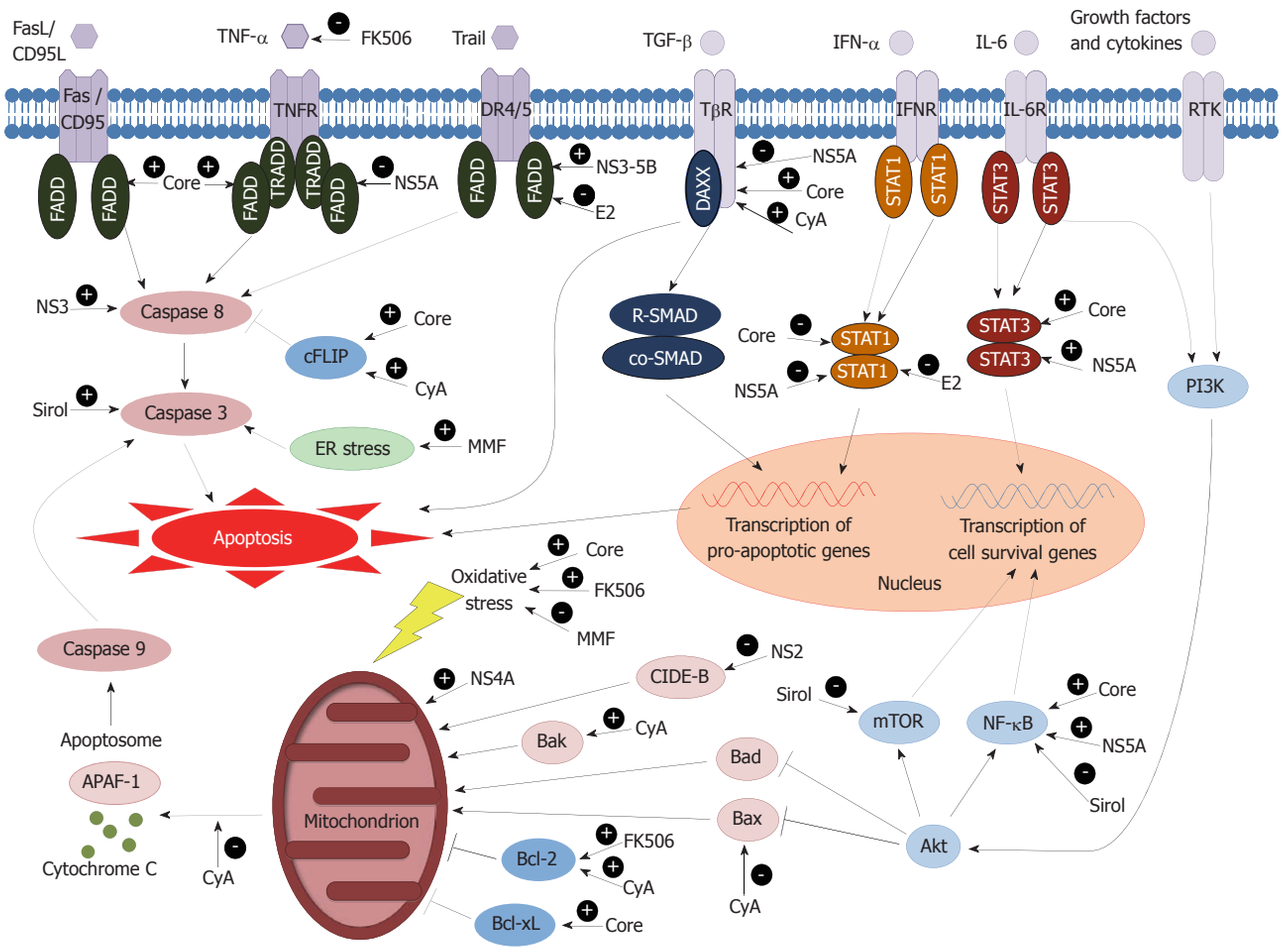

Figure 1 Where the hepatitis C proteins and immunosuppressants are thought to interact with the apoptotic pathways within the hepatocyte. CyA: Cyclosporine; FK506: Tacrolimus; MMF: Mycophenolate mofetil; Sirol: Sirolimus; TNF-α: Tumor necrosis factor-alpha.

to increase in parallel with the severity of inflammation and disease progression[21].

There is also considerable experimental evidence that HCV structural proteins can directly influence hepatocyte apoptosis. HCV core protein has been reported to sensitize hepatocytes to TNF-α-[22] and FasL/CD95L-[23] mediated apoptosis, by interacting with the cytoplasmic domains of TNFR1 and Fas/CD95 to enhance downstream signaling events. It also induces oxidative stress, enhances mitochondrial-mediated hepatocyte apoptosis[24] and upregulates *TGF-β1* gene expression, thereby promoting apoptosis and fibrogenesis. However, the expression of core protein has also been shown to have a number of possible anti-apoptotic effects. These include inhibition of TNF-α- and Fas/CD95-mediated apoptosis through the upregulation NF-κB[25], and interaction with cFLIP, an endogenous caspase-8 inhibitor[26]. Core protein has also been reported to promote the anti-apoptotic Bcl-xL expression, inhibit interferon-α-mediated STAT1 signaling and activate STAT3, thereby protecting infected hepatocytes from T-cell-mediated apoptosis[27]. Both the E1 and E2 glycoproteins of HCV have been shown to induce hepatocyte apoptosis[28], with the E2 protein noted to activate the mitochondrial caspase pathway. However, E2 protein has also been shown to inhibit interferon-α-mediated STAT1 signaling and TRAIL-induced apoptosis, as well as enhance the proliferation of transfected Huh7 human hepatoma cells[29]. The data on the effect of HCV on caspase-independent apoptosis are lacking. One study showed that core protein expression promoted apoptosis-like caspase-independent cell death in osteosarcoma-derived cells[30], but the effect in liver cells is unknown.

The non-structural proteins of HCV have also been shown to affect hepatocyte apoptosis. By using a NS3-5B subgenomic replicon of HCV, Huh7.5 human hepatoma cells were shown to be sensitized to TRAIL-induced apoptosis[31]. Accumulation of NS4A on mitochondria has been found to promote mitochondrial-mediated apoptosis[32]. Similarly, the HCV protease NS3, can induce apoptosis in a caspase 8-dependent manner. On the other hand, NS2 has been found to inhibit the mitochondrial release of cytochrome c, thereby inhibiting mitochondrial-mediated apoptosis[33]. NS5A inhibits interferon-α-mediated STAT1 signaling[34] and protects hepatocytes against interferon-α- and TNF-α-mediated apoptosis. NS5A also prevents apoptosis by activating NF-κB, inhibiting TGF-β, and upregulating STAT3 expression to

promote hepatocyte proliferation[35].

Thus HCV proteins have been shown to have a number of both pro- and anti-apoptotic effects in cultured hepatocytes but the net of contribution of these changes to hepatocyte apoptotic rates and liver fibrosis *in vivo* remains unclear. The discrepancies in these effects may be partly explained by differences in experimental conditions, cell types, apoptotic stimuli and HCV genotype-specific proteins expressed in various *in vitro* systems that may not mimic the true *in vivo* situation. Our current understanding of how the HCV proteins interact with apoptotic pathways within the hepatocyte is summarized in Figure 1.

HEPATITIS C AND APOPTOSIS OF OTHER LIVER CELL TYPES

Activated HSCs are the key cell type promoting fibrogenesis in the liver. HSC activation is increased in patients with chronic HCV infection and the degree of activation correlates with necroinflammatory grade and fibrosis stage[36]. Interestingly, patients with chronic HCV infection have elevated plasma levels of TGF-β1 and increased expression of TGF-β1 in the liver, while the clearance of HCV infection with anti-viral treatment is associated with normalization of plasma TGF-β1 levels[37]. This argues for an important role of TGF-β in HCV-mediated HSC activation and liver fibrogenesis.

Normally, hepatocytes do not express TGF-β, but hepatocytes exposed to HCV non-structural proteins upregulate TGF-β expression, resulting in the activation of HSCs[38]. HSCs express CD81 and LDL receptor, the putative receptors for HCV, and may perhaps be infected by HCV *in vivo*[39]. Expression of HCV core and non-structural proteins in HSCs was found to activate HSCs, resulting in upregulation of TGF-β and procollagen 1 expression[39]. The interaction of HCV E2 glycoprotein with HSCs is noted to upregulate HSC expression of matrix metalloproteinase 2, thus facilitating hepatic fibrogenesis.

Activated HSCs are primarily cleared by apoptosis, a process that would normally restrict the fibrogenic response within an inflamed liver. However, in patients with chronic HCV and advanced fibrosis, HSC apoptosis is reduced compared to patients with mild fibrosis[40]. This suggests that the inhibition of HSC apoptosis by HCV may contribute to the progression of liver fibrosis in this disease. Also, HCV-infected patients who are noted to have a high number of activated HSCs in liver biopsies done several months after liver transplantation developed advanced fibrosis within 2 years of transplantation, indicating that the degree of HSC activation may be an early predictor of post-transplant rapid fibrosis[41].

Kupffer cells have an integral role in the development of chronic liver inflammation in response to hepatocyte injury. Activated Kupffer cells contribute to HSC activation and thereby promote liver fibrosis. The interaction between HCV core protein and toll-like receptor (TLR) 2 on human Kupffer cells has been shown to upregulate cell surface programmed death-ligand 1 (PD-L1). The binding of Kupffer cell PD-L1 to PD-1 receptors on T-cells promotes T-cell apoptosis, thereby impairing the host adaptive anti-viral response[42]. HCV core protein has also been shown to inhibit TLR3-mediated induction of interferon-α, interferon-β and TRAIL, and this may impair the anti-viral activity of Kupffer cells[42]. HCV has not been shown to affect Kupffer cell apoptosis.

IMMUNOSUPPRESSIVE DRUGS AND APOPTOSIS

The aim of post-liver transplant immunosuppression is to dampen the adaptive immune response and prevent graft rejection. However, robust CD4+ and cytotoxic CD8+ T-cell responses play a central role in controlling HCV replication. The experimental evidence that the increased HCV viraemia that occurs post-transplant may directly drive higher rates of apoptosis suggests a likely link between immunosuppressive drug therapy, the resultant loss of immune control of HCV replication, and apoptosis-induced liver injury and fibrosis.

It is has been suggested that the overall level of immunosuppression, rather than the individual agent, is associated with the level of HCV viraemia and the degree of hepatic injury on liver biopsy in patients with post-transplant HCV recurrence[43]. Thus the use of pulse methylprednisolone for the treatment of acute graft rejection has been shown to dramatically elevate HCV viral load[43], while OKT3, another highly potent immunosuppressant used to treat steroid-refractory acute rejection, has been shown to accelerate HCV-associated liver fibrosis.

However, there is emerging evidence that individual immunosuppressive drugs used in long-term maintenance therapy may also have individual specific effects on both HCV replication and HCV-mediated liver injury. Some groups have shown that cyclosporine therapy is associated with less severe histological recurrence and improved graft survival post-liver transplantation compared to tacrolimus[44]. One possible explanation for this effect is that cyclosporine is known to inhibit HCV replication *in vitro* by the inhibition of NS2 and NS5A[45]. Tacrolimus, on the other hand exhibits no anti-viral effect *in vitro* and in fact impairs interferon-α activity by interfering with STAT-1 phosphorylation, and thus, may promote viral replication and persistence[46]. Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), inhibits HCV replication in Huh7 human hepatoma cells without inhibiting cell proliferation or inducing apoptosis[47]. A synergistic inhibition of viral replication has also been shown when MPA was combined with cyclosporine or interferon-α[48].

In addition to their possible effects on viral replication, there is increasing evidence that some of the immunosuppressive agents may also directly contribute to apoptosis. Figure 1 summarizes our current understand-

ing of where individual immunosuppressants interact with intracellular apoptotic pathways.

Cyclosporine has been shown to prevent hepatocyte necrosis in mice exposed to concanavalin A[49], but data on its effect on hepatocyte apoptosis are lacking. Cyclosporine is noted to cause apoptosis of renal vascular endothelial cells via endoplasmic reticulum stress, as well as fibrosis of the renal tubulointerstitium by upregulating TGF-β expression[50]. These findings raise concerns that similar effects may occur within the liver. Indeed, cyclosporine has been found to promote hepatocyte expression of pro-apoptotic Bak in a rat model of liver injury[51]. On the other hand, cyclosporine has also been shown to prevent apoptosis of human gingival fibroblasts by inhibiting Bax and upregulating anti-apoptotic Bcl-2[52], as well as reducing mitochondrial permeability and inhibiting cytochrome c release in human platelets and rat vascular endothelial cells *in vitro*[53]. In an animal model of colitis, cyclosporine was found to have a protective role against epithelial apoptosis through the upregulation of anti-apoptotic cFLIP and inhibition of caspase-8 activity[54].

Tacrolimus has also been shown to have both pro-apoptotic and anti-apoptotic effects in various cell lines in culture. Treatment with tacrolimus promotes Jurkat T-cell G0/G1 phase cell cycle arrest and the generation of reactive oxygen species, mitochondrial dysfunction and thereby apoptosis[55]. In contrast, in human islet cells exposed to pro-inflammatory cytokines such as IL-1 and interferon-γ, tacrolimus has an anti-apoptotic effect, causing a reduction in TNF-α and down-regulation of caspase-3, -8 and -9[56]. Tacrolimus has also been shown to promote hepatic expression of anti-apoptotic Bcl-2 in a rat model of liver injury[51]. However the effect of tacrolimus on apoptosis in human liver is unknown.

After solid organ transplantation, treatment with MMF has been associated with increased mucosal apoptosis in the upper gastrointestinal tract and colon, producing an appearance similar to graft-*vs*-host disease[57]. While MMF has been shown to induce apoptosis via promoting endoplasmic reticulum stress and increasing caspase-3 activity in human pancreatic islet cells[58], the opposite effect has been observed in renal transplant recipients, where reduced apoptosis of renal tubular epithelial, glomerular and interstitial cells was noted[59]. MMF has also been shown to reduce pancreatic β-cell apoptosis in a rodent model of diabetes, and reduce hepatocyte oxidative stress and apoptosis in a rat model of ischaemia/reperfusion injury[60]. The effect of MMF on human hepatocyte apoptosis is currently unknown.

Sirolimus has been found to induce apoptosis in acute lymphoblastic leukemia cells by inhibiting the PI3K/Akt pathway[61]. It also induces apoptosis in vascular smooth muscle cells by activating caspase-3 and inhibiting NF-κB nuclear translocation[62]. However, sirolimus is known to inhibit HSC proliferation *in vitro*, reduce TGF-β expression and inhibit collagen deposition, thereby reducing hepatic fibrosis in a rat model of liver injury[63]. Indeed, sirolimus has also been shown to reduce liver fibrogenesis, improve liver function and enhance survival in rats with established cirrhosis[64]. Huh7 hepatoma cells transfected with the HCV-1b genome have upregulated PI3K-Akt-mTOR signaling[65], possibly rendering HCV-infected cells more resistant to apoptosis. Sirolimus, by inhibiting the mTOR pathway, has been shown to inhibit NS5A phosphorylation, thereby inhibiting HCV replication[66]. Sirolimus-based maintenance immunosuppression has been associated with lower HCV RNA levels at 12 mo following liver transplantation and improved patient survival at 6 years compared to calcineurin inhibitors[67].

THERAPEUTIC IMPLICATIONS

Understanding the role of hepatocyte apoptosis in the pathogenesis of post-transplant HCV-mediated liver injury and the likely contributing role of the immunosuppressive agents has a number of important therapeutic implications. It is hoped that increased knowledge of the pro- or anti-apoptotic effects of different immunosuppressive agents and whether they exacerbate HCV-induced apoptosis may allow the development of immunosuppressive regimes that minimize this aspect of HCV-mediated liver injury. In this regard, sirolimus is of particular interest given its possible anti-apoptotic and anti-fibrotic effects both *in vitro* and in animal models.

These findings also suggest a possible therapeutic role for apoptosis inhibitors in post-transplant HCV. There is increasing experimental and clinical experience with the use of this class of compounds in liver disease. The pan-caspase inhibitor IDN-6556 was found to reduce hepatocyte apoptosis and liver fibrosis in bile duct-ligated mice[16], and improve liver function tests in patients with hepatic dysfunction[68]. VX-166, another pan-caspase inhibitor, has been shown to reduce hepatocyte caspase-3 expression and apoptosis, thereby decreasing hepatic fibrosis in a murine model of non-alcoholic steatohepatitis[69]. Given the evidence linking HCV-induced hepatocyte apoptosis with liver fibrosis, 2 randomized, double-blind, placebo-controlled studies have been conducted using pan-caspase inhibitors in patients with chronic HCV, one using PF-03491390[70] and the other using IDN-6556[71]. In both studies, the orally administered pan-caspase inhibitors were well tolerated with minimal adverse effects and showed significant reductions in serum transaminases. Besides directly targeting caspases, compounds that inhibit other components of the apoptotic pathway upstream to caspases are currently in development. There are currently no drugs that inhibit the caspase-independent apoptotic pathway in the literature.

Conversely, the promotion of HSC apoptosis may also act to reduce hepatic fibrosis. Cortex Dictamni extract was noted to induce apoptosis of activated HSCs, resulting in decreased hepatic collagen deposition and attenuated fibrosis in a murine model of liver injury[72]. Another compound, 2',4',6'-tris(methoxymethoxy) chalcone, is noted to induce apoptosis of activated HSCs by enhancing FasL/CD95L expression without affecting

hepatocyte apoptosis[73]. The tyrosine kinase inhibitor sorafenib has also been found to increase HSC expression of caspase-3 and induce HSC apoptosis resulting in reduced hepatic collagen deposition and fibrosis in bile duct-ligated rats[74]. These compounds raise the possibility of treatment to reduce the population of activated HSCs within the transplanted liver in HCV-recurrence.

In conclusion, the management of post-liver transplant HCV disease remains one of the major challenges in transplant medicine. Enhanced hepatocyte apoptosis appears to contribute to much of the liver injury that drives rapid liver fibrosis in this disease, and in the near future clinically useful serum biomarkers of apoptosis may be available to monitor for this. The precise mechanisms that drive this accelerated hepatocyte apoptosis post-transplant require further study, but it appears that both HCV itself and immunosuppressants play contributory and possibly synergistic roles. In the future as the effects of various immunosuppressive agents on HCV-induced liver cell apoptosis are clarified, a combination of fine-tuning immunosuppressive regimens as well as the manipulation of apoptosis within the liver represents novel therapeutic possibilities for the management of this complex disease.

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ORIGINAL ARTICLE

Side-stream smoking reduces intestinal inflammation and increases expression of tight junction proteins

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Abstract

AIM: To investigate the effect of side-stream smoking on gut microflora composition, intestinal inflammation and expression of tight junction proteins.

METHODS: C57BL/6 mice were exposed to side-stream cigarette smoking for one hour daily over eight weeks. Cecal contents were collected for microbial composition analysis. Large intestine was collected for immunoblotting and quantitative reverse transcriptase polymerase chain reaction analyses of the inflammatory pathway and tight junction proteins.

RESULTS: Side-stream smoking induced significant changes in the gut microbiota with increased mouse intestinal bacteria, *Clostridium* but decreased *Fermicutes* (*Lactoccoci* and *Ruminococcus*), *Enterobacteriaceae* family and *Segmented filamentous baceteria* compared to the control mice. Meanwhile, side-stream smoking inhibited the nuclear factor-κB pathway with reduced phosphorylation of p65 and IκBα, accompanied with unchanged mRNA expression of tumor necrosis factor-α or interleukin-6. The contents of tight junction proteins, claudin3 and ZO2 were up-regulated in the large intestine of mice exposed side-stream smoking. In addition, side-stream smoking increased c-Jun N-terminal kinase and p38 MAPK kinase signaling, while inhibiting AMP-activated protein kinase in the large intestine.

CONCLUSION: Side-stream smoking altered gut microflora composition and reduced the inflammatory response, which was associated with increased expression of tight junction proteins.

Key words: Inflammation; Microbiota; Tight junction protein; Side-stream smoking; Intestine

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INTRODUCTION

Cigarette smoking is a remarkable etiological factor in the pathogenesis of cardiovascular diseases, hypertension, pulmonary diseases and gastroenterological diseases[1-4]. Meanwhile, passive smoking (second-hand smoking) is also a contributing factor for the development of coronary artery disease[5-7], lung cancer[7] and Crohn's disease[8], which pose a substantial health risk to non-smoking adults and young children worldwide[9]. It was estimated in 2004 that more than 600 thousand deaths were due to

second-hand smoke, which accounted for about 1% of worldwide mortality[9]. On the other hand, it was reported that smoking had a protective effect in reducing ulcerative colitis mostly based on the epidemiologic studies[8,10,11].

Chronic inflammatory bowel diseases, mainly Crohn's disease and ulcerative colitis, are characterized by chronic inflammation of the intestines[8]. Recent studies clearly show that gut epithelial integrity and barrier function are the central predisposing factors in inflammatory bowel diseases, autoimmune and related allergic diseases[12-16]. The intestinal epithelium is composed of tightly assembled intestinal epithelial cells which form a protective barrier against pathogenic and commensal bacteria, preventing their penetration from the lumen to initiate inflammatory responses in the mucosal system[17]. Impairment of the tight junction barrier is associated with chronic diseases such as inflammatory bowel diseases, obesity and type 1 diabetes[18-21]. Epithelial cells form an integrated web through interaction of tight junction proteins including intracellular proteins, zona occludens (ZO)-1, (ZO)-2 and (ZO)-3, cingulin, 7H6 and ZA-1, and membrane proteins, occludin, claudin and junctional adhesion molecules[22,23]. The tight junction functions are affected by extracellular stimuli such as the microbial components, pro-inflammatory cytokines and stress[24,25].

Inflammation disrupts tight junctions. Inflammatory cytokines such as interleukin (IL)-13, and IL-6, increase tight junction permeability through increasing claudin 2 expression[26,27]. The activation of the inflammatory pathway nuclear factor (NF)-κB by TNF-α, down-regulates *ZO-1* gene expression and induces its relocation in Caco-2 cells[28]. Therefore, local inflammation impairs the barrier function of gut epithelium.

The "microflora hypothesis" suggests that gut microflora composition plays an important role in the immunological response of the gut[29]. Lactic acid bacteria are known to have an anti-inflammatory effect[30-34], and alteration of microflora composition is linked to the incidence of inflammatory bowel diseases[35,36]. Up to now, there is no published studies assessed gut microflora changes due to smoking.

We hypothesized that side stream smoking may possess a potent anti-inflammatory effect on the gut mucosal immune system which promotes the expression of tight junction proteins in the intestine, exerting beneficial effects on the prevention of ulcerative colitis.

MATERIALS AND METHODS

Animal care and experiment design

C57BL/6 female mice at 6 mo of age were housed in a temperature-controlled room with a 12 h light and 12 h darkness cycle and were given food and water *ad libitum*. Mice were placed in an exposure box and exposed to side-stream smoke for 1 h daily for 40 d. Commercial cigarettes (golden monkey, tar: 13 mg; nicotine: 1.1 mg; CO: 15 mg) were used at a dose equivalent to one commercial cigarette's smoke per day[37]. The animal care procedures described in this study was approved by the University of Wyoming Institutional Animal Use and Care Committee.

Tissue collection

On the day of necropsy, mice were anesthetized intraperitoneally with tribromoethanol (250 mg/kg body wt). Blood samples were collected from the orbital sinus while mice were under general anesthesia. Mice were then sacrificed by cervical dislocation. Large intestines were dissected, flushed with phosphate-buffer saline and then frozen in liquid nitrogen for immunoblotting and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses. Cecal contents from each mouse were collected and frozen for microflora analyses.

Reagents and antibodies

Antibodies against ZO1, ZO2, Claudin3 and Occludin were purchased from Invitrogen (Camarillo, CA). Antibodies against phospho- c-Jun N-terminal kinase (SAPK/JNK) (Thr183/Tyr185), SAPK/JNK, phospho-NF-κB p65 (ser536), NF-κB p65, phospho-IκB kinase (IKK) α/β (Ser176/180), IKKβ, phospho-IκBα, IκBα, phosho-p38 MAP kinase and p38 MAP kinase, phospho-AMP-activated protein kinase (AMPK) α and AMPKα were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against xanthine oxidase (XO), heat shock protein (HSP) 60 and superoxide dismutase (SOD) 1 were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Affinity BioReagents (Golden, CO).

Quantitative reverse transcription PCR

Total RNA was extracted from powdered large intestine using Trizol®Reagent (Sigma, St. Louis, MO), treated with DNase I (Qiagen, Valencia, CA) and purified with RNeasy Mini kit (Qiagen). cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). qRT-PCR was conducted on a Bio-Rad CFX96 machine and SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) was used for all qRT-PCR reactions. Mouse GAPDH was used as the housekeeping gene. Primer sequences are listed in Table 1. The final primer concentration was 200 nmol for each gene. The amplification efficiency was 0.90-0.99. The qRT-PCR conditions were 95 ℃, 3 min, and 35 cycles of 95 ℃ for 10 s, 58 ℃ for 20 s and elongation step at 72 ℃ for 20 s. At the end of each run, dissociation melting curve was obtained to confirm the purity of PCR products[38].

Microflora analyses

The frozen caecal contents (0.1 g) were homogenized and bacterial genomic DNA was extracted using a QIAamp DNA stool mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The abundance of specific intestinal bacterial groups was measured by qPCR using Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA) as described above. Group specific

Table 1 Primer sets used for quantitative reverse transcriptase polymerase chain reaction of mouse large intestine tissue

Gene name	Accession no.	Product size	Direction	Sequence (5′→3′)	Source
IL-6	NM_031168.1	107 bp	Forward	GCTGGTGACAACCACGGCCT	This study
			Reverse	AGCCTCCGACTTGTGAAGTGGT	
TNF-α	NM_013693.2	67 bp	Forward	TGGGACAGTGACCTGGACTGT	[58]
			Reverse	TTCGGAAAGCCCATTTGAGT	
Claudin 3	NM_009902.4	132 bp	Forward	CAGGGGCAGTCTCTGTGCGAG	This study
			Reverse	GCCGCTGGACCTGGGAATCAAC	
Occludin	NM_008756.2	308 bp	Forward	ATGTCCGGCCGATGCTCTC	[58]
			Reverse	TTTGGCTGCTCTTGGGTCTGTAT	
ZO-1	NM_009386.2	403 bp	Forward	ACCCGAAACTGATGCTGTGGATAG	[58]
			Reverse	AAATGGCCGGGCAGAACTTGTGTA	
ZO-2	AF113005.1	106 bp	Forward	CCCAGCACCAAGCCACCTTTTCA	This study
			Reverse	TCGGTTAGGGCAGACACACTCCC	
GAPDH	NM_008084.2	132 bp	Forward	AACTTTGGCATTGTGGAAGG	This study
			Reverse	GGATGCAGGGATGATGTTCT	

IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; ZO: Zona occludens; GPADH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Primer sets used for quantitative polymerase chain reaction of 16S rRNA of specific bacterial species or genus

Target organism	Primer set	Sequence (5′ to 3′)	Product size	Annealing temp (℃)	Reference
Bacteroides	BactF285	GGTTCTGAGAGGAGGTCCC	53	61	[59]
	UniR338	GCTGCCTCCCGTAGGAGT		61	
Clostridium butyricum	Cbut825F	GTGCCGCCGCTAACGCATTAAGTAT	213	72	[60]
	Cbut1038R	ACCATGCACCACCTGTCTTCCTGCC		72	
Clostridium clostridiiforme	Cclos99F	AATCTTGATTGACTGAGTGGCGGAC	148	62	[60]
	Cclos247R	CCATCTCACACTACCGGAGTTTTTC		62	
Clostridium perfringens	Cperf165F	CGCATAACGTTGAAAGATGG	104	61	[59]
	Cperf269R	CCTTGGTAGGCCGTTACCC		61	
Enterobacteriaceae	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	195	63	[60]
	Eco1652R	CTCTACGAGACTCAAGCTTGC		63	
Enterococcus	Ec-ssu1F	GGATAACACTTGGAAACAGG	115	60	[61]
	Ec-ssu1R	TCCTTGTTCTTCTCTAACAA		60	
Eubacteria	UniF340	ACTCCTACGGGAGGCAGCAGT	210	63	[62]
	UniR514	ATTACCGCGGCTGCTGGC		63	
Faecalibacterium prausnitzii	Fprau223F	GATGGCCTCGCGTCCGATTAG	199	58	[60]
	Fprau420R	CCGAAGACCTTCTTCCTCC		58	
Lactoccoci	LabF362	AGCAGTAGGGAATCTTCCA	315	56	[59]
	LabR677	CACCGCTACACATGGAG		56	
Mouse intestinal Bacteria	Uni516F	CCAGCAGCCGCGGTAATA	161	58	[59]
	MIBR677	CGCATTCCGCATACTTCTC		58	
Segmented filamentous bacteria	SFB736F	GACGCTGAGGCATGAGAGCAT	108	58	[59]
	SFB844R	GACGGCACGGATTGTTATTCA		58	
Ruminococcus albus	Ralb561F	CAGGTGTGAAATTTAGGGGC	246	63	[60]
	Ralb807R	GTCAGTCCCCCCACACCTAG		63	

or kingdom specific 16S rRNA gene primers were listed in Table 2. Eubacteria 16S rRNA was used as the housekeeping gene.

Immunoblotting analyses

Immunoblotting analyses were conducted as previously described[39,40]. Briefly, protein extracts from the mouse large intestine were separated by 5%-15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels and transferred to nitrocellulose membranes for immunoblotting analyses. Band density was normalized according to the GAPDH content[39,40].

Statistical analysis

Statistical analyses were conducted as previously described[41-43]. Data were analyzed as a complete randomized design using General Linear Model of Statistical Analysis System (2000). Mean ± SEM are reported. Mean difference was separated by a least significant difference multiple comparison test. Statistical significance is considered as $P < 0.05$.

RESULTS

Effect of side-stream cigarette smoking on the gut microflora composition

Quantitative PCR analysis of 16S rRNA showed that exposure of C57BL6 mice to side-stream cigarette smoking increased the amount of *Clostridium clostridiforme* and mouse intestinal bacteria (MIB) in the cecal microflora, while decreasing the content of *Lactoccoci, Ruminococcus albus*, *Enterobacteriaceae* and segmented filamentous bacteria

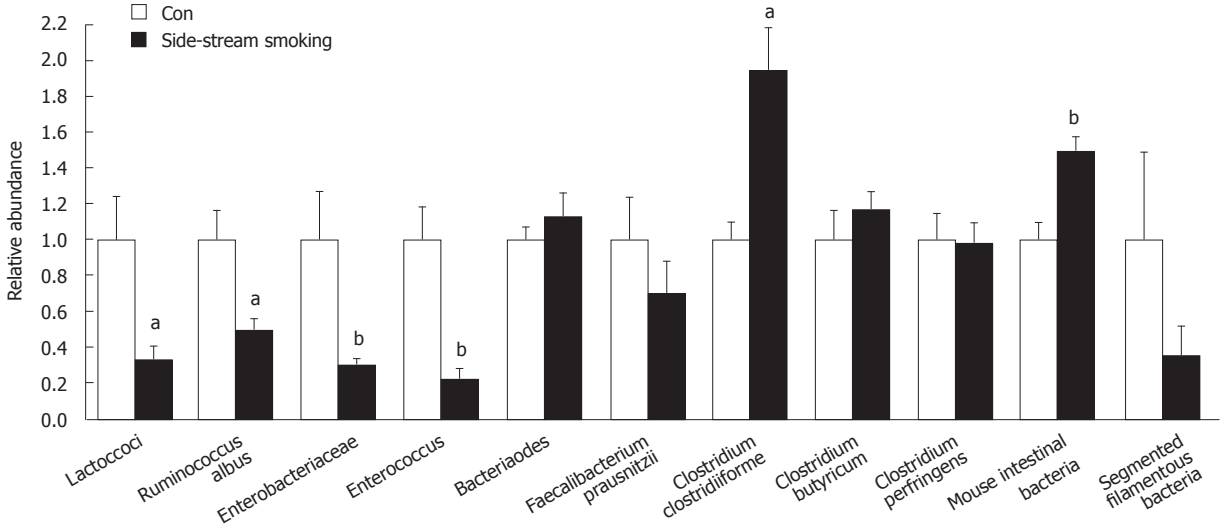

Figure 1 Cecal microflora composition of Con and side-stream smoking mice. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group).

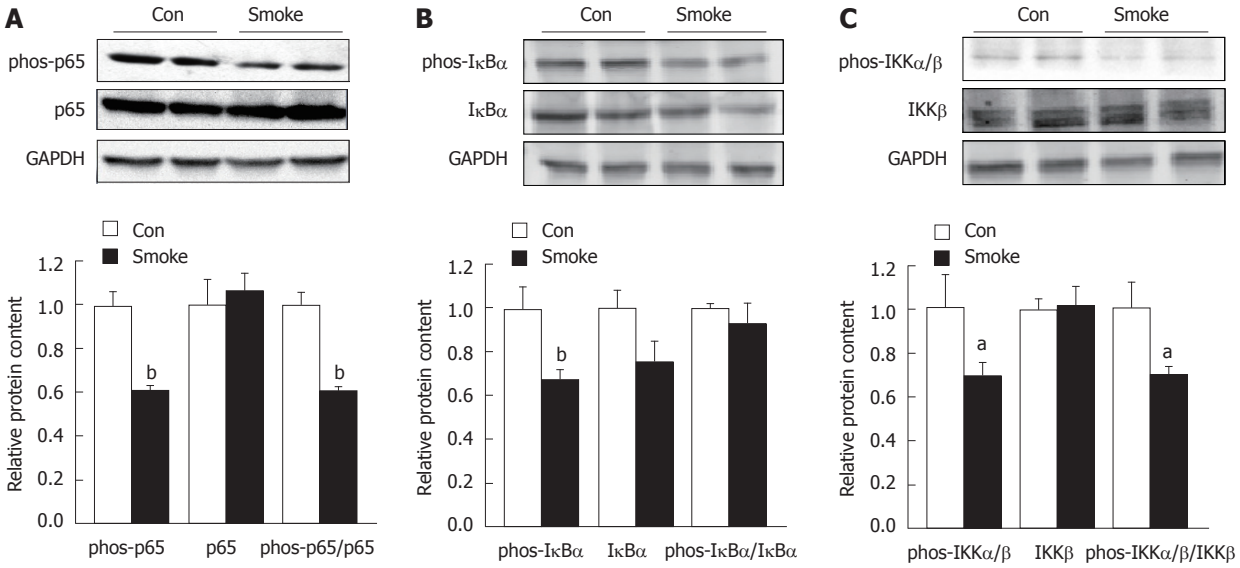

Figure 2 NF-κB signaling pathway in large intestine of Con and side-stream smoking mice. A: Phos-p65 and p65; B: Phos-IκBa and IκBa; C: Phos-IKKα/β and IKKβ. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group).

(SFB) compared with those of control mice (Figure 1).

Intestinal inflammatory responses of gut to side stream smoking

Side-stream smoking decreased phosphorylation of NF-κB p65, a key mediator of the NF-κB inflammatory signaling pathway. Consistently, phosphorylation of IκBα and IKKα/β were also down-regulated in mice exposed to side-stream smoking, indicating that smoking is capable of reducing inflammation in the gut (Figure 2). qRT-PCR analysis indicated that mRNA expression of the two main inflammatory cytokines, TNFα and IL-6, were not changed (data not shown).

Side-stream smoking induced oxidative stress in large intestine

There was an enhanced oxidative stress in side-stream smoking mice compared to that of control mice, as indicated by increased XO ($P = 0.06$) and decreased SOD1 ($P < 0.01$) protein content in the side-stream smoking mice (Figure 3). Meanwhile, the heat shock protein 60 (HSP60) decreased in the side-stream smoking mouse large intestine when compared to that of control mice (Figure 3). Consistently, the phosphorylation of stress signaling mediators, JNK and p38 MAP kinase, were increased in the large intestine of side-stream smoking mice (Figure 4). However, the phosphorylation of another kinase related to stress, AMPK, was reduced in response to side-stream smoking (Figure 5).

Tight junction protein expression

Both mRNA expression and protein content of selected tight junction proteins were further analyzed. Protein content of claudin3 ($P < 0.01$) and ZO2 ($P < 0.05$) were

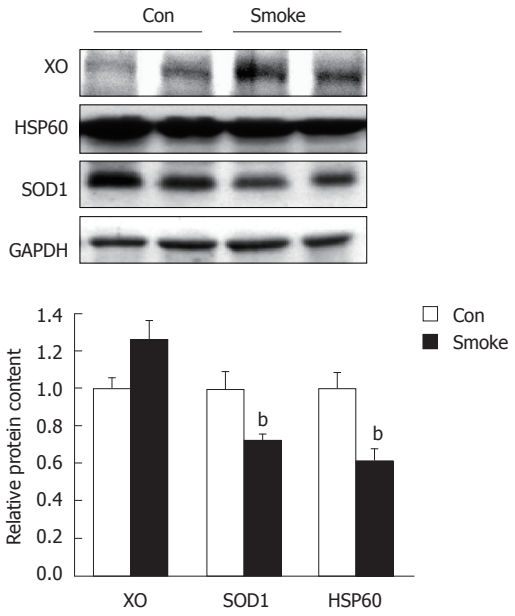

Figure 3 Xanthine oxidase, superoxide dismutase 1 and heat shock protein 60 content in large intestine of Con and side-stream smoking mice. $^{b}P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group). XO: Xanthine oxidase; SOD1: Superoxide dismutase 1; HSP60: Heat shock protein 60.

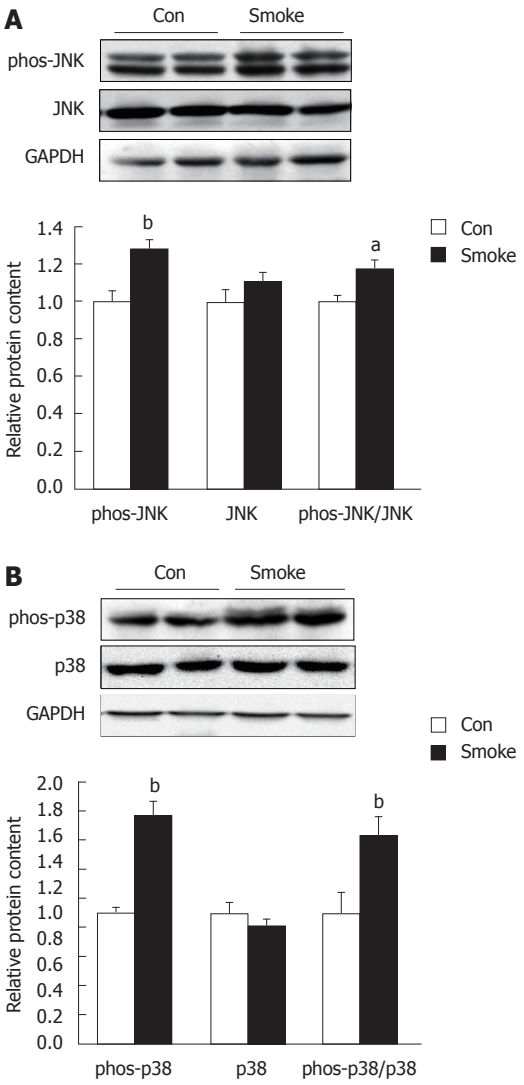

Figure 4 MAP kinase signaling pathways in large intestine of Con and side-stream smoking mice. A: JNK; B: MAP kinase p38. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group).

increased in the large intestine of side-stream smoking mice (Figure 6B), while there is no difference in their mRNA expression (Figure 6A).

DISCUSSION

Epidemiology studies have shown that smoking, including passive smoke inhalation, reduces the incidence of ulcerative colitis, which may be due to the reduction of epithelial permeability[44]. Intestinal permeability was reduced in healthy smokers compared to the non-smokers[45,46].

Mechanisms by which side-stream smoking improves intestinal tight junctions are not well understood. Previous studies suggest that activation of NF-κB signaling increases intestinal permeability[47]. In this study, we observed that the NF-κB signaling was down-regulated in mice exposed to side-stream smoking. This indicates that side-stream smoking negatively regulates NF-κB signaling which might be a contributing factor to the reduction of intestinal permeability. We also observed that side-stream smoking increased Claudin3 and ZO-2 content without affecting Occludin and ZO-1. In summary, our data revealed that side-stream smoking up-regulated the expression of tight junction proteins and inhibited NF-κB signaling, which may be responsible for the preventive effect of smoking on ulcerative colitis.

Smoking generates reactive oxygen species and nitrogen species in blood, resulting in oxidative stress[48-50]. In this study, we also observed that oxidative stress related enzymes such as xanthine oxidase and superoxide dismutase 1 were altered in the large intestine due to side-stream smoking. Consistent with altered oxidative stress, two pivotal stress signaling mediators, the activation of JNK and p38 signaling were enhanced in the large intestine of mice exposed to side stream smoking. Previously, it was reported that oxidative stress related signaling promotes tight junction protein claudin1 expression in hepatocytes and Sertoli cells[51,52].

A recent published study in gut epithelial cells shows that AMPK is related to the impairment of tight junction and barrier properties of gut induced by inflammation[53]. Our data showed that AMPK activity was dramatically inhibited in the gut tissue of side-stream smoking mice, which may provide an additional mechanism for the association between passive smoking and gut epithelial barrier function.

Furthermore, we found that microflora were altered due to the side-stream smoking. The "microflora hypothesis" suggests that gut microflora composition plays an important role in the immunological response of the gut[29]. Up to now, there have been no published studies assessing changes in gut microflora due to smoking. Our data showed that exposure to side-stream smoking altered the composition of cecal microflora, reducing *Fermicutes*

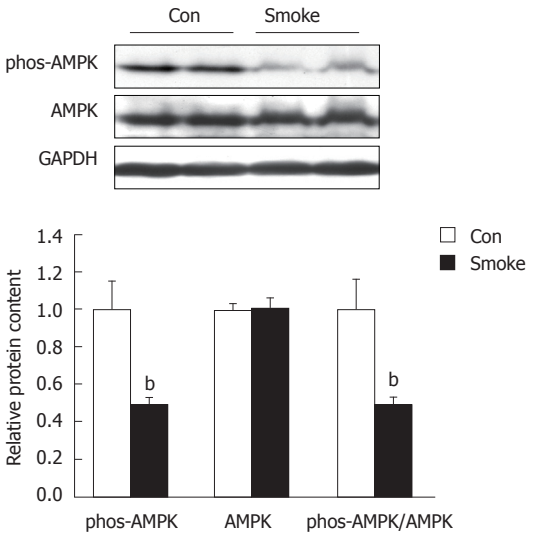

Figure 5 Total AMP-activated protein kinase α subunit content and its phosphorylation at Thr 172 in large intestine of Con and side-stream smoking mice. $^{b}P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group). AMPK: Total AMP-activated protein kinase.

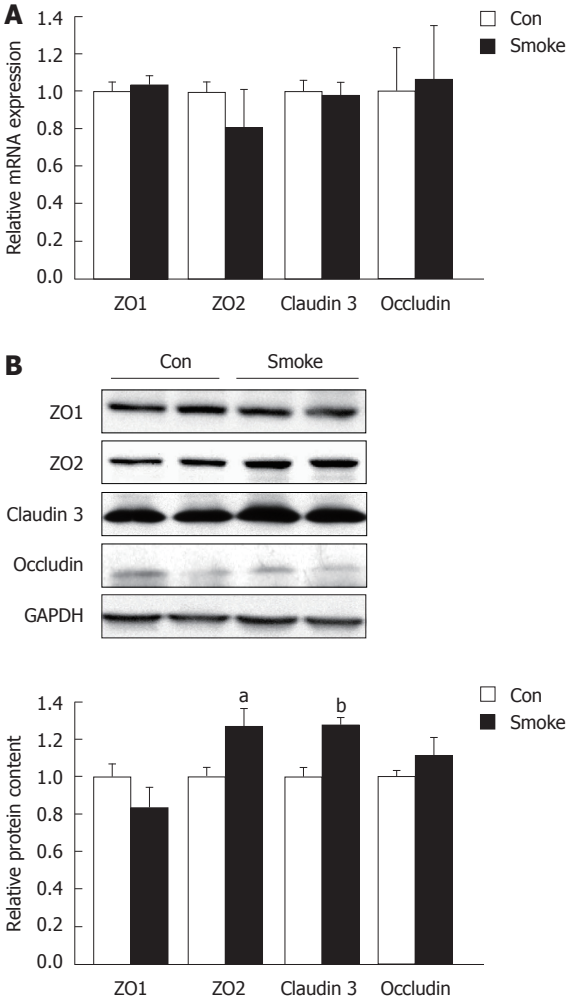

Figure 6 Tight junction protein content in large intestine of Con and side-stream smoking mice. A: mRNA expression; B: Protein content. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group).

and *Enterobacteriaceae*. Both *Fermicutes* and *Enterobacteriaceae* belong to a group of bacteria contributing to fermentation and nutrient intake. *Lactoccoci* and other lactic acid bacteria are known to have anti-inflammatory effects[30-34]. The dramatic reduction of *Lactoccoci* in side-stream smoking mice indicates that *Lactoccoci* might not be responsible for the reduced inflammation in the gut of side-stream smoking mice. The reason for the reduction of *Lactoccoci* in cecal microflora due to smoking is unclear, but might be related to oxidative stress. Many *Lactoccoci* lack catalase and are sensitive to oxidative stress[54], which may render them less competitive in the oxidative environment induced by smoking. We also observed that MIB was increased while SFB was decreased in smoking mice. Because SFB is known to have important roles in maturation of the gut immune system, its reduction in smoking mice could be associated with the adverse effect of smoking on Crohn's disease[55]. MIB refers to a group of bacteria called *Cytophaga-Flavobacter-Bacteroides* phylum[56], and their abundance in the gut is known to be altered by environmental factors[57]. The biological effect of MIB alteration due to smoking is unclear.

In conclusion, data from our present study demonstrated that exposure to side-stream smoking inhibited mucosal inflammation and enhanced the expression of tight junction proteins in the large intestine. Further, side-stream smoking increased oxidative stress and altered gut microflora composition.

COMMENTS

Background

Despite its apparent harmful effects, side-stream smoking reduces the risk of inflammatory gastrointestinal diseases. Gut epithelial integrity and barrier function is a central predisposing factor to inflammatory bowel diseases. Local inflammation impairs the barrier function of gut epithelium. We hypothesized that side stream smoking may possess potent anti-inflammatory effects, which promote the expression of tight junction proteins in the intestine, exerting beneficial effects on the prevention of ulcerative colitis.

Research frontiers

Epidemiologic studies indicate that smoking had a protective effect on ulcerative colitis though the underlying mechanisms remain elusive. In this study, we demonstrated that exposure to side-stream smoking inhibited mucosal inflammation, improved gut tight junction protein expression, and altered gut microflora composition in mice, which could partially explain the preventive effects of smoking on ulcerative colitis.

Innovations and breakthroughs

Recent epidemiologic studies have highlighted the preventive effect of smoking on ulcerative colitis. This is the first study to report that side-stream smoking has anti-inflammatory effect on gut mucosal, improving gut tight junction protein expression and altering gut microflora composition.

Applications

By understanding how side-stream smoking affects gut mucosal immune response and tight junction protein expression, the authors can develop alternative strategies to reduce the risk of ulcerative colitis and possibly other inflammatory bowel diseases without the harmful effects of smoking.

Terminology

Inflammatory bowel diseases are characterized by chronic inflammation in the intestine. Side-stream smoking, mimicking secondhand smoking, has anti-inflammatory effect, which may be responsible for its beneficial effects against ulcerative colitis.

Peer review

The authors address the observation that passive smoking decreases inflammatory response in large intestine. The authors are to be commended for excel-

lent work in performing a very important and informative study. The experimental methods are well summarized and explained. The statistics are appropriate for this study.

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ORIGINAL ARTICLE

Agmatine induces gastric protection against ischemic injury by reducing vascular permeability in rats

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Abstract

AIM: To investigate the effect of administration of agmatine (AGM) on gastric protection against ischemia reperfusion (I/R) injury.

METHODS: Three groups of rats (6/group); sham, gastric I/R injury, and gastric I/R + AGM (100 mg/kg, i.p. given 15 min prior to gastric ischemia) were recruited. Gastric injury was conducted by ligating celiac artery for 30 min and reperfusion for another 30 min. Gastric tissues were histologically studied and immunostained with angiopoietin 1 (Ang-1) and Ang-2. Vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) were measured in gastric tissue homogenate. To assess whether AKt/phosphatidyl inositol-3-kinase (PI3K) mediated the effect of AGM, an additional group was pretreated with Wortmannin (WM) (inhibitor of Akt/PI3K, 15 μg/kg, i.p.), prior to ischemic injury and AGM treatment, and examined histologically and immunostained. Another set of experiments was run to study vascular permeability of the stomach using Evan's blue dye.

RESULTS: AGM markedly reduced Evan's blue dye extravasation (3.58 ± 0.975 μg/stomach *vs* 1.175 ± 0.374 μg/stomach, $P < 0.05$), and VEGF (36.87 ± 2.71 pg/100 mg protein *vs* 48.4 ± 6.53 pg/100 mg protein, $P < 0.05$) and MCP-1 tissue level (29.5 ± 7 pg/100 mg protein *vs* 41.17 ± 10.4 pg/100 mg protein, $P < 0.01$). It preserved gastric histology and reduced congestion. Ang-1 and Ang-2 immunostaining were reduced in stomach sections of AGM-treated animals. The administration of WM abolished the protective effects of AGM and extensive hemorrhage and ulcerations were seen.

CONCLUSION: AGM protects the stomach against I/R injury by reducing vascular permeability and inflammation. This protection is possibly mediated by Akt/PI3K.

Key words: Ischemia reperfusion injury; Agmatine; Wortmannin; Vascular permeability; Monocyte chemoattractant protein-1; Stomach; Vascular endothelial growth factor

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INTRODUCTION

Agmatine (AGM), 4-(aminobutyl) guanidine, is a natural biogenic endogenous dicationic amine metabolite mainly present in the deprotonated form at physiological pH and produced by decarboxylation of L-arginine *via* arginine decarboxylase in bacteria, plants, invertebrates, and mammals[1-5]. It is not supplied by nutritional com-

ponents or bacterial colonization. AGM is metabolized by two distinct pathways depending on the tissue where it is contained: by "agmatinase" (AGM uryl hydrolase) to putrescine with cleavage of urea, mainly in the brain, and by "diamineoxidase" (DAO), in peripheral tissues, to 4-guanidinobutyraldehide, then dehydrogenated and hydrolyzed by specific enzymes and excreted out of the body. The heterogeneous location of DAO suggests that certain tissues or organs may have the capacity to regulate local AGM levels[6,7]. AGM is transported to organs by an energy-dependent mechanism which is inhibited by dose-dependent administration of putrescine, suggesting a correspondence between the transport mechanism of polyamines and AGM, probably using a carrier[8,9].

After its discovery in the brain, AGM was demonstrated in nearly all organs of rats, with organ-specific distribution. Its highest concentrations were found in the stomach (71 ng/g wet weight), followed by the aorta, small and large intestine, and spleen[10,11]. AGM was also shown in vascular smooth muscle and endothelial cells[12], and in plasma of rats at a concentration of 0.45 ng/mL, which is similar to that of catecholamines[10]. The source of circulating AGM remains undefined. In humans, higher plasma concentrations (47 ng/mL) were determined in comparison to rats[13]. The reasons underlying this large difference remain to be clarified.

It is becoming clear that AGM has multiple physiological functions in the body. It acts as a potential neurotransmitter in the brain[14,15], and a regulator of polyamine concentration[16] by acting on different enzymes involved in the polyamine pathway. It inhibits all isoforms of nitric oxide synthase (NOS), providing evidence of its important role in modulating NO production as an endogenous regulator[17]. In particular, AGM irreversibly inhibits the endothelial NOS and downregulates the inducible form (iNOS), and exhibiting a neuroprotective role since NO contributes to ischemic brain injury[18].

It has been reported that AGM is protective against ischemia reperfusion (I/R) injury in different organs including the brain, retina, kidney and heart[19-22]. However, no previous reports on its protective effect in gastric reperfusion injury have been investigated. Despite the fact that AGM is a strong base[23] and is found in mucous-secreting cells and in parietal cells where it localizes in the canaliculi, it was reported to be deleterious in ethanol-induced gastric lesions[5] as well as in gastric stress-induced lesions[24,25]. Therefore, the aim of the present study was to investigate whether or not the administration of AGM is protective to rat stomach subjected to I/R injury, and the mechanisms involved.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 170-210 g were obtained from the College of Medicine Animal House at King Saud University. Rats were maintained on standard rat chow and tap water *ad libitum*. Rats were kept in an air-conditioned room with a 12 h day/light cycle. Animals were fasted 12 h prior to the experimental procedure. All studies were approved by the Ethics Committee of King Saud University.

Experimental design

Rats were divided into 3 experimental groups (6 rats/group): (1) control sham-operated group; (2) a gastric I/R group; and (3) I/R + AGM (100 mg/kg) group, administered AGM 15 min prior to I/R injury induction. Rats were anesthetized by i.p. injection of urethane at a dose of 125 mg/100 g body weight (BW). To investigate whether or not the protection induced by AGM is mediated by the Akt/IPK3 pathway, wortmannin (WM), an inhibitor of this pathway, was given at a dose of 15 μg/kg, i.p.[26], 15 min prior to AGM treatment in an additional I/R injury group. The stomach was observed macroscopically for hemorrhages and ulceration. The dose of AGM, selected for the current study was based on the previously published doses used in models of brain[19] and myocardial I/R injury[22]. AGM was dissolved in normal saline and controls received saline in an equivalent volume. WM was dissolved in dimethyl sulfoxide (10%).

Experimental model

Gastric IR lesions were produced as described by Yoshikawa *et al*[27]. Briefly, the stomach was exposed and the esophagus and the pylorus were occluded using bulldog clamps. The celiac artery was clamped and 100 mmol hydrochloric acid (HCL, 1 mL/100 g BW) was placed in the stomach to maintain acid levels during ischemia. The acid was then removed 25 min after ischemia and clamps were removed 30 min after ischemia. The tissues were allowed to re-perfuse for 30 min and then the stomach was removed and examined. AGM 100 mg/kg was found to be effective depending on histological examination and Evans blue (EB) dye extravasation.

At the end of the experiment gastric tissues were collected. Gastric samples were snap-frozen in liquid nitrogen and stored at -80 ℃ for subsequent assays of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1). A piece of each stomach was fixed in 4% phosphate-buffered formalin, embedded in paraffin, and cut. Paraffin sections were hydrated and stained with hematoxylin and eosin (HE) for assessment of mucosal damage or stained with sera specific for angiopoietin-1 (Ang-1) and Ang-2, (R and D Systems, United States).

Determination of vascular permeability

EB dye, an azo dye, is widely used as an indicator of increased capillary permeability[28-30]. Systemic administration of EB leads to the formation of a dissociable complex with serum albumin, and when there is microvascular tissue damage, EB extravasates. In another three experimental groups, 1 mL of EB (0.5% v/w) was injected i.v. after reperfusion or sham operation. The amount of EB that accumulated in the stomach within

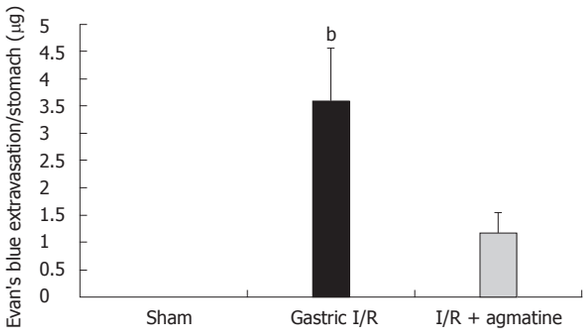

Figure 1 Evan's blue extravasation as a measure of vascular permeability in the studied groups. Gastric ischemia reperfusion (I/R) injury induced extravasation of Evan's blue dye due to increased vascular permeability. Agmatine (AGM) pre-treatment (100 mg/kg, i.p.) significantly attenuated the vascular leakage (gastric I/R *vs* I/R + agmatine, $^{b}P < 0.001$). Results are expressed as mean ± SD.

the reperfusion period was measured. Briefly, the animals were killed and the stomach was removed. After collecting the gastric content carefully by lavage with 5 mL cold distilled water, the stomach was opened along the greater curvature and the corpus mucosa was scraped off and put into a tube containing 5 mL distilled water. The EB was extracted by a modified method of Lange *et al*[29] and its concentration was spectrophotometrically quantified. The EB present in the gastric contents and mucosa was extracted by adding 5 mL formamide to each tube and kept in a shaking water bath at a temperature of 50 ℃ for 24 h. This was followed by centrifugation at 3000 *g* for 10 min and the absorbance of supernatant was measured at 612 nm (Lambada 5, Perkin-Elmer, Pomona, CA, United States). The amount of EB was calculated from a previously prepared standard curve and expressed as μg per stomach.

Histological study

Gastric tissues from the studied groups were fixed in 10% phosphate-buffered formalin, embedded in paraffin and 4 μm sections were made, followed by staining with HE and were examined histologically for mucosal damage.

Enzyme-linked immunosorbent assay

VEGF and MCP-1 were assayed in a supernatant of gastric tissue homogenate and calculated according to protein concentration in each sample. Protein was determined in each sample using Bradford Reagent (Biorad, United States). Concentrations of VEGF and MCP-1 were measured using an ELISA kit according to the manufacturer's instructions (R and D Systems, United States).

Immunohistochemistry

Immunostaining was performed using formalin fixed, paraffin-embedded sections (4 μm) after dewaxing and rehydration. Endogenous peroxidase was quenched with 3% H_2O_2 for 30 min and sections were blocked with 10% normal goat serum (Sigma). Sections were incubated with Ang-1 and Ang-2 (Santa Cruz, Biotech., United States) at a concentration of 1:200 and were kept at room temperature for 2 h. Sections were then washed and incubated with secondary antibody, and immunoperoxidase staining was carried out using the Vectastain ABC Elite reagent kit (Vector Laboratories, CA, United States). Di-aminobenzidine was used as a chromogen. All slides were counterstained with HE.

Chemicals and reagents

All chemicals were purchased from Sigma (St, Louis, MO, United States) unless otherwise specified.

Statistical analysis

All values are expressed as the mean ± SD. Statistical significance of differences was determined using one-way analysis of variance. Further statistical analysis for *post hoc* comparisons was carried out using the Tukey test. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of AGM on gastric tissue vascular permeability

Gastric perfusion for 30 min following 30 min of ischemia induced a marked leakage of EB dye in the stomach lumen (3.58 ± 0.975 μg/stomach). The administration of AGM 100 mg/kg prior to induction of ischemia attenuated the leakage by about 60% when compared with no treatment (1.173 ± 0.374 μg/stomach, $P < 0.05$) (Figure 1).

Histological assessment

The histological features of gastric I/R injury included hemorrhage, and ulceration of the mucosa with inflammatory cell infiltration (Figure 2B). Gastric mucosa of normal rats showed intact mucosa and sub-mucosa (Figure 2A). The administration of AGM at a dose of 100 mg/kg attenuated the mucosal damage by some of 80% of the surface exposed (Figure 2C) with reduction in hemorrhage, ulceration and cellular infiltration. WM prevented the protective effect resulting in extensive ulceration, edema, and hemorrhage (Figure 2D).

Effect of AGM on VEGF gastric tissue level

Following ischemic injury to the stomach, VEGF demonstrated an increase in gastric tissue homogenate (48.4 ± 6.53 pg/100 mg protein) compared with normal gastric tissue (32.725 ± 37.7 pg/100 mg protein). AGM treatment significantly reduced this increase (36.87 ± 2.71 pg/100 mg protein *vs* 48.4 ± 6.53 pg/100 mg protein, $P < 0.05$) (Figure 3).

Effect of AGM on MCP-1 gastric tissue level

As shown in Figure 4, the I/R injury of the gastric mucosa induced an increase in the level of MCP-1 compared with the mucosa in control sham rats (41.17 ± 10.3 pg/100 mg protein *vs* 18.3 ± 7.4 pg/100 mg protein, $P < 0.01$). AGM pretreatment markedly reduced the MCP-1

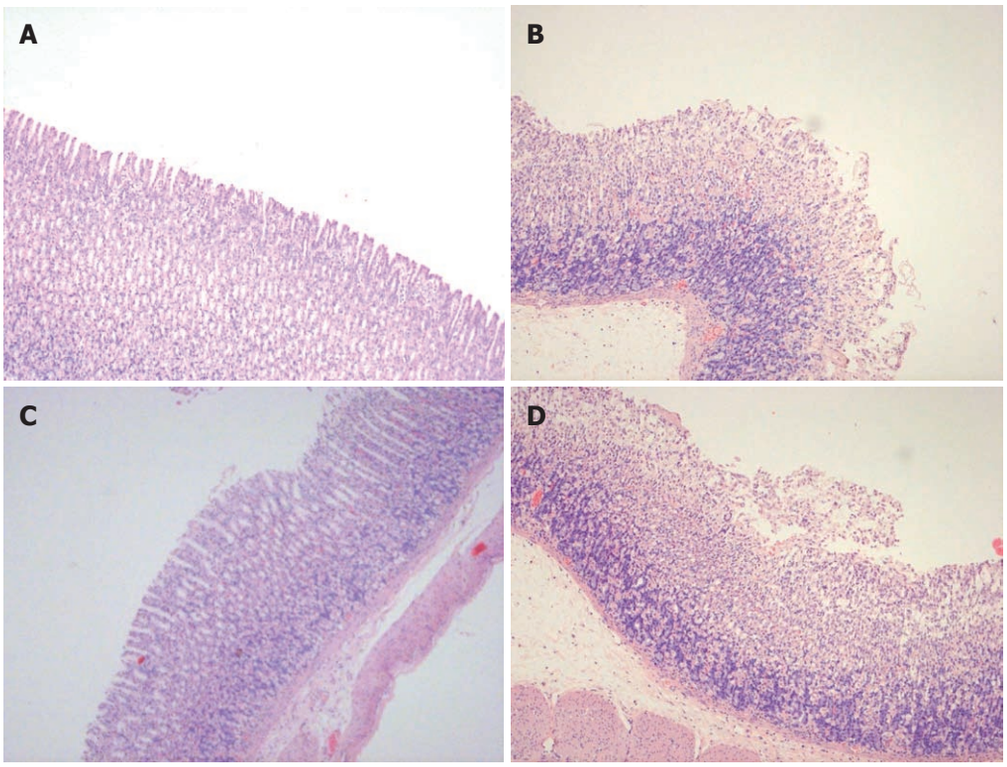

Figure 2 Histological appearance of the gastric mucosa of HE stained sections of the studied groups. A: Sham-operated showing intact mucosa; B: Gastric ischemia reperfusion (I/R) showing hemorrhages, edema and ulceration; C: Agmatine (AGM) treated group (100 mg/kg, i.p. prior to I/R) with marked preservation of gastric mucosa and disappearance of ulceration and hemorrhages; D: Effect of inhibition of Akt/phosphatidyl inositol-3-kinase (PI3K) (wortmannin 15 μg/kg, i.p.) prior to AGM treatment, showing extensive lesions and salvage of gastric mucosa into the lumen (× 20 magnification).

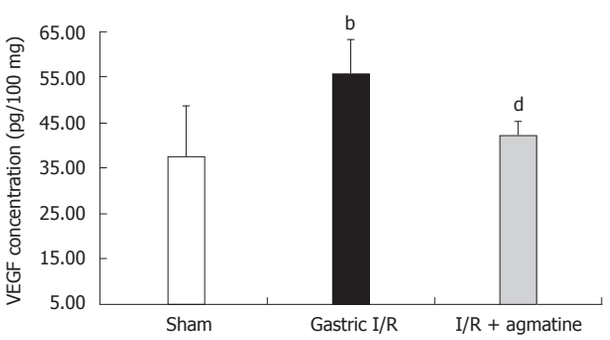

Figure 3 Effect of administration of agmatine on gastric vascular endothelial growth factor tissue level. Gastric vascular endothelial growth factor (VEGF) protein significantly increased after ischemia reperfusion (I/R) injury, $^{b}P < 0.01$ *vs* sham group. Administration of agmatine administration (100 mg/kg, i.p.) 15 min prior to gastric I/R reduced the VEGF level in gastric tissue homogenate, $^{d}P < 0.01$ *vs* I/R group. Results are expressed as mean ± SD.

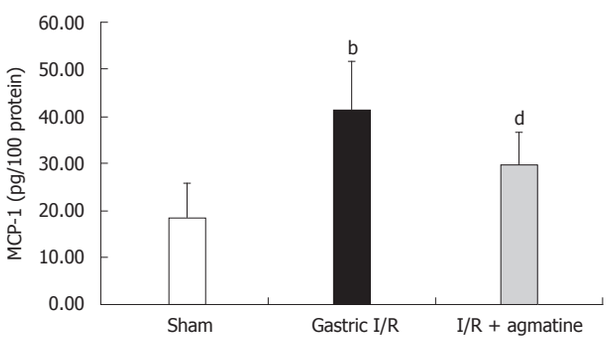

Figure 4 Effect of AGM administration on gastric monocyte chemoattractant protein-1 tissue level. Gastric monocyte chemoattractant protein-1 (MCP-1) protein significantly increased after ischemia reperfusion (I/R) injury, $^{b}P < 0.01$ *vs* sham group. Administration of agmatine (100 mg/kg, i.p.) 15 min prior to gastric I/R reduced the MCP-1 level in gastric tissue homogenate, $^{d}P < 0.01$ *vs* I/R group. Results are expressed as mean ± SD.

levels relative to I/R injury (29.5 ± 7 pg/100 mg protein *vs* 41.17 ± 10.4 pg/100 mg protein; $P < 0.01$).

Ang-1 and Ang-2 immunostaining

A very faint staining for Ang-1 was seen in normal rat stomach (Figure 5A), while it was extensively expressed in areas where congestion and damage occurred in ischemia reperfused stomach (Figure 5B). However, AGM pretreatment (100 mg/kg) markedly attenuated the expression of Ang-1 (Figure 5C). Ang-2 expression of normal rat stomach (Figure 6A), was almost undetected. Extensive expression of Ang-2 was seen mostly in the mucosa of ischemia reperfused stomach (Figure 6B). AGM markedly attenuated Ang-2 expression and protected the mucosa from injury (Figure 6C).

Effect of blocking the phosphatidyl inositol-3-kinase pathway on protection induced by AGM

AKt/phosphatidyl inositol-3-kinase (PI3K) is described as an important component of cell survival pathways in many cell types[26]. Pretreatment of rats with WM (15 μg/kg per i.p.), an inhibitor of AKt/PI3K, 15 min prior to

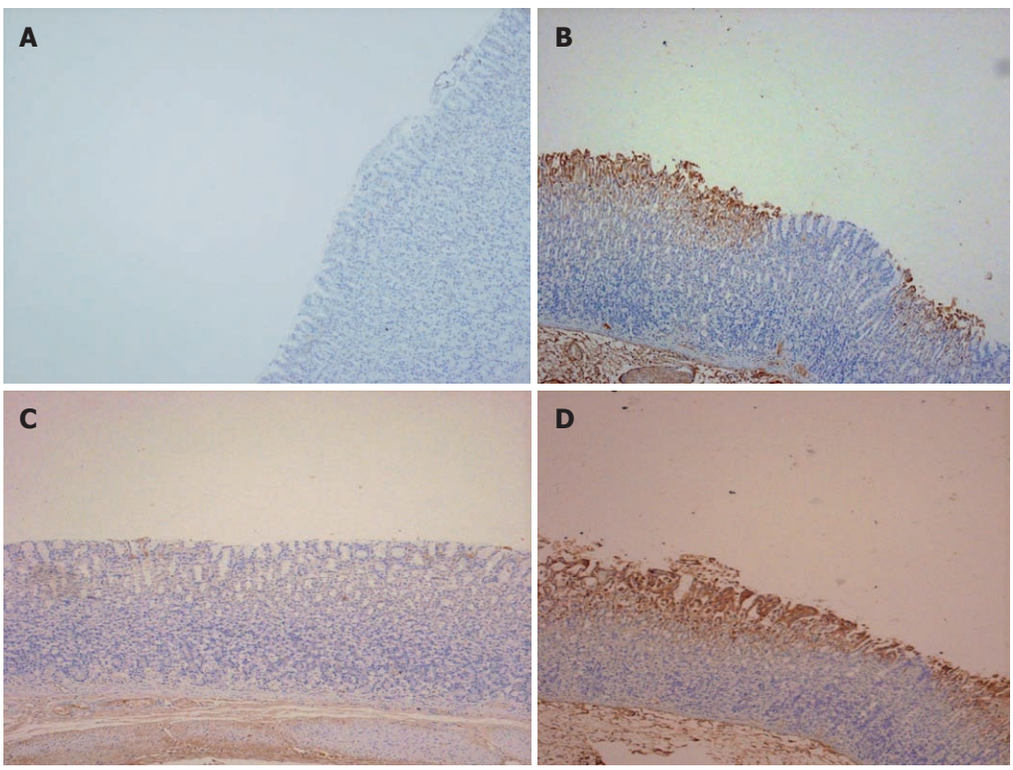

Figure 5 Photomicrographs representative of angiopoietin-1 immunostaining of gastric sections of the studied groups. A: Sham-operated showing lack of expression of angiopoietin-1 (Ang-1); B: Gastric ischemia reperfusion (I/R) group showing extensive cytoplasmic expression of Ang-1 particularly in areas of congestion and damage; C: Agmatine (AGM) treated group (100 mg/kg, i.p.), with marked attenuation of Ang-1 expression in intact mucosa; D: Wortmannin (15 μg/kg, i.p.) treated group prior to AGM administration abolished the effect of AGM and the expression of Ang-1 is marked (× 20 magnification).

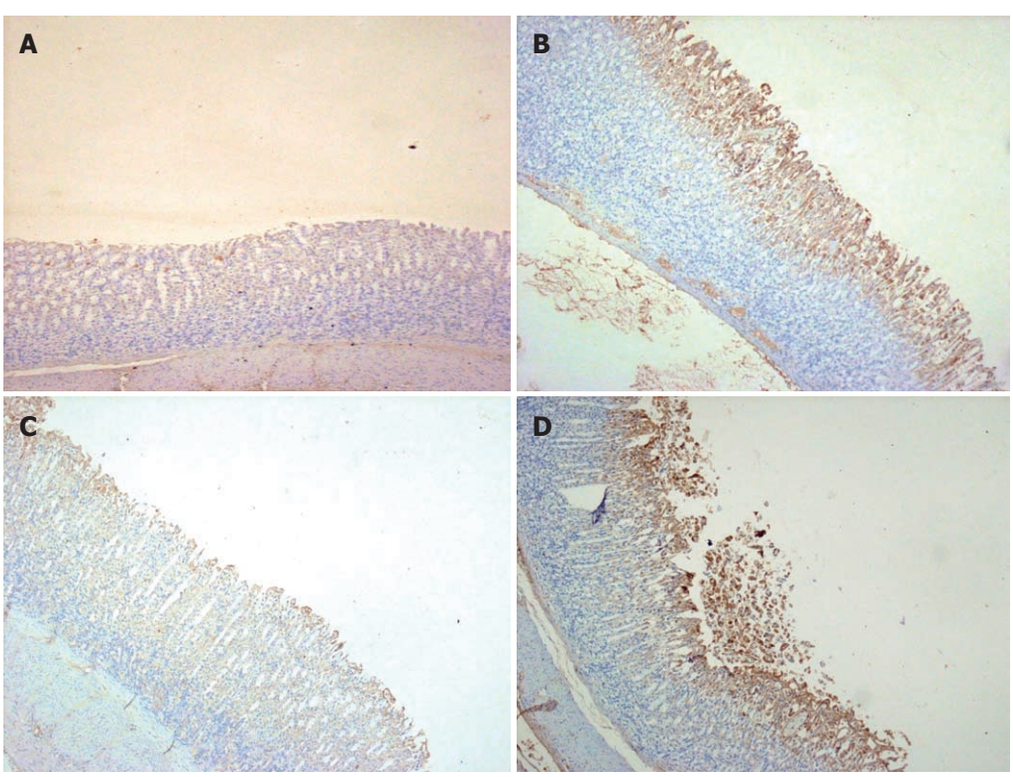

Figure 6 Photomicrographs representative of angiopoietin-2 immunostaining of gastric sections of the studied groups. A: Sham-operated showing faint expression of angiopoietin-2 (Ang-2); B: Gastric ischemia reperfusion (I/R) group showing extensive cytoplasmic expression of Ang-2 in areas of congestion and damage, and in the submucosa; C: Agmatine (AGM) treated group (100 mg/kg, i.p.), with marked attenuation of Ang-2 expression in intact mucosa; D: Wortmannin (15 μg/kg, i.p.) treated group prior to AGM administration abolished the effect of AGM and the expression of Ang-2 is marked in mucosa and sloughed tissues (× 20 magnification).

AGM administration, markedly prevented its protective effect and extensive hemorrhages and ulceration were demonstrated microscopically (Figure 2D), suggesting that AGM probably acted on this signaling pathway to protect the stomach from I/R injury. The administration of WM prior to AGM also induced marked expression of both Ang-1 and Ang-2 in the mucosa and even in the submucosa (Figures 5D and 6D).

DISCUSSION

As mentioned earlier, the present study examined whether or not the administration of AGM prior to gastric I/R injury has a protective effect. Our results revealed a gastroprotective effect of AGM by reducing vascular permeability of the gastric mucosa as evidenced by reduction of EB dye extravasation, Ang-1 and Ang-2 protein expression in gastric sections, as well as VEGF and MCP-1 concentrations in gastric tissue homogenate. This effect is probably mediated by the Akt/PI3K pathway.

The gastric injury model we used in the present study is a gastric ischemia reperfusion model. In this model we injected 100 mmol HCL (1 mL/100 g BW) to maintain the gastric acid level during the procedure. The acid was then removed 25 min after ischemia and clamps were removed 30 min after ischemia. I/R injury was demonstrated macroscopically and microscopically in the form of congestion, hemorrhages, and mucosal ulceration. With prior administration of AGM, hemorrhages, blood clots and ulceration were attenuated. This protective effect, while supported by some studies, was opposed by others.

Circumstantial evidence was provided in a recent study that AGM is protective to the gastric mucosa[23]. That study, utilizing immunohistochemistry, showed that AGM is found in the mucus-secreting cells of the stomach and in the parietal cells where it is localized in the lumen of the canaliculi. Biochemically, basic amino acids are ideally suited to act as a source of carbon dioxide in the stomach, and the most basic amino acid is arginine (isoelectric point 11.15). A significant source of arginine is found in the stomach[23]. CO_2 can be produced from the decarboxylation of arginine. The activation segment of pepsinogen in the parietal cell canaliculi can provide a source of arginine[31]. AGM is the decarboxylation product of arginine. Interestingly, the highest concentration of AGM is found in the stomach[10]. The fact that AGM is such a strong base and its cellular localization is in the gastric mucosa makes AGM a strong candidate for a protective role against HCL formed in the stomach[23]. In addition, *Helicobacter pylori* (*H. pylori*) infection is associated with a decrease in the amount of mucous-secreting cells in the stomach. This change is associated with a decrease in the amount of AGM in these mucous-secreting cells[32]. It was speculated that such a decrease in the amount of AGM in the epithelium of the *H. pylori*-infected stomach would make this epithelium more vulnerable to damage by gastric acid[23]. On the other hand, a group of investigators reported higher AGM concentrations in gastric juice from *H. pylori*-positive patients than from *H. pylori*-negative patients, and concluded that AGM is deleterious to the stomach and may be involved in the pathogenesis of gastroduodenal lesions[33]. However, these results suggest an association rather than a causal link. Furthermore, these results might implicate AGM as a counter-regulatory molecule to *H. pylori*. Supporting our speculation, a recent report demonstrated that bacteria such as *Escherichia coli* and *H. pylori* utilize AGM to survive the highly acidic medium of the stomach and even prevent AGM being taken up by stomach cells[34]. This explains the high levels of AGM previously reported in gastric juice of *H. pylori* patients, suggesting a compensatory mechanism.

The pattern of gastric response to AGM in our study is opposite to what has been reported by some other previous studies[24,25], which showed that AGM augments gastric acid and pepsin secretion, decreases gastric adherent mucus and worsens experimental gastric mucosal injury in rats in a pylorus-ligated ischemic stress model. Similar results demonstrating exacerbation of gastric mucosal injury by AGM pretreatment in ethanol-induced stress model in rats have been reported[5]. The inconsistency of these results with ours could be attributed to the models used and the duration of gastric stress. In our model, the rats were exposed to 30 min of ischemia and 30 min of reperfusion, while these investigators exposed the animals to 4 h of stress. In addition, these studies administered AGM at a dose of 10 mg/kg[24] or 20 mg/kg[5], which is much lower than that used in the present study or other studies. For example in a rat model of brain I/R injury AGM was given at a dose of 100 mg/kg[19].

The mechanisms by which AGM induced gastric protection were the focus of the present study. Increased vascular permeability occurs after insult to the gut[35] and hence, reduction of hyper-permeability can induce tissue protection. The current work provides evidence that AGM works by reducing vascular permeability of the stomach in response to I/R injury. This was investigated using EB dye extravasation as a measure of vascular permeability. Also, we measured VEGF concentration in gastric tissues and Ang-1 and Ang-2 distribution in gastric sections. The present study showed an elevation of VEGF content in the stomach following 30 min of ischemia and 30 min of reperfusion. This increase is most probably due to increased blood to the tissue upon reperfusion, but the possibility of increased expression of VEGF protein could be a factor. The prevention of this increase by AGM could be explained by the capability of AGM to reduce vascular permeability as was seen in the present study by the EB dye experiment. Interestingly, previous studies showed that transgenic mice overexpressing VEGF induced hyper-permeable vessels[36], providing evidence that VEGF increases vascular permeability. VEGF has been implicated in the pathophysiology of liver I/R injury, and its increase during reperfusion injury was seen to mediate leukocyte trafficking and ischemic injury response early after reperfusion, whereby

VEGF acts *via* MCP-1[37]. Interestingly, the administration of VEGF antibodies was reported to block reperfusion injury[37]. The proinflammatory functions of VEGF have been demonstrated by previous studies[37-39]. Therefore, agents attenuating VEGF during the early period of reperfusion could possibly indirectly reduce the inflammatory response resulting from reperfusion injury.

Other vascular molecules studied in the current work were Ang-1 and Ang-2. We demonstrated the attenuation of both Ang-1 and Ang-2 expression in rats receiving AGM prior to induction of gastric I/R, compared with untreated rats. Ang-2, in contrast to Ang-1, was shown to increase vascular permeability, and to promote vascular leakage from blood vessels *in vivo*[40]. AGM seems to reduce vascular permeability at least partly by reducing VEGF and Ang-2 in gastric tissues.

MCP-1 is an inflammatory mediator molecule[41] whose upregulation is responsible for recruitment of inflammatory cells after I/R injury[42]. Importantly, the interaction of MCP-1 with its receptor CCR2 has been attributed a central role in experimental cardiac, renal and cerebral I/R models[43]. Therefore, decreased MCP-1 production will attenuate attraction and recruitment of monocytes and subsequently ameliorate the post-ischemic inflammatory responses. The present study demonstrated an increase in gastric tissue content of MCP-1 after I/R which was suppressed by AGM pre-treatment. Indeed, we provided evidence for the protective anti-inflammatory effect of AGM, whereby it reduced gastric MCP-1 in the ischemic group. In support of our observation, previous reports also showed an anti-inflammatory effect of AGM[44,45], a mechanism by which AGM can attenuate I/R injury. Furthermore, AGM was shown to inhibit the production of NO in macrophages, thus providing a molecular basis for the anti-inflammatory actions of AGM[42]. We, previously demonstrated that iNOS is upregulated in the gastric mucosa in response to I/R injury[46]. Interestingly, Mu *et al*[18] reported that iNOS was also upregulated in brain in response to I/R injury and was significantly downregulated by AGM. With these data in mind we may speculate that AGM could contribute to gastric protection against I/R injury by reducing NO production, possibly by inhibiting iNOS. Other suggested mechanisms that might be involved in the protection offered by AGM could be *via* preservation of endothelial dysfunction[47] and inhibition of matrix metalloproteinase-9, which is known to be upregulated in ischemic injury and degrades the basement membrane of blood vessels[48].

The signaling pathway by which AGM induced gastric protection is possibly mediated *via* Akt/PI3K. The present study provided indirect evidence, as inhibition of this pathway by WN, prior to AGM administration, prevented protection offered by AGM. A limitation to this study is that we did not measure Akt/PI3K activity in gastric tissues.

There are many reasons making AGM a good candidate for gastric protection. First, it is a strong base[22]; second, the highest concentration of AGM is found in the stomach[10]; third, AGM is localized in the mucus-secreting cells[22]. In the parietal cells, it is localized to the lumen of the canaliculi. Finally, a decrease in AGM concentration in mucus-secreting cells in *H. pylori* infection of the stomach is also associated with a decrease in the amount of mucus in the mucus-secreting cells of the stomach[32]. Recently, it has been reported that bacteria such as *E-coli* and *H. pylori* utilize AGM to survive the highly acidic medium of the stomach and even prevent AGM being taken up by stomach cells[34].

In conclusion, the present study revealed that AGM protects the rat stomach exposed to I/R injury at least for brief periods by reducing vascular permeability possibly mediated *via* Akt/PI3K pathway.

COMMENTS

Background

Gastric ischemia reperfusion (I/R) injury which can occur after major surgeries, may lead to extensive gastric ulceration and even perforation if it is not prevented/treated. Although there are therapies that treat gastric lesions, the search for novel molecules is needed. Agmatine (AGM) an endogenously produced amine has its highest concentration in the stomach with alkaline pH. Many studies reported that AGM protected rat kidneys and brains against ischemic injury. Its effectiveness in a model of gastric ischemia reperfusion injury needs investigation.

Research frontiers

AGM is a biological amine found in most cells with the highest concentration in the stomach. The study focused on the effectiveness of AGM in protecting the stomach against I/R injury.

Innovations and breakthroughs

Previous application of AGM to rats in which gastric injury was performed by ethanol administration was found to be unprotective. It was given at a dose of 20 mg/kg. The present study used a model of gastric I/R injury by clamping the gastric vessels for 30 min followed by declamping. AGM was administered at 100 mg/kg before reperfusion. I/R injury induced gastric ulceration and hemorrhages and increased gastric permeability. AGM treatment was accompanied by preservation of gastric histology and vascularity. Also this protection was produced by an anti-inflammatory effect. AGM was demonstrated to be effective in preventing gastric injury induced by I/R.

Applications

The study results suggest that the AGM can be a potential therapeutic option that could be used in preventing gastric injury induced by I/R injury.

Terminology

Ischemia reperfusion (I/R) injury: Injury induced in tissue when blood supply to a tissue/organ stops for a while and then resumes. During reperfusion some injurious substances are released from the tissues including inflammatory, vasodilator and oxidative stress molecules. Therefore it is important to protect tissues against reperfusion injury. Agmatine (AGM): A naturally occurring amine found in our body organs, with the highest concentration in the stomach. It is also found in bacteria and animals. It has diverse functions that deserve future investigations.

Peer review

The authors demonstrated the protection against gastric ischemia-reperfusion injury in rats by agmatine. This study has an interesting finding.

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BRIEF ARTICLE

Protective effects of 5-methoxypsoralen against acetaminophen-induced hepatotoxicity in mice

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Abstract

AIM: To investigate the hepatic protective effects of 5-methoxypsoralen (5-MOP) and to learn if 5-MOP causes hepatotoxicity at protective doses.

METHODS: C57BL/6J mice were administrated orally with 5-MOP at doses of 12.5, 25 and 50 mg/kg body weight respectively every morning for 4 d before given acetaminophen (APAP) subcutaneously at a dose of 500 mg/kg. The 5-MOP alone group was treated with 5-MOP orally at a dose of 50 mg/kg body weight for 4 d without APAP. Twenty-four hours after APAP administration, blood samples of mice were analyzed for serum enzyme alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) levels, and malondialdehyde (MDA), reduced glutathione (GSH) and oxidized glutathione (GSSG) of liver tissues were measured and histopathologic changes of the liver were observed.

RESULTS: Compared with the vehicle control group, the serum levels (IU/L) of ALT, AST and LDH were all increased significantly in APAP group (8355 ± 3940 *vs* 30 ± 21, $P < 0.05$; 6482 ± 4018 *vs* 146 ± 58, $P < 0.05$; 24627 ± 10975 *vs* 1504 ± 410, $P < 0.05$). Compared with APAP group, the serum ALT levels (IU/L) (1674 ± 1810 *vs* 8355 ± 3940, $P < 0.05$; 54 ± 39 *vs* 8355 ± 3940, $P < 0.05$; 19 ± 9 *vs* 8355 ± 3940, $P < 0.05$), AST levels (IU/L) (729 ± 685 *vs* 6482 ± 4108, $P < 0.05$; 187 ± 149 *vs* 6482 ± 4108, $P < 0.05$; 141 ± 12 *vs* 6482 ± 4108, $P < 0.05$) and LDH levels (IU/L) (7220 ± 6317 *vs* 24 627 ± 10 975, $P < 0.05$; 1618 ± 719 *vs* 24 627 ± 10 975, $P < 0.05$; 1394 ± 469 *vs* 24 627 ± 10 975, $P < 0.05$) were all decreased drastically in the three-dosage 5-MOP pretreatment groups. Pretreatment of 5-MOP could attenuate histopathologic changes induced by APAP, including hepatocellular necrosis and infiltration of inflammatory cells, and the effect was dose-dependent. MDA levels (nmol/mg) were decreased by 5-MOP in a dose-dependent manner (0.98 ± 0.45 *vs* 2.15 ± 1.07, $P > 0.05$; 0.59 ± 0.07 *vs* 2.15 ± 1.07, $P < 0.05$; 0.47 ± 0.06 *vs* 2.15 ± 1.07, $P < 0.05$). The pretreatment of 5-MOP could also increase the GSH/GSSG ratio (3.834 ± 0.340 *vs* 3.306 ± 0.282, $P > 0.05$; 5.330 ± 0.421 *vs* 3.306 ± 0.282, $P < 0.05$; 6.180 ± 0.212 *vs* 3.306 ± 0.282, $P < 0.05$). In the group treated with 5-MOP but without APAP, the serum enzyme levels, the liver histopathologic manifestation, and the values of MDA and GSH/GSSG ratio were all normal.

CONCLUSION: 5-MOP can effectively protect C57BL/6J mice from APAP-induced hepatotoxicity and possesses an antioxidative activity, and does not cause liver injury at the protective doses.

Key words: 5-Methoxypsoralen; Protection; Acetaminophen; Hepatotoxicity; Antioxidation

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INTRODUCTION

Acetaminophen (APAP), a widely used antipyretic and analgesic drug, could induce hepatotoxicity and even acute liver failure (ALF) when taken at overdose[1]. APAP overdose is a common cause of adult and children ALF in the United States and other countries[2-5]. APAP can be metabolized by cytochrome P450 enzymes (CYPs) to N-acetyl-P-benzoquinoneimine (NAPQI)[6]. At overdoses of APAP, a large number of NAPQIs is generated, which can deplete reduced glutathione (GSH) and then bind to mitochondrial proteins to cause mitochondrial dysfunction and oxidant stress[7,8], leading to hepatocellular damage and centrilobular hepatic necrosis. In this process, APAP can increase the level of malondialdehyde (MDA) both in the liver and plasma[9] and NAPQI is capable of lowering GSH/oxidized glutathione (GSSG) ratio by oxidizing the thiol group of GSH[10]. Oxidant stress plays a central role in the hepatic damage induced by APAP[11].

5-methoxypsoralen (5-MOP), a furocoumarin found in many medicinal plants, possesses slight antioxidative activity evidenced from researches *in vitro*[12,13]. 5-MOP has been used in combination with UV radiation in skin photochemotherapy for decades[14], and some studies also found that it has anticancer[15-19], antidepressant[20-24], anticonvulsion[25] and anti-inflammatory effects[26,27], but none of previous studies have shown that 5-MOP could prevent hepatotoxicity.

In addition, some patients suffered from toxic hepatitis induced by 5-MOP when it was used as photochemotherapeutic agent[28,29], and one animal experiment demonstrated that high doses of 5-MOP can induce hepatotoxicity in mice[30]. So it is essential to examine if 5-MOP can cause liver injury at therapeutic doses.

This study was designed to determine the protective effects of 5-MOP in APAP-induced hepatotoxicity using mouse hepatotoxic models, and to investigate if 5-MOP can cause hepatotoxicity in mice at effective doses.

MATERIALS AND METHODS

Chemicals

5-MOP was purchased from Tokyo Chemical Industry (Tokyo, Japan), and APAP from Jiaozuo Xin'An Science and Technology Company (Henan, China). Tween 80, which was used to prepare 5-MOP suspension, was bought from Biodee Biotechnology Company (Beijing, China). APAP was dissolved in normal saline before use. GSH and N-ethylmathione gained from Lizhudongfeng Biotechnology Company (Shanghai, China), GSSG from Hongxing Biotechnology Company (Beijing, China), o-phthalaldehyde (OPT) from Jinlong Chemical Company (Beijing, China), and thiobarbituric acid (TBA) from Acros (United States).

Animals and treatment

Male C57BL/6J mice, 18-22 g in weight, were purchased from Peking University Laboratory Animal Department, Beijing, China. They were housed in a well-ventilated room and the room temperature was controlled at 21 ℃ -23 ℃ and humidity at 65%-70% with a 12 h light-12 h dark cycle. All the mice were fed adaptively for three d before experiment, and they had free access to water and were fed with forage supplied by Laboratory Animal Center of Military Medical Science Academy.

5-MOP was suspended in 1% Tween 80 at different concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL, and all of these suspensions were administrated to mice at 10 mL/kg body weight; that is, mice were administrated with 5-MOP at doses of 12.5 mg/kg, 25 mg/kg and 50 mg/kg, respectively. A 5-d experiment was performed with 36 mice which were randomly divided into 6 groups by weight. Group 1 was the vehicle control group and group 2 was APAP alone group, both groups were orally treated with 1% Tween 80 (10 mL/kg body weight) every morning for 4 d. Groups 3, 4 and 5 were 5-MOP multiple-dose groups administered with oral 5-MOP at doses of 12.5, 25 and 50 mg/kg body weight respectively every morning for 4 d. Group 6 was 5-MOP alone group treated with oral 5-MOP at a dose of 50 mg/kg body weight also for 4 d. Thirty minutes after the administrations, all mice except those in the vehicle control group and 5-MOP alone group were subcutaneously administrated with APAP (500 mg/kg body weight). Twenty hours after APAP administration, blood samples were collected from orbital venous plexus of the mice. After the mice were sacrificed, their livers were dissected out immediately and washed with normal saline, dried on a filter paper and weighted. Then the livers were prepared immediately for further examinations.

The animal care and surgical procedures were performed in compliance with the Guidelines for Animal Care and Use of Peking University.

Biochemical test

The blood samples were collected to determine serum enzyme [alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH)] levels by HITACHI-7170A automatic analyzer. The liver tissues were homogenized with potassium chloride (KCl) solution (0.15 mol/L) on ice to yield a 5% (w/v) homogenates for MDA test. The hepatic MDA levels were determined as thiobarbituric acid reactive substances levels using a published colorimetric method[31].

The liver tissues were homogenized with phosphate buffered solution on ice to yield a 5% (w/v) homogenates for glutathione test. The GSH and GSSG levels in liver tissues were measured by the improved Hission method[32], a fluorometric method that uses OPT as a fluorescent reagent. Then GSH/GSSG ratio was calculated.

Table 1 Effects of acetaminophen alone, 5-methoxypsoralen multi-dose and 5-methoxypsoralen alone on alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase activities, hepatic malondialdehyde and reduced glutathione/oxidized glutathione ratio in mice (mean ± SE)

Treatments groups	ALT (IU/L)	AST (IU/L)	LDH (IU/L)	MDA (nmol/mg)	GSH/GSSH
Vehicle control	30 ± 21	146 ± 58	1504 ± 410	0.18 ± 0.11	6.045 ± 0.629
APAP alone at 500 mg/kg	8355 ± 3940[a]	6482 ± 4018[a]	24 627 ± 10975[a]	2.15 ± 1.07[a]	3.306 ± 0.282[a]
5-MOP at 12.5 mg/kg	1674 ± 1810[c]	729 ± 685[c]	7220 ± 6317[c]	0.98 ± 0.45	3.834 ± 0.340
5-MOP at 25 mg/kg	54 ± 39[c]	187 ± 149[c]	1618 ± 719[c]	0.59 ± 0.07[c]	5.330 ± 0.421[c]
5-MOP at 50 mg/kg	19 ± 9[c]	141 ± 12[c]	1394 ± 469[c]	0.47 ± 0.06[c]	6.180 ± 0.212[c]
5-MOP alone at 50 mg/kg	37 ± 20	138 ± 22	1471 ± 191	0.15 ± 0.09	6.858 ± 0.678

[a]$P < 0.05$ *vs* vehicle control group; [c]$P < 0.05$ *vs* acetaminophen (APAP) alone group. 5-MOP: 5-methoxypsoralen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; GSH: Reduced glutathione; GSSG: Oxidized glutathione.

Histopathologic examination

The left liver lobes were scissored out and fixed in 10% formalin solution for 48 h. The liver samples were then cut into thin transverse sections with the help of microtome and permanent slides were prepared with HE staining. Liver histopathologic changes were examined under an optical microscope with the original magnification × 200.

Data treatment and statistical analysis

The experimental results were expressed as mean ± SE (standard error). Statistical comparison between groups was performed by one-way analysis of variance with SPSS 13.0 statistical software. A P value < 0.05 was indicated as a statistically significant difference.

RESULTS

Effects of APAP alone, 5-MOP multiple-dose and 5-MOP alone on serum enzyme levels

The hepatocellular damage induced by a toxic dose (500 mg/kg) of APAP and the effects of pretreatment with 5-MOP were investigated by measuring the serum levels of ALT, AST and LDH. As shown in Table 1, APAP significantly increased the serum ALT, AST and LDH levels compared with the control group**,** and the multiple-dose 5-MOP pretreatment significantly prevented the increases of serum enzyme levels. The effect of 5-MOP was dose-dependent, and in the highest dose group, serum levels of ALT, AST and LDH were close to the normal levels as compared with the vehicle control group ($P > 0.05$).

The influences of 5-MOP alone on serum enzyme levels were also observed. There were no statistically significant differences in the serum levels of ALT, AST and LDH between 5-MOP alone group (50 mg/kg) and the vehicle control group ($P > 0.05$).

Effects of APAP alone, 5-MOP multiple-dose and 5-MOP alone on liver tissue MDA and GSH/GSSG ratio

As seen in Table 1, compared with the vehicle control group, a toxic dose of APAP elevated liver MDA and lowered the hepatic GSH/GSSG ratio. With the escalating dose of 5-MOP (12.5, 25 and 50 mg/kg), the content of MDA decreased and ratio of GSH/GSSG increased.

In the 5-MOP alone group, the MDA level in liver was as low as that in the vehicle control group (0.15 ± 0.09 *vs* 0.18 ± 0.11, $P > 0.05$). In addition, the hepatic GSH/GSSG ratio in the 5-MOP alone group was not significantly changed as compared with that in the vehicle control group (6.858 ± 0.678 *vs* 6.045 ± 0.629, $P > 0.05$).

Effects of APAP alone, 5-MOP multi-dose and 5-MOP alone on histopathologic changes

The liver histopathologic changes of mice in the six groups are shown in Figure 1. The liver sections displayed the representative hepatocellular morphological changes of each group.

In the vehicle control group, hepatocytes, presenting normal morphology, arranged around the central vein in a radical pattern, and liver lobule structures were clear and regular (Figure 1A). Normal liver lobule structures were damaged and collapsed in the APAP alone group. Large areas of hepatocellular necrosis and infiltration of inflammatory cells were also observed (Figure 1B). 5-MOP administration could alleviate the pathological injury induced by APAP in a dose-dependent manner. 5-MOP at a dose of 12.5 mg/kg could slightly relieve the pathological injury. In this group, no hepatocellular necrosis and infiltration of inflammatory cells were observed, but hepatocellular hydropic degeneration and sinusoidal dilation occurred (Figure 1C). There was no necrosis and hydropic degeneration of hepatocytes, no sinusoidal dilation and infiltration of inflammatory cells in the 25 mg/kg 5-MOP dose group. However, liver lobule structures were still not clear in this group (Figure 1D). 5-MOP at a dose of 50 mg/kg could significantly prevent APAP-induced hepatotoxicity with an almost normal lobular structure comparable to the vehicle control group (Figure 1E). There were no significant liver histopathologic changes in the 5-MOP alone group (Figure 1F).

DISCUSSION

The protective effect of 5-MOP against hepatocellular injury and oxidative stress, and the potential toxic effect of 5-MOP on the liver were investigated in this study. C57BL/6J mice were used because our previous research found that C57BL/6J mice were more suscep-

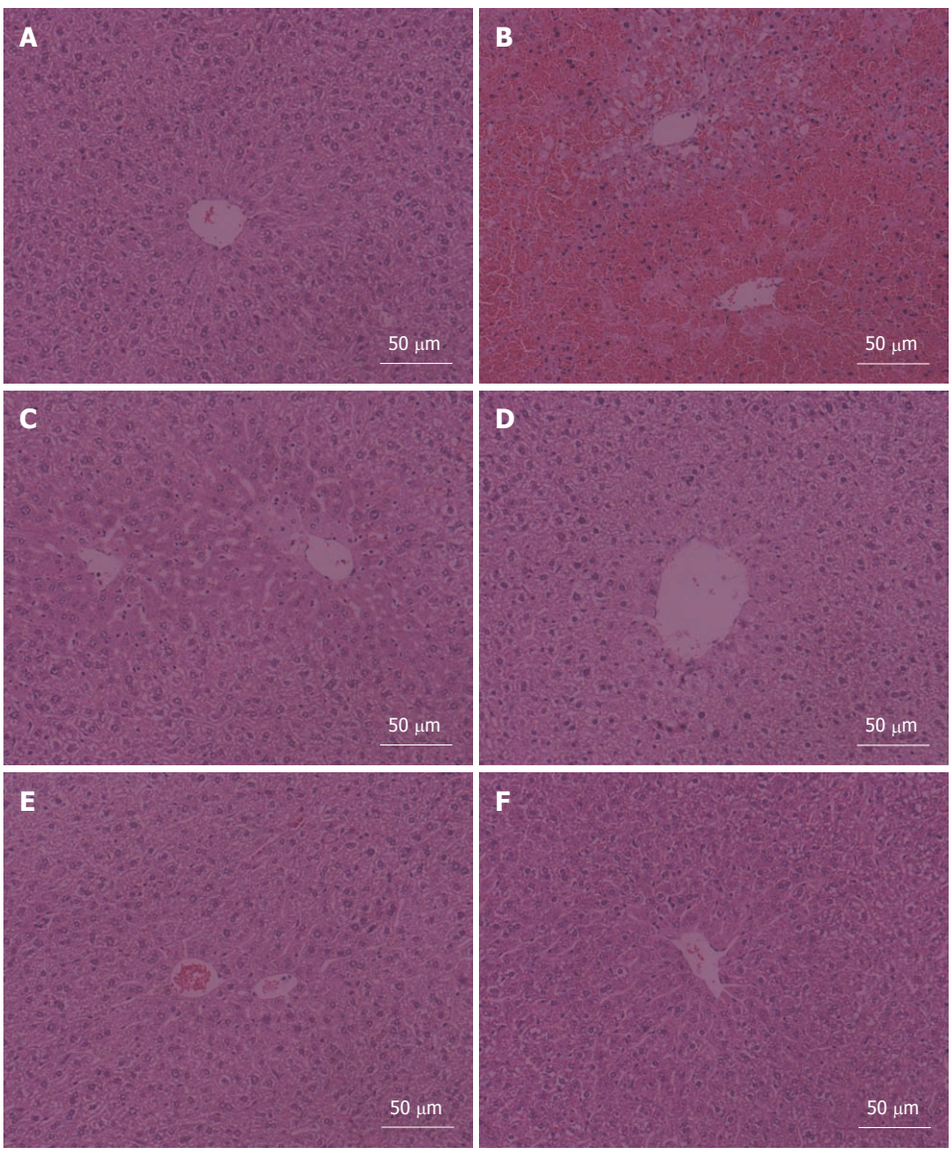

Figure 1 Representative pathological changes of liver section of the six groups (original magnification × 200). A: Section of liver from the vehicle control group showing a normal lobular structure; B: Section of liver from acetaminophen alone group showing large areas of centrilobular necrosis with inflammatory cell infiltration; C: Section of liver from the 12.5 mg/kg 5-methoxypsoralen (5-MOP) dose group showing absence of hepatocellular necrosis and infiltration of inflammatory cells but presence of hepatocellular hydropic degeneration and sinusoidal dilation; D: Section of liver from the 25 mg/kg 5-MOP dose group showing normal hepatocellular morphology but liver lobule structure damage; E: Section of liver from the 50 mg/kg 5-MOP dose group showing a significant alleviation of liver pathological injury with an almost normal lobular structure; F: Section of liver from 5-MOP alone group (50 mg/kg) showing presence of normal lobular structure.

tible to APAP[33]. The serum levels of ALT, AST and LDH are main indices of liver injury[34] and the levels of MDA, GSH and GSSG can be used as indices of oxidative stress[9]. We evaluated the hepatic protective effect of 5-MOP based on these indices. It is well known that any chemical can be toxic if its dose is high enough, so a 5-MOP alone group was designed to see if the highest therapeutic dose of 5-MOP could cause hepatotoxicity.

APAP used alone can significantly increase the serum levels of ALT, AST and LDH and cause pathological changes as compared with the vehicle control group. Oxidative stress also took place as shown by the increase of MDA level and decrease of GSH/GSSG ratio. The model of APAP-induced hepatotoxicity was successfully established in this experiment.

5-MOP can protect mice from APAP-induced acute liver injury based on the fact that it can decrease the serum ALT, AST and LDH levels in a dose-dependent manner and alleviate the liver histopathologic alterations. Moreover, 5-MOP decreased the MDA level and increased the GSH/GSSG ratio in a dose-related manner, which reflected that 5-MOP could significantly attenuate the oxidative stress induced by APAP and suggested that the hepatoprotective effect of 5-MOP may be associated with its antioxidative activity.

However, besides antioxidant activity, 5-MOP also possesses biological activities to inhibit the mouse and human CYPs both *in vivo* and *in vitro*[14]. And CYPs-catalyzed formation of NAPQI is the key mechanism in APAP-induced hepatotoxicity[35]. So we presume that

inhibition of CYPs of 5-MOP may also account for the protective mechanism against APAP-induced hepatotoxicity, which should be further investigated.

In the 5-MOP alone group, the serum enzyme (ALT, AST and LDH) levels and histopathologic changes were as normal as in the vehicle control group, which indicated that 5-MOP could not cause liver injury at a dose of 50 mg/kg (the highest therapeutic dose used in this study). The MDA level and the GSH/GSSG ratio were not significantly changed as compared with the vehicle control group, which showed that 5-MOP did not influence the normal oxido-reduction levels.

In conclusion, 5-MOP could protect against APAP-induced hepatotoxicity in mice and had an antioxidative activity, and caused no hepatotoxicity at protective doses.

COMMENTS

Background

Overdose of acetaminophen (APAP) can induce hepatotoxicity and oxidative stress plays a central role in the hepatic damage. Though 5-methoxypsoralen (5-MOP) possesses antioxidative activity suggested by researches *in vitro*, none of previous studies has found that 5-MOP could prevent APAP-induced hepatotoxicity.

Research frontiers

It is important to search for effective methods to protect human from APAP-induced hepatotoxicity. Despite the various applications of 5-MOP, no research has been conducted to determine if 5-MOP could prevent APAP-induced hepatotoxicity. In addition, although the antioxidative activity of 5-MOP has been evidenced from researches *in vitro*, this activity was not manifested *in vivo*. Besides, 5-MOP may also cause hepatotoxicity when taken at high doses.

Innovations and breakthroughs

This study manifested that 5-MOP could protect mice from APAP-induced hepatotoxicity *in vivo*, and this hepatoprotective effect was associated with its antioxidation activities. In addition, 5-MOP caused no hepatotoxicity at protective doses.

Applications

This study has suggested that 5-MOP can be used at appropriate doses as a drug against APAP-induced hepatotoxicity in human. However, before clinical use, more researches are needed to confirm the safety of APAP administration at protective doses.

Peer review

The authors investigated the protective effects of 5-MOP against APAP-induced hepatotoxicity and whether 5-MOP could cause hepatotoxicity in mice. The results suggested that 5-MOP resisted APAP-induced hepatotoxicity, reduced APAP-induced oxidative stress, and did not cause liver injury at protective doses.

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BRIEF ARTICLE

Effect of soy protein supplementation in patients with chronic hepatitis C: A randomized clinical trial

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Abstract

AIM: To evaluate the effects of soy supplementation on insulin resistance, fatty liver and alanine aminotransferase (ALT) levels in non-diabetic patients with chronic hepatitis C (CHC).

METHODS: In a prospective, randomized and single-blinded clinical trial, we compared patients with CHC who had casein as a supplement (n = 80) (control group), with patients who consumed a soy supplement diet (n = 80) [intervention group (IG)]. Both groups received 32 g/d of protein for 12 wk.

RESULTS: Patients' baseline features showed that 48.1% were overweight, 43.7% had abdominal fat accumulation, 34.7% had hepatic steatosis and 36.3% had an homeostasis model assessment index of insulin resistance (HOMA-IR) $\geqslant$ 3.0. Descriptive analysis showed that protein supplementation diet reduced hepatic steatosis in both groups; however, significant reductions in ALT levels occurred in the soy group. Multiple regression modeling indicated that in the presence of severe fibrosis (F3/F4), γ glutamyl transferase elevation and high density lipoprotein (HDL) reduction, the intervention group had 75% less chance of developing hepatic steatosis (OR= 0.25; 95% CI: 0.06-0.82) and 55% less chance of presenting with an ALT level $\geqslant$ 1.5 × the upper limit of normal (ULN) (OR = 0.45, 95% CI: 0.22-0.89). Soy treatment did not have any effect on insulin resistance (OR = 1.92; 95% CI: 0.80-4.83), which might be attributed to the fact that the HOMA-IR values at baseline in most of our patients were in the normal range. Advanced hepatic fibrosis, an ALT level > 1.5 × ULN and visceral fat were predictors of an HOMA-IR $\geqslant$ 3. The IG group had a reduced risk of an ALT level > 1.5 × ULN. An HOMA-IR $\geqslant$ 3.0 and HDL < 35 mg/dL were also risk factors for increased ALT.

CONCLUSION: Soy supplementation decreased ALT

levels and thus may improve liver inflammation in hepatitis C virus (HCV) patients; it also reduced hepatic steatosis in a subgroup of patients but did not change insulin resistance. It should be considered in the nutritional care of HCV patients.

Key words: Chronic hepatitis C; Soy supplementation; Insulin resistance; Hepatic steatosis; Hepatitis C virus

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INTRODUCTION

Hepatitis C virus (HCV) infection is considered an important public health problem[1] and is the leading cause of liver transplantation in the Western world. Chronic HCV infection increases the risk for hepatic steatosis, insulin resistance, glucose intolerance and type 2 diabetes[2-4].

The pathophysiology of nonalcoholic fatty liver disease (NAFLD) involves histology ranging from fat alone (hepatic steatosis) to fat plus inflammation (nonalcoholic steatohepatitis, NASH) to fat plus hepatocyte injury (ballooning degeneration) with or without fibrosis or Mallory's bodies which can lead to liver failure[5]. NAFLD and NASH have been associated with insulin resistance resulting in glucose intolerance and hyperglycemia[6]. Insulin resistance also contributes to increased lipolysis, which reduces fat uptake and oxidation by peripheral tissues. Both mechanisms lead to fat influx and accumulation in hepatic tissue[7].

Insulin resistance induced by HCV may involve several mechanisms, such as an immune response mediated by Th1 lymphocytes, the action of pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-6), the degradation of intracellular components that participate in the insulin signaling system, including insulin receptor substrates (IRS-1 and IRS-2), and the reduced activation of phosphatidyl inositol 3-kinase (PI3-K) and protein kinase B (AKT)[8-12].

Considering these factors, appropriate nutrition becomes an essential tool to minimize HCV's comorbidities such as fatty liver, inflammation and insulin resistance. Recently, functional foods have been considered essential for promoting and maintaining health. According to some reports, soy protein and its derivatives might be able to lower insulin resistance in patients with chronic liver diseases due its constituents, such as fiber, isoflavones and high biological value protein[13], and modulation of hepatic lipid metabolism[14,15]. The aim of this study was to evaluate the influence of soy protein supplementation on insulin resistance, liver fat content and alanine transaminase (ALT) levels in non-diabetic patients with chronic hepatitis C.

MATERIALS AND METHODS

Subjects

Non-diabetic patients with chronic hepatitis C were recruited from a reference ambulatory unit of the Federal University of Bahia's Hospital between June 2008 and December 2009. The diagnosis of HCV infection was made by the presence of serum anti-HCV, which was confirmed by qualitative determination of HCV RNA. Inclusion criteria were the following: patients aged over 18 years, with or without liver cirrhosis; patients with ethanol consumption below 20 g/d; patients with normal liver function (Child-Pugh A); and patients who were not under antiviral therapy or who had discontinued antiviral therapy for at least three months.

Patients co-infected with HIV and/or HBV with renal failure as well as those with heart disease, decompensated cirrhosis, pregnancy, any malignancy, diabetes mellitus or obesity (BMI > 30 kg/m^2) were excluded. The subjects gave written informed consent before participating in the study. The Ethics Committee of the Federal University of Bahia approved the study.

Study design

The study was a prospective, randomized and single-blinded clinical trial. Patients who were regularly followed in the Hepatology outpatient clinic were informed about the protocol and referred to the Nutrition clinic. Subjects who met the inclusion criteria of the study were randomly allocated into one of two study groups. The study was single-blinded (only blinded for patients). The estimated sample size was 160 patients.

The 160 patients were equally divided into two groups (n = 80), and each group received isonitrogenous protein supplementation with 32 g of protein per day for twelve weeks. The control group (CG) was supplemented with animal protein (casein), and the intervention group (IG) was supplemented with vegetable protein (soy). The nutritional composition of the supplements used in this study is reported in Table 1.

Patients were instructed to dissolve the protein supplement in water, juice, soup, porridge or to consume it with fruits. Additionally, considering their nutritional status and dietary habits, patients received dietary guidelines to promote healthy eating and weight control. Diet counseling aimed to promote the ingestion of a normocaloric, normoglycidic and high protein (1.5 g/kg per day) diet by both groups. Patients returned monthly to receive their supplements.

Table 1 Nutritional content per 100 g of supplement

	Whole soy powder[1]	Calcium caseinate
Energy (Kcal)	392	371
Carbohydrate (g)	14	0.2
Protein (g)	40	97.4
Total fat (g)	18.8	2.4
Alpha linolenic acid (mg)	1252	0
Linoleic acid (mg)	9332	0
Dietary fiber (g)	18	0
Isoflavones (mg)	53.82	0

[1]Soyos® ingredients.

Clinical parameters

Clinical survey data such as clinical diagnosis, viral genotype, necroinflammatory activity index and fibrosis (METAVIR classification) were either collected from medical records or from patient examinations.

Patients underwent ultrasonography of the upper abdomen with a team of three examiners using a single piece of equipment at the University Hospital's radiology service. Hepatic steatosis was graded as mild, moderate or severe according to the classification of Saverumuttu *et al*[16].

Measurement of waist circumference was performed according to the World Health Organization recommendations using an inelastic tape measure (TBW Import Ltd.) that was 0.5 cm wide and 200 cm in length. Waist circumference was measured at a level midway between the superior aspect of the iliac crests and the lower lateral margins of the ribs. The cutoff points adopted for classifying central obesity and increased risk of metabolic complications were above 80 cm for women and 94 cm for men[17]. Socio-demographic and lifestyle information was also collected using a structured questionnaire during the first appointment of follow-up (baseline).

Patients underwent follow-up visits once a month with registered dietitians to elucidate the adherence to the diet prescription and protein supplementation. Schedule monitoring also included weekly telephone calls in the first month and biweekly thereafter. After 12 wk of supplementation, physical, biochemical and anthropometric tests as well as a questionnaire were applied to evaluate possible changes during the intervention program. Ultrasound was also performed in patients with a diagnosis of hepatic steatosis at baseline. All procedures were performed within a maximum interval of ten days after the nutrition counseling.

Laboratory measurement

After a 12-h fast, a blood sample was collected for the determination of aspartate aminotransferase (AST), ALT, gamma glutamyl transferase (γGT), alkaline phosphatase, plasma glucose, insulin, total cholesterol and cholesterol fractions. Analyses were performed on a Beckman Coulter LX-20 PRO and CX-9 equipment. Serum insulin was measured using an electrochemiluminescence method with an Elecsys 2010 device.

The insulin resistance index was predicted according to the homeostasis model assessment index of insulin resistance (HOMA-IR). The formula was as follows: insulin resistance (HOMA-IR) = fasting insulinemia (microU/mL) × fasting glycemia (mmol/L)/22.5[18]. We considered $\geqslant$ 3.0 as the cutoff point to define insulin resistance.

Virological tests

An enzyme linked immunosorbent assay was performed on all serum samples to detect the presence of anti-HCV using third generation commercial kits (anti-HCV Hepatitis C® Wiener Lab.) following the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on all samples for qualitative determination of HCV RNA. The method used was the nested PCR (HCV-RNA detectable using the COBAS® AMPLICOR HCV Test, v2.0, Roche). HCV genotyping was performed using the technique of restriction fragment polymorphism (RFLP-PCR).

Histological analysis

The stages of fibrosis and inflammation were determined according to the METAVIR scoring system: F0 = no fibrosis, F1 = expansion of fibrosis in portal areas without septa, F2 = fibrous portal expansion with septa, F3 = numerous septa or fibers with nodular transformation, and F4 = cirrhosis[19].

Statistical analysis

Descriptive analysis was performed to characterize the population. Mann-Whitney *U* and Wilcoxon's rank sum tests were used to compare the biochemical values in the intervention and control groups and to evaluate the differences obtained at baseline and after intervention. Logistic regression analysis was used to evaluate risk predictor factors for hepatic steatosis, insulin resistance (HOMA-IR $\geqslant$ 3.0) and changes in ALT levels (1.5 above the upper limit of normal). Confounding and interaction analyses were performed to select the final models. Multiple regression analysis was performed after the intervention considering the groups had similar demographic, clinical and laboratory features before protein supplementation.

The sample size was calculated using an estimated 20% loss to follow-up, a confidence level of 95% and 80% power. A statistical significance was inferred at $P < 0.05$. In some biochemical analyses, the Bonferroni method was applied to adjust the multiple comparisons *P*-value between the groups at the significance level of 0.007. Statistical analysis was performed with the statistical package R version 2.12[20].

RESULTS

Characteristics of patients

The characterization of the study population is presented in Table 2. Males (63.8%) were predominant. The prevalence of HCV genotype 1 infected patients was 83.5%.

Table 2 Characterization of the study population *n* (%)

	CG-casein	IG-soy	Total	*P* value
Demographic characteristics				
Gender				
Male	49 (61.2)	53 (66.2)	102 (63.8)	0.62[1]
Female	31 (38.8)	27 (33.8)	58 (36.2)	
Marital Status				
Married	56 (70.0)	55 (68.7)	111 (69.4)	1.00[1]
Single, widowed or divorced	24 (30.0)	25 (31.3)	49 (30.6)	
Anthropometric data				
Body mass index				
< 25 kg/m^2	40 (50.0)	43(53.8)	83 (51.9)	0.751[1]
⩾ 25 kg/m^2	40 (50.0)	37(46.2)	77 (48.1)	
Waist circumference				
Adequate	43 (53.7)	46 (59.0)	89 (56.3)	0.525[1]
Inadequate	37 (46.3)	32 (41.0)	69 (43.7)	
Clinical data				
Genotype				
1	66 (88.0)	61 (79.2)	127 (83.5)	0.189[1]
2/3	9 (12.0)	16 (20.8)	25 (16.5)	
Necroinflammatory activity				
A0	26 (41.3)	29 (46.8)	55 (44.0)	0.792[1]
A1	26 (41.3)	22 (35.5)	48 (38.4)	
A2 e A3	11 (17.4)	11 (17.7)	22 (17.6)	
Stage of fibrosis				
F0/F1/F2	35 (55.6)	40 (63.5)	75 (59.5)	0.468[1]
F3/F4	28 (44.4)	23 (36.5)	51 (40.5)	
Hepatic steatosis				
Yes	24 (40.0)	19 (29.7)	43 (34.7)	0.260[1]
No	36 (60.0)	45 (70.3)	81 (65.3)	
HOMA-IR				
< 3.0	55 (53.9)	47 (46.1)	102 (63.7)	0.25[1]
⩾ 3.0	25 (43.1)	33 (56.9)	58 (36.3)	
Biochemical data	Median (iq r)	Median (iq r)		
Glucose (mg/dL)	91.0 (14.2)	91.5 (14.2)		0.70[2]
HOMA-IR	2.35 (2.24)	2.26 (2.59)		0.99[2]
Triglycerides (mg/dL)	96.5 (60.2)	96.0 (67.2)		0.57[2]
Total cholesterol (mg/dL)	149.0 (49.7)	160.0 (44.0)		0.42[2]
HDL (mg/dL)	44.0 (14.2)	44.0 (15.5)		0.30[2]
LDL (mg/dL)	90.5 (40.7)	90.0 (43.0)		0.94[2]
AST (U/L)	59.5 (40.7)	52.0 (44.5)		0.14[2]
ALT (U/L)	76.5 (54.5)	73.5 (53.0)		0.38[2]
γGT (U/L)	102.0 (118.0)	87.0 (114.0)		0.71[2]
Alkaline phosphatase (U/L)	79.0 (33.5)	80.0 (53.2)		0.49[2]

[1]Fisher's exact test; [2]Mann-Whitney; iq r: Interquartile range. *n* = 160. CG-casein: Control group-casein; IG-soy: Intervention group-soy; Adequate waist circumference: ⩽ 80 cm for women and ⩽ 94 cm for men. HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; LDL: Low density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transferase.

Advanced fibrosis (Metavir F3/F4) was detected in 40.5% of patients. The average age was 52.2 (± 10.5) years, 48.1% were overweight and 51.9% had a BMI < 25 kg/m^2; 43.7% individuals had abdominal fat accumulation. It was also observed that 34.7% of the patients had hepatic steatosis, and 36.3% had an HOMA-IR ⩾ 3.0 at baseline.

Biochemical data presented in Table 2 show that glucose and lipid profiles were not altered; however, high transaminase levels were observed in both groups. It is also observed that the median value for γGT is greater in the CG than in the IG (102.0 U/L *vs* 87.0 U/L); however, no significant difference was detected between the groups. Median alkaline phosphatase was in the normal range for both groups. Baseline demographic, anthropometric, clinical and laboratory data show that the study population had a homogeneous distribution between the groups.

Table 3 Comparison of the distribution of hepatic steatosis before and after intervention between groups of patients with hepatitis C virus

Hepatic Steatosis	*n*	Yes	No	*P* value
Before				0.2604[1]
IG-soy	64	19 (29.7)	45 (70.3)	
CG-casein	60	24 (40.0)	36 (60.0)	
After				0.1850[1]
IG-soy	64	10 (15.6)	54 (84.4)	
CG-casein	60	16 (26.7)	44 (73.3)	
IG-soy: before *vs* after				0.0076[2]
With hepatic steatosis	19	10 (52.6)	9 (47.4)	
Without hepatic steatosis	45	0 (0.0)	45 (100.0)	
GC-casein: before *vs* after				0.0133[2]
With hepatic steatosis	24	16 (66.7)	8 (33.3)	
Without hepatic steatosis	36	0 (0.0)	36 (100.0)	

[1]Fisher's exact test: Comparison between groups before and after intervention; [2]Paired analysis: McNemar's test: Comparison in each group. CG-casein: Control group-casein; IG-soy: Intervention group-soy.

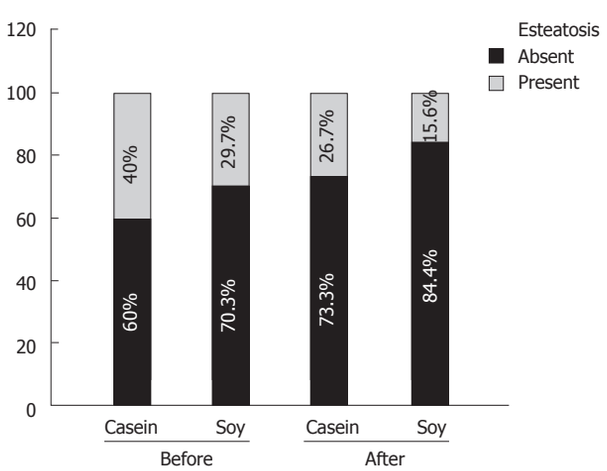

Figure 1 Hepatic esteatosis before and after intervention between groups of patients with hepatitis C virus.

Prevalence of steatosis at baseline and after intervention

Table 3 shows the comparison of the prevalence of hepatic steatosis before and after the intervention in the IG and CG. The prevalence of hepatic steatosis was different between the groups at baseline (29.7% *vs* 40.0%) and remained different after the interventions (15.6% *vs* 26.7%), but without statistical significance in both instances (Figure 1).

Paired analysis, within groups (before and after protein supplementation), showed the reduction in hepatic steatosis was significant for both groups. The IG showed a reduction from 19 to 10 cases (47.4% reduction, *P* =

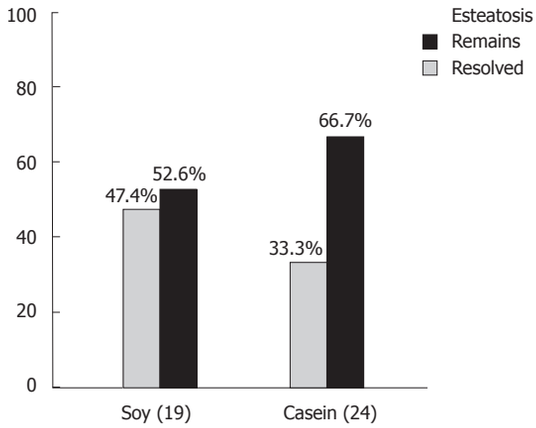

Figure 2 Prevalence of hepatic steatosis condition after intervention for those who begin with hepatic steatosis.

Table 4 Median values of biochemical tests in patients with hepatitis C virus after protein supplementation

	CG-casein		IG-soy		Treatment difference
Exams	*n*	median (iq r)	*n*	median (iq r)	*P* value[1]
Total cholesterol (mg/dL)	80	152.0 (61.5)	80	153.0 (44.7)	0.54
HDL (mg/dL)	80	46.0 (17.0)	80	43.5 (15.2)	0.21
LDL (mg/dL)	80	89.5 (45.0)	80	84.5 (41.5)	0.39
AST (U/L)	78	61.5 (40.5)	80	49.5 (44.2)	0.02
ALT (U/L)	78	73.0 (65.7)	79	64.0 (50.0)	0.007
γGT (U/L)	75	108.0 (120.5)	73	84.0 (107.0)	0.09
HOMA-IR	79	2.4 (2.7)	80	2.6 (2.2)	0.91

[1]Mann-Whitney test ($P < 0.05$): comparison between groups; Bonferroni correction ($P < 0.007$); CG-casein: Control group-casein; IG-soy: Intervention group-soy; iq r: Interquartile range; $n < 80$: When an examination was not performed; HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; LDL: Low density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transferase.

0.0076) and the CG decreased from 24 to 16 cases (33.3% reduction, $P = 0.0133$) (Figure 2 and Table 3).

Biochemical data before and after intervention

After protein supplementation, a significant reduction was observed in the transaminase levels of the IG *vs* the CG (AST: 49.5 U/L *vs* 61.5 U/L, $P = 0.02$; ALT: 64.0 U/L *vs* 73.0 U/L, $P = 0.007$). A reduction in the levels of γGT was observed in the IG compared to the CG (84.0 U/L *vs* 108.0 U/L, $P = 0.09$) but without statistical significance (Table 4).

The IG had a significant reduction in ALT levels (73.5 U/L *vs* 64.0 U/L; 12.92% change, $P = 0.006$) (Figure 3) and γGT levels (87.0 U/L *vs* 84.0 U/L; 3.45% change, $P = 0.007$) after 12 wk of intervention. Reductions were also observed in total cholesterol (160.0 mg/dL *vs* 153.0 mg/dL; 4.37% change), but without statistical significance (Table 5). It is noteworthy that the CG did not present improvements in these biochemical tests.

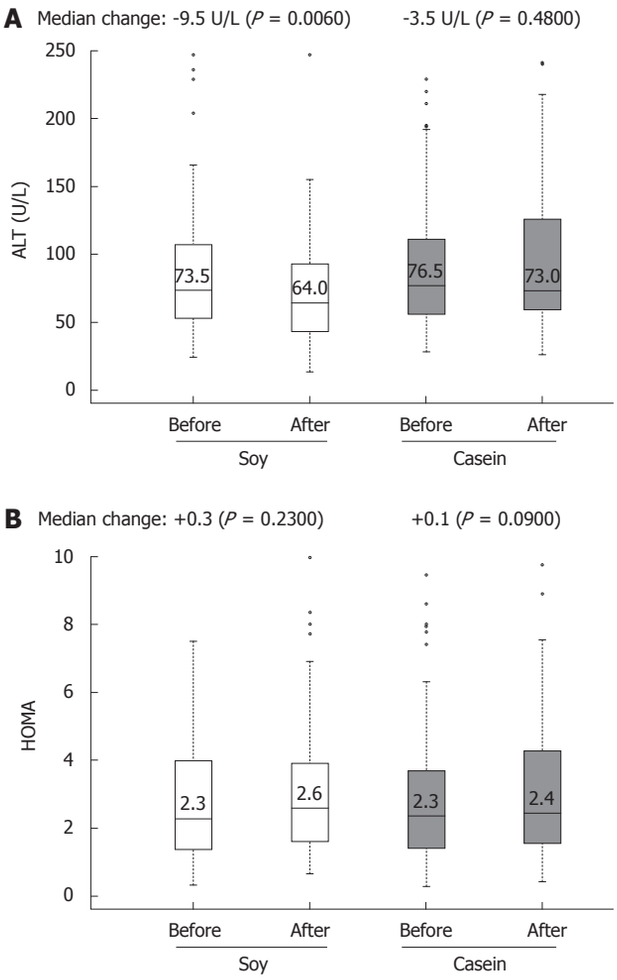

Figure 3 Distribution of alanine aminotransferase and homeostasis model assessment according to intervention groups and time.

Predictors of hepatic steatosis after intervention

Table 6 presents the logistic regression analysis for predictive factors of hepatic steatosis. Multiple regression modeling indicates that, in the presence of severe fibrosis (F3/F4), γGT elevation and HDL reduction, the IG had a 75% less chance of developing hepatic steatosis (OR = 0.25; 95% CI: 0.06-0.82). Nevertheless, in the IG, those with an HOMA-IR ⩾ 3 were three times more likely to develop hepatic steatosis (OR = 3.49; 95% CI: 1.10-11.90) (Table 6). The bivariate analysis also revealed that an age ⩾ 60 years (crude OR = 3.81; 95% CI: 1.50-9.70), abdominal fat accumulation (crude OR = 3.87; 95% CI: 1.57-10.29) and BMI ⩾ 25.0 kg/m^2 (crude OR = 1.32; 95% CI: 1.12-1.61) were independent risk factors for hepatic steatosis (data not shown).

Predictors of insulin resistance (HOMA-IR ⩾ 3.0) after intervention

Soy treatment did not have any effect on insulin resistance (OR = 1.92; 95% CI: 0.80-4.83). In patients with advanced fibrosis, an ALT level ⩾ 1.5 times upper limit of normal (ULN) and increased abdominal fat accumulation were independent risk factors for insulin resistance.

Table 5 Median values of biochemical tests in patients with hepatitis C virus at the beginning of monitoring and after protein supplementation

	IG-soy						CG-casein					
		Baseline	12 wk					Baseline	12 wk			
Exams	*n*	Median (iq r)	Median (iq r)	change	% change	*P* value[1]	*n*	Median (iq r)	Median (iq r)	change	% change	*P* value[1]
Total cholesterol (mg/dL)	80	160.0 (44.0)	153.0 (44.7)	-7.0	-7.37	0.05	80	149.0 (49.7)	152.0 (61.5)	+3.0	+2.01	0.63
HDL (mg/dL)	79	44.0 (15.5)	43.5 (15.2)	-0.5	-1.14	0.69	80	44.0 (14.2)	46.0 (17.0)	+2.0	+5.54	0.13
LDL (mg/dL)	78	90.0 (43.0)	84.5 (41.5)	-5.5	-6.11	0.03	80	90.5 (40.7)	89.5 (45.0)	-1.0	-1.10	0.66
AST (U/L)	80	52.0 (44.5)	49.5 (44.2)	-2.5	4.81	0.32	78	59.5 (40.7)	61.5 (40.5)	+2.0	+3.36	0.04
ALT (U/L)	76	73.5 (53.0)	64.0 (50.0)	-9.5	-12.92	0.006	78	76.5 (54.5)	73.0 (65.7)	-3.5	-4.57	0.48
γGT (U/L)	73	87.0 (114.0)	84.0 (107.0)	-3.0	-3.45	0.007	75	102.0 (118.0)	108.0 (120.5)	+6.0	+5.88	0.19
HOMA-IR	80	2.3 (2.6)	2.6 (2.2)	+0.3	+13.04	0.23	79	2.3 (2.2)	2.4 (2.7)	+0.1	+4.35	0.09

[1]Paired analysis: Wilcoxon test ($P < 0.05$): Change in the group, the Bonferroni correction ($P < 0.007$). HDL: High density lipoprotein; LDL: Low density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transferase; CG-casein: Control group-casein; HOMA-IR: Homeostasis model assessment index of insulin resistance; IG-soy: Intervention group-soy; iq r: Interquartile range; $n < 80$: When an examination was not performed.

Table 6 Logistic regression model for factors predictive of hepatic steatosis in patients with hepatitis C virus after 12 wk of protein supplementation

	Bivariate analysis		Multivariate analysis	
	Crude OR (95% IC)	*P* value	Adjusted OR[1] (95% IC)	*P* value
Groups				
Control-casein	1		1	
IG-soy	0.51 (0.20-1.22)	0.135	0.25 (0.06-0.82)	0.032
HOMA-IR				
< 3.0	1		1	
⩾ 3.0	1.97 (0.82-4.77)	0.129	3.49 (1.10-11.90)	0.037
Stage of fibrosis				
F0/F1/F2	1		1	
F3/F4	1.78 (0.68-4.69)	0.239	1.59 (0.50-5.40)	0.436
γGT				
< 85 U/L	1		1	
⩾ 85 U/L	2.07 (0.81-5.64)	0.135	1.52 (0.47-5.18)	0.487
HDL-C				
⩾ 35 mg/dL	1		1	
< 35 mg/dL	1.25 (0.41-3.40)	0.677	2.18 (0.56-8.21)	0.247

[1]Adjusted for the other variables shown in the table; γGT: 85 U/L upper limit of normal. γGT: Gamma glutamyl transferase; HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; IG-soy: Intervention-soy; OR: Odd ratio; 95% IC: 95% confidence interval.

Table 7 Logistic regression model for factors predictive of homeostasis model assessment of insulin resistance in patients with hepatitis C virus after 12 wk of protein supplementation

	Bivariate analysis		Multivariate analysis	
	Crude OR (95% IC)	*P* value	Adjusted OR[1] (95% IC)	*P* value
Groups				
Control-casein	1		1	
Intervention-soy	0.90 (0.47-1.70)	0.745	1.92 (0.80-4.83)	0.15
Stage of fibrosis				
F0/F1/F2	1		1	
F3/F4	4.21 (1.99-9.19)	0.0002	5.25 (2.17-13.67)	0.0004
Alanine aminotransferase				
1.5 times below ULN	1		1	
1.5 times above ULN	2.22 (1.16-4.30)	0.017	3.26 (1.30-8.71)	0.014
Marital status				
Married	1		1	
Single, widowed or divorced	0.52 (0.24-1.07)	0.081	0.24 (0.08-0.65)	0.007
Waist circumference[2]				
Adequate	1		1	
Inadequate	2.62 (1.37-5.16)	0.004	5.57 (2.23-15.27)	0.0004

[1]Adjusted for the other variables shown in the table; [2]Adequate waist circumference: ⩽ 80 cm for women and ⩽ 94 cm for men; ULN: Upper limit of normal; OR: Odd ratio; 95% IC: 95% confidence interval.

Logistic regression analysis showed that the presence of severe fibrosis promoted a five-fold increase in the chance (OR = 5.25; 95% CI: 2.17-13.67) for an HOMA-IR ⩾ 3.0 as well as accumulation of abdominal fat (OR = 5.57; 95% CI: 2.23-15.27). Those with an ALT level ⩾ 1.5 × ULN had a three times greater chance of developing insulin resistance. Being single, widowed or divorced was a protective factor (OR: 0.24; 95% CI: 0.08-0.65) for an HOMA-IR ⩾ 3.0 (Table 7).

Predictors of elevated an ALT level after intervention

The independent predictive factors for changes in ALT levels (⩾ 1.5 × ULN) were an HOMA-IR ⩾ 3.0, HDL < 35 mg/dL and being a male subject (Table 8). Severe fibrosis and alterations in AST and γGT levels were also independent predictors of an increased ALT level (⩾ 1.5 × ULN). Multivariate analysis showed that supplementation with soy protein *per se* represents a protective factor; the IG had a 55% less chance of presenting with an ALT level ⩾ 1.5 × ULN (OR = 0.45, 95% CI: 0.22-0.89), and subjects with an HOMA-IR ⩾ 3.0 were three times more likely to have an increased ALT level (OR = 3.16, 95% CI: 1.51-6.93). However, females had 72% less chance to have an increased ALT level (OR = 0.28, 95% CI: 0.12-0.60) (Table 8).

DISCUSSION

Our population was predominantly male, infected with HCV genotype 1, overweight and presented abdominal

Table 8 Logistic regression model for factors predictive of alanine aminotransferase levels in patients with hepatitis C virus after 12 wk of protein supplementation

	Bivariate analysis		Multivariate analysis	
	Crude OR (95% IC)	*P* value	Adjusted OR[1] (95% IC)	*P* value
Groups				
Control-casein	1		1	
IG-soy	0.55 (0.29-1.04)	0.068	0.45 (0.22-0.89)	0.024
HOMA-IR				
< 3.0	1		1	
⩾ 3.0	2.22 (1.16-4.30)	0.017	3.16 (1.51-6.93)	0.001
Gender				
Male	1		1	
Female	0.39 (0.20-0.77)	0.007	0.28 (0.12-0.60)	0.003
HDL-C				
⩾ 35 mg/dL	1		1	
< 35 mg/dL	3.15 (1.37-7.76)	0.008	2.85 (1.18-7.40)	0.024

[1]Adjusted for the other variables shown in the table. HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; IG-soy: Intervention-soy; OR: Odd ratio; 95% IC: 95% confidence interval.

fat accumulation. Both studied groups had similar characteristics. These clinical conditions increase the chances of developing insulin resistance and hepatic steatosis, which have a negative impact in patients with chronic hepatitis C[21]. In patients infected with HCV genotype 1, steatosis is frequently associated with metabolic syndrome and insulin resistance and is also called "metabolic steatosis"[4,8,21].

A large proportion of our patients had increased liver enzymes (i.e., ALT, AST, γGT) and had not yet been subjected to antiviral treatment. In our population, at baseline, there was a 34.7% prevalence of hepatic steatosis and a 36.3% prevalence of an HOMA-IR ⩾ 3.0. The prevalence of hepatic steatosis associated with HCV varies widely in the literature[22,23] and may differ depending on the population profiles[24].

In this study, protein supplementation caused a significant reduction of hepatic steatosis in both groups; however, this reduction was not significant between the groups. The probable mechanism is not associated with the quality of protein supplementation (animal or vegetable) but likely the nutritional care offered to both groups, which promoted changes in eating habits and consequently improved the overall quality of the diet. Of note, there was no change in body mass index (BMI) or in the pattern of physical activity of these patients.

It is controversial whether insulin resistance is a cause or consequence of steatosis, however the literature suggests that it seems to work more like a cause than a consequence of steatosis in patients infected with HCV genotype 1[2]. In the present study, the regression model showed that an HOMA-IR ⩾ 3 increased the chances of hepatic steatosis more than three-fold. The regression model also revealed that advanced age (⩾ 60 years) and a higher waist circumference and BMI were independent predictor factors for hepatic steatosis. These results are in agreement with other studies which have observed a direct correlation between BMI, visceral obesity and liver steatosis[25-27].

Clinical and experimental studies suggest that soy protein and isoflavones can synergistically act to promote a greater benefit in controlling hypercholesterolemia, hypertriglyceridemia, insulin resistance and steatosis[13,28,29]. We observed that consumption of soy protein had a protective effect and was associated with 75% less chance of having hepatic steatosis.

In an experimental study with obese Zucker rats, a diet with isolated soy protein favored reduced triglycerides in the liver. The proportions of AST/ALT, alkaline phosphatase, bile acids in plasma and pro-inflammatory cytokines (TNF-α and IL1) were also reduced. The authors suggested that soy protein enriched with isoflavones has a favorable effect on the inflammatory status of obese mice, which may promote a favorable outcome in NAFLD patients[28]. It is known that oxidative stress is a decisive factor in the progression of steatosis[30,31]; thus, if isoflavones can act as an antioxidant, then they may minimize the negative progression of steatosis[32,33]. The morbidity of hepatic steatosis is increasing and has been recognized as a liver component of the metabolic syndrome, which also has a negative effect on HCV treatment[8,26,34-36].

Our data revealed a significant decrease in ALT values after supplementation with soy protein compared to the control patients who consumed casein. These findings agree with experimental studies, which have found that soy protein enriched with isoflavones reduces plasma aminotransferase levels[32] and the proportion of AST/ALT[28]. However, a reduction in HOMA-IR levels after supplementation with soy was not observed in our study, which can be attributed to the fact that the HOMA-IR values at baseline in most of our patients were in the normal range. In contrast, Jayagopal *et al*[13] in a study conducted with diabetic women in which the mean value of the HOMA-IR was 5.54 in the intervention group and 5.14 in the control group, supplementation with soy protein enriched with isoflavones significantly reduced serum insulin and the HOMA-IR.

We found that insulin resistance (HOMA-IR ⩾ 3.0) and lower HDL values were predictors for increased ALT. Soy protein intervention in female subjects presented *per se* as a protective factor for increased ALT levels. Our data are in agreement with a recent study that showed higher levels of ALT were significantly associated with gender, a low HDL level and a high HOMA-IR[25]. When evaluating patients with HCV with and without changes of ALT levels and healthy controls, Addel-Azziz *et al*[37] found a higher value of HOMA-IR in patients with abnormal ALT levels compared with those with no change in ALT levels and healthy controls (3.98 *vs* 2.69 *vs* 1.92, respectively), with a significant difference between those with abnormal ALT levels and controls (3.98 *vs* 1.92).

In our study, abdominal fat concentration, an ALT level ⩾ 1.5 the upper limit of normal and the presence of advanced fibrosis were independent predictors of insulin resistance, and even in a multivariate model, they

remained significant. Addel-Azziz *et al*[37] also detected a positive correlation between the HOMA-IR and fibrosis. We detected that marital status was a predictor for insulin resistance as well; single, widowed or divorced patients were less susceptible to inadequacy of the HOMA-IR. This could be associated with the fact that a higher prevalence of overweight and abdominal fat accumulation has been described among married subjects.

In patients with hepatitis C, the presence of a high BMI, insulin resistance and high cholesterol are important predictors for mortality. Multivariate analysis has shown increased mortality associated with metabolic disorders such as diabetes, hypertension and a higher BMI[36,38]. Mehta *et al*[39] found that individuals with HCV and an age above 40 years had a three-fold higher chance of presenting with type 2 diabetes. Therefore, it is recommended that all patients with chronic hepatitis C avoid excess weight and maintain blood glucose, cholesterol levels and blood pressure within normal ranges[40].

Soy supplementation decreased ALT levels and thus may improve liver inflammation in HCV patients. It also reduced hepatic steatosis in a subgroup of individuals with advanced fibrosis, insulin resistance, increased γGT levels and low HDL. On the other hand, soy supplementation did not change insulin resistance, which might be attributed to the fact that the HOMA-IR values at baseline in most of our patients were in the normal range. To our knowledge, this is the first study to show that soy protein supplementation reduces hepatic steatosis and decreases ALT levels in chronic hepatitis C patients. Control of insulin resistance, hepatic steatosis, abdominal obesity and body weight seems to play an essential role in nonpharmacological therapies for chronic hepatitis C treatment. These practices should therefore be encouraged by a multidisciplinary team. Supplementation with soy protein should be considered as an important choice of nutritional management of patients with chronic hepatitis C.

COMMENTS

Background

Hepatitis C virus (HCV) infection is an important public health problem and is the leading cause of liver transplantation in the Western world. Chronic HCV infection increases the risk for hepatic steatosis, insulin resistance, glucose intolerance and type 2 diabetes. The improvement of these comorbidities may benefit the clinical course of the patients.

Research frontiers

Several studies have shown that soy protein may stimulate peroxisome proliferator-activated receptors-α and thus might increase liver fatty oxidation and decrease hepatic steatosis. It also may inhibit sterol regulatory element-binding transcription factor 1 and decrease hepatic lipogenesis. Clinical studies have previously demonstrated that soy consumption may reduce plasma lipid levels, promote insulin resistance reduction and maintain normal glucose levels. HCV infection may be associated with hepatic steatosis and increased insulin resistance. The morbidity of hepatic steatosis is increasing and it has been recognized as a liver component of the metabolic syndrome, which also has a negative effect on HCV treatment. The role of soy supplementation in the improvement of liver diseases is still a matter of debate and there are no studies that have evaluated its effect on insulin resistance, liver fat content and alanine transaminase (ALT) levels in non-diabetic patients with chronic hepatitis C.

Innovations and breakthroughs

Our work is characterized by its originality since it evaluated the impact of soy nutritional intervention in a population of patients infected with hepatitis C. To our knowledge, this is the first study to show that soy protein supplementation decreases ALT levels and reduces hepatic steatosis in a subgroup of individuals with advanced fibrosis, insulin resistance, increased γGT levels and low HDL in chronic hepatitis C. In an experimental study with obese Zucker rats, a diet with isolated soy protein favored reduced triglycerides in the liver. The AST/ALT ratio, alkaline phosphatase, bile acids in plasma and pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1) were also reduced. However, there are no clinical studies that have evaluated the role of soy supplementation on liver enzymes levels, insulin resistance and hepatic steatosis of patients with chronic HCV infection. The authors emphasize the need for further clinical trials to confirm the soy effects on ALT levels and hepatic steatosis of patients with chronic HCV infection.

Applications

This study showed that soy supplementation may improve liver inflammation (decrease ALT level), and may improve steatosis in a sub-group of patients with HCV. Therefore, supplementation with soy protein should be considered in the nutritional management of patients with chronic hepatitis C. However, further clinical trials are necessary to confirm our results.

Terminology

HOMA-IR: Homeostasis model assessment index of insulin resistance; PPARs: Peroxisome proliferator-activated receptors. These are nuclear receptors that function as transcription factors regulating the expression of genes. PPARs play essential roles in the regulation of cellular differentiation and metabolism (carbohydrate, lipid, protein); SREBP-1: Sterol regulatory element-binding transcription factor 1 is a transcription factor involved in sterol biosynthesis.

Peer review

The topic of the study is interesting and based on rational logic. The design of the study is appropriate, but to be sure of that we need to know some more information about the recruitment, randomization and blinding method. In a clinical trial design the conclusions are based only in the analysis performed for the main objectives of the study, otherwise the conclusion from sub-analysis must be biased.

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BRIEF ARTICLE

Satisfaction with patient-doctor relationships in inflammatory bowel diseases: Examining patient-initiated change of specialist

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Abstract

AIM: To assess the reasons for, and factors associated with, patient-initiated changes in treating specialist in inflammatory bowel diseases (IBD).

METHODS: Prospectively identified IBD patients (n = 256) with $\geqslant$ 1 encounter at a metropolitan hospital were surveyed, including whether they had changed treating specialist and why. Negative reasons included loss of confidence, disagreement, and/or personality clash with the specialist.

RESULTS: Of 162 respondents, 70 (43%) had ever changed specialists; 30/70 (43%) for negative reasons, 52/70 (74%) in the preceding year. Patients with negative reasons for changing (n = 30) were younger (median, 35.2 years *vs* 45.3 years) ,had higher IBD knowledge (median, 5.0 years *vs* 4.0 years), yet had lower medication adherence and satisfaction scores (median, 19.0 years *vs* 22.0 years, 14.0 years *vs* 16.0 years respectively, Mann-Whitney tests, all $P < 0.05$), compared to all other responders (n = 132). Patients with a recent change (for any reason) were more likely to have Crohn's disease, currently active disease, previous bowel resection and recent hospitalization [OR 2.6, 95% CI (1.3-5.4), 2.2 (1.0-4.7), 5.56 (1.92-16.67), 2.0 (1.3-3.0), each $P < 0.05$].

CONCLUSION: Changing specialist appears associated with patient- related (age, nonadherence) and contemporaneous disease-related factors (recent relapse) which, where modifiable, may enhance patient-doctor relationships and therefore quality of care.

Key words: Inflammatory bowel disease; Patient-doctor relationship; Quality of care; Disease outcomes; Quality of life

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INTRODUCTION

In chronic inflammatory bowel diseases (IBD), a positive patient-doctor relationship (PDR) appears integral to optimizing quality of care (QoC). Although specific IBD data are lacking, a positive PDR is associated with improved patient satisfaction and health outcomes in other chronic diseases like diabetes and hypertension[1]. In a consumer-driven society, patient-centered care appears essential for a positive PDR[2], and thus assessing patients' satisfaction with their specialist is important[3]. A positive PDR should facilitate the development of trust essential

to the therapeutic process and ensure continuity of care, thus consolidating high-quality care[4,5].

Conversely therefore, PDR discord may compromise QoC, leading to adverse outcomes[6]. Discord may result from personality clashes, lack of rapport, misunderstandings, patient disenfranchisement over management decisions, or adverse disease outcomes. Yet, quantifying PDRs is difficult. Patient self-report instruments to rate PDRs are emerging, but understanding of their relevance to QoC is limited[7,8].

In Australia, as in many countries worldwide, there is significant variation in care models available to IBD patients; ranging from those managed solely by their primary care practitioner; those seen by a colorectal or general surgeon, general physician/internist, or by a specialist gastroenterologist either in private rooms or within a local hospital clinic setting; to those seen in the secondary/tertiary care setting within a dedicated hospital-based "IBD service" with multiple gastroenterologists and clinicians co-located with specific interests in IBD[9]. Each model has inherent strengths and weaknesses and may be attractive to different patients for various reasons. Moreover, with the multiple options potentially available, patients are theoretically able to select and change treating specialist (either in the government-funded public or private sectors), pending appropriate referral from their primary care practitioner and subject to regional availability. Thus one may theoretically assume the patient, as the consumer, may change their treating IBD specialist if their perceived PDR discord "threshold" was overcome and a suitable alternative existed (i.e., "voting with their feet").

Hence, we aimed to explore PDRs in an IBD cohort with the simple, tangible measure of whether patients changed treating specialists and whether this had occurred recently and/or for negative reasons, as a marker of patient satisfaction with the PDR and their medical care. We also explored potential factors associated with patients switching specialist for their care. Although patients logically may report changing specialists for innocuous reasons (e.g., relocation), instances where change occurs, especially for negative reasons from the patients' perspective, represent vulnerable moments in QoC delivery, but also provide an insight into the importance of the PDR, and its continuity[10].

MATERIALS AND METHODS

Subjects and recruitment

All patients with confirmed IBD diagnoses who had an inpatient or outpatient encounter for any reason at the Royal Adelaide hospital (RAH), in a 6-mo period from November 1 2007 to April 30 2008, were prospectively identified as previously described[9].

Subsequently, medical record review was performed to verify diagnoses, and extract further data including demographics, psychological comorbidity, previous surgery and healthcare utilization data. A contemporaneous (± median 14 d), physician global assessment[11] of disease activity (0 = inactive through to 4 = severe) was performed based on all information available, including clinical data (Harvey-Bradshaw criteria)[12], pathology and histological/endoscopic activity grading.

Patient satisfaction with their medical care' questionnaire
(four questions used in study)

In response to each of the statements below, please mark on the line exactly where you think most accurately describes your own feelings:

1. I have a good relationship with my inflammatory bowel diseases (IBD) doctor and look forward to my appointments with him/her.

Never Rarely Sometimes Often Very often

2. The hassle of taking medications for IBD makes me wonder if it is worthwhile

Never Rarely Sometimes Often Very often

3. I wonder if there was another doctor available who could manage my IBD better.

Never Rarely Sometimes Often Very often

4. Overall I am satisfied with the treatment I am taking for IBD

Never Rarely Sometimes Often Very often

Figure 1 Scoring system. Maximum 5 points per question, maximum total of 20 indicates complete, 100% satisfaction with medical care. Score less than 16 indicates "suboptimal" satisfaction. Before scoring, responses to questions 2 and 3 must be inverted (i.e., never = 5, rarely = 4, *etc.*) prior to calculating absolute scores.

Patient survey

Surveys were distributed to each patient comprising multiple components; IBD patient knowledge was assessed using two validated questionnaires[13,14], health-related quality of life was assessed using the shortened inflammatory bowel disease questionnaire (sIBDQ10)[15], the medication adherence report scale (MARS-5) assessed medication adherence[16], and the hospital anxiety depression scale screened for anxiety and depression[17]. Patient satisfaction with medical care was measured using a novel instrument (Figure 1), yet to be validated but the use of which has previously been reported[9]. This comprises four questions with a total score of 20 indicating 100% patient satisfaction. A score < 16 arbitrarily indicates suboptimal satisfaction.

Endpoints relating to change in treating specialist

IBD patients were asked "Have you ever changed your treating specialist," and "If yes, why?" Reasons for changing were deemed negative if the patients' response included at least one of: (1) loss of confidence; (2) disagreement/dissatisfaction regarding management; (3) personality clash with specialist; or (4) other (including "specialist too busy", "waiting time too long"). Alternative reasons including "doctor retired", "doctor/patient moved" or "financial reasons" were not deemed negative responses. The endpoint "Change within 12 mo of survey completion, for any reason" was used to determine contemporaneous disease- and patient-related associations with chang-

Table 1 Clinical and disease characteristics of inflammatory bowel diseases patients who responded to survey (*n* = 162)

Patient variable	*n* (%)
Female sex	85 (52.5)
Crohn's disease	95 (58.6)
Ulcerative colitis	65 (40.1)
Previous bowel resection surgery (ever)	50 (30.9)
Recent inpatient admission[1]	80 (49.4)
Active disease[2]	69 (42.6)
Current stoma	19 (11.7)
Current perianal disease	29 (18.0)
Current cigarette smoker	31 (19.1)
Documented history of psychological comorbidity	41 (25.3)
Currently unable to work due to illness	39 (24.1)
Proportion of lowest socioeconomic group[3]	16 (9.9)

[1]Inpatient admission in period between January 1, 2007-April 31, 2008; [2]as determined by physician global assessment at time of survey completion; [3]according to Social Health atlas, Central Northern Adelaide Health Service, Department of Health, SA 2004.

ing specialist. At the time of survey, 108 (66.6%) of the cohort saw their current treating specialist at an outpatient clinic at RAH, whereas 54 (33.3%) had a current treating specialist based externally to the hospital (either in public or private sector).

Analysis

Statistical analyses were performed using SPSS 15.0.1.1 (Chicago, IL, United States). Bivariate correlations were conducted between variables and changing endpoints. Subsequently, exploratory logistic regression analyses assessed variables for inclusion in the final multivariable model. Those of definite clinical relevance were retained in the model regardless of statistical significance or fit; continuous variables remained unchanged wherever possible.

Ethics

The study was approved by the RAH Research Ethics Committee. Return of a completed survey was accepted as implied patient consent.

RESULTS

Patient characteristics

Two hundred and fifty-six confirmed IBD patients were prospectively identified over 6 mo, and 162 (63.3%) returned a completed survey. Responders had a median age of 43 years (range, 18-90), median IBD duration of 7 years (range, 0-47) (Table 1). As reported elsewhere, survey responders and non-responders did not differ significantly[9].

Changing specialist for any reason

Overall, 70/162 (43.2%) respondents had ⩾ 1 change in specialist for any reason since IBD diagnosis. Of these, the median number of changes per patient since diagnosis was 2.0 (range: 1-6, Figure 2). Thirty of 70 patients who changed specialists (42.9%) gave a negative reason, and 52/70 patients changing specialist (74.3%) had done so within the prior 12 mo (for any reason). The total number of changes per patient correlated weakly positively with IBD duration (r_s = 0.19), and when controlling for disease duration, positively with IBD knowledge (r_s = 0.20) and negatively with age (r_s = -0.22) (Spearman's partial correlations, all $P < 0.02$).

IBD patients with four or more changes in specialists had lower median quality of life scores (sIBDQ10, median score 37 *vs* 48 respectively, $P = 0.01$) and higher disease activity scores (median score 2.75 *vs* 2.28 respectively, $P = 0.04$) than those with a lower number of changes over their total duration of IBD.

Recent change in specialist

In order to identify temporal associations with changing specialist, bivariable (Table 2) then multivariable logistic regression analyses were conducted with the endpoint of specialist change within 12 mo prior to survey completion. The multivariable model (incorporating associations from bivariable analyses where $P \leqslant 0.05$ plus age) showed those with a recent change were more likely to have Crohn's disease, had recent hospitalization, had a past bowel resection, and trended towards having currently active disease (Table 3).

Patients reporting negative reasons for changing specialist

Thirty patients gave 34 negative reasons for changing specialist, including dissatisfaction with management (*n* = 23), lost confidence (*n* = 10) and personality clash (*n* = 1) (Figure 3). These 30 patients were generally younger (median, 35.2 years *vs* 45.3 years), had higher IBD knowledge (median, 5.0 score *vs* 4.0 score respectively), yet had lower medication adherence and satisfaction scores (median, 19.0 score *vs* 22.0, respectively, 14.0 score *vs* 16.0, respectively, Mann-Whitney, all $P < 0.05$) compared to all other responders (*n* = 132). There were no other statistically significant differences including no difference in disease duration, IBD-related characteristics, hospitalization outcomes, or QoL scores between patients changing for negative reasons and other responders (data not shown). However, the frequency of changing specialist [i.e. the duration (years) since IBD diagnosis divided by the total number of changes in specialist over the same period] trended towards being higher in those with one or more negative reasons for changing specialist, compared to other responders (median, 2.8 *vs* 4.0 years between each change, $P = 0.06$, Mann-Whitney).

Finally, in order to identify factors associated with changing specialist for a negative reason, bivariable (Table 2) then multivariable logistic regression analyses were conducted where a negative reason for change in specialist was the dependent variable. The multivariable model (incorporating associations from bivariable analyses with $P \leqslant 0.05$, plus sex) showed those with a negative reason had poorer medication adherence and trended towards being of male sex (Table 4).

Table 2 Bivariable logistic regression analyses of relevant clinical and demographic factors potentially associated with a change in treating specialist

	Associated with change in treating specialist within 12 mo of survey completion		Associated with change in specialist for a "negative" reason at any time	
Variable	**OR [95% CI]**	***P* value**	**OR [95% CI]**	***P* value**
Age under 30	1.13 [0.52, 2.47][5]	0.01[5]	0.60 [0.25, 1.46]	0.33
Female gender	1.15 [0.59, 2.24]	0.73	1.57 [0.71, 3.49]	0.31
Crohn's as inflammatory bowel diseases diagnosis	2.03 [1.03, 4.03][5]	0.04[5]	0.79 [0.35, 1.78]	0.68
Recent inpatient admission[3]	1.70 [0.86, 3.35][5]	0.13[5]	0.82 [0.37, 1.82]	0.69
Previous bowel resection surgery[1]	3.23 [7.81, 1.41][5]	0.005[5]	0.61 [0.27, 1.38]	0.28
Moderate/severe disease activity[4]	2.09 [1.06, 4.12][5]	0.04[5]	0.96 [0.43, 2.15]	0.93
Polypharmacy[2]	1.09 [0.51, 2.36]	0.85	1.50 [0.57, 3.97]	0.49
Living alone	1.06 [0.54, 2.07]	1.0	1.59 [0.71, 3.57]	0.31
Low socioeconomic status	1.62 [0.75, 3.48]	0.26	1.33 [0.53, 3.36]	0.66
Limited employment status	1.09 [0.52, 2.28]	0.85	0.65 [0.29, 1.49]	0.37
Poor inflammatory bowel diseases knowledge	0.39 [0.18, 0.88][5]	0.03[5]	2.04 [0.90, 4.55][5]	0.12[5]
Poor medication adherence	1.22 [0.62, 2.40]	0.60	2.22 [0.99, 4.95][5]	0.06[5]
Poor HRQoL	1.20 [0.57, 2.49]	0.71	0.91 [0.37, 2.24]	1.0
Possible psychological disorder (HADS > 7)	1.13 [0.58, 2.20]	0.74	0.75 [0.34, 1.68]	0.55
Dissatisfaction with medical care	0.95 [0.47, 1.90]	1.0	2.39 [1.05, 5.42][5]	0.04[5]

[1]At any time in the past; [2]taking ⩾ 6 prescription medications currently; [3]in observation period between January 1, 2007-April 31, 2008; [4]as determined by physician global assessment at time of survey; [5]items included in multivariate model (see Tables 3 and 4). OR: Odd ratio; 95% CI: 95% confidence interval; HADS: Hospital anxiety and depression scale; HRQoL: Health-related quality of life.

Table 3 Factors associated with change of treating specialist within 12 mo of survey completion - multivariable logistic regression analysis results

Variable	OR [95% CI]	*P* value
Crohn's disease as IBD diagnosis	2.60 [1.25, 5.41]	0.01
Age[1]	0.98 [0.97, 0.99]	0.01
Previous bowel resection surgery[2]	5.56 [1.92, 16.67]	0.002
Recent inpatient admission[3]	1.97 [1.29, 3.01]	0.002
Moderate/severe disease activity[4]	2.16 [0.99, 4.71]	0.05

[1]For every one year increase in age; [2]at any time in the past; [3]within observation period between 2007-April 31, 2008; [4]as determined by physician global assessment at time of survey. OR: Odd ratio; 95% CI: 95% confidence interval; IBD: Inflammatory bowel diseases.

Table 4 Factors associated with change in specialist for a negative reason - multivariable logistic regression analysis results

Variable	OR [95% CI]	*P* value
Female gender[1]	0.36 [0.13, 1.01]	0.053
Suboptimal satisfaction with medical care	1.22 [0.40, 3.75]	0.73
Poor disease knowledge	0.54 [0.20, 1.50]	0.24
Poor medication adherence[1]	3.49 [1.12, 10.89]	0.03

[1]Variables indicate those statistically, significantly associated with a negative reason for change in specialist. OR: Odd ratio; 95% CI: 95% confidence interval.

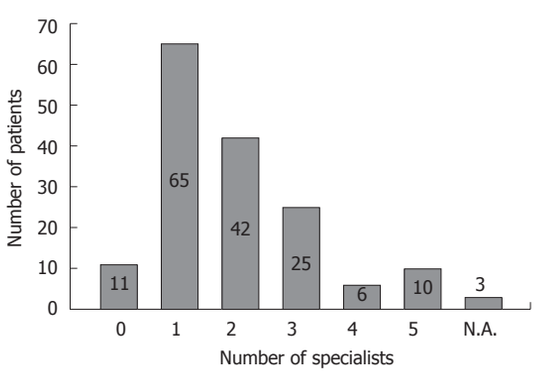

Figure 2 Number of changes in specialist ever by each patient. N.A.: Not available.

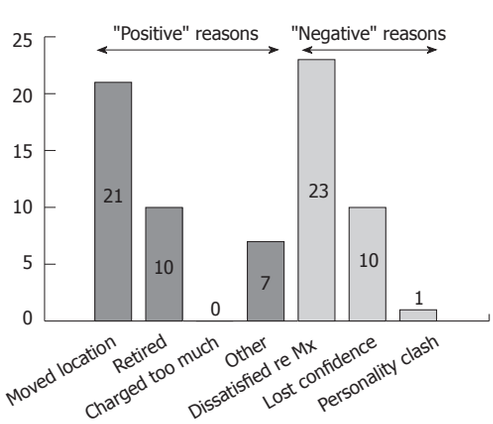

Figure 3 Reasons stated for patient change of treating specialist (72 reasons from 70 patients).

DISCUSSION

Accepted IBD dogma dictates that expert specialist care is vital in optimizing outcomes, as per recent IBD consensus guidelines[18,19]. However, these guidelines do not specifically address continuity of care or patient satisfaction with their PDR. Moreover, the United Kingdom IBD Standards Group emphasizes the importance of maintaining patient-centered care (Standard C), "offering personalized and responsive healthcare so that any patient can migrate between models of care according to activity and

complexity of disease, local facilities and personal preference[20]. In this prospectively encountered IBD cohort, we showed that patients frequently changed treating specialist (43.2% of responders) and of these, many changed (42.9%) for negative reasons, which appears to represent a high prevalence of discordant/poor PDR, which has not previously been documented. Suboptimal PDRs and/or discontinuity of care appear to impair QoC[18], therefore, clinicians (for both the sake of the patient and retaining their own practice) should be cognizant of and, wherever possible, endeavor to address when and why this discord exists[21].

Quantifying PDRs is inherently difficult, however assessing past and hence, risk of future changes in treating specialist *via* patient self-report is a novel, tangible method of surrogately measuring patient satisfaction with the PDR and their medical care. Increasingly, health providers are utilizing patient feedback *via* satisfaction questionnaires as a means to establish a patient-centered approach and for evaluating and improving quality performance[22,23]. In IBD, this has led to development of a limited number of disease-specific satisfaction questionnaires such as the comprehensive QUOTE-IBD survey[24] and others such as the Treatment Satisfaction Questionnaire for Crohn's disease[25]. Given the lack of brief but validated surveys focusing on specialist care in IBD, an unvalidated four-item survey was used in this study[9]. Further refinement and validation of this and similar surveys are needed, whereupon specialists may utilize these in routine practice as a means to identify and address problems with patient dissatisfaction and thus potentially enhance the PDR, and hence quality of care[26].

We thus examined the data to identify characteristics of patients likely to opt for change. Patient factors temporally associated with a recent change, accepting these associations may not be causal, although nevertheless potentially modifiable, include higher disease activity, diagnosis of Crohn's disease, and previous surgery. We believe that these adverse disease factors may jeopardize patient satisfaction and the PDR. In this context, subsequent hospitalization may be the "final straw" in an already vulnerable PDR, prompting a patient-initiated change[10]. Additionally, given all patients were identified *via* a recent hospital encounter at the study centre (RAH), this encounter may have resulted in a sample biased towards those provided with an opportunity to change IBD specialist, given that their inpatient care may have been conducted by a different treating specialist than their usual doctor. However as mentioned, one-third of the cohort continued seeing a treating specialist external/unaffiliated to the study hospital at the time of survey.

Indeed, the data may also reflect societal changes. First, patient care is increasingly often disjointedly administered across multiple primary care, hyper-specialized and provider-specific boundaries[5]. This frequently tests the ability of health systems to ensure a seamless flow of clinical information and correspondence responsive to patient transitions between health providers, especially where larger institutions are involved[27,28]. For instance, a gastroenterologist with a solely community-based practice caring for a patient who suffers an IBD flare may remain unaware when their patient is admitted to a tertiary hospital, and conversely the hospital staff may not be aware of the treating specialist's long-term relationship with the patient. Post-discharge follow-up may then be routinely arranged in the hospital-based IBD clinic instead of the private gastroenterologist's rooms. Hence, depending on the patient's own initiative, a "system-induced change" in specialist may occur, resulting in a loss of continuity of care and potentially, reduced patient satisfaction and jeopardized QoC. Second, consumer expectations of doctors and health outcomes, congruent with medical technological advances, continue to escalate, thus conceivably, patients do (and will) change specialists more readily than ever before[5,29]. Finally, there is a consumer perception that larger entities (e.g., large department stores analogous to large hospitals) offer better products, more conveniently at a lower cost which may also drive patient-initiated changes[30].

Interestingly, we also found that patients with superior IBD knowledge appeared more likely to change specialists. Potentially, knowledgeable IBD patients who expect to participate in management decisions, desire a patient-centered emphasis within the PDR, otherwise this unmet need may drive patients to change specialist[10,31]. Moreover, given those with negative reasons generally exhibited lower adherence again underlines potential disadvantages of a discordant PDR, although these data cannot ascertain whether this discord elicits nonadherence or vice versa. Hence, these patients may be deemed at risk of future adverse disease outcomes in the context of nonadherence, which is unlikely to be salved by changing specialist[21,32].

Regardless in many ways, the patient's self-reported reasons for changing specialist may over-simplify the complex interplay of patient beliefs and expectations, their underlying illness and the PDR, resulting in the change. Hence the fact that the change occurred, rather than the stated reason may be more relevant to consider. For instance, we showed that patients with four or more changes for any reason had lower QoL scores, yet there was no statistically significant difference between patients stating a negative reason for changing specialist and other responders. Thus, the prevalence and frequency of changes (regardless of reason or timing) warrant attention so as to determine whether these lead to increased risk of adverse outcomes *via* disjointed care and underlying dissatisfaction, and whether these outcomes are potentially preventable. Possible avenues begin with the specialist, including engendering patient involvement in clinical decision making and self-empowerment[33], patient-friendly doctor-patient communication, and regular opportunities for patients to provide feedback on their care received[34,35]. Furthermore, patients suspected of medication nonadherence must be sensitively confronted and efforts made to rectify this as previously documented elsewhere[36,37]. Also, fail-safe systems of timely referral and correspondence between health providers must be instituted in order to prevent

loss to treating specialist follow-up and discontinuity of care, which often may occur during times of disease deterioration where continuity and high QoC may in fact be most needed[20,38]. Indeed, in occasional scenarios where a discordant PDR is irreconcilable, and upon mutual agreement between doctor and patient, patients may ultimately benefit from referral on to alternative colleagues or services for ongoing care[39].

In conclusion, in this novel study, we demonstrated that a patient-initiated change in treating specialist in IBD occurs frequently and appears temporally associated with adverse disease traits. Continuity of care, within a positive PDR, is an important element of high-quality care, thus, we recommend that treating specialists should monitor their patients for history and future risk of changing specialists. In view of the recent genesis of national standards in the United Kingdom and United States[20,40], we recommend that continuity of care and institution of efficient, fail-safe referral mechanisms are included as markers of quality in IBD. Adopting a patient-centered approach to IBD management, regularly surveying patient satisfaction and maintaining best practice therapeutic strategies, may result in durable benefits to both patients and doctors alike, although proven formulae to minimize avoidable change in specialist and maintain positive PDRs require further evaluation.

COMMENTS

Background

Inflammatory bowel diseases (IBD) is typically an unpredictable, relapsing- remitting condition and thus patient satisfaction and a robust patient-doctor relationship (PDR) are fundamental to high quality care. Conversely however, relational discord between patient and doctor may compromise quality of care (QoC), leading to adverse outcomes. Thus, in this study, we aimed to explore whether patients changed treating specialists as a tangible marker of patient satisfaction with the PDR and their medical care.

Research frontiers

In the 21st century, patient-centered care is integral in chronic disease management. Measuring patient satisfaction is an important component of ensuring a high standard of care delivery. A compromised PDR, and therefore, QoC, may result in inferior disease outcomes.

Innovations and breakthroughs

A patient-initiated change in treating specialist represents a vulnerable moment in delivery of care but also is a surrogate, tangible measure of patient satisfaction. By establishing factors associated with a change in specialist, one may better understand to what extent these are preventable, and/or how these are best identified so as to minimize disruptions to QoC, thus avoiding adverse outcomes.

Applications

Monitoring patient satisfaction is important to maintain continuity of care and therefore quality. Ensuring failsafe referral and follow-up mechanisms, especially in times of IBD relapse may reduce contemporaneous changes in specialists, thus ultimately improving outcomes for patients.

Peer review

This is an interesting study that sheds some light on the nature and complexity of the PDR in IBD patients. Given the importance of a positive PDR in IBD, such a study is necessary to understand the full dynamics of that relationship. The manuscript should be a good addition to the existing literature on the subject.

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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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]

Issue with no volume

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No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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

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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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

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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