(Original paper)

 Rabeprazole is effective for bile reflux oesophagitis after total gastrectomy in a rat model.

Running title: Rabeprazole for reflux oesophagitis

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ABSTRACT

(Background/Aims) Reflux of duodenal content into the oesophagus has a role in the pathogenesis of oesophageal inflammatory lesions. Although medications such as PPIs are thought to be efficacious in the treatment of reflux oesophagitis, the mechanism of the curative effect of such drugs remains unclear. We studied the effect of proton pump inhibitor (PPI) therapy on oesophageal bile reflux in oesophagitis after total gastrectomy. The purpose of this study is to clarify the effect of a PPI (rabeparazole) (Eisai, Tokyo, Japan) on reflux oesophagitis.

(Methodology) Sixteen 8-week-old male Wistar rats underwent total gastrectomy and oesophagoduodenostomy to induce oesophageal reflux of biliary and pancreatic juice. In 5 rats, the sham operation included a midline laparatomy alone (Sham). One week after surgery, they were treated with saline (Control) (n=8) or PPI (rabeprazole, 30 mg/kg/day ip) (n=8) for 2 weeks. Three weeks after the operation, all rats were sacrificed, and each oesophagus was evaluated histologically. Oesophageal injury was evaluated by macroscopic and microscopic findings as well as the expression of COX2. We measured bile acid in the oesophageal lumen and the common bile duct.

(Results) At 3 weeks after surgery, a histological study analysis revealed an increase in the thickness of the oesophageal mucosa, hyperplasia of the epidermis and basal cells, ulcer formation, and marked infiltration of the inflammatory cells. The macroscopic ulcer score and microscopic ulcer length were significantly reduced in the rabeprazole-treated group. The enhanced expression of COX2 in the control group was also markedly inhibited in the rabeprazole-treated group. Although there was no difference between the control and PPI groups in the total bile acid in the common bile duct, the bile acid activity in the oesophageal lumen was significantly decreased in the rabeprazole-treated group due to augmentation of the duodenal motor complex.

(Conclusion) With this model, we have demonstrated that rabeprazole is an effective therapy for reflux oesophagitis after total gastrectomy from bile reflux. These results indicate that bile acid plays an important role in the mucosal damage induced by duodenal reflux and that it can be a therapeutic target in patients with reflux oesophagitis.

KEY WORDS: Oesophagoduodenostomy, Reflux oesophagitis, PPI, Rabeprazole, ABBREVIATIONS: Cyclooxygenase-2 (COX2)

Proton pump inhibitors (PPIs) Barrett’s oesophagus (BO)

Core tip

The purpose of this study was to clarify the effect of PPI (Rabeparazole) on reflux oesophagitis. Sixteen 8-week-old male Wistar rats underwent total gastrectomy and oesophagoduodenostomy to induce oesophageal reflux of biliary and pancreatic juice. In 5 rats, the sham operation involved a midline laparatomy alone (Sham). One week following the surgery, the rats were treated with saline (Control) (n=8) or PPI (rabeprazole, 30 mg/kg/day ip) (n=8) for 2 weeks. Three weeks after the operation, all rats were sacrificed, and each oesophagus was evaluated histologically. Oesophageal injury was evaluated by macroscopic and microscopic findings as well as by the expression of COX2. We measured the bile acid in the oesophageal lumen and common bile duct. The macroscopic ulcer score and microscopic ulcer length were significantly reduced in the rabeprazole-treated group. The enhanced expression of COX2 in the control group was also markedly inhibited in the rabeprazole-treated group. Although there was no difference between the control and PPI groups in the total bile acid in the common bile duct, the bile acid activity in the oesophageal lumen was significantly decreased in the rabeprazole-treated group due to augmentation of the duodenal motor complex.

With this model, we have demonstrated that rabeprazole is an effective therapy for reflux oesophagitis after total gastrectomy from bile reflux.

(Introduction)

Reflux of duodenal content contributes to the development of oesophageal mucosal damage and inflammation[1]. Oesophagitis after total gastrectomy has been associated with biliary and pancreatic reflux into the oesophagus.

Camostat mesilate has been reported as a serine protease inhibitor of various proteases, especially trypsin, kallikrein and plasmin[2].

Camostat mesilate is commonly used in medical therapy for reflux oesophagitis after total gastrectomy. However, camostat mesilate therapy alone may not result in complete recovery of reflux oesophagitis after total gastrectomy.

More than 10% of patients are reported to have relapses of oesophagitis, even if camostat mesilate is used for maintenance therapy. Proton pump inhibitors (PPIs) are considered the medical therapy of choice for reflux oesophagitis. Although medications such as PPIs are thought to be efficacious in the treatment of reflux oesophagitis, the mechanism of the curative effect of such drugs remains unclear.

The purpose of this study was to clarify the effect of a PPI (rabeparazole, Eisai, Tokyo, Japan) on reflux oesophagitis.

Materials and Methods

Eight-week-old male Wistar rats, weighing 200-250 g, were used in this study. The rats were housed four per cage under standard laboratory conditions (room temperature 22±2°C, 　relative humidity 55±5% and a 12-h light/dark cycle). The animal care and use committee of Kinki University prospectively approved all procedures.

Surgical Procedures

The rats were permitted to acclimate for 2 weeks before surgery. Prior to surgery, the animals were fasted for 24 h. An oesophagoduodenal anastomosis was performed under general anaesthesia (pentobarbital 50 mg/kg body wt, intraperitoneal injection) through an upper midline incision. The gastroesophageal junction was ligated, and the distal oesophagus was transected 2 mm above the ligature. Moreover, the gastroduodenal junction was also ligated, and the proximal duodenum was transected 3 mm distal to the pylorus. A total gastrectomy was performed with the removal of the entire stomach and end-to-end anastomosis of the oesophagus and duodenum. The abdominal incision was closed in two layers. In the sham group, five rats underwent a sham operation, with a midline laparotomy alone without further surgical intervention.

Postoperatively, the rats were allowed to drink water after 6 hours and were fed the following day. Feeding with commercial chow (Oriental Co Ltd) was resumed on day 2.

Study Design and Drug Administration (Fig 1)

Seven days postoperatively, 16 operated rats with reflux oesophagitis were allocated into two groups, a control group (n=8) that was treated with intraperitoneal injection of vehicle (physiological saline)/per day for 2 weeks and a PPI group (n=8) that was given rabeprazole sodium (Eisai, Tokyo, Japan) by intraperitoneal injection at a dose of 30 mg/kg per day for 2 weeks. The sham group (n=5) was followed without any interventions and were kept on regular rat chow to obtain normal, control tissue. Fujisaki et al 3 reported that the subcutaneous injection of Rabeprazole at doses of 1~30 mg/kg to rats was effective for reducing reflux oesophagitis induced by ligation of both the boundary regions between the forestomach and the glandular portion as well as between the pylorus and the duodenum in Sprague-Dawley rats. Rabeprazole, at a dose of 30 mg/kg, inhibited reflux oesophagitis that had been induced by 10 hours of ligation. In the present study, we selected a sufficient dose (30 mg/kg/day) of rabeprazole against rat oesophagitis.

Tissue Preparation

Rats were evaluated 21 days postoperatively under general anaesthesia. Special care was taken to separate the oesophagus from the duodenum according to the suture line. All the oesophagi were cut longitudinally; half were quickly frozen on dry ice and then stored at -80°C for the analysis of prostaglandin E2 (PGE2) and the other half were fixed in 10% buffered formalin. The formalin–fixed oesophagus was Swiss-rolled, processed and embedded in paraffin. Five-micron sections were mounted onto glass slides and were used for the pathological and immunohistochemical analysis.

Macroscopic examination

The oesophagus was opened and gently rinsed with saline. A person who was blinded to the treatment scored the macroscopic ulcer lesions as follows: normal glistening mucosal appearance (score 0), oedematous mucosa with focal haemorrhagic spots (score 1), multiple erosions with haematins attached (score 2), linear ulcerations with yellowish exudates (score 3) or haemorrhagic coalesced ulcerations (score 4).

Microscopic examination

The entire area of damage was collected and fixed in 10% formalin for the histological evaluation. The degree of epithelial loss was measured by micrometre as the ulcer length (the ulcer length of each rat was calculated as the average ulcer length of each section).

Immunohistochemistry

COX2: Localisation of COX2 protein was determined by immunohistochemical staining using specific antibodies. The DAKO EnVision system (Dako Cytomation Japan Co. Ltd., Kyoto, Japan) was used with autoclave acceleration. After blocking with endogenous peroxidase, deparaffinised sections covered with a protein block and serum-free media (Dako) were incubated overnight at 4°C with individual primary antibodies, including antimouse COX2 (1:50, mouse monoclonal; BD Transduction Laboratories, San Jose, Calif). Sections were treated with a secondary biotinylated antibody (Dako). 3,3’-diamonobenzidine tetrahydrochloride was used as the chromogen, and the sections were counterstained with haematoxylin.

Measurement of Bile acid in the oesophageal lumen

The oesophagus was removed and lavaged with 0.5 ml of saline. The saline used for the lavage was centrifuged at 1500 ×*g* at 4°C for 5 min. The supernatant was frozen and stored. The total bile acid concentration was measured with an ENZa BILE kit (Daiichi Chemical, Tokyo).

Measurement of the total bile acid in bile juice aspirated from the common bile duct

Bile juice was directly collected from the common bile duct using a 24-G indwelling needle. The total bile acid concentration was measured with an ENZa BILE kit (Daiichi Chemical, Tokyo).

Statistical Analysis

Data are expressed as the mean±SD of each group. The Mann-Whitney U test was used to compare each group. Differences were considered significant when the P value was<0.05.

Result

1. Macroscopic findings (Fig 2,)

In control rats, the oesophageal wall had longitudinal ulcerations that were located primarily in the middle and lower thirds of the oesophagus. However, the gross appearance of the oesophagus from the PPI group showed only scattered erosions or mild haemorrhage spots along the oesophagus. The ulcer score was significantly (p<0.05) decreased by treatment with a PPI (score 1: 2, score 2: 3, score 3: 3, and score 4: 0) compared with control (score 1: 0, score 2: 0, score 3: 1, and score 4: 7).

1. Microscopic findings (Fig 2)

The control group had evident thickening of the epithelium, elongation of the lamina papillae, and basal cell hyperplasia in the oesophageal mucosa. Histological examination revealed severe oesophagitis in the control group. However, the ulcer length, degree of inflammatory cell infiltration, and degree of hyperplasia were significantly decreased in the PPI group. The microscopic ulcer length was significantly (p<0.05) increased in the control group (8±1 mm) compared with the PPI group (5±1 mm).

1. Total bile acid in the oesophageal lumen (μmol/L)

The total bile acid in the oesophageal lumen was significantly higher in the control group (175±50) compared with the sham operated rats (35±5). The treatment with a PPI (45±5) significantly (p<0.05) inhibited the increase in the total bile acid activity in the oesophageal lumen.

1. The total bile acid (mmol/L) of bile juice aspirated from the common bile duct

There is no difference between the control group (26.5±2.5) and the PPI group (22.9±1.7). However, the control and PPI groups have significantly (p<0.05) higher levels than the sham group (18.2±1.3).

1. Expression of COX2 (Fig 3)

Bile reflux can cause tissue injury. Based on our in vitro findings, it was of interest to determine whether COX2 would be induced in an established surgical model of duodenoesophageal reflux. COX2 was not detected in the normal oesophagus. However, COX2 was abundantly expressed in the inflamed oesophageal mucosa of rats that were exposed to chronic duodenoesophageal reflux. The expression of COX2 was significantly (p<0.05) increased according to immunostaining in the control group compared with the PPI group.

**(Discussion)**

It is well accepted that bile reflux plays an important role in the pathogenesis of oesophageal lesions. In patients with gastroesophageal reflux disease, the concentration of bile acids in the oesophageal refluxate correlates with the degree of oesophageal mucosal injury[4]. In experimental animals, the creation of a duodenoesophageal anastomosis led to oesophagitis[5]. Bile acids can induce mucosal injury and stimulate cell proliferation[6].

Helsingen[7] performed total gastrectomy and oesophagoduodenostomy on rats, examined the rats for changes in oesophageal mucosa from 4 days through 4 months after the operation and reported that the mucosal epithelium was destroyed and inflammation occurred inside the lamina muscularis mucosae relatively early. In our experiment on rats that underwent total gastrectomy and oesophagoduodenostomy, erosion and ulceration of the oesophagus were noted 2 weeks after the operation. Erosion,　ulceration, inflammation cell infiltration and hyperplasia of the mucosal epithelium were the pathologic features of reflux oesophagitis in rats, which were consistent with the Helsingen’s descriptions.

This study provides concrete evidence that bile acid is involved in oesophageal mucosal injury and inflammation in rats by oesophagoduodenal reflux as well as that treatment with RPZ, a PPI, is excellent, decreasing the gross and histopathological findings of reflux oesophagitis as well as decreasing the expression of COX2 in affected oesophageal mucosa compared with saline treatment.

The most striking finding in the present study was that RPZ attenuated oesophageal mucosal injury that was induced by duodenoesophageal reflux. The significant role of bile acid in the pathogenesis of reflux oesophagitis has previously been demonstrated. As discussed above, bile acids represent one of the important constituents of duodenal fluid that has been implicated in oesophageal mucosal injury. Based on our histological findings, an animal model was used to determine whether duodenoesophageal reflux is caused induction of COX2. We observed markedly enhanced expression of COX2 in inflamed oesophageal mucosa that was obtained from rats in which an oesophagoduodenal anastomosis had been created. In contrast, COX2 was undetectable in normal oesophageal mucosa. Bile acid is harmful to the squamous epithelium of the oesophagus, causing reflux oesophagitis even without gastric acid reflux.

The precise mechanisms by which bile acid causes oesophageal injury remain unclear. Bile acids induce COX2 by both transcriptional and post-transcriptional mechanisms[8.9] . Previously, protein kinase C was implicated as important for the bile acid-mediated induction of COX2. Bile acids can also stimulate PI-3K activity[10]; this finding suggests that PI-3K could be involved in mediating the induction of COX2. In support of this notion, we found that 2 inhibitors of PI-3K activity blocked the induction of COX2 by bile acids. ERK1/2MAPK is downstream of PI-3K and has been implicated in the regulation of COX2[11]. In fact, ERK1/2MAPK is involved in regulating both the transcription of COX2 and stability of COX2 mRNA. Treatment with bile acid induced ERK1/2 activity, and inhibiting the activation of ERK1/2 blocked the induction of COX2 by bile acid. Taken together, it seems likely that the bile acid-mediated induction of COX2 involves a signalling cascade that consists of PKC, PI-3K and ERK1/2MAPK. Data on how RPZ reduces the degree of bile reflux are scarce.

Champion et al found a reduction in the percentage time of bilirubin absorbance>0.14 from 32.8% to 4.7% with 40 mg of omeprazole daily in nine patients (three with GERD and six with Barrett’s oesophagus)[12]. Administering the same dose of omeprazole to 11 BO patients, Marshall et al observed a decrease in the oesophageal bilirubin exposure from a median of 28.9% to 2.4%[13].

 There are two possible explanations for the reduction of DGER with acid suppressant therapy. First, PPIs generally reduce gastric secretion by approximately 40%, decreasing the volume of refluxate[14]. Second, PPIs have been shown to augment the antral and duodenal phase Ⅲ migrating motor complex in healthy individuals, accelerating antroduodenal passage of gastric contents, which should reduce duodenogastric reflux[15] . These findings show that PPIs can reduce the reflux of bile acids into the oesophagus. In our total gastrectomy model, gastric secretion was not affected by PPI. There was no difference between the control (26.5±2.5 mmol/L) and PPI groups (22.9±1.7 mmol/L) in bile acid concentration from the common bile duct. PPIs do not inhibit the secretion of bile acid from the common bile duct. Therefore, we speculate that PPIs accelerate the duodenal phase Ⅲ migrating motor complex, accelerating the duodenal passage of duodenal contents (bile acids), which should reduce duodenoesophageal reflux.

Recent studies have indicated that PPIs have effects beyond acid suppression and have revealed many types of inflammatory cytokines in the oesophageal mucosa of GERD patients[16]. Additionally, histological improvement may also implicate the cytoprotective properties of Rabeprazole against bile-induced oesophageal damage[17].

This evidence suggests that PPIs not only inhibit acid secretion but also reduce inflammation in the oesophageal mucosa.　In our study, rabeprazole was an effective therapy for reflux oesophagitis after total gastrectomy due to bile reflux. Further non-clinical and clinical investigations into the mechanism of the anti-inflammatory actions of PPI are needed.

In conclusion, we have demonstrated, with our model, that rabeprazole is an effective therapy for reflux oesophagitis after total gastrectomy due to bile reflux. These results indicate that bile acid plays an important role in the mucosal damage induced by duodenal reflux and that it can be a therapeutic target in patients with reflux oesophagitis.

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Legend

Fig 1 Study design and Drug Administration

Fig 2 Macroscopic and microscopic findings

Fig 3 Expression of COX2