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ORIGINAL ARTICLES

Characteristic pathological findings and effects of ecabet sodium in rat reflux esophagitis

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Abstract

AIM: To explore the pathological findings in the entire esophagus in rats with reflux esophagitis, and the effects of ecabet sodium (ES).

METHODS: A rat model of chronic acid reflux esophagitis was used. In the treatment group, ES was administered after surgery (n = 16). No drug was administered postoperatively to the esophagitis group (n = 9). Sham-operated rats were used as a control group (n = 5). Rats were sacrificed on day 7 after the operation. The epithelial thickness and leukocyte infiltration were examined in the upper, middle and lower areas of the esophagus. The survival rate, incidence of esophageal ulcer, and mean surface area and number of esophageal ulcers were determined in the esophagitis and ES groups. Esophageal histology was assessed in all three groups.

RESULTS: Leukocyte infiltration in the esophagitis group was 26.3 ± 22.0 in the middle esophagus and 8.2 ± 4.9 in the upper esophagus, which was significantly greater than that in the controls (1.3 ± 1.1 and 1.4 ± 1.0, respectively) (P < 0.05). The thickness of the epithelium in the esophagitis group was 210.8 ± 47.7 μm in the lower esophagus and 204.2 ± 60.1 μm in the middle esophagus, which was significantly greater than that in the controls (26.0 ± 5.5 and 21.0 ± 6.5 μm, respectively) (P < 0.05). The mean number of ulcers per animal in the ES group in the entire esophagus was 5.4 ± 2.5, which was significantly less than that in the esophagitis group (9.0 ± 3.5) (P < 0.05). The epithelial thickness in the ES group was 97.5 ± 32.2 μm in the lower esophagus, which was decreased compared with that in the esophagitis group (210.8 ± 47.7 μm) (P < 0.05).

CONCLUSION: Mucosal inflammation extended to the upper esophagus close to the hypopharynx. Our study suggested that ES may have a useful defensive role in reflux esophagitis.

Key words: Gastroesophageal reflux disease; Upper esophagus; Laryngopharyngeal reflux disease; Extraesophageal syndrome

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INTRODUCTION

Against the background of an aging society and changing dietary habits that now include western-style food, and despite a low rate of infection with *Helicobacter pylori* (*H pylori*), the number of patients with gastroesophageal reflux disease (GERD) has increased recently in Japan[1,2]. The symptoms of GERD decrease quality of life[3], and long-term reflux of gastric acid is known to increase the risk of Barrett's esophagus and Barrett's adenocarcinoma. As a result of the global ground swell

of GERD, a worldwide consensus definition of GERD has been developed recently[4]. Laryngopharyngeal reflux disease (LPRD) is a common condition in the primary care setting and is one of the extraesophageal syndromes regarded as secondary to GERD[5,6]. Exactly how reflux of gastric juice influences the esophagus and/or extraesophageal structures is unknown, and the causal association between gastric-juice reflux and pathogenesis of reflux esophagitis is a controversial subject.

There are limitations associated with the investigation of the pathophysiology of GERD in humans, thus, experiments using animal models are fundamental to this investigation. Proton pump inhibitors (PPIs) and histamine-2 receptor antagonists are likely to be used for reflux esophagitis, but there have been few reports about using mucosal-protective drugs. We previously induced chronic acid-reflux esophagitis in a rat model and investigated the underlying mechanism of reflux esophagitis, with a focus on the mechanism of esophageal mucosal resistance[7,8].

In the current study, we used a rat model of chronic acid-reflux esophagitis to explore the esophageal mucosal damage macroscopically and microscopically throughout the entire esophagus, including the upper esophagus close to the hypopharynx, and to investigate the protective effects of ecabet sodium (ES) on the esophageal mucosa.

MATERIALS AND METHODS

Chronic acid-reflux esophagitis model

Specific-pathogen-free male Wistar rats aged 9 wk were purchased from SLC (Tokyo, Japan). They were used after acclimatization for 1 wk in an animal room with a controlled temperature (23 ± 2℃). The rats were fed a standard diet but fasted for 12 h prior to the surgical induction of chronic acid-reflux esophagitis, which was induced by modifying the method of Omura *et al*[9]. Anesthesia was induced by inhalation of isoflurane. After laparotomy, duodenal stenosis was accomplished by wrapping the duodenum near the pylorus with a piece of 18 Fr Nelaton catheter (length: 3.0 mm, diameter: 4.0 mm; Terumo Inc., Tokyo, Japan). To prevent dislodgement, we sutured the edge of the catheter to the serosa of the pylorus using 4-0 nylon thread. The transitional zone between the fore-stomach and the glandular portion (i.e. the limiting ridge) was ligated with 2-0 silk thread. The animals were fasted for 48 h after the operation but were allowed free access to drinking water. In those allocated to the ES treatment group (n = 16), ES (65 mg/kg) was intragastrically administered only once immediately after surgery, and drinking water including ES (21.39 ± 2.74 mg/kg per day) was given from the day after surgery until day 7. ES was obtained from Tanabe Seiyaku Co. Ltd. (Osaka, Japan). No drug was administered after surgery to the animals in the esophagitis group (n = 9). Sham-operated rats were used as a control group (n = 5). The animals were sacrificed on day 7 after the operation. All the procedures performed on laboratory animals were approved by the Institutional Animal Care and Use Committee of Juntendo University School of Medicine (Tokyo, Japan), and all the animal experiments were carried out in compliance with the guidelines for animal experimentation of Juntendo University School of Medicine.

Measurement of gross esophageal lesions

In the chronic acid-reflux esophagitis and ES groups, the survival rate and incidence of esophageal ulcers were determined. The esophagus was resected up to the upper segment close to the hypopharynx. After taking photographs of esophageal lesions, the numbers of ulcers were measured in the upper, middle and lower segments of the esophagus. The surface areas of ulcers in these three segments of the esophagus were measured using a high-resolution computerized image analyzer (KS 400; Carl Zeiss Imaging Solutions GmbH, Hallbergmoos, Germany).

Histological assessment

After being fixed in 10% buffered formalin, the tissues were embedded in paraffin, and 3-μm sections were prepared and stained with HE. The epithelial thickness was assessed in the upper (approximately 5 mm below the cricopharyngeus), middle [midpoint between the cricopharyngeus and the esophagogastric (EG) junction] and lower (approximately 0.5 mm above the EG junction) segments of the esophagus, using light microscopy (high-power fields). The numbers of leukocytes that infiltrated each high-power field were counted in these three segments of the esophagus.

Statistical analysis

All data are presented as mean ± SD. Student's t test and χ^2 test were used, and $P < 0.05$ was regarded as statistically significant.

RESULTS

Modified chronic acid-reflux esophagitis model vs ES model

In the esophagitis group, the 7-d survival rate after the operation was 66.7% (6/9 rats), and the incidence of ulcers in the survivors was 100%. In the ES group, the 7-d survival rate after the operation was 62.5% (10/16), and the incidence of ulcers was 90.0% (9/10 rats). There were no statistically significant between-group differences in the survival rate and incidence of ulcers.

Macroscopic assessment

In both the esophagitis and ES groups, ulcers were noted in the mucosa, but none occurred in the control group. Also in the esophagitis and ES groups, macroscopic esophageal ulcers were found in the lower and middle parts of the esophagus in most cases (Figure 1). The mean number of ulcers per animal in the ES group in the

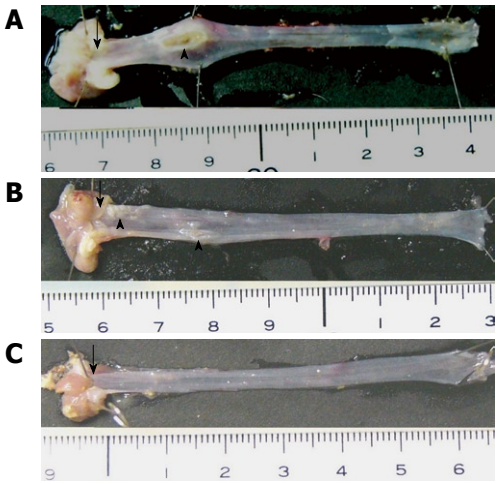

Figure 1 Macroscopic findings in the three groups. A: Esophagitis group on day 7 after operation; B: ES group on day 7 after operation; C: Control group on day 7 after operation. In the esophagitis and ES groups, ulcers were noted in the mucosa, but none occurred in the control group. The arrows indicate the EG junction. The arrowheads indicate ulcers.

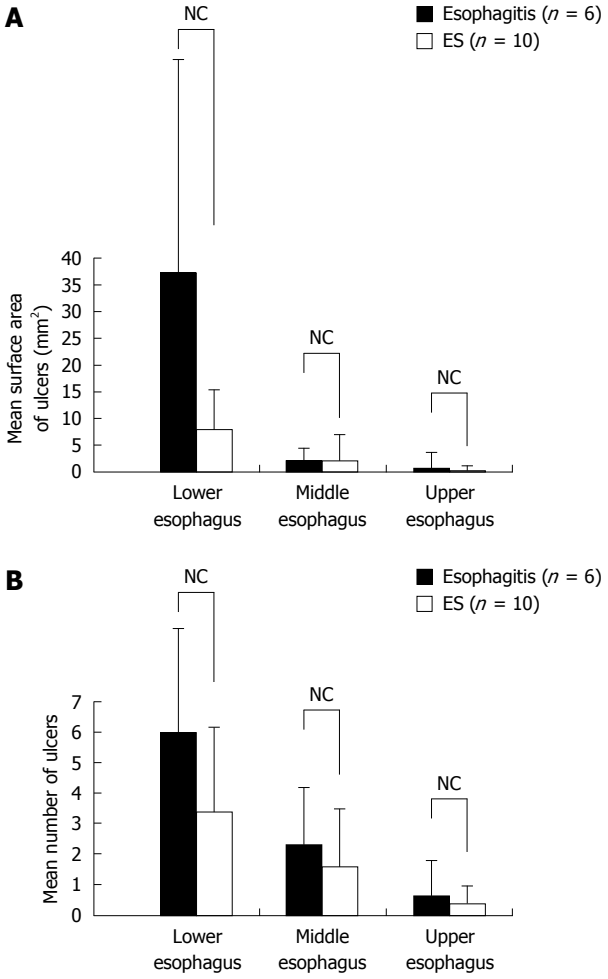

Figure 3 Mean surface area and number of ulcers in each of the three individual segments of the esophagus. A: Mean surface area of ulcers in each part of the esophagus in the esophagitis and ES groups; B: Mean number of ulcers in each part of the esophagus in the esophagitis and ES groups. There were no significant between-group differences in terms of the number and mean area of ulcers in each of the three individual segments of the esophagus.

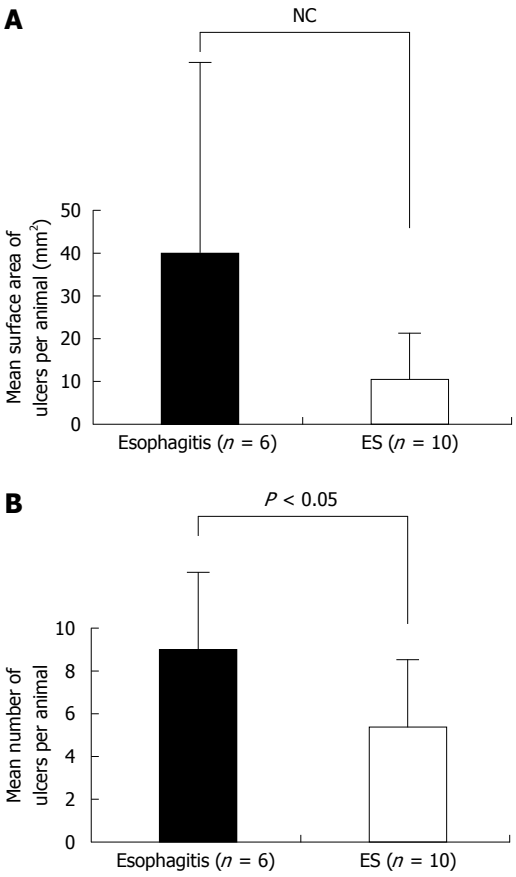

Figure 2 Mean surface area and number of ulcers per animal. A: Mean surface area of ulcers per animal in the esophagitis and ES groups. There were no significant differences in the mean area of ulcers between the two groups; B: Mean number of ulcers per animal in the esophagitis and ES groups. In the ES group, the number of ulcers was significantly less than in the esophagitis group (5.4 ± 2.5 *vs* 9.0 ± 3.5, $P < 0.05$).

entire esophagus was 5.4 ± 2.5, which was significantly less than the number in the esophagitis group (9.0 ± 3.5) ($P < 0.05$) (Figure 2). The mean surface area of ulcers per animal was 40.0 ± 56.8 and 10.5 ± 8.6 mm^2 in the esophagitis and ES groups, respectively ($P = 0.120$) (Figure 2). No significant between-group differences were found in terms of the number and area of ulcers in each of the three individual segments of the esophagus (Figure 3).

Histological assessment

Histologically, the esophagus showed a thin epithelial layer with squamous cells in the control group. In the esophagitis group, the epithelium was thickened markedly, and elongation of the lamina propria papillae into the epithelium was noted. Basal cell hyperplasia and inflammatory cell infiltration were marked in the lamina propria (Figure 4). The thickness of the epithelium in the lower esophagus was the most severely affected of the three areas. The thickness of the epithelium in the esophagitis group was 210.8 ± 47.7 μm in the lower esophagus and 204.2 ± 60.1 μm in the middle esophagus, which was significantly greater than that in the controls (26.0 ± 5.5 and 21.0 ± 6.5 μm, respectively) ($P < 0.05$)

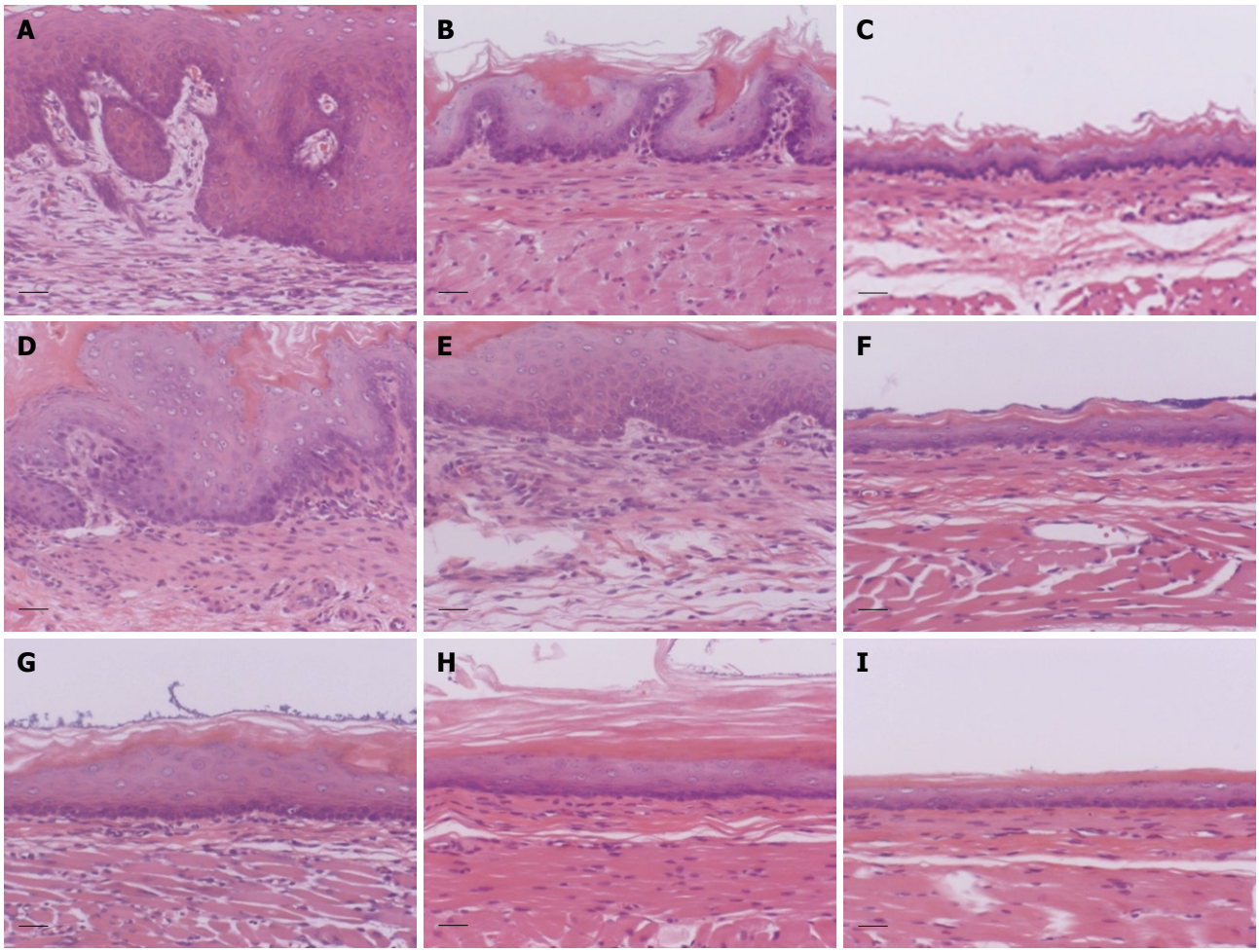

Figure 4 Histological findings in each part of the esophagus in the three groups (HE staining). Lower esophagus: esophagitis (A), ES (B) and control (C) groups. Middle esophagus: esophagitis (D), ES (E) and control (F) groups. Upper esophagus: esophagitis (G), ES (H) and control (I) groups. In the ES group, the thickness of the epithelium in the lower esophagus was significantly decreased compared with that in the esophagitis group. Scale bar, 30 μm.

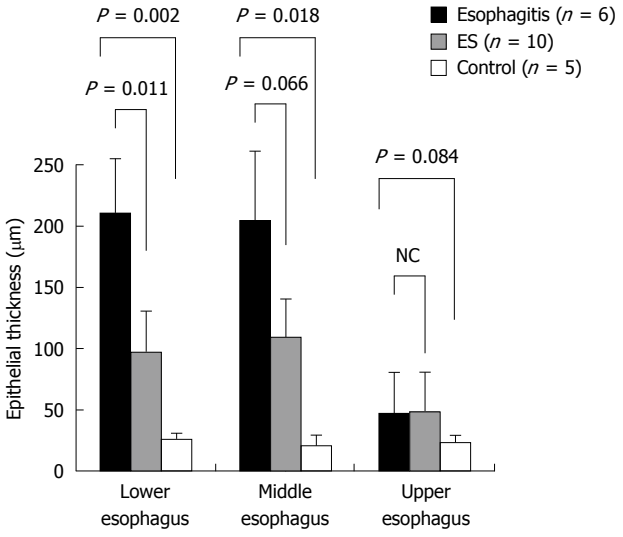

Figure 5 Thickness of the esophageal epithelium in the three groups. In the esophagitis group, the thickness of the epithelium in the lower and middle esophagus was significantly greater than that in the controls ($P < 0.05$). In the ES group, the thickness of the epithelium in the lower esophagus was significantly less than that in the esophagitis group ($P < 0.05$), and the thickness of the epithelium in the middle esophagus tended to be less than that in the esophagitis group ($P = 0.066$).

(Figure 5). Also, the epithelial thickness in the ES group was 97.5 ± 32.2 μm in the lower esophagus, which was decreased compared with that in the esophagitis group (210.8 ± 47.7 μm) ($P < 0.05$), and the epithelial thickness in the ES group in the middle esophagus also tended to be less than that in the esophagitis group.

Leukocyte infiltration in the esophagitis group was 26.3 ± 22.0 in the middle esophagus and 8.2 ± 4.9 in the upper esophagus, which was significantly greater than that in the controls (1.3 ± 1.1 and 1.4 ± 1.0, respectively) ($P < 0.05$) (Figure 6).

DISCUSSION

We revealed that epithelial thickening occurs at the same time as inflammatory cell infiltration in the middle to lower esophagus in chronic acid-reflux esophagitis. An imbalance between offensive factors (e.g. gastric acid and pepsin) and defensive factors (e.g. mucin, saliva and the esophageal epithelial lining) results in the development of reflux esophagitis[7,10,11]. However, there have been few reports about the pathological findings in the esophageal squamous epithelium, and there are differing opinions among pathologists about the findings considered

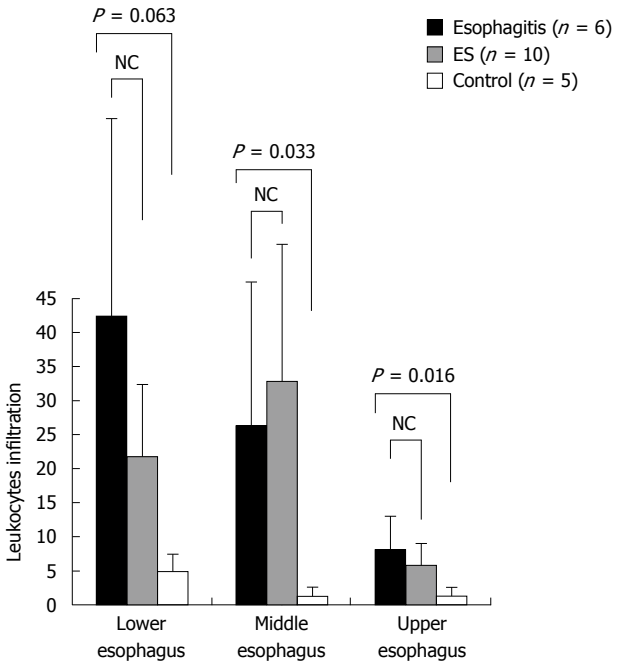

Figure 6 Leukocyte infiltration in the esophageal epithelium on day 7 after operation in the three groups. In the esophagitis group, leukocyte infiltration in the middle and upper esophagus was significantly greater than that in the controls ($P < 0.05$).

characteristic of chronic reflux esophagitis[12-14]. Some authors have reported the relationship between chronic inflammation and epithelial changes in other parts of the gastrointestinal tract. Yasunaga *et al*[15] have suggested that increased interleukin 1β and hepatocyte growth factor production caused by *H pylori* infection may contribute to fold thickening of the stomach by stimulating epithelial cell proliferation and foveolar hyperplasia in patients with enlarged fold gastritis[15]. In patients with erosive reflux disease, the thickness of the basal cell layer and length of the papillae were associated with the severity of esophagitis. After esomeprazole treatment, the basal layer thickness and length of papillae were reduced in both non-erosive and erosive reflux disease[16]. However, these studies were performed in human subjects, so detailed pathological investigation in chronic reflux esophagitis was not performed.

Recently, the mechanism by which acid may induce inflammation has been proposed by Tobey *et al*[17]. They clarified that the permeability of the esophageal epithelium to acid is increased by dilatation of the intracellular space, and PPIs may decrease inflammation of the esophageal epithelium[17,18]. These results suggest that acid may diffuse through the esophageal epithelium and induce infiltration of inflammatory cells. This increases the release of proinflammatory cytokines, which may induce thickening of the esophageal epithelium. Furthermore, we demonstrated that inflammatory cells infiltrated the epithelium of the upper esophagus close to the hypopharynx, where there was no evidence of ulcers. It has been reported previously that inflammation mainly occurs in the lower esophagus near the EG junction in cases of reflux esophagitis[8], but how the reflux of gastric juice influences esophageal and/or extraesophageal symptoms is unknown. Recently, GERD-related extraesophageal syndromes have attracted attention and it has been suggested that gastric-juice reflux can extend to the upper esophagus close to the hypopharynx. In a study of the pathogenesis of LPRD, which is one of the extraesophageal syndromes, Tokashiki *et al*[19] have found that patients with LPRD show a significantly longer acid reflux time in the upper esophagus than healthy volunteers do.

In our chronic acid-reflux esophagitis model, gastric juice passed through the EG junction and diffused directly into the esophagus. This model resembles the reflux esophagitis that is seen in the clinical setting. The direct acid injury to the hypopharynx appeared to be reflected in the microscopic pathological changes in the upper esophagus.

Secondarily, we demonstrated that ES inhibited the epithelial thickening of the lower esophagus, which was the most severely inflamed segment of the esophagus, and ES also tended to inhibit the epithelial thickening of the middle esophagus. ES, a dehydroabietic acid derivative from pine resin, has been used clinically in the treatment of gastritis and gastric ulcer, and is believed to exert its effects through various mechanisms[20-24]. ES binds directly to the gastric mucosa, thereby protecting the mucosa against ethanol binding, and it has been shown to inhibit pepsin activity in rat and human gastric juices.

In the present study, ES may decrease the number of ulcers in the entire esophagus by binding directly to the esophageal mucosa and inhibiting pepsin activity. ES was shown to be useful in preventing inflammation from the lower to the middle esophagus. ES may inhibit the increase in cytokines which are released as part of the inflammatory process and induce epithelial thickening. However, the relationship between the occurrence of ulcers and epithelial thickening is unknown, and further study about this relationship is necessary.

In conclusion, this study revealed that mucosal inflammation extended to the upper esophagus close to the hypopharynx, even where there was no evidence of ulcers. This finding of inflammation suggested that direct injury to the hypopharynx may occur as a result of the reflux of gastric juice. Our study also suggested that ES may play a useful defensive role in the prevention of reflux esophagitis. Further studies are necessary to explore the factors that are responsible for the protective effects of ES in reflux esophagitis.

COMMENTS

Background

Recently, the number of patients with gastroesophageal reflux disease (GERD) has increased in Japan. Although GERD-related extraesophageal syndromes have attracted attention, how gastric-juice reflux influences the esophagus and/or extraesophageal structures is unknown. In the present study, the authors explored the pathological findings in the entire esophagus and the effects of ecabet sodium (ES).

Innovations and breakthroughs

The authors revealed that epithelial thickening occurred at the same time as

inflammatory cell infiltration in the middle to lower esophagus in chronic acid-reflux esophagitis. Furthermore, they demonstrated that inflammatory cells infiltrated the epithelium of the upper esophagus close to the hypopharynx, where there was no evidence of ulcers. These findings suggested that the reflux of gastric juice can extend to the upper esophagus close to the hypopharynx.

Terminology

Laryngopharyngeal reflux disease is a common condition in the primary care setting and is one of the extraesophageal syndromes regarded as secondary to GERD.

Peer review

The authors evaluated the pathological findings in rat chronic acid-reflux esophagitis and the defensive effects of ES on esophageal mucosal injury. This an interesting, well-executed study.

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