ORIGINAL ARTICLE

Bile-induced Adenosine Triphosphate Depletion and Mucosal Damage During Reflux Esophagitis

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Background: This study was designed to investigate the role of bile in a large animal model of acute esophageal reflux disease. **Methods:** An agar electrode was used to measure the transmucosal potential difference of the esophagus in anaesthetized dogs. The vascular permeability index and the epithelial permeability index of the mucosa were evaluated by means of the Evans blue and the sodium-fluorescein clearance method, respectively. The tissue adenosine triphosphate (ATP) level and the myeloperoxidase activity were determined from tissue biopsies, while the degree of mucosal damage was evaluated histologically on a grade 0–100 scale. Group 1 ($n = 8$) served as saline-treated control; groups 2 ($n = 8$), 3 ($n = 5$) and 4 ($n = 5$) were exposed for 4 h to canine bile alone, to hydrochloric acid + bile, or to hydrochloric acid alone, respectively. **Results:** In Groups 2, 3 and 4 the degree of mucosal damage was significantly increased, and a 4-fold elevation in myeloperoxidase activity was observed. The transmucosal potential difference was decreased significantly below the control level, while the vascular and epithelial permeability indices were significantly increased compared with the control values. Bile, but not hydrochloric acid, evoked a significant (40%) decrease in the ATP level of the esophageal tissue. **Conclusions:** We propose that mucosal dysfunction, structural damage and leukocyte invasion during hydrochloric acid-induced esophageal injury are exacerbated by bile-induced changes in tissue ATP concentrations during experimental esophageal reflux disease.

Key words: Bile reflux; mitochondrial dysfunction; mucosal injury; esophagus

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Gastroesophageal reflux disease (GERD) is responsible for a high proportion of dyspeptic/digestive symptoms in the European population (1). Although the pathogenesis has been extensively studied, the primary cause of the mucosal barrier damage leading to the clinico-pathologic complications in the affected patients is still unknown. The presence of refluxed material could conceivably contribute to mucosal inflammation and injury in patients with GERD through either or any of at least two potential components: (i) by reflux and abnormal clearance of gastric acid; (ii) by reflux of bile and/or other noxious compounds in the duodenal content into the stomach and the esophagus. Most investigations have found an apparent association between gastric acid reflux and esophageal inflammation (2). However, esophagitis is a frequent finding in GERD patients with acid-suppressive therapy or even after total gastrectomy. Similarly, it has been shown that bile-induced mucosal changes per se may contribute significantly to esophageal barrier lesions and the development of gastroesophageal reflux disease (3).

Although the exact pathomechanism of GERD is not fully understood, several lines of indirect evidence suggest that a mitochondrial dysfunction may play a role in the reflux-induced esophageal mucosal responses. The hepatic mitochondria are one of the main targets of bile-induced hepatocyte damage, and the intracellular accumulation of hydrophobic bile salts during cholestasis causes hepatocyte necrosis by inducing a mitochondrial permeability transition (4, 5). However, the in vivo effects of intraluminal bile exposure on the mitochondrial functions of the esophagus are still unclear.

Our aim was to obtain an insight into the initial processes leading to tissue injury during GERD. We hypothesized that an esophageal reflux may be directly associated with a mitochondrial dysfunction and secondary functional and structural changes in the esophageal mucosa. Accordingly, the consequences of exposure to bile with or without gastric acid, as possible luminal damaging agents, were characterized separately in a large animal model of acute GERD.

Material and Methods

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was

approved by the Ethics Committee for the Protection of Animals in Scientific Research of the University of Szeged.

Surgical preparation

Four separate groups of experiments were performed on a total of 31 mongrel dogs (average weight 11.4 ± 2 kg) under sodium pentobarbital anesthesia (30 mg kg^{-1} i.v.). Small supplementary doses of pentobarbital were administered when necessary. The left femoral artery and vein were cannulated for the recording of mean arterial pressure and for blood sampling, respectively. The animals were placed in a supine position on a heating pad for the maintenance of body temperature between 36 °C and 37 °C, and received an infusion of Ringer's lactate at a rate of 10 ml^{-1} kg^{-1} h during the experiments.

The esophageal lumen was inspected by endoscopy (Olympus GIF-E) prior to the experiments. Following a collar incision, the cervical esophagus with intact neurovascular connections was dissected free, and an approximately 8–10-cm segment of the middle portion was then occluded at both ends with atraumatic clips. An agar electrode was placed surgically into the esophagus lumen for continuous measurement of the transmucosal potential difference. A second polyvinyl tube (0.5 mm i.d.) was secured with purse-string suture for the administration of test compounds.

Experimental protocol

Surgery was followed by a 20-min recovery period for cardiovascular stabilization, and 7 ml of isotonic saline (pH 7.4) was injected into the lumen of the dissected esophagus for 30 min in order to determine the baseline variables. The esophagus segment was next filled with test solution (7 ml) for 4 h. At the end of the experiment, a biopsy was taken from the esophageal segment, along with a tissue sample from the aboral, intact part of the esophagus, with the freeze-clamp technique, for determination of the tissue adenosine triphosphate (ATP) concentrations, and additional biopsies were obtained for measurement of the tissue myeloperoxidase activity and the vascular permeability index, and establishment of the severity of tissue damage.

The animals were randomized to one or other of the following groups: group 1 ($n = 8$) – saline-treated (pH 7.4) control; group 2 ($n = 8$) – bile-treated (pH 6.5); group 3 ($n = 5$) – hydrochloric acid (HCl) + bile-treated (pH 2.5); and group 4 ($n = 5$) – HCl-treated (pH 2.0). Canine bile was obtained from three healthy dogs before the experiments, pooled and stored at −20 °C. The intraluminal volume load was identical in all groups studied, and all animals received a continuous infusion of Ringer's lactate at a rate of 10 ml kg^{-1} h^{-1} during the experiments.

Measurements

Central venous pressure and mean arterial pressure were measured continuously with Statham P23Db transducers. Arterial blood gases were measured with an AVL Compact 2 blood gas analyzer.

Transmucosal potential difference

An exploring electrode was inserted into the lumen of the esophagus, and the reference electrode was placed into the paraesophageal space. The electrodes were filled with 3% agar solution dissolved in saturated potassium chloride solution. The agar bridges were connected to a calomel electrode (Radelkis, Budapest, Hungary) and a potentiometer, and the transmucosal potential difference changes (in mV) were recorded continuously on a polygraph.

Epithelial permeability index

One-hundred-and-eighty minutes after the insult, lumen-to-blood directional epithelial permeability changes were detected by the Na-fluorescein (NaFl) clearance method (6). Briefly, 40 mg of NaFl (MW 376, Sigma Chemicals) (5 mg ml^{-1}) was added to the esophageal lumen. Blood samples were taken from the femoral vein in 10-min periods, and the NaFl concentration of the plasma was determined. The blood samples (2 ml) were collected in prechilled tubes containing 250 IU heparin, and immediately centrifuged at 1000*g* at 4 °C for 5 min. The samples were stored at 0 °C in the dark for a maximum of 120 min. NaFl concentrations were measured with an F-2000 Hitachi fluorescence spectrophotometer (ex: 455 nm, em: 515 nm). The lumen-to-plasma clearance of NaFl was calculated according to the following equation: inward NaFl clearance = [NaFl concentration]serum × 100/ [NaFl concentration]test solution × volume.

Vascular permeability index

The vascular permeability index was determined using the azo dye Evans blue (Sigma Chemicals), which binds rapidly to albumin and migrates with it (7). At 210 min in the experiments, 20 mg Evans blue ml^{-1} kg^{-1} was given i.v. in a bolus injection, and 30 min later a blood sample was taken from the femoral vein, together with tissue samples from the intact and exposed sections of the esophagus. The mucosal layer was scraped off, rapidly placed in 5 ml of formamide and homogenized for 1 min in a glass Potter homogenizer. The homogenate was incubated at room temperature for 20 h and then centrifuged at 2500*g* for 30 min. The absorbance of the supernatant was determined at 650 nm against a formamide blank with a UV-1601 spectrophotometer (Shimadzu, Japan). The concentration of Evans blue was determined from a standard curve. The protein contents of the samples were determined by the procedure of Lowry et al. (8). Similarly, blood samples were centrifuged at 600*g* at 4 °C for 10 min and the absorbance of the 100-fold-diluted plasma was measured. Vascular permeability index was defined as the ratio of the tissue and plasma concentrations of Evans blue: Vascular permeability index = [Evans blue concentration]tissue/[Evans blue concentration]plasma < 100.

Myeloperoxidase activity

The mucosal tissue myeloperoxidase activity, as a marker of tissue leukocyte infiltration, was measured from mucosal biopsies by the method of Kuebler et al. (9). Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 2000*g*. The myeloperoxidase activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan), and the data were referred to the protein content.

ATP measurement

A whole-thickness sample was taken from the esophagus with a Wollenberg forceps cooled in liquid nitrogen, and the tissue was stored at −70 °C. The sample was weighed, placed in a 3-fold volume of trichloroacetic acid (6% w/v), homogenized for 1 min and centrifuged at 5000*g*. The supernatant was neutralized with saturated potassium carbonate solution. The ATP concentration was measured spectrophotometrically according to Lamprecht et al. (10). The method is based on the principle that beta-nicotinamide adenine dinucleotide phosphate is used up in an enzymatic reaction catalyzed by glucose-6-phosphate dehydrogenase and hexokinase.

Histology and light microscopy

Biopsies for light microscopy were obtained from the intact and treated parts of the esophagus of each animal. The samples were fixed in 10% phosphate-buffered formalin solution for 24 h, embedded in paraffin, sectioned (6 μm) and stained with hematoxylin-eosin. Histological analysis was performed in coded sections by one investigator (L.T.). Mucosal injury was graded on the 0–100 esophageal mucosal damage score of Lanas et al. (11), with the following criteria: epithelial changes (epithelial splitting, erosion and ulceration): maximal score 40; inflammation (intraepithelial leukocytes and cellular hyperplasia): maximal score 40; vascular lesions (edema, congestion and hemorrhage): maximal score 20.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Non-parametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures, median values and 75th and 25th percentiles are given. P values <0.05 were considered significant.

Results

The baseline values of the macrohemodynamic variables did not differ significantly in the different groups and there were no significant hemodynamic changes compared with the baseline values during the experimental period (data not shown). In the control group, saline administration did not significantly influence the mucosal morphology as compared with that observed in the untreated part of the esophagus. In this group, the baseline mucosal permeability, the transmucosal potential difference, the ATP level and the intramucosal leukocyte accumulation remained essentially constant throughout the observation period.

Intraluminal bile resulted in an approximately 60% decrease in transmucosal potential difference (M = 29.5%; 25p = 18.8%; 75p = 31.9%), and in significant increases in the vascular permeability index (M = 0.953; 25p = 0.84; 75p = 1.05) and transmucosal NaFl clearance (M = 4.54; 25p = 4.1; 75p = 5.01) (Figs 1–3).

Similar changes were observed in the bile + HCl-treated group: transmucosal potential difference was significantly decreased, with parallel increases in vascular permeability index and NaFl clearance (Figs 1–3).

HCl alone induced a decrease in transmucosal potential difference and an increase in the lumen-to-blood direction mucosal permeability, similar to those observed following bile and bile + HCl administration. However, the change in vascular permeability index remained statistically non-significant throughout the experiments (Figs 1–3).

The leukocyte accumulation was significantly increased in the mucosa in groups 2, 3 and 4 as compared with the intact part of the esophagus, or with the saline-treated group 1.

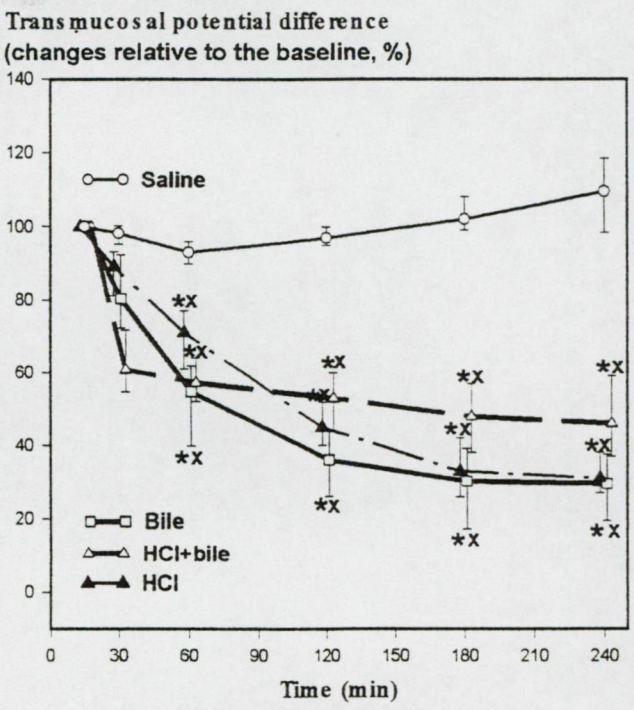

Fig. 1. Changes in transmucosal potential difference between intra- and extraluminal esophageal spaces. Thin line with empty circles: saline-treated control, thick line with empty squares: bile-treated group, broken line with empty triangles: HCl + bile-treated group, broken line with filled triangles: HCl-treated group. * $P < 0.05$ versus baseline, × $P < 0.05$ versus saline-treated control group.

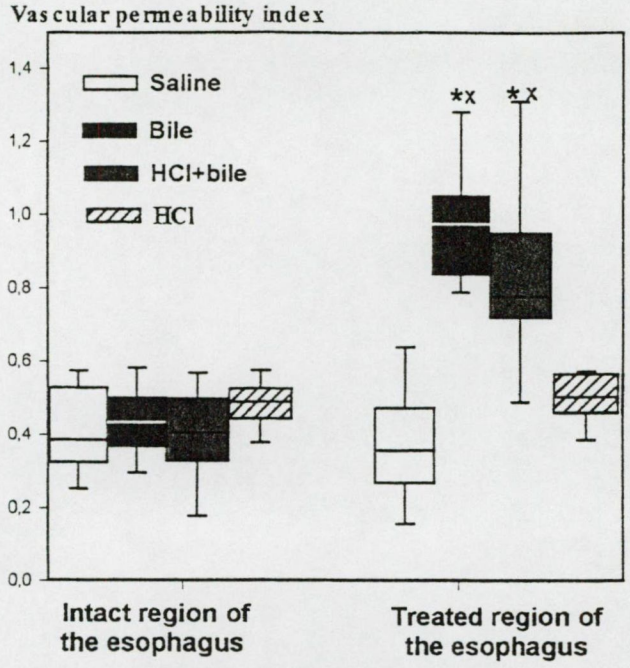

Fig. 2. Vascular permeability index in the intact and treated regions of the esophagus at 240 min in the experiments. Saline-treated control group = white box; bile-treated group = black box, HCl-bile-treated group = gray box; HCl-treated group = hatched box. * $P < 0.05$ versus baseline, × $P < 0.05$ versus saline-treated group.

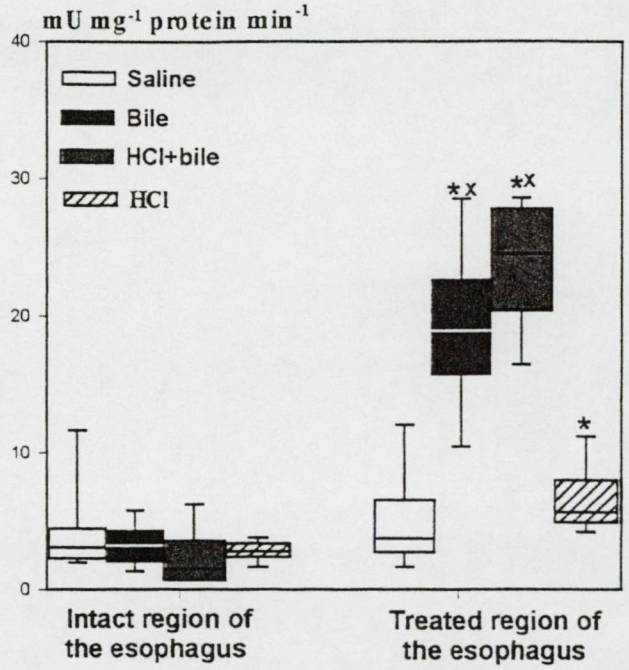

Fig. 4. Myeloperoxidase activity in the intact and treated parts of the mucosa of the esophagus at 240 min in the experiment. Saline-treated control group = white box; bile-treated group = black box; HCl + bile-treated group = gray box; HCl-treated group = hatched box. * $P < 0.05$ versus baseline, × $P < 0.05$ versus saline-treated control group.

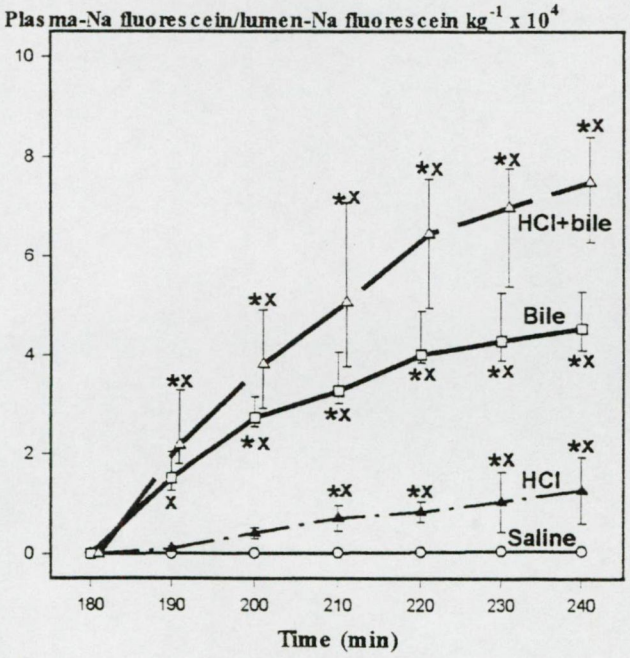

Fig. 3. Changes in epithelial permeability index (Na-fluorescein clearance) during the last 60 min of the experiments (between 180 and 240 min). Saline-treated control group = thin line with empty circles; bile-treated group = thick line with empty squares; HCl + bile-treated group = broken line with empty triangles; HCl-treated group = broken line with filled triangles. * $P < 0.05$ versus baseline, × $P < 0.05$ versus saline-treated group.

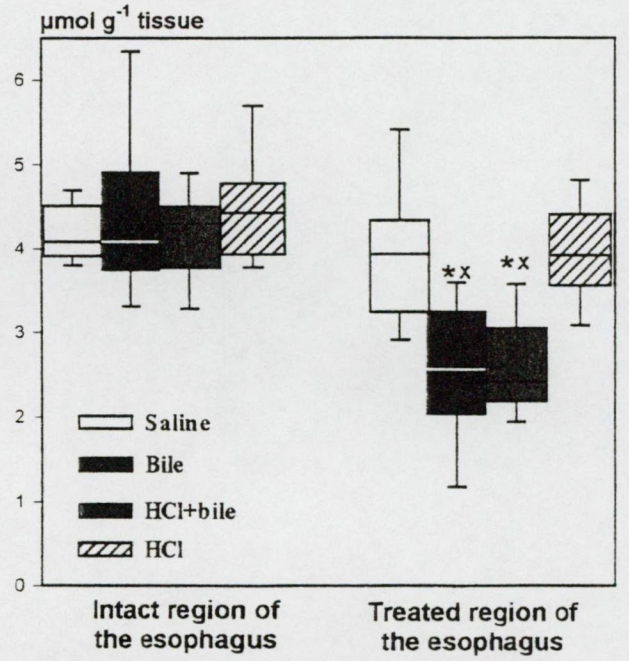

Fig. 5. Tissue ATP contents in the intact and treated parts of the esophagus at the end of the experiments. Saline-treated control group = white box; bile-treated group = black box; HCl + bile-treated group = gray box; HCl-treated group = hatched box. * $P < 0.05$ versus baseline, × $P < 0.05$ versus saline-treated control group.

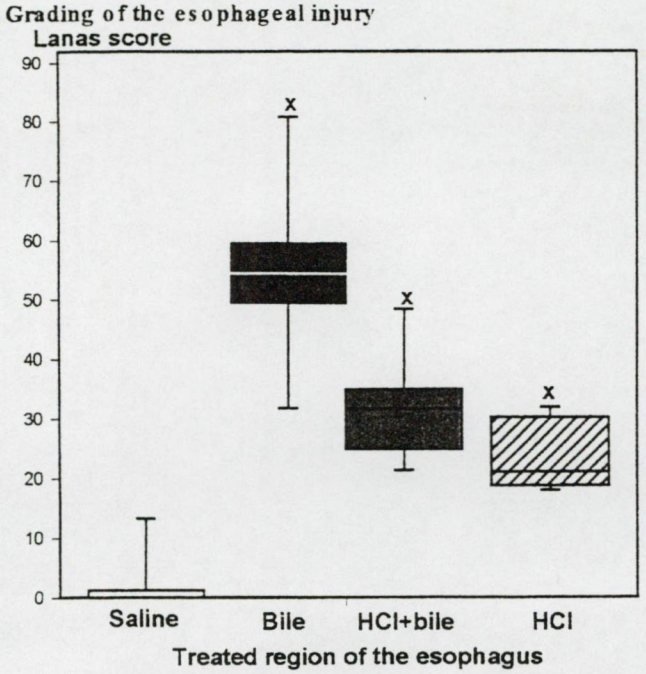

Fig. 6. Scores of histological evaluation of mucosal damage of the esophagus, with grading performed according to Lanas et al. (see Methods for description). Saline-treated control group = white box; bile-treated group = black box; HCl + bile-treated group = gray box; HCl-treated group = hatched box. $^{\times} P < 0.05$ versus saline-treated control group.

Bile, bile + HCL, or HCl administration alone resulted in a 6-fold (M = 18.1; 25p = 15.7; 75p = 22.6), an 8-fold (M = 24.6; 25p = 20.4; 75p = 27.8) and a 2.5-fold (M = 6.65; 25p = 4.9; 75p = 8,05) increase in myeloperoxidase activity, respectively (Fig. 4).

Data on the esophageal ATP content are presented in Fig. 5. There were no significant differences in tissue ATP levels between the intact and treated parts of the esophagus in the saline-treated control group. Intraluminal bile and bile + HCl resulted in an approximately 40% decrease in esophageal tissue ATP level (bile: M = 2.66; 25p = 2.04; 75p = 3.25; bile-HCl: M = 2.42; 25p = 2.19; 75p = 3.05) by the end of the observation period. In these groups, the ATP levels were significantly reduced compared with the saline-treated group. Administration of HCl alone did not influence the esophageal ATP content (Fig. 5).

Tissue samples taken from the intact and saline-treated parts of the esophagus of the control group exhibited an average grade of injury of 2.57 (range of scores 0–18) (Fig. 6). Control samples from the bile, bile + HCl and HCl-treated groups revealed the structure of the normal esophageal mucosa. In these sections, the luminal surface was lined by a continuous layer of epithelial cells.

Semiquantitative evaluation of samples from bile-treated animals revealed a significant exacerbation of the mucosal injury in all cases ($P < 0.01$) and an injury score of 62.61 (range 42–90). Moderate and severe lesions were commonly observed, with epithelial desquamation and necrosis, intra-epithelial and subepithelial leukocytosis, basal cell hyperplasia and subepithelial connective tissue damage (Fig. 7b).

Less severe lesions were observed following bile + HCl treatment (Fig. 7c). In this group, mild and moderate lesions were usually seen within the same section. Disruption of the epithelial layer, desquamation, minor necrosis, intraepithelial leukocytosis, edema of the subepithelial connective tissue and subepithelial leukocytosis were generally observed, but the deeper tissue layers were less strongly involved. The injury score was 38.66 (range 21–70) (Fig. 6).

In HCl-treated group 4, the reactive epithelial changes were different from those observed in the bile alone or bile + HCl-treated groups (Fig. 7d). The injury was apparently less severe in this environment (average score 29.7, range 18–42; Fig. 6). The subepithelial inflammation was milder, and petechiae were rarely observed. In general, there was no evidence of deep submucosal damage, though transmural lesions could occasionally be observed.

Discussion

The importance of duodenal components in the progression of GERD is well known. In a number of cases, the bile content of the regurgitated fluid has been demonstrated by a fiberoptic probe (Bilitec) in the esophagus, and it has been confirmed that a mixed reflux is more harmful than gastric juice alone (12, 13). Similarly, it is assumed that bile can play a decisive role in the development of Barrett's metaplasia (14). However, the pathomechanism of reflux-induced mucosal injury is still unclear and the significance of the individual components is controversial.

Our in vivo study has provided a comparison of the separate responses to HCl and bile in the canine esophagus. The results reveal that intraluminal bile may be involved in the mechanism of esophageal ATP reduction and mucosal impairment barrier simultaneously. Bile and HCl mixed together decreased the ATP content of the exposed tissues, increased the epithelial and vascular permeability indices, evoked a rise in leukocyte accumulation, and induced severe structural alterations in the esophageal mucosa. The reduction in the mucosal ATP level was attributed exclusively to bile.

Bile acid toxicity has been repeatedly demonstrated in the hepatic tissues. It has recently been confirmed that the intrahepatic accumulation of toxic bile salts is directly connected with a mitochondrial dysfunction (15, 16). Similarly, it has been shown that bile salts cause hepatocyte death by inducing mitochondrial permeability transition, Fas-dependent hepatocyte apoptosis or necrosis. Bile salts at low concentrations inhibit the activities of complexes I and III of the mitochondrial respiratory chain (17). Although the disturbance of mitochondrial energy production could be an important factor in the pathomechanism of acute damage of the canine esophagus, the sequence of events is still unclear. Bile-induced ATP depletion may be an important component of the esophageal damage, but the different sensitivities of the

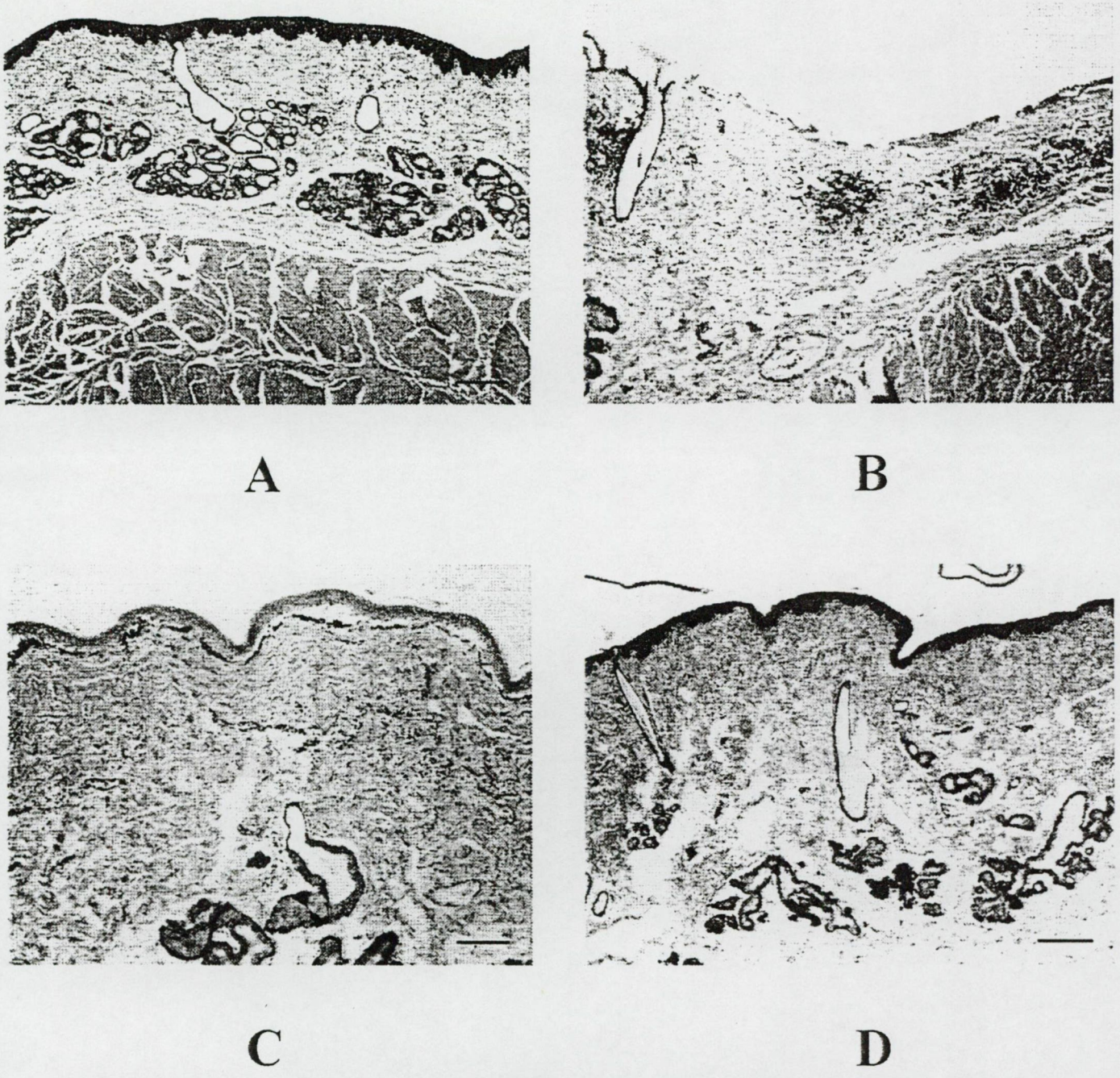

Fig. 7. A. Histological picture of normal esophagus in saline-treated group (group 1). The epithelium is essentially of normal thickness. The submucosa, the esophageal glands and the muscularis layer show no significant pathological lesions (H & E staining, original magnification 112×). B. Severe damage in group 2 (bile-treated). Extensive epithelial loss (erosion) with transmural inflammation, edema and vasodilatation (H & E, original magnification 112×). C. Intra- and subepithelial inflammatory cells in group 3 (HCl + bile treated). The inflammatory cells in the submucosa are mainly around the esophageal gland ducts (H & E, original magnification 112×). D. Mainly epithelial damage displayed in group 4 (HCl-treated). The basal cell hyperplasia is considerable, and the subepithelial lesions are negligible (H & E, original magnification 112×).

esophageal and intestinal mucosal surfaces toward bile remains an interesting problem. Moreover, non-specific detergent effects of bile, such as bile-induced oxidative damage, have to be considered.

At first sight, a biliary reflux alone cannot evolve in GERD patients. However, HCl secretion can be completely blocked by novel proton-pump inhibitor therapy. With long-term continuous proton-pump inhibitor therapy, HCl suppression results in a pH >5 and an 'iatrogenic bile reflux' may occur if a duodenogastro-esophageal reflux is also present. Further, achlorhydria or gastric acid suppressive therapy might lead to bacterial colonization in the upper gastrointestinal tract (18). As a result of the bacterial flora, deconjugated bile acids appear in a higher proportion, inducing severe mucosal damage in the esophagus (19). Thus, in patients on proton-pump therapy with concomitant bile regurgitation, a 'bile danger zone' of higher pH (pH >5) has to be considered.

The link between gastric pH and esophageal injury has

been the source of controversy. Previous studies have demonstrated a synergistic effect of pepsin and acid in mucosal damage at low pH; and these results have led the authors to suggest that gastric acid exerts an indirect effect on esophageal damage by determining peptic activity (20). However, it has recently been shown that the presence of pepsin did not exacerbate the severity of acid-induced esophageal injury (21). Similarly, it is important to note that the solubility of bile salts may be dependent on luminal pH (22). Although the importance of this phenomenon remains to be investigated, it seems that in our study the degree of bile-induced mucosal injury was not influenced by pH.

In line with these data, there is good evidence that a 4 h bile exposure results in a process which significantly affects the permeability characteristics of the canine esophagus. The falling level of tissue ATP described here was accompanied by an increased mucosal permeability in the blood-to-lumen and lumen-to-blood directions, and by a significant decrease in transmucosal potential difference. The transmucosal potential difference maintained across the healthy mucosa is thought to be due to asymmetric ion pumping combined with resistance to the back-diffusion of the separated charge (23). A decreased transmucosal potential difference is indicative of a loss of mucosal integrity and also points to a disturbed mitochondrial energy turnover (24). The degree of impairment of the protective mucosal barrier may range from reversible permeability changes to complete transmural necrosis. The extent of permeation across the mucosal barrier depends on the solubility and size of the molecule and on the blood flow. Equilibration of small molecules such as NaFl requires only a short period, and subtle structural changes might therefore result in rapid permeation changes in the mucosa. Although the overall results of our study suggest that the permeability alterations in the esophageal mucosa may be dependent on bile-induced ATP depletion, this possibility should be treated with caution. It is clear that the early mucosal responses to luminal damaging agents involve a multiplicity of factors, and the mechanism of chronic bile salt-induced mucosal dysfunction remains an area of active investigations. However, it is tempting to speculate that bile salts may play similar triggering roles in GERD after prolonged or intermittent bile exposure.

In our model, exposure to either HCl or bile was accompanied by a significant rise in tissue myeloperoxidase activity. The contact of the neutrophil leukocyte with a chemoattractant triggers a complex metabolic event, with the production of oxygen intermediates. Once the leukocytes have entered the tissues, they might induce further endothelial and epithelial damage and interfere with the aggressive action of intraluminal agents. Although these data suggest that reflux-induced leukocyte activation and tissue accumulation might be secondary components of the pathomechanism of mucosal destruction, more direct approaches are needed to identify the roles of bile and HCl precisely in the microvascular changes and leukocyte reactions. In this regard, our results emphasize the importance of monitoring functional parameters of esophageal injuries in order to obtain valid conclusions from studies involving tissue salvage therapies.

It has been found that the intraluminal administration of HCl induces increases in esophageal permeability and myeloperoxidase activity with the same efficacy as observed for HCl mixed with bile or bile alone. Similarly, the evolved injury of the esophageal mucosa could be directly connected to the 'topical' effect of HCl. On the other hand, exogenous HCl had no effect on the mucosal ATP content. It is therefore conceivable that a synergy exists between the two components, and biliary reflux may play a key role in the HCl-induced loss in mucosal integrity or significantly exacerbate the manifestation of structural mucosal damage.

It appears that there is a need for adequate medical therapy in GERD patients. During a long-term follow-up, a pathologic reflux in the esophagus was detected more than 10 years after effective anti-reflux procedures (25, 26). Several studies have described esophagitis or the aggravation of esophageal inflammation after 'effective' medical or surgical therapy. Similarly, it has been demonstrated that the grade of Barrett's metaplasia can progress, or an even more malignant transformation can occur after seemingly appropriate surgical therapy (27–29). It has recently been reported that a 'duodenal switch' or biliary diversion gives good results when surgery is indicated (30–32). However, our study introduces the possibility that inhibitors of mitochondrial permeability transition may also be beneficial for the treatment of GERD.

In conclusion, we have demonstrated that bile induces ATP depletion, contributes to the early mucosal permeability alterations and barrier lesions during experimental reflux, and plays an important role in intramucosal leukocyte accumulation and structural injury. Our data suggest that severe bile-induced tissue injury may evolve when the pH of the refluxed material is 6 or above. Monitoring of the biliary components of the regurgitated fluid and determination of the rate of bile reflux episodes is therefore warranted before a decision on therapy is made. With regard to the deleterious consequences of biliary exposure, bile-targeted diagnostic and treatment regimens could have appreciable therapeutic effects in GERD patients.

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A nyelőcső nyálkahártya mikrokeringési változásai akut kísérletes reflux alatt – Az epés komponens kóroki szerepe

Changes in microcirculation of mucosa during experimental esophageal reflux. The role of biliary component

Vizsgálataink célja a nyelőcső nyálkahártya mikrokeringésének megfigyelése volt in vivo körülmények között kutyákon, kísérletes reflux oesophagitis alatt. Összehasonlítottuk a 3 órán át fenntartott savas, kevert (savas és epés), valamint epés reflux következményeit, és meghatároztuk a konstitutív és induktív nitrogén monoxid szintáz (cNOS és iNOS) enzim aktivitásában bekövetkező változásokat.

Módszerek: A nyaki nyelőcső mikrokeringését intravitális videomikroszkóppal vizsgáltuk, meghatároztuk a funkcionális kapilláris denzitást (FCD), a relatív perfundált érterületet (RVA) és a vörösvértestek áramlási sebességét (RBCV). A mucosa barrier integritását a vascularis és epitheliális permeabilitási index alapján vizsgáltuk. Szövetmintákból meghatároztuk a leukocyta akkumulációt (myeloperoxidáz aktivitást), a cNOS és iNOS aktivitását és a szöveti károsodás mértékét.

Eredmények: Az RBCV és az RVA minden kezelt csoportban szignifikánsan nőtt a kontroll csoporthoz képest, az FCD csak a savval kezelt csoportban csökkent. Minden kezelt csoportban jelentősen fokozódott az epitheliális permeabilitás, az MPO és az iNOS aktivitás. A cNOS aktivitás az epével kezelt csoportban szignifikánsan csökkent, a sav és a kevert kezelés a cNOS aktivitását nem befolyásolta. Az epe súlyosabb laesiót okozott, a savas és kevert kezelés esetén a károsodás mértéke enyhébb volt.

Következtetés: Kísérletes modellünk segítségével igazoltuk az epés reflux nyálkahártya mikrokeringést károsító hatását. Eredményeink arra utalnak, hogy a nyelőcső nyálkahártya károsodásban és leukocyta invázióban szerepet játszik az epe hatására kialakuló cNOS gátlás.

Kulcsszavak: mikrokeringés, nyelőcső nyálkahártya károsodás, epe, nitrogén monoxid

Our aims were to examine microcirculation during experimental reflux esophagitis in dogs. We compared the effects of microcirculation of the mucosa to 3-hr exposure with acid, mixed acid and bile, we measured the changes in constitutive and inducible nitric oxide synthase activity (cNOS and iNOS).

Methods: The microcirculation of the upper esophagus was investigated by intravital videomicroscopy. The functional capillary density (FCD), relative vessel area (RVA) and red blood cell velocity (RBCV) were measured. Mucosal barrier integrity was examined by vascular and epithelial permeability indices. Myeloperoxidase (MPO) enzyme activity, cNOS, iNOS activities and microscopic damage were examined in biopsies.

Results: The vascular permeability index, the RBCV and the RVA increased significantly in the treated groups, the FCD significantly decreased after acid exposure. The MPO and iNOS activities were significantly elevated in all treated groups. The cNOS activity did not change after exposure to acid + bile or acid, but significantly decreased after sole bile treatment. Severe mucosal damage was observed after bile exposure.

Conclusion: Bile induced characteristic microcirculatory changes during experimental reflux esophagitis. Tissue damage and leukocyte infiltration could be exacerbated by bile-induced cNOS inhibition.

Keywords: microcirculation, esophageal mucosal damage, bile, nitric oxide

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Bevezetés

A nyelőcső leggyakoribb gyulladásos megbetegedése a reflux oesophagitis: hosszabb rövidebb reflux epizód az átlagos populáció 20–30%-ában jelentkezik [1]. A gastro-oesophagealis reflux (GERD) betegség leggyakoribb oka az alsó nyelőcső sphincter (LES) inkompetenciája, a csökkent nyelőcső clearance, gyengült nyelőcső motilitás, hiatus hernia, valamint a késleltetett gyomorürülés lehet. Az eredeti elképzelések szerint a pathomechanizmusban a savas komponens játssza a főszerepet, s ennek megfelelően a „savcsökkentő" gyógyszerek jelentik a konzervatív terápia egyik legfontosabb eszközét. A későbbi vizsgálatok ugyanakkor azt igazolták, hogy a III.–IV. stádiumú oesophagitisek esetében a savszuppresszió mintegy 40–50%-ban hatástalan. Feltételezhető tehát, hogy a gyomorsav mellett egyéb tényezők is károsítják a nyelőcső nyálkahártyát. Emellett szól az is, hogy atrophiás gastritis, anaemia perniciosa – sőt gastrectomia után is írtak le oesophagitist [2]. Az utóbbi évtizedek kísérletes munkái, nyelőcső pH-metriás, aspirációs, Bilitec vizsgálatok után az alkáli-, illetve epés reflux mint külön entitás is megjelent [3]. Klinikai vizsgálatok igazolták, hogy GERD alatt fellépő epés reflux esetén az oesophagitis foka súlyosabb, és a szövődmények megjelenése (nyelőcső fekély, strictura, Barrett metaplasia) is gyakoribb.

Bár a mucosa mikrokeringési reakciói jelentős szerepet játszanak a gasztrointesztinális traktus gyulladásos folyamatainak kialakulásában és progressziójában, a reflux epizód alatt *in vivo* körülmények között bekövetkező mikrocirkulációs változások nem ismertek. Jelen vizsgálataink fő célja a nyelőcső mikrokeringési változásainak megfigyelése volt kísérletes reflux oesophagitis alatt. Modellünkben jellemeztük az epe hatására kialakuló mikrokeringési és morfológiai elváltozásokat és ezeket összevetettük a sav által okozott funkcionális és strukturális károsodással. Emellett megvizsgáltuk a nyelőcső nyálkahártya vérátáramlás szabályozásában központi szerepet játszó nitrogén monoxid (NO) szintézisért felelős konstitutív és az indukálható NO szintáz (cNOS és iNOS) enzim aktivitás változásait is [4, 5]. Eredményeink arra utalnak, hogy az epés reflux hatására kialakuló cNOS gátlás meghatározó módon hozzájárulhat a nyálkahártya mikrokeringési zavarához kísérletes GERD alatt.

Anyag és módszer

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Kísérleteinket 30 keverék kutyán végeztük (átlagos testsúly 12,5±2 kg) Na-pentobarbital anaesthesiában (30 mg kg^{-1} iv.). A bal femoralis vénát és artériát kipreparáltuk és kanüláltuk a folyamatos artériás vérnyomás, illetve a laboratóriumi vizsgálatok és a parenterális folyadékpótlás céljából. A kísérletek alatt az állatok folyamatosan 10 ml $tskg^{-1}$ h^{-1} Ringer-laktát infúziót kaptak.

A fej jobbra fordítása mellett a m. sternocleidomastoideus elülső élének megfelelően hosszanti-ferde metszést ejtettünk. Ebből a behatolásból tártuk fel a nyaki nyelőcsövet, melyen egy kb. 8–10 cm-es szakaszt kirekesztettünk, s így egy ép neurovascularis ellátással bíró, lezárt nyelőcső szakaszt nyertünk. A lumenbe egy A-s jelű kanült rögzítettünk dohányzacskó öltéssel a teszt folyadékok bevitele céljából. A kirekesztett nyelőcső szakasz felett a nyaki bőrt néhány szituáló öltéssel zártuk.

Vizsgálati állatainkat véletlenszerűen 4 csoportba osztottuk. Az 1. csoport (n = 8) fiziológiás sóoldatot (pH 7,4), a 2. csoport (n = 8) epét (pH 6,5), a 3. csoport (n = 7) sósav (HCl) és epe keverékét (pH 2,5), a 4. csoport (n = 7) HCl oldatot (pH 2,0; 0,01 nHCl) kapott. A 2. és 3. csoport kezeléséhez három intakt kutyából az epehólyag nyitott punkciója során nyert (felhasználásig –20 °C-on tárolt) epét használtunk fel.

A kísérletek menete

A sebészi beavatkozást követően 20 percig (a cardiovascularis állapot stabilizációjáig) további beavatkozást nem végzünk. Ezt követően a zárt nyelőcső szakaszt kiöblítettük, majd 30 percig feltöltöttük fiziológiás sóoldattal, s eközben az alapállapotot jellemző mérési adatokat rögzítettük. Ezt követően a nyelőcsövet a csoportnak megfelelő teszt folyadékkal (7 ml össz-volumenben) feltöltöttük, 3 óra időtartamig. A kezelés végén a nyelőcső kezelt és intakt szegmenséből is szöveti mintavétel történt. Az artériás középnyomást és a centrális vénás nyomást Statham P23Db transducerrrel mértük. Az artériás vérgázt óránként AVL Compact 2 vérgáz analizátorral detektáltuk.

Vasculáris Permeábilitási Index (VPI)

A VPI-t az albuminhoz gyorsan kapcsolódó és áramló Evans kék (Sigma Chemicals) festékkel állapítottuk meg [6]. A kísérlet 150. percében 20 mg ml^{-1} kg^{-1} Evans kék oldatot adtunk bolusban iv., majd 30 perccel később (a kísérlet végén) vénás vérmintát vettünk (v. femoralis kanülön), és szövetmintát vettünk a kezelt nyelőcső szakaszból. A nyálkahártya réteget az izomról lepreparáltuk, azonnal 5 ml formamidba helyeztük, majd homogenizáltuk. Az így nyert szuszpenziót inkubáltuk, majd 30 percig centrifugáltuk. A felülúszó abszorpcióját, 650 nm-en, formamid vakkal szemben határoztuk meg UV-1601 spektrofotométerrel (Shimadzu, Japán). A vérmintákat 10 percig centrifugáltuk, majd a 100-szorosra higított plazma abszorpcióját mértük. A VPI-t az Evans kék szöveti és plazma koncentrációjának arányában határoztuk meg.

Epitheliális Permeábilitási Index (EPI)

A kísérlet 120. perce után megmértük a „lumen – plazma" irányú EPI változásait Na-fluoreszcein (NaFL) clearence módszer segítségével [7]. Röviden: a kísérlet 120. percében 40 mg NaFL-t (Mw 376, Sigma Chemicals, 5 mg ml^{-1}) adtunk be a kirekesztett nyelőcső lumenébe. Ezt követően 10 percenként vénás vérmintákat vettünk 250 NE heparint tartalmazó csövekbe, majd 5 percig centrifugáltuk. A plazmát maximum 120 percig 0 °C-on sötétben tároltuk, majd a NaFL koncentrációt spektrofotométerrel (F-2000 Hitachi, Japan, ex: 455 nm, em: 515 nm) megmértük. A „lumen – plazma" NaFl clearence számítását a plazma NaFL koncentráció – lumen NaFL koncentráció arányában számítottuk ki.

Myeloperoxidáz (MPO) enzimaktivitás mérés

Az MPO enzim aktivitást Kuebler és munkatársai metodikája alapján határoztuk meg [8]. A szövetmintát pH 7,40-es Trisz-HCl pufferrel homogenizáltuk, majd centrifugáltuk. A kapott felülúszót pH 6,0-os 0,05 M-os K-foszfát + 0,5%-os hexadecil-trietil-ammonium-bromid pufferrel feltártuk. A feltárt anyag felülúszójának MPO tartalma a hozzáadott hidrogén-peroxidból hidroxil gyököt szabadít fel, amely a tetrametil-benzidinnel reagálva 450 nm-nél fotometrálható színreakciót ad. Az MPO enzimaktivitást az ugyanabból a mintából mért fehérjére vonatkoztatva adtuk (sec. Lowry).

Mikrokeringési vizsgálatok

A nyelőcső nyálkahártya mikrocirkulációjának változásait orthogonális polarizációs spektrális (OPS) technika segítségével intravitális video-mikroszkóp alkalmazásával (Cytoscan A/R, Cytometrics Co, PA,

USA) folyamatosan monitoroztuk. Ez a módszer alkalmas minden haemoglobin tartalmú anyag vizualizálásához, festékek alkalmazása nélkül [9]. A mikrocirkulációs paraméterek kvantitatív mérése *off-line* módon „*frame-to-frame*" analízissel történt a rögzített felvételek alapján. A funkcionális kapilláris sűrűséget (FCD; a perfundált kapilláris hosszának és a vizsgált terület aránya $\mu m/\mu m^2$), relatív érterületet (RVA: a perfundált érterület és a vizsgált terület aránya), és a vörösvértest áramlási sebességet (RBCV; μm/sec) vizsgáltuk egy adott időben 3 különböző területen, számítógépes képanalízissel (IVM Pictron, Budapest).

Szöveti NOS aktivitás meghatározása

A szöveti NOS enzim aktivitásának meghatározásához a ^{3}H-arginin-^{3}H-citrullin konverzión alapuló metodikát használtuk [10]. A szövetet pH 7,4-es Trisz-HCl pufferben homogenizáltuk, majd centrifugáltuk. A reakcióelegy a NOS működéséhez szükséges kofaktorokat, valamint a homogenizátumból 70 μl-t tartalmazott. A cNOS aktivitást 2 mM kalcium jelenlétében, az iNOS aktivitást kalcium mentesített reakcióelegyben határoztuk meg. A NOS aktivitást a mintából mért protein tartalomra vonatkoztatva adtuk meg, amelynek meghatározása Lowry és mtsai módszerével történt [11].

Szövettani vizsgálatok

A biopsziák 24 órán keresztül 10%-os pufferolt formalinban fixálódtak. Legalább 2–2 reprezentatív hosszanti kimetszést végeztünk, s ezeket rutin hisztológiai módszerekkel a kezelési csoportok ismerete nélkül dolgoztuk fel. Az elkészült metszeteken haematoxilin-eozin festést alkalmaztunk. A mikroszkópos elváltozásokat Lanas és mtsai 0–100 közötti nyelőcső károsodás „score"-ja szerint osztályoztuk [12]. A feldolgozás során az epithel elváltozásait (felrostozódás, hasadás, erosio, fekély, intraepithelialis leukocytosis, basalis sejt hyperplasia, acanthosis, parakeratosis stb.), a subepithelialis kötőszövet, az izomréteg és az adventitia károsodását (oedema, vérbőség, vérzés, gyulladás) és ezek kiterjedését és intenzitását vizsgáltuk. A mucosa károsodás mértékét a leírtak szerint összegezve adtuk meg az epithelialis károsodás, a gyulladásos jelek és vascularis eltérések alapján.

Statisztikai vizsgálat

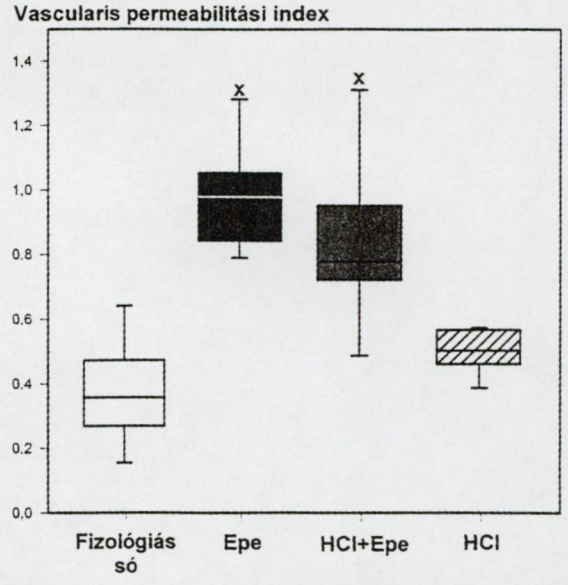
Az adatok statisztikai feldolgozásához SigmaStat for Windows (Jandel Scientific, Erkrath, Germany) software-t és nem paraméteres tesztet használtunk. A statisztikai különbségeket a csoportokon belül Friedman és Dunn próbával, a csoportok között Kruskal-Wallis és Dunn próbával vizsgáltuk. Az ábrákon a medián, a 25. és 75. percentilis értékeket tüntettük fel, $p<0,05$ esetén tekintettük szignifikánsnak az adott különbséget.

Eredmények

A kiindulási makrohemodinamikai paraméterek a kísérleti csoportokban közel azonosak voltak, és a kísérlet során nem mutattak szignifikáns változást. A fiziológiás sóoldattal kezelt kontroll csoportban egyik vizsgált paraméterben sem volt eltérés a kísérlet során, a műtéti preparálás, kezelési metodika nem okozott nyelőcső károsodást.

VPI

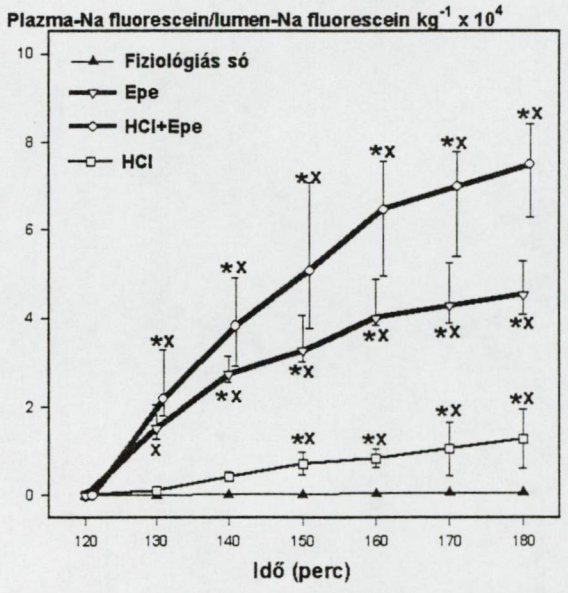
Az epés kezelés hatására szignifikánsan emelkedett a VPI (M = 0,953; 25p = 0,84; 75p = 1,05). Ehhez hasonló eredményeket találtunk a kevert (epe + savas) kezelés után (M = 0,801; 25p = 0,72; 75p = 0,945). Savas kezelés hatására nem alakult ki szignifikáns változás *(1. ábra)*.



1. ábra. A vasculáris permeábilitási index (VPI) értékének változása 180 perces kezelés után a különböző csoportokban. Üres doboz: fiziológiás sóoldattal történő kezelés; fekete: epés kezelés; szürke: sav + epe kezelés; vonalazott: a tiszta savas kezelés hatását ábrázolja. x $p<0.05$ a fiziológiás só oldattal kezelt kontroll csoporthoz képest. * $p<0,05$ kiinduló értékhez viszonyítva

EPI

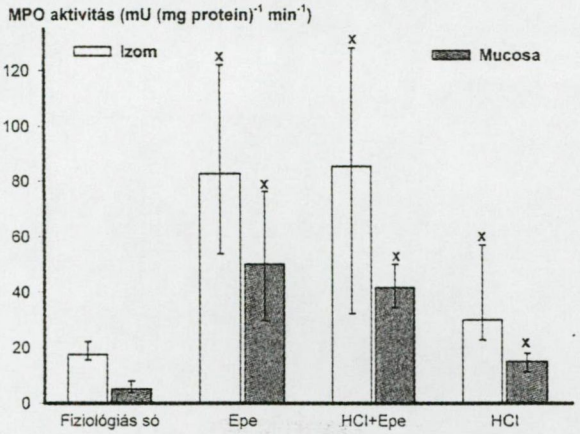
A nyelőcső lumenébe adott Na-fluoreszcein az epével és az epe + savval kezelt csoportokban már 10 perc múlva jelentős koncentrációban jelent meg a keringésben. A savas kezelés nem ilyen jelentős mértékű, de a kontroll csoporthoz és a kiindulási értékekhez képest is szignifikáns emelkedést eredményezett *(2. ábra)*.



2. ábra. Az epitheliális permeábilitási index (EPI) változása a kísérlet során. Vékony vonal fekete háromszögekkel: fiziológiás sóoldat kezelés; vastag vonal fehér háromszögekkel: epés kezelés; vastag vonal fehér körökkel: sav + epe kezelés; vékony vonal fehér négyzetekkel: savas kezelés. x $p<0.05$ a fiziológiás só oldattal kezelt kontroll csoporthoz képest; * $p<0,05$ kiinduló értékhez viszonyítva

MPO

A leukocyta akkumuláció minden kezelt csoportban szignifikánsan emelkedett a kontroll csoporthoz képest. Az MPO aktivitás epés kezelés hatására közel 10-szeresére (M = 50,0; 25p = 29,5, 75p = 76,1), savas kezelés után háromszorosára emelkedett (M = 15,0; 25p = 11,2, 75p = 17,9), epe + savas kezelés után nyolcszoros emelkedést észleltünk (M = 41,5; 25p = 34,3, 75p = 49,9). Bár a nyelőcső fal izomszövetében mért átlagos MPO értékek kissé nagyobbak voltak mint a mucosában, az MPO aktivitás a két szövetrétegben statisztikailag azonosnak mutatkozott: ötszörös emelkedés alakult ki a 2. és 4. csoportban, és kétszeres emelkedés a 3. csoportban *(3. ábra)*.



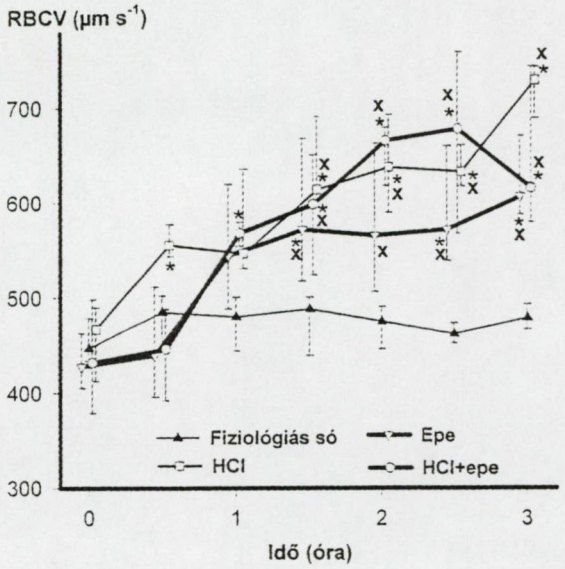
3. ábra. A nyelőcső nyálkahártya (szürke oszlop) és a nyelőcső izomréteg (fehér oszlop) myeloperoxidáz (MPO) enzimaktivitása a különböző kezelések hatására. x $p<0,05$ a fiziológiás sóoldattal kezelt kontroll csoporthoz képest

Mikrokeringési paraméterek

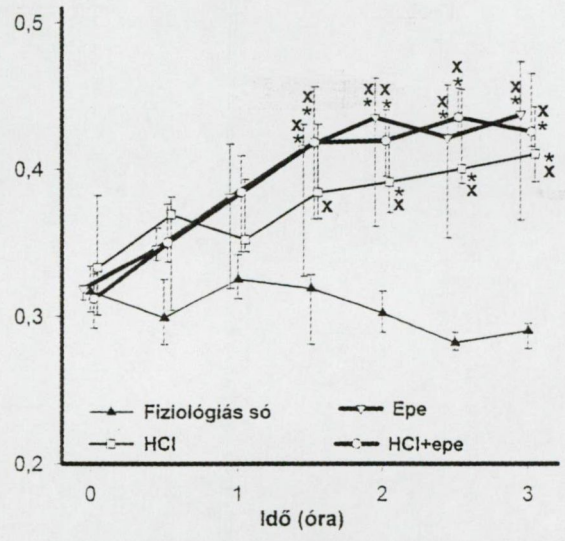
Az RBCV alapértékei mindegyik csoportban a 430–470 μm/s közötti tartományba estek. A tesztoldatok 3 órás alkalmazása után az RBCV átlagos értéke a 2. csoportban 607 μm/s; a 3. csoportban 730 μm/s, a 4-ben 620 μm/s volt, a kontroll csoportban változatlan maradt *(4. ábra)*. Az RVA értéke a 2., 3. és a 4. csoportban is szignifikánsan emelkedett a kontrollhoz képest *(5. ábra)*. Az FCD értéke a sósavval kezelt 3. csoportban szignifikánsan csökkent (0,0377 → 0,0292 μm/μm^2) míg a többi csoportban nem változott a kezelés során *(6. ábra)*.

NOS aktivitás változások

A *7. ábra* a cNOS és az iNOS aktivitás változását szemlélteti. Az epével kezelt csoportban a cNOS aktivitása szignifikánsan csökkent, míg az iNOS aktivitás ezzel egyenlő mértékben (kb. ötszörösére) emelkedett. A sósavval kezelt állatokban a cNOS aktivitás nem változott, az iNOS közel 2,5-szeresére emelkedett az álműtött csoport értékeihez képest. Epe + sósav kezelés hatására a cNOS aktivitás nem változott, míg az iNOS izoenzim aktivitása szignifikánsan emelkedett, közel ugyanolyan mértékben, mint tisztán epés kezelés hatására.



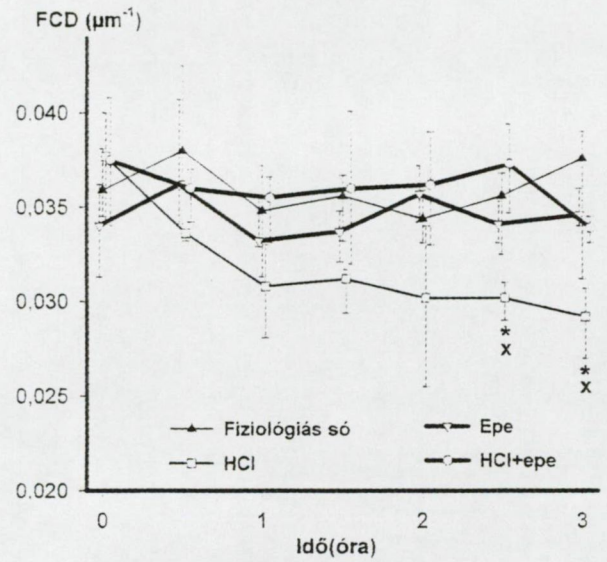
4. ábra. A nyelőcső nyálkahártya kapilláris vörösvérsejt sebesség (RBCV) alakulása a kísérlet során. Vékony vonal fekete háromszögekkel: fiziológiás sóoldat kezelés; vastag vonal fehér háromszögekkel: epés kezelés; vastag vonal fehér körökkel: sav + epe kezelés; vékony vonal fehér négyzetekkel: savas kezelés. x $p<0.05$ a fiziológiás só oldattal kezelt kontroll csoporthoz képest; * $p<0,05$ kiinduló értékhez viszonyítva



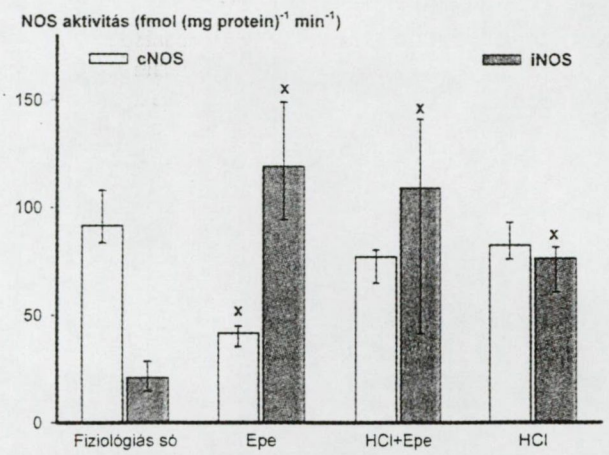
5. ábra. A nyelőcső nyálkahártya relatív perfundált terület (RVA) alakulása a kísérlet során. Fiziológiás sóoldat kezelés; vastag vonal fehér háromszögekkel: epés kezelés; vastag vonal fehér körökkel: sav + epe kezelés; vékony vonal fehér négyzetekkel: savas kezelés. x $p<0,05$ a fiziológiás só oldattal kezelt kontroll csoporthoz képest; * $p<0,05$ kiinduló értékhez viszonyítva

Szövettani eredmények

Az 1. csoportban az ép és a kezelt falrészlet között gyakorlatilag nem volt eltérés, a Lanas score 0–18 között alakult (átlag = 2,57). A 2. csoportban a kezelés hatására szignifikáns nyálkahártya sérülés jelentkezett: itt a felületi hám elvékonyodott, desquamatio, necrosis, intraepithelialis leukocytosis (IEL), subepithelialis kötőszövet fellazulása, oedema, rostok struktúra változása figyelhető

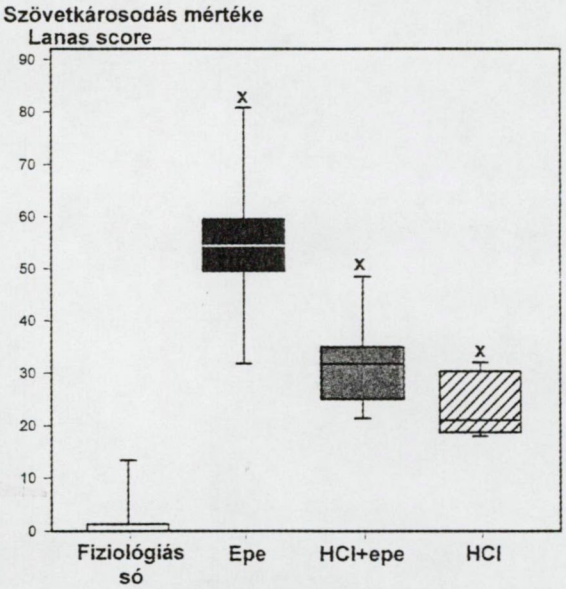


6. ábra. A nyelőcső nyálkahártya funkcionális kapilláris denzitás (FCD) változása a kísérlet során. A savas kezelés hatására a kísérlet végére szignifikáns csökkenést észlelhető. Vékony vonal fekete háromszögekkel: fiziológiás sóoldat kezelés; vastag vonal fehér háromszögekkel: epés kezelés; vastag vonal fehér körökkel: sav + epe kezelés; vékony vonal fehér négyzetekkel: savas kezelés. x $p<0,05$ a fiziológiás só oldattal kezelt kontroll csoporthoz képest; * $p<0,05$ kiinduló értékhez viszonyítva



7. ábra. A nyelőcső szövet cNOS és iNOS aktivitása a kísérletes protokoll végén. A fehér oszlop a szöveti cNOS aktivitást, míg a szürke oszlop a szöveti iNOS aktivitást mutatja a különböző kezelések után. x $p<0,05$ a fiziológiás sóoldattal kezelt kontroll csoporthoz képest

meg. Subepithelialis leukocytosis (SEL) és basal-sejtes hyperplasia (BSH) alakult ki. A nyelőcső teljes falvastagsága érintett a degeneratív folyamatokban, a Lanas score 42–90 között alakult (átlag = 62,61). A 3. csoportban az epés kezelés hatására létrejövő károsodásokhoz hasonló, de annál kevésbé súlyos nyálkahártya laesiók alakultak ki. Felületi hám elvékonyodás, desquamatio, ritkán necrosis, intraepithelialis leukocytosis, subepithelialis kötőszövet fellazulás, oedema, SEL volt látható, de a mélyebb rétegek kevésbé voltak érintettek, a Lanas score 21–70 között volt (átlag = 38,66). A 4. csoportban a reaktív epitheliális eltérések különböztek az epés, illetve a kevert csoportban leírtakhoz képest. A subepithelialis gyulladás mérsékeltebb volt, petechia ritkán alakult ki. Összességében a mély submucosális rétegek nem voltak involválva a degeneratív folyamatokban, transmurális laesio csak elvétve alakult ki savas kezelés után. Lanas score átlagosan 29,7 (18–42 között) volt *(8. ábra)*.



8. ábra. A kezelések hatására kialakuló szöveti károsodás a Lanas score szerinti feldolgozásban. A legsúlyosabb károsodást az epés kezelés okozta. Üres oszlop: fiziológiás sóoldattal történő kezelés; fekete oszlop: epés kezelés; szürke oszlop sav + epe kezelés; míg a vonalazott oszlop a tiszta savas kezelés hatását ábrázolja. x $p<0,05$ a fiziológiás sóoldattal kezelt kontroll csoporthoz képest

Megbeszélés

Az alkáli reflux nyelőcső nyálkahártya károsító hatását már korábbi klinikai és kísérletes munkák is igazolták [13–15], s eredményeink is megerősítik az epés expositio kifejezetten káros hatását. A folyamat csaknem mindig a nyelőcső teljes falát érintette, gyakran felületi necrosist hozott létre. Bár klinikai körülmények között valószínűleg nem alakul ki kizárólag epés reflux, a gyakorlatban alkalmazott tartós proton pumpa inhibitor (PPI) kezeléssel a sav szekréció csaknem teljesen kikapcsolható. Duodeno-gastro-oesophagealis reflux esetén a hosszantartó PPI kezelés és sav szuppresszió mellett „iatrogen epés refluxot" és pH 4,0 feletti tartományt alakíthatunk ki, s így ezekben a GERD betegekben epés regurgitáció esetén epés „danger zone"-nal (pH 6,0–8,0 között) is számolni kell. Emellett az is ismert, hogy a felső gasztrointesztinális traktusban achlorhydria (vagy savszuppresszió) esetén bakteriális kolonizáció alakulhat ki [16]. A megváltozott flóra következtében nagyobb arányban alakulnak ki dekonjugált epesavak, és ezek súlyosabb nyelőcső mucosa károsodást okozhatnak [17].

Korábbi vizsgálatainkkal igazolni tudtuk, hogy a biliáris expozíció a nyelőcső nyálkahártya energiaforgalom zavarát, jelentős ATP csökkenést, MPO enzimaktivitás fokozódást eredményezett. A mucosa integritás károsodását jelezte a transzmucosális potenciál különbség jelentős csökkenése is, mely szintén utal a mitochondrialis energiaforgalom zavarára [18]. Az epének a cNOS enzimaktivitásra gyakorolt hatását kísérletes epevezeték lekötés során és biliaris fibrosisban írták le [19]. Az is bebizonyosodott, hogy az alacsony cNOS és magas iNOS aktivitás felelős endotoxemia során a gyomorfal károsodás súlyosbodásáért [20]. Figyelemre méltó az is, hogy több szerző a GERD-ben jelentkező krónikus gyulladást tartja a carcinogenesis lehetséges kiindulási pontjának, míg mások a mitogen hatású epesavak glicin és taurin amidok N-nitrozó vegyületekké való alakításában játszott szerepének tulajdonítanak jelentőséget [21, 22]. Az NO rövid félélet ideje miatt csak képződése helyén fejti ki hatását, így az ér átmérő változása a lokális NO szintézis mértékén múlik. Az NO-nak kulcsszerepe van a nyelőcső mucosa normális mikrokeringésének fenntartásában is. A fokozott NO képződés révén megnövekvő mucosalis véráramlásnak protektív szerepe lehet a károsító hatású gyomortartalom, vagy más noxákkal szemben [23]. Feltételezhető, hogy a megnövekedett iNOS aktivitás a gyulladásos válasz része lehet, az iNOS gyors aktiválódása rövid távon megvédheti a szöveteket a mikróbás infekciókkal szemben. Az NO gyulladásos túlprodukciójának viszont egyenes következménye lehet az N-nitrozó vegyületek képződése, s így az epés reflux az iNOS aktiválásán keresztül hosszú távon szerepet játszhat a mutagén folyamatok elindításában is.

Fontos kiemelni, hogy a sav önmagában nem befolyásolja a cNOS aktivitást, ami azt is jelenti, hogy az iNOS aktivitás másodlagosan, a gyulladásos reakció hatására növekszik meg. Bár az NO produkció mértékét nem ismerjük, az iNOS mRNS expressziója a gyulladásos stimulust követő 30 percen belül megnő, és ezt követi az NO metabolitok plazmaszintjének emelkedése néhány órával később [24].

A kevert reflux köztes állapotot hozott létre, és a szövettani feldolgozás azt igazolta, hogy a kevert (sav + epe) csoportban enyhébb strukturális eltérések mutathatók ki. A kevésbé savas pH hatására az FCD nem csökkent, így a sav által indukált mikrocirkulációs változás kevésbé volt számottevő, s emellett a cNOS aktivitása sem gátlódott. Így – meglepő módon – az epe károsító hatása mérséklődött a tisztán epés refluxhoz képest. Egy újabb keletű hipotézis szerint a GERD-ben általánosan alkalmazott savtermelést csökkentő terápia alkalizálja a gyomor pH-t, ami töltést von el a konjugált epesavaktól, s ezáltal lehetővé válik áthaladásuk az epitheliumon keresztül [25]. Eredményeink megerősítik ezt a feltételezést, és arra engednek következtetni, hogy gyomorsav jelenlétében a tisztán epés reflux által okozott mucosa károsodás kevésbé súlyos mértékű.

Állatkísérletes modellünk elsőként vizsgálta a nyelőcső mikrokeringését *in vivo* körülmények között. Összegezve megfigyeléseinket megállapíthatjuk, hogy epés regurgitáció mellett súlyos szöveti károsodás alakul ki, jelentősen fokozódik az epithelialis és a vascularis permeabilitás, sérül a mucosa integritása. A mucosában megjelenő leukocyták összefüggésbe hozhatók a nyálkahártya laesio kialakulásával. Véleményünk szerint az epe a cNOS aktivitás gátlása és az iNOS aktivitás növekedése útján az NO túltermelésével járó elváltozásokat (mutagenitás, metaplázia) is okozhat a reflux betegség késői szakaszában. Vitathatatlan, hogy a GERD terápiájának alappillérét továbbra is a sav szuppresszió képezi, viszont így az epés reflux nyelőcső mucosa károsító hatása előtérbe kerülhet. A klinikai körülmények között alkalmazott gyógyszeres terápia általában nem véd az epés regurgitációtól, és a sav szuppresszió csak ritkán teljes mértékű [26]. Bár nehéz lehet az állatkísérletekből nyert adatok humán extrapolálása, eredményeink megerősítik az epe szerepét a mucosa károsodás fokozásában. Ezek után felmerülhet, hogy a GERD kivizsgálása során meg kell-e határozni a biliáris komponens, illetve az epés reflux epizódok arányát – és ennek megfelelően esetleg módosítani kell-e terápiás taktikánkat. A sebészi kezelésben elsősorban a jó eredményeket mutató „*duodenal switch*" beavatkozásra gondolhatunk [27–29], de emellett a gyógyszeres kezelés megfelelő beállítása, illetve esetleges új gyógyszerek igénye is felmerülhet, melyek befolyásolhatják az epe topikus hatását és a mitochondriális energiaforgalmat. Emellett szól, hogy effektív antireflux műtétek késői, 10 éves utánkövetése során is detektálható pathologiás reflux, Barrett-oesophagus, vagy akár malignus átalakulás a nyelőcsőben [30–34].

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ORIGINAL ARTICLE

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Microcirculatory Changes in the Canine Oesophageal Mucosa During Experimental Reflux Oesophagitis: Comparison of the Effects of Acid and Bile

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Szentpáli K, Erös G, Kaszaki J, Tiszlavicz L, Lázár G, Wolfárd A, Balogh Á, Boros M. Microcirculatory changes in the canine oesophageal mucosa during experimental reflux oesophagitis: comparison of the effects of acid and bile. Scand J Gastroenterol 2003;38:1016–1022.

Background: The response of the oesophageal microcirculation to luminal damaging agents may play an important role in reflux-induced mucosal injury. We characterized the microcirculatory consequences of exposure to bile with or without hydrochloric acid, and determined the changes in the constitutive nitric oxide synthase and inducible nitric oxide synthase activities in a canine model of acute reflux oesophagitis. **Methods:** Group 1 served as a saline-treated control, while groups 2–4 were exposed for 3 h to bile alone, to hydrochloric acid, or to bile + hydrochloric acid, respectively. The mucosal microcirculation was observed continuously by means of intravital videomicroscopy with an orthogonal polarization spectral imaging technique. Myeloperoxidase, constitutive and inducible nitric oxide synthase activities were measured via tissue biopsies, while the degree of mucosal damage was evaluated histologically. **Results:** Bile evoked deep tissue damage and leucocyte accumulation in the mucosa and muscle layer. The capillary red blood cell velocity and the relative vessel area increased significantly ($P < 0.05$). The constitutive NO synthase activity was decreased, and the inducible NO synthase activity was increased significantly. In the hydrochloric acid-treated group the functional capillary density decreased, the mucosal damage was less severe, the constitutive NO synthase activity did not change, whereas the inducible NO synthase activity was increased significantly. The constitutive NO synthase activity did not change after the bile + hydrochloric acid treatment either. **Conclusion:** Reflux components induce characteristic microcirculatory alterations. The structural damage and leucocyte invasion are accompanied by bile-induced constitutive NO synthase inhibition when hydrochloric acid production is suppressed.

Key words: Gastro-oesophageal reflux disease; mucosa; microcirculation; orthogonal polarization spectral imaging

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Regurgitation of the stomach content may lead to gastro-oesophageal reflux disease (GORD), the most common inflammatory disorder of the foregut (1). Although the importance of gastric acid in the progression of GORD is indisputable, the significance of other components of the refluxed material is still controversial. In particular, regurgitated bile can often be detected in the oesophagus, and is assumed to trigger the development of Barrett's metaplasia and oesophageal adenocarcinoma (2–4). Accordingly, it is clear that appropriate treatment or prevention of these complications presupposes an understanding of the nature of reflux-induced barrier lesions.

The available clinical and experimental evidence suggests that microvascular injury is a causative factor in secondary detrimental reactions, including permeability changes and bacterial or endotoxin translocation in the gastrointestinal tract (5, 6). In contrast, the in vivo response of the oesophageal microcirculation to acute regurgitation has not been as thoroughly studied. Our main objective was therefore to characterize the reactive microcirculatory changes in the oesophageal mucosa in a large-animal model of acute GORD. To this end, the mucosal microcirculatory changes after a standardized biliary or acidic challenge were observed continuously by means of intravital videomicroscopy with orthogonal polarization spectral (OPS) imaging. This novel non-invasive diagnostic method utilizes reflected polarized light to visualize haemoglobin-containing structures without the additional use of a fluorochrome (7).

A further aim was to outline those elements which could be linked to microcirculatory alterations and could participate in the evolving mucosal dysfunction. Nitric oxide (NO) plays a central role in the maintenance of the normal resting oesophageal mucosal blood flow (8, 9), and it has been suggested that local changes in NO release may be critically

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involved in gastrointestinal inflammatory reactions (10). Hence, our second objective was to determine the consequences of acute reflux on the activities of the constitutive NO synthase (cNOS) and inducible NOS (iNOS) isoforms. In line with this goal, bile-induced morphological changes were also evaluated and compared with acid-caused structural damage.

The results indicate that bile per se causes a severe mucosal microcirculatory derangement, with decreased cNOS activity and structural damage, and thus reflux-caused oesophageal injury could be exacerbated by bile-induced constitutive NO synthase inhibition when hydrochloric acid production is suppressed.

Materials and Methods

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethics Committee for the Protection of Animals in Scientific Research at the University of Szeged.

Surgical preparation

Four separate groups of experiments were performed on a total of 23 mongrel dogs (average weight 15.5 ± 2 kg) under sodium pentobarbital anaesthesia (30 mg kg^{-1} i.v.). Small supplementary doses of pentobarbital were administered when necessary. The animals were placed in a supine position on a heating pad for the maintenance of body temperature between 37 °C and 38 °C. In aseptic techniques, the left femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and for fluid and drug administration, respectively. All animals received a continuous infusion of Ringer's lactate at a rate of 10 ml kg^{-1} h^{-1} during the experiments. The oesophagus was inspected by endoscopy (Olympus GIF-E) prior to the experiments to exclude major mucosal lesions. Following a collar incision, the cervical oesophagus with intact neurovascular connections was dissected free, and an approximately 8–10-cm segment of the middle portion was then occluded at both ends with atraumatic clips. Through a small incision, the objective of the intravital microscopic device was introduced into the middle portion of the segment. A plastic tube (0.5 mm i.d.) was inserted distally into the lumen and secured with a purse-string suture for the administration of test compounds.

Study protocol

Surgery was followed by a 20-min recovery period for cardiovascular stabilization, and 7 ml isotonic saline (pH 7.4) was then introduced into the oesophageal segment for 30 min to determine the baseline microcirculatory parameters. After this period, the oesophagus was filled with different test solutions (7 ml) for 3 h. At the end of the experiments, full-thickness biopsies were taken from the oesophagus for histology, and NOS and myeloperoxidase (MPO) activity measurements.

The animals were randomly allocated into four groups. Group 1 ($n = 5$) received 0.9% saline (pH 7.4), group 2 ($n = 8$) canine bile (pH 6.5), group 3 ($n = 5$) hydrochloric acid (HCl) (pH 2.0), and group 4 ($n = 5$) bile + HCl solution (pH 2.5). The bile was obtained from 3 healthy dogs before the experiments, pooled and stored at −20 °C. The intraluminal volume load was identical in all groups. MAP and central venous pressures were measured with Statham P23Db transducers and registered with a computerized data-acquisition system (Haemosys 1.17; Experimetria Ltd., Budapest, Hungary). Arterial blood gases were measured with a blood gas analyzer (AVL, Graz, Austria).

Intravital videomicroscopy

An intravital videomicroscope was used with an OPS imaging technique (Cytoscan A/R, Cytometrics, Pa., USA) to monitor microvascular perfusion changes in the oesophageal mucosa. The OPS imaging technique involves the use of reflected polarized light at 548 nm, which is the isosbestic point of oxy- and deoxyhaemoglobin. Since polarization is preserved in reflection, only photons scattered from relatively deep inside the tissue contribute to the images. In this way, a virtual light source is created in the tissues, so that the vessels appear black (7). A 10× objective was connected to a light source with a flexible cable; in this way, the oesophageal segment was not exteriorized during the study. Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. The functional capillary density (FCD; the length of perfused nutritive capillaries per observation area; μm^{-1}), the relative vessel area (RVA; the perfused vessel area per observation area), and the red blood cell velocity (RBCV; $\mu m\ sec^{-1}$) were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron®, Budapest, Hungary). All data were expressed as the means of three measurements at each time-point.

Histology and light microscopy

Biopsies for light microscopy were obtained from the treated part of the oesophagus of each animal. The samples were fixed in ice-cold 10% phosphate-buffered formalin solution for 24 h, embedded in paraffin, sectioned (6 μm) and stained with haematoxylin eosin. Histological analysis was performed in coded sections by one investigator (LT). Mucosal injury was graded on the 0–100 oesophageal mucosal damage scale of Lanas et al. (11) with the following criteria: epithelial changes (epithelial splitting, erosion and ulceration): maximal score 40; inflammation (intraepithelial leucocytes and cellular hyperplasia): maximal score 40; vascular lesions (oedema, congestion and haemorrhage): maximal score 20. In parallel, the degree of damage was evaluated with the 0–16 scoring system of Geisinger et al. (12): basal cell hyperplasia: maximal score 4; intraepithelial leucocytes: maximal score 4; subepithelial leucocytes: maximal score 4; presence of ulceration: maximal score 4.

NOS activity measurements

NO formation in oesophageal tissues was measured by the conversion of (^{3}H)L-citrulline from (^{3}H)L-arginine according to the method of Szabó et al. (13). Briefly, tissue biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM tris-(hydroxymethyl)-aminomethane-HCl (Reanal, Budapest, Hungary), 0.1 mM ethylenediaminetetra-acetic acid (Serva Feinbiochemica GmbH, Heidelberg, Germany), 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μg ml^{-1} soybean trypsin inhibitor. The homogenate was centrifuged at 4 °C for 20 min at 24000 *g* and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100000 MW cut-off ultra-filter). The tubes were centrifuged at 1000 *g* for 150 min and the concentrated supernatant was washed out from the ultrafilter with 300 μl homogenizing buffer. The samples were incubated with a cation-exchange resin (DOWEX AG 50W-X8, Na^+ form) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation (1500 *g* for 10 min) and the supernatant containing the enzyme was assayed for NOS activity.

For the Ca^{2+}-dependent NOS (cNOS) activity, 50 μl enzyme extract and 100 μl reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM NADPH, 10 μM tetrahydrobiopterin, 1.5 mM $CaCl_2$, 100 U ml^{-1} calmodulin and 0.5 μCi (^{3}H)L-arginine (ICN Biomedicals, specific activity 39 Ci mmol^{-1}) were incubated together for 30 min at 37 °C. The reaction was stopped by the addition of 1 ml ice-cold HEPES buffer (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. Measurements were performed with boiled enzyme and with the NOS inhibitor N-ω-nitro-L-arginine (3.2 mM) to determine the extent of (^{3}H)L-citrulline formation independent of the NOS activity. Ca^{2+}-independent NOS activity (iNOS) was measured without Ca-calmodulin and with EGTA (8 mM).

Reaction mixture (1 mL) was applied to DOWEX cation-exchange resin (AG 50W-X8, Na^+ form) and eluted with 2 mL distilled water. The eluted (^{3}H)L-citrulline activity was measured with a scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2100TR/2300TR, Packard Instrument Co, Meriden, Ct., USA). Protein contents of samples were determined by the method of Lowry et al. (14).

MPO activity

The MPO activity, as a marker of the tissue leucocyte infiltration, was measured via mucosal and muscle biopsies by the method of Kuebler et al. (15). Briefly, the mucosa was scraped off the underlying muscle tissue and homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 2000 *g*. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan), and the data were referred to the protein content.

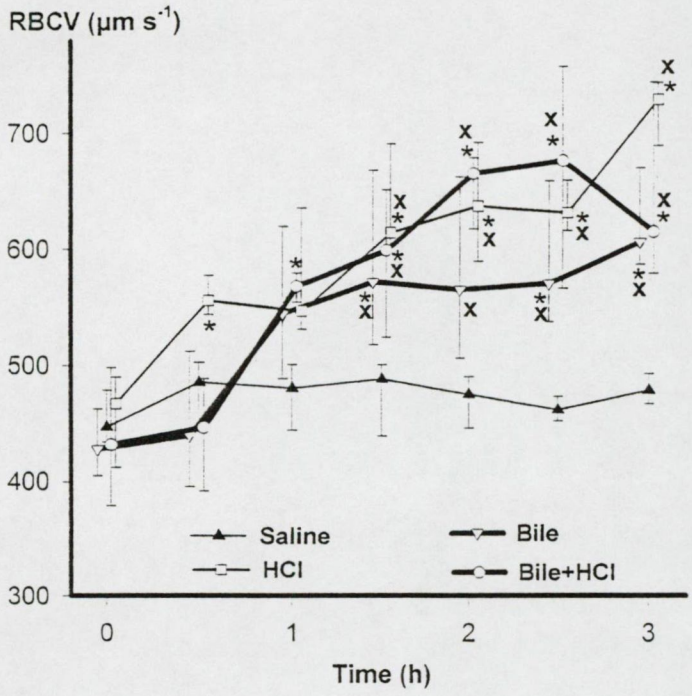

Fig. 1. Effects of various intraluminar treatments on the red blood cell velocity (RBCV) of the oesophageal mucosa (μm s^{-1}) after 1, 2 and 3 h of exposure. Saline treatment (thin line with filled triangles), bile treatment (thick line with empty triangles), HCl treatment (thin line with empty squares) and bile + HCl-treated (thick line with empty circles) groups. Median values and 75th and 25th percentiles are given. *$P < 0.05$ within groups versus baseline values; X $P < 0.05$ between groups versus saline-treated control group values.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Non-parametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analysed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. Median values and 75th and 25th percentiles are given in the Figures. *P* values <0.05 were considered significant.

Results

The baseline values of the macrohaemodynamic variables did not differ significantly in the different groups and there were no significant haemodynamic changes as compared with the baseline values during the experimental period (data not shown). In the control group, saline administration did not significantly influence the histology scores of the mucosal morphology.

The baseline level of the capillary RBCV ranged between 430 and 470 μm s^{-1} in the various groups. In the control group, the RBCV in the capillaries of the oesophagus did not change during the experiments. However, the RBCV was

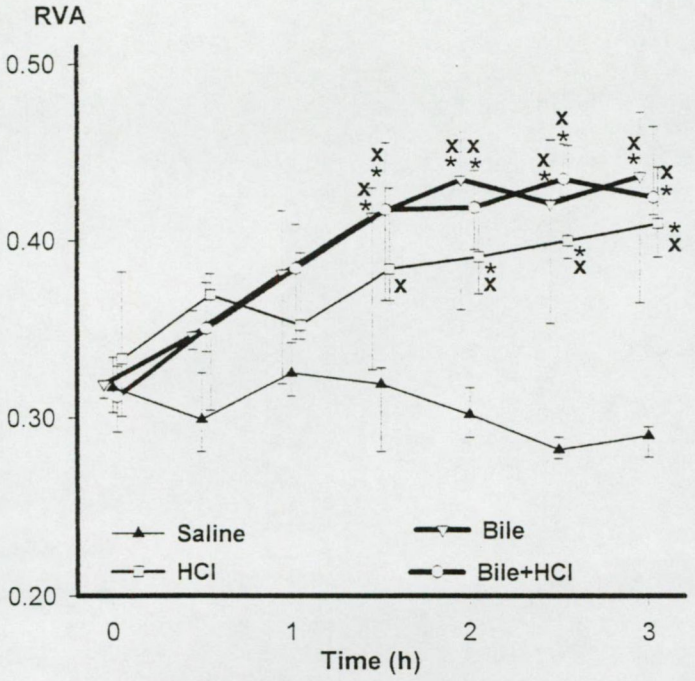

Fig. 2. Changes in the relative vessel area (RVA) in the saline-treated (thin line with filled triangles), bile-treated (thick line with empty triangles), HCl-treated (thin line with empty squares) and bile + HCl-treated (thick line with empty circles) groups. Median values and 75th and 25th percentiles are given. *$P < 0.05$ within groups versus baseline values; X $P < 0.05$ between groups versus saline-treated control group values.

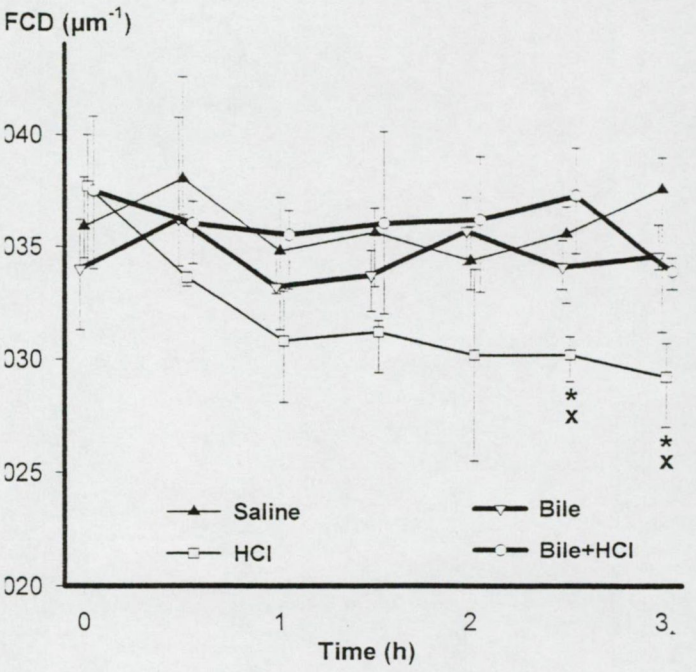

Fig. 3. Changes in the functional capillary density (FCD) values (μm^{-1}) in the saline-treated (thin line with filled triangles), bile-treated (thick line with empty triangles), HCl-treated (thin line with empty squares) and bile + HCl-treated (thick line with empty circles) groups. Median values and 75th and 25th percentiles are given. *$P < 0.05$ within groups verusus baseline values; X $P < 0.05$ between groups versus saline-treated control group values.

increased significantly after the 3-h exposure to the bile or HCl-containing test solutions and average values of 607, 730 and 620 $\mu m\ s^{-1}$ were measured after bile, HCl and bile + HCl treatment, respectively (Fig. 1).

The RVA was significantly elevated in all treated groups compared with the sham-operated group or with the baseline. Administration of bile, HCl alone or bile + HCl resulted in a rise from the baseline values 0.319, 0.333 and 0.312 to 0.437 (0.365; 0.473), 0.41 (0.391; 0.442) and 0.425 (0.415; 0.465), respectively (Fig. 2).

The FCD was not influenced by lumenal bile or HCl + bile treatment for 3 h. However, HCl treatment alone was followed by a significant decrease from 0.0377 μm^{-1} (0.0342; 0.040) to 0.0292 μm^{-1} (0.027; 0.0307) at the end of the experimental period (Fig. 3).

The effects of bile, HCl and bile + HCl on the mucosal morphology are presented in Fig. 4. The structural damage to the mucosa was quantified by histology using two different scoring systems; in consequence of the highly characteristic tissue lesions, there was no significant difference between the results of the evaluation methods. The biopsy samples from the saline-treated control group exhibited an average grade of injury of 2.25 (range of scores 0–18) on the Lanas scale, and of 0.37 (range of scores 0–3) on the Geisinger scale. The 3-h bile exposure induced severe mucosal damage, with median values of 65 (range 50–70) on the Lanas scale, and 12 (range 8–14) on the Geisinger scale ($P < 0.01$). Deep lesions were commonly observed, with extensive epithelial loss, desquamation and necrosis, intraepithelial and subepithelial leucocytosis, basal cell hyperplasia and subepithelial connective tissue damage. In most cases, transmural inflammation, oedema and vasodilatation were apparent.

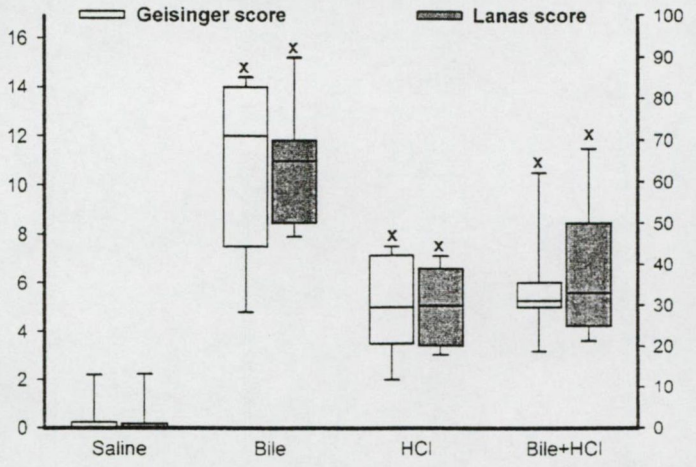

Fig. 4. Box plot diagrams presenting scores of histological evaluation of the mucosal damage of the oesophagus, with grading performed according to Lanas et al. (shaded box) and Geisinger et al. (empty box). See Methods for description. Horizontal lines of boxes show (from top) first quartile, median and third quartile, respectively. The capped bars indicate the 5th and 95th percentiles. X $P < 0.05$ between groups versus saline-treated control group values.

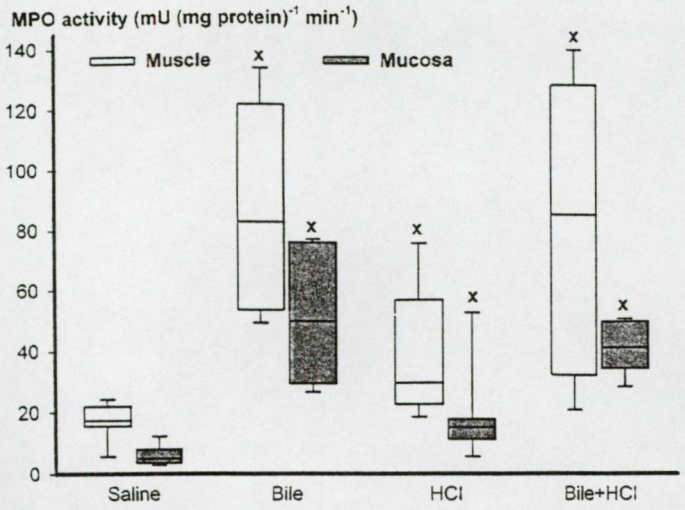

Fig. 5. Myeloperoxidase (MPO) activity in the mucosa (empty column) and the muscle tissue (shaded column) of the oesophagus 180 min after treatment. Horizontal lines of boxes show (from top) the 25th percentile, median and the 75th percentile, respectively. The capped bars indicate the 5th and 95th percentiles. X $P < 0.05$ between groups versus saline-treated control group values.

In HCl-treated group 3, the reactive mucosal changes were different from those observed in the bile-treated groups. HCl administration induced severe epithelial damage with considerable basal cell hyperplasia. Petechiae and deep transmural lesions were only observed occasionally. The median value of the Lanas scores was 30 (range 19–40), and that was of the Geisinger scores 5.0 (range 3–7). Following treatment with bile + HCl, the histology revealed less severe (mild to moderate) morphological changes. Although disruption of the epithelial layer, desquamation, intra- and subepithelial inflammatory cells and subepithelial connective tissue oedema were generally observed, the deeper tissue layers were less involved. The degree of injury was 33 (range 25–50) on the Lanas scale, and 5 (range 5–6) on the Geisinger scale.

The MPO data demonstrated that the leucocyte accumulation was significantly increased in the mucosa in groups 2, 3 and 4 as compared with saline-treated group 1 (Fig. 5). Bile, HCl alone or bile + HCl administration resulted in a 10-fold (M = 50.0; 25p = 29.5; 75p = 76.1), 3-fold (M = 15.0; 25p = 11.2; 75p = 17.9) and 8-fold (M = 41.5; 25p = 34.3; 75p = 49.9) rise in MPO activity, respectively. Although the average MPO values were usually higher within the muscle, the MPO activities in the two tissue layers were not significantly different. Both bile and bile + HCl administration resulted in a 5-fold elevation, while HCl administration alone resulted in an approximately 2-fold increase in muscle MPO activity (Fig. 5).

Fig. 6 depicts the changes in the oesophageal cNOS and iNOS activities. The activity of cNOS was significantly depressed after bile treatment, and this change was accompanied by a significant, approximately 6-fold increase in iNOS activity. HCl treatment did not influence the oesophageal cNOS activity, while it resulted in a somewhat lower, 2.5-fold increase in iNOS activity, as compared with the value for the sham-operated group. Similarly, the cNOS activity was not influenced by bile + HCl administration for 3 h, whereas the activity of iNOS was increased significantly, similarly as observed after bile treatment alone.

Discussion

Despite the continuously growing body of information, the exact pathophysiology of the reflux-induced mucosal dysfunction leading to clinicopathologic complications is still unknown. It appears that several major pathways contribute to the development of mucosal damage, including topical irritation, barrier derangement and interference with mucosal repair. Most investigations have found an apparent association between the excessive reflux of the acidic gastric content into the oesophagus and symptomatic irritation, but oesophagitis is a frequent finding in GORD patients with total gastrectomy (16). Bile can also reach the oesophagus by the reflux of gastroduodenal juice, and in recent decades duodenogastroesophageal reflux or alkaline reflux has become an autonomic entity (17). Most importantly, oesophageal pH-metry and fiberoptic Bilitec investigations have proved the correlation between this type of regurgitation and the presence of severe complications, e.g. oesophageal ulceration and Barrett metaplasia (18).

The microcirculatory consequences of biliary exposure became apparent quickly after the start of the treatment. The RBCV and the RVA were significantly increased at 90 min, while the FCD was unchanged until 180 min. The RBCV is determined primarily by the blood flow and the cross-section

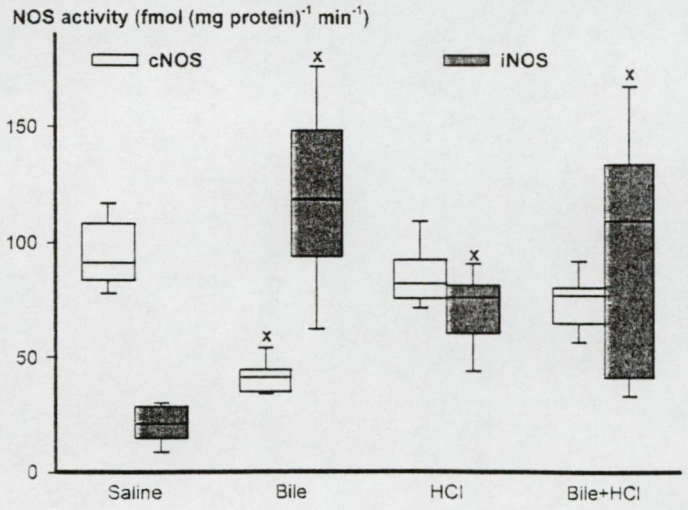

Fig. 6. Changes in constitutive (empty box) and inducible (shaded box) NOS activities (fmol (mg protein)$^{-1}$ min^{-1}) in oesophageal tissue from saline-treated ($n = 5$), bile-treated ($n = 8$), HCl-treated ($n = 5$) and bile + HCl-treated ($n = 5$) animals. Horizontal lines of boxes show (from top) the 25th percentile, median and the 75th percentile, respectively. The capped bars indicate the 5th and 95th percentiles. X $P < 0.05$ between groups versus saline-treated control group values.

of the circulatory area and this suggests that significant vasodilation evolves in the mucosa. The MPO data implied that leucocytes accumulated in the mucosa, and the enzyme activity was high in the muscle layer too. The histology also revealed the destructive effects of bile: in almost all cases, a deep, almost transmural injury was observed. These data together demonstrate that the noxious agent penetrates deep into the oesophageal wall.

NO, formed in vivo by the continuously active cNOS, is involved in the physiological regulation of the peripheral vascular tone (19). In inflammation, endotoxaemia or sepsis, a distinct isoform, the inducible, Ca^{2+}-independent iNOS is activated, thereby leading to an excessive production of NO (20). In our study, the bile-induced microcirculatory alterations were accompanied by inverse changes in cNOS and iNOS activities. Although the disturbance of mitochondrial energy production could be an important factor in the acute decrease in cNOS activity in the canine oesophagus, the molecular mechanism of the events is still unclear. Previously, we have shown that the multifactorial ulcer-producing actions of bile are initiated by the lowering of adenosine triphosphate (ATP) generation in the affected oesophageal tissues (21). The mitochondria, which play a crucial role in maintaining the cell ATP-dependent processes, are considered to be potential targets of bile-induced toxicity. It has been shown that bile salts cause hepatocyte death by inducing Fas-dependent hepatocyte apoptosis or necrosis and inhibit the activities of complexes I and III of the mitochondrial respiratory chain (22, 23).

The effects of bile on the cNOS activity have been described in biliary fibrosis (24) and after experimental bile-duct ligation (25, 26). Recently, it has also been reported that a low cNOS and an increased iNOS activity are responsible for the exacerbation of gastric injury from luminal irritants during endotoxaemia (27). The increased iNOS activity could be a compensatory phenomenon of cNOS inhibition or an aftermath of inflammatory stimuli. It should be noted that a proposed cause of carcinogenesis in GORD is chronic inflammation (28), while others have suggested that the mechanism of the mitogenic effects of bile acids may involve the N-nitrosation of glycine and taurine amides, leading to the production of carcinogenic N-nitroso amides (29). Because of its short half-life, the effects of NO are limited to the site of its production; thus, NO-related vascular changes mostly depend on local NO synthesis. NO plays a central role in the maintenance of the normal resting oesophageal mucosal blood flow, and an increased oesophageal blood flow may be a protective response against damaging gastric juice or other noxious stimuli (8, 9). Similarly, the rapid induction of iNOS may in the short run protect the tissue against microbial infection. However, excess NO generation in the inflamed tissue is likely to lead directly to the formation of N-nitroso compounds, and it is tempting to speculate that the bile-induced overproduction of NO through the activation of iNOS may in the long run play a role in the mutagenic process (30).

HCl induced a more superficial injury compared with bile, and the intensity and extent of leucocyte accumulation were also more moderate. The consequence of HCl exposure on the microcirculation were different too. Vasodilatation and an increased flow rate were again present, but concomitantly the FCD was decreased. It has been reported that the adaptive increase in flow after HCl treatment is dependent on the paracrine effect of NO, but mast cell-derived histamine, substance P and calcitonin gene-related peptide, also play roles in this response (9, 31, 32). The acute reduction in the FCD is usually due to an imbalance in the Starling forces or intravascular cellular events influencing the circulation in the subepithelial capillary network. A decreased FCD is a characteristic of low-flow ischaemia, but this condition is regularly observed with blood acidosis in both haemorrhagic shock and intentional acid infusion (33).

It is important that the instillation of HCl did not change the cNOS activity, which suggests that the increase in iNOS activity is a consequence of a separate, secondary inflammatory reaction. Although the amount of bioavailable NO is usually unknown, it has been shown that the iNOS mRNA expression increases within 30 min after an inflammatory challenge, and this is followed several hours later by increased plasma levels of the NO metabolites nitrate and nitrite (34).

It seems that the mixture of HCl and bile induced an intermediate condition. The alkaline pH improved the microcirculatory consequences of the acid-induced damage the FCD did not change, and the detrimental effects of the bile were also mitigated, as the cNOS activity was not inhibited in this case. It was recently suggested that HCl-suppressive therapy in GORD patients increases the stomach pH and that this will remove the charges on the conjugated bile acids, allowing entry into the epithelium (35). Our results strengthen this hypothesis and suggest that the reactive response of the oesophageal mucosa to a pure biliary reflux is indeed alleviated in the presence of gastric acid.

In conclusion, our experimental model allowed the first in vivo visualization of the canine oesophageal microcirculation. Bile impairs the cNOS activity and enhances the iNOS activity, and may therefore induce NO overproduction-related changes in the later phases of reflux. Medical therapy usually does not stop bile from regurgitating to the oesophagus, and acid suppression is often incomplete (36). While acid suppressive therapy remains a cornerstone of treatment for GORD, the deleterious consequences of biliary exposure on oesophageal mucosal NO synthesis could be manifested in the absence of acid. Although the extrapolation of findings of animal studies to humans could be difficult, the results underline the significance of bile in the pathogenesis of mucosal injury of GORD.

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