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RAPID COMMUNICATION

Reactive oxygen species and chemokines: Are they elevated in the esophageal mucosa of children with gastroesophageal reflux disease?

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Abstract

AIM: To determine the role of inflammatory cytokines and reactive oxygen species (ROS) in childhood reflux esophagitis.

METHODS: A total of 59 subjects who had complaints suggesting GERD underwent esophagogastroduodenoscopy. Endoscopic and histopathologic diagnosis of reflux esophagitis was established by Savary-Miller and Vandenplas grading systems, respectively. Esophageal biopsy specimens were taken from the esophagus 20% proximal above the esophagogastric junction for conventional histopathological examination and the measurements of ROS and cytokine levels. ROS were measured by chemiluminescence, whereas IL-8 and MCP-1 levels were determined with quantitative immunometric ELISA on esophageal tissue. Esophageal tissue ROS, IL-8 and MCP-1 levels were compared among groups with and without endoscopic/histopathologic esophagitis.

RESULTS: Of 59 patients 28 (47.5%) had normal esophagus whereas 31 (52.5%) had endoscopic esophagitis. In histopathological evaluation, almost 73% of the cases had mild and 6.8% had moderate degree of esophagitis. When ROS and chemokine levels were compared among groups with and without endoscopic esophagitis, statistical difference could not be found between patients with and without esophagitis. Although the levels of ROS, IL-8 and MCP-1 were found to be higher in the group with histopathological reflux esophagitis, this difference was not statistically significant.

CONCLUSION: These results suggest that the grade of esophagitis is usually mild or moderate during childhood and factors apart from ROS, IL-8 and MCP-1 may be involved in the pathogenesis of reflux esophagitis in children.

Key words: Gastroesophageal reflux disease; Reflux esophagitis; Reactive oxygen species; Interleukine-8; Monocyte chemoattractant protein-1

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of

the most frequently encountered gastrointestinal pathologies in children with a prevalence of 8% during infancy. When the presence of heartburn as a presenting symptom was considered, the prevalence rises up to 40% among adults[1-4]. Early diagnosis and treatment is of importance as GERD results in serious problems that have negative influences on the quality of life. Since there is a wide range of symptoms regarding GERD and the results of GER questionnaires (GERQ) are controversial together with the incoherencies among other investigative methods; diagnosis of the disease is difficult[5].

Oxidative stress has been associated with several disease states like atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases and aging[6-7]. Furthermore, reactive oxygen species (ROS) are reported to play a role in the pathogenesis of several gastrointestinal diseases such as inflammatory bowel disease and peptic ulcer[8-11]. In studies carried out on animal models of esophagitis as well as those on human esophageal tissue, ROS that are generated in the process of reflux esophagitis were found to be responsible for the esophageal tissue damage, and this finding was further supported by the studies showing that tissue damage could be prevented with the use of antioxidants[12-19].

Several chemokines have been shown to increase significantly in inflammatory disease states like pulmonary diseases, viral meningitis, asthma, rhinitis, a topical dermatitis, ulcerative colitis and Crohn's disease[20-24]. While IL-8 has chemotactic activity for neutrophils, monocyte chemoattractant protein-1 (MCP-1) is effective in the activation of monocytes, macrophages as well as lymphocytes[25]. In studies conducted in adults, chemokine levels were found to be significantly high in the esophageal tissues of the patients with esophagitis as compared to controls; furthermore a significant correlation was reported between the severity of the reflux esophagitis and chemokines levels[26-30]. However, there is only limited data on the levels of ROS and tissue cytokines in the pathogenesis of reflux esophagitis in children. In a study including 10 children with reflux symptoms, IL-6 levels of the esophageal tissue was found to be higher than that of the normal resulting in a claim by the researchers that cytokines could be important in the pathogenesis of the reflux disease[31].

The aim of this study is to investigate the roles of chemokines and ROS in reflux esophagitis in children by measuring the levels of ROS, IL-8 and MCP-1 in the esophageal tissues of children having endoscopic and histopathological esophagitis.

MATERIALS AND METHODS

Patient selection, endoscopy and histopathology

Consecutive children who underwent upper gastrointestinal endoscopy between September 2005 and January 2006 were prospectively considered for the study. Patients who had complaints related to GER such as vomiting, epigastric pain, regurgitation, retrosternal pain, dysphagia and persistent wheezing were included in this study. Patients with a history of non-steroidal anti-inflammatory drug, proton pump inhibitor, H2 receptor antagonist or antibiotics use or those having severe chronic co-morbidities like diabetes mellitus, renal diseases or neurological diseases were excluded from the study. The study was approved by the local ethics committee of Marmara University School of Medicine, and informed consent forms were obtained from first degree relatives of all the patients.

Same endoscopy team performed the upper gastrointestinal system endoscopy in all patients with a pediatric fiberoptic gastroscope having an inner diameter of 2.8 mm (Fujinon EG 200 HR, Japan). Following an appropriate fasting time based on age of the patients, sedation was established by iv administration of meperidine (2-4 mg/kg) and midazolam (0.2-0.4 mg/kg) and endoscopy was performed. The degree of endoscopic esophagitis was evaluated according to Savary-Miller classification[32]. Patients with and without endoscopic esophagitis formed the first group (Group Ⅰ). During endoscopy, 4 esophageal biopsy samples were obtained from 20% proximal part of esophagogastric junction for conventional histopathological examination and the measurements of ROS and cytokine levels. At the end of the study, histopathological examination of the biopsy samples was carried out at the same time by the same pathologist who was blind for the clinical and laboratory findings of the patient. The diagnosis of histopathological esophagitis was based on the classification by Vandenplas[33] and the cases with or without histopathological esophagitis formed the second group (Group Ⅱ). For simplifying histopathological evaluation; Stage 1a, 1b and 1c were grouped as mild, Stage 2 and 3 as moderate and finally Stage 4 and 5 as severe esophagitis.

The third tissue sample was rinsed with 0.9% physiological saline and placed in eppendorf tubes for measurement of ROS levels in fresh tissues in the biochemistry laboratories. Other tissue samples were stored at -80℃ for the measurement of tissue IL-8 and MCP-1 at the end of the study.

For conventional histopathological examination, 3 samples from the antrum, 2 from the corpus and 2 from the duodenum were obtained from all patients, and these were placed in 10% formaldehyde and sent to pathology for examination. The presence of *H pylori* was confirmed with a positive rapid urease test and histopathological identification of *H pylori*. Gastric biopsy samples were evaluated with a modified Sydney scoring system that allowed for identifying the presence of gastritis, its severity and the density of *H pylori*.

Biochemical measurements

For the measurement of free oxygen radicals on esophageal tissue, chemiluminescence method was employed. The measurements were carried out with Mini Lumat LB 9506 Luminometer (EG&G, Berthold, Germany) at room temperature on fresh tissue samples. In the method employed, lucigenin corresponds to superoxide radical, whereas luminol identifies the total

value for hydroxyl radical (OH^-), hydroperoxyl radical (HO2') and peroxyl (RO2') radical. The tissues were first placed into 3 mL of PBS solution, on top of the tissues luminol or lucigenin (Sigma Chemicals, USA) were added at a concentration of 4 mmol/L as enhancers and measurements were obtained.

IL-8 and MCP-1 levels were measured by the quantitative immunometric sandwich enzyme linked-immunosorbent assay (ELISA) method. For IL-8 (Diaclone Research, France) and MCP-1 (Biosource, California, USA) measurements, the tissue samples were placed into eppendorf tubes and kept at -80℃. Cytokine measurements were performed on the same day with IL-8 and MCP-1 from the same samples. The tissues obtained were homogenized with phosphate buffered physiological saline to prepare 10% homogenates. After the homogenates were centrifuged at 1000 × *g* for 10 min, the supernatant obtained was used for measuring IL-8 and MCP-1 levels. As IL-8 and MCP-1 levels were to be calculated based on total amount of tissue protein, protein measurements were also performed on biopsy samples. The measurement of protein in esophageal homogenates was performed according to the "Bicinchoninic acid" method. For the procedure; bicinchoninic acid solution (Sigma B 9643, Sigma Chemicals) and 4% $CuSO_4$, protein coloring reagent (0.2 mL 4% $CuSO_4$ on 10 mL bicinchoninic acid solution) were used. Ten μL of homogenate was added onto 200 μL of protein coloring reagent. After mixing, the tube was kept at 37℃ for 30 min. It was brought back to room temperature and the absorbance of the coloured complex was measured at 562 nm. The values were expressed as pg/mg tissue protein. IL-8 and MCP-1 measurements were carried out with EL × 800 BIO-TEK Instruments, Inc./USA brand ELISA device in line with the instructions provided in the commercial kits.

Statistical analysis

In the statistical evaluation of the findings obtained from the study SPSS (Statistical Package for Social Sciences) for Windows 10.0 program was used. The Kolmogorov Smirnov test was used to compare the distributions of luminol, lucigenin, MCP-1 and IL-8 to parameters with normal distribution. As IL-8 and MCP-1 parameters had limit values, logarithmic conversion was used and the values were identified accordingly. In the comparisons of two groups with normal distributions, Student-*t* test was used. In the evaluation of the parameters that do not have a normal distribution, the Kruskal Wallis test was used. Chi-square test, McNemar test, Kappa analysis and diagnostic screening tests (sensitivity, specificity) were used to compare qualitative data. *P* level of < 0.05 was evaluated as being statistically significant.

RESULTS

A total of 152 children underwent endoscopy during the study period and 59 out of 152 subjects who had complaints suggesting GERD included in the study. ROS levels were measured in 54 and cytokine levels in 55 out of 59 patients. Mean age of the patients in the study was 8.9 ± 4.4 years (age range 1.5-17 years). Of 59 patients 28 (47.5%) had normal esophagus whereas 31 (52.5%) had endoscopic esophagitis according to Savary Miller classification. In histopathological evaluation, 80% of the cases had mild or moderate degree of esophagitis. Of 31 patients having endoscopic esophagitis, 29 (93.5%) also had histopathological esophagitis whereas of 28 patients not having endoscopic esophagitis 18 (64.3%) had histopathological esophagitis. The sensitivity of endoscopic esophagitis for prediction of histopathological esophagitis was significantly high (93.6%, $P = 0.0083$). However, endoscopy had a low specificity in the diagnosis of histopathological esophagitis in children. Kappa correlation rate between the 2 methods was 30.1%: PPV, 61.7% and NPV, 83.3%.

When esophageal tissue ROS and chemokine levels were compared among groups with and without endoscopic esophagitis, statistical difference could neither be found between the stages of endoscopic esophagitis nor between the patients with and without esophagitis (Table 1).

When a comparison was made in terms of luminol and lucigenin levels of cases with and without histopathological esophagitis, there was a difference of statistical significance between the two groups ($P = 0.049$ and $P = 0.044$, respectively). While luminol levels did not differ among normal and patients with mild esophagitis, luminol levels of patients with moderate esophagitis were significantly higher than the normal patients (Table 2). Since there was not a statistically significant difference in luminol levels between children with mild and moderate esophagitis, two groups were combined, however, groups with and without histopathological esophagitis did not differ for either luminol or lucigenin measurements ($P > 0.05$).

Patients with histopathological esophagitis and normal were compared for MCP-1 and IL-8 levels. The highest cytokine levels were identified in patients with moderate esophagitis while lowest levels were found in normal children. However, this difference did not reach a statistical evaluations ($P > 0.05$, Table 3).

In order to investigate the effect of presence of *H pylori* gastritis, the patients having esophagitis were classified and patients with and without *H pylori* gastritis were compared. Patients with *H pylori* gastritis and those not having gastritis did not show any statistically significant difference between their levels of ROS and chemokines.

DISCUSSION

Gastroesophageal reflux when untreated in children may be related to some serious complications such as esophagitis, failure to thrive, esophageal strictures, Barrett esophagus and adenocarcinoma[34]. Delineation of the pathogenesis of GERD will allow for the development of more effective treatment strategies while making it possible to prevent complications. In

Table 1 The comparison of luminol, lucigenin, MCP-1 and IL-8 levels between endoscopic esophagitis and normal groups (mean ± SD)

	Esophagitis	Normal	*P* value[1]
Luminol (rlu/mg)	175.2 ± 98.5	152.7 ± 71.3	0.349
Lucigenin (rlu/mg)	154.1 ± 74.9	155.7 ± 79.9	0.939
MCP-1 (pg/mg)	39.8 ± 1.7	47.8 ± 1.7	0.248
IL-8 (pg/mg)	331.1 ± 2.9	323.6 ± 2.3	0.898

[1]Student-*t* test.

Table 2 The comparison of luminol and lucigenin levels according to the severity of histopathological esophagitis (mean ± SD)

Vandenplas classification	Normal (*n* = 12)	Mild esophagitis (*n* = 43)	Moderate esophagitis (*n* = 4)	*P* value[1]
Luminol (rlu/mg)	129.9 ± 80.9	164.4 ± 76.3	236.8 ± 142.8	0.049
Lucigenin (rlu/mg)	156.8 ± 91.7	145.4 ± 65.4	244.8 ± 94.6	0.044

[1]Kruskal Wallis test (normal *vs* moderate esophagitis).

Table 3 The comparison of MCP-1 and IL-8 levels according to the severity of histopathological esophagitis (mean ± SD)

Vandenplas classification	Normal (*n* = 12)	Mild esophagitis (*n* = 43)	Moderate esophagitis (*n* = 4)	*P* value[1]
MCP-1 (pg/mg)	38.9 ± 1.7	44.5 ± 1.7	53.7 ± 2.7	0.804
IL-8 (pg/mg)	262.4 ± 2.3	337.3 ± 2.5	630.9 ± 5.6	0.614

[1]Kruskal Wallis test.

recent years, the role of ROS and chemokines in the pathogenesis of GERD and reflux esophagitis are being investigated both in experimental esophagitis models and in humans[12,17,19,27,29,35].

The relationship between reflux esophagitis and free oxygen radicals was elaborated in this study, no statistical difference could be identified between normal cases and those having different degrees of endoscopic and histopathologic esophagitis. However, although it was statistically not significant, ROS levels were found to be increased in patient groups compared to the normal group.

Free oxygen radicals in general and superoxide radical in particular were shown to increase in animals with esophagitis and it was claimed that free radical scavengers like SOD and DA-9601 as well as anti-inflammatory agents like ketotifen could prevent the tissue damage[12,13,15,17,35]. However, in the study by Soteras *et al*[36] free oxygen radicals were found to play a role mostly in severe esophagitis and it was concluded that free oxygen radicals did not increase in the mild histopathological esophagitis model and thus ROS might not be influential on the pathogenesis of mild esophagitis. Studies performed in adults with reflux esophagitis are in support of the experimental esophagitis models showing that free oxygen radicals do play a role in the pathogenesis of reflux esophagitis[18,19]. Olyaee *et al*[37] have identified a significant correlation between the degree of esophagitis and the levels of free oxygen radicals in the tissue; under the light of this finding they stipulated that in mucosal epithelial cells affected by the injury, the production of ROS was increased. After examining mucosal biopsies of cases with erosive gastritis and Barrett esophagus; another group of researcher reported that the main oxidant product responsible for the development of reflux esophagitis was superoxide anion[38].

Although there are no studies investigating the role of free oxygen radicals in children with reflux esophagitis, our findings are different than the results obtained from adult patients with reflux esophagitis. In our study, neither lucigenin which showed superoxide radical production nor luminol reflecting the productions of other free radicals were found to be increased in children with esophagitis compared to controls. Despite not reaching the level of statistical significance, free oxygen radical levels were found to be higher in children with esophagitis when compared to the controls. The reason behind not identifying a statistically significant difference between the cases of esophagitis and normal may be attributed to the fact that in most of the cases with histopathological reflux esophagitis was of mild degree.

Likewise, the levels of IL-8 and MCP-1 that are chemokines in relation with neutrophil and mononuclear cell migration were found to be higher in the group with histopathological reflux esophagitis compared to normal, however this difference did not reach a level of statistical significance either. In studies performed in adults, tissue chemokine levels are reported to be higher in cases with esophagitis than normal and the increase is reported to be in parallel with the severity of the histopathological esophagitis concerned. Fitzgerald *et al*[26] have identified 3-10 fold higher levels of tissue cytokines in the esophageal mucosa of adults with esophagitis when compared to patients with Barrett esophagus and normal controls. Furthermore, adult patients with nonerosive esophagitis were reported to have higher levels of tissue cytokines when compared to normal[27-30]. Similarly, in a study comparing 10 children with reflux esophagitis to normal patients, esophageal tissue IL-6 levels were reported to be significantly higher in patients with esophagitis[31]. In our study, the lowest levels were measured in normal cases whereas the highest levels were identified in patients with a moderate degree of esophagitis. The difference between these two groups was not statistically significant, this can be explained by the fact that the number of individuals who were normal and who had mild and moderate esophagitis were quite different from each other.

In *H pylori* gastritis, it has been shown that levels of IL-8, MCP-1 and other cytokines increased in gastric mucosa but we do not know the effect of this infection on esophageal tissue which is not normally colonized by the bacteria[39-41]. In our study, we could not identify any

effects of *H pylori* gastritis on esophageal tissue ROS and chemokine levels.

One drawback of this study is the lack of a true control group. All the study group was selected from the patients who had GER symptoms. The subjects who had either endoscopic or histopathological reflux esophagitis were compared to their nonreflux counterparts. The subjects who were included in the normal group according to the endoscopic findings might have a nonerosive reflux disease. Similarly, the subjects included in the normal group according to the histopathological examination might have an increased tissue level of proinflammatory substances before the establishment of the well-known histopathological changes. This might be another explanation for the lack of a clear cut statistical significance between the groups. However it is not possible to find a true control group for these kind of studies because it is not ethical and possible to perform endoscopy in totally normal children.

In parallel with the results of the studies on adult GERD or esophagitis, the patients in our study had higher levels of tissue ROS and chemokines; however, this increase did not reach a level of statistical significance. The number of studies aiming at identifying the pathogenesis of reflux esophagitis in children is very limited. Studies to be performed with higher numbers of patients with the aim of identifying the pathogenesis will allow for the development of further diagnostic and therapeutic opportunities for GERD which is a significant cause of morbidity.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is one of the most frequently encountered gastrointestinal pathology not only in adults but also in children. Since it may be related to some serious complications such as esophagitis, failure to thrive, esophageal strictures, Barrett esophagus and adenocarcinoma, early diagnosis and treatment of reflux disease is crucial. In recent years, it has been reported that reactive oxygen species (ROS) and inflammatory chemokines play an important role in the pathogenesis of GERD in adults but there is scarce data regarding children with reflux esophagitis.

Research frontiers

Inflammatory chemokine levels were found to be significantly high in the esophageal tissues of the patients with esophagitis and a significant correlation was found between the severity of the reflux esophagitis and chemokines levels in adults. The aim was to investigate the role of chemokines and ROS in children with reflux esophagitis. Since the duration of reflux disease might be shorter in children compared to adults, the potential role of chemokines and ROS was interrogated in early or mild cases of reflux esophagitis.

Innovations and breakthroughs

In this study we showed that the level of ROS and chemokines in esophageal tissue were higher in children with esophagitis compared to the subjects without esophagitis. However the difference was not statistically significant. Hence, factors apart from ROS, IL-8 and MCP-1 might be important in the pathogenesis of reflux esophagitis in children.

Applications

It was found that ROS and chemokines increased in children with reflux esophagitis. Opposite to the adults, the exposure of the noxious agents to the esophagus is not long enough in children, endoscopic or histopathological esophagitis might be obscure. Hence tissue level of ROS or/and chemokines might be an important finding in early diagnosis of childhood esophagitis. Furthermore, the use of antioxidants or antiinflammatory agents might be alternative treatment modalities to the established treatment regimens.

Peer review

This manuscript showed that ROS and chemokines are increased in esophageal tissue in children with reflux esophagitis, though not statistically significant. This finding might be important for the delineation of the pathogenesis of reflux esophagitis in children, and therapeutic alternatives targeting these chemokines or ROS could be an option in the future.

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