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Original article

Evaluation of systemic oxidative status and mononuclear leukocytes DNA damage in children with caustic esophageal stricture

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SUMMARY. **Esophageal stricture (ES) due to accidentally caustic digestions is a common problem in children. Mucosal damage and repeated dilatations lead to chronic inflammation and finally ES. We investigated the oxidative status and DNA damage of children with ES. Five children with ES were compared with the same age- and sex-matched healthy subjects. Oxidative status of plasma was evaluated by measuring myeloperoxidase (MPO) activity, and total peroxide (TP) level. Anti-oxidative status of the plasma was evaluated by measuring catalase (CAT) activity, and total antioxidant response (TAR). We used the Single Cell Gel Electrophoresis (also called Comet Assay) to measure DNA strand break in peripheral blood mononuclear leukocytes. Mean MPO activity and TP levels in the ES group were significantly higher than the control group (0.83 ± 0.35, 0.09 ± 0.03 and 0.98 ± 0.38, 0.34 ± 0.20, $P = 0.009$ and $P = 0.047$ respectively). There was no significant difference in CAT activity and TAR levels between the two groups ($P = 0.347$). DNA damage in patients with ES was increased compared to control subjects (108.8 ± 51.2 and 57.6 ± 31.2 arbitrary units, respectively), but this difference was not significant statistically ($P = 0.09$). This study shows that systemic oxidative stress and alteration at the nuclear level occur in patients with ES, as a result of multiple dilatations and tissue injury. On the other hand, these results support that patients with ES may benefit from antioxidant treatment.**

KEY WORDS: **caustic esophageal strictures, DNA damage, oxidative stress.**

INTRODUCTION

Children often accidentally swallow caustic agents, which can cause deep circumferential burns of the esophagus. Stricture formation with inability to swallow food after the injury is inevitable in some cases. The traditional treatment of esophageal strictures (ES) in children consists of different dilatation procedures, and it may be repeated many times.[1–3] Multiple dilatations can also increase the risk of chronic inflammation and irritation of the esophagus.

The association between chronic inflammation and oxidative stress is well documented. Elevated levels of reactive oxygen species (ROS), such as hydroxyl radicals, superoxides, and peroxides in inflammatory conditions have been reported previously.[4,5] Several experimental and clinic studies have also shown increased oxidative stress in chronic esophageal inflammation.[6,7] Since chronic inflammation is known to induce persistent oxidative stress, it would be reasonable to postulate that oxidative stress is a driving force of damage at nuclear level.

The aim of this study was to evaluate DNA damage and oxidative imbalance at the nuclear level in peripheral blood mononuclear leukocytes of patients with ES.

MATERIALS AND METHODS

Patients

Fifty-two children with esophageal caustic burns were admitted to our department between January 2000 and December 2004. Eight patients who were unable to swallow were assumed as ES. In these patients, the barium swallow study was performed to evaluate the length and degree of esophageal strictures. A Stamm gastrostomy was performed in

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patients with severe stricture, and they were put on esophageal dilatation programs using retrograde Tucker's dilators.[8] All dilatations were performed under general anesthesia starting with a dilator smaller than the lumen diameter and going up gradually to the desired number of dilator suitable with the diameter of the patient's esophagus. The dilatations were initially done at 2-week intervals. Depending on the response, there was an attempt to reduce the frequency of dilatations. Five children with ES, who received dilatations over at least one year, were included into the study (patient group). The samples were taken at least one month after the dilatation procedure. There were three boys and two girls; three patients had ingested acid while two had injuries with strong alkalis. The control group consisted of five healthy patients of inguinal hernia. The study was carried out according to the Helsinki Declaration, and written consent to conduct these 'investigations for esophageal strictures' were obtained from the parents or legal guardians of the children involved in this study.

Sample preparation

After an overnight fasting, the blood samples were obtained before dilatation procedure or herniectomy. The blood samples (total 5 mL) were withdrawn from an antecubital vein into heparinized tubes, stored at 10°C, kept in the dark to prevent further DNA damage, and were processed within 2 h. Mononuclear leukocytes isolation for the comet assay was performed using Histopaque 1077 (Sigma Aldrich, Washington DC, USA). An amount of 1 mL heparinized blood was carefully layered over 1 mL Histopaque and centrifuged for 35 min at 1500 rpm and 25°C. The interface band containing mononuclear leukocyte was washed with phosphate buffered saline (PBS) and then collected by 15 min-centrifugation at 1300 rpm. The resulting pellets were resuspended in PBS and the cells were counted in a Neubaer chamber. Membrane integrity was assessed by means of Trypan Blue exclusion method. The remaining blood was centrifuged at 1500 rpm for 30 min to obtain the plasma. The separated plasma was then stored at −80°C for further analysis of plasma myeloperoxidase (MPO) activity, catalase (CAT) activity, total peroxide (TP) level and total antioxidative response (TAR) level.

Comet assay

To assess DNA damage, comet assay was performed according to Singh *et al.*,[9] with the following modifications: 10 μL of resuspended pellet (≈ 20 000 mononuclear cells) were mixed with 80 μL of 0.7% low-melting agarose in PBS at 37°C. Subsequently 80 μL of mixture was layered onto a slide precoated with thin layers of 1% normal melting point agarose (NMA), and immediately covered with a coverslip. Slides were left for 5 min at 4°C to allow the agarose to solidify. After removing the coverslips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 mol/L NaCl, 100 mmol EDTA-2Na; 10 mmol Tris-HCl, pH 10–10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH, and 1 mmol/L Na_2EDTA, pH > 13) at 4°C for unwinding (40 min) and then electrophoresed (25V/300 mA, 25 min). All the steps were carried out under minimal illumination. After electrophoresis, the slides were neutralized (0.4 mole Tris-HCL, pH 7.5) for 5 min. The dried microscope slides were stained with ethidium bromide (2 μ/mL in distilled H_2O; 70 μL/slide), covered with a coverslip and analyzed using a fluorescence microscope (Nikon). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed visually from each subject. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either 0, 1, 2, 3 or 4 (from undamaged class 0 to maximally damaged class 4), so that the total score of slide could be between 0 and 400 arbitrary units (AU).

Biochemical analyses

To evaluate oxidative status of blood plasma, plasma MPO activity, which is main oxidative enzyme, was determined by the method of Bradley *et al.*,[10] which based on kinetic measurement of the formation rate of the yellowish-orange product of the oxidation of *o*-dianisidine with MPO in the presence of H_2O_2, at 460 nm. MPO activity was expressed as units per litre of plasma (U/l). TP concentrations, as another oxidative parameter, were determined using the 'FOX2' method,[11] with minor modifications. TP levels were expressed as μmol H_2O_2/L.

CAT activity, as an index of antioxidative status, was measured by the spectrophotometric method of Aebi,[12] which based on the decomposition of H_2O_2 (IU/mL). TAR status of the plasma was measured using a novel automated colorimetric measurement method developed by Erel.[13] The results were expressed as mmol Trolox equivalent/l.

Statistical analysis

Biochemical parameters were analyzed with a computer based SPSS software, version 11.0 for Windows. All data were expressed as mean ± standard deviation. Differences between the groups were examined using Kruskal–Wallis and Mann–Whitney *U*-test as appropriate. A P-value < 0.05 denoted the presence of a statistically significant difference.

Table 1 Comparative analysis of oxidative, antioxidative parameters, and levels of DNA damage in patients with caustic esophageal stricture and healthy subjects

Parameters	Patients† (n = 5)	Control† (n = 5)	P-value
Plasma MPO activity (U/L)	0.83 ± 0.35	0.09 ± 0.03	0.009
Plasma TP (μmol H_2O_2/L)	0.98 ± 0.38	0.34 ± 0.20	0.047
CAT activity (IU/mL)	0.31 ± 0.03	0.30 ± 0.03	0.347
TAR (Trolox equivalent/L)	1.95 ± 0.13	1.60 ± 0.70	0.251
Mononuclear leukocyte DNA damage (AU)	108.8 ± 51.2	57.6 ± 31.2	0.09

†Mean ± SD; MPO, myeloperoxidase; TP, total peroxide; CAT, Catalase; TAR, total antioxidant response.

RESULTS

At the time of the study, patients with ES were aged 4–7 years (5 ± 1.4 years, mean ± SD). Patients in two groups were similar with regard to age and gender, and the frequency of dilatation in patient group was 10 times per year in average (ranging 9–15 times/year).

As shown in Table 1, Fig. 1, and Fig. 2, analysis of oxidative markers showed that both mean MPO activity and TP levels in ES patients were significantly higher than the control group (P = 0.009 and P = 0.047, respectively), whereas the values of plasma CAT and TAR levels in the plasma of patient group did not differ significantly from the control group (P = 0.347 and P = 0.251, respectively).

Table 1 and Fig. 3 show the endogenous levels of DNA damage (mean AU ± S.E) in mononuclear leukocytes of patients with ES compared to control subjects. Although the mean levels of DNA damage in ES patients were higher than the control patients (108.8 ± 51.2 AU and 57.6 ± 31.2 AU respectively), there was no significant difference between the two groups (P = 0.09).

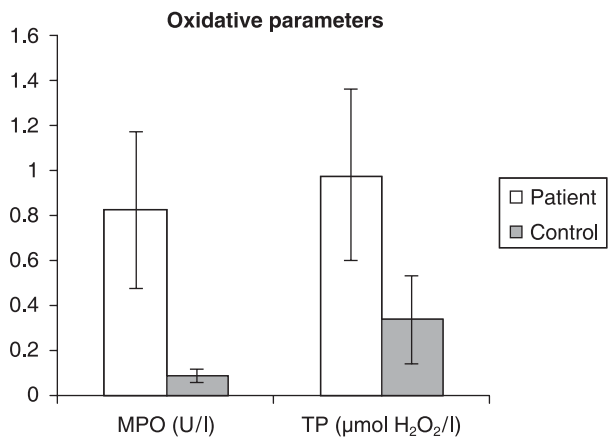

Fig. 1 Mean values ± SD of MPO activity and TP level according to groups. Both two parameters in patient group were found significantly higher than control group (P < 0.05). MPO, myeloperoxidase; TP, total peroxide.

DISCUSSION

Several researches of the caustic esophageal stricture are focused on the systemic effect of stricture and chronic inflammatory processes of the esophagus. It is known that chronic inflammation and irritations promote local or systemic oxidative stress characterized by the generation of ROS and the cellular damage.[5,7,14,15] In this respect, our main focus is on systemic oxidative/antioxidative status of the patients with ES, and the effect of this situation on peripheral mononuclear leukocyte DNA.

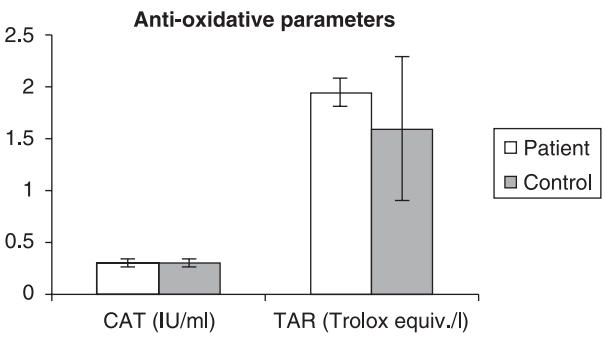

Fig. 2 Mean values ± SD of CAT activity and TAR level as antioxidative parameters are shown. No significant difference was found between groups (P > 0.05). CAT, Catalase; TAR, total antioxidant response.

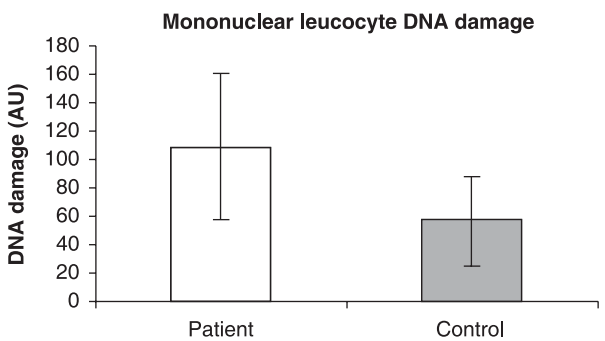

Fig. 3 DNA damage measured by comet assay in the mononuclear leukocytes of children with esophageal stricture *versus* control subjects. The figure shows mean values ± SD of the results of comet assay. DNA damage in patients is higher than control, but difference is not significant (P > 0.05).

We found that plasma MPO activity and TP level were significantly higher, as markers of ROS, in patients with ES than controls. On the other hand, in the evaluation of plasma antioxidative parameters, no difference was noted between two groups. These data suggest that the level of oxidants exceeds the antioxidants, and a systemic oxidative stress occurs in children with ES. It may be speculated that ES accompanied with repeating dilatations or ES itself may play an important role in oxidative stress. Unfortunately it is not known which one is predominant. On the other hand, as shown in our study, children with severe caustic ES often need a large number of dilatations over long periods of time. Inflammation initiates releasing of a variety of cytokines and chemokines that alert the vasculature to release inflammatory cells and factors into the tissue environment, thereby causing oxidative stress.

It has been demonstrated that oxidative stress in chronic inflammation can lead to DNA damage, including DNA adducts, strand breaks and other lesions.[16] The molecular basis for the increased risk is thought to be two-fold: generation by inflammatory macrophages of ROS leads to DNA damage in the surrounding epithelial cells and enhanced proliferative signals mediated by cytokines released by inflammatory cells increase the number of cells at risk for mutations. Several studies have also demonstrated that a consistent link is present between chronic esophagitis and free radical-induced DNA damage in the esophageal mucosa.[7,15,17–20] Chronic inflammation resulting from esophageal reflux gives rise to gastroesophageal reflux disease (GERD) and Barrett's esophagus. In the case of Barrett's, chronic inflammation leads to the production of ROS.[15,17–19] Laboratory experiments suggest that the presence of activation and detoxification enzymes in the esophagus may play an important role in determining the susceptibility of the esophagus to the carcinogenic effect of oxidative DNA damage.[7,15,20] Although the scarred and damaged esophagus might have an increased incidence of carcinoma, limited evidence has been reported.[4,6,17,19,21] Kim *et al.*,[21] have reported that cicatrical carcinoma developed in 13% of the patients with ES who ingested lye. In the same study the interval between the time of injury and the time when the cancer was found ranged from 29 to 46 years.

To investigate the cellular changes in nuclear level in ES, we evaluated by means of the comet assay to measure the levels of DNA damage in mononuclear leukocytes from fresh whole blood of patients with ES. As a result, a higher level of DNA damage in ES compared with blood plasma from the normal healthy controls was demonstrated; however, this analysis revealed that difference was not significant. The medical mechanisms are still unclear, but there is a lot of evidence that oxidative stress contribute to DNA damage, and not just in patients who have already an underlying condition. Although, the relationship between oxidative stress and DNA damage has not been proven, oxidative DNA damage in the patients with ES may play a role in cancer development.

Although our results appear to be encouraging, more studies are necessary to investigate whether the numbers of dilatations or clinical course of any of these patients correlated with a higher level of MPO or total peroxide level. In addition, future assessment of DNA oxidation in the systemic circulation might shed further light on the role of oxidative stress in esophageal malignancy. Further study should be focus on oxidative DNA damage in esophageal tissue of the patient with ES.

In conclusion, this study supports that a systemic oxidative stress in patients with ES exists and that systemic effect can be demonstrated at the nuclear level. These results promise the benefit of adjuvant antioxidant treatment in patients with ES.

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