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The effect of low dose aspirin intake on gastro-duodenal mucosa, and protective effects of GSH-vitamin C-E

[Gastro-duodenal mukoza üzerine düşük doz aspirin alımının etkisi ve GSH-vitamin C-E'nin koruyucu etkileri]

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ABSTRACT

Objectives: Neutrophil-mediated inflammation and oxidative stress may be the important events that lead to gastroduodenal mucosal damage induced by aspirin. It is hypothesized that GSH-vitamin C-E may protect to the upper gastrointestinal mucosa against low dose aspirin's (L-ASA) adverse effects.

Material and methods: Male rats were fed regular diets and maintained for 40 days in the control group (n=8), the L-ASA group (n=8), which was given aspirin (1.44 mg/kg/day) administered intragastrically by feeding tube to rats, or the L-ASA with antioxidant supplement group (n=8), to whom 1.44 mg of aspirin/kg/day + a solution that contained 50 mg vitamin C, 25 mg vitamin E and 25 mg GSH was administered intragastrically by feeding tube to rats every day. After the treatments, blood, stomach and duodenum were taken for pathological and myeloperoxidase (MPO) activity, heat shock protein (Hsp) 70, lipid and protein oxidation analyses.

Results: The stomach and duodenum of the L-ASA group rats had higher scores of pathological findings compared with the control group, whereas the severity of the lesions was found low in the antioxidant-supplemented group according to the L-ASA group. In addition, the gastric mucosal MPO activity and Hsp 70 levels in the L-ASA group were significantly higher than control, but antioxidant supplementation lowered the values of MPO and Hsp 70 in the antioxidant supplemented group (P=0.009, P=0.04).

Conclusion: A simultaneous intake of GSH-vitamin C-E along with aspirin attenuated the gastric injury. GSH-vitamin C and E could play a protective role in the stomach against gastric damage resulting from low dose daily long-term aspirin use.

Key Words: Low dose aspirin, gastro-duodenal pathology, antioxidants, oxidative stress

Conflict of Interest: The authors have no conflict of interest to declare.

ÖZET

Amaç: Aspirin kullanımı üst gastrointestinal mukozada nötrofil-aracılı inflamasyon ve oksidatif strese neden olabilir. Bu çalışmada GSH-vitamin C ve E'nin, düşük doz aspirin alımının yan etkilerine karşı üst gastrointestinal mukozayı koruyucu etkisinin araştırılması amaçlandı.

Materyal ve metod: Erkek sıçanlar normal sıçan diyetiyle beslendi ve kontrol grup (n=8), L-ASA grup (n=8, ratlara beslenme tüpü ile 1.44 mg/kg/gün aspirin intragastrik verildi) ve antioksidan ilaveli L-ASA grup (n=8, 50 mg vitamin C, 25 mg vitamin E ve 25 mg GSH içeren solusyon + 1.44 mg/kg/gün aspirin sıçanlara beslenme tüpü ile intragastrik her gün verildi) olarak 40 gün deneysel süreç devam etti. Muamele işlemleri sonrası; ratların kan, mide ve duodenumları patalojik ve myeloperoksidaz (MPO) aktivitesi, ısı-şok protein (Hsp) 70, lipid ve protein oksidasyon analizleri için alındı.

Bulgular: L-ASA grup ratların mide ve duodenumları kontrol gruba kıyasla yüksek patolojik bulgu skorlarına sahipti, ancak lezyonların şiddeti L-ASA gruba göre antioksidan ilaveli grupta düşük bulundu. Ayrıca gastrik mukozal MPO aktivitesi ve Hsp 70 düzeyleri L-ASA grupta kontrol grubuna kıyasla anlamlı olarak yüksekti, fakat antioksidan takviyesi antioksidan ilave grupta MPO ve Hsp 70 düzeyleri azalttı (P=0.009, P=0.04).

Sonuç: Aspirin ile birlikte alınan GSH-vitamin C-E histolojik gastrik hasarı hafifletti. GSH-vitamin C ve E günlük uzun-dönem düşük doz aspirin kullanımı sonucu gastrik hasara karşı midede koruyucu bir rol oynayabilir.

Anahtar Sözcükler: Düşük doz aspirin, gastro-duodenal patoloji, antioksidanlar, oksidatif stres

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Low dose aspirin (L-ASA) is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) for primary or secondary prevention of ischemic heart disease and ischemic cerebrovascular disease. NSAIDs cause gastrointestinal injury through both topical and systemic effects. The latter is mediated mainly by blocking prostaglandin synthesis through inhibition of the cyclooxygenase (COX) enzymes. The integrity of gastric mucosal defense depends on continuous generation of prostaglandin E_2 and prostacyclin I_2, mediated by COXs. In addition to prostaglandin depletion, topical injury initiates the initial mucosal damages or erosions by disrupting the gastric epithelial cell barrier [1]. This results in enhancement of acid back-diffusion and microvascular injury accompanied by the activation of neutrophils. However, neutrophils not only destroy the foreign substances or the bacteria that exist in the gastrointestinal mucosa, but also compounds produced by activated neutrophils themselves may be potentially harmful for normal tissue. Also, current evidences suggest that NSAID ingestion is a causative factor in the pathogenesis of gastric mucosal injury in humans [1,2]. In response to NSAID, neutrophils are recruited to the site of inflammation and generate excessive reactive oxygen and nitrogen species and proteases. Reactive free radicals cause lipid peroxidation, protein oxidation and DNA injury and then cell or tissue damage when antioxidant defense mechanisms are not always adequate to neutralize the production of free radicals, resulting in a condition termed oxidative stress [3,4,5]. Hsp 70 expression is up regulated upon exposure to oxidative damage. On the other hand, L-ASA is usually needed for a long time, and sometimes other anti-platelet and anticoagulant drugs are co-prescribed on the treatment of disease. It is known that daily low-dose aspirin induces gastric bleeding and gastroduodenal mucosal injury or erosions (40-50%) as an adverse effect similar to other NSAID [6,7]. Because the number of L-ASA prescriptions is considerably high, the total number of gastroduodenal mucosal damages should not be ignored even if the frequency of mucosal injury is small [8]. Low dose ASA-induced mucosal injury may lead to a sudden bleeding peptic ulcer without pain, and it might be fatal as well.

It is well known that GSH, vitamins C and E are the potent anti-oxidants. The present study was designed to investigate low dose aspirin-induced oxidative stress on the gastro-duodenal mucosa and the antioxidant and/or protecting capacity of GSH-vitamin C and E mixture in the state of gastro-duodenal mucosa injury of the rats treated with low dose daily long-term aspirin.

Material and Methods

The study had the approval of the local ethical committee for animal studies. This study was performed in the Experimental Research Center, Ondokuz Mayıs University, Samsun /Turkey. A total of 24 Sprague-Dawley male rats, weighing 250-300 g, were allowed to adapt to laboratory environment for 1 week before the onset of the experiment. The rats were housed in per cage with wood chip bedding and fed on standard laboratory chow and water ad libitum. They were maintained on a 12 h light: dark cycle with a constant room temperature at 22 ± 1°C.

Animal Treatment: The rats were randomized into three groups (n=8): I, the control group, receiving isotonic sodium chloride (0.9% NaCl). II, the L-ASA group, receiving aspirin (suspended in 0.9% NaCl, 1.44 mg/kg/day). III, the L-ASA with antioxidant supplement group, receiving aspirin (1.44 mg/kg/day) + a solution containing up to 50 mg vitamin C, 25 mg vitamin E and 25 mg GSH kg^{-1} body wt./day. L-ASA, 0.9% NaCl and antioxidant mixture (GSH-vitamin C and E, suspended in 0.9% NaCl) solution (1 ml of the solution) were administered intragastrically by feeding tube to rats every day. Treatments were carried out for 40 days. Note that one rat in the group III died at the last day of study.

Laboratory Analyses: At the end of experimental study, the rats were fasted overnight. The next morning, under light ether anesthesia, the heart was punctured using an injector and blood was collected into biochemical test tubes. These test tubes were centrifuged at 2000 g for 10 minutes at room temperature, and the serum was separated. After removing the blood samples, the rats were killed by decapitation and their peritoneal cavity was opened and the stomach and duodenum were removed. These tissues were washed in a cooled 0.9% NaCl solution, dried with filter paper and samples were stored until examination at -70°C. Gastric and duodenal mucosal erosions or hyperemic areas were examined macroscopically. The areas with the most likely mucosal damages on gastro-duodenum were removed by scraping with a blunt knife, weighed and a part of it was minced and homogenized well in liquid nitrogen, and then the homogenate was sonicated for 1 minute at 220 V (Fisher, Sonic Dismembrator; Model 300) in each appropriate cold assay buffer. Homogenates were centrifuged by using a refrigerator centrifuge at 4°C. Then, the supernatants were used for the determination of biochemical analyses. The markers of oxidative stress marked in this study include protein carbonyls, a marker of protein oxidation; malondialdehyde, a marker of lipid peroxidation; and myeloperoxidase, a neutrophil enzyme that produces oxidants. All chemicals used for the assays were of analytical grade and purchased from Sigma Chemical Co.

In the supernatants of gastric and duodenal mucosal homogenate and serum, lipid peroxidation was estimated spectrophotometrically by the thiobarbituric acid-reactive substance (TBARS) method with slight modifications and expressed in terms of TBARS/mg protein [9]. Measurements of protein (carbonyl)

oxidation in the supernatant samples were made by the method of Evans et al. [10] with slight modifications. Carbonyl concentration (nmol/mg of protein) = nmol/ml of carbonyl groups/protein concentrations in mg/ml. The usefulness of measuring MPO activity to assess neutrophil infiltration has been previously reported [11]. MPO activity levels in the supernatants were determined by using hydrogen peroxide as a substrate for the enzyme. One unit of MPO activity is defined as that degrading 1 μmol of peroxide per minute at 25°C and is expressed in units per gr protein. The protein concentration was determined by the method of Lowry et al. [12]. Gastric and duodenal Hsp 70 levels in the supernatant samples were measured with ELISA kit (EKS-700B, Stressgen).

Histopathological Study: For histopathological studies, samples of the stomach and duodenum from the control and experimental group animals were fixed in 10% neutral formalin for subsequent light microscopic examination. Ulcerated stomachs and duodenums were processed by routine methods for subsequent histopathological evaluation. The total number of lesions per stomach and duodenum were counted and each lesion was scored by a pathologist blinded to the experimental groups according to the following scheme [13]. Normal (0); damage to luminal surface and gastric pit cells only (1); superficial gastric gland damage (2); deep gastric gland damage (3); and ulcer (4).

Statistical Analysis

All statistical analysis was performed with SPSS software (SPSS, release 15.0, Inc, Chicago, ILL). Data was tested for normality assumption. They were not normally distributed. Therefore non-parametric statistical analyses were used for all comparisons. Kruskal-Wallis test was used to determine the statistical significance of the differences in the groups. Then Mann-Whitney-U test (with Bonferroni correction) was used for comparisons between groups. Total lesion scores between any two groups compared by Fisher exact test for proportions in the stomachs and duodenums. Values of $P < 0.05$ were accepted as being statistically significant.

Results

Biochemical parameters: Tissue levels of MPO directly correlate with the number of neutrophils in any given tissue. Intragastrically the administration of L-ASA for 40 days resulted in an increase in the MPO activity, an index of neutrophil infiltration in the gastric and duodenal mucosa. MPO activity in the gastric mucosa significantly increased in L-ASA-treated rats compared with that of control rats not given L-ASA (P=0.042). The elevation in MPO activity in gastric mucosa after the treatment of L-ASA was significantly suppressed by the administration of antioxidant mixture as shown in Figure 1 (P=0.009).

As shown in Figure 2, the levels of gastric Hsp 70 were significantly raised in the L-ASA-treated rats compared to control rats (P=0.038). Besides, its levels were reduced in the antioxidant supplemented group when compared to L-ASA-treated group (P=0.04). The results obtained in the present study indicate an effect of low dose daily long-term aspirin intake on the oxidative damage to proteins on the gastric and duodenal mucosa with or without GSH-vitamin C-E. As shown in Table 1, the level of protein carbonyls in gastric tissue was slightly, but insignificantly, increased in the treated groups compared with the control group. Likewise, there was no statistically significant difference between the treated and control groups in terms of TBARS, protein carbonyls, MPO activity and Hsp 70 levels in the duodenal tissue and TBARS levels in gastric tissue (P>0.05).

Histopathology: As indicated in Table 2, 3 and Figure 3, compared with the control group, L-ASA administration induced gastric lesions including damage to luminal surface and gastric pit cells, and superficial gastric gland damage assessed histologically. The former only observed for duodenal tissue. Total lesion scores in the stomach and duodenum were statistically higher in L-ASA treated group than in the control group (P=0.041, P=0.01 respectively). The severity of lesions in gastric tissue was attenuated in antioxidant supplemented group with respect to L-ASA group.

Discussion

Treatment with low dose aspirin (50-325 mg/d) has been permitted frequently as a medicinal remedy for the prevention of thrombus and embolus in patients suffering from ischemic heart disease or ischemic cerebrovascular disease. Nevertheless human and animal studies have indicated that use of L-ASA causes to mucosal damages in the gastrointestinal system, which might lead to oxidative stress, gastro-duodenal erosion, ulceration, and bleeding [6-8,13-16]. In the present study, we observed histologically gastric and duodenal mucosal injuries induced by use of L-ASA daily for 40 days in agreement with previous reports [13,16]. The severity of the lesions was found high on gastric mucosa with respect to duodenal tissue. However, antioxidant supplementation (GSH-vitamin C and E) ameliorated mucosal damages in the gastric tissues as shown in Table 2 and Figure 3.

As cited in literature, protein (carbonyl) oxidation and lipid peroxidation are commonly used as biomarkers of tissue damage or oxidative stress [17,18]. In addition to these, the stomach mucosal damage caused by aspirin appears to involve oxidative stress [19]. In this experimental study, protein carbonyls in gastric and duodenal tissue were higher by a median value in long-term L-ASA-treated animals compared to control as shown in Table 1. As a result of these findings, the administration of L-ASA resulted in gastric and duodenal protein damage. It might suggest that oxidative stress is involved in the production of low dose aspirin-induced

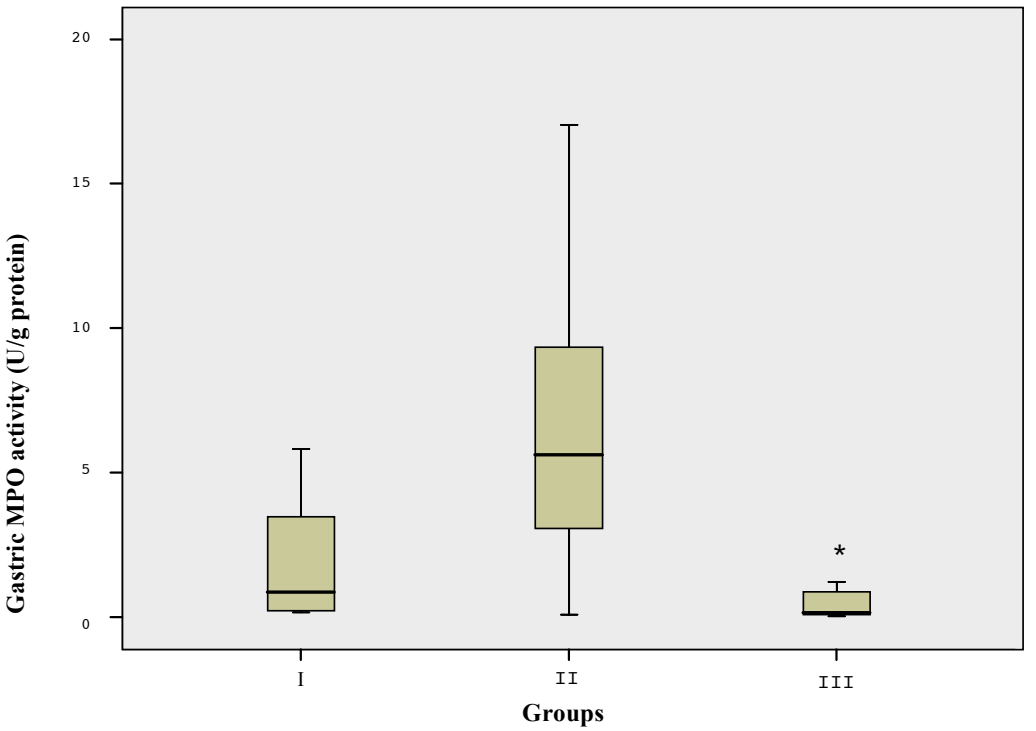

Figure 1. Gastric MPO activity after 40 days of L-ASA treatment and antioxidant mixture combined with L-ASA. GSH-vitamin C and E caused a significant decrease in MPO levels compared with the L-ASA (*P=0.009). Group I: Control, Group II: L-ASA, Group III: L-ASA + GSH-Vit.C-E.

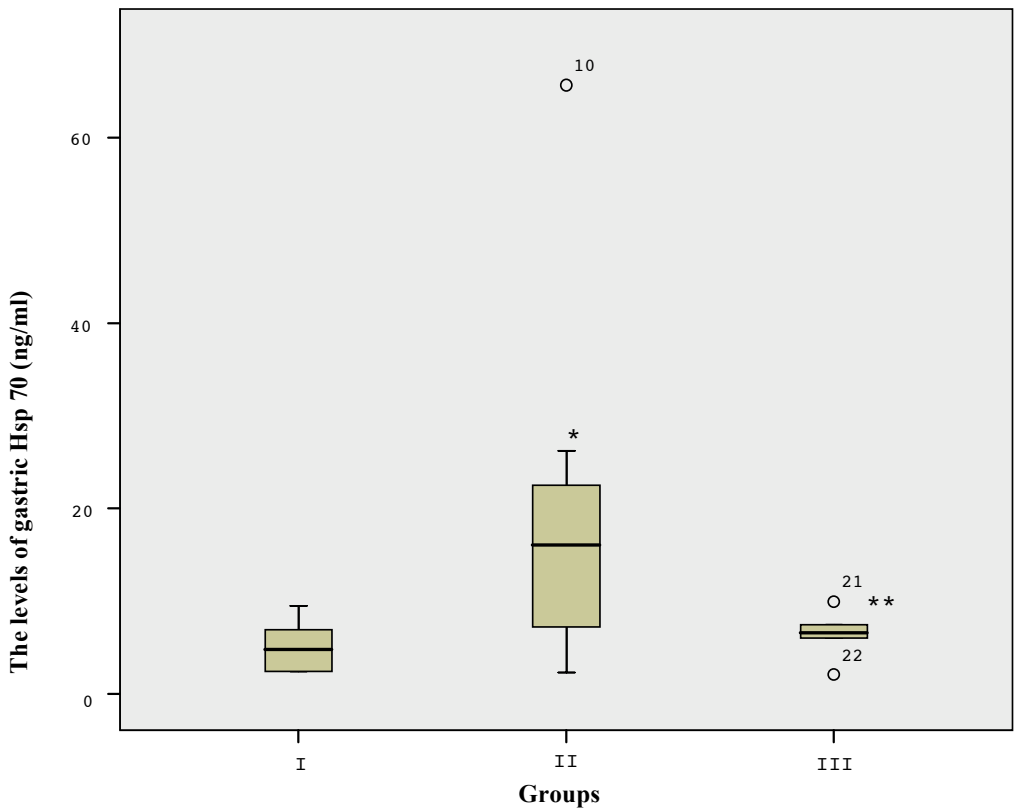

Figure 2. The levels of gastric heat shock protein 70 in the experimental groups. *P=0.038 vs. control values. **P=0.04 vs. L-ASA values. Group I: Control, Group II: L-ASA, Group III: L-ASA + GSH-Vit.C-E. ○[10], ○[21], ○[22]; observations with extreme values.

Table 1. The levels of biochemical parameters in the experimental groups

Parameters	*Groups*		
	Control (n=8)	L-ASA (n=8)	L-ASA+GSH-Vit.C-E (n=7)
Serum TBARS (μmol/L)	0.20 (0.14-0.4)	*0.49 (0.19-2.9)	0.35 (0.1-3.7)
Serum protein carbonyls (nmol/mg protein)	5.02 (0.1-7)	3.3 (2.5-5)	2.3 (0.74-5.34)
Gastric TBARS (μmol/mg protein)	0.03 (0.01-0.09)	0.04 (0.02-0.09)	0.025 (0.02-0.09)
Gastric protein carbonyls (nmol/mg protein)	4.1 (2.8-6.7)	5.9 (1.9-11.5)	4.34 (2.28-9.8)
Duodenal TBARS (μmol/mg protein)	0.4 (0.12-1.54)	0.33 (0.03-1.02)	0.28 (0.08-2.02)
Duodenal protein carbonyls (nmol/mg protein)	3.02 (1.61-7.19)	5.5 (1.57-1.11)	3.8 (0.1-9.6)
Duodenal MPO activity (U/gr protein)	2.6 (0.04-7.7)	2.86 (0.35-13.9)	2.4 (0.28-11.45)
Duodenal Hsp 70 (ng/ml)	3.6 (1.03-9.4)	4.3 (1.34-10)	3.2 (1.7-13.5)

Data are expressed as median (min-max). *Serum TBARS is statistically different in L-ASA group compared to control (P=0.007).
TBARS: Thiobarbituric acid-reactive substance, MPO: Myeloperoxidase, Hsp: Heat shock protein.

Table 2. The scores of histopathology findings in the stomachs of study group rats

The findings of stomach histopathology	*Groups*		
	Control (n=8)	L-ASA (n=8)	L-ASA+ GSH-Vit.C-E (n=7)
*Damage to luminal surface and gastric pit cells	2	4	3
*Superficial gastric gland damage	-	3	-
*Deep gastric gland damage	-	-	-
*Ulcer	-	-	-
*Total Lesion Scores x (%)	2 (0.25)	7 (0.88)	3 (0.43)

(0): lesion absent, (1): damage to luminal surface and gastric pit cells only, (2): superficial gastric gland damage,
(3): deep gastric gland damage, (4): ulcer. L-ASA: Low dose aspirin.

Table 3. The scores of histopathology findings in the duodenums of study group rats

The findings of duodenum histopathology	*Groups*		
	Control (n=8)	L-ASA (n=8)	L-ASA+GSH-Vit.C-E (n=7)
*Damage to luminal surface and gastric pit cells	1	7	5
*Superficial gastric gland damage	-	-	-
*Deep gastric gland damage	-	-	-
*Ulcer	-	-	-
*Total Lesion Scores x (%)	1 (0.13)	7 (0.88)	5 (0.71)

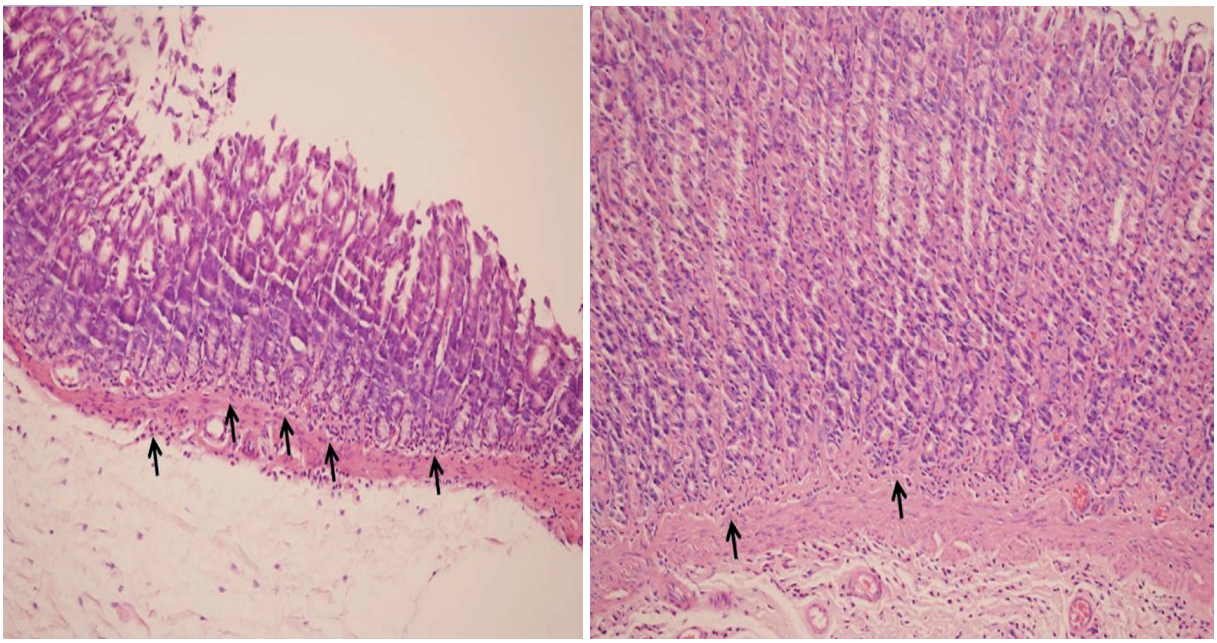

Figure 3. Morphologic changes of gastric tissue at 40th day after the administration of low dose aspirin hematoxylin-eosin stained slides. Representative photo of gastric mucosa: a) demonstrates increased polymorphonuclear cells (arrows) and decrease in mucosal layer in low dose aspirin treated group (x400), b) shows scarce of neutrophils in the antioxidant supplemented group (x200).

gastric and duodenal mucosal injury. In relation to this issue, mucosal pro-inflammatory mediators like TNF-α, IL-1β, IL-8 and PAF are up regulated following NSAIDs included aspirin. These are key mediators of the ensuing gastric injury and healing. TNF-α, IL-8 and PAF strongly promote inflammation and subsequent tissue injury via recruiting leucocytes, particularly neutrophils and monocytes, by inducing adhesion molecules on the vascular endothelium (ICAM-1) and neutrophils (CD11b/CD18), leading to their activation [1,2,20]. Activated neutrophils can cause microvascular and gastric mucosal injury by producing reactive free radicals and proteases [2]. MPO activity is an index of neutrophil recruitment [4]. In our study, gastric MPO activity was measured high in L-ASA treated group compared with the control. From this finding, it may be concluded that activation of neutrophils seems to be important for the development of L-ASA damage, and that neutrophils act as a major source of free radicals in L-ASA treated gastric tissue. In addition to this, administration of GSH-vitamin C and E to rats treated with L-ASA reduced significantly gastric MPO activity as indicated in Figure 1. GSH (glutathione: glycyl-glutamyl-cysteine) is crucial in the body's defense against reactive free radicals as well as the other functions. The oxidation of GSH to GSSG is catalyzed by glutathione peroxidase which is an antioxidant enzyme. The GSH is predominant, normally constituting more than 90%. Normally GSH is not degraded intracellulary but is exported out of the cell [21,22]. It is a scavenger of $O_2^{\cdot-}$ and protects thiol groups from oxidation. The role of GSH as an endogenous antioxidant is to protect gastric mucosa as well [4]. Likewise, vitamin C is a powerful antioxidant that is eliminated reactive free radicals. Pohle et al. [4] have reported that significantly lower gastric juice concentration of vitamin C was found in subjects treated with aspirin. Vitamin E functions as a lipid-soluble antioxidant, scavenging the free radicals protecting the integrity of the cell membrane against oxidant agents [23]. Along with these statements, we hypothesized whether administration of glutathione-vitamin C and E as upper gastrointestinal-protective agents to rats exposed to L-ASA on a long-term basis could be diminished or prevented gastro-duodenal mucosal injuries. As last scientific reports have implied that vitamin C and E possess an inhibitory effect on the activation of neutrophils and oxidative stress in aspirin-induced gastric damage [3,4,24].

The induction of Hsp in the case of oxidative stress, oxidative damage to proteins could be the activating signal [14]. Heat shock proteins are induced by various stressors, including protein damage, and NSAIDs. From these, Hsp 70 provides cellular resistance to NSAIDs and previous reports suggest that it protects gastric mucosa under various pathological conditions including NSAID-induced gastric lesions [14,25,26]. In the current study, the levels of Hsp 70 were found high in L-ASA treated gastric mucosa with respect to control. Also, gastric protein carbonlys were measured high by an average of 43.9 % in this group according to control. So, it may suggest that Hsp 70 in gastric mucosa can up regulate in the state of oxidative stress or protein oxidation caused by L-ASA. However, the values of Hsp

70 in gastric tissues were estimated lower statistically in the antioxidant supplemented group when compared to L-ASA treated group as shown in Figure 2. In this condition, it can suggest that Hsp 70 is down regulated in relation to an oxidative stress reduced with antioxidant mixture in the gastric tissue.

In conclusions, our study has an advantage on a long-term basis investigated firstly in the view of upper gastrointestinal mucosal damage induced by L-ASA intake experimentally. The increase in oxidative stress after long-term L-ASA ingestion depends likely upon neutrophil activation and involves the myeloperoxidase pathway. L-ASA damage may be associated with a significant decrease in antioxidants agents including anti-oxidizing enzymes and non-enzymatic such as glutathione, vitamins C and E that are the non-toxic substances. These substances combined with L-ASA may be useful as gastro-protective agents against gastric mucosal damage. However, the study is limited by the number of animals. The mechanism of the inhibitor effect of antioxidant mixture on the MPO activity was not clarified well in the present study, and further investigation is needed.

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Conflict of Interest: The authors have no conflict of interest to declare.

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