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Effect of fulvic acid on gastric mucosa damage caused by chronic water avoidance stress

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ABSTRACT

We investigated the antioxidant and anti-ulcerogenic effects of fulvic acid (FA) on oxidative damage caused by water avoidance stress (WAS) in rat gastrointestinal mucosa. Three experimental groups were established: control (C), chronic stress (CS), and chronic stress + FA (CS + FA). After WAS, a single dose of FA was administered for 10 days to the CS + FA group. Samples of the pyloric region of the stomach were stained with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS). Immunohistochemical staining was performed for inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). Total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) levels were measured biochemically. By light microscopy, we observed loss of gastric epithelial cells and greater polymorphonuclear cell migration into the mucosa in the CS group compared to the C group. We found intact epithelial cell structure and a thick superficial mucus layer in the CS + FA group compared to the CS group. These findings in the CS + FA group were similar to those for group C. iNOS staining was stronger in the CS group compared to the C group. TOS and OSI levels in the CS + FA group were decreased compared to the CS group, but TAS, SOD, GPx and CAT levels were increased. We found that WAS caused damage to epithelium and connective tissue of the stomach mucosa and that this damage was prevented by FA. Therefore, administration of FA appears to prevent stress induced damage to rat stomach.

KEYWORDS

Antioxidant; fulvic acid; gastric mucosa; inflammation; oxidative stress; rats; stomach; stress; water avoidance

Stress adversely affects one's daily life both physiologically and psychologically (Konturek et al. 2011). The chronic water avoidance stress (WAS) is an appropriate model for mimicking anxiety experienced by people suffering from stress related diseases (Nozu et al. 2014). WAS in rats produces gastrointestinal inflammation, breaks tight connections between intestinal epithelial cells and increases oxidative stress (Ersoy et al. 2008). Stress induced gastric lesions are associated with imbalance between the production and degradation of reactive oxygen species (ROS), i.e., oxidative stress (Oyenihi et al. 2015). ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and peroxynitrite molecules ($ONOO^-$). Reactive nitrogen species (RNS), e.g., nitric oxide (NO^-), participate in generating free radicals. Nitric oxide (NO) is a reactive species with a normal physiological role, but under conditions of oxidative stress, NO potentially is harmful due to reactions with superoxide anion radicals to produce peroxynitrite. Excess production of RNS is called nitrosative stress. Organisms possess enzymatic defenses including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), which are antioxidants that reduce ROS and RNS levels. Under normal conditions, NO in the gastric mucosa protects the mucosa, but under pathological conditions, increased NO becomes a ROS. Increased synthesis of NO increases expression of iNOS and eNOS, which damage the gastric mucosa (Mittal et al. 2014). Other gastrointestinal system (GIS) diseases including peptic gastric ulcer, infectious enteritis and ulcerative colitis are characterized by reduced mucus secretion (Suyama et al. 2018). Damage to the mucosal barrier causes GIS diseases such as Crohn's disease, inflammatory bowel syndrome, gastritis and ulcers (Ma et al. 2004; Farhadi et al. 2005). Proton pump inhibitors and antibiotic combinations are the most common treatments, but they exhibit serious side effects and are costly (Ortac et al. 2018).

Humic substances are high molecular weight heterogeneous substances formed by decomposition of

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plant and animal residues in soil. Humic matter is composed of a variety of humified substances, e.g., humic acid, hymatomelanic acid and fulvic acid (FA) (Tan 2014). FA, MW = 500–5,000 Da, is smaller than humic matter and can pass easily through normal morphological barriers. It has been used in traditional medicine for more than 3,000 years to treat inflammatory diseases (Schepetkin et al. 2002). FA acts as an antioxidant by removing free radicals (Yudina et al. 2011; Winkler and Ghosh 2018). Other investigators have reported that FA is an effective antioxidant; oral and intravenous administration is useful for treating diseases including gastritis, diarrhea and gastric ulcers. (Schepetkin et al. 2003; Agarwal et al. 2007). Although FA appears to be a useful antioxidant, its mechanism remains unclear. We investigated the antioxidant mechanism of FA and its healing effect in cases of GIS damage caused by chronic WAS.

Material and methods

Animals

Our study conformed to ethical standards and national and international guidelines; it was approved by the Ethics Committee of Istanbul Medipol University (Permission/date-number 24/02/2016-27). We used 18 250–300 g male Spraque-Dawley rats procured from the Istanbul Medipol University, Medical Research Center (MEDİTAM). Animals were housed in standard cages at 22–24 °C and 55% relative humidity with a 12 h light:12 h dark cycle. Rats were permitted free access to standard rat pellets and water.

FA preparation

FA as "Pahokee peat fulvic acid standard" (International Humic Substance Society, Denver, CO) was prepared 150 mg/kg in distilled water. The FA solution was stored at room temperature.

WAS

Pools, 50 x 50 × 50 cm, were made from plexiglass. The pools had a 6 × 4 cm platform in the center on which animals can stand and the pools are filled with water to the level of the platform. Stress is created by the animals' aversion to the surrounding water. Animals were exposed to WAS daily for 1 h between 08:00 and 10:00 for 10 days.

Experimental design

Rats were distributed into three experimental groups of six: control (C), chronic stress (CS) and chronic stress + FA (CS + FA). For the CS group, animals were exposed to WAS for 1 h/day for 10 days. For the CS + FA group, 150 mg/kg/day FA was injected intraperitoneally (i.p.) after WAS for 1 h/day each day for 10 days. At the end of the tenth day, animals were sacrificed by decapitation under isoflurane anesthesia (Aerrane Isofluran Volatil).

Histology

Following laparotomy, the pyloric region of the stomach was excised and fixed with 10% neutral buffered formalin for 24 h at room temperature. Specimens were dehydrated through ascending alcohols, cleared with xylene and embedded in paraffin. Sections were cut at 4 µm and affixed to slides. Sections then were deparaffinized, rehydrated and stained with either hematoxylin & eosin (H & E) (Dey 2018) for general morphology or with periodic acid-Schiff (PAS) to examine carbohydrate content of the gastric mucus layer following the supplier's protocol (04–130808A; Bio-Optica, Milano, Italy)

Ten sections were obtained randomly and five similar areas were analyzed semiquantitatively at x 200. Pyloric damage was scored as: 0, normal tissue; 1, mild degeneration of surface epithelium; 2, severe degeneration of surface epithelium and empty mucous cells; 3, loss of surface epithelium and surface mucus layer.

Immunohistochemistry

Endogenous peroxidase activity in some rehydrated sections was blocked with 3% H_2O_2 in methanol for 30 min, then the sections were rinsed with PBS. Sections then were placed in sodium citrate buffer in a de-cloaking chamber (DC2008; Bicare Medical, Pacheco, CA) for 40 min, then sections were cooled at room temperature in the same sodium citrate buffer. Nonspecific labeling was blocked by incubation in a blocking solution (Super Block, 37960; ScyTek Laboratories Logan, UT) for 30 min. Sections then were incubated with iNOS antibody (rabbit polyclonal, QD213201;Thermo Fischer Scientific, Waltham, MA) diluted 1:200 and eNOS antibody, QJ214409; Thermo Fischer Scientific) diluted 1:500 overnight at 4 °C. For the negative control, the primary antibody step was omitted for both iNOS and eNOS. The following day, sections were washed with PBS, then incubated with an anti-polyvalent biotinylated secondary antibody (41865; ScyTek Laboratories) for 20 min. Sections were incubated with diaminobenzydine (DAB) for 5 min and counterstained with hematoxylin, dehydrated through ascending alcohols and cleared with xylene and mounted with Entellan. Sections were examined using an Olympus BX53 and photographed using an attached DP2-SAL digital camera (Tokyo, Japan). Immunohistochemical

staining of pyloric mucosa was scored as: 0, weak; 1, moderate; 2, strong; 3, very strong.

Biochemistry

Samples of pylorus were frozen in liquid nitrogen and stored at −80 °C for biochemical analysis. For analysis, a sample of tissue was weighed, then homogenized in ice cold 0.15 M potassium chloride (KCl) solution using an Ultra Turrax T10 homogenizer (IKA, Wilmington, NC). The homogenate was centrifuged at 600 x g for 10 min at 4 °C and the supernatants were used to measure total antioxidant status (TAS) (mmol Trolox equiv/l), total oxidant status (TOS) (μmol hydrogen peroxide (H_2O_2) equiv/l), and oxidative stress index (OSI) (arbitrary units) using commercial colorometric kits, all from Rel Assay Diagnostics (Gaziantep, Turkey). Other portions of supernatants were used to measure SOD (ng/dl), CAT (ng/dl) and GPx (ng/dl) expression using commercial ELISA kits, all from Bioassay Technology Laboratory (Shanghai, China).

Statistical analysis

Data are means ± SE. Statistical analyses were performed using the GraphPad Prism program. For data with normal distribution, the analysis of variance (ANOVA) test using the *post hoc* Tukey test was applied, while for data for which the distribution was not normal, the Kruskal–Wallis ANOVA with the *post hoc* Dunn's multiple comparison test was used. Values for $p \leq 0.05$ were considered significant.

Results

Histology

H & E staining of group C epithelial tissue and connective tissue exhibited normal morphology (Figure 1A). Connective tissue damage, polymorphonuclear cell migration into the mucosal connective tissue and loss of epithelial cells was observed in the WAS group (Figure 1B). H & E staining for the CS + FA group exhibited more intact epithelial tissue than the CS group. Cell borders were intact without significant cell loss. The morphology of the connective tissue was similar to the control group. Areas of blood leakage appeared in the mucosa near the surface; no areas of blood leakage were observed in the basal and intermediate areas (Figure 1C).

PAS staining for group C revealed a thick and uninterrupted mucus layer on the surface of the gastric mucosa (Figure 1D). We found decreased superficial mucus in the CS group. Despite thinning of the mucous layer, surface mucous cells were stained more intensely by PAS than for group C (Figure 1E). In the CS + FA group, we observed that the superficial mucous layer was interrupted in the damaged areas, but generally was thicker than for the CS group (Figure 1F). We found that mucosal damage in the CS group was increased compared to the C and CS + FA groups; mucosal damage was decreased compared to the CS group ($p < 0.001$ and 0.01, respectively) (Table 1).

Immunohistochemistry

iNOS immunostaining was weak but widespread in group C (Figure 2A). iNOS staining was observed in the superficial regions of the CS group (Figure 2B). Expression was weak in CS + FA group, but more frequent than for group C (Figure 2C). No iNOS expression was found in the negative control sections (Figure 2D). The iNOS expression of CS group was greater than for group C ($p < 0.01$) and for group CS + FA; expression was decreased compared to the CS group, but the difference was not statistically significant (Table 1).

Immunostaining of eNOS was weak, but widespread in group C (Figure 3A). A strong and broad reaction was observed in the CS group (Figure 3B). Expression was weak in the CS + FA group, but more common than for group C (Figure 3C). We found no eNOS expression in the negative control section (Figure 3D). In the CS group, eNOS expression was increased compared to the CS and CS + FA groups. Areas stained for eNOS were decreased compared to the CS group, but differences were not statistically significant (Table 1).

Biochemistry

The CS + FA group exhibited a significant increase in TAS compared to the CS group ($p < 0.05$). The CS + FA groups exhibited significantly decreased TOS and OSI compared to the CS group ($p < 0.05$). The CS + FA groups exhibited significantly increased SOD, CAT and GPx levels compared to the CS group ($p < 0.05$, 0.01, 0.05, respectively). Differences between the C and the CS groups were not statistically significant for any of the biochemical variables (Table 2).

Discussion

Prolonged stress increases ROS production and oxidative stress due to imbalance with the body's antioxidant defense mechanisms (Kwiecien et al. 2014). Oxidative stress causes many gastrointestinal diseases (Chapple et al. 2017). FA is an effective hydroxyl and superoxide radical scavenger that

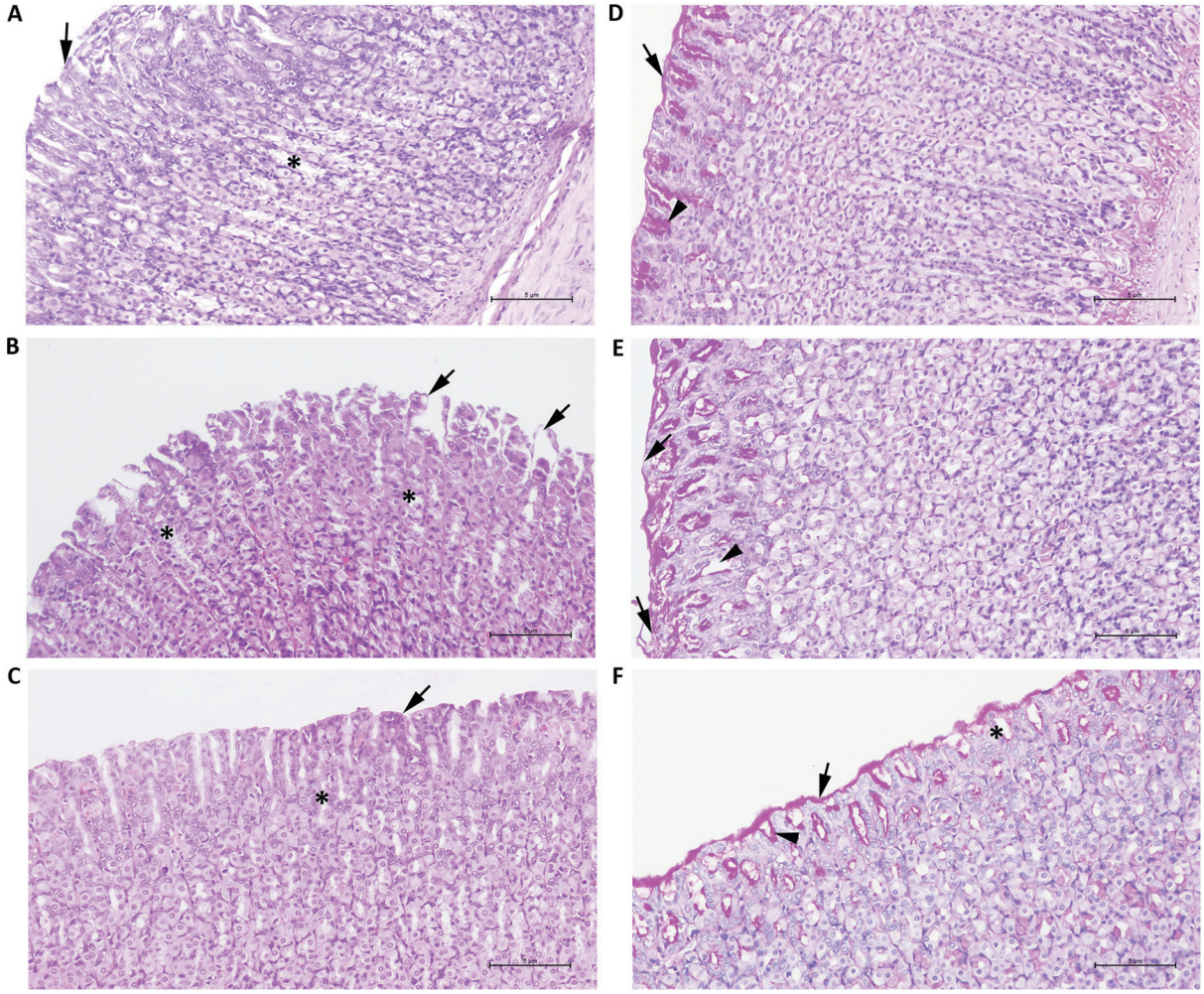

Figure 1. H & E and PAS staining. H & E staining. A) Control group. *Undamaged epithelium; arrow, mucosa). B) CS group. *Dilated epithelium; arrow, loss of surface epithelial cells. C) CS + FA group. *Reorganized mucosa; arrow, intact surface epithelium. Scale bars = 5 µm. PAS staining. D) Control group. Arrow, undamaged, continuous surface mucus layer; arrowhead, mucus filled cells. E) CS group. Arrow, thin surface mucus layer; arrowhead, empty mucus cells. F) CS + FA group. Arrow, restored, thick mucus layer, arrowhead, filled mucus cells, mucus filled cells; *small number of empty cells. Scale bars = 5 µm.

Table 1. Histological evaluation scores.

	Groups			
	C	CS	CS + FA	*p*
Light microscopy	0.33 ± 0.21	2.33 ± 0.33	0.66 ± 0.21	C vs. CS*** CS vs. CS + FA**
iNOS	0.16 ± 0.16	2.5 ± 0.22	1.5 ± 0.34	C vs. CS**
eNOS	1.16 ± 0.30	2.16 ± 0.30	1.33 ± 0.21	ns

Data are means ± S.E; n = 6. C, control; CS, chronic stress; CS + FA, chronic stress + FA; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase. **$p < 0.01$; ***$p < 0.001$; ns, not significant.

reduces the effects of ROS (Rensburg et al. 2001). FA, 150 mg/kg, exerts a protective effect against oxidative stress (Afify and Konswa 2017). We found that FA reduced the harmful effects of WAS on gastric mucosa and increased TAS, SOD, GPx and CAT levels in the CS + FA group compared to the CS group. FA also reduced the mucosal damage induced by WAS. We also found significantly decreased levels of iNOS and eNOS in the CS + FA group compared to CS group.

Rats subjected to WAS for 1 h/day exhibited morphological changes in the intestinal mucosa and inflammatory cell infiltration in the lamina propria by day 10 (Overman et al. 2012; Sun et al. 2013). Goel et al. (1990) reported that administration of 100 mg/kg

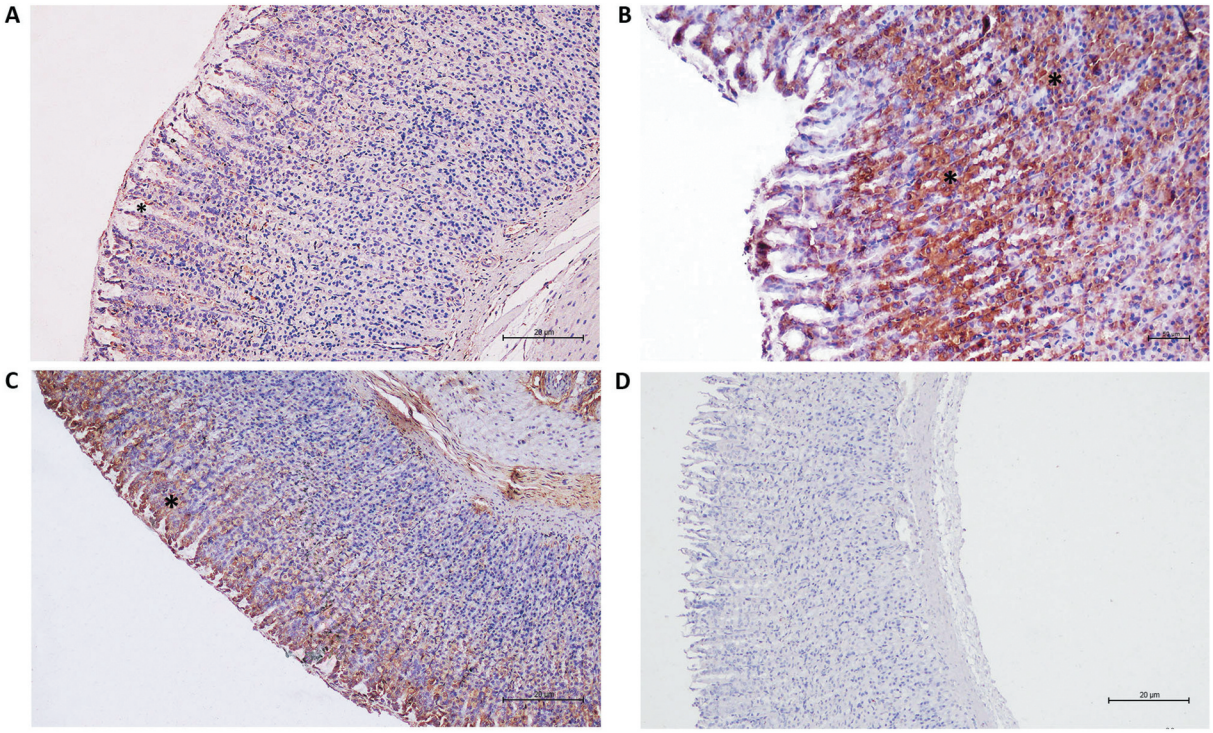

Figure 2. iNOS immunohistochemistry. A) Control group. Weak iNOS immunostaining. B) CS group. Greater iNOS immunostaining area of superficial regions. C) CS + FA group. Less iNOS staining. D) Negative control. No iNOS staining. *iNOS immunostained areas. Scale bars: A, C, D, 20 µm; B, 50 µm.

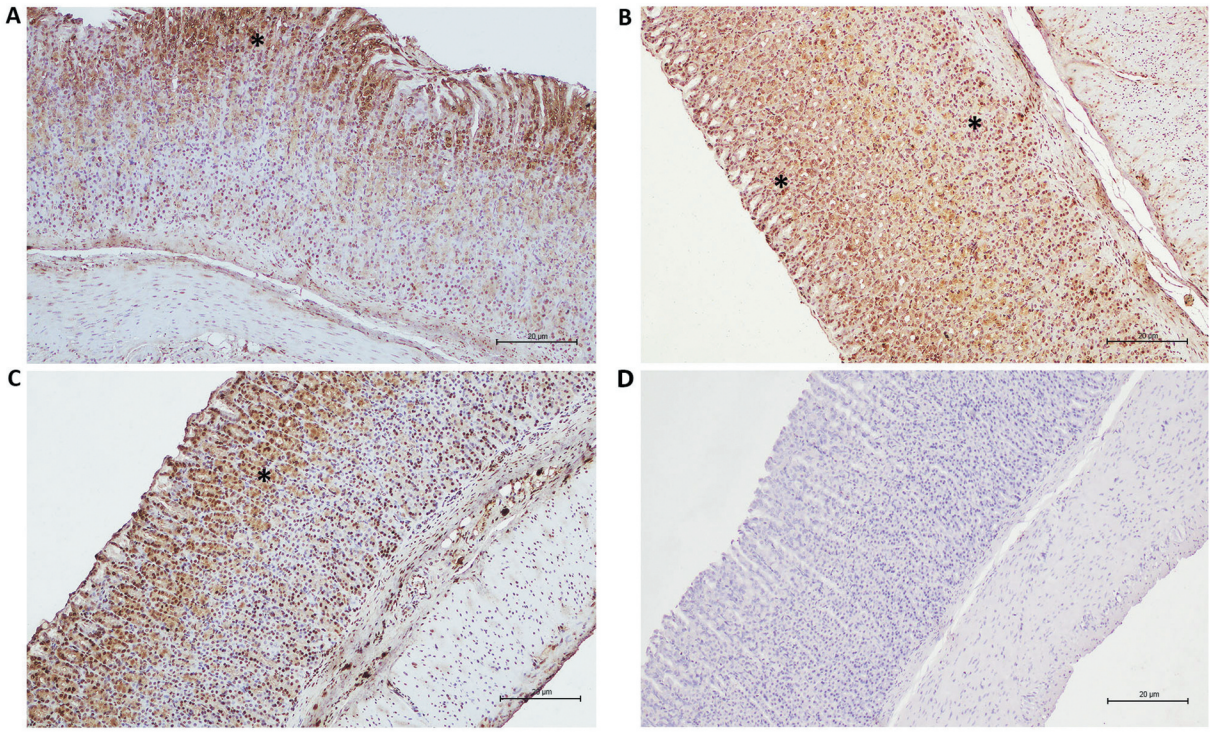

Figure 3. eNOS immunohistochemistry. A) Control group. Weak eNOS immunostaining. B) CS group. Widespread eNOS immunostaining. C) CS + FA group. eNOS immunostained regions. D) Negative control section. No eNOS staining. *eNOS immunostained area. Scale bars = 20 µm.

Table 2. Biochemistry.

Variables	Groups C	CS	CS + FA	p
TAS (mmol Trolox/l)	6.19 ± 0.83	5.29 ± 0.64	8.30 ± 0.73	CS vs. CS + FA*
TOS (μmol H_2O_2 equiv/l)	1.81 ± 0.21	2.95 ± 0.55	1.39 ± 0.27	CS vs. CS + FA*
OSI (arbitrary units)	0.03 ± 0.006	0.07 ± 0.02	0.02 ± 0.004	CS vs. CS + FA*
SOD (ng/dl)	1.09 ± 0.22	0.44 ± 0.13	1.16 ± 0.18	CS vs. CS + FA*
CAT (ng/dl)	8.56 ± 1.27	6.43 ± 1.30	12.90 ± 0.57	CS vs. CS + FA**
GPx (ng/dl)	4.01 ± 0.62	3.10 ± 0.41	5.62 ± 0.84	CS vs. CS + FA*

Data are means ± SE; n = 6. C, control; CS, chronic stress; CS + FA, chronic stress + FA; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase. *$p < 0.05$, **$p < 0.01$.

FA to rats produced anti-inflammatory effects. We found that chronic stress caused significant damage to the gastric mucosa, and FA injected i.p. immediately following WAS prevented this damage due to its antioxidative and anti-inflammatory properties. FA exhibits antioxidative, anti-inflammatory and anti-allergic activity (Nergard et al. 2004; Gandy et al. 2012).

Gastric epithelial cells, epithelial immune cells and mucous layer constitute a mucosal barrier (Blikslager et al. 2007). Disruption of this barrier facilitates passage of bacteria from the lumen to the lamina propria, while increasing nutrient absorption, ion transfer, secretion, motility and visceral sensitivity (Zhu et al. 2014). The mucus barrier between the lumen and epithelium protects the stomach during stress (Laine et al. 2008). We found that the mucosa thickness was decreased by WAS. We found decreased epithelial damage and a thickened mucus layer in the FA treated group compared to the CS group.

The mucosal defense system produces stress-related changes in the mucosa (Repetto Mal 2002). SOD, GPx and CAT are intracellular enzymatic antioxidants that provide defense against oxidants (Odabasoglu et al. 2006). NO normally is responsible for protecting the gastric mucosa from intracellular and extracellular damage. Chronic stress causes conversion of NO to RNS by reducing the amount of SOD, GPx and CAT, which increases the amount of antioxidants in the tissues. We measured TAS and TOS in gastric tissue and calculated OSI by comparing these. The CS + FA group exhibited significantly decreased OSI compared to the CS group. Our biochemical findings are consistent with our morphological findings and suggest that FA supplementation may be useful for restoring oxidant-antioxidant balance under oxidative stress conditions. The relation between ROS and inflammatory reactions has been demonstrated by earlier investigators (Ersoy et al. 2008). ROS can cause harmful oxidative reactions in physiological pathways; ROS can be destroyed by enzymatic and non-enzymatic antioxidant mechanisms in the organism (Cikler-Dulger and Sogut 2020). NO reacts with superoxide (O_2^-) to produce peroxynitrite. Peroxynitrite is an oxidant that causes cellular damage (Lowenstein et al. 1994). Under normal homeostatic conditions, SOD and GPx scavenge O_2 radicals, which increases cellular use of NO and prevent its conversion into ROS (Sahin et al. 2018). An important functions of NO is to protect the stomach mucosa. Under pathological conditions, eNOS dysfunction causes O_2^- production instead of NO, then reduces NO and increases oxidative stress, which causes endothelial dysfunction. Large amounts of iNOS and eNOS are produced under pathological conditions, (Banerjee and Vats 2014). Suo et al. (2015) reported increased expression of iNOS and eNOS due to gastric mucosal damage caused by HCl/ethanol in mice. Kato et al. (2009) reported that iNOS and eNOS expression was observed in the vasculature of the gastric mucosa of normal rats, but that levels increased significantly in arthritic rats. We found that iNOS expression of the CS group was increased due to increased oxidants in the stomach following chronic stress. iNOS immunostaining in the gastric tissue of the FA supplemented group exhibited a weaker reaction than for the CS group. Weaker immunostaining eNOS was observed in the FA treated group compared to the CS group. Therefore, FA controls NO production by decreasing oxidative stress in the cell. In this way, the cells are protected against damage caused by high levels of NO. In the CS group, iNOS and eNOS were increased and SOD, CAT and GPx levels were decreased. Our biochemical oxidative damage results support our morphological findings. The antioxidant, anti-inflammatory, anti-allergic and anti-apoptotic properties of FA have been reported earlier (Schepetkin et al. 2002). It has been suggested also that FA reduces lipid oxidation and increases CAT and SOD levels caused by RNS due to oxidative stress (Stohs 2014). We found that SOD, GPx and CAT activities for the CS + FA group were increased significantly compared to the CS group. In the FA treated group, we found a significant increase in the levels and cellular use of SOD,

CAT and GPx. This results from decreased conversion of NO to RNS, thereby decreasing the amount of oxidant in the environment.

All tissues, especially the gastrointestinal system, are affected adversely by long term stress. Mucosal damage by WAS may be caused by increased ROS and NOS activity and consequent disruption of the balance of antioxidant systems. Antioxidant and anti-ulcer agents may be useful for clinical treatment of stress induced gastric diseases. FA may be useful as an alternative prophylactic for stress induced gastrointestinal damage.

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Disclosure statement

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