

The Effects of Fulvic Acid Against Water Avoidance Stress-Induced Damage of Rat Colon Mucosa

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ABSTRACT

Objective: Chronic stress plays an important role in the etiology of many inflammatory diseases. Reactive oxygen species (ROS), a source of free radicals, act as signaling molecules in the progression of stress-related inflammatory diseases. Oxidative stress occurs as a result of an increase in free radicals in the tissues. The damage caused by oxidative stress can be reduced by antioxidant replacement. In our study, the effect of fulvic acid, a powerful antioxidant, on the damage caused by the water avoidance stress model in the rat colon was investigated morphologically and biochemically.

Methods: Experimental groups (n=6, Sprague-Dawley male rats, 300 g): control (C), water avoidance stress (WAS), and water avoidance stress+fulvic acid (WAS+FA). Rats in the WAS + FA group were given a single dose of FA (150 mg/kg i.p.) immediately after exposure to water avoidance stress. The colons were stained with hematoxylin-eosin and toluidine blue. Total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) were analyzed biochemically.

Results: Compared to the C group, the WAS group showed epithelial damage, a few empty goblet cells, inflammatory cell infiltration, and many active mast cells in the connective tissue. Mucosal integrity, the number of goblet cells, and mast cell activity improved in the WAS+FA group as compared to the WAS group. Biochemically, as compared to the C group, TAS levels decreased, and TOS and OSI levels increased in the WAS group. In the WAS+FA group, TAS levels increased, and TOS and OSI levels decreased with respect to those in the WAS group.

Conclusion: Our findings indicated that fulvic acid reduced the damage caused by chronic oxidative stress in the colon.

Keywords: Fulvic acid; water avoidance stress; oxidative stress; inflammation; colon

1. INTRODUCTION

Stress is an impaired homeostasis that must be balanced by the adaptive stress response (1). When an organism is exposed to internal and external stressors, it develops molecular, cellular, psychological, and behavioral adaptations to maintain homeostasis (2). Stress, which can have physical and emotional types, is a global problem in the modern age. Various experimental animal models have been developed to study the stress-related effects. Chronic water avoidance stress exposure in rodents mimics the experience of stress in humans (3). In experimental animals exposed to chronic water avoidance stress, inflammation in their gastrointestinal systems, damage to epithelial cells, and an increase in oxidative damage in their tissues have been reported (4).

Stress, which is frequently experienced in daily life, stimulates inflammatory responses in multiple bodily systems and causes and aggravates diseases of unknown etiology (5,6). In cases of chronic stress, the formation of reactive oxygen species (ROS) increases in tissues (7). Under normal conditions, ROS

is compensated for by endogenous antioxidants, whereas under stress conditions, antioxidants are insufficient against increased levels of oxidants, and oxidative stress occurs in the tissues. Oxidative stress first causes damage to the lipid and protein components of the cell membranes, then to DNA, and ultimately to the entire cell (8).

Gastrointestinal system disorders are in the foreground of diseases triggered by stressors, such as economic and social pressure, that intensely affect daily life of adults (2,9). The gastrointestinal barrier consists of intestinal epithelial cells, subepithelial immune cells, and mucus layer. Under conditions that increase oxidative stress, such as chronic stress, pro-inflammatory cytokines secreted by mucosal mast cells disrupt this barrier (10). Cell death and tissue damage resulting in oxidative stress in the inflammation area are caused by insufficient endogenous antioxidants against the increased levels of oxidants. When the body's antioxidant

system is inadequate, supplementation with external antioxidants is favored.

Humic substances, which have antioxidant and anti-inflammatory properties, have been used as supplements against inflammatory diseases in conventional medicine for more than 3000 years (11). Humic substances are divided into two forms, humic acid and fulvic acid, depending on their solubility in acids and bases (12). Because the average molecular weight of fulvic acid is 500–5000 Da, it can cross all morphological barriers. Because it has an organic structure, the body does not perceive fulvic acid as an antigen and can easily enter the targeted area (13). In this study, we investigated the protective effects of fulvic acid against the oxidative damage caused by daily stress in the colon.

2. METHODS

2.1. Animals

Eighteen male Wistar albino rats weighing 250–300 g were used in this study. Animals were housed in standard cages with a 12 hours light/dark cycle at 22°C and 55% humidity during the experiment. They were fed standard pellets and tap water (*ad libitum*). Ethics committee approval was obtained from İstanbul Medeniyet University (Decision date and number: 20/08/2020-42), and experimental studies were conducted at this institution.

2.2. Fulvic Acid Preparation

Fulvic acid (“Pahokee Peat Fulvic Acid Standard II”) (International Humic Substance Society, Denver, CO, USA; cat no:2S103F) was homogeneously dissolved in distilled water at a ratio of 150 mg/kg. The fulvic acid solution was maintained at room temperature (14).

2.3. Water Avoidance Stress Protocol

Plexiglas pools of dimensions 50 cm × 50 cm × 50 cm were used for chronic water avoidance stress. The pools had a platform of size 4 cm × 6 cm in the center. They were filled with warm water, up to 1 cm below the platform. The rats were left on the platform for 1 h a day. The stress protocol was repeated for 10 consecutive days and was applied to all rats between 08.00–10.00 am (15). On the last day of the stress protocol, the rats were sedated with 4% isoflurane. The colons were obtained and fixed in Bouin’s solution for histological examination. Colons reserved for biochemical analysis were stored at – 80 °C until analysis.

2.4. Experimental Design

The rats were divided into three groups of six animals each: control (C), chronic stress (WAS), and chronic stress+fulvic acid (WAS+FA). The C group did not receive any treatment for 10 days, the WAS group was subjected to the water

avoidance stress protocol for 10 days, and the WAS+FA group was subjected to water avoidance stress followed by intraperitoneal (i.p.) injection of 150 mg/kg fulvic acid (International Humic Substance Society, Denver, CO; cat no: 2S103F) for 10 days.

2.5. Histological Analysis

In the colon, the general tissue morphology was examined with hematoxylin-eosin (H&E), and the morphology of the goblet cells was evaluated by applying the periodic acid-Schiff (PAS) reaction. In addition, morphometric evaluation of the amount and morphology of mast cells in the mucosa was performed using toluidine blue staining (TB).

The colon tissues obtained for histological examination were fixed in Bouin’s solution, dehydrated in an alcohol series starting from 70% to 100%, and cleared in xylene. Tissues were kept in paraffin overnight in an incubator at 60°C and embedded in paraffin blocks at room temperature. For histological evaluations, 5 µm-thick sections were prepared from paraffin blocks with a microtome (Leica RM, IL, USA) and stained with H&E, PAS, and TB. For the morphometric evaluation of mast cells, a total of 10 sections were taken, one in every five sections, and mast cell counts were performed in five different areas of each section using a light microscope (Zeiss PrimoStar, Oberkochen, Germany). After counting the granulated and degranulated mast cells at x400 magnification, the microscope field was adjusted to x100 magnification and the section was photographed.

2.6. Biochemical Analysis

For biochemical analysis, the total antioxidant status (TAS) and total oxidant status (TOS) were determined in tissues stored at – 80 °C, and the oxidative stress index (OSI) was determined as the ratio of TOS to TAS (16).

For biochemical studies, colons were homogenized in 0.15 N potassium chloride (KCl) solution using Ultra Turrax T10 (IKA, Wilmington, NC, USA). TAS and TOS levels were determined in the supernatant obtained after centrifugation of the colon homogenates using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). The oxidative stress index (OSI) was calculated as the ratio of TOS to TAS (16).

2.7. Statistical Analysis

GraphPad Prism 5.03 (GraphPad Software Inc.) program was used for statistical analysis. One-way ANOVA and post-hoc Tukey test were performed for data suitable for normal distribution. Kruskal-Wallis test and post-hoc Dunn’s comparison test were used for data not suitable for normal distribution. Results were given as mean ± standard error (SE) or mean (minimum-maximum). A p value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Histological Results

In the C group, H&E staining of the colonic mucosa exhibited a normal morphology. PAS staining in the C group revealed that the glycocalyx continued uninterrupted; the goblet cells were generally filled with mucus and they showed intense PAS (+) staining. Few mast cells were found in the submucosal layer of some of the TB-stained colons (Figure 1 a, b, and c).

In the WAS group, H&E staining revealed that there were losses of goblet cells and enterocytes in the epithelial tissues and openings in the connective tissue of the colon. In addition, extensive inflammatory cell migration was observed in the connective tissues. The superficial mucus layer of the colon sections stained with PAS was thin, and the PAS staining was weak in the goblet cells. In the sections stained with TB, no mast cells were found in the mucosa, whereas an increased number of granulated and degranulated mast cells was observed in the submucosa (Figure 1 d, e, and f).

In the WAS+FA group, the staining with H&E showed reduced cellular loss in epithelial tissues, and the morphology was intact in most areas, similar to the C group. In addition, similar to the C group, the continuity of the glycocalyx layer was preserved in the sections stained with PAS and goblet cells showed intense PAS (+) staining. Few granular mast cells were observed in the submucosa of TB-stained colon sections, but no mast cells were found in the mucosa (Figure 1 g, h, and i).

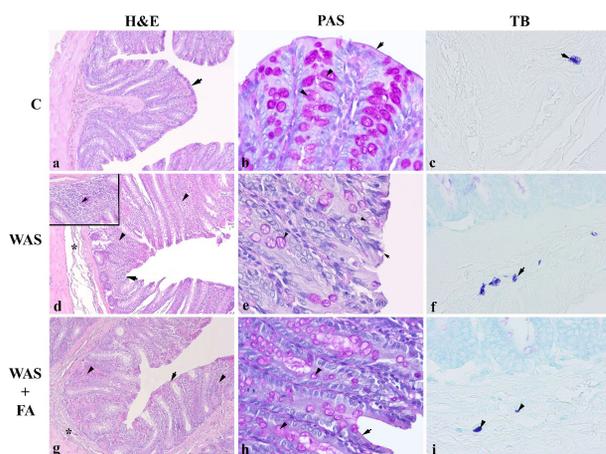


Figure 1. C: Intact colonic epithelium of the control group (arrow), (a); Goblet cells (arrowhead) and intact glycocalyx (arrow), (b); Few mast cells in connective tissue (arrow), (c). WAS: Damaged epithelium (arrow), increased inflammatory cells in the lamina propria (arrowhead), and detachments in submucosa (asterix), (d); Discontinued glycocalyx (arrow), few and low-intensity PAS (+) reacted goblet cells (arrowhead), (e); Numerous active, degranulated mast cells (arrow) in connective tissue, (f). WAS+FA: Intact colonic epithelium (arrow), inflammatory cells persisting in the lamina propria (arrowhead), integrity in connective tissue organization (asterix), (g); Intact glycocalyx (arrow), numerous PAS (+) reacted goblet cells (arrow head), (h); Few granular mast cells in connective tissue (arrow), (i). H&E staining, x100 microscope magnification (a, d, g) and H&E staining, x400 microscope magnification (d, insert); PAS reaction, x400 microscope magnification (b, e, h); TB staining, x400 microscope magnification (c, f, i).

3.2. Biochemical Findings

TAS (mmol Trolox/L) levels in the colon samples were lower in the WAS group than in the C group and higher in the WAS+FA group than in the WAS group. However, these changes in the TAS levels were not statistically significant (n.s.); (Table 1). TOS ($\mu\text{mol H}_2\text{O}_2$ eq/L) and OSI (arbitrary units) levels increased significantly in the WAS group compared to the C group and decreased significantly in the WAS+FA group compared to the WAS group ($p < .05$) (Table 1).

Table 1. Comparison of TOS, TAS and OSI values of C, WAS and WAS+FA groups.

Groups → Values ↓	C	WAS	WAS+FA	p
TOS	0.469 ±0.13	0.929 ±0.22	0.537 ±0.13	C-WAS* WAS-WAS+FA*
TAS	3.165 (2.43-3.99)	2.491 (2.00-2.99)	3.918 (3.20-4.83)	ns
OSI	17.05 ±7.90	39.37 ±13.28	14.56 ±6.45	C-WAS* WAS-WAS+FA*

Data are means ± SE; n=6. C, control; WAS, water avoidance stress; WAS+FA, water avoidance stress+fulvic acid; TOS, total oxidant status; TAS, total antioxidant status; OSI, oxidative stress index. * $p < 0.05$, ns: not statistically significant.

4. DISCUSSION

In this study, it was shown that prolonged water avoidance stress, which mimics daily life stress, increases ROS production in colon tissues, leading to oxidative damage. Stress causes the formation of reactive oxygen species (ROS) in tissues of several systems (7). In this study, we hypothesized that the harmful effects of oxidative stress can be minimized by supplementation with fulvic acid, a powerful antioxidant and anti-inflammatory agent. Chronic stress is a cognitive, behavioral, physiological, and psychological response to long-term internal and external stressors. Chronic stress leads to the deterioration of homeostasis in organisms (17). Anxiety and depression, which increase under the influence of chronic stress, significantly reduce the quality of life of individuals and even cause an increase in mortality (18). Animal models developed to mimic life stress, which causes depression and anxiety in humans, have shown that stress is an important risk factor for the occurrence or accumulation of diseases in various organ systems. (5,19). The organism develops cognitive, behavioral, physiological, and psychological adaptations to adapt to chronic stress conditions; however, under repetitive or long-term stress conditions, homeostasis is impaired and cellular damage and inflammation occur due to increased oxidative stress in tissues. The gastrointestinal tract is highly sensitive to stress factors (2). Irritable bowel syndrome (IBS) is an example of a gastrointestinal tract disease that is associated with stress-induced symptoms such as fatigue, anxiety, and depression, and significantly reduces the quality of life of individuals (20). Studies have reported that, similar to the clinical findings of IBS, under chronic stress conditions, mucosal barrier

permeability increases in the colon, the mucus layer becomes thinner, epithelial cell hyperplasia and neutrophil and mast cell infiltration into connective tissue increases (21,22).

The water avoidance stress model, which experimentally mimics physiological and psychological stress, mimics life stress when applied for ten days (19). This stress model stimulates stress-related mechanisms in the brains of rats and mice. In the presence of chronic stress, rats exhibit behaviors such as anxiety and major depression (23). In this study, it was observed that compared with the other groups, the animals in the WAS group showed more grooming and aggression. In addition to the behavioral responses of the animals in the WAS group, epithelial damage in the colon tissues and an increase in inflammatory cells in the connective tissue were observed. At the same time, an increase in the number and activity of mast cells was observed in the colon tissues of the WAS group compared with the control group. The increase in the number and activity of mast cells, which play a key role in the pathophysiology of stress, causes increased permeability of the colon tissue (24).

Mast cells play a critical role in the pathogenesis of inflammatory diseases by secreting mediators that stimulate inflammatory cell migration from the blood to connective tissue (6). In our study, we observed that histological damage in the colon tissues of the WAS group increased mast cell activation, inflammation, and oxidative stress. In our study, we observed that the total oxidant (TOS) amount and oxidative stress index (OSI) increased as a result of damage to the colon tissues of rats in the chronic stress group. The increase in the amount of TOS and OSI in the colon tissues of the WAS group and the decrease in the amount of TAS indicated that oxidative stress in the colon tissues increased and the endogenous antioxidants in the body were depleted. This suggests that pro-inflammatory cytokines secreted by mast cells migrate from the blood to connective tissue against oxidative stress, increase mucosal permeability, and stimulate inflammation. Similar to our results, Sun et al. reported morphological changes in the intestinal mucosa and increased inflammatory cell infiltration in experimental animals exposed to a 10-day chronic water avoidance stress (25). Zeybek et al. reported that a 5-day chronic water avoidance stress caused mucosal degeneration, inflammatory cell infiltration, and mast cell activation in the rat gastrointestinal tract (26). In our study, it was observed that the number of goblet cells in the colon tissues in the WAS group decreased compared to that in the C group and showed a weaker PAS (+) staining. The decrease in the number of goblet cells and mucus secretion under stress conditions suggests that goblet cells are also damaged by oxidative stress like other cells, correspondingly mucus secretion is reduced. Similar to our findings, Söderholm et al. reported that the number of goblet cells containing mucus in the ileum and colon tissues decreased as a result of a 10-day WAS (21).

Supplementation with exogenous antioxidants can minimize the effects of oxidative stress caused by long-term stress in tissues (27). It has been reported that inflammation induced

by oxidative damage caused by ethanol in the gastrointestinal tract can be reversed with natural compounds with antioxidant effects (28,29). It is known that substances such as peat, spropel, and shilajit containing fulvic acid as well as several agents such as antioxidant compounds, vitamins A and E, melatonin, and alpha-lipoic acid have been used in traditional medicine for more than 300 years (11). Due to its antioxidant, anti-inflammatory, anti-allergic, and anti-apoptotic properties, fulvic acid is an important agent that has been studied for many years in the field of medicine (11).

A study by Goel et al. in rats reported that treatment with 100 mg/kg/d fulvic acid played an antiulcerogenic and anti-inflammatory role (30). Bahçivan et al. concluded that 150 mg/kg fulvic acid could be used as a therapeutic agent against testicular damage caused by chronic stress (15). In our study, fulvic acid was administered to rats via intraperitoneal (i.p.) injection immediately after the chronic stress protocol. The epithelial cell morphology of the group administered fulvic acid after chronic stress was similar to that of the control group. The number of goblet cells and the level of PAS (+) staining increased. In the same group, it was observed that the number and activation of mast cells in the connective tissue decreased compared to that in the chronic stress group. In parallel with the findings on mast cells, the significant decrease in leukocyte infiltration in the connective tissue in the WAS+FA group as compared to the stress group suggests that fulvic acid contributes to anti-inflammatory mechanisms and may have a protective effect. Fulvic acid may have decreased the mast cell activation via antiallergic activities mediated by the biphenyl-type hydrocarbons present in it (11). According to the biochemical results obtained from the group administered fulvic acid after stress, TOS and OSI values decreased compared to those in the stress group, while TAS values increased. According to these findings, since fulvic acid has an antioxidant effect, the TAS ratio in the colon tissues increased compared with that in the chronically stressed group, and the TOS and OSI ratios decreased. As a result of the reduction in oxidative stress, the damage caused by stress in tissues, mast cells, and inflammation were also reduced. Our biochemical results, which are compatible with our morphological findings, support our hypothesis that fulvic acid supplementation may be beneficial for restoring the oxidant-antioxidant balance that is disrupted under oxidative stress conditions.

5. CONCLUSION

In conclusion, fulvic acid can be used as an alternative preventative agent against stress-induced colonic damage owing to its antioxidant and anti-inflammatory properties. The findings of this study may serve as the basis for future experimental studies.

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Author Contributions:

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Design of the study: E.Ç.

Acquisition of data for the study: İ.S., S.G.A., S.K.

Analysis of data for the study: E.Ç., İ.S., S.G.A, S.K.

Interpretation of data for the study: E.Ç., İ.S., C.H.

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