Research Article

Acacetin ameliorates acetylsalicylic acid-induced gastric ulcer in rats by interfering with oxidative stress, inflammation, and apoptosis

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Abstract

Objectives: Gastric ulcer (GU) is a benign lesion in which excessive acid and pepsin activity affects the mucosal epithelium and is common worldwide. Gastrointestinal disturbances come to the fore among the side effects observed in the treatment with drugs such as aspirin. Acacetin is a plant-derived flavonoid with intriguing properties such as anti-inflammatory, antioxidant, and anticancer. The aim of the study is to investigate the effects of acacetin in GU model caused by aspirin active ingredient acetylsalicylic acid.

Methods: Thirty-two Wistar albino rats were divided into four groups: Control, GU, acacetin, and GU +acacetin. Acetylsalicylic acid (150 mg/kg) and acacetin (25 mg/kg) were administered intraperitoneally as a single dose. Gastric lesions were examined microscopically and macroscopically. TNF-a, cyclooxygenase-2 (COX-2), and nuclear factor kappa B (NF-kB) for inflammation; Caspase-3 and Bcl-2 for apoptosis, total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) for oxidative stress were analyzed.

Results: Bcl-2 and TAS values were decreased, while Tumor necrosis factor-alpha (TNF-α), COX-2, NF-kB, Caspase-3, TOS, and OSI values were increased in the GU group compared to the control group. Bcl-2 and TAS values were increased and TNF-α, COX-2, NF-kB, Caspase-3, TOS, and OSI values were decreased in the GU +acacetin group compared to the GU group. The GU index (GUI) detected in the GU group decreased significantly with the administration of acacetin.

Conclusion: High doses of ASA contributed to the formation of GU in the stomach tissue by increasing the levels of inflammation, oxidative stress, and apoptosis, whereas ACA reduced the ulcer damage by reducing the increase in all these pathways.

Keywords: Acacetin, acetylsalicylic acid, apoptosis, gastric ulcer, inflammation, oxidative stress

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Gastric ulcer (GU) is a benign lesion in which excessive acid and pepsin activity affect the mucosal epithelium [1]. GU is common worldwide, affecting approximately 10% of the world's population [2, 3]. Frequent use of non-steroidal anti-inflammatory drugs (NSAIDs) is prominent in the formation of GU; in addition, stress, alcohol, spicy foods, and smoking are among other causes [1].

Aspirin (acetylsalicylic acid), one of the NSAID drugs, is widely used for preventing cardiovascular thrombosis, fever, and pain [4]. Gastrointestinal (GI) disorders are one of the most common side effects observed in treatment with NSAIDs such as aspirin. Stomach lesions and GI bleeds are the best-known of these side effects [5]. Although many drugs are used for treating GU, different treatment options are required because these drugs have side effects and do not provide treatment at the desired level [6].

Flavonoids, one of the natural phenolic compounds found in many fruits and vegetables has many beneficial effects, among which anti-inflammatory, antioxidant, anti-bacterial,

and anti-cancer properties come to the fore [7]. Flavonoids play a key role both in disease and in the management of vigor and hearty health [8].

Acacetin (5,7-dihydroxy-4'-methoxyflavone, ACA) is a plant-derived flavonoid that has recently gained worldwide attention [9]. The reason for this interest is that ACA has anti-inflammatory, anti-cancer, diabetes, and anti-obesity effects and as neuroprotective and cardioprotective effects [10]. It has been determined that ACA inhibits nuclear factor kappa B (NF-kB), which is the active agent in inflammation, and accordingly decreases cyclooxygenase-2 (COX-2) gene expression [11]. Since ACA is a newly focused active substance, its potential effects on GU have not yet been clearly understood.

The purpose of the present study is to determine the effects of ACA on key parameters effective in oxidative stress, inflammation, and apoptosis in GU induced by ASA, the active ingredient of aspirin, which is frequently used for treating diseases such as pain and fever. For this aim, biochemical, molecular, and histopathological methods were used.

Materials and Methods

Groups

Thirty-two male *Wistar albino* rats (220–250 g, 3 months) were used in the experiments. Experimental animals were obtained from Aksaray University Experimental Animals Center (Aksaray, Türkiye). Ethical approval was obtained by Aksaray University Animal Experiments Local Ethics Committee (No: 2020/3-7, Date: December 30, 2020). Rats were housed under standard conditions. The rats were deprived of chow for 24-h before the experiment procedures but were provided ad libitum access to water. The animals were randomly divided into four groups (n=8) as control (CNT), GU, acacetin (ACA), and gastric ulcer+acacetin (GU+ACA).

Experimental protocol

ASA (Sigma, ≥99.0%, St. Louis, USA) was dissolved in distilled water (37°C) then given as 1 ml (150 mg/kg) intragastric gavage to create a GU model [3]. ACA (Cayman, ≥98%, Michigan, USA) was dissolved in normal saline and administered intraperitoneally at 25 mg/kg [12]. ACA treatment was performed 2 h after ASA administration. After 2 h, blood, and stomach tissues were collected (total of 4 h).

Blood and tissue collection

Four hours after ASA administration, rats were decapitated under ketamine/xylazine anesthesia, and gastric tissues and blood samples were collected. To be used in biochemical analyses, blood samples were separated into serum by centrifugation at 3000 rpm for 5 min and the serums were stored at −80°C until analysis. Stomach tissues were photographed for macroscopic analysis. Then, part of the stomach tissue was stored at −80°C for use in molecular analysis, and the other part was used for histopathological examination.

Measurement of inflammation markers

NF-κB and tumor necrosis factor-alpha (TNF-α) levels and COX-2 activity were analyzed from serums using commercial ELISA kits (BT Lab, Shanghai, China). Concentration values were determined at 450 nm absorbance using the Elisa Plate Reader (Chromate, Florida, USA).

Oxidative stress index

The total antioxidant status (TAS) and total oxidant status (TOS) of serums were measured with commercial kits (Rel Assay Diagnostic, Gaziantep, Türkiye). Measurement with these commercial kits is an automatic measurement method developed by Erel. TAS as mmol Trolox Equiv./L and TOS as μmol H_2O_2 Equiv./L was expressed [13, 14]. The calculation method of Zeren et al. [3] was used to determine the oxidative stress index (OSI).

Molecular analysis

After all procedures were completed, RNA isolation from the gastric tissues was performed with a commercial kit (Hydra Biotechnology, Van, Türkiye). cDNA was obtained from RNAs using a commercial kit (Atlas Biotechnology, Ankara, Türkiye). The transcription levels of the apoptotic Caspase-3 (F:5'-GACTGCGGTATTGAGACAGA-3'; R:5'-CGAGTGAGGATGTGCATGAA-3') and anti-apoptotic Bcl-2 (F:5'-GTGGATGACTGAGTACCTGAAC-3'; R:5'-GCCAGGAGAAATCAAACAGAGG-3') genes and the expression level of the inflammatory NF-kB gene (F:5'-CACTGTCAACAGATGGCCC-3'; R:5'-GATAACCTTTGCAGGCCCCA-3') were measured from cDNAs. β-actin (F:5'-CTATCGGCAATGAGCGGTTCC-3'; R:5'-TGTGTTGGCATAGAGGTCTTTACG-3') was used as the housekeeping gene. PCR mixtures (7 μl) were electrophoresed on a 2% agarose gel, which was subsequently stained with SafeView Classic (ABM, Richmond, Canada). Gels were scanned with a UV scanner, and densities were measured with the ImageJ program [15].

Macroscopic examination

After the gastric tissues were taken and cleaned with physiological saline, they were stretched and examined. The number and area of bleeding foci in gastric tissues were determined for GU index (GUI). The criteria and method determined by Hladkykh were used for ulcer severity scoring [16]. 0 points: No damage; 1 point: Presence of one or more of the features in the list: edema, bleeding(s), ulcer(s) up to 1 mm in diameter, not more than three; 2 points: More than three ulcers up to 1 mm in diameter or one ulcer up to 3 mm in diameter; 3 points: Presence of at least one ulcer up to 4 mm in diameter; 4 points: several ulcers up to 4 mm in diameter; and 5 points: Perforated ulcer [16]. GUI was determined according to the formula GUI= UN+USS+AGU×10^{-1}. UN: total number of ulcers/number of animals (per group), USS: ulcer severity score, and AGU: number of animals with GU per group [17].

Light microscopy examination

At the end of the experiment, gastric tissues were fixed in a 10% formalin solution to evaluate histological changes. Tissues fixed after 24 h were passed through an ascending series of alcohol. Then, the tissues cleared with xylene were placed in paraffin and blocks were prepared. Sections of 5 μm thickness were taken from paraffin blocks using microton and stained with hematoxylin and eosin (H&E) dyes. Images under the light microscope (Olympus Cx 43; Japan) were transferred to digital media and evaluated.

Statistical analysis

SPSS 26.0 (SPSS Inc., Chicago, IL, USA) package program was used to evaluate the data obtained for this study. Data were given as mean±standard error (mean±SE). The normal distribution of the data was assessed using the Shapiro–Wilk test. Data with a normal distribution were evaluated using analysis of one-way ANOVA and non-normally distributed data were assessed with the Mann–Whitney U test. P<0.05 was considered statistically significant.

Results

Inflammation findings

The levels of TNF-α, COX-2, and NF-kB parameters were measured to determine the serum inflammation level. A significant increase was found in TNF-α (p=0.028), COX-2 (p<0.001), and NF-kB (p<0.001) parameters in the GU model group compared to the CNT group. These parameters, which increased in the GU group, decreased with the ACA treatment and the decrease became significant at the NF-kB (p<0.001) level (Fig. 1).

Oxidative stress findings

OSI was determined by measuring TOS for the oxidant level and TAS for the antioxidant level of serum samples. TAS (p=0.022) decreased, while TOS (p<0.001) and OSI (p<0.001) increased in the GU group compared with the CNT group. With the ACA implementation, this situation was reversed. There was a decrease in TOS (p=0.007) and OSI (p<0.001) values in the GU+ACA group compared to the GU group. Although the TAS (p=0.764) level increased, it did not gain significance (Fig. 2).

mRNA transcript findings

In the gastric tissue, the apoptotic factor Caspase-3 and the antiapoptotic factor Bcl-2, and the inflammatory factor NF-kB mRNA transcript levels were measured. Bcl-2 (p<0.001) decreased, whereas Caspase-3 (p<0.001) and NF-kB (p<0.001) increased in the GU group compared with the CNT group. With the ACA implementation, this situation was reversed. While Bcl-2 (p<0.001) increased, Caspase-3 (p<0.001) and NF-kB (p=0.026) were decreased in the GU+ACA group compared with the GU group (Fig. 3).
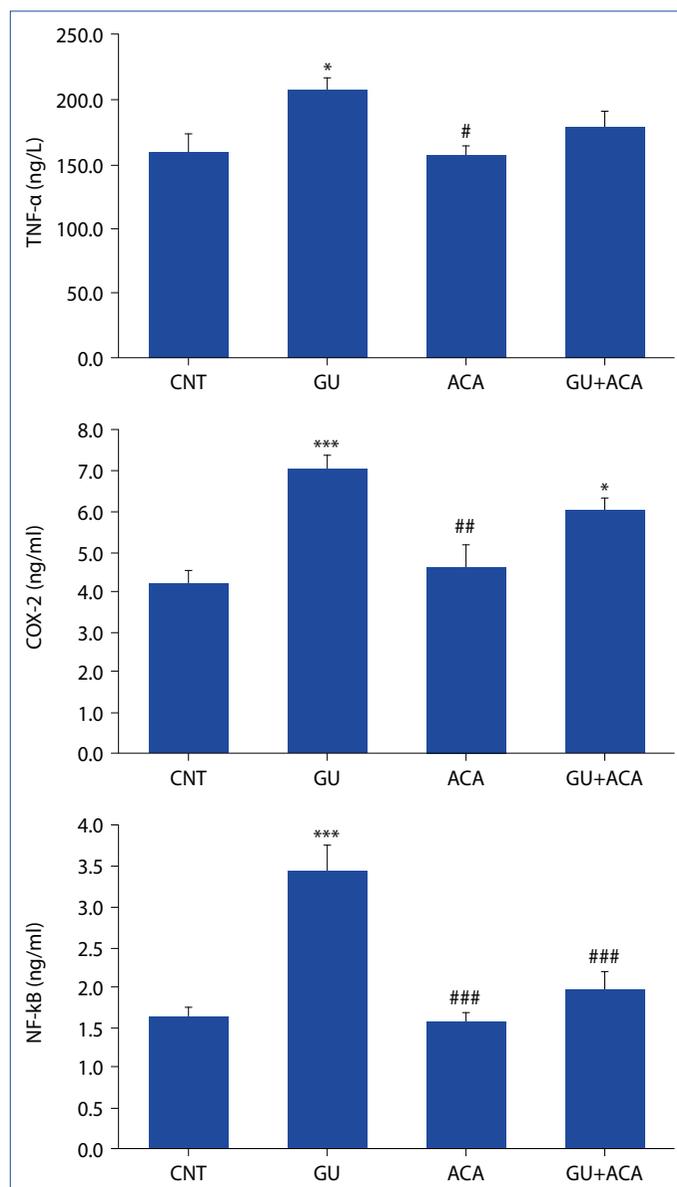

Figure 1. Serum levels of TNF-α, COX-2 and NF-kB. All data were expressed as mean±SE.

CNT vs others: *p<0.05; ***p<0.001; GU vs. others: #p<0.05; ##p<0.01; ###p<0.001) was analyzed using One Way ANOVA (TNF-α, COX-2) and Mann–Whitney U (NF-kB). CNT: Control; GU: Gatric Ulcer; ACA: Acacetin; GU+ACA: Gatric ulcer+Acacetin; SE: Standard error.

Macroscopic examination

The data obtained as a result of the macroscopic examination (Fig. 4) are shown in Table 1. According to the results, lesions were seen in all animals in the ASA group and the area and numbers were determined as 7.545±0.450 and 6.750±0.250. With the ACA implementation, this situation was reversed and area and numbers decreased to 1.855±0.152 and 2.125±0.226.

Histopathological examination

In this study, the effect of ACA on the histological changes caused by an ulcer in the gastric tissue is shown in Figure 5 by performing H&E staining. When the gastric tissues of the CNT
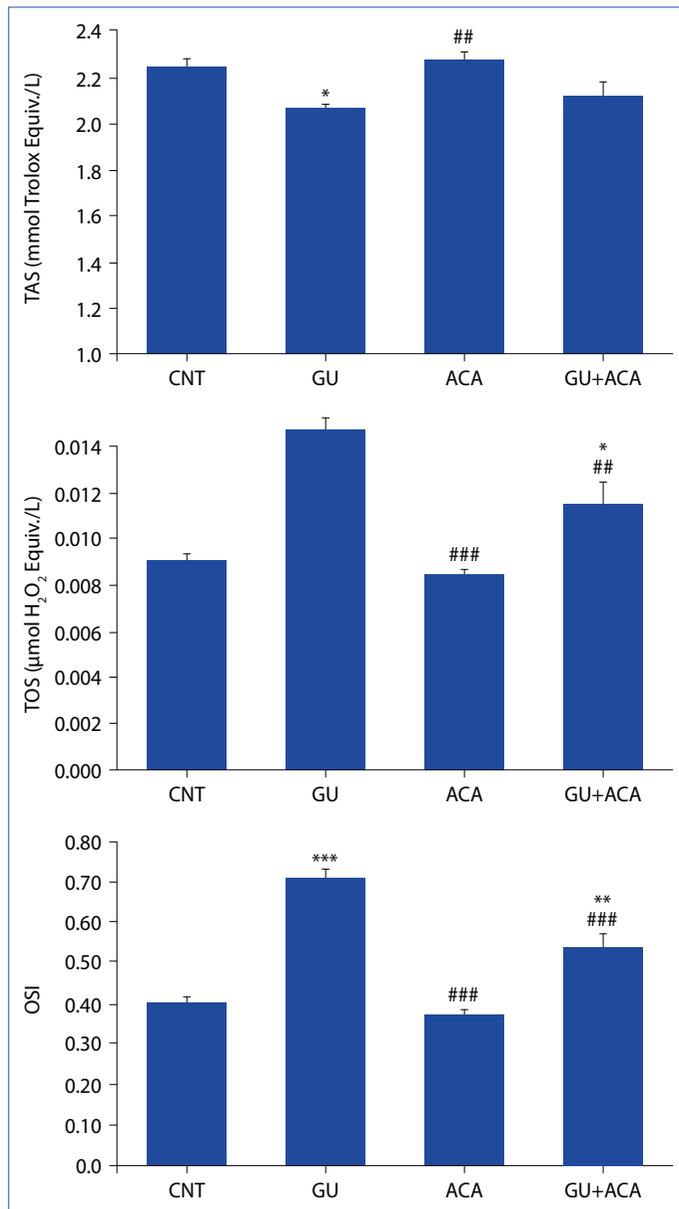
Figure 2. Serum levels of TAS, TOS and OSI. All data were expressed as mean±SE.

CNT vs. others: *p<0.05; **p<0.01; ***p<0.001; GU vs. others: ##p<0.01; ###p<0.001) was analyzed using One Way ANOVA. CNT: Control; GU: Gatric Ulcer; ACA: Acacetin; GU+ACA: Gatric ulcer+Acacetin; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; SE: Standard error.

Figure 3. The mRNA transcript levels of Bcl-2, Caspase-3 and NF-kB in the gastric tissue of rats. All data were expressed as mean±SE.

Control vs others: *p<0.05; ***p<0.001; GU vs others: #p<0.05; ###p<0.001) was analyzed using One Way ANOVA (NF-kB) and Mann-Whitney U (Bcl-2, Caspase-3). CNT: Control; GU: Gatric Ulcer; ACA: Acacetin; GU+ACA: Gatric ulcer+Acacetin; SE: Standard error.

and ACA groups were examined histologically, it was observed that their architectural structures had a normal appearance. The mucosal layer, submucosa, gastric glands, and muscular layer of histological layers had a healthy appearance (Fig. 5a, c). In the GU, especially deterioration in villi, shedding and erosions in the mucosal epithelium exhibited a remarkable appearance. In the GU, especially deterioration in villi, shedding and erosions in the mucosal epithelium exhibited a remarkable appearance. Inflammatory cell infiltration was observed in the gastric submucosa of this group of rats. The nuclei of the cells forming the mucosal epithelium were seen as more pyknotic. Bleeding foci were observed in the mucosa (Fig. 5b). It was observed that
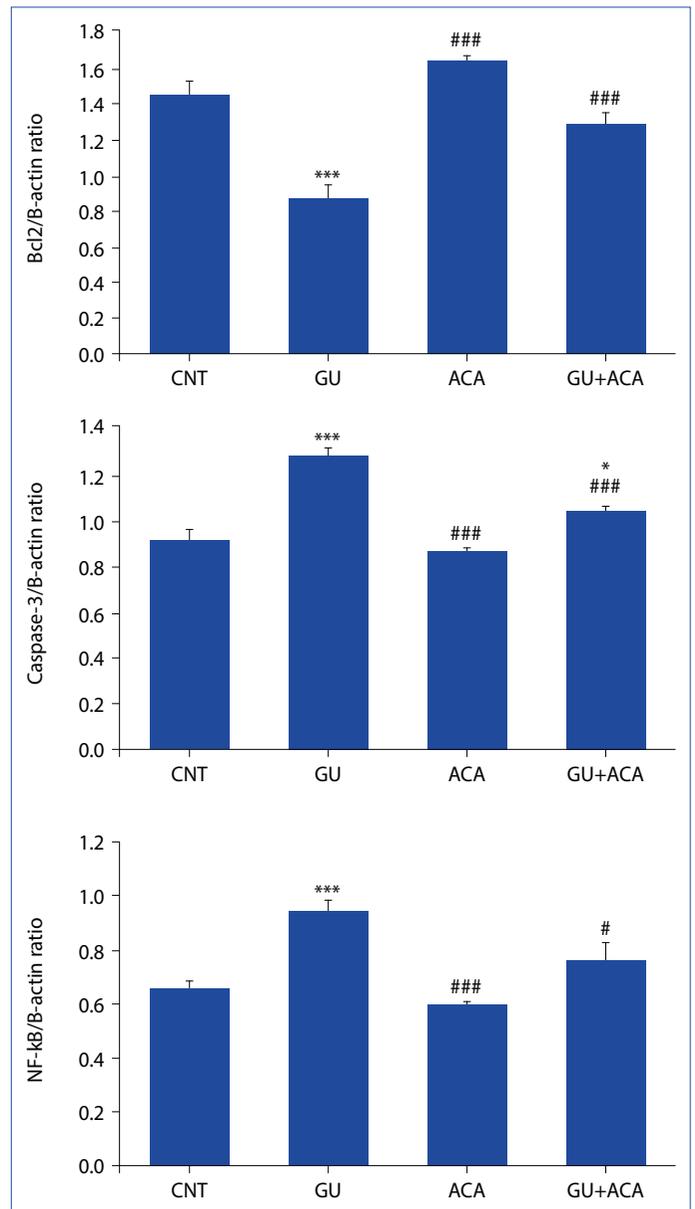
ulcer-induced damage was reversed with ACA treatment. The gastric mucosa appeared regular and the gastric pits appeared normal. In the GU+ACA, inflammatory cell infiltration and bleeding of gastric tissues decreased. It was observed that the general histological pattern was closer to that of the CNT (Fig. 5d).

Discussion

GU is one of the most common diseases of the GI system worldwide (20–60 per 100,000 population) and it is 3–4 times more common in men than in women and mostly occurs at advanced ages [18, 19]. Many factors such as stress,
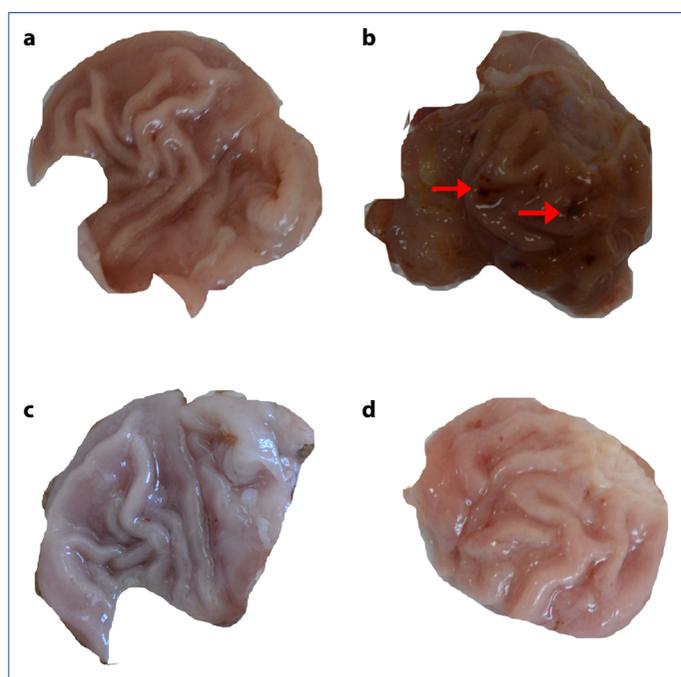
Figure 4. Macroscopic examination of gastric tissues. (a) CNT group, (b) GU group, (c) ACA group, (d) GU+ACA group. Arrow: lesion.

CNT: Control; GU: Gatric Ulcer; ACA: Acacetin; GU+ACA: Gatric ulcer+Acacetin

smoking, bad eating habits, and NSAID ingestion may contribute to the formation of GU [20]. ASA is one of the most highly consumed NSAID worldwide and causes gastric mucosal damage, increasing acid release and back-diffusion of H+ ions and resulting in the collapse of the mucosal barrier [17]. Although there are prescription antibiotics, proton pump inhibitors (PPI-omeprazole), prostaglandin analogs, and H2 receptor blockers (cimetidine, ranitidine, and famotidine) in the treatment of GU, new drugs with lower cost and fewer side effects need to be discovered [21]. ACA is a naturally abundant flavonoid found in fruits such as acacia honey and citrus fruits. It has various biological and pharmacological effects, such as anti-inflammatory, anti-oxidative, and anti-tumor effects [22]. This study demonstrated the protective effects of ACA in the gastric tissue by regulating some parameters effective in inflammation, oxidative stress, and apoptosis in the GU model created with ASA in rats.

GU induced with ASA is a GU model frequently used in experiments. Due to the acidic nature of gastric juice, ASA

becomes highly deionized and easily affects the mucosal barrier. As a result, large erosions and bleeding foci occur in a very short time. The pathophysiological changes that occur in ASA-induced GU are comparable to human GUs [3]. Inflammation and neutrophil infiltration play an important role in the pathogenesis of NSAID-induced GUs [23]. According to the data obtained from histopathological evaluations in our study, ASA caused damage to the gastric mucosa. This was clearly observed in both ulcer scoring and HE staining images. Inflammatory cell infiltration, deterioration in gastric mucosa, the pyknotic appearance of cell nuclei before apoptosis or necrosis, and hemorrhage foci are evidence of this situation. ACA treatment resulted in reductions in both ulcer score and tissue damage. ACA has shown a therapeutic effect on ulcer-induced tissue damage.

While aspirin triggers inflammation in the stomach tissue, it also increases the level of TNF-α [23]. Pro-inflammatory cytokines play a role in the formation of inflammation and TNF-α plays an active role in this [24]. NF-KB is a transcription factor that is stimulated when inflammation occurs in tissues during GU and triggers the accompanying production of cytokines, chemokines, and growth factors [18]. With the activation of NF-kB, activation of pro-inflammatory cytokine genes such as TNF-α and COX-2 begins and an inflammatory response occurs [25]. COX-2 is an inflammatory agent and is expressed by the NF-kB gene and is associated with reactive oxygen species (ROS) production in normal tissues [26]. Previously studies showed that increases in COX-2 mRNA and protein levels are observed with the exposure of gastric mucosa to harmful factors or ischemia-reperfusion injury. These observations support the concept that COX-2 represents another line of defense for the GI mucosa that is essential for the maintenance of mucosal integrity and ulcer healing [27]. In this study, TNF-α, COX-2, and NF-kB serum levels and gastric tissue NF-kB mRNA transcription levels were increased in rats with the GU model. In rats treated with ACA, these levels, which increased due to GU, decreased. Similar to our study, studies have shown that the application of ASA to the gastric mucosa causes up-regulation in COX-2 [4, 28]. Although ASA should inhibit COX enzyme activity by acetylating the serine residue in the active site of the COX enzyme, in contrast, acetylation of the serine residue in the COX-2 active site, its ability to metabolize arachidonic acid and produce prostaglandin has been reported as the cause

Table 1. Macroscopic examination results

Groups	Area of lesion (mm)	Number of lesions	UN	USS	AGU	GUI
CNT	0	0	0	0	0	**0**
GU	7.545±0.450	6.750±0.250	6.750	4	8	**1.875**
ACA	0	0	0	0	0	**0**
GU+ACA	1.855±0.152	2.125±0.226	2.125	1.250	8	**1.137**

UN: Total number of ulcers/number of animals (per group); USS: Ulcer severity score; AGU: Number of animals with gastric ulcer per group; GUI: Gastric ulcer index; CNT: Control; GU: Gatric Ulcer; ACA: Acacetin; GU+ACA: Gatric ulcer+Acacetin
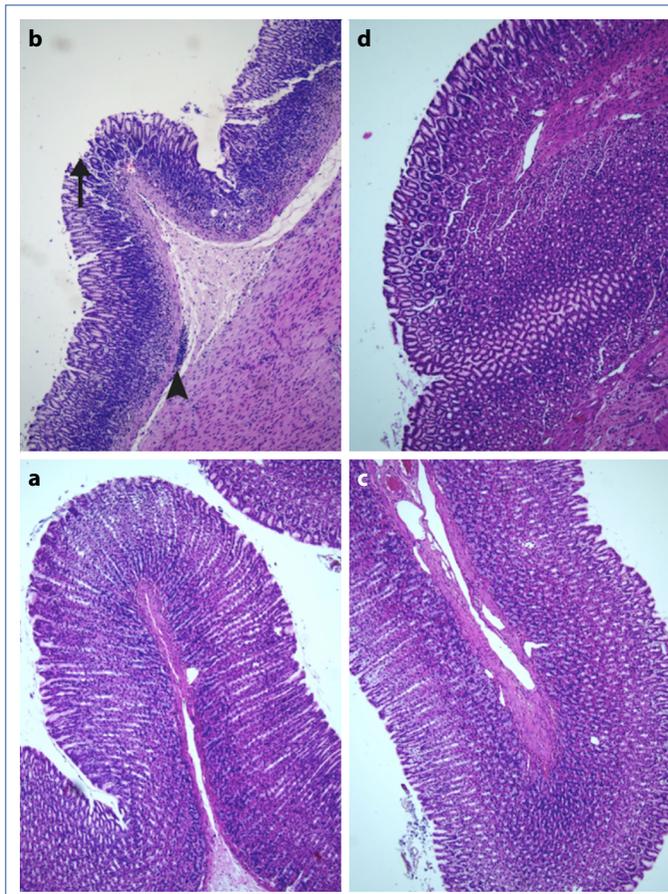
Figure 5. Photomicrographs of histological changes in gastric tissues (H&E staining). (a) CNT group, (b) GU group; arrow: Mucosal tissue defect, arrowhead: increased inflammatory cell infiltration, (c): ACA group, (d) GU+ACA group.

CNT: Control; GU: Gatric Ulcer; ACA: Acacetin; GU+ACA: Gatric ulcer+Acacetin.

of this situation [28]. Similarly to our study, Liou et al. [29] found that ACA decreased COX-2 expression in different tissues in their study. ACA may come to the fore as a potent agent in reducing inflammation, which is effective in the formation of GU.

Oxidative stress is one of the most effective factors in the pathological processes of GU [30]. Oxidative stress is expressed as the deterioration of the balance between the oxidant and antioxidant systems in favor of the oxidant system. Studies on drug toxicity have generally focused on oxidative stress. Lipid, protein, and DNA damage occurs due to oxidative stress [31, 32]. Free radicals are among the mucosal invasive factors that play an important role in the pathophysiological changes of GU by causing oxidative damage to gastric mucosal cells [30]. The removal of free radicals from the body is provided by enzymatic and non-enzymatic antioxidants against free radicals [33]. Antioxidants are naturally found in plants and stand out in terms of their effectiveness in reducing tissue and organ toxicity against various drugs. Endogenous and exogenous antioxidants, called "free radical scavengers," can prevent and repair damage due to

ROS [34]. In our study, while serum TAS decreased in the GU group, TOS and OSI increased. This situation was reversed when ACA was applied to GU. ACA caused a significant reduction in OSI by increasing the antioxidant mechanism and decreasing the oxidant mechanism. ACA can be an effective agent in reducing the occurrence of oxidative stress.

ROS is largely produced in mitochondria and has an important effect on the development of apoptosis [35]. Apoptosis is the programmed and regular death of cells controlled by various genes to maintain body homeostasis [36]. While apoptosis is a protective mechanism that removes damaged or dangerous cells in the body, it also creates cellular stress or damage in healthy cells [37, 38]. Excessive production of ROS mediates gastric mucosal injury and mucosal barrier destruction by causing apoptosis in gastric mucosal epithelial cells [39]. In this study, caspase-3 mRNA transcript level, which is one of the apoptotic factors, increased in the gastric tissues of GU group rats, while the antiapoptotic factor Bcl-2 decreased. In the gastric tissues of rats administered ACA, this situation had the opposite effect. Therefore, in tissue damage caused by increased apoptosis due to GU, ACA may be an effective agent against apoptosis.

Conclusion

Conclusively, it was determined that high doses of ASA contributed to the formation of GU in the stomach tissue by increasing the levels of inflammation, oxidative stress, and apoptosis, whereas ACA reduced the ulcer damage by reducing the increase in all these pathways. In terms of all these pathways, ACA may be effective in preventing gastric tissue damage and increasing the patient's quality of life. The limitation of the study is that acacetin was not compared with the PPI used in the treatment of GU. Therefore, further studies are needed for its clinical use.

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