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Protective effect of peroral capsaicin administration in ethanol-induced gastric ulcer model in rats

Sıçanlarda etanol kaynaklı gastrik ülser modelinde peroral kapsaisin uygulamasının koruyucu etkisi

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ABSTRACT

Aim: Capsaicin is a compound that possesses antioxidants and anti-inflammatory properties, and it is found in almost all bitter fruits. The objective of our study was to investigate the protective effect of capsaicin in an ethanolic ulcer model.

Materials and Methods: The male *Wistar albino* rats were divided into four groups, comprising a control group and three experimental groups (n=8). The control group (C) administered a single dose of distilled water subcutaneously. The Capsaicin (CAP) group was administered a single dose of 1 mg/kg CAP via gavage. The ulcer group administered a single dose of 1ml absolute alcohol via gavage, 30 minutes following the administration of a single dose of distilled water via gavage. The U+CAP group administered 1ml of absolute alcohol 30 minutes following the administration of 1 mg/kg CAP. Macroscopic and microscopic ulcer scores, as well as mucosal barrier integrity, were evaluated in the gastric tissues that had been removed. The levels of total oxidant status (TOS) and total antioxidant status (TAS) were determined in the tissue samples, and an oxidative stress index (OSI) was calculated. **Results:** In comparison to the U group, the macroscopic and microscopic mucosal lesions, TOS, OSI levels and IL-1, TNF-α, NFκB and Caspase 3 expressions were found to be decreased in the U+CAP group, while TAS levels were observed to be increased.

Conclusion: The results of the study demonstrated that capsaicin protects mucosal integrity through its antioxidant, anti-inflammatory and anti-apoptotic properties in the ethanol-induced gastric ulcer model.

Keywords: Ethanol, ulcer, capsaicin, caspase 3, NF-κB.

ÖZ

Amaç: *Kapsaisin, hemen tüm acı biberlerin yapısında bulunan anti-oksidan ve anti-inflamatuar özelliklere sahip bir bileşiktir. Çalışmamızda etanolik ülser modelinde kapsaisin'in koruyucu etkisi araştırılmıştır.*

Gereç ve Yöntem: *Erkek Wistar albino sıçanlar biri kontrol üçü deney grubu olmak üzere dört gruba ayrılmıştır (n=8). Kontrol (C) grubuna tek doz distile su subkutan uygulanmıştır. Kapsaisin (CAP) grubuna tek doz 1 mg/kg CAP gavaj yoluyla uygulanmıştır. Ülser (U) grubuna tek doz distile suyun gavaj yoluyla uygulanmasından 30 dakika sonra tek doz 1 ml mutlak alkol gavaj yoluyla uygulanmıştır. U+CAP grubuna 1 mg/kg CAP uygulanmasından 30 dakika sonra 1 ml mutlak alkol uygulanmıştır. Çıkarılan mide dokularında makroskobik ve mikroskobik ülser skorları ve mukozal bariyer bütünlüğü değerlendirilmiştir. Dokuda total oksidan status (TOS) ve total anti-oksidan status (TAS) seviyeleri ölçülmüş ve oksidatif stres indeksi (OSI) hesaplanmıştır.*

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Bulgular: *U grubuyla karşılaştırıldığında, makroskobik ve mikroskobik mukozal lezyonlar, TOS, OSI seviyeleri ve IL-1, TNF-α, NFκB ve Kaspaz 3 ekspresyonlarının U+CAP grubunda azaldığı, ancak TAS seviyesinin arttığı görülmüştür.*

Sonuç: *Çalışma, kapsaisin'in etanol kaynaklı gastrik ülser modelinde anti-oksidan, anti-inflamatuar ve anti-apoptotik özellikleriyle mukozal bütünlüğü koruduğunu göstermiştir.*

Anahtar Sözcükler: *Etanol, ülser, kapsaisin, kaspaz 3, NF-κB.*

INTRODUCTION

Peptic ulcer disease (PUD) represents a significant and pervasive public health concern, with an estimated incidence of approximately 1 in 1,000 cases per year in the general population across the globe (1). Histopathologically, PUD is defined as damage that reaches the submucosal layer in the stomach, resulting from the disruption of the balance between the factors that form the mucosal barrier, such as bicarbonate secretion, adequate mucosal blood flow, the regeneration capacity of epithelial cells, and the production of gastric acid and pepsin (2). Today, Helicobacter pylori infection is held primarily responsible for the etiology of peptic ulcer disease, but it is well known that factors such as nonsteroidal anti-inflammatory drug (NSAID) use, stress, smoking and alcohol are also causative agents of peptic ulcer (3). Ethanol has been frequently used to create ulcer models in animal studies because it disrupts the mucosal barrier by reducing mucosal microcirculation and triggers inflammation by increasing free oxygen radicals in the tissue (4, 5).

Capsaicin (CAP) is a compound that is present in almost all varieties of chili peppers and is responsible for the spicy aroma that is characteristic of these peppers (6). *In vivo* and *in vitro* studies have demonstrated that CAP and related but non-pungent capsinoids (capsiate, dihydrocapsiate, nordihydrocapsiate) possess the capacity to elicit a range of beneficial effects, including analgesic, antioxidant, anti-inflammatory and anti-carcinogenic properties (7). In a sepsis model created in rats, CAP administered subcutaneously at a dose of 1 mg/kg was shown to reduce plasma interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels and malondialdehyde (MDA) levels in liver, lung and heart tissues (8). Li et al. showed that CAP reduced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), TNF-α and nitric oxide (NO) levels in a lipopolysaccharide-induced inflammation model in BALB/c mice (9).

Although CAP has been shown to have a mucosal protective effect in NSAID, stress and ethanol-induced ulcer models by desensitizing afferent nerve fibers via transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors, no studies were found demonstrating the efficacy of orally administered CAP in an ethanol-induced ulcer model (10, 11, 12).

The aim of our study was to demonstrate the efficacy of CAP in the ethanol-induced gastric ulcer model in *Wistar albino* rats through histopathological, biochemical and immunohistochemical methods.

MATERIALS and METHODS

Male *Wistar albino* rats (n=32, weighing 300-350 g each) from the Animal Care and Research Unit of Bülent Ecevit University (Zonguldak, Turkey) were used in the present study. The appropriate environmental conditions for the care of the animals (room temperature of 20 ± 1°C, humidity of 60 ± 10%, and a 12/12 hour light/dark cycle) were provided throughout the experiment. The subjects were permitted free access to food and water. The study was conducted in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals, published by the US Public Health Service. Prior to the commencement of the study, approval was obtained from the Institutional Animal Ethics Committee of Bülent Ecevit University (Zonguldak, Turkey; 2020-06-27/02).

Experimental Design

A total of four groups were constituted, comprising one control group and three experimental groups, each with eight rats. The control group administered a single dose of CAP solvent (distilled water) subcutaneously. The CAP group administered a single dose of 1 mg/kg CAP via gavage. The ulcer (U) group administered a single dose of 1ml absolute alcohol via gavage, 30 minutes following the administration of a single dose of CAP solvent via gavage. The U+CAP group administered 1 ml of absolute alcohol for 30 minutes following the administration of 1 mg/kg CAP.

Macroscopic Ulcer Score

One hour after the administration of absolute alcohol, the animals were euthanized, and their stomach tissues were excised. The gastric tissues were incised along with the greater curvature and washed with isotonic NaCl solution. They were then flattened on paraffin plates to allow for the examination of the mucosal folds. The dimensions of the lesion area and the total stomach surface area were determined macroscopically by affixing transparent millimeter-scale paper templates to the stretched stomach surface. The macroscopic ulcer score was calculated using the following formula: lesion area/total stomach surface area x 100.

Histopathological Evaluations

Following the application of a 10% formalin fixative, paraffin blocks were obtained from stomach tissues that had undergone the requisite tissue processing steps and were embedded in paraffin. In order to determine the histological features of the stomach, 5µ thick sections were taken from the paraffin blocks and subjected to staining with either hematoxylin-eosin (H&E) and periodic acid Schiff (PAS) dyes. The stained tissues were imaged using the Axio Lab A1 microscope (Zeiss, Germany). Each 1 cm section was subdivided into three areas, and a microscopic ulcer score was calculated for each area based on the following criteria:

0: normal mucosa, 1: epithelial damage, 2: glandular disruption, vasocongestion or oedema of the upper parts of the mucosa, 3: vasocongestion or oedema extending to the middle parts of the mucosa, 4: mucosal damage involving the entire mucosa (13). The mean score for each area was deemed to represent the microscopic ulcer score for that particular section.

Evaluation of TAS, TOS and OSI in Tissue

Stomach tissue homogenates were prepared and total antioxidant status (TAS) and total oxidant status (TOS) measurements were performed in accordance with the protocol provided with the commercially purchased Rel Assay (Rel Assay Diagnostics kit, Mega Medicine, Gaziantep, Turkey) (14). Oxidative stress index was calculated with the formula TOS/TAS × 100 (15).

Immunohistochemical Evaluations

In gastric tissues, expressions of inflammatory cytokines interleukin1-β (IL-1β), TNF-α, caspase 3, a common pathway caspase in apoptotic cell death, and NF-κB, a transcription factor, were demonstrated by immunohistochemistry. Stomach sections with a thickness of 5 microns were taken from paraffin-embedded samples. The sections were initially incubated with rabbit anti-NF-κB (1:200 dilution, Novus Biologicals, USA), anti-caspase-3 (1:200 dilution, Sigma-Aldrich, Germany), anti-TNF-α (1:200 dilution, Sigma-Aldrich, Germany) and anti-IL-1-β antibodies (1:100 dilution, Novus Biologicals, USA) at +4°C for 24 hours. Subsequently, immune complexes formed on tissues incubated with a biotinylated goat anti-rabbit secondary antibody (Thermo Fisher Scientific, USA) were detected by incubation with 3,3'-diaminobenzidine tetrachloride (Vector Laboratories, DAB Substrate Kit, Peroxidase (HRP), with Nickel, USA). Subsequently, a counterstain was applied using hematoxylin. The H-score was conducted on the sections using the Axio Lab A1 microscope (Zeiss, Germany) in accordance with the following criteria: 0: no staining, 1+: weak but detectable staining, 2+: medium or prominent staining, 3+: intense staining. The H-score value for each section was calculated by multiplying the percentage of stained cells for each density category by its density. The scoring was conducted under a light microscope at 40x objective magnification on 20 randomly selected fields on each section, with the mean scores employed for statistical analysis. H-score = ∑i i xPi, i; density score, Pi; cell percentage (16).

Statistical Analysis

The data analysis was conducted using the Jamovi 2.3.21 package program. The immunohistochemical H-score index was calculated as the median (minimum-maximum). Firstly, the suitability of the data for normal distribution was evaluated using the Shapiro-Wilk test. Subsequently, one-way analysis of variance was employed to compare the normally distributed quantitative values. The Kruskal–Wallis test was employed for the comparison of normally distributed qualitative variables. A Mann-Whitney U test with Bonferroni correction was employed for the purpose of conducting pairwise comparisons. $p<0.05$ was considered significant.

RESULTS

Effect of CAP on Ethanol-Induced Gastric Mucosal Injury

In Groups C and CAP, the gastric mucosa exhibited no macroscopic abnormalities. There

was no statistically significant difference between the ulcer scores observed at the macroscopic level in the C and CAP groups (p>0.05). The width of erosion areas and macroscopic ulcer scores in the gastric tissues of rats in the U+CAP group were significantly lower compared with those in the U group (p<0.01) (Figure-1 and Figure-2).

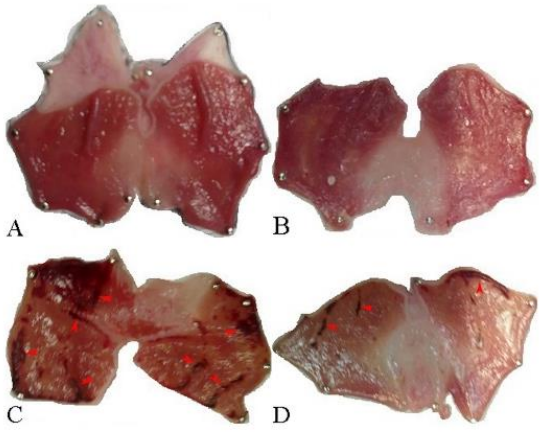

Figure-1. Macroscopic view of gastric lesions in all groups. No lesions were observed in the gastric mucosa of rats in the C (A) and CAP (B) groups. The rats in the U group (C) have extensive hemorrhagic lesions on the mucous membrane of the stomach. Rats in U+CAP group (D) have limited gastric mucosal hemorrhagic lesions. Red arrowheads indicate ulcerated areas.

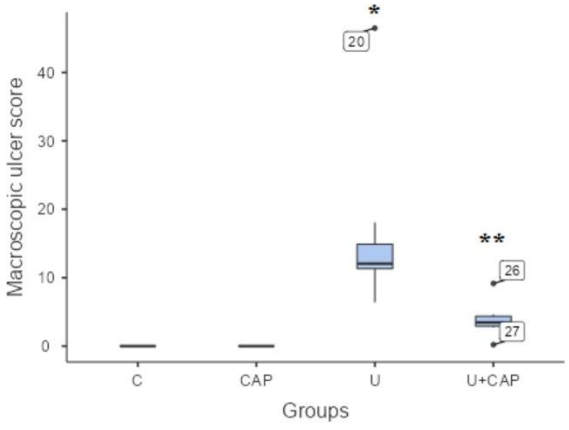

Figure-2. *Significant when compared with C, CAP and U+CAP groups (P<0.01). **Significant when compared with C, CAP and U groups (p<0.01).

The gastric mucosa of the subjects belonging to the C and CAP groups showed a normal histological appearance microscopically, and there was no statistically significant difference between the microscopic ulcer scores of these two groups (p>0.05). The microscopic ulcer score of the C and CAP groups showed a statistically significant difference compared to both the U (p<0.01 for both) and U+CAP (p<0.01 for both) groups. The microscopic evaluation of H&E-stained sections of the stomach tissues of subjects belonging to the U group revealed denudation in both surface epithelium and glandular epithelium, as well as vasocongestion, erythrocyte extravasation, localized areas of mucosal necrosis and inflammatory cell infiltration. In contrast, both the lesion width and depth were significantly reduced in the U+CAP group (p<0.01). Furthermore, it was observed that the submucosal thickness was markedly increased in the U group, whereas the submucosal thickness was within the normal range in the U+CAP group (Figure-3 and Figure-4).

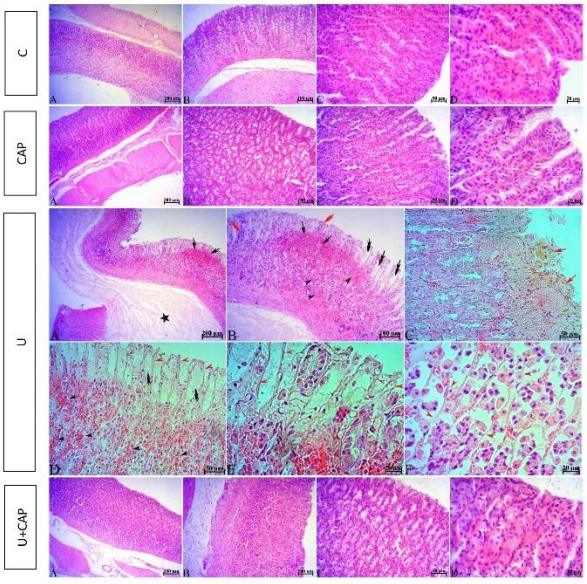

Figure-3. Histological evaluation of rat stomach mucous membranes in all groups. Epithelial desquamation (red arrowhead), mucosal hemorrhage (black arrow), vascular congestion (black arrowhead), inflammatory cell infiltration (red double arrow), mucosal necrosis (red arrow), oedema in lamina propria (black double arrow) and submucosa (black star). H&E staining. Scale bar; In C, CAP and U+CAP groups A; 200µm, B; 100 µm, C; 50 µm, D; 20 µm. In U group A; 200 µm, B; 100 µm, C, D; 50 µm, E, F; 20 µm

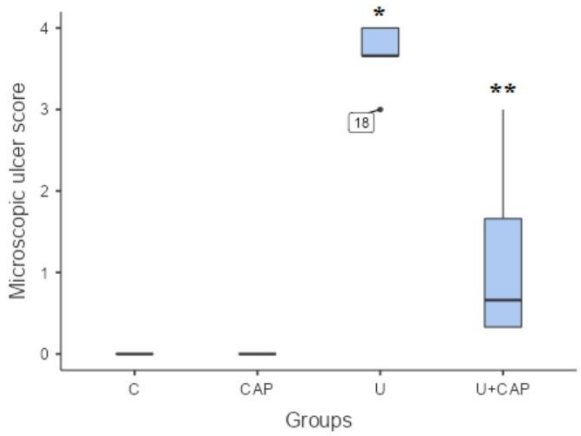

Figure-4. *Significant when compared with C, CAP and U+CAP groups (P<0.01). **Significant when compared with C, CAP and U groups (p<0.01).

The PAS staining method was applied for the purpose of evaluating the mucus barrier that covers the stomach surface. The intense magenta staining observed on the mucosal surface in the U+CAP group in comparison to the U group indicated that the glycoprotein mucus barrier was intact (Figure-5).

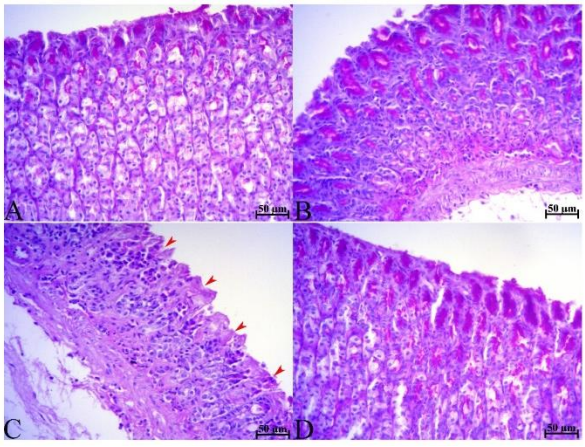

Figure-5. Histological assessment of the integrity of the mucus barrier in the gastric mucosa of rats in all groups. A: C group, B: CAP group, C: U group, D: U+CAP group. Disruption of the mucus barrier in group U, manifested by loss of magenta staining on the mucosal surface (red arrowhead). PAS staining. Scale bar; 50µm.

Effect of CAP on TAS, TOS and OSI Levels

A comparison of the TAS, TOS and OSI values in stomach tissue revealed no statistically significant difference between the C and CAP groups (p>0.05 for all). TOS value was higher in the U group compared to the C, CAP and U+CAP groups (p<0.01, p<0.01 and p<0.05, respectively). TAS value was lower in the U group compared to the C, CAP and U+CAP groups (p<0.01 for all). In the intergroup comparison in terms of OSI, a statistically significant difference was found between the U group and the C, CAP and U+CAP groups (p<0.01 for all) (Figure-6).

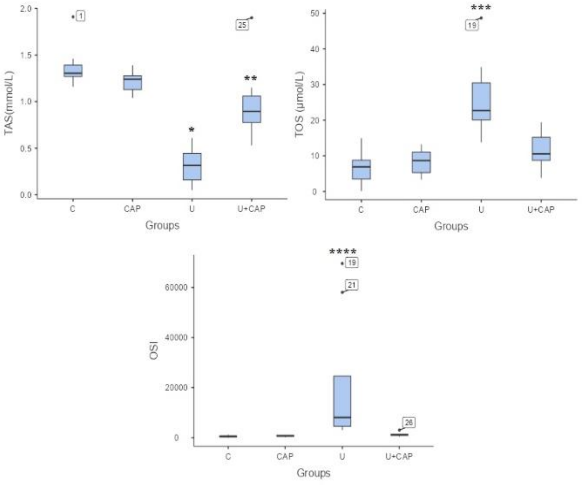

Figure-6. *Significant when compared with C, CAP and U+CAP groups (P<0.01). **Significant when compared with C and U groups (p<0.05 and p<0.01 respectively. ***Significant when compared with C, CAP and U+CAP groups (p<0.01, p<0.01 and p<0.05 respectively). ****Significant when compared with C, CAP and U+CAP groups (p<0.01 for all).

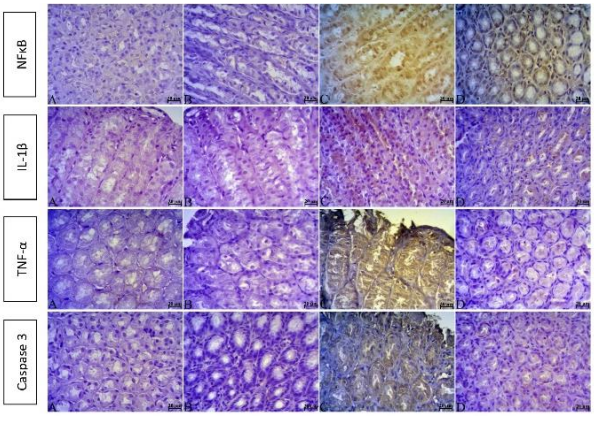

Figure-7. Results of immunohistochemical staining for IL-1, TNF-α, NF-κB and caspase 3 in stomach tissues obtained from subjects in all groups. Scale bar; 20 µm.

Effect of CAP on Immunohistochemical Staining Results

The expression of IL-1-β, TNF-α, caspase-3 and NF-κB in the mucosa was evaluated by immunohistochemical staining of gastric sections. Immunohistochemical staining of gastric tissues from groups C and CAP showed that all protein expressions were very weak. When the h-scores of these two groups were compared, no statistically significant difference was found ($p>0.05$). A statistically significant difference was found between the U group and the C, CAP and U+CAP groups for all protein expressions evaluated ($p<0.01$ for each) (Figure-7 and Figure-8).

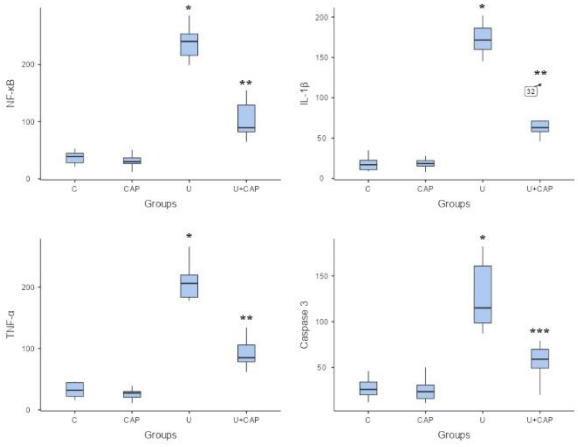

Figure-8. *Significant when compared with C, CAP and U+CAP groups ($p<0.01$). **Significant when compared with C, CAP and U groups ($p<0.01$). ***Significant when compared with C, CAP and U groups ($p<0.05$, $p<0.05$ and $p<0.01$ respectively).

DISCUSSION

Our study is the first to show that oral administration of capsaicin has a protective effect on ethanol-induced gastric ulcers.

Alcohol consumption is a common cause of peptic ulcer. Ethanol has been demonstrated to exert a detrimental effect on the gastric mucosa through a number of mechanisms, including direct damage, increased gastric acidity, decreased mucosal prostaglandin levels, disruption of mucosal microcirculation, and the induction of oxidative stress and inflammation (17, 18). In experimental studies in which gastric ulcers were induced through the use of ethanol, a range of histopathological changes were frequently observed. These included epithelial necrosis, hemorrhage, mucosal and submucosal oedema, inflammatory cell infiltration and loss of the surface mucus layer. Additionally, large ulcer areas were commonly observed at the macroscopic level (19, 20). The results of our study showed that subjects in the ulcer group had macroscopic and histopathological features similar to findings described in the literature. However, it was observed that the macroscopic and microscopic ulcer scores in the stomach tissues of the subjects who were administered capsaicin in conjunction with ethanol exhibited a notable decline.

The alteration in the equilibrium between oxidants and antioxidants within the tissue, with a predominance of oxidants, is referred to as oxidative stress (21). The primary targets of oxidant agents are cellular lipids, proteins, and nucleic acids. The peroxidation of cellular lipids by oxidants gives rise to the formation of MDA. In addition, reactive oxygen species (ROS) have the potential to cause damage to nucleic acids, proteins and other macromolecules in the cell (22). Sarıyer et al. observed an increase in myeloperoxidase (MPO) and MDA levels in the gastric mucosa of *Sprague-Dawley* rats subjected to an ethanol-induced ulcer model. The administration of astaxanthin, an antioxidant, to the diet resulted in a notable reduction in MDA and MPO levels, accompanied by a decline in macroscopic and microscopic ulcer scores (23). Zhao et al. demonstrated that ethanol administration resulted in a reduction in superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and total antioxidant status (TAS) levels in the gastric mucosa of *Wistar albino* rats, concomitant with an increase in MDA levels. However, Dan-Shen-Yin treatment, an antioxidant phenolic compound mixture employed in traditional Chinese medicine, was observed to restore the antioxidant balance (24). The current study revealed a significant difference in TAS and TOS levels between subjects who were administered ethanol and those who were treated with capsaicin and ethanol. These findings are in accordance with those reported in the existing literature.

A significant consequence of oxidative stress at the cellular level is the induction of an inflammatory response, which itself represents a source of ROS production (25). The pivotal molecule that establishes the interconnection between ROS and inflammation is NF-κB, which serves as a principal regulator of proinflammatory genes. A variety of cytokine responses, including those associated with IL-1, IL-6 and TNF-α, are

primarily regulated by NF-κB (26). In experimental ulcer models induced by ethanol, in which Hsuan et al. used aibika flower flavonoid extract (27) and Fan et al. administered *Dendrobium officinale flos* water extract as therapeutic agents (28), they demonstrated that these antioxidant agents markedly reduced the elevated ROS levels in the tissue resulting from ethanol exposure, concurrently with a reduction in the expression of NF-kB and proinflammatory cytokines.

Apoptosis is a well-controlled and extremely specialized process of cell death in which a cell destroys itself and it is vital for cellular homeostasis that the process of apoptosis is properly regulated (29). Both ROS and inflammatory stimuli have been demonstrated to trigger apoptosis. At low levels of ROS, the cell can undergo DNA repair and survival through p53 activation. Conversely, at high levels of ROS, p53 directs the cell to the intrinsic apoptotic pathway by regulating the levels of pro-apoptotic (Bcl-2-associated X protein (Bax), BH3 interacting-domain death agonist (Bid), Apoptotic protease activating factor 1 (Apaf-1)) and anti-apoptotic [B cell lymphoma/leukemia type 2 (Bcl-2), B-cell lymphoma-extra-large ($Bcl\text{-}X_L$), surviving] proteins and activating caspase 9 (30). In addition to the NF-κB-mediated increase in proinflammatory cytokines during the inflammatory process, elevated levels of ROS inhibit phosphatases, resulting in enhanced phosphorylation of the Inhibitor of κB (IκB) (31). This, in turn, leads to its degradation, thereby facilitating NF-κB activation. Consequently, an augmented proinflammatory cytokine response, particularly TNF-α, instigates the extrinsic apoptotic pathway via by binding to tumor necrosis factor receptor 1 (TNF-R1) and activating caspase 8 (29). Irrespective of whether the process of apoptosis is initiated by intrinsic or extrinsic mechanisms, the activation of caspase 3 ultimately leads to cellular demise (32). Recent studies have demonstrated the involvement of apoptosis in the pathogenesis of ethanol-induced gastric ulceration. Alamoudi et al. observed an increase in ROS, NF-kB, proinflammatory cytokines and caspase 3 in ethanolic ulcers in rats. They also demonstrated that hesperidin caused a significant decrease in these parameters and a significant improvement in the histological appearance of the gastric mucosa, which they attributed to its antioxidant and anti-inflammatory effects (33). Similarly, Lin et al. demonstrated that galangin, which possesses antioxidant and anti-inflammatory properties, led to a reduction in the levels of MDA, NO, NF-κB, pro-inflammatory cytokines IL-1, IL-6, IL-8, TNF-α as well as caspase 3 in ethanolic ulcer models induced in BALB/c mice (34). Our study yielded similar results to those observed by other researchers, indicating a correlation between the decline in caspase 3 expression and the reduction in ROS, NF-κB, IL-1 and TNF-α levels in the gastric mucosa of subjects who were administered capsaicin.

CONCLUSION

The results of our study revealed that capsaicin exerts its protective effect on the gastric mucosa in the ethanol-induced ulcer model through its antioxidant, anti-inflammatory and anti-apoptotic properties.

Conflict of interest: There is no conflict of interest among the authors.

Acknowledgement

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