ABSTRACT

BACKGROUND: Inflammatory bowel disease (IBD) is an important health problem. The most important hypotheses for the pathogenesis of this disease are the deterioration of immune responses and loss of tolerance against bacteria in the enteric flora. Although IBD has been widely investigated, its treatment remains difficult. This study aims to investigate the effects of garlic oil (GO) on an experimental colitis model.

METHODS: Twenty-eight rats were randomly divided into four equal groups as follows: group 1 (sham), group 2 (control), group 3 (topical treatment) and group 4 (topical and systemic treatment). An acetic acid-induced colitis model was produced in groups 2, 3 and 4 and was administered normal saline, topical GO and topical and systemic GO, respectively.

RESULTS: Hydroxyproline levels were lower in the treatment groups than in the control group. TNF-α levels were significantly lower in group 3 than in group 2. Macroscopic scores were significantly lower in group 4 than in group 2. Significant differences were observed between the treatment and control groups according to their epithelial loss.

CONCLUSION: GO can reduce colonic damage and inflammation in the acetic acid-induced colitis model, with effects on both local and systemic treatments, but with a more pronounced effect in local treatment.

Keywords: Acetic acid; colitis; diallyl sulphide; garlic oil; inflammatory bowel disease; TNF-α.

INTRODUCTION

Inflammatory bowel disease (IBD), mainly includes ulcerative colitis (UC) and Crohn’s disease (CD), is an inflammatory disease primarily involving the gastrointestinal tract. While various infectious agents, allergens, nutrition habits, psychosomatic factors and immune responses to auto-antigens take part in the etiology of IBD, changes in the balance of mucosal protective factors and excessive bacterial proliferation, as well as changes in cytokine and mediator synthesis, can be responsible for the pathogenesis of this disease.[4–5] The most important hypotheses for the pathogenesis of IBD are both deterioration of immune reactions and lack of tolerance to bacteria in the enteric flora.

The fundamental changes seen in the immune reaction are an increase in macrophage counts and activation of dendritic cells in the lamina propria, which causes nuclear factor κB (NFκB) stimulation, and proinflammatory cytokines (interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-6 and IL-8) production, as well as increased expression of adhesion molecules.[4–5] Therefore, the development of colitis can be reduced with many experimental agents, which cause changes in the immune response as well as the release of inflammatory molecules.
Hydroxyproline is an important constituent of the major structural protein and plays a key role in the synthesis and stability of the collagen. A normal and stable cellular structure of the extracellular matrix is a sign of a complete wound healing process after a tissue injury. Increased hydroxyproline content in granulation tissue is the indicator of increased collagen turnover, which indicates better maturation and proliferation of collagen during wound healing. Therefore, IBD shows both acute and chronic wound healing, measurement of tissue hydroxyproline levels contributes to revealing wound healing.[8]

Garlic oil (GO) is beneficial to human health and contains effective natural organosulfur compounds, such as allicin, allium, diallyl sulphide (DAS) and diallyl trisulphide (DATS).[7] Because GO has a broad diversity of pharmacological properties including anticancer, antibacterial, anti-inflammatory, fibrinolytic, wound-healing, antioxidant and anti-adhesive, it has been used in many different areas of clinical medicine.[8] Studies have shown that purified garlic compounds inhibit NFκB activation and TNF-α production.[10] Another study found that DAS inhibits both pro- and anti-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-10, in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophages.[11] In this study, we presented the effects of GO on cellular damage and inflammation in an acetic acid-induced colitis rat model.

MATERIALS AND METHODS

Study Design

Twenty-eight female adult Wistar–Albino rats, weighing 260 ± 20 g, were enclosed at room temperature (22 °C ± 1 °C) with 12 h:12 h light/dark cycle and fed a standard diet and water. Diet and water were stopped 12 h and 2 h, respectively, before inducing anaesthesia. No antibiotics were administered during the experiment. The experimental protocol was accepted by the Animal Ethics Committee of Bülent Ecevit University (Date/Number: 2014/13b).

Induction of the colitis model

The rats were anaesthetised with intramuscular ketamine (50 mg/kg) and xylazine (6 mg/kg). After inducing anaesthesia, 1 mL of 5% acetic acid (AA) was instilled using a soft 6-Fr paediatric catheter inserted into the anus up to 6–8 cm. Eventually, after 15–20 seconds of exposure, the fluid was withdrawn. Before the withdrawal of the catheter, 2 mL of air was applied to distribute AA into the colon. In the treatment groups, the GO was administered one hour after induction. This experimental model has been shown to have similar histopathological features in human colitis.[12]

Experimental Groups

Twenty-eight rats were randomly divided into four equal groups. The colitis model was produced in all groups except the sham group.

Group 1 (Sham): After each rat was rectally administered saline, 1 mL of saline was intraperitoneally applied.

Group 2 (Control): After the colitis model was produced, 1 mL of saline was intrarectally applied. No treatment was administered.

Group 3 (Topical treatment): One hour after the colitis model was produced, 5 mL/kg of GO (Oleum Allii Sativi®, Arifoglu, Avcilar, Istanbul) was rectally applied. The rats were incubated for 10 min in a Trendelenburg position to provide sufficient mucosal contact with the drug. Subsequently, 1 mL of saline was intraperitoneally administered.

Group 4 (Topical + systemic treatment): One hour after the colitis model was produced, 5 mL/kg of GO was rectally administered. The rats were kept in Trendelenburg position for 10 minutes to ensure adequate drug-mucosal interaction. Subsequently, 5 mL/kg of GO was intraperitoneally applied as a single daily dose for 10 days.

On the tenth day, the abdomen and thorax were opened by a midline incision under anaesthesia. Blood samples were taken by cardiac puncture method for biochemical analysis and rats were sacrificed by the same method. Later, the distal 8 cm of the colon was extracted for histopathological evaluation.

Histopathological Examination

After cleaning the mucosa with saline, the mucosal injury was macroscopically evaluated using the grading scale of Morris et al.[13] (Table 1). A single well-experienced pathologist, who was also blind for the study design, examined each specimen. For this examination, 8 cm distal colon samples were obtained. The colon samples were fixed using 10% formaldehyde, and 4-µm-thick tissue samples in paraffin blocks were obtained and stained with haematoxylin and eosin (H&E) and then analysed under a light microscope (Olympus BX53, Tokyo, Japan). The degree of inflammation of the colon was semiquantitatively graded from 0 to 11, according to the criteria defined by Özgün et al.[14] (Table 2).

Table 1. Variables used for macroscopic evaluation[13]

<table>
<thead>
<tr>
<th>Score</th>
<th>Macroscopic variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No damage</td>
</tr>
<tr>
<td>1</td>
<td>Localized hyperemia, but no ulcers</td>
</tr>
<tr>
<td>2</td>
<td>Linear ulcers with no significant inflammation</td>
</tr>
<tr>
<td>3</td>
<td>Linear ulcer with inflammation at one site</td>
</tr>
<tr>
<td>4</td>
<td>Two or more sites of ulceration and/or inflammation</td>
</tr>
<tr>
<td>5</td>
<td>Two or more major sites of inflammation and ulceration or one more site of inflammation and ulceration extending &gt;1 cm along the length of the colon</td>
</tr>
</tbody>
</table>
Biochemical Analysis

Blood
Blood was collected into tubes at the time of death. Blood samples were centrifuged at 1000 g for 10 minutes at 4°C to remove plasma. Aliquots of the samples were transferred into polyethylene tubes to be used in the assay of biochemical parameters and were stored at -80°C until analysis. TNF-α levels were measured in the serum using a rat TNF alpha ELISA kit (Eastbiopharm, Hangzhou) on fully automatic devices.

Tissue
All tissues were washed twice with cold saline solution, placed into glass bottles, labeled, and stored in a deep freezer (-80°C) until processing. Colon tissues were homogenized in 10 volumes of 150 mM ice-cold KCL using a glass Teflon Homogenizer (Ultra Turrax IKA T18 Basic; IKA, Wilming-ton, NC, USA) after cutting the tissues into small pieces with scissors (for two minutes at 5000 rpm). The homogenate was then centrifuged 5000 g for 15 minutes. The supernatant was used for analysis. Later, spectrophotometry was used to detect tissue hydroxyproline concentrations, and the results were denoted as micrograms per milligram of tissue.[15] First, the samples, standards, and streptavidin-HRP were added to the well. The antibodies were labeled with an enzyme, and the plate was incubated for 60 minutes at 37°C. The plate was washed five times, and chromogen solutions were added. The plate was incubated for 10 minutes at 37°C, and the stop solution was added into the wells. The optical density (OD) was measured under 450 nm wavelengths with a microplate reader. According to standards concentration, the corresponding OD values were calculated using the standard curve linear regression equation to calculate the corresponding sample’s concentration.

Statistical Analysis
Results were analysed using the SPSS (Statistical Package for Social Science) for Windows 19.0 package programme. The normality of the data was determined using the one-sample Kolmogorov–Smirnov test. Continuous variables were analysed using the Kruskal–Wallis variance analysis. While continuous variables were expressed as mean ± standard error, non-continuous variables were expressed as the median (min–max). The chi-square test was used to compare non-continuous variables. A P-value of <0.05 was considered to be a statistically significant difference for all tests.

RESULTS
Hydroxyproline and TNF-α results of all groups are summarised in Table 3. Hydroxyproline levels in the treatment groups were significantly lower than the control group (p=0.002, group 3; p=0.015, group 4). There was no difference in the TNF-α levels between groups 2 and 4; however, they were significantly lower in group 3 than TNF-α levels in the control group (p=0.008).

The distribution of the histopathological results is summarised in Table 4 and is shown in Fig. 1. The method described by Morris et al.[13] was used to assess macroscopic damage in the colon samples. The results of the macroscopic scores in group 4 were significantly lower than those in the control group (p=0.015). Microscopic damage was also evaluated according to criteria defined by Özgün et al.[14] The sham group had histopathological findings similar to the normal colonic mucosa. There was a significant difference between the treatment groups and the control group as it relates to epithelial loss (p=0.001, group 3; p=0.021, group 4). There was no difference between the groups in terms of inflammatory infiltrate, the integrity of crypts and stress to goblet cells. The total damage score was significantly lower in the treatment groups compared to the control group (p=0.001).

<table>
<thead>
<tr>
<th>Score</th>
<th>Epithelial loss</th>
<th>Inflammatory infiltrate</th>
<th>Integrity of crypts</th>
<th>Stress to goblet cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>Intact</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>&lt;5%</td>
<td>Mild</td>
<td>&lt;10%</td>
<td>Present</td>
</tr>
<tr>
<td>2</td>
<td>5–10%</td>
<td>Moderate</td>
<td>10–20%</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>&gt;10%</td>
<td>Severe</td>
<td>&gt;20%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2. Variables used in the classification of the inflammatory histological score[14]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hydroxyproline</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Grup 1 (Sham)</td>
<td>3.55±0.48</td>
<td>26.93±6.35</td>
</tr>
<tr>
<td>Grup 2 (Control)</td>
<td>4.98±0.59</td>
<td>60.82±26.05</td>
</tr>
<tr>
<td>Grup 3 (Topical treatment)</td>
<td>2.95±0.70</td>
<td>45.99±8.97</td>
</tr>
<tr>
<td>Grup 4 (Topical+ systemic treatment)</td>
<td>3.17±1.11</td>
<td>50.16±24.33</td>
</tr>
</tbody>
</table>

TNF-α: Tumor necrosis factor alpha; SD: Standard deviation.
*p=0.002, p=0.015, and p=0.008 vs. control groups.

Table 3. Comparison of groups according to biochemical results
DISCUSSION

The present study indicates that systemic and local administration of GO reduces the inflammation and cellular damage in an AA-induced colitis model.

IBDs, including UC and CD, are inflammatory diseases that primarily affect the gastrointestinal tract. The etiopathogenesis of the disease is multifactorial. While various infectious agents, allergens, nutrition habits, psychosomatic factors and immune response to auto-antigens take part in the etiology of IBDs, changes in the balance of mucosal protective factors and excessive bacterial proliferation as well as changes in cytokine and mediator synthesis can be responsible for the pathogenesis of the disease.[1–3] The most important hypotheses for the pathogenesis of IBD include oxidative stress and reactive oxygen products. Reactive oxygen products that are formed due to oxidative stress cause mucosal damage along with proinflammatory mediators. Therefore, the effects of various antioxidant and anti-inflammatory agents have been the subject of research in IBD.[18,19]

Garlic has been used in many fields of medicine for centuries. The part of the garlic that is responsible for its biological activity is the active organosulphuric compounds, which include allicin, alliin, DAS and DATS. Many studies have shown that garlic compounds have antioxidant, antimicrobial, antimutagenic and anticancer properties.[20,21] Ho et al.[21] found that DAS reduces TNF-α in rat aortic smooth muscles and suggested that DAS could prevent oxidative stress in inflammation. Furthermore, immunohistochemical studies have indicated that DAS reduces inflammatory biomarkers, such as TNF-α and IL-1β, by affecting inducible nitric oxide synthase (iNOS) and activating NFκB.[21,22] Another study demonstrated that allicin inhibits NFκB activation as well as TNF-α and iNOS production.[10] Likewise, it was shown that DAS

Many proinflammatory cytokines are upregulated in IBD, of which TNF-α and IL-1 are the most important. TNF-α is the main mediator of intestinal inflammation. It is synthesised by many inflammatory cells and activates many genes in the NFκB pathway. Other important mechanisms accused in the pathogenesis of IBD include oxidative stress and reactive oxygen products. Reactive oxygen products that are formed due to oxidative stress cause mucosal damage along with proinflammatory mediators. Therefore, the effects of various antioxidant and anti-inflammatory agents have been the subject of research in IBD.[18,19]
inhibits both pro- and anti-inflammatory cytokines in LPS-stimulated macrophages.[11]

Very few studies that investigate the effects of garlic on intestinal diseases are available in the literature. Balaha et al.[24] showed that GO reduces inflammation in the UC model. Blackler et al.[25] in their studies investigating the protective effects of hydrogen sulphide on encephalopathy associated with naproxen, found that the co-administration of DAS reduced naproxen-induced intestinal injury and inflammation. In another study, Voltes et al.[26] found that topically applied hydrocortisone, pectin/alginate and olive oil combination may be effective against inflammatory infiltration in the colitis model. In our study, we found that TNF-α levels in the topical treatment group were lower than those in the control group (p=0.008). Although TNF-α levels were also lower in systemic + topical treatment, the results were not significant.

Hydroxyproline is an important constituent of the major structural protein and plays a key role in the synthesis and stability of the collagen. Hydroxyproline is an important indicator of collagen accumulation. Deficiency of protein during wound healing may diminish new capillary development, fibroblast proliferation, collagen and proteoglycans synthesis and remodeling and contraction of the wound. A normal and stable cellular structure of the extracellular matrix is a sign of a complete wound healing process after a tissue injury. Breakdown of collagen releases free hydroxyproline and its peptides. Thus, hydroxyproline as a biochemical marker during wound healing is extensively used to evaluate the tissue collagen content and as an indicator for collagen turnover after wound-healing. Increased hydroxyproline content in granulation tissue is the indicator of increased collagen turnover, which indicates better maturation and proliferation of collagen during wound healing. There are numerous reasons to support the use of hydroxyproline as a biomarker of the collagen content within tissues after the wound-healing process as it is abundantly found in collagen and plays a vital role in wound healing.[8] Sadar et al.[27] found that increased hydroxyproline levels significantly correlated with accumulated collagen in the colonic tissue after trinitrobenzene sulfonic acid (TNBS) administration, whereas ferulic acid treatment significantly reduced colonic hydroxyproline activity. Motawi et al.[28] also found similar results. In our study, we found that hydroxyproline levels were significantly higher in the control group and significantly lower in the treatment groups, which was in accordance with the literature (p=0.002, topical treatment group; p=0.015, topical + systemic treatment group).

Macroscopic and histopathological examinations are the gold standard for evaluating inflammatory in the colon. Appleyard and Wallace[29] found that total histopathological damage score was higher in the colitis group compared to the saline group in the original colitis studies in which they underwent histopathological evaluation. Balaha et al.[24] found that GO ameliorated the marked macroscopic and microscopic changes of colonic mucosa in a dose-dependent manner. In our study, we found that the total damage score was significantly lower in the treatment groups compared to the control group (p=0.001).

Conclusion

In conclusion, GO able to inhibit AA-induced colitis in rats, maybe through its anti-inflammatory and immunomodulatory properties. This effect is seen in both local and systemic treatments; however, the effect is more pronounced in local treatment. Moreover, further investigations are currently in progress to determine the precise underlying protective mechanism of GO on AA-induced colitis. Therefore, GO can be used both to reduce cellular damage and disease severity during the exacerbation period and to be recommended as a protective agent recommended for UC patients.

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Peer-review: Internally peer-reviewed.


Conflict of Interest: None declared.

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REFERENCES

Pharmacol Ther 2014;142:183–95. [CrossRef]