The Effect of N. Acetylcysteine and Ginger on Acetic Acid Induced Colitis in Adult Male Albino Rat: Histological, Immunohistochemical and Morphometric Study

Tarek I Abd El-Galil, Tarek A ElGhamrawy* and Abir O El Sadik
Department of Anatomy and Embryology, Faculty of Medicine, Cairo University, Egypt

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Introduction

Idiopathic Inflammatory bowel diseases (IBD), mainly Crohn’s disease and ulcerative colitis are increasingly worldwide in their incidence and prevalence, posing a significant morbidity and poor quality of patient life [1]. They are chronic progressive diseases with persistent inflammation of the bowel. The aetiology of IBD is multifactorial, involving bacterial translocation through the injured mucosa. In addition, genetic, immunological factors and oxidative stress factors are involved [2,3]. Several experimental IBD in vivo studies have showed increased antioxidant enzymes to metabolize the oxidative toxic intermediates [4,5]. Currently, there is no curative treatment and conventional pharmacological drugs had partial efficacy, being unable to maintain a long-term clinical remission [4,6,7]. New therapeutic alternatives are in need. In addition, the long-term use of the pharmaceutical drugs results in numerous side effects [8]. So, new therapies such as the nutritional ones, especially those with antioxidant properties are suggested for the treatment of such disorders.

Ginger (Zingiber officinale) is a frequently used herbal medicine throughout the world, especially in most of the Asian countries, and one of the most commonly used dietary substances [9,10]. Native Americans have also used wild ginger rhizome to regulate menstruation and heart beats [11]. It has been used traditionally as carminative, diaphoretic, antispasmodic and antiemetic [12]. It has analgesic effects [13], antineoplastic effects [14,15] and interestingly ginger possesses antioxidant activity [16], as well as significant anti-inflammatory property [17]. It inhibits the structural changes in diabetes induced brain damage [18]. It also has a potential ability to scavenge a number of oxygen free radicals and to protect cell membrane lipids from oxidation [19]. The anti-inflammatory properties of ginger are mediated by inhibiting the production of nitric oxide, inflammatory cytokines and arachidonic acid [20]. It also modulates the lymphocytes function, so it might exert beneficial effects in a number of clinical conditions such as chronic inflammation and autoimmune diseases [21].

N. acetylcysteine has been used in the treatment of acetaminophen toxicity [22] and as a mucolytic therapy [23]. It is a potent antioxidant, acting by raising the intracellular concentration of cysteine and glutathione and scavenging reactive oxygen species [24]. It replenishes hepatic glutathione depleted by drug conjugation [25]. Recently, clinical studies reported its potential antioxidant activity in protection against contrast induced nephropathy [26] and thrombosis [27]. Also, it has anti-inflammatory properties reported in several tissues like in cerebral and pulmonary inflammation [28,29].

The present study utilized acetic acid-induced colitis model, for investigating the protective effects of both ginger, used as a nutritional supplement, and NAC, used as a pharmaceutical drug and...
a nutritional supplement, using histological, immunohistochemical and morphometric techniques. They have been selected due to their anti-inflammatory and anti-oxidant properties aiming to find new therapeutic approaches for the treatment of IBD.

Material and Methods

Materials

Acetic acid (4% concentration, ready-made, VACSERA, Egypt) is used to induce colitis in the experimental groups [30].

N. acetylcysteine (NAC) (SEDICO Pharmaceutical Company, 6th of October city, Egypt) was prepared by dissolving 500 mg powder in 1 ml saline.

Ginger preparation and extraction: Limed peeled ginger rhizomes were purchased from herbal stores in Beni Suef, Egypt. Identity and purity was confirmed by microscopical examination of the powdered drug in the Department of Pharmacognosy, Faculty of Pharmacy, Beni Suef University. The dry powdered plant (0.7 Kg) was subjected to exhaustive extraction by percolation with 70% ethanol for 24 hours (3 times 1.5 L each) at room temperature. The crude ethanol extract was filtered by Whatman No.1 filter paper and concentrated under vacuum at a temperature not exceeding 45° C. The dried total crude extract gave 252 mg and was used for the next step.

Induction of experimental colitis: Acute colitis was induced by acetic acid. After 36 hours fasting, with access to water ad libitum, each rat was lightly anesthetized by an intramuscular injection of ketamine hydrochloride (10 mg/kg body weight). The abdomen was opened and 1 ml saline was injected in the ascending colon. The abdomen was closed and the rats were maintained in a head down position for 5 minutes to prevent leakage [32].

Experimental design

Animals: Fifty adult male albino rats with an average weight of 200-250 g were used in this study. They were locally bred at the animal house at Faculty of Medicine, Cairo University, Egypt. The animals were housed at room temperature and had access to food and water ad libitum. The animals were given two weeks acclimatization period before starting the experiment. The study was approved by the Ethics Committee, Faculty of Medicine, Cairo University. All animal procedures followed the ethical guidelines of experimental animals and all the efforts to ensure minimal animal sufferings were taken.

The animals were equally and randomly divided (n=10) into the following groups.

Group I (Control group): The rats received 0.5 ml saline followed by 2 ml air intrarectally by Nelaton catheter (size 8).

Group II (Colitis model group): Induction of experimental colitis with acetic acid was performed.

Group III (NAC treated group): Rats, with acetic acid-induced colitis, were treated with NAC (1 g/kg/day) by orogastric gavage [33].

Group IV (Ginger treated group): Rats, with acetic acid-induced colitis, were treated with ginger (500 mg/kg body weight) by orogastric gavage [18].

Group V (Combined NAC and ginger treated group): Rats, with acetic acid-induced colitis, were treated by both NAC and ginger with the same route and dose given to the preceding two groups.

Both NAC and ginger were given one day after induction of colitis. Two weeks from the beginning of the experiment, all animals were euthanized by intraperitoneal injection of sodium pentobarbital (120 mg/kg body weight). Segment of the distal colon 6 cm length and 3 cm from the anus was excised for histological and immunohistochemical studies.

Light microscopic examination: The colon was removed and rapidly fixed in 10% formal saline solution, processed, embedded to obtain paraffin blocks and cut at 5-6 micron thickness sections. Sections were subjected to the following stains:

1. Routine Haematoxylin and Eosin (H & E) stain [34].
2. Periodic acid-schiff (PAS) reaction to demonstrate the polysaccharides in goblet cells [35].
3. Masson’s trichrome stain to show the collagen fibers [36].

Immunohistochemical staining: Sections were cut at 5-6 micron thickness and collected on poly-L-lysine coated slides, deparaffinized in two changes of xylene, 10 minutes each, then hydrated through graded washes of ethanol in water, ending in a final rinse in pure water. Non-specific endogenous peroxidase activity was blocked by treatment with 0.9% hydrogen peroxide in absolute methanol for 10 min. Antigen retrieval was performed by heating the sections in 10 mM sodium citrate buffer, in a water bath at 95–100°C for 30 minutes. Sections were rinsed twice in PBS Tween 20 for 2 minutes, then blocked with 5% normal goat serum for 30 minutes at room temperature. They were incubated with the following primary antibodies for 30 minutes: 1. Proliferating cell nuclear antigen (PCNA), rabbit polyclonal IgG (FL-261; catalog# sc-7907, 200 µg/ml, dilution 1:50, Santa Cruz Biotechnology, USA) which is a cofactor of DNA polymerase delta involved in DNA replication. 2. Nitric oxide synthase 2 inducible (i.NOS), rabbit polyclonal antibody (Product GTX15323; dilution 1:100, Gene Tex, USA) which is expressed in activated macrophages. Then, a biotinylated goat anti-polyvalent secondary antibody (Catalog # TP-060-BN, Thermo Scientific, USA) was applied for 60 min at room temperature. Immunodetection was carried out with the horseradish peroxidase-avidin-biotin complex method using a VECTASTAIN Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) and DAB substrate kit. Localization was visualized with DAB and counter-stained in Mayer’s hematoxylin, dehydrated, and mounted. Negative control sections were performed with the same procedure mentioned before except that the primary antibody was replaced by non-immune rabbit serum [35].

Morphometric study and statistical analysis: The following parameters were measured in different fields with fixed field area 4 x 10^4 µm²:

1. The number of goblet cells per field in PAS stained sections.
2. The area percent of colonic collagen fibers and iNOS immunopositive expression. They were measured by selecting a positive localized area from the field required to be measured and the software system detected its colour and then scanned the whole field for the areas with the same colour and calculated them out of total field area.
3. PCNA proliferation index was measured as the percentage number of PCNA positively labelled cells to the total number of cells per field.

The measurements were done in ten non overlapping fields per specimen at a magnification of 400, using Leica LAS V3.8 image analyzer computer system (Switzerland). The data obtained for all groups were expressed as mean and standard deviation (± SD) and subjected to statistical analysis using “SPSS 22” software. One-way analysis of variance (ANOVA) for comparison between the different groups was done. Results were considered significant when p value was ≤0.05 [37].

Results

Histological results

Haematoxylin and Eosin stained sections: Sections in the colon of control group showed normal histological structure of the mucosa which was arranged into deep narrow spaced intestinal crypts, lined with both simple columnar epithelium and goblet cells, resting on a thin continuous layer of muscularis mucosa. Narrow submucosal layer separated the mucosa from the muscle layer and extended in the core of the colonic mucosal folds (Figures 1a and 1b).

Acetic acid induced colitis group exhibited extensive microscopic damage with disturbed histological architecture of the mucosa and submucosa of the colon. The mucosa showed pyknotic nuclei, sloughed epithelial cells with widened submucosa and capillary congestion (Figures 2a and 2b). Irregular intestinal surface epithelium with shallow and dilated intestinal crypts was lined by cells with vacuolated cytoplasm. The mucosa showed inflammatory cellular infiltration (Figures 2c and 2d). Dilated congested lymphatic vessels and submucosal inflammatory infiltration were seen (Figure 2e).

NAC treated group showed dilated intestinal crypts, lined by vacuolated cells with areas of sloughed epithelium among normal surface epithelium and submucosal inflammatory cellular infiltration (Figures 3a and 3b). Ginger treated group expressed intact intestinal epithelium and few inflammatory cells (Figures 3c and 3d).

NAC and ginger treated group showed normal appearance of colonic mucosa with few inflammatory cells, preservation of columnar epithelium and intact mucosal surface (Figures 4a and 4b).

1. PAS stained sections: Sections in the colon of control group revealed large number of goblet cells within the intestinal mucosa, however, their number was markedly decreased in colitis model group, then it was increased in all treated groups, especially in ginger treated and both NAC and ginger treated groups (Figure 5).

Figure 1: Photomicrographs of sections in the colon of control group showing: (a) the mucosa arranged into deep narrow spaced intestinal crypts, lined by simple columnar epithelial cells (arrows), resting on a thin continuous layer of muscularis mucosa (thick arrow). Narrow submucosal layer (sm) separates the mucosa from the muscle layer (M) and extends in the core of the colonic mucosal folds. (b) The intestinal crypts are lined with both simple columnar epithelium (arrow) and Goblet cells (arrow heads). (H & E, (a) 100X; (b) 400X).

Figure 2: Photomicrographs of acetic acid colitis group showing: (a, b) sloughed epithelial cells (arrowheads), pyknotic nuclei (encircled in b) and widened submucosa (sm) with capillary congestion (thick arrow). (c) Irregular intestinal surface (arrows) and dilated intestinal crypts lined by vacuolated cells (thick arrows). The mucosa shows inflammatory cellular infiltration (arrow heads). (d) Shallow intestinal crypts (arrow) and mucosal inflammatory cellular infiltration (arrow heads). (e) Dilated congested lymphatic vessels (arrows) within widened submucosa (sm). (H & E, 400X).

Figure 3: Photomicrographs of NAC treated group (a,b) and ginger treated group (c,d) showing: (a) dilated intestinal crypts (arrows) and areas of sloughed and interrupted epithelium (arrow heads) among normal surface epithelium. (b) Dilated intestinal crypts lined by vacuolated cytoplasmic cells (arrow) and submucosal inflammatory infiltration (arrow head). (c,d) Intact intestinal epithelium (arrow heads) and dilated crypts (arrow). There are few inflammatory cells (wavy arrows). (H & E, (a,b,c) 400X; (d) 1000X).
2. Masson’s trichrome stained sections: Sections of the colon of control group showed minimal fibrous tissue arranged within the lamina propria of the mucosa and within the submucosa. Increased fibrous tissue deposition in both mucosa and submucosa was seen in acetic acid colitis and NAC treated groups. However, ginger treated group exhibited reduced fibrous tissue deposition. NAC and ginger treated group had minimal fibrous tissue similar to control group (Figure 6).

Immunohistochemical results

NOS stained sections: Control group revealed a small number of iNOS immunostained cells dispersed throughout the mucosa and within the fibrous tissue lamina propria with diffuse cytoplasmic staining. In acetic acid induced colitis group the number of positive cells propagated sharply. Both NAC treated and ginger treated groups had a marked decrease in immunopositive cells; more in ginger group. NAC and ginger treated group showed few number of immunopositive cells among all the treated colitis groups (Figure 7).
PCNA immunostaining: Sections in the colon of the control group showed a large number of PCNA stained nuclei throughout the mucosa. Acetic acid colitis group had weakly stained few PCNA cells in the basal cells of intestinal crypts. NAC treated group revealed increasing number of densely stained PCNA cells within the basal and mid-regions of intestinal crypts and in the fibrous tissue within the lamina propria. Similarly, ginger treated group showed increasing number of PCNA stained cells in all parts of intestinal crypts from the base up to mucosal surface. NAC and ginger treated group had the largest distribution of positive cells (Figure 8).

Morphometric results and statistical analysis

The number of goblet cells compared to the control group was significantly reduced in acetic acid colitis, NAC treated and ginger treated groups but increased to show no difference in NAC and ginger treated group (Table 1 and Bar chart 1). The area percent of fibrous tissue significantly increased in both acetic acid colitis and NAC treated groups and was reduced markedly to show nonsignificant difference in ginger treated and combined NAC and ginger treated groups (Table 2 and Bar chart 2). Similarly, the area percent of the inflammatory mediator iNOS was significantly increased in both acetic acid colitis and NAC treated groups and declined to show nonsignificant difference in ginger treated and both NAC and ginger treated groups (Table 3 and Bar chart 3). The PCNA proliferation index significantly declined in acetic acid colitis group but increased to nonsignificant difference from control in the rest of treated groups (Table 4 and Bar chart 4).

Discussion

The chronic and intermittent nature of IBD and inability of the current drugs to sustain long term remission made a continuous need to testify new drugs aiming to minimize the morbidity and to improve the quality of patient life. Two agents, NAC and ginger, known for their anti-oxidant and anti-inflammatory properties were tested here for their potential curative effects in IBD. The acetic acid rat model applied in the current study is commonly used to produce acute colitis mimicking the inflammatory response in human IBD. The model was validated by using several drugs applied in treatment of IBD and finding similar profile effects in murine and human colonic specimens [38-40].

The present work demonstrated characteristic histological features in untreated colitis, essentially loss of intestinal crypt architecture and sloughing of intestinal cells, reduced goblet cell number and presence of different inflammatory cell infiltration. These results concord with recent findings of Lean et al. [41]. The submucosa was widened due to increased collagen fibres deposition and probably from oedema. Ginger treatment obviously restored the mucosal epithelium integrity and diminished mucosal and submucosal inflammatory cells; however NAC had marginal mucosal regenerative effects but no notable reduction of inflammatory cellular infiltration. The combined ginger and NAC treatment gave results similar to ginger alone.

The current study found decreased goblet cell number in the colitis model. Only in combined NAC and ginger treated group, it increased to normal values. The combined effect of both exceeded the protective effect of each one alone. The cell number insignificantly increased in ginger treatment compared to control group. The increased goblet number was concomitantly associated with intestinal epithelial regeneration and restoring the distorted intestinal crypts. Goblet cells continually produce mucins to replenish and maintain the mucus barrier. It keeps intestinal microbial flora separated from the epithelium and is involved in immune functions such as antigen presentation.
and tolerance [42]. Recently, the compromised mucus barrier role in pathogenesis of inflammatory bowel disease was highlighted [43]. In consistent with the present results, goblet cell depletion has been observed in ulcerative colitis patient biopsies [44]. Also, lower levels of trefoil factor 2, peptides abundantly secreted by goblet cells on the lumen surface, were reported in mucosal biopsy of children with IBD [45]. Recently, impaired differentiation of goblet cells from intestinal stem cells was found in inflammatory bowel diseases and this resulted in defective mucus layer [46,47]. Contrary to the present results, other investigators found increased number of goblet cells in dextran sulfate sodium-colitis rats [48]. The authors explained their findings by the heterogeneity of colonic inflammation with depleted goblet cell areas and increased in another areas with mucus overproduction previously suggested by Nakano et al. [49].

The colitis model showed increased fibrous tissue deposition within the lamina propria of mucosa and in the submucosa and ginger treatment managed to reduce the fibrotic changes towards control values. In accordance with the present results, increased pro-collagen mRNA expression and collagen protein deposition were reported to occur in experimental colitis [50-53]. Recurrent inflammation met in inflammatory bowel diseases led to extensive tissue fibrosis and stiff colon, unable to carry out fluid reabsorption or peristalsis [54]. Also, ginger expressed ability to decrease fibrotic changes in other conditions like liver fibrosis [55,56].

Nitric oxide expressed by the inducible nitric oxide pathway was identified in the epithelium of normal human colonic mucosa in low levels [57]. Also, it was localized in the inflammatory and normal mucosa of patients with ulcerative colitis [58]. Schreiber et al. [48] reported that increased i.NOS mediates an increase in mucus layer

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**Table 2:** Mean values, standard deviation and significance of area percent of collagen fibres in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>p value</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.92 ± 0.62</td>
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<tr>
<td>Acetic acid induced colitis</td>
<td>29.05 ± 2.19</td>
<td>0.001*</td>
</tr>
<tr>
<td>NAC treated colitis</td>
<td>19.95 ± 1.56</td>
<td>0.001*</td>
</tr>
<tr>
<td>Ginger treated colitis</td>
<td>10.40 ± 1.14</td>
<td>0.06</td>
</tr>
<tr>
<td>NAC and ginger treated colitis</td>
<td>8.15 ± 0.74</td>
<td>0.920</td>
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</tbody>
</table>

* = statistically significant compared to control (p<0.05).

**Table 3:** Mean values, standard deviation and significance of area percent of i.NOS in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>p value</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.76 ± 0.56</td>
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<tr>
<td>Acetic acid induced colitis</td>
<td>31.33 ± 1.16</td>
<td>0.001*</td>
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<tr>
<td>NAC treated colitis</td>
<td>28.59 ± 0.67</td>
<td>0.001*</td>
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<tr>
<td>Ginger treated colitis</td>
<td>4.52 ± 1.09</td>
<td>0.837</td>
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<tr>
<td>NAC and ginger treated colitis</td>
<td>4.20 ± 0.77</td>
<td>0.826</td>
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* = statistically significant compared to control (p<0.05).

**Table 4:** Mean values, standard deviation and significance of PCNA proliferation index per field in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>p value</th>
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<tbody>
<tr>
<td>Control</td>
<td>46.96 ± 19.27</td>
<td>--</td>
</tr>
<tr>
<td>Acetic acid induced colitis</td>
<td>26.03 ± 8.49</td>
<td>0.016*</td>
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<tr>
<td>NAC treated colitis</td>
<td>39.74 ± 12.50</td>
<td>0.788</td>
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<tr>
<td>Ginger treated colitis</td>
<td>54.17 ± 10.30</td>
<td>0.739</td>
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<tr>
<td>NAC and ginger treated colitis</td>
<td>67.86 ± 6.09</td>
<td>0.160</td>
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</tbody>
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* = statistically significant compared to control (p< 0.05).
thickness. The current results found marked up-regulation of i.NOS immunoeexpression in induced colitis model accompanied by dense inflammatory cellular infiltration, mucosal and submucosal. This was in agreement with the previously reported data at mRNA levels [59,60]. Interestingly, this up-regulation was reversed with ginger treatment and not in NAC treated group. It could be perceived from these findings that ginger could reduce the inflammatory process via inhibition of i.NOS production. In addition to intestinal epithelial and inflammatory restoration found with ginger treatment. Moreover, it was found that i.NOS could be a potential prognostic marker for ulcerative colitis. Neutrophil infiltration and inflammatory oedema were partially decreased using i.NOS inhibitors in colitis rat model [61]. Nitric oxide played a crucial role in oxidative related disorders. Ginger suppressed nitrites generation by scavenging oxygen free radicals and protected cell membrane lipids from oxidation [14]. This in turn, led to suppression of proinflammatory cytokines released from inflammatory cells [62].

Intestinal mucosa possesses rapidly proliferating epithelial cells producing an intact, physical barrier against stresses ranging from digestive trauma to severe insults like infection [3]. The standard method of evaluation of cell proliferation is by using 5-bromodeoxyuridine (BrdU) immunohistochemistry but has several disadvantages, however PCNA proved to be highly sensitive and can substitute it [63]. PCNA is essential in both DNA replication and repair [64]. In the present work PCNA proliferation index was suppressed in the colitis model and up-regulated in both ginger and NAC treated groups. The proliferating effect of both ginger and NAC could be linked to their antioxidant effects and modulating the intracellular redox environment of the injured colonic cells. In accordance with these results, ginger enhanced skin cell proliferation [65] and collagen production [66]. It stimulated the suppressed neurogenesis in diabetic rats [18]. Recently, Wu et al. [67] reported that NAC reversed the antiproliferative effects of Icariside II, a metabolite derived from Herba Epimedi, in human melanoma cell lines and resumed cell-division cycle by scavenging reactive oxygen species. The current beneficial effects of ginger were evident after short-term administration, however long-term study is needed. This is to be sure that these effects are sustainable in treatment of IBD.

Overall, the novelty of this work is: (1) to provide immunohistochemical evidence of the anti-inflammatory effect of ginger and the mechanism of its action by suppressing nitric oxide production in the treatment of experimentally induced colitis. (2) To show the superior results of ginger than NAC on structural restoration of colonic mucosa. It enhanced mucosal cell proliferation and suppressed inflammatory cell recruitments. This conclusion encourages clinical studies on ginger use as a promising nutritional therapeutic alternative in the management of inflammatory bowel diseases.

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