A histological and immunohistochemical study of different therapeutic modalities for experimentally induced ulcerative colitis in rats

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Background
Ulcerative colitis (UC) is a recurrent bowel disease. Several medications are used to treat patients with active UC. However, these are associated with side effects that add to the disease-related complications.

Aim
The aim of the study was to evaluate the therapeutic effect of pentoxifylline, nanocurcumin, and mesenchymal stem cells on experimentally induced UC in rats.

Materials and methods
Sixty-five adult male rats were divided into six groups: group I was the control group; group II (UC) consisted of rats that received a single intrarectal injection of 2 ml of 4% acetic acid; group III consisted of rats that received a daily oral dose of pentoxifylline starting 3 days after induction of colitis, for 2 weeks; group IV consisted of rats that received a daily oral dose of nanocurcumin starting 3 days after induction of colitis, for 2 weeks; group V comprised rats that received a single intrarectal injection of bone marrow-derived mesenchymal stem cells through the tail vein 3 days after induction of colitis; and group VI consisted of rats that received a single intrarectal injection of 2 ml of 4% acetic acid and were left without treatment for 2 weeks. Colonic samples were processed and examined using histological and immunohistochemical techniques.

Results
Group III showed improvement in the histopathological picture of colitis. Group IV showed a near-normal histological picture, except for a few areas of surface simple columnar cells that showed discontinuity and a few distorted crypts. Group V showed a histological picture that was nearly similar to that of the control group. Group VI showed a picture that was nearly similar to that of the UC group. There was a significant increase in asialo blue-positive reaction and a significant decrease in the amount of collagen fiber deposition and in tumor necrosis factor-α expression (P<0.05) in groups III, V, and VI compared with group II, but these parameters were nonsignificant in the recovery group.

Conclusion
Bone marrow-derived mesenchymal stem cells and nanocurcumin are more effective than pentoxifylline in the treatment against UC in rats. Nanocurcumin is cheaper and hence more cost-effective.

Keywords:
nanocurcumin, pentoxifylline, stem cell, ulcerative colitis

Introduction
Inflammatory bowel disease (IBD) is a chronic inflammatory condition with sudden remissions and exacerbations in its clinical course [1]. IBD has a high incidence in industrialized nations [2]. Ulcerative colitis (UC) is considered one of the IBDs that affect the lining of the colon and the rectum. Its symptoms vary and include abdominal pain, cramping, bloody diarrhea, pus stool, fever, rectal pain, weight loss, nausea, vomiting, arthritis, mouth sores, and growth retardation in children [3]. The inflammatory process is accompanied by immunological cell infiltration to the lamina propria of the gut and by untagulated production of proinflammatory mediators (tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, IL-17, and IL-21) and reactive oxygen species (ROS) in colonic tissues [4]. These mediators have been
suggested to contribute to the development of mucosal damage of the colon and lead to a chronic inflammatory process [5]. ROS can induce membrane damage and DNA adducts and can eventually cause the degeneration of tissues and cellular transformation [6].

Pentoxifylline (PTX) is a xanthine derivative with the chemical name 1-(5-oxohexyl)-3,7-dimethylxanthine (7). PTX is a phosphodiesterase inhibitor that is widely used in peripheral vascular disease treatment and for which a wide range of immunomodulatory activities have been reported and described [8]. The therapeutic use of PTX has been studied alone or as an adjuvant therapy in different conditions, including in infectious processes, and positive results have been observed. PTX prevents murine cerebral malaria and improves the prognosis of this disease in humans. It also decreases leukocyte recruitment into the cerebral fluid in experimental bacterial meningitis, decreases organ damage, and improves survival in humans and in animals with sepsis [9].

Curcumin, derived from the rhizome of the plant Curcuma longa, is a yellow-orange polyphenol compound. It is widely used as a spice, food preservative, and flavoring and coloring agent [10]. Over the last century, curcumin has been found to possess antineoplastic, antimicrobial, anti-inflammatory, antioxidant, and wound-healing activities [11]. Despite displaying multiple beneficial pharmacological effects, curcumin suffers from extremely low aqueous solubility in water at physiological pH. The poor solubility of curcumin limits its absorption and scatters in low bioavailability [12]. Similar to many other compounds of limited aqueous solubility, curcumin delivery may benefit from nanotechnological approaches [13]. Formulating curcumin nanoparticles encapsulated with a biodegradable polymer can significantly improve curcumin's solubility, stability, and bioavailability to extend [14]. Curcumin-encapsulated nanoparticle formulations were proven to be safe, suggesting its usage in oral drug delivery applications [15].

Mesenchymal stem cells (MSCs) are self-renewing progenitor cells with the capacity to differentiate into various cell types under specific conditions [16]. Adult stem cells are derived from different sources, including bone marrow, adipose tissue, or postnatal tissues such as umbilical cord and placenta, and have been shown to possess regenerative, anti-inflammatory, or immunomodulatory potential in a variety of diseases [17]. Of all the adult stem cell types, MSCs are of considerable interest because of their easy isolation, multipotency, and high proliferative potential in vitro [18]. Indeed, bone marrow-derived mesenchymal stem cells (BMSCs) can not only act in the hematopoietic system but also migrate into damaged tissues and organs and induce differentiation into corresponding cells [19]. As the traditional and most well-characterized source of human MSCs, BMSCs are increasingly being used in clinical applications because of the availability of a large amount of data regarding their biological characteristics and their safety in clinical studies [20].

The present study was conducted to evaluate the therapeutic effect of PTX, nanocurcumin and BMSCs on experimentally induced UC in rats.

Materials and methods

Reagents

Acetic acid was purchased from Merck Chemicals GmbH (Darmstadt, Germany). PTX (Trental) tablets (400 mg) were purchased from Sanofi Aventis (Cairo, Egypt). Tablets were dissolved in distilled water and were given (100 mg/kg/day) orally through a gastric tube for 2 weeks [21].

Nanocurcumin (250 mg veg caps) was purchased from One Planet Nutrition (Palmco, Florida, USA). Each capsule of powder was dissolved in 10 ml of distilled water and given to the animals at a dose of 100 mg/kg/day orally through a gastric tube for 2 weeks [22].

BMSCs labelled with iron oxide were purchased from the Biochemistry Department, Faculty of Medicine, Cairo University. The BMSCs were injected intravenously into the tail vein of each rat at a single dose of 3×10^6 cells suspended in 0.5 ml PBS [23].

Animals

Sixty-five healthy adult male albino rats weighing 180–230 g were obtained from the Animal House, Monashor Faculty of Veterinary Medicine, Benha University. The rats were fed a balanced diet and had free access to water. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. They were kept under observation for 1 week before the beginning of the experiment to acclimatize.

Experimental procedure

The animals were divided into six groups:

1. Group I (the control group): 15 rats were subdivided equally into three groups.
   a. Subgroup Ia: the rats in this group received a single intraperitoneal injection of 2 ml saline.
   b. Subgroup Ib: the rats in this group received 1 ml of distilled water orally daily through a gastric tube for 2 weeks.
   c. Subgroup Ic: the animals in this group received a single dose of 0.5 ml PBS intravenously through the tail vein.

2. Group II (UC group): 10 rats received a single intraperitoneal injection of 2 ml of 4% acetic acid and were sacrificed 3 days after induction of UC.

3. Group III (UC with PTX): 10 rats received a daily oral dose of PTX starting 3 days after induction of colitis, for 2 weeks.

4. Group IV (UC with nanocurcumin): 10 rats received a daily oral dose of nanocurcumin starting 3 days after induction of colitis, for 2 weeks.

5. Group V (UC with BMSCs): 10 rats received a single injection of BMSCs through the tail vein 3 days after induction of colitis.

6. Group VI (the recovery group): 10 rats received a
single intrarectal injection of 2 ml of 4% acetic acid and were left without treatment for 2 weeks after induction of colitis.

**Induction of ulcerative colitis**

Colitis was induced according to the method described by some authors [24]. Rats were anesthetized with ether after a 24-h fast, and a 2.7-mm-diameter soft pediatric catheter was lubricated with gel and then inserted 8 cm proximal to the anus. A solution of 2 ml of acetic acid (4%, v/v) was instilled into the lumen of the colon and maintained in a supine Trendelenburg position for 30 s to prevent leakage of the intracolic instillation. Seventy-two hours after a single dose of acetic acid UC was induced.

**Light microscopic studies**

The fasted rats were anesthetized with ether and sacrificed by means of cervical decapitation. Rats in group II were sacrificed 3 days after intrarectal injection of acetic acid (induction of UC). Rats in other groups were sacrificed 2 weeks after induction of UC. The abdominal cavities were rapidly opened and the distal colon was excised. The specimens were fixed in 10% buffered formal saline and processed for paraffin sections of 5-7 μm thickness, and mounted on glass slides for H&E, Mal- lory’s iron-hematoxylin, and alizarin blue stain [25]. Other sections were mounted on positively charged slides for immunohistochemical staining using avidin-biotin immunohistochemistry staining for TNF-α [26].

**Immunohistochemical staining for tumor necrosis factor α**

Immunohistochemical detection of TNF-α in the cells of the colon was carried out using a standard avidinbiotin-peroxidase complex system as per the instructions on the kit used (Dako, Glostrup, Denmark). Monoclonal mouse anti-human antibody was used as the primary antibody against TNF-α. Sections were mounted on positively charged glass slides. The paraffin sections were deparaffinized, hydrated, and kept in 10% H2O2 to block endogenous peroxidase activity, and the slides were treated in running water and PBS. The primary specific antibody was diluted 1 : 500 with PBS-0.1% saponin. The site of antibody immunostaining was visualized after incubation with the avidin-biotin-peroxidase complex (ABC reagent) for 30 min and after washing with PBS. Freshly prepared diaminobenzidine was used as chromogen. The sections were incubated with diaminobenzidine for 10 min and then washed with tap water counterstained with hematoxylin, dehydrated, and mounted. For negative control, the primary antibody was replaced with PBS (phosphate buffered solution).

**Immunohistochemical staining for CD4 in colon mesenchymal stem cells**

Immunohistochemistry was performed as per the instructions on the kits. Paraffin sections were deparaffinized and hydrated. After blocking the endogenous activity of peroxidase using 10% H2O2, the sections were incubated with primary antibody: CD44 antibody is a mouse monoclonal antibody clone 2A4 (Lab Vision Corporation, Neomarkers, Westinghouse, Thumont, Frenqent, California, USA). After washing with PBS, the secondary antibody was applied (biotinylated goat anti-rabbit). The slides were incubated with labeled avidin-biotin-peroxidase, which binds to the biotin on the secondary antibody. The site of antibody binding was visualized after adding diaminobenzidine as chromogen, which is converted into a brown precipitate by peroxidase. Sections were counterstained with Mayer’s hematoxylin.

**Prussian blue staining for iron oxide-labelled mesenchymal stem cells in the colon**

The Nova Ultra Prussian Blue Stain Kit (HC World Llc, Ellicott City, Maryland, USA) was used for the procedure. Prussian blue reaction involves the treatment of sections with acid solutions of ferrocyanides. Any ferric ion (Fe3+) present in the tissue combines with the ferrocyanide and results in the formation of a bright blue pigment called Prussian blue, or ferrocyanide (Fe3+).

**Morphometric and statistical study**

The mean area% of alicia blue-positive reaction, collagen fiber deposition, and TNF-α expression was quantified in 10 images from 10 rats of each group using Image-Pro Plus program (version 6.0, Media Cybernetics Inc., Bethesda, Maryland, USA). The alicia blue-positive reaction, collagen fiber deposition, and TNF-α expression in group III (UC with PTX group), group IV (UC with nanosilver group), group V (UC with MSCs group), and group VI (recovery group) were compared with those of group II (UC group) using the t-test, with P value less than 0.05 as the level of statistical significance. Statistical analyses were carried out using IBM SPSS statistics software for Windows (version 20; IBM Corp., Armonk, New York, USA).

**Results**

Bone marrow-derived mesenchymal stem cells tracking

MSCs were identified by means of a light microscope in colon sections from group V after immunohistochemical staining for the CD44 antigen (Fig. 1) and after staining with Prussian blue stain (Fig. 2).

**H&E stain**

Group I (subgroups Ia, Ib, and Ic) showed the same histological picture. They showed a normal shape for the mucosa, submucosa, muscularis externa, and serosa (Fig. 3). The mucosa was intact and continuous, and had many folds with surface simple columnar cells and regularly arranged closely related crypts. Crypts appeared tubular in structure with numerous goblet cells (Fig. 4). Group II (the UC group) showed absence of mucosal folding, loss of surface columnar epithelial lining, and distorted crypts with a few goblet cells. The mucosa and submucosa demonstrated heavy mononuclear
cellular infiltration. Amoeboid muscularis mucosa and submucosal congested blood vessels were observed (Fig. 5). Group III (the UC+PTX group) showed some areas of disrupted muscular surface and surface columnar cells. Many crypts were distorted. Some apparently normal crypts with goblet cells were observed (Fig. 6). Group IV (UC+nano-carcinulin) showed near-normal surface simple columnar cells with discontinuity in certain areas and a few distorted crypts (Fig. 7). Group V (UC+stem cells) showed a picture similar to that of the control group (Fig. 8). Group VI (recovery) showed a picture nearly similar to that of group II (Fig. 9).

**Aldan blue stain**

The control group showed colonic mucosa with numerous goblet cells in the crypts with alcian blue-positive reaction (Fig. 10). Group II showed a few goblet cells in the crypts with reduced intensity of alcian blue-positive reaction (Fig. 11). Group III showed many goblet cells in the crypts with alcian blue-positive reaction (Fig. 12). Groups IV and V showed numerous goblet cells in the crypts with alcian blue-positive reaction (Figs. 13 and 14). Group VI (recovery) showed a few goblet cells in the crypts with alcian blue-positive reaction (Fig. 15).

**Mallory’s trichrome stain**

The control group showed fine collagen fibers between crypts and in the submucosa (Fig. 16). Group II showed dense collagen fiber deposition between crypts and in the submucosa (Figs. 17). The amount of collagen fiber deposition decreased in groups III (Fig. 18), IV (Fig. 19), and V (Fig. 20) compared with group II, whereas group VI showed dense collagen fiber deposition (Fig. 21).

**Immunohistochemistry stain for tumor necrosis factor**

Positive immunohistochemical staining of TNF-α was demonstrated as brown cytoplasmic reaction in the cells of the epithelial lining and in the connective tissue cells of the lamina propria (index for inflammation). The control group showed minimal TNF-α expression (Fig. 22). Group II showed dense expression of TNF-α (Fig. 23). The expression of TNF-α decreased in groups III (Fig. 24), IV (Fig. 25), and V (Fig. 26) compared with group II, whereas group VI showed dense expression of TNF-α (Fig. 27).

**Morphometric results**

The mean area% of alcian blue-positive reaction, collagen fiber deposition, and TNF-α expression for all groups is presented in Tables 1, 2, and 3 in Histograms 1, 2, and 3. There was a significant increase in alcian blue-positive reaction and a significant decrease in collagen fiber deposition and in TNF-α expression (P<0.05) in groups III, IV, and V compared with group II, whereas these increases and decreases were nonsignificant in group VI (recovery group).

### Table 1. The mean area, SD of collagen fiber deposition in groups I, II, III, IV, V, and VI

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean area%</th>
<th>SD</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.3</td>
<td>0.2860</td>
<td>0.000</td>
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<tr>
<td>II</td>
<td>13.0</td>
<td>0.4211</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>III</td>
<td>2.66</td>
<td>0.4704</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>IV</td>
<td>2.66</td>
<td>0.5910</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>V</td>
<td>2.2</td>
<td>0.3648</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>VI</td>
<td>13.48</td>
<td>0.427</td>
<td>0.134</td>
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</table>

Groups III, V, and VI compared with group II.

### Table 2. The mean area, SD of alcian blue-positive goblet cells in groups I, II, III, IV, V, and VI

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean area%</th>
<th>SD</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
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<tr>
<td>II</td>
<td>5.08</td>
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<tr>
<td>III</td>
<td>10.63</td>
<td>0.6233</td>
<td>0.000</td>
<td>S</td>
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<tr>
<td>IV</td>
<td>14.14</td>
<td>0.5163</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>V</td>
<td>14.14</td>
<td>0.5074</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>VI</td>
<td>6.44</td>
<td>1.0633</td>
<td>0.141</td>
<td>NS</td>
</tr>
</tbody>
</table>

Groups III, V, and VI compared with group II.

### Table 3. The mean area% and SD of tumor necrosis factor α expression in groups I, II, III, IV, V, and VI

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean area%</th>
<th>SD</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
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<td>0.0620</td>
<td>0.000</td>
<td>S</td>
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<tr>
<td>II</td>
<td>7.77</td>
<td>0.6540</td>
<td>0.000</td>
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<tr>
<td>III</td>
<td>2.29</td>
<td>0.2911</td>
<td>0.000</td>
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<tr>
<td>IV</td>
<td>0.35</td>
<td>0.2565</td>
<td>0.000</td>
<td>S</td>
</tr>
<tr>
<td>V</td>
<td>0.50</td>
<td>0.1151</td>
<td>0.000</td>
<td>S</td>
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<tr>
<td>VI</td>
<td>0.90</td>
<td>1.5507</td>
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<td>NS</td>
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Groups III, V, and VI compared with group II.

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