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Phoenixin-14 ameliorates acetic acid-induced ulcerative colitis in rats via antioxidant, anti-inflammatory and anti-apoptotic mechanisms

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ABSTRACT

Phoenixin (PNX), first discovered in the rat hypothalamus, was initially identified as a reproductive peptide. PNX-14 (14 amino acid isoform) has also been shown to function in cardiovascular regulation, neuroprotection, glucose metabolism, appetite, anxiety, and memory. We aimed to investigate the potential therapeutic role of PNX-14 in acetic acid (AA)-induced ulcerative colitis. Rats were given intrarectally 1 ml saline (control) or 5 % AA (colitis groups). The control group was treated intraperitoneally with saline, while the colitis groups were treated intraperitoneally with saline or PNX-14 (50 μg/kg/d) or gonadotrophin-releasing hormone (GnRH)-antagonist cetrorelix (CTX; 100 μg/kg/d) or CTX and PNX-14 or sulfasalazine as a positive control (100 mg/kg/d) instantly and once a day for 3 days following colitis induction. Colonic samples were evaluated histologically and biochemically [malondialdehyde (MDA), glutathione (GSH), myeloperoxidase (MPO), chemiluminescence (CL), pro-inflammatory cytokines (tumor necrosis factor-α, interferon-γ, interleukin (IL)-1β, IL-6, IL-8), caspase-3, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) measurements] on the 3rd day. Elevated damage scores (macroscopic and microscopic), MPO, MDA, caspase-3, cytokines, and CL values, and decreased GSH levels of the colitis group were reversed by PNX-14 treatment ($p < 0.05$–0.001). CTX or CTX plus PNX-14 reduced damage scores, caspase-3, 8-OHdG, cytokines, and CL values ($p < 0.05$–0.001). Sulfasalazine treatment improved all parameters except MDA and GSH. PNX-14, which alleviates macroscopic, histological and biochemical parameters, can be considered as a potential therapeutic agent in ulcerative colitis with its anti-inflammatory, antioxidant and anti-apoptotic actions. Furthermore, despite its effects as an GnRH-antagonist, CTX has also revealed a similar beneficial role as PNX-14 in this ulcerative colitis model.

1. Introduction

Inflammatory bowel disease (IBD) includes a group of idiopathic diseases of the gastrointestinal tract, characterized by an intense immune response to various antigens or environmental factors in genetically predisposed individuals and manifested by periods of exacerbation and remission [1]. Ulcerative colitis (UC) is one of two major types of IBD with widespread mucosal inflammation involving the rectum and colon. The incidence of the disease has increased in industrialized countries in recent years due to changes in dietary habits as well as environmental and social factors [2]. Since the cause of the disease is not known for certain, the drugs used in treatment (such as, nonsteroidal anti-inflammatory drugs, immunosuppressants, corticosteroids and biological therapeutics) are administered to provide regression of inflammation, control symptoms, and improve quality of life [2]. In recent years, the effects of different peptides have been intensively

Abbreviations: PNX, phoenixin; CTX, cetrorelix; SS, sulfasalazine; AA, acetic acid; GnRH, gonadotrophin-releasing hormone; ROS, reactive oxygen species; RNS, reactive nitrogen species; MPO, myeloperoxidase; MDA, malondialdehyde; GSH, glutathione; CL, chemiluminescence; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; TNF-α, tumor necrosis factor-α; IL, interleukin; IFN-γ, interferon-γ; NO, nitric oxide; $ONOO^-$, peroxynitrite; AUC, area under the curve; Rlu, relative light units; ELISA, enzyme-linked immunosorbent assay.

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investigated in various experimental studies in order to elucidate the pathophysiology of UC and to suggest different treatment strategies.

Phoenixin (PNX), first discovered in the rat hypothalamus in 2013, is a peptide produced by the degradation of small integral membrane protein 20 (Smim20) [3,4]. It is a highly conserved peptide among vertebrates with two active isoforms: PNX-14 (14 amino acid) and PNX-20 (20 amino acid) [3]. Both of these isoforms have shown similar functions in most studies and they exhibit biological activities on central (such as, hypothalamus, spinal cord, anterior and posterior pituitary gland) and peripheral (such as, pancreatic islets, heart, gastrointestinal tract, skin, thymus, adipose tissue, and ovaries) tissues by binding to transmembrane G-protein-coupled receptor 173 (GPR173) [4]. Initially, PNX was identified as a reproductive peptide, therefore the first studies investigating the effects of PNX focused on its relationship with gonadotrophin-releasing hormone (GnRH) [3,5]. In later studies, cardioprotective [6], neuroprotective [7], anti-inflammatory [4], antioxidant [8], memory-enhancing [9], anxiolytic [10] and orexigenic [11] effects of PNX-14 were also shown. However, the number of studies examining the effects of PNX-14 in different *in vivo* and *in vitro* models is quite limited. For instance, in the duodenal ulcer model induced by indomethacin in rats, it was found that PNX-14 showed stronger anti-inflammatory and antioxidant effects than famotidine used in current ulcer treatment [12]. Furthermore, PNX-14 alleviated acetic acid (AA)-induced gastric damage in rats [13]. In the experimental diabetes model induced by streptozotocin in mice, PNX-14 was shown to lead to improvement in cardiac damage by demonstrating anti-inflammatory and antioxidant effects [14]. Additionally, the actions of PNX-14 treatment in a model of high fat diet-induced non-alcoholic fatty liver disease in mice were revealed to reduce inflammation, cytokine levels and oxidative stress [15]. In a different study in rats, PNX-14 was shown to reduce sepsis-induced liver damage [16]. It has also been shown that PNX-14 regulates glucose-stimulated secretion of insulin in rat pancreatic beta cell line [17]. Recently, decreased levels of PNX-14 have been detected in patients with type 2 diabetes, suggesting that the peptide may be associated with the pathophysiology of diabetes [18]. Thus, when taken together, experimental and clinical studies have revealed that PNX-14 has pleiotropic effects on several tissues, and exerts anti-inflammatory, antioxidant and anti-apoptotic actions.

Anxiolytic and memory-enhancing effects of intracerebroventricularly administered PNX-14 was antagonized by the selective GnRH receptor (GnRHR) antagonist cetrorelix (CTX), suggesting that the role of central actions of PNX-14 are mediated via GnRHR [9,10]. However, apart from its antagonistic effects on GnRHR, CTX has been shown to improve ovarian function and fibrosis in aged mice [19], to reduce post-operative adhesion formation in female rats [20], and to exert anti-apoptotic effects on the chemotherapy-induced rat ovarian damage [21]. In addition, in a study conducted in patients with rheumatoid arthritis, CTX was reported to show anti-inflammatory effects, which was suggested to occur by its antagonistic effect on GnRH [22]. Besides its wide distribution in the reproductive tissues, GnRH/GnRHR system has been found in several systems that include the gastrointestinal tract [23,24]. We firstly hypothesized that PNX-14 treatment would alleviate intracolonic AA-induced injury via its anti-inflammatory, antioxidant, and anti-apoptotic features. Similarly, based on the previous studies conducted in the reproductive organs, GnRHR antagonist CTX is also hypothesized to exert therapeutic effects on AA-induced colonic injury. Moreover, we also aimed to evaluate the possible synergistic effects of PNX-14 and CTX in alleviating colitis-induced inflammation and oxidative damage. Therefore, the present study aimed to investigate the possible therapeutic effects of PNX-14, CTX or their combination on the AA-induced colitis model using histological and biochemical evaluations.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (200–250 g) were obtained from the University of Health Sciences Experimental Animal Implementation and Research Center. Temperature (22 ± 2°C) controlled room and standardized light/dark cycles (12/12 h) with relative humidity (50 ± 5 %) environment were ensured for the rats. They were given tap water ad libitum and fed with standard rat pellets. This study with all protocols was approved by the University of Health Sciences Animal Care and Use Committee (no: 2022–04/03) and were established in accordance with Guidance for the Care and Use of Laboratory Animals.

2.2. Induction of colitis and experimental design

Following an overnight fasting and under light ether anesthesia, 5 % (pH: 2.3; v/v) 1 ml acetic acid (AA; Merck, Darmstadt, Germany) was administered to the rats intrarectally 8 cm proximal to the anus, using a polyethylene catheter [25]. Where as, control rats were administered saline intrarectally. All rats were maintained in the Trendelenburg position for a duration of 30 s to prevent the risk of leakage.

The rats were divided into 6 randomized groups with 8 rats per group: (a) control group, (b) saline-treated AA group, (c) phoenixin (PNX)-treated AA (AA+PNX) group, (d) cetrorelix (CTX)-treated AA (AA+CTX) group, (e) CTX plus PNX-treated AA (AA+CTX+PNX) group, and (f) sulfasalazine (SS)-treated AA (AA+SS) group. Control group injected intraperitoneally saline (0.1 ml/100 g rat); AA groups injected intraperitoneally either saline (AA group) or PNX-14 (50 μg/kg/d; Phoenix, USA; AA+PNX group) or a synthetic decapeptide CTX (100 μg/kg/d; Sigma-Aldrich, St. Louis, MO, USA; AA+CTX group) or CTX and PNX-14 (AA+CTX+PNX group) or SS (100 mg/kg/d; Pfizer; AA+SS group; positive control) immediately and once a day for 3 days following AA-induced colitis. PNX, CTX and SS doses were determined according to the reference studies [12,26–28]. SS, widely used in the clinics to maintain remission in UC, was given as a positive control drug to determine the efficacy of PNX and CTX.

On the 3rd day, the rats were sacrificied with a rodent guillotine following light ether anesthesia. Colonic tissue samples were harvested and stored at −80∘C for the biochemical assessments. Additional tissue samples were taken for histogical evaluation. The experimental design of the study is given in Fig. 1.

2.3. Evaluation of severity of colitis

2.3.1. Macroscopic examinations

After decapitation, the distal 8 cm of the colons were removed, cut longitudinally and rinsed with saline. The colonic specimens were weighed to obtain tissue wet weights and mucosal lesions were examined using macroscopic damage scoring [29] (Table 1). The colonic tissues' wet weight index values were recorded for each rat after macroscopic damage scoring and corrected for body weight (g/100 g bw).

2.3.2. Histological evaluation

The tissue samples taken from distal colons were placed in 10 % neutral buffered formalin (Merck, Darmstadt, Germany) for fixation process. They were dehydrated in rising alcohol series (70–100 %) and embedded in paraffin. Paraffin tissue 5 μm), cut by a rotary microtome (Microm HM 325, Thermo Scientific, Germany), were stained with hematoxylin and eosin (H&E). An experienced histologist blinded to treatment examined all these sections under a photomicroscope (Zeiss Primostar, UK).

Microscopic scoring of colonic injury was done by the criteria as follows: 0–3 scale (0 = none, 1 = mild, 2 = moderate, 3 = severe) was used for submucosal edema, ulceration or necrosis, inflammatory cell

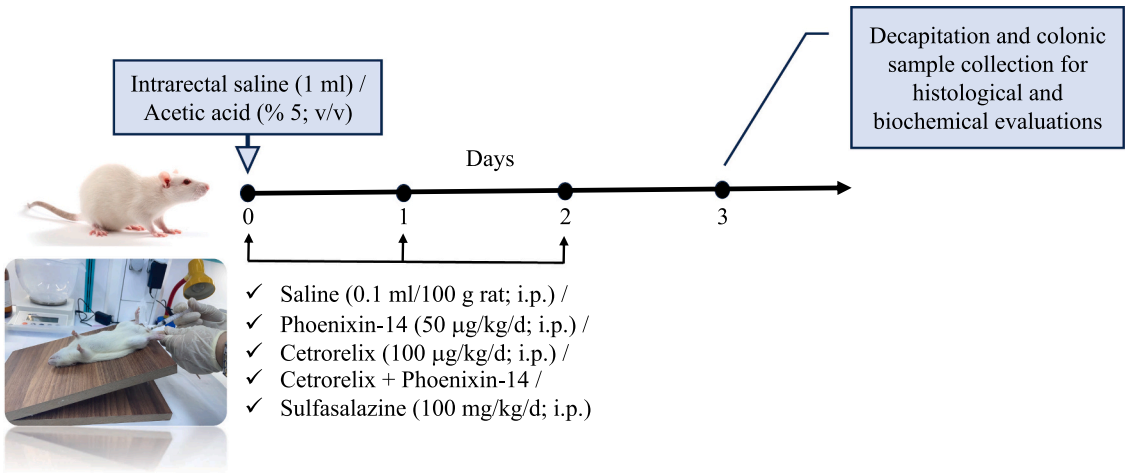

Fig. 1. The experimental design of the study.

Table 1
Macroscopic scoring of colonic injury.

Score	*Appearance*
0	Normal
1	Focal hyperemia with no ulcers
2	Linear ulceration without hyperemia or thickening of colon wall
3	Linear ulceration with inflammation at one site
4	Ulceration and inflammation at two or more sites
5	Major site of injury extending more than 1 cm of colon
6–10 (Max. score 10)	Score is increased by one for each additional cm of injury beyond 2 cm

infiltration, and vasculitis. Furthermore, the presence of perforation was evaluated (0 = none, 1 = present). Total maximum score was 13 [30].

2.3.3. Myeloperoxidase (MPO), malondialdehyde (MDA) and glutathione (GSH) assays

Measurement of tissue-associated MPO activity indicates the neutrophil accumulation to the injured tissue. MPO activity was assessed spectrophotometrically by measuring the hydrogen peroxide-dependent oxidation of o-dianisidine.2HCl. Results were expressed as U/g tissue [31]. Tissue MDA measurements were done as an indicator of lipid peroxidation, and results were determined as nmol MDA/g tissue [32]. Antioxidant GSH was measured by Ellman procedure, and results were expressed as µmol GSH/g tissue [33].

2.3.4. Chemiluminescence (CL) assays

The tissue levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were determined by CL method. For this purpose, luminescence of the colonic specimens was measured at room temperature using luminometer (EG & G Berthold Junior LB 9509, Germany) in the presence of luminol (measures hydroxyl radical, hydrogen peroxide, hypochlorite, lipid peroxyl radicals) or lucigenin (selective for superoxide anion) probes (Sigma-Aldrich, St. Louis, MO, USA) [34].

Nitric oxide (NO) detection was made by the purified luminol-hydrogen peroxide system. Carboxy-PTIO (Sigma-Aldrich, St. Louis, MO, USA) was used as a NO scavenger for peroxynitrite ($ONOO^-$) determination [35]. Area under the curve (AUC) of the data was stated as relative light units (rlu)/mg colonic tissue.

2.3.5. Enzyme-linked immunosorbent assay (ELISA) for pro-inflammatory cytokines, caspase-3 and 8-hydroxy-2′-deoxyguanosine (8-OHdG)

The colonic tissue levels of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6 and IL-8), caspase-3, an important marker of apoptosis, and 8-OHdG, indicator of oxidative DNA damage, were measured using commercially available ELISA kits specific for rats. All ELISA kits were purchased from BT-LAB, China.

2.4. Statistical analysis

Analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison tests was performed to compare the groups of data by using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). All data were expressed as mean ± standard error of the mean (SEM). Values of $p < 0.05$ were regarded as significant.

3. Results

3.1. PNX-14, CTX, CTX plus PNX-14 and SS improved macroscopic appearance, damage scores and tissue wet weights in tissues of rats with AA-induced colitis

The macroscopic appearance of the colon belonging to the control group was healthy with no damage in the mucosa. On the other hand, a severe edematous mucosal ulceration was evident in saline-treated AA group. This observation was reduced upon all treatments (Fig. 2a-f).

The colonic tissue wet weight indices of the saline-treated AA colitis group was found to be significantly rised compared to the control group ($p < 0.001$). These high values were decreased by PNX-14, CTX, CTX plus PNX-14 and SS treatments ($p < 0.05$–0.001) (Table 2).

While intrarectal AA instillation significantly elevated macroscopic lesion scores of the saline-treated AA colitis group compared to the control group ($p < 0.001$), this elevation was reduced significantly in all treatment groups ($p < 0.001$) (Fig. 3a). In the same way, increased microscopic damage scores of the saline-treated AA colitis group were significantly diminished in all treated AA colitis groups ($p < 0.05$–0.001) (Fig. 3b).

3.2. PNX-14, CTX, CTX plus PNX-14 and SS attenuated submucosal edema, ulceration/necrosis, inflammatory cell infiltration, and/or vasculitis in colonic tissues of rats with colitis

In the light microscopic examination of the colonic tissues, healthy mucosa and submucosa morphology was observed in the control group (Fig. 4A). On the other hand, intense epithelial damage, inflammation and edema were evident in the saline-treated AA colitis group. Also, loss of epithelial cells adjacent to the inflamed lamina propria was detected. Perforation areas were observed in the mucosa. The presence of intense inflammatory cells and vasculitis in the submucosa layer was remarkable (Fig. 4B). In AA+PNX group, it was observed that edema in the

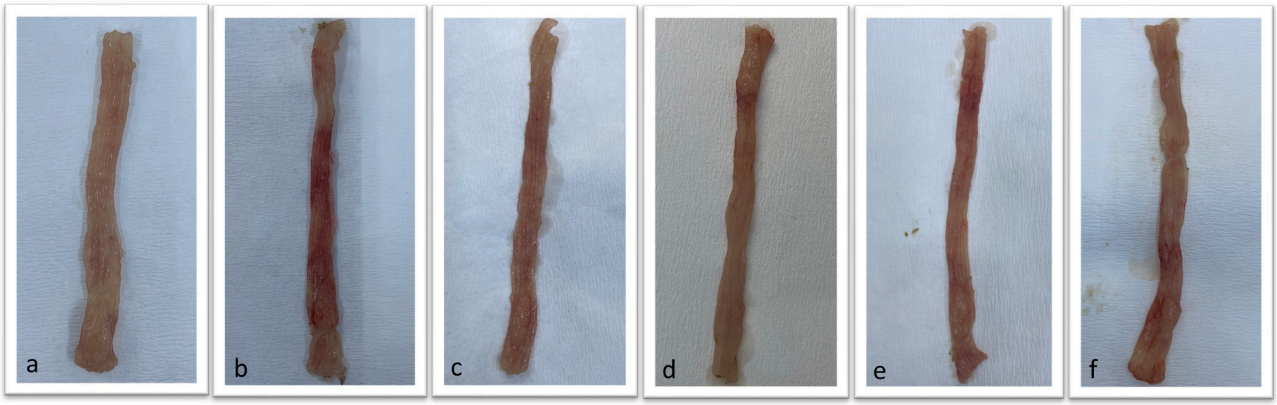

Fig. 2. Macroscopic images of the excised distal 8 cm of the colons from different experimental groups. **(a)** Control group, **(b)** AA group, **(c)** AA+PNX group, **(d)** AA+CTX group, **(e)** AA+CTX+PNX group and **(f)** AA+SS group. AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine.

Table 2
Colonic tissue wet weight index.

Groups	Tissue wet weight (g per 100 g body weight)
Control	0.35 ± 0.01
AA	$0.54 \pm 0.02^{***}$
AA+PNX	$0.44 \pm 0.01^{+}$
AA+CTX	$0.40 \pm 0.02^{+++}$
AA+CTX+PNX	$0.41 \pm 0.02^{+++}$
AA+SS	$0.42 \pm 0.03^{++}$

AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine. $^{***}p < 0.001$ compared to control group; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to saline-treated AA colitis group.

lamina propria diminished compared to the saline-treated AA colitis, AA+CTX and AA+SS groups. Additionally, inflammation decreased in the submucosa. No mucosal perforation was observed in this group (Fig. 4C). In the AA+CTX group, mucosa and submucosa showed edema and inflammation of varying severity and extent. A small amount of hemorrhage and vasculitis were observed in the mucosal connective tissue. Edema and vasculitis in the submucosal area were more evident than in the mucosa (Fig. 4D). Reduced inflammation and edema were observed in the lamina propria of the AA+CTX+PNX group compared to saline-treated AA colitis, AA+CTX and AA+SS groups. Loss of epithelial cells adjacent to the areas with decreased inflammatory cell density was detected (Fig. 4E). Compared to the saline-treated AA colitis group, mild mucosal damage and inflammation with reduced edema in the submucosal area were observed in AA+SS group (Fig. 4F).

3.3. Effects of treatments on MPO activity (an indicator of neutrophil infiltration), MDA (an indicator of lipid peroxidation) and GSH (antioxidant) levels in tissues of rats with colitis

MPO activity in the colonic tissues of rats with colitis (saline-treated AA colitis group) was significantly increased compared to the control group ($p < 0.001$). PNX-14 treatment (AA+PNX group) resulted in a significant reduction in MPO level compared to saline-treated AA colitis group ($p < 0.01$). The MPO activity value of the AA colitis group in which CTX was administered alone (AA+CTX group) was not found to be different from the saline-treated AA colitis group, whereas the MPO value of the AA+CTX+PNX group was significantly reduced ($p < 0.05$). Similarly, SS treatment (AA+SS group) also significantly diminished the MPO value compared to the saline-treated AA colitis group ($p < 0.05$) (Fig. 5a).

Tissue MDA level was found to be significantly higher in the saline-treated AA colitis group compared to the control group ($p < 0.05$), and these high levels decreased significantly in rats treated with PNX-14 (AA+PNX group) ($p < 0.05$). However, in AA+CTX, AA+CTX+PNX and AA+SS groups, MDA levels were not different from saline-treated AA colitis group (Fig. 5b).

While the antioxidant GSH level in the saline-treated AA colitis group was found to be significantly lower compared to the control group ($p < 0.05$), the level was increased in the AA+PNX group ($p < 0.01$). On the other hand, GSH levels in AA+CTX, AA+CTX+PNX and AA+SS groups did not differ compared to saline-treated AA colitis group (Fig. 5c).

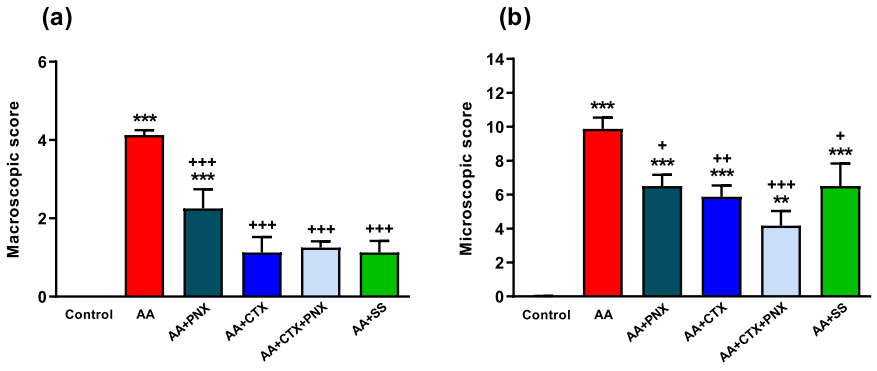

Fig. 3. (a) Macroscopic and **(b)** microscopic scores in the experimental groups. AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control group; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to saline-treated AA colitis group.

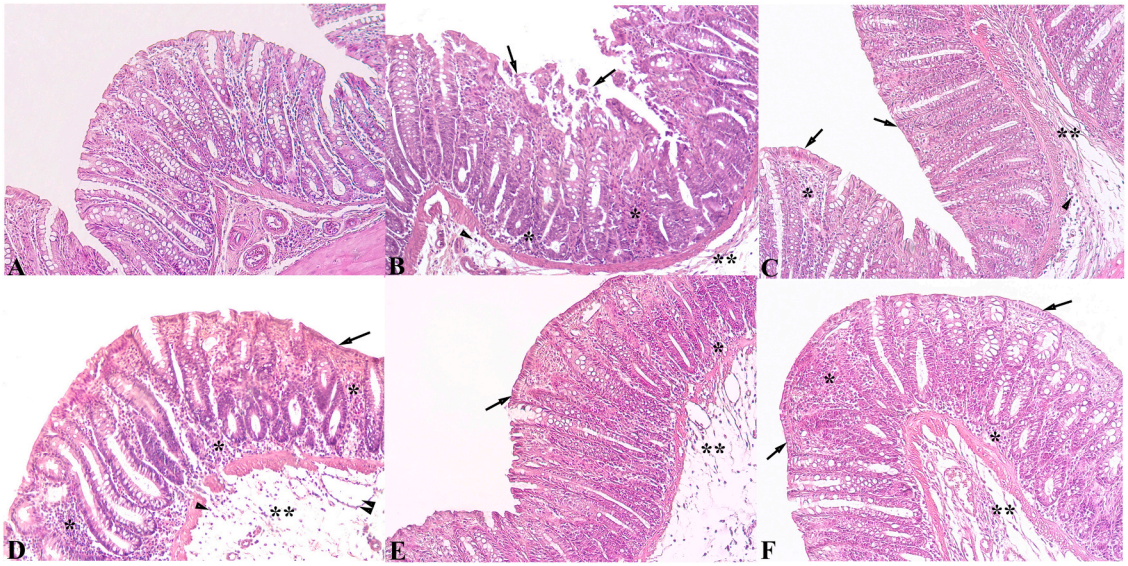

Fig. 4. Histological evaluation of the colonic tissue. **(A) Control group:** Normal morphology, **(B) AA group:** Epithelial damage in the mucosa (arrow), severe inflammation (*), inflammatory cells in the submucosa (arrowhead) and disruption of connective tissue integrity (**), **(C) AA+PNX group:** Healthy epithelial cells in the mucosa (arrow), local inflammatory areas (*), few inflammatory cells in the submucosa (arrowhead) and largely intact connective tissue (**), **(D) AA+CTX group:** Morphological changes in the epithelium (arrow), inflammation in the lamina propria (*), inflammatory cells in the submucosa (arrowhead), connective tissue damage and edema (**), and impaired capillary morphology (double arrowhead), **(E) AA+CTX+PNX group:** Healthy epithelial cells in the mucosa (arrow), local inflammatory areas with decreased intensity (*), scattered inflammatory cells and edema in the submucosa (**), **(F) AA+SS group:** Healthy epithelial cells in the mucosa (arrow), local inflammatory areas (*), and connective tissue in the submucosa that largely maintains its integrity (**). AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine. H&E staining, magnifications: 200x.

3.4. PNX-14, CTX, CTX plus PNX-14 and SS abolished tissue ROS and RNS levels in rats with colitis

Compared to the control group, the significant increments in luminol- and lucigenin-enhanced CL measurements observed in the saline-treated AA colitis group ($p < 0.001$), and these high values significantly abolished in all treatment groups ($p < 0.01$–0.001) (Fig. 6a and b).

The NO and $ONOO^-$ CL values measured in the saline-treated AA colitis group was significantly higher than the control group ($p < 0.001$). On the other hand, both of these CL values in tissue samples of rats receiving PNX-14 treatment (AA+PNX group), CTX treatment (AA+CTX group), CTX plus PNX-14 treatments (AA+CTX+PNX group) or SS treatment (AA+SS group) showed a statistically significant decrease compared to saline-treated AA colitis group ($p < 0.001$) (Fig. 6c and d).

3.5. Effects of treatments on tissue 8-OHdG (an indicator of oxidative DNA damage) and caspase-3 (a marker of apoptosis) levels on the 3rd day of colitis

Tissue 8-OHdG levels in the saline-treated AA colitis group were significantly higher than in the control group ($p < 0.05$). While the 8-OHdG values observed in AA+CTX, AA+CTX+PNX and AA+SS groups were significantly decreased compared to saline-treated AA colitis group ($p < 0.05$), PNX-14 treatment did not change the 8-OHdG value although it showed a decreasing trend (Fig. 7a).

While the tissue caspase-3 level of the saline-treated AA colitis group was significantly elevated compared to the control group ($p < 0.001$), caspase-3 levels in colonic tissue samples of all treatment groups showed a significant reduction compared to the saline-treated AA colitis group ($p < 0.01$–0.001) (Fig. 7b).

3.6. Impacts of treatments on pro-inflammatory cytokine levels in tissues of rats with colitis

The levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8 and IFN-γ) in colonic tissue samples of the saline-treated AA colitis group were found to be significantly higher than the cytokine levels of the control group ($p < 0.05$–0.001). These elevated tissue pro-inflammatory cytokine levels (except IL-6) were significantly decreased in all treatment groups compared to the saline-treated AA colitis group ($p < 0.05$–0.001). While, high IL-6 levels of the saline-treated AA colitis group was significantly decreased in PNX-14 and SS treated AA colitis groups ($p < 0.05$ and $p < 0.001$, respectively), CTX alone and CTX plus PNX-14 treatments did not show a significant change compared to the saline-treated AA colitis group (Table 3).

4. Discussion

The results of the study demonstrate that PNX-14 and/or CTX has therapeutic effects against AA-induced UC model in rats via anti-inflammatory, antioxidant and anti-apoptotic properties. Colitis model induced by intrarectal administration of AA was chosen due to its histopathological and biochemical resemblance to human UC with lesions that are mucosal, acute and non-transmural [36]. When compared with other models, AA-induced colitis model is an easy, cheap and reproducible experimental UC model. PNX-14 reduced high macroscopic and microscopic damage scores, tissue wet weight indices, MPO, MDA, caspase-3, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8 and IFN-γ), and CL values, and increased low levels of GSH. Similarly, CTX alone (AA+CTX group) or CTX before PNX-14 treatment (AA+CTX+PNX group) improved inflammatory (except MPO for AA+CTX group), oxidant (except MDA and GSH) and apoptotic parameters. These beneficial effects were found to be similar to those of sulfasalazine, a medicine used in clinics to treat UC.

Since PNX-14 has been shown to increase of GnRHR expression and GnRH secretion, it was initially described as a peptide active on reproductive organs [3]. However, based on the presence of PNX-14 and

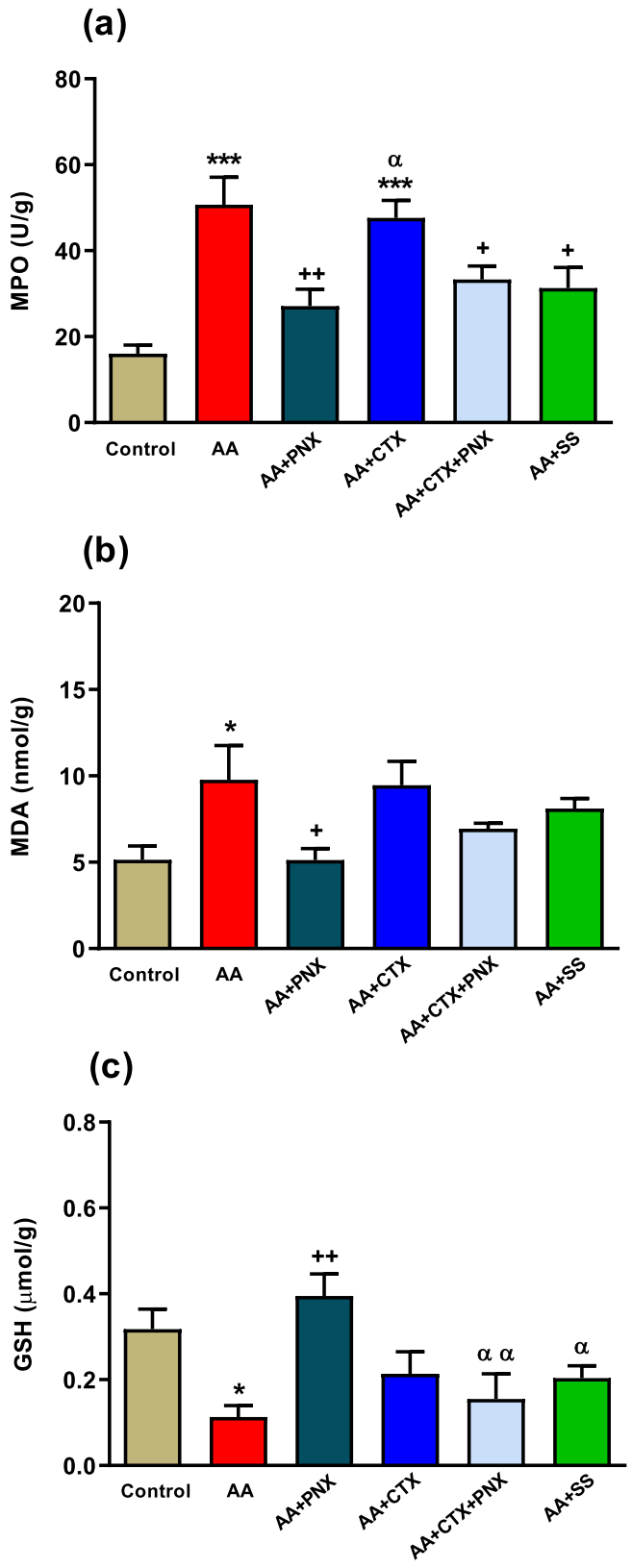

Fig. 5. (a) Myeloperoxidase (MPO), **(b)** malondialdehyde (MDA), and **(c)** glutathione (GSH) levels in the experimental groups. AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control group; $^{+}p < 0.05$ and $^{++}p < 0.01$ compared to saline-treated AA colitis group; $^{\alpha}p < 0.05$ and $^{\alpha\alpha}p < 0.01$ compared to AA+PNX group.

GnRH receptors in the gastrointestinal tract [3,24,37], and their common antioxidant and anti-inflammatory actions in several other tissues, we have postulated that PNX-14 or CTX or their combination could have beneficial effects in AA-induced colonic injury. Our present results reveal that pretreatment with CTX or treatment with PNX-14 has anti-inflammatory, antioxidant and anti-apoptotic effects suggesting that these effects of PNX-14 on colonic tissue are not likely due to GnRHR and the related GnRH signaling pathways. On the other hand, many of the functions of PNX-14 have been shown to be mediated by GRP173, a highly conserved G-protein-coupled receptor that plays an important role in the intracellular signaling cascade of PNX [4]. Presumably, binding of PNX-14 by GPR173 and activation of the PNX-14/GPR173 signaling pathway may be responsible for these effects. Moreover, GnRH and GnRHR immunoreactivity were previously demonstrated in the rat enteric neurons, smooth muscle cells, and epithelial cells [24], implicating that the colon-protective actions of CTX could be via GnRHR. Our results suggest that PNX-14 and CTX induce anti-inflammatory, antioxidant and anti-apoptotic responses via different pathways, but addition of their actions does not result in a synergistic effect.

It is well documented that intestinal inflammation in IBD is accompanied by excessive production of ROS and RNS, which in turn causes oxidative and nitrosative stress, respectively [38]. These two types of stress causes degradation of the intestinal mucosa and infiltration of the inflammatory mediators into the inflamed areas. Neutrophils are one of well-known sources of ROS and RNS in UC [38,39]. MPO levels, as an indicator of neutrophil infiltration to the inflamed area, were found to be elevated in the colonic tissues of rats exposed to AA in this study. PNX-14 treatment alone (AA+PNX group) or with CTX administration (AA+CTX+PNX group) significantly abolished these high MPO activities which may also be associated with the improvement in oxidative/nitrosative stress in injured colonic tissue by PNX-14 as confirmed by CL data. These results were also supported by our histological findings. Similarly, PNX-14 has been reported to decrease MPO levels in indomethacin-induced duodenal ulcer model in rats [12]. Although the MPO activity in the AA+CTX group was not found to be different than AA colitis group, histologically evident extensive neutrophil infiltration in the saline-treated colitis group was significantly reduced by CTX treatment as confirmed by microscopic damage score.

Oxidative/nitrosative stress is an important pathophysiologic factor involved in the development and progression of IBD [38]. Simple and reproducible CL measurement technique allowed us to quantitate the ROS and RNS generation in the present study. Once they are formed, this highly reactive species begins to interact with the molecular complexes inducing cellular damage in the gastrointestinal mucosa [40]. Our CL data showed that PNX-14 and/or CTX treatments effectively abolished these unstable free radical generations and ameliorates their seriously devastating actions in AA-induced UC model. Similarly, PNX-14 treatment alleviated high luminol- and lucigenin-enhanced CL levels of pancreatic and ileal tissues in streptozotocin-induced diabetic rats [41]. On the contrary, 15 days of the i.p. CTX treatment at dose of 300 μg/kg per day has been shown to decrease the antioxidant effects of Ephedra herb in testicular cells by increasing ROS levels in a rat model of adriamycin-induced testicular toxicity [42]. The difference between these disparate results could be due to the high dose of CTX, long period of the experiment, and the tissue difference examined. Nitric oxide synthase (NOS), thought to be the major producer of NO in UC, was found in high amounts in experimental colitis models and in the intestinal mucosa of patients with IBD [40]. One mechanism through which RNS contributes to UC pathophysiology is the ONOO- formation, a highly reactive tissue damage mediator formed by the reaction of NO with superoxide anion [38,39]. Continuously elevating NO and superoxide anion levels increased the rate of ONOO- formation in colonic tissues of the saline-treated AA colitis group, but high RNS (NO and ONOO-) CL levels markedly decreased in all treatment groups. These findings suggest that PNX-14 and/or CTX treatments could have the

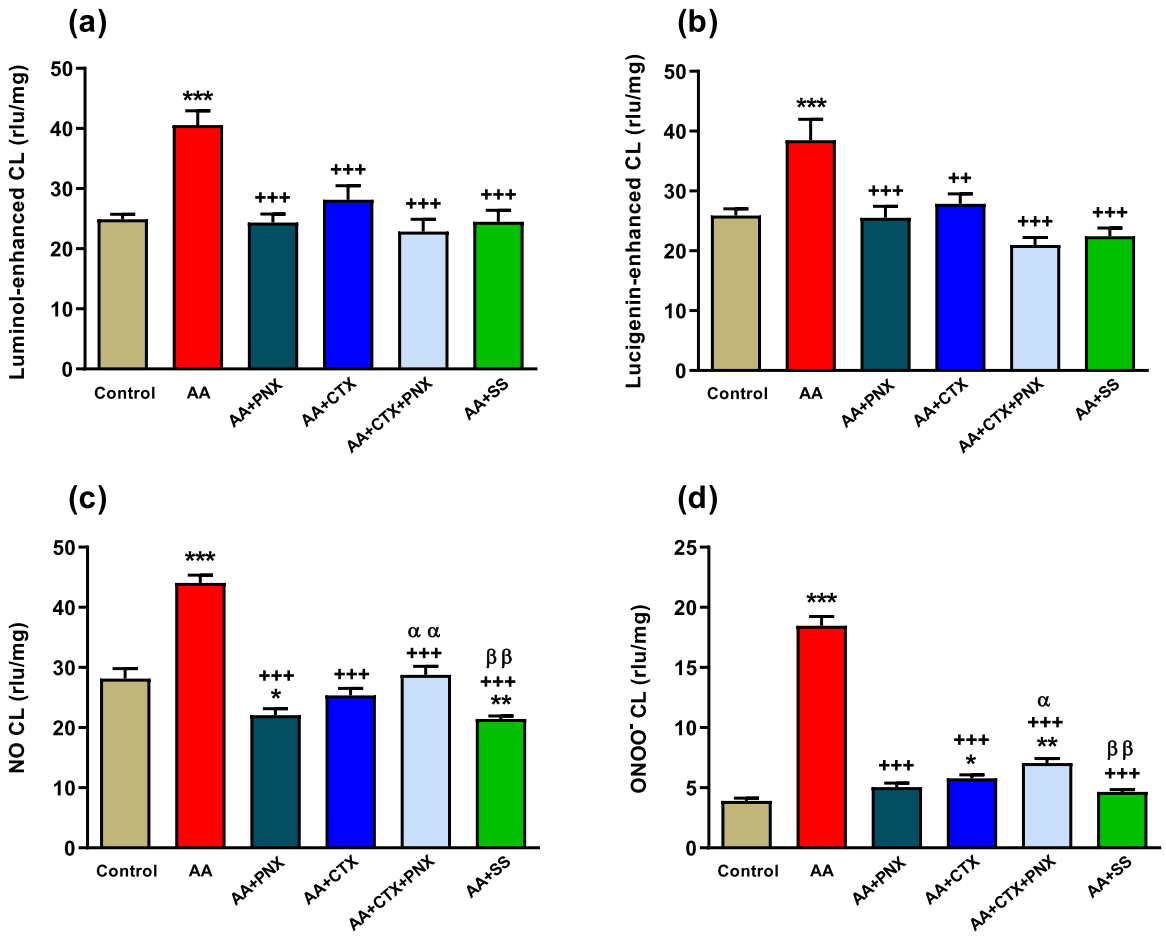

Fig. 6. **(a)** Luminol, **(b)** lucigenin **(c)** nitric oxide (NO) and **(d)** peroxynitrite ($ONOO^-$) chemiluminescence (CL) levels in the experimental groups. AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine; rlu=relative light units. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control group; $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to saline-treated AA colitis group; $^{\alpha}p < 0.05$ and $^{\alpha\alpha}p < 0.01$ compared to AA+PNX group; $^{\beta\beta}p < 0.01$ compared to AA+CTX+PNX group.

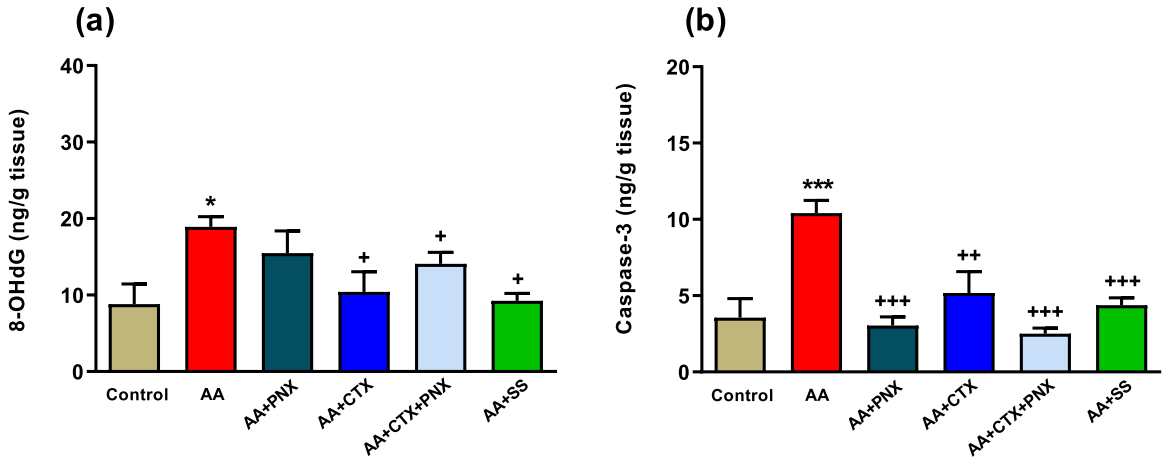

Fig. 7. **(a)** 8-Hydroxy-2'-deoxyguanosine (8-OHdG) and **(b)** caspase-3 levels in the experimental groups. AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control group; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to saline-treated AA colitis group.

potential to scavenge free radicals in injured colonic tissue.

In the present study, PNX-14 not only inhibited the MDA production which implies a reduction in the peroxidation of the lipids and damage to cellular the components, but also rescued the expression of the antioxidant GSH in the inflamed colonic tissue. Since PNX-14 treatment completely prevented the colonic lipid peroxidation and GSH depletion, it appears that the therapeutic effect of PNX-14 involves the maintenance of antioxidant capacity in protecting the colonic tissue against oxidative stress. The antioxidant properties of PNX-14 were also demonstrated in previous studies [13,15], consistent with our study findings. On the other hand, CTX alone or CTX plus PNX-14 administration for three subsequent days did not significantly affect MDA and GSH levels during our study period in male rats. A possible explanation for this finding is that CTX treatment (AA+CTX group) may not have

Table 3
Tissue tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and interferon (IFN)-γ levels in the experimental groups.

	Control	AA	AA+PNX	AA+CTX	AA+CTX+PNX	AA+SS
TNF-α (ng/g)	2545.00 ± 237.40	4161.00 ± 588.80*	2468.00 ± 219.30^{+}	1322.00 ± 45.01^{+++}	1987.0 ± 369.70^{+++}	2676.00 ± 228.50^{+}
IL−1β (pg/g)	36.15 ± 10.83	79.13 ± 6.60*	38.81 ± 4.74^{+}	22.28 ± 4.67^{++}	48.09 ± 11.40^{+}	32.70 ± 11.44^{++}
IL−6 (ng/g)	16.41 ± 0.14	61.05 ± 4.60**	40.19 ± 7.92^{+}	69.30 ± 8.56***	39.11 ± 11.50$^{\&}$	13.69 ± 1.46$^{+++,\&\&\&}$
IL−8 (ng/g)	1073.00 ± 4.04	2624.00 ± 6.75***	1370.00 ± 126.20^{+++}	1138.00 ± 101.60^{+++}	793.10 ± 186.20$^{+++,\alpha}$	1361.00 ± 156.40$^{+++,\beta}$
IFN-γ (ng/g)	13.37 ± 0.98	373.40 ± 62.28***	108.30 ± 21.97^{+++}	126.80 ± 9.27^{+++}	172.60 ± 5.49$^{+++,**}$	61.13 ± 21.11^{+++}

AA, Acetic acid; PNX, Phoenixin; CTX, Cetrorelix; SS, Sulfasalazine. *$p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control group; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to saline-treated AA colitis group; $^{\alpha}p < 0.05$ compared to AA+PNX group; $^{\beta}p < 0.05$ compared to AA+CTX+PNX group; $^{\&}p < 0.05$ and $^{\&\&\&}p < 0.001$ compared to AA+CTX group.

enough time to replenish GSH stores and CTX treatment with PNX-14 (AA+CTX+PNX group) may effect the antioxidant capacity of PNX-14 through different mechanism that needs to be clarified. CTX may also boost the activity of another powerful antioxidants such as superoxide dismutase or catalase to counteract the damaging effects of free radicals in this UC model. In support of that interpretation, CTX increased antioxidant enzymes (catalase and glutathione peroxidase) in luteal cells against luteal regression in the late pregnant rats [43].

In the pathophysiology of UC, reactive species react with DNA and this interaction causes to increase 8-OHdG levels which represent the biological footprints of the oxidative stress. 8-OHdG is an important marker widely used in ROS-related injury and indicates oxidative DNA damage [44]. 8-OHdG has been found to be elevated in biopsies taken from UC patients [45] and in colonic tissues of rats with experimentally induced UC [39,46]. Accordingly, tissue levels of 8-OHdG were significantly increased in our study, implicating that DNA is one of the targets for colonic oxidative damage in rats. Moreover, elevated 8-OHdG levels were markedly decreased in AA-induced UC models in rats by different compounds or agents that have antioxidant properties and free radical scavanger abilities [39,46]. In the present study, although PNX-14 treatment alone did not decrease 8-OHdG levels significantly, 8-OHdG levels were reduced in the AA+CTX+PNX group. Also, CTX administration alone markedly negated the oxidative DNA damage. Consistent with our results, CTX reduced DNA damage in developing follicles of mice treated with cyclophosphamide to induce ovarian follicular destruction [47]. During inflammation and apoptotic cell death, caspases (a family of cysteine-protease enzyme) play fundamental roles, and caspase-3 as a marker of apoptosis, has been demonstrated to elevate in AA-induced UC [39]. In a study, PNX-14 protected the rat myocardium against ishemia/reperfusion injury by switching off apoptosis through reduced active caspase-3 expression [6]. Similarly, in our study, one of the therapeutic effects of PNX-14 in alleviating colonic injury in rats is suppression of the tissue caspase-3 activity. Also, similar results were obtained with CTX alone (AA+CTX group) or CTX plus PNX-14 (AA+CTX+PNX group) treatment in colitis groups. In line with these findings, it has been shown that CTX significantly reduced caspase-3 activity in different experimental models [21,48]. Under inflammatory conditions, the colonic mucosa is exposed to high levels of reactive species (ROS and RNT), which increases epithelial apoptosis [49]. Therefore, supression of apoptosis by treatments in our study can be attributed to suppression of oxidative and nitrosative stress.

A huge amount of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α are produced locally in UC patients and experimental UC model [39,50]. However, a newly discovered peptide PNX-14 has been shown to decrease pro-inflammatory mediators in different experimental models [8,12,51]. Similarly, in this study, PNX-14 presented anti-inflammatory activity by diminishing or abolishing pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-8, and IFN-γ, thus protecting the intestinal epithelial barrier functions and maintaining the immunoregulatory balance. Also, our results showed that treatment of colitic rats with CTX alone (AA+CTX group) or CTX plus PNX-14 (AA+CTX+PNX group) inhibited the pro-inflammatory cytokines (except IL-6). In accordance with our data, CTX directly reduced gene expression of various pro-inflammatory cytokines through GnRH receptors in rats [52] and produced rapid anti-inflammatory effects by reducing TNF-α and IL-1β in rheumatoid arthritis patients with high gonadotrophin levels [22]. The reduction of pro-inflammatory cytokines and disease activity by CTX administration in our study, might be explained, at least partially, through GnRHR blockade on peripheral immune cells that can secrete GnRH.

Although we have shown that PNX-14 and/or CTX treatment mitigates experimentally induced colonic injury via their anti-inflammatory, antioxidant and anti-apoptotic effects, further studies are needed to elucidate the cellular and/or molecular signaling pathways that include the GPR173 and GnRH receptor activation and additional inflammatory mediators in the ameliorative actions of PNX-14 and/or CTX on colonic inflammation.

5. Conclusion

In conclusion, our data suggests that PNX-14 exhibits anti-inflammatory, antioxidant, and anti-apoptotic actions in AA-induced colonic injury by inhibiting neutrophil migration, recovering antioxidant status, abolishing reactive species formation and regulating inflammatory and apoptotic processes. Despite its effects as an GnRH-antagonist, CTX has also revealed a similar beneficial role as PNX-14 in this UC model. On the other hand, the combination of PNX-14 and CTX treatments did not have a synergistic effect in ameliorating colonic injury.

Understanding the complex interaction between inflammatory mediators and oxidative/nitrosative stress in UC is very important for developing novel therapeutic strategies including agents with anti-inflammatory and antioxidant properties. The interplays caused and maintained by the excess production of free radicals in the pro-inflammatory environment may lead to a self-reinforcing vicious cycle, while PNX-14 and/or CTX treatments can be considered to disrupt this cycle by their anti-inflammatory and antioxidant qualities. From this point of view, their therapeutic potential in UC should be evaluated in clinical trials after their efficacy and safety have been established.

CRediT authorship contribution statement

Hülya Buzcu: Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Meral Yüksel:** Investigation, Formal analysis. **Seda Kırmızıkan:** Investigation, Formal analysis. **Esra Çikler:** Investigation, Formal analysis. **Berna Karakoyun:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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Conflict of Interest

The authors all declare that there is no existing conflict of interest.

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Data availability

Data will be made available on request.

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