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ANTI-INFLAMMATORY EFFECTS OF NESFATIN-1 IN RATS WITH ACETIC ACID - INDUCED COLITIS AND UNDERLYING MECHANISMS

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Mucosal balance impairment, bacterial over-proliferation, cytokines, inflammatory mediators are known as responsible for inflammatory bowel disease. Besides known anorexigenic, neuroprotective, and anti-apoptotic effects, the major effect of nesfatin-1 on colitis is unknown. Our aim was to investigate the possible anti-inflammatory effects of nesfatin-1 in acetic acid induced colitis model and potential underlying mechanisms. Male Spraque-Dawley rats were anesthetized by intraperitoneal ketamine (100 mg/kg) and chlorpromazine (0.75 mg/kg). For nesfatin-1 and antagonist applications some of the rats were intracerebroventricularly (i.c.v.) cannulated. In colitis group, intrarectally (i.r.) 4% acetic acid solution (1 ml) and 10 minutes later i.c.v. nesfatin-1 (0.05 µg/5 µl) or vehicle (5 µl) were administered.Treatments continued for 3 days. In control group, physiological saline solution was used intrarectally. To identify the underlying effective mechanism of nesfatin-1, rats were divided into 3 subgroups, 5 minutes following colitis induction; i.c.v. atosiban (oxytocin receptor antagonist), SHU9119 (melanocortin receptor antagonist) or GHSR-1a antagonist (ghrelin receptor antagonist) were administered, 5 minutes later nesfatin-1 was administered for 3 days. On the fourth day, rats were decapitated, and colon tissues were sampled. Macroscopic and microscopic damage scores of distal colon, and colonic tissue malondialdehyde, glutathione, myeloperoxidase, superoxide dismutase, catalase, luminol and lucigenin chemiluminescence measurements were analysed. The increased myeloperoxidase activity, malondialdehyde levels, luminol and lucigenin chemiluminescence measurements, macroscopic and microscopic damage scores with colitis induction ($P < 0.05 – 0.001$) were decreased with nesfatin-1 treatment ($P < 0.05 – 0.001$). Nesfatin-1 may show this effect by inhibiting neutrophil infiltration through tissues and by decreasing formation of free oxygen radicals. Atosiban and GHSR-1a administration alleviated the protective effect of nesfatin-1 from microscopic and oxidant damage parameters and lipid peroxidation ($P < 0.05 – 0.001$). The results of the study suggest that nesfatin-1 had a protective effect from colitis induction, and the anti-inflammatory and antioxidant effects of nesfatin-1 on colitis might occur *via* oxytocin and ghrelin receptors.

Key words: *nesfatin-1, acetic acid-induced colitis, ghrelin receptor, oxytocin receptor, melanocortin receptor, lipid peroxidation, myeloperoxidase*

INTRODUCTION

Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease (IBD) with recurrent, diffuse inflammation of the colonic and rectal mucosa, which is predominantly described by cycles of acute inflammation, ulceration and bleeding (1). The pathogenesis of disease involves an interaction between immune status, genetics, and environmental factors (2). The etiology of UC is not clearly understood, but excessive production of reactive oxygen species (ROS) by the inflamed mucosa have been suggested to contribute significantly to the development of tissue injury (3). These molecules are known to play a role not only in initiation of UC but also in progression of the disease. Furthermore, the production of these metabolites that are known to be the common mediators of inflammation which has been shown to occur in the gastrointestinal tract (4).

The possible therapeutic effects of peptides on IBD are frequently experimentally investigated. Nesfatin-1 is a recently described 82-amino-acid polypeptide (5) and nesfatin-1-containing proteins have been shown to be located in several brain areas related with feeding and metabolic regulation, including the hypothalamic paraventricular nucleus, arcuate nucleus, supraoptic nucleus, lateral hypothalamic area, and nucleus tractus solitarius in the brain stem (6, 7). In addition to the wide distribution of the peptide in the central nervous system, the messenger RNA of amino-terminal fragment was shown to be widely expressed also in the peripheral organs such as stomach, pancreas, testis, colon and adipose tissue in rodents and goldfish (8, 9). Further studies have shown 20-fold higher amino-terminal fragment mRNA expression in the rat stomach compared with the brain (10). Moreover, studies have suggested that the presence of inflammatory status might have a relation with nesfatin-1 production and release and the neurons that

produce nesfatin-1 respond to peripheral inflammatory signals (11). Recently, the anti-inflammatory and gastroprotective effects of nesfatin-1 on oxidative gastric damage and (12) anti-inflammatory and anti-apoptotic effects in subarachnoid hemorrhage (SAH) were demonstrated (13).

Nesfatin-1 firstly was shown to be a potent inhibitor of both food and water intake *via* a leptin-independent, melanocortin receptor-dependent mechanism in paraventricular nucleus (PVN) of hypothalamus (5, 14, 15). In PVN, nesfatin-1 co-localizes most extensively with oxytocin (16, 17). Further studies have reported its close relationship with regulation of blood pressure and its increasing effect on mean arterial pressure (MAP) when injected into the lateral cerebroventricle (18). Moreover, pretreatment with the melanocortin receptor antagonist (SHU9119), and oxytocin receptor antagonists block its well-known anorexigenic, anti-dipsogenic and the hypertensive effects (14, 15, 18). Although nesfatin-1 acts on melanocortin pathway and oxytocin receptors during these effects, it is not known if it may use these receptor families for the anti-inflammatory actions or not.

Another peptide related to regulation of food intake is ghrelin, which is known as fasting hormone (19, 20). Although the effective mechanism of peptide is not clear yet, it is postulated that the increased motility, amelioration in inflammation, increased appetite, and improved colonic blood flow might have a role (21). In the literature, the anti-inflammatory role of ghrelin has been shown in variant types chronic inflammation such as colitis (20, 23). Moreover, nesfatin-1 has been co-localized with ghrelin in the stomach and hypothalamus of rats. Also, nesfatin-1 has been shown to increase expression of ghrelin (22). While ghrelin and nesfatin-1 are released from brain tissue and have common anti-inflammatory effects, it draws attention if nesfatin-1 acts on ghrelin receptors for its possible anti-inflammatory effects in colitis.

Based on the aforementioned evidence, the study was designed to examine the possible anti-inflammatory effects of nesfatin-1 in acetic acid induced colitis model and potential underlying mechanisms. Firstly, we assessed the effect of nesfatin-1 on experimental colitis model. Secondly, we tested whether nesfatin-1 effected on melanocortin pathway, oxytocin receptors or ghrelin receptors in its anti-inflammatory effect.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (250 – 300 g, n = 48) were supplied from the Marmara University Animal Center (DEHAMER; approval no: 26.2012.mar) and were housed in a temperature-controlled (22 ± 2°C) room and standardized light/dark (12/12 hour) cycles. Rats were fed with standard rat pellets and tap water *ad libitum*. All experimental protocols were approved by the Marmara University Animal Care and Use Committee.

Experimental design

In the beginning of the experimental period for nesfatin-1 and antagonists applications some of the rats were intracerebroventricularly (i.c.v.) cannulated under intraperitoneal (i.p.) ketamine (100 mg/kg) and chlorpromazine (0.75 mg/kg) anesthesia. Following a recovery period, the rats were divided in to two groups as control and colitis. In colitis groups, intrarectally 4% acetic acid solution (1 ml) and 10 minutes later i.c.v. nesfatin-1, 0.05 µg/5 µl, (Enzo life sciences, catalogue number:ALX-522-116-C010) (24) or vehicle (5 µl) were administered. Treatments continued for 3 days. In control group, intrarectally physiological saline solution was given. In the second part of the study, to identify the underlying effective mechanism of nesfatin-1, 5 min after colitis induction; i.c.v. atosiban, oxytocin receptor antagonist, 3 µg/rat, (Tractocile, Ferring, Switzerland) (25), SHU9119, melanocortin receptor antagonist, 0.3 nmol/rat, (Phoenix peptide, catalogue number: 043-24) (14), or GHSR-1a, ghrelin receptor antagonist, 3 nmol/rat, (BACHEM, catalogue number: H-3108.0005) (26) were administered. Following a 5 min interval nesfatin-1 administrations were made. On the fourth day, rats were decapitated, colon tissues were sampled (27). Samples from distal colon were taken and stored at –80°C for later measurements of tissue myeloperoxidase (MPO) activity, malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) levels. Formation of reactive oxygen species in colonic samples were monitored by using chemiluminescence method and luminol and lucigenin chemiluminescence measurements were made. Tissue samples were placed in 10 % formaldehyde for histological evaluation, macroscopic and microscopic scoring.

Intracerebroventricularly cannulation

The rats were anesthetized (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine, i.p.) and body temperature was kept at ~37°C, using a heating pad placed under the rat. The animals were fixed in a stereotactic apparatus (Stoelting, standard stereotaxic instrument, Wood Dale, Illinois) with the head flat, and holes were drilled for bilateral implantation of cannula guides (22-gauge; Plastic Products, Roanoke, VA). The cannulas were inserted to within 1 mm above the target location (anterior/posterior [A/P], –3.30 mm; lateral [L], ± 0.0 mm; dorsal/ventral [D/V], –3.8 mm) and secured with acrylic dental cement (Croform acrylic powder and liquid) stabilized by two skull screws. A dummy cannula (Plastics One Canula C313DC) was placed in each of the guides to prevent clogging. Rats were allowed to recover for at least 5 days before experiments (28). All intracerebroventricular injections were made in a 5 µl volume over a period of 100 s using a Hamilton syringe. After each experiment, correct placement of the cannula was verified by injection of methylene blue.

Induction of colitis

After an overnight fasting, colonic inflammation was induced under light ether anesthesia by intrarectal administration of 1 ml of 4% (v/v) acetic acid in 0.9% NaCl solution (pH: 2.3) or saline (for control group) with a 8 cm long cannula. Rats were kept in Trendelenburg position for 30 seconds to prevent leakage during acetic acid and saline solution administration process. At the end of this process, 1.5 ml of phosphate buffer solution (PBS) of pH 7.4 was administered (3).

Measurement of colon myeloperoxidase activity

Since tissue myeloperoxidase (MPO) activity was shown to correlate significantly with the number of neutrophils determined histochemically, it is frequently used to estimate tissue neutrophil accumulation in the inflamed tissues (29). The method used for the assay of MPO activity was similar to that was previously described by Bradley *et al.* (29). Colon samples (0.2 – 0.5 g) were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mM K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). The homogenate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was discarded. The pellet was then re-homogenized with an equivalent volume of 50 mm

K_2HPO_4 containing 0.5% (w/v) HETAB and 10 mm EDTA (Sigma Chemical Co., St. Louis, MO, USA). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianisidine. 2HCl. One unit of enzyme activity was defined as the amount of MPO present per gram of tissue weight that caused a change in absorbance of 1.0 min^{-1} at 460 nm and 37°C. MPO activity was expressed as U/g tissue.

Measurement of colon malondialdehyde and glutathione levels

Samples of colon tissues were homogenized in 10 volumes of ice-cold 10% trichloracetic acid in an Ultra Turrax tissue homogenizer. Homogenized tissue samples were centrifuged at 3,000 g for 15 min at 4°C. The supernatant was removed and recentrifuged at 15,000 g for 8 min. GSH measurements were performed using a modification of the Ellman procedure (30). Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid-reactive substances as previously described (31). Lipid peroxide levels were expressed in terms of malondialdehyde (MDA) equivalents using an extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$. Results were expressed in nmol glutathione (GSH)/g tissue.

Measurement of superoxide dismutase and catalase activity in the colon

Superoxide dismutase (SOD) activity in colon samples was measured according to a previously described method (32). Briefly, measurements were performed in cuvettes containing 2.8 ml 50 mM potassium phosphate (pH = 7.8) with 0.1 mM EDTA, 0.1 mM 0.39 mM riboflavin in 10 mM potassium phosphate (pH 7.5), 0.1 ml of 6 mM o-dianisidin.2 HCl in deionized water, and tissue extract (50, 100 ml). Cuvettes with all their components were illuminated with 20-W SlylvaniaGro-Lux fluorescent tubes (Sylvania GRO-LUX F18W/GRO, Erlangen, Germany) that were placed 5 cm above and to one side of cuvettes maintaining a temperature of 37°C. Absorbance was measured at 460 nm with a Schimadzu UV-02 model spectrophotometer (Schimadzu, Tokyo, Japan). A standard curve was prepared routinely with bovine SOD (S-2515-3000 U; Sigma Chemical Co, St Louis, MO, USA) as reference. Absorbance readings were taken at 0 and 8 min of illumination and the net absorbance was calculated. The method for the measurement of catalase (CAT) activity is based on the catalytic activity of the enzyme that catalyzes the decomposition reaction of H_2O_2 to give H_2O and O_2 (33). Briefly, the absorbance of the tissue samples containing 0.4 ml homogenate and 0.2 ml H_2O_2 was read at 240 nm and 20°C against a blank containing 0.2 ml phosphate buffer and 0.4 ml homogenate for about 1 min. With the help of the standard graphic and considering the dilutions, the SOD activity of supernatant was calculated in the form of U/mg protein (34). Catalase measurements were expressed as U/ml/min.protein.

Measurement of colon luminol and lucigenin chemiluminescence levels

Chemiluminescence (CL) assay is a direct noninvasive method for measuring reactive oxygen species. Due to limitations, i.e., potential variability and low intensity of native CL, luminol and lucigenin can be used as enhancers. Lucigenin is more specific for superoxide radical, while luminol detects a group of reactive species such as OH, H_2O_2, HOCl radicals (12). Due to their high quantum efficiency after oxidation, they function as bystander-substrates for oxygenation and form high levels of excited-state products and CL, when added to an *in vitro* biological system. The excited electrons in these compounds revert to their ground state with the emission of energy as light (CL) and can be detected by a luminometer.

Luminescence of the colon samples was recorded at room temperature using Mini Lumat LB 9509 luminometer (EG&G Berthold, Germany) in the presence of luminol or lucigenin 0.2 mM each. All counts were obtained at 15 s intervals for 5 min and the results were expressed as area under the curve (AUC) of relative light unit (rlu) for 5 min per mg tissue. The calculation was based on the integration of the curve by the trapezoidal rule (a linear approximation) (35). Results were expressed in rlu/mg tissue.

Macroscopic scoring

On the fourth day of colitis, following decapitation the last 8 cm of the colon was excised, opened longitudinally, and rinsed with saline solution. Then, the distal colon was weighed and the mucosal lesions were scored macroscopically using the criteria outlined in *Table 1* (36).

Histological evaluation

For light microscopic investigations, samples from distal colon were placed in 10% formaldehyde, dehydrated in ascending alcohol series (70%, 90%, 96% and 100%), cleared in toluene and embedded in paraffin. For each animal, four randomly taken tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) and examined under an Olympus BX51 photomicroscope. All tissue sections were examined by experienced histologists who were unaware of the treatments. Histological scoring was performed by the criteria shown in *Table 2* (37).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as means ± S.E.M. Groups of data were compared with an analysis of variance followed by Mann-Whitney U non-parametric tests and Student's t test. Values of $P < 0.05$ were regarded as significant.

Table 1. Criteria for macroscopic scoring of colonic lesions.

Score	Appearance
0	No damage
1	Focal hyperemia, no ulcers
2	Hyperemia or bowel wall thickening without linear ulceration
3	Ulceration with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Major sites of damage extending more than 1 cm along the length of colon
6 – 10	If damage extends more than 2 cm along the length of colon, the score is increased by one for each additional 1 cm.

RESULTS

Acetic acid application increased both macroscopic ($P < 0.001$) and microscopic ($P < 0.001$) lesion scores of colitis group significantly compared to control group (*Fig. 1A* and *1B*). Although the increased macroscopic and microscopic lesion scores of colitis group were reduced by nesfatin-1 treatment ($P < 0.01 – 0.001$), atosiban application prior to nesfatin-1 treatment significantly increased both damage scores ($P < 0.05 – 0.001$) and GHSR-1a application prior to nesfatin-1 treatment significantly increased microscopic damage score ($P < 0.01$).

In histologic analysis, massive epithelial loss, severe inflammatory cell infiltration, vasculitis and submucosal edema were evident in colitis group (*Fig. 2B*). Although a regular epithelium, moderate inflammation, vasculitis and moderate submucosal edema were observed in only nesfatin-1 or SHU9119 treated groups, severe inflammation, vasculitis and edema were prevalent in GHSR-1a or atosiban treated groups (*Fig. 2C-2F*).

Colonic MPO activity, the indicator of neutrophil migration to injured tissue, showed a marked increase in colitis group compared to control group ($P < 0.001$) (*Fig. 3A*). While nesfatin-1 treatment ameliorated the increased MPO activity ($P < 0.01$), SHU9119 application prior to nesfatin-1 reversed its effect by increasing the neutrophil infiltration through the tissue. Additionally, MPO activity remained decreased in GHSR-1a and atosiban applicated groups suggesting that these receptors may not have a role in the effect of nesfatin-1 on neutrophil infiltration ($P < 0.001$) (*Fig. 3A*).

As expected, a significant increase in colonic MDA level ($P < 0.01$) with a concomitant decrease in antioxidant GSH content ($P < 0.001$) was observed in the colitis group (*Fig. 3B* and *4A*). The

Table 2. Criteria for the microscopic scoring of colonic lesions.

Score	Appearance
0 none; 1 mild; 2 moderate; 3 severe	Submucosal edema
0 none; 1 localized; 2 moderate; 3 severe	Damage/necrosis
0 none; 1 mild; 2 moderate; 3 severe	Inflammatory cell infiltration
0 none; 1 mild; 2 moderate; 3 severe	Vasculitis
0 –; 1+	Perforation

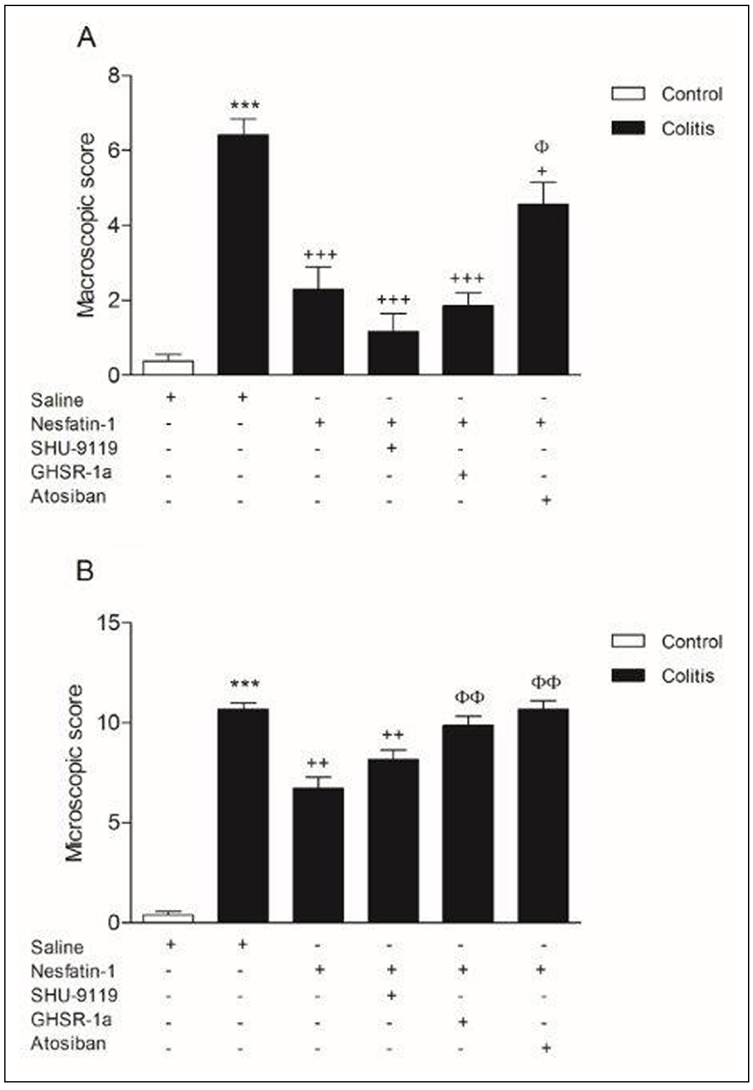

Fig.1. Colonic macroscopic (A) and microscopic (B) damage scores. ***P < 0.001 compared to control group; $^{++}$P < 0.01; $^{+++}$P < 0.001 compared to colitis group; $^{\varphi}$P < 0.05; $^{\varphi\varphi}$P < 0.01 compared to nesfatin-1-treated (without any other drugs) group.

increase in colonic MDA level of the colitis group was reduced by nesfatin-1 treatment, underlying the lipid peroxidation inhibiting effect of nesfatin-1 ($P < 0.01$) (*Fig. 3B*). Besides, SHU9119, GHSR-1a and atosiban treatments prevented the beneficial effect of nesfatin-1 on MDA level ($P < 0.01$) (*Fig. 3B*). Although nesfatin-1 treatment did not affect the antioxidant GSH levels compared to colitis group, SHU9119 and GHSR-1a treatments increased GSH levels compared to colitis group ($P < 0.01$) and compared to only nesfatin-1 treated group ($P < 0.05$) (*Fig. 4A*).

SOD activity was tended to decrease in colitis group ($P = 0.02$) and increased in GHSR-1a and atosiban treated groups compared to colitis group ($P < 0.05$) (*Fig. 4B*). Although antioxidant colonic CAT activity in the colitis group showed a decrease compared to control group ($P < 0.05$), it was not different than control in nesfatin-1 treated group (*Fig. 4C*). Furthermore CAT levels of atosiban treated group were also declined compared to control group ($P < 0.01$), suggesting an inhibitor effect on protective role of nesfatin-1 (*Fig. 4C*).

While luminol- and lucigenin-enhanced CL levels showed significant increases in the colitis group as compared with control values ($P < 0.001$), both parameters were decreased in nesfatin-1 treated group compared to colitis group ($P < 0.05 – 0.001$) (*Fig. 5A* and *5B*). As luminol levels were tended to increase in GHSR-1a and atosiban treated groups, lucigenin levels were risen back in all treatment groups ($P < 0.05 – 0.001$), suggesting that the applications of GHSR-1a and atosiban prior to nesfatin-1 inhibited its protective effect and nesfatin-1 may have a role on ghrelin and oxytocin receptors in its reactive oxygen metabolite decreasing effect. (*Fig. 5A* and *5B*).

DISCUSSION

Colitis is defined as the inflammation of the layer that covers the inner surface of the colon and a disease causing wounds with bleeding in the mucosa, and affecting the patient's quality of life. In this study, we aimed to show the colonic tissue damage induced by acetic acid application, and to demonstrate the probable recovery in the colon tissue with nesfatin-1 therapy and the possible underlying mechanism nesfatin-1 acts on.

The anti-apoptotic, neuroprotective (13), antihyperglycemic (38), anxiogenic, anorexigenic (39) effects of nesfatin-1 and in addition, the role of nesfatin-1 in delaying gastric emptying (24) and in the cardiovascular control (40) were revealed by previous studies. This is the first study that shows the anti-inflammatory effect of nesfatin-1 in an experimental colitis model by ameliorating inflammatory status and enhancing the biochemical and histological parameters. Previously, the anti-inflammatory

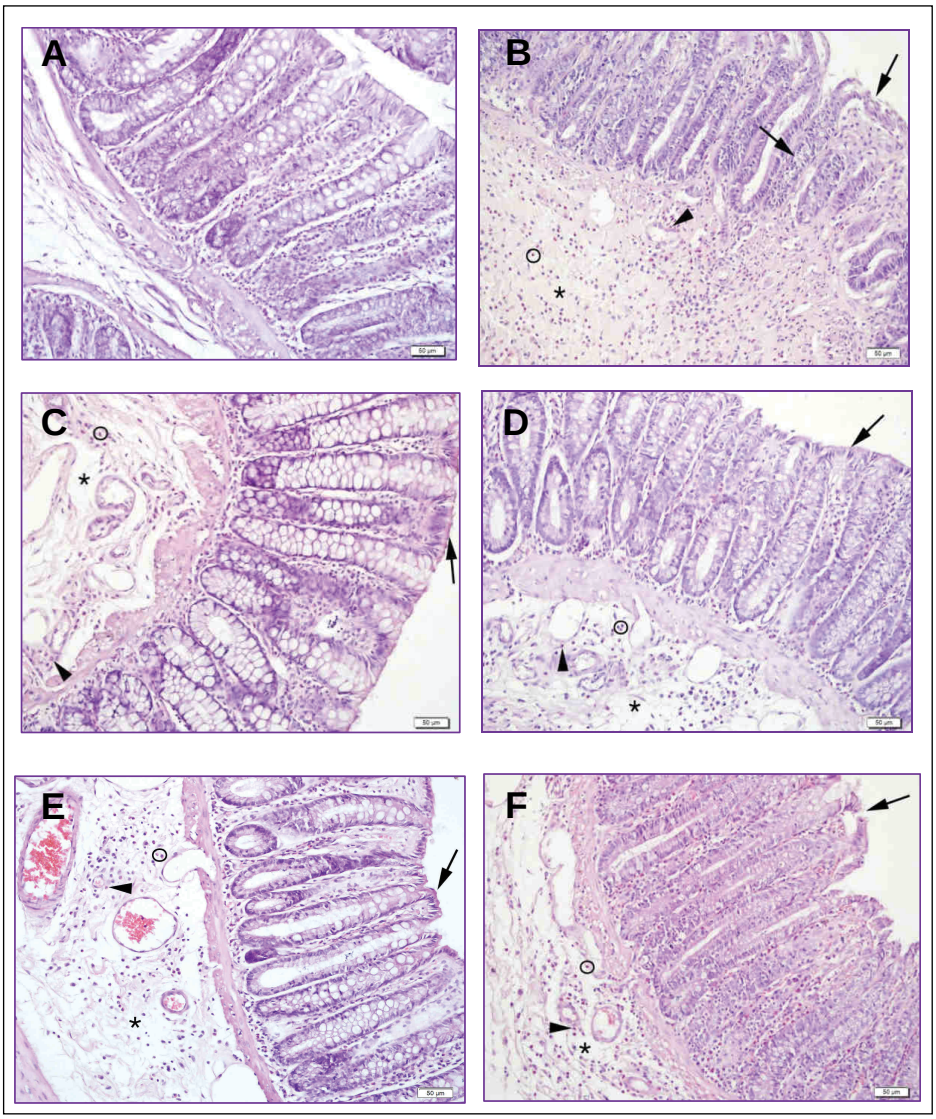

Fig. 2. Light microscopic examination of the colonic mucosa in experimental groups. (**A**) Control group, describes normal morphology, regular epithelial line and submucosa. (**B**) Colitis group characterized by degenerated surface epithelium (➔), severe submucosal edema (*), inflammation (o), vasculitis (▶) (**C**) Nesfatin-1 treated colitis group, describes regular epithelial line (➔), moderate submucosal edema (*), inflammation (o), vasculitis (▶). (**D**) SHU9119 treated colitis group, describes quite regular surface epithelium (➔), moderate submucosal edema (*), inflammation (o), vasculitis (▶). (**E**) GHSR-1a treated colitis group, describes regular epithelial line (➔), severe submucosal edema (*), moderate inflammation (o), vasculitis (▶). (**F**) Atosiban treated colitis group, is characterized by degenerated surface epithelium (➔), severe submucosal edema (*), inflammation (o), moderate vasculitis (▶). Hematoxylin and eosin (H&E) stain.

effect of intraperitoneally administrated nesfatin-1 was shown in an acedic acid induced gastritis model by Kolgazi *et al.* (12). Additionally, in a recent study its essential role in healing process of chronic gastric ulcers *via* activating the gastric blood flow at ulcer margin and also its inducing role in the mucosal regeneration was reported (41). As the possible beneficial effects of peptides on inflammation models attrack attention and are frequently experimentally investigated, recently, the reparative and healing activity of another intraperitoneally administrated peptide such as stable gastric pentadecapeptide BPC157 supported the nature of certain endogenous peptides in amelioration of colitis (42). However, our study focuses on the central administration of nesfatin-1 and peripheral anti-inflamatory effects in a colitis model. Furthermore, neuroprotective effects of nesfatin-1 in a brain injury model induced by subarachnoid hemorrhage and improved sensitivity of central nesfatinergic system to peripheral inflammatory stimuli was reported (11, 13). Bonnet *et al.* conveyed the association between nesfatin-1 neurons and peripheral inflammation and pointed out activation of central nesfatin-1-expressing neurons during an inflammatory stimulus such as LPS stimulated endotoxaemia and reported that the neurons of the central nesfatinergic system were sensitive to peripheral inflammatory stimulus and were belong to a specific immunosensitive neurocircuitry (11). Similarly, peripheral inflammatory status such as colitis may stimulate central nesfatinerjic system to ameliorate the inflammatory process. Thus, we aimed to demonstrate the possible protective effect of i.c.v. administered nesfatin-1 in a colitis model. However, additional studies are required to analyze improved central nesfatin-1 activity in colitis.

The macroscopic and microscopic damage scores of colonic tissue was evaluated histopathologically and increased in rats with colon injury significantly and was consistent with the results of a previous study on an acetic acid-induced colitis model (43). Although application of nesfatin-1 ameliorated the increased scores in the colitis group, the atosiban application has reduced both macroscopic and microscopic damage inhibitory effect of nesfatin-1. This result suggests that the reducing effect of nesfatin-1 on damage scores might be *via* oxytocin receptors. Previously, nesfatin-1 has been shown to use the oxytocinergic signaling pathway while regulation of food intake. Similar to its well known satiety controlling effect, the interaction with oxytocin might be an intermediate pathway for nesfatin-1 in its anti-inflammatory effect (15). Although the macroscopic damage reducing effect of nesfatin-1 continued with the application of GHSR-1a, the microscopic damage reducing effect was eliminated. This situation suggests that ghrelin mediates the microscopic damage reducing effect of nesfatin-1. Similarly, in the light microscopic histological examination, the damage was continued in the groups treated with GHSR-1a and atosiban, as nesfatin-1 was not able to show healing effect in the groups treated with these antagonists prior to nesfatin-1 application.

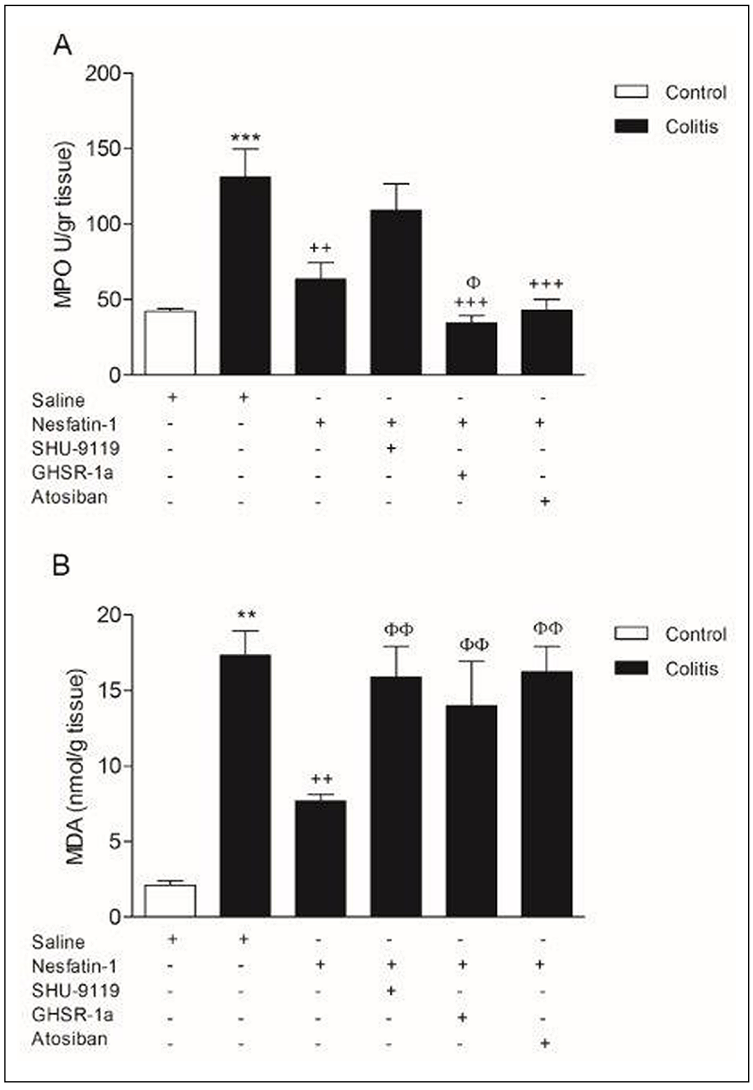

Fig. 3. Colonic tissue myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) levels (B).
**P < 0.01, ***P < 0.001, compared to control group; $^{++}$P < 0.01, $^{+++}$P < 0.001 compared to colitis group; $^{\varphi}$P < 0.05, $^{\varphi\varphi}$P < 0.01 compared to nesfatin-1-treated (without any other drugs) group.

Myeloperoxidase is an enzyme especially presented in neutrophils, and also in monocytes and macrophages in a very small amount. Therefore, MPO activity is directly proportional to the quantity of neutrophils in inflamed tissues, and measurement of MPO activity is considered to be a sensitive and quantitative index of acute intestinal inflammation (44). In our study, an increase in colonic MPO activity was observed *via* acetic acid administration that suggests increased neutrophil infiltration in the colon tissue following damage induction. Although there is no previous study showing the effect of nesfatin-1 on colitis, it has been shown that intraperitoneal administration of nesfatin-1 inhibited the increase in myeloperoxidase activity of the brain tissue in a SAH model (13) and also of the gastric tissue with acetic acid induced ulcer model (12). Similarly, our MPO results suggest that the application of nesfatin-1 may prevent colonic tissue damage by reducing the neutrophil infiltration. The

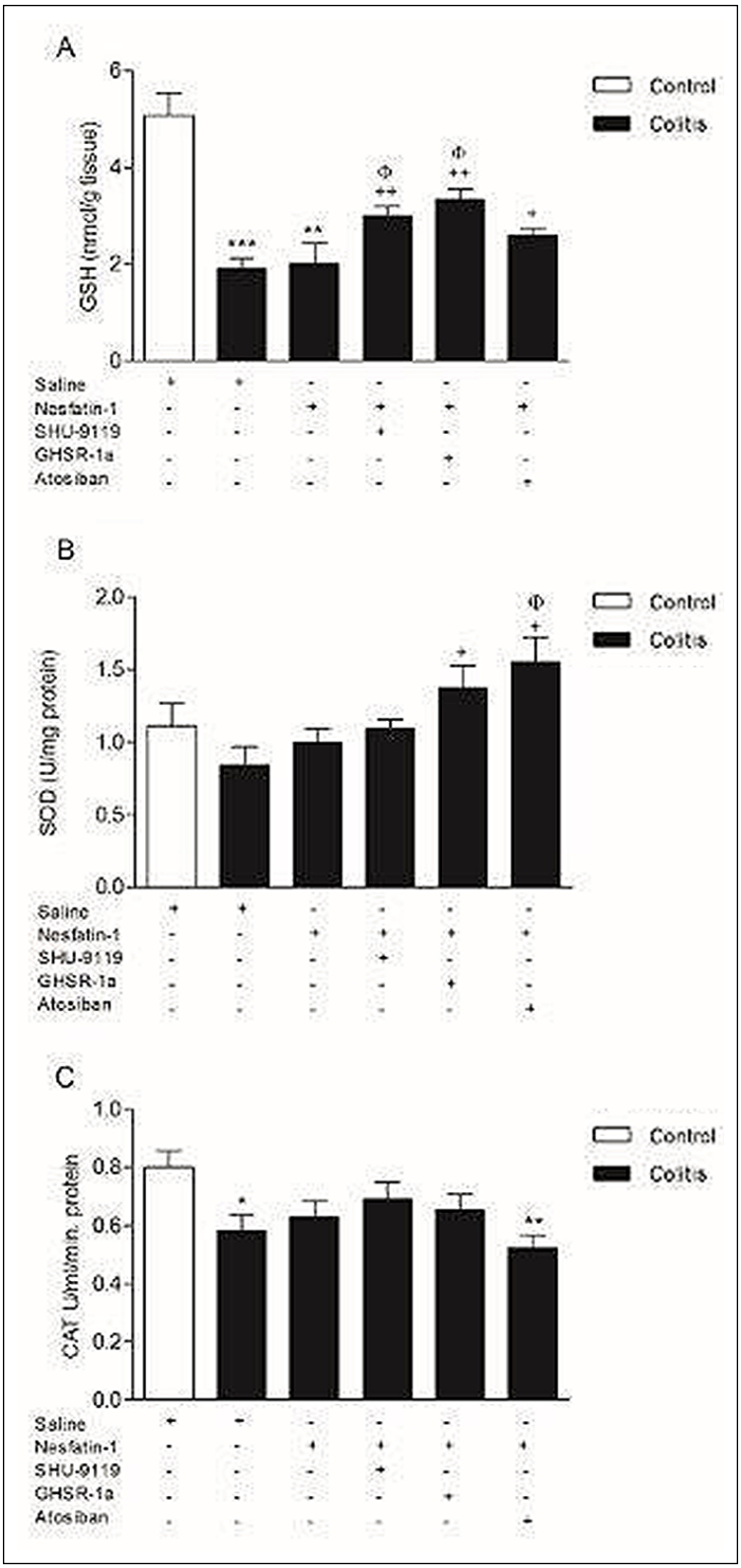

Fig. 4. Colonic tissue glutathione (GSH) levels (A), superoxide dismutase (SOD) (B) and catalase (CAT) (C) activities.
*P < 0.05, **P < 0.01, ***P < 0.001 compared to control group; $^{+}$P < 0.05, $^{++}$P < 0.01 compared to colitis group; $^{\varphi}$P < 0.05 compared to nesfatin-1-treated (without any other drugs) group.

reduction in MPO activity continued also in groups receiving antagonists, GHSR-1a and atosiban, while SHU9119 application prior to nesfatin-1 prevented this reduction. These results indicate that melanocortin receptors may mediate the effect of nesfatin-1 on neutrophil infiltration. Furthermore, it is known that alpha-melanocyte-stimulating hormone causes anti-inflammatory effects on colitis and melanocortin-1 receptor (MC-1R) has role in this anti-inflammatory function (45). In the study conducted by Maaser *et al.* on C57BL / 6 and MC-1R mutant mice, the protective effect of MC-1R was demonstrated in colitis model induced by dextran sodium sulfate (44). Although the relationship between tissue MPO activity and melanocortin receptors have been pointed out in previous studies, our study suggests that the effect of nesfatin-1 on MPO activity may be *via* melanocortin-1 receptors.

It is well known that malondialdehyde, the end product of lipid peroxidation, is detected in increasing amounts with damage (43) and in our study this damage were clearly manifested in the groups treated with acetic acid. Nesfatin-1 has been demonstrated to ameliorate the MDA levels in a few inflammatory processes (12, 13). Consistent with previous studies, in this study MDA levels were reduced with nesfatin-1 treatment, moreover were elevated with all antagonist applications. Eventually, nesfatin-1 may be effective in restoring continuity of cells by reduction of lipid peroxidation *via* suppression of malondialdehyde production and may play a role on ghrelin, oxytocin and melanocortin receptors in this function.

Glutathione molecule is an important antioxidant for the protection against free oxygen radicals and is oxidized with peroxidase enzyme and eliminates the hydrogen peroxide radical (30, 46). Consistent with a previous study, serious depletion in the antioxidant glutathione levels was observed in the colitis group with colonic damage (43), however in our study nesfatin-1 treatment was not able to increase antioxidant glutathione levels. According to our results nesfatin-1 may not effect on antioxidant glutathione levels during its anti-inflammatory action.

SOD is in a class of enzymes that catalyze the dismutation of the oxygen and hydrogen peroxide, and is primary protective against oxyradicals such as superoxide that is a major reactive oxygen specy in the cells (47). The effect of nesfatin-1 treatment on the activity of SOD in colitis is not known yet. According to our results, although nesfatin-1 treatment did not create significant change in SOD activity compared to the colitis group, in previous inflammatory models nesfatin-1 increased the SOD activity (12, 13). The reason for lack of significant change in SOD activity of nesfatin-1-treated group compared to colitis group may be due to the non-significant decrease in SOD levels of colitis group, although there was a tendency to decrease (P = 0.22). If the SOD levels were decreased significantly in colitis group, the levels would be increased to the control levels with the treatment by

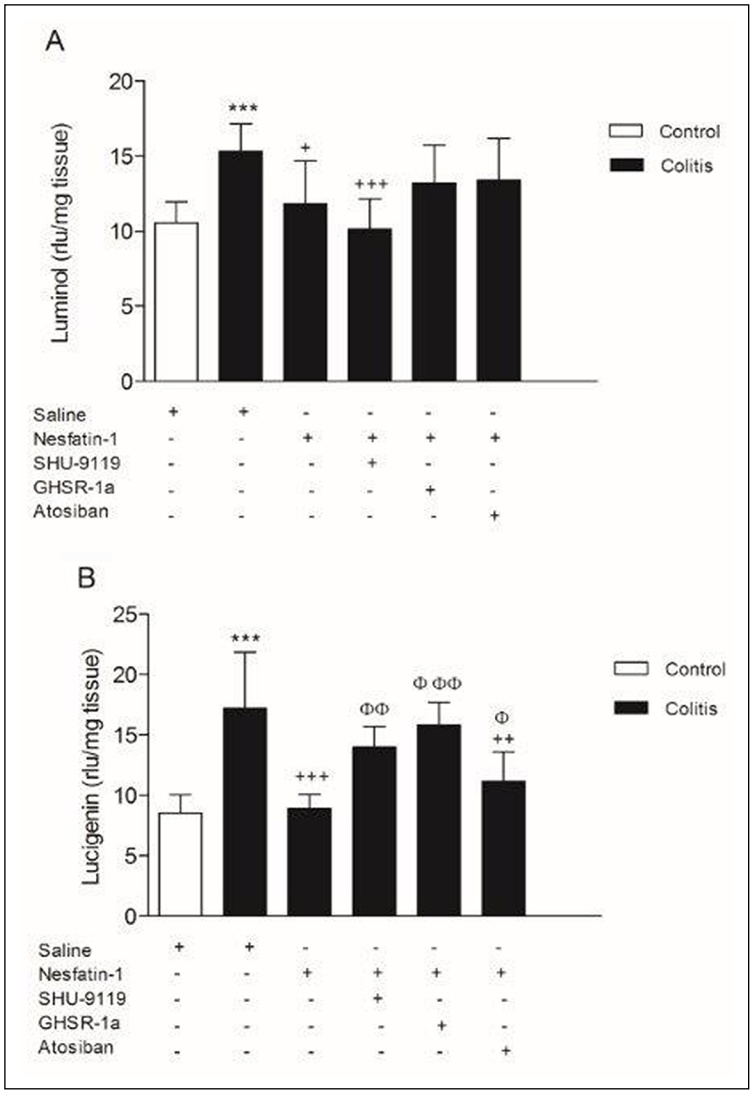

Fig. 5. Luminol- (A) and lucigenin- (B) enhanced tissue chemiluminescence levels.
***P < 0.001 compared to control group; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 compared to colitis group; $^{\varphi}$P < 0.05, $^{\varphi\varphi}$P < 0.01, $^{\varphi\varphi\varphi}$P < 0.001 compared to nesfatin-1-treated (without any other drugs) group.

nesfatin-1. Eventually, nesfatin-1 treatment inhibited the decrease tendency that occurs in SOD levels with the colitis induction.

The main antioxidant effect of catalase is breaking down H_2O_2 into water and molecular oxygen. Although H_2O_2, is formed *via* SOD, is not a radical, is a precursor of OH- radical which is the most reactive free oxygen radical (47). In this study, antioxidant catalase activity was ameliorated *via* colitis induction, and was not significantly different than control with nesfatin-1 treatment. Similarly, catalase activity of colonic tissue was reduced in ulcerative colitis models (48, 49). Additionally, our results suggest that nesfatin-1 may inhibit the decrease in antioxidant catalase activity observed *via* colitis induction. Moreover, the catalase activity of the group treated with atosiban showed a trend of decrease which was not significantly different compared to the nesfatin-1-treated group and decreased significantly compared to the control group. Although the inhibitory effect of nesfatin-1 on reduction of catalase activity observed in the colitis group is not clear, the additional studies are needed to determine whether it occurs *via* oxytocin receptors.

In accordance with previous data, along with the significant increase of tissue myeloperoxidase activity and the alleviation of lipid peroxidation in colitis, the rise in luminol and lucigenin chemiluminescence levels point out the production of reactive oxygen metabolites in the tissue (43). The increase in luminol and lucigenin chemiluminescence were reduced in the group treated with nesfatin-1, and the reduction of luminol chemiluminescence disappeared in the groups treated with GHSR-1a and atosiban, although the reduction of lucigenin chemiluminescence inhibited by all receptor antagonists. This data suggests that the applications of GHSR-1a and atosiban inhibited the effect of nesfatin-1 and nesfatin-1 may have a role on ghrelin and oxytocin receptors in its reactive oxygen metabolite decreasing effect. As lucigenin is more specific to superoxide, nesfatin-1 may show its superoxide decreasing effect *via* all these antagonists especially GHSR-1a or by interfering with other pathways. Although effect of ghrelin and oxytocin receptors in luminol and lucigenin chemiluminescence levels of inflammatory processes is not known, previously reducing effect of ghrelin and oxytocin on these reactive oxygen parameters were demonstrated (50, 51). Addition to previous data, our results suggest that nesfatin-1 may use these pathways in its reactive oxygen metabolite reducing effect.

Consequently, it was observed that the oxidative damage developing in colitis model induced by acetic acid administration recovered with application of intracerebroventricular nesfatin-1 and this peptide reduced the microscopic and macroscopic damage with its anti-inflammatory effects. Our results suggest that nesfatin-1 may demonstrate these anti-inflammatory effects by preventing neutrophil infiltration to the tissue and by suppressing the free radical formation. The application of atosiban and GHSR-1a prevented the effect of nesfatin-1 on microscopic damage, lipid peroxidation, luminol and lucigenin chemiluminescence levels. At the same time, atosiban application also caused a regression in the macroscopic damage. These findings indicate that nesfatin-1 may show its anti-inflammatory and antioxidant effects on colitis *via* oxytocin and ghrelin receptors. Additionally, melanocortin receptor antagonist inhibited the protective effect of nesfatin-1 on MPO activity, lipid peroxidation, lusigenin chemiluminescence and glutathione levels. Although it has been shown previously that nesfatin-1 has used the melanocortin signaling pathway for its effects on food intake, this is the first study suggesting that nesfatin-1 may also use the receptors which mediate its anorexigenic effects in terms of the anti-inflammatory effects.

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