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NESFATIN-1 PROTECTS FROM ACUTE PANCREATITIS: ROLE OF MELANOCORTIN RECEPTORS

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Nesfatin-1, a recently discovered peptide, was shown to have anti-inflammatory effects. Acute pancreatitis (AP) is a life-threatening condition caused by various reasons. Although the etiology of AP is well-known, its pathogenesis is not clear. The aim of this study is to investigate the possible anti-inflammatory role of nesfatin-1 and its probable protective underlying mechanisms in an acute pancreatitis model. Caerulein was applied intraperitoneally to induce acute pancreatitis in Sprague-Dawley female rats. Nesfatin-1 was administered 5 minutes before the application of caerulein to determine its potential anti-inflammatory role on AP. Five minutes before nesfatin-1 injection, in order to investigate the underlying mechanism, oxytocin receptor antagonist (atosiban), melanocortin receptor antagonist (HS024), or ghrelin receptor antagonist (cortistatin) were administered. Five minutes after nesfatin-1 administration, two doses of caerulein were applied one hour apart. The rats were sacrificed 12 hours after the first caerulein dose for serum and pancreatic tissue sampling. Microscopic damage scoring, malondialdehyde and glutathione levels, myeloperoxidase activity, luminol and lucigenin chemiluminescence levels in pancreatic tissue and amylase, lipase, trypsinogen-2 levels in serum were evaluated. Oxidative damage was decreased with nesfatin-1 treatment in the acute pancreatitis model ($P < 0.05 - 0.001$). The administration of HS024 reversed the effect of nesfatin-1, *via* increasing lipase, amylase, trypsinogen-2, malondialdehyde (MDA), myeloperoxidase (MPO) and lucigenin levels ($P < 0.05 - 0.01$). Atosiban pre-treatment elevated MPO activity, luminol and lucigenin chemiluminescence levels ($P < 0.01 - 0.001$) and cortistatin increased lucigenin and luminol chemiluminescence ($P < 0.05 - 0.01$). Although receptor antagonists reversed the effect of nesfatin-1 on related biochemical parameters, no significant difference was found in histological scoring. Our results indicated that nesfatin-1 had an anti-inflammatory effect on acute pancreatitis *via* mainly effecting melanocortin receptors.

Key words: *nesfatin-1, pancreatitis, oxytocin, melanocortin, ghrelin, reactive oxygen species, oxytocin, lipid peroxidation, nucleobindin-2*

INTRODUCTION

Acute pancreatitis (AP) is inflammation of the pancreas that may be mild or life threatening. Although the etiology of AP has been well identified little is known about its pathogenesis (1). AP is the activation of enzymes that are inactive in the pancreas under normal conditions and causes pancreatic acinar cell damage under the effect of various reasons (2). For instance, the most common etiological factors are gallstones and alcohol (3). The disease is classified as mild, moderate and severe acute pancreatitis, according to the intensity of inflammation and development of organ failure. Although there are no local and systemic complications of organ failure in mild form, transient organ failure and complications are seen in moderate type, while persistent organ failure develops in the severe form of the disease (4). Symptoms include severe abdominal pain, nausea-vomiting, jaundice, anorexia, fever and shortness of breath. For the diagnosis of the disease increased serum amylase and lipase

levels and abdominal imaging are used (5). Though there is no specific treatment for the disease yet, supportive treatments such as antibiotics, fluid replacement, and analgesics are preferred (4).

The potential therapeutic effects of variant peptides on AP are frequently experimentally investigated. Nesfatin-1 is a newly identified anorexigenic peptide that was produced by the nucleobindin-2 (NUCB2) precursor (6). It was discovered in 2006, and consist of 82 amino acids (6). Its specific receptor has not been identified yet. Nesfatin-1 releasing neurons are found in the hypothalamic paraventricular nucleus, supraoptic nucleus, arcuate nucleus, lateral hypothalamic area, and nucleus tractus in the brain stem (7). Nesfatin-1 may cross the blood-brain barrier in both directions (8). In addition to brain tissue, NUCB2 mRNA was shown to be present in gastrointestinal system tissues such as gastric mucosa, pancreatic beta cells and colonic tissue (9-12). Besides its anorexigenic role, nesfatin-1 has variant effects such as, anti-inflammatory, antiapoptotic, hypertensive and gastric emptying delaying effect, on these numerous tissues (9, 13).

Although its specific receptor is not known yet, nesfatin-1 has been shown to inhibit neutrophil migration to tissues, and to have cytoprotective and anti-inflammatory effects in a rat colitis model *via* directing oxytocin and ghrelin receptors (13). Its interaction with oxytocin receptors was also reported in a previous study that investigated anorexigenic effects of it (14). Additionally, a functional relationship between ghrelin and nesfatin-1 has been shown, that points out its suppressing role on ghrelin receptor expression while directing its anorexigenic effect (15). It represents its different effects not only by oxytocin or ghrelin pathways, but also by melanocortin system, thus it reduces food intake by suppressing the fasting center in the paraventricular nucleus of the rat hypothalamus by melanocortin pathway (16). Although previous data has shown anti-inflammatory role of nesfatin-1 on gastrointestinal system, the effects of nesfatin-1 on acute pancreatitis and probable underlying mechanism is not known and takes attention.

Caerulein is an analog of cholecystikinin that causes pancreatitis, causing relaxation of the oddi sphincter and contraction of the gallbladder, by this way stimulation of exocrine pancreatic secretion and causes early proteolysis of zymogens (17). The resulting activated trypsin converts other proteolytic enzymes into active form to digest the pancreatic tissue. Acute pancreatitis induced by caerulein is histologically very similar to early acute pancreatitis in humans (18). As rat models are

frequently chosen for acute pancreatitis induction (19), and in our laboratory, different types of rat acute pancreatitis models have been performed, in this study a caerulein-induced rat acute pancreatitis model was preferred (20, 21).

Based on the aforementioned evidence, the aim of this study was to investigate the possible anti-inflammatory effects of nesfatin-1 on a caerulein-induced acute pancreatitis rat model and its potential underlying mechanism. Secondly, we examined whether nesfatin-1 affected on oxytocin or ghrelin receptors, or melanocortin pathway in its anti-inflammatory effect.

MATERIALS AND METHODS

All the experiments were undertaken in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and all experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee (17.2017.mar). The study was supported by the Marmara University Scientific Animals Research Projects Commission (SAG-C-YLP- 090517-0251).

Female Sprague-Dawley rats (250 – 300 g, 10 – 12 weeks old) were acquired from Marmara University Experimental Animals Research and Implementation Centre (DEHAMER) (Istanbul, Turkey). In a previous study of our group, we showed

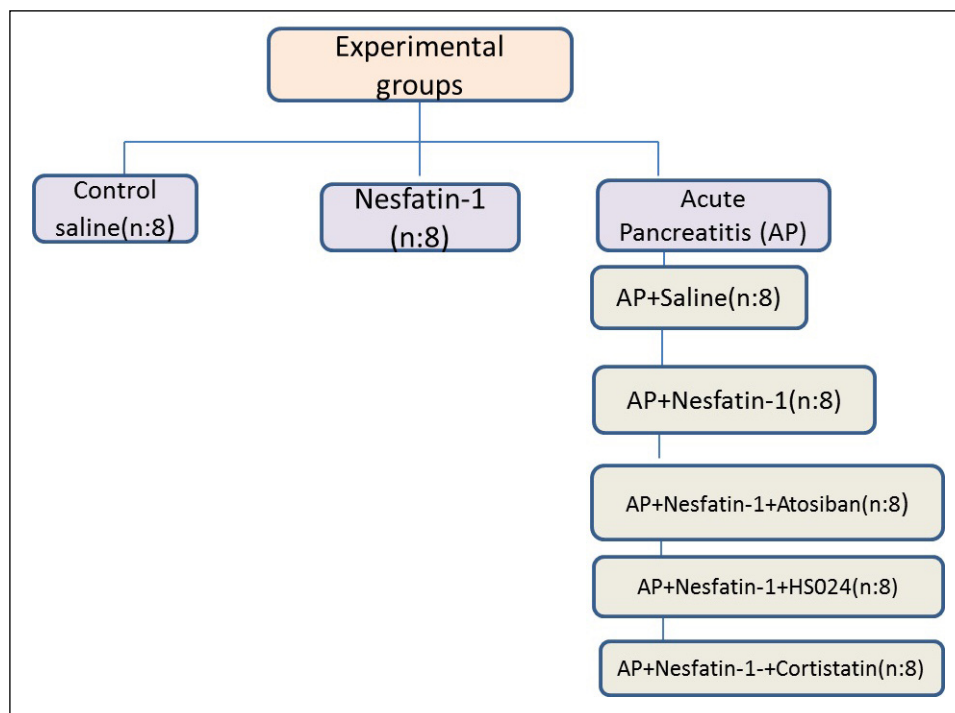


Fig. 1. The groups scheme.

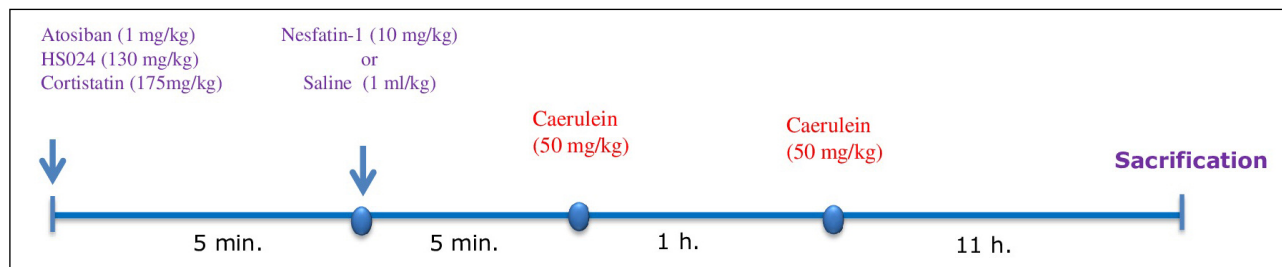


Fig. 2. Time table that shows atosiban (1 mg/kg), HS024 (130 µg/kg) or cortistatin (175 µg/kg) pre-treatments, nesfatin-1 (10 µg/kg) or saline (1 mg/kg) treatments and acute pancreatitis induction *via* caerulein administration.

oxidative and inflammatory changes in an acute pancreatitis rat model (20). There were no differences according to the gender. Considering our previous experience, in this study, we preferred to work on females. The rats were housed in a 12 h light:12 h dark photoperiod under controlled temperature ($22 \pm 1^\circ\text{C}$) and humidity (65–70%). Feeding were performed with standard rat pellets and tap water *ad libitum*.

Experimental design

The rats were divided into three main groups as saline-treated control, nesfatin-1-treated and acute pancreatitis (AP) groups. AP group was subsequently divided into subgroups as saline-treated (AP) or nesfatin-1-treated (10 $\mu\text{g/kg}$) AP groups. To evaluate the possible underlying mechanisms that nesfatin-1 may have a role, nesfatin-1-treated AP group was separated into subgroups as atosiban (1 mg/kg) and nesfatin-1-treated, HS024 (130 $\mu\text{g/kg}$) and nesfatin-1-treated or cortistatin (175 $\mu\text{g/kg}$) and nesfatin-1-treated AP group, randomly (Fig. 1). All groups were consisted of 8 rats. G power analysis was performed to determine the number of rats for each group. Nesfatin-1 and the receptor antagonists were given intraperitoneally (i.p.). According to previous studies, the anti-inflammatory dose of nesfatin-1, and the efficient doses of antagonists were used (22–25). Melanocortin receptor antagonist HS024 (Tocris, Minneapolis, MN, USA), ghrelin receptor antagonist cortistatin (Tocris, Minneapolis, MN, USA), oxytocin receptor antagonist atosiban (Tocris, Minneapolis, MN, USA), nesfatin-1 (PHOENIX Mannheim, Germany) and caerulein were purchased from Sigma (Sigma-Aldrich Co. LLC, Germany). All drugs were freshly dissolving in 0.9% sterile saline.

To induce acute pancreatitis, rats were administered two i.p. injections of caerulein (50 $\mu\text{g/kg}$) at hourly interval (26). Nesfatin-1 was given 5 min, and antagonists were applied 10 min before the first caerulein injection. A pilot study was done to test the AP model, since the rats were sacrificed 6 or 12 hours after last caerulein injection and pancreatic tissue was examined macroscopically to go on with the more effective AP model. Twelve hour AP model was selected to move on next experiments considering the pancreatic edema, hyperemia and increase in tissue weight. Twelve hours after last caerulein or saline injections, the animals were sacrificed by cardiac puncture under anesthesia (ketamin, 100 mg/kg; xylazine, 10 mg/kg) (Fig. 2). Blood was collected and centrifuged for the assessment of amylase, lipase and trypsinogen-2 in sera. Pancreatic tissue samples were taken and stored at -80°C for the determination of malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and luminol and lucigenin chemiluminescence (CL). For histological analysis, the pancreatic tissue samples were fixed in 10% (vol/vol) phosphate buffered.

Measurement of pancreatic malondialdehyde activity and glutathione level

Malondialdehyde levels, used as an indicator of peroxidation on membrane lipids, were determined in pancreas tissues as previously described by Casini *et al.* (27). Glutathione, as a cellular antioxidant, was measured *via* modification of the Ellman procedure (28). The pancreatic samples were homogenized (Ultra Turrax) in 10 volumes of ice-cold 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 mins at 4°C . The supernatant was removed and re-centrifuged at 10000 rpm for 8 min for the subsequent determination of MDA and GSH levels. Lipid peroxide levels were calculated by using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ at 532 nm. Lipid peroxide levels were expressed as nmol MDA/g tissue. GSH levels were calculated by using an extinction coefficient of $1.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 412 nm. Results were expressed as $\mu\text{mol GSH/g tissue}$.

Measurement of pancreatic myeloperoxidase activity

MPO is an enzyme that is found in the azurophilic granules of polymorphonuclear leukocytes (PMNL). Measuring MPO activity is a marker for neutrophil infiltration through the tissue. MPO activity was measured in 0.2–0.3 g pancreatic tissue samples by using a previously described method (20). Pancreatic tissues 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 rpm for 10 min at 4°C , and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mM K_2HPO_4 containing 0.5% (w/v) HETAB and 10 mM ethylenediaminetetraacetic acid. MPO activity was assessed by measuring the H_2O_2 -dependent oxidation of o-dianizidine $\cdot 2\text{HCl}$. One unit (U) of enzyme activity was defined as the amount of MPO present that causes a change in absorbance of 1.0 unit/min at 460 nm and 37°C and was signified in units per g tissue (29).

Measurement of amylase, lipase and trypsinogen-2 levels in sera

Amylase, lipase and trypsinogen-2 levels were measured by ELISA (enzyme linked immunosorbent assay) method. All samples were assayed using the commercial kits (SUNRED rat amilase Elisa kit 201-11-0719, SUNRED rat lipase elisa kit 201-11-5135, ELABSCIENCE rat trypsinogen-2 elisa kit E-EL-R1186), in accordance with the instructions.

Chemiluminescence assay in pancreatic tissue

The chemiluminescence measurements of luminol and lucigenin that representing the reactive oxygen species were determined by a light emission method. The luminol and lucigenin CL levels were measured by using a Junior LB 9509 luminometer (EG & G Berthold Mini Lumat LB 9509, Germany). Pancreatic tissue samples were cut and added into the tubes including HEPES (20 mM; pH 7.2) solution in phosphate buffered saline (PBS; 0.5 M). Following the addition of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridiniumnitrate) probes (final concentration at 0.2 mM) CL levels were tested. While lucigenin probe is sensitive to superoxide anion, luminol probe detects hydroxyl, hypochlorite, peroxyxynitrite, hydrogen peroxide, and peroxy radicals. Luminol (0.2 mM) and lucigenin (0.2 mM) were counted for 5 min at 1 min intervals by the luminometer. The results were expressed as area under curve (AUC) and relative light units (rlu) per mg of pancreatic tissue (30).

Histological analysis

For light microscopic evaluations, pancreatic tissue samples were fixed in 10% phosphate buffered formaldehyde and dehydrated in ascending ethanol series (70%, 90%, 96% and 100%) following cleared in xylene and embedded in paraffin. Samples were evaluated by an experienced histologist who was blind to the study design and the groups. The paraffin-embedded pancreatic tissues were cut at 5 μm thick by a rotary microtome (Leica RM2125RT, Wetzlar, Deuchland), transversally and then were stained with Hematoxylin & Eosin (H&E) and were examined and photographed under an Olympus BX-51 photomicroscope (Olympus BX51 attached with Olympus DP72, Tokyo, Japan). At least 4 sections from each group were histopathologically scored by a modified method from Moreno *et al.* (31). From each sections, five different areas at $\times 200$ magnification were evaluated for histological scoring. The scores

were graded from 0 to 3 (0: none, 1: mild, 2: moderate, 3: severe), according to the following criteria, (a) vascular congestion, (b) interstitial edema, (c) vacuolization in acinar cell (d) inflammatory cell (PMN) infiltration; and (e) damaged Langerhans islets (31).

Statistical analysis

Statistical analysis was done using a Graph Pad Prism statistical package program (Version 6.0) (San Diego, CA, USA). All data were expressed as means $\pm$ SEM. In the comparison of multiple groups, *post hoc* Tukey's multiple test was used after

one-way analysis of variance (ANOVA). Values of two groups were compared by Student's *t* test. Values of $P < 0.05$ were considered as significant.

RESULTS

Activity levels of serum amylase, lipase and trypsinogen-2

Although serum amylase, lipase and trypsinogen-2 activity levels were significantly increased in the AP group compared to

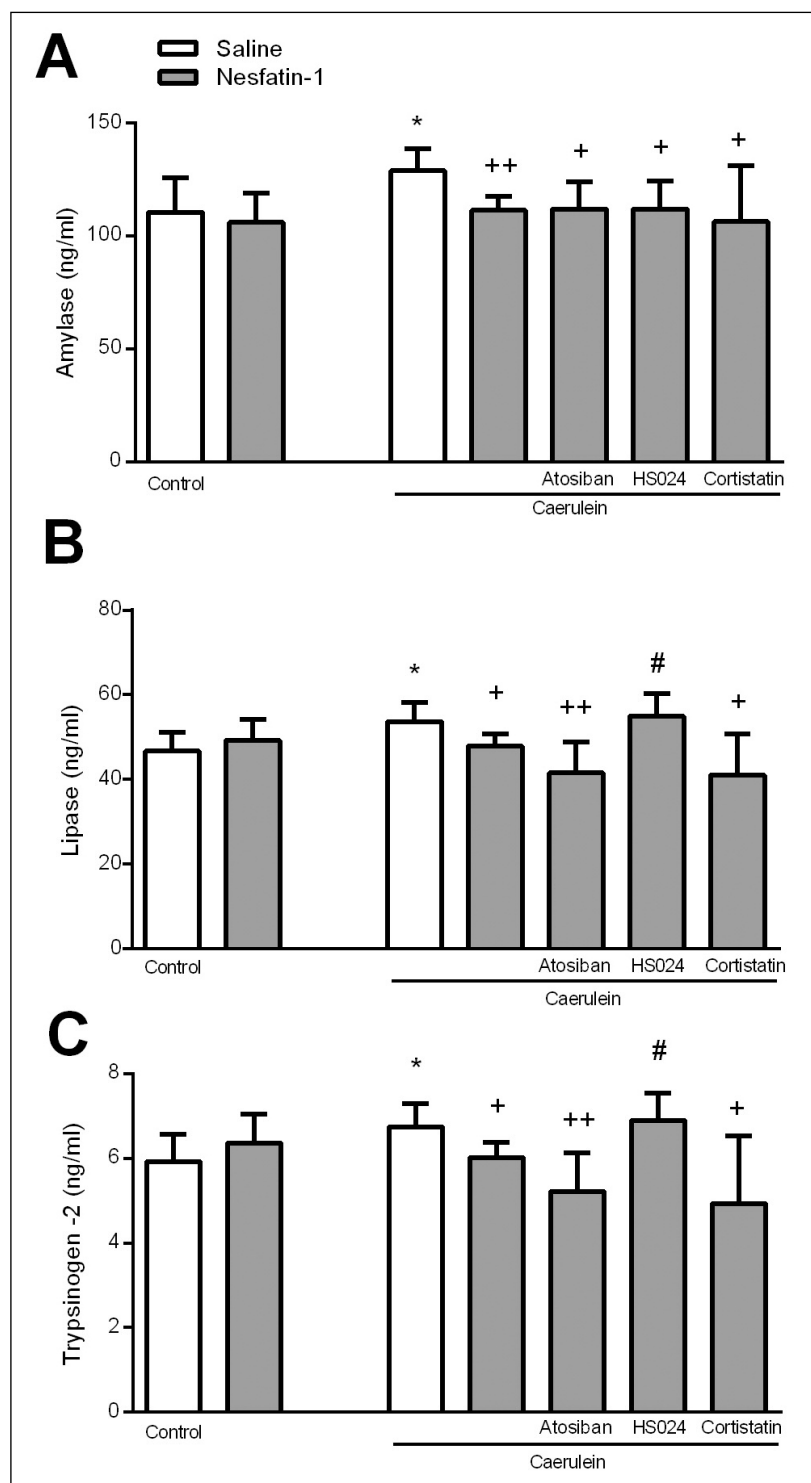


Fig. 3. Amylase (A), Lipase (B), and trypsinogen-2 activity levels in pancreas tissues of saline-treated control rats, nesfatin-1 treated rats and caerulein-induced acute pancreatitis (AP) groups which were saline- or nesfatin-1-treated, and which were atosiban, HS024, or cortistatin pre-treated.

* $P < 0.05$, compared to saline-treated control group; + $P < 0.05$, ++ $P < 0.01$ compared to caerulein-induced acute pancreatitis group; # $P < 0.05$, compared to nesfatin-1-treated acute pancreatitis group.

the control group ($P < 0.05$), they were all decreased significantly back to the control levels in nesfatin-1-treated AP group ($P < 0.05 - 0.01$). Moreover, HS024 pre-treatment increased lipase and trypsinogen-2 activities to the levels of AP group ($P < 0.05$) (Fig. 3).

Myeloperoxidase activity, malondialdehyde and glutathione levels of pancreatic tissue

The MPO activity and MDA levels were increased with AP induction ($P < 0.01 - 0.001$), however nesfatin-1 application

decreased MPO activity in the pancreatic tissue (0.001) (Fig. 4). Atosiban, HS024 and cortistatin pre-treatments lead to higher MPO activities when compared with nesfatin-1 applied acute pancreatitis group ($P < 0.05 - 0.001$). Although there was an insignificant decrease in MDA levels of nesfatin-1 treated AP group ($P = 0.51$), HS024 pre-treatment exacerbated MDA levels when compared to nesfatin-1 treated AP group ($P < 0.05$) (Fig. 4).

The antioxidant GSH levels were significantly suppressed in the AP group compared to the control group ($P < 0.01$), conversely nesfatin-1 application led to an increase in the

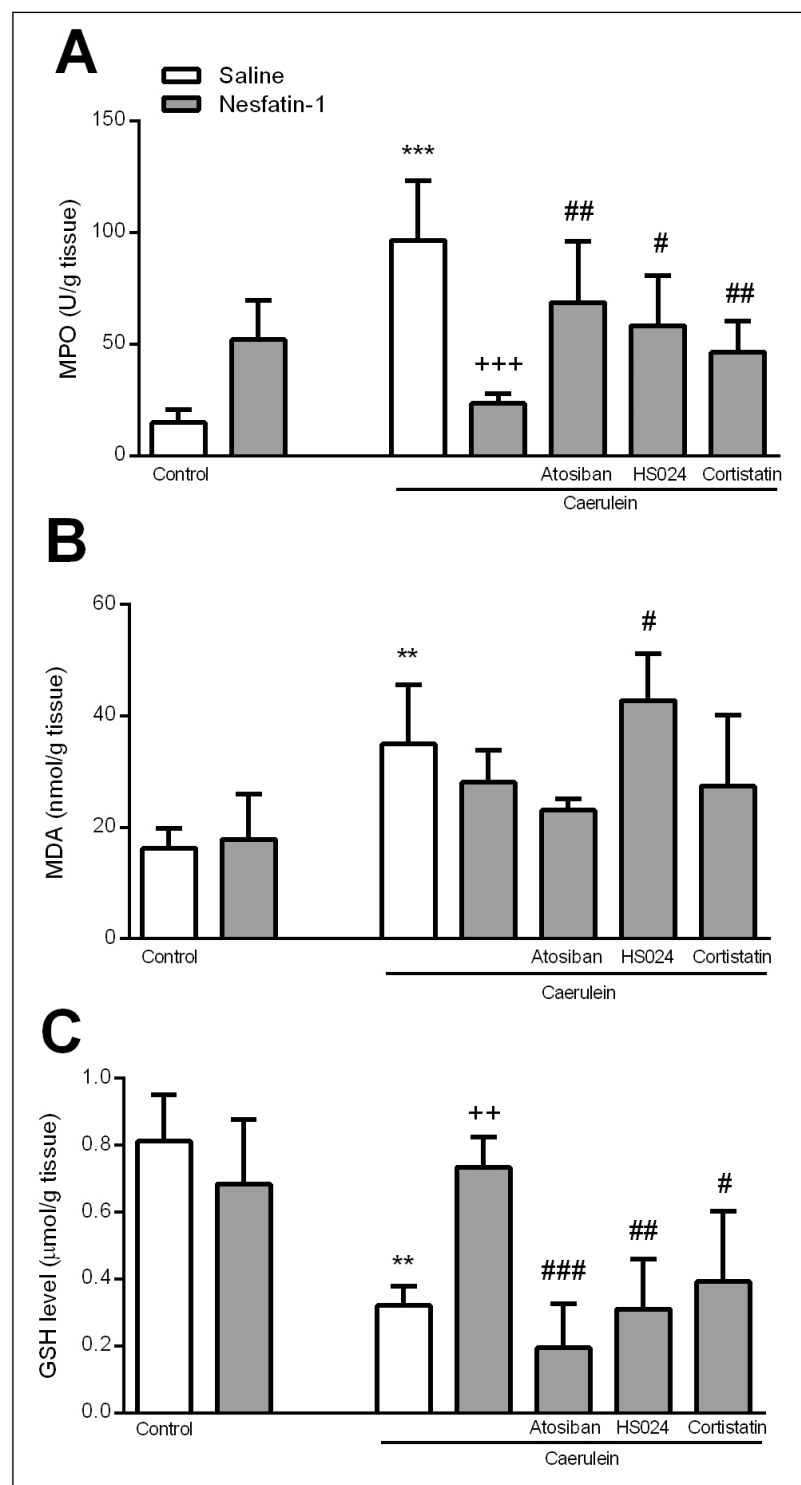


Fig. 4. Myeloperoxidase (MPO) (A), malondialdehyde (MDA) (B) and glutathione (GSH) levels in pancreas tissues of saline-treated control rats, nesfatin-1 treated rats and caerulein-induced acute pancreatitis (AP) groups which were saline- or nesfatin-1-treated, and which were atosiban, HS024, or cortistatin pre-treated.

** $P < 0.01$, *** $P < 0.001$, compared to saline-treated control group; ++ $P < 0.01$, +++ $P < 0.001$, compared to caerulein-induced acute pancreatitis group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to nesfatin-1 treated acute pancreatitis group.

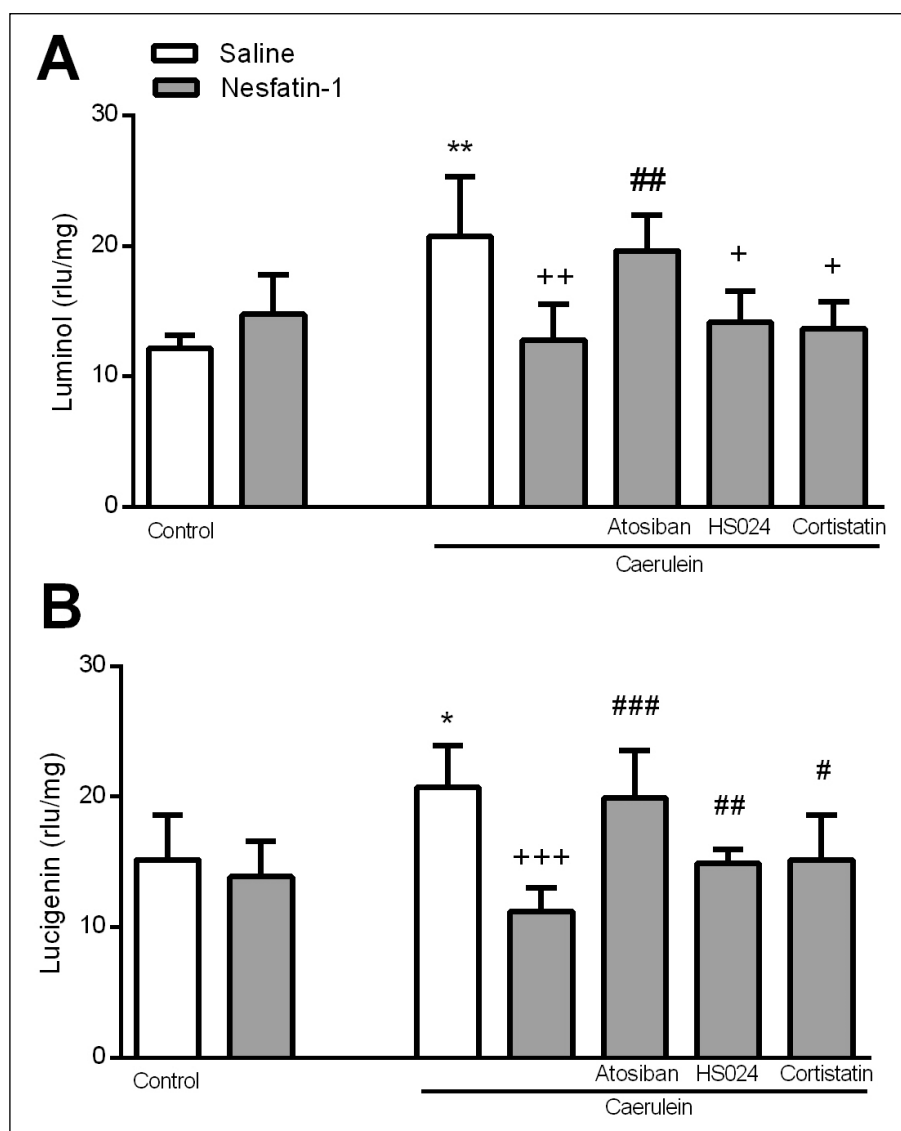


Fig. 5. Luminol (A), and lucigenin chemiluminescence (B) levels in pancreas tissues of saline-treated control rats, nesfatin-1 treated rats and caerulein-induced acute pancreatitis (AP) groups which were saline- or nesfatin-1-treated, and which were atosiban, HS024, or cortistatin pre-treated.

* $P < 0.05$, ** $P < 0.01$ compared to saline-treated control group; + $P < 0.005$, ++ $P < 0.01$ +++ $P < 0.001$ compared to caerulein-induced acute pancreatitis group; # $P < 0.05$, ### $P < 0.01$, ### $P < 0.001$ compared to nesfatin-1-treated acute pancreatitis group.

GSH levels ($P < 0.01$). All of the antagonist pre-treatments repressed the antioxidant GSH levels when compared with nesfatin-1 treated AP group ($P < 0.05 - 0.001$) (Fig. 4).

Lucigenin and luminol chemiluminescence levels

The lucigenin and luminol chemiluminescence levels that indirectly show reactive oxygen species were significantly aggravated in the AP group compared to the control group ($P < 0.05 - 0.001$), however nesfatin-1 administration significantly inhibited this increase ($P < 0.01 - 0.001$). Furthermore, all of the antagonist pre-treatments amplified the lucigenin chemiluminescence levels ($P < 0.05 - 0.001$). Following atosiban pre-treatment, the luminol chemiluminescence levels were significantly higher than the levels of the nesfatin-1 treated AP group ($P < 0.01$) (Fig. 5).

Histopathological alterations in rat pancreas tissue

In control group, well-organized acini and Langerhans islets were seen in parenchyme with regular morphology. In nesfatin-1 treated group without AP induction, the histological morphology was similar to control group. In saline-treated acute

pancreatitis group, acinar cells show severe cytoplasmic degeneration, additionally, vacuolization, vascular congestion, degenerative Langerhans islets and inflammatory cell infiltration were observed. In nesfatin-1 treated AP group, the findings that were represented by saline-treated acute pancreatitis group were decreased and the pancreatic tissues were shown to have a control-like appearance. In all antagonist pre-treated groups, the histologic findings were not significantly different than nesfatin-1 treated AP group (Fig. 6).

In the AP group, pancreatic tissue damage scores were significantly increased compared to the control group ($P < 0.001$), however nesfatin-1 application remarkably reduced tissue damage scores ($P < 0.001$). Atosiban, HS024 and cortistatin pre-treatments did not cause a significant change in the effect of nesfatin-1 statistically in terms of histological scoring (Fig. 7).

DISCUSSION

The present study aimed to investigate the possible protective effects of nesfatin-1 on an acute pancreatitis rat model that was induced by caerulein administration and to examine the

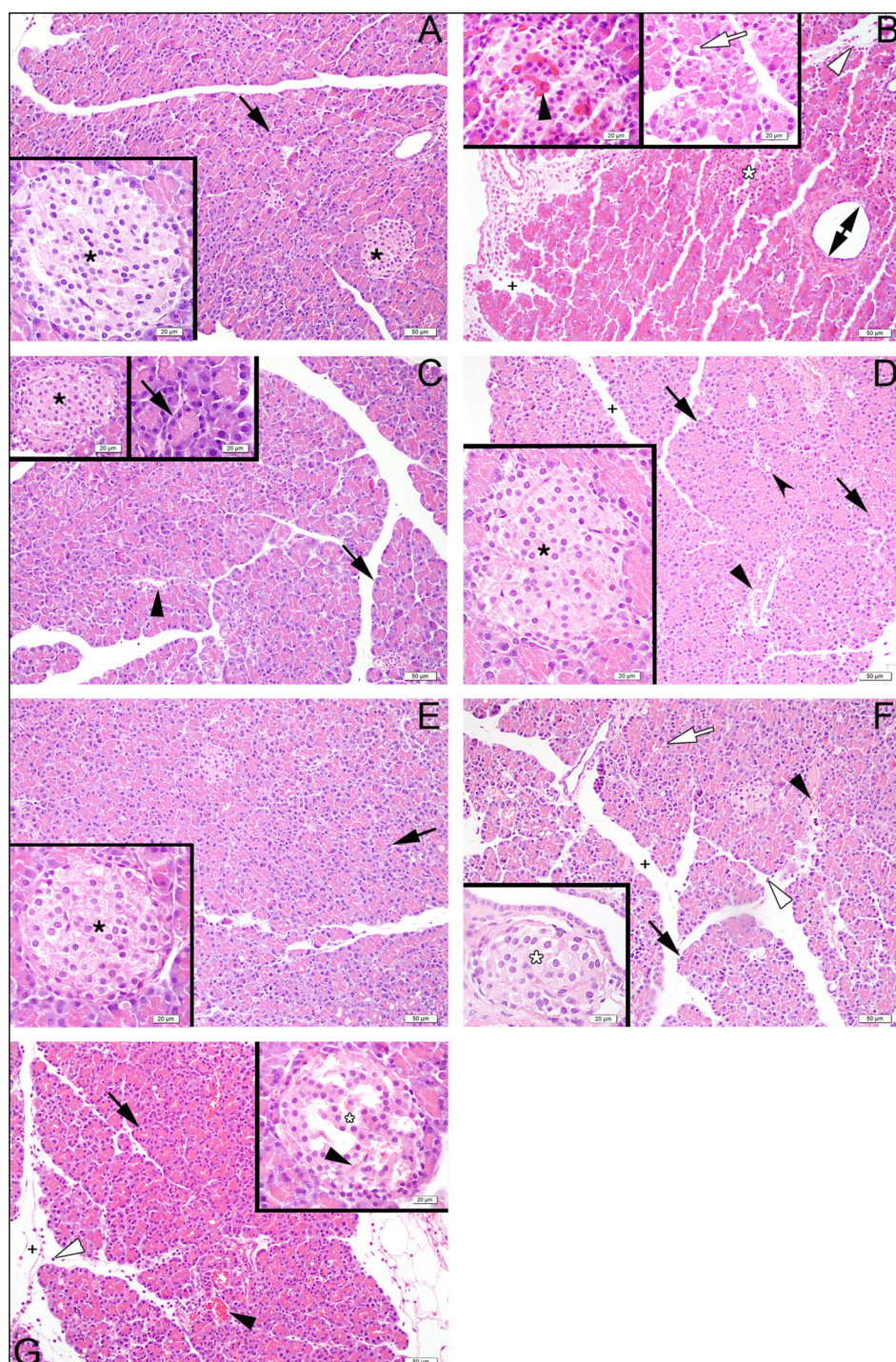


Fig. 6. Representative micrographs of saline-treated control (A), nesfatin-1-treated (B), saline-treated acute pancreatitis (C), nesfatin-1-treated acute pancreatitis (D), HS024 pre-treated and nesfatin-1-treated acute pancreatitis (E), atosiban pre-treated and nesfatin-1-treated acute pancreatitis (F), cortistatin pre-treated and nesfatin-1-treated acute pancreatitis (G) groups. Arrow: acinus with regular morphology. Asterisk: Langerhans islet with normal appearance. Arrowhead: vascular congestion. Notched arrowhead: duct with regular morphology. Plus (+): interstitial edema. Haematoxylin and eosin staining.

probable underlying mechanisms *via* using antagonists of melanocortin, ghrelin and oxytocin receptors, that are thought to mediate the effects of nesfatin-1. These findings reveal that exacerbated inflammatory process and increased oxidative damage with acute pancreatitis induction was suppressed *via* nesfatin-1 administration, moreover melanocortin receptor antagonist pre-treatment reversed the effect of nesfatin-1 by aggravating neutrophil infiltration, increasing lipid peroxidation, elevating lucigenin activities, reducing GSH content, and rising lipase and trypsinogen-2 activity levels.

The pathogenesis of acute pancreatitis has not been fully elucidated, it is an inflammatory disease involving different mediators. Especially in severe acute pancreatitis, experimental

studies are more important than clinical studies to understand the mechanism of this complex disease and to try new pharmacological agents for treatment.

According to aforementioned evidence, serum amylase, lipase and trypsinogen-2 activity levels are used as biochemical parameters to show occurrence of acute pancreatitis (32, 33). These enzyme levels are known to be increased following the digestive process and the cellular damage in acute pancreatitis (34). Similar with the previous data, in the present study, the increased amylase, lipase and trypsinogen-2 activity levels were determined with acute pancreatitis induction. There is no data regarding the effect of nesfatin-1 on acute pancreatitis, therefore this is the first study to show the suppressing effect of nesfatin-

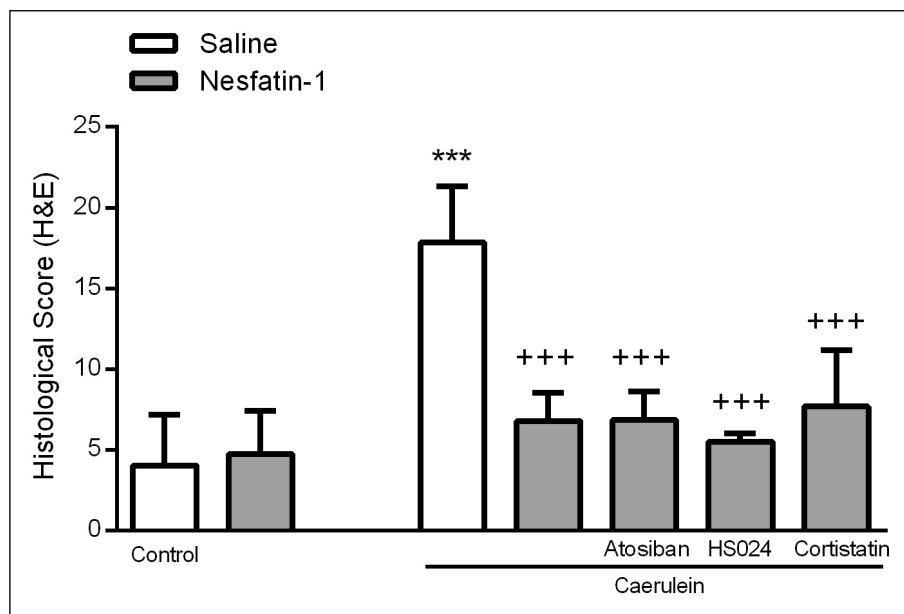


Fig. 7. Histological damage scores of pancreas tissues of saline-treated control rats, nesfatin-1 treated rats and caerulein-induced acute pancreatitis (AP) groups which were saline- or nesfatin-1-treated, and which were atosiban, HS024, or cortistatin pre-treated. *** $P < 0.001$, compared to saline-treated control group; +++ $P < 0.001$, compared to caerulein-induced acute pancreatitis group.

1 on these biochemical enzyme activity levels and these results suggest its possible protective role on cellular damage which is prevalent in acute pancreatitis. Our histological findings also support the defensive role of nesfatin-1 on the cellular damage that is prevalent in AP. Atosiban and cortistatin pre-treatments did not cause a significant alteration in the effect of nesfatin-1 on enzyme activity levels, however HS024 pre-treatment reversed its effect on lipase and trypsinogen-2 activities, suggesting the melanocortin receptors as an underlying mechanism. Although HS024 pre-treatment affected those biochemical parameters, it did not change the histological scores. According to our experimental design, the rats were sacrificed 12 hours after last caerulein injection. The histological changes may take longer time.

According to previous data, pancreatic endothelial cell damage and edema formation goes on with the polymorphonuclear leucocyte (PMNL) infiltration in acute pancreatitis (35). In this injury, besides the direct damaging effects of free oxygen radicals on tissues, it is well-known that they induce the attraction, adhesion and migration of PMNLs through the pancreatic tissues (36). PMNLs worsen the tissue damage by activating cytotoxic enzymes, including myeloperoxidase, proteases, elastase, and lactoferrin (20, 37). Therefore, in the present study MPO activity levels were evaluated as a marker of tissue neutrophil infiltration. As expected, the elevated MPO activity levels were observed with acute pancreatitis induction. This result was parallel with the previous studies that investigated the PMNL infiltration in the pancreatic tissue or the far organs that are affected from the injury in AP (21, 38). Previously, nesfatin-1 was reported to reduce inflammation and decrease the MPO activity in different inflammatory models (13, 39, 40), however this is the first study that shows its inhibiting effect on MPO activity levels in an AP protocol. The present results illustrate that nesfatin-1 attenuates PMNL infiltration through tissue. Moreover, pre-treatment with receptor antagonists inhibited the effect of nesfatin-1 on MPO levels, leading to an increase in its activity. This result suggest that nesfatin-1 may use these receptors in its PMNL suppressing role, as ghrelin and nucleobindin-2/nesfatin-1 co-localization in gastric tissue while directing its regulatory effects on food intake and the interaction of nesfatin-1 with ghrelin and oxytocin

receptors in its anti-inflammatory effects on a colitis model was reported (13, 41). This the first study to show a possible interaction with nesfatin-1 and melanocortin receptors while inhibiting PMNLs.

Antioxidants contribute to protecting cells against toxic effects of inflammation and preventing diseases by reducing and inactivating free radicals, the toxic by-products of normal cell metabolism. Glutathione acts as an antioxidant and has protective roles by neutralizing free radicals that have variant harmful effects on cellular functions. Previous studies showed depleted GSH levels with acute pancreatitis induction *via* different models, such as pancreaticobiliary duct ligation or caerulein application, in pancreatic tissue and also in distant organs (20, 21). Consistent with those reports, in our study, glutathione levels were significantly reduced with acute pancreatitis, in addition to previous evidence, nesfatin-1 administration significantly improved glutathione levels. In those previous studies, different peptides were used as an anti-inflammatory agent, although improved GSH levels were reported in distant organs, such as lung and kidney, which were affected from acute pancreatitis, the endogenous antioxidant GSH levels were consumed and were not improved by treatments in pancreatic tissue. The severity of inflammation which was developed in the pancreatic tissue was addressed as a reason. However, in the present study, nesfatin-1 administration enhanced GSH levels of pancreatic tissue, supporting the findings that nesfatin-1 may have an antioxidant role in preventing the severe tissue damage mediated by acute pancreatitis. Moreover, atosiban, cortistatin and HS024, inhibited the effect of nesfatin-1, resulting in a reduction in glutathione levels. This result suggests that all receptor antagonists may inhibit the protective effect of nesfatin-1 on antioxidant GSH content.

Oxidative stress is caused by disruption of the balance between free radical production and antioxidant defense. Malondialdehyde, a lipid peroxidation product, has negative consequences by changing ion permeability of cell membrane and altering enzyme activities. As we have shown previously, current data confirm the exaggerated lipid peroxidation levels in pancreatic tissue with induction of acute pancreatitis (20, 21). In those studies, we used a pancreaticobiliary duct ligation model.

In addition to these previous data, increased MDA levels were also reported in different acute pancreatitis models such as taurocholate and caerulein induced models (18, 33). Not only our previous data but also the other outcomes support the aggravated lipid peroxidation levels in acute pancreatitis regardless from the reason or the model used for induction. As mentioned above, increased levels of neutrophil enzyme activity indicate activation of neutrophils, and might lead to the generation of reactive oxygen series that will stimulate lipid peroxidation. Together with the risen MPO activity levels, the increased MDA levels underlie the oxidant damage that goes on with acute pancreatitis.

Previously, the inhibiting effects of nesfatin-1 on increased MDA levels that were observed in different inflammation models other than pancreatitis were reported (13, 40). In the present study, although nesfatin-1 administration protected the pancreatic tissue from neutrophil infiltration, suppressed the production of reactive oxygen series, and improved the antioxidant GSH content, showed only a tendency to suppress the increased MDA levels. The severe inflammation that was observed in the pancreatic tissue may be the reason for this result. Furthermore, according to our data, the MDA levels were augmented with HS024 pre-treatment when compared with the levels of nesfatin-1 treated group. In a previous study of our group, not only a melanocortin receptor antagonist, but also ghrelin and oxytocin receptor antagonists augmented the tissue MDA levels when applied before nesfatin-1 in a colitis model (13). Together with the previous data, these consequences suggest mainly role of melanocortin pathway on lipid peroxidation that goes on in pancreatic tissue.

Systemic inflammatory response and oxidative stress play a major role in pathophysiological events in the development of acute pancreatitis. O_2 radicals and other reactive O_2 derivatives have been implicated in the pathogenesis of acute pancreatitis (37). Luminol is mainly sensitive to hydrogen peroxide, hydroxyl, hypochlorite, peroxynitrite and lipid peroxide radicals, whereas lucigenin is selective to superoxide radical. Consistent with literature, in the present study, increased luminol and lucigenin levels due to caerulein induced acute pancreatitis were demonstrated (42, 43). Similar with our results, in gastric injury and acute colitis models, nesfatin-1 has been shown to reduce luminol and lucigenin chemiluminescence levels (13, 40), therefore it was supposed to suppress reactive oxygen species production. The pre-treatment with atosiban, an oxytocin receptor antagonist, reversed this decrease in luminol chemiluminescence. This result suggests that the effect of nesfatin-1 on luminol levels might be mediated by oxytocin receptors. Furthermore, regarding lucigenin chemiluminescence levels, nesfatin-1 had a suppressive role that might involve all these receptors, thus the antagonist pre-treatments increased the chemiluminescence levels not only in our study but also in that colitis study (13). As lucigenin is more specific to superoxide radical, nesfatin-1 may display its superoxide reducing effect *via* all these antagonists especially atosiban or by interfering with other pathways.

Eventually, our findings indicate that intraperitoneal nesfatin-1 treatment prevents or ameliorates the oxidative damage in caerulein induced acute pancreatitis. According to our results the oxidative damage that was induced *via* acute pancreatitis and the underlying mechanisms appear to involve neutrophil migration, lipid peroxidation, reactive oxygen species generation, and depletion of antioxidant GSH. Furthermore, our results suggest for the first time that nesfatin-1 may have anti-oxidant and anti-inflammatory effects on acute pancreatitis by its neutrophil infiltration suppressing role, and by its free radical scavenging efficacy. Nesfatin-1 was reported to have healing effects on acetic-acid induced gastric ulceration and colitis models (13, 40). Taken together with our results, these data

underlie the anti-inflammatory role of nesfatin-1 in the gastrointestinal tract. Although the melanocortin signaling pathway is well-known to direct the effect of nesfatin-1 on food intake, there is only one study that reported this pathway for the anti-inflammatory effects of nesfatin-1 on an inflammation model (13). Consequently, the present study suggests mainly melanocortin signaling pathway for the protective anti-oxidant and anti-inflammatory effects of nesfatin-1 on acute pancreatitis.

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