

Nesfatin-1 alleviates extrahepatic cholestatic damage of liver in rats

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ABSTRACT

Obstructive jaundice (OJ) can be defined as cessation of bile flow into the small intestine due to benign or malignant changes. Nesfatin-1, recently discovered anorexigenic peptide derived from nucleobindin-2 in hypothalamic nuclei, was shown to have anti-inflammatory and antiapoptotic effects. This study is aimed to investigate the therapeutic effects of nesfatin-1 on OJ in rats. Twenty-four adult male Wistar-Hannover rats were randomly assigned to three groups: sham (n = 8), control (n = 8), and nesfatin (n = 8). After bile duct ligation, the study groups were treated with saline or nesfatin-1, for 10 days. Afterward, blood and liver tissue samples were obtained for biochemical analyses, measurement of cytokines, determination of the oxidative DNA damage, DNA fragmentation, and histopathologic analyses. Alanine aminotransferase and gamma-glutamyl transferase levels were decreased after the nesfatin treatment; however, these drops were statistically non-significant compared to control group ($p = 0.345$, $p = 0.114$). Malondialdehyde levels decreased significantly in nesfatin group compared to control group ($p = 0.032$). Decreases in interleukin-6 and tumor necrosis factor- α levels from the liver tissue samples were not statistically significant in nesfatin group compared to control group. The level of oxidative DNA damage was lower in nesfatin group, however this result was not statistically significant ($p = 0.75$). DNA fragmentation results of all groups were similar. Histopathological examination revealed that there was less neutrophil infiltration, edema, bile duct proliferation, hepatocyte necrosis, basement membrane damage, and parenchymal necrosis in nesfatin compared to control group. The nesfatin-1 treatment could alleviate cholestatic liver damage caused by OJ due to its anti-inflammatory and antioxidant effects.

KEY WORDS: Obstructive jaundice; oxidative stress; hepatic damage; cholestasis; DNA fragmentation

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INTRODUCTION

Cholestasis is defined as a reduction of bile flow leading to the accumulation of bile acids and other toxic compounds within the liver, which induce progression to hepatocellular injury and fibrosis [1]. Obstructive jaundice (OJ) is caused by a mechanical obstruction of the extrahepatic or intrahepatic bile ducts due to stones, tumor, inflammation, or other causes [2]. This obstruction results in hepatic damage induced by reactive oxygen species (ROS). In the acute phase, bile duct obstruction is characterized by increased lipid peroxidation as well as by a marked decrease of glutathione (GSH), which is a major antioxidant in cells [3,4]. Excessive ROS generated

during cholestasis can cause oxidative damage to hepatocytes and the biliary tract, so consumption of antioxidants can have beneficial effects on cholestatic liver injury. Based on this assumption, a number of antioxidants have been studied in experimental bile duct obstruction models [5-7].

Nesfatin-1 is a novel 82-amino acid anorexigenic peptide derived from the precursor peptide nucleobindin-2 (NUCB2) in hypothalamic nuclei. It is widely distributed in the central nervous system and also in peripheral tissues such as pancreas, adipose tissue, duodenum, and stomach [8,9]. Recent studies have shown that it has antioxidant and antiapoptotic properties [10,11]. It exerts its anti-inflammatory effects via the maintenance of the intracellular antioxidant pools [12].

We aimed to investigate the potential healing effects of nesfatin-1 on hepatic oxidative stress that occurs during cholestasis.

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MATERIALS AND METHODS

Animals

Twenty-four male Wistar-Hannover rats (300-400 g), obtained from Bağcilar Training and Research Hospital Animal Center, were housed in cages (one per cage) under controlled room temperature ($21 \pm 2^\circ\text{C}$), humidity (60-70%) with 12 hours light-dark schedule and were fed with standard pellet, *ad libitum*. All experimental procedures were approved by the Bağcilar Training and Research Hospital Animal Care and Use Committee (2015/90).

Animal grouping and induction of OJ

The rats were randomly assigned to three groups: Sham ($n = 8$), control ($n = 8$), and nesfatin ($n = 8$). Surgical OJ was achieved in the control and nesfatin groups. The rats were anesthetized by isoflurane (5% for the induction and 2% for the maintenance, Isoflurane®; Baxter, Puerto Rico, USA). Under aseptic conditions, 3-cm midline abdominal incision was performed. In the sham group, only laparotomy and common bile duct (CBD) exploration were conducted. After laparotomy, dissociation of CBD along the hepatoduodenal ligament was performed in the other groups. In the control and nesfatin groups, the proximal end of the CBD was double ligated above the pancreas just beneath the liver with surgical threads (4/0 silk sutures, Dogsan, Trabzon, Turkey), CBD was cut off, and abdominal cavity was closed with separated 2/0 silk sutures (Dogsan, Trabzon, Turkey) (Figure 1).

The study groups were treated with 1 ml/kg/day of saline (control group) or 10 µg/kg/day of nesfatin-1 (nesfatin group) for 10 days. Following the operation, intraperitoneal acetaminophen (Fentanyl Citrate®, Abbott, IL, USA; 0.1 mg/kg) was injected for analgesia. After recovery from anesthesia, rats were housed individually in disinfected cages for 10 days. The dose of nesfatin-1 was increased according to the previous studies [13,14].

Biochemical parameters

On the 10th day of the experiment, all rats were euthanized under anesthesia, and blood was collected by cardiac puncture,

centrifuged, and stored at -80°C for the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, and albumin. Liver function tests and protein levels of clear supernatants were studied on a Cobas® 6000 (Roche Diagnostics, Tokyo, Japan) analyzer.

Measurement of serum malondialdehyde (MDA), GSH peroxidase (GPx), and superoxide dismutase (SOD) levels

Blood samples were centrifuged at 4000 g for 10 minutes. Measurement of serum MDA, GPx, SOD, interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) levels were analyzed with micro-enzyme-linked immunosorbent assay (ELISA) method. IL-6 and TNF- α levels of supernatants were determined with eBioscience ELISA® (San Diego, USA) kits. GPx, MDA, and SOD levels of both, supernatants and serum samples, were determined with Sunred ELISA kits (Shanghai, China).

Measurement of tissue IL-6 and TNF- α levels

Postmortem livers were taken and stored in an ice bath until homogenization. The tissues were homogenized in serum physiologic solution, then centrifuged in an ultracentrifuge at 14,000 g for 10 minutes. Upper clear supernatants were used in the assays. All the procedures were performed at 4°C throughout the experiment.

The liver tissues were kept in serum physiologic solution and homogenized with phosphate buffer at 1/20 (w/v) percent after weighing. Thereafter, the tissues were homogenized with phosphate-buffered saline (pH 7.2) in a Velp® Scientifica vortex (Usmate Velate, Italy). The homogenates were centrifuged at 14,000 g for 10 minutes. The protein content of homogenates was measured at Cobas® 6000 (Tokyo, Japan) analyzer by turbidimetric method. IL-6 and TNF- α measurements were performed by micro-ELISA method.

Oxidative DNA damage detection

Freshly excised liver samples from all groups were directly placed on ice at -20° to freeze, and then stored at -80° until

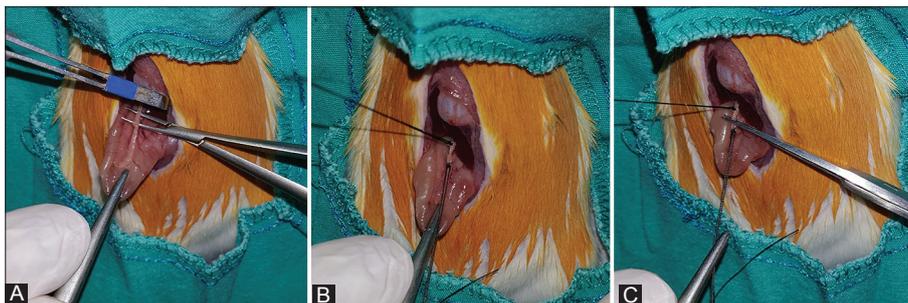


FIGURE 1. Illustration of obstructive jaundice model performed in the study. Dissection and dissociation of common bile duct along the hepatoduodenal ligament (A), double ligation of the common bile duct above the pancreas just beneath the liver with surgical threads (B), and finally common bile duct was cut off (C).

oxidative DNA damage detection with ELISA and DNA fragmentation analysis.

Isolation of DNA and 8-hydroxy-2'-deoxyguanosine (8-OHdG) identification by using SunRed Rat (8-OHdG) ELISA kit

The levels of 8-deoxyguanosine were determined with standard ELISA after DNA isolation, using PureLink Genomic DNA Kit (Invitrogen™, Life Technologies, USA). Briefly, the samples were homogenized in the presence of digestion buffer and proteinase K. After being treated with ribonuclease (RNase), the nucleic acid was concentrated with absolute ethanol and bound to colons before being eluted and preserved at -20° until ELISA analysis.

According to the instructions, 50 µL of standards (0.75, 1.5, 3, 6, and 12 ng/mL) and 40 µL of sample DNA were incubated at 37° for 1 hour in the 96-well ELISA plate ready to use with capture antibody immobilized in the wells, with 10 µL of 8-OHdG antibody and 50 µL of streptavidin-horseradish peroxidase. After serial washings and stopping the reaction, absorption was measured at 450 nm in ELISA reader.

DNA fragmentation

The tissue samples kept at -80° were cut on ice to obtain tissues of 125-150 mg, which were homogenized in ×10 (v/w) lysis buffer of 1 M Tris, 0.5 M ethylenediaminetetraacetic acid (EDTA), and 0.5% t-octylphenoxypolyethoxyethanol (Triton X-100). After centrifugation at 25,000 g for 30 minutes, the supernatant was separated, and the pellet was resuspended in Tris/EDTA 10 mM:1 mM buffer. The samples were then studied according to modified version of diphenylamine protocol by Burton [15,16]. Briefly, 25% trichloroacetic acid (TCA) was used to precipitate nucleic acids overnight, which were then treated in a hot bath with 5% TCA to dissolve DNA. The solution was then incubated with diphenylamine dissolved in acetic and sulfuric acids at 37° for 4 hours. The absorption of the color reaction product was measured at 595 nm.

Histological preparation and analysis

Excised liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Four-micron-thick paraffin sections were obtained and stained with hematoxylin and eosin (H&E), Masson's trichrome, and periodic acid-Schiff (PAS) dyes. Masson's trichrome stain is among the most common special stains applied to liver specimens. The stain imparts a blue color to collagen against a red background of hepatocytes and other structures. It is used for staging of chronic liver diseases and helps to delineate patterns of injury. In addition, Masson's trichrome stain is very useful in evaluating portal tract structures (e.g., bile

duct structures, arteries, and veins) in inflamed liver. The PAS stain is useful for identifying glycogen, but removing glycogen with diastase digestion enhances the detection of non-digested material, including debris within macrophages. The PAS stain is helpful for the demonstration of bile duct basement membrane injury.

Statistical analysis

Statistical analysis was performed using the Number Cruncher Statistical System 2007 Statistical Software (Utah, USA). Besides descriptive statistical methods (mean and standard deviation), the Kruskal-Wallis test was used for the comparison of groups and Dunn's multiple comparison test was used for subgroup analysis. All *p* < 0.05 were accepted as statistically significant.

RESULTS

Liver function tests

Liver function tests of the control and nesfatin groups were slightly increased after bile duct ligation (BDL) and were significantly higher compared to the sham group, which indicated that BDL was successfully performed in both groups. The AST, ALP, GGT, total bilirubin, direct bilirubin, LDH, and albumin values of the nesfatin group were not significantly different compared to the control group (Tables 1 and 2). The ALT levels in the nesfatin group were lower compared to the control group; however, this result was not statistically significant (*p* = 0.345).

TABLE 1. Values of biochemical parameters in serum of sham, control, and nesfatin groups after bile duct ligation and 10 days of the nesfatin-1 treatment in the nesfatin group

Parameters	Sham (n=5)	Control (n=8)	Nesfatin (n=8)	<i>p</i>
AST (U/L)	101.6±7.8	309.1±104.3	356.01±228	0.004
ALT (U/L)	54.6±6.06	104.8±25.85	96.5±47.5	0.008
ALP (U/L)	298.6±53.5	573.6±177.5	573.7±140	0.006
GGT (U/L)	1.02±0.1	26.75±10.9	19.63±10.2	0.002
T. Bilirubin (mg/dL)	0.06±0.01	6.01±0.47	6±1.65	0.003
D. Bilirubin (mg/dL)	0.05±0.01	5.8±0.27	5.9±1.72	0.002
Albumin (g/dL)	4.3±0.12	3.6±0.27	3.6±0.39	0.004

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; T. Bilirubin: Total bilirubin; D. Bilirubin: Direct bilirubin; BDL: Bile duct ligation

TABLE 2. Comparison of liver function tests by Dunn's multiple comparison test

Groups	AST	ALT	ALP	GGT	Total bilirubin	Direct bilirubin	Albumin
Sham/control	0.003	0.003	0.005	0.002	0.003	0.002	0.003
Sham/nesfatin	0.003	0.019	0.003	0.004	0.002	0.003	0.003
Control/nesfatin	0.834	0.345	0.916	0.114	0.074	0.066	0.601

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase

Serum and tissue cytokine levels

The GPx levels of nesfatin group were higher compared to the control group; however, this result was not statistically significant ($p = 0.359$). The nesfatin group had significantly lower levels of MDA than the sham and control groups ($p = 0.031$). The SOD levels of nesfatin group were significantly higher compared to the sham group ($p = 0.019$). The serum oxidative stress parameter results are summarized in Figure 2. The TNF- α levels of the liver tissue of nesfatin group were lower than in the sham and control groups, but this result was not statistically significant. The IL-6 levels of the liver tissue were decreased after the nesfatin-1 treatment, and the results were similar to those obtained in the sham group (Figure 3).

Oxidative DNA damage results

Oxidative DNA damage emerged due to the cholestatic damage was measured by 8-OHdG identification with the ELISA method. The nesfatin-1 treatment caused a reduction of DNA damage after 10 days of biliary obstruction (Figure 4), but this result was not statistically significant.

DNA fragmentation results

DNA fragmentation ratios were similar between the sham, nesfatin, and control groups, with averages of 2.9% (± 0.35), 2.4% (1.13), and 2.3% (0.95), respectively.

Histopathological analysis results

Histopathological examination was performed by three staining techniques: H&E, Masson's trichrome, and PAS dyes. In the histopathological evaluation, there were no pathological changes in the liver tissue of the sham group. H&E staining revealed an edema in the portal area, neutrophil infiltration, and marked bile duct proliferation. The nesfatin-1 treatment significantly reduced these changes. Similarly, there was less hepatocyte necrosis, basement membrane damage, and parenchymal necrosis revealed by PAS staining in the nesfatin group compared to the control group. Masson's trichrome staining showed that there was less edema, inflammation, and ductal proliferation in the nesfatin group compared to the control group (Figure 5).

DISCUSSION

Cholestasis appears when the bile drainage to intestine is impaired due to benign or malignant conditions. This causes bile regurgitation to the blood with the consequent retention of bile constituents in the liver [17]. After cessation of bile flow, dilatation of extrahepatic and intrahepatic bile ducts and

alteration of portal tracts occur in a few days. Consequently, accumulation of toxic hydrophobic bile salts within hepatocytes may cause hepatocyte toxicity, liver failure, immunodeficiency, coagulation disorders, renal failure, impaired wound healing, and sepsis [18-20]. The mechanism of hepatocyte injury in cholestasis is believed to be due to inflammatory response of pro-inflammatory mediators created by inflammagen bile acids [21]. This response is augmented by an increased production of IL-6 and TNF- α [22].

Nesfatin-1 is a recently discovered 82-amino-acid satiety peptide that reduces food intake when injected intracerebroventricularly or peripherally in rats in a dose-dependent manner [10]. It is derived from post-translational processing of the nucleobindin 2 (*NUCB2*) gene that is expressed in the hypothalamic nuclei in rats [23,24]. Nesfatin-1 is also expressed in

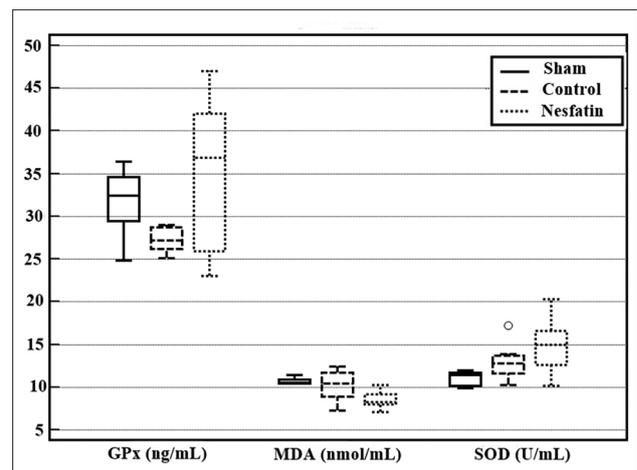


FIGURE 2. Serum glutathione peroxidase (GPx), malondialdehyde, (MDA) and superoxide dismutase (SOD) levels of sham, control, and nesfatin groups. After bile duct ligation and 10 days of the nesfatin-1 treatment in the nesfatin group, nesfatin-1 caused significant reduction of MDA levels as well as an increase of GPx and SOD levels.

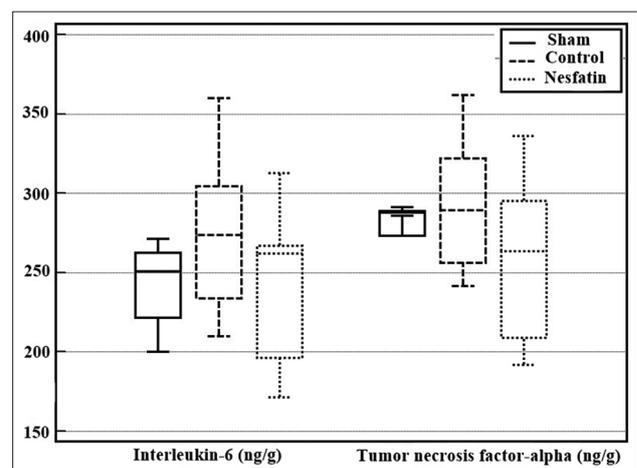


FIGURE 3. Tissue interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) levels of sham, control, and nesfatin groups. After bile duct ligation and 10 days of the nesfatin-1 treatment in the nesfatin group, nesfatin-1 caused a decrease of IL-6 and TNF- α levels.

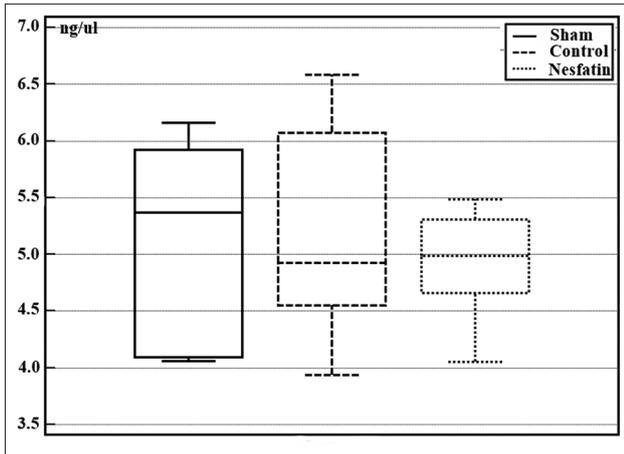


FIGURE 4. Oxidative DNA damage results measured by 8-hydroxy-2'-deoxyguanosine identification with the enzyme-linked immunosorbent assay method, in sham, control, and nesfatin groups. After bile duct ligation and 10 days of the nesfatin-1 treatment in the nesfatin group, nesfatin-1 decreased the oxidative DNA damage of the liver tissue.

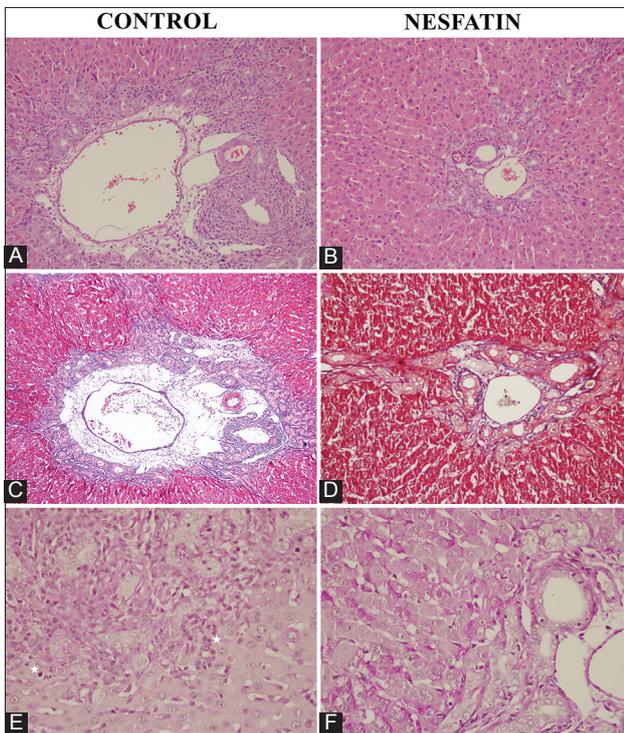


FIGURE 5. (A) Edema, neutrophil infiltration, and marked bile duct proliferation can be observed (H&E, $\times 200$); (B) Nesfatin treatment reduced these changes (H&E, $\times 200$). (D) Similarly, there is less edema, inflammation, ductal proliferation, and dilatation of portal vein (Masson's trichrome, $\times 100$); compared to (C) the control group (Masson's trichrome $\times 200$). (E) Significant hepatocyte necrosis (white stars), parenchymal, and basement membrane damage can be observed (Periodic acid-Schiff [PAS], $\times 200$); (F) Nesfatin treatment caused regression of these changes (PAS, $\times 200$).

extrahypothalamic tissues such as gastrointestinal tissue (gastric oxyntic mucosa, pancreatic beta cells, and liver), skeletal muscle, and adipose tissue [25-28].

Previous studies with nesfatin-1 revealed its anti-inflammatory, antiapoptotic, and gastroprotective effects [12,20,25].

Özsavcı et al. [11] demonstrated its anti-inflammatory feature by inhibiting the neutrophil infiltration in brain damage model in rats. Tang et al. [13] also used nesfatin-1 in brain damage related to subarachnoid hemorrhage model in rat, and they also showed suppression of inflammation with a dose of 20 $\mu\text{g}/\text{kg}$. Inhibition of neutrophil infiltration by decreasing the formation of free oxygen radicals with the use of nesfatin-1 was demonstrated in rats [29]. Dong et al. [14] used nesfatin-1 in diabetic rats and the authors claimed that fatty acid oxidation is stimulated by the use of 10 $\mu\text{g}/\text{kg}$ of nesfatin in mice. Similarly, we showed anti-inflammatory feature of nesfatin-1 in wound healing model in diabetic and non-diabetic rats. We found that nesfatin-1 improves wound healing by suppressing the neutrophil recruitment [25].

In this study, the effects of nesfatin-1 on cholestatic hepatic damage were investigated. For this purpose, treatment with saline or nesfatin-1 was continued for 10 days after BDL in rats. Afterward, liver function tests and cytokine levels were measured. Oxidative DNA damage was evaluated by 8-OHdG ELISA kit, DNA fragmentation was interpreted by spectrophotometric measurement, and finally histopathologic analyses were performed with H&E, Masson's trichrome, and PAS dyes.

In our study, the liver function test results decreased non-significantly in the nesfatin group compared to the control group. This may be due to the short follow-up time of the treatment after BDL.

MDA is a product of lipid peroxidation in the cells. Among the major factors that give rise to hepatic damage in cholestasis are increased levels of lipid peroxidation products and the depletion of cellular antioxidants such as GSH and SOD [6,30-33]. It has been demonstrated that the accumulation of lipid peroxides in tissues is correlated with the impairment of antioxidant defense systems and leads to cellular necrosis [31,32]. The ligation of CBD caused an increase of MDA and decrease of GPx in our study, which is similar to the results reported in the literature [33-35]. However, the nesfatin-1 treatment induced the decrease of MDA and increase of GPx. Previous experimental studies of cholestatic jaundice have demonstrated that substances that have anti-inflammatory properties also reduce the damage of toxic bile acids to hepatocytes [36,37].

It was demonstrated that TNF- α , primarily released by activated macrophages, plays a critical role in promoting the release of other pro-inflammatory mediators and in the movement of leukocytes to the inflamed tissues [38]. The results of our study demonstrated that nesfatin-1 reduces the recruitment of neutrophils and also decreases serum levels of proinflammatory cytokines (TNF- α and IL-6). These results confirm the antioxidant effect of nesfatin-1. Dalia et al. [39] reported that nesfatin-1 contributes to decrease of TNF- α in gastric ulcer model in rats.

Although DNA is a stable molecule, it may undergo oxidative damage like other organic molecules (carbohydrates, proteins, and lipids). It has been shown that oxidative base modifications (e.g., 8-OHdG) can be observed even in newborn rats [40]. Increase of ROS, with the decrease of antioxidant enzyme levels, leads to oxidative DNA damage [41]. Due to the balance between damage and repair of DNA, oxidative DNA damage is, in normal conditions, always observed at very low levels. In the present study, the nesfatin-1 treatment was continued for 10 days, and this treatment caused a decrease of DNA damage to some extent. The oxidative DNA damage might have been more severe, if the duration of the experiment was longer than 10 days.

DNA fragmentation is the separation or breaking of DNA strands into pieces, which is the evidence of cell death. It was firstly reported by Williamson in 1970 when he observed the discrete oligomeric fragments occurring during cell death in primary neonatal liver cultures [42]. After 10 days of the nesfatin-1 treatment, the amount of DNA fragmentation slightly changed. These results might have been more obvious if the duration of experiment was longer.

The main histopathological changes observed after BDL depend on the duration and severity of the underlying cause [43]. Histopathologically, edema and neutrophil infiltration of the portal area of liver are first observed after BDL. If the obstruction continues, bile duct proliferation occurs. After that, necrosis and fibrosis can be observed [20]. Although the results of oxidative DNA damage and DNA fragmentation analyses were not as expected, the histopathological results supported the beneficial effects of nesfatin-1 treatment on liver tissue after common BDL. In the present study, the nesfatin-1 treatment demonstrated a decrease of edema in the portal area, neutrophil infiltration, and bile duct proliferation on the H&E sections; hepatocyte necrosis, basement membrane damage, and parenchymal necrosis on the PAS sections; and inflammation and ductal proliferation on the Masson's trichrome sections.

To conclude, the nesfatin-1 treatment in biliary cholestasis alleviated the oxidative damage and provided a hepatoprotective effect after common BDL, through its anti-inflammatory and antioxidant potential. Nevertheless, further experimental and clinical studies with longer follow-up are needed to obtain exact results.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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