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Investigation of the effects of N-acetylcysteine on asprosin hormone activity and liver tissues in rats with experimentally-induced diabetes

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ABSTRACT

Objective: To investigate the possible effect of N-acetylcysteine (NAC) treatment on rat diabetes-induced liver damage and immune reactivity of asprosin hormone in the liver.

Material-Method: Twenty-eight Wistar albino male rats were used in the study. They were separated into 4 groups as Control (n=7), Diabetes (n=7), Diabetes+NAC (n=7), and NAC (n=7). The rats in all groups were dissected after the treatment, and liver tissues were taken for pathological examination. Tissue sections were stained with immunohistochemistry for detecting asprosin immunoreactivity, hematoxylin-eosin and picrosirius red staining were performed to determine the changes in the tissues.

Result: In the microscopical examination of hematoxylin-eosin-stained sections normal histological hepatic tissues were seen in the Control and NAC groups. Pathological examination of liver tissue from diabetic rats showed marked dissociation, fibrosis, degeneration, inflammation, necrosis, Kupffer cells activation, bile duct proliferation, and congestion. A significant decrease in these lesions was observed in the DM+NAC group. Immunohistochemical studies showed that asprosin immunoreactivity was increased in the DM group in a significant manner. Asprosin expression was observed to be significantly reduced in the DM+NAC group in comparison to the DM group.

Conclusion: Our findings show that NAC administration reduces liver damage in diabetic rats and can be used to reduce/eliminate the negative effects of diabetes in rat liver tissue..

Keywords: *Asprosin, Diabetes mellitus, Liver, N-acetylcysteine, Rat*

INTRODUCTION

Nowadays, Diabetes mellitus (DM), which is reported to be a risk factor for Covid-19 disease (Lim et al., 2021; Sarkar et al., 2021; Shang et al., 2021), is common all over the world (WHO, 2021). DM is defined by an increase in blood sugar level (hyperglycemia) caused by insulin hormone not being synthesized or insufficiently synthesized. Insulin deficiency and resistance to insulin production also cause DM and, accordingly, changes in protein, carbohydrate, and lipid metabolism can occur (Hasselbaink et al., 2003). Hyperglycemia causes an increase in hepatic production of glucose and a decrease in peripheral

use of muscle and fat cells due to insufficient glucose intake and an increase in blood glucose levels (Yaman and Doğan, 2016). This situation causes oxidative stress by increasing reactive oxygen species (King and Loeken, 2004). It has been reported that as a result of oxidative stress caused by hyperglycemia, marked swelling, chromatin concentration, apoptotic bodies, and necrosis occur in hepatocytes (Manna et al., 2010). In other words, DM causes different structural and functional disorders that influence the metabolism of glycogen and lipid in the liver (Sanchez et al., 2000). Degenerated hepatocytes in form of cloudy swelling, vacuolar or fatty changes were observed in rats with STZ-induced diabetic liver injury during histopathological examination (Al-Ani et al., 2017; Sharkawi et al., 2020). In addition, severe dilatation and congestion of the central vein along with diffuse Kupffer cells proliferation (Sharkawi et al., 2020), and inflammatory cell infiltration (Begum and Mahboob, 2020) were observed. These changes can lead to liver fibrosis and are similar to the modifications observed in the human liver. Asprosin is a fasting-induced glycogenic protein that targets the liver to increase glucose release and plasma glucose levels synthesized from the C-terminal portion of pro-fibril. The main release site of asprosin is white adipose tissue and it circulates in the plasma at nanomolar levels. Studies on this newly discovered hormone have shown that asprosin increases the insulin resistance in humans and mice, and is thought to be linked to diabetes and metabolic syndrome (Romere et al., 2016). It has been observed that the balance between the antioxidant defense system and free radicals is disrupted by the effect of pro-oxidant and oxidant substances, it has paved the way for the development of oxidative stress in patients with diabetes (Rahal et al., 2014). N-acetylcysteine (NAC) is a mucolytic drug that contains a sulfhydryl group. By interacting with disulfide bonds in mucus, NAC breaks down mucoproteins and reduces the viscosity of mucus. Mucolytic drugs cause an increase in the amount of cysteine in the cell. It has been discovered that the increased cysteine in the cell has antioxidant properties by increasing glutathione synthesis over time (Ivanova et al., 2020; Muftakhov and Shchukin, 2020). Some antioxidants have been used against the negative effects of diabetes on various tissues and positive results have been reported at different levels (Bajaj and Khan, 2012).

In the present study, considering the positive effects of antioxidants, the effects of NAC to reduce oxidative damage and possible release sites of asprosin hormone other than white adipose tissue have been investigated.

MATERIALS and METHODS

The ethical guidelines for the care of laboratory animals (Kahramanmaraş Sütcü İmam University (KSÜ) Faculty of Medicine) Animal Experiments Local Ethics Committee (Ethics committee dated December 06, 2017, session number 2017/05, decision number 02) were followed throughout the experimental period. The rats were kept at 22-25°C room temperature for 12 hours of light and 12 hours of darkness and were fed in specially constructed cages. Standard rat food was given to all groups and add-libitum water was supplied, and the animals were cared for on daily bases by cleaning their bottoms. Twenty-eight male Albino rats (Wistar strain), 8-10 weeks old, weighing 200-210 g, were separated into 4 groups with 7 rats in each group.

Preparation of Streptozotocin (STZ)

STZ was dissolved in distilled water and then added to one drop of 0.1 M citrate buffer to obtain STZ solution with a pH of 4.5 and stored at 4°C.

Groups

Group I (Control group); No action was taken throughout the experiment period of 8 weeks. Glucose levels and body weights were measured and noted at the beginning and end of the study.

Group II (Diabetic group); A single dose of streptozotocin (STZ) at 50 mg/kg (Gajdosik et al., 1999) was administered intraperitoneally (i.p.). Those with blood sugar levels above 250 mg/dL in blood taken from the tail vein after 72 hours were approved to have diabetes, and glucose levels and body weights were measured and recorded at the beginning and end of the study (Shanmugam et al., 2011).

Group III (Diabetes+NAC group); A single dose of STZ at 50 mg/kg was administered i.p. After 72 hours, those with a blood sugar level above 250 mg/dL in the blood were taken from the tail vein was approved as diabetic. After inducing experimental diabetes, 100 mg/kg NAC i.p. was administered (Hong et al., 2009) every day for 8 weeks (Mahajan et al., 2020).

Group IV (NAC group); rats were treated with NAC at 100 mg/kg (i.p.) on daily basis for 8 weeks. Glucose levels and body weights were measured

and recorded at the beginning and at the end of the study. Rats in all groups were anesthetized by i.p. administration of ketamine (75 mg/kg)+xylazine (10 mg/kg) and decapitated.

Histopathological Method

The liver tissues were collected and fixed with 10% buffered formaldehyde, followed by histological follow-up series and embedded in paraffin. Tissue sections of 5 μm thickness were obtained from paraffin blocks and were stained with hematoxylin-eosin, picrosirius red, and also immunohistochemical techniques were applied for asprosin.

The histoscore, that reflects the prevalence of asprosin expression on the liver tissue was calculated according to Yalcin et al. (2017). Rating scale: 0.1, < 25%; 0.4, 26–50%; 0.6, 51–75%; 0.9, 76–100%, and intensity of expression: 0, unstained; 0.5, little staining; 1, some staining; 2, moderate staining; 3, strong staining. The histoscore = prevalence x intensity.

Immunohistochemical Analyzes

Sections taken from paraffin blocks were used to determine the immune reactivity of asprosin. For this purpose, 5 μm thick sections were transferred to Poly-L-Lysine slides. These slides were deparaffinized with xylene and cleared with graded alcohol. Endogenous enzyme activity was quenched by treating the tissues with 10% hydrogen peroxide solution for 10 minutes. After that, the tissues were boiled in a microwave oven (750W) for 7+5 minutes for antigen retrieval. It was incubated with primer (anti-asprosin antibody, FNab09797, Fine Test, China) for 60 minutes after treatment with Ultra V Block solution to prevent background stain. Slides were treated with secondary antibody (30 minutes), Streptavidin Alkaline Phosphatase (TS–125-HR, Lab Vision Corporation, USA) (30 minutes), and Fast Red Substrate System. The tissues that were counterstained with Mayer's hematoxylin were passed through PBS (Phosphate Buffered Saline) solution and distilled water and covered with a suitable sealing solution (Su-Ming et al., 1981). The slides were evaluated and photographed under a light microscope.

Statistical Methods

Statistical analysis of histopathological and immunohistochemical findings was performed using the SPSS 25.0 version (SPSS for Windows®) package program. Normal distribution analysis of the obtained data was performed using the Kolmogrov-Smirnov test. In addition, the homogeneity of variances was controlled by the Levene test. Normally distributed data were first evaluated using the one-way ANOVA test. Next, post-hoc Duncan analysis was performed to determine the differences between groups. Results were reported as mean±standard error ($\bar{X}$ ± SE) and $p<0.05$ was approved significant (Özdamar, 2004).

RESULTS

Histopathological Findings

During the microscopical examination of hematoxylin-eosin (HE) stained sections normal histological hepatic tissues were seen in the Control (Figure 1a) and NAC (Figure 1b) groups. In comparison to the Control group, markedly dissociated Remark cords, congestion, degenerated and necrotic hepatocytes, kupffer cell activation, fibrosis, scarce inflammation, and bile duct proliferation were observed in the DM group (Figure 1c). In comparison to the DM group, a marked decrease of these lesions was observed in the DM+NAC group (Figure1d) ($p<0.001$) (Table 1).

Table 1. Histopathological findings *

	Control	DM	NAC	DM+NAC
Congestion	0.33 ± 0.21^{a}	2.66 ± 0.21^{c}	0.83 ± 0.16^{ab}	1.33 ± 0.21^{b}
Fibrosis	0.16 ± 0.16^{a}	2.16 ± 0.16^{c}	0.33 ± 0.21^{a}	1.33 ± 0.21^{b}
Degeneration	0.16 ± 0.16^{a}	2.00 ± 0.00^{c}	0.33 ± 0.21^{a}	1.33 ± 0.21^{b}
Inflammation	0.16 ± 0.16^{a}	1.66 ± 0.21^{c}	0.50 ± 0.22^{ab}	0.83 ± 0.16^{b}
Necrosis	0.00 ± 0.00^{a}	1.33 ± 0.21^{b}	0.33 ± 0.21^{a}	0.33 ± 0.21^{a}
Kupffer cell activation	0.16 ± 0.16^{a}	2.33 ± 0.21^{c}	1.83 ± 0.16^{bc}	1.33 ± 0.21^{b}
Bile duct proliferation	0.16 ± 0.16^{a}	2.66 ± 0.21^{c}	0.50 ± 0.22^{a}	1.66 ± 0.21^{b}
Dissociation	0.33 ± 0.21^{a}	2.00 ± 0.25^{c}	0.83 ± 0.16^{ab}	1.33 ± 0.21^{b}
	p<0.001	p<0.001	p<0.001	p<0.001

* The values represent the mean ±SE; $^{a-c}$ Values in rows without common superscripts differ significantly, $p<0.01$ (One-way ANOVA post-hoc Duncan Test).

Table 2. Immunohistochemical findings *

	Control	DM	NAC	DM+NAC
Asprosin	0.33±0.21[a]	2.66±0.21[c]	0.83±0.16[a]	1.50±0.22[b]
	p<0.001	p<0.001	p<0.001	p<0.001

* The values represent the mean ±SE; [a-c] Values in rows without common superscripts differ significantly, p<0.01 (One-way ANOVA post-hoc Duncan Test).

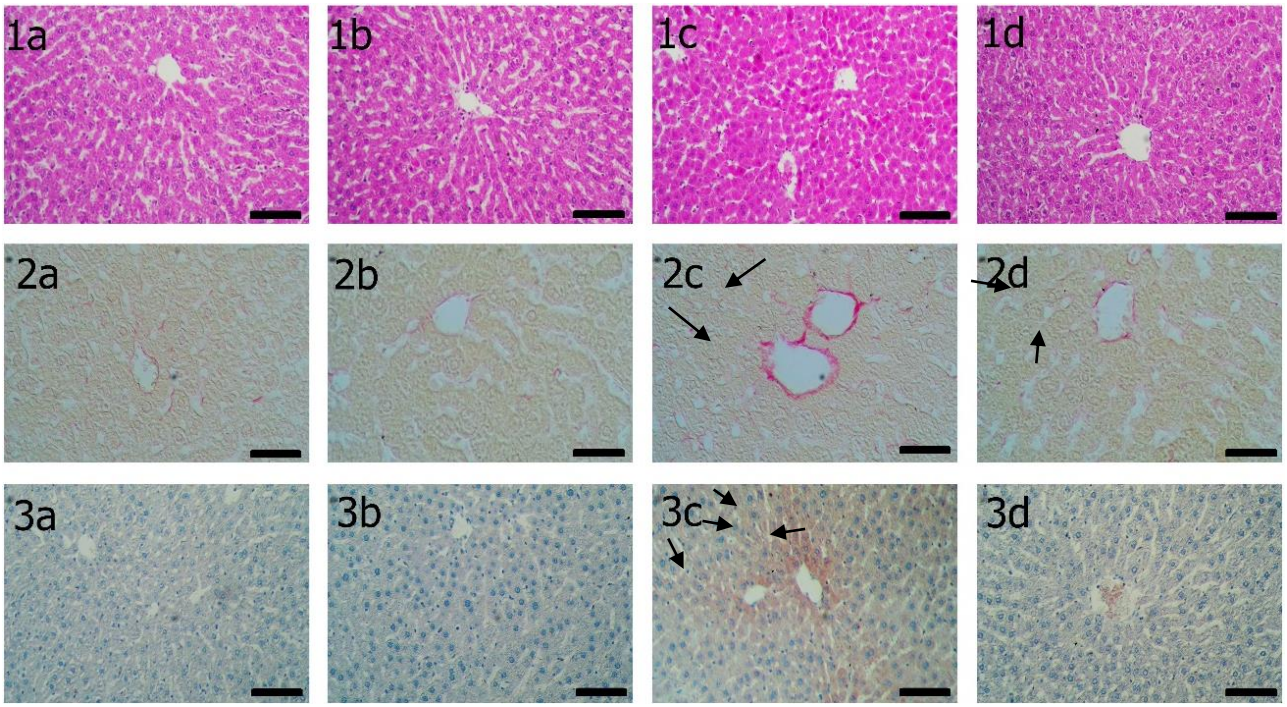

Figure 1. 1a-d; HE stained liver. Scale bars represent 200 μm. 1a- Control group, normal histological appearance of the liver. 1b- NAC group, normal histological appearance of the liver. 1c- DM group, dissociated Remark cords, degenerated hepatocytes, and inflammatory cells in the liver. 1d- DM+NAC group, compared to the DM group, pathological lesions are significantly less. **2a-d;** Picrosirius red staining for collagen in liver tissue. Scale bars represent 200 μm. 2a- Control group, no collagen staining. 2b- NAC group, no collagen staining. 2c- DM group, specific collagen staining (arrows). 2d- DM + NAC group, less specific staining of collagen (arrows). **3a-d;** Asprosin expression in liver tissue. Scale bars represent 200 μm. 3a-Control group, no immunoreactivity of asprosin. 3b-NAC group, no immunoreactivity of asprosin. 3c- DM group, highly pronounced immunoreactivity of asprosin (arrows). 3d- DM + NAC group, indistinct immunoreactivity of asprosin.

Collagen Content

In picrosirius red-stained liver tissues examination there were no histopathologic findings in the Control (Figure 2a), and NAC (Figure 2b) groups in terms of collagen content (p>0.05). Notably, significantly increased collagen fibers staining was determined in liver tissue of the DM group (Figure 2c) (p<0.05). In the DM+NAC group (Figure 2d), significantly decreased collagen content was observed in comparison to the DM group (p<0.05). These findings provide compelling evidence for the therapeutic use of NAC in DM-induced liver fibrosis.

Immunohistochemical Findings

Similar asprosin expressions were determined in the Control (Figure 3a) and NAC (Figure 3b) groups. Asprosin expression was significantly upregulated in the DM group (Figure 3c) in comparison to the Control group (p<0.001) while significantly reduced in the DM+NAC group (Figure 3d) in comparison to the DM group (p<0.001) (Table 2).

DISCUSSION

In the current study, DM led to severe histopathological damage in liver tissues described as dissociation and hemorrhages, degenerated

hepatocytes, and inflammatory cells infiltration. These findings are consistent with the previously reported data (Alqasim et al., 2017; Atta et al., 2020; Samadi-Noshahr et al., 2021) on liver injury induced by DM. In our study, the pathological examination of liver tissue from diabetic rats clearly indicates an association between liver damage and DM.

Oxidative stress has been reported to cause diabetic pathology, including diabetic liver injury (Arthur, 2000). The hepatocytes are injured and Kupffer cells are activated during oxidative stress. The inflammatory cells along with platelets release growth factors and cytokines that lead to fibrogenesis (Friedman, 2000). Fibrosis is the accumulation of connective tissue by the liver in response to liver injury (Komolkriengkrai et al., 2019). It has been reported that collagen accumulation is an initiating factor that triggers the formation of fibrosis in the liver tissue (Mabuchi et al., 2004). Concerning diabetic experiments, diabetes has been reported to increase collagen deposition in the liver tissue (Lo et al., 2011; Yang-en et al., 2016; Komolkriengkrai et al., 2019; Samadi-Noshahr et al., 2021). In our study, to evaluate fibrosis development in the liver, tissue sections were stained with picrosirius red. In line with previous reports (Lo et al., 2011; Yang-en et al., 2016; Komolkriengkrai et al., 2019), DM led to a significant increase in liver collagen content. However, collagen was observed to reduce after NAC administration in diabetic rats, implying that the liver tissue reorganization regained its normal histologic appearance. This restorative effect of NAC on liver tissue could be attributed to its anti-oxidative (Lei et al., 2012; Yalçın and Gürel, 2021) and anti-fibrotic effects (Morsy et al., 2012; Nagai et al., 2014).

Previous research has shown a linear link between blood glucose levels and asprosin hormone levels in experimental studies on animals (Romere et al., 2016). Accordingly, in our experimental study on animals, asprosin levels were found to be below in the livers of the control and NAC-treated groups compared to the DM group in immunohistochemical analyzes. In the experimental study conducted by Kocaman and Kuloğlu (2020), tissue asprosin hormone expressions decreased in group livers that developed diabetes with streptozotocin, unlike our study. This difference is because of the cytoprotective feature of asprosin. We think that the amount of asprosin increases in order to protect the tissue in the early stages of diabetes and may decrease in the late period of diabetes (after the 8 months) because of burnout. Therefore, long-term experimental studies are required to indicate the effect of asprosin on the liver with diabetes. Rosa et al. (2018) reported the beneficial effect of NAC on the liver due to its anti-oxidative properties against DM, it is thought that asprosin expression was decreased in the liver tissue of the DM+NAC group in the current study.

CONCLUSION

Our findings show that NAC administration reduces liver damage in diabetic rats and can be used to reduce/eliminate the negative effects of diabetes on rat liver tissue.

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