RESEARCH ARTICLE / *Araştırma Makalesi*

The Effects of N-Acetylcysteine on MMP-2 and MMP-9 Immune Activities in Testicular Tissue of Streptezotocin Induced Diabetic Rats

N-Asetilsistein'in Streptozotosin İle Oluşturulan Diyabetik Ratların Testis Dokusundaki MMP-2 ve MMP-9 İmmun Aktivitelerine Etkileri

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Abstract

Objective	This study was performed to investigate the effects of N-Acetylcysteine (NAC) on matrix metalloproteinases (Mmp-2 and Mmp-9) immunoreactivity in testicular tissue of diabetic rats. **(Sakarya Med J 2019, 9(1):59-67)**
Materials and Methods	28 male rats were allocated into four groups n (7); No treatment was applied to control group. Animals in the NAC alone group was treated with i.p.100 mg/kg NAC daily. Diabetes was induced upon injection of a single dose streptozocin 50 mg/kg intraperitoneally on diabetes group (DM). Following diabetes development, Diabetic + NAC groups were treated with i.p.100 mg/kg NAC daily. Oxidative damage was evaluated with Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) activities while the testicular damage was determined by histopathological evaluation and immunohistochemical assessment of MMP-2 and MMP-9 at the testicular tissues.
Results	TAS levels were found to be increased in diabetic NAC-treated group animals whereas TOS, MMP-2 and MMP-9 levels were decreased in the same group. For the histological findings, there were no testicular changes in the NAC alone and control group whereas the alterations such as marked degeneration, vacuole formation and basement membrane thickening of tubules seminiferus contortus were observed in the testicular tissues of the DM group. But, in the treatment group, DM+NAC, these alterations were found to be comparatively decreased.
Conclusion	Our findings suggest that administration of NAC minimize testicular damage in diabetic rats and might be a potential candidate to reduce/eliminate the negative effects of diabetes on the testicular tissue.
Keywords	NAC, MMP2, MMP9, Testes, Diabetes

Öz

Amaç	*Bu çalışma N-Asetilsistein'nin (NAS), diyabetik ratların testis dokusunda metalloproteinazların (Mmp2-Mmp9) immun reaktivitesi üzerine olan etkilerini araştırmak amacıyla yapıldı. (**Sakarya Tıp Dergisi 2019, 9(1):59-67**).*
Gereç ve Yöntemler	*28 adet erkek rat 4 gruba ayrıldı (n:7). Kontrol grubu hayvanlarına herhangi bir uygulama yapılmadı. NAS grubundaki hayvanlara 30 günlük deney süresince her gün i.p. 100 mg/kg NAS verildi. Diyabet grubundaki (DM) hayvanlarda diyabeti oluşturmak için intaperitonel (i.p.) tek doz 50 mg/kg streptozotosin uygulandı. Diyabet oluşumundan sonra DM + NAS grubuna 27 gün sure ile her gün i.p. 100 mg/kg NAS verildi. Oksidatif hasar, toplam antioksidan ve toplam oksidan aktivitelerinin ölçümü ile; testis hasarı ise testis dokusunun histopatolojisi ve Mmp2-Mmp9 immun reaksiyonlarının immunhistokimyasal değerlendirmesi ile yapıldı.*
Bulgular	*Diyabetik ratlardaki düşük olan TAS seviyesinin, NAS uygulanan diyabetik ratlarda artmış olduğu saptandı. Diyabetik ratlarda artmış olan TOS, Mmp2 ve Mmp9'un NAS uygulaması ile azaldığı gözlendi. NAS uygulanan grupta testis dokusunun olağan histolojik yapısına sahip olduğu gözlendi. Kontrol grubu ile karşılaştırıldığında, DM grubunda göze çarpar bir şekilde dejenerasyon, vakuol oluşumu ve seminifer tübüllerin bazal membranında kalınlaşma gözlendi. DM ile karşılaştırıldığında, DM + NAS grubunda dokudaki hasarın belirgin şekilde azaldığı gözlendi.*
Sonuç	*Bulgularımız NAS uygulamasının diyabetik ratlarda testis hasarını azalttığını ve diyabetin testis dokusu üzerindeki negatif etkilerini azaltma/elimine etmekte kullanılabileceğini göstermektedir.*
Anahtar Kelimeler	*NAS, MMP2, MMP9, Testis, Diyabet*

INTRODUCTION

DM is a metabolic condition that causes any deficiency or disability in the mechanism of insulin.[1] In addition to disruption of the insulin mechanism, there are common disorders in carbohydrate, fat, and protein metabolism [2,3] and therefore problems in other organs and systems.[4–6] Male infertility is a serious complication of DM in addition to the other major organ and/or organ systems disorders. [6,7] Adverse effects of diabetes mellitus on fertility occur at several different ways. [8,9] Altered spermatogenesis, degenerative and apoptotic testicular changes, inconsistent glucose and testosterone levels, and also insufficient sexual physical behaviors were described both in diabetic men and animal models.[10]

The blood-testes barrier is essential for the development and maturation of germ cells.[11] MMPs are endopeptidases and may degrade the most proteinous components of the extracellular matrix (ECM) and basement membranes.[12] MMP-2 (gelatinase A) and MMP-9 (gelatinase B) involved in the functioning of the blood-testes barrier [13,14] that are secreted by Sertoli and peritubular cells and these two cell types cooperate for deposition of ECM components in the basement membrane.[15,16]

Besides endocrine disorders,[17] oxidative stress is also an important factor in the pathogenesis of many chronic complications of diabetes.[5,18–23] Among the targets of oxidative stress in diabetes are MMP-2 and MMP-9, which are susceptible to oxidative stress.[24] Changes in the blood-testis barrier in streptozotocin-induced diabetic rats adversely affect spermatogenesis.[25]

NAC, a precursor of reduced glutathione (GSH), has been used in therapeutic practices.[26] Experimental studies suggest that NAC showed increased antioxidant capacity and depression of reactive oxygen species rate associated with increases of GSH levels.[27] GSH is the most important intracellular antioxidant.[28] NAC showed the ability to prevent the toxic effects of oxidative stress during diabetes and have been proposed as a complementary treatment.[29–32] The mechanisms responsible for the beneficial effects of NAC have been associated to its antioxidant properties.[33–35] Antioxidant treatments that relied on the effects of oxidative stress have reduced glycemic index and also complications of DM.[36,37] Therefore, in this study, whether the contribution of NAC to prevent pathology in the blood-testis barrier, which is known to be damaged in diabetic rats was investigated by immunohistochemical, pathological and biochemical methods.

MATERIALS and METHODS

Chemicals and Test kits

Streptozotosin (STZ) (Sigma Chemical Co Louis Missouri), acetylcysteine (NACR, Basel Pharmaceutical Inc. Sakarya-Turkey), Total Oxidant and Antioxidant Status Test Kits (Rel Assay DiagnosticR, Gaziantep, Turkey), and analytical reagents and solvents (Sigma Aldrich® and Merck®) which used in all procedures were commercially purchased.

Animals, diets and experimental protocols

A total of 28 male Wistar Albino Rat (200-220 gr), 8-10 weeks of age, supplied by Adıyaman University Experimental Animal Production and Research Center were divided into four groups (n=7). After seven-days acclimatization in a room condition at which maintained 12 h light/12 h dark cycle at room temperature (25±3°C) with ad libitum standard rodent pellet diet and water, the experiment was started. First group was named as Control group and animals were maintained on rodent standard pellet diet and water ad libitum, without any treatment during 30 days of experimental period. The second group was called as DM group. Streptozotocin was dissolved in 0.1 M sodium citrate buffer (pH: 4.5) and a single dose of STZ in 50 mg/kg ratio was administered to animals via i.p. route at the first day of the experiment. After 72 hours, blood samples were taken from the tail veins. Animals with glucose levels above 250 mg/dl were considered as diabetic. The third group was called DM+NAC group. Streptozotocin was dissolved

in 0.1 M sodium citrate buffer (pH: 4.5) and a single dose of STZ in 50 mg/kg ratio was administered to animals via i.p. route. After 72 hours, animals with blood glucose levels above 250 mg/dl were considered diabetic in samples taken from the tail vein. Following experimental diabetes, NAC was administered daily at a dose of 100 mg/kg via i.p. route until the end of the experiment. The animals in the fourth group, defined as the NAC group, were administered daily 100mg/kg NAC via i.p. route for the duration of the experiment. Glucose levels and body weights of all groups were measured and recorded regularly at the onset and at the end of the experiment. At the end of the experiment, rats in all groups were decapitated under ketamine (75 mg/kg) + xylazine (10 mg/kg) anesthesia.

Throughout the experiment, animals were processed according to the suggested ethical guidelines for the care of laboratory animals (Laboratory Animal Care Committee of Adiyaman University, protocol number: 2018/008). Blood samples were collected by cardiac puncture before decapitation under the anesthesia.

Tissue preparation and histopathologic examination

Animals were sacrificed after collecting the blood samples. Testes tissues were removed and fixed in buffered 10% formalin solution. Tissue samples were embedded in paraffin after routine procedures and then sectioned and stained with hematoxylin-eosin (HxE) and then stained sections were blindly analyzed by two experts. Mainly marked degeneration, vacuole formation and basement membrane thickening were scanned under a light microscope (Leica DM500 attached Leica DFC295 Digital Image Analyze System).

Immunohistochemical methods:

Streptavidin-biotin-peroxidase complex method was used with Thermo Scientific™ TP-015-HA commercial kit. Antibodies against Matrix Metalloproteinase-2 (MMP-2, Rabbit Polyclonal H-029-30, Phoenix Pharmaceuticals, Inc., California, USA) and Matrix Metalloproteinase-9 (MMP-9, Rabbit Polyclonal, BS-4593R Bioss Inc., Massachusetts) with 1/200 dilutions. Positive and negative controls were made as recommended by the manufacturers. Sections were visualized with 3-amino-9-ethylcarbazole (AEC) chromogens and background colorized with Mayer's Hematoxylin. Sections were scanned under a light microscope (Leica DM500 attached Leica DFC295 Digital Image Analyze System). A histological score was created based on the prevalence of immunopositivity as 0.1:<25%, 0.4:26-50, %0.6:51-75%, 0.9:76-100%) and severity (0: no lesion, +0.5: very little lesion, +1: little lesion, +2: mild lesion, +3: moderate lesion). Histological scorer= Distribution x severity.

Biochemical analysis:

After the experimental period, blood samples were taken from animals by intracardiac puncture under general anesthesia and then centrifuged at 2500 rpm to separate sera for 5 minutes. The obtained sera were stored at -200C until analyzed. To evaluate the degree of damage, Serum Total Antioxidant Level (TAS), 38 and Total Oxidant Level (TAS), 39 were measured for judging the degree of damage based on previous studies, by using the Total Antioxidant Status Assay Test Kit (Rel Assay DiagnosticR, Gaziantep, Turkey) and the Total Oxidant Status Assay Test Kit (Rel Assay DiagnosticR, Gaziantep, Turkey) with auto analyzer (Olympus AU2700).

Statistical Methods

Statistical analysis was performed in SPSS 15.0 program. The normal distribution of the TAS, TOS and immune variables in the groups was evaluated by Kolmogorov Smirnov test. One-way variance-analysis was used for TAS, TOS and Immune variables between groups. Levene statistics were used for homogeneity test of variances. Tukey dual comparison test was used to determine the differences of groups of significant variables. Results were given as mean ± SD. Significance level was accepted at least $P < 0.05$.

RESULTS

Beginning and final body weights of animals

When compared the beginning and final body weights of rats in all groups were evaluated; the final body weights of the Control and NAC groups were statistically higher than the beginning ($p < 0.05$). However, the body weights of DM and DM+NAC groups were statistically decreased compared to the beginning ($p<0.05$). (Table 1).

Biochemical Findings

Blood-glucose levels: The beginning and final blood-glucose levels of the rats in all groups were compared and no changes were observed in the Control and NAC groups. However, blood-glucose levels in DM and DM+NAC groups were found to be significantly increased compared to the beginning ($p < 0.05$). (Table 2).

TAS and TOS levels: The level of TOS, which was significantly elevated in the DM group ($p < 0.05$), was close to each other in the Control, NAC, and NAC + DM groups ($p < 0.05$). A significant TAS level decrease was observed in DM group compared to DM + NAC group ($p < 0.05$) and also in DM+NAC group compared to Control and NAC groups ($p < 0.05$) (Table. 3).

Histopathological Findings

In microscopical examination of HE stained sections, normal histological testicular tissues were seen in the Control (Figure 1a) and NAC (Figure 1b) groups. When compared with the Control group, marked degeneration, vacuole formation and basement membrane thickening of tubules seminiferus contortus were observed in the DM group (Figure 1c). Compared with DM group, a marked decrease of

Table 1. Beginning and final body weights of animals (g).

	Control	NAC	DM	DM+NAC
Beginning body weights (g)	211.96±14.08	201.02±15,20	250.73±11.10	222.05±15.88
Final body weights (g)	268,17±8.68a	284,63±9,07a	179,27±6,48a	178,25±15,75a

Values are given as mean ± standard deviation.
a According to the beginning body weight ($p < 0.05$).

Table 2. Beginning and final blood-glucose levels of animals (mg/dl).

	Control	NAC	DM	DM+NAC
Beginning blood-glucose levels (mg/dl)	102,94±4.31	105,41±2.45	105.37±2.24	102.93±2.13
Final blood-glucose levels (mg/dl)	103.30±5.06	107,09±3.78	384.76±54.43a	374.44±53.77a

Values are given as mean ± standard deviation.
a According to the beginning blood-glucose levels, $p < 0.05$).

Table 3: Serum TAS and TOS levels and immunohistochemical localizations of MMP-2 and MMP-9 in testes tissues of animals.

	N	TOS, P* 0.000	TAS, P* 0.000	MMP-2, P* 0.008	MMP-9 P* 0.002
Control	7	17,41b±0,55	1,68a±0,10	1,41b±0,37	1,20b±0,30
NAC	7	17,63b±0,79	1,77a±0, 07	1,45b±0,33	1,20b±0,30
DM	7	22,29a±1,20	1,27c±0, 07	2,22a±0,62	1,97a±0,50
DM+NAC	7	18,53b±0, 92	1,49b±0, 09	1,84ab±0,43	1,45ab±0,32

[abc:] Means within the same column with differing superscripts are significantly different ($p < 0.05$)
*:One Way Anova

these lesions were observed in the DM+NAC group (Figure 1d).

Immunohistochemical Findings

Immunohistochemical investigations showed that both MMP-2 (Figure 2a-2d) and MMP-9 (Figure 3a-3d) immunoreactivity were especially localized in the seminiferous tubules in the testicular tissue. Granular-type cytoplasmic staining was observed both sertoli and in germ cells.

A significant elevation of both MMP-2 and MMP-9 levels were detected in DM group compared with the Control and NAC groups (P<0. 05). but this value was statistically insignificant. Although no statistically significant difference between DM and DM+NAC groups were observed. (P<0. 05) (Table 3), both MMP-2 and MMP-9 levels were decreased at DM + NAC groups compared to DM.

DISCUSSION

Arrangement of intercellular junctions and associated proteins are critically important in the movement of germ cells across the seminiferous epithelium in the unique design of spermatogenesis.[40] Sertoli cells are indispensable in supporting developing germ cells, and any damage to them leads to reduced support capabilities.[41,42] Because of the deterioration of adhesion between the Sertoli cells and germ cells, the movement of germ cells within the seminiferous epithelium disrupt and early release of immature germ cells occur during differentiating of germ cells moving across the BTB and this is likely to cause infertility.[40,43]

The permeability of BTB is affected by the cytokine-mediated, [44] and/or by protease-mediated corruption of junction proteins. MMPs, a group of proteases, can disrupt or regulate the different blood barriers (including blood-testes

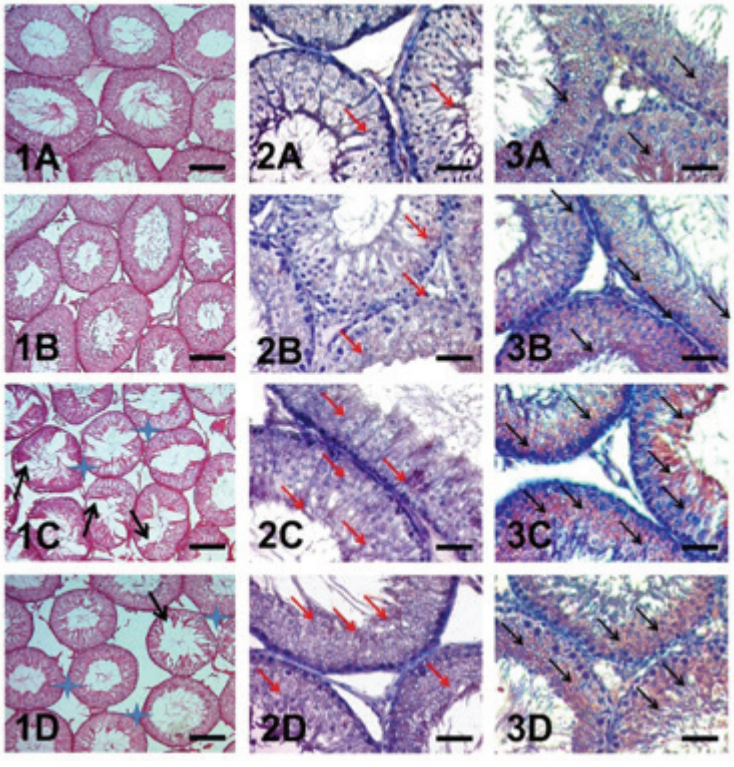

Figure 1A-D: Hematoxylin & Eosin stained testes. The scale bars represent 100 μm. A: Microscopical view of Control group. Normal testicular tissues. B: Microscopical view of NAC-treated group. Normal testicular tissue view. C: Microscopical view of DM group. Marked degeneration (black arrow) and basement membrane thickening (blue asterix) of seminiferous tubules were observed in the DM group. D: Microscopical view of DM + NAC. A significant decrease of degeneration (black arrow) and basement membrane thickening (blue asteriks) of seminiferus tubules.

Figure 2A-D: MMP-2 immunoreactivity of testes tissues. The scale bars represent 25 μm. Tissues were stained with Streptavidin biotin peroxidase complex method with Mayer's Hematoxylin counterstain. AEC chromogen was used for visualization. A: Control group. B: NAC group. C: DM group. D: DM+NAC group.

Figure 3A-D: MMP-9 immunoreactivity of testes tissues. The scale bars represent 25 μm. Tissues were stained with Streptavidin biotin peroxidase complex method with Mayer's Hematoxylin counterstain. AEC chromogen was used for visualization. A: Control group. B: NAC group. C: DM group. D: DM+NAC group.

barrier) by degrading tight junction proteins.[45–47]

Matrix metalloproteinase-9 is essential for assessing semen quality [48] and MMP-2 regulates the migration of spermatogonia and spermatocytes.[49] MMP-2 activation in the testis contributes to the decreased supportive capacity of Sertoli cells by altering junctional connections between Sertoli cells and germ cells[41,50] and such disruptions will cause initiating germ cell detachment.[41] In an in vivo study MMP-2-induced germ cell detachment inhibited by pretreatment with a MMP-2 inhibitor.[42]

Decreases in tissue inhibitor of metalloproteinase-2 (TIMP-2) expression in Sertoli cells led to MMP-2 activation.[42] Activated MMP-2 may alter the microenvironment in the adluminal compartment and further lead to the remodeling of tight junctions at the BTB between adjacent Sertoli cells. Finally, activated MMP-2 may directly breaks laminin/integrin complexes at apical ectoplasmic specializations (ESs) between Sertoli cells and spermatids and further contribute to the release of these cells into the lumen.[41]

Immunoreactivity of MMP-2 and MMP-9 explained by researchers in mice,[51] rats[52] and dogs.[48] MMP-2 has been reported to be localized in apical ESs that are mainly associated with the heads of prolonged spermatids.[52] In dogs, MMP-2 immunoreactivity was described in head of elongate spermatid, residual body and the Sertoli cell and MMP-9 immunoreactivity was defined in cytoplasm of spermatocyte, round spermatid and residual body.[48] Same to researchers intra- or extra tubular immunoreactivity were detected testes tissues of rats in immunohistochemical staining. These more frequently cytoplasmic immunopositivity were seen more intense in diabetic animals. Although they did not decrease to the extent of the control levels, these values were significantly decreased in NAC-treated diabetic animals.

According to the results obtained in the study, the high MMP-2 and MMP-9 levels in diabetic animals are not compatible with the results of some studies, but there is no discrepancy. Because, in experimental and in field studies in which MMP levels are detected, especially in diabetics, differences are observed in serum and tissue levels, in different tissues, in active and passive form, and in the method used to determine. For example, significantly elevated MMP-9 was measured in the sera of diabetic patient 53 and increased levels of activated MMP-2 and MMP-9 from the retinas of diabetic patients had been reported.[4] A significant reduce was described the activity of latent MMP-2, active MMP-2 and MMP-9 in diabetic testes by using different techniques and decreases in MMP-2 and MMP-9 have been associated with testopathy.[54] When the literature on the subject is viewed collectively, the detrimental effects of both increases and decreases of MMPs levels are damaging for the total health of the organism. Therefore, the steady-state balance levels are essential for leading a healthy life.

Oxidative stress associated testicular damages has been described both in diabetic rats and humans. Testicular oxidative stress, induced by both oxygen and nitrogen free radicals, cause MMPs activation and this irregular MMPs and TIMPs are adversely affect the construction of the multi-layered epithelium and cytoskeleton of germ cells.[24,55,56] In our study, elevated TOS and decreased TAS levels in diabetic animals returned to normal course with NAC treatment. In parallel with the increase of oxidative stress, MMP-2 and MMP-9 levels were high in the diabetic animals and these levels decreased with NAC administration. TAS levels increased with NAC administration, whereas MMP-2 and MMP-9 immunoreactivities decreased.
In conclusion; in our study, it was observed that the evident decrease in body weights of diabetic animals were not prevented by NAC treatments and similarly increased blood glucose levels in diabetic animals were not affected by NAC applications. But, NAC application caused a decrease in elevated MMP-2 and MMP-9 levels which elevated with diabetes. These findings suggest that NAC might be a po-

tential candidate to reduce/eliminate the negative effects of diabetes especially on male fertility on the testes health.

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