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Original article

The effect of silymarin on diabetes mellitus-induced male rats reproductive impairment: Evidences for role of heat shock proteins 70 and 90

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Abstract

Male fertility is adversely influenced by diabetes. The beneficial effects of antioxidant bioflavonoids in improving fertility have been reported. This study was conducted to evaluate the effects of silymarin on diabetes mellitus-induced male reproductive impairment in rats by investigating its role in Hsp70 and Hsp90 expression. To conduct this study, 18 mature male Wistar rats were divided into three groups: control (Con), experimental diabetes type 1 (T1D-sole)-induced, and silymarin (SMN, 120 mg/kg, orally)-treated T1D-induced groups. The testicular total antioxidant capacity (TAC) and malondialdehyde (MDA) levels were evaluated. Tubular differentiation index (TDI), repopulation index (RI), spermiogenesis index (SPI), and the Johnson score were also investigated. The DNA Ladder test was used to evaluate testicular DNA fragmentation, and RNA damage was assessed through fluorescent staining. Immunohistochemical and RT-PCR analyses were performed for Hsp70 and Hsp90. Oral administration of SMN significantly ($p<0.05$) increased the TAC ratio and decreased the MDA content in testicles compared to the T1D-sole group. The results showed that T1D increased the percentage of seminiferous tubules with negative TDI, RI, and SPI and reduced the Johnson score compared to the Con group ($p<0.05$). However, treatment with SMN ameliorated the T1D-induced damages to TDI, RI, SPI, and the Johnson score ($p<0.05$) compared to the T1D group ($p<0.05$). The staining intensities and the number of Hsp70+ and Hsp90+ cells were significantly higher in the Con group compared to the T1D-sole animals ($p<0.05$). However, rats treated with SMN showed an increased number of Hsp70+ and Hsp90+ cells per mm² of tissue compared to the T1D-sole group ($p<0.05$). Diabetes caused DNA fragmentation and RNA damage, but silymarin reduced its negative effects.

In conclusion, SMN ameliorates T1D-suppressed spermatogenesis by upregulating testicular antioxidant status and Hsp70 and Hsp90 expression in testicular tissue. Consequently, it can be considered a potential complementary medication for male patients with T1D.

Keywords: antioxidant, diabetes, rat, silymarin, testis

Introduction

Diabetes mellitus (type 1 diabetes [T1D]) is one of the chronic metabolic disorders that negatively affect several tissues (Kozakova et al. 2019). It is well-documented that T1D results in hyperglycemia, and hyperglycemia, in turn, increases reactive oxygen species (ROS) production in various tissues, such as the testicles, via mitochondria-related excessive ROS production (Stolf et al. 2018). Several clinical characteristics have been reported for T1D, including erectile dysfunction, ejaculatory failure, diminished semen volume, sperm count, sperm motility, and abnormal sperm morphology (Alves et al. 2013).

Accordingly, a significant increase in highly potent ROS production and advanced glycation end products (AGEs) has been detected in the testicular tissue of patients with diabetes mellitus (Vlassara and Uribarri 2014). In this context, the suppressed antioxidant status, as well as the increasing levels of highly potent ROS in testicular tissue, can result in severe DNA damage, lipid peroxidation, and protein peroxidation at the germ cell level, which consequently leads to spermatogenesis arrest (Hosen et al. 2015). The most important advantage of SMN is that it not only scavenges ROS but also inhibits free radical production, reduces inflammatory responses, and amplifies the optimal redox balance in the cell (Surai 2015). The administration of SMN has been shown to increase body and testis weight, normalize sperm count, and improve sperm viability in male rats (Hamid et al. 2018). Therefore, SMN was selected as an exogenous bioflavonoid in the current study, focusing on its impact on heat-shock protein expression as a novel area in T1D research. Heat shock proteins (HSPs) are known as stress-responsive proteins that are involved in the folding and unfolding of DNA and proteins (De Maio 1999), and any disruption in their expression can suppress spermatogenesis or result in spermatogenesis arrest (Minami et al. 2001). Their critical roles in refolding active proteins in germ cells (Zuo et al. 2016), stabilizing mRNA content in haploid cells during spermatogenesis, and supporting antioxidant status (Agarawal et al. 2012) have been previously demonstrated. Specifically, Hsp70 is known as a key HSP subtype involved in spermatogenesis, and it is expressed in specific germ cell types during spermatogenesis in mouse and human testes (Held et al. 2011). Another member of this family, Hsp90, has been identified in human and rat testes (Terada et al. 2005) and is expressed in spermatogonia, spermatocytes, Sertoli, Leydig, and myoid cells in normal human and rat testicular tissues (Held et al. 2011). Considering the antioxidant properties of SMN and the important roles of Hsp70 and Hsp90 in the progression of spermatogenesis, the present study was conducted to evaluate the effect of SMN on T1D-induced male reproductive impairment in rats by investigating its role in Hsp70 and Hsp90 expression. To date, no study has been conducted to investigate the effect of silymarin on diabetic infertile rats by exploring its impact on Hsp70 and Hsp90.

Materials and Methods

Materials

Streptozotocin (STZ) was purchased from Sigma Chemical Company (St. Louis, MO, USA), while other chemicals were of analytical grade and obtained from standard commercial suppliers. Silymarin was prepared by Goldaru Company (Isfahan, Iran).

Animals

All procedures used in this study were approved by the Ethical Committee for Animal Use of the Islamic Azad University, Tabriz Branch, East-Azarbaijan-Iran (Certificate no. 1106). Eighteen mature male rats were grouped and housed at a temperature of 22±2°C under a 12-hour light/12-hour dark cycle. The animals had free access to food and water.

T1D induction

The animals were fasted for 12 hours and then received an intraperitoneal injection of STZ (80 mg/kg), diluted in citrate buffer (pH 4.5; Invitrogen, Carlsbad, CA, USA), or vehicle (10 mM citrate buffer, pH 4.5) (Guimaraes et al. 2013). Blood glucose levels were measured 72 hours after diabetes induction using a glucometer (Accu-Chek® Active, Roche Diagnostics, Mannheim, Germany) via tail puncture in a fasting state. Animals with blood glucose >250 mg/dL were considered T1D-induced, while those with lower glucose levels received a second dose after 48 hours. The glucose concentration was 130±10 mg/dL prior to diabetes induction.

Animal grouping

For this purpose, the animals were randomly divided into 3 groups (n=6). Groups included: 1) control group (Con); with no intervention, 2) T1D-induced group and 3) SMN (120 mg/kg daily, for 60 days)-treated T1D-induced group (Tuorkey et al. 2015).

Testicular total antioxidant capacity (TAC) and MDA analyses

After 60 days, testicular samples were collected and weighed. Tissue samples (0.3 g) were homogenized in ice-cold 50 mM phosphate buffer solution (PBS,

pH 7.4). The ferric reduction antioxidant power (FRAP) method was used to evaluate the TAC at 593 nm, with results reported as nMol/mg protein (Pant and Srivastava 2003). The thiobarbituric acid (TBA) reaction was used to measure MDA content at 532 nm (Niehaus et al. 1968). The results for MDA were reported as nMol/mg protein. Protein content was determined using the Lowry method (Lowry et al. 1951).

Immunohistochemistry (IHC) staining

All the protocols were conducted based on a previous study (Farjah and Farahpour 2020). For the IHC staining technique, the tissue sections (5 µm) were prepared, heated at 60°C for 25 minutes (Venticell, MMM, Einrichtungen, Germany), de-paraffinized , and finally rehydrated and then subjected to the staining process. A volume of 10 mM sodium citrate buffer (pH 7.2) and peroxidase blocking solution (0.03% hydrogen peroxide) were used for antigen retrieval and blocking endogenous peroxidases. Phosphate-buffered saline (PBS, pH 7) was used to wash the sections. Slides were incubated with Hsp70 (E-AB-40208, rabbit, anti-rat/mouse/human) and Hsp90 (E-AB-60131, rabbit, anti-rat/mouse/human) at 4°C in a humidified chamber following the blocking stage. A volume of streptavidin-HRP (streptavidin conjugated to horseradish PBS containing an anti-microbial agent for 20 minutes) was applied for incubation. DAB chromogen was applied to the tissue sections and incubated for 10 minutes. Following gentle rinsing with ammonia (0.037 mM) and rinsing with distilled water, sections were coverslipped. Brown stains were considered positive reactions, which were observed as brown stains under a light microscope. The mean changes in the percentage of Hsp-70-2a+, Hsp90+, and PCNA+ cells per mm² of tissue were recorded by an unbiased examiner and compared between groups. The seminiferous tubules per mm² of tissue were counted according to the same criterion. Moreover, to minimize visual errors and evaluate the positive reactions, the photomicrographs of cross-sections were analyzed with Image Pro-Insight software (Media Cybernetics, USA, version: 9.00). For this purpose, 20-megapixel photos were randomly taken from cross-sections of testicular slides and the pixel-based intensity of positive reactions (brown-stained pixels/total pixels) for target proteins was analyzed in photomicrographs from 20 random microscopic fields (3000 µm × 3000 µm). Next, the mean percentage of the pixel-based intensities from three cross-sections for each animal was calculated in each group (n=10 animals in each group) and compared between groups.

DNA ladder

To investigate DNA fragmentation, the Cina Pure-DNA extraction Commercial Kit (Sinaclon, Iran) was used to test the DNA ladder. In this study, 100 µL of protease buffer was added to 35 mg of testicular tissue in a 1.50 mL micro-centrifuge tube. Microtubes were incubated at 55°C for 2 hours, and the samples were then completely homogenized. A 100 µL volume of homogenized sample was transferred into a new microtube. A lysis solution (400 µL) was added, followed by 300 µL of precipitation solution (isopropanol-based), and the mixture was vortexed for 5 minutes. Following centrifugation at 12,000 g for 10 minutes, 1 mL of ethanol-based wash buffer was added to the pellets; vortexing was conducted, and the samples were centrifuged at 12,000 g for 5 minutes (twice). The wash buffer was completely poured off, and the pellets were dried at 65°C for 5 minutes. Residual pellets were gently mixed with distilled water. Any unsolved materials were centrifuged for precipitation at 12,000 g for 30 seconds, and the DNA-containing supernatant was used in further steps. A NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA) was used for evaluating DNA content, quality, and purity. The eluted DNA was directly applied. A volume of elute corresponding to 2 µg DNA (15-17 µL of eluted DNA) was mixed with loading buffer (50% glycerol, 2 mm ethylenediaminetetraacetic acid, and 0.40% bromophenol blue), and the DNA solution was run in a 1% agarose gel for 70 minutes at 70 V constant voltage. The size of the DNA fragments was identified using PST1 as a marker. Ethidium bromide was used for staining gels, which were visualized using the Gel Doc 2000 system (ATP, Tehran, Iran).

mRNA extraction, cDNA synthesis and qRT-PCR

TRIZOL and 0.2 mL chloroform (per mL of TRIzol reagent) (Invitrogen, Carlsbad, California, USA) were used for isolating total RNA. The sample was incubated for 5 minutes at room temperature (RT) and then centrifuged at 12,000 g for 15 minutes at 4°C. Isopropanol (0.5 mL/1 mL TRIZOL used for initial homogenization) was added to extract the aqueous phase. The samples were precipitated at RT for 10 minutes. The RNA pellet was centrifuged at 12,000 g for 10 minutes at 4°C and washed with 1 mL of 70% ice-cold ethanol. The samples were again centrifuged at 7,500 g for 5 minutes at 4°C, air-dried, and the pellets were dissolved in RNase-free water. Quantity and quality of RNA were investigated using a spectrophotometer, assessing the absorbance ratio (260/280=1.8-2.0). The cDNA synthesis was performed in a 20 µL reaction

Table 1. Primers pair's sequences for rat individual genes.

Genes	Forward	Reverse
Hsp70	ACCGTGGAGCCCGGGAGAAG	TTGGTGGGGATGGTGGAGTTG
Hsp90	TGGACAGCAAACATGGAGAG	TGTAACCCATTGTTGAGTTGTCT
GAPDH	CTGCACCACCAACTGCTT	GCCATCCACAGTCTTCTGR

Table 2. Scoring based on Johnson's score in the rat.

Score	Status
1	No germ cells or Sertoli cells present
2	No germ cells present
3	Only spermatogonia present
4	Only a few spermatocytes present
5	No spermatozoa or spermatids but presence of spermatocytes
6	Only a few spermatids present
7	No spermatozoa but many spermatids present
8	Only a few spermatozoa present
9	Many spermatozoa present but disorganized spermatogenesis
10	Complete spermatogenesis

mixture containing 1 µg RNA, oligo (dT) primer (1 µL), 5×reaction buffer (4 µL), RNAse inhibitor (1 µL), 10 mM dNTP mix (2 µL), and M-MuLVReverse Transcriptase (1 µL), according to the producer's guidelines (Pars Tous, Iran). The PCR reaction contained: 0.5 µL (about 5-10 ng) of cDNA template, 10 µL 1X SYBR GREEN master mix (High ROX, Noavaran Teb-Beinolmelal, Iran), and 0.5 µL (600 nM) of reverse and forward primers of the target genes. PCR conditions included: general denaturation at 95°C for 5 minutes, annealing temperature at 60°C for 30 seconds, elongation at 72°C for 1 minute, and final elongation at 72°C for 5 minutes. For product size investigation, the PCR products were resolved on 2% agarose gel containing 0.5 µg/mL ethidium bromide. Normalization of target PCR threshold cycle (CT) values was conducted by subtracting the mean CT value of GAPDH. The relative expression level of the target mRNA was evaluated using the equation 2−(Ct target − Ct GAPDH). Table 1 shows the primer pair sequences for individual genes.

Histomorphometric analyses

To investigate the repopulation index (RI), the relative number of spermatogonia type B cells (cells with lightly stained nuclei) to spermatogonial type A cells (cells with darkly stained nuclei) was evaluated, and comparisons between groups were performed. The spermiogenesis index (SPI) was investigated, and tubules with intact spermiogenesis and arrested spermiogenesis were classified as positive and negative SPI, respectively. For the estimation of the tubular differentiation index (TDI), tubules with more than three layers of germ cells were counted as tubules with positive TDI, whereas those with fewer than two layers were classified as seminiferous tubules with negative TDI. The obtained results were reported as the percentage of seminiferous tubules with negative RI, SPI, and TDI. Scoring based on Johnson's score is shown in Table 2.

Fluorescent staining for RNA damage

In the present study, Darzynkiewicz's method was chosen to investigate RNA damage (Darzynkiewicz, 1990). Briefly, testicular tissues were washed with ether-alcohol and then cut by cryostat (8 µm). Different levels of ethanol (90%, 80%, 70%) were used to fix the tissue for 15 minutes. The sections were subsequently washed in 1% acetic acid and distilled water. The samples were stained with acridine orange for 3 minutes and destained in phosphate buffer (pH=6.85). The slides were then incubated in calcium chloride to differentiate the fluorescent dye. Necrotic germ cells were identified by the loss of RNA or weak red staining of RNA. Bright red staining was considered indicative of normal cells.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were conducted using SPSS 22 and Prism 6.0 software (GraphPad, San Diego, CA, USA). A p-value < 0.05 was considered significant. Normality was confirmed using the Shapiro-Wilk test, and parametric tests were applied. Between-group comparisons were performed using one-way ANOVA with Tukey's post-hoc test.

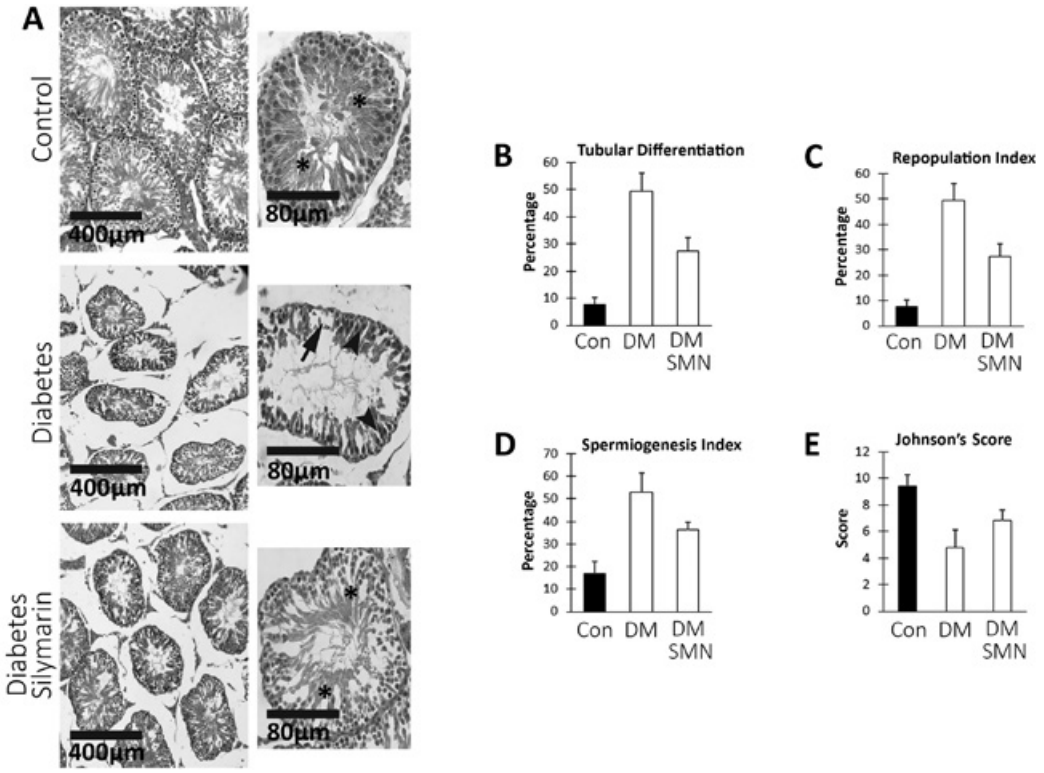

Fig. 1. Cross-sections of the rat testicular tissue from different groups; see intact spermiogenesis in higher magnifications (*) from control and silymarin-treated groups. Note, the arrested spermatogenesis (head arrow) and germ cells dissociation (arrows) in diabetes-sole group (hematoxylin and eosin staining), (B) negative tubular differentiation (TDI), (C) negative repopulation index (RI), (D) negative spermiogenesis index (SPI) and (E) the Johnson's score in different groups. All data are presented as Mean±SD. Different letters are indicating significant differences between groups (N=6 rats in per subgroup).

Results

SMN Ameliorated the T1D-Suppressed RI, TDI, and SPI Ratios

The results of the histomorphometric analyses are shown in Fig. 1. Tubular RI and TDI were analyzed, and observations revealed that SMN significantly ameliorated the T1D-suppressed repopulation and differentiation indices. Accordingly, animals in the SMN-treated groups showed a remarkable ($p<0.05$) reduction in the percentage of tubules with negative RI and TDI compared to the T1D-sole group. In addition to the RI and TDI analyses, the results showed that T1D increased the percentage of tubules with negative SPI compared to the Con group ($p<0.05$). Treatment with SMN significantly ($p<0.05$) mitigated the effects of T1D on the spermiogenesis ratio by reducing the percentage of seminiferous tubules with negative SPI. However, there was a significant difference between the SMN-treated and Con groups ($p<0.05$). Finally, the Johnson score was evaluated to determine the general effect of T1D and SMN on the spermatogenesis process. Observations revealed a significantly higher Johnson score in the SMN-treated group compared to the T1D-sole animals.

The results of fluorescent staining

Special fluorescent staining for RNA damage is shown in Fig. 2. The results showed that germ cells had light yellowish-red staining reactions, and the SMN-treated group showed a lower level of RNA damage.

DNA fragmentation

The DNA ladder test was used to investigate the diabetes-induced DNA fragmentation. The results showed that diabetes resulted in increased DNA fragmentation, but silymarin alleviated its negative effects (Fig. 3).

SMN increased the T1D-diminished Hsp70+ and Hsp90+ cells distribution in testicle

The data for IHC are shown in Fig. 4. Observations indicated a significant ($p<0.05$) reduction in Hsp70+ and Hsp90+ cells distribution per mm² of testicular tissue in the T1D-sole group. Meanwhile, animals in the SMN-treated group demonstrated a remarkable ($p<0.05$) enhancement in the number of Hsp70+ and Hsp90+ cells per mm² of tissue compared to the T1D-sole group. To reduce analysis errors, pixel-based intensity measurements for Hsp70 and Hsp90 proteins (represented as brown reactions) were conducted, and the results

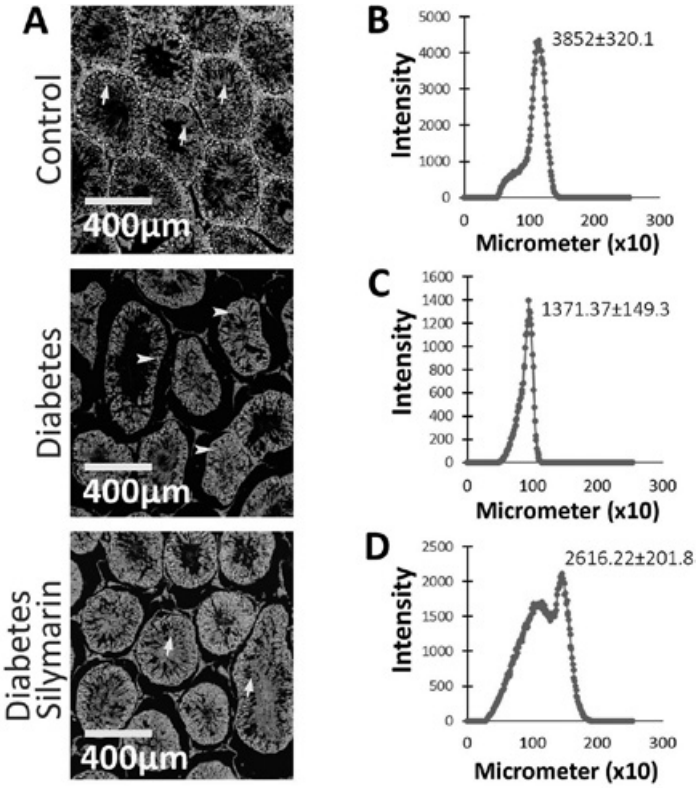

Fig. 2. Special fluorescent staining for RNA damage. The control group, representing germ cells with intact RNA content (bright red stained). The diabetes group: the germ cells are presented with light yellowish-red staining reactions. (note the DNA in green). The SMN-treated group: note the ameliorated mRNA damage in SMN-treated cross-section, presented in light red reactions.

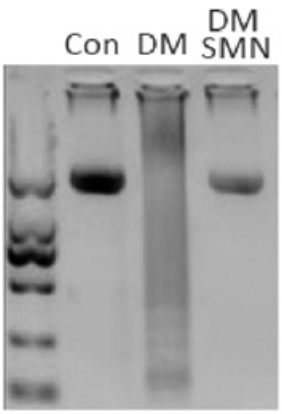

Fig. 3. DNA laddering test for DNA fragmentation; See intact DNA content in the control and silymarin-treated groups. The lane of the diabetes-sole group is presenting severe DNA fragmentation.

were compared between groups. The highest and lowest intensities of Hsp70 and Hsp90 were observed in the Con and T1D-sole groups, respectively. Rats in the SMN-treated group demonstrated higher staining intensity compared to the T1D-sole group but lower staining intensity compared to the Con group ($p<0.05$). The results for PCR (Fig. 5) confirmed our findings for IHC.

SMN amplified the T1D-decreased mRNA expression of Hsp70 and Hsp90

To assess the effect of T1D and SMN on mRNA content, qRT-PCR analyses were conducted. Observations revealed the highest and lowest Hsp70 and Hsp90 mRNA levels in the Con and T1D-sole groups, respectively (Fig. 5). However, SMN-treated animals exhibited a remarkable ($p<0.05$) increment in mRNA levels of Hsp70 and Hsp90 compared to the T1D-sole animals.

The levels of TAC and MDA

The lowest and highest (Fig. 6) testicular TAC ratio and MDA content were found in T1D-sole animals ($p<0.05$). Accordingly, animals in the T1D-sole group exhibited a remarkable ($p<0.05$) reduction in TAC ratio

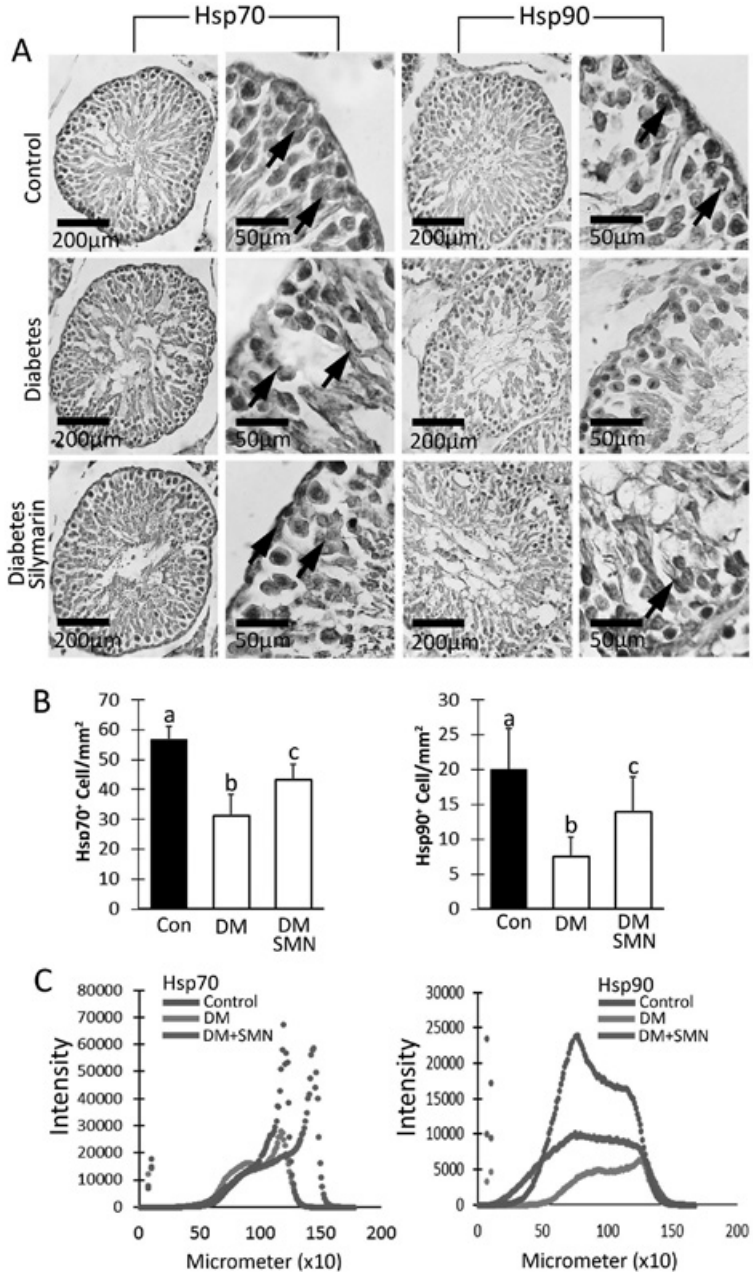

Fig. 4. (A) Immunohistochemical staining for Hsp70 and Hsp90; the positive reactions for Hsp70 and Hsp90 are marked with arrows. Note decreased expressions of Hsp70 and Hsp90 in the diabetes-sole group and increased in the silymarin-treated group, (B) mean number of $Hsp70^{+}$ and $Hsp90^{+}$ cells in different groups, all data are presented in Mean ± SD and different letters are representing significant differences ($p<0.05$) between marked groups (N: 6 rats in each group), (C) Software analyses of pixel-based intensity for brown reactions (representing Hsp70 and Hsp90 proteins) in 2000×2000 micrometer of the tissue. Note decreased pixel-based intensities of Hsp70 and Hsp90 in the diabetes-sole group that is significantly increased in the silymarin-treated group.

and an increase in MDA content of testicles compared to the Con and SMN-treated groups. In contrast, SMN significantly ($p<0.05$) increased the TAC ratio and reduced the MDA content compared to the T1D-sole animals.

Discussion

The results showed that T1D negatively affects spermatogenesis. Our findings align with previous studies, which have reported that spermatogenesis is negatively influenced in diabetic men (Han et al. 2019). Indeed, glucose metabolism significantly impacts spermatogenesis because glucose is transported from the blood-testis barrier through Sertoli cells to maintain the necessary energy supply for developing germ cells (Ding et al. 2015). Moreover, T1D disrupts spermatogenesis by preventing the effect of insulin in controlling the levels of serum FSH (Omolaoye Temidayo and Plessis Stefan 2018). Thus, diabetes has adverse effects on spermatogenesis through its impact on insu-

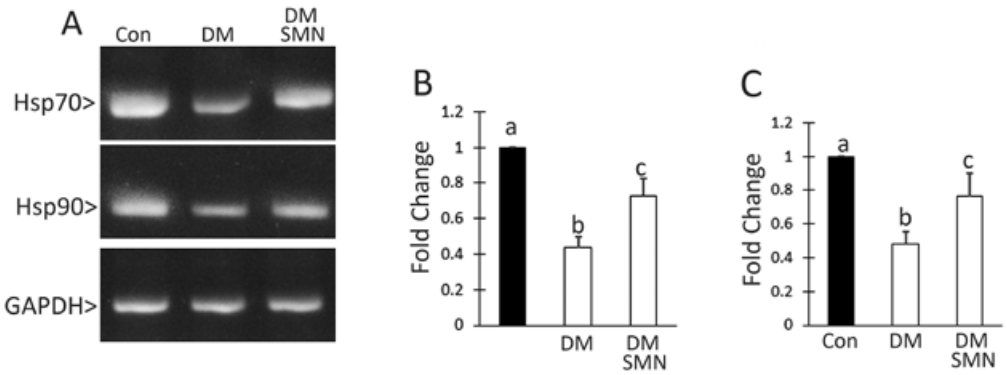

Fig. 5. (A) The results for PCR electrophoresis gel in the different groups, (B) qRT-PCR results of mRNA fold change in different groups for Hsp70 and (C) qRT-PCR results of mRNA fold change in different groups for Hsp90, all data are presented in Mean ± SD and different letters are representing significant ($p<0.05$) differences between each other (N: 6 rats in each group).

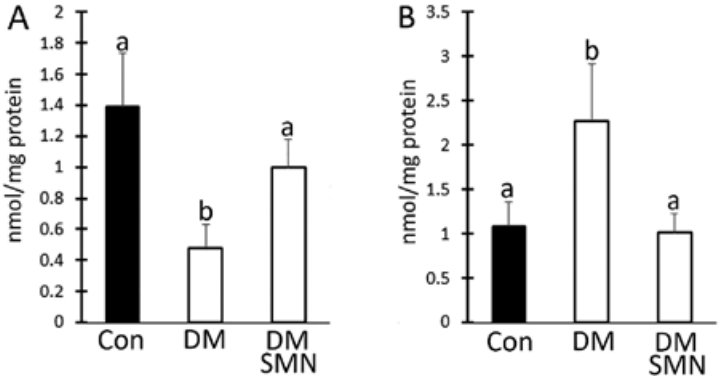

Fig. 6. (A) Tissue total antioxidant capacity (TAC), (B) tissue malondialdehyde (MDA) levels in different groups, all data are presented in Mean ± SD and different letters are representing significant ($p<0.05$) differences between each other (N= 6 rats in each group).

lin and glucose metabolism. Our findings showed that the administration of SMN significantly improved spermatogenesis. These results regarding the effects of SMN on spermatogenesis align with previous studies (Fatehi et al. 2018). As mentioned, diabetes negatively affects spermatogenesis by disrupting glucose metabolism. Studies have reported the effects of SMN in decreasing glucose levels in diabetic subjects through its involvement in the expression of cytochrome P450 enzymes (Hüttl et al. 2021) and glucose metabolism (Xiao et al. 2020). In addition, SMN demonstrated antioxidant properties in this study, a mechanism for decreasing glucose under diabetic conditions (Sun et al. 2021). On the other hand, increased lipid peroxidation and decreased antioxidant enzymes positively correlate with spermatogenesis arrest (Adewoyin et al. 2017). The improvement of antioxidant mechanisms by SMN can be considered one of the mechanisms for enhancing antioxidant properties. Thus, SMN exerts its effects through glucose metabolism and antioxidant properties, positively influencing spermatogenesis under diabetic conditions. Our findings showed the negative effects of diabetes on TAC and MDA. T1D-induced hyperglycemia increases ROS production as well as consequent ROS-induced DNA damage (Kaludercic et al. 2020). Studies have reported that diabetes significantly affects sperm viability by inducing lipid peroxidation in sperm (Simas et al. 2021). The results also showed that administration of SMN significantly decreased MDA content (a lipid peroxidation marker) and increased the TAC level of the testicular tissue. The results of SMN's effects on MDA and TAC are consistent with those of previous studies conducted under diabetic conditions (Mirzaei et al. 2021, Pourheydar et al. 2021). The antioxidant activity of silymarin is attributed to its compounds, including silybin, silydianin, silychristin, and flavonolignans (Pourheydar et al. 2021). We previously mentioned some relationships between antioxidants, spermatogenesis, and SMN, but several additional mechanisms also exist. Studies have reported the antioxidant role of SMN in preventing free radical production, reducing inflammatory responses, and maintaining optimal redox balance in the cell (Surai 2015). These findings suggest that SMN can significantly ameliorate diabetes-induced derangements by upregulating the testicular antioxidant status, which in turn reduces lipid peroxidation levels (marked by MDA in the testicles). In sum, it can be concluded that SMN improves spermatogenesis and other reproductive parameters in diabetic male rats by enhancing antioxidant proper-

ties. In line with this, previous reports have indicated a significant relationship between semen MDA and abnormal sperm morphology (Alyethodi et al. 2021). Palani et al. (2020) reported a positive relationship between TAC and MDA in seminal plasma from normozoospermic samples. The DNA laddering test was used to uncover the possible effects of diabetes on DNA fragmentation and DNA damage-dependent cell cycle arrest. The DNA ladder test showed increased DNA damage in the T1D-sole group. To understand the importance of DNA damage, it is essential to note that DNA damage can individually initiate the checkpoint machinery of genes involved in cell cycle progression during spermatogenesis. In addition, the results showed that SMN decreased RNA damage. The results from RT-PCR and IHC confirmed that diabetes reduces the expression and staining intensities of Hsp70 and Hsp90. HSPs are widely found on the sperm surface of various animals (Purandhar et al. 2014). Studies have reported the expression of Hsp70 protein in specific spermatogenic cell types during spermatogenesis (Huang et al. 2005). Defective Hsp70-2 in mice leads to arrest of spermatogenesis in meiotic prophase I and causes infertility (Yaman et al. 2018). Wu (1995) showed that the expression pattern of Hsp90 is similar to that of Hsp70. Consistent with these findings, we demonstrated that SMN administration significantly improved the spermatogenesis ratio (marked with RI, TDI, and SPI). Moreover, Hsp70 has been shown to play a crucial role in refolding proteins involved in germ cell development (Witkin et al. 2017). Thus, considering the stress responder role of Hsp70 and Hsp90, we suggest that SMN can remarkably protect essential proteins, maintain their functionality, and promote spermatogenesis by upregulating the expression of Hsp70 and Hsp90. It can be implied that higher expression levels of Hsp70 and Hsp90 during spermatogenesis help diabetic rats achieve better reproductive parameters compared to diabetic rats with lower expression levels of Hsp70 and Hsp90. On the other hand, Hsp70 is known to regulate the mitochondrial pathway of caspase-mediated cell death, preventing it at different stages by inhibiting stress-inducing signaling during mitochondrial processes (Purandhar et al. 2014). In addition, HSPs protect cells against oxidative stress (Purandhar et al. 2014). Thus, it can be concluded that SMN reduces oxidative stress, increases TAC, and decreases MDA, ultimately improving reproductive parameters in males affected by stress and diabetes.

Conclusion

This study was conducted to evaluate the effect of SMN, an antioxidant and anti-inflammatory agent, on T1D-induced male reproductive impairment by investigating its impact on the antioxidant status of the testicles as well as Hsp70 and Hsp90 expression levels. The results showed a positive correlation between Hsp70 and Hsp90 expression and staining intensity with the levels of TAC, and a negative correlation between Hsp70 and Hsp90 expression and staining intensities with spermatogenesis and the level of MDA. Based on the results, it can be concluded that the administration of SMN significantly reduced MDA and the adverse effects of diabetes on reproductive parameters by increasing the expression levels of Hsp70 and Hsp90, enhancing TAC levels, and decreasing MDA levels. We recommend the use of SMN in commercial agents for the treatment of diabetes as a safe compound with fewer side effects, pending future clinical studies.

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