

Moderate-Intensity Exercise Training in Association with Insulin Promotes Heat Shock Proteins 70 and 90 Expressions in Testicular Tissue of Experimental Type 1 Diabetes

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Received: 12/March/2020, Accepted: 15/June/2020

Abstract

Objective: The current research was designed to analyze the effect of moderate-intensity exercise training (MEXT), solely and simultaneous with insulin, on the network between oxidative stress and Hsp70 and Hsp90 chaperones after experimental type I diabetes (DM) induction in rats.

Materials and Methods: In the experimental study, 36 mature Wistar rats were assigned into control and experimental type I DM-induced groups, and then the diabetic animals were categorized to sedentary type I DM-induced (SDM), exercise training-sole without DM (E), exercise training DM-induced (EDM), insulin-treated sedentary DM-induced (ISDM), and exercise training insulin-treated DM-induced (EIDM) groups. After 6 weeks, Johnson's score was evaluated to analyze the spermatogenesis ratio.

Results: The Hsp70 and Hsp90 expression levels, testicular total antioxidant capacity (TAC), protein peroxidation ratio, testicular DNA fragmentation ratio, and mRNA damage were investigated. The animals in EDM and EIDM groups (solely and simultaneously) represented a significant ($P < 0.05$) improvement in Johnson's score, spermatogenesis, and TAC ratios versus SDM animals. Moreover, the DM-induced DNA and mRNA damage and protein peroxidation ratio were significantly ($P < 0.05$) recovered in EDM and ISDM groups, which was more remarkable in the EIDM group. The EDM and EIDM groups exhibited significant ($P < 0.05$) increment in Hsp70 and Hsp90 expression levels versus the control and SEDT1 animals. However, the EIDM group exhibited no significant changes compared to the control animals.

Conclusion: The EX could ameliorate the EDT1-induced detrimental impact by up-regulating Hsp70 and Hsp90 expressions. Meanwhile, it exerts potentially more effective impact, when it is considered simultaneously with insulin therapy.

Keywords: Diabetes, Exercise, Heat Shock Proteins, Oxidative Stress, Spermatogenesis

Cell Journal (Yakhteh), Vol 23, No 6, November 2021, Pages: 708-716

Citation: Samadian Z, Tolouei Azar J, Rezaei Aghdam H, Tofighi A, Razi M. Moderate-intensity exercise training in association with insulin promotes heat shock proteins 70 and 90 expressions in testicular tissue of experimental type 1 diabetes. Cell J. 2021; 23(6): 707-716. doi: 10.22074/cellj.2021.7502.

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Introduction

Diabetes is known as a metabolic disorder that impairs the carbohydrates, fats, and proteins metabolism which is clinically detectable with chronic hyperglycemia (1). Diabetes is characterized into two types. The insulin-producing disability of beta cells results in chronic autoimmune disease, named type I diabetes. Type II diabetes is known as insulin-dependent and/or independent type (2). Type I DM is known as a more prevalent type, which is reported in a wide range of ages, from children and teenagers to adults (3). According to previous animal studies, diabetes suppresses the reproductive potential of both males and females. There are various meta-analyses and clinical trials, which represent the DM-induced temporal and/or complete infertility in male individuals (4, 5). Based on previous findings, DM adversely affects spermatogenesis which consequently decreases

sperm quality (6-9). Indeed, studies on many fertility and infertility factors such as testosterone deficiency, severe oxidative and nitrosative stresses, and increased mitochondria-related apoptosis revealed a direct link between levels of diabetes-induced impairments and male fertility issues in animal models (8).

Considering the well-known adverse impacts of oxidative stress and testosterone withdrawal on the germ cell population, it is important to understand how the DM-induced effect on sperm parameters. As different kinds of stressors such as including oxidative stress are in association with testosterone withdrawal, they can potentially influence the expression of several proteins like heat shock proteins (HSPs) (10, 11). HSPs are known as the most important stress responder chaperone involved in cellular homeostasis through controlling the process of cellular protein and DNA folding processes

(12). HSPA family is the largest member of HSPs family contains about 13 members in humans and rodents. The Hsp70, specific HSPA in testes, is expressed at high levels in the germ cells during spermatogenesis (11). The Hsp90, another HSP family member has been shown in human and rat testicular tissue (13). In addition to DNA-related interactions among HSPs members, Hsp70 and Hsp90 are able to interact with pro-apoptotic elements and act as anti-apoptotic molecules during spermatogenesis (11). Besides, both of these proteins (directly and/or indirectly) are involved in the chromatin-histone replacement process during late spermiogenesis (14). Considering the DM-induced detrimental impact on the sperm chromatin condensation process, it seems that any changes in expression of Hsp70 and Hsp90 may significantly affect both spermatogenesis and sperm nuclear maturation process.

It is well-known that physical activities and exercises could potentially promote health benefits in various tissues. For instance, according to a previous study, physically active individuals are able to produce higher percentages of sperm with high quality *versus* sedentary controls (15). In line with these findings, Gaskins et al. (16) showed that students with higher physical activities exhibit higher sperm count and concentration compared to those intact sedentary ones. In another study, moderate-intensity exercise training (MEXT) also results in 43% higher sperm concentration in men with infertility disorders (17). Likewise, the beneficial effect of MEXT on the antioxidant status of various tissues is shown previously (18). Interestingly, this kind of exercise training is shown to diminish the age-related sperm DNA fragmentation, testicular atrophy, and inflammation in animal models versus the control (18, 19). On the other hand, some other reports have shown that intense or exhaustive exercise training and activities-induced could have adverse impacts on male reproductive potential. For example, prolonged intensive exercise training suppresses the hormonal balance in male individuals which consequently affects spermatogenesis (20, 21). More clinical phenotypes, including elevated abnormal sperm morphology and motility, and enhanced seminal oxidants are reported for intensive exercise training/activities-induced detrimental impacts. Considering these reports, the exercise training, albeit based on its intensity, is able to fairly boost and/or suppress the fertilization potential in male individuals.

Therefore, considering the unavoidable role of Hsp70 and Hsp90 in germ cell survival and development during spermatogenesis, and boosting effect of MEXT on male reproductive potential, the present research was designed to explore MEXT and insulin-induced ameliorative impacts insole and simultaneous forms against experimentally-induced type 1 diabetes (DM). For this purpose, the cross-links between Hsp70 and Hsp90 expression levels, oxidative stress, and germ cells' survival and development during spermatogenesis were assessed.

Materials and Methods

Chemicals

Streptozotocin (STZ) was obtained from Sigma Co (Sigma Chemical, Saint Louis, MO, USA). Acridine-orange and aniline-blue staining dyes were purchased from Setareh-Asia-Derakhseh-Teb Co, (Tehran, Iran). Primary antibodies for Hsp70 (Cat NO: E-AB-40007) and Hsp90 (Cat NO: E-AB-10353) were from Elabsciences, USA.

Animals and grouping

To perform this original experimental research, thirty-six mature rats (Wistar, 180-220 g, eight weeks of age) were considered. Following adaptation, the rats were assigned into the control (Con) and experimental groups. The experimental DM was induced and the rats were categorized in sedentary type I DM-induced (SDM), exercise training-sole without DM (E), exercise training DM-induced (EDM), insulin-treated sedentary DM-induced (ISDM), and exercise training insulin-treated DM-induced (EIDM) groups (Fig.1). The standard environmental conditions of 12 hours/12 hours light and dark, humidity, temperature, and ad libitum food and water were considered. All experimental innervations were considered in accordance with The University of Urmia "Animal Care and Use" Committee's approval (IR.UMSU.REC.1396.161).

Before 6 weeks of exercise training, the rats in E, EDM, and EIDM groups got familiar with a treadmill (5-canal treadmill special for the rat; DaneshYakhte, Iran) running for one. Meanwhile, no exercise condition was considered for Con, SDM, and ISDM groups. Moreover, 0.9 IU/100 g from insulin, at approximately zone-time (ZT) 8 to ZT 10, once-daily doses (0.9 U per 100 g) was administrated in the ISDM and EIDM groups, for 6 weeks (22). Following 6 weeks of training termination, the high-dose (100 mg kg⁻¹) ketamine (Alfasan, Utrecht, The Netherlands) were administrated intraperitoneally and the rats were euthanized. The left-hand side testicles were fixed in Bouin solution for at least 72 hours and the right-hand side testicles were dissected and stored at -80°C for additional molecular analyses.

Diabetes induction induction

In order to induce DM, no food animals were considered as SEDM, EDM, ISDM, and EIDM groups (8 hours). Following a single dose of STZ (55 mg kg⁻¹ body weight, 0.1 M, pH=4.5) was injected into the study groups (23). The animals in the Con group received sodium citrate buffer (0.4 ml, pH=4.5), as a solvent of STZ in the same volume. Following 48 hours, the levels of blood glucose were evaluated for them using a glucometer (Accu-Chek Advantage; Mannheim, Germany). The rats with a blood glucose level of 200 mg/dl or above were considered diabetic.

MEXT protocol

The intensity of exercise training was organized based

on running speed. The speed of 18m/min (an incline of 5%) for 30 minutes was considered per day on the treadmill. This condition of running was included 5 days a week with a maximum 65% consumption of oxygen (23). Please see the schematic Figure 1 for the principle of gradual overloading, intensity, and duration in 6 weeks.

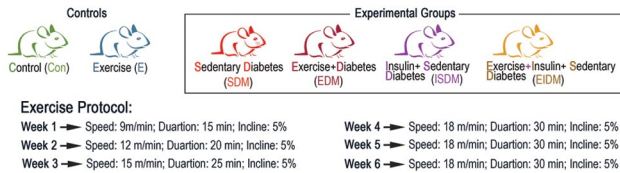


Fig.1: Schematic view for animal grouping and exercise protocol. Min; Minutes.

Histological analysis

After fixation, the routine tissue passage procedure, embedding, and cutting (4-5 μ m, LKB microtome 2218, UK) were conducted. Johnson's scoring method was used to evaluate spermatogenesis development and quality (24). Thereafter, 20 cross-sections from seminiferous tubules in each slide were examined for each rat. Finally, A scores were compared between all groups.

Immunohistochemistry staining

The number of Hsp70⁺ and Hsp90⁺ cells/mm² of the testicular tissue was examined. The IHC staining was performed for this purpose. The tissue slides (5 μ m) were pre-heated at 60°C (25 minutes), de-paraffinized in xylene, rehydrated by ethanol, and the antigen retrieval process was considered (10 mM sodium citrate buffer, pH=7.2). The endogenous peroxidases were blocked by using sodium acid containing 0.03% hydrogen peroxide. Following the blocking process, the histological sections were incubated with primary Hsp70 and Hsp90 biotinylated antibodies (1:500 for Hsp70, and 1:300 for Hsp90), 18 hours at 4°C. Next step, the slides were incubated with streptavidin conjugated to horseradish phosphate buffer solution (PBS) containing an anti-microbial agent (20 minutes), and continued with DAB chromogen (10 minutes). The hematoxylin was used for counterstaining the nuclei (10 seconds). The Hsp70 and Hsp90 proteins were revealed in a brown reaction.

Special fluorescent mRNA damage staining

The mRNA damage was analyzed by special fluorescent staining. In brief, the sections were cut by cryostat microtome (8 μ m, Heidelberg HM5000M, UK), fixed by ethanol (90-70%, every 15 minutes), transferred to acetic acid (1%, 30 seconds) and consequently, washed with distilled water. Next, the slides were incubated with acridine-orange (3 minutes), destained in PBS (pH=6.8,

3 minutes), and differentiated for fluorescent color in calcium chloride (25).

Western blot analysis

The western blot technique was conducted as previously conducted (19). In brief, the testicular samples were homogenized using RIPA lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich S8820, USA). The extracted protein's concentration levels were evaluated based on the Lowry method (26). The extracted protein was diluted, heated at 95°C (5 minutes), and electrophoresed on sodium dodecyl sulphate-polyacrylamide (SDS-PAGE, 120 V), continued with a polyvinylidene fluoride (PVDF) membrane (at 100 V for 1-2 hours). The 5% non-fat milk buffer (overnight) was used to incubate the PVDF membrane in order to block endogenous peroxidases. After washing the membranes with Tris-buffered saline (pH=7.2, containing 0.1% Tween 20, x3, 15 minutes each time) the membranes were incubated with anti-Hsp70, anti-Hsp90, and β -Actin (at 4°C, E-AB-40208, E-AB-10353, E-AB-40338) antibodies for 2 hours. After removing the unbound antibodies, the membranes were incubated for 1 hour (at room temperature) with the HRP-conjugated secondary antibody (E-AB-1003). Finally, the blots were visualized using an enhanced chemiluminescence detection kit (ECL, Thermo Scientific, USA). The protein intensity was evaluated using enhanced laser densitometer software (Arash-Teb-Pishro, Iran).

RNA isolation and cDNA synthesis

The total mRNA of testicular tissues was isolated using TRIZOL (Invitrogen, Carlsbad, California, USA) and chloroform (per ml of TRIZOL reagent). Then, the mixture was incubated at room temperature (5 minutes), centrifuged (12000 \times g, 15 minutes at 4°C) and incubated with 0.5 ml of isopropanol to extract the RNA in the aqueous phase. After precipitation at room temperature (10 minutes), the RNA pellets were separated by centrifugation (12000 \times g at 4°C), and thereafter washed with 70% chilled ethanol, centrifuged (7500 \times g, 5 minutes at 4°C) and air-dried. Finally, the extracted RNA was dissolved in RNase free water. The extracted RNA's concentration and quality were evaluated by measuring the absorbance ratios at 260 nm and 260/280=1.8-2.0. The 20 μ l reaction mixture of cDNA was synthesized using commercial cDNA easy synthesis kit (Pars tous, Iran).

Primer design and real-time quantitative reverse transcription assay

A primer set was designed for each gene. Briefly, the Multiple alignment program for amino acid or nucleotide sequences (MAFFT), version7 (<https://mafft.cbrc.jp/alignment/server/>) was used to align the sequences for each target gene, including

Hsp70-

F: ACCGTGGAGCCCGGGAGAAG
R: TTGGTGGGGATGGTGGAGTTG

Hsp90-

F: TGGACAGCAAACATGGAGAG
R: TGTAACCCATTGTTGAGTTGTCT

GAPDH-

F: GCAAGAGAGAGGCCCTCAG
R: TGTGAGGGAGATGCTCAGTG

and consequently, the primers were designed as explained previously (27). The NCBI BLAST software was used to confirm the specificity of the designed primer sequences for each gene. To determine the expression level of genes at different experimental conditions, diluted cDNAs were used for qPCR assays in triplicate.

Each qPCR reaction contained 0.5 μ l of 5-10 ng cDNA template, 10 μ l from 1X SYBR GREEN master mix (High ROX, Noavaran Teb-Beinolmelal, Iran), and 0.5 μ l from each reverse and forward primers. PCR condition was designed as; denaturation: 95°C for 5 minutes (1 cycle), followed by 45 cycles of 95°C (20 seconds); annealing temperature (60°C, 30 seconds); elongation: 72°C (1 minute) and final elongation: 72°C (5 minutes). Moreover, in order to check the product size, the products were transferred and resolved on 2 % agarose gel. The means generated from three threshold cycle (CT) values for each sample was normalized by the mean CT value of GAPDH for the same sample, as the internal control gene (ICG), and the relative expressions were determined using the equation: $2^{-(Ct_{target} - Ct_{ICG})}$ as explained previously (27).

Protein peroxidation

To assess the protein peroxidation ratio, the carbonyl contents of testicles were evaluated. For this purpose, the tissue homogenates were prepared, then 2, 4- dinitrophenylhydrazine (DNPH) and protein carbonyls reaction was evaluated (28). In brief, a chilled phosphate buffer containing 1 mM EDTA (50 mM, pH=6.7) was used to homogenize 0.1-0.2 g of the samples, then centrifuged at 9300 g (10 minutes at 4°C), and 0.2 ml of supernatant from each individual sample was set as test and a control samples. Each set was incubated with 0.8 ml of DNPH and 2 M HCl solution. The samples then were incubated in a dark room (1hr at room temperature) and 0.5 ml of trichloroacetic acid (30%) was included into the tube and mixed by vortexing (30 seconds). Following centrifugation (9300 g for 3 minutes), the supernatant of samples was collected, resuspended in 1 ml of (1:1) ethanol/ethyl acetate solution (15 minutes) and then, the supernatant was removed after centrifugation. Next, the precipitates were dissolved with 0.6 ml guanidine hydrochloride solution (15 minutes at 37°C). For each sample, the optical density (OD) against 6 M guanidine hydrochloride solution was recorded at 370

nm. The carbonyl content was determined as follows: Carbonyl (nmol/ml)=[(CA)/(0.011 mm)] (600 μ l/200 μ l); where CA: corrected absorbance and computed as the average OD for each control sample was subtracted from average OD of the test sample at 370 nm. The extinction coefficient for DNPH at 370 nm was 2200 M⁻¹ cm⁻¹. The protein levels were measured at 280 nm in each sample to determine the carbonyl content per mg of protein.

Testicular total antioxidant capacity

In order to evaluate the tissue total antioxidant capacity (TAC) level, the testicles were weighed, and 0.6 gr of each testicular tissue was homogenized using 50 mM PBS (pH=7.4). The testicular TAC level was evaluated based on ferric reduction antioxidant power (FRAP). The tissue TAC level was recorded at 593 nm, and the testicular TAC level was presented as nmol/mg protein (29, 30).

DNA laddering

The DNA fragmentation was assessed by the laddering test. To extract necessary DNA content, the commercial kit (Sinaclon, Iran) was used. Inline, 35 mg of testicular tissue was transferred to 1.50 μ l microtube, protease buffer (100 μ l) was included in the tube, and thereafter, incubated at 55°C (2 hours). Following complete homogenization, 100 μ l of samples were collected and moved into a new microtube, containing of lysis solution (400 μ l) and precipitation solution (300 μ l, isopropanol based) and vortexed (5 minutes), and continued with centrifugation (12,000 g, 10 minutes). The supernatant was discarded and the pellets were mixed with 1 ml ethanol (5 seconds) and centrifuged [12,000 g, 5 minutes (twice)]. The remained pellet was dried at 65°C (5 minutes). Following final centrifugation (12000, 30 seconds), the residual pellets and supernatant containing DNA remained. Then, the DNA was collected and after analyzing the DNA's quality and concentration/ μ l, the eluted DNA (15-17 μ l of eluted DNA) was transferred and run on the 1% agarose gel for 70 minutes at -70 V constant voltage. The electrophoresis gel was stained with ethidium bromide and visualized by Gel Doc 2000 system (ATP, Tehran, Iran).

Photographs, image presentation, and statistical analyses

The samples of the mRNA staining technique were analyzed by a fluorescence microscope (Nikon, Japan), equipped with an ApoTome optical-sectioning device and SONY on-board camera (Zeiss, Cyber-Shot, Japan), using a filter sets of Ex 538-562 nm, Em 570-640 nm for DsRed. A SONY onboard camera (Zeiss, Cyber-Shot, Japan) was used to capture the light microscopic images. The images were resized, reprocessed, and presented using the Adobe Photoshop CS10 software (Adobe System Inc., Mountain View,

CA, USA). To reduce the examination errors and investigate the Hsp70⁺ and Hsp90⁺ reactions in the IHC-stained sections, pixel-based intensities of positive brown reactions (representing the target proteins) were reanalyzed in photomicrographs in 20 random microscopic visions (500 μm×500 μm) by using an Image pro-insight software (version: 9.00). To check the normality and homogeneity of data, the Kolmogorov-Smirnov and Levene's tests were considered, respectively. The one-way ANOVA with the appropriate post hoc (Tukey's multiple comparisons) was considered by SPSS software (version 11.00, California, USA) to statistically analyze the quantitative histological and molecular data. A P<0.05 was reported as a statistical difference of data and the results were reported in mean ± SD.

Results

General histological and biochemical findings

In order to check DM in different groups, the blood glucose levels were assessed every week until the 6th week. The animals in insulin-treated (ISDM and EIDM) groups represented a remarkable decrement in the blood glucose level compared to other experimental groups (Fig.2A). The animals in EDM, ISDM, and EIDM groups represented a significant amelioration in DM-induced pathogenesis. To evaluate histological alterations, Johnson's score was considered. Light microscopic analyses exhibited a significant (P<0.05) decrement in the score of the SDM group in comparison with the Con and E groups (Fig.2B). The highest Johnson score was observed in the EIDM group versus those in the EDM and ISDM rats. Seminiferous tubules atrophy, edema in connective tissue, germ cells dissociation, and tubular deformation were observed in the SDM group. No histopathological alterations were revealed in both E and Con groups (Fig.2C).

Hsp70-related changes

The Hsp70-related changes were examined using qRT-PCR, IHC, and western blot techniques. The results of qRT-PCR represented a significant (P<0.05) decrement in the level of Hsp70 mRNA for SDM animals in comparison with those animals in the Con and other experimental groups. However, the rat in the E, EDM, and ISDM groups showed a remarkable (P<0.05) enhancement in Hsp70 mRNA level versus the Con animals. The animals in the EIDM group showed no significant (P>0.05) changes in the level of Hsp70 mRNA compared to the Con group (Fig.3A, B). According to the IHC analyses, the SDM group represented a diminished percentage of Hsp70⁺ cells, while the EIDM group showed no significant changes compared to the Con group (Fig.4A, B). To minimize the examination errors, the pixel-based intensity analysis of Hsp70⁺ was conducted. The results from software analyses confirmed the quantitative data of

Hsp70⁺ cell percentages. Accordingly, the pixel-based intensities for Hsp70 were increased in E, EDM, ISDM groups compared to the Con, SDM, and EIDM animals (Fig.4C). Similar results were obtained from western blot analyses. The animals in the E, EDM, ISDM groups exhibited a significant enhancement in Hsp70 protein contents, and the animals in, the EIDM group showed no statistically different result versus the Con animals (Fig. 4D, E).

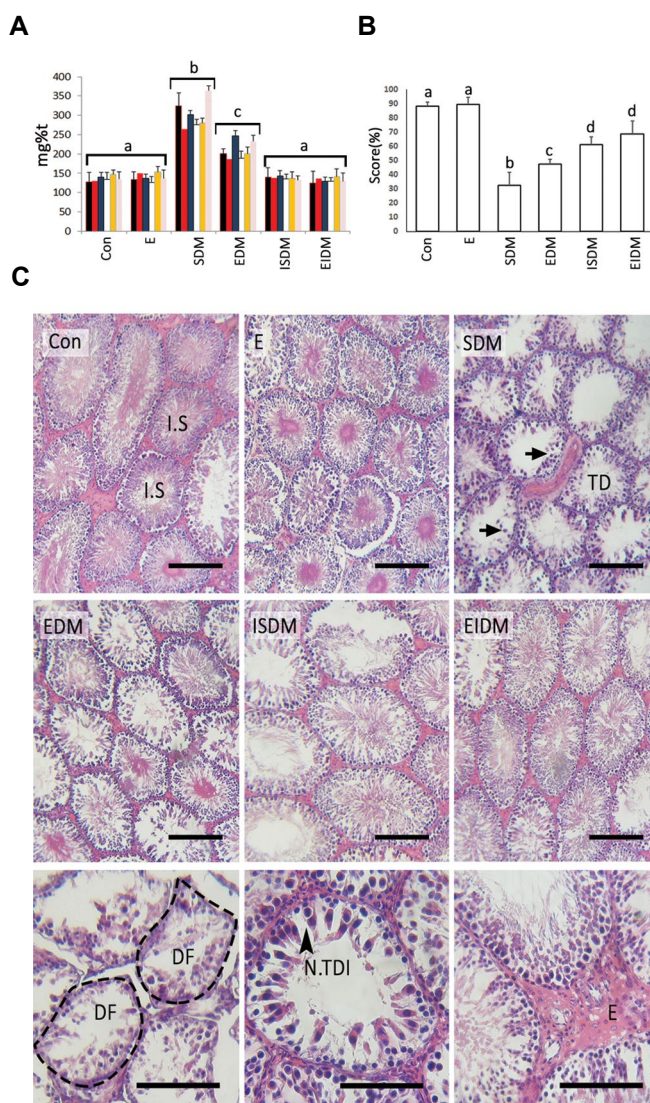


Fig.2: Histomorphometric changes and serum glucose levels. **A.** Mean blood glucose level in different groups, all data are presented in mean ± SD. Different letters are representing significant statistical differences, a vs. b, c, d; P<0.001, b vs. c; P<0.02, b vs. d; P<0.01, c vs. d; P<0.01 (n=6 rats in each group), **B.** Johnson's scores of different groups, **C.** Cross sections of seminiferous tubules in different groups. See intact spermatogenesis (I.S) in the control group, tubular depletion (TD) and germ cell dissociation (arrows) in SDM group, and ameliorated experimental diabetes type 1-induced histological changes in different experimental groups. The cross section of EIDM is representing the histological structure close to control and exercise-sole (E) group. The cross sections with higher magnification are representing deformed seminiferous tubules (DF), negative tubular differentiation (N.TDI, head arrow) and edema in the connective tissue of SDM group (H&E staining, scale bars: 150 μm). Con; Control, SDM; Sedentary type I DM-induced, E; Exercise training-sole without DM, EDM; Exercise training DM-induced, ISDM; Insulin-treated sedentary DM-induced, and EIDM; Exercise training insulin-treated DM-induced.

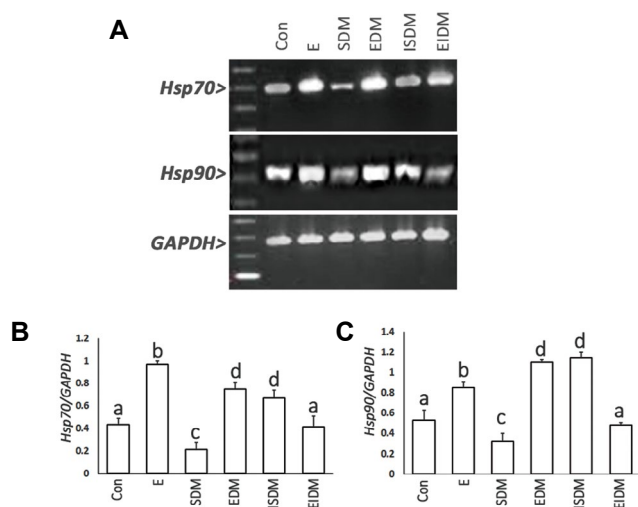


Fig.3: mRNA levels of *Hsp70* and *Hsp90*. **A.** Photomicrograph of PCR electrophoresis for *Hsp70*, *Hsp90* and *GAPDH* in different groups, **B.** mRNA levels of *Hsp70*, **C.** *Hsp90* in different groups, all data are presented in mean \pm SD. Different letters are representing significant statistical differences, a vs. b; $P < 0.001$, a vs. c; $P < 0.03$, a vs. d; $P < 0.01$, b vs. c; $P < 0.001$, b vs. d; $P < 0.02$, c vs. d; $P < 0.001$ ($n = 6$ rats in each group), Con; Control, SDM; Sedentary type I DM-induced, E; Exercise training-sole without DM, EDM; Exercise training DM-induced, ISDM; Insulin-treated sedentary DM-induced, EIDM; Exercise training insulin-treated DM-induced, and PCR; Polymerase chain reaction.

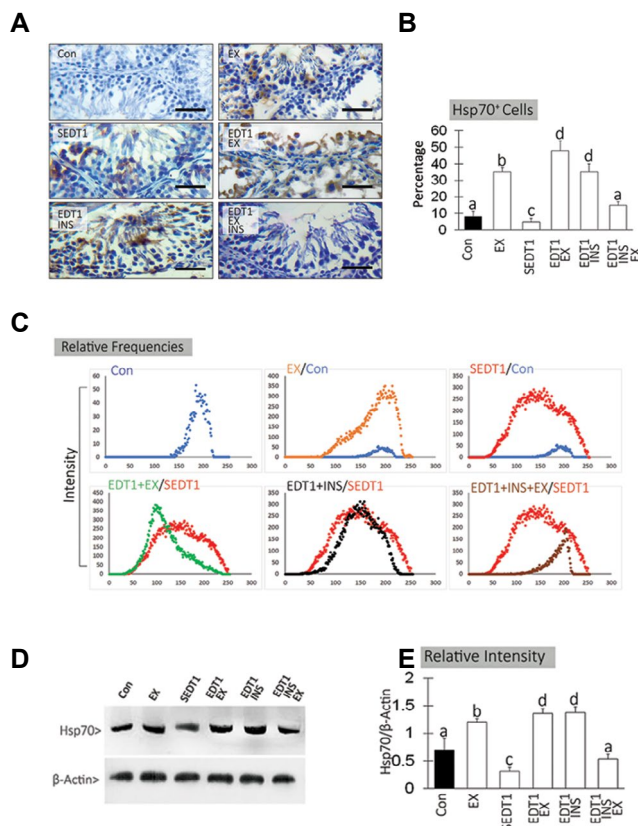


Fig.4: Effect of MEXT and insulin on *Hsp70* protein expression. **A.** Immunohistochemical staining for *Hsp70* (scale bar: 50 μ m), **B.** Mean percentages of *Hsp70*⁺ cells in different groups, **C.** Software analyses for pixel-based intensity of positive reaction for *Hsp70* versus control group, **D.** Western blot analysis for *Hsp70* and **E.** Mean intensities for *Hsp70* relative to β -Actin in different groups, all data are presented in mean \pm SD. Different letters are representing significant statistical differences, a vs. b; $P < 0.01$, a vs. c; $P < 0.02$, a vs. d; $P < 0.01$, b vs. c; $P < 0.001$, b vs. d; $P < 0.04$, c vs. d; $P < 0.001$ ($n = 6$ rats in each group), Con; Control, SDM; Sedentary type I DM-induced, E; Exercise training-sole without DM, EDM; Exercise training DM-induced, ISDM; Insulin-treated sedentary DM-induced, and EIDM; Exercise training insulin-treated DM-induced.

Hsp90-related changes

Similar to *Hsp70*, the relative changes in expression levels of *Hsp90* were investigated using qRT-PCR, IHC, and western blot analyses. The SDM group exhibited a significant decrement in *Hsp90* mRNA level (Fig.3A, C). However, the animals in the E, EDM, and ISDM groups showed a significant enhancement in the mRNA level of *Hsp90* versus the Con group. No remarkable difference was demonstrated between the EIDM and Con groups ($P > 0.05$). The same pattern was demonstrated for the *Hsp90*⁺ cell percentage and *Hsp90* protein contents in comparison with other groups. The pixel-based intensity analysis was conducted to minimize the visual errors of IHC staining. The animals in the E, EDM, and ISDM groups presented a significant increment in the pixel-based intensity of brown reactions (marking the *Hsp90* protein) versus the Con group (Fig.5).

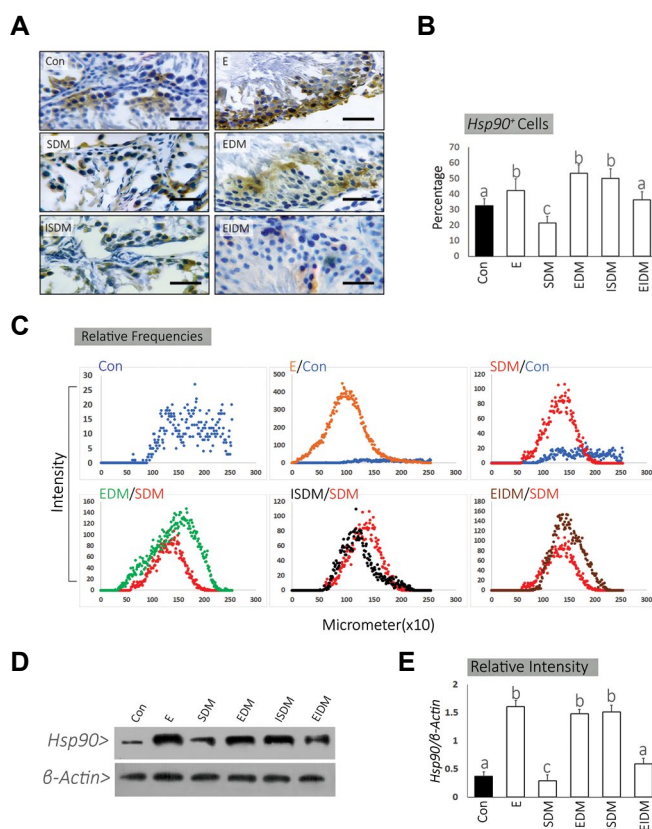


Fig.5: Effect of MEXT and insulin on *Hsp90* protein expression. **A.** Immunohistochemical staining for *Hsp90* (scale bars: 50 μ m), **B.** Mean percentages of *Hsp90*⁺ cells in different groups (positive reactions are presented in brown), **C.** Software analyses for pixel-based intensity of positive reaction for *Hsp90* versus control group, **D.** Western blot analysis for *Hsp90* and **E.** Mean intensities for *Hsp90* relative to β -Actin in different groups, all data are presented in mean \pm SD. Different letters are representing significant statistical differences, a vs. b; $P < 0.001$, a vs. c; $P < 0.01$, b vs. c; $P < 0.03$ ($n = 6$ rats in each group), Con; Control, SDM; Sedentary type I DM-induced, E; Exercise training-sole without DM, EDM; Exercise training DM-induced, ISDM; Insulin-treated sedentary DM-induced, and EIDM; Exercise training insulin-treated DM-induced.

Relative changes in mRNA content and damage

Fluorescent microscope analyses showed remarkable damage for mRNA in the SDM group. No damage was observed in mRNA of the E and Con groups. The animals in EDM, ISM, and EIDM groups exhibited

ameliorated mRNA damage (Fig.6A). To minimize the examination errors, the pixel-based intensity of mRNA damage was performed, as well. The lowest and highest pixel-based intensity for red fluorescent reaction (representing intact mRNA) was revealed in SDM and EIDM groups, respectively (Fig.6B).

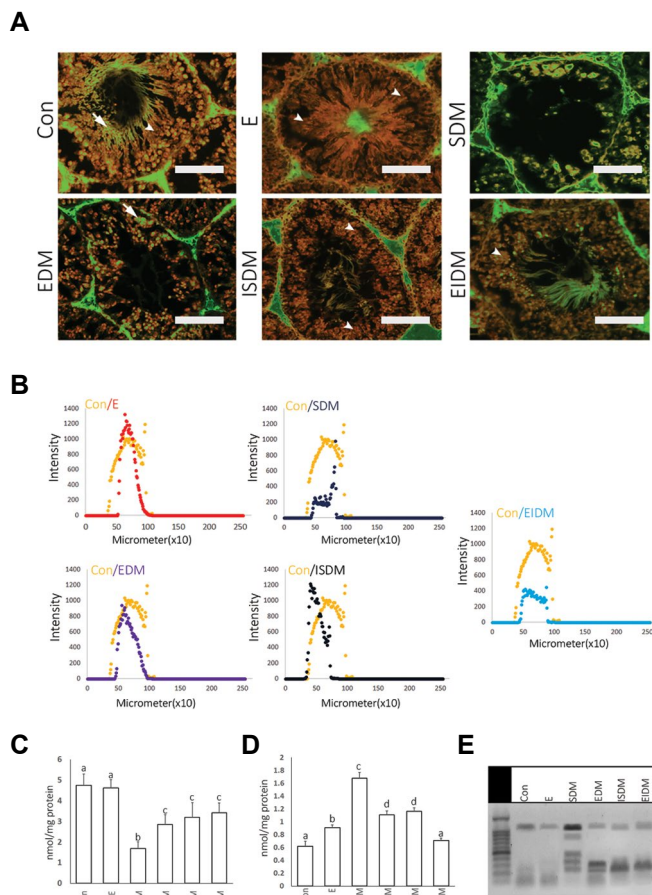


Fig.6: Effect of MEXT and insulin on testicular mRNA and DNA damage and biochemical changes. **A.** Special fluorescent staining for mRNA damage: the cells with intact mRNA are presented with red fluorescent reaction (head arrow) and the DNA is presented in light green fluorescent (head arrow). See the cross section from sedentary experimental diabetes type 1-induced (SDM) group for faint red reaction and ameliorated cells in moderate-intensity exercise (E, EDM), insulin (ISDM) and moderate-intensity exercise+insulin-treated groups (EIDM), (scale bar: 50 μ m). **B.** Pixel-based intensity analyses for red fluorescent reaction of different groups versus the control group, **C.** Mean changes in testicular total antioxidant capacity (TAC) and **D.** Carbonyl groups in different groups, all data are presented in mean \pm SD. Different letters are representing significant statistical differences, a vs. b; $P < 0.001$, a vs. c; $P < 0.03$, a vs. d; $P < 0.03$, b vs. c; $P < 0.02$, b vs. d; $P < 0.04$, c vs. d; $P < 0.01$ ($n = 6$ rats in each group), **E.** DNA ladder test for DNA fragmentation: The lane of experimentally-induced diabetes type 1 (SDM) represents severe DNA damage which is significantly diminished in moderate-intensity exercise training and insulin-treated groups. Con; Control.

Total antioxidant capacity and protein peroxidation

Biochemical analyses exhibited a significant ($P < 0.05$) decrement in testicular TAC level of SDM animals versus those in the Con and other experimental groups. However, excluding the Con and E groups, as controls, the animals in EDM, ISDM, and EIDM groups showed remarkably ($P < 0.05$) higher TAC levels versus SDM animals (Fig.6C). The SDM group showed intensive protein peroxidation,

which was significantly ($P < 0.05$) decreased in EDM, ISDM, and EIDM groups. No statistically ($P > 0.05$) difference was revealed between the EIDM and Con groups (Fig.6D).

DNA damage

In order to analyze the ameliorative effect of MEXT and insulin on diabetes-induced DNA damage, the DNA ladder test was performed. The SDM rats represented significant DNA damage, which was not revealed in Con and E groups. The EDM, ISDM, and EIDM groups represented diminished DNA damage versus the SDM group (Fig.6E).

Discussion

Further to several roles of heat shock proteins in different cell types, they are specifically involved in protein and DNA-related homeostasis, including DNA folding and refolding processes, protein-related translation, translocation, and refolding of denatured proteins (12, 31). Although the Hsp70 and Hsp90 are referred to as “heat shock protein”, the oxidative and nitrosative stresses activate these proteins expressions in various tissues such as testicles. Indeed, diabetes-related oxidative stress is shown to interfere with various biological interactions in germ cells during spermatogenesis, by inducing severe damages at cellular DNA, RNA, and protein backbones (8, 32, 33). On the other hand, MEXT is shown to promote the antioxidant statuses of different tissues. For instance, the healthy subjects, who regularly practice moderate, represent better fertility markers, such as sperm parameters, and exhibit amplified antioxidant capacity, at the same time (15, 18, 34). Considering the aforementioned facts and minding the crucial role of Hsp70 and Hsp90 in maintaining cellular DNA and protein contents, here in the current study the MEXT-induced effects (solely and simultaneous with insulin) against DM-induced histological and molecular damages were investigated by focusing on the Hsp70 and Hsp90 expression levels.

Our findings showed that DM, significantly, diminished the Hsp70 and Hsp90 expression levels in SDM animals compared to the Con group. In contrast, the animals in EDM and ISDM groups represented increased Hsp70 and Hsp90 levels versus the SDM group. Indeed, two major suggestible hypotheses can be considered for this outcome. First, the DM resulted in a severe cellular depletion in the seminiferous tubules. The MEXT and insulin (in EDM and ISDM groups) could also maintain the testicular cellular population. Thus, due to higher cellularity, the EDM and ISDM groups represented higher Hsp70 and Hsp90 contents. Second, the DM could reduce the level of Hsp70 and Hsp90 resulted in severe DNA and protein damage (marked with DNA ladder and protein peroxidation tests) which is consequently may result in intensive cell loss in testicular tissue. In contrast, the MEX and/or insulin, in EDM and ISDM groups, could recover/maintain cellular DNA and protein contents via up-regulating the Hsp70 and Hsp90 expression levels. To estimate the first hypothesis (cellular population), Johnson’s score was evaluated. As expected, the animals

in the SDM group represented the lower score compared to EDM and ISDM groups. This finding clearly shows the impaired cellular development, as well as cellular depletion in the SDM group. Thus, it is logical to conclude that MEXT and insulin in sole forms (in EDM and ISDM groups) could fairly maintain/protect the cellular population, and because of the higher cellular population in the testicles, we found higher Hsp70 and Hsp90 protein contents.

Regarding the second hypothesis in this study, it has been clearly revealed simultaneously diminished DNA damage and protein peroxidation ratios in EDM and ISDM groups due to the high level of Hsp70 and Hsp90. It means that MEXT and insulin, when considered solely, maintain the cellularity of testicles by forcing the Hsp70 and Hsp90 expressions. Through this mechanism, they would maintain the cellular DNA and protein contents. As a natural result of the last event, the testicles exhibit a higher cellular population. However, with a little more precision on the results, it comes clear that the Hsp70 and Hsp90 expression levels in EDM and ISDM groups were higher than that of the control animals, and the EIDM group (groups with simultaneous consideration of MEXT and insulin) represented no significant difference in comparison with the Con animals. Based on the aforementioned hypothesis, it could be suggested that the MEXT and insulin in sole forms may enhance the Hsp70 and Hsp90 level of expressions even more than the sedentary Con group, while it remains, why their combination exhibits an opposite outcome. To understand the subject, one should note that the expression levels of different heat shock proteins (especially Hsp70 and Hsp90) in different tissues alter depending on sex (35), age (36), type of tissue, intensity, and condition of exercise training (37). For instance, heavy physical activities/exercises increase the ROS generation in different tissues and high ROS generation consequently is able to promote the Hsp27, Hsp60, Hsp70, Hsp90 expressions (38). Moreover, MEXT and insulin are shown to amplify the testicular HSPs expression, despite their effect on antioxidant status.

On the other hand, DM (especially in long-term conditions) is shown to negatively impact the balance between antioxidant status and ROS generation in testicular tissue (8, 33). Thus to answer this question, we had to consider the oxidative stress, addition to Hsp70 and Hsp90 expressions. To assay this subject, the testicular TAC level was evaluated as an indirect marker of oxidative stress. The result showed that in the DM-sole condition (in the SDM group), the testicular TAC was decreased versus the Con animals, while in EDM and ISDM groups, although it was lower than Con group, the TAC levels were higher versus the SDM animals. It means the lower TAC in SDM animals could be the result of oxidative stress production (due to withdrawal of antioxidant agents and/or imbalance between antioxidant elements/free radicals). Besides, the higher TAC ratios in EDM and ISDM groups were demonstrated could be because of the compensatory

effects of MEXT and insulin, which showed the TAC data higher versus SDM animals. In other words, the high level of ROS generation in EDM and ISDM groups could be control by MEXT and insulin sole effects in promoting the antioxidant defense system and Hsp70-90 related homeostasis reactivity (showed by increased Hsp70 and Hsp90). Regarding the EIDM group, the story seems to be completely different, which can be considered as a limitation for this study. This group represented TAC approximately close to that in EDM and ISDM, while it presents lower Hsp70 and Hsp90 expression levels versus EDM and ISDM groups but close to that of the Con group. Indeed, the animals in the EIDM group showed lower DNA, RNA, and protein damages versus EDM and ISDM groups. Minding the independent changes in Hsp70 and Hsp90 expressions despite TAC level, the DNA, RNA, and protein integrity could influence the Hsp70 and Hsp90 expression more effectively rather than any changes in TAC ratio. Because, these animals (in the EIDM group) represented lower DNA, RNA, and protein damages versus EDM and ISDM groups, and at the same time, exhibited lower Hsp70 and Hsp90. In line with this conclusion, the damaged germ cells with denatured/misfolded proteins are shown as the main stimulators of HSPs expression in the testicular microenvironment (39). Moreover, the Hsp70, more effectively, and Hsp90 with lower impact, assemble/fold the special RNA-binding proteins involving in the mRNA stability for up to 7 days in haploid cells (40).

Conclusion

Data in this study revealed that MEXT can up-regulate the expression of Hsp70 and Hsp90 in sole from (without diabetes), and when it is considered with diabetes. However, this up-regulation was much higher, even more than Con rats, when it was considered without diabetes. Moreover, MEXT without diabetes and solely in intact rats, exerts no effect on testicular TAC, while enhances the protein peroxidation ratio. In contrast to its sole effect in normal animals, MEXT may enhance the testicular TAC and down-regulate the protein peroxidation ratio in diabetic rats. About MEXT-induced effect simultaneous with insulin, it was shown that it could amplify the insulin-induced ameliorative effects mainly by maintaining the cellular DNA, RNA, and protein contents as well as stabilizing the expression of the Hsp70 and Hsp90.

Acknowledgments

The authors wish to thank the Department of Exercise Physiology and Corrective Exercises, Faculty of Sport Sciences, Urmia University and Departments of Basic Sciences and Faculty of Veterinary Medicine, Urmia University for scientific supports, and deeply appreciate RASTA Co. for laboratory and technical bits of help. This study has been financially supported by Urmia University and there are no conflicts of interest between the authors.

Authors' Contributions

J.T.A., M.R., Z.S.; Participated in study design, data collection and evaluation, drafting, and statistical analysis. Z.S., J.T.A., A.T.; Performed exercise training intervention of the study. M.R., J.T.A., A.T.; Contributed extensively in the interpretation of the data and the conclusion. H.R.; Conducted molecular experiments and RT-qPCR analysis. All authors participated in the finalization of the manuscript and approved the final draft.

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