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Forskolin Improves Male Reproductive Complications Caused by Hyperglycemia in Type 2 Diabetic Rats

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Abstract

Background: In many diabetic patients, spermatogenesis complications are frequent causing infertility problems. This study aimed to demonstrate the effect of Forskolin on male reproductive dysfunction caused by type 2 diabetes.

Materials and Methods: In this experimental study, type 2 diabetes was induced by a high-fat diet (HFD) for one month and then a low single dose injection (35 mg/kg) of streptozotocin (STZ) in Wistar rats. After 72 hours, rats with more than 200 mg/dl of blood glucose were considered type 2 diabetic rats. Forty rats (200-250 g) were divided into four groups (n=10) including group 1 (G1): rats with normal diet and buffer citrate (STZ solvent) injection, group 2 (G2): control type 2 diabetic rats with HFD and STZ injection, group 3 (G3): type 2 diabetic rats received phosphate buffer saline (PBS) as Forskolin solvent, and group 4 (G4): Forskolin treated diabetic rats (10 mg/kg) for 1 month.

Results: In comparison to control group, in diabetic groups (G2 and G3) some parameters are increased significantly: The blood glucose (P=0.00078), testicular malondialdehyde (MDA) level and body weight (P=0.00009) and *Bax* gene expression (P=0.00007). Unlike, some parameters are decreased significantly: The serum level of testosterone (P=0.0009), testicular superoxide dismutase (SOD, P=0.00007) and glutathione peroxidase (GPX) levels (P=0.00008), sperm concentration (P=0.00008), motility (P=0.00009), normal morphological sperm (P=0.00008) and *Bcl-2* gene expression (P=0.00009). However, in Forskolin treated group (G4) the parameters stayed close to control values that was significantly (P=0.00007) higher than in G2 and G3 groups. Therefore, treatment with Forskolin significantly improved these abnormal changes in Forskolin-treated group.

Conclusion: Our study demonstrates that Forskolin is an effective antidiabetic agent, which significantly improves sperm concentration, testosterone levels, and antioxidant activity in diabetic rats.

Keywords: Forskolin, Male Infertility, Oxidative Stress, Testicular Dysfunction, Type 2 Diabetes

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Introduction

Diabetes mellitus is an endocrine and metabolic disorder. There are two general types of diabetes: type 1 and type 2 diabetes. Type 2 diabetes accounts for almost 90% of all cases accompanying by hyperglycemia. Hyperglycemia is caused by the destruction of beta cells or insulin resistance of cells, which in general, causes complications such as inflammatory issues, oxidative stress, and obesity. Statistics show that there are more than 415 million diabetic people in the world, which is predicted to approximate 642 million by 2040 (1, 2). It is also more prevalent in men than women (3).

Complications of type 2 diabetes include retinopathy, nephropathy, neuropathy, cardiovascular problems, and reproductive disorders. Type 2 diabetes is often associated with decreased levels of testosterone (T) and gonadotropin-releasing hormones (GnRH) (4). Diabetic testicular disorders include decreased spermatogenesis, testicular germ cell destruction, decreased testosterone and estradiol synthesis, and abnormal semen parameters; they generally lead to diminished reproductive ability or even infertility. It has been shown that reactive oxygen species (ROS) are the main destructive players in hyperglycemia of type 2 diabetes. Prolonged hyperglycemia could produce

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large amounts of ROS, which can upset the balance of the antioxidant system in the body. Following the oxidative stress, testicular germ cells become apoptotic and the spermatogenesis is disrupted (5, 6). Therefore, one of the main therapeutic approaches in diabetic patients is reducing ROS using antioxidants. In this regard, the application of herbal and natural antioxidants has been well established (7). Forskolin, an herbal medicine, is extracted from an Indian plant called Coleus forskohlii. The chemical formula of Forskolin is $C_{22}H_{34}O_7$ and it has been used to treat and prevent cancers, cardiovascular diseases, asthma, glaucoma, obesity, and high blood pressure (8). Interestingly, Forskolin has also documented as an effective antidiabetic drug that increases insulin production from pancreatic beta cells and consequently decreases blood sugar levels (9). It has been demonstrated that cyclic AMP (cAMP) is the mediator that makes Forskolin a highly potent medication (10). In this regard, it has been reported that Forskolin-induced cAMP could reduce cytotoxicity and apoptosis. According to In vitro studies, Forskolin protects cells against H_2O_2 by increasing the antioxidant levels approximately two times (11). The antioxidant effect of Forskolin in different diseases has been documented (12), but its effect on the male reproductive disorders following type 2 diabetes has not been addressed yet. It seems clarifying the potential effects of Forskolin on hormonal status, spermatogenesis, and testicular tissue of diabetic patients might open new ways for the application of this herbal medicine. Therefore, the present study aims to evaluate the potential positive effects of Forskolin on diabetes-induced male reproductive dysfunction.

Materials and Methods

Animals and study design

All steps of the experiment were conducted according to the protocols and guidelines of the Ethical Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1399.080). In this study, forty Wistar rats of 8 weeks' age and weight of 200 to 250 g were recruited. Animals were maintained under standard laboratory conditions including 50% humidity, 21-25°C temperature, and 12 hours. dark/light cycle. The animals were randomly divided into 4 groups including 10 animals in each group (n=10) as the following:

- Control group (G1): rats with a standard diet that were intraperitoneally treated with 0.5 ml of buffer citrate solution (0.05 M and pH=4.5) as Streptozotocin (STZ) solvent.
- Diabetic control group (G2): rats were fed a high-fat diet (HFD) for 4 weeks and then type 2 diabetes was induced with a single dose of 35 mg/kg STZ.
- Vehicle control group (G3): rats with type 2 diabetes that were treated with phosphate buffer saline (PBS), the solvent of Forskolin, using a gavage syringe.
- Diabetic treated group or experimental group (G4): rats with type 2 diabetes that received Forskolin (>96% pure, HPLC detected, Abcam. Cat. No. 66575-29-9) 10 mg/kg body weight/day for 30 days with a gavage syringe (13).

The HFD was continued for another 30 days after the first 4 weeks in G2, G3 and G4 groups to maintain the type 2 diabetic model. Forskolin was administrated orally to prevent other injuries such as peritoneal and hematologic infections as suggested by previous studies (9, 11, 13). The weight and blood sugar of animals in different groups were measured regularly after HFD, just before STZ injection, after induction of diabetes, just before starting the Forskolin treatment, and after Forskolin treatment.

Induction of type 2 diabetes

Groups 2, 3, and 4 were fed with an HFD consisting of 35% carbohydrates, 45% fat, and 20% protein for 4 weeks (14). At the end of the fourth week, the rats intraperitoneally received a single dose of STZ (35 mg/kg body weight) to induce type 2 diabetes (15). A single and low dose of STZ could affect some of the beta cells causing partial destruction of pancreatic beta cells which could result in type 2 diabetes development (16). The STZ solution was freshly prepared by dissolving STZ (Sigma, USA. Cat. No. S0130-1G) in 0.05 M citrate buffer solution (pH=4.5) (17, 18). To confirm diabetes development, the blood glucose was measured through tail vein 72 hours after STZ injection using a glucometer (Roche Diagnostics, Basel, Switzerland. Cat. No. 05213509001). The rats with stable blood sugar above 200 mg/dl were considered diabetic (15).

Measurement of blood glucose and body weight

In the present study, we measured the blood glucose four times: i. Before healthy rats grouping, ii. After HFD, iii. After STZ injection, iv. After treatment with Forskolin. In this way, we measured the fasting blood glucose using a glucometer (Roche Diagnostics, Basel, Switzerland. Cat. No. 05213509001). Also, we measured the body weight of rats 3 times: i. Before HFD, ii. After HFD and STZ injection, and iii. After treatment with Forskolin. The body weight was measured by digital scale (HL-200, Japan).

Sample collection

At the end of the drug-intervention period, fasting blood samples were collected from the animals, and then all animals sacrificed for sampling. During the animal sacrifice, for the minimum pain and stress in rats as well as the best sampling mode the blood samples were taken from the rats' hearts (cardiac puncture) under deep anesthesia 24 hours after the last treatment of Forskolin (Intraperitoneal, ketamine: 100 mg/kg and xylazine: 10 mg/kg) (19). Subsequently, serum samples were extracted from the blood by centrifugation (20 minutes at 3500 rpm) and kept at -80°C for antioxidant and hormonal analyses.

Semen evaluation

The caudal part of the rat epididymis was separated, chopped into smaller pieces and placed in 1.5 ml of PBS

(pH=7.2) for sperm counting (25). For this purpose, a drop of the solution containing the sperm was transferred to the Neubauer slide chambers (HBG, Germany) and the sperm were counted manually using a light microscope (Olympus CX31, Japan). The results of sperm count were reported as sperm/mL (20). To evaluate sperm motility, the number of motile sperms was counted under a light microscope (Olympus CX31, Japan) and the percentage of motile sperms (number of motile sperms/ the total number of counted sperms) calculated. Sperm motility was classified into three groups: progressive, non-progressive, and immotile (21). To examine sperm morphology, a sperm-contained droplet was placed on the slides to be air-dried and then fixed with 96% alcohol and stained with H&E. For each slide, 100 sperms were evaluated and the percentage of normal and abnormal sperms was determined (18, 22).

Hormonal measurements

Serum levels of luteinizing hormone (LH, mIU/mL), follicle-stimulating hormone (FSH, mIU/mL), and T (nmol/L) were measured by the Enzyme-Linked Immunosorbent Assay (ELISA) method using commercial kits (DRG Instruments GmbH, Marburg, Germany).

Biochemical analysis (lipid peroxidation and antioxidant enzymes)

Homogeneous testicular tissue samples were used to evaluate the levels of antioxidant enzymes and lipid peroxidation. Potassium chloride solution (1.5%) was applied to homogenized testicular tissue (26). To evaluate lipid peroxidation, the amount of malondialdehyde (MDA) was measured according to Nair and Turner's method using Biocore Diagnostik (ZellBio) MDA assay Kit. Briefly, the reaction of the testicular sample with the Thiobarbituric Acid (TBA) solution was used to measure MDA. The mixture was then centrifuged after heating, and the resulting supernatant was placed in a spectrophotometer to measure the amount of MDA (23).

To measure superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes commercial kits were used (Randox Laboratories, UK for SOD and Biocore Diagnostik, ZellBio for GPx). In brief, to measure SOD, a solution of hydrochloride, EDTA, and Triton X-100 was incubated with a homogeneous testicular sample and the amount of SOD enzyme measured as units/g protein with a spectrophotometer (24). To evaluate the amount of GPx enzyme, an enzymatic reaction with NaNO3, EDTA, glutathione reductase, cumene hydroperoxide, and NADPH was conducted. The resulting mixture was transferred to a spectrophotometer and the amount of enzyme obtained based on units/g protein (25).

Real-time quantitative polymerase chain reaction analysis

After Forskolin treatment, the samples were taken from the left testis of all rats, and the expression level of *Bax* and *Bcl-2* was evaluated. For this purpose, total RNA was extracted with TRIzol reagent solution (Invitrogen, Paisley, UK Cat. No. 15596026) (26). The RNA extraction was performed with the RNeasy Micro kit (Invitrogen Life Technologies, USA). We used DNase I enzyme to remove DNA contamination. After the determination of RNA density using an absorption ratio of 260 and 280 nm, the RNA was washed with RNase-free water and exactly adjusted to a concentration of 0.7 µg/ml. Then, the cDNA was synthesized in a volume of about 20 µl by applying a commercial Kit (Thermo Scientific, EU Cat. No. FERK1622) (27, 28).

The real-time quantitative polymerase chain reaction analysis (qRT-PCR) reaction was done in a 48-well plate. Each well contained 1 µl of each primer (forward and reverse primers), 1 µl of cDNA, 10 µl of SYBR Green, and 7 µl DNase/RNase free water (Sigma-Aldrich, Germany. Cat. No. 7732-18-5). For the thermal cycle, Biosystems (UK) sequence detection system was used according to the manufacturer's protocol. To determine the fold change expression of each gene, Pfaffl method ($2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct=\Delta Ct$ Sample-ΔCt Control) was utilized (27, 28).

Histological examination

The rats' testes were placed in Bowen's fixation solution for 48 hours and then embedded into paraffin blocks. Afterward, paraffin blocks with a thickness of 5 µm were sectioned and deparaffinized and the slides stained with H&E. A slide was taken from the top, middle, and bottom of each testicle, respectively. Then, the slides were observed under a light microscope (Olympus, Japan).

Ten round seminiferous tubules in each slide were randomly selected. Factors such as the Seminiferous Tubule Diameter (STD), the height of the germinal epithelium layer (HE), the overall shape, and internal spaces of the tubules were evaluated with image J software. To determine testicular damage, the process of spermatogenesis was examined histopathologically by the Mean Johnsen Score test (MJS) with scores from 1 to 10 (26).

Statistical analysis

The homogeneity test (Kolmogorov-Smirnov test), to check the normal distribution of data, was performed to ensure data normality. To analyze data, one-way and two-way ANOVA and also post hoc Tukey tests were performed. $P<0.05$ was considered statistically significant. Statistical analysis was performed using Prism GraphPad software (ver.7.0, Graph-Pad, San Diego, CA, USA). Data presented with mean ± SD.

Results

Blood sugar changes in different animal groups

Blood glucose was reported in the study groups in four time points, including: i. Before HFD, ii. After a HFD without STZ injection, iii. After HFD and STZ injection, and just before Forskolin treatment initiation, and iv.

After Forskolin treatment course. In G1 group, the rats had a normal blood glucose level in all four time points because of normal diet and the absence of any intervention (Fig.1A). In G2 group, a significant increase (P<0.00091) was observed in blood sugar after HFD which was noted as pre-diabetic status but not the diabetic phase. After the STZ injection, the blood sugar increased (P=0.00078) more than 200 mg/dl, and after a month, it continued to rise (P=0.00056, Fig.1A). In G4 or Forskolin treatment group, the blood glucose status was almost the same as in the G2 and G3 after HFD with slight increase (P=0.00086) but no in the diabetic range. After the STZ injection, the animals' blood sugar significantly rose again to higher than 200 mg/dl (P=0.00071). In this group, after one month of Forskolin treatment, a significant decrease was found (P=0.00016) in blood sugar which was although higher than the G1 group in the same time point, it was still very close to normal range; particularly when it was compared with the G2 at the same time that had blood sugar above 300 mg/dl (Fig.1A).

Effect of Forskolin on body weight in diabetic rats

The rats' weight was evaluated in three time points, including: i. Before HFD, ii. Three days after STZ injection (after induction of diabetes), and iii. After a one-month treatment of rats with Forskolin (after the treatment with Forskolin) (Fig.1B).

In the G1 group, there was no sharp change in the weight of rats at different time points and there was just a slight difference in the rats' weight after induction of diabetes compared to after the treatment (P=0.00032). After diabetes induction, a significant (P=0.00009) weight gain was observed in diabetic control group when compared to the healthy control group. In we also found significant weight gain (P=0.0008) after the induction of diabetes (before starting Forskolin treatment) the G4 group, but the weight of the animals decreased after treatment with Forskolin (P=0.00085) (Fig.1B).

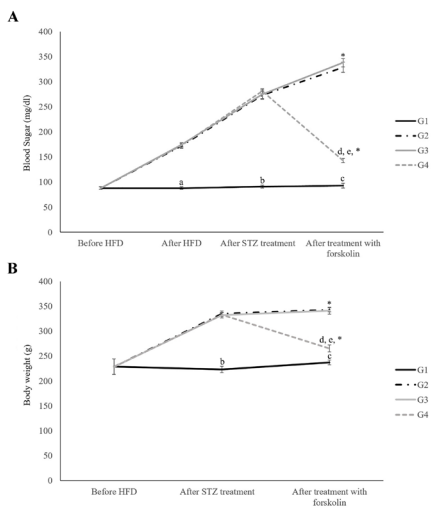


Fig.1: Effects of forskolin on blood sugar and body weight indexes in different time periods. Comparision of **A.** Blood sugar and **B.** Body weight in different experimental groups: control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin-treated diabetic group (G4). There are ten rats in each group. One-way ANOVA test was used and all values were presented as mean ± SD. Values with various superscripts are significantly different. a; indicates significant difference between the G1 with G2, G3, and G4 groups after HFD (P<0.001), b; Indicates significant difference between the G1 with G2, G3, and G4 groups after STZ treatment (P<0.001), c; Indicates significant difference between the G1 with G2, G3, and G4 groups after Forskolin treatment (P<0.001), d; Indicates significant difference between the G4 with G2 after Forskolin treatment (P<0.001), e; Indicates significant difference between the G4 with G3 after Forskolin treatment (P<0.001), and *; Indicates significant difference among different treatment of the G2, G3, and G4 groups (P<0.001), HFD; High-fat diet, and STZ; Streptozotocin.

Effect of Forskolin on sperm parameters

Figure 2 shows the sperm parameters of different study groups. Sperm concentration (×10^6 /ml) (P=0.00008), percentage of normal morphology (P=0.00087) and percentage of sperm motility (P=0.00096) decreased in the diabetic group compared to the healthy control group. The treatment of diabetic rats with Forskolin could increase the sperm concentration, normal morphology, and motility when compared to the untreated group. The difference in sperm concentration of the G1 and G4 groups decreased but it was still significant (Fig.2A, P=0.00009). The comparison of morphology and motility in the G1 and G4 groups showed a decrease in the difference and a progression toward normalization, but the difference was still significant (P=0.00093, Fig.2).

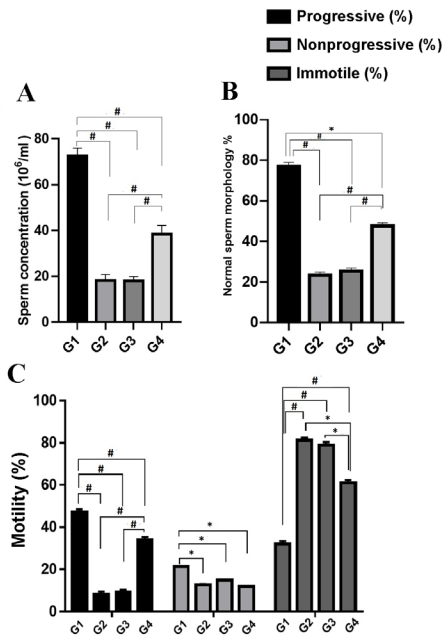


Fig.2: Effects of Forskolin on sperm parameters in different experimental groups. **A.** Sperm concentration (×10^6 /ml), **B.** Normal sperm morphology (%), and **C.** Sperm motility (%). Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin-treated diabetic group (G4). There were ten rats in each groups. One-way ANOVA test was used. Data represented as mean ± SD. Significant difference with *; P<0.001, #; P<0.0001, HFD; High-fat diet, and STZ; Streptozotocin.

Effect of Forskolin on serum levels of gonadotropins and testosterone

As shown in Figure 3, the serum levels of LH (P=0.0009), FSH (P=0.0008), and testosterone (P=0.0009) in the G2 group were significantly lower than the G1 group. administration of Forskolin in the G4 increased the levels of these hormones when comparing to the G2 group (P=0.0008). Moreover, we found significantly

higher levels of LH (P=0.0077) in serum of the G4 rats in comparison with the G1 group (Fig.3A). FSH and testosterone levels were significantly different between the G1 and G4 groups (Fig.3B, C, P=0.0009).

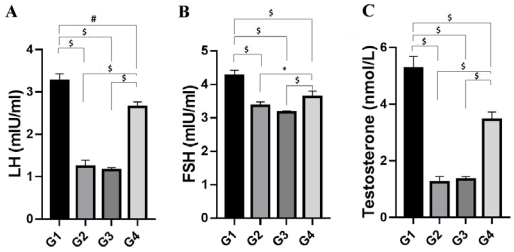


Fig.3: Effect of Forskolin on serum levels of gonadotropins and testosterone. Comparison of **A.** LH, **B.** FSH, and **C.** Testosterone hormones among different groups. Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin treated diabetic group (G4). There were ten rats in each groups. One-way ANOVA test was used. Data represented as mean ± SD. Significant difference with *; P<0.05, #; P<0.01, $; P<0.001, LH; Luteinizing hormone, and FSH; Follicle-stimulating hormone.

Effect of Forskolin on testicular levels of oxidative markers

The results are illustrated in Figure 4. Induction of type 2 diabetes (group G2) caused a significant decrease in the levels of SOD (P=0.0007) and GPx (P=0.0008) when compared to the control group (G1). On the other hand, Forskolin treatment significantly increased SOD and GPx enzymes levels when compared to the G2 (P=0.0009). Furthermore, the levels of MDA were higher in diabetic group (group G2) than the healthy group (P=0.0009). Forskolin treatment could significantly reduce MDA levels as we found significant difference in MDA levels between the G2 (P=0.0006) and G4 groups (P=0.0009, Fig.4C).

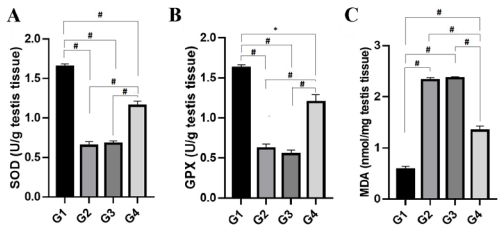
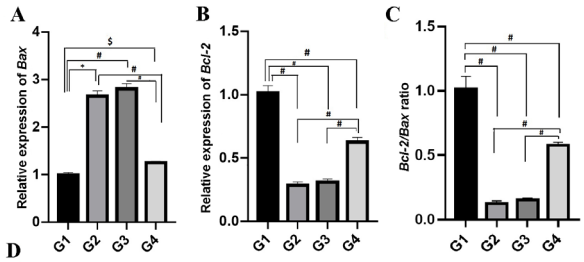


Fig.4: Effect of Forskolin on testicular levels of oxidative markers. Comparison of the **A.** SOD, **B.** GPx, and **C.** MDA levels in the testis tissue among study groups. Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin treated diabetic group (G4). There were ten rats in each groups. One-way ANOVA test was used. Data represented as mean ± SD. Significant difference with *; P<0.001, #; P<0.0001, SOD; Superoxide dismutase, GPx; Glutathione peroxidase, and MDA; Malondialdehyde.

Effect of Forskolin on the expression of *Bax* and *Bcl-2* genes

The effect of Forskolin on the expression of *Bax* (pro-apoptotic) and *Bcl-2* (anti-apoptotic) genes in testicular tissue of rats was evaluated by RT-PCR. Our results demonstrated that diabetes could cause a significant increase in mRNA levels of Bax when compared to the healthy groups (Fig.5A, P=0.00007). In addition, *Bax* expression was decreased in the G4 compared to the G2 group (P=0.0008). On the other hand, induction of type 2 diabetes reduced the expression level of *Bcl-2* gene when compared to healthy untreated rats (Fig.5B, P=0.0009). While treatment with Forskolin increased *Bcl-2* expression when compared to the diabetic rats (P=0.0009) and there was no significant difference between the G1 and G4 in the expression of *Bax* but there was significant difference between the G1 and G4 in the expression of *Bcl-2* (P=0.00007) genes.

Regarding the ratio of *Bcl-2* and *Bax* genes, the results show a significant decrease in the G2 (P=0.00007) and G3 (P=0.00007) groups, but with Forskolin treatment in the G4 group, an increase (P=0.00008) was observed in the ratio (Fig.5C). The primers sequences for *Bax*, *Bcl-2* , and *GAPDH* (housekeeping) genes are shown in Figure 5D (26).



D

Genes	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Annealing Temperature	Accession No	Product Size
BAX	TGGCAGCTGACATGTTTTCTGAC	TCACCCAACCACCCTGGTCTT	42°C	NM_017059	195 bp
Bcl-2	TCGCCCTGTGGATGACTGA	CAGAGACAGCCAGGAGAAATCA	42°C	NM_016993	134 bp
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG	42°C	NM_017008	160 bp

Fig.5: Effect of Forskolin on the expression of *Bax* and *Bcl-2* genes. The mRNA expression of **A.** *Bax*, **B.** *Bcl-2*, **C.** Their ratio in different groups as well as **D.** Applied primer sequences. Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin treated diabetic group (G4). There were ten rats in each groups. Expression level of genes (*Bax* and *Bcl-2*) were evaluated regarding the housekeeping gene (*GAPDH*). One-way ANOVA test was used. Significant difference with *; P<0.001, #; P<0.0001, and $; Non-significant.

Testicular histology and seminiferous tubular indices

Figure 6A shows histological structures of the testicular tissue in different study groups. Figure 6A1 is a testicular section from the control group demonstrating a quite regular lumen of tubules and a normal thickness of the germinal epithelium covering most diameter of the tubules. No congestion and edema were observed in this group. As shown in Figure 6B1, the tubular Lumina were irregular and the thickness of germinal epithelium was clearly declined in the diabetic control group. Figure 6A3, a section of vehicle control group, shows a reduced germinal epithelium thickness and vast tubular Lumina. The spaces between tubules indicates the presence of edema. Figure 6A4 is a section of Forskolin treated diabetic group. The thickness of the germinal epithelium and tubular Lumina in Forskolin treated group were almost similar to the control group. There was no edema and congestion between tubules.

Seminiferous tubular diameter (STD) are shown in Figure 6B. The STD in diabetic control group (G2) and vehicle control group (G3) were significantly lower

in comparison to the control group (G1, P<0.00067). In Forskolin treated diabetic group (G4), the STD was significantly lower than the control group (P=0.0091) and higher than the G2 and G3 groups (P=0.0074).

Figure 6B1 shows the thickness or height of the germinal epithelium (HE) in study groups. As the graph shows, HE in the G2 and G3 groups was significantly lower than the G1 (P=0.0009) and G4 (P=0.0008) groups. The HE in the G4 group was significantly lower than control group (P=0.0008). Figure 6B3 shows the Mean Johnsen Score (MJS) in study group. As the graph shows the MJS in the G2 and G3 groups was significantly lower than the G1 and G4 groups (P=0.0009). The MJS in the G4 group was slightly lower than the control group (P=0.043).

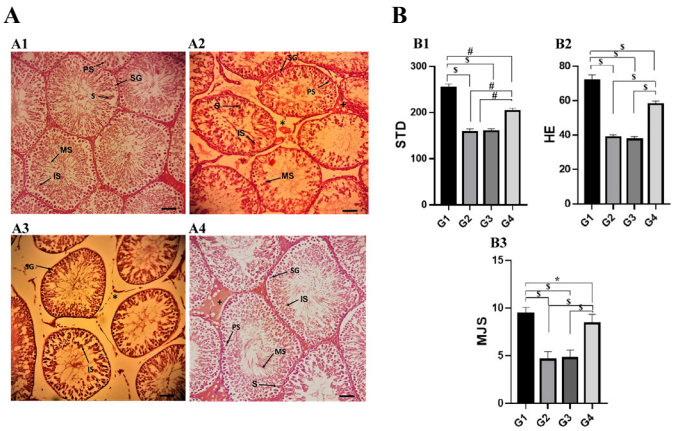


Fig.6: The histological finding in testicular sections and seminiferous tubular indices. **A.** Photomicrograph of testicular tissue sections showing morphological characteristics of seminiferous tubules in different groups. **A1.** Control group, **A2.** Diabetic control group, **A3.** Vehicle control group, and **A4.** Forskolin treated diabetic group. **B1.** Seminiferous tubule diameter (STD), **B2.** Height of germinal epithelium (HE), and **B3.** Mean Johnsen score (MJS)] in different groups. G1; Control group, G2; Diabetic control group, G3; Vehicle control group, G4; Forskolin treated diabetic group. There were ten rats in each group. One-way ANOVA test was used and data represented as mean ± SD. S; Sertoli cell, SG; Spermatogonia, PS; Primary spermatocyte, IS; Immature spermatid, MS; Mature spermatid, *; Space and +; Hemorrhage. H&E staining (scale bar: 50 µm, magnification: 400x). Significant difference with *; P<0.05, #; P<0.01 and $; P<0.001.

Discussion

To induce type 2 diabetes, rats were fed with a HFD and after a one-month feeding, low-dose STZ was injected. Diabetic rats showed: weight gain, hyperglycemia, and increased oxidative stress factors. The increase in body mass in diabetic rats was mostly due to fat accumulation of abdominal areas. Obesity and its problems is a type 2 diabetes complication (29). The body weight changes results in the present study are similar to a study conducted in 2019 that examined the therapeutic effect of Forskolin in diabetes models (30).

Diabetes-caused hyperglycemia leaves negative effects on testicular function such as decreased levels of testosterone and gonadotropins, the number of spermatogonia, Sertoli cells and Leydig, and finally reduced sperm production (31).

In a study by Singh et al. (30) by using Forskolin to evaluate its effect on the improvement of type 2 diabetic nephropathy, it was observed that Forskolin reduced blood sugar in therapeutic groups. Parallel to their study, a decrease in blood sugar levels was also observed in rats treated with Forskolin in the present study.

In another study in 2018, using Forskolin at a similar dose (10 mg/kg body weight/day) to the present study, in a diabetic model after one month with a report of blood sugar levels a significant reduction in blood sugar was reported, that is in line with the findings of the present study (9).

Type 2 diabetes-produced hyperglycemia causes an increase in ROS levels and then, inflammation through the polyol pathway. cAMP has been reported to be involved in regulating antioxidant, anti-inflammatory, and lipid metabolism activities. Increased production of cAMP due to the use of Forskolin, inhibits the activity of tumor necrosis factor (TNF)-α and nuclear factor-kappa B (NF-κB), which play role in causing tissue inflammation and ROS. NF-κB is a factor that is produced in almost all types of cells and its activation that will be due to different types of cellular stresses such as: hyperglycemia, oxidative stress, increased plasma fatty acids as a result of obesity, etc.

In the study, diabetic rats showed a significant decrease in sperm factors such as number, motility, and rapid increase in morphological abnormalities of sperm. In addition, in HFD/STZ diabetic rats, a significant reduction in the number of germ cells and destruction of the germinal layer of the tubules was observed.

Forskolin is very effective in sperm production and semen quality, in general, due to its antioxidant properties, scavenging properties of free radicals, and inhibition of lipid peroxidation. Girish and Reddy (13) found that the use of Forskolin (5 mg/kg bwt/day) as gavage in male Wistar rats of the infertility toxicity model induced by mancozeb for 65 days, increased sperm count, sperm viability, and motility.

Our studies showed that the induction of type 2 diabetes in rats significantly reduced the levels of testosterone, LH, and FSH hormones. Studies in mice with knocked out insulin receptor genes showed an association between fertility and insulin signaling. The linking mechanism behind is that the insufficient insulin in diabetic patients has a negative effect on the activity of the pituitary gland that reduces both the hormonal output of this gland and the levels of LH and FSH. The reduction of LH decreases the effect of LH on Leydig cells and consequently reduces the production of T from these cells and also reduces the production of FSH and less effect on Sertoli cell decreases the amount of sperm production (32). It has been found that one third of all men with type 2 diabetes have lower amounts than normal testosterone levels (33). Consistent with these findings, in animal models of type 2 diabetes induced by HFD/STZ and nicotinamide/STZ, the levels of LH, FSH, and testosterone hormones, sperm count, and motility were significantly reduced (34).

The positive effect of Forskolin on the process of spermatogenesis happens in two ways. First (indirect effect) is the healing effect of Forskolin on diabetes itself and the second (direct effect) is the positive effect on

the disorder in the spermatogenesis pathway caused by diabetes.

In the first case (indirect effect), Forskolin increases the level of insulin and eventually lowers blood sugar. In this pathway, by increasing the production of cAMP, due to Forskolin, cAMP probably increases the production of insulin from the protein kinase (PKA), guanine nucleotide, and beta cells viz. pathways (9).

In the second case (direct effect), the research has also shown that cAMP stimulates Leydig cells to produce testosterone hormone needless of LH hormone (35). Therefore, therapeutic use of Forskolin can change cAMP levels and thus, increase testosterone production. An investigation with the similar findings reported that the use of Forskolin increased the testosterone production in the mancozeb-induced toxicity model in rats (13).

Comparing rats in the diabetic group with rats in the healthy group revealed that the level of MDA in this group increased significantly and the amount of antioxidant enzymes (SOD and GPx) decreased sharply. The results in other studies showed the same changes (13, 36). It has been reported that the increased levels of ROS due to hyperglycemia can lead to infertility complications in diabetic males (37). When type 2 diabetic rats were treated with Forskolin, they showed an increase in the level of antioxidant enzymes (SOD and GPx) and a decrease in the level of MDA resulting in the process of spermatogenesis to resume naturally and increase in the production of testosterone. The results of the present study were similar to those research that reported the protective effect of Forskolin against ROS (13). Parallel to the present study Venkatachalapathi et al. (9) reported the similar changes in oxidative factors levels (SOD, GPx and MDA).

Hyperglycemia-produced ROS disturbs the balance of expression of pro- and anti-apoptotic genes (*Bax*, *Bcl-2*) (38). As a result, in male testes, ROS cause protein and gene damage, which causes cellular damage and subsequent cell apoptosis (39). Although our results showed a significant increase in *Bax* expression in the diabetic group when compared to the healthy control group, in the Forskolin-treated diabetic group, *Bax* levels were significantly lower than the untreated diabetic group. On the other hand, the expression of *Bcl-2* gene in the diabetic group decreased significantly when compared to the healthy group and in the treatment group with Forskolin, the expression of this gene increased compared to the untreated diabetic group.

According to the results, from a histopathological point of view, caused destructive changes in testicular tissue that can be recognized because of the disease. In G2 of diabetic rats, the diameter of the seminiferous tubules decreased sharply and their internal structure disrupted. There was also a significant decrease in mean Johnsen score for this group. These unhealthy tissue changes were greatly reduced by Forskolin treatment.

In fact, it is not possible to include all the items in one study, and according to the cases studied in this research, as well as comparing the results and data obtained from this study with that of other researches, cases such as: longer-term treatment with Forskolin, applying different doses of Forskolin, applying Forskolin in combination with other drugs, the study of specific genes involved in the process of spermatogenesis and etc. are among the items that should be considered in future studies related to the use of Forskolin in the field of spermatogenesis.

In general, type 2 diabetic rats (29, 31) showed advanced destructive tissue effects of testicular tissue and a sharp decrease in the number of spermatogonia following a decrease in the number of germ cells and a decrease in testosterone levels. Treatment of type 2 diabetic rats with Forskolin in 30 days resulted in a significant improvement in testicular tissue and sex hormones (LH, FSH and testosterone), especially testosterone, which were completely consistent with the results of other studies.

Conclusion

The present study showed that the use of Forskolin improves the sperm quality and sex hormones in diabetic rats. In addition, this study showed that Forskolin as a powerful antioxidant decreased the level of MDA and increased the level of antioxidant enzyme and antiapoptotic gene (*Bcl2*) also reduced the proapoptotic gene (*Bax*) in testis of type2 diabetic rats. Consequently, Forskolin can be considered an effective antidiabetic agent for improving sperm quality and fertility power of type2 diabetic rats.

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Authors' Contributions

M.N.; Designed the study, performed all experimental work, manuscript preparation, editing, and critical review. H.T.N., J.S.R.; Contributed extensively in interpretation of the data and the conclusion. M.S.Gh.F.; Contributed to all experimental work, and data analysis. D.M.; Supervised the study, performed acquisition, data and statistical analysis, and interpretation of data. A.G.; Monitoring the preparation and administration of drugs to animals. All authors read and approved the final manuscript.

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