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Investigation the Spermatogenesis and Testis Structure in Diabetic Rats After Treatment With *Galega officinalis* Extract

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Abstract

Objectives: This study aimed to evaluate the anti-oxidative potential of *Galega officinalis* extract on oxidative damages in the testes and sperm parameters of diabetic rats.

Materials and Methods: In this experimental study, 32 male Wistar rats were segregated in 4 groups: Control, Diabetic control, Diabetic treated with *G. officinalis* extract and healthy group that received *G. officinalis* extract. An instillation of distilled water was performed in the control and diabetic groups. Also, treatment groups received *Galega* extract (50 mg/kg body weight) for 8 weeks. After treatment period all of subjects were anesthetized, their blood samples were taken, the serum level of insulin and glucose were measured then the testicles and epididymis were removed and sperm parameters and oxidative stress markers were assessed.

Results: Treatment of diabetic rats with *G. officinalis* extract significantly increased the Johnson score and diameter of seminiferous tubule as well as reduced the glucose plasma levels ($P=0.001$) and increased the insulin levels ($P=0.001$). Furthermore, during diabetes an upsurge in the level of malondialdehyde (MDA) and a decrease in the levels of superoxide dismutase (SOD) and catalase (CAT) enzymes activity were observed in the testes. Administration of *G. officinalis* extract (50 mg/kg BW) significantly rectified these parameters ($P<0.05$). Moreover, the sperm parameters decreased in the diabetic group, while the use of *G. officinalis* significantly improved the mentioned disorders in the treatment groups ($P<0.05$).

Conclusions: The results of this study confirm the antioxidant role of hydroalcoholic extract of *G. officinalis* in the improvement of the testicular oxidative damage caused by diabetes.

Keywords: Oxidative Stress, Diabetes, *Galega officinalis*, Testis, Sperm parameters

Introduction

Diabetes is one of the most common metabolic disorders worldwide, which is related to increased serum levels of glucose (1). Diabetes ensued either from defects of insulin production (insulin dependent diabetes) or owing to hormone resistance in peripheral tissues (non-insulin dependent diabetes) with a decrease in the secretion of hormone from β-cells of the pancreatic islets. The prevalence of diabetes is increasing globally and it is expected that by 2025 the number of people with diabetes would even rise to approximately 300 million people worldwide (2). One of the important complications of diabetes in men is sexual dysfunction as well as decreased testicular weight, semen quality parameters, testosterone level, increased abnormal sperm and infertility (2,3).

During diabetes, hyperglycemia causes an increased advanced glycated end products (AGEs), changes in the activity of protein kinase C, a disruption in the balance of prostanoids and increased production of mitochondrial superoxide; these effects lead to increased oxidative stress due to excess free radicals (4-7). Some studies have shown that reinforcing antioxidant system can reduce the complications of diabetes. Herbal remedies prescribed since ancient times have hardly been efficacious for treating diabetes (8).

Galega officinalis is a natural plant that grows in southeastern parts of Europe and the Middle East (9,10). It was used as a traditional treatment for diabetes in the medieval period (10,11). The hydroalcoholic extract of *G. officinalis* includes flavonoids, tannins, saponins, glycosides, resins, and steroids (12,13). Alkaloid galegine, as one of the main components of *G. officinalis*, showed not only hypoglycemic properties in animal models but also a hypoglycemic effect in vivo (14). Furthermore, it was shown that *G. officinalis* has a remarkable effect on body weight loss and insulin (15). The latter discovery yielded to the expansion of metformin, which is a biguanide used to treat diabetes mellitus type 2 (16). In fact, metformin

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extracted from *G. officinalis* is the exclusive approved antidiabetic drug, which has been extending from an herbal source with a long history of use for diabetes (17). Hence, the application of this herb to the treatment of diabetes may increase the sensitivity of tissues to insulin and reduce the tissue damage (15). In this study the impact of hydro-alcoholic extract of *G. officinalis* on oxidative damages in the testes and sperm parameters of diabetic rats has been investigated.

Materials and Methods

Preparation of Extract

First, the plant of *Galega officinalis* was purchased from Dineh Company (Iran). A half kilogram of this plant was dissolved in 2 L of alcohol 50% and distilled water to obtain the extract of *G. officinalis*. Afterwards, it was kept on a shaker (Thermo Fisher) at room temperature. After 48 hours, the solution was filtered and centrifuged at 3000 rpm for 5 minutes. Then, it was poured into an open-top container and the solvent was evaporated. Finally, the extract was dissolved in normal saline to obtain an appropriate concentration.

Study Design and Experimental Groups

In this experimental, 32 male Wistar rats (10 weeks old and weighing 250 ± 20 g) were kept at 22 ± 2°C and 12 hours light/dark cycle at 7:00 AM. These specimens were randomly divided into 4 collections and treated based on the experimental protocol: (1) Control, (2) Diabetic, (3) Diabetic+ *G. officinalis* 50 mg/kg BW (Diabetic+ *G. officinalis*), (4) *G. officinalis* 50 mg/kg BW (*G. officinalis*). The animals of groups 3 and 4 were administered orally once daily with 50 mg/kg BW of the *G. officinalis* extract for the period of 8 weeks.

Diabetes was induced in groups 2–4 by a single intraperitoneal (i.p) injection of streptozotocin (50 mg/kg). Subjects with serum glucose higher than 250 mg/dL at 3 days after streptozotocin injection were included in the study. After 8 weeks the rats were anesthetized. Their right testes were removed and then kept at a temperature of -80°C for biochemical (malondialdehyde [MDA], catalase [CAT] and superoxide dismutase [SOD]) measurements. The doses of *Galega officinalis* were selected on the basis of previous studies that demonstrated significant changes in glucose plasma level.

Histopathological Examination of Testis

To appraise the histologic changes in "seminiferous tubules" of testis after fixation, the testis was dehydrated, then cleared, and next embedded in paraffin. Spermatogenesis in the seminiferous tubules examined with "Johnson's score". To this end, 50 of "seminiferous tubules" were indiscriminately selected from each section and Johnson's score (scale of 1–10 based on the level of spermatogenesis) was measured for each tubule. Then, the mean of Johnson's score was calculated. The "seminiferous tubules diameter" from the base membrane of one side of tubule to the other side of the tubule was calculated at 400x magnification (18).

Biochemical Assays

In order to measure the changes in the glucose plasma level, blood samples were obtained from the rats' ophthalmic veins in 3 phases including before the injection, the third and the sixth week after the beginning of the study. All the blood sampling phases were performed 12 hours after the fast. Immediately after sampling, blood samples were centrifuged and the serum was stored at -8 °C until analysis. The concentration of glucose level was measured by Iran Pars Azmoon kit using glucose oxidase method via the auto-analysis device. Plasma levels of insulin were measured by the ELISA method using the commercial kit of Rat Insulin (Mercodia). The intra assay variation was 4.9%. The level of insulin in serum was expressed in mIU/mL.

Measurement of Lipid Peroxidation

The level of lipid peroxidation was indicated by the amount of MDA in the testis tissue. In this regard, 375 mg of thiobarbituric acid (TBA) was dissolved in 2 mL of hydrochloric acid (HCL) to provide a solution of TBA-TCA-HCL. Afterwards, it was added to 100 mL of 15% trichloroacetic acid (TCA). A 50°C water bath was utilized to finalize the sediment dissolution. In order to achieve a 10% homogenized mixture, the tissue was weighed and homogenized immediately with a solution of potassium chloride 5.1%. Later, 1 mL of homogenized tissue mixture was mixed with 2 mL of TBA-TCA-HCL solution and heated in boiling water for 45 minutes (pink- orange solution). It was centrifuged at 1000 rpm for 10 minutes after cooling. A spectrophotometer was used to read the absorption (A) at 535 nm. Finally, it was calculated using the following formula:

TBARS concentration: C (m) =Absorbance/1.65×10^5

Determination of Superoxide Dismutase Activity

SOD activity assays were based on Madesh method (7). In brief, the reaction mixture contained 0.65 mL of phosphate buffered saline (PBS) (pH 7.4), 30 µL of MTT (1.25; mM), 75 µL of pyrogallol (100 µmol), and 10 µL of tissue homogeneity. The mixture was kept in a laboratory incubator at room temperature for 5 minutes. The reaction was stopped by adding 0.75 mL of dimethyl sulfoxide. Absorbency of the solution was read using an ELISA plate reader at 570 nm. The enzyme activity was achieved by matching the inhibition percentage with the standard curve and was reported, based on unit/mg of total protein.

Catalase Activity Assays

The catalase activity assays were based on Aebi method (7). Briefly, the activity was determined by measuring the

decrease in absorbance of a reaction mixture consisting of 30 mM H2O2, in sodium phosphate buffer (pH = 7), and requisite volume of tissue homogenized at 240 nm. The specific activity was calculated and was expressed as units per mg of total protein.

Evaluation of Sperm Parameters

The epididymis from both testes was removed and minced in 5 mL phosphate buffered saline (PBS, pH 7.2). Afterwards, they were put in the incubator of 37°C CO2 for 30 minutes and removed the 100 µL of the solution and dissolved in 900 µL from PBS. This process was repeated for new solutions. One drop of the solution was mixed carefully and it was added to Neubauer's chamber. According to the standard Protocol, the sperm count was conducted in 8 squares of 0.1 cm^2 each, except the central erythrocyte area. Finally, the total sperm count was multiplied by correction factor, 5 x 10^6 m (19).

Morphometry of Sperm

After preparing the smears of sperm, the slides were dried in exposed to the air and fixed with alcohol 96%. This enabled to access the morphometry of sperm. Afterwards, the slides were stained with H & E. In this regard, 100 sperm were counted in each slide of each sample. Finally, the percentages of normal and abnormal sperms were determined (19).

Statistical Analysis

The SPSS software (SPSS version 19) was used to carry out all statistical analyses. The data were expressed as mean ± standard error (SE). The one-way analysis of variance (ANOVA) followed by Tukey range test were used to analyze the data. *P* values ≤0.05 were considered statistically significant.

Results

The Histological Finding

The histological examinations contained Johnson's score, "seminiferous tubules diameter", and were compared in the experimental groups that are presented in Table 1 and Figure 1. The "mean Johnson's score" (MJS), "seminiferous tubule diameter" (STD) notably declined in the diabetic groups as compared with the control group, as well as the MJS and STD in treated group that received 50 mg/kg of *G. officinalis* extract significantly enhanced compared to diabetic control group ($P < 0.05$).

The Oxidative Stress Markers Levels in the Testis Tissue

A highly significant increase ($P = 0.001$) in the MDA levels was recorded in testes of the diabetic group compared to the control group. Treating diabetic rats with 50 mg/kg of *G. officinalis* notably reduce increasing of testicular MDA level caused by diabetes ($P = 0.001$). However, treating with 50 mg/kg of *G. officinalis* in healthy group declined the MDA level compared to untreated diabetic rats, significantly ($P = 0.001$). These results demonstrated that diabetes caused a significant decrease in the activity of the catalase enzyme in contrast to the control group ($P = 0.001$). Treating diabetic rats with 50 mg/kg of *G. officinalis* has made significant differences in comparison with the untreated diabetic group ($P = 0.001$). The results showed that SOD activity has a significant decrease in the diabetic group ($P = 0.001$) than the control group. Comparison between the treatment group (50 mg/kg) and the diabetic group showed a significant increase in SOD enzyme activity ($P = 0.001$) (Table 2).

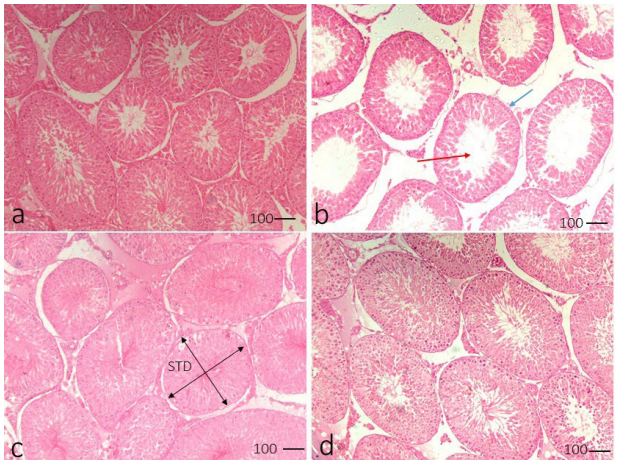

Figure 1. Seminiferous Histopathological Findings in the Experimental Groups (H&E staining)
The blue arrow shows seminiferous tubule and the red arrow shows lumen seminiferous tubule.
A: control, tthe lumens of tubules are regular and they have a normal thickness of the germinal epithelium; (B) Diabetic group: The germinal epithelium thickness was substantially reduced; (C) Diabetic + *G. officinalis*. prevented from reduction in the germinal epithelium thickness. (D)*G. officinalis*: The thickness of "seminiferous tubules" is normal.

Table 1. Histological Assessment of Testis Tissue in the Study Group

Groups	MJS Mean ± SE	STD Mean ± SE
Control	9.6 ± 0.011	254.12 ± 2.69
Diabetic	5.4 ± 0.015[a]	176.80 ± 3.34[a]
Diabetic + *G. officinalis*	7.4 ± 0.041[ab]	200.65 ± 7.42[ab]
G. officinalis	9.8 ± 0.010[b]	261.62 ± 6.30[b]

[a] In comparison with control group ($P = 0.001$); [b] In comparison with diabetic group ($P = 0.001$).

The Sperm Count, Morphology and Motility

The number of sperms was significantly decreased in the diabetic group as compared with the control group ($P = 0.001$). In both therapeutic groups, the number of sperms was significantly increased when comparing with the diabetic group ($P = 0.001$). Diabetes led to increase the number of abnormal sperm when comparing with the control group ($P = 0.001$). Also, treatment with *G. officinalis* was significantly decreased the number of

Table 2. The Concentration of MDA, CAT, and SOD in rat's Testes Tissues of 4 Groups

Groups	MDA (ng/mg) Mean ± SE	CAT (U/mg) Mean ± SE	SOD (U/mg) Mean ± SE
Control	64.78 ±1.13	5.85 ± 0.057	23.18 ± 1.38
Diabetic	140.20 ± 3.04[a]	3.62 ± 0.050[a]	13.05 ± 1.33[a]
Diabetic + *G. officinalis*	85.27 ± 1.42[b]	4.32± 0.072[b]	18.25± 1.31[b]
G. officinalis	60.53± 1.22[b]	6.05 ± 0.059[b]	24.17 ± 1.59[b]

[a] In comparison with control group (P =0.001); [b] In comparison with diabetic group (P =0.001).

abnormal sperms compared with the diabetic group (P=0.001). On the other hand, the motility of sperm was significantly decreased in the diabetic group compared with the control group (P=0.001). Treatment of diabetic rats with *G. officinalis* significantly improved the sperm motility in both therapeutic groups (Table 3).

Serum Glucose Level

A significant increase has been observed in serum glucose level during the third and sixth weeks of the study in the diabetic group compared with the control group (P=0.001). Additionally, a significant decrease was observed in serum glucose levels in the third and sixth weeks in the diabetic group that was under treatment with 50 mg/kg of hydro-alcoholic extract of *G. officinalis* compared to the untreated diabetic group (P=0.001) (Table 4).

Serum Level of Insulin

Serum insulin level between the subject groups showed that diabetes causes a significant decrease in the level of serum insulin in contrast to the control group (P=0.001). Treatment of diabetic rats with 50 mg/kg of *G. officinalis* extracts improved the decrease of serum insulin level when compared with the diabetic group (P=0.03) (Figure 2).

Discussion

The present study assessed the ameliorative effect of *G. officinalis* extract against damages induced by diabetes in the male reproductive system. Diabetes causes testicular dysfunctions in male reproductive system and *G. officinalis* extract treatment improves these functional deficits by antioxidant and anti-diabetic roles. The results of this study showed that hydro-alcoholic extract of *G. officinalis* changed blood glucose levels and also the level of oxidative stress enzymes in the testes of diabetic rats. *G. officinalis* extracts reduced blood glucose levels in treated diabetic rats. The hypoglycemic activity of *G. officinalis* is due to the increasing the sensitivity of tissues to insulin (20).

During diabetes, in addition to increased amount of glucose, the balance between production and elimination of free radicals is also disrupted. As a result, free radicals increase and cause oxidative stress (21,22). Oxidative stress causes cell damage through mechanisms such as lipid peroxidation and DNA and protein oxidative damage (23). The results of this study showed that diabetes significantly increases the levels of MDA (as Lipid peroxidation marker) in the testis of diabetic rats compared with the control group. This result corresponds to the finding of several previous studies on oxidative stress in the testis of diabetic rats (24,25). Therefore, MDA levels increasing in the testis of diabetic group emphasize the enhancement of lipid peroxidation. In the present study, treatment of diabetic rats with *G. officinalis* extract showed significant reduction of MDA level in testis tissue. Previous studies have suggested that flavonoids in *G. officinalis* scavenge the free radicals produced during lipid peroxidation (26). Thereby testis MDA level reduction in the treated groups with the *G. officinalis* extract may be related to antioxidant effects of compounds present in *G. officinalis* including

Table 3. The Comparison of Sperm Parameters After Treatment Period

Groups	Sperm Parameters				
	Sperm Count Mean ± SE x 10^6	Sperm Morphology Mean (%) ± SE		Sperm motility Mean (%) ± SE	
		Normal	Abnormal	Motile	Immotile
Control	75. 5 ± 5.48	76.2%±1.07	24.8%±1.07	75.2%±1.03	24.8±1.03
Diabetic	19.45 ± 3.98[a]	36.5%±.50[a]	63.5%±1.17[a]	24.45%±1.15[a]	75.55±1.15[a]
Diabetic + *G. officinalis*	45.5 ± 6.34[ab]	55.8%±.70[ab]	44.2%±.70[ab]	54.35±.84[ab]	46.65±.84[ab]
G. officinalis	78.35 ± 4.52[ab]	78.2%±1.3[ab]	22.8%±1.3[ab]	75.05±1.04[ab]	24.95±1.04[ab]

[a] Shows significant difference between control group and diabetic group and [b] shows significant difference between diabetic group and diabetic treatment with *T. polium* (P<0.05).

Table 4. The Comparison of Serum Glucose Level Before the Test, Third and Sixth Week

Groups	Glucose (mg/dL)		
	Baseline (mean ± SE)	Third week (mean ± SE)	Sixth week (mean ± SE)
Control	99.78 ±4.13	96.91 ± 3.57	104.29 ± 4.38
Diabetic	349.00 ± 6.54	362.55 ± 7.50[a]	413.45 ± 7.33[a]
Diabetic + *G. officinalis*	344.37 ± 9.02	207.31± 6.07[b]	158.22± 8.31[b]
G. officinalis	97.57 ± 6.87	93.38 ± 4.59[b]	94.17 ± 4.59[b]

[a] Shows significant difference between control group and diabetic group and [b] shows significant difference between diabetic group and diabetic treatment (P<0.05).

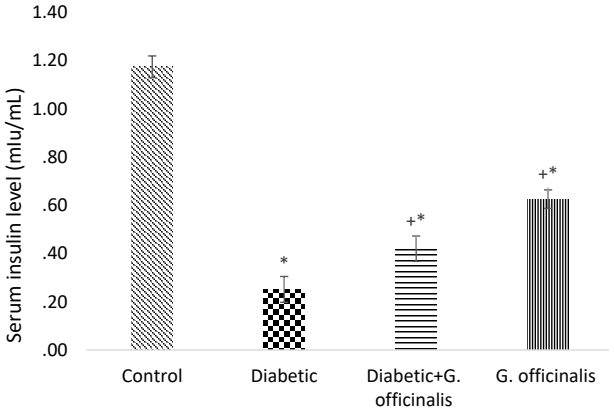

Figure 2. Comparison percentage changes of serum level of insulin between groups before the test in week 3 and 6. * sign shows significant difference with the control group ($P=0.001$) and + sign shows significant difference with the diabetic group ($P=0.03$).

flavonoids.

Our study indicated that SOD activity was considerably reduced in diabetic rats in comparison with control group. These findings correspond to previous studies (27). SOD is counted as one of the most significant enzymes of the antioxidant system while its main action is decomposition of superoxide anion radicals to H_2O_2. Through this process toxicity of superoxide fades and no free radicals from superoxide are produced (28). On the other hand, in this study, SOD activity had a significant increase in testes of diabetic rats being under treatment with extract of *G. officinalis* in contrast to diabetic group.

CAT is another antioxidant enzyme that has detoxification effects against free radicals (23,24). In the present study, the activity of CAT enzyme in diabetic group had more significant decline than the control group, whereas in the group treated with *G. officinalis* extract it showed more significant increase than thr diabetic group. A decrease in CAT activity in our study can be resulted from the increase in H_2O_2 production because of glucose autoxidation and non-enzymatic protein glycation that cause the generation of oxygen-free radicals (29,30). It is known that the administration of antioxidants causes an increase in CAT activity, as confirmed in the present study too.

The results of present research indicated that the diabetes decreased the sperm parameters (count, motility and morphology) and treatment with *G. officinalis* extract can increase the number of sperm and improve the motility and morphology of sperm in diabetic rat. Its maybe due to *G. officinalis* extract contain flavonoids as an antioxidant that can counteract free radicals. In confirmed with our results, previous study indicated that plants with flavonoids compounds can improve the sperm parameters and testosterone level in diabetic rats (31,32).

Possible mechanisms involved in amelioration of testicular oxidative stress in diabetic rats by *G. officinalis* extract can be expressed as follows: *G. officinalis* has anti-oxidant properties; it decreases blood glucose levels and increases insulin secretion (26,33,34). During diabetes, free radicals cause lipid peroxidation and consequently, damages to the testes. The antioxidant properties of *G. officinalis* enhance the antioxidant system and can reduce testicular damages. It has also been demonstrated that the administration of a hydroalcoholic extract of *G. officinalis* increase the sensitivity of tissues to insulin and reduce the tissue damage (20,35,36).

Conclusions

The present findings reveal that diabetes has a negative effect on testis and sperm parameters through oxidative stress. *G. officinalis* has a potent antioxidant effect in reducing the oxidative stress induced by diabetes. However, many studies are necessary to clarify these results.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

Animal handling and all related procedures conducted in this study were confirmed by the Ethical Committee of Tabriz University of Medical Sciences.

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