ORIGINAL ARTICLE

Veterinary Research Forum. 2021; 12 (4) 497 – 503
doi: 10.30466/vrf.2020.108502.2576
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Protective effects of *Equisetum arvense* methanolic extract on testicular tissue disorders in streptozotocin-induced diabetic murine model

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Article Info

Article history:

Received: 25 May 2019
Accepted: 14 June 2021
Available online: 15 December 2021

Keywords:

Diabetes mellitus
Field horsetail
Spermatogenesis
Spermiogenesis
Testosterone

Abstract

Diabetes in a long period can damage the testicular tissue and impair the male fertility potential. Recently, different herbal treatments have been used for the prevention of type I diabetes and its pathological effects. Methanolic extract of *Equisetum arvense* has anti-oxidant and hypoglycemic properties. Thus, the current study aimed to evaluate the protective effects of *Equisetum arvense* methanolic extract (EE) on diabetes-induced detrimental effects in mice testicular tissue. Thirty-two adult male mice were randomly divided into four groups including control-sham, diabetic (induced by streptozotocin, 50.00 mg kg^{-1} for five days), diabetic + EE 250 (250 mg kg^{-1}) and diabetic + EE 500 (500 mg kg^{-1}). After 45 days, all animals were euthanized and their testicles were dissected out and undergone histological analyses. Moreover, the serum level of testosterone was evaluated. Analyses showed that seminiferous tubules diameter, Leydig cells number per mm^2 of the connective tissue, Sertoli cells number per tubule, serum level of testosterone and percentage of seminiferous tubules with positive tubular differentiation, repopulation and spermiogenesis indices were significantly decreased in the diabetic group in comparison with control-sham group. The administration of EE in test groups significantly decreased the adverse effects of diabetes (especially 500 mg kg^{-1}). The results of this study revealed that diabetes disturbs spermatogenesis and spermiogenesis processes in mice. Meanwhile, the EE prevents diabetes-induced damages in mice testicular tissue, which may be associated with its hypoglycemic and antioxidative activities.

Introduction

Diabetes mellitus is a metabolic disorder affecting the carbohydrates, proteins and fats metabolism.[1] It is accompanied by a defect in insulin secretion or decreased sensitivity of tissues to insulin and leads to hyperglycemia.[2] The major complications in diabetes mellitus are neuropathy, retinopathy and accelerated macro- and microvascular complications.[3] Thus, it is thought that neuropathy and vascular insufficiency may lead to impotence, decreased libido and ejaculation disorder.[4] On the other hand, diabetes alters spermatogenesis, steroidogenesis and sperm maturation.[5] The consequences of these alterations are sperm quantity, motility and viability reductions.[6,7] Hypogonadism is one of the complications of diabetes reducing testosterone secretion and damaging testicular tissue leading to erectile dysfunction.[8,9]

Apoptosis is a form of cell death eliminating dying cells from the proliferating or differentiating cell population; this process is thought to play a pivotal role in the pathogenesis of hypogonadism.[10] In laboratory animals, experimental diabetes can be induced using various chemical compounds such as streptozotocin (STZ) and alloxan.[11] Evaluation of histopathological changes in the testis in alloxan-induced diabetic model in mice has showed that this disease leads to a reduction in the number of spermatogenic cells, thickness of germinal epithelium and diameter of seminiferous tubules. Also, multinucleated giant cells have been observed in some seminiferous tubules.[11] Moreover, diabetes affects the different stages of spermatogenesis and spermatogonia are the primary target cells of diabetes.[12,13]

The testicular function is principally controlled by pituitary gland hormones including follicle-stimulating

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hormone (FSH) and luteinizing hormone (LH). The FSH regulates spermatogenesis and LH controls Leydig cells activity. Decreased serum levels of FSH and LH have been reported in diabetes. On the other hand, some studies have showed that diabetes decreases testosterone production. This effect may be due to a reduction in the number of Leydig cells and a decline in testosterone biosynthesis.[12] The activity of the hypothalamic-pituitary-gonadal axis is sensitive to adverse metabolic conditions.[14]

Although chemical drugs used to control diabetes have desired effects, they can cause significant side effects.[15] Thus, the use of herbal remedies has become popular in the treatment of diseases such as diabetes because of their low cost and fewer side effects. There are many kinds of medicinal plants with hypoglycemic properties.[16] One of these herbs is *Equisetum arvense* (Equisetaceae, sub-genus Equisetum; traditional name: Horsetail). The *E. arvense* is a famous plant distributed throughout the northern hemisphere[17] and grows in Europe, Asia (Turkey, Iran, The Himalayas, China, Korea and Japan) and the United States.[18] This plant has different compounds such as vitamins,[19] minerals,[20] flavonoids, phenolic glycosides, alkaloids and triterpenoids.[18] According to previous studies, in the extraction of *E. arvense* with hydromethanol using the Soxhlet apparatus, various phytochemicals such as flavonoids, alkaloids and glycosides have been detected.[21] The *Equisetum arvense* methanolic extract (EE) has antioxidative and hepatoprotective effects.[22] Moreover, the anti-diabetic effect of *E. arvense* and *E. myriochaetum* has been reported formerly.[18,23,24]

In line with that, the purpose of the present study was to investigate the protective effects of *E. arvense* on testicular tissue of STZ-induced diabetic murine model.

Materials and Methods

Animals. In this experimental study, 8-week-old male mice weighing 20.00 to 25.00 g were used. They were provided from Animal House of Faculty of Veterinary Medicine, Urmia University, (Urmia, Iran), and maintained under controlled environmental condition (12 hr dark/12 hr light cycle and temperature of 22.00 ± 2.00 ˚C with humidity of 50.00 ± 10.00%). This project was carried out in accordance with the international guidelines for the care and use of laboratory animals and approval of the Ethics Committee of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran (IR-UU-AEC-992/AD/3).

Experimental design. Thirty-two animals were allocated into the following groups: 1) Control-sham group: single injection of 0.10 mL of sodium citrate (Merck, Darmstadt Germany) buffer which is the STZ solvent, 2) Diabetic group: after induction of diabetes by intra-peritoneal injection of 50.00 mg kg^{-1} body weight STZ (Sigma-Aldrich, St. Louis, USA), they were kept for 45 days, 3) Diabetic group treated with EE at a dose of 250 mg kg^{-1} body weight and 4) Diabetic group treated with EE at a dose of 500 mg kg^{-1} body weight. In the third and fourth groups, after induction of diabetes, the mice were daily received extract orally for 45 days (re-solubilized extract in normal saline). Also, the mice of the first and second groups were daily received normal saline orally for 45 days. The doses of the methanolic extract were selected according to the previous studies.[18]

Induction of experimental diabetes. In order to induce diabetes, the overnight fasted mice were injected intraperitoneally with 50.00 mg kg^{-1} body weight STZ in freshly prepared citrate buffer (0.10 M; pH: 4.50) for five days. Five to seven days later, their blood glucose levels were measured. The mice having blood glucose levels above 250 mg kg^{-1} were considered diabetic and selected for present study.[1]

Extraction. Dried aerial parts of *E. arvense* were powdered by an electrical grinder. The powder (80.00 g) was packed in a filter paper and then placed into a Soxhlet apparatus (Fisher Scientific, Loughborough, UK). At first, one liter of n-hexane (Merck, Germany) was poured into Soxhlet balloon (48 hr at 68.00 ˚C). Then, the n-hexane was emptied from the Soxhlet balloon and replaced with one liter of dichloromethane (72 hr at 39.60 ˚C). Finally, one liter of methanol was poured into the balloon instead of dichloromethane (48 hr at 65.00 ˚C). At all stages, the Soxhlet apparatus was covered with aluminum foil to prevent radiation of light to the extract. Then, the methanol was evaporated in a rotary evaporator and the obtained extract was stored in the refrigerator below 10.00 ˚C until use.[18,23,24]

Histological analyses. For these purposes, after 45 days, the mice were anesthetized with ketamine at a dose of 25.00 mg kg^{-1} for blood sampling and then euthanized with ketamine at a dose of 100 mg kg^{-1}. The testes were dissected out and fixed in 10.00% formalin fixative for 48 to 72 hr. Then, the fixed samples were embedded in paraffin and thin sections (6.00 µm) were cut using a rotary microtome (Microm, Walldorf, Germany) and stained with Hematoxylin and Eosin (Merck) for histological investigations using a light microscope (Olympus, Tokyo, Japan).

Testosterone assay. The blood samples were collected from animals into the dry and clean micro-tubes. Then, the serum samples were separated by centrifuging at 1,000 *g* for 15 min and stored at –70.00 ˚C. Finally, the serum level of testosterone was measured using the electrochemiluminescence method (Roche Diagnostics, Mannheim, Germany).

Histomorphometrical analyses. In order to measure the diameter of the seminiferous tubules, about 100 round or nearly round cross-sections of seminiferous tubules were evaluated randomly for each group. The large and small diameters of each cross-section were measured

using a calibrated, graded objective lens (Olympus) and then the mean diameter was calculated. Moreover, latticed objective lens (Olympus) was used to count the number of Leydig cells in one mm^2 of testicular interstitial connective tissue.

Assessment of spermatogenesis in the testicular tissue. To estimate spermatogenesis in the seminiferous tubules, tubular differentiation index (TDI), repopulation index (RI) and spermiogenesis index (SPI) were used. For the determination of TDI, the percentage of seminiferous tubules with more than four layers of differentiated germinal cells from type A of spermatogonia was recorded. These tubules were considered as positive TDI. To evaluate the RI, the ratio of active spermatogonia cells (spermatogonia type B were seen with dark nuclei) to inactive spermatogonia cells (spermatogonia type A were seen with light nuclei) in the seminiferous tubules was calculated. Also, the ratio of the percentage of seminiferous tubules contained sperm to empty tubules was calculated for assessment of SPI. Seminiferous tubules contained sperm were considered as positive SPI.[25]

Statistical analysis. All data were analyzed by SPSS software (version 16.0; SPSS Inc., Chicago, USA) using one-way ANOVA. For determination of significant differences between groups, Tukey's post hoc test with $p < 0.05$ was used. All results were presented as the mean ± standard deviation.

Results

Histological observations of testicular tissue. In histological evaluations of testicular tissue in all groups, severe edema in the interstitial connective tissue was observed in the diabetic group. The severity of edema in extract-treated groups was decreased compared to the diabetic group. The interstitial connective tissue was without edema in the control-sham group. Also, in the diabetic group, the cellular attachment of germinal epithelium cells was abnormal and some depleted seminiferous tubules were seen. Whereas, the degree of these changes was decreased in extract-treated groups compared to the diabetic group. In the control-sham group, the germinal cells had standard attachment and more than four cell layers were seen in seminiferous tubules (Fig. 1).

Blood glucose level. Evaluation of blood glucose levels showed a significant increase in the diabetic group compared to the control-sham group. While, there was a significant decrease in blood glucose levels in the extract-treated groups compared to the diabetic group. No significant difference was seen between extract-treated groups (Table 1).

Assessment of spermatogenesis. The assessment of testicular tissue showed that the percentage of seminiferous tubules with positive TDI was decreased significantly in the diabetic group compared to the control-sham group ($p < 0.05$). Whereas, this parameter was increased significantly in extract-treated groups compared to the diabetic group ($p < 0.05$).

Also, a significant difference was observed between extract-treated group at dose of 250 mg kg^{-1} and control-sham group ($p < 0.05$). There was no significant difference between extract-treated groups ($p > 0.05$; Table 2). The RI was decreased significantly ($p < 0.05$) in the diabetic group compared to the control-sham group. This parameter was significantly higher ($p < 0.05$) in extract-treated groups than that of the diabetic group. There was no significant difference between extract-treated groups and also between extract-treated groups and control-sham group ($p > 0.05$; Table 2). The percentage of seminiferous tubules with positive SPI was significantly lower ($p < 0.05$) in the diabetic group than that of the control-sham group. While, this parameter was increased significantly in extract-treated groups in comparison with the diabetic group ($p < 0.05$). There was no significant difference between extract-treated groups and also between extract-treated groups and control-sham group ($p > 0.05$; Table 2).

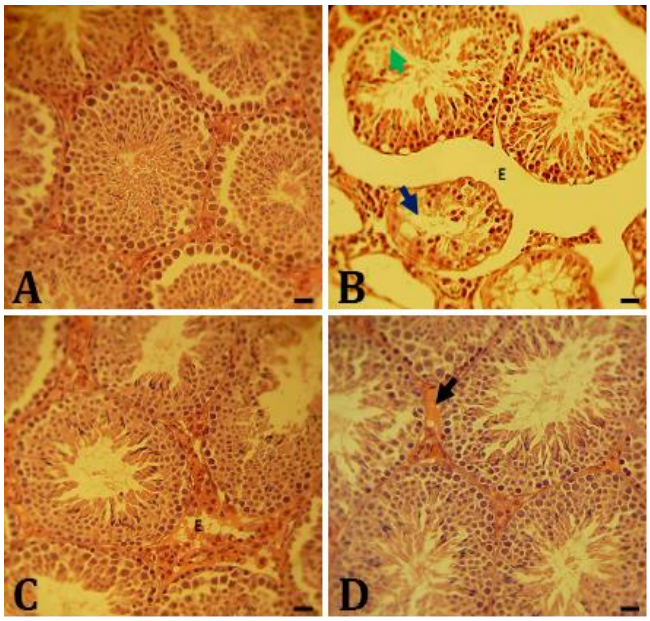

Fig. 1. Cross-section of mice testicular tissue. A) Control-sham group: Seminiferous tubules are normal and interstitial connective tissue is without edema. B) Diabetic group: Interstitial edema and increased interstitial space (E) in the interstitial connective tissue can be seen. The negative tubular differentiation index (TDI; green arrow) and germ cells depletion (blue arrow) are obvious. C) Extraxt-treated group at dose of 250 mg kg^{-1}: The severity of edema is lesser. Positive TDI is seen in the tubules. D) Extract-treated group at dose of 500 mg kg^{-1}: Black arrow shows the decreased severity of edema compared to the other extract-treated group, (H&E; scale bars = 25.00 µm).

Table 1. Effects of *Equisetum arvense* methanolic extract (EE) on blood glucose concentration in streptozotocin-induced diabetes in mice.

Duration of treatment (Days)	Control-sham	Diabetic	Diabetic + 250 mg kg^{-1} EE	Diabetic + 500 mg kg^{-1} EE
0	47.57 ± 18.74	474.60 ± 49.68^{a}	347.40 ± 53.49^{ab}	324.40 ± 49.99^{ab}
15	56.80 ± 3.34	421.40 ± 36.53^{a}	222.80 ± 48.94^{ab}	186.40 ± 43.27^{ab}
30	58.40 ± 3.43	403.00 ± 56.08^{a}	223.80 ± 39.82^{ab}	194.20 ± 46.98^{ab}
45	53.00 ± 1.58	479.60 ± 108.10^{a}	178.40 ± 13.92^{ab}	108.60 ± 29.33^{b}

^{ab} indicate significant difference compared to the control-sham group and diabetic group, respectively at $p < 0.05$.

Table 2. Effects of *Equisetum arvense* methanolic extract (EE) on spermatogenesis indices and diameter of seminiferous tubules in streptozotocin-induced diabetes in mice.

Parameters	Control-sham	Diabetic	Diabetic + 250 mg kg^{-1} EE	Diabetic + 500 mg kg^{-1} EE
TDI (%)	87.19 ± 2.42	56.63 ± 2.73^{a}	76.65 ± 5.23^{ab}	82.83 ± 3.64^{b}
RI (%)	74.00 ± 3.00	57.63 ± 4.39^{a}	70.51 ± 1.92^{b}	71.18 ± 1.59^{b}
SPI (%)	86.36 ± 1.77	70.55 ± 2.76^{a}	82.27 ± 5.93^{b}	80.77 ± 1.50^{b}
STD	202.80 ± 3.63	130.40 ±19.12^{a}	189.20± 7.29^{b}	189.40 ±18.75^{b}

TDI: Tubular differentiation index; RI: Repopulation index; SPI: Spermiogenesis index; STD: Seminiferous tubule diameter.
^{ab} indicate significant difference compared to the control-sham group and diabetic group, respectively at $p < 0.05$.

Histomorphometrical analyses. The results of this study showed that the diameter of seminiferous tubules was decreased significantly in the diabetic group compared to the control-sham group ($p < 0.05$). However, there was no significant difference between extract-treated groups and control-sham group ($p > 0.05$). In extract-treated groups, the diameter of seminiferous tubules was increased significantly ($p < 0.05$) when compared to the diabetic group. Also, there was no significant difference between extract-treated groups ($p > 0.05$; Table 2).

Histological observations also showed that the number of Leydig cells per one mm^2 was decreased significantly ($p < 0.05$) in the diabetic group compared to the control-sham group. On the other hand, there was no significant difference between extract-treated groups and control-sham group ($p > 0.05$). This parameter was significantly higher ($p < 0.05$) in extract-treated groups than that of the diabetic group. There was no significant difference ($p > 0.05$) between extract-treated groups (Table 3).

The number of hypertrophic Leydig cells was increased significantly ($p < 0.05$) in the diabetic group compared to the control-sham group. While, in extract-treated groups, in comparison with the diabetic group, the number of hypertrophic Leydig cells was decreased significantly ($p > 0.05$). There was no significant difference between extract-treated groups and control-sham group ($p > 0.05$; Fig. 2 and Table 3).

Our results showed that the number of Sertoli cells was significantly lower ($p < 0.05$) in the diabetic group compared to the control-sham group. While, in extract-treated groups, in comparison with the diabetic group, the number of Sertoli cells was significantly higher ($p < 0.05$). There was no significant difference ($p > 0.05$) between extract-treated groups and also between extract-treated groups and control-sham group (Table 3).

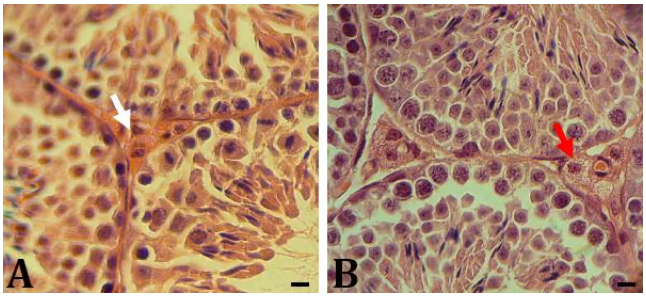

Fig. 2. Cross-section of mice testicular tissue. **A)** Normal Leydig cell in interstitial connective tissue (white arrow) of control-sham group. **B)** Hypertrophic Leydig cell with vacuolated cytoplasm (red arrow) in diabetic group, (H&E; scale bars = 20.00 µm).

Serum testosterone level. Serological analyses revealed that serum testosterone level was decreased significantly ($p < 0.05$) in the diabetic group compared to the control-sham group. While, treated groups showed a significant increase ($p < 0.05$) in serum testosterone level compared to the diabetic group. There was no significant difference ($p > 0.05$) between treated groups (Fig. 3).

Table 3. Effects of *Equisetum arvenese* methanolic extract (EE) on total number of Leydig and Sertoli cells in streptozotocin-induced diabetes in mice.

Parameters	Control-sham	Diabetic	Diabetic + 250 mg kg^{-1} EE	Diabetic + 500 mg kg^{-1} EE
Leydig cells in 1.00 mm^2	70.60 ± 5.02	52.20 ± 4.81^{a}	67.20 ± 5.58^{b}	66.20 ± 8.92^{b}
Hypertrophic Leydig cells in 1.00 mm^2	3.52 ± 1.04	26.12 ± 1.32^{a}	18.24 ± 1.10^{b}	23.40 ± 1.42^{bc}
Percentage of hypertrophic Leydig cells	4.98 ± 1.47	50.03 ± 2.53^{a}	27.14 ± 1.64^{b}	35.34 ± 2.15^{bc}
Sertoli cells per tubule section	20.80 ± 1.48	12.60 ± 1.51^{a}	18.20 ± 1.78^{b}	19.80 ± 2.16^{b}

^{abc} indicate significant difference compared to the control-sham group, diabetic group, and diabetic + 250 mg kg $^{-1}$ extract group respectively at $p < 0.05$.

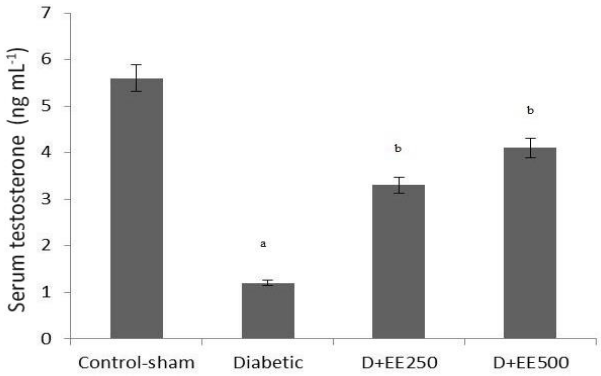

Fig. 3. Comparison of serum testosterone levels in the different groups. [ab] indicate significant difference compared to the control-sham group and diabetic group, respectively ($p < 0.05$). D: Diabetic; EE: *Equisetum arvenese* methanolic extract.

Discussion

Nowadays, diabetes mellitus is one of the common diseases in human societies.[26] The results of the present study indicated that diabetes has detrimental effects on spermatogenesis indices including TDI, RI and SPI, diameter of seminiferous tubules, number of Leydig and Sertoli cells and serum testosterone level. While, oral administration of the extract at doses of 250 mg kg^{-1} and 500 mg kg^{-1} decreased diabetes-induced detrimental effects.

Some studies have shown that the serum levels of LH and FSH reduce considerably in diabetic patients.[27] It is well known that these hormones play a pivotal role in the spermatogenesis process. The LH affects Leydig cells through adenylyl cyclase enzyme activation and regulates steroidogenesis.[27,28] It is noteworthy to mention that Sertoli cells are the main target of FSH and this hormone also activates adenylyl cyclase enzyme in these cells. Sertoli cells stimulated by FSH release androgen-binding protein. Therefore, FSH must stimulate the Sertoli cells for spermatogenesis initiation.[29] On the other hand, testosterone is produced by Leydig cells to regulate the Sertoli cells physiological function.[29] In the present study, serum testosterone levels were evaluated and it was found that the level of this hormone was reduced significantly in the diabetic group; while, testosterone levels were significantly higher in extract-treated groups. Thus, it was possible that the serum levels of LH and FSH were higher in extract-treated groups and this may be due to the low complication of diabetes in the pituitary gland. Also, more testosterone was produced in extract-treated groups protecting the Sertoli cells physiological activity. This hypothesis is confirmed well by higher serum testosterone levels and higher number of Sertoli cells in extract-treated groups. Accordingly, the high level of testosterone reduced Sertoli cells atrophy and degeneration.

Our results showed that the number of Leydig cells was decreased significantly in the diabetic group compared to extract-treated groups and a higher percentage of these cells was hypertrophic in the diabetic group. These findings were in accordance with prior reports. Some studies have shown that diabetes causes a significant decrease in the number of Leydig cells and in several experimental cases, these cells were hypertrophic in animal models.[30,31] Based on these findings, it can be concluded that the number of Leydig cells is retained considerably in extract-treated groups. The high level of testosterone in these groups is the evidence of this claim. Therefore, these cells produce testosterone stimulating Sertoli cells to continue the physiological activity. This hypothesis is demonstrated by low blood glucose and higher number of Sertoli cells in extract-treated groups.

Some studies have shown that diabetes can impair the spermatogenesis process in men.[32,33] Sertoli cells are contributed to germ cells junction and control spermatogenesis in the testicular tissue.[30] Histological results of the present study showed that the number of Sertoli cells is significantly decreased in the diabetic group compared to extract-treated groups. Also, the marked germ cells disintegration was observed in diabetic group. Moreover, the percentage of tubules with positive TDI, RI and SPI was significantly lower in the diabetic group. So, the protective effects of EE lead to the Sertoli cells maintenance in the seminiferous tubules. Thus, the spermatogenesis and the percentage of seminiferous tubules with positive TDI, RI and SPI increased significantly in treated groups.

Based on our results, it can be concluded that diabetes influences the spermatogenesis and spermiogenesis processes and ultimately, leads to reduced fertility in mice. The *Equisetum arvense* methanolic extract inhibits the diabetes complications in mice testicular tissue and can be used to control diabetes and its complications.

Acknowledgments

This study was supported by the Research Council of Urmia University, Urmia, Iran.

Conflict of interest

There is no conflict of interest in this work.

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