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Effect of saponin on spermatogenesis and testicular structure in streptozotocin-induced diabetic mice

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Abstract

About a third of human infertility is related to male factors. Of these, idiopathic-related infertility is not curable. Diabetes mellitus is a metabolic disorder affecting male impotence and fertility by increased production of free radicals and oxidative stress. Saponin, a glycosidic compound found in many plants, improves sperm parameters. The present study investigated the effect of saponin on sperm oxidative stress and testicular structure in streptozotocin (STZ)-induced diabetic mice. The diabetes was induced by the administration of 150 mg kg^{-1} STZ via a single intra-peritoneal injection. All experimental mice were allocated to the following groups: Control group, diabetic control group, diabetic group administrated 100 mg kg^{-1} saponin daily and one healthy group administrated saponin daily for 56 days. At the end of the treatment period, serum levels of insulin, glucose and oxidative stress markers were measured. A histological evaluation of testicles was performed. Treatment of diabetic mice with saponin ameliorated testicular tissue damage as well as serum glucose and insulin concentrations. Furthermore, in the diabetic group, the serum concentration of malondialdehyde was increased; while, the activity of superoxide dismutase and glutathione peroxidase enzymes was reduced. The mean Johnsen's score and the diameter and thickness of seminiferous tubules were lower in the diabetic mice than control ones. However, these parameters were higher in the saponin-treated mice than controls. Overall, saponin administration rectified all examined parameters. The anti-oxidant role of saponin improves sperm parameters and diabetes-induced testicular oxidative damage.

Introduction

According to the report of the World Health Organization, infertility is a disorder occurring in 10.00 - 15.00% of couples, of which 30.00 - 40.00% are associated with the male factor.[1,2] Abnormal semen parameters due to the factors other than idiopathic reasons can be improved; while, treatment for poor idiopathic semen quality is not promising. [3]

Diabetes or diabetes mellitus is a chronic and endocrine disease, causing numerous concerns worldwide. Diabetes mellitus is a heterogeneous metabolic disorder caused by the lack of insulin production in the body or insulin resistance impairing male sexual ability and fertility.[4,5] Testicular dysfunction decreases the testicular weight along with sperm count and motility and changes the morphology of the seminiferous tubules. Testosterone levels are also reduced.[6] Diabetes increases the apoptosis rate (pro-apoptotic genes such as *Bax* up-regulation) in germ cells and also interrupts the spermatogenesis process.[5] In about 90.00% of diabetic patients, defects in sexual activity are seen as decreased libido and reduced fertility.[7]

Although the exact mechanism of diabetes mellitus is not well understood, the increase in the production of free radicals and increased oxidative stress are its major proposed damaging mechanisms.[7,5]

The presence of anti-oxidants such as vitamins or flavonoids in the diet can exert protective effects in diabetic patients.[8] Reactive oxygen species (ROS) over-production damages the mitochondrial membrane causing cytochrome C release, resulting in the apoptosis induction in testicular tissue cells.[7]

Saponins are glycosidic chemical compounds being abundant in many plants. Saponin is involved in protecting

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the plant against germs and fungi. Although high doses of this substance are very toxic, several reports have indicated that saponin increases sperm motility and viability and hormone levels.[9,10]

This study aimed to investigate the effects of saponin on spermatogenesis, testicular tissue damage and blood biochemical and hormonal parameters in diabetic mice.

Materials and Methods

Sixty-four male mice weighing 25.00 to 30.00 g were obtained from the Animal House of Tabriz University of Medical Sciences, Tabriz, Iran, and kept for 2 weeks in standard conditions with 12 hr of light and adequate humidity. All procedures performed in studies involving animals were in accordance with the ethical standards of Tabriz University, Tabriz, Iran (Ethical code: 1398.027).

The animals were randomly divided into 4 groups of 16 and treated as follows:

Group 1: The control group (no injections); Group 2: The diabetic control group received a single intra-peritoneal injection of 150 mg kg^{-1} streptozotocin (STZ);[11] Group 3: The healthy control group received 100 mg kg^{-1} per day saponin via intra-peritoneal injection for 8 weeks;[2] Group 4: Treatment group receiving 150 mg kg^{-1} STZ (one injection) and 100 mg kg^{-1} per day saponin intra-peritoneally for 8 weeks.

At first, the glucose levels of all mice in both experimental and control groups were determined by a glucometer (Easy-Gluco 2657A; Complete Medical Supplies Inc., New York, USA). Then, to induce diabetes, 150 mg kg^{-1} per day of STZ was administered intra-peritoneally to groups 2 and 4. After 72 hr, blood glucose levels were measured again. After confirming that the mice were diabetic (blood glucose levels above 250 mg dL^{-1}), they received 100 mg kg^{-1} of saponin via intra-peritoneal injection once a day for 56 days.[12] At the end of the treatment period, all mice were anesthetized with a combination of 50.00 mg kg^{-1} ketamine (Panpharma, Luitré-Dompierre, France) and 10.00 mg kg^{-1} xylazine (Alfasan, Woerden, The Netherlands). Then, 2.00 to 3.00 mL of the blood samples were taken from the hearts of animals for biochemical assays.

In order to isolate sera, immediately after sampling, blood samples were centrifuged at 3,000 rpm for 10 min, and the harvested sera were stored at – 80.00 ˚C until used. The glucose concentration was measured by a commercial kit (Iran Pars Azmoon, Tehran, Iran). Serum concentrations of insulin were measured by the enzyme-linked immunosorbent assay (ELISA) using a standard commercial kit for mice (Mercodia Inc, Uppsala, Sweden) and reported as μg L^{-1}.

The lower abdominal area was incised under sterile conditions, and both testicles and epididymides were bilaterally removed and weighed. For histological examination, the right testicle was fixed in Bouin's fixative for 72 hr. Then, 5.00 μm sections were prepared,[13,5] and stained with the Hematoxylin and Eosin staining method. About 50 round seminiferous tubules were randomly examined by a light microscope (CX22; Olympus, Tokyo, Japan) with 400× magnification to determine the seminiferous tubule diameter, germinal epithelium height and spermatogenesis alterations.

Serum testosterone concentration was measured using a commercial ELISA kit (Demeditec Diagnostics, Kiel, Germany). Briefly, serum samples (25.00 μL) were incubated with 200 μL enzyme conjugate in pre-coated wells for 60 min at room temperature. Then, the wells were washed three times with 300 μL diluted irrigation solution and incubated with 200 μL substrate solution for 15 min at room temperature. The enzymatic reaction was ended by adding 100 μL stop solution, and the optical density of the solution in each well was recorded at 450 nm. The testosterone concentration was calculated using six standard concentrations and a four-parameter logistic curve fitting. The final testosterone concentration was obtained from each set of duplicates and expressed as ng mL^{-1}.

The superoxide dismutase (SOD) activity of serum samples was measured using a commercial kit (Ransod, Randox Laboratories Ltd., Crumlin, UK) according to the Arthur and Boyne.[14] In summary, this method is based on the generation of superoxide radicals by adding xanthine and xanthine oxidase to the sample and its reaction with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by the inhibition power of this reaction and expressed as U of SOD per 10.00 mg of protein. Protein was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, USA) according to the method described by Bradford.[15]

Glutathione peroxidase (GPx) activity was measured by a diagnostic kit (Randox) according to the Paglia and Valentine.[16] The oxidation of glutathione (GSH) is catalyzed by cumene hydroperoxide in this method. The oxidized GSH is immediately converted into the reduced form with concomitant oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP+ (oxidized form of NADPH) in the presence of glutathione reductase. Then, the decline in absorbance at 340 nm is calculated in a spectrophotometer (Thermo Fisher Scientific) and expressed as U L^{-1}.

To measure serum malondialdehyde (MDA) levels, first, 0.20 mL of serum was added to a microtube containing 3.00 mL of glacial acetic acid, following which 1.00% thiobarbituric acid (in 2.00% NaOH) was added to the microtube. The tube was then placed in the boiling water for 15 min. After cooling, the adsorption of the resulting solution was read in a spectrophotometer (Thermo Fisher Scientific) as pink at 532 nm.[17]

Statistical analysis. All statistical analyses were carried out using the SPSS software (version 19.0; IBM Corp., Armonk, USA). After ensuring the normal distribution of the variables, they were compared using a one-way analysis of variance. Tukey's *post hoc* test was applied to determine the differences between groups. The results were expressed as mean ± standard deviation. For all data, $p < 0.05$ was considered statistically significant.

Results

A significant increase was found in serum glucose levels in group 2 compared to the group 1 at the end of the study ($p < 0.05$). Additionally, a significant decrease was observed in serum glucose levels in group 3 in contrast to group 2 at the same time ($p < 0.05$; Table 1). This was true for one week before and one week after diabetes induction. The administration of the saponin to healthy mice (group 4) did not significantly alter glucose concentrations at any time of sampling.

Serum insulin evaluation indicated that group 2 (0.29 ± 0.02) had lower ($p < 0.05$) concentrations than group 1 (0.54 ± 0.02). Treatment of the diabetic mice with saponin (group 3) (0.37 ± 0.01) and healthy saponin-treated (group 4) (0.52 ± 0.02) improved ($p < 0.05$) the serum insulin level compared to the group 2 (0.52 ± 0.02 *versus* 0.37 ± 0.01; Fig. 1A).

The results of the histological evaluation showed that the mean Johnsen's score (MJS) was decreased ($p < 0.05$) in group 2 compared to the group 1 Table 1 and Fig. 2).

On the other hand, the MJS was higher ($p < 0.05$) in group 3 and group 4 than group 2. Histopathological examination showed that the diameter of seminiferous tubules was decreased ($p < 0.05$) in the group 2 compared to the group 1. Similarly, the thickness of seminiferous tubules was decreased ($p < 0.05$) in the group 2 compared to the group 1.

In addition, the diameter of seminiferous tubules was increased ($p < 0.05$) in the group 3 and group 4 compared to the group 2. In the same manner, the thickness of the seminiferous tubules was higher ($p < 0.05$) in the group 3 and group 4 than group 2 (Table 1).

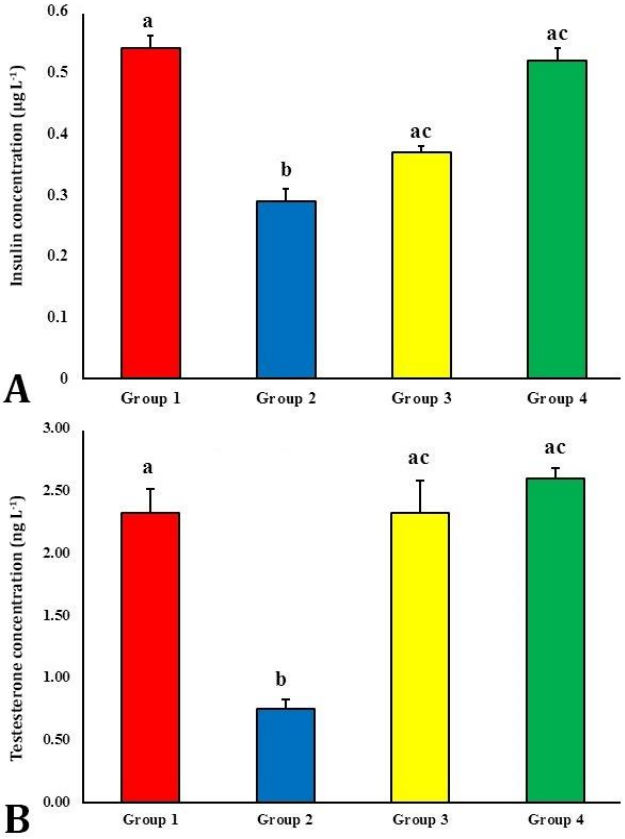

Fig. 1. The serum concentrations of **A)** insulin and **B)** testosterone in the group 1 (control), group 2 (diabetic control), group 3 (diabetic treated with 100 mg kg^{-1} saponin) and group 4 (healthy saponin treated mice). [abc] Indicate significant difference between control (Group 1) and other groups ($p < 0.05$).

Table 1. Concentrations of malondialdehyde, glutathione peroxidase, superoxide dismutase, and histological parameters of the testicles and glucose concentrations in week -1, week +1 and week +8 in testicular tissue, in the group 1 (control), group 2 (diabetic control), group 3 (diabetic treated with 100 mg kg^{-1} saponin) and group 4 (healthy saponin-treated mice).

Parameters	Group 1	Group 2	Group 3	Group 4
Malondialdehyde (U per 10 mg protein)	0.63 ± 0.06	2.30 ± 0.05*	1.54 ± 0.17*†	0.80 ± 0.05*†
Glutathione peroxidase (mmol L^{-1})	2.15 ± 0.01	0.84 ± 0.02*	1.72 ± 0.03*†	2.43 ± 0.06*†
Superoxide dismutase (U per 10 mg protein)	1.67 ± 0.03	0.79 ± 0.03*	1.38 ± 0.02*†	1.55 ± 0.25*†
Mean Johnsen's score	9.62 ± 0.36	4.35 ± 0.17*	7.35 ±0.54*†	9.55 ± 0.24*†
Seminiferous tubule diameter (µm)	262.42 ± 4.25†	140.11± 2.57*	190.50± 3.23*†	260.22 ± 1.70*†
Height of germianl epithelium (µm)	64.50 ± 1.23†	33.50 ± 2.03*	54.50 ± 2.15†	64.03 ± 1.05*†
Glucose week -1 (mg dL^{-1})	94.50 ± 5.34	100.20 ± 2.60*	95.70 ± 5.89*†	96.25 ±3.40†
Glucose week +1 (mg dL^{-1})	102.42 ± 7.25†	320.25 ± 4.07*	291.50 ± 4.03*†	93.80 ± 4.70†
Glucose week +8 (mg dL^{-1})	92.40 ± 9.34†	367.60 ± 38.73*	179.20 ± 3.20†	92.30 ± 8.05†

*† Indicate significant differences between the treatment versus control and diabetic groups, respectively ($p < 0.05$).

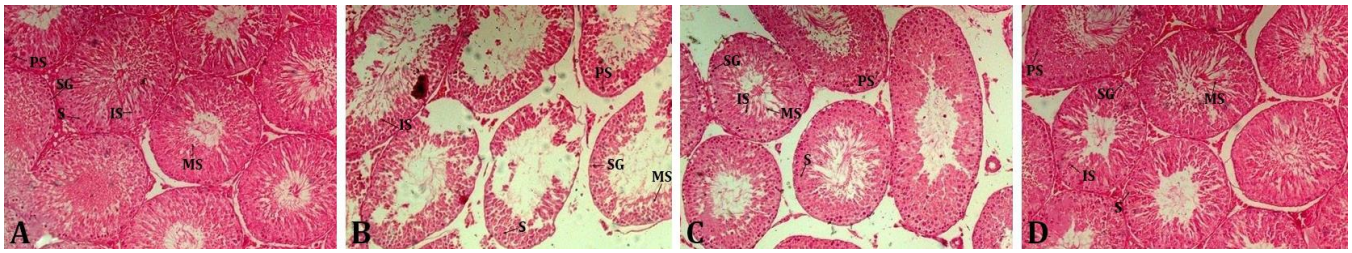

Fig. 2. The diameter and the thickness of the seminiferous tubules shown in the histological sections from **A)** group 1 (control, **B)** group 2 (diabetic control, **C)** group 3 (diabetic treated with 100 mg kg^{-1} saponin, and **D)** group 4 (healthy saponin treated mice. (Hematoxylin and Eosin, × 400). IS: Immature spermatid; MS: Mature spermatid; PS: Primary spermatocyte; S: Sertoli cell, and SG: Spermatogonium.

The serum testosterone concentrations were decreased ($p < 0.05$) in group 2 (0.75 ± 0.05) compared to the group 1 (2.31 ± 0.16). Moreover, the group 3 (2.32 ± 0.15) and group 4 (2.60 ± 0.18) showed higher ($p < 0.05$) serum testosterone concentrations compared to the group 2 (Fig. 1B).

As shown in Table 1, a substantial increase in the MDA levels was observed in the testes of group 2 compared to the group 1 ($p < 0.05$). The group 3 (and group 4 showed a dramatic decline in serum MDA levels compared to group 2 ($p < 0.05$). The SOD activity was decreased in group 2 compared to the group 1 ($p < 0.05$). The treatment of the diabetic group with saponin (group 3) elevated the activity of SOD enzyme in comparison with group 2 ($p < 0.05$). The SOD activity was also increased in group 4 compared to the group 2. The activity of the GPx enzyme was also decreased in group 2 compared to the group 1 ($p < 0.05$). Furthermore, group 3 and group 4 indicated higher GPx enzyme activity compared to the group 2 ($p < 0.05$).

Discussion

The present study examined the ameliorative effect of saponin on diabetes-induced injuries in male mice reproductive system. The findings of the present study showed that saponin declined the blood glucose and oxidative stress markers in the testes of diabetic mice. Diabetes produces testicular dysfunctions and reportedly, treatment with saponin improves these functional deficiencies via its anti-oxidant and anti-diabetic properties.[9,10] Accordingly, some studies have reported that treatment of STZ-induced diabetic mice with saponin reduces the blood glucose levels and increases the tissue sensitivity to insulin.[18,19] In another study, the saponin-contained fraction of the *Momordica charantia* plant stimulated insulin secretion in an *in vitro*, static incubation assay.[20] The hypoglycemic effect of saponin is related to its ability to increase the sensitivity of tissues to insulin.[19,21]

In diabetic patients, in addition to an enhanced amount of blood glucose, the balance between the generation and resolution of free radicals is also suspended. As a result, free radical levels increase and cause oxidative stress.[7,12] Oxidative stress results in cell injury via mechanisms such as lipid peroxidation and DNA and protein oxidative damages.[22] The results of the present study showed that diabetes remarkably incremented the MDA (a lipid peroxidation marker) levels in the testicular tissue of diabetic mice, indicating that lipid peroxidation had been elevated. This finding corresponds to the results of previous research on the effects of oxidative stress on the testis of diabetic mice.[5,7] Several studies in this context have reported an increase in lipid peroxidation and MDA level in the diabetic patients.[23] Other studies have reported that saponin scavenges the free radicals generated during lipid peroxidation.[24] Hence, the decline in testis MDA concentrations in the saponin-treated group may be related to the anti-oxidant effects of saponin. Akbarizare *et al*.,[25] have showed that saponin decreases the MDA level probably due to its anti-oxidant properties.

The activity of SOD dramatically declined in the diabetic mice in this study. These results confirm the findings of previous studies. The SOD is known as one of the most important enzymes of the anti-oxidant system. It mainly catalyzes the conversion of superoxide anion radicals to H_2O_2. Through this procedure, the toxicity of superoxide is decreased and no free radicals from super-oxide are produced.[22] The activity of SOD was remarkably enhanced in the serum of diabetic mice being treated with saponin in contrast to the diabetic control group in the present study. This is in line with the related literature. Hu *et al*.,[26] have showed that saponin increases the serum SOD levels and the protection against cisplatin-evoked intestinal injury via multiple ROS-mediated mechanisms.

In the present research, the GPx enzyme activity was intrinsically reduced in the diabetic mice compared to the control group. However, it was notably increased in the saponin-treated group compared to the diabetic control group. The GPx, an anti-oxidant enzyme, is another enzyme with detoxification effects against free radicals.[27] A decline in the activity of GPx in this study can be due to the increment in H_2O_2 generation because of glucose autoxidation and non-enzymatic protein glycation, causing oxygen free radicals production.[28] It is well-known that anti-oxidant therapy increases GPx activity.[29]

In the present study, the STZ-induced diabetes in mice resulted in alterations in the histological indices of testicular tissue. The treatment of the diabetic mice with saponin ameliorated most of the diabetes-induced deficits

as well as spermatogenesis. These alleviating effects in the treated animals were almost similar to those of the healthy control group (group 4).

The reduction or absence of insulin can also decrease testosterone concentrations causing testicular atrophy. Insulin itself, is known as an anti-apoptotic factor that can control testicular apoptosis and reproductive malfunction resulted from diabetes.[30]

In line with the findings of this study, previous studies have indicated that medicinal plants containing flavonoids can improve sperm quality and testosterone levels.[5,7,31] In a similar study, the increased rate of testicular germ cell death through apoptosis in STZ-induced diabetic rats was protected by *Dracaena arborea* aqueous extract containing saponins.[32] Feasible mechanisms complicated in the recuperation of testicular oxidative stress by saponin in diabetic mice can be described by its anti-oxidant property, decreased blood glucose and enhanced insulin secretion.[33]

The treatment of diabetic mice with saponin ameliorated diabetes-induced histological alterations in the seminiferous tubules. In this regard, the MJS and diameter and thickness of seminiferous tubules were decreased in the diabetic mice in the present study. These alterations are often important indicators of spermatogenic dysfunction alongside to the decreases in sperm production.[34] All these alterations could be due to the toxic effect of STZ on male reproductive system via decrease in testosterone concentrations and consequently interrupting testicular function.[35] This late event could resulted in the reduction and death of germ cells.[36] The oxidative-induced free radicals are proposed to explain the etiology and pathophysiology of the biological effects of diabetes mellitus. In this regard, the free radicals generated by STZ metabolism can damage DNA and chromosomes resulting in the cell death via apoptosis or necrosis.[35]

Moreover, the serum testosterone levels were decreased in diabetic mice, which may be related to the testicular tissue damage and Leydig cell injuries. However, treatment with saponin was able to ameliorate these damages. In this regard, Shoorei *et al*.,[7] have reported that diabetes induces testicular tissue damage and decreases testosterone levels in diabetic rats.

In conclusion, diabetes exerts a negative effect on the testis and sperm quality through oxidative stress. Saponin has a potent effect on the anti-oxidant system activation in reducing the oxidative stress induced by diabetes. However, further detailed researches are required to confirm these results.

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Conflict of interest

The authors declare no conflict of interest.

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