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Therapeutic potential of Bee gomogenat against testicular damage in a streptozotocin- induced type I diabetic mouse model

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ABSTRACT

Diabetes Mellitus (DM) is a metabolic disorder that has serious effects for male fertility. Additionally, DM mediates impairment of spermatogenesis; decrease in sperm count, viability, and motility; and apoptosis in both somatic and germ cells. This study aimed to investigate the dietary effect of bee gomogenat (BG) supplementation on the architecture of testis in a mouse model of streptozotocin (STZ)-induced type 1 diabetes (TID). Mice were allocated to three different groups: control non-diabetic, diabetic, and BG-treated diabetic mice. STZ-induced diabetes was associated with pathological alterations in the architecture of the testis, decreased distribution of YAP+ Leydig cells in testis sections and decreased level of testosterone. Furthermore, diabetic mice showed perturbed alteration in the expression of PPAR-γ, Nrf2 and 3β-HSD in testis as compared to control animals. Interestingly, supplementation of diabetic mice with BG displayed an improvement in the architecture of testis, restored the distribution of

YAP+ Leydig cells and the testosterone level. Furthermore, Treatment of diabetic mice with BG significantly restored the expression levels of PPAR-γ, Nrf2 and 3β-HSD nearly to control animals.

Conclusion: Our data revealed, for the first time, the beneficial impacts of BG supplementation on the architecture and functions of testis during T1D.

INTRODUCTION

Diabetes mellitus (DM) is one of the main causes of reproductive disorders such as testicular tissue malfunction and pituitary-gonadal axis dysfunction, sperm quality and count reduction [1, 2], testosterone levels decrement [3] and apoptosis induction in both the somatic and germ cells [4-7]. Indeed, circulatory and testicular levels of all sex hormones had been changed, including testosterone, luteinizing hormone (LH), and follicular stimulating hormones (FSH) in both diabetic patients and experimental animal models [7, 8]. Oxidative stress impairs spermatogenesis and causes loss of germ cells [9, 10]. Diabetes-related male infertility has been studied in both animal and human models [11]. Due to decreased sexual functions (erection, ejaculation, and libido), testicular structural, and spermatogenesis disorders, approximately 90% of diabetic individuals are infertile or sub-fertile [12]. STZ-induced diabetic rat could be considered one of the most successful models for studying the effect of diabetes on male infertility [13].

The nuclear factor erythroid 2-related factor 2 (Nrf-2) is found in mammalian testes and sperms, and it plays a critical role in avoiding oxidative damage and maintaining normal spermatogenesis [14-16]. Additionally, Nrf-2 is found in cytoplasm and translocates to the nucleus upon exposure to oxidative and inflammatory signals, where it connects with enhancer, antioxidant response element (ARE), mediating the activation of genes coding for antioxidant proteins, particularly the glutathione (GSH). Nrf-2 plays a vital role against ROS and regulate metabolism of lipid [17]. Peroxisome proliferator-activated receptors-γ (PPAR-γ) plays a key function in the regulation of energy homeostasis, which modulates the hypothalamic-pituitary-gonadal (HPG) axis. The

PPAR-γ protein can be found in spermatozoa, germ cells, and Sertoli cells in humans. [18].

3β-hydroxysteroid dehydrogenase (3β-HSD) is a critical enzyme in the production of androgens, as well as practically all other biologically active steroids. Therefore, increase activity of 3β-HSD in the testes is necessary for normal steroidogenesis and hence for reproduction [19].

Recently, researchers have paid more attention to diabetes and its associated complications. Thus, novel and more effective therapeutic agents for treating diabetic complications are urgently needed and should be developed.

Bee gomogenat (BG) of trutnevy larvae has a nutritious structure such as proteins, vitamins (A, B, E, D, B-carotene), amino acids, minerals (potassium, magnesium, calcium, iron, phosphorus, zinc), different enzymes and steroid hormones (an estradiola, progesterone). Nevertheless, there is no available published works describing the impacts of BG on the architectures of testis. Therefore, the current study aimed to investigate the impact of dietary supplemtation of BG on the architectures of testis in mouse model of streptozotocin (STZ)-induced T1D. Moreover, we registered a patent for our preliminary studies monitoring the influence of BG on the immune system of heat stress animals and diabetic mouse model.

MATERIALS AND METHODS

Bee gomogenat Preparation

BG was obtained from Etman hives for honey bee products, Tanta, Egypt. The active chemical components of BG were analyzed, using GC-MS (The Analytical Chemistry Unit at chemistry department, Faculty of Science, Assiut University). BG is a creamy substance when stored at -20 °C. Based on our preliminary experiments, we found no adverse effects of high-dose oral BG supplementation on mice up to 4 g/kg body weight, and the optimal dose was 1 g/kg body weight. In the present study, the optimal dose of B

G that was prepared by dissolving 1 g of BG in a final volume of 10 ml of distilled H_2O (100 mg/ml). Then, 250 µl of diluted BG (25 mg) was orally administered to each mouse weighing 25 g (1 g/kg body weight/day for 30 days).

Chemicals

STZ was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The STZ was freshly prepared for immediately use (within 5 min) by dissolving in cold 0.01 M citrate buffer (pH 4.50).

Experimental design and doses

45 BALB/c adult male mice (25–30 g) were obtained from the Institute of Theodor Bilharz, Cairo, Egypt. Mice were housed in cages and kept at a room temperature at 25 ± 5 °C under a normal 12 h light/12 h dark cycle. They were fed a grain- and water-based diet for one week to acclimatize. All animal experiments were carried out according to the Institutional Animal Care laws and to the International Guidelines for Animal Care (Council of European Communities 1986), and then were approved by the Ethical Committee of the Faculty of Medicine at Assiut University (Ethics approval number163/2204-2020). We minimize animal distress and keep their number to a minimum as previously described [20]. The mice were divided into three groups of 15 mice each after one week of acclimatization: control (cont.), diabetic (diab.), and diabetic treated with BG (diab.+BG). Diabetes was induced in mice of group 2 and 3 by three intraperitoneal injections (i.p.) of STZ (60 mg/kg body weight) in 0.01 M citrate buffer (pH 4.5). Control group of mice was injected with only the vehicle (0.01 M citrate buffer, pH 4.5). After 4 days, glucose levels were measured and the animal were considered diabetic when the glucose levels became >220 mg/dl. After two weeks of intraperitoneal injection with STZ, control non-diabetic mice were orally supplemented with distilled water (250 µl/mouse/day for one month by oral gavage); group 2 diabetic mice were orally supplemented with distilled water (250 µl/mouse/day for one month by oral gavage); and group 3 diabetic mice were orally supplemented with BG (1 g/kg body weight/day for one month by oral gavage).

Histopathological and Immunohistochemistry study

Testis samples were fixed immediately in formal alcohol until processed as previously described [21]. Samples were then dehydrated, embedded and thin sections (5 μm) were prepared. For histopathological examination, sections were stained with H&E and Sirius red staining. For immunohistochemistry tissue sections were processed according to [22]. We stained the tissue sections with the primary antibody (anti-YAP (Yes associated protein).

Western blot analysis

RIPA buffer was used to prepare lysates from the tissues of testis organ. The concentrations of protein were measured using a protein assay kit (Bio-Rad, Hercules, CA). Fifty micrograms of protein lysate were separated by SDS-PAGE prior to transfer onto nitrocellulose membranes. The membranes were then blocked for I h using non-fat milk (50 g/L) in TBS, after which they were incubated overnight with primary antibodies specific for PPAR-γ, Nrf-2, 3β-HSD and β-actin (1:1000; Santa Cruz Biotechnology). Then, HRP-conjugated species-matched secondary antibody was used to detect the protein bands with enhanced chemiluminescence (ECL, Super Signal West Pico Chemilumines-cent Substrate, Perbio, Bezons, France), and the ECL signals were recorded using LI-COR scanner. ImageJ software was used to quantify the protein band intensities as previously described [23].

Statistical analysis

Statistical analysis was performed based on normality difference data which are expressed as the means ± standard error of the mean (SEM) using graph Pad Prism software version 5. One-way ANOVA was used to examine the significant differences between the three groups, followed by Tukey's post-test.

RESULTS

Treatment of diabetic mice with BG repairs histopathological changes in the testis.

The histopathological alterations in the testis tissues of the three animal groups after induction of diabetes with STZ were examined. Pictures of the control, diabetic, and diabetic treated with BG groups were taken at x400 magnification using the H&E staining method, and Photographs of one representative are displayed. The testis sections of control group revealed the normal histological appearance of seminiferous tubule (**Fig. 1A**). However, Examination of testis sections from diabetic animals showed degeneration in spermatocytes with the presence of marked spermatid giant cells associated with incomplete spermatogenesis inside seminiferous tubule (**Fig. 1B**). BG treatment showed partial restoration of histological structure in testis of diabetic animals similar to control mice (**Fig. 1C**).

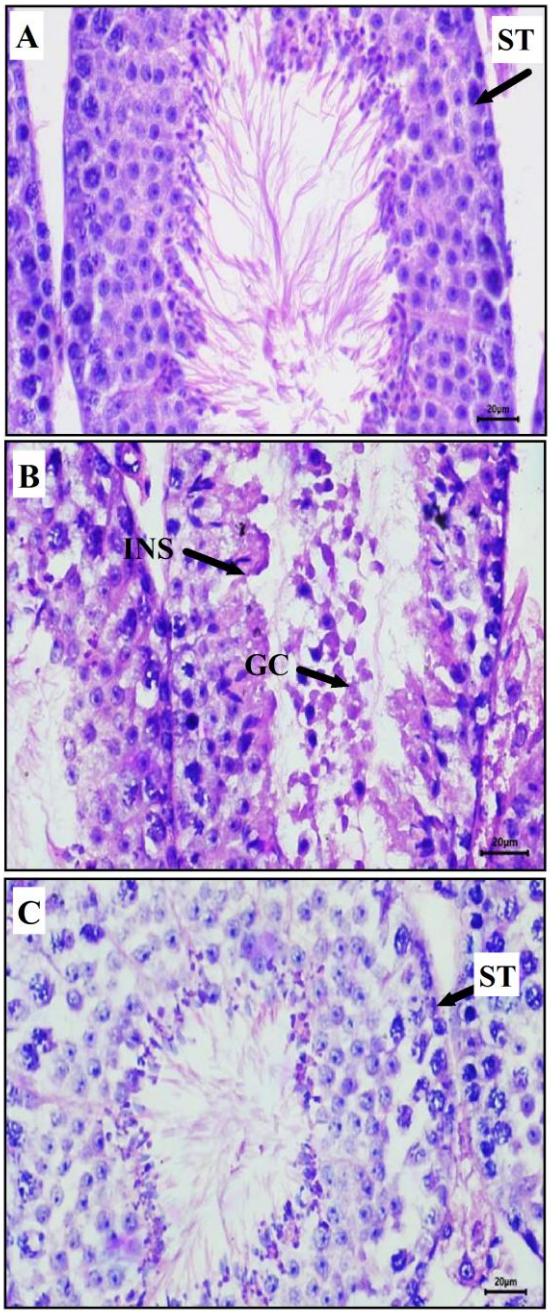

Fig. 1. **Impact of BG boosts the histological structure of testis in T1D.**

Photomicrograph of testis sections from control mice **(A)** showing normal histological appearance of seminiferous tubule (ST). **(B)** Diabetic group showing incomplete spermatogenesis (INS) inside seminiferous tubule and marked spermatids giant cells (GC). **(C)** Diabetic mice treated with BG showing more or less complete spermatogenesis inside seminiferous tubule (ST) (H&E staining; 400x).

Oral supplementation of BG reduces the deposition of collagen in the testis of diabetic mice.

The deposition of collagen fibres in the testis sections of diabetic animals was monitored using the Sirius red staining technique. Photomicrographs of testis sections from the three animal groups were taken at x400 magnification. The Sirius red staining of control group revealed the normal collagen deposition in testicular capsule (**Fig. 2A**). In diabetic mice, Sirius red stain showed that marked increase in collagen deposition in the testicular capsule (**Fig. 2B**). BG-treated diabetic animals established moderate amounts of collagen deposition in testicular capsule which was nearly to control (**Fig. 2F**). Five animals from each group were used for the quantification of the collagen deposition using image J software. Results elucidated that diabetic animals displayed a significant increase in the deposition of collagen as compared to control non-diabetic animals. BG-treated diabetic animals significantly restored the deposition of collagen nearly to those found in control group (**Fig. 2D**).

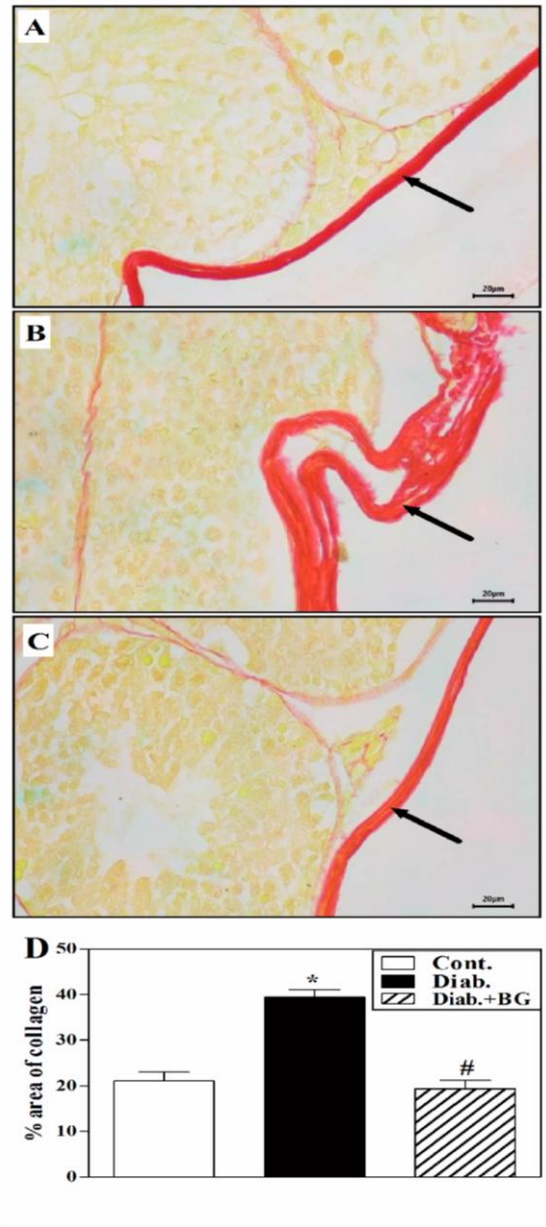

Fig. 2. BG treatment increases collagen deposition in testis of T1D.

Photomicrograph of testis section from control mice **(A)** showing normal collagen fibers deposition (arrow) in testicular capsule. **(B)** Diabetic group showing increase collagen fibers deposition (arrow) in testicular capsule. **(C)** Diabetic mice treated with BG showing moderate collagen fibers deposition in testicular capsule (arrow). Accumulated data from five mice from each group are expressed as the mean ± SEM (n = 5). $^{*}P < 0.05$ for diab. vs. cont.; $^{+}P < 0.05$ for diab.+BG vs. cont.; and $^{\#}P < 0.05$ diab.+BG vs. diab.(ANOVA with Tukey's post-test).

BG treatment of diabetic mice enhances the distribution of YAP+ Leydig cells in testis sections.

The influence of diabetes on distribution of YAP+ Leydig cells in testis sections of three animal groups using anti-YAP+ was investigated. At x400 magnification, the control testis exhibited normal distribution of YAP^+ Leydig cells (brown colour) between the seminiferous tubules (**Fig. 3A**). However, diabetic mice displayed decrease in the distribution of YAP^+ Leydig cells between the seminiferous tubules **(Fig. 3B)**. Most importantly, treatment of diabetic animals with BG obviously restored the distribution of YAP^+ Leydig cells between the seminiferous tubules as in control testis (**Fig. 3C**). Using image J we quantified the number of Leydig cells and pooled data from five animals from each group demonstrated that diabetic animals showed a significant decrease in the number of Leydig cells as compared to control non-diabetic animals. BG-treated diabetic mice significantly restored the number of Leydig cells nearly to those found in control group (**Fig. 3D**).

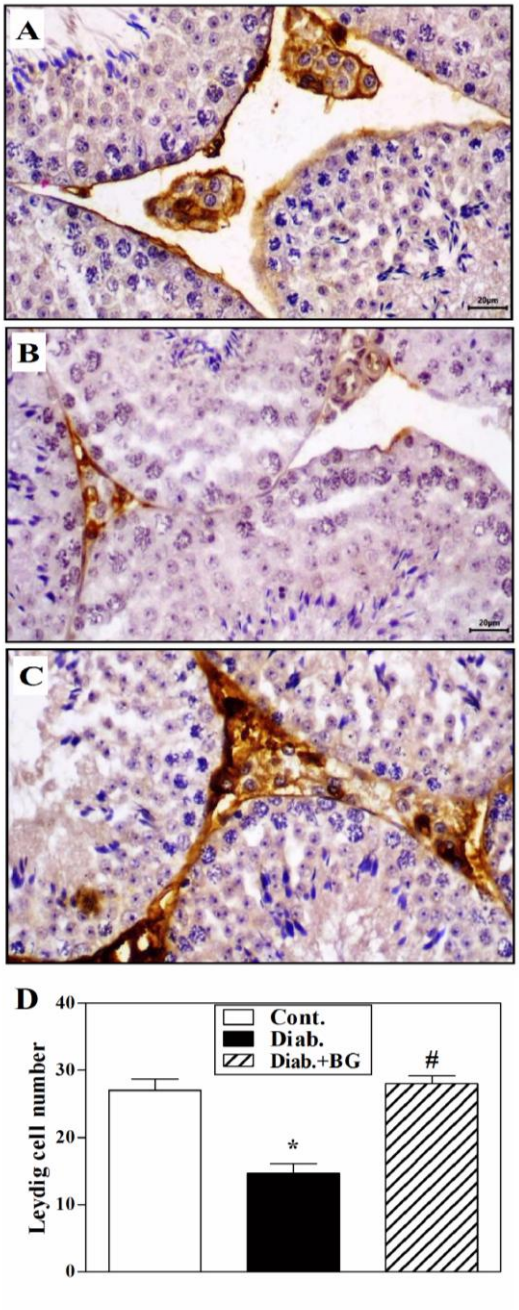

Fig.3. Effect of BG on the distribution of YAP+ Leydig cells during T1D.

Photomicrographs of testis sections from the cont. **(A)**, Diab. **(B)**, and Diab.+ BG **(C)** mice that were stained with antibody recognizing YAP and were then detected by immunohistochemical analysis (Immunoperoxidase 400×). **(D)** Pooled data from five mice from each group are expressed as the mean ± SEM (n = 5). $^{*}P < 0.05$ for diab. vs. cont.; $^{+}P < 0.05$ for diab.+BG vs. cont.; and $^{\#}P < 0.05$ diab.+BG vs. diab.(ANOVA with Tukey's post-test).

BG supplementation decreases the testosterone level in diabetic mice.

Fig. 4 shows a significant increase in the level of plasma testosterone in diabetic mice compared to the control non-diabetic mice. Interestingly, treatment of diabetic animals with BG revealed a significant restoration of testosterone level compared to diabetic animals.

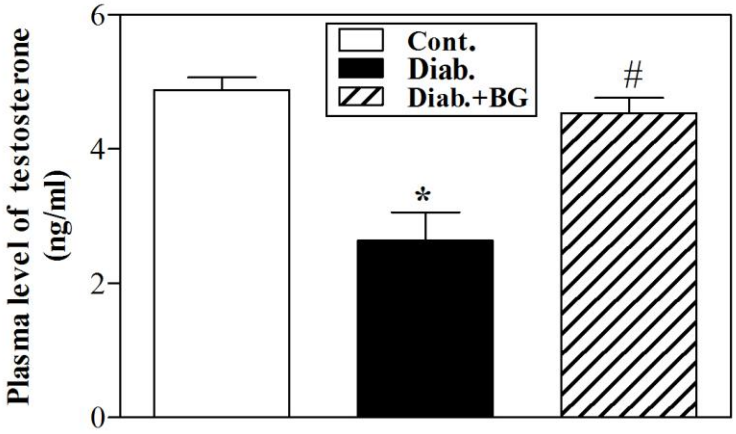

Fig.4. BG treatment decreases the level of testosterone in T1D.

Testosterone level was measured for the three groups of mice. The collected data for five mice from each group are presented as the mean ± SEM (n = 5). $^{*}P < 0.05$ for diab. vs. cont.; $^{+}P < 0.05$ for diab.+BG vs. cont.; and $^{\#}P < 0.05$ diab.+BG vs. diab.(ANOVA with Tukey's post-test).

BG treatment repairs the architecture of testis through restoring the expression of PPAR-$_{\gamma}$, Nrf2 and 3β-HSD in diabetic animals.

The expression of PPAR-**γ**, Nrf2 and 3β-HSD in testis tissues of the three animal groups using Western blot analysis was assessed. **Fig. 5** shows immunoblots for PPAR-**γ** (**Fig. 5A**), Nrf2 (**Fig. 5C**) and 3β-HSD (**Fig. 5E**) and β-actin (loading control) in the testis of control, diabetic, and Diab.+BG mice. The expression level of PPAR-**γ**, Nrf2 and 3β-HSD were normalized to the expression level of total β-actin and accumulated data from five individual mice from control, diabetic, and Diab.+BG in PPAR-**γ** (**Fig. 5B**), Nrf2 (**Fig. 5D**) and 3β-HSD **(Fig. 5F**) are presented as the means ± SEM of the normalized values. Diabetic mice displayed a significant down-regulation in the expression of PPAR-**γ** and 3β-HSD, and up-regulation in the expression of Nrf2 compared to control mice. However, orally supplemented diabetic mice with BG significantly restored the expression of PPAR-**γ**, 3β-HSD and Nrf2 nearly to the control mice.

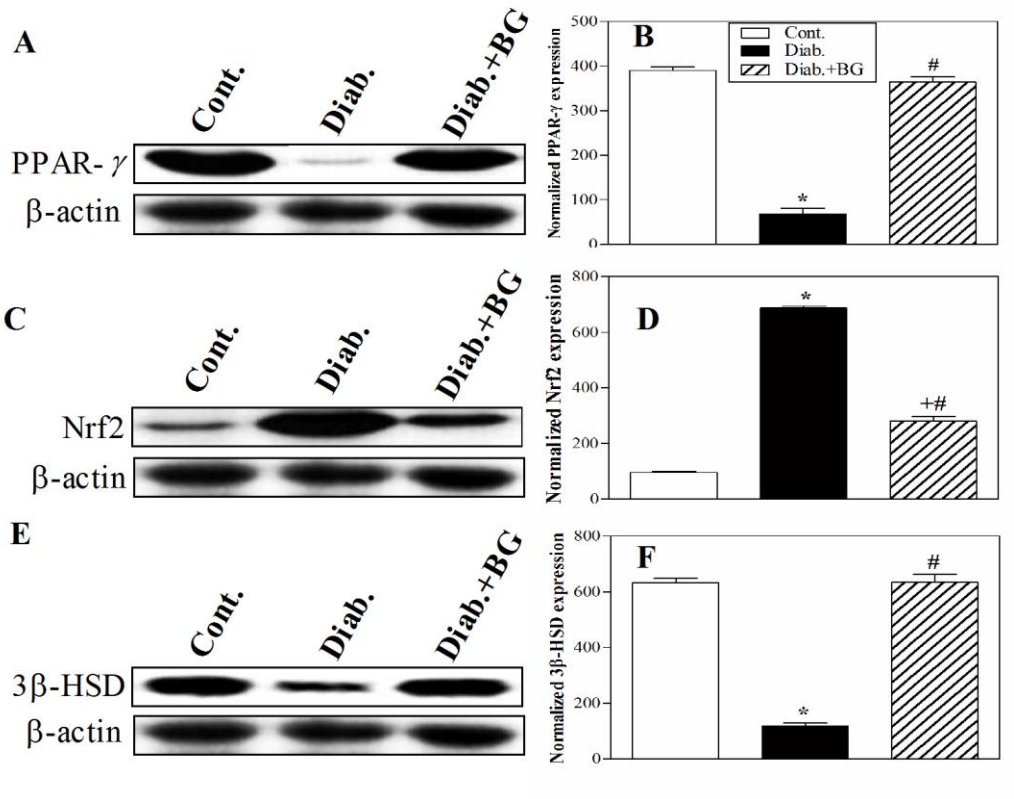

Fig.5. Impact of BG supplementation on the expression of expression of PPAR-γ, Nrf2 and 3β-HSD in testis tissues of diabetic mice.

Immunoblots of PPAR-γ **(A)**, Nrf2 **(C)**, and 3β-HSD **(E)** and β-actin, from one representative experiment are shown for cont., diab. and diab.+BG mice in testis. **(B)** Quantification of normalized PPAR-γ, **(D)** quantification of normalized Nrf2 and **(F)** quantification of normalized 3β-HSD expression levels to total actin determined by western blotting for each of the three experimental groups.

DISCUSSION

Hyperglycemia is a hallmark of diabetes which is accused for increasing the oxidative stress related to male infertility [24]. In our study, the induction of diabetes affects the testicular organ and causes degenerative changes represented by degeneration in spermatocytes with the presence of marked spermatid giant cells, incomplete spermatogenesis inside seminiferous tubule and fibrosis in testicular capsule which in turn mediates dysfunction of testicular tissues, in line with the results of previous studies [25, 26]. Nevertheless, treatment of diabetic animals with BG exhibited improvement in the histological architecture of testis and reduction of fibrosis which in turn lead to increase fertility.

YAP is a non-receptor protein tyrosine kinase that regulates cell growth and survival, as well as apoptosis, adhesion, cytoskeletons, and differentiation [27]. In rat testes, YAP is present in the blood-testis barrier and arranges actin filaments [28]. Because testosterone regulates all component of erectile function, decrease level of testosterone in diabetic rat lead to decrease of sexual performance [29]. Reduced insulin secretion is associated with decreased Leydig cell (testosterone-producing cell) and sertoli (spermatogenesis) function [30]. In our study, the testosterone level and distribution of YAP^+ decreased in diabetic mice as a result of decease of Leydig cell, these results in line with previous study [31]. BG-treated diabetic animals restored the level of testosterone and distribution of YAP^+ Leydig cells in testis sections.

3-HSD is a catalytic enzyme that converts its steroid substrates into testosterone [32]. 3β-HSD is closely linked to the synthesis and secretion of testosterone, this explain the decreased testosterone level in T1D group [7, 33]. PPAR-γ is located in germ cells and Sertoli cells where it controls Sertoli cells lipid metabolism [34]. The present study demonstrated that diabetic animal decreased the expression level of 3β-HSD and PPAR-γ, which were in agreement with previous study [35]. Oral supplementation of diabetic animals with BG partially restored the expression of 3β-HSD and PPAR-γ similar to nearly that found in control animals. Nrf-2 plays a key function in avoiding the formation of oxidative stress through up-regulation of the Nrf2-related antioxidants [36, 37]. The

current study revealed that the expression level of Nrf-2 increased in diabetic animals. However, treatment of diabetic animals with BG restored the expression level of Nrf-2 nearly to control animals.

Because diabetic complications are life-threatening, different new strategies should be applied to reduce complications associated with diabetes. Since BG repairs the architecture of testis organ, restored the level of testosterone, the distribution of YAP and the expression of Nrf-2, PPAR-γ and 3β-HSD without any side effects, it could be considered a new approach to overcome diabetic complications (testicular damage) .

CONCLUSION

BG improves fertility by regulating testicular function through restoring testosterone level, and, hence, restoring the normal distribution of YAP between seminiferous tubules. BG also regulated the expression of Nrf-2, PPAR-γ, and 3β-HSD which, in turn, repairs the pathological alterations that were mediated by diabetes in testes.

These findings demonstrated that BG supplementation had a significant positive impact on testis architecture and function after STZ-induced type 1 diabetes.

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