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Research Article
Adenium obesum Flowers Extract Mitigates Testicular Injury and Oxidative Stress in Streptozotocin-induced Diabetic Rats

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Abstract

Background and Objective: Diabetes Mellitus (DM) is a major healthcare problem worldwide and considerable evidence proved its negative impact on the male reproductive system. *Adenium obesum* is an interesting medicinal plant with a wide range of bioactivities. The current study examined the protective effects of *A. obesum* flower extract (AOE) on testicular injury in streptozotocin (STZ)-induced type I diabetic rats. **Materials and Methods:** Diabetes was induced by a single injection of 50 mg kg^{-1} STZ. Diabetic rats received 250 and 500 mg kg^{-1} AOE for 21 days and samples were collected for analysis. **Results:** As compared to the diabetic control rats, treatment with AOE increased serum testosterone, Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) levels, decreased testicular thiobarbituric acid reactive substances (TBARS) content, effectively enhanced reduced glutathione (GSH) content and superoxide dismutase (SOD) activity. Additionally, AOE effectively inhibited diabetes-induced testicular tissue injury and prevented inflammatory and apoptotic responses manifested by decreased TNF-α, IL-6 and Bax and increased Bcl-2. **Conclusion:** These results demonstrated that AOE mitigates testicular injury, oxidative stress, inflammatory response and apoptotic cell death in STZ-induced diabetic rats.

Key words: Desert rose, oxidative injury, inflammation, antioxidants, phenolics, diabetes, testosterone, apoptosis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic disorder and represents a major healthcare problem worldwide. Chronic hyperglycemia and macromolecular metabolic defects are the main characteristics of DM. Several literatures reported that DM can affect male reproduction and may lead to male infertility[1,2]. The testis play a key role in the reproductive system and several studies demonstrated that DM can cause different harmful effects such as testis and epididymis weight reduction[3]. Although, the exact mechanism behind testicular damage in diabetes is not fully understood, oxidative stress[4,5], inflammation[6,7] and apoptosis[8,9] have been postulated to play significant roles. Oxidative stress is a state where excessive Reactive Oxygen Species (ROS) can't be antagonized effectively by the body antioxidant defense system, leading to testicular injury in hyperglycemic conditions. Given the high metabolic rate of testicular tissue, oxidative injury can be destructive to the testis[2,5]. Additionally, inflammation is another contributing factor of tissue injury caused by hyperglycemia and manifested by significant elevations in the pro-inflammatory cytokines Tumor Necrosis Factor-alpha (TNF-α) and interleukin-6 (IL-6)[10-15]. Both oxidative stress and inflammation are well-acknowledged to work in concert and promote cell death via apoptosis[16,17]. Therefore, mitigating oxidative stress and inflammatory responses in diabetes could represent an effective strategy to attenuate testicular tissue injury.

Increasing evidence has demonstrated that plants have gained much thoughtfulness as a source of therapeutic agents for the treatment of human diseases[18]. In this context, different plants as well as their active constituents have shown great benefits for the amelioration of testicular injury in streptozotocin (STZ)-induced diabetes[1,19,20]. *Adenium obesum* is a plant belongs to family Apocynaceae, commonly known as desert rose[21]. It possesses anti-oxidant[22,23], anti-cancer[24,25], anti-microbial[26] and anti-viral[27] activities. In traditional and complementary medicine, different parts of *A. obesum* are utilized for the treatment of various diseases, such as; skin diseases, wounds and joint pain[23]. Although, it possesses several pharmacological activities, the potential protective effect of *A. obesum* flower extract (AOE) against testicular injury in diabetes has not been investigated. Therefore, the current study investigated the possible protective effects of AOE against testicular injury, oxidative stress, inflammatory response and apoptotic cell death in STZ-induced diabetic rats.

MATERIALS AND METHODS

Collection of *A. obesum* flower and extract preparation: This study was conducted during the period from January, 2019-2020. The flowers of *A. obesum* were collected from Riyadh city (Saudi Arabia) and were identified and authenticated by an expert taxonomist. The flowers were washed, dried in shade, pulverized and macerated with 80% methanol for 72 h at 4°C. The mixture was filtered and the filtrate was concentrated by using rotary evaporator and kept at -20°C until used.

Determination of total phenolics and flavonoids: Total phenolics content was determined using Folin Ciocalteu method[28] and flavonoids content was assayed using aluminum trichloride method[29].

Animals and treatment protocol: Thirty male Wistar albino rats with a body weight of 160-180 g were used in the current investigation. These rats were kept in a controlled environment (12/12 h light/dark cycle, humidity 60±10% and temperature 23±2°C) with free access to water and food. The experimental procedures were conducted in accordance with the guidelines for the care of laboratory animals and approved by the local ethical committee.

Type I DM was induced by intraperitoneal (i.p.) injection of 50 mg kg^{-1} STZ (Sigma, USA)[30] dissolved in freshly prepared cold citrate buffer (pH = 4.5). Three days after STZ injection, blood glucose level was measured using a digital glucometer and rats with fasting glucose levels $\geq$250 mg dL^{-1} were considered diabetic and included in the study.

The rats were allocated randomly into five groups, each comprising 6 animals as follows:

- **Group I:** Rats received a single i.p. injection of citrate buffer and 0.5% carboxymethyl cellulose (CMC) orally for 21 days
- **Group II:** Rats received a single i.p. injection of citrate buffer and 500 mg kg^{-1} AOE dissolved in 0.5% CMC orally for 21 days
- **Group III:** Diabetic rats received 0.5% CMC orally for 21 days
- **Group IV:** Diabetic rats received 250 mg kg^{-1} AOE dissolved in 0.5% CMC orally for 21 days
- **Group V:** Diabetic rats received 500 mg kg^{-1} AOE dissolved in 0.5% CMC orally for 21 days

Collection of samples: At the end of the experiment, overnight fasted rats were sacrificed under anesthesia and blood samples were collected for serum separation. The animals were dissected and testes were excised and washed with cold Phosphate Buffered Saline (PBS). Samples from the testis were homogenized (10% w/v) in cold PBS, centrifuged and the supernatant was separated for the assessment of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and superoxide dismutase (SOD). Other samples were fixed in 10% neutral buffered formalin for histological processing.

Determination of reproductive hormones: Serum testosterone was measured using ELISA kit (Cusabio, China), and Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) were measured by using ELISA kits (Novos Biologicals, USA) according to the manufacturers instructions.

Histopathology assessment: Samples from the testis fixed in 10% neutral buffered formalin for 24 h were dehydrated, cleared and embedded in paraffin. About 5 µm sections were cut, mounted, deparaffinized, rehydrated and stained with Hematoxylin and Eosin (H&E) as previously described[31].

Assessment of oxidative stress biomarkers: TBARS, a marker of lipid peroxidation were assayed according to the method of Ohkawa *et al*[32]. The GSH was estimated according to the method described by Ellman[33] and SOD activity was assayed based on the method of Nishikimi *et al*[34].

Determination of cytokines: Serum levels of TNF-a and IL-6 were determined by using commercial kits (Cusabio, China) according to the manufacturer's instructions.

Gene expression analysis by quantitative real time-PCR (qRT-PCR): Total RNA was isolated from testis samples using TRIzol reagent (Invitrogen, USA) and its quantity were determined using a nanodrop. Samples with A260/A280 higher than 1.7 were immediately reverse transcribed into cDNA. For gene expression analysis, qRT-PCR was employed using QuantiFast SYBR Green RT-PCR kit (Qiagen, Germany) and the following primers: Bax: F: 5'-AGGACGCAT CCACCAAGAAG-3' and R: 5'-CAGTTGAAGTTGCCGTCTGC-3', BCL-2: F: 5'-ACTCTTCAGGGATGGGGTGA-3' and R: 5'-TGACATCTCCC TGTTGACGC-3' and GAPDH: F: 5'-AACTTT GGCATCGTGGAAGG-3' and R: 5'-TACATTGGGGGTAGGAACAC-3'. qRT-PCR reactions were performed using ViiA™ 7 System (Thermo Fisher Scientific, CA, USA) in duplicates. The transcript number was determined using the $2^{\Delta\Delta C}$t method[35].

Statistical analysis: The results are given as Means±Standard Error of the Mean (SEM) and all statistical comparisons were made by means of the one-way ANOVA test followed by Tukey's test *post hoc* analysis using GraphPad Prism 7 (GraphPad Software, CA, USA). A p-value less than 0.05 was considered significant.

RESULTS

Total phenolic and flavonoids in AOE: Phytochemical analysis of AOE revealed the presence of 3.02±0.01 mg gallic acid equivalent/g total phenolics and 1.21±0.04 mg quercetin equivalent/g total flavonoids.

AOE improves serum testosterone, LH and FSH levels in diabetic rats: As illustrated in Fig. 1, the diabetic rats showed a significant ($p<0.001$) decrease in serum levels of FSH (Fig. 1a), LH (Fig. 1b) and testosterone (Fig. 1c) hormones. On the contrary, treatment with AOE, either 250 or 500 mg kg^{-1}, increased the levels of these hormones significantly. Normal rats received the higher dose of AOE showed non-significant changes in the levels of these hormones.

AOE prevents histopathological alterations in the testis of diabetic rats: Figure 2 shows representative light microscopy images of testicular tissues from each group. The control rats showed regular morphology with no evidence of histopathological changes (Fig. 2a). In contrast, diabetic rats showed a reduction in the number of spermatogonia and spermatozoa, degenerative changes and vacuolations (Fig. 2b). These pathological changes were significantly reduced in diabetic rats received 250 mg kg^{-1} (Fig. 2c) and 500 mg kg^{-1} AOE (Fig. 2d).

AOE prevents testicular oxidative stress in diabetic rats: The testicular content of TBARS (Fig. 3a) showed a significant increase ($p<0.001$) in diabetic rats while GSH (Fig. 3b) and SOD activity (Fig. 3c) exhibited significant decrease when compared to the normal control group. Administration of AOE to diabetic rats significantly decreased the testicular content of TBARS along with a dramatic increase in GSH and SOD. The high dose of AOE didn't alter the levels of TBARS and antioxidant in the testis of normal rats.

AOE attenuates inflammation in diabetic rats: The effect of AOE on serum levels of pro-inflammatory cytokines TNF-α and IL-6 was investigated in normal and diabetic rats. While the

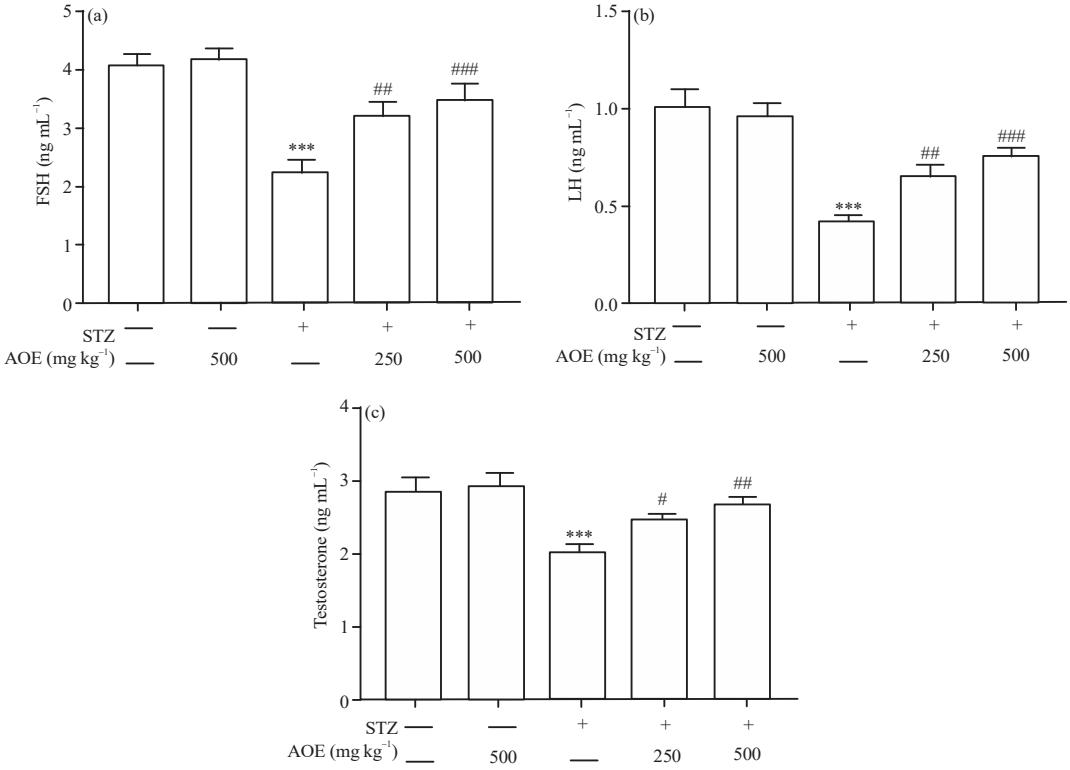

Fig. 1(a-c): AOE increased serum (a) FSH, (b) LH and (c) Testosterone in diabetic rats.

Data are Mean±SEM (n = 6), ***p<0.001 compared to control. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to diabetic, AOE: *Adenium obesum* flowers extract, STZ: Streptozotocin, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, +: Rats received STZ and -: Rats didn't receive either STZ or AOE

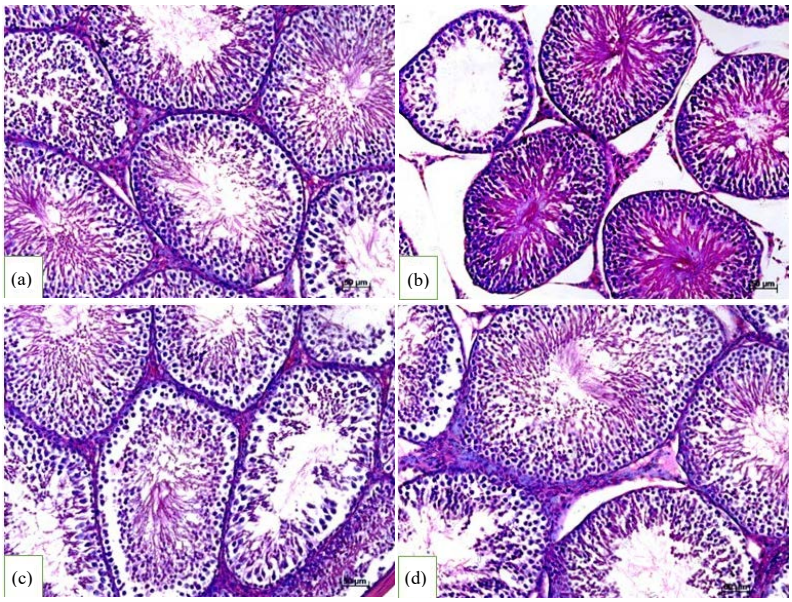

Fig. 2 (a-d): Photomicrographs of H and E-stained sections in the testis of (a) Control rats showing normal histological structures, (b) Diabetic rats showing reduced number of spermatogonia and spermatozoa, degenerative changes and vacuolations and (c-d) Diabetic rats treated with 250 and 500 mg kg^{-1} AOE showing significant improvement in the histological appearance

Scale bar = 50 µm

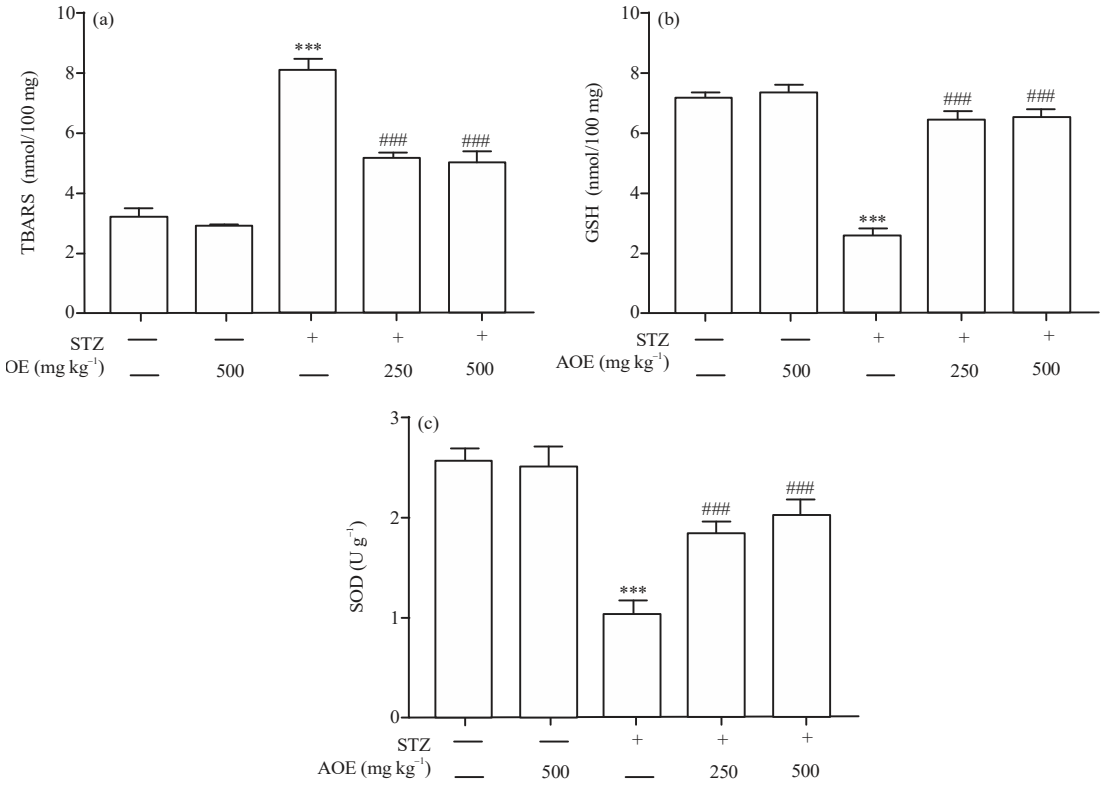

Fig. 3(a-c): AOE decreased testicular (a) TBARS and increased (b) GSH and (c) SOD in diabetic rats

Data are Mean±SEM (n = 6), ***p<0.001 compared to control and ###p<0.001 compared to diabetic, AOE: *Adenium obesum* flowers extract, STZ: Streptozotocin, TBARS: Thiobarbituric acid reactive substances, SOD: Superoxide dismutase, GSH: Reduced glutathione, +: Rats received STZ and -: Rats didn't receive either STZ or AOE

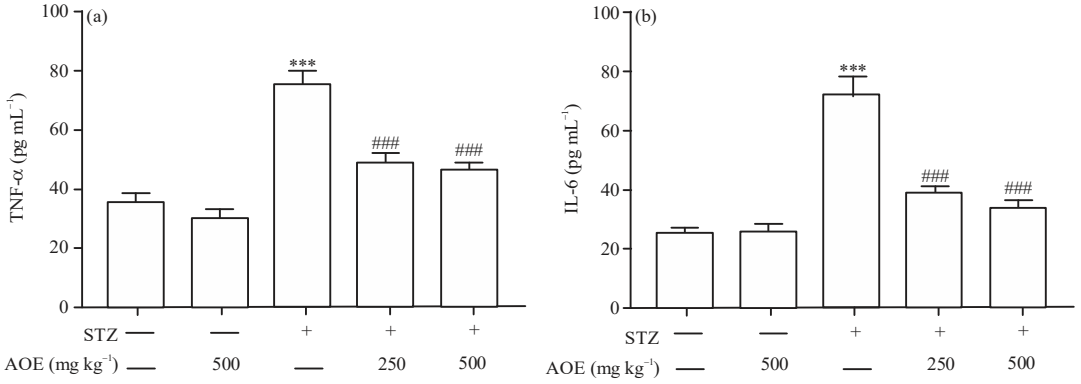

Fig. 4(a-b): AOE reduced serum (a) TNF-α and (b) IL-6 in diabetic rats

Data are Mean±SEM (n = 6), ***p<0.001 compared to control and ###p<0.001 compared to diabetic, AOE: *Adenium obesum* flowers extract, STZ: Streptozotocin, TNF-α: Tumor necrosis factor alpha, IL-6: Interleukin-6,+: Rats received STZ and -: Rats didn't receive either STZ or AOE

500 mg kg^{-1} AOE exerted no effect, the obtained results showed a significant increase in TNF-α (Fig. 4a) and IL-6 (Fig. 4b) in diabetic rats. Treatment with AOE markedly decreased the circulating levels of both TNF-α and IL-6 as compared to diabetic control rats.

AOE inhibits testicular apoptosis in diabetic rats: To investigate the effect of AOE on diabetes associated testicular apoptosis in rat, the gene expression levels of Bax (Fig. 5a) and Bcl-2 (Fig. 5b) as well as the Bax/Bcl-2 ratio (Fig. 5c) were determined. The results demonstrated that AOE

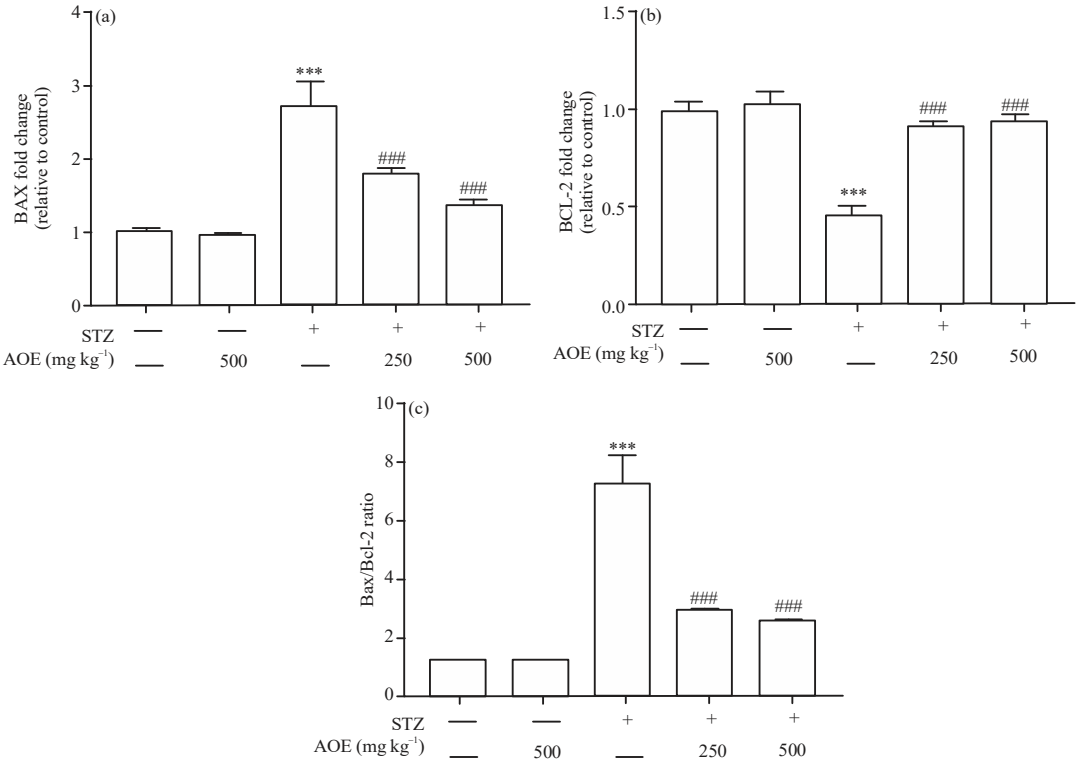

Fig. 5(a-c): AOE suppressed testicular (a) Bax gene, (b) Increased Bcl-2 gene (B) and (c) Decreased Bax/Bcl-2 ratio in diabetic rats
Data are Mean±SEM (n = 6), ***p<0.001 compared to control and ###p<0.001 compared to diabetic, AOE: *Adenium obesum* flowers extract, STZ: Streptozotocin, Bcl-2: B-cell lymphoma 2, Bax: Bcl-2 associated X, +: Rats received STZ and -: Rats didn't receive either STZ or AOE

administration, dose independently, resulted in a significant inhibition of apoptosis through up-regulation of the gene expression levels of Bcl-2 while the level of pro-apoptotic gene Bax was significantly down-regulated in diabetic rats.

DISCUSSION

Diabetic complications are the major health problems that cause pathological and functional damage to different body systems. Diabetes is significantly associated with infertility in males[36,37]. Therefore, the development of efficient approaches to attenuate or delay these complications is a great area of research interest. The present study explored the protective effect of AOE against testicular dysfunction and injury in diabetic rats, pointing to its modulatory effect on oxidative stress, inflammation and apoptosis. *A. obesum* has been reported to exert several beneficial effects, including antioxidant, anti-cancer, antimicrobial and anti-viral activities[22-27]. Additionally, different parts of *A. obesum* have been traditionally used in the treatment of skin diseases, wounds and joint pain[23]. Thus, *A. obesum* represents a promising agent for the prevention of testicular dysfunction in diabetes. Herein, type 1 diabetes mellitus was induced by STZ and the diabetic rats were treated with AOE for 21 days.

Testicular dysfunction in diabetic rats has been evidenced by the significant decrease in serum levels of FSH, LH and testosterone along with multiple histological alterations, including reduced numbers of spermatogonia and spermatozoa and vacuolations and degenerative changes. Accordingly, several studies have shown that diabetes diminishes sex hormones as indicated by lower levels of testosterone, LH and FSH[1,2,38,39]. The present study revealed that treatment with AOE increased serum FSH, LH and testosterone and effectively prevented histological alterations in the testis of diabetic rats. Thus, AOE is an effective agent against sexual dysfunction triggered by diabetes in rats. Given the free-radical scavenging activity of *A. obesum* flowers, it is noteworthy assuming that attenuation of oxidative stress improved the pituitary-gonadal axis and consequently serum levels of sex hormones.

Oxidative stress is triggered in diabetes through the excessive generation of ROS provoked mainly by hyperglycemia[40-42]. Reduction in the antioxidant status and/or increase in ROS lead to cellular damage, mitochondrial

dysfunction, disruption of the DNA and triggers the inflammatory response, resulting in activation of the apoptotic signaling pathway[43-45]. In the current study, the testicular content of TBARS showed a marked increase in diabetic rats while GSH and SOD enzymatic activity were decreased. On the other hand, treatment with AOE resulted in a significant decrease in the testicular content of TBARS along with a dramatic increase in GSH and SOD. In this context, Hossain *et al.*[46] reported that methanolic extract of *A. obesum* stem has a remarkable antioxidant activity and different crude extracts from the stems exhibited strong free radical scavenging activity which were attributed to the high quantity of polyphenolic compounds. Accordingly, the methanolic extract of *A. obesum* flowers used in this study contains considerable content of phenolics and flavonoids. The antioxidant and radical scavenging activities of phenolics and flavonoids have been well-acknowledged in different animal's models including diabetic rats[11,47-49].

Hyperglycemia-mediated oxidative stress can also provoke inflammation and elevated pro-inflammatory cytokines have been reported in diabetic rats[12,50]. Notably, inflammation has a significant impact on the male reproductive system of diabetic animal models[2,51]. In the present study, the diabetic group showed a significant elevation in serum TNF-α and IL-6 demonstrated the triggered inflammatory response. The AOE administration resulted in a significant decline in serum levels of TNF-α and IL-6. The amelioration of inflammation could be a direct consequence of the attenuation of oxidative stress following treatment with AOE.

Given the role of oxidative stress and inflammation in promoting cell death via apoptosis, the protective effect of AOE against testicular injury could be explained through its anti-apoptotic effect. Although, testicular apoptosis occurs at low levels during normal spermatogenesis, it increased markedly in diabetes[9,52]. Bcl-2 family plays a vital regulatory role in the intrinsic apoptotic pathway caused by mitochondrial dysfunction. The pro-apoptotic protein Bax stimulated the release of cytochrome c from the mitochondria, resulting in caspase-3 activation and cell death. The antiapoptotic protein Bcl-2 prevented apoptosis via inhibition of Bax activity[53]. Several studies have demonstrated that testicular apoptosis in diabetic experimental rodent models occurs mainly through the activation of the mitochondrion-mediated cell death pathway[52,54]. These studies revealed that oxidative injury plays a key role in testicular cell death in diabetes. In accordance, diabetic rats in the present study showed a significant increase in testicular Bax and decreased Bcl-2. Interestingly, AOE administration resulted in a significant inhibition of apoptosis through up-regulation of Bcl-2 while the level of Bax was significantly down-regulated. These results supported that testicular apoptotic cell death could be significantly mitigated by AOE treatment.

CONCLUSION

This study provides the first evidence that AOE improves the pituitary-gonadal axis and mitigates testicular injury, oxidative stress and inflammatory response in STZ-induced diabetic rats. The AOE suppressed lipid peroxidation and pro-inflammatory cytokines and enhanced testicular antioxidant defenses in diabetic rats. Consequently, AOE prevented testicular cell death manifested by ameliorating the balance between Bax and Bcl-2. Therefore, *A. obesum* flowers represent a promising candidate for the development of an effective therapeutic agent against testicular injury in diabetes.

SIGNIFICANCE STATEMENT

This study shows for the first time that the extract of *Adenium obesum* flowers (AOE) can prevent testicular dysfunction and injury in diabetic rats. The extract effectively ameliorated serum levels of sex hormones and attenuated histological alterations in the testis of diabetic rats. The AOE suppressed oxidative stress, inflammation and apoptosis provoked by hyperglycemia in testicular tissue. Given these beneficial effects, AOE might represent a potential candidate for preventing sexual dysfunction in diabetes.

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