Introduction

Diabetes mellitus, which is characterized by high glucose serum levels, is one of the most common metabolic disorders and is categorized as a chronic non-communicable disease [1]. Diabetes mellitus can lead to sexual dysfunction, retinopathy, neuropathy, nephropathy, visual impairment, physical disability, renal injury, and illnesses in the nervous and cardiovascular systems [2,3]. Elevated glucose levels lead to oxidative stress, cell apoptosis, and subsequent structural and functional disorders in organs such as the testes, ultimately resulting in infertility [4,5]. Although insulin and hypoglycemic drugs are effective treatments for diabetes, they are associated with adverse effects, including alcohol flush, vomiting, sickness, dysentery, swelling, migraine, malignant, faintness, and anemia [6]. Medicinal plants and their derivatives, which are widely available and known to have low side effects, are used as another strategy for diabetes treatment. Their primary therapeutic mechanism in diabetes management is the inhibition of free radicals [7]. Thus, antioxidants play a major role in overcoming male infertility induced by diabetes.

Diabetes can affect male reproductive performance at various levels by inducing spermatogenic disorders, morphological changes in the testicles, and changes in the metabolism of glucose in Sertoli
cells. It also causes decreased testosterone levels, ejaculation disorders, and low libido. The oxidative stress induced by diabetes is the main cause of its chronic harmful effects on tissues such as the testis in streptozotocin-induced animal models of diabetes [8-12].

Harmine (Figure 1) is found in the *Peganum harmala* L. and *Banisteria caapi* plants. This β-carboline alkaloid has diverse therapeutic and pharmacological effects, including antioxidant, anti-inflammatory, anti-apoptotic, anti-hypertensive, antidepressant, and anti-parasitic properties [13-16]. In the context of diabetes mellitus, harmine affects β-cell proliferation and induces adipocyte thermogenesis [17,18]. Other studies have revealed that harmine could relieve streptozotocin-induced cognitive dysfunction in diabetic rats [19,20].

Considering that harmine is effective in reducing oxidative stress and inflammation, it is reasonable to hypothesize that it can decrease the chronic damage caused by oxidative stress in testicular tissue in a streptozotocin-based rat model of diabetes. To date, no academic research has investigated harmine’s effects on the testicular damage caused by diabetes. Therefore, the goal of this study is to assess the impact of harmine on testicular injury in diabetic rats, with evaluations focusing on related histological alterations, induction of apoptosis, oxidative/antioxidant balance, and levels of sex hormones.

**Methods**

1. **Experimental animals**

   The study received ethical approval and was carried out at Kermanshah University of Medical Sciences on 2-month-old male 32 Wistar rats (250±5 g) according to the World Medical Association Helsinki Declaration (IR.KUMS.REC.1398.906). The rats were kept under normal environmental conditions with a 12/12-hour light-dark cycle, *ad libitum* feeding, a temperature of 22 °C, and a 1-week adaptation period before starting the experiment.

2. **Diabetic induction**

   Rats with serum glucose levels lower than 90 mg/dL were injected with streptozotocin (S0130 500MG; Sigma Aldrich) at 50 mg/kg/day intraperitoneally. One week after streptozotocin treatment, blood samples were taken from the animals’ tails, and a blood glucose level of more than 300 mg/dL was considered as the selection criteria for diabetic rats [8].

3. **Design of experiments**

   32 mature male Wistar rats were randomly divided into four groups (n=8), as shown in Figure 2. The doses of streptozotocin and harmine (Merck; 286044) were determined based on previous studies [8,14]. On the 36th day, the animals were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and euthanized. Subsequently, the testes were collected, and heart blood was drawn. The blood samples were centrifuged at 3,000 rpm for 15 minutes, and the resulting serum samples were stored at −20 °C for the subsequent determination of insulin, glucose, and sex hormones.

4. **Histopathology examinations**

   After fixation in 10% buffered formaldehyde, testes from the groups were randomly selected for analysis. Subsequently, five histological sections, each 5 μm thick and spaced by four sections, were prepared from a paraffin-embedded block of each sample. These sections were then stained with hematoxylin and eosin (H&E). To minimize observational bias, histopathological evaluations were conducted on 25 zigzag photographs taken from each sample, encompassing five fields per section. Measurements were then taken of the seminiferous tubules’ diameter, and the Johnsen score, which is a qualitative scale of spermatogenesis, was assessed [21,22].

5. **Measurement of sex hormone levels**

   The blood serum sample levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were calculated using the enzyme-linked immunosorbent assay (ELISA) method, following the manufacturers’ instructions for specific kits (Accubind, Monobind; and Immunotec, Beckman Coulter Co.).

6. **Estimation of testis levels of nitric oxide**

   Nitric oxide (NO) in homogenized testicular tissue was evaluated using the Griess method (sulfanilamide and N-1-naphthylethenediamine dihydrochloride). Briefly, 6 mg of zinc sulfate was added to 400 μL of the specimen, and the supernatant was transferred to the separate well. Next, vanadium chloride (100 μL), sulfanamide (50 μL), and naphthylethenediamine dihydrochloride (50 μL) were added to 100 μL of the solution. The wells were stored at 30 °C in the dark, and the opacity or optical density of each specimen was estimated with an ELISA reader (450 nm).

![Figure 1](https://doi.org/10.5653/cerm.2023.06254)
7. Estimation of testis levels of malondialdehyde

To quantify lipid peroxidation levels, we estimated malondialdehyde (MDA) levels in testicular tissue. In brief, the samples were homogenized in a homogenization buffer containing 1.15% KCl and centrifuged for 10 minutes. Subsequently, a reaction mixture consisting of thiobarbituric acid, acetic acid (pH 3.5), sodium dodecyl sulfate, and distilled water was added, and the samples were heated at 95°C for 1 hour. After heating, the samples were centrifuged at 3,000×g for 10 minutes. The absorbance of the resulting supernatant was measured using a spectrophotometer (550 nm).

8. Assessment of total antioxidant capacity in the testes

We assessed total antioxidant capacity (TAC) with a kit (TAC 96A) from ZellBio GmbH using the oxidation colorimetric resuscitation method. In 96-well microplates, the reagent and buffer were mixed and processed following the kit's instructions. We used a spectrophotometer to evaluate the antioxidant capacity of the specimens at a wavelength of 490 nm. The outcomes were compared to ascorbic acid results as an internal standard.

9. Estimation of serum levels of insulin, and fasting blood glucose

Serum insulin was estimated using the ELISA method via the Accubind kit (Monobind). The serum samples were thawed (collected at three time points, following the protocol used in the blood glucose study). Prior to initiating the study, the kits were acclimated to laboratory temperature. To each vial containing 10 mL of contents, 2 mL of distilled water was added. The samples were then transferred to the wells coated with conjugate and mixed with 100 μL of insulin solution. The wells were covered with plate adhesive to prevent evaporation, and the contents were subsequently discarded. After this, 350 μL of diluted wash solution was added. Following the addition of 6 μL of substrate to each well and a 10-minute incubation period, 50 μL of stop solution was dispensed into all the wells. The absorbance was then measured at 540 nm using an ELISA reader [23].

10. In situ detection of apoptosis in seminiferous tubules

We applied the terminal deoxynucleotidyl transferase-mediated dNTP nick end labeling (TUNEL) method for detecting apoptosis using an in situ method (Cell Death Detection Kit AP; Roche Diagnostics) according to the manufacturer's instructions. In brief, the sections were washed with phosphate-buffered saline and then permeabilized with 0.1% Triton X-100 (Sigma) for 5 minutes on ice. This was followed by incubation with 50 μL of terminal deoxynucleotidyl transferase end labeling solution for 60 minutes at 37°C in a humidified chamber in the dark. Finally, the sections were stained with 1% methylene green.

Photomicrographs of apoptotic cells with nuclei stained with methyl green were examined under a fluorescent microscope (with ×400 magnification), and the apoptosis index was measured based on the following formula:

\[ \text{Apoptotic index} = \frac{\text{number of apoptotic cells}}{\text{total number of cells counted}} \times 100 \]

[24].

11. Semen analysis

Sperm were obtained from the cauda epididymis for sperm analysis, which included assessments of count, motility, morphology,
viability. The samples were placed in a Petri dish containing 5 mL of Dulbecco’s Modified Eagle Medium/F12 medium with 10% fetal bovine serum and then incubated in a CO₂ environment (5%) at 37 °C for 20 minutes. Sperm counting was performed by mixing an equal volume of the medium containing sperm with an equal volume of 10% formalin and spreading the mixture onto Neubauer slides for examination under a light microscope (×100 magnification). The sperm count was calculated by counting the sperm in five out of the 25 central squares, with adjustments made for all groups. The mean sperm count was then multiplied by 2 to account for the formalin dilution and by 0.05×10⁶ for the dilution factor. Sperm motility was assessed by light microscopy (×400 magnification), examining 10 randomly selected zigzag fields for each specimen. The viability and morphology of the sperm were determined by mixing an equal volume (typically 10 μL) of 1% trypan blue dye with the sperm-containing medium. Smears were then prepared, and a total of 100 sperm per case were evaluated for morphology and viability by observing randomly selected fields in a zigzag pattern under a light microscope (×400 magnification) [25].

12. Statistical analysis
We used SPSS ver. 16 software (SPSS Inc.) for data analysis. The normal distribution of the variables was tested by applying the Kolmogorov-Smirnov test. One-way analysis of variance followed by the Tukey post hoc test was run to determine the statistical significance between groups in multiple comparison testing. The reported data were presented as mean±standard deviation of the mean. The significance level of p<0.05 was adopted for all statistics.

Results

1. Histological evaluation of the testes
Microscopic images of testicular tissue are displayed in Figure 3A. The control group and the harmine-treated groups exhibited well-arranged seminiferous tubules with minimal inter-tubular spaces. Conversely, the diabetic group showed shrinkage of seminiferous tubules and increased inter-tubular spaces. Quantitative analysis of the testicular tissue images revealed that both the seminiferous tubule diameter and the Johnsen score were significantly reduced in the diabetic group compared to the control group (p<0.01). The apoptotic index showed a significant increase in the diabetic and diabetes+harmine groups compared to the control group (p<0.01) (Figure 5A, 5B). Conversely, the TAC level in testicular tissue was significantly lower in the diabetic group than in the control (p<0.01). Compared to the diabetes group, there was a significant increase in the TAC level in testicular tissue in both the harmine and diabetes+harmine groups (p<0.01) (Figure 5C).

4. Insulin and fasting blood glucose serum levels
 Serum insulin levels were significantly lower in the diabetes group than in the control group (p<0.01) (Figure 6A). Conversely, fasting blood glucose levels were significantly higher in both the diabetes and diabetes+harmine groups than in the control group (p<0.05) (Figure 6B). Significantly higher serum insulin levels were observed in the harmine and diabetes+harmine groups than in the diabetes group (p<0.01) (Figure 6A). Additionally, fasting blood glucose levels in the harmine and diabetes+harmine groups were significantly lower than those in the diabetes group (p<0.01) (Figure 6B).

5. Effects of diabetes and harmine on the apoptotic index in the testes
TUNEL-stained sections were used to estimate the levels of apoptotic cells in the testicular tissue (Figure 7A). Quantitative data related to the apoptotic index are presented in Figure 7B. The levels of the apoptotic index showed a significant increase in the diabetes and diabetes+harmine groups compared to the control group (p<0.05). The apoptotic index was significantly lower in the harmine and diabetes+harmine groups than in the diabetes group (p<0.01).

6. Effects of diabetes and harmine on semen analysis parameters
Sperm parameters were significantly reduced in the diabetes and diabetes+harmine groups compared to the control group (p<0.01). Treatment with harmine significantly improved sperm parameters compared to the diabetes group alone (p<0.01) (Figure 8A). Figure 8B illustrates the morphological characteristics of sperm, highlight-
Figure 3. (A) Histopathologic testicular tissue sections in rats following harmine administration and diabetes induction under light microscopy (×100 and ×400 magnification, stained with hematoxylin and eosin) (n=8). According to histological slides shown in (A), The diameter of seminiferous tubules (B) and Johnson’s score (C) were calculated. \(^*p<0.01\), \(^*p<0.05\) compared to the control group; \(^*p<0.01\) compared to the diabetes group.

Figure 4. The effect of harmine administration and diabetes induction on serum sex hormone levels in male rats (n=8). (A) Testosterone. (B) Luteinizing hormone. (C) Follicle-stimulating hormone. \(^*p<0.01\), \(^*p<0.05\) compared to the control group; \(^*p<0.01\), \(^*p<0.05\) compared to the diabetes group.
Figure 5. The effect of harmine administration and diabetes induction on oxidative stress-related parameters in rats (n=8). (A) Testicular tissue nitric oxide. (B) Testicular tissue malondialdehyde. (C) Testicular tissue total antioxidant capacity. \( p < 0.01 \) compared to the control group; \( p < 0.01 \) compared to the diabetes group.

Figure 6. The effect of harmine administration and diabetes induction on serum levels of (A) insulin and (B) fasting blood glucose in rats (n=8). \( p < 0.01 \), \( p < 0.05 \) compared to the control group; \( p < 0.01 \) compared to the diabetes group.

Discussion

The present study initially established that diabetes-induced by streptozotocin was characterized by elevated fasting blood glucose serum levels. In diabetic rats, increased damage to testicular tissue and sperm was observed, along with decreased serum levels of insulin and male sex hormones. Additionally, oxidative stress was induced in the testes, leading to apoptosis in the testicular tissue of rats. However, the administration of harmine was found to mitigate these adverse effects.

In our study, the diabetic group exhibited a significant reduction in the diameter of the seminiferous tubules and the Johnsen scores were significantly improved in the diabetes+harmine group. These findings may be attributed to the oxidative defense properties of harmine, which protect the cytoarchitecture and function of the seminiferous tubules.

In the current study, consistent with previous findings [26,27], we observed a significant decrease in serum levels of FSH, LH, and testosterone in the diabetic group. Diabetes interferes with the male reproductive system by impacting the hypothalamus-pituitary-gonadal axis or the testes [28]. FSH and LH are pivotal in stimulating the production of androgens such as testosterone. Ballester et al. [29] reported that diabetic rats exhibited reduced serum concentrations of testosterone, LH, and FSH. They suggested that the diminished testosterone levels in diabetic animals might be linked to a reduction in Leydig cells or a disruption in androgen biosynthesis [29]. Based on the histological examination of testicular tissue and sperm parameters in our study, we propose that diabetes has a direct adverse effect...
Figure 7. Apoptosis induction in the testes of rats following harmine administration and diabetes induction. (A) Terminal deoxynucleotidyl transferase-mediated dNTP nick end labeling (TUNEL)-stained sections under fluorescence microscopy (×400 magnifications) (n=8). Left, middle, and right show cytoplasm staining, nuclei staining, and merged images, respectively. The yellow arrows point to the shiny green nuclei of apoptotic cells. (B) Apoptotic index-related quantitative data. *p<0.01, **p<0.05 compared to the control group; ***p<0.01 compared to the diabetes group.

Diabetes is known to directly affect the testes, but it can also impact the histology of the adenohypophysis. In our study, the administration of harmine to diabetic groups proved effective in elevating levels of testosterone, LH, FSH, and testosterone, as well as ameliorating histological changes in diabetic rats. These findings support the notion that harmine mitigates oxidative stress in the testicular tissue of diabetic rats and bolsters the hypothesis that diabetes has a direct effect on testicular tissue. Our results are consistent with other studies demonstrating the beneficial impact of antioxidants such as those found in *Nigella sativa* and *Ferulago angulata* on sex hormone levels in diabetic male rats mediated by improvements in testicular tissue damage [9,31,32].

Diabetes-induced oxidative stress has been reported to cause atrophy of tubules, as observed by the reduced diameter of tubules and loss of spermatogenic cells. The increase in tissue NO and MDA levels, along with the decrease in TAC levels in the current study, confirms this notion. Furthermore, harmine appears to alleviate testicular damage in the studied rats by reducing tissue levels of NO and MDA and increasing TAC levels. To the best of our knowledge, there have been no reports on the protective effects of harmine against diabetes-induced damage. However, the antioxidant effects of harmine on testicular tissue observed in this study may explain its beneficial impact on sperm [33]. Considering the role of oxidative stress in the progression of diabetes, it seems that the development of oxidative stress conditions in these patients is due to increased production of reactive oxygen species in the blood plasma and a weakened antioxidant defense system [11]. Thus, we suggest that the mechanism of diabetes-related injury is linked to the depletion of the antioxidant support system. The present study has revealed that harmine administration may counterbalance lipid peroxidation and enhance the antioxidant capacity of the testis, thereby reducing oxidative stress. Consequently, it could be posited that the antioxidant properties of harmine, through its significant free radical scavenging capacity, could improve TAC and reduce NO and MDA levels in the studied groups. In line with these findings, Komelli et al. [19] concluded that the administration of *P. harmala* hydroalcoholic ex-
Diabetes and harmine administration. Apoptosis, characterized by the induction of DNA fragmentation, leads to increased oxidative stress. Therefore, the generation of free radicals and the subsequent oxidative stress may be one of the primary causes of tissue apoptosis, reflecting the pervasive impact of oxidative stress [36]. Numerous studies support the conclusion that free radical generation and oxidative stress are key factors in the onset of diabetes [11,26,31]. This stress triggers apoptosis not only in the pancreas [37] but also in distant organs such as the testes, as demonstrated in our study. This vicious cycle is perpetuated by apoptosis, making the disease chronic, progressive, and, to date, incurable. Furthermore, we emphasize the crucial role of antioxidants in disrupting these detrimental processes and their potential in the treatment of diabetes. A study have also highlighted the role of autophagy in reducing oxidative stress, thereby decreasing apoptosis [38]. The quantitative relationship between antioxidant capacity, oxidative stress, and apoptosis presents an intriguing area for future research, with the potential to treat diseases such as diabetes, neurodegenerative diseases, heart failure, stroke, cancer, arthritis, and more. Conversely, in our study, harmine administration was found to protect against apoptosis in the testicular tissue of diabetic rats, as evidenced by a lower apoptotic index. Kajbaf et al. [39] reported the anti-apoptotic effect of harmine in the renal tissue of diabetic rats through the downregulation of caspase-3 gene expression. Additionally, our previous studies have shown that the antioxidant properties of harmine can inhibit renal tissue apoptosis induced by mercuric chloride and cisplatin [14,40].

One limitation of this study was the absence of a simultaneous evaluation of pituitary tissue, which would have confirmed whether diabetes directly affects pituitary tissue, thereby reducing the secre-

Figure 8. (A) The quantitative estimation of sperm parameters in rats after diabetes induction and harmine administration. (B) The spermatozoa’s morphology and viability, shown by trypan blue staining and photographed using a light microscope (×400): (a) normal and alive sperm, (b) amorphous head, (c) bent neck, (d) bent head, (e) no head, and (f) banana head. *p<0.01, **p<0.05 compared to the control group; ††p<0.01 compared to the diabetes group.
tion of gonadal hormones. This aspect was not explored in the harmine plus diabetic group. Another limitation is that we did not evaluate autophagy in testicular tissue to establish a quantitative relationship between oxidative stress, autophagy, apoptosis, and antioxidant capacity.

We concluded that diabetes can induce oxidative stress and apoptosis in the testes of rats, disrupting their cytoarchitecture and affecting the cells produced, including sperm. In contrast, harmine has the potential to mitigate or prevent this damage by ameliorating histopathological changes and reducing oxidative stress and apoptosis. Therefore, harmine treatment may be effective in scavenging free radicals to alleviate and halt complications in the testicular tissue of people with diabetes.

**Conflict of interest**

No potential conflict of interest relevant to this article was reported.

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