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Antidiabetic and Protective Effects of *Ferula assa-foetida* L. oleo Gum Resin Ethanolic Extract on the Testis of Streptozotocin-Induced Diabetic Rats: A Histopathological Study

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ABSTRACT

Diabetes is one of the most common metabolic diseases worldwide which affects all organs, including the reproductive system. Today, many researchers use medicinal plants instead of chemical agents to achieve fewer side effects. *Ferula assa-foetida* L. is one of the medicinal plants used to treat many diseases traditionally for years. The present study evaluated the antidiabetic and protective effects of *Ferula assa-foetida* L. on the testis of streptozotocin-induced diabetic male rats. The histomorphologic study of diabetic rats treated with Ferula assa-foetida L. extract showed a significant improvement in testes. Histological studies revealed that treatment with the Ferula assa-foetida L. extract significantly increased sperm count in the seminiferous tubules and reduced fibrosis. Our study confirmed the improving effects of *Ferula assa-foetida* L. on histomorphometric and biochemical parameters in diabetes and related testicular damage, which are partially attributed to the bioactive compounds and antioxidants in *Ferula assa-foetida* L.

Keywords

Ferula assa-foetida, Rat, Diabetes, Reproductive system, Spermatogenesis, Medicinal plants

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Abbreviations

FAE: Ferula assa-foetida L. oleo gum resin ethanolic extract
SOD: Superoxide Dismutase
Met: Metformin
STZ: Streptozotocin
FSH: Follicle-Stimulating Hormone
LH: Luteinizing Hormone

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Introduction

One of the common metabolic and endocrine diseases that is a serious threat to public health, especially in developing countries, is diabetes [⁋ 1]. Diabetes is caused by a decrease in insulin secretion or sensitivity [2]. Disturbances in carbohydrate, lipid, and protein metabolism affect the secretion and function of insulin which is very worrying for the health of society [3]. Therefore, improper lifestyle and nutrition leading to obesity and overweight have an effective role in the prevalence and occurrence of diabetes [4].

An increase in blood glucose level is one of the clear symptoms of diabetes, which results in structural and functional changes in various tissues and organs, such as the reproductive system [5]. Abnormal feedback of sex steroids in the hypothalamus-pituitary axis, which is observed in diabetic rats, is the result of abnormal transfer of steroids or decreased sensitivity of the pituitary gland [6, 7]. Moreover, various studies have shown that hyperglycemia in diabetes negatively affects male and female fertility [8,9]. Testes are sensitive to hyperglycemia [10]. Weight loss[11], abnormal germinal epithelium [5, 12], and disruption of the testicular blood barrier are among the complications of diabetes [13]. When the blood glucose level rises, glucose autoxidation causes excessive production of free radicals and finally oxidative stress [14]. A medication with fewer side effects to treat diabetes is Met which is used to control hyperglycemia and inhibits gluconeogenesis in hepatocytes. Its mechanism of action is inhibiting mitochondrial respiration and reducing cellular energy levels, which decreases glucose production by hepatocytes [15]. Treatment of male diabetic mice with Met preserves the structure and function of the testis [16]. In order to reduce the negative effects of free radicals on the reproductive system and testes, many investigations have evaluated the impact of antioxidant compounds on this system. Among these antioxidant compounds, natural antioxidants found in medicinal plants have attracted the attention of scientists due to fewer side effects than chemical antioxidants on living organisms. Some medicinal plants, such as curcumin[17], Ficus Carica [18], Telferia Occidentalis [19], and Ginger [20], have been investigated. Another medicinal plant with antioxidant properties is Ferula assa-foetida L. [21,22,⁋ 23] which has been used to treat many diseases for centuries. This plant, which is native to Iran, is also called Anghuzeh, a member of the Umbelliferae (Apiaceae) family [24]. Components of Ferula assa-foetida L. oleogum resin are Ferulic acid, esters, coumarins, other terpenoids [24], umbelliferone [25], bisabolol, and quercetin[23]. It has been effective and available for the treatment of neurological disorders, stomachache, intestinal parasites, weak digestion, asthma, bronchitis, influenza, infertility, and diabetes for many years [24, 26, 27]. Research has shown that the use of Ferula assa-foetida L. is effective in the treatment of liver and kidney diseases, hyperglycemia, and hyperlipidemia⁋ [28,⁋ 29]. In addition, the anti-obesity impacts of Ferula assa-foetida L. were investigated and the results showed that leptin and blood glucose levels decreased after consuming Ferula assa-foetida L. [30]. Therefore, Ferula assa-foetida L. can be a good candidate for the treatment of diabetes because of its availability and natural antioxidant properties. As the number of people with diabetes is increasing rapidly [1], the age of diabetes is decreasing[31] which raises the number of people with diabetes in reproductive age. Therefore, identifying the mechanisms that destroy the testes in diabetes, discovering effective substances and medications, and preventing infertility and reproductive disorders are important issues. With this background, we decided to investigate the protective effects of FAE on the testis of STZ-induced diabetic rats.

Result

Morphometric Data

According to Figure 1 and Table 1, the thickness of the epithelium of seminiferous tubules in group 5 (diabetic rats treated with 250 mg/kg FAE) decreased significantly compared to other groups (Figure 1).

The examination of the size of the seminiferous tubules in different experimental groups showed that the size of the seminiferous tubules in groups 1, 3, and 4 increased significantly compared to groups 2 and 5, also in groups 3 and 4 compared to group 2 (Figure 1; Table. 1).

Johnson, s Score

According to Figure 1 and Table 1, Johnson's Score decreased in groups 2 and 5 compared to other groups.

Biochemical Evaluation

The comparison between groups 1 and 2 showed that diabetes caused a significant decrease in testosterone levels in rats. Furthermore, a significant increase in testosterone levels in group 4 compared to group 2 indicated the positive effect of FAE on testosterone levels in diabetes (Figure 2.d).

As shown in Figure 3, the level of blood glucose increased in diabetic control group rats compared to non-diabetic control group rats. Moreover, the lack of significant difference between groups 1 and 3 showed that the low dose of FAE in non-diabetic rats did not

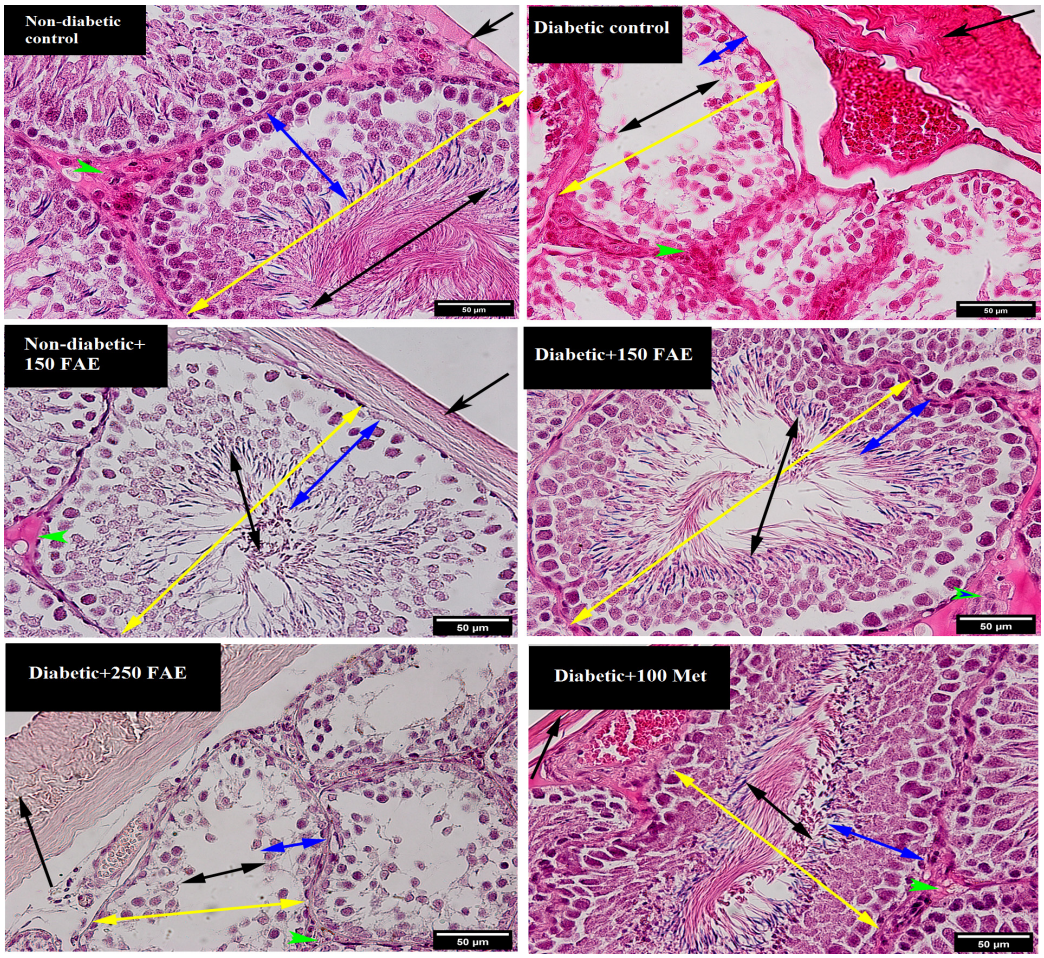

Figure 1.
Cross section of the testis from different groups showing Tubular Diameter (TD) and different parts of the testis. The yellow arrows showing tubular diameter (TD); the black double-sided arrow indicate lumen diameter (LD); green arrow heads showing interstitial tissue (IT); black single-side arrows show tunica albuginea (TA); the blue double-sided arrow indicate epithelium thickness (ET).

reduce blood sugar levels. A significant decline in blood glucose levels was observed in group 4 compared to groups 2 and 5, which shows that a lower dose of FAE reduces blood sugar levels in diabetes (Figure 2. b).

The results of evaluating blood insulin levels in different groups showed that insulin secretion in group 3 rose compared to groups 1, 2, 5, and 6. The levels of insulin in groups 3 and 4 did not differ significantly. The high levels of insulin in group 3 compared to other groups indicated the positive effect of a lower dose of FAE on insulin levels in non-diabetic subjects (Figure 2. c).

Table 1.
FAE improves the morphometric features of the testis tubules in diabetes-induced rats.

	Johnson, s score	Tubule Diameter	Epithelium thickness
Group1	$9.58 \pm 0.54^{\ddagger\#}$	$168.83 \pm 9.06^{\#\ddagger}$	46.16 ± 1.19
Group2	$5.57 \pm 0.49^{\dagger\pm\text{¤}}$	$114.66 \pm 84^{\dagger\pm\text{¤}}$	$33.83 \pm 1.30^{*\dagger\pm}$
Group3	9.89 ± 0.32	$174.40 \pm 6.72^{\ddagger}$	$47.60 \pm 1.72^{\ddagger}$
Group4	$9.51 \pm 0.17^{\ddagger}$	$176.80 \pm 3.18^{\ddagger}$	47.40 ± 1.07
Group5	$5.34 \pm 1.22^{\text{¤}\dagger}$	88.50 ± 12.40	$21.66 \pm 4.79^{*\#\pm\text{¤}}$
Group6	9.41 ± 0.40	$163.00 \pm 8.61^{\ddagger}$	40.80 ± 2.78

Data showing the tubular diameter, epithelium thickness and Johnson's Score in different experimental groups.
Statistics: Data are mean values ± SEM; One-way ANOVA with Tukey test; $P < 0.05$ was considered as significant; *, significant in comparisons with non-diabetic control; #, significant in comparison with diabetic control; †, significant in comparison with non-diabetic+150 mg/kg b.w FAE ; ±, significant in comparison with diabetic rats treated with 150 mg/kg b.w FAE; ‡,significant in comparison with diabetic rats treated with 250 mg/kg b.w FA; ¤, significant in comparison with diabetic rats treated with 100 mg/kg b.w Met.

Enzyme Activity

As shown in Figure 3, SOD enzyme activity decreased in diabetes and low dose of FAE increased the activity of this enzyme in diabetic and non-diabetic rats.

Weight Evaluation

A significant difference in body weight was observed between group 5 and other groups (Figure 4. c). As shown in Fig. 4, the study groups were not significantly different in terms of the relative weight of the testes neither in the right testis nor in the left testis (Figure 4.g&h). According to Figure 5, there was a significant difference between groups 1, 2, 5, and 6,

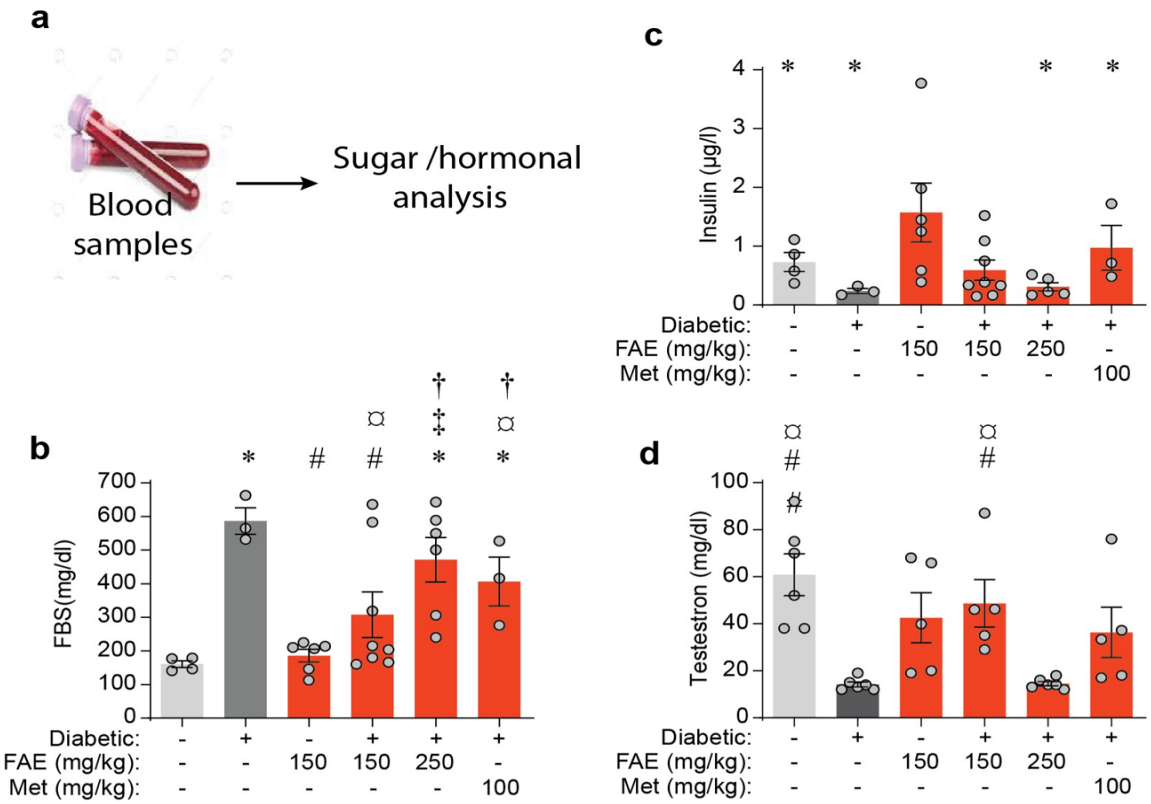

Figure 2.
FAE (150 mg/kg b.w) corrects sugar and hormonal level in the blood of treated rats and its positive effect reduces at higher doses.
a, collected blood samples from tail vein of the animals before organ collcetion. b, bar graph showing the FBS level in different experimental groups. c, bar graph showing the serum insulin level in different experimental groups. d, bar graph showing the testestrone level in different experimental groups.
Statistics: bar graphs are mean values ± SEM; One-way ANOVA with Tukey test; $P < 0.05$ was considered as significant; d & b, *, significant in comparisons with non-diabetic control; #, significant in comparison with diabetic control; †, significant in comparison with non-diabetic+150 mg/kg b.w FAE; ‡, significant in comparison with diabetic rats treated with 150 mg/kg b.w FAE; ¤, significant in comparison with diabetic rats treated with 250 mg/kg b.w FAE.C, *; significant in comparison with non-diabetic+150 mg/kg b.w FAE.

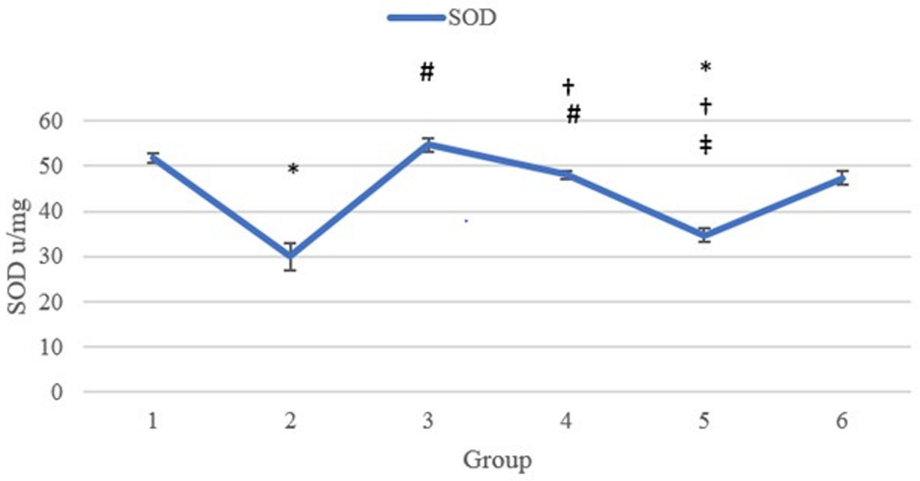

Figure 3.
FAE increases antioxidative enzyme (SOD): super oxide dismutase in diabetes-induced rats.
Statistics: bar graphs are mean values ± SD; One-way ANOVA with Tukey test; $P < 0.05$ was considered as significant; *, significant in comparisons with non-diabetic control (Group1); #, significant in comparison with diabetic control (group2); †, significant in comparison with non-diabetic+150 mg/kg b.w FAE (group3); ‡, significant in comparison with diabetic rats treated with 150 mg/kg b.w FAE (group4).

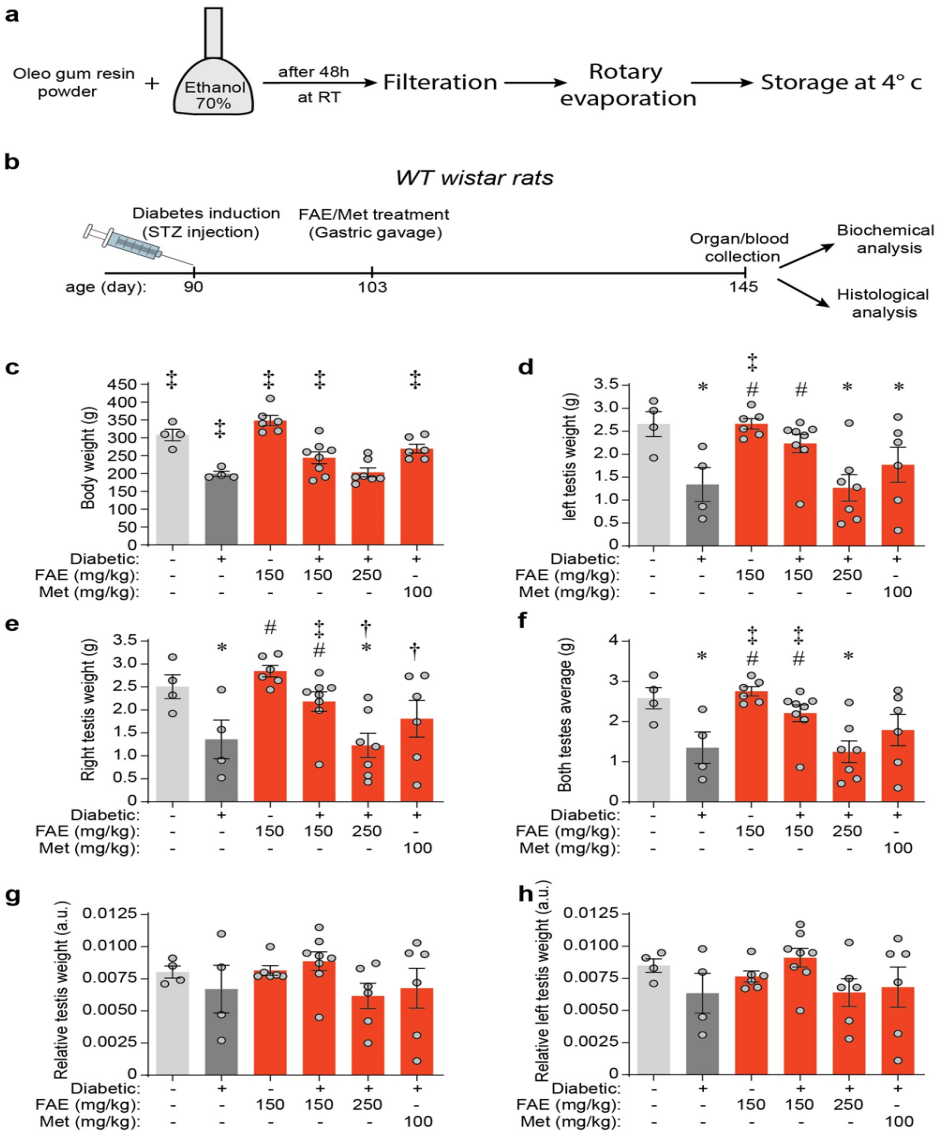

Figure 4.
FAE balances the body and testis weight in diabetic wistar rats.
a, Schematic drawing of the used protocol for FAE extraction. b, a drawing of the protocol used for induction of diabetes in rat and organ/blood collection from treated/untreated animals. c, representing the weight of the animals at the time of organ collection. d-f, showing the weight of the left, right and the average mass of both testes respectively. g & h, are the bar graphs showing the relative testis weight obtained by dividing the mass of each testis to the weight of the body. Statistics: bar graphs are mean values ± SEM; One-way ANOVA with Tukey test; $P < 0.05$ was considered as significant; *, significant in comparisons with non-diabetic control; #, significant in comparison with diabetic control; †, significant in comparison with non-diabetic+150 mg/kg b.w FAE; ±, significant in comparison with diabetic rats treated with 150 mg/kg b.w FAE; ‡, significant in comparison with diabetic rats treated with 250 mg/kg b.w FAE.

which shows that diabetes had a negative effect on testis weight. Moreover, the weight of testicles in group 2 was reduced compared to group 4, which confirmed the positive effect of FAE on increasing testis weight (Figure 4.d). The decrease in testis weight in groups 2 and 5 compared to group 1 indicated the negative effect of diabetes on the absolute weight of the testis. The weight of the testicle in group 4 increased compared to groups 2 and 5, which emphasizes the positive effect of FAE as a treatment for diabetes. Groups 4 and 5 showed that a low dose of FAE has an increasing effect on the weight of the testis compared to its high dose. In addition, the absence of a significant difference between the untreated diabetic group and the diabetic group treated with a high dose of FAE indicated the destructive effect of high amounts of FAE on the testis tissue (Figure 4. e).

Testis Tissue Changes

H&E Staining

Histological findings of testes (Figure 1) by H&E staining showed that in groups 1 and 3, the structure and shape of tubules and sperm cells were normal compared to group 2. In group 2, the structure and shape of the seminiferous tubules changed. The number of cell layers and spermatozoa reduced and secondary spermatids were not seen. Degenerated spermatic cells and apoptotic cells with pyknotic nuclei were visible.

As seen in images (Figure 1), histopathological changes in the diabetic groups treated with FAE (group 4) and Met (group 6) were less than in the diabetic control group (group 2) and the arrangement and quality of sperm cells and the structure of the tubules improved (Figure 1).

In groups 2 and 5, the number of sperm cells decreased and secondary spermatids were not seen in the seminiferous tubules. In addition, the number of cell layers decreased (Figure 1). In group 3 (Figure 1),

good and normal spermatogenesis was observed in most of the tubules. We found that in group 4, spermatogenesis was good and normal, and Ferula prevented the effect of diabetes on the tubules (Figure 1). Good and normal spermatogenesis was observed in group 6 (Figure 1).

Masson's Trichrome Staining

Masson's Trichrome staining was used in order to show the changes in the connective tissue. The results showed that the connective tissue was normal in groups 1 and 3 (Figure 5). In group 2, the seminiferous tubules in the testes were degenerated. The extension of connective tissue into interstitial tissue, called fibrosis, increased significantly (Figure 5). Fibrosis in the group receiving the lower dose of FAE was less than in other diabetic groups (Figure 5), in group 5 was similar to group 2, and it was less in group 6 than in group 2 (Figure 5).

Discussion

Many studies have shown that diabetes has complex effects on the male reproductive system and spermatogenesis [⁋ 5, 6, 8-10, 12, 17, ⁋ 38-40]. Some of these influences include decreasing testosterone and insulin levels and increasing blood glucose. These changes were observed in the current investigation (Figures 1, 2, and 5). These biochemical alterations lead to decreased protein synthesis and increased cell apoptosis [40]. In the current study, the effects of diabetes on testicular tissue included a reduction in the number of germinal epithelial cell layers and a change in the number of germinal cells. These alterations are a result of apoptosis. The increase in testosterone levels in group 4 compared to groups 2 and 5 shows the positive effect of a lower dose of FAE and the negative effect of a higher dose of FAE on the testosterone level in diabetic rats (Figure 2.d). Diabetes affects pituitary gonadotropins and causes ultrastructural changes in the Sertoli and Leydig cells, and these changes disrupt normal spermatogenesis [⁋ 41]. Moreover, low and high doses of FAE increase and decrease testosterone levels in male rats, respectively [26]. We indicated that these hormonal alterations are along with tissue changes, including alterations in the number of cells and thickness of germinal epithelium in diabetic

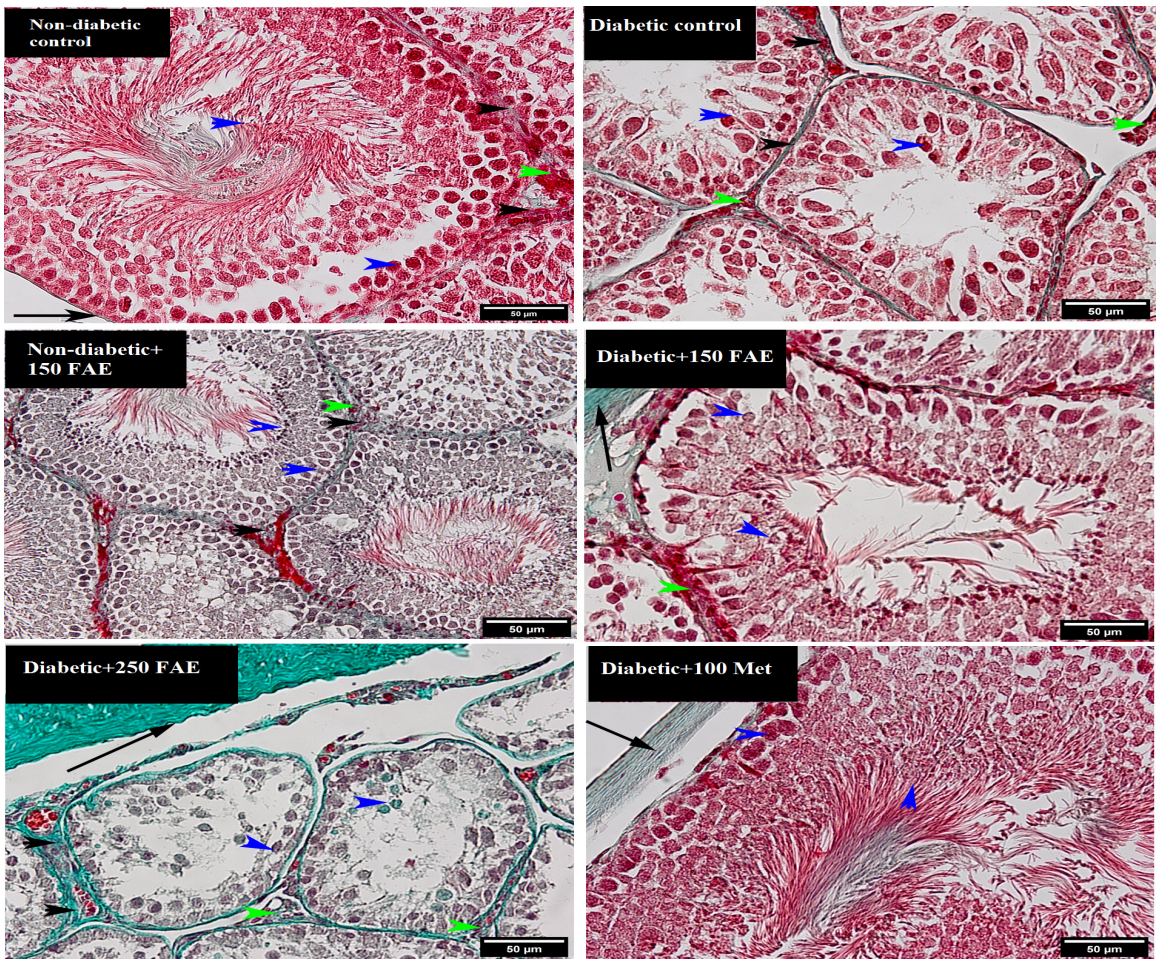

Figure 5.
Cross section of the testis from different groups showing the semineferous tubules with fibrosis. Arrows showing connective tissue and cells in different groups. The green arrow heads showing interstitial tissue (IT); the black single-side arrows show tunica albuginea (TA); the blue arrow head indicate germinal cell; the black arrow heads show connective tissue.

testis. A low dose of FAE improves the disruptive effects of diabetes on the testis, while a high dose does not have such effects. According to the previous and present research, seminiferous tubule diameter and germinal epithelium thickness declined in diabetic subjects [5]. These morphometric alterations are attributable to the apoptotic and oxidative effects of diabetes [5] and more interestingly, FAE improved the morphometric features of the testis tubules in diabetic rats. It seems that these restorative effects of FAE are due to the presence of antioxidant and anti-apoptotic compounds in FAE.

The effect of diabetes on Johnson's score was evaluated and the obtained data were consistent with the results of the previous study [41]. In addition, the positive effect of a low dose of FAE on Johnson's score and the negative effect of a high dose of FAE on Johnson's score in diabetic and non-diabetic animals were observed in this study (Table 1).

The effect of FAE, as a compound containing natural antioxidants, on spermatogenesis in diabetic rats was investigated. The results were consistent with previous studies. For example, one previous study showed that FAE has positive effects on spermatogenesis and by increasing the dose of FAE, spermatogenesis increases, although tissue damage such as vacuolation of Leydig cells were observed . [26]. There are many natural active compounds in FAE, which make it a good candidate for the treatment of diabetes and infertility. Ferulic acid, quercetin, and umbelliferon are the three important compounds found in FAE [23-25]. Ferulic acid and quercetin have antioxidant and anti-apoptotic properties [43].

Park et al. showed that the effects of ferulic acid on increasing testosterone levels result from inhibiting testosterone-reducing enzymes in the liver' [44]. Furthermore, quercetin increases the level of testosterone, FSH, and LH, while diabetes negatively affects these parameters. These changes in diabetic conditions are attributed to the production of reactive oxygen species that reduce the secretion of LH and FSH, and these events lead to a decrease in the number of Leydig cells and testosterone levels [45]. In our study, a low dose of FAE, as a rich source of ferulic acid and quercetin, reduced blood glucose levels (Figure 2. b) and increased insulin (Figure 2. c) and testosterone (Figure 2.d) levels. In the current study, the treatment of diabetic and non-diabetic rats with a low dose of FAE corrected the weight loss of the testis (Figure 4.d and 4. e). This weight loss is due to insulin deduction which leads to decreasing structural protein synthesis [46, 47]. Ferulic acid and quercetin reverse these weight changes by hyperglycemia control and insulin levels increase [43]. SOD and glutathione peroxidase are two key enzymes that neutralize free radicals and clean the testis from reactive oxygen species, thus reducing oxidative stress [48]. These enzymes decline in diabetic subjects, as in our study, the amount of SOD decreased (Figure 3). The activity of the SOD enzyme (Figure 3) rose in group 4 compared to group 2. These results emphasize that the presence of antioxidant compounds in FAE prevents the destruction and apoptosis of the testis. Our study showed that the effects of FAE can be attributed to the antioxidant compounds, including ferulic acid and quercetin. Umbelliferon is another antioxidant component found in FAE with many useful properties. Reduction of insulin resistance, hyperglycemia, and hyperlipidemia in diabetic rats are the effects of umbelliferon. Moreover, the increase of FSH, LH, and testosterone, and the upregulation of FSH, LH, and Peroxisome Proliferator-Activated Receptor γ (PPAR-γ) in the testes of rats are other effects of umbelliferon. PPAR-γ increases insulin sensitivity [49]. Umbelliferon reduces oxidant factors, including reactive oxygen species, malondialdehyde, and nitric oxide, and augments antioxidant factors, namely SOD, glutathione, and catalase [50]. As can be seen in our study, in Figure 2, the increase in testosterone in group 4 compared to group 2 shows the beneficial effects of FAE and its natural antioxidant compounds, including umbelliferon. The mammalian testis is very sensitive to lipid peroxidation due to the presence of unsaturated fatty acids. Therefore, treatment with umbelliferon before testicular ischemia prevents the harmful effects of oxidative stress [50, 51].

As shown in previous studies, antioxidants have ameliorating effects on diabetes and reproductive system disorders. Furthermore, our study emphasizes the antioxidant and improving effects of FAE, as a compound rich in antioxidants, in the treatment of

Materials and Methods

Ethical Considerations

All the experimental procedures were performed in compliance with the policies of the Animal Care and Ethics Committee (ACEC) of Ferdowsi University of Mashhad (No. 41,391). According to ACEC recommendations, we tried our best to minimize research animal pain and suffering.

Animals

All the Wistar rats used in this study were wild-type. A total of 42 male Wistar rats at 3 months of age (weighting 270±20 grams) were obtained from the animal house. To adapt animals to the new environment they were kept in the laboratory for two weeks before the experiments. The rats were housed under the standard conditions at 23°C±1°C with a 12:12h light: dark cycle and had access to food and water ad libitum.

Medications and Materials

In this study, we used STZ (Sigma Aldrich, USA) for diabetes induction and Met (Merck, Germany) as a reference for the

treatment of diabetes. A rat insulin enzyme-linked immunosorbent assay kit (Merccodia, Sweden) was used for insulin measurement. Testosterone and glucose were also measured by standard kits (testosterone was measured by rat testosterone ELIZA kit (Cayman Chemical, USA) and glucose were measured by Pars Azmoon glucose kit (Pars Azmoon, Iran)).

Plant Collection, Specimen Voucher, and Ethanolic Extract of Ferula assa-Foetida Preparation

Ferula assa-Foetida L. was collected from Bastak desert in Hormozgan province at latitude 27° 16' 25" N and longitude 54°21' 51"E in the height of 1650 meters. The plant was identified by Ferdowsi University of Mashhad Herbarium with a voucher specimen (accession number: E-1165 FUMH) in 2020. The plant name was checked with http://www.theplantlist.org.

Ferula assa-Foetida L. ethanolic extract was prepared as previously reported [28]. Briefly, the dried oleo gum resin was collected and powdered by a grinder. A total of 100 grams of the powder was dissolved in 1 liter of ethanol 70° and after 48 h at room temperature, the solution was filtered four times using Whatman filter paper (grade 40). The filtered solution was dried using a rotary evaporator and the product was frozen on dry ice before storage at 4°C for further use.

Diabetes Induction and Experimental Groups

Diabetes was induced by injecting a single dose of STZ (55 mg/kg b.w, intraperitoneally) as reported [32]. All non-diabetic groups in this study (including the control) received the same volume of citrate buffer (0.01 M, pH: 4.5) as the vehicle. Fasting blood sugar was monitored after STZ injection for 10 consecutive days, and animals with a constant fasting blood sugar level upper than 250 mg/dl [33] were considered diabetic [34] and were used in our study. For studying the effect of Ferula assa-Foetida L. ethanolic extract on the reproductive organs of male rats, animals were divided into six groups including 1) non-diabetic control group that did not receive any treatment, 2) diabetic control group that was injected with only a single dose of STZ for diabetes induction, 3) non-diabetic treatment group treated with FAE (150 mg/kg b.w, gavage), 4 and 5) diabetic treatment groups treated with FAE (150 and 250 mg/kg b.w, respectively, gavage, and 6) diabetic positive control group that received Met (100 mg/kg b.w, intraperitoneally). Both FAE and Met were dissolved in distilled water and the final volume used for treating the animals via gastric gavage was 1 ml.

Organ Collection, Tissue Processing, and Microscopy

The animals were anesthetized with ether and then euthanized with CO_2 gas for organ collection 42 days after treatment (28). The testes were separated, weighed, and washed in normal saline before fixation in 10% neutral buffered formalin and Bouin-Hollande's. Subsequently, tissue sections of 5 µm thickness were prepared and stained routinely by Hematoxylin & Eosin (H&E) (Merck) and Masson's Trichrome (Merck) [35]. The stained tissue sections were studied by light microscope and the acquired images were used for further quantifications.

Histomorphometric Analysis

To study the histomorphology of animal testes, the obtained images were opened with Image J software (version 1.44 p), and some parameters, such as the diameter of the seminiferous tubules and germinal epithelium thickness, were measured. To measure the diameter of the seminiferous tubule, two opposite points were considered in the circumference of the tubules from the location of the connective tissue in the basement membrane. The basement membrane was defined based on the connective tissue and myoid cells. To measure the germinal epithelium thickness, the distance between the round spermatid and the basement membrane was reported as epithelium thickness (Figure 1) (Table 1). In total, 20 tubules were analyzed in each tissue section and their average was reported as a single data point.

Spermatogenesis Evaluation

Johnson's score is a measure for evaluating spermatogenesis in the seminiferous tubules [42]. For studying the FAE effect on spermatogenesis, Johnson's score values were calculated and analyzed. In this way, Johnson's score in each seminiferous tubule was determined based on a score of 1 to 10. At each tissue section, 50 tubules were studied and their average was considered a data point (Table 1).

Biochemical Evaluation

Insulin, testosterone, and glucose were evaluated by standard kits. SOD activity was determined by the Marklund method [37].

Data Analysis

For each experimental group, 5-7 rats were analyzed. The means of the calculated values for each rat were reported as single data points and were used for making the graphs. Graphs were drawn with GraphPad Prism, Adobe Illustrator, and Microsoft Excel. Data were statistically analyzed by the SPSS software (version 22). One-way analysis of variance and Tukey post hoc test were used and significance levels were considered at $p \leq 0.05$. The error bars on the graph present the mean ± SEM.

Authors' Contributions

Design and conception: ZA, AAM and EL. Methodology validation, Preparation and chemical constituent analysis: EL, AAM and HN. Diabetes induction and daily treatment: EL and AAM. Preparation of samples for biochemical analysis: ZA, EL and AAM. Data analysis and manuscript drafting: ZA, and EL. Data validation and manuscript revision: ZA, EL and HN.

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Competing Interests

The authors declare that there is no conflict of interest.

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