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Eugenol Reduces Oxidative Stress and Modulates BAX/BCL-2 in Testes of Diabetic Rats

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Abstract

Objectives: Diabetes is a prevalent and chronic condition that has been found to have adverse effects on male fertility. Recent studies have explored the potential of eugenol in preventing diabetes-related complications. This research aimed to investigate whether eugenol has any protective effects against testicular tissue damage in diabetic rats induced by streptozotocin (STZ).

Materials and Methods: In this experimental study, 32 male Wistar rats aged 8 weeks and weighing between 200-250 g were randomly divided into four groups: a control group, a diabetic group, a diabetic group treated with 4 mg/kg of eugenol, and a control group receiving the same dose of eugenol. Diabetes was induced in the appropriate groups using a 50 mg/kg intraperitoneal (IP) dose of STZ. The treatment lasted for eight weeks, after which testicular tissue samples were collected.

Results: Histopathological analysis showed that eugenol treatment resulted in a notable decrease in testicular damage. The eugenol-treated group exhibited an elevation in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) within the testicular tissue. The level of malondialdehyde (MDA) was significantly reduced in the eugenol-treated group. In terms of apoptosis, eugenol treatment led to decreased protein and mRNA expression of the pro-apoptotic protein BAX, a reduced BAX/BCL2 ratio, and decreased germ cell apoptosis ($P < 0.01$). Conversely, eugenol treatment increased the expression of the anti-apoptotic protein BCL2 at both the gene and protein levels.

Conclusions: The administration of eugenol has the potential to mitigate testicular tissue damage in diabetic rats due to its antioxidant and anti-apoptotic properties.

Keywords: Diabetes Mellitus, Eugenol, Testes, Oxidative stress, Apoptosis

Introduction

Diabetes mellitus (DM) is a prevalent metabolic condition characterized by chronic hyperglycemia, which is associated with elevated mortality and morbidity rates (1). The International Diabetes Federation (IDF) has reported that since 2010, approximately 300 million people have been identified as having diabetes worldwide, and this number is predicted to reach nearly half a billion by 2030 (1,2).

DM has the potential to generate severe consequences in various organs, including the heart, retina, and reproductive system (1,3). Several studies on both human and animal models of diabetes have documented various changes in spermatogenesis, such as degenerative and apoptotic modifications in the testis. These studies have also observed altered glucose metabolism in Sertoli cells, reduced synthesis and secretion of testosterone, as well as instances of ejaculatory dysfunction and diminished libido (2,4,5). Studies have shown that insulin-dependent diabetes can cause a decline in Leydig cell function and testosterone production (6,7). Recent studies suggest that males with DM are prone to varying degrees of testicular dysfunction due to oxidative stress (OS) (8,9). Hyperglycemia is known to cause cellular OS due to increased reactive oxygen species (ROS) generation and impaired antioxidant defenses (10). ROS production has a considerable adverse impact on the quality and function of sperm (11). The overproduction of ROS leads to these detrimental effects, which can result in abnormal sperm morphology, apoptosis of germ cells, and lipid peroxidation, ultimately leading to male infertility (12). It appears that OS is closely related to every signaling pathway involved in diabetes-related reproductive issues. Therefore, strategies that reduce OS in the testis can be useful in mitigating problems of infertility caused by diabetes (13).

Given that oxidative stress is an important and influential factor in reproductive system problems and that diabetes is one of the factors causing oxidative stress in testicular tissue, the use of antioxidant compounds helps prevent this damage (8). While there are many chemical medications available for the treatment of diabetes, there is growing interest in using herbal ingredients as safe therapies. Eugenol, a naturally occurring phenolic compound synthesized in various plants such as basil, cinnamon, and clove, is regarded as the primary component of cloves

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Key Messages

- Diabetes led to oxidative stress and testis damage
- Diabetes led to over expression of Bax and Apoptosis in testicles.
- Eugenol led to reduction of oxidative stress and testis damage related to diabetes.

(14). Eugenol has been investigated in both in vitro and in vivo experiments to determine whether it functions as an antioxidant against oxidative radicals, enhances lipid peroxidation, and affects the activity of enzymes like SGOT, Cyt P450, and glucose-6-phosphatase (14). Studies have shown that eugenol acts as an antioxidant compound against oxidative damage and can help prevent tissue damage related to oxidative stress (15).

The existing body of literature, however, provides limited insights into the effects of eugenol on drug-associated gonadal toxicity or disease. Further investigation into the molecular mechanisms underlying these effects is necessary to achieve a comprehensive understanding. Consequently, the purpose of this research was to determine whether eugenol could protect male rodents from testicular injury and oxidative stress caused by complications of diabetes induced by streptozotocin (STZ).

Materials and Methods

Sample Size

The number of samples used in the present study was estimated based on previous studies and the following formula, where 8 rats were considered for each group.

$$n = 1 + 2C\left(\frac{s}{d}\right)^2 = 1 + 2 * 7.85\left(\frac{0.212}{0.556}\right)^2 \approx 4$$

$$\acute{n}=n\sqrt{g-1}=4\sqrt{5-1}=8$$

Study Design

A total of 32 adult male Wistar rats (200-250 g) prepared from the Animal Lab of Tabriz University of Medical Sciences were utilized for this experimental study. They had free access to food and water while being housed in a standard environment with respect to temperature, humidity, and light/dark cycles. The experiment consisted of the random allocation of rats into four distinct groups: control group (G1), diabetic group (G2), diabetic group receiving a daily dose of eugenol (4 mg/kg) (G3), and control group receiving a daily dose of eugenol (4 mg/kg) (G4) (the dose of eugenol was selected based on previous studies) (16,17). The treatment lasted for a period of eight weeks. To induce diabetes, STZ (Sigma-Aldrich, Germany) solution in 0.01 M citrate buffer (pH = 4.5) was injected intraperitoneally at a dose of 50 mg/kg. Three days after the injection, blood glucose levels were measured by drawing blood from the tail vein. Rats with blood glucose levels higher than 250 mg/dL were considered diabetic. The rats in G3 and G4 received eugenol to evaluate its protective effect on the testis against OS and diabetes-induced testis damage (3). A control group was administered normal saline, which served as the solvent for STZ. The administration of eugenol commenced ten days subsequent to the confirmation of the induction of the diabetic model (18).

Sample Collection and Surgical procedure

After 8 weeks, the rats were subjected to anesthesia via intraperitoneal injection of ketamine/xylazine (10/1 mg/kg) (Sigma Aldrich, Germany). The left testes were then dissected and immediately immersed in Bouin's solution (Sigma Aldrich, Germany) for a duration of 48 hours, then immersed in formalin 10% in preparation for histopathological examinations. The blood sample and right testis were subjected to cryopreservation at -80 °C for gene expression and examination of oxidative stress markers in testis tissue.

The cryopreservation of the right testes allowed for these important molecular and biochemical analyses to be carried out on the samples.

The Histopathological Assessment of Tissue Specimen

The rat testes were fixed in Bouin's solution, embedded in paraffin, and cut into 5-µm sections. The sections were then processed to remove the paraffin and stained with hematoxylin and eosin (H&E). Three slides from different regions of each testicular tissue were evaluated by two impartial, blinded evaluators. They assessed the mean seminiferous tubule diameter (MSTD) and height of the germinal epithelium (HE/HST) by examining 10 circular seminiferous tubules per slide. The evaluators also determined the Johnson's score, a measure of testicular injury, by ranking each seminiferous tubule on a scale of 1-10 based on previous studies. All evaluations were performed using a Nikon light microscope (19,20).

Biochemical Factors Analysis

The Bradford method was used to determine the concentration of protein in the samples. This technique involves adding a dye, Coomassie Brilliant Blue G-250, to the sample, which causes a shift in the dye's absorbance spectrum and a color change. The intensity of the resulting color is then measured using a spectrophotometer and compared to a standard curve of known protein concentrations to determine the sample's protein concentration (17,21). The thiobarbituric acid reactive substances (TBARS) assay was used to measure the levels of malondialdehyde (MDA) as a marker of lipid peroxidation. In this method, the homogenized tissue samples are mixed with thiobarbituric acid (TBA) reagent and heated. The TBA reacts with the MDA produced during lipid peroxidation, forming a pink chromophore that can be measured spectrophotometrically at 532 nm. The concentration of MDA is determined using a molar

extinction coefficient of the chromophore. A modified version of this assay, the Uchiyama and Mihara method, uses trichloroacetic acid (TCA) as a protein precipitant to remove interfering substances from the tissue homogenate (22). Furthermore, SOD and GPx levels were determined using commercial kits (Ransod and Ransel, Randox Com, UK).

Quantitative Real-Time RT-qPCR Analysis

The mRNA expression of BAX and BCL-2 genes in the left testis of the rats was analyzed using the reverse transcription quantitative PCR (RT-qPCR) method. First, total RNA was isolated from the testicular tissues using the TRIzol reagent kit. Any genomic DNA contamination was eliminated by DNase I treatment (23). The concentration of the extracted RNA was determined, and cDNA was generated using a commercial kit. The RT-qPCR reaction was carried out in a 48-well plate, with each well containing the following components: DNase/RNase-free water, forward and reverse primers, cDNA, and SYBR Green. The thermal cycling was performed on the Applied Biosystems sequence detection system. The Pfaffl method was used to determine the fold change in the expression of each target gene (22,24).

These are the primer sequences used for the analysis of gene expression:

BAX forward: 5'-GGCGAATTGGAGATGAACTG-3'
BAX reverse: 5'-TTCTTCCAGATGGTGAGCGA-3'
BCL-2 forward:5'-CTTTGCAGAGATGTCCAGTCAG-3'
BCL-2 reverse: 5'-GAACTCAAAGAAGGCCACAATC-3'
GAPDH forward: 5'-GCAGCTCCTTCGTTGCCGGT-3'
GAPDH reverse: 5'-CCCGCCCATGGTGTCCGTTC-3'

Immunofluorescence Analysis

The investigation employed a commercially available immunoperoxidase kit (Santa Cruz Biotechnology, Inc. Germany) to assess the levels of BAX and BCL-2 protein expressions. The experimental protocol encompassed the inactivation of endogenous peroxidase activity, subsequent washing of sections with phosphate-buffered saline (PBS), retrieval of antigens through the application of the boiling method, further washing of sections with PBS, and incubation with Triton to augment cell membrane permeability. Subsequently, the samples were subjected to incubation with primary polyclonal antibodies targeting BAX (Santa Cruz, sc-493), BCL-2 (Santa Cruz, sc-783), and GAPDH (Santa Cruz, sc-25778) for an extended period of time. Following the washing procedure, the samples were subjected to incubation with a fluorescent secondary antibody (FITC-conjugated IgG) for a duration of 90 minutes. The samples underwent a washing procedure using PBS, followed by staining with propidium iodide (PI) (Sigma Aldrich, Germany). Subsequently, the samples were washed two additional times with PBS. The sections were mounted and subsequently examined using an Olympus fluorescence microscope. The investigation conducted by Banerjee and Chaturvedi involved the utilization of ImageJ software to determine the mean density of BAX and BCL-2 (25).

TUNEL Staining

The evaluation of apoptosis in testicular germ cells was performed using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) method with the In Situ Cell Death Detection Kit from Roche, Germany. First, the tissue sections were deparaffinized and dehydrated. Then, they were treated with proteinase K and a permeation solution. Next, the TUNEL dye solution was applied to the sections and incubated for 1 hour at 37 °C. After washing with PBS, the sections were observed under a fluorescence microscope (Olympus BX51, Japan). The number of TUNEL-positive apoptotic cells was counted in randomly selected microscopic fields. The measurement of apoptosis was quantified by calculating the apoptotic index, which represents the proportion of TUNEL-positive cells per field. The nuclei of the cells were stained with DAPI to aid in the identification and counting of the apoptotic cells (26).

Statistical Analysis

The data analysis for this study was performed using SPSS software version 18. To determine if there were any significant differences between the experimental groups, the researchers conducted an analysis of variance (ANOVA) test. This was followed by a least significant difference (LSD) post hoc test to identify which specific groups differed from one another. To confirm the accuracy and reliability of the findings, the statistical tests were conducted at least three times.

Results

The Total Weight of the Body and Testes

The results presented in Table 1 suggest that there were significant differences in both testis weights and body weights among all the groups of rats included in the study. Specifically, the data show that the diabetic group had significantly lower testis weights and body weights compared to the control group ($P<0.001$ and $P<0.05$, respectively). However, when the diabetic group was treated with eugenol, it resulted in a significant increase in both testis weights and body weights compared to the untreated diabetic group ($P<0.01$ and $P<0.05$, respectively). Interestingly, there were no significant differences in either testis weights or body weights between the control group and the treated-control group (i.e. G1 and G4) ($P>0.05$).

Histopathological Testes Evaluations

The histological analysis revealed that the administration of eugenol improved the morphological structure of the seminiferous tubules in the diabetic rats. The treated diabetic group showed decreased degeneration in the

Table 1. Body Weight and Testis Weight in Different Groups of the Study

Groups	Body weight (g) (Mean ± SD)	Testis weight (g) (Mean ± SD)
Control	252.5 ± 11	1.65 ± 0.025
Diabetic	176.45 ± 8*	0.91 ± 0.032*
Diabetic + Eugenol	225.5 ± 12*#	1.42 ± 0.028*#
Eugenol	255.3 ± 10	1.67 ± 0.023

The ANOVA (analysis of variance) test was used for statistical analyses
SD: standard deviation.
* $P < 0.001$ compared to the control group.
# $P < 0.001$ compared to the diabetic group.

seminiferous tubules compared to the untreated diabetic group.

Specifically, the data analysis showed that the mean Johnson's score, MSTD, and height of the seminiferous epithelium were all significantly increased in the eugenol-treated diabetic group compared to the untreated diabetic group ($P<0.05$). Interestingly, these testicular structural parameters were also comparable between the treated control group and the untreated control group (i.e. G1 and G4) ($P>0.05$), as shown in Figure 1. These findings suggest that the eugenol treatment may have had a protective effect on preserving the normal testicular structure in the diabetic rats (Table 2).

Oxidative Stress Markers Status

The results suggest that eugenol has a potential protective effect against OS induced by diabetes in testis tissue. The levels of SOD and GPx in the diabetic group was remarkably decreased compared to the control ($P<0.05$). The activity of these enzymes in the eugenol-treated group was significantly increased ($P<0.05$). Additionally, the MDA level in diabetic group was notably enhanced compared to control ($P<0.05$). Also, decreased levels of MDA in the eugenol-treated group were observed ($P<0.05$). It is worth noting that there was no significant difference in the biochemical parameters between the control and eugenol-treated control groups ($P>0.05$) (Table 3).

Gene and Protein Levels of BAX and BCL-2

The study employed RT-qPCR and immunofluorescence techniques to examine the impact of eugenol on the protein and gene expression of two key apoptotic regulators, BAX and BCL-2, in the testis tissue of diabetic rats. The findings revealed a notable elevation in both the mRNA expression and protein levels of the pro-apoptotic BAX gene in the diabetic rats compared to the control group ($P<0.001$). In contrast, the eugenol-treated diabetic group showed a significant reduction in the mRNA and protein expression of BAX compared to the untreated diabetic group ($P<0.05$). Conversely, the diabetic rats exhibited a significant reduction in both the mRNA and protein levels of the anti-apoptotic BCL-2 gene when compared to the control group ($P<0.002$). However, the administration

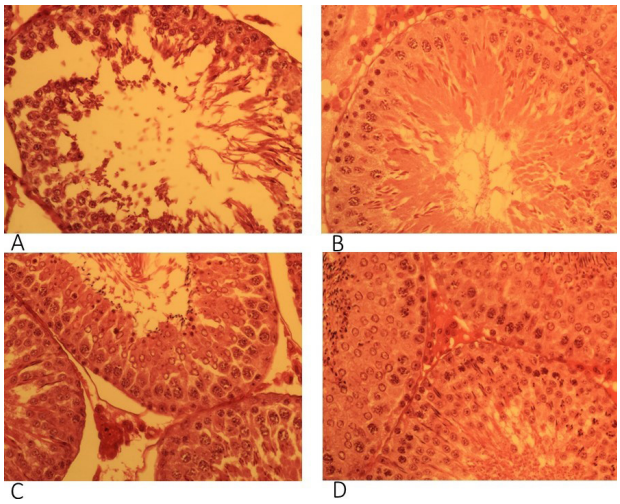

Figure 1. Testis Histology Stained With Hematoxylin and Eosin (H&E) in Different Groups of the Study. (A): diabetic group in which severe testicular damage was noted (B): control group in which normal testicular architecture was seen; (C): diabetic group treated with eugenol in which there was an improvement in the seminiferous tubule structure; (D): Normal group treated with eugenol in which normal testicular architecture was seen. Scale bar: ×200 (50 µm).

of eugenol resulted in an increase in both the mRNA and protein concentrations of BCL-2 relative to the untreated diabetic group ($P<0.05$). Importantly, the expression of both BAX and BCL-2 genes did not differ between the

Table 2. The Comparison of the Testicular Mean Johnson's Score, the Mean Seminiferous Tubule Diameter and the Height of Epithelium in Each Group

Groups	MJS (Mean ± SD)	MSTD (Mean ± SD)	HST (Mean ± SD)
Control	9.70 ± 0.46	252.42 ± 4.25#	65.5±1.25#
Diabetic	4.40 ± 0.17*	145.11 ± 2.57*	31.5 ± 2.13*
Diabetic + Eugenol	7.25 ± 0.44#*	188.5 ± 3.23#*	52.5 ± 2.15#
Eugenol	9.50 ± 0.34	155.22 ± 1.70#	66.03 ± 1.05#

The ANOVA (analysis of variance) test was used for statistical analyses
MJS: mean Johnson's score; MSTD: mean seminiferous tubule diameter; HST: the height of seminiferous epithelium (HE). SD: standard deviation.
* $P < 0.001$ compared to the control group.
# $P < 0.001$ compared to the diabetic group.

Table 3. Effect of Eugenol on the Levels of SOD, GPx, and MDA in the Rat Testicles After STZ-Induced Diabetes

Groups	SOD (U/g protein) (Mean ± SD)	GPx (U/g protein) (Mean ± SD)	MDA (nmol/g tissue) (Mean ± SD)
Control	1.63 ± 0.21	33 ± 3.21	0.78 ± 6.5
Diabetic	0.60 ± 0.11*	12.35 ± 2.3*	1.88 ± 7.35*
Diabetic + Eugenol	1.16 ± 0.18*#	22.75 ± 3.6*#	0.97 ± 5.7*#
Eugenol	1.65 ± 0.25#	35.65 ± 2.25	0.68 ± 3.5

The ANOVA (analysis of variance) test was used for statistical analyses.
SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde. SD: standard deviation.
* $P < 0.001$ compared to the control group.
# $P < 0.001$ compared to the diabetic group.

control and the eugenol-treated control groups ($P > 0.05$), suggesting that eugenol did not have any adverse effects on these apoptotic regulators in the normal testicular environment. The diabetic group had a higher BAX/BCL-2 ratio than the control group ($P < 0.001$), indicating a pro-apoptotic state. In contrast, the eugenol-treated diabetic group had a lower BAX/BCL-2 ratio compared to the untreated diabetic group ($P \leq 0.001$) (Figures 2 and 3; Table 4).

Testicular Germ Cell Apoptosis Index (TUNEL Staining)

To determine the impact of eugenol on testicular germ cell apoptosis, TUNEL staining was performed. Results showed that the testis of diabetic rats had a large number of TUNEL-positive cells compared to the control group ($P < 0.01$). Conversely, in the diabetic group treated with eugenol, the number of apoptotic cells significantly decreased compared to the diabetic group ($P < 0.05$). However, there was no significant difference between control and treated-control groups ($P > 0.05$). The TUNEL staining of testis sections was illustrated in Figure 4.

Discussion

Previous studies have established the antioxidant effects of eugenol in regulating the balance between pro-oxidants and antioxidants. However, it was unknown whether eugenol could reduce the oxidative damage caused by diabetes to the testes. Therefore, this study aimed to investigate the antioxidant properties of eugenol against testicular tissue damage. The findings revealed that STZ-induced diabetes increased OS, leading to various degenerative changes in the testis, such as the deterioration of seminiferous tubules and loss of spermatogenic cells. Treatment with eugenol was found to attenuate these degenerative changes.

The findings of our study indicated a statistically significant variation in the weights of the testicles and body of the rats. However, the administration of eugenol helped in preventing the reduction in weight of both the testicles and body. These results may be related to the effect of eugenol on controlling blood glucose. A prior investigation highlighted that eugenol treatment prevented the reduction in testis weight in a group with reproductive toxicity induced by cisplatin (27).

In the diabetic group, our study observed significant

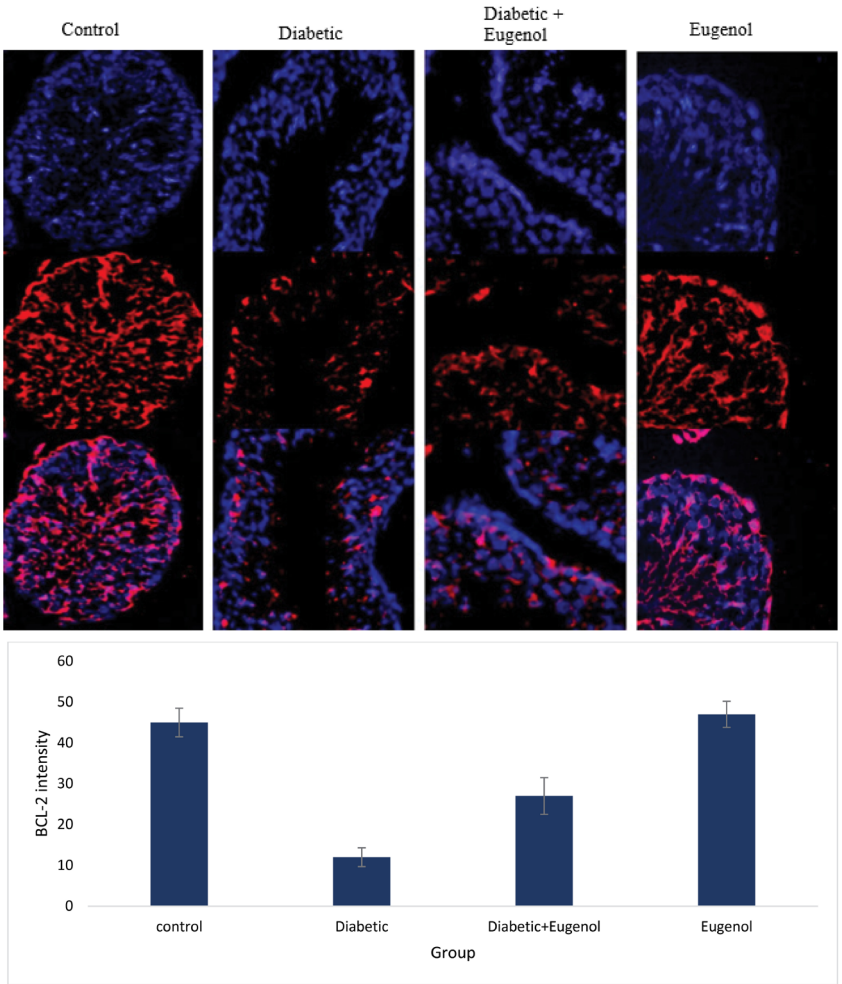

Figure 2. BCL-2 Protein Immunofluorescence Staining in the Testis of Rats in Study Groups: (A) Control group exhibited normal seminiferous tubules. (B) Diabetic group. (C) In Diabetic rats that received 4 mg/kg of eugenol, (D) normal rats that received 4 mg/kg of Eugenol. (×400).

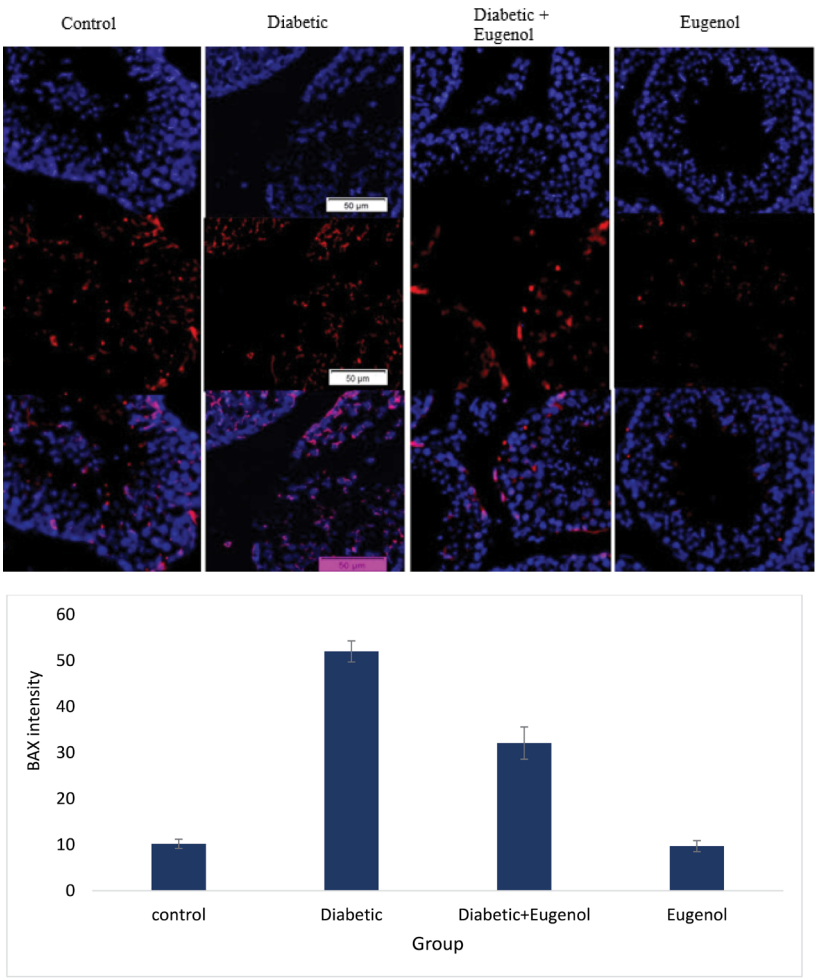

Figure 3. BAX Protein Immunofluorescence Staining in the Testis of Rats in Study Groups: (A) Control group exhibited normal seminiferous tubules. (B) Diabetic group. (C) In Diabetic rats that received 4 mg/kg of eugenol, (D) normal rats that received 4 mg/kg of Eugenol (×400).

degeneration in testicular tissue compared to the control group, resulting in a higher testis weight loss rate. Histopathological analysis showed that the diabetic group had a marked reduction in the diameter of seminiferous tubules and disruption in their structure. Moreover, the mean Johnson's scores were significantly reduced. In the diabetic group, eugenol treatment was observed to alleviate the aforementioned histological changes. However, eugenol did not show any negative effects on the control group, and normal histological features were observed. These findings are consistent with a study by Ekinci Akdemir et al, which also demonstrated that treatment with eugenol could decrease the degeneration of seminiferous tubules and reproductive damage induced by cisplatin (27).

Table 4. Relative mRNA Expression of BAX and BCL-2 Genes Analyzed by RT-qPCR

Groups	BAX (Mean ± SD)	BCL-2 (Mean ± SD)
Control	0.32 ± 0.022	1.0 ± 0.034$^{\#}$
Diabetic	1.03 ± 0.032*	0.41 ± 0.038*
Diabetic + Eugenol	0.64 ± 0.04$^{\#}$	0.68 ± 0.014$^{\#}$
Eugenol	0.35 ± 0.014$^{\#}$	1.12 ± 0.028$^{\#}$

Relative expression levels normalized to the amount of GAPDH as the internal control.
SD: standard deviation.
* $P < 0.001$ compared to the control group.
$^{\#}$ $P < 0.001$ compared to the diabetic group.

The results of our biochemical analysis revealed that diabetes caused an increase in the level of MDA, indicating OS, while decreasing the levels of SOD and GPx when compared to the control group. Diabetes is known to induce lipid peroxidation, protein glycation, and glucose auto-oxidation, which results in the production of free radicals such as superoxide, hydroxyl, and peroxyl radicals (28). The elevated free radicals deplete endogenous antioxidant enzymes such as SOD and GPx, resulting in reproductive impairment (17,22). Therefore, the negative impact of diabetes on reproductive health is mainly due to the disruption of the pro-oxidant-antioxidant balance or OS generated by free radicals (29). Our study showed that the higher level of MDA in the testis tissue of diabetic rats is an indication of lipid peroxidation and OS. It is noteworthy that the assessment of MDA levels can provide valuable insights into the degree of testicular tissue impairment (28). In our study, treatment with

eugenol was observed to significantly decrease the lipid peroxidation induced by diabetes and also enhance the activity of antioxidant enzymes SOD and GPx, thereby supporting the natural antioxidant defense system of the body's cells. These biochemical results are consistent with another study that demonstrated the beneficial effects of eugenol in mitigating reproductive damage induced by ovarian torsion, by decreasing the oxidative stress marker MDA and increasing levels of the antioxidant GSH, as well as the activities of GPx, SOD, and CAT (14).

It has been reported that in male testicles affected by diabetes, the damage to DNA and proteins caused by ROS can result in significant cellular injury and apoptosis (8). The initiation of cell death can be stimulated by the activation of death receptors (TNFR1 and Fas) located outside the cell. When these receptors bind to their corresponding ligands (TNF-α and FasL, respectively), it triggers the activation of initiator caspase 8. This caspase cleaves and activates downstream effector pro-caspases, such as caspases 3, 6, and 7, which ultimately lead to the death of the cell (8,23). Indeed, BCL-2 has been shown to have antioxidant properties and its overexpression can protect cells from OS-induced apoptosis. This is achieved through various mechanisms, such as the inhibition of ROS production, the maintenance of mitochondrial function, and the upregulation of antioxidant enzymes. In addition, it has been reported that BCL-2 can directly scavenge ROS, such as superoxide anion and hydrogen peroxide, and prevent oxidative damage to cellular components (23). Therefore, the increase in BCL-2 mRNA levels and protein fluorescence intensity observed in our study, following treatment with eugenol, may have contributed to the reduction of OS and apoptosis in the diabetic rats' testicular tissue. On the other hand, we found that eugenol dramatically reduced BAX mRNA levels, protein fluorescence intensity, and the apoptosis index of testicular germ cells. BAX is a pro-apoptotic factor that belongs to the BCL-2 family and is a key regulator of the intrinsic apoptotic pathway. The findings of Ekinci Akdemir et al suggest that eugenol may have

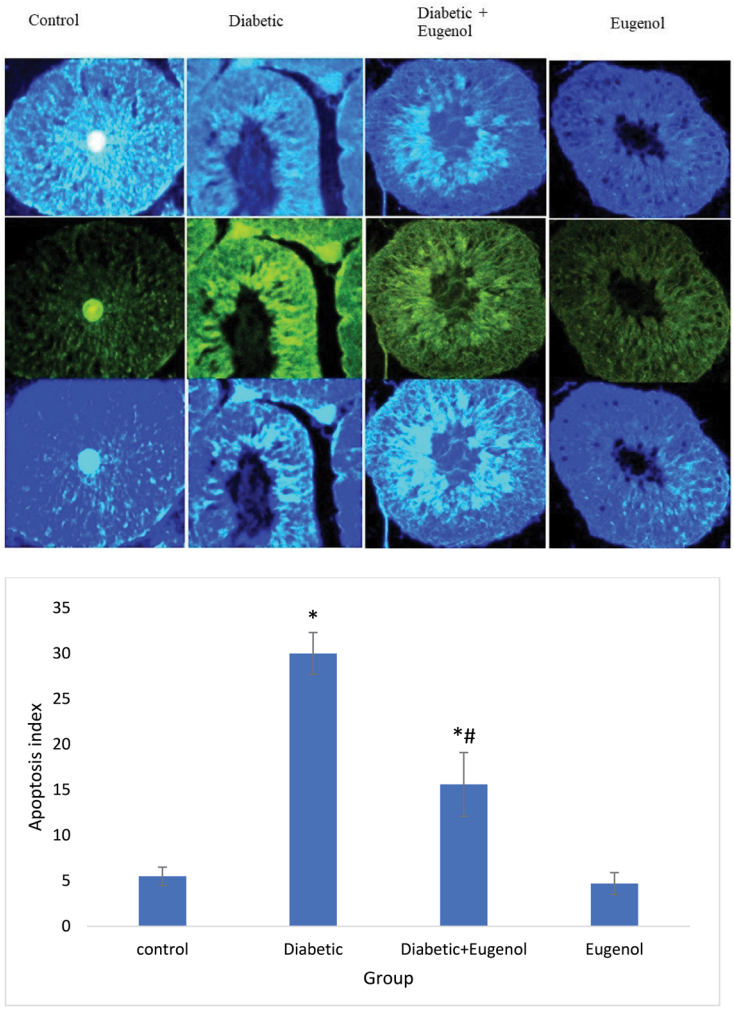

Figure 4. Representative Photograph of TUNEL Staining Showing the Effects of Diabetes and Eugenol on Germ Cell Apoptosis in Different Groups of the Study. In the column relative to diabetic group (the second column), increased TUNEL positive cells can obviously be observed. In the third column (diabetic group treated with eugenol) compared to the second column, it can be observed the number of TUNEL positive cells significantly decreased. Arrows show TUNEL positive cells. Scale bar: ×200 (20 μm). Apoptotic index (*$P < 0.02$ and #$P < 0.05$). DAPI: 4′,6-diamidino-2-phenylindole.

an anti-apoptotic effect by inhibiting caspase-3 activity and protecting the testis tissue from cisplatin-induced reproductive damage. Caspase-3 is a key executioner caspase that plays a crucial role in apoptosis, and its activity is often used as a biomarker for the measurement of apoptosis (30,31). Eugenol has been reported to have anti-oxidative and anti-apoptotic properties, which can help protect against testicular damage. In a study on rats subjected to testicular damage, treatment with eugenol was found to down-regulate the activity of caspase-3 and decrease the expression of BAX, which is a pro-apoptotic gene, while increasing the expression of BCL-2, which is an anti-apoptotic gene. These findings suggest that eugenol may have a protective effect on the testes by inhibiting apoptosis and reducing OS (30). Stevens and Allred conducted an exhaustive review that underscored the significance of eugenol in modulating the activity of numerous crucial regulatory enzymes implicated in glucose metabolism processes. This suggested that eugenol may have an effect on diabetic conditions and associated secondary complications (32). It is possible that the protective effect of eugenol against testicular dysfunction and oxidative damage caused by ROS is due to its antioxidant and antiapoptotic effects. Apoptosis is a complex process involving various signaling pathways, including ROS-mediated pathways. Antioxidants can scavenge ROS and inhibit the apoptotic signaling cascade. Therefore, it is plausible that eugenol's antioxidant activity may play a role in its ability to decrease apoptosis in testicular cells of treated-diabetic rats. However, further studies are required to elucidate the exact mechanism underlying eugenol's protective effects in the context of testicular dysfunction and oxidative damage.

Study Limitations

While Western blot analysis represents a valuable method for detecting and quantifying protein expression levels, its absence in this investigation may have constrained the detailed examination of apoptosis-related protein expression. The lack of access to the requisite equipment and resources serves as a valid limitation in the conducting of this research project. Future studies should consider incorporating this technique to enhance and complement the findings presented in the current study.

Conclusions

The results of the present research show that eugenol administration led to a reduction in MDA levels and an increase in antioxidant activity, regulates the expression of pro-apoptotic and anti-apoptotic genes, and reduces testicular injury and apoptosis, thereby protecting testicular function against diabetes-induced damage. These results support the use of eugenol as a potential therapeutic agent for male infertility associated with diabetes.

Authors' Contribution

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Writing–review & editing: Maryam Shayeghi Rad, Nasim Hayati Roodbari, Amir Afshin Khaki, Fardin Amidi, Akram Eidi.

Conflict of Interests

Authors declare that they have no conflict of interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Issues

The present study was part of the research project of Dr. Amir Afshin Khaki, which was approved by the ethics committee of Tabriz University of Medical Sciences. All the steps of working with laboratory animals were carried out in a standard environment and based on the ethical protocol of working with laboratory animals of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1397.020).

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