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Potential of *Stevia rebaudiana* Bertoni Aquatic Extract in Treating Diabetes-Induced Infertility: Effects on Autophagy and Apoptosis

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ABSTRACT

Backgrounds: Infertility is one of the major complications of diabetes. Diabetic-mediated oxidative stress could destroy autophagy in testes, leading to damaged sperm and male infertility. *Stevia rebaudiana* Bertoni is a medicinal herb with various health-beneficial effects, such as improving diabetes-induced infertility. However, till now, no exact mechanism is known for its effects on diabetic testes. This study aimed to investigate the anti-infertility role of *S. rebaudiana* aqueous extract in diabetic rats induced by STZ by focusing on autophagy and apoptosis pathways and antioxidant activity.

Methods: Male rats were divided into four groups of five, including a healthy control group, a diabetic control (STZ) group, and MET-treated (500 mg/kg) and stevia-treated (400 mg/kg) diabetic groups for 30 days. After the experimental period was over, rats were sacrificed. Testes tissue and blood samples were prepared for biochemical, molecular, and histopathological analyses.

Results: Treatments with stevia and MET significantly increased body weight and testosterone levels and decreased FBS compared to the STZ group. Moreover, both treatments decreased the mRNA expression of *Akt1*, *Mtor*, *Sqstm1/p62*, *Nfkb1* and *caspase-9* and increased the expression of *Becn1*, *Atg3*, *Atg5*, *Atg7*, *Map1lc3b/LC3B*, *Nrf2* and *Bcl2*, suggesting a possible improvement in autophagy- and apoptosis-related responses in diabetic rats. Histopathological changes and sperm characteristics also showed improvement following stevia and MET treatments.

Conclusion: Stevia aqueous extract could exert potential therapeutic effects on diabetes-induced infertility in diabetic rats, possibly through the modulation of autophagy, apoptosis, and antioxidant activity.
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Keywords: Antioxidants, Apoptosis, Autophagy, Diabetes mellitus, Stevia

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List of abbreviations

Akt1: AKT serine/threonine kinase 1; ***Atg*:** autophagy-related gene; ***Bcl2*:** B-cell leukemia/lymphoma 2; ***Becn1*:** *Beclin-1*; **CHOP:** CCAAT/enhancer-binding protein (C/EBP) homologous protein; **ER:** endoplasmic reticulum; **FBS:** fasting blood sugar; ***GAPDH:*** glyceraldehyde-3-phosphate dehydrogenase; **H&E:** hematoxylin-eosin staining; ***Map1lc3b/LC3B*:** microtubule-associated protein 1 light chain 3 beta; **MDA:** malondialdehyde; **MET:** metformin; **Mtor:** mechanistic target of rapamycin kinase; **Nfkb1:** nuclear factor kappa B subunit 1; ***Nrf2*:** nuclear factor erythroid 2-related factor 2; **PPARγ:** peroxisome proliferator-activated receptor-γ; **qRT-PCR:** quantitative real-time polymerase chain reaction; ***S. rebaudiana:*** *Stevia rebaudiana*; **SM:** diabetes mellitus; **STZ:** streptozotocin; **TAC:** totalantioxidant capacity

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INTRODUCTION

Diabetes mellitus is one of the most prevalent metabolic endocrine disorders, resulting from impaired regulation of blood glucose[1]. Patients with type 2 DM are at high risk of serious complications, including cardiovascular disease[2], premature mortality[3], retinopathy[4], kidney dysfunction[5], and depression[6]. Reproductive system impairment is another important complication of diabetes. Almost 90% of diabetic men and women experience some forms of sexual dysfunction, including reduced libido, erectile dysfunction, and infertility[7]. Chronic hyperglycemia damages the testicular tissue, sperm quality, and overall reproductive health in men. Insulin resistance, as a hallmark of type 2 diabetes, can further contribute to erectile dysfunction, low testosterone levels, and poor sperm parameters[8]. Persistent hyperglycemia also induces oxidative stress, which plays a major role in the development of diabetic complications, including infertility. Cellular exposure to oxidative stress activates ER stress and signaling pathways such as the unfolded protein response and autophagy[9]. The unfolded protein response restores ER homeostasis by removing the unfolded and misfolded proteins from the cell. However, under prolonged or severe stress, this compensatory mechanism becomes insufficient, and the autophagy pathway is activated[10]. The autophagy signaling pathway breaks down and recycles the damaged and nonfunctional proteins and organelles to maintain cellular homeostasis and survival[11]. During this process, cellular components that need to be destroyed are engulfed into vesicular autophagosomes and destroyed after fusion with lysosomes containing degrading enzymes. When oxidative or ER stress exceeds the protective capacity of autophagy, apoptosis (programmed cell death) is initiated[12].

Recent evidence suggests that dysregulation of autophagy and apoptosis contributes significantly to testicular dysfunction in diabetes. Impaired autophagy can result in the accumulation of damaged mitochondria, testicular inflammation, and loss of germ cells, ultimately leading to reduced sperm quality and infertility[13]. Conversely, maintaining a balance between autophagy and apoptosis can protect germ cells and preserve spermatogenesis. Therefore, elucidating the molecular interplay between autophagy and apoptosis in the diabetic testis is critical for identifying novel therapeutic targets to improve male fertility[14,15]. Enhancement of autophagy has been proposed as a potential therapeutic strategy to alleviate diabetes-induced infertility. In this regard, autophagy inducers such as MET[16-18] and rapamycin[19] have shown beneficial effects on spermatogenesis and fertility in diabetic models.

Many efforts have been conducted till now to reduce diabetes complications[20]. Insulin, which is routinely used in diabetic patients, mediates some side effects such as daily injection pain and hypoglycemic reactions[21]. MET is also clinically used to treat type 2 diabetes. However, there are some gastrointestinal symptoms such as diarrhea and nausea, metallic taste, and impairment of vitamin B12 absorption[22]. Therefore, other potential compounds have been introduced for the treatment of diabetes, such as herbal remedies[23].

A native plant of Paraguay and Brazil, *Stevia rebaudiana* (Bertoni), has recently received increasing scientific attention[24]. This natural, plant-based sweetener has been used for centuries in India and South America[25]. The main sweet compounds of this plant are dulcoside, stevioside, rabadioside C, and rabadioside A[26]. Considering that stevia leaves have no calories but their sweetness is more than 200-300 times than that of sucrose, it has been noticed all over the world today[27]. Beyond its sweetening capacity, stevia exhibits antioxidant, antidiabetic, anti-inflammatory, and antihypertensive properties[28]. It has also been shown to stimulate insulin secretion and improve glucose metabolism[28]. Our research team conducted an animal study in 2019, which found that stevia, with its anti-diabetic properties, was able to improve the complications of diabetes and infertility caused by it. Stevia also had positive effects on steroidogenesis and spermatogenesis and improved the testicular function of affected rats[29].

According to the above descriptions, there is a relationship between diabetes and infertility in men, as well as the role of autophagy in both of the above diseases. However, despite the known antidiabetic effects of stevia, the underlying molecular mechanisms, particularly the involvement of autophagy and apoptosis, remain unclear. Understanding these mechanisms is essential, as it could provide valuable insight into how stevia or its bioactive compounds might protect reproductive function and serve as a potential adjunct therapy for diabetic infertility. Therefore, the present study was designed to investigate the effects of *Stevia rebaudiana* on autophagy signaling, oxidative stress, and apoptosis in diabetic male rats, to elucidate its potential role in improving diabetes-induced infertility.

MATERIALS AND METHODS

Animals

Twenty male Sprague-Dawley rats with an average weight of 250 ± 20 g and age of three months were prepared from the Animal Laboratory of Shiraz University of Medical Sciences, Shiraz, Iran. The

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acclimatization of animals was performed one week before the initiation of the experiments. All animals were housed in standard conditions (a balanced 12-hour light-dark cycle) and fed with the standard diet.

Materials

MET and STZ were prepared from Sigma-Aldrich (Germany). Stevia leaves were bought from Golsaran Company (Rasht, Iran). The RNA extraction kit (total RNA extraction, KEX505, KiyanZOL, Iran) and primers were obtained from Methabion Company (Germany). The cDNA synthesis kit was acquired from SinaClon (SinaClon First Strand cDNA synthesis Kit, RT5201, Iran). The RealQ Plus 2× Master Mix SYBR Green High ROX was prepared from Ampliqon (California, USA).

Preparation of aqueous extract of stevia leaf

The stevia leaves were washed, followed by drying at the temperature <50 °C, and then powdering. The material extraction was performed using the soxhlet apparatus. Following drying, the obtained material was evaporated to dryness with a vacuum rotatory evaporator. Then 100 g of the above powder was suspended in distilled water (1200 ml) and stirred in a shaker-incubator at 24 ˚C for 24 hours. Afterwards, the solid part was removed by a filtration process at 40-50 °C. The remained humidity was removed using a vacuum desiccator at 50 °C. Eventually, 35 g of extract was obtained from 100 g of powder of stevia leaves[29,30].

Study design

Diabetes induction was performed by a single-dose intraperitoneal injection of STZ (60 mg/kg body weight) in a citrate buffer solution (0.l M, pH 4.5)[31]. The blood sample was obtained after seven days from the rat tail veins to determine the FBS. The FBS level of more than 300 mg/dl, along with other symptoms of diabetes, including polydipsia, weight loss, and polyuria, was considered a confirmation of the diabetic model. All 20 rats were distributed into four groups of five, including a healthy control group (without any treatment), a diabetic control group induced by STZ, a diabetic group treated with a standard drug MET (500 mg/kg), for diabetes, and a stevia-treated diabetic group (400 mg/kg)[31]. All treatments were administered by oral gavage every morning for 30 days. After the 30-day treatment period, a mixture of 10% ketamine and 2% xylazine (80 and 5 mg/kg, respectively; Alfasan, the Netherlands) was used to anesthetize animals. Then, after extraction of the blood sample (5 ml) from the rat heart and serum isolation by centrifugation (10 minutes at 1200 ×g), the obtained serum was stored at -70 °C for the next assessment of biochemical markers. Subsequently, a portion of testicular tissue weighing 50-100 mg was extracted for investigation of gene expression. The other testis was extracted and stored in a 10% formaldehyde solution for further histopathological analysis.

Serum glucose and testosterone profile analysis

The blood samples were collected from overnight fasted rats after a 30-day treatment period. After serum isolation from blood, the biochemical parameters, including FBS, were measured using diagnostic colorimetric kits (Biosystem, Spain) and a biochemistry auto-analyzer (Hitachi Japan). The assessment of testosterone ng/ml was conducted using an ELISA kit, which was sourced from the Bioassay Technology Laboratory (China).

Assessment of the oxidative stress in the testis tissue

To measure oxidative stress markers, homogenization of a small portion of the testis tissue (50 mg) was performed in sodium phosphate buffer. After 15 min centrifugation at 10,000 ×g, the supernatant was applied for colorimetric evaluation of MDA (KPG-MDAK) and TAC (KPG-FRAPK) using the kits of the Karmania Pars Gene Company (Iran).

Analysis of gene expression using qRT-PCR

To identify the impact of stevia on specific genes of the autophagy and apoptosis signaling pathways in testis tissues, we used the RT-PCR[32]. First, based on the manufacturer's instructions, the testis total RNA was isolated using an RNX-Plus RNA extraction kit (KiyanZOL). To determine the purity concentration of the isolated RNA, the optical density ratio 260:280 nm was measured by a Nanodrop spectrophotometer (Thermo Scientific, USA). To assess the integrity of the obtained RNA, denaturing gel electrophoresis was applied. The cDNA synthesis was also conducted using a cDNA Synthesis Kit. Eventually, genes were amplified using the SYBR green-based RT-qPCR (Amplicon, USA) and specific primers (Methabion) by the ABI real-time PCR 7500 system. The sequences of gene-specific primers are mentioned in Table 1.

Sperm analysis

The sperm analysis was performed based on a protocol used in a previous study[33]. The sperm samples were taken by an excision of a small piece (1 cm) from the ductus deferens of rats. The obtained samples were then placed in Petri dishes containing Hank's Balanced Salt Solution, and gently shaked at 37 °C for 15 min. Finally, the microscopic examination determined the sperm count and motility.

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Table 1. The primer sequences of the evaluated genes

Gene	Accession number	Sequences (5'->3')	Product size (bp)
Akt1	NM_033230.3	F: TTTATTGGCTACAAGGAACG R: AGTCTGAATGGCGGTGGT	213
Mtor	NM_019906.2	F: TGCCTTCACAGATACCC R: TAAACTCGGACCTCACC	131
Becn1	NM_001034117.1	F: TTCAAGATCCTGGACCGAGTGAC R: AGACACCATCCTGGCGAGTTTC	142
Atg3	NM_134394.3	F: GCTCAGTGCTGTGCGATGAAG R: AGCCGTGGCGTCTGGTAGTA	229
Atg5	NM_001014250.2	F: TGACGCTGGTAACTGACAAAGTG R: TGATGTTCCAAGGCAGAGCTGAG	168
Atg7	NM_001012097.1	F: GTCTGTCAAGTGCCTGCTGCT R: TGCCTCACGGGATTGGAGTAG	132
Sqstm1/p62	NM_175843.4	F: CCATGGGTTTCTCGGATGAA R: GGAGGGTGCTTTGAATACTGG	106
Map1lc3b/LC3B	NM_022867.2	F: AGAGCGATACAAGGGTGAGAAG R: AGGAGGAAGAAGGCTTGGTTAG	141
Bcl2	NM_016993.1	F: GGAGGATTGTGGCCTTCTTT R: GTCATCCACAGAGCGATGTT	100
Caspase-9	NM_031632.3	F: ACATCTTCAATGGGACCGGC R: TCTTTCTGCTCACCACCACAG	85
Nrf2	NM_001399173.1	F: TGTCAGCTACTCCCAGGTTG R: AGGGCAAGCGACTGAAATGT	138
Nfkb	NM_001415012.1	F: CCAGCACCAAGACCGAAGCAA R: CGCCAGCAGCATCTTCACATC	170
GAPDH	NM_017008.4	F: GCTGCCTTCTCTTGTGAC R: TTGAACTTGCCGTGGGTA	110

Tissue sample preparation for histopathological evaluation

Testis tissues were separated and fixed in buffered formalin (10%) before tissue processing steps using a tissue processor (model DS2080/H, Did Sabz Co., Iran) and paraffin blocks were prepared. Microtome sections of 5 µm thickness were prepared and exposed to H&E for pathological assessment. A pathologist assessed the prepared slides using a microscope (Nikon E200, Japan) and the modified Johnson method to determine testicular tissue changes (Table 2). After that, photomicrographs were taken at 400 times magnification.

Statistical analysis

The SPSS software (version 23.0; SPSS Inc., Chicago, USA) was applied for data analysis. The statistical analysis was conducted using one-way Analysis of Variance (ANOVA) test and Tukey's post-hoc test. The p values less than 0.05 were statistically considered significant.

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Table 2. Evaluation of the maturity of the spermatogenesis process based on Johnson's scoring

Score	Description
10	Complete spermatogenesis and perfect tubule
9	Many late spermatids present but disorganized tubular epithelium
8	Only a few late spermatids
7	No late spermatids but many early spermatids
6	Few early spermatids, arrest of spermatogenesis at the spermatid stage, disturbance of spermatid differentiation
5	No spermatids, many spermatocytes
4	Few spermatocytes, arrest of spermatogenesis at the primary spermatocyte stage
3	Only spermatogonia
2	No germ cells, Sertoli cells only
1	No germ cells or Sertoli cells, tubular sclerosis

RESULTS

Body weight, FBS, and testosterone in the experimental groups

As shown in Table 3, a significant decline in body weight and an increase in FBS levels were observed in all diabetic groups, regardless of treatment regimen, compared to the healthy control group ($p < 0.001$). Treatment with MET (STZ + Met) or stevia (STZ + stevia) significantly increased the body weight and reduced the FBS levels in comparison with the STZ group ($p < 0.05$ and $p < 0.01$, respectively). Testosterone was also significantly diminished in all diabetic groups, regardless of treatment regimen, compared to the healthy control group ($p < 0.001$ for the STZ group and $p < 0.05$ for STZ + Met and STZ + Stevia groups). Both treatments with MET or stevia (STZ + Met and STZ + stevia) significantly enhanced the testosterone level compared to the STZ group ($p < 0.05$).

Improvement of testis antioxidant capacity after stevia treatment

As depicted in Figure 1, all diabetic groups revealed significant enhancement of MDA levels in comparison with the healthy group ($p < 0.001$ for STZ, $p = 0.015$ for STZ + Met, and $p = 0.026$ for STZ + stevia), which confirmed the increased oxidative stress in diabetes. However, the administration of Met or stevia in diabetic animals significantly decreased the MDA level compared with the STZ group ($p = 0.009$ for STZ + Met, and $p = 0.005$ for STZ + stevia). The TAC level significantly decreased in the STZ group in comparison with the healthy group ($p < 0.001$), which showed decreased antioxidant activity in diabetes. No significant difference was observed between the TAC levels of Met/stevia treated groups in comparison with the healthy control group ($p\ b>0.05$). On the other hand, the STZ + Met ($p = 0.002$) and STZ + stevia ($p < 0.001$) groups showed significant enhancement in TAC levels compared with the STZ group.

Improvement of sperm quality after stevia treatment

As shown in Figure 2, the results of sperm analysis indicated a significant enhancement in sperm non-progressive movement ($p = 0.049$) and immotile sperms ($p < 0.001$) in the STZ group in comparison with the healthy group. A significant reduction in sperm progressive movement and sperm count ($p < 0.001$) was also observed in comparison to these two groups. All these findings indicated a decline in the quantity of sperm and finally infertility as a consequence of diabetes. The sperm analysis showed a significant role of Met or stevia treatment in sperm improvement. Accordingly, sperm progressive movement ($p < 0.001$ for both treatments) and sperm count ($p = 0.003$ for both treatments) were significantly enhanced in comparison with the diabetic control group.

Histopathological changes and modified Johnson's score in experimental groups

According to Johnson's scoring system, as shown in Figure 3, the score of the control group is greater than 9 (9.60 ± 0.547), showing completely regular lumens and typical cell types, including round and elongated spermatids, spermatogonia, spermatocytes, and Sertoli. Additionally, it displays excellent tubules and full spermatogenesis, along with a large number of late spermatids but a disorganized tubular epithelium. Active spermatogenesis was detected in the control group, which also had normal germinal epithelium thickness without connective tissue congestion or edema. Conversely, a significant reduction in Johnsen scores below 5 (4.4 ± 0.547) was detected in the STZ group when compared to the healthy group. No spermatids, a large number of spermatocytes, a small number of spermatocytes, and an arrest of spermatogenesis at the primary spermatocyte stage were also observed. In comparison with the STZ group, the modified Johnsen score was significantly higher in the treated groups ($p < 0.001$ for MET [7.20 ± 0.836]; and

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Table 3. The biochemical parameters in different treated groups

Parameters	Control	STZ (60 mg/ml)	STZ-Met (500 mg/ml)	STZn+Stevia (400 mg/ml)
Body weight (g)	326.0 ± 9.27†††	190.20 ± 29.23***	230.20 ± 16.97***†	235.60 ± 14.36***†
FBS (mg/dl)	147.40 ± 14.40†††	383.20 ± 59.30***	294.00 ± 25.75***††	267.60 ± 37.40***††
Testosterone	5.58 ± 2.32†††	0.17 ± 0.03***	2.59 ± 0.82*†	2.58 ± 1.0*†

STZ: STZ-induced diabetic control group (60 mg/kg), STZ + Met: metformin-treated diabetic rats (500 mg/kg); STZ + Stevia: diabetic rats treated with aquatic extract of stevia (400 mg/kg). All values are provided as mean ± SD (n = 6). *statistically significant with the control group; †statistically significant variance with the STZ group ($^{*,\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{***,\dagger\dagger\dagger}p < 0.001$).

$p = 0.001$ for stevia [6.20 ± 0.447]). Despite the increase of modified Johnsen score in both treated groups, this score was still significantly low compared with the control group ($p < 0.001$). According to Figure 4, the control group exhibited normal tubules and germ cells without any pathological damage. The negative effects of diabetes on testicular tissue were demonstrated by the diabetic group's tubular atrophy and shrinkage, the germinal epithelium disintegration, a sharp decline in the germ cells, and the presence of empty spaces within the tubules. Additionally, the thickness of the germinal epithelium was significantly reduced in this group (Fig. 4B). Although it did not reach the level detected in the control group, a significant increase in the germ cells was noted in the diabetes groups treated with stevia extract and MET (Fig. 4C and 4D, respectively). Also, cell-free tubules and tubular shrinkage, indicated in Figure 4B, were observed in the diabetic group. In contrast, the treated group demonstrated signs of improvement (Fig. 4C and 4D). Figure 2B illustrates a significant reduction in Leydig cells in the diabetic group, while this decline was prevented in the treated groups.

Differential expression of the autophagy-related genes in experimental groups

To determine the effect of stevia extract on autophagy signaling in testicular tissues, quantitative gene expression was performed by real-time PCR. As shown in Figure 5, the expression of negative regulators of autophagy, including *Mtor* and *Akt1*, was enhanced in the STZ group compared with the control group ($p < 0.05$ for *Akt1*). In contrast, important players in the autophagy pathway including, *Becn1* and *Map1lc3b/LC3B*, as well as *Atg3*, *Atg5*, and *Atg7* were decreased, and *Sqstm1/p62* was also enhanced in diabetic groups in comparison with the control group ($p < 0.05$ for *Atg5*, *Sqstm1/p62*, and *Map1lc3b/LC3B*, $p < 0.01$ for *Becn1*, and $p < 0.001$ for *Atg3*). These results showed that autophagy inhibition is a consequence of diabetic disease. Treatment with stevia or Met reversed these consequences in diabetic patients. Accordingly, both Met- and stevia-treated diabetic groups decreased the expression of autophagy-negative regulators in comparison with the STZ group ($p < 0.001$ for *Akt1* and *Mtor*). The treatment with Met or stevia

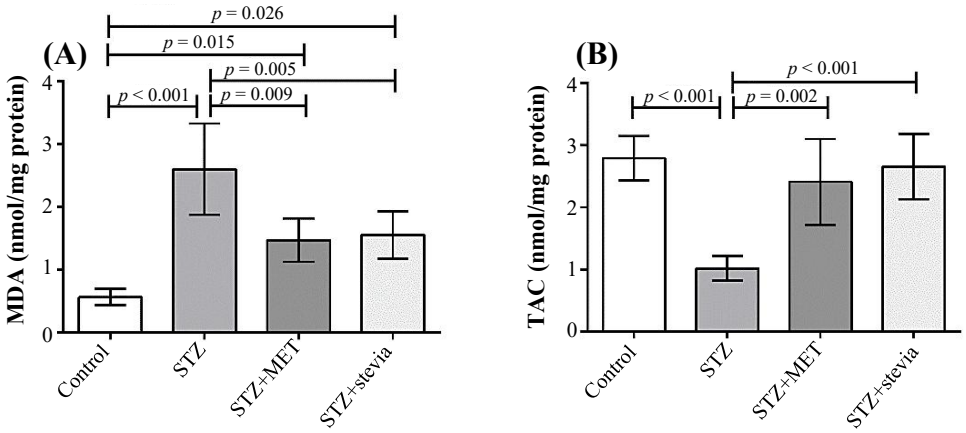

Fig. 1. Evaluation of the oxidative stress and antioxidant activity in the experimental groups by measuring the (A) MDA and (B) TAC markers, respectively. STZ: streptozotocin-induced diabetic control group (60 mg/kg); STZ + Met: metformin-treated diabetic rats (500 mg/kg); STZ + Stevia: diabetic rats treated with stevia (400 mg/kg).

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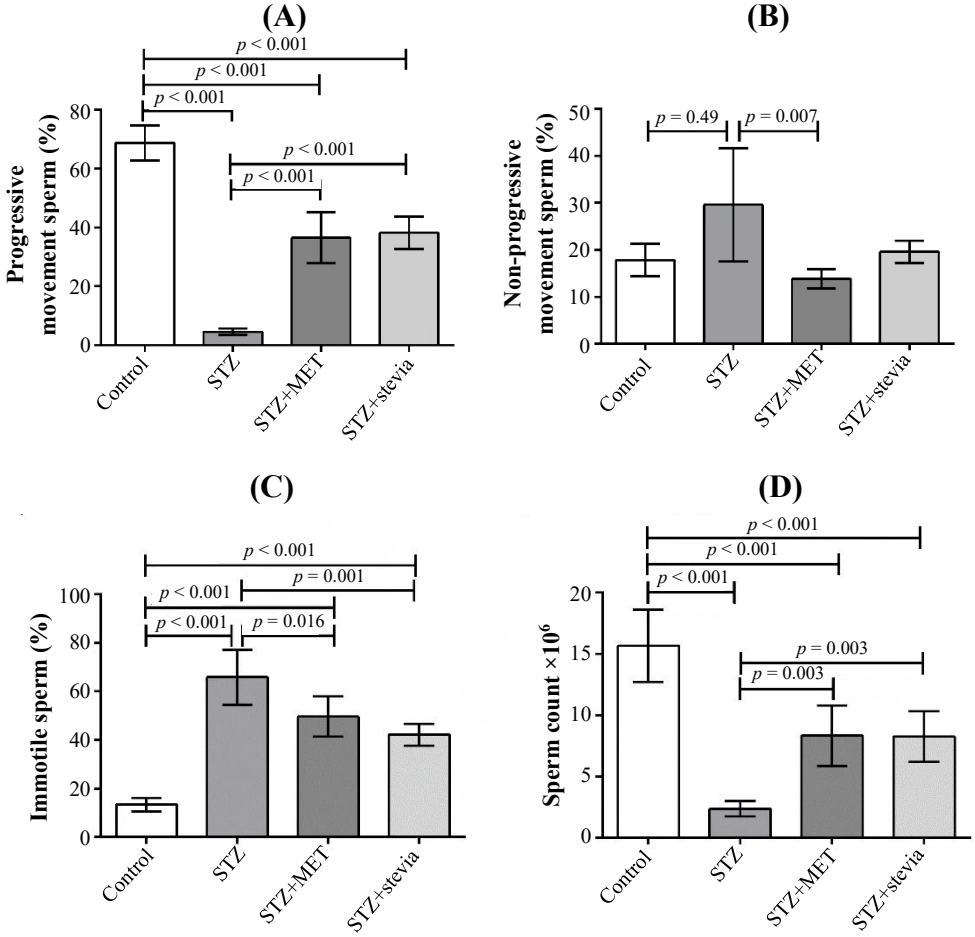

Fig. 2. The sperm analysis in experimental groups by measuring parameters, including (A) progressive movement, (B) non-progressive movement, (C) immotile sperm, and (D) sperm count. STZ + Met: metformin-treated diabetic rats (500 mg/kg); STZ + Stevia: diabetic rats treated with stevia (400 mg/kg).

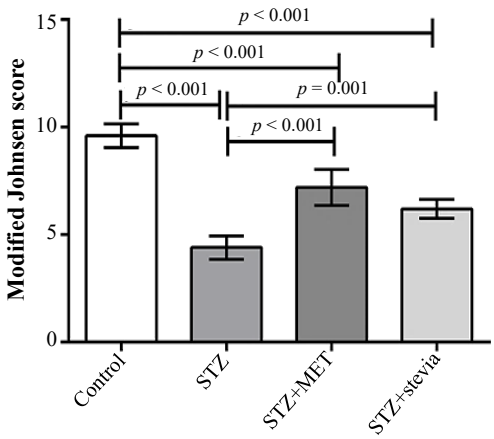

Fig. 3. Evaluation of sperm morphology including, size, shape and structural abnormalities by measuring the modified Johnsen score in experimental groups. STZ: streptozotocin-induced diabetic control group (60 mg/kg); STZ + Met: metfromin-treated diabetic rats (500 mg/kg); STZ + Stevia: diabetic rats treated with stevia (400 mg/kg).

also significantly enhanced the expression of autophagy-related genes ($p < 0.05$ for *Becn1* in both groups, $p < 0.001$ for *Atg3* and *Atg5* of stevia-treated group, $p < 0.01$ for *Atg7* and *Map1lc3b/LC3B* of stevia-treated group, and $p < 0.05$ for *Atg7* of Met-treated group) and decreased the expression of *Sqstm1/p62* ($p < 0.001$) of stevia-treated group. The obtained results indicated significant enhancement of autophagy-related genes, including *Atg3*, *Atg5*, and *Map1lc3b/LC3B* and also a decrease of *Sqstm1/p62* in stevia-treated versus Met-treated diabetic groups ($p < 0.001$ for *Atg3*, $p < 0.01$ for *Sqstm1/p62*, and $p < 0.05$ for *Map1lc3b/LC3B* and *Atg5*).

Differential expression of the apoptotic and oxidative related genes in experimental groups

The expression of apoptotic-related genes, including (*Bcl2*, and *Caspase-9*, and oxidative stress-related genes, including *Nrf2* and *Nfkb1*, was considered to

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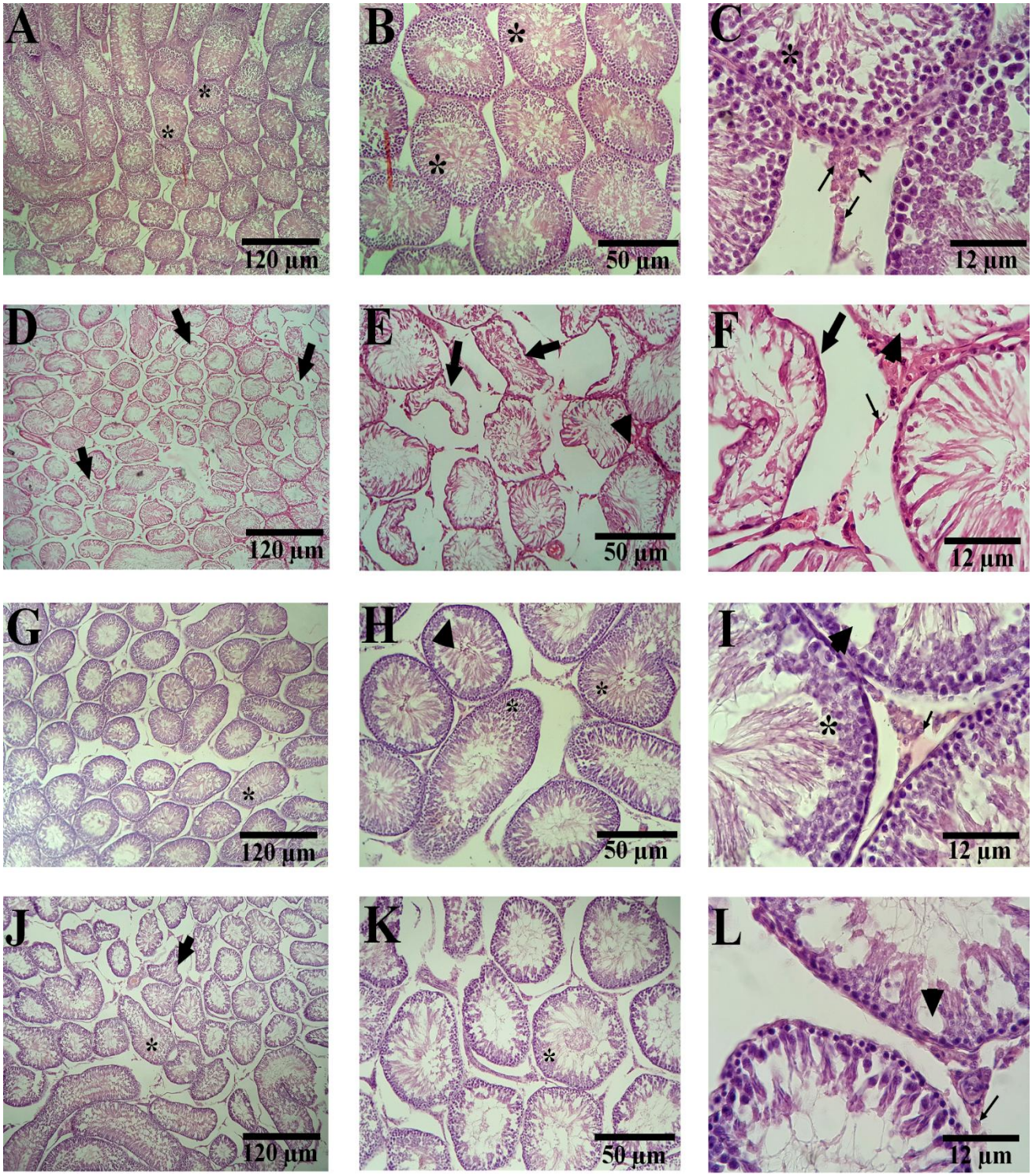

Fig. 4. H&E-stained photomicrograph of the histopathological alterations in testicular tissue, showing the following groups: (A, B, and C) control, (D, E, and F) STZ, (G, H, and I) STZ + Met, and (J, K, and L) STZ + stevia. A portion of the seminiferous tubule with many layers of germinal epithelium is shown in a control rat testicular slice (star). According to Johnson's scoring system, this group scored higher than 9. A photomicrograph of a diabetic group's testicular section reveals tubule atrophy and shrinkage, germinal epithelium destruction (arrows), a significant drop in the number of germ cells, and the presence of empty areas inside the tubules (arrow heads). The stevia- and MET-treated diabetic group exhibited a notable increase in germline sexual cells within the germinal epithelium (star; however, this enhancement was not as great as that of the healthy control group. Leydig cells, indicated by a thin arrow, were significantly reduced in the diabetic group, but this reduction was effectively prevented in the treated groups. Magnifications: A, D, G, and J (×40); B, E, H, and K (×100); C, F, I, and L (×400).

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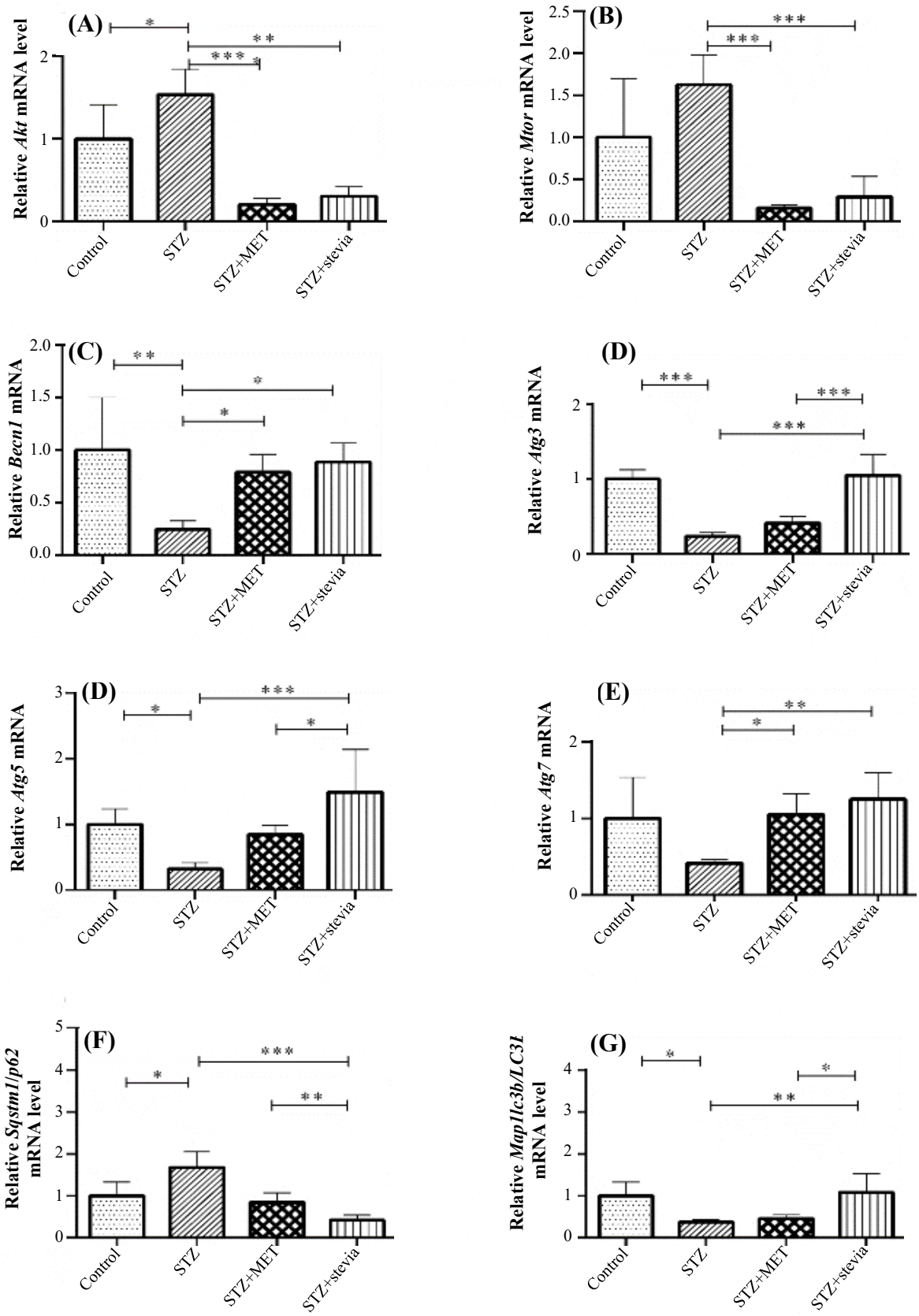

Fig. 5. Gene expression analysis of autophagy-related genes, including *Akt1*, *Mtor*, *Becn1*, *Atg3*, *Atg5*, *Atg7*, *Sqstm1/p62*, *Map1lc3b/LC3B* in experimental groups by qRT-PCR ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

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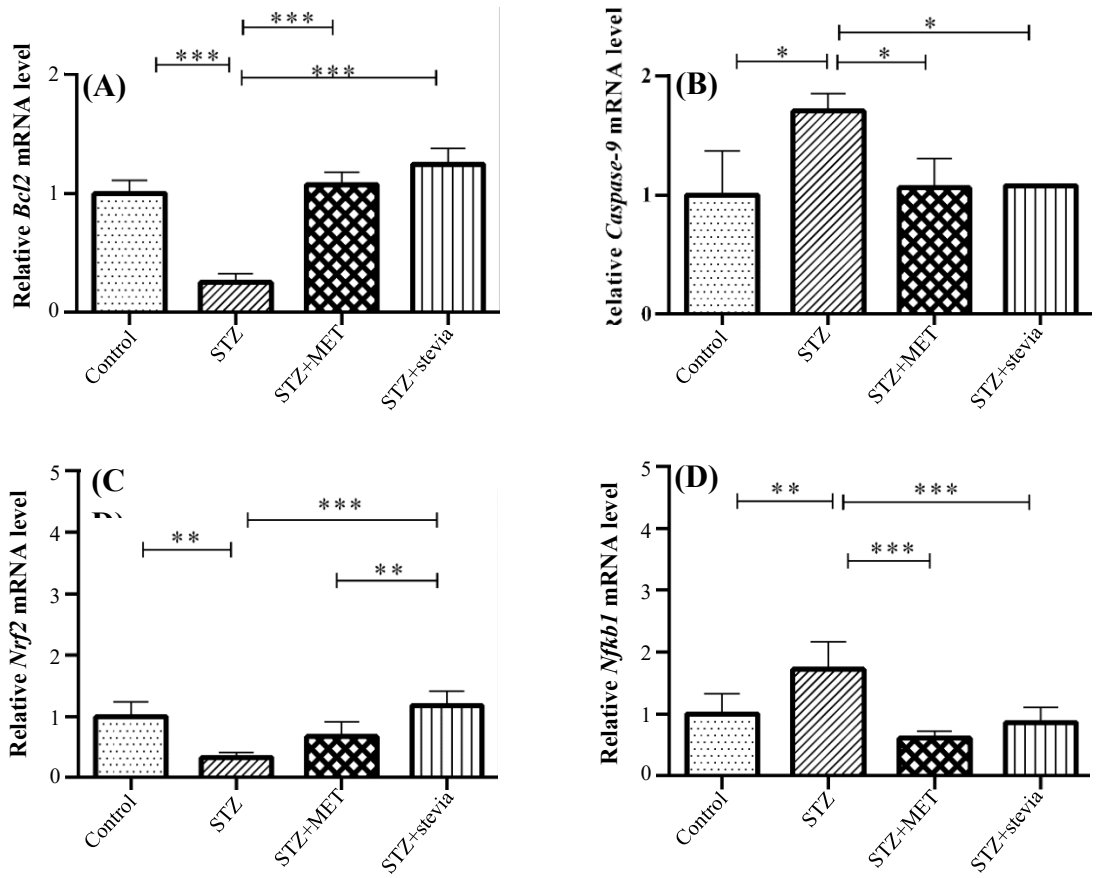

Fig. 6. Gene expression analysis of apoptosis-related genes including (A) *Bcl2* and (B) *Caspase-9* and also oxidative stress-related genes including (C) *Nrf2* and (D) *Nfkb1* in experimental groups by qRT-PCR ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

detect the effect of stevia extract on the apoptosis signaling and oxidative stress response in diabetes compared with the control. As described in Figure 6, apoptosis increased in the STZ group compared with the healthy control group, with a significant decline in expression of anti-apoptotic marker *Bcl2* and a significant increase in expression of pro-apoptotic marker *caspase-9* ($p < 0.001$ for *Bcl2* and $p < 0.05$ for *caspase-9*). On the other hand, the oxidative stress response was decreased in the STZ group compared with the healthy control group by a significant decline in oxidative stress response marker *Nrf2* and a significant increase in oxidative stress marker *Nfkb1* ($p < 0.01$ for *Nrf2* and *Nfkb1*). Both Met and stevia treatments reversed these changes in diabetic groups compared with the STZ group by increasing the expression of *Nrf2* and *Bcl2* and decreasing the expression of *Nfkb1* and *caspase-9* genes ($p < 0.05$ for caspase-9, $p < 0.001$ for *Nfkb1* and *Bcl2*, and $p < 0.001$ for *Nrf2* of the stevia-treated group). A significant enhancement of *Nrf2* expression was observed in the stevia-treated group versus the Met-treated group ($p < 0.01$), which indicated that stevia was more effective in increasing the *Nrf2* factor.

DISCUSSION

Diabetes and its complications lead to a great burden to both patients and the healthcare system. Therefore, various medications, including herbal remedies, have been proposed to decrease the complications of this disease[23]. Our study focused on *Stevia rebaudiana* (Bertoni) as one of these plant-based medications. Previous reports have revealed many useful health effects of this plant, including reduction in blood glucose, improvement in kidney function, decrease in blood pressure, and enhancement of antioxidant activity and also antitumor and anti-inflammatory effects[28].

Our research team has previously investigated the positive effects of stevia in STZ-induced diabetic rats. Our study revealed that stevia increased insulin levels in

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STZ-induced diabetic rats[30]. In addition, stevia showed anti-hyperglycemic effects by mechanisms such as antioxidant activity and a PPARγ-dependent pathway[30]. In another study by our team, Dastghaib et al. found that stevia controlled blood glucose levels and reduced oxidative stress. It also had protective effects on pancreatic islets and increased the number and volume of pancreatic β-cells in diabetic rats. Therefore, stevia was introduced as a potential antidiabetic therapeutic candidate[34]. Oudbor et al. also demonstrated that stevia extract improved the antioxidative defense system by enhancing the expression of related genes and suppressing the death-signaling pathways through the downregulation of cell death-related genes in pancreatic tissues of diabetic rats and murine pancreatic cell lines[35].

The failure of the reproductive system and infertility are the main diabetic complications. Therefore, our research team focused on this issue and demonstrated that stevia administration in STZ-induced diabetic rats improved infertility by decreasing the FBS level, increasing the luteinizing hormone level, and increasing sperm quantity and quality[29]. Similarly, Abdi et al. indicated the significant ameliorative effect of stevia on testis weight, blood testosterone levels, sperm parameters, and in vitro fertilization success in an STZ-induced mouse model of diabetes[36]. Ghaheri et al. reported stevia as a potential treatment for erectile and intromission dysfunction in STZ-induced diabetic male rats[37]. The diabetic-induced testicular injury was also ameliorated by stevia in diabetic rats[38]. In line with these findings, we found protective effects of stevia, including increasing the body weight and testosterone level, decreasing the FBS level, improving the sperm quality and quantity in STZ-induced diabetic rats. We also observed the enhancement of TAC levels and reduction of MDA levels following the treatment with stevia in STZ-induced diabetic rats, which indicates the protective effect of stevia against oxidative stress by increasing antioxidant activity. The gene expression analysis also showed the enhancement of oxidative stress response gene *Nrf2* and the decrease of oxidative stress marker *Nfkb1*, which indicate the important role of stevia in decreasing oxidative stress by improving oxidative stress response in diabetic rats.

Evidence suggest that autophagy is disrupted in pancreatic beta cells of patients with type 1 and type 2 diabetes[39,40]. Elevated apoptosis is another mechanism of beta-cell death in diabetic patients[41]. Although the beneficial effects of stevia on male diabetic infertility have been previously reported, to the best of our knowledge, there is still no study that evaluates its effect on the autophagy pathway in the testes of diabetic rats. Therefore, to better understand the exact mechanism of diabetes-induced infertility and the role of stevia in improving this problem, we focused on the autophagy signaling pathway as an oxidative stress-dependent pathway and also apoptosis. According to the available evidence**,** diabetic hyperglycemia leads to high oxidative stress[42], inflammation[43], vascular and nerve damage[44], and hormonal imbalances[45], which promote diabetic male infertility. Elevated ROS and oxidative stress are considered the initiating factor of all diabetic-mediated reproductive dysfunction[43]. In this regard, various signaling pathways are affected by enhanced oxidative stress, including ER stress, autophagy, apoptosis, angiogenesis, and inflammation[43]. Among these pathways, few data are available about autophagy changes in the testicular tissue of diabetic animal models. Li et al reported diabetic hyperglycemia as the cause of autophagy downregulation in epididymal tissues of diabetic rats, which may lead to epididymis impairment. They have found that insulin treatment attenuates epididymal injury and prevents reproductive dysfunction by improving autophagy[46]. Shi et al. indicated the protective effect of rapamycin on the testes of diabetic rats through the induction of autophagy and inhibition of ER stress and apoptosis[19]. Their study showed reduced expression of *Becn1*, *LC3* (autophagy activator genes), *Bcl-2* (apoptosis inhibitor gene) and *Nrf2* (oxidative stress-related gene) in the testis of diabetic rats, along with increased expression of C/EBP CHOP; ER-stress marker), *caspase-12*, *Bax* (apoptosis activator genes) and *Sqstm1/p62* (autophagy-related gene). They found that rapamycin, as an activator of autophagy, significantly reversed all the above alterations in the testicular tissue of diabetic rats[19]. Similarly, we observed an increase in *Akt1*, *Mtor*, *Sqstm1/p62*, and *caspase-9* and a decrease in *Atg3*, *Atg5*, *Atg7*, and *Map1lc3b/LC3B* mRNA levels, which suggest a possible reduction in autophagy and enhancement of apoptosis in STZ-induced diabetic rats. We indicated that treatment with stevia or MET reversed all the mentioned changes, similar to treatment with rapamycin, as an autophagy activator. Therefore, the autophagy induction and the apoptosis inhibition may be considered as the potential mechanisms by which both stevia and Met improve diabetes-induced infertility in STZ-induced diabetic rats.

In the current study, the evaluation of the histopathological changes and the maturity of the spermatogenesis process based on Johnson's scoring indicated negative effects of diabetes on testicular tissue and spermatogenesis. Treatment with stevia or MET improved diabetic-mediated testicular tissue damage and spermatogenesis. Therefore, it seems that, like MET, stevia treatment improved the diabetes-mediated

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testicular damage through potential mechanisms such as autophagy induction and apoptosis inhibition. Notwithstanding the emphasis of the current study on the stevia's potential effect on STZ-induced infertility in diabetic rats through the potential modulation of autophagy and apoptosis pathways, some limitations must be recognized. Future investigation is required to clarify the protective effect of stevia-derived stevioside and other active glycosides in the testis of diabetic rats. The present study employed qRT-PCR to evaluate expression levels of autophagy- and apoptotic-related genes. However, additional confirmation is advisable through evaluation of the related protein concentrations using methods such as Western blotting, which would contribute to yielding a more thorough comprehension and assist in validating the results.

CONCLUSION

The obtained data suggest that enhanced antioxidant activity, autophagy activation, and apoptosis inhibition might be among the mechanisms through which stevia helps alleviate diabetes-induced infertility in diabetic rats. Further studies will contribute to confirming these results.

DECLARATIONS

Acknowledgments

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Ethics approval

This study was first reviewed and endorsed by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran before being carried out (ethical Code: IR.SUMS.AEC.1403.004).

Consent to participate

Not applicable

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

MZ: contributed to study conception and design and to analysis and interpretation of results and wrote the manuscript. MZ received funding; FK: performed the experiments and data collection and wrote the manuscript. MZ received funding; AR: performed the experiments and data collection; contributed to analysis and interpretation of results; SD: contributed to study conception and design, to analysis and interpretation of results, and wrote the manuscript. MZ received funding.

Data availability

The raw data supporting the conclusions of this article are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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