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Original article

# Ellagic acid improved diabetes mellitus-induced testicular damage and sperm abnormalities by activation of Nrf2

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ABSTRACT

Diabetes mellitus induces testicular damage, increases sperm abnormalities, and impairs reproductive dysfunction due to induction of endocrine disturbance and testicular oxidative stress. This study evaluated the reproductive protective effect of ellagic acid (EA) against testicular damage and abnormalities in sperm parameters in Streptozotocin (STZ)-induced diabetic rats (T1DM) and examined some possible mechanisms of protection. Adult male rats were segregated into 5 groups (n = 12 rat/each) as control, control + EA (50 mg/kg/day), T1DM, T1DM + EA, and T1DM + EA + brusatol (an Nrf-2 inhibitor) (2 mg/twice/week). All treatments were conducted for 12 weeks, daily. EA preserved the structure of the seminiferous tubules, prevented the reduction in sperm count, motility, and viability, reduced sperm abnormalities, and downregulated testicular levels of cleaved caspase-3 and Bax in diabetic rats. In the control and diabetic rats, EA significantly increased the circulatory levels of testosterone, reduced serum levels of FSH and LH, and upregulated Bcl-2 and all steroidogenic genes (StAr, 3β-HSD1, and 11β-HSD1). Besides, it reduced levels of ROS and MDA but increased levels of GSH and MnSOD and the transactivation of Nrf2. All these biochemical alterations induced by EA were associated with increased activity and nuclear accumulation of Nrf2. However, all these effects afforded by EA were weakened in the presence of brusatol. In conclusion, EA could be an effective therapy to alleviated DM-induced reproductive toxicity and dysfunction in rats by a potent antioxidant potential mediated by the upregulation of Nrf2.


## 1. Introduction

Diabetes mellitus (DM) is classified into type 1 (T1DM) and type 2 (DM) that result from lack of insulin and/or reduced peripheral insulin sensitivity, respectively (Zaccardi et al., 2016). DM is associated with structural and functional abnormalities in several organs, which ends up with cardiomyopathy, retinopathy, neuropathy, and nephropathy (Cade, 2008). Besides, it is currently well-established that both types of DM lead to testicular damage and male's infertility/subfertility by acting through pre-testicular testicular, and post-testicular mechanisms including altering the hypothalamic-pituitary-gonadal axis hormone (HPGA), inducing testicular damage testis, and altering sperm count and quality (Rehman et al., 2001; Amaral et al., 2008; Condorelli et al., 2018).

Indeed, altered circulatory and testicular levels of all sex hormones, including testosterone, luteinizing hormone (LH), and follicular stimulating hormones (FSH) were observed in diabetic individuals and experimental animals (Chen et al., 2016; Nna et al., 2019). Besides, DM impairs spermatogenesis, reduces sperm count, viability, and motility, and induces apoptosis in both the somatic and germ cells (Oksanen, 1975; Guneli et al., 2008; Rashid and Sil, 2015; Long et al., 2018; Nna et al., 2019; Shoorei et al., 2019). Also, DM can progressively affect penile erectile function (De Young et al., 2004). Yet, hyperglycemia-induced reactive oxygen species (ROS), form mitochondria and non-mitochondrial resources, as well as the concomitant reduction in antioxidant cellular defense systems, in the testes, sperms, and epididymis, is the

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most accepted theory to explain DM-induced male infertility (Amaral et al., 2008; Long et al., 2015; Condorelli et al., 2018; Shoorei et al., 2019). Further explanation of these mechanisms is demonstrated in excellent studies and reviews (Ahmed, 2005; Yu et al., 2006; Condorelli et al., 2018).

On the other hand, the transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is the master transcription factor responsible for the synthesis of phase II antioxidant enzymes including heme oxygenase-1 (HO-1), catalase, NAD(P)H-quinone oxidoreductase (NQO-1), glutamate-cysteine ligase (GCL), and manganese superoxide dismutase (MnSOD), through the antioxidant response element-dependent pathway (Ma, 2013). Nrf2 is widely expressed in the testes and the sperms of mammals and plays crucial roles in preventing oxidative damage and maintaining normal spermatogenesis (Nakamura et al., 2010; Chen et al., 2012; Pan et al., 2017). Levels of Nrf2, as well as glutathione (GSH) and other antioxidant enzymes (i.e. SOD and catalase), are significantly diminished in the testes, epididymis, and sperms of the diabetic rats and were correlated with impaired reproductive function, testicular damage, and reduced sperm count (Wang et al., 2014; Atta et al., 2017; Zha et al., 2018; Shoorei et al., 2019; Abdel-Aziz et al., 2020). However, activation of Nrf2 or increasing cell antioxidant levels, effectively, prevented testicular damage and preserved sperm parameters in diabetic rats (Omu et al., 2014; Heeba and Hamza, 2015; Maremanda et al., 2016, Pan et al., 2017; Mirzaei Khorramabadi et al., 2019; Laleethambika et al., 2019; Sahu et al., 2020).

Nevertheless, several plant phytochemicals were reported to protect against DM-induced testicular damage and reproductive dysfunction with many thanks to their potential to improve the gonadal antioxidant stores and restore the impaired endocrine disturbance (Oliveira et al., 2016; Atta et al., 2017; Zha et al., 2018). Ellagic acid (EA) a common polyphenol derived from the metabolism of gallic acid, which is abundantly found in pomegranates, and berries (González-Sarrías et al., 2015). During the last decades, extensive research has been conducted to reveal the health beneficial effects of EA, and several studies have confirmed its ability to treat several chronic disorders including cancers, DM, neurodegeneration, and cardiovascular disorders (CVDs) by exerting antioxidant properties (Ahmed et al., 2016). Also, EA prevented testicular damage, restored sperm count, preserved sperm motility and morphology, and attenuated the alteration in sex hormones in several animal models of reproductive toxicity including those induced by monosodium glutamate, arsenic, tobacco smoke, polychlorinated biphenyl, and sodium valproate (Türk et al., 2008; Girish et al., 2014; Kaya et al., 2017; Mehrzadi et al., 2018; Hamza and Al-Baqami, 2019). EA also prevented erectile dysfunction in diabetic rats (Goswami et al., 2014). All these reproductive protective effects were attributed to the ability of EA to stimulate testicular antioxidant levels including glutathione (GSH), SOD, and catalase.

Despite these findings, the protective effect of EA against DM-induced testicular damage and alterations in sperm parameters were never tested before in diabetic rats of either T1DM or T2DM. Therefore, this study was conducted to evaluate the effect of chronic administration of EA, at a previously reported therapeutic dose (50 mg/kg) against STZ-induced testicular damage and abnormalities in sperm parameters in a T1DM rat model, established by a single administration of streptozotocin (STZ). Besides, we have examined the possible mechanisms of protection effect by focusing on the effect of EA on the endocrine function (levels of FSH and LH), steroidogenesis-related enzymes, markers of oxidative stress, and intrinsic (mitochondria-mediated) apoptosis, levels of endogenous antioxidants. In addition, using brusatol, an Nrf-2 inhibitor, we have evaluated if this protection is mediated by the transactivation of Nrf2.

## 2. Materials and methods

### 2.1. Animals

Adult male Wistar rats were supplied form from the Experimental Animal Care Center at King Saud University, Riyadh, Saudi Arabia. Always, the rats were had free access to their diet and drinking water and were kept under controlled stable ambient conditions (22 ± 2 °C/12-h light/dark cycle). All procedures, protocols, treatments, sampling, and euthanasia were approved by the Official Review Board at Princess Nourah University, Riyadh, KSA (IRB Number 20-0096), Riyadh, KSA.

### 2.2. Induction of DM

T1DM was induced to rats using an i.p. single bolus of STZ (65 mg/kg) dissolved in 0.5 M citrate solution (cat Ab142155, Abcam, UK) as described by others (Wang-Fischer and Garyantes, 2018). To prevent the death from adverse sudden hypoglycemia, all rats were orally supplemented with 0.5% glucose. Three days later, plasma glucose levels were measured, and rats having levels higher than 340 mg/dl were considered having T1DM and were included in this study.

### 2.3. Experimental design

The rats (130 ± 10 g) were classified into 5 groups (n = 12/group) as **1) control rats**: received only 0.1 M NaOH (vehicle) orally and daily; **2) EA-treated rats**: control rats which were only treated with EA dissolved in 0.01 M NaOH at a final dose of 50 mg/kg, orally and daily (cat E2250, Sigma Aldrich, St Louis, MO, USA); **3) T1DM rats:** with pre-established T1DM and received a daily oral dose of 0.1 M NaOH; **4) T1DM + EA rats:** diabetic rats which were orally and daily treated with EA (50 mg/kg); **5) T1DM + EA + brusatol-treated rats**, were diabetic rats which were orally administered EA (50 mg/kg) and i.p. injected with brusatol, an Nrf2 inhibitor (Cat. No. SML1868, Sigma Aldrich, MO, USA), dissolved in 0.1% DMSO and diluted in normal saline at a final dose of 2 mg/kg/once every 3 days. All treatments were given for 12 weeks on their regimen schedule. All oral treatments were conducting by gavage using a feeding cannula. In our preliminary data, the administration of 0.1% DMSO did not affect testes structure and semen parameter in control rats, thus this group was not included. Food intake and body weights were recorded twice per week. During this protocol, we had 3 deaths in the T1DM and 1 death in DM + EA-treated rats, only.

### 2.4. Selection of doses

Both the dose and treatment regimen of EA were adopted from the study of Goswami et al. (2014) who have shown to prevent erectile dysfunction in diabetic rats. A similar dose of EA was used in rats to prevent reproductive toxicity induced by in sodium valproate (Girish et al., 2014). A simailr dose of EA also prevented DM-induced neurodegeneration in rats (Farbood et al., 2019). The dose and route of administration of brusatol is used by Huang et al. (2017), in vivo.

### 2.5. Plasma collection

All rats were fasted overnight by the end of the last day of the experiment and then anesthetized (1.9 mg/kg ketamine/xylazine hydrochloride solution). Blood samples were directly collected by cardiac puncture protocol (2 ml) and drained into plain or

EDTA-tubes, centrifuged (1300*g*/10 min) to isolate the serum or plasma. These were further stored at −20 °C until analysis.

### 2.6. Testis and epididymis collection

The rats were ethically authenticated by cervical dislocation and their testes and the right epididymis were directly removed on ice and weighted. The right epididymis was directly minced by a needle in normal saline (5 ml/37 °C), kept for 5 min, and then was used freshly for semen analysis as shown below. However, the right testes of some rats (n = 5/group) were placed in buffered formalin and forwarded to the histology laboratory at the College of Science at KSU for routine processing and staining. All other testes from all groups were cut into smaller pieces and kept at −80 °C for further use.

### 2.7. Tissues processing

To prepare total cell homogenates for the biochemical analysis or western blotting, parts of each frozen testis (100 mg) were homogenized in 400 μl of either PBS (pH = 7.4) or radioimmunoassay buffer (RIPA) (cat 89900, TherfoFisher), respectively, in the presence of protease inhibitor (cat 78430, ThermoFisher Scientific). In both cases, the supernatants containing the proteins were isolated after being centrifuged (11,200*g*/10 min/4 °C). To prepare the nuclear fractions from the other frozen testicular tissues, parts of testes (50 mg) were prepared using a commercially available kit (Cat K266, BioVison, CA, USA) as per the manufacturer's instructions. In all cases, protein levels in the prepared samples were measured using an assay kit (cat 5000002, BioRad, USA). All supernatants and fractions were stored at −80 °C until the time of use. These procedures were performed for n = 6 samples/group.

### 2.8. Semen analysis

This has been done following previously established methods (Eleawa et al., 2014). A drop of the minced epididymis was fixed with 10% formalin/PBS (pH 7.4) solution and placed on a hemocytometer. The sperm count was performed under a light microscope. Another drop was placed on a glass slide and sperm motility was calculated under a light microscope. For morphological assessment, an aliquot of the sperm suspension was mixed with a similar volume of 1% eosin and investigated under the 200 x. The abnormalities considered were the headless, tailless, curved head, and coiled tail sperms. To calculate viability, a drop of the sperm aliquot was mixed was 2 drops of eosin (1%) for 30 sec and then incubated with 3 drops of nigrosin (10%). The number of dead sperms that normally stained pink was counted per the observed field. All observations were performed under a magnification of 200x. Sperm motility and viability, and abnormalities were presented as percentages. Data were calculated for n = 9/group.

### 2.9. Measurements in the plasma and serum

Levels of plasma glucose and insulin, as well as serum levels of testosterone, FSH, and LH, were measured using special rats' ELISA kits (cat MBS7233226, cat MBS724709, cat MBS727352, cat MBS2021901, MBS2509833, respectively (MyBioSource, CA, USA). These measurements were done for n = 6 samples/group.

### 2.10. Biochemical measurements in the total cell homogenates

Testicular levels of ROS were determined using an assay kit (cat STA-347; Cell Biolabs, CA, USA). Testicular levels of glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA) were measured using special rat's ELISA Kits (cat MBS265966, cat MBS738685, and cat MBS268427 respectively (MyBioSource, CA, USA). (No. KA3736, Abnova, Taiwan). The activities of Nrf2 (binding to ARE) in the nuclear fraction of the testicular tissues of all groups of rats were measured using a special (Cat 50296, TransAM, Active Motif, Tokyo, Japan). All parameters were analyzed for n-=6/group and measurments were done per the manufactures' instructions.

### 2.11. Western blotting

Nuclear and total cell protein were prepared in 0.5 ml of the loading buffer to a final concentration of 2 μg/μl. All samples were then boiled for 5 min and separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a final concentration of 40 μg/well. All gels were then transferred on nitrocellulose membranes, washed, and blocked by 5% of skimmed milk for 1 h. Then the membranes were washed again and incubated with the mouse monoclonal antibodies primary antibodies against Nrf2, Bcl2, cleaved caspase-3, Bax, StAR, 17β-hydroxysteroid dehydrogenases (17β-HSD3), 3β-HSD1, lamin A (nuclear marker), and β-actin (total cell marker) (all from Santa Cruz Biotechnology). After washing, the membranes were then incubated with the 2nd horse radish-peroxidase (HRP-conjugated antibody. Incubations with the primary or the 2nd antibodies were done at room temperature for 2 h with rotation. Washing between the steps was done 3 times each of 10 min with 1X TBST (Tris-buffered saline-tween 20) buffer. Bands of the interactions between the primary and 2nd antibodies on each blot were developed using a 5 min incubation with ECL pierce west kit substrate reagents (cat 32109, ThermoFisher) and were captured using the C-Di Git blot scanned and associated software (LI-COR, USA). Nuclear expression of Nrf2 was normalized against lamin A whereas all other proteins were normalized against β-actin.

### 2.12. Light microscope

Freshly collected tested were placed in 10% buffered formalin. After 24 h, all samples were dehydrated in ascending concentration of alcohol (70–100%). All tissues were then cleared in xylene, embedded in paraffin, and cut by the microtome at a thickness of 3 μm. Then, all the slides were routinely stained with hematoxylin and eosin (H&E) and examined under a light microscope (Model Nikon eclipse E200, Japan) by a blind observer.

### 2.13. Statistical analysis

Version 8 of the GraphPad Prism statistical software package (Australia) was used to perform the statistical analysis in this study using the one-way ANOVA test. Tukey's *t*-test was used in all analyses as a post hoc. Data were considered significantly varied at $p < 0.05$. All data were presented as mean ± standard deviation (SD).

## 3. Results

### 3.1. Effect of EA on the body, testes, and epididymis weights

In this study, we had 3 deaths in the 12 used STZ-induced T1DM rats and one death in the T1DM + EA-treated rats (data not shown). No death was observed in all other groups. No significant variation was observed in the final body, both testes, and right epididymis weights between the control and control + EA-treated rats **(Table)**. However, a significant reduction in the final body, testes, and epididymis weights was observed in T1DM-induced rats as compared to control rats (Table 1). On the contrary, a significant increase in

**Table 1**
Final Body, testis and epididymis weights in all groups of rats.

| Parameter | Control | Control + EA | T1DM | T1DM + EA | T1DM + EA + brusatol |
|---|---|---|---|---|---|
| Body weights (g) | 497 ± 22.1 | 489 ± 25.7 | 343 ± 13.2 [ab] | 475 ± 31.1[c] | 331 ± 15.4 [abd] |
| Testes weight (g) | 3.76 ± 0.42 | 3.94 ± 0.63 | 2.98 ± 0.38 [ab] | 3.84 ± 0.54[c] | 3.01 ± 0.49 [abd] |
| Right epididymis weight (mg) | 0.83 ± 0.14 | 0.93 ± 0.29 | 0.43 ± 0.12 [ab] | 0.71 ± 0.16[c] | 0.47 ± 0.14 [abd] |
| Testes index (%) | 0.75 ± 0.11 | 0.83 ± 0.21 | 0.82 ± 0.24 | 0.81 ± 0.14 | 0.83 ± 0.13 |
| Epididymis index (%) | 0.167 ± 0.05 | 0.18 ± 0.04 | 0.12 ± 0.02[ab] | 0.15 ± 0.02[c] | 0.11 ± 0.02 [abd] |

Data are presented as mean ± SD of n = 9 rats/groups. Values were considered significantly different at $p < 0.05$. [(a)]: versus control rats; [(b)]: versus control + ellagic acid (EA) treated rats; [(c)]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [(d)]: versus T1DM + EA-treated rats. **Brusatol:** is a selective Nrf2 inhibitor.

rats' final body, testes, and epididymis weights was observed in the T1DM + EA-treated rats as compared to T1DM rats (Table 1). No significant variation in the body, testes, epididymis weights were observed between the T1DM and T1DM + EA + brusatol-treated rats (Table 1).

### 3.2. Effect of EA on plasma glucose and insulin levels and serum levels of sex hormones

Insulin levels were not significantly changed but glucose levels were significantly reduced in the sera of the control + EA-treated rats as compared to control rats (Table 2). However, testosterone levels were significantly increased and levels of FSH and LH were significantly reduced in sera of control + EA-treated rats as compared to control rats (Table 2). Plasma insulin levels were significantly reduced and plasma glucose levels were significantly increased in T1DM-induced rats as compared to control rats (Table 2). Concomitantly, serum testosterone levels were significantly decreased but serum levels of FSH and LH were significantly increased in T1DM-induced rats as compared to control rats. A significant increase in plasma insulin levels and serum testosterone levels with a significant reduction in plasma glucose and serum levels of LH and FSH were observed in T1DM + EA-treated rats as compared to T1DM-induced rats (Table 2). Besides, no significant variation in the plasma or serum levels of all these markers was noticed when a comparison was made between T1DM-induced rats and T1DM + EA + brusatol-treated rats (Table 2).

### 3.3. Effect of EA on sperm parameters

Administration of EA to control rats (control + EA) only and significantly increased sperm count without affecting all other sperm parameters including motility, viability, and morphology (Table 3). A significant reduction in sperm count, motility, and viability with an increased number of tailless, headless, coiled neck, and coiled tail sperms were observed in T1DM-induced rats as compared to control rats (Table 3). All these parameters were significantly reversed in T1DM + EA-treated rats but didn't significantly alter in T1DM + EA + brusatol-treated rats as compared to T1DM-induced rats (Table 2).

### 3.4. Effect of EA on testicular morphology

Testicular morphological images of both the control and control + EA-treated rats showed normal testicular features that are characterized by intact seminiferous tubule structure and Leydig cells (Fig. 1**A&B**). All spermatogenic cells including the spermatogonia, primary spermatocytes, and early spermatids were well-preserved and abundantly seen in the testes of both groups and the lumens of their seminiferous tubules were filled with mature sperms (Fig. 1**A&B**). On the other hand, testis of the T1DM rats showed severe edema around the seminiferous tubules. Loss of the epithelium surrounding the seminiferous tubules, and severe vacuolization within seminiferous tubes (Fig. 1**C**). There was an obvious decrease in the number of all spermatogenic cells, as well as the accumulation of mature sperms in the lumen of the seminiferous tubules (Fig. 1**C**). Besides, the spermatogonia look abnormally large and deeply stained (Fig. 1**C**). However, almost normal seminiferous tubules structure with intact spermatogenic cells and no edema were observed in the testes of T1DM + EA-treated rats (Fig. 1**D**). However, some vacuolization, loss of spermatocytes, and some damage in the epithelium surrounding the seminiferous tubules remained seen in the testes of T1DM + EA-treated rats (Fig. 1**E**). Similar pathological changes like those observed in T1DM-induced rats have also been observed in the testes of T1DM + EA + brusatol-treated rats (Fig. 1**F**).

### 3.5. Effect of EA on testicular markers of oxidative stress

Levels of ROS and MDA were significantly but total levels of GSH and MnSOD, as well as the nuclear activity and protein levels of Nrf2, were significantly reduced in the testis of T1DM-induced rats as compared to control rats (Fig. 2**A-D,** Fig. 3**A&B**). On the opposite, levels of ROS and MDA were significantly reduced but total levels of GSH and MnSOD, as well as nuclear protein level and activity of Nrf2, were significantly increased in the testes of both the control + EA and T1DM + EA-treated rats as compared to control and T1DM-induced rats, respectively treated rats as compared to control rats (Fig. 2**A-D,** Fig. 3**A&B**). No significant variations in the testicular levels of all these biochemical endpoints were observed when T1DM + EA + brusatol-treated rats were compared to T1DM-induced rats (Fig. 3**A-D,** Fig. 4**A&B**).

**Table 2**
Serum circulatory sex hormone levels, as well plasma glucose and insulin levels in all groups of rats.

| Hormones | Control | Control + EA | T1DM | T1DM + EA | T1DM + EA + brusatol |
|---|---|---|---|---|---|
| Testosterone (ng/ml) | 7.4 ± 2.1 | 11.3 ± 2.6 [a] | 3.2 ± 0.83[ab] | 6.8 ± 1.7[c] | 3.7 ± 1.1 [abd] |
| FSH (ng/ml) | 193 ± 11.5 | 163 ± 12.2[a] | 293 ± 15.8[ab] | 201 ± 13.1[c] | 311 ± 22.1[abd] |
| LH (U/ml) | 2.4 ± 0.41 | 1.43 ± 0.46[a] | 4.4 ± 0.42 [ab] | 1.65 ± 0.43[c] | 4.7 ± 0.73 [abd] |
| Plasma glucose (mg/dl) | 118 ± 11.4 | 93 ± 7.6 [a] | 336 ± 15.4 [ab] | 152 ± 9.4[abc] | 352 ± 26.5[abd] |
| Plasma insulin(ng/ml) | 6.4 ± 1.2 | 6.9 ± 4.5 | 2.5 ± 0.38 | 4.6 ± 0.83 | 2.1 ± 0.43 |

Data are presented as mean ± SD of n = 12 rats/groups. Values were considered significantly different at $p < 0.05$. [(a)]: versus control rats; [(b)]: versus control + ellagic acid (EA) treated rats; [(c)]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [(d)]: versus T1DM + EA-treated rats. **Brusatol:** is a selective Nrf2 inhibitor.

**Table 3**
Semen analysis in all groups of rats.

| Parameter | Control | Control + EA | T1DM | T1DM + EA | T1DM + EA + brusatol |
|---|---|---|---|---|---|
| Epididymis Sperm count (million) | 24.3 ± 1.6 | 29.5 ± 2.1[a] | 11.3 ± 1.2[ab] | 23.4 ± 1.4[bc] | 12.5 ± 1.5[abd] |
| Epididymis sperm viability (% of total count) | 89.3 ± 7.6 | 91.3 ± 8.3 | 42.4 ± 8.6[ab] | 84.5 ± 7.3[c] | 44.7 ± 4.5[abd] |
| Epididymis sperm motility (% of total count) | 71.4 ± 8.1 | 73.2 ± 7.4 | 39.5 ± 4.8[ab] | 57.5 ± 5.7[c] | 36.5 ± 5.8[abd] |
| Sperm morphology | | | | | |
| Headless sperm (% of total count) | ± 0.15 | 1.2 ± 0.25 | 4.6 ± 0.42[ab] | 1.5 ± 0.11[abc] | 3.9 ± 0.64[abd] |
| Tailless sperm (% of total count) | 3.4 ± 0.47 | 3.7 ± 4.2 | 15.2 ± 3.2[ab] | 5.4 ± 1.2[abc] | 13.5 ± 2.8[abd] |
| Coiled neck (% of total count) | 3.7 ± 0.68 | 3.3 ± 0.71 | 14.8 ± 2.9[ab] | 5.6 ± 1.1[abc] | 13.9 ± 3.7 [abd] |
| Coiled tail (% of total count) | 2.6 ± 0.58 | 2.1 ± 0.53 | 13.8 ± 2.7 [ab] | 3.2 ± 0.68 [abc] | 11.9 ± 1.9 [abd] |

Data are presented as mean ± SD of n = 9 rats/groups. Values were considered significantly different at $p < 0.05$. [(a)]: versus control rats; [(b)]: versus control + ellagic acid (EA) treated rats; [(c)]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [(d)]: versus T1DM + EA-treated rats. **Brusatol:** is a selective Nrf2 inhibitor.

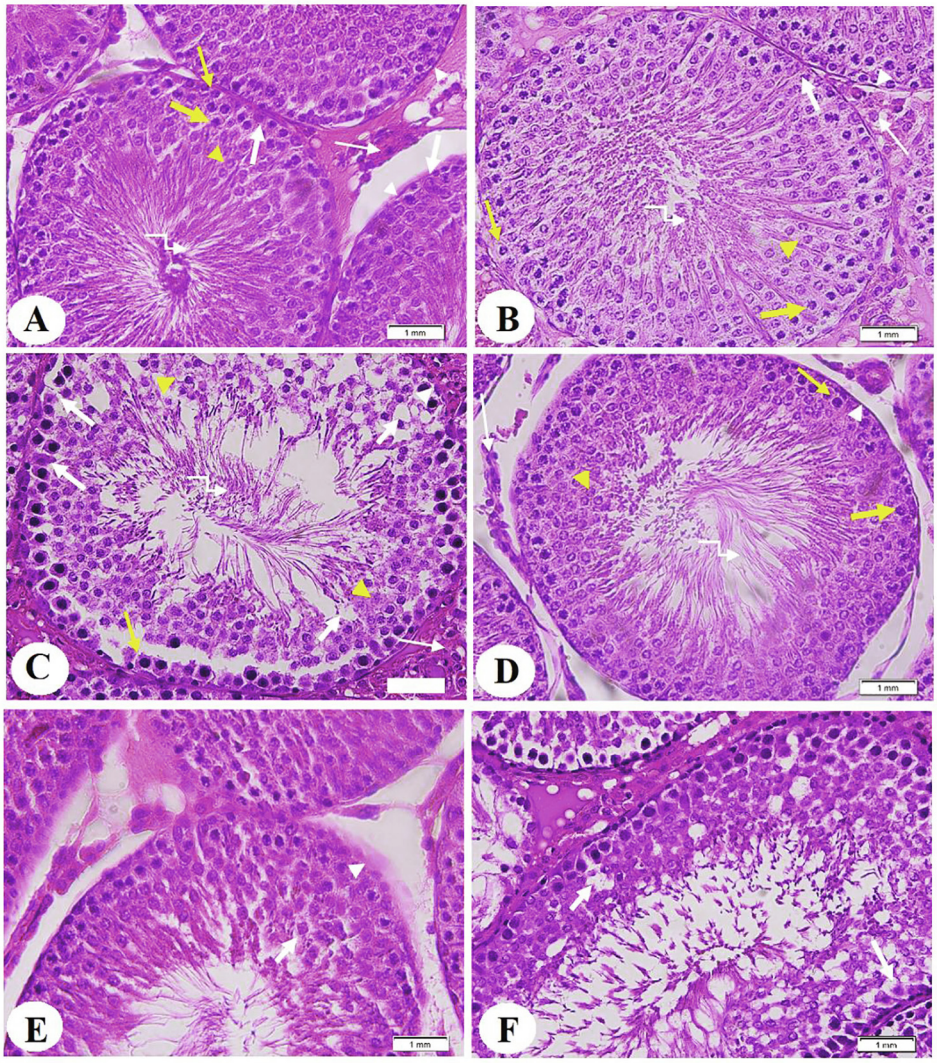


**Fig. 1. Histological pictures of the samples testis from all groups of rats.** **A and B:** were taken from control and control + EA-treated rats and showed normal testicular architectures. Both testicular tissues showed normal circular seminiferous tubules with regular contour and intact spermatogonia (thin long yellow arrow), primary spermatocytes (thick yellow arrow), round early spermatid (yellow arrowhead, and mature sperm (curved white arrow). The Sertoli cells appear normal (Thick white arrow) and interstitial looked intact and contains delicate loose C. T and Leydig cells (long thin arrow). Note the intact epithelium surrounding the seminiferous tubes (white arrowhead). **C:** was taken from a T1DM-induced rat and showed severe edema (thin white arrow) and severe damage in the rats' seminiferous tubules. These include damaged epithelium (white arrowhead) and Sertoli cells (white thick arrow). The spermatogonia seemed enlarged and deeply stained (thin yellow arrow) with an obvious and abnormal shape of the early spermatid. Also, increased vacuolization and spaces between the spermatogenic cells were evidenced. **D and E:** was taken form a T1DM + EA-treated rats and showed much improvement in the structure of the seminiferous Sertoli cells, and all spermatogenic cells. However, some damage in the epithelium (white arrowhead) and little vacuolization between the early spermatid (white arrow) were still observed. **F:** was taken from T1DM + EA + brusatol (an Nrf2 inhibitor)-treated rats and showed similar pathological changes to those observed in the T1DM-treated rats.

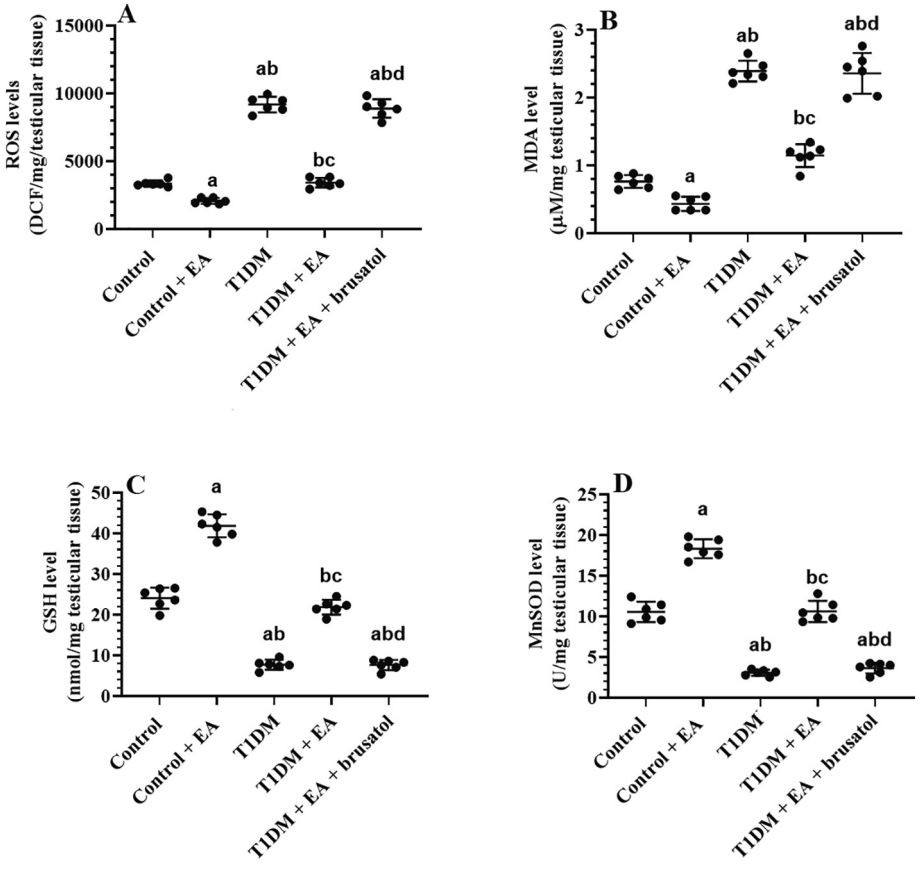


**Fig. 2. Markers of oxidative stress levels in the testes of all groups of rats.** Data are presented as mean ± SD of n = 6 rats/groups. Values were considered significantly different at $p < 0.05$. [(a)]: versus control rats; [(b)]: versus control + ellagic acid (EA) treated rats; [(c)]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [(d)]: versus T1DM + EA-treated rats. **Brusatol:** is a selective Nrf2 inhibitor.

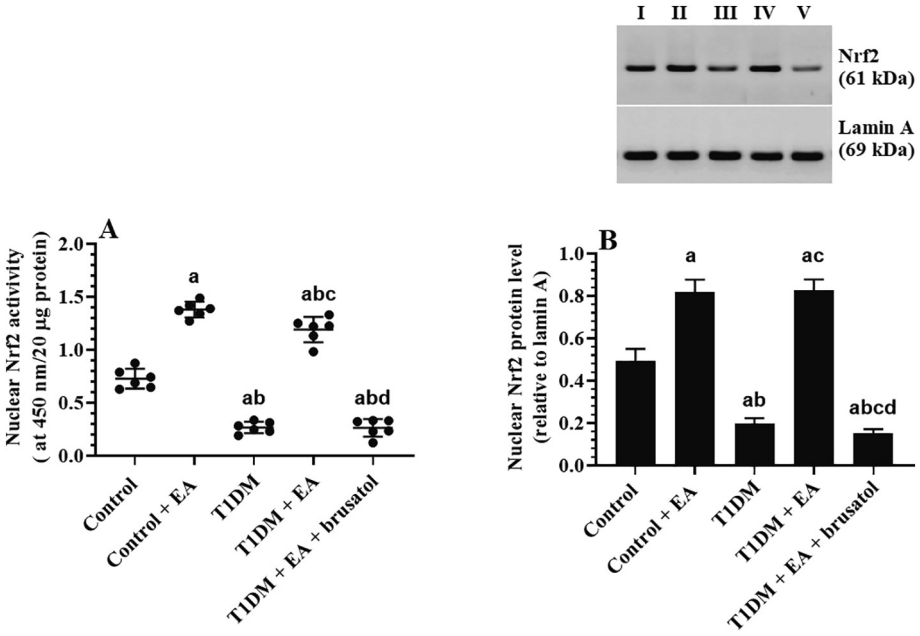


**Fig. 3. Activity and protein levels of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in the testes of all groups of rats.** Data are presented as mean ± SD of n = 6 rats/groups. Values were considered significantly different at $p < 0.05$. [(a)]: versus control rats; [(b)]: versus control + ellagic acid (EA) treated rats; [(c)]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [(d)]: versus T1DM + EA-treated rats. **Brusatol:** is a selective Nrf2 inhibitor.

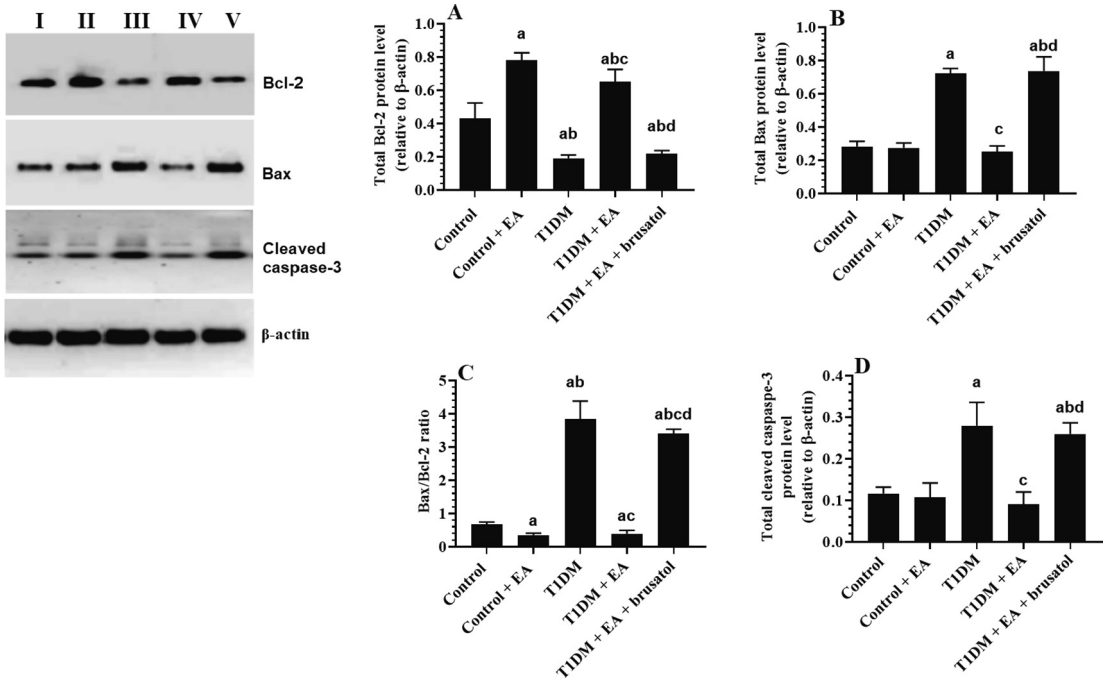


**Fig. 4. Protein levels of some markers of mitochondria-mediated (intrinsic) cell apoptosis in the testes of all groups of rats.** Data are presented as mean ± SD of n = 6 rats/groups. Values were considered significantly different at $p < 0.05$. [(a)]: versus control rats; [(b)]: versus control + ellagic acid (EA) treated rats; [(c)]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [(d)]: versus T1DM + EA-treated rats. **Brusatol**: is a selective Nrf2 inhibitor.

### *3.6. Effect of EA on testicular markers of apoptosis*

With no effect on the protein levels of Bax and cleaved caspase-3, the testes of control + EA-treated rats showed a significant increase in the protein levels of Bcl-2 and a significant reduction in Bax/Bcl-2 ratio as compared to control rats (Fig. 4**A-D)**. However, protein levels of Bcl-2 were significantly decreased but protein levels of Bax and cleaved caspase-3, as well as Bax/Bcl-2 ratio were significantly reduced in the testes of T1DM-induced rats as compared to control rats **(Figure A-D)**. On the other hand, levels of Bcl-2 were significantly increased and protein levels of Bax and cleaved caspase-3, as well the ratio of Bax/Bcl-2 was significant decreased in the testes of T1DM + EA-treated rats as compared to T1DM-induced rats (Fig. 4A-D). The protein levels of all these markers as well as the ratio of Bax/Bcl-2 were not significantly varied in the testes of T1DM-induced rats and T1DM + EA + brusatol-treated rats **(**Fig. 4**A-D).**

### *3.7. Effect of EA on the expression of some testicular steroidogenesis-related genes*

Levels of StAR, 3β-HSD1, and 11β-HSD1 were significantly reduced in the testes of T1DM-induced rats as compared to control rats **(**Fig. 5**A-C)**. However, administration of EA to both the control and T1DM-induced rats significantly upregulated the testicular protein levels of all these steroidogenic markers **(**Fig. 5**A-C)**. Of note, no significant variations in the testicular protein levels of StAR, 3β-HSD1, and 11β-HSD1 were seen when T1DM-induced rats were compared with T1DM + EA + brusatol-treated rats **(**Fig. 5**A-C)**.

## 4. Discussion

In this study, we are showing a potent protective effect of EA against testicular damage and alterations in sperm parameters in STZ-induced diabetic rats. Accordingly, chronic administration of diabetic rats for consecutive 12 weeks not only preserved testicular weights and structure in diabetic rats but also stimulated steroidogenesis, increased testosterone levels, and enhanced sperm count, and preserved sperm viability, motility, and normal morphology in both the control and diabetic rats. These effects have coincided with increased antioxidant levels and transactivation of Nrf2. Of note suppressing Nrf2 by brusatol abolished all the observed reproductive beneficial effect of EA, thus confirm that EA reproductive effect is mediated by an antioxidant potential

In the control and diabetic rats, administration of EA lowered the plasma levels of fasting glucose. However, it only stimulated fasting plasma insulin levels and stimulated HOMA-β levels, a clinically preferred marker that is correlated with the functional β-cells in diabetic rats only (Song et al., 2010). Such an increase in insulin levels in diabetic rats may be explained by increasing the release of insulin from the survival β-cells or increasing peripheral insulin sensitivity. On the other hand, the hypoglycaemic effect of EA could be attributed to the suppression of hepatic gluconeogenesis. In support, EA anti-diabetic effect was shown in all previously published studies in diabetic animals and was associated with hypoglycaemic and insulin-releasing effects (Fatima et al., 2017; Nankar and Doble, 2017; Polce et al., 2018; Farbood et al., 2019). Also, EA can control hyperglycemia in diabetic animals by several independent mechanisms including suppressing hepatic β-cells apoptosis and hepatic gluconeogenesis, as well as stimulating peripheral insulin activity (Nankar and Doble, 2017; Fatima et al., 2017; Polce et al., 2018; Farbood et al., 2019). Besides, it could be also possible that EA enhances insulin release from the survival pancreatic cells by altering the activity of the pancreatic $K^+$ channels in a similar action afforded by many sulfonylurea antidiabetic drugs (Proks et al., 2002; Sola et al., 2015). Therefore, it seems reasonable that the observed reduction in rat's body weight of the STZ- diabetic rats is due to increase peripheral tissue lipolysis due to insulin deficiency (Enoksson et al., 2003).

On the other hand, hyperglycemia induces testicular damage, impair spermatogenesis, decreases sperm count by inducing large quantities of ROS in most gonads including the sperms, epididymis,

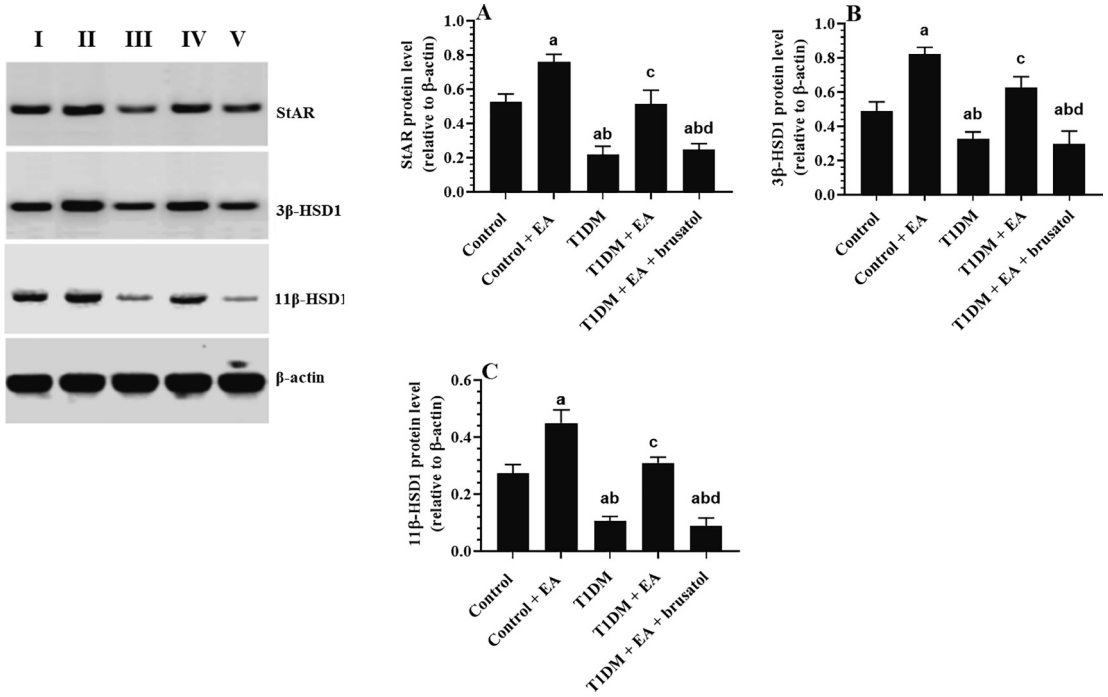


**Fig. 5. Protein levels of some steroidogenesis-related enzymes in the testes of all groups of rats.** Data are presented as mean ± SD of n = 6 rats/groups. Values were considered significantly different at $p < 0.05$. [a]: versus control rats; [b]: versus control + ellagic acid (EA) treated rats; [c]: significantly versus type 1 diabetes mellitus (T1DM)-induced rat; [d]: versus T1DM + EA-treated rats. **Brusatol:** is a selective Nrf2 inhibitor.

and testes, which leads to induction of apoptosis of both the somatic and germ cells (Zhao et al., 2011; Rashid and Sil, 2015; Condorelli et al., 2018; Nna et al., 2019; Shoorei et al., 2019). Besides, ROS generated in the sperms can stimulate lipid peroxidation, oxidizes protein, and damage the nuclear and mitochondria DNA leading to reduce sperm motility, viability, and count and increasing sperm abnormalities (Alahmar, 2019; Laleethambika et al., 2019). Of note, mitochondria-mediated cell death (intrinsic cell death) is the most common cell modality in sperms and reproductive organs of diabetic animals and is always characterized by higher expression of Bcl2 and a reduction in Bcl2 (Long et al., 2015; Rashid and Sil, 2015; Nna et al., 2019). This has been also confirmed in this study where the testes of the diabetic rats showed a higher level of ROS and MDA, higher protein levels of Bax and cleaved caspase-3, and less expression of Bcl2. Therefore, it seems logical that DM induces testicular damage, reduces testes weights, and disturbs sperm count and quality by induction of oxidative stress and apoptotic.

Besides, low levels of the cellular defense antioxidants, GSH, catalase, and SOD, were observed in the testes of the STZ-diabetic rats. These data also support many other previous studies that have shown hyperglycemia-induced testicular damage in diabetic rats by overwhelming and/or downregulation of the cellular antioxidants (Aybek et al., 2008; Laleethambika et al., 2019; Abdel-Aziz et al., 2020). On the other hand, the reduction in the expression of Nrf2 in the testicular tissues of the diabetic rats supports the observations of many authors who have shown that the inhibitory effect of hyperglycemia of the testicular levels of the antioxidant systems is due to suppression and inactivation of this transcription factors (Jiang et al., 2014; Zhao et al., 2016; Pan et al., 2017).

Antioxidant therapy showed promising results and alleviated DM-induced induced reproductive dysfunction in both experimental and clinical trials (Imamovic Kumalic and Pinter, 2014; Alahmar, 2018). In this study, the first interesting observation the ability of EA to mitigate the damage in the testes of the diabetic rats and improve all sperm parameters. These effects could be attributed to the suppressing ROS and upregulation of GSH and SOD. This is supported by the observation that EA also increased sperm count and stimulated the levels of all these antioxidant markers in the testes of the control rats also treated with this compound. Although the stimulatory effect of EA on the antioxidant markers is the first to be shown in the testes of this diabetic animal model, all the other previous studies which were held in other animal model have shown that EA prevents testicular damage and improve sperm parameter by enhancing the testicular levels of GSH, SOD, and catalase. (Girish et al., 2014; Ateşşahin et al., 2010; Kaya et al., 2017; Mehrzadi et al., 2018; Hamza and Al-Baqami, 2019). Besides, by reducing the generation of ROS, EA prevented erectile dysfunction in diabetic rats (Goswami et al., 2014). Also, EA prevented improved erection in diabetic rats by reducing the generation of ROS and the advanced glycation product (AGEs) (Goswami et al., 2014).

Next, it was of our interest to figure out the precise mechanism by which EA stimulates the cellular antioxidant potential in the testes of the control and diabetic rats. Therefore, we targeted the transactivation of Nrf2 given its role as a master transcription factor responsible for the synthesis of the cellular antioxidant enzymes (Ma, 2013), and the observed reduction in its nuclear activation in the testes of diabetic rats. Also, some older studies have shown that EA can stimulate Nrf2 in other extra-testicular tissues. Indeed, EA protected from rotenone-induced astroglia cell damage, oxidant-induced endothelial dysfunction and atherosclerosis, methotrexate-induced, hepatocytes apoptosis via upregulation and activation of Nrf2 (Ding et al., 2014; Ebrahimi et al., 2019; Wei et al., 2020). Besides, we have also shown that EA prevents DM-induced nephropathy by activation of Nrf2 (in press). As expected, EA also stimulated the nuclear activation and accumulation of Nrf2 in the testes of both the control and STZ-induced diabetic rats of this study. Therefore, we could strongly argue that the reproductive protective effect of EA is presented by brusatol, a selective Nrf2 inhibitor, which completely abolished all the

observed reproductive beneficial effects of EA on all measured testicular and sperm parameters. However, since this has been measured in the whole testicular homogenates, we still unable to determine if this mechanism occurs also in the sperms which could be a limitation of this study.

Under normal conditions, testosterone is essential for testicular cell survival, development, and spermatogenesis (Ramaswamy and Weinbauer, 2015) in the adult testes, two cell populations regulate this namely the Leydig and Sertoli cells. in this regard, FSH and LH hormones regulate spermatogenesis by acting on both cell types to testosterone and germ cell production respectively (Ramaswamy and Weinbauer, 2015). In this view, the binding of LH to its receptors on the Leydig cells stimulates the uptake of cholesterol and subsequent activation of several steroidogenic biosynthetic enzymes including StAR, 3β-HSD1, 11β-HSD1, CYP17A1; and 17β-HSD3 (Smith and Walker, 2014; Ramaswamy and Weinbauer, 2015; Wu et al., 2017).

In diabetic rats and humans, testosterone is significantly depleted (Kanter et al., 2013; Bansal et al., 2017; Rovira-Llopis et al., 2017; Condorelli et al., 2018; Zha et al., 2018; Abdel-Aziz et al., 2020). Although the mechanism by which DM suppresses testosterone production is still unclear, some authors have shown this to be due to reduced hormonal levels of FSH and LH, increase activation of aromatase, and oxidant-mediated cell apoptosis of the Leydig cells (Heeba and Hamza, 2015); Chen et al., 2016). In addition to apoptosis, such reduction in testosterone levels may participate significantly in the reduction in testes weight in diabetic rats. Besides, the protein expressions of StAR, 3β-HSD1, 11β-HSD were significantly reduced in the testes of diabetic rats (Ding et al., 2016; Reddy et al., 2016). This has been also confirmed in the diabetic rats of this study which showed a reduction in the circulatory levels of testosterone, a reduction in testicular protein levels of StAR, 3β-HSD1, 11β-HSD1, and an increase in the circulatory levels of FSH and LH. These data support many other authors who have shown that the increase in FSH and LH is a negative feedback regulation due to increase testosterone production (Nna et al., 2019). However, the effect of DM on FSH and LH is still contradictory. In this regard, the levels of FSH and LH were either decreased (Chen et al., 2016), increased (Himabindu et al., 2015), or remained not altered in diabetic animals (Guo et al., 2016).

Another interesting observation in this study is the ability of EA to stimulate testosterone synthesis which seems through upregulation of testicular steroidogenesis genes. Indeed, administration of EA to STZ-diabetic rats significantly increased circulatory levels of testosterone, FSH, and LH, and upregulated the protein levels of StAR, 3β-HSD1, 11β-HSD1.

Similar effects were also observed in the control rats of this study which were also treated with EA. Interestingly, EA failed to achieve these effects in the presence of brusatol, thus confirming that DM-suppresses steroidogenesis by an oxidative stress-related mechanism, and the protection afforded by EA is mediated by the upregulation of Nrf2. Also, these data implicate that EA stimulates testosterone synthesis by activation and upregulation of steroidogenic-related genes. However, some authors also suggested that EA stimulates testosterone synthesis and secretion by suppressing the aromatase which normally metabolizes testosterone to other androgens (Hamza and Al-Baqami, 2019). Nonetheless, it seems reasonable that such an increase in testosterone levels after EA treatment reduced the circulatory levels of FSH and LH in the serum of the control and diabetic rats, again, by a negative feedback mechanism. Supporting our data, EA also stimulated testosterone levels in the serum of control rats, as well as in rats intoxicated with monosodium glutamate, Polychlorinated Biphenyl, sodium arsenite, treated rats (Ateşşahin et al., 2010; Mehrzadi et al., 2018; (Hamza and Al-Baqami, 2019).

Overall, these data suggest that daily supplementation with EA alleviated DM-induced testicular damage and alterations in sperm parameters in rats by several mechanisms including hypoglycaemic and insulin-releasing effects, as well as suppression of oxidative stress-mediated by upregulation of Nrf2 and subsequent upregulation of antioxidant defense systems. Despite these findings, this study still has some limitations. Importantly, even EA stimulated Nrf2 and antioxidant in control rats, it is still beyond our ability to indicate if the stimulatory effect of EA on this transcription factor of Nrf2 is a direct effect or secondary to its hypoglycaemic effect. This is correct given the observed hypoglycaemic effect of EA in the control rats. Therefore, further studies using cultured cells may confirm this mechanism. Besides, it is well established that the regulation of Nrf2 is regulated by cytoplasmic and nuclear factors such as keap1 and PI3K/Akt/GS3K/Fyn pathway (Bitar, 2012). Therefore, further studies targeting the effect of EA on keap1 expression and the activity of Fyn should be further in more extended research.

In conclusion, the exclusive finding of this study supports the protective effect of EA against DM-induced testicular damage and alterations in sperm parameters in rats. Given the high tolerance and safety of EA, even at higher doses, the findings presented in this study encourage further large-scale clinical trials in infertile or sub-fertile male individuals.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Further Reading

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