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Tempol improves oxidant/antioxidant parameters in testicular tissues of diabetic rats

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ABSTRACT

Aims: Oxidative stress induced by diabetes mellitus (DM) is considered as one of the main causes of infertility in diabetic patients. The aim of the present study was to assess the effect of Tempol – as a synthetic antioxidant- on the testis oxidative stress and sperm parameters in type 2 diabetic (T2D) rats.
Main methods: Twenty male Wistar rats were divided into 4 groups. Control groups (C) and diabetic groups (D); the control and diabetic groups received Tempol (100 mg/kg) for one month. Sperm parameters and oxidative stress biomarkers were evaluated in testicular tissue.
Key findings: The results demonstrated that administration of Tempol in diabetic rats improved sperm motility and viability and decreased the count of abnormal sperms. Also Tempol decreased the fasting blood sugar (FBS) and lipid peroxidation (LPO). In addition, Tempol significantly increased total antioxidant capacity (TAC) levels in testis tissue of T2D rats. Histopathological changes were also improved in the diabetic treated group.
Significance: Taken together, the results indicated that Tempol improved fertility parameters in a diabetic rat through reducing oxidative stress.

1. Introduction

Diabetes mellitus (DM) is a common multifactorial metabolic disorder characterized by chronic hyperglycemia and defect in the metabolism of macromolecules [1]. DM is a global health challenge; based on reports from the International Diabetes Federation (IDF), about 415 million people suffered from DM worldwide in 2017, expected to rise to 645 million people by 2040 [2,3]. Etiologically, DM might result from defect in synthesize, secretion, and function of insulin. DM is usually classified as: type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes (GD) and other types [4]. The most common form of DM is the type 2 diabetes, usually affecting adults, which mainly results from systemic resistance to insulin [7]. The main feature of DM is a chronic elevated blood sugar (hyperglycemia) which in long term damages various body organs, resulting in multiple physiological complications such as nephropathy, retinopathy, and neuropathy [5].

Insulin regulates the metabolism of glucose in sperm cells through various glucose transporters (GLUTs) [7]. Not surprisingly, therefore, the reproductive system is significantly affected in male diabetic patients as a result of defect in glucose metabolism and elevated reactive oxygen species (ROS) [8,9].

Oxidative stress is also a common feature of DM pathogenesis that reflects an imbalance between production and degradation of free radicals [6]. The sperm cells membrane has a high content of unsaturated fatty acids, turning them vulnerable to oxidative damage by free radicals [10]. DM mediated oxidative stress thus affects both the quality and quantity of semen which causes infertility problems [9] [11,12].

About 45% of infertility is related to male factors, including gene mutations, infectious diseases, ejaculatory duct obstruction, testicular varicose, and radiation. However, reasons for almost half of the infertility cases in men are unknown (idiopathic) [13]. Recent studies show that oxidative stress is one of the factors that might mediate the male infertility [14]. Excessive amounts of ROS alongside a reduced antioxidant capacity in the spermatozoa and seminal plasma cause defects in the membrane and damage to the DNA of sperm cells [15–17].

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Antioxidant supplements might be helpful in empowering the antioxidant defense system against oxidative damages caused by free radicals. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a synthetic antioxidant that mimics the role of superoxide dismutase (SOD), for example, has been shown that reduces the oxidative stress mediated cell damage in several rodent models [18]. Nevertheless, its antioxidant role in many diseases associated with oxidative stress has not yet been studied. Therefore, to further evaluate the efficiency of Tempol in reducing the DM related oxidative damage, we investigated the effect of Tempol on sperm and oxidant/antioxidant parameters in testicular tissue of T2D rats.

2. Material and methods

2.1. Animals and work design

In this study, twenty male Wistar rats weighing 220 ± 10 were used. The rats were maintained in an animal house at the Hamadan University of Medical Sciences in standard conditions (in cages, in a ventilated room at 22 ± 2 °C and on a 12-h light/12-h dark cycle). The animals were randomly divided into 4 groups (n = 5), including; C: control group receiving normal saline, D: T2D group, C + T: control group receiving Tempol 100 mg/kg and D + T: T2D group receiving Tempol 100 mg/kg. The study was approved by the Medical Ethics Review Board of Hamadan University of Medical Sciences (IR.UMSHA.REC.1394.163).

For induction of T2D, streptozotocin (STZ) (65 mg/kg) and nicotinamide (110 mg/kg) were injected intraperitoneally to rats [19–21]. After 72 h, fasting blood glucose was measured using glucometer (Accuchek; Roche, Germany). Animals with fasting blood glucose equal to and > 150 mg/dl were considered as T2D rats [22]. Treatment was carried out by IP injection for 30 days. After the treatment period, fasted rats were anesthetized with ketamine, their testis tissue were removed and stored for further evaluation.

2.2. Epididymal sperm preparation

The left testis cauda epididymis of each rat was removed and minced in 2 ml of Ham's F10 medium (pre-warmed in 37 °C and 5% CO_2). The cauda epididymis was cut into small pieces to release more sperm. The sperm suspension was then incubated in 37 °C for 15 min [23]. The sperm parameters including sperm count, morphology, motility and viability were evaluated.

The sperm counting was performed using the hemocytometer. One ml of sperm suspension was diluted with 9 ml normal saline 3% to keep sperms completely immobilized for counting. Then one drop of the diluted suspension was transferred to the Neubauer slide. After 5 min, the intact sperms were counted under a light microscope according to the World Health Organization (WHO) manual. For evaluation of morphology, sperm suspension was smeared on a microscopic slide, allowed to dry, then fixed and stained. The slides were viewed under a light microscope. Abnormalities in sperm morphology, including head, tail and head-neck connection were analyzed. Sperm motility was tested under 400× magnification. Sperms with different motility characteristics including motile, immobile, hyperactive, and sperms with the progressive movement were counted in each field. To determine the percentage of viable sperm, one drop of the sperm suspension was placed on a microscopic slide, then stained with Eosin B (Sigma-Aldrich, Germany). Viable sperms had a white color while non-viable sperms appeared as purple to red. At least 100 spermatozoa were counted. Sperm viability was expressed as the percentage of live sperms. The volume of sperms was calculated by cylinder and their color was determined by ocular assessment.

2.3. Biochemical assessments

2.3.1. Lipid peroxidation (LPO) assay

Thiobarbituric acid reactive substances (TBARS) assay was used to measure lipid peroxidation which is based on the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) in the glacial acetic acid medium. Samples were first mixed with 1.5 ml tri-chloro-acetic acid (20% w/v), then centrifuged at 3000*g*. The taken pellets were re-suspended with 1.5 ml 2-thiobarbituric acid (0.2% w/v) and 1.5 ml H_2SO_4 (0.05 M), following by incubation in boiling water for 45 min. Afterwards, samples were first mixed with 2 ml n-butanol, allowed to cool, then centrifuged and their absorption was read at 532 nm. The tetraethoxypropan standard curve was used to determine the concentrations [23].

2.3.2. Total antioxidant capacity (TAC) assay

Total antioxidant capacity (TAC) was measured using the ferric reducing ability of plasma (FRAP) method which is based on the ability of biological antioxidants in reducing Fe^{3+} to Fe^{2+} in the presence of TPTZ (2,4,6 tripyridyl-s-triazine). This reaction results in a blue color complex with maximum absorbance in the 593 nm [24].

2.3.3. Total thiol groups (TTG) assay

Plasma TTG was evaluated using DTNB as a reagent. Briefly, 1 ml of tris buffer (250 mM tris, 2 mM EDTA, pH = 8) was mixed with 50 μL of each sample. By adding DTNB, the reaction with thiol molecules resulted in the formation of a yellow complex with a maximum optical absorbance at 412 nm [25].

2.3.4. Total protein assay

The protein content was quantified by the Bradford method. 750 uL diluted coomassie blue was mixed with 50 μL of samples. After 10 min incubation in room temperature, absorbance of samples was measured in 595 nm. A standard curve was created using bovine serum albumin ranging from 0.25 mg/ml to 1 mg/ml [24].

2.4. Histopathology assessments

The testis samples were fixed in neutral 10% formalin for 24 h at room temperature, then dehydrated in ethanol, cleared in xylene and finally embedded in paraffin. Slices of 4–6 μm thickness were prepared and stained with hematoxylin (5 min at room temperature) and eosin (2 min at room temperature). The sections were examined under a light microscope.

2.5. Statistical analysis

The analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago-USA). Results were expressed as mean ± SD. The statistical mean difference between groups was evaluated using one-way test followed by Tukey test. Differences were considered significant if the p value was < 0.05.

3. Results

3.1. Tempol effect on fasting blood sugar

FBS levels was measured in the first day and at the end of the treatment. At the beginning of the treatment, FBS was significantly higher in T2D and treated T2D groups compared to the healthy control group ($p < 0.001$). However, Treatment of T2D group with Tempol 100 mg/kg caused a dramatic decrease in FBS compared to diabetic group ($p < 0.001$) did not reduce FBS to the normal range ($p < 0.001$) (Fig. 1).

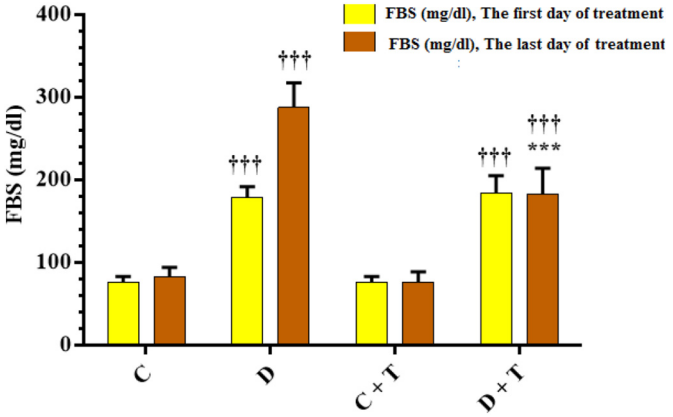

Fig. 1. Tempol effect on FBS. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg, FBS: fasting blood sugar. †Significantly different compared to untreated control group (†††$p < 0.001$). *Significantly different compared to untreated diabetic group (***$p < 0.001$).

3.2. Tempol effect on body weight

Final body weight significantly decreased in the T2D group ($p < 0.001$) compared to the normal controls. Diabetic rats treated with Tempol, however, showed significant improvement in their body weight ($p < 0.001$) (Fig. 2).

3.3. Tempol effect on sperm count

The evaluation of sperm counts indicated that the number of sperms in the T2D group was strikingly decreased compared to the control group ($p < 0.001$). Treatment of diabetic rats with Tempol significantly increased the sperm count compared to the untreated T2D group ($p < 0.05$), but did not restore the sperm count to the range of the healthy group ($p < 0.01$) (Fig. 3).

3.4. Tempol effect on sperm morphology

Morphological observation showed that spermatozoa with abnormal morphology significantly increased in T2D rats compared to the control

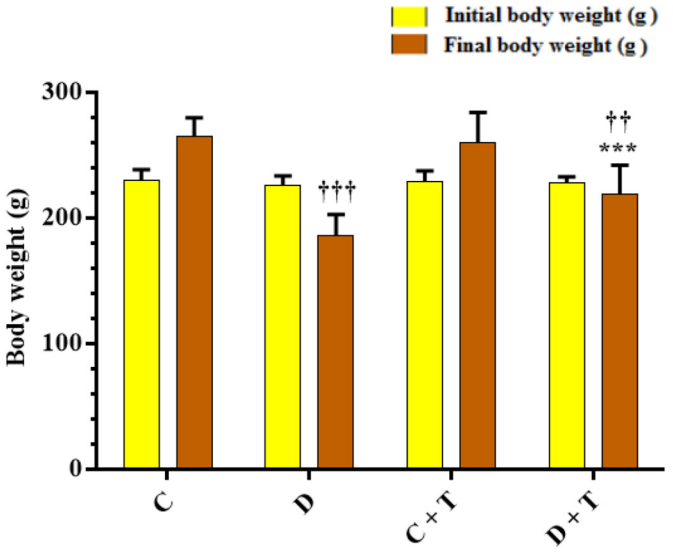

Fig. 2. Tempol effect on body weight. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg. †Significantly different compared to untreated control group (††$p < 0.01$, ††† $p < 0.001$). * Significantly different compared to untreated diabetic group (***$p < 0.001$).

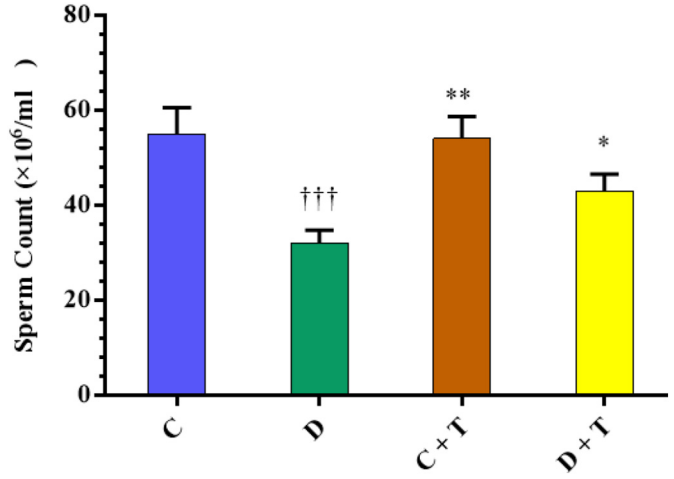

Fig. 3. Tempol effect on sperm count. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg. † Significantly different compared to untreated control group (††† $p < 0.001$). * Significantly different compared to untreated diabetic group (*$p < 0.05$, ** $p < 0.01$).

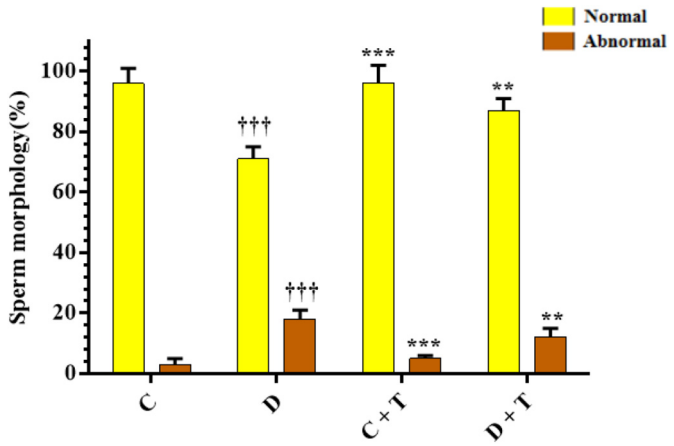

Fig. 4. Tempol effect on sperm morphology. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg. † Significantly different compared to untreated control group (†††$p < 0.001$). * Significantly different compared to untreated diabetic group (**$p < 0.01$, *** $p < 0.001$).

group ($p < 0.001$). Tempol treatment decreased the number of abnormal sperm in T2D ($p < 0.01$) and control ($p < 0.001$) rats compared to untreated T2D rats (Fig. 4).

3.5. Tempol effect on sperm motility

The results of sperm motility showed that the number of motile ($p < 0.001$), forward movement ($p < 0.001$) and hyperactive ($p < 0.01$) sperms in T2D rats significantly decreased and the number of immobile sperm ($p < 0.001$) significantly increased compared to the control group. Treatment of diabetic rats with Tempol increased the motile ($p < 0.01$), forward movement immobile ($p < 0.01$), and hyperactive sperms immobile ($p < 0.01$), but reduced the number of immobile sperm in comparison to the untreated diabetic group ($p < 0.05$) (Fig. 5).

3.6. Tempol effect on sperm viability

The viability test showed that the number of viable sperms in T2D rats far lower compared to the control group immobile ($p < 0.001$). Treating with Tempol increased viable sperm compared to the

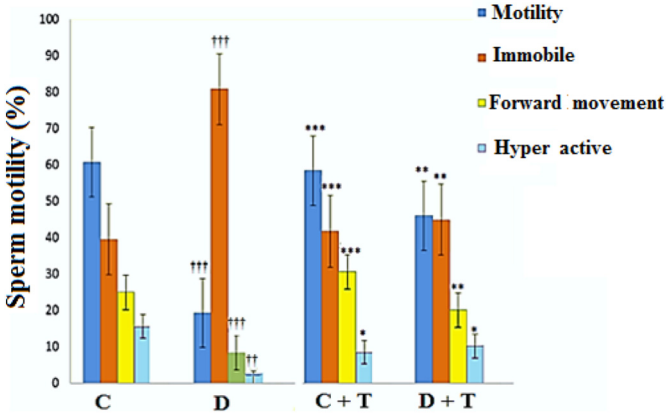

Fig. 5. Tempol effect on sperm motility. The data are presented as Mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg. † Significantly different compared to untreated control group (†††p < 0.001, ††p < 0.01). * Significantly different compared to untreated diabetic group (*p < 0.05, ** p < 0.01, *** p < 0.001).

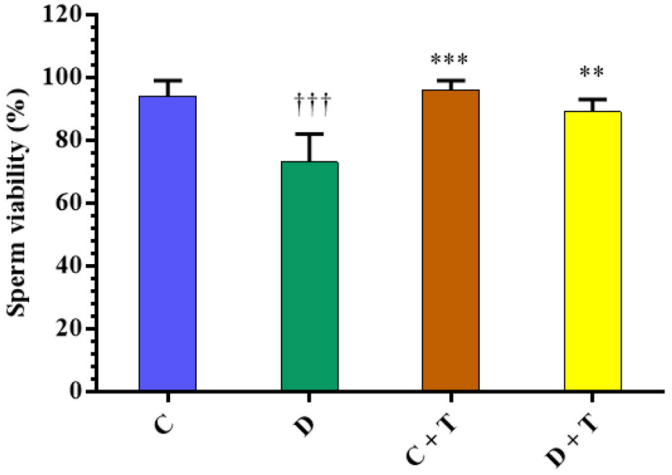

Fig. 6. Tempol effect on sperm viability. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg. † Significantly different compared to untreated control group (†††p < 0.001). * Significantly different compared to untreated diabetic group (**p < 0.01, ***p < 0.001).

untreated diabetic group (p < 0.01) (Fig. 6).

3.7. Tempol effect on lipid peroxidation (LPO)

The levels of malondialdehyde (MDA), as an LPO index, rose in the T2D group compared to the control group (p < 0.001). Treatment of diabetic rats with Tempol reduced MDA levels compared to the untreated diabetic group (p < 0.01) (Fig. 7).

3.8. Tempol effect on total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) in the diabetic group was significantly reduced compared to the control group (p < 0.001). However, in the diabetic group receiving Tempol, the TAC restored significantly in comparison to the diabetic group (p < 0.01) (Fig. 8).

3.9. Tempol effect on total thiol groups (TTG)

The results of this study showed that the levels of TTG in the studied groups were not statistically significant (p > 0.05) (Fig. 9).

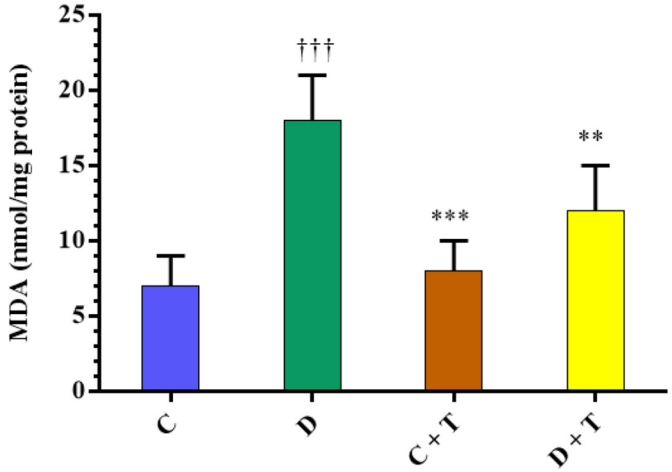

Fig. 7. Tempol effect on MDA levels of testis tissue. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg, MDA: malondialdehyde. † Significantly different compared to untreated control group (†††p < 0.001). * Significantly different compared to untreated diabetic group (** p < 0.01, *** p < 0.001).

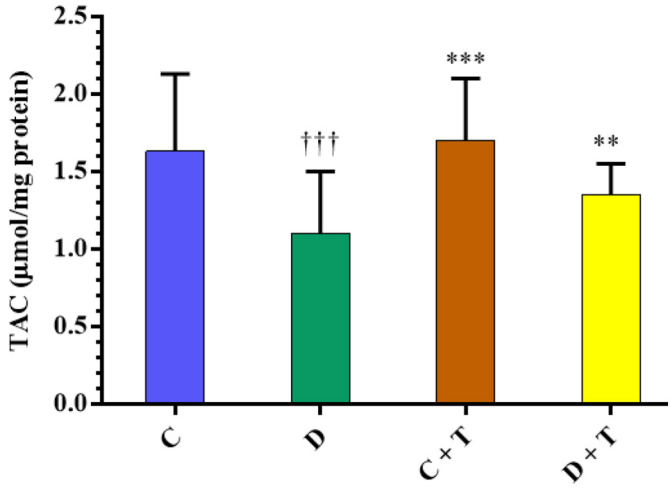

Fig. 8. Tempol effect on TAC levels in testis tissue. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg, TAC: total antioxidant capacity. † Significantly different compared to untreated control group (†††P < 0.001). * Significantly different compared to untreated diabetic group (**p < 0.01, *** P < 0.001).

3.10. Histopathological findings

Histopathological evaluation of right testis is presented in Table 1. T2D mellitus caused a dramatic decrease in the count of spermatogonia (p < 0.001), primary spermatocytes (p < 0.001), late spermatids (p = 0.01) and leydig cells (p < 0.001) compared to normal controls. Treatment of T2D rats with Tempol did not improve these parameters (p > 0.05).

The number of early spermatid in T2D group showed reduction in comparison with the normal group, but this difference was not significant (p > 0.05). The number of sertoli cells was not significantly different between groups (p > 0.05).In the microscopic evaluation of tissue sections from control group no abnormal structure was observed; arrangement of cells inside the seminiferous tubules, their number, and the thickness of membrane and tubules were normal.

Histopathological studies in the diabetic group indicated that seminiferous tubules altered. Thickening of the basal membrane, disrupting in a cellular arrangement on the base membrane, decreasing all

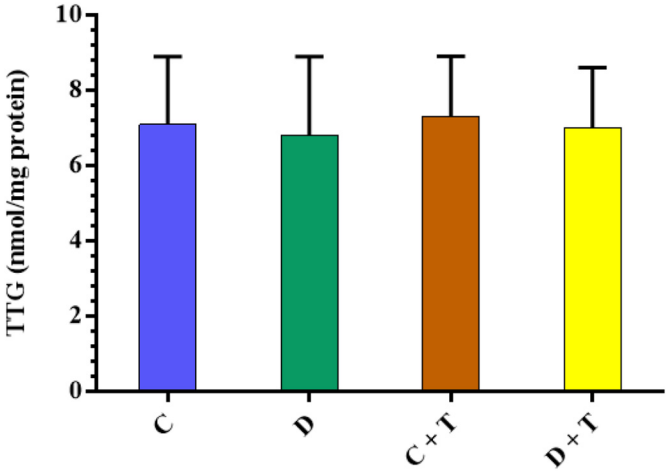

Fig. 9. Tempol effect on TTG levels in testis tissue. The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg, TTG: total thiol groups.

cell types, increasing vacancy and the presence of giant cells in some of the seminiferous tubules were observed in these examinations. The severity of the lesions in the diabetic group treated with Tempol reduced.

4. Discussion

T2D is one of the most common type of metabolic disorder that affects male fertility capability [25]. The present study revealed that Tempol as a synthetic antioxidant modulates FBS, increases antioxidant capacity in testis tissue and also improves fertility related parameters in T2D model.

The destructive effect of T2D on male fertility have been indicated by previous studies [26]. Indeed, T2D caused disruption of seminiferous tubular morphology and thereby decreased the serum levels of gonadotropins and testosterone, number of leydig and sertoli cells and sperm production that finally affect the fertility capacity [27].

Chronic hyperglycemia is one of the key factors that link DM to the infertility. It has been shown that hyperglycemia and insulin resistance induce the free radicals formation and reactive oxygen species [28].

Our results revealed that T2D decreased the levels of some sperm parameters such as; sperm count, motility and viability and also increased the count of sperm with abnormal morphology. Consistent with our results, defective effects of DM on the sperm parameters have been documented by previous studies [29,30].

The research findings indicated that the DM can lead to alter in histopathology of testis [31]. Furthermore, scientific evidences show in diabetes, in addition to degeneration and vacuolization of spermatogonia, spermatocytes and spermatids, the size of the seminiferous tubules is also decreased [32,33]. The findings of the present study were consistent with these studies, emphasizing damaging effects of DM on testis.

Destructive effects of hyperglycemia on male reproductive system are interpreted by various mechanisms. It has been established that hyperglycemia induces generation of ROS and reactive nitrogen species (RNS) [34]. Oxidative stress induced by diabetes is correlated with the vascular disorders, endothelial dysfunction and neuropathy in erectile tissue [35]. Furthermore, oxidative stress has several harmful effects such as testicular atrophy, decrease in body weight and sexual organs and also reduction in sperm count [29].

The membrane of sperm cells is enriched with poly unsaturated fatty acids (PUFA), this makes them very vulnerable to T2D-oxidative damage [10]. The reaction of free radicals membrane fatty acids can then lead to decrease in sperm motility, inactivation of glycolytic, DNA oxidation and ultimately sperm failure in oocyte fertilization [36]. Therefore, it can be concluded that antioxidant supplementing might be one of the effective therapeutic strategies to counteract effects of T2D on fertility [29]. There is evidences that the administration of antioxidants can protect the sperm cell DNA against ROS-induced damage, thereby improve sperm quality [37,38]. Tempol provides anti-oxidant effects by mimicking the activity of SOD enzyme, it might therefore have protective effects against associated complication of oxidative stress [18]. Murawski et al. revealed a positive correlation between SOD activity in seminal plasma and semen quality parameters [39]. In another study, Negri et al. evaluated the effectiveness of SOD-based antioxidant supplementation in the reducing of sperm DNA fragmentation. Their results showed that this supplementation has a beneficial effects on integrity of sperm DNA [40].

The present study showed that the Tempol has a modulated effect on the FBS. In line with our results, Jabbarpour et al. showed that Tempol reduced FBS in diabetic rats [41]. The proposed mechanism for this issue is that Tempol improved pancreas function by induction of insulin production [42].

The current study showed that STZ caused a significant decrease in body weight but treatment with Tempol modulated it. Insulin promotes glycogen and lipid synthesis in muscle cells, therefore, insulin deficiency under diabetes condition decreases the uptake of glucose by muscles, increases lipolysis and gluconeogenesis and subsequently lead to muscle atrophy and weight loss [43]. In agreement with our findings, previous studies have shown that Tempol is able to recover weight and improves glucose and insulin levels in diabetic rats [44].

On the other hand, our results showed that the TAC and MDA levels significantly decreased and increased, respectively, in T2D rats compared to the control which is consistent with previous studies [16,45]. It has been established that excessive generation of ROS under hyperglycemia condition induces damage to macromolecules which is associated with the development and progression of T2DM [36].

In the present study, Tempol modulates the effects of T2D-induced oxidative stress through the mimicking the activity of SOD. Furthermore, our recent study on diabetic nephropathy also showed that Tempol administration might increase the glutathione peroxidase activity [46].

Overall, Tempol seems to play an important role in protecting the sperm membrane against ROS-induced lipid peroxidation, thereby improvement the quality of sperm parameters.

Table 1
Count of typical germ cells right testis of studied groups.

Group	Spermatogonia	Primary spermatocytes	Early spermatids	Late spermatids	Sertoli cells	Leydig cells
C	30.35 ± 1.74	40.10 ± 1.98	56.60 ± 3.02	19.25 ± 0.82	6.95 ± 0.27	15.55 ± 0.59
D	21.50 ± 1.22*	24.30 ± 1.18*	42.70 ± 3.16	12.05 ± 2.94*	5.65 ± 0.29	10.25 ± 0.39*
C + T	30.90 ± 1.67	41.95 ± 1.87	59.20 ± 2.76	20.75 ± 0.90	6.80 ± 0.27	12.20 ± 0.40
D + T	22.10 ± 1.07	27.20 ± 0.94	47.85 ± 3.58	14.40 ± 0.82	6.05 ± 0.30	12.55 ± 0.45

The data are presented as mean ± SD. C: control group, D: type 2 diabetic group, T: Tempol 100 mg/kg.
* Significantly different compared to control group ($p < 0.05$).

5. Conclusion

Therefore, it can be concluded that Tempol may be a candidate antioxidant to counteract the harmful effects of T2D on fertility capability. Finally, this study has few limitations including: evaluation of sperm DNA fragmentation, expression of GLUT genes, increasing the duration of study and evaluation of sperm fertilizing capacity under in vitro condition.

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Conflict of interest

The authors have declared no conflicts of interests.

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