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Trimetazidine has protective effects on spermatogenesis in a streptozotocin-induced diabetic rat model

M. F. Ozcan[1] | E. R. Hekimoglu[2] | K. Ener[1] | M. Namuslu[3] | R. Altintas[4] | H. T. Celik[3] | Z. Akbulut[1] | S. Altinova[1]

[1]Department of Urology, Ankara Ataturk Education and Research Hospital, Ankara, Turkey

[2]Department of Histology and Embryology, Faculty of Medicine, Kastamonu University, Kastamonu, Turkey

[3]Departments of Biochemistry, Faculty of Medicine, Turgut Ozal University, Ankara, Turkey

[4]Department of Urology, Faculty of Medicine, Inonu University, Malatya, Turkey

Correspondence
Muhammet Fuat Ozcan, Department of Urology, Ankara Ataturk Education and Research Hospital, Ankara, Turkey.
Email: mfuatozcan@hotmail.com

Summary

The aim of this study was to investigate the protective effects of trimetazidine (TMZ), as an antioxidant agent, on streptozotocin (STZ)-induced diabetic rats. A total of 50 male Sprague Dawley rats were randomly classified into five groups as follows: Group 1 (control), Group 2 (STZ-induced diabetic rats), Group 3 (STZ-induced diabetic rats treated orally with 1 cc/day isotonic saline), Group 4 (diabetic rats treated orally with 10 mg/kg/day TMZ) and Group 5 (diabetic rats treated orally with 20 mg/kg/day TMZ). After 8 weeks, orchiectomy was carried out. Histopathological and electron microscopic examinations were performed in all groups. In groups 1 and 5, the structural and ultra-structural findings of the testicular tissue and spermatogenesis were found normal. In groups 2, 3 and 4, similar results were obtained in terms of the impaired testicular architecture and degeneration of spermatogenesis. The administration of an optimal dose of TMZ protects against the harmful effects of diabetes mellitus on spermatogenesis in rats. TMZ therapy can be used to maintain normal spermatogenesis in diabetic rats.

KEYWORDS

diabetes, rats, spermatogenesis, streptozotocin, trimetazidine

1 | INTRODUCTION

The prevalence of diabetes mellitus (DM) is rapidly increasing worldwide and it is expected that around 600 million people will suffer from diabetes by 2,035 (Guariguata et al., 2014). Another major health problem is infertility, the prevalence of which has been reported significantly higher in patients with diabetes compared with nondiabetic patients (La Vignera et al., 2012).

Infertility is a common complication in diabetic humans and experimental animals, mainly due to the loss of germ cells by apoptotic cell death (Mallidis, Agbaje, McClure, & Kliesch, 2011; Ricci et al., 2009). DM in both humans and animals leads to structural and ultra-structural changes in the spermatogenic process (La Vignera et al., 2012; Ricci et al., 2009). Oxidative stress and peroxidative damage are believed to be the most harmful factors (Boujbiha et al., 2009; Mohasseb, Ebied, Yehia, & Hussein, 2011). Excessive reactive oxygen species (ROS) also have an important role in the induction of apoptosis, and a prominent reduction in germ cell apoptosis has been found in diabetic rats supplemented with antioxidants. These findings suggest that antioxidants have a protective effect against the significant testicular oxidative damage and germ cell apoptosis in diabetes (Mohasseb et al., 2011).

Type 1 diabetes can be induced by the injection of streptozotocin (STZ), which causes β-cell death in the pancreas and is commonly used to induce type 1 diabetes in experimental models (King, 2012). STZ-induced diabetic rats have shown morphological testicular lesions that practically affect all the structures of the seminiferous tubules (ST), including Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa (Ricci et al., 2009; Schöffling, Federlin, Schmitt, & Pfeiffer, 1967). It is important to maintain the balance between ROS production and antioxidant defence systems in the pathogenesis of diabetic complications (Ceriello, 2003). Therefore, regulating ROS via antioxidant treatment can also have a beneficial, therapeutic effect on infertility.

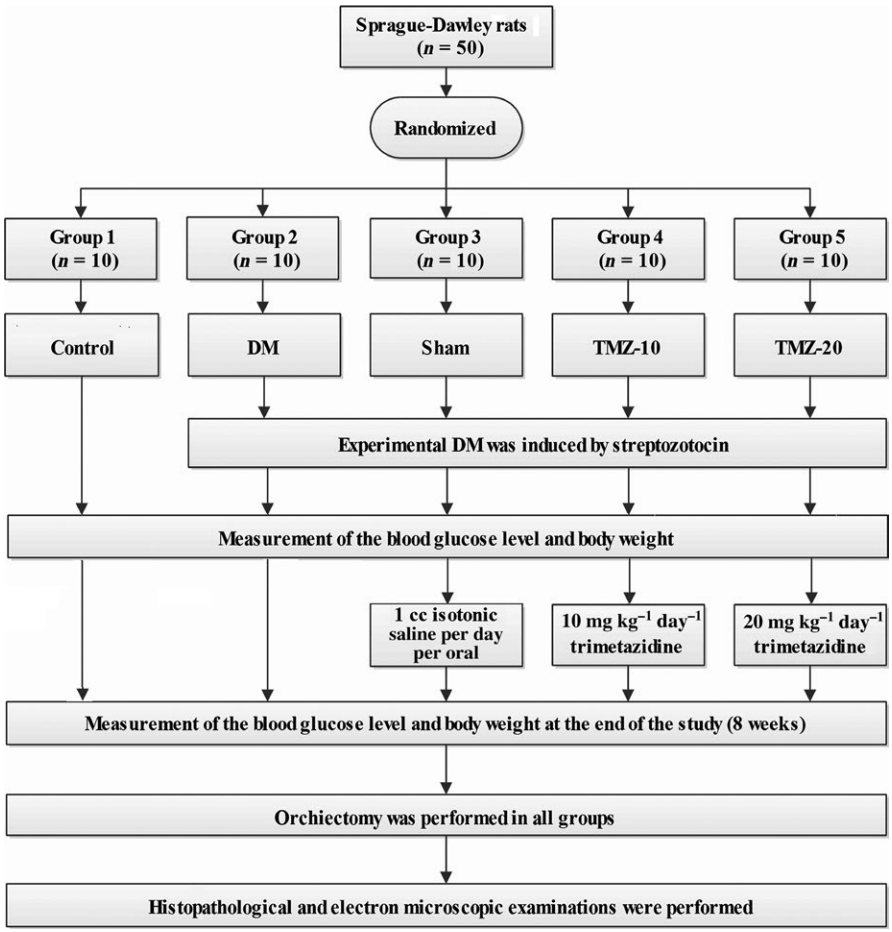

FIGURE 1 Randomisation of 50 male Sprague Dawley rats into five groups. Group 1: The non-diabetic control group. In the remaining groups, diabetes was induced by a single dose of STZ. Rats were considered diabetic if their blood glucose values were higher than 250 mg/dl. Group 2: Diabetic control group, receiving no treatment. Group 3: The sham group treated orally with 1 cc/day isotonic saline. Groups 4 and 5: Treated orally with TMZ, 10 mg/kg/day and 20 mg/kg/day respectively

Trimetazidine [TMZ; 1-(2, 3, 4-trimethoxybenzyl)-piperazine dihydrochloride] is an anti-ischaemic, the effects of which have been experimentally assessed in various models including cell cultures, isolated and perfused organs as well as in vivo (Baumert et al., 1999; Gabryel, Adamek, & Trzeciak, 2001; Hauet et al., 1998). In addition, TMZ has antioxidant properties. This study was designed to investigate the protective effect of different doses of TMZ, as an antioxidant agent, on STZ-induced rats with DM.

2 | MATERIALS AND METHODS

This study conformed to the recommendations of the Declaration of Helsinki for care and use of laboratory animals and was approved by the local ethics committee (approval number 2013/64). A total of 50 male Sprague Dawley rats aged 5.5–6 months were randomly and equally allocated to five groups. Group 1 was the nondiabetic control group. In the remaining groups, diabetes was induced by a single dose of STZ. Group 2 (diabetic control group) did not receive any treatment. Group 3 was the sham group treated orally with 1 cc/day isotonic saline. Groups 4 and 5 were treated orally with 10 mg/kg/day TMZ and 20 mg/kg/day TMZ respectively. The study design was summarised in Figure 1.

Experimental diabetes was induced in overnight-fasted rats by a single dose of 45 mg/kg STZ (Sigma, St. Louis, MO) administered intraperitoneally, dissolved in cold 0.1 M sodium citrate buffer, pH 4.5. The blood glucose levels of all rats were checked 48 hr after the injection using an Accu-Chek Compact Meter (Roche Diagnostics, Indianapolis, IN, USA), and rats were considered diabetic if their blood glucose values were higher than 250 mg/dl.

The experimental animals were housed at 18-22°C throughout the study period (8 weeks) and placed in Plexiglas cages on a 12-hr light/dark cycle with standard food and water ad libitum. Body weight and blood glucose levels were monitored every 2 weeks. Over the 8-week period, isotonic saline and TMZ were administrated daily by oral gavage followed by orchiectomy. All surgical procedures were performed under ketamine anaesthesia and full sterile conditions. Histopathological and electron microscopic (EM) examinations were performed in all groups. For light microscopy examination, orchiectomy samples were fixed in 4% paraformaldehyde and then paraffin-embedded. Using a microtome, 5-μm sections were cut. Tissues were stained with haematoxylin and eosin and examined under light microscopy. For EM examination, orchiectomy samples were fixed in a 2.5% glutaraldehyde solution in phosphate buffer at pH 7.4 for 4 hr. After further post-fixation, slicing and staining procedures, the sections were examined under a JEOL-JEM 1400 and 2100F electron microscope and photographed using a charge-coupled device camera (Gatan Inc., Pleasanton, CA, USA).

TABLE 1 Johnsen score system modified by Erdemir et al. (2014)

Johnsen score	
1	No seminiferous epithelium
2	No germinal cells, Sertoli cells only
3	Spermatogonia only
4	No spermatozoa or spermatids, few spermatocytes
5	No spermatozoa or spermatids, many spermatocytes
6	No spermatozoa, no late spermatids, few early spermatids
7	No spermatozoa, no late spermatids, many early spermatids
8	Less than five spermatozoa per tubule, few late spermatids
9	Slightly impaired spermatogenesis, many late spermatids, disorganised epithelium
10	Full spermatogenesis

TABLE 2 Modified Johnsen scores of the groups

	Modified Johnsen score
Group 1	10 (n = 10)
Group 2	5 (n = 4), 6 (n = 6)
Group 3	5 (n = 4), 6 (n = 6)
Group 4	7 (n = 10)
Group 5	9 (n = 4), 10 (n = 6)

The sections stained with haematoxylin and eosin were studied under a light microscope to evaluate spermatogenesis. The seminiferous tubules diameter (STD) and the germinal epithelium of tubules were assessed for each testis, and Johnsen score modified by Erdemir et al. was calculated for each rat (Erdemir et al., 2014). Johnsen's criteria were used to categorise spermatogenesis. This system describes the preservation of spermatogenesis, on a scale from of 1 to 10, according to the absence or presence of the main cell types arranged in order of maturity. A Johnsen score of 9 or 10 indicates normal histology, a score of 8 signifies hypospermatogenesis, a score of 3-7 implies maturation arrest, a score of 2 indicates germinal cell aplasia (Sertoli cells only), and a score of 1 represents tubular fibrosis (Table 1). The Johnsen scores of the rats are summarised in Table 2.

For the ultrastructural examination of the ST, the aspects of spermatogonia, spermatids, the Sertoli and Leydig cells and mitochondria were evaluated in relation to the presence or absence of vacuolisation, accumulation of waste materials and anomalous corpuscles in the cytoplasm and/or membranes cells, alterations in nuclear chromatin, cell degeneration and cell death.

The fragments obtained for EM were fixed in fresh tissue samples fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.4) for 4 hr, and post-fixed for 1 hr in 1% osmium tetroxide solution in 0.1 M phosphate buffer. After washing in phosphate buffer, the samples were dehydrated in a graded series of ethanol to absolute ethanol, treated with propylene oxide and embedded in Araldite/Epon812 (Cat No: 13940, EMS, Hatfield, PA, USA).

The sample size was calculated as 50 (the power of the study was 80% and the effect size was (f): 0.55). The distribution of the variables was analysed by the Shapiro–Wilk test. The variables with normal distribution were expressed as mean ± standard deviation. Interquartile range (IQR) was used to show the variables with skewed distribution. Statistical analysis and calculations were performed by SPSS Statistics for Windows version 21.0 (IBM Corp. Released 2012.). Statistical significance was defined as $p < .05$.

3 | RESULTS

Table 3 and Figure 2 present the weights and blood glucose levels of rats respectively. The body weights of the rats were measured before and after the study and compared within the groups. Before the study (week 0), body weights were similar in the groups ($p > .05$). No statistical significance was observed in Group 3; however, at the end of the study, there was a significant increase in body weight in Group 1 and a significant decrease in groups 2, 4 and 5 (Table 3). Furthermore, in Group 1, the mean blood glucose level was within a normal range, while it was above 300 mg/dl in the other groups (Figure 2).

The findings of light microscopy are summarised in Table 4 and Figure 3. The light microscopy findings of groups 1 and 5 were normal. In groups 2 and 3, degeneration and vacuolation of the ST epithelium were observed, the interstitial area was oedematous, and spermatogenesis was abnormal. In Group 4, there was less vacuolisation in the

TABLE 3 Body weight of the rats

	Body weight, mean ± SD (g)			
	Before the study	At the end of the study	t	p
Group 1	342.4 ± 17.1	390.1 ± 29.9	4.101	.003
Group 2	374.1 ± 23.8	306.5 ± 39.5	4.509	.001
Group 3	374.5 ± 29.5	348.9 ± 48.5	1.492	.170
Group 4	386.4 ± 30.3	330.4 ± 36.5	5.482	<.001
Group 5	368.0 ± 38.1	315.6 ± 48.3	5.430	<.001

Before the study: Week 0. At the end of the study: Week 8.

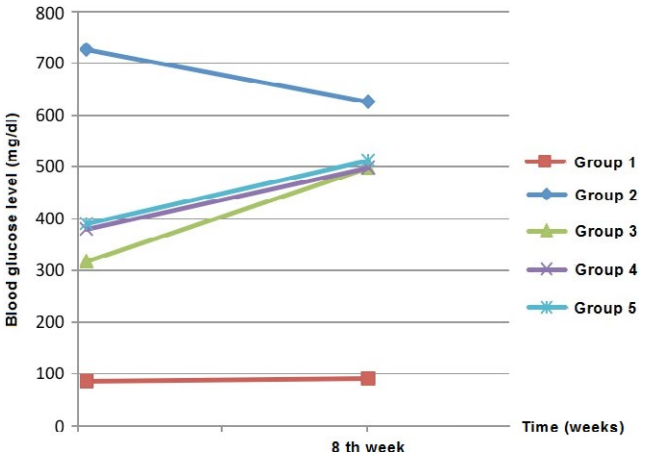

FIGURE 2 Blood glucose levels of the groups after STZ treatment. In Group 1, the mean blood glucose level was within a normal range, while it was above 300 mg/dl in the other groups

ST and less oedema in the interstitial area; however, degeneration of spermatogenic cells was observed.

The EM sections of the groups are presented in Table 5. In Group 1, the spermatogonia, primary spermatocytes, spermatids, the Sertoli and Leydig cells were normal. Myoid (contractile) cells and collagen fibrils were detected in the tunica propria of the ST (Figure 4a). In STs where spermatogenesis was found to be permanent, acrosomal vesicles were present in round spermatids that connected to the gap junctions of the surrounding developing cells. In elongated spermatids that were in progressive development stages, the cell chromatin was condensed (Figure 4b). In the Sertoli cells located on the basal lamina, the euchromatin nucleus could be distinguished from the distinct nucleolus with several mitochondria and electron-dense granules being present (Figure 4b). The organelles and chromatin were normally distributed in the nucleus of the interstitial connective tissue, and the nuclei of Leydig cells were normal and located close to the capillaries (Figure 4c). These findings indicated a normal process of spermatogenesis.

In Group 2, the cell layers in the ST epithelium were reduced in number and desquamated. Myoid cells and collagen fibrils were detected in the tunica propria of the ST (Figure 4d). Several vacuoles of various sizes were present in the cytoplasm of primary spermatocytes and round spermatids (Figure 4e). In the STs, primary spermatocytes and round spermatids were observed, but no elongated spermatids and spermatozoa were detected. The cytoplasm of the round spermatids contained centrally located spherical nuclei and peripherally located mitochondria (Figure 4e). There were gaps between the round spermatids (Figure 4g). The cytoplasm of the Leydig cells had several vacuoles in the interstitial connective tissue but no lipid droplets (Figure 4f). The findings of Group 3 were similar to those of Group 2.

Group 4 had similar EM findings to Group 2 in terms of myoid cells and fibroblast being detected in the tunica propria of the ST, the ST epithelium being reduced and the STs having primary spermatocytes and round spermatids but no elongated spermatid and spermatozoon (Figure 4g). In addition, in the Sertoli cells located on the basal lamina, the euchromatin nucleus could be distinguished from the distinct nucleolus, and mitochondria, several electron-dense granules and vacuoles were present in the cytoplasm (Figure 4h). The cytoplasm of the Leydig cells located in the interstitial connective tissue contained multiple vacuoles but no lipid droplets (Figure 4i).

The EM findings of Group 5 were similar to those of Group 1 and better than those obtained from Group 4. Even though vacuoles were detected in the cytoplasm of the Sertoli and Leydig cells, spermatogonia, primary spermatocytes and spermatids, they were significantly decreased in number than those in groups 2, 3 and 4 (Figure 4j). Myoid cells and fibroblast were present in the tunica propria of the ST. In STs where spermatogenesis was normal, there were acrosomal vesicles in round spermatids that connected to the gap junctions in the surrounding developing cells, but there were fewer vacuoles (Figure 4k). In the spermatids that were in the advanced phases of development, the chromatin of the nucleus was condensed and vacuoles were detected in their cytoplasm (Figure 4k). The euchromatin nucleus and distinct nucleolus of the Sertoli cells located on the basal lamina were visible, and their cytoplasm contained mitochondria and vacuoles (Figure 4j). In the interstitial connective tissue, the Leydig cells had normally distributed chromatin in their nucleus and nucleolus, and normal-shaped organelles. The cytoplasm of these cells contained lipid droplets and vacuoles (Figure 4l), although fewer in number compared with Group 4.

4 | DISCUSSION

Over the 8 weeks, the body weight of the control rats gradually increased, whereas the body weight of the diabetic rats decreased. Once the rats became diabetic, their serum glucose levels were maintained over 300 mg/dl for the remainder of the study.

The mean blood glucose levels of Group 2 were significantly higher than the groups 3, 4 and 5 after STZ treatment. Thereafter, repeated measurements demonstrated that the blood glucose levels were similar between groups 2, 3, 4 and 5 at week 8 ($p > .05$). In groups 2 and 3, similar results were obtained in terms of the impaired testicular architecture and degeneration of spermatogenesis. It can be concluded that any blood glucose level which exceeds 250 mg/dl has the same harmful effect and leads to structural and ultra-structural changes.

DM increases ROS activity and oxidative stress and can lead to infertility. Oxidative stress is determined by hyperglycaemia, which reduces the endogenous antioxidants and increases the production of superoxide, hydroxyl and peroxyl by damaging the cellular DNA and proteins (Sinha Hikim & Swerdloff, 1999). The antioxidant effect of TMZ prevents excessive production of free oxygen radicals. It decreases intracellular acidosis in the cells exposed to hypoxia or ischaemia by directly affecting the energy metabolism, and precludes the reduction in intracellular adenosine triphosphate and phosphocreatine. It has been shown that antioxidants can protect against the adverse effects of DM on fertility (Boujbiha et al., 2009). The current study evaluated the protective effects of an antioxidant and anti-ischaemic agent, TMZ, on spermatogenesis.

TABLE 4 Light microscopy findings of the testicular tissue in all groups

	Group 1	Group 2	Group 3	Group 4	Group 5
Seminiferous tubule	Normal	Reduction and desquamation of the germinal epithelium Irregular ST shape	Reduction and desquamation of the germinal epithelium Irregular ST shape	Reduction and desquamation of the germinal epithelium (less than Group 2) Irregular ST shape	Normal
Vacuolisation	Absent	+	+	+ (less than Group 2)	Absent
Degeneration of epithelium	Absent	+	+	+	Absent
Spermatozoa	+	Absent	Absent	Absent	+
Early Spermatid	+	Few	Few	Many	+
Late spermatid	+	Absent	Absent	Absent	+
Location	Normal	Irregular[a]	Irregular[a]	Irregular[a]	Similar to Group 1
Germinal epithelium					
Basement membrane	Intact	Intact	Intact	Intact	Intact
Tunica propria	Normal	Normal	Normal	Normal	Normal
Spermatogenesis	Normal	Abnormal	Abnormal	Abnormal	Normal
Interstitial area	Normal	Oedema	Oedema	Partly Normal, partly oedema	Larger than Group 1
Spermatogenesis	Normal	Degeneration of the spermatogenic cells	Degeneration of the spermatogenic cells	Degeneration of the spermatogenic cells	Normal

[a]Seminiferous tubules with irregular shape; DM, diabetes mellitus; ST, seminiferous tubule.

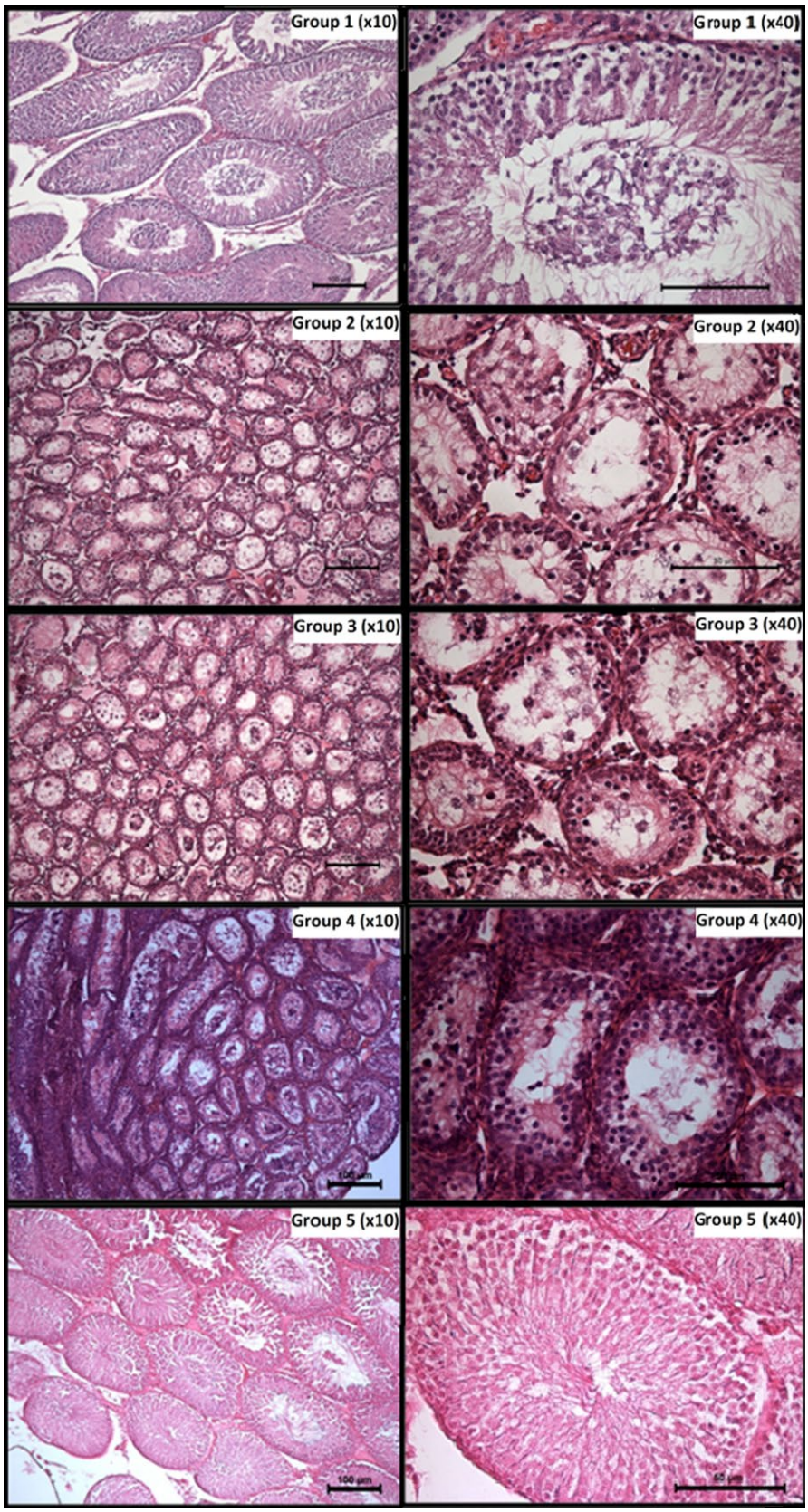

FIGURE 3 Light microscopy findings of the groups: tissues were stained with haematoxylin and eosin and examined under light microscopy (×10 and ×40)

Infertility may occur due to DM. The process of spermatogenesis normally takes about 52–53 days in rats. The development of the seminiferous epithelium cycle or the spermatogenetic cycle is segmental in rats, consisting of 14 stages, and takes place over 12 days (Wankeu-Nya et al., 2013). It has been demonstrated that one or more stages of the spermatogenic process can be altered during diabetes

TABLE 5 Electron microscopy findings of the testicular tissue

	Group 1	Group 2	Group 3	Group 4	Group 5
Seminiferous (germinal) epithelium	Normal ultrastructure	Reduced epithelial thickness	Reduced epithelial thickness	Reduced epithelial thickness	Normal ultrastructure
Spermatogonium	Normal ultrastructure	Normal ultrastructure	Normal ultrastructure	Normal ultrastructure	Normal ultrastructure
Primary spermatocytes	Normal ultrastructure	Large and small lipid droplets in the cytoplasm, some cells have no intercellular junction	Large and small lipid droplets in the cytoplasm, some cells have no intercellular junction	Large lipid droplets in the cytoplasm, some cells have no intercellular junction	Normal ultrastructure, small lipid droplets in the cytoplasm
Early (round) Spermatid	Normal ultrastructure	Few	Few	Many	Normal ultrastructure
Late (elongated) spermatid	Normal ultrastructure	Absent	Absent	Absent	Normal ultrastructure
Acrosomal vesicles in spermatids	+	–	–	Few +	+
Sertoli cell	Normal ultrastructure	Normal ultrastructure and vacuoles in the cytoplasm	Normal ultrastructure and vacuoles in the cytoplasm	Normal ultrastruc-ture and vacuoles in the cytoplasm	Normal ultrastructure and rare vacuolisation.
Leydig cell	Contains numerous membrane-bound lipid droplets and a large amount of SER	Contains few membrane-bound lipid droplets, SER and a large number of vacuoles.	Contains few membrane-bound lipid droplets, SER and a large number of vacuoles.	Contains few membrane-bound lipid droplets, SER and a large number of vacuoles.	Contains numerous membrane-bound lipid droplets and a large amount of SER. Few vacuoles.
Interstitial Area	Normal	Decreased vascularisation	Decreased vascularisation	Increased vascularisation	Increased vascularisation
Spermatogenesis	Normal	Abnormal	Abnormal	Abnormal	Normal

SER, smooth endoplasmic reticulum.

(Wankeu-Nya et al., 2013). In the current study, by evaluating the light microscopy and EM findings after the administration of TMZ for 56 days, we found that the STZ-induced DM leads to dramatic changes in the testicular morphology and altered the spermatogenic process in rats. In the group treated with 10 mg/kg/day TMZ (Group 4), the spermatogenesis improved and there was mild improvement in structural and ultra-structural of the testicular tissue findings while almost all the findings were normal in Group 5 treated with 20 mg/kg/day TMZ. These findings confirmed that the optimal doses of TMZ protect against the harmful effects of DM on spermatogenesis.

The light microscopy results of the control group revealed a standard architecture of the testicular tissue, with normal ST, germinal epithelium, interstitial area and spermatogenesis. Diabetic rats demonstrated reduction and desquamation of the germinal epithelium, degeneration of the epithelium, vacuolisation, oedema of the interstitial area and degeneration of the spermatogenic cells. The sham group (Group 3) showed no visible differences from the diabetic DM group (Group 2). The diabetic rats treated with high-dose TMZ had an improved testicular architecture that was almost the same as that of the nondiabetic control group.

In the nondiabetic rats, the morphometric measurements of the ST varied due to the various stages of the cycle of spermiogenesis (Wing & Christensen, 1982). Testicular size was decreased in the DM group, although this atrophy of the testes is mainly due to the decrease in the testosterone level associated with the absence or diminution of the serum insulin levels as insulin acts as an anti-apoptotic factor capable to regulate testicular apoptosis induced by diabetes (Ballester et al., 2004; Cai et al., 2000; Schoeller, Albanna, Frolova, & Moley, 2012). In the EM examination of the diabetic control group, spermatogenetic dysfunction was revealed by the atrophy of the seminiferous tubules characterised by the decreased STD and the thickness of the basement membrane diameter of the ST; the reduction in the spermatogenic cell series; the disorganisation and degeneration of the spermatocytes, spermatids and sperm germ cells. The ST atrophy and decrease in the spermatogenic cells are important indicators of a spermatogenesis failure. Moreover, diabetes increases the thickening of the basement membrane of the ST, which is accompanied by decreased sperm production and an overall reduction in the size or diameter of the ST (Cameron, Murray, & Drylie, 1985; Rohrbach & Martin, 1982; Sainio-Pollanen, Henriksen, Parvinen, Simell, & Pöllänen, 1997).

One month after the induction of diabetes, vacuolisation was detected in the Sertoli cells. These cells are necessary for producing substances for the survival of the germ cells and creating an essential and adequate environment for spermatogenesis (Griswold, 1995). The vacuolisation of these cells may impair their role in providing germs with nutrients and regulatory factors, thus leading to the alteration in spermatogenesis resulting in a reduced number of spermatozoa with increased number of defects. In the current study, vacuolisation was still observed in Group 4 that received 10 mg/kg/day TMZ, but no vacuolisation was detected in Group 5 that received 20 mg/kg/day TMZ. In addition, spermatogenesis was diminished in the rats in Group 4, while the regular progress of spermatogenesis was maintained in Group 5. This finding shows that vacuole formation may be an indicator of impaired spermatogenesis.

The results of the current study show that the administration of 20 mg/kg/day TMZ improves the testicular tissue. This raises the idea

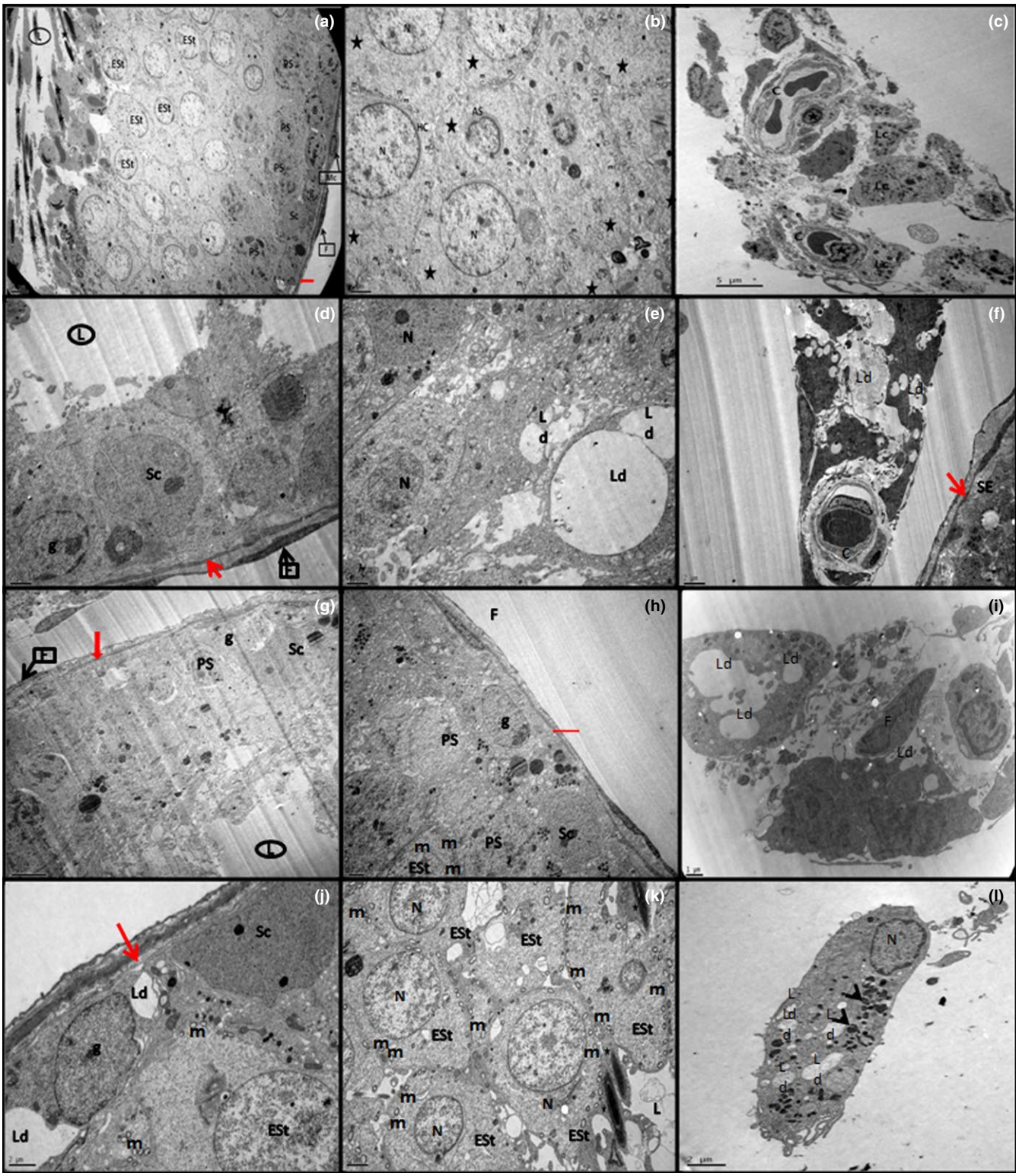

FIGURE 4 (a–c) Group 1; (d–f) Group 2; (g–i) Group 4; (j–l) Group 5. (a, b, d, e, g, h, j, k) germinal epithelium; (c, f, i, l) interstitial area. MC, myoid cell; F, fibroblast; Sc, sertoli cell; g, spermatogonia; PS, primary spermatocyte; ESt, early spermatids; star, late spermatids; red arrow, basal lamina; black arrow, electron-dense body; L, lumen. ★ (b) Spermatid with acrosome. AS, acrosomic system; N, nucleous; HC, head cap; m, mitochondria; C, blood vessel. ★ (c) Endothelial cell. Lc, leydig cell; Ld, lipid droplet (Uranyl acetate, lead citrate, a: ×2,000, b: ×6,000, c, g: ×3,000, d, e j, l: ×6,000, f: ×15,000, h: ×4,000, i: ×10,000, k: ×8,000)

that changing the testicular architecture with sufficient amounts of TMZ can improve fertility in rats. Furthermore, it is clear that TMZ reduces the negative effect of DM on the testicular tissues. The protective impact of TMZ is even greater when the dose is 20 mg/kg/day TMZ.

Our results demonstrate that in the STZ-induced diabetic rats, the decrease in the germ cell death was regulated by the administration of 20 mg/kg/day TMZ without leading to a major antihyperglycaemic activity. Therefore, TMZ can be successfully used in preventing and treating diabetes-induced testicular dysfunction.

The current study has certain limitations. First, the study period was relatively short, being only 8 weeks. The changes in the structural and ultrastructural findings may be better observed over a longer period of using TMZ. In addition, evaluating the presence of vacuoles under light microscopy over a longer period can provide more information about the long-term effects of TMZ. Finally, higher doses of TMZ should be investigated to determine the optimal dose for the prevention of the negative effects of DM.

5 | CONCLUSION

In conclusion, the administration of TMZ in sufficient amounts protects against the harmful effects of DM on the process of spermatogenesis in rats. Therefore, in diabetic rats, normal spermatogenesis can be maintained by TMZ therapy. In the future, TMZ can be an effective option for treating infertility in humans due to diabetes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This study conformed to the recommendations of the Declaration of Helsinki for care and use of laboratory animals and was approved by the local ethics committee (approval number 2013/64).

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