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Research Paper

Protective effects of metformin and vitamin C on diabetes-induced testicular dysfunction

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In brief

Diabetes mellitus negatively impacts reproductive health in males. This study demonstrates the protective effects of a combination of metformin and vitamin C on diabetes-induced testicular damage.

Abstract

This study aimed to investigate the potential protective effects of combined metformin (Met) and vitamin C (VitC) therapy on diabetes mellitus (DM)-induced testicular damage, sperm parameters, and the regulation of gene and protein expression associated with reproductive function. 36 C57BL mice were divided into six groups: control, VitC, DM, DM+VitC, DM+Met, and DM+VitC+Met. Diabetes mellitus was induced with streptozotocin, and treatment with Met (250 mg/kg/day) and VitC (100 mg/kg/day) for 40 days. Sperm parameters and serum oxidative stress markers were also evaluated. Sperm viability and apoptosis were assessed using Muse Cell Analyzer. *Dnah7* and *Tomm20* gene expression in the testis was determined by real-time PCR. Spermatogenesis was evaluated using the Johnsen score. Caspase-3 and proliferating cell nuclear antigen levels in the testes were assessed using western blotting and immunohistochemistry. Hyperglycaemia and decreased superoxide dismutase levels were observed in the DM group. Combination therapy resulted in higher sperm viability, Johnsen score, and testicular *Tomm20* and *Dnah7* mRNA expression levels than monotherapy. The combined administration of Met and VitC has significant potential for managing DM-associated reproductive dysfunction by maintaining sperm viability, Johnsen scores, and testicular protein profiles. Our results suggest that combination therapy holds promise for protecting testicular function and modulating key molecular markers, thereby mitigating DM-induced testicular damage at the molecular level.

Keywords diabetes mellitus, testis, metformin, vitamin C, combination therapy

Diabetes mellitus (DM) is a public health concern characterized by chronic hyperglycaemia and its associated complications (Huang et al., 2024; Lotti & Maggi, 2023). Streptozotocin (STZ) is a widely used diabetogenic agent that induces DM—most commonly Type 1—in rodents by destroying pancreatic islet β-cells. Chronic hyperglycaemia resulting from insulin deficiency can, over time, induce secondary insulin resistance (Furman, 2021). Beyond hyperglycaemia, increased oxidative stress leads to systemic complications of DM (Huang et al., 2024). At the cellular level, oxidative stress plays a key role in the pathogenesis of hyperglycaemia-related damage (Lavin-Perez et al., 2021). Diabetes mellitus and oxidative stress impair male fertility by disrupting the hypothalamic–pituitary–gonadal axis, erectile function, and sperm quality (He et al., 2021; Huang et al., 2024).

The primary goal of DM treatment is to prevent hyperglycaemia. Blood glucose levels can be lowered through lifestyle modifications and antidiabetic agents (Lotti & Maggi, 2023; Tavares et al., 2018). Metformin (Met) is particularly effective in regulating carbohydrate and lipid metabolism, and reduces inflammation and oxidative stress (Shpakov, 2021). However, the influence of Met on human sperm parameters remains controversial, as its effect on sperm count appears to be associated with fluctuations in oxidative stress, despite its recognized role in improving metabolic control (Service et al., 2023). In animal models, Met has been shown to restore fertility by modulating testicular steroidogenesis and spermatogenesis (Tseng, 2022).

Increased oxidative stress is a component of various diseases, including DM and male infertility. Elevated reactive oxygen species (ROS) levels can induce DNA fragmentation in spermatozoa and reduce sperm viability and motility (Kaltsas, 2023). Several biomarkers, such as enzymes and metabolites, indicate oxidative stress. Glutathione (GSH) acts as a key intracellular antioxidant in redox reactions (Zhai et al., 2022). Superoxide dismutase (SOD) neutralizes free radicals by converting superoxide anions into the

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less reactive hydrogen peroxide (H_2O_2) (Wang et al., 2025). The protective effects of Vitec have been investigated in animal models of elevated oxidative stress. Testicular GSH levels decrease in STZ-induced diabetes in mice, and VitC offsets STZ-induced oxidative damage (Shrilatha & Muralidhara, 2007). Intraperitoneal VitC treatment restores sperm motility and viability in mice with DM (Talebi et al., 2014). The coadministration of VitC and dexamethasone ameliorates the detrimental effects of dexamethasone on spermatogenesis in mice (Sadeghzadeh et al., 2019). In this context, VitC reduces the detrimental effects of DMs on male reproductive system.

Male fertility assessment analyses sperm count, motility, and morphology, reflecting testicular and epididymal function (Ozkocer & Konac, 2021; Shrilatha & Muralidhara, 2007). Mitochondria play a key role in providing the energy required for motility. Abnormalities in mitochondrial function or structure can impair both sperm motility and morphology, and cause infertility in mice. TOMM20 is a component of the mitochondrial import machinery, and altered TOMM20 expression has been associated with mitochondrial dysfunction and impaired energy metabolism in sperm cells (Wang et al., 2020). Sperm motility is also dependent on the structural and functional integrity of the axoneme within the sperm flagellum. The dynein arms located along the axoneme generate force for movement via ATP hydrolysis. DNAH7 encodes an axonemal dynein heavy chain essential for the proper function of inner dynein arms. Mutations or dysregulation of DNAH7 expression can disrupt axonemal structure, resulting in reduced motility and asthenozospermia (Morohoshi et al., 2020; Neesen et al., 2001). Although Met and VitC play important roles in the management of diabetic complications, the therapeutic role of this combination on mitochondrial and axonemal proteins in the male reproductive system remains to be fully explored.

In this study, we aimed to investigate whether combined Met and VitC treatment could support sperm energy metabolism and structural integrity in STZ-induced diabetes. By examining the molecular changes associated with diabetic reproductive dysfunction, this work seeks to clarify how metabolic regulation and antioxidant support may protect testicular function and sperm health, providing insight into potential therapeutic strategies for preserving male fertility.

Materials and methods

Animal experiments

Animal experiments were conducted at the KOBAY Experimental Animal Laboratory with the ethical approval of KOBAY DHL A.Ş. Local Ethics Committee (No: 559, July 3, 2021). Eight- to 10-week-old male C57BL/6 mice, were randomly separated into six groups ($n = 6$ per group). A single dose of STZ (120 mg/kg) dissolved in citrate buffer (pH = 4.5) was intraperitoneally (i.p.) injected to induce DM (Yeo et al., 2018). To confirm DM, fasting blood glucose levels was measured after 72 hr of a single dose of STZ (120 mg/kg, i.p.) (Kottaisamy et al., 2021). Animals with glucose levels higher than 200 mg/dl were diagnosed with DM. Animals were treated by oral gavage for the following 40 days according to their group's treatment regimens (Table 1). The control group was treated with a single dose of citrate buffer (i.p.) and water as a solvent of Met and VitC (orally, p.o.) for 40 days. The VitC group received a single dose of citrate buffer (i.p.) and VitC (100 mg/kg/day, p.o.) for 40 days. The DM group was treated with water (p.o.) while DM+VitC was treated with VitC (100 mg/kg/day, p.o.) for 40 days. The DM+Met group received Met (250 mg/kg/day, p.o.) and the DM+VitC+Met group was treated with a combination of Met (250 mg/kg/day) and VitC (100 mg/kg/day) for 40 days. The weights and fasting blood glucose levels of the animals were monitored weekly. At the end of 40 days, the animal weights were measured. Following sacrifice, cardiac blood, testis, and epididymis tissues were collected for further evaluation.

Table 1 Experimental groups, dosage regimens, and routes of administration.

Group	Induction (day 0, single i.p.)	Treatment (days 3–42, daily oral gavage)
Control	Citrate buffer	Water
VitC	Citrate buffer	VitC (100 mg/kg)
DM	STZ (120 mg/kg)	Water
DM+VitC	STZ (120 mg/kg)	VitC (100 mg/kg)
DM+Met	STZ (120 mg/kg)	Met (250 mg/kg)
DM+VitC+Met	STZ (120 mg/kg)	VitC (100 mg/kg) + Met (250 mg/kg)

Note. STZ dissolved in citrate buffer (pH = 4.5); Met and VitC dissolved in water. i.p. = intraperitoneal; VitC = vitamin C; DM = diabetes mellitus; Met = metformin; STZ = streptozotocin.

Evaluation of sperm count, motility, and morphology

Spermatozoa were obtained from the cauda of the epididymis by making incisions in phosphate-buffered saline (PBS) and incubated at 37 °C for 30 min (Abdelmonem et al., 2024). Then the supernatant PBS was collected to evaluate sperm concentration and motility using a Makler chamber maintained at 37 °C, under 400× magnification. Spermatozoa were classified as motile or immotile, and the results were expressed as a percentage of total sperm counted. Morphology assessments were conducted using the Spermac stain (SPS50, FertiPro, Belgium) according to the manufacturer's instructions. A total of 200 spermatozoa were evaluated under 1,000× magnification (oil immersion) for morphological assessment. Spermatozoa with no detectable head or tail abnormalities were recorded as the percentages of morphologically normal sperm.

Immunofluorescent staining of TOMM20 and DNAH7 proteins in sperm

After evaluating the sperm count, motility, and morphology, the remaining samples were fixed with 4% paraformaldehyde (PFA). Smears were prepared on poly-L-lysine-coated glass slides for immunofluorescence analysis. Heat-induced antigen retrieval was performed using citrate buffer solution, followed by blocking with 1% bovine serum albumin (BSA) to prevent nonspecific binding. The slides were incubated overnight at +4 °C with primary antibodies DNAH7 (Novus Biologicals, NBP1-93613,1:200)

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or TOMM20 (Thermo Fisher Scientific, PA5-52843 0.1 mg/ml). After washing with PBS, the slides were incubated with Alexa Fluor488-conjugated secondary antibody (LOT: 342973-1, 1:100) for 1 hr. Nuclei were stained with DAPI (ab104139) for 5 min and coverslips were mounted. Negative controls were included by performing staining without the primary antibody to confirm specificity and exclude nonspecific binding. Immunopositive spermatozoa were quantified by counting 200 sperm cells per slide under a fluorescence microscope (LEICA, Germany). Quantification of immunopositive spermatozoa was conducted by a histologist blinded to sample identity to minimize observer bias.

Determination of oxidative stress from serum

Blood samples were centrifugated to evaluate oxidative stress parameters. The protein concentrations in obtained serum samples were measured using the bicinchoninic acid method. Glutathione (BTLAB, Cat. No. EA01004Mo) and SOD (BTLAB, Cat. No.: E2608Mo) levels were evaluated with a spectrophotometer in accordance with the manufacturer's enzyme-linked immunosorbent assay kit protocols. Hydrogen peroxide concentrations were assessed using a colorimetric assay kit (Elabscience, Cat. No. E-BC- K102-S) according to the manufacturer's protocol.

Evaluation of sperm viability and apoptosis

The percentage of apoptotic sperm in mice was determined using the Muse™ Annexin V & Dead Cell Kit. Sperms were collected and concentrated to 5×10^5 cells/ml, and washed twice within cold PBS. The suspension was stained by 100 µl of Muse® Annexin V Dead (Merck Millipore, Germany) and incubated for 30 min in the dark. Following incubation, the stained sperms were analysed using Muse Cell Analyzer (Merck Millipore, Germany).

Gene expression analysis

Total RNA was isolated from the testicular tissues using a total RNA isolation kit (PureLink, 12183018A) according to the manufacturer's protocol. The quality and concentration of the obtained RNA were measured at 260 nm using Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The changes in mRNA levels of selected genes were measured by the StepOne Plus Real Time PCR (Applied Biosystems Invitrogen, USA). The commercial primer sets of DNAH7 (Mm01254595_m1, Thermo Fisher Scientific, USA), TOMM20 (Mm02601883_g1, Thermo Fisher Scientific, USA), and ACTB (Mm02619580_g1, Thermo Fisher Scientific, USA) were used. β-Actin was accepted as an endogenous reference gene. The thermocycling conditions were as follows: denaturation at 95 °C for 10 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min for 40 cycles. The experiments were conducted in triplicate.

Evaluation of Tomm20, Caspase-3, and PCNA protein levels

The amount of protein obtained from the testis using RIPA buffer was determined using a Qubit 4.0 Flurometer (Invitrogen, USA). Each sample was prepared by mixing NuPAGE LDS Sample Buffer (4X) and NuPAGE Sample Reducing Agent (10X) to obtain 30 µg of protein. The samples were loaded onto Bolt 4%–12%, Bis-Tris, 1.0 mm, Mini Protein Gels. Proteins were run at 200 V for 22 min in a mini-gel tank system (Thermo Fisher Scientific, USA). At the end of the run, the transfer was performed using an iBlot Transfer Stack with an iBlot 2 gel transfer system (Thermo Fisher Scientific, USA). Primary antibodies including anti-Tomm20 (0.04 µg/mL), anti-proliferating cell nuclear antigen (PCNA, 1:2,000, Novus Biologicals NB500-106), anti-Caspase-3 (1:1,000, Novus Biologicals NB100-56113), and anti-ACTB (1:1,000, sc47778, Santa Cruz, USA), and secondary antibody (1:1,000, 31430, 31460, Thermo Fisher Scientific, USA) were incubated for 4 hr in an iBind Flex (Thermo Fisher Scientific, USA) device. The membranes were evaluated in a chemiluminescence imaging system (Syngene, UK) using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA).

Histopathological examination of testis

Unilateral testes were fixed in Bouin solution for 24 hr (Kaneda et al., 2023). Tissue samples were washed and dehydrated with increasing concentrations of ethanol. The samples were clarified with xylene. After tissue processing, the specimens were embedded in paraffin. Sections (5 µm thick) were stained with haematoxylin and eosin. Histological evaluations were conducted by investigators blinded to the treatment groups. Johnsen score (Table S1) was used for the testicular evaluation (Guo et al., 2023; Johnsen, 1970; Nguyen-Thanh et al., 2022).

PCNA and Caspase-3 immunohistochemical examination

To verify the protein expression levels in the seminiferous tubules, immunohistochemical staining was performed on paraffin sections. The testis sections were rehydrated, and antigen retrieval was performed using citrate buffer solution. Endogenous peroxidase activity was inhibited with H_2O_2 and antigen blocking was performed with ultraviolet block solution. The preparations were incubated with primary antibodies overnight at +4 °C (anti-PCNA [1:1,000, Novus Biologicals NB500-106] and anti-Caspase-3 [1:1,250, Novus Biologicals NB100-56113]). After incubation with the secondary antibody and streptavidin peroxidase (UltraVision Detection System Large Volume Anti-Polyvalent, HRP, TP-125-HL, Thermo Fisher Scientific, USA), aminoethyl carbazole was used as the chromogen. The counter stain was completed with haematoxylin. Negative control in immunohistochemistry was performed by processing tissue sections without the addition of the primary antibody. Caspase-3 positivity in seminiferous tubules was calculated using the area fraction of ImageJ (National Insitutes of Health) and PCNA positivity calculated with the ratio of the positive nuclei to all nuclei within the seminiferous tubules

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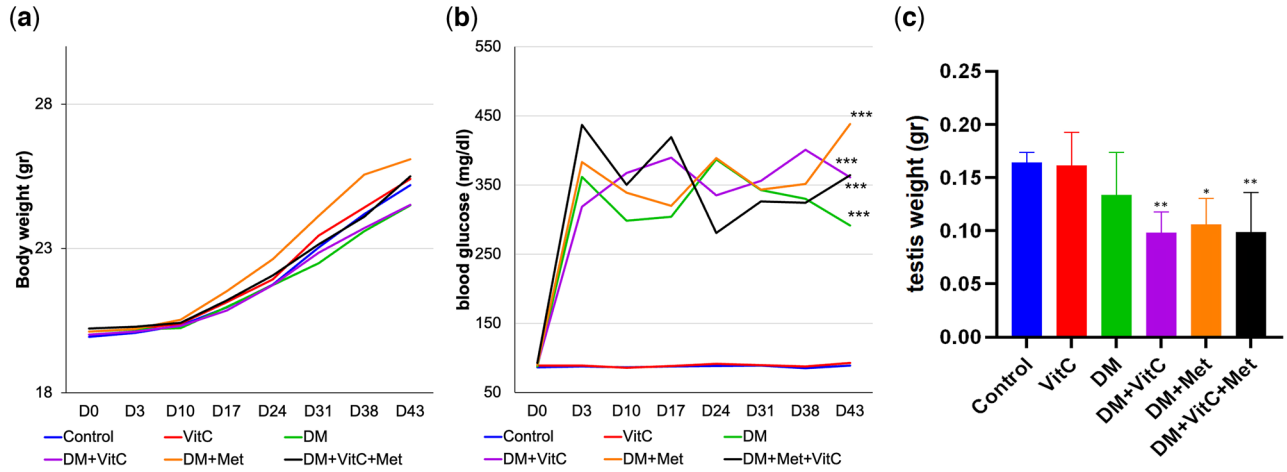


Figure 1 Body weight (A) and blood glucose (B) concentration changes of groups during the experiments. All groups gained weight and differences between groups were insignificant. Blood glucose levels of diabetic animals remained significantly higher than control group after diabetes induction with STZ. Data are presented as mean values. Testis weights (C) of diabetic groups reduced compared with control group. Bar plot represents mean ± standard deviation. Statistical significance is indicated by asterisks relative to the control group (*$p<0.05$, **$p<0.01$, ***$p<0.001$). *Note*: VitC = vitamin C group; DM = diabetes mellitus group; DM+VitC = diabetes mellitus with vitamin C treatment group; DM+Met = diabetes mellitus with metformin treatment group; DM+VitC+Met = diabetes mellitus treatment with vitamin C and metformin combination group; D = day; STZ = streptozotocin.

(Nofal et al., 2023). Quantitative assessment of immunopositivity was carried out by observers blinded to experimental groups to ensure objective analysis.

Statistical analysis

Statistical analyses were performed with SPSS (version 26.0, IBM) and Prism (version 10.5.0, GraphPad). For multiple comparisons against the control group, a one-way analysis of variance followed by Dunnett's test was used. A nonparametric Kruskal–Wallis test was performed to compare Johnsen scores across groups. An adjusted *p*-value <0.05 was considered statistically significant. Gene expression was determined using data analysis programs (REST [2009 V2.0.13]), and changes in the expression levels of genes were identified as fold changes.

Results

Animal and testis weights, and blood glucose measurement

Weekly blood sugar and weight measurements were performed to monitor the systemic effects of DM and treatments. The body weights (mean ± standard deviation [SD]) of the groups at day zero were 19.94 ± 0.23, 20.13 ± 0.44, 20.12 ± 0.3, 20.01 ± 0.56, 20.12 ± 0.63, and 20.23 ± 0.29 g for the control, VitC, DM, DM+VitC, DM+Met, and DM+VitC+Met groups, respectively. The difference in initial body weight among the groups was statistically insignificant ($p>0.05$). Body weight increased in all groups during the experiment. The body weight differences among the groups were not statistically significant on the last day (control [25.19 ± 1.21 g], VitC [25.19 ± 1.19 g], DM [24.5 ± 0.73 g], DM+VitC [24.5 ± 1.91 g], DM+Met [26.09 ± 1.26 g], and DM+VitC+Met [25.5 ± 0.82 g], $p>0.05$). Mean body weight changes are shown in Figure 1A. Weekly measurements and *p*-value were presented in Table S1.

Blood glucose levels of the DM groups were above 200 mg/dl from day 3 to day 43, while the blood glucose levels of the control and VitC groups were below 100 mg/dl (Figure 1B). Weekly measurements from day 3 to day 43 revealed that mean blood glucose levels of the control group were significantly lower than those of the DM, DM+VitC, DM+Met, and DM+VitC+Met groups ($p<0.05$, Table S1).

Testis weight measurements (mean ± SD) were significantly decreased in the DM+VitC (0.10 ± 0.02 g, $p=0.007$), DM+Met (0.12 ± 0.03 g, $p=0.024$), and DM+VitC+Met (0.08 ± 0.01 g, $p=0.007$) groups compared with the control group (0.16 ± 0.01 g). The differences between the DM (0.13 ± 0.04 g), VitC (0.16 ± 0.03 g) and control groups were statistically insignificant ($p>0.05$). Testis weights are shown in Figure 1C.

Sperm count, motility, and morphology

The effect of DM on fertility was further investigated by evaluating the total sperm count, motility, and morphology (Figure 2A). The total sperm count (mean ± SD) changes among groups were not statistically significant ($p>0.05$, control [16 ± 6.9 × 10^6], VitC [13.5 ± 6.4 × 10^6], DM [13.8 ± 6.8 × 10^6], DM+VitC [11.9 ± 6.7 × 10^6], DM+Met [13.5 ± 5.7 × 10^6], and DM+VitC+Met [13.7 ± 3.9 × 10^6]). The motile sperm percentage (mean ± SD) changes among groups were not statistically significant ($p>0.05$, control [59 ± 5], VitC [59 ± 7], DM [55 ± 3%, DM+VitC [56 ± 3], DM+Met [55 ± 3], and DM+VitC+Met [57 ± 5]). Normal sperm morphology percentage (mean ± SD) changes among groups were not statistically significant ($p>0.05$, control [51.2 ± 7.9], VitC [47.7 ± 6.8] DM [45.9 ± 8.4], DM+VitC [39.8 ± 4.6], DM+Met [41.3 ± 7.4], and DM+VitC+Met [44.8 ± 4.5]).

TOMM20 and DNAH7 immunofluorescence staining in sperm

TOMM20 is part of the mitochondrial membrane and DNAH7 is found in the flagella of spermatozoa (Figure 2B). Immunofluorescence analysis revealed that TOMM20 was localized in

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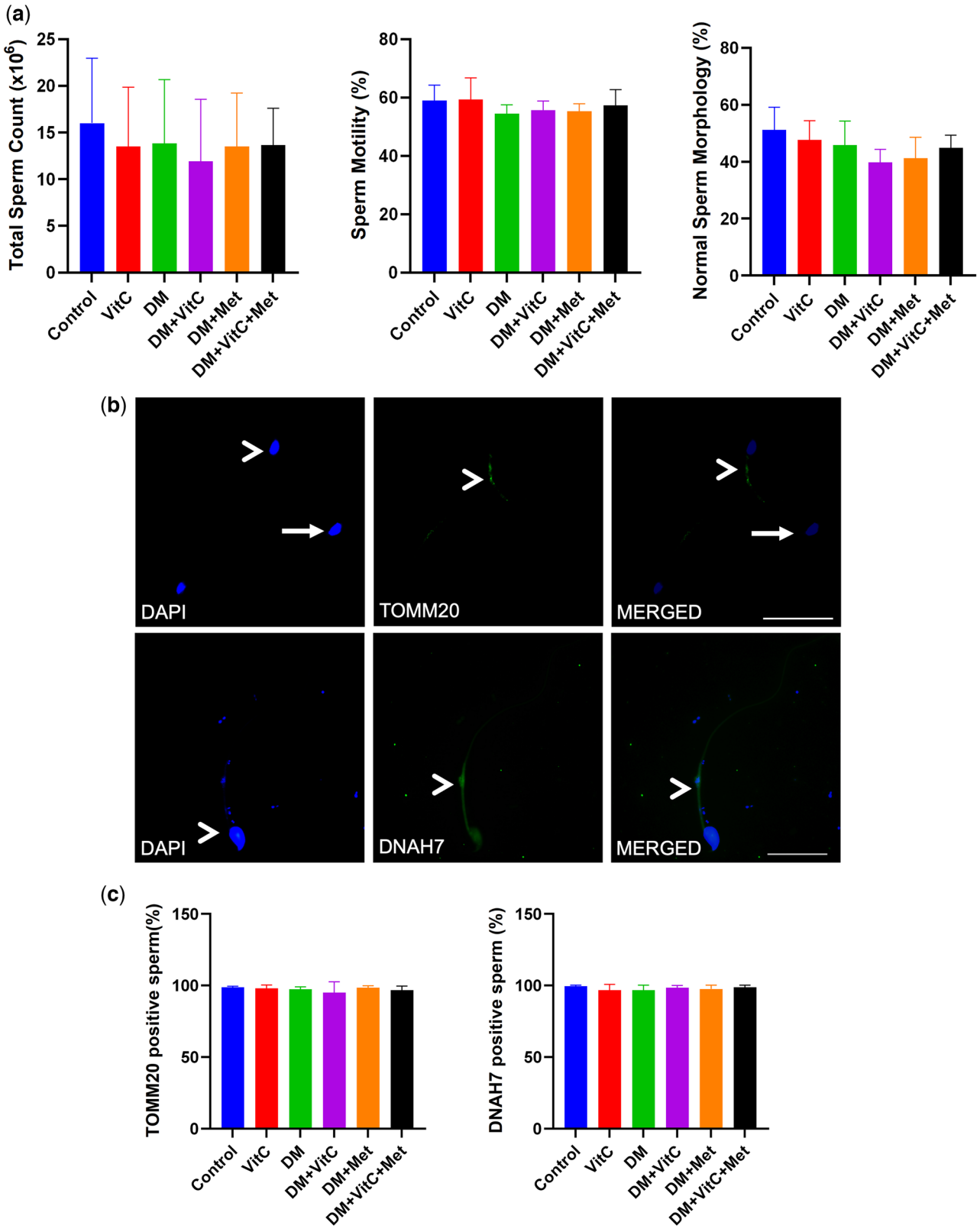


Figure 2 Total sperm count, sperm motility, and normal sperm morphology reduced in diabetic groups (A). Vitamin C and metformin combination treatments slightly increase sperm motility. The first row of micrographs (B) shows DAPI, TOMM20, and MERGED; TOMM20-positive sperm is shown with an arrowhead while TOMM20-negative sperm is shown with an arrow. The second row of micrographs represent DAPI, DNAH7, and merged images. DNAH7-positive sperm is shown with arrowhead. (C) TOMM20-positive sperm percentages and DNAH7-positive sperm percentages among groups did not change significantly. Scale bars represent 20 µm. Micrographs were captured using a 100× objective. Bar plots represent mean ± standard deviation. *Note*: VitC = vitamin C group; DM = diabetes mellitus group; DM+VitC = diabetes mellitus with vitamin C treatment group; DM+Met = diabetes mellitus with metformin treatment group; DM+VitC+Met = diabetes mellitus treatment with vitamin C and metformin combination group.

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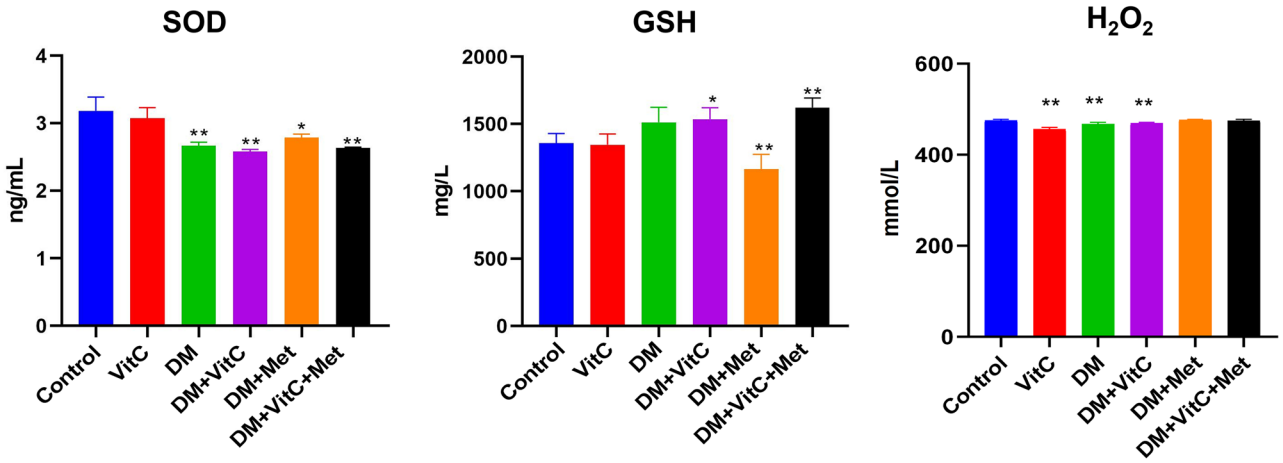


Figure 3 Serum oxidative stress markers show SOD levels significantly reduced in diabetic groups while GSH levels significantly increased in DM+VitC and DM+VitC+Met groups. Significant reduction of GSH level was detected in DM+Met group. The H_2O_2 levels significantly decreased in VitC, DM and DM+VitC groups compared with control. Data are presented as mean ± standard deviation. Statistical significance is indicated by asterisks relative to the control group (*$p<0.05$, **$p<0.01$). *Note*: SOD = superoxide dismutase; GSH = glutathione; H_2O_2 = hydrogen peroxide VitC = vitamin C group; DM = diabetes mellitus group; DM+VitC = diabetes mellitus with vitamin C treatment group; DM+Met = diabetes mellitus with metformin treatment group; DM+VitC+Met = diabetes mellitus treatment with vitamin C and metformin combination group.

the midpiece region of spermatozoa. The mean percentage of TOMM20-positive sperm (Figure 2C) was 98.75% in the control group, compared with the VitC (98.08%), DM (97.42%), DM+VitC (95.08%), DM+Met (98.41%), and DM+VitC+Met (96.75%) groups ($p>0.05$, Figure 2C). Furthermore, DNAH7 protein was localized in the flagella of the spermatozoa. The mean percentage of DNAH7-positive sperm was 99.6% in the control group, while the mean percentage of DNAH7 was 96.75%, 96.83%, 98.42%, 97.5%, and 98.75% in the VitC, DM, DM+VitC, DM+Met, and DM+VitC+Met groups, respectively ($p>0.05$, Figure 2C).

Evaluation of changes in the oxidative stress markers

To evaluate systemic oxidative stress biomarkers in the serum, the levels of SOD, GSH, and H_2O_2 were determined (Figure 3). The mean SOD level in the control group (3.2 ng/ml) was significantly higher than in the DM (2.67 ng/ml), DM+VitC (2.58 ng/ml), and DM+VitC+Met (2.63 ng/ml) groups ($p<0.01$). The decrease of SOD level in DM+Met (2.79 ng/mL) was statistically significant compared with the control group ($p<0.05$). Mean GSH levels were 1,357.7, 1,345.5, 1,511.8, 1,536.6, 1,163.7, and 1,622.6 ng/ml in the control, VitC, DM, DM+VitC, DM+Met, and DM+VitC+Met groups, respectively. The increased GSH levels in the DM+VitC and DM+VitC+Met groups were statistically significant ($p<0.05$). The DM+Met group had significantly lower levels than the control group ($p<0.01$). The mean H_2O_2 level in the control group (475.6 mmol/L) was significantly higher than in the VitC (456.3 mmol/L), DM (468 mmol/L), and DM+VitC (469.3 mmol/L) groups ($p<0.01$).

Sperm cell death assessment

Spermatozoa viability and apoptosis were evaluated using Annexin V analysis (Figure 4A). Sperm viability (Figure 4B) was significantly lower in VitC (80.82%), DM+VitC (87.2%), and DM+Met (79.08%) groups than in the control group (96.97%) ($p<0.01$). In the control, VitC, DM, DM+VitC, DM+Met, and DM+VitC+Met groups, early apoptotic death rates (Figure 4B) were 1.75%, 14.57%, 0.483%, 1.933%, 1.283%, and 3.15%, respectively, while late apoptotic death (Figure 4B) rates were 0.80%, 2.917%, 3.183%, 7.15%, 13.77%, and 3.117%, respectively. Although early apoptosis was significantly increased in the VitC group compared with the control ($p<0.01$), a significant increase in late apoptotic death was observed in the DM+VitC ($p<0.05$) and DM+Met ($p<0.01$) groups.

Tomm20 and Dnah7 expression levels in the testis

Spermatogenesis occurs within the seminiferous tubules of the testes; therefore, the mRNA levels of *Dnah7* and *Tomm20* were measured to evaluate gene expression, and the results are summarized in Figure 5A. Compared with the control group, *Dnah7* gene expression was significantly increased by 7.7-, 13.9-, and 26.2-fold in the DM+VitC, DM+Met, and DM+VitC+Met groups, respectively ($p<0.01$). *Dnah7* gene expression in VitC (1.2-fold) and DM (1.5-fold) showed no statistically significant changes compared with the control. *Tomm20* expression significantly increased in the DM+VitC+Met group (5.07-fold) compared with the control group (*pV*<C0.01). *Tomm20* expression levels in the VitC, DM, DM+VitC, and DM+Met groups showed no statistically significant changes (0.77-, 0.96-, 1.76-, and 2.13-fold, respectively; $p>0.05$) compared with the control group.

Assessment of protein levels in the testis

TOMM20, PCNA, and Caspase-3 protein levels in the testis were evaluated using western blot analysis (Figure 5B, C). TOMM20 levels in the VitC (3.1-fold, $p<0.01$), DM (3.08-fold, $p<0.05$), and

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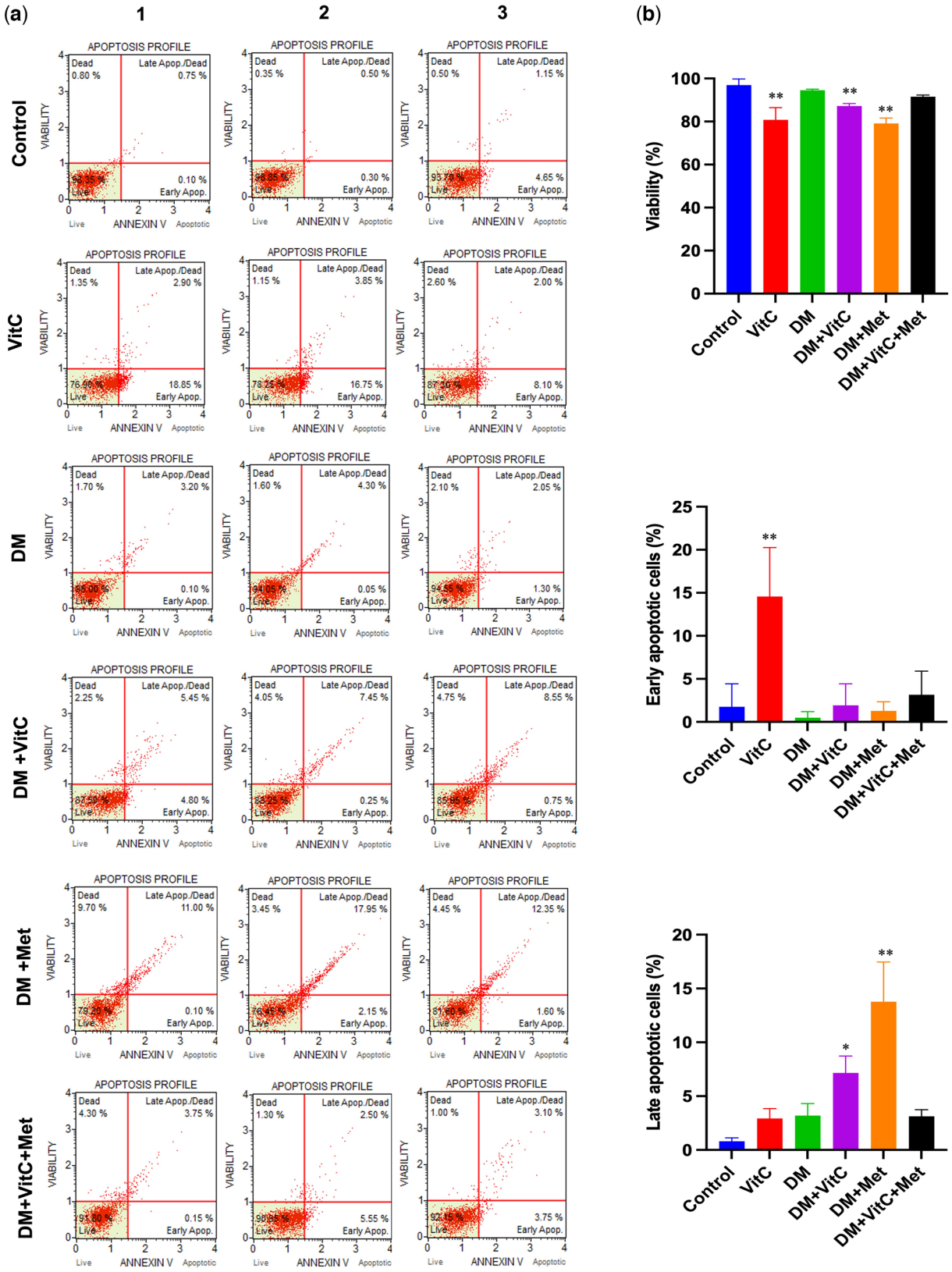


Figure 4 Apoptosis analysis with Muse Analysis graph examples from each group are presented in section (A). Sperm viability (B) significantly decreases in VitC, DM+VitC, and DM+Met groups. Early apoptotic spermatozoa were significantly increased in VitC, whereas in DM+VitC and DM+Met late apoptotic cells were prominent. Data are presented as mean ± standard deviation. Statistical significance is indicated by asterisks relative to the control group (*$p < 0.05$, **$p < 0.01$). *Note*: VitC = vitamin C group; DM = diabetes mellitus group; DM+VitC = diabetes mellitus with vitamin C treatment group; DM+Met = diabetes mellitus with metformin treatment group; DM+VitC+Met = diabetes mellitus treatment with vitamin C and metformin combination group.

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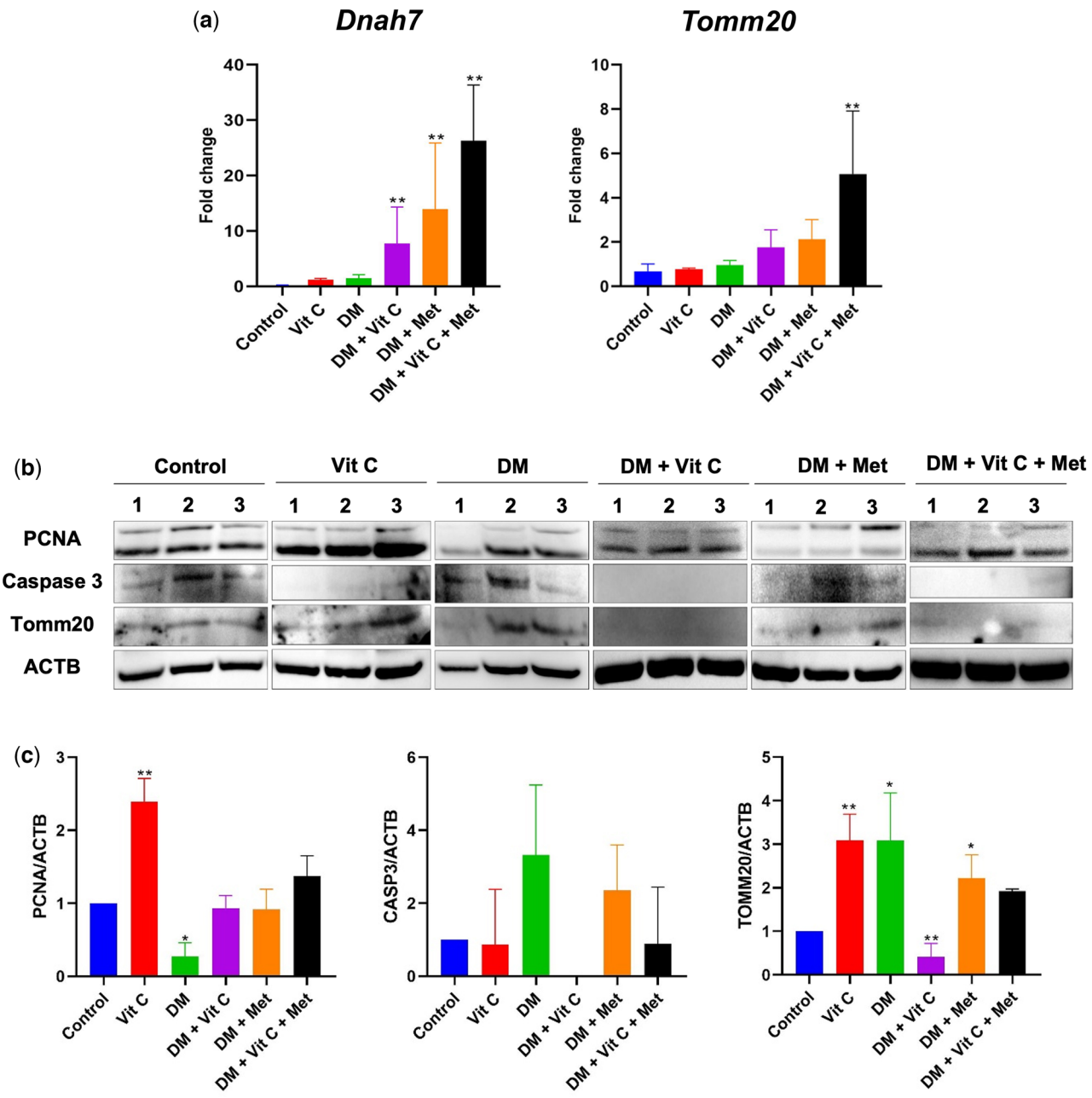


Figure 5 *Dnah7* and *Tomm20* mRNA fold changes in testis among groups (A). *Dnah7* gene expression significantly increased in diabetic groups with vitamin C and metformin treatment either alone or in combination. *Tomm20* gene expression significantly increased in combination treatment. (B) Western blot gel images of PCNA, Caspase-3, TOMM20, and actin proteins of testis are presented. (C) According to western blot analysis, PCNA protein expression significantly increased with vitamin C treatment, but significantly decreased in the untreated DM group. Caspase-3 protein expression increased in untreated DM group. TOMM20 expressions significantly increased in the VitC, DM and DM+Met groups, while there was a significant decrease in the DM+VitC group. Data are presented as mean ± standard deviation. Statistical significance is indicated by asterisks relative to the control group (*$p<0.05$, **$p<0.01$). *Note*: VitC = vitamin C group; DM = diabetes mellitus group; DM+VitC = diabetes mellitus with vitamin C treatment group; DM+Met = diabetes mellitus with metformin treatment group; DM+VitC+Met = diabetes mellitus treatment with vitamin C and metformin combination group; PCNA = proliferating cell nuclear antigen.

DM+Met (2.22-fold, $p<0.05$) groups were significantly increased compared with the control group. Significantly reduced TOMM20 protein levels were detected in DM+VitC (0.41) compared with the control ($p<0.05$). The TOMM20 protein level in DM+VitC+Met (1.92) did not differ significantly from the control group ($p>0.05$).

Cell proliferation in the testis was evaluated using PCNA protein levels (Figure 5B, C). Compared with the control group, PCNA protein level in the VitC group (2.39) significantly increased ($p<0.01$), while a considerable reduction was detected in the DM group (0.27) ($p<0.05$). Changes in PCNA protein levels in the DM+VitC (0.94), DM+Met (0.92), and DM+VitC+Met (1.37) groups were statistically insignificant compared with the control group ($p>0.05$).

Apoptosis in the testis was evaluated by measuring Caspase-3 protein levels (Figure 5B, C). Changes in Caspase-3 protein levels among VitC (0.87), DM (3.32), DM+VitC (0), DM+Met (2.36), and DM+VitC+Met (0.89) groups were statistically insignificant ($p>0.05$).

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Morphological and immunohistochemical evaluations of the testis

Structural changes in the seminiferous tubules were evaluated using haematoxylin and eosin staining. Histological examination of the seminiferous tubules revealed the presence of spermatozoa in the lumen, as well as spermatogenic cells such as late and early spermatids and spermatogonia in all samples (Figure 6A). Compared with control group (median: 8, quartile 1–quartile 3: 8.75–9.25), Johnsen scores showed a statistically significant difference in the DM (8, 8–8, $p<0.01$), DM+VitC (8, 8–8, $p<0.01$), and DM+Met (8, 8–8.25, $p<0.05$). However the VitC (8, 8–9) and

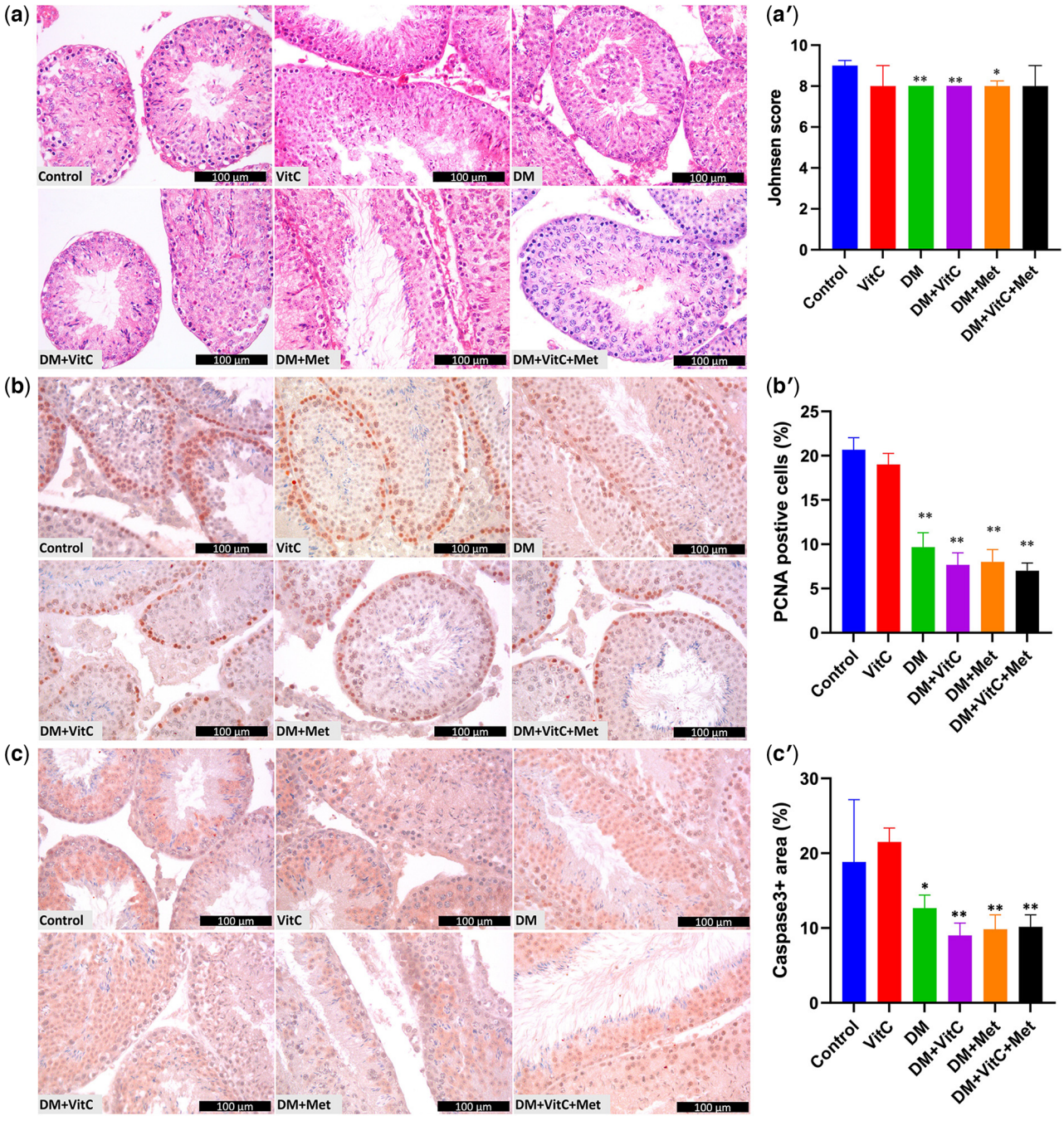


Figure 6 Haematoxylin and eosin-stained micrographs showing seminiferous tubules (A). Johnsen score was significantly reduced in DM, DM+VitC, and DM+Met groups compared with the control. Bar plot represents median and interquartile range (A). PCNA-positive cell nuclei are shown in red in the seminiferous tubules (B). The percentages of PCNA-positive cells in seminiferous tubules were significantly reduced in the diabetic groups. Bar plot represents mean ± standard deviation (C). Caspase-3-positive area in seminiferous tubules is presented in red (C); the area fraction was significantly reduced in diabetic groups (C). Bar plot represents mean ± standard deviation. Statistical significance is indicated by asterisks relative to the control group (*$p<0.05$, **$p<0.01$). Scale bars represent 100 µm. Micrographs were captured using a 40× objective. *Note*: VitC = vitamin C group; DM = diabetes mellitus group; DM+VitC = diabetes mellitus with vitamin C treatment group; DM+Met = diabetes mellitus with metformin treatment group; DM+VitC+Met = diabetes mellitus treatment with vitamin C and metformin combination group; PCNA = proliferating cell nuclear antigen.

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DM+VitC+Met (8, 8–9) groups showed no significant difference ($p > 0.05$, Figure 6A).

Cell proliferation within the seminiferous tubules was evaluated using PCNA expression. In the seminiferous tubules, PCNA-immunopositive cells were primarily identified as spermatogonia and primary spermatocytes (Figure 6B). The mean percentage of immunopositive cells in the seminiferous tubules was significantly higher in the control (20.7%) than DM (9.7%), DM+VitC (7.7%), DM+Met (8.0%), and DM+VitC+Met (6.8%) groups ($p < 0.01$, Figure 6B).

Apoptosis within the seminiferous tubules was evaluated using Caspase-3 staining. In the seminiferous tubules, Caspase-3 immunopositive cells were predominantly identified in the adluminal area (Figure 6C). The mean percentage of immunopositive cells in the seminiferous tubules was significantly higher in the control (18.8%) than DM (12.6%, $p < 0.05$), DM+VitC (9.0%, $p < 0.01$), DM+Met (9.83% $p < 0.01$), and DM+VitC+Met (10.17%, $p < 0.01$) groups (Figure 6C).

Discussion

This study investigated the protective effects of VitC and Met treatment against DM-associated reproductive dysfunction. The combination of VitC and Met for the treatment of STZ-induced DM was more effective than monotherapy, as it increased sperm viability, *Dnah7* and *Tomm20* gene expression in the testis, and the Johnsen score. Thus, the combination of VitC and Met ameliorated the detrimental molecular effects of DM on testicular tissue. Diabetes induction with STZ is a common method in animal studies (Kottaisamy et al., 2021). In our study, all groups with STZ-induced diabetes exhibited elevated blood glucose levels, indicating the successful establishment of the experimental model. Consistent with Shrilatha and Muralidhara (2007), we demonstrated that VitC treatment of DM did not significantly reduce glucose levels. The persistence of hyperglycaemia in Met-treated DM groups compared with the control is aligned with a previous study of Liu et al. (2019) and Behdarvand-Margha et al. (2021). These findings suggest that the glucose-lowering effect of Met is limited in DM models characterized by destruction of pancreatic β-cells. Despite persistent hyperglycaemia, the treatments promoted weight gain in the DM groups, suggesting that they may exert systemic effects independent of their glucose-lowering efficacy. The diabetic mice treated with Met exhibited lower testicular weights than the control mice. Behdarvand-Margha et al. (2021) reported that a 21-day Met treatment (250 mg/kg) significantly decreased fasting glucose levels in methylglyoxal-induced diabetic NMRI mice, although no significant changes in testicular weight. Similarly, Khorasani et al. (2021) found that 14-day Met treatment (150 mg/kg) significantly reduced blood glucose levels in methylglyoxal-induced diabetic NMRI mice, although no significant changes were observed in body or testicular weights. Liu et al. (2019) demonstrated that prolonged hyperglycaemia, lasting >2 months, decreased testicular weight. In our study, lower testicular weights than those of the control group were associated with different Met treatment protocols, including dose and duration. Additionally, preserved Johnsen scores, despite reduced testicular weights in the DM+VitC+Met group, suggest that combined treatment effectively mitigated the progression of testicular atrophy.

Oxidative stress exerts paradoxical effects on male fertility: while physiological levels of reactive oxygen species ROS are essential for sperm capacitation and fertilization, excessive accumulation triggers mitochondrial dysfunction and DNA fragmentation (Moustakli et al., 2024). The results of our study align with the existing literature, indicating that hyperglycaemia is associated with decreased serum SOD activity (Behdarvand-Margha et al., 2021; Khorasani et al., 2021; Soetan et al., 2024; Zhai et al., 2022). We propose that the concurrent reduction in H_2O_2 levels, alongside decreased activity in the DM group, indicates an increase in deleterious superoxide ($O_2^{\cdot-}$) radicals. Previous research has demonstrated that Met treatment effectively maintains glycaemic control while preventing a reduction in SOD activity (Behdarvand-Margha et al., 2021; Khorasani et al., 2021). However, the observed decline in SOD and GSH in our DM+Met group suggests that the antioxidant capacity of Met may be insufficient under conditions of persistent hyperglycaemia. Previous research has established that the decrease in serum GSH levels caused by diabetes can be ameliorated through antioxidant supplementation (Soetan et al., 2024; Zhai et al., 2022). In alignment with these findings, the observed increase in GSH levels in our DM+VitC and DM+VitC+Met groups, despite the persistence of hyperglycaemia, indicates the antioxidant effectiveness of the combination treatment.

Exogenous oxidative stress increases mitochondrial membrane potential in human spermatozoa, leading to apoptosis due to mitochondrial dysfunction (Bravo et al., 2021, 2024). In our study, we observed an increase in early apoptotic sperm and a decrease in sperm viability within the VitC-treated group. Similarly, Talebi et al. (2014) reported reduced sperm viability in mice treated with VitC. This phenomenon may be attributed to the disruption of the oxidative balance essential to normal sperm function, caused by excessive antioxidant intake (Moustakli et al., 2024). Furthermore, decreased sperm viability was accompanied by an increased number of late apoptotic sperm in both the DM+VitC and DM+Met groups. In this context, a higher percentage of late apoptotic cells in human spermatozoa is associated with DNA fragmentation and subfertility (Shen et al., 2002) and thus further studies associated with the protective effects of Met or VitC alone for the reversal of DM induced sperm viability are required. However, the combination effects of Met and VitC maintained the sperm viability and did not induce apoptotic death. In this context, the synergism between Met and VitC restored the DM-induced damage to the sperm.

Diabetes mellitus has different effects on sperm parameters. Lotti and Maggi (2023) stated that DM has a negative effect on sperm morphology, while having contradictory effects on other semen parameters in humans. In our study, differences in total sperm count, sperm motility, and normal sperm morphology among the groups were not statistically significant. Our findings aligned with the study of Pouriayevali et al. (2024). They reported that STZ-induced diabetes does not have a significant effect on sperm motility. The reduction in sperm count observed by Khorasani et al. (2021) in diabetic mice may be attributed to the use of a different mouse strain and to the induction of diabetes via 30 days of methylglyoxal administration. Furthermore, the significant decline in sperm count reported by Liu et al. (2019) at 2 months and beyond can be explained as a consequence of the chronic complications of diabetes. Therefore, a decrease in the

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sperm count could relate to the treatment protocol for DM and the mouse strain. In this context, further studies should be conducted to determine each difference involved in the development of STZ-induced diabetes.

Additionally, mitochondrial and axonemal structures are closely linked to sperm motility and morphology. In the literature, the loss or dysfunction of key structural proteins, such as TOMM20 and DNAH7, is directly associated with male infertility. For instance, biallelic loss of the *SEPTIN4* gene in humans reduces TOMM20 positivity in sperm (Wang et al., 2022), while DNAH7 dysfunction in mice leads to mitochondrial displacement and axonemal defects (Gao et al., 2022). Neesen et al. (2001) demonstrated that the targeted deletion of an inner dynein arm heavy chain gene in mice resulted in male infertility due to immotile sperm. In our study, TOMM20 was localized to the midpiece, whereas DNAH7 was observed along the flagella in all groups. The stability observed in mitochondrial and flagellar structures indicated the consistent motility and morphological parameters across groups. We propose that a robust antioxidant defence system within the epididymal lumen effectively neutralizes ROS, thereby protecting maturing spermatozoa from oxidative damage (Chabory et al., 2009). This enzymatic protection likely serves as a critical compensatory mechanism for preserving the structural and functional integrity of the sperm flagellum and mitochondria, despite the systemic hyperglycaemic stress observed in our model. Additionally, the effects of DM and the subsequent combination treatment were more prominent in the testis. We observed a significant upregulation of *Dnah7* mRNA levels in the DM+VitC, DM+Met, and DM+VitC+Met groups, indicating an active molecular response to ensure the production of essential flagellar proteins. This response supports the continuity of spermatogenesis despite the diabetic environment.

In the testis, the axoneme is restricted to germ cells and the ciliated epithelia of the rete testis and efferent ducts (Linck et al., 2016), whereas mitochondria are prevalent in both germ and various somatic cells (Ramalho-Santos et al., 2009). In the current study, significant upregulation of *Dnah7* was observed in DM+VitC, DM+Met, and DM+VitC+Met, whereas a significant increase in *Tomm20* mRNA expression was detected only in DM+VitC+Met. On the other hand, testicular TOMM20 protein levels were significantly elevated in the VitC, DM, and DM+Met groups compared with the control group. The differences between mRNA and protein levels of TOMM20 could be associated with transcriptional and posttranscriptional modifications, epigenetic regulations and the activation of the ubiquitin-proteasome system (Sarkar et al., 2017; Wu et al., 2022; Xiong et al., 2022). Therefore, further molecular investigations could be performed to examine the role of epigenetic mechanisms in the reversal of DM-induced testicular damage.

Diabetes mellitus has a detrimental impact on spermatogenesis, resulting in a marked reduction in Johnsen scores, which assess the integrity and completion of the spermatogenic process (Abdelmonem et al., 2024; Badejogbin et al., 2025; Sayed et al., 2023). In our study, reduced Johnsen scores were observed in the DM, DM+VitC, and DM+Met groups compared with the control, whereas the Johnsen score in the DM+VitC+Met group remained preserved. Our data demonstrated that combination therapy effectively counteracts the negative impact of hyperglycemia on spermatogenesis. Immunohistochemical analyses revealed prominent PCNA expression in spermatogonia and primary spermatocytes. The PCNA-positive cell percentages of seminiferous tubules significantly decreased in the DM groups with or without treatments, indicating a decrease in cell proliferation within seminiferous tubules. The observed reduction in PCNA levels within the seminiferous tubules of the DM group was consistent with previous findings (Annie et al., 2020; Das et al., 2021; Ranjan et al., 2020; Nna et al., 2020). Therefore, PCNA expression could be increased in extratubular compartments, such as Leydig, vascular, or peritubular cells, due to no significant differences in total testicular PCNA protein levels among the DM+VitC, DM+Met, and DM+VitC+Met groups compared with the control. Furthermore, Caspase-3 levels were significantly lower in the seminiferous tubules of the DM groups compared with the control, indicating diminished apoptosis. In our study, differences in protein levels in the testis and seminiferous tubules reflected changes in cell proliferation and apoptosis in nonseminiferous tubule cells. Alterations in the balance between proliferation and apoptosis during spermatogenesis may lead to changes in sperm output (Sharma et al., 2023). In DM groups, reduced PCNA and Caspase-3 positivity in seminiferous tubules may reflect impaired hormonal feedback, decreased cell proliferation, and inadequate apoptosis, possibly resulting in an increased proportion of abnormal sperm (Andlib et al., 2023). These results suggest that combination therapy may ameliorate the effects of DM on Leydig cells and steroidogenesis.

In conclusion, DM leads to systemic hyperglycaemia, a significant reduction in Johnsen scores, and suppression of testicular cell proliferation. The combined administration of Met and VitC upregulated the expression of *Dnah7* and *Tomm20* in the testis, while preserving sperm viability, Johnsen scores, and testicular cell proliferation at levels comparable to those in the healthy control. These findings suggest that the coadministration of Met and VitC may offer a promising therapeutic strategy for mitigating DM-related reproductive dysfunction. Additionally, differential gene and protein levels may reflect the involvement of complex regulatory pathways, including epigenetic mechanisms. Future research should investigate these pathways and their effects on steroidogenesis, spermatogenesis, and male fertility in DM. Long-term studies assessing fertility outcomes and dose-dependent effects of combination therapies are warranted.

Supplementary material

Supplementary material is available at *Reproduction* online.

Conflicts of interest

The authors declare no conflict of interest.

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Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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