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Red rice bran extract attenuates oxidative stress and testicular damage in high-fat diet/streptozotocin-induced diabetic rats

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ABSTRACT

Type 2 diabetes (T2D) is a chronic metabolic disorder known to impair male reproductive function through mechanisms involving insulin resistance, hyperglycemia, and oxidative stress. This study aimed to investigate the potential of red rice bran extract (RBE), a dietary antioxidant, alone or in combination with metformin (Met), in alleviating reproductive dysfunction in a T2D rat model. Male Wistar rats were divided into five groups: non-diabetic control, untreated diabetic, RBE-treated diabetic, Met-treated diabetic, and RBE+Met-treated diabetic. Diabetes was induced by a high-fat diet followed by a low-dose streptozotocin injection. After confirmation of diabetes, treatments were administered orally once daily for 12 weeks. Reproductive and oxidative stress markers were evaluated, including serum testosterone, sperm quality, Johnsen score (a histological index of spermatogenesis), antioxidant enzyme activities, and testicular histology. Diabetic rats showed significant reductions in testosterone levels, sperm parameters, Johnsen score, and testicular morphology, along with elevated malondialdehyde and reduced superoxide dismutase and glutathione levels. Treatment with RBE and/or Met improved sperm concentration, Johnsen score, and testicular architecture, and attenuated oxidative stress. However, sperm motility and testosterone levels did not return to control levels. These findings suggest that RBE may serve as a natural adjunctive therapy for diabetes-induced male reproductive impairment and warrant further investigation.

1. INTRODUCTION

Type 2 diabetes (T2D) accounts for approximately 96% of all diabetes cases and is primarily driven by obesity, sedentary lifestyles, and poor dietary habits [1]. The pathogenesis of T2D involves β-cell dysfunction and insulin resistance, leading to chronic hyperglycemia and metabolic disturbances [2,3]. Persistent hyperglycemia contributes to oxidative stress, inflammation, and cellular damage via apoptosis and necrosis, exacerbating disease complications [3–5]. Notably, obesity significantly increases the risk of developing T2D, with obese individuals being up to 80 times more likely to develop the condition than those with a normal body mass index [6]. Beyond metabolic dysfunction, chronic hyperglycemia is a key driver of diabetes-related comorbidities, including cardiovascular disease, neuropathy, retinopathy, and nephropathy [7]. Emerging evidence suggests that T2D also adversely affects male reproductive health, yet the underlying mechanisms remain incompletely understood. Metabolic imbalances associated with obesity and T2D have been implicated in testicular dysfunction, contributing to reduced fertility [8,9]. Oxidative stress plays a central role in diabetes-induced reproductive damage, leading to impaired steroidogenesis, disrupted spermatogenesis, and structural degeneration of the seminiferous tubules [10–14]. These alterations collectively exacerbate infertility risks, emphasizing the importance of antioxidants in mitigating diabetes-induced reproductive dysfunction. A well-established experimental model for studying T2D is the high-fat diet (HFD) combined

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with a low dose of streptozotocin (STZ), which mimics human T2D by inducing insulin resistance followed by selective β-cell impairment [15–17]. This model effectively reproduces metabolic disturbances observed in diabetic patients, making it valuable for investigating both disease pathophysiology and potential treatment strategies.

Red rice (*Oryza sativa* L.), a pigmented rice variety, has gained attention for its bioactive compounds, particularly in its bran, which contains potent antioxidants such as proanthocyanidins, catechins, γ-oryzanol, vitamin E, and coenzyme Q10 [18,19]. Red rice bran extract (RBE) has been shown to exert antioxidant, anti-inflammatory, and anti-apoptotic properties [20,21]. Given its strong antioxidant capacity, RBE may counteract oxidative stress-induced testicular damage and restore male reproductive function under diabetic conditions. Metformin (Met), a first-line anti-diabetic drug, is well known for its metabolic and antioxidant benefits. It has been shown to improve glycemic control, insulin resistance, and pancreatic morphology in HFD/STZ-induced diabetic models [18]. Additionally, Met has been reported to restore testicular function by reducing oxidative stress, improving mitochondrial function, and modulating hormonal balance in diabetic rats [13]. However, despite the well-established metabolic benefits of RBE, its potential role in mitigating diabetes-induced testicular dysfunction remains largely unexplored.

This study aims to evaluate the impact of RBE, alone or in combination with Met, on testicular function in HFD/STZ-induced diabetic rats. Key reproductive parameters such as oxidative stress markers, testosterone levels, sperm quality, the Johnsen score (as an indicator of spermatogenesis), and testicular histology will be assessed to determine the therapeutic potential of RBE in restoring male reproductive health under diabetic conditions.

2. MATERIALS AND METHODS

2.1. Red rice bran aqueous extract preparation

Red glutinous rice bran (RB) was sourced from a Thai farmer in Ban Dok Bua, Phayao, Thailand. The RBE was prepared by boiling 100 g of dried red RB in 1,000 ml of distilled water at 100°C for 10 minutes. The extract was subsequently filtered and freeze-dried using a Scanvac freeze dryer (Lillerød, Denmark), yielding approximately 20% (w/w) [18]. The phenolic composition of RBE was previously analyzed using high-performance liquid chromatography by Ontawong *et al.* [18] in accordance with ISO/IEC 17025 standards. The analysis identified epicatechin as the predominant compound (15.5 mg/kg), followed by protocatechuic acid (11.2 mg/kg), vanillic acid (5.32 mg/kg), and caffeic acid (0.55 mg/kg). The total phenolic content of RBE was 871.22 ± 12.24 mg GAE/kg RBE, while the anthocyanin content was 6.20 ± 0.24 mg catechin/g RBE [18].

2.2. Animal model and experimental design

The experimental procedures were approved by the Ethics Committee of the Laboratory Animal Research Center, University of Phayao, Thailand (Protocol no. 640104013 and Protocol no. 2-002-65), with all efforts made to minimize animal suffering. A total of 30 male *Wistar rats* (170–220 g) were obtained from Nomura Siam International, Bangkok, Thailand. The rats were housed in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle and acclimatized for 1 week prior to the experiment. During the acclimatization and experimental periods, rats had free access to water and food. As shown in Figure 1, the rats were divided into two dietary groups: one receiving a standard control diet (19.77% fat by energy, CP Mice Feed no. 082, Bangkok, Thailand) and

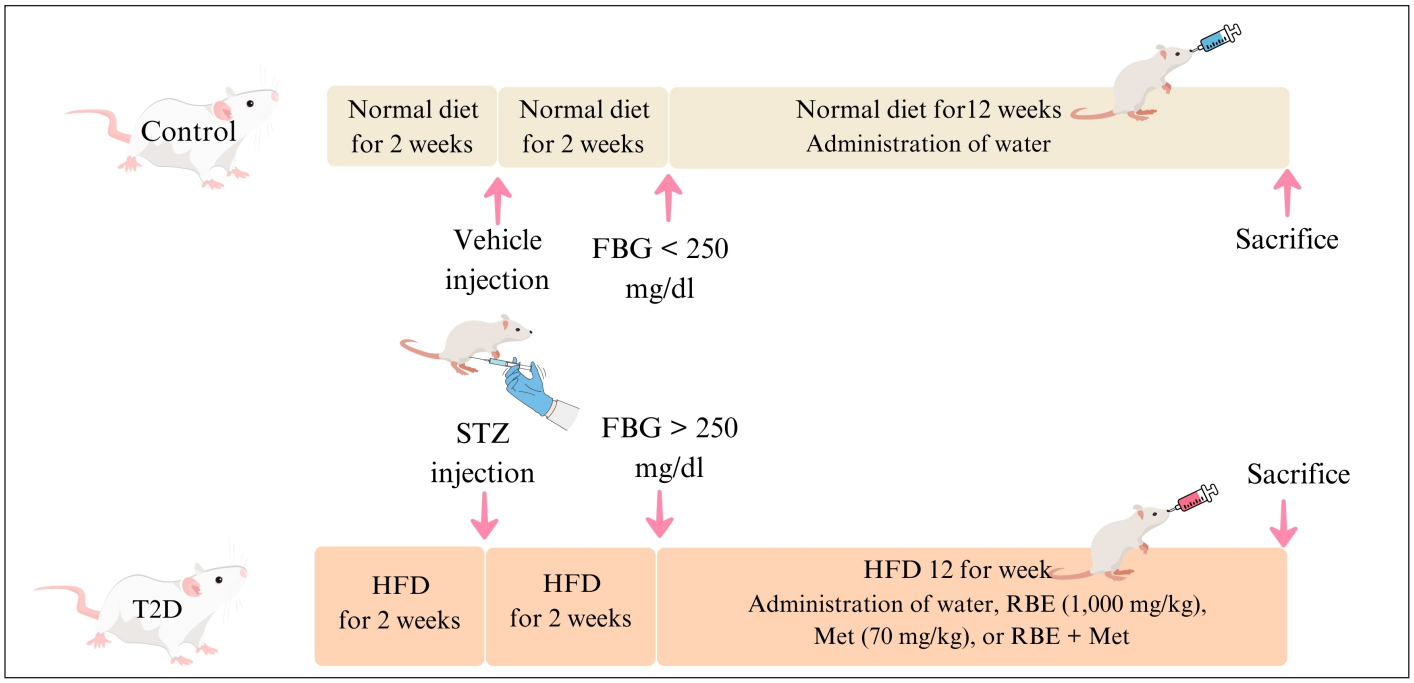

Figure 1. Experimental design for induction and treatment. T2D = type 2 diabetes; FBG = fasting blood glucose; STZ = streptozotocin; HFD = high-fat diet; RBE = red rice bran extract; Met = metformin; RBE = red rice bran extract.

the other a HFD (60% fat by energy). After 2 weeks, the HFD group received a single intraperitoneal (i.p.) injection of STZ (40 mg/kg BW; Sigma-Aldrich, USA) dissolved in 0.09 M citrate buffer (pH 4.5), while the control group received only the buffer. Two weeks after STZ injection, fasting blood glucose (FBG) levels were measured. Rats with FBG levels exceeding 250 mg/dl were classified as diabetic [22]. The diabetic rats were then randomly divided into four treatment groups (n = 6 per group) as follows:

- Group 1 (Control Diet; CD): Non-diabetic rats fed a standard control diet without STZ injection.
- Group 2 (Diabetic Model; DM): Diabetic rats with no treatment.
- Group 3 (DM + RBE): Diabetic rats treated with RBE (1,000 mg/kg BW, orally).
- Group 4 (DM + Met): Diabetic rats treated with Met (70 mg/kg BW, orally).
- Group 5 (DM + RBE + Met): Diabetic rats treated with both RBE (1,000 mg/kg BW, orally) and Met (70 mg/kg body weight, orally). All treatments were administered via oral gavage once daily for 12 weeks. Dosages were based on previous studies [18].

2.3. Sample collection

Upon completion of the study, rats were euthanized using gradual CO_2 asphyxiation. Blood samples were collected via cardiac puncture for serum testosterone analysis. Following blood collection, the cauda epididymis and vas deferens were carefully dissected for sperm evaluation. The left testis was immediately frozen at −80°C for the biochemical analysis of malondialdehyde (MDA) and glutathione (GSH) levels, along with the activity of superoxide dismutase (SOD) and catalase (CAT). The right testis was fixed in 10% buffered formalin for histological examination.

2.4. Biochemical analysis

Testicular tissue was first homogenized (10% w/v) in ice-cold phosphate-buffered saline (PBS, 1 M, pH 7.4; P4417, Sigma-Aldrich, St. Louis, MO) containing a protease inhibitor (Calbiochem, Sigma-Aldrich, St. Louis, MO) using a Handheld Homogenizer D-160 (BioLogics, Manassas, VA). The homogenates were centrifuged at 4,000 × g for 15 minutes at 4°C, and the resulting supernatants were used for MDA analysis. For antioxidant enzyme activity and GSH measurements, the samples were further centrifuged at 14,000 × g for 15 minutes at 4°C. The MDA, GSH, and CAT were normalized to total protein content, which was determined using the Pierce® BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Rockford, IL). The following biochemical parameters were analyzed.

- MDA concentration was determined using a modified thiobarbituric acid reactive substances (TBARS) assay based on Jeefoo *et al.* [23]. Briefly, 40 µl of either the sample or standard (1,1,3,3-Tetramethoxypropane) was mixed with 80 µl of 8.1% sodium dodecyl sulfate, 600 µl of 20% acetic acid (pH 3.5), and 600 µl of 0.8% thiobarbituric acid. The mixture was incubated at 95°C for 60 minutes, then cooled and centrifuged at 10,000 × g for 5 minutes at 4°C. Absorbance was measured at 540 nm using a microplate reader (Synergy H1; BioTek; Agilent Technologies, Inc.).
- SOD activity was measured using a commercial assay kit (S19160, MilliporeSigma), following the manufacturer's protocol. Results were expressed as the percentage inhibition of WST-1 formazan formation.
- CAT activity was determined based on the degradation of H_2O_2. In brief, 20 µl of the sample was mixed with 100 µl of 6 mM H2O2 and incubated at 37°C for 1 minute. The reaction was terminated by adding 100 µl of 32.4 mM ammonium molybdate, and absorbance was recorded at 405 nm using a microplate reader. Results were expressed as U/mg protein.
- GSH levels were quantified using a commercially available kit (Bioassay Systems, Hayward, CA), following the manufacturer's protocol.

2.5. Testosterone analysis

Blood samples were centrifuged at 3,500 rpm for 20 minutes at 4°C to separate serum. Total testosterone concentrations were determined using a fluorescent immunoassay (FIA), following the manufacturer's protocol (Biotime FIA Analyser, Xiamen Biotime Biotechnology Co., Ltd, China). Each sample was analyzed in duplicate, and results were expressed in ng/ml.

2.6. Semen evaluation

Semen samples were collected from both the cauda epididymis and vas deferens and diluted in 10 ml of 1 M PBS, followed by incubation at 36°C–37°C for 15 minutes. Sperm concentration was assessed using an improved Neubauer haemocytometer. A 10 µl aliquot of the sperm suspension was placed onto the haemocytometer and allowed to settle for 5 minutes to ensure uniform distribution. Sperm cells in five designated squares were counted, and the total concentration was calculated by applying the dilution factor. Sperm motility was evaluated under a light microscope at 400× magnification. The percentage of motile sperm was calculated by dividing the number of motile sperm by the total sperm counted in a given field of view, multiplied by 100.

2.7. Histological examination

Following fixation, tissues were washed in running tap water for 10 minutes and then rinsed twice in PBS (pH 7.4) for 10 minutes each. The samples were dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin blocks using standard histological techniques. Testis sections were cut at 5 µm, stained with haematoxylin (05-06002/L, Bio-Optica) and eosin (05-10003/L, Bio-Optica), and mounted under a cover glass. Histological examination and imaging were conducted using a light microscope (Nikon, Japan). For morphometric analysis, 20 seminiferous tubules with an approximately round shape were randomly selected per animal from different regions of each cross-section. The seminiferous tubule diameter (STD) was measured as the average of the shortest and longest diameters, while the height of the germinal epithelium (HE) was also assessed. ImageJ software (v1.53; National Institutes of Health) was used for all measurements. Additionally, the quality of spermatogenesis was evaluated using the Johnsen scoring system. For each animal, at least 50 seminiferous tubules were randomly selected from different regions of the

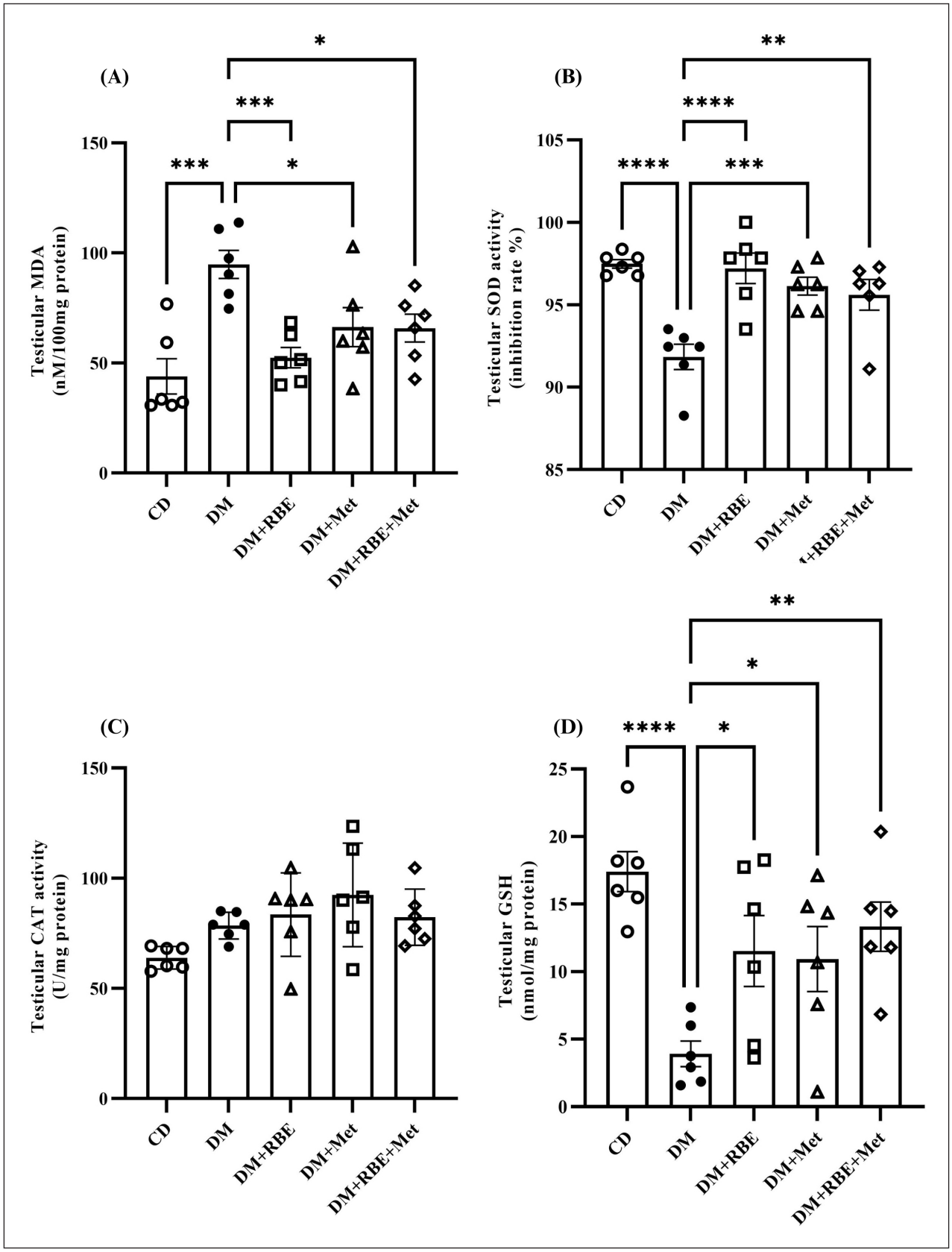

Figure 2. RBE and Met effects on oxidative stress markers in testes. (A) MDA levels; (B) SOD activity; (C) CAT activity; (D) GSH levels. Values are mean ± SEM ($n = 6$). One-way ANOVA and Dunnett's post hoc test vs. DM group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. MDA = malondialdehyde; SOD = superoxide dismutase; CAT = catalase; GSH = glutathione; RBE = red rice bran extract; Met = metformin; DM = diabetic model; CD = control diet.

testicular cross-section. Each tubule was scored from 1 to 10 based on the most advanced germ cell type and the organization of the seminiferous epithelium, following the criteria described by Johnsen [24]. The mean Johnsen score was then calculated for each animal and used as an index of spermatogenic activity.

2.8. Statistical analysis

All data are presented as mean ± SEM. One-way analysis of variance was performed to determine overall group differences. Dunnett's post-hoc test was used to compare all treatment groups against the DM group. Additionally, Tukey's post-hoc test was applied to compare differences among treatment groups (DM + RBE, DM + Met, and DM + RBE + Met). A p-value <0.05 was considered statistically significant. Statistical analyses were conducted using GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Oxidative stress markers in testicular tissue

As shown in Figure 2, MDA levels were significantly elevated in the DM group compared to the CD group ($p < 0.001$), indicating enhanced lipid peroxidation. Treatment with RBE, Met, or their combination significantly reduced MDA levels ($p < 0.001$, $p < 0.05$, and $p < 0.05$, respectively) (Fig. 2A). SOD activity was markedly decreased in the DM group compared to the CD group ($p < 0.0001$), suggesting compromised antioxidant defense, while RBE, Met, and their combination significantly restored SOD activity ($p < 0.0001$, $p < 0.001$, and $p < 0.01$, respectively) (Fig. 2B). Although CAT activity was slightly elevated in the DM group, the change was not statistically significant, and no significant differences were observed in the treatment groups (Fig. 2C). GSH levels were significantly reduced in the DM group compared to the CD group ($p < 0.0001$), whereas all treatment groups exhibited significant improvements in GSH levels ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) (Fig. 2D).

3.2. Serum testosterone levels

As shown in Figure 3A, serum testosterone levels were significantly reduced in the DM group compared to the CD group ($p < 0.001$). Although a trend toward increased testosterone levels was observed in the DM + RBE, DM + Met, and DM + RBE + Met groups, the differences were not statistically significant when compared to the DM group ($p > 0.05$).

3.3. Sperm parameters

Sperm concentration and motility were significantly lower in the DM group compared to the CD group ($p < 0.0001$ and $p < 0.05$, respectively). Sperm concentration was significantly increased in the DM + Met and DM + RBE + Met groups compared to the DM group ($p < 0.01$ and $p < 0.05$, respectively). However, no significant changes in sperm motility were observed among the treatment groups. These findings are illustrated in Figure 3B and C, showing the differential effects of treatments on sperm quality parameters in diabetic rats.

3.4. Histological changes in testicular tissue

Representative histological images of testicular tissue from different groups are shown in Figure 4A. The DM group exhibited a significant reduction in STD compared to the CD group ($p < 0.0001$) (Fig. 4B). However, treatment with RBE, Met, or their combination significantly increased STD compared to the DM group ($p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). Similarly, HE was significantly lower in the DM group than in the CD group ($p < 0.0001$) (Fig. 4C). In contrast, all treatment groups (DM + RBE, DM + Met, and DM + RBE +

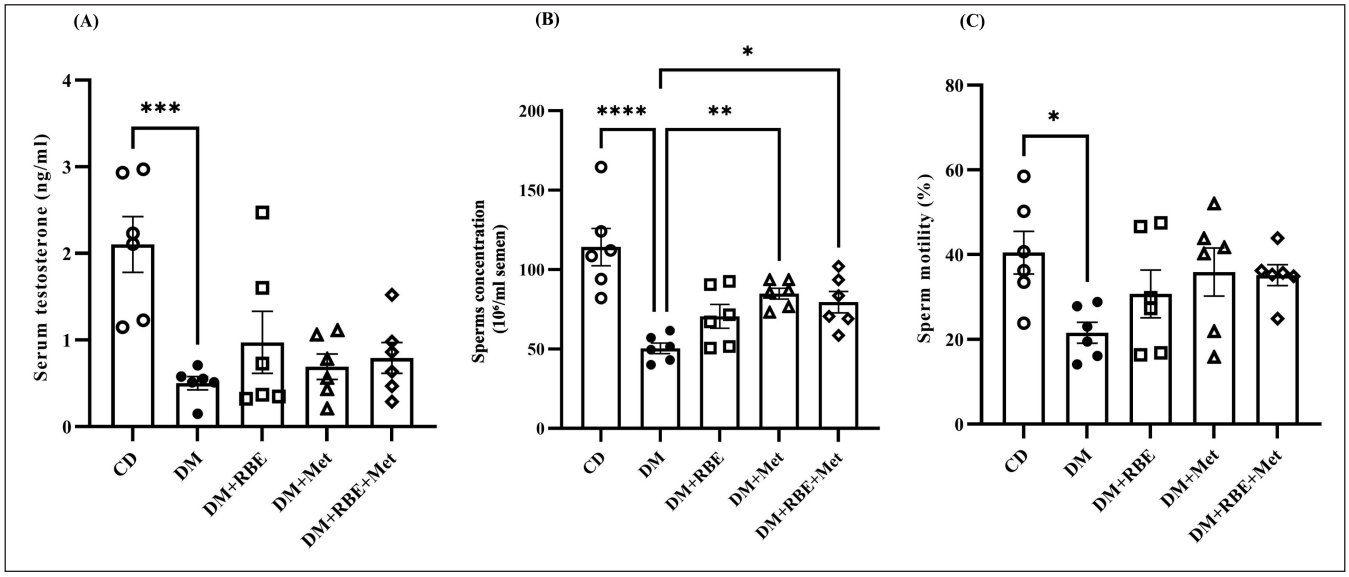

Figure 3. Serum testosterone levels and sperm parameters in diabetic rats. (A) Serum testosterone levels; (B) Sperm concentration; (C) Sperm motility. Values are presented as mean ± SEM ($n = 6$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test vs. DM group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. RBE = red rice bran extract; Met = metformin; DM = diabetic model; CD = control diet.

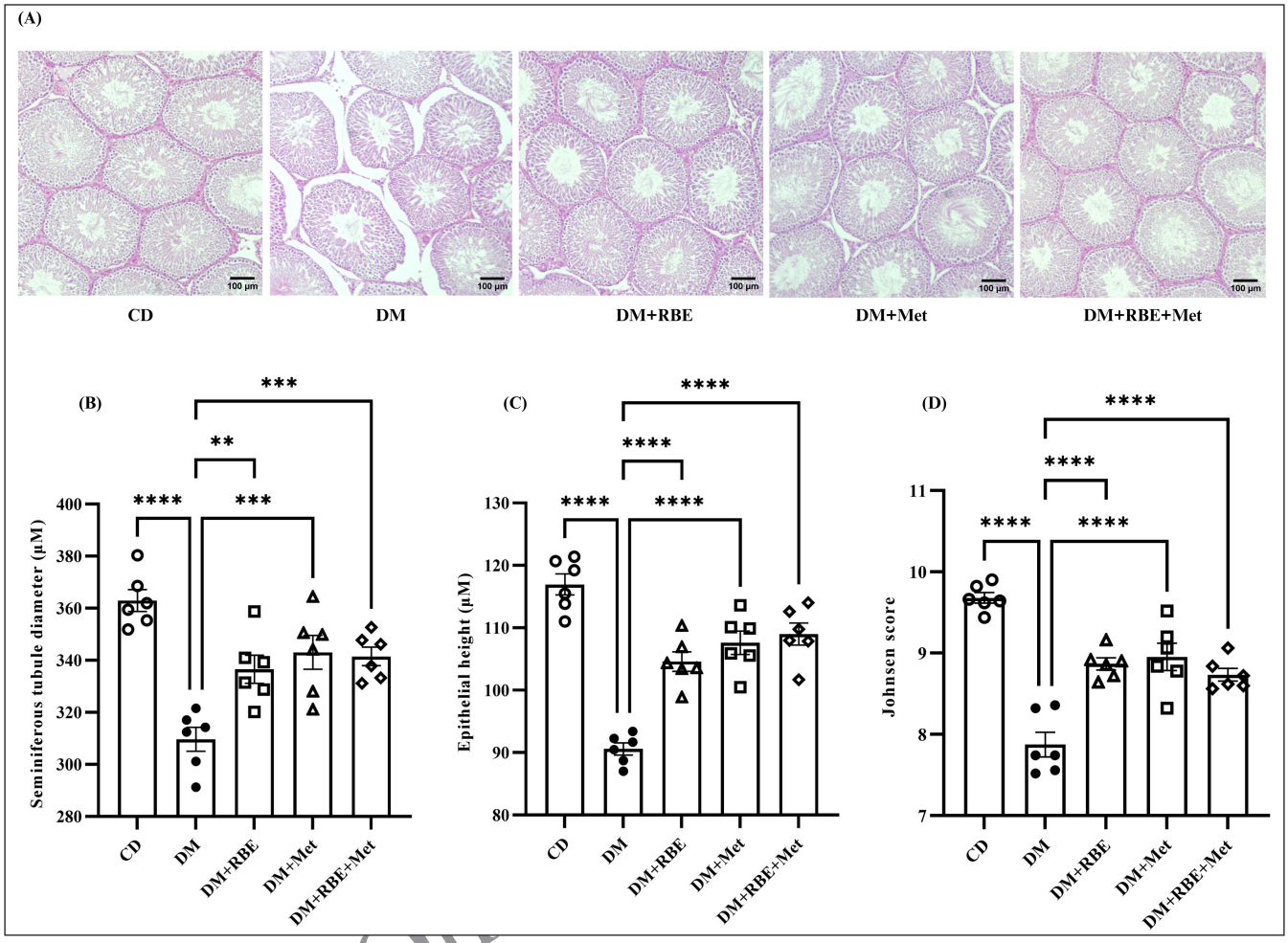

Figure 4. Histological changes in testicular tissue. (A) H&E-stained sections (scale bar = 100 µm); (B) Seminiferous tubule diameter; (C) Epithelium height; (D) Johnsen score. Values are mean ± SEM ($n = 6$). One-way ANOVA and Dunnett's post hoc test vs. DM group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. RBE = red rice bran extract; Met = metformin; DM = diabetic model; CD = control diet.

Met) showed a significant increase in HE compared to the DM group ($p < 0.0001$ for all groups). In addition, the Johnsen score, a marker of spermatogenic activity, was markedly decreased in the DM group relative to the CD group ($p < 0.0001$) (Fig. 4D). Treatment with RBE, Met, or their combination significantly elevated the Johnsen score compared to the DM group ($p < 0.0001$ for all treatments). These findings indicate that RBE and Met may ameliorate diabetes-associated impairment of spermatogenesis.

4. DISCUSSION

This study demonstrates that RBE and Met, whether administered individually or in combination, ameliorate diabetes-induced testicular dysfunction in a well-established rat model of T2D. The protective effects observed included reductions in oxidative stress, restoration of testicular structure, and improvements in spermatogenic activity, suggesting that RBE and Met exert their benefits primarily through antioxidant pathways.

The diabetic condition induced by an HFD combined with STZ reliably replicated key features of human T2D, including chronic hyperglycaemia and compensatory hyperinsulinaemia. These metabolic disturbances are major contributors to diabetes-related complications. In particular, persistent hyperglycaemia promotes excessive reactive oxygen species (ROS) production, which plays a central role in testicular dysfunction. Insulin resistance exacerbates metabolic dysregulation by impairing glucose uptake and disrupting hormonal signaling, particularly within the hypothalamic–pituitary–gonadal (HPG) axis [25,26]. These pathological changes contribute to reduced testosterone levels, impaired spermatogenesis, and degeneration of testicular tissue [8–10,12,13,27,28].

To evaluate the involvement of oxidative stress in T2D-induced testicular dysfunction, we measured MDA, SOD, CAT, and GSH levels, consistent with several previous studies [29–31]. Diabetic rats exhibited significantly elevated MDA, a marker of lipid peroxidation, along with reduced levels of SOD and GSH, indicating a disruption of redox homeostasis and impaired antioxidant defenses. These findings are consistent with earlier reports demonstrating that chronic hyperglycemia promotes excessive ROS production, which

contributes to oxidative damage in testicular tissue [8,32,33]. This redox imbalance may be mechanistically linked to the dysregulation of oxidative stress-responsive transcription factors. Specifically, T2D has been shown to impair the activation of Nrf2, a key regulator of antioxidant defense, while simultaneously promoting NF-κB signaling, which drives pro-inflammatory gene expression. The resulting suppression of antioxidant capacity and persistent inflammation may establish a self-perpetuating cycle of testicular damage in diabetes [33].

Given this mechanistic insight into oxidative disruption, we evaluated whether antioxidant-based interventions could reverse redox imbalance in diabetic testes. In this context, both RBE and Met significantly improved markers of oxidative stress, indicating a restoration of redox homeostasis. Of particular interest, the combination treatment elicited the most pronounced increase in GSH levels. This enhanced GSH response may reflect differential regulatory mechanisms, as GSH synthesis is largely dependent on Nrf2-mediated gene expression, while SOD activity is primarily enzymatic. Previous studies have shown that RBE exerts antioxidant and anti-inflammatory effects through modulation of Nrf2 and NF-κB signaling pathways, particularly in brown adipose tissue under HFD-induced obesity [34]. However, no prior studies have investigated these mechanisms in testicular tissue. In contrast, the role of Met in regulating Nrf2 and NF-κB pathways has been well documented in testicular tissue under diabetic conditions, as evidenced by studies demonstrating increased Nrf2 expression and reduced oxidative stress following treatment [33,35].

These observations suggest that both agents may mitigate testicular oxidative stress, at least in part, through activation of Nrf2 signaling, contributing to reduced MDA levels and improved antioxidant defenses. Additionally, RBE is rich in bioactive phytochemicals such as epicatechin, protocatechuic acid, anthocyanins, and γ-oryzanol [18], all of which are reported to scavenge ROS, inhibit lipid peroxidation, and enhance endogenous antioxidant systems. These properties likely underlie the observed protective effects of RBE in this study and reinforce its potential as a natural antioxidant and anti-inflammatory compound. Of note, CAT activity is commonly reported to decline in diabetic conditions due to oxidative inactivation [8,36,37]. However, we did not observe a significant reduction in CAT activity in the present model. This discrepancy may be attributed to tissue-specific antioxidant responses or compensatory mechanisms that maintained enzyme function despite persistent oxidative stress.

While these findings support a plausible role for Nrf2 and NF-κB signaling in mediating the observed effects of RBE and Met, it is important to acknowledge that this study did not include direct molecular or protein-level assessments of these pathways. Therefore, the mechanistic interpretations provided here remain speculative and are derived from prior literature rather than direct experimental evidence. Future investigations should incorporate targeted analyses of Nrf2 and NF-κB activity to confirm their involvement in testicular protection under diabetic conditions.

While molecular mechanisms remain hypothetical, histological findings provide direct evidence of testicular impairment and therapeutic restoration at the tissue level. Histological evaluation revealed substantial testicular damage in diabetic rats, including reduced STD and thinning of the germinal epithelium. These alterations reflect impaired Sertoli cell support and germ cell degeneration, consistent with previous findings that chronic hyperglycaemia and oxidative stress impair spermatogenic cell integrity, disrupt Sertoli cell function, and induce germ cell apoptosis leading to testicular atrophy [38]. T2D impairs male fertility through both direct and indirect mechanisms. Oxidative stress is a key contributor to diabetes-related testicular dysfunction, promoting lipid peroxidation, mitochondrial dysfunction, sperm DNA damage, and apoptosis [29,30,39]. Excessive ROS particularly damages germ cells and spermatozoa due to their high polyunsaturated fatty acid content [40–42]. Additionally, diabetes disrupts the HPG axis by impairing insulin and leptin signaling in the hypothalamus, reducing GnRH secretion and consequently decreasing LH and FSH levels, ultimately compromising Leydig cell testosterone synthesis [25,30,36]. Consistent with these mechanisms, our findings suggest that oxidative damage likely contributed to both Leydig and Sertoli cell dysfunction in diabetic rats, reflected by reduced testosterone production and impaired germ cell development and maturation.

This structural damage is further compounded by endocrine dysfunction, as reflected in serum testosterone levels. Despite these structural improvements, serum testosterone levels remained significantly suppressed in diabetic rats and did not return to control levels following treatment, although a trend towards improvement was observed. While testosterone deficiency serves as a key indicator of Leydig cell dysfunction, it should be noted that this study did not measure gonadotropins such as FSH or LH, representing a limitation that precludes a complete assessment of hypothalamic–pituitary involvement. Moreover, Sertoli cell dysfunction may result not only from direct oxidative damage but also from reduced testosterone-mediated support, further compounding impaired spermatogenesis.

To further assess spermatogenic function beyond hormonal changes, we evaluated histological and functional sperm parameters. The Johnsen score, a quantitative marker of spermatogenic activity, was markedly reduced in diabetic rats, indicating impaired germ cell development and maturation. This reduction corresponded with a significant decline in both sperm concentration and motility. Treatment with RBE, Met, or their combination led to a significant improvement in Johnsen scores, reflecting a partial restoration of spermatogenesis. Notably, sperm concentration was significantly increased in the Met and combination groups; however, sperm motility did not improve significantly in any treatment group. These findings suggest that while antioxidant therapy may enhance spermatogenic output, sperm motility may be governed by additional factors beyond oxidative stress, such as mitochondrial function, energy metabolism, and ATP production. These factors should be the subject of further investigation.

The comparable improvements observed across treatment groups and the absence of statistically significant differences suggest overlapping mechanisms of action for RBE and Met, particularly their antioxidant and anti-inflammatory

properties. Polyphenolic compounds present in RBE, such as epicatechin and protocatechuic acid, as well as anthocyanins, have been shown to enhance insulin sensitivity and reduce oxidative stress, while Met also promotes Nrf2 activation and mitochondrial protection. These shared mechanisms likely account for the similar protective effects observed in this study.

Several limitations of this study should be acknowledged. First, although the potential involvement of Nrf2 and NF-κB pathways was discussed, no direct molecular or protein-level analyses were performed; thus, mechanistic conclusions remain speculative. Second, only total testosterone levels were measured, without assessment of sex hormone-binding globulin, which limits accurate interpretation of androgen status. Third, the use of fixed treatment doses and a 12-week intervention period may not reflect the optimal therapeutic regimen. Additionally, the sample size, while sufficient for detecting most outcomes, may have been underpowered to detect subtle changes, particularly in testosterone recovery and sperm motility. Finally, although the Johnsen score provided a semi-quantitative assessment of spermatogenesis, more detailed histological parameters such as the spermatid-to-Sertoli cell ratio were not evaluated.

In summary, RBE and Met significantly ameliorated diabetes-induced oxidative stress, preserved testicular architecture, and improved spermatogenic activity, as evidenced by histological recovery and improved Johnsen scores. However, neither intervention fully restored testosterone levels or sperm motility, indicating persistent endocrine dysfunction. These findings highlight the potential of RBE as a natural adjunct to existing treatments for diabetes-associated male reproductive impairment. Future research should explore optimised dosing strategies, extended treatment durations, and detailed molecular analyses to elucidate mechanisms and maximise therapeutic efficacy.

5. CONCLUSION

In summary, this study demonstrates that both RBE and Met offer protective effects against testicular damage associated with T2D. Improvements were evident in oxidative stress markers, testicular histology, and spermatogenic activity, particularly through reductions in MDA levels, restoration of SOD and GSH, and increased Johnsen scores. Among the treatments, RBE alone demonstrated the most consistent overall effect in mitigating oxidative stress, showing the greatest reduction in MDA levels and the most significant improvement in SOD activity. Although the combination treatment yielded the highest increase in GSH levels, RBE's broader antioxidant impact suggests it is the most effective single intervention for restoring redox balance in diabetic testicular tissue.

The underlying mechanisms are likely related to antioxidant actions, possibly involving modulation of the Nrf2 and NF-κB signaling pathways, although these were not directly examined in the present study. Importantly, testosterone levels and sperm motility did not return to normal following treatment, indicating that some aspects of endocrine and functional recovery may require further intervention.

Overall, the findings support the potential role of RBE as a natural adjunct to existing therapies for diabetes-induced reproductive complications. Further studies are needed to confirm molecular mechanisms, optimize treatment parameters, and assess long-term outcomes in both preclinical and clinical settings.

6. ACKNOWLEDGMENTS

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

Ethical approvals detail are given in the 'Materials and Methods' section.

11. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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