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Protective effect of *Dioscorea zingiberensis* ethanol extract on the disruption of blood–testes barrier in high-fat diet/streptozotocin-induced diabetic mice by upregulating ZO-1 and Nrf2

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Abstract

Testicular injury is the primary pathogenesis of diabetes-induced male infertility. *Dioscorea zingiberensis* (DZ), a traditional Chinese medicine (TCM) including saponins, flavonoids and cellulose, is used to treat diseases in the reproductive system. But the protective effects of DZ on diabetes-induced testicular injury remain poorly understood. In this study, the therapeutic effects of chronic oral DZ treatment on testis impairment in a diabetic mouse model were explored by assessing sperm morphology, blood–testes barrier (BTB) integrity and testicular histological examination. Our results showed that DZ significantly reversed BTB disruption, testicular tissue injury and abnormal sperm morphology in diabetic mice. Interestingly, diabetes-induced disruption of the BTB was associated with a decrease in the tight junction (TJ) protein zonula occludens-1 (ZO-1). *Dioscorea zingiberensis* effectively increased ZO-1 expression in testis tissue to restore the integrity of the BTB. Moreover, DZ treatment significantly reduced hyperglycaemia-induced increases in malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels. Further mechanistic studies revealed that DZ substantially enhanced the expression of Nrf2, NOQ1 and HO-1, which indicated that DZ exerts potential antioxidant effects against testicular tissue damage via the activation of Nrf2. In conclusion, the protective effects of DZ rely on repairing the integrity of the BTB and on reducing oxidative stress damage by mediating ZO-1 and Nrf2. The study contributes to discovering the DZ possible mechanism of action.

KEYWORDS

blood–testes barrier, *Dioscorea zingiberensis*, diabetes, Nrf2, ZO-1

1 | INTRODUCTION

Infertility is defined as the failure to conceive within one year of unprotected sexual intercourse, which may affect 8%–15% of couples (Carp, Selmi, & Shoenfeld, 2012). Approximately 20%–30% of infertility is caused by male factors (Isidori et al., 2006). Previously, attention has been focused on the adverse effects of some malignancies on male fertility. In a retrospective cohort study, Eisenberg ML et al. noted that the incidence of male infertility caused by diabetes is higher than that of male infertility

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caused by other chronic nonmalignant diseases (Eisenberg, Li, Cullen, & Baker, 2016). In another survey, a higher incidence of male infertility was closely associated with diabetes (Glazer et al., 2017). With the increasing incidence of diabetes, there is an urgency to prevent and treat male infertility induced by diabetes.

The blood–testes barrier (BTB) is one of the tightest blood–tissue barriers in mammals. The BTB is composed of basal ectoplasmic specialisations, tight junctions (TJs), desmosomes near the gap junctions (GJs) and basement membrane. The BTB provides a reliable microenvironment for spermatogenesis, particularly in meiosis I/II and post-meiotic spermatid development (Li et al., 2019; Wen et al., 2018). It is an essential problem that BTB structural failure or dysfunction is closely associated with testicular injury and male infertility. Recent studies have shown that diabetes-induced testicular injury by first inducing BTB disruption led to the germ cells adhesion, apoptosis, reduced sperm count and infertility (Alves, Martins, Cavaco, Socorro, & Oliveira, 2013). The destruction of BTB integrity is the primary target in testicular injury and male infertility. Tight junctions between adjacent Sertoli cells play a crucial role in establishing the BTB, which divides the seminiferous epithelium into an adluminal compartment and a basal compartment. Zonula occludens-1 (ZO-1), as a ubiquitous peripheral protein localised at junctional sites, was the first TJ-associated protein (Herve, Derangeon, Sarrouilhe, & Bourmeyster, 2014) to be identified in the BTB. The abnormal distribution and expression of ZO-1 are essential pathological mechanisms in which a cholesterol-enriched diet leads to sperm damage and male infertility due to BTB disruption (Morgan, Ghribi, Hui, Geiger, & Chen, 2014). Therefore, regulating the ZO-1 protein could be a preferred treatment strategy to rescue BTB disruption to further reverse diabetes-induced male infertility.

In addition, recent research has indicated that excess reactive oxygen species (ROS) and altered antioxidant defence induced by diabetes mellitus (DM) in the male reproductive system lead to BTB disruption, cellular apoptosis, abnormal spermatogenesis and infertility in animal models and diabetic patients (La Vignera et al., 2009; Wang et al., 2014). The research thus indicated that oxidative stress is likely to be intimately involved in diabetes-induced testicular injury and male infertility. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), an essential protein in cellular antioxidant defence, is also expressed in germ cells, Leydig cells and Sertoli cells. Generally, Nrf2 is usually located in the cytoplasm, where it is tagged and degraded via ubiquitination by Kelch-like ECH-associated protein 1 (Keap1) (Wajda et al., 2016). After Nrf2 is activated and translocated into the nucleus, it binds to the antioxidant response element (ARE), to further mediate the expression of many downstream intracellular antioxidants and phase II detoxification enzymes, including NAD(P)H:quinone oxidoreductase1 (NQO1), haem oxygenase-1 (HO-1) and glutamate–cysteine ligase catalytic subunit (GCLC) (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). Deficiency or suppression of Nrf2 in Sertoli cells has deleterious effects on the testis and spermatogenetic function (Nakamura et al., 2010). More importantly, the induction of Nrf2 activity was found to be a potential therapeutic target for testicular injury and male infertility in vitro and in vivo.

Dioscorea zingiberensis C.H. Wright (DZ), as an essential medicinal plant and a source of diosgenin, is a wild woody climber found in northern subtropical and subtropical regions in China. The biological properties of DZ are well established including antithrombosis, antiaging, antioxidant, and anthelmintic activities (Du et al., 2016) and antitumor activity towards various human cancer cells (Zhang et al., 2018). The active ingredients of DZ have been used to treat a variety of diabetic complications and male reproductive system diseases. Oxidative stress (Zhang et al., 2018) induced by hyperglycaemia is a primary pathological mechanism for the occurrence of diabetic male infertility (Faid, Al-Hussaini, & Kilarkaje, 2015). Previous studies have reported that *DZ* had strong antioxidant effects and reversed the potency of male reproductive system disease (Song et al., 2019; Zhang et al., 2018). Moreover, a toxicity study showed that total steroidal saponin (TSSN) extracts from DZ had proper safety and toleration in a toxicological evaluation (Zhang et al., 2017). The toxicological studies ensure that patients can tolerate DZ treatment for extended periods. Unfortunately, the potential pharmacological actions and molecular mechanisms of DZ in male infertility remain unclear.

Therefore, in this study, the protective effect of DZ against the disruption of the BTB and testis injury was first investigated using a high-fat diet (HFD)/streptozotocin (STZ)-induced mouse model. Moreover, further studies revealed whether DZ reduces oxidative stress by inducing Nrf2 signalling pathways and restores the integrity of the BTB by regulating TJ proteins involved in the underlying pharmacological mechanisms of this protective effect.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

FITC–dextran was obtained from Xi`an Ruixi Biological Technology Co., Ltd. *Dioscorea zingiberensis* was obtained from Jiangsu Huanghe Pharmaceutical Co., Ltd. DAPI, diaminobenzidine (DAB), hematoxylin and bovine serum albumin (BSA) were purchased from Beyotime (Beyotime). Purchase, dilution, catalog numbers and application condition of primary antibodies and second antibodies were listed in Table 1.

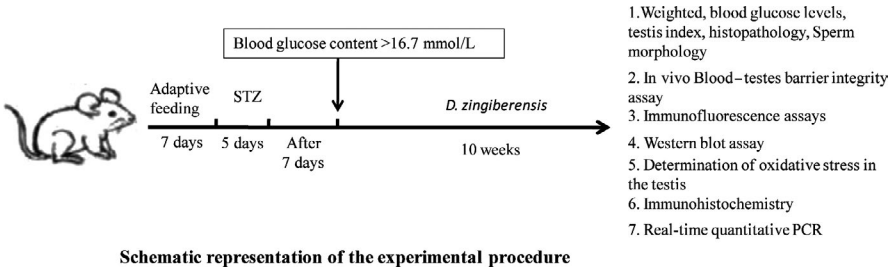
2.2 | Animals

The C57BL/6 mice, 18–22 g, male, were obtained from the Comparative Medicine Centre of Yangzhou University. The mice were maintained in an animal room at a constant 22 ± 1°C and 50% humidity with a 12-hr light/dark cycle. The animal procedures were carried out according to the Guidelines for the Animal Ethics Committee (NO. 20180307) of Nanjing Tech University (Nanjing, China).

TABLE 1 Purchase and dilution condition of primary antibodies and second antibodies

Antibody	Corporation	The catalog numbers	Corporation	Dilution factor	
				WB	IHC
ZO-1	Proteintech Group		Proteintech Group	1:1,000	1:400
Nrf2	Proteintech Group		Proteintech Group	1:1,000	1:400
NQO1	Affinity		Affinity	1:1,000	–
HO-1	Affinity		Affinity	1:1,000	–
8-OHdG	Bioss		Bioss	–	1:600
GAPDH	Beyotime		Beyotime	1:1,000	–
HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L)	Affinity		Affinity	1:5,000	–
CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG (H + L)	Proteintech Group		Proteintech Group	–	1:250

FIGURE 1 Schematic representation of the experimental procedure



Schematic representation of the experimental procedure

2.3 | Experimental design and sample collection

After adaptive feeding for one week, animal models of diabetes were induced by feeding an HFD for four weeks and then were given an intraperitoneal injection of 50 mg kg^{-1} day^{-1} STZ for five consecutive days. After seven days, mice blood was collected via tail prick and blood glucose levels were checked using glucose colorimetric assay kit (Sigma). Moreover, mice with blood glucose content >16.7 mmol/L were used in the study. The control mice were fed a standard diet and injected with citrate buffer for five days. In addition to the control group, twenty-four diabetic mice were randomly divided into the following three groups (n = 8, each group): model group and DZ treatment groups (medium and high-dose groups) in which animals were treated with 125 and 250 mg kg^{-1} day^{-1} DZ (dissolved in 0.5% sodium carboxymethyl cellulose, CMC-Na) by oral administration respectively. Additionally, the mice from the control group and the model group were given a 0.5% CMC-Na aqueous solution for ten weeks. At the end of the experiment, all of the mice were weighed, blood samples were collected from the tail vein, and blood glucose levels were detected via a blood glucose meter. After all animals were sacrificed, testicular tissues were removed and calculated the testis index (testis weight/body weight) as well as histopathology was performed. The tissues were stored in liquid nitrogen for further studies. Storing tissue in liquid nitrogen must be detected within two months (Figure 1).

2.4 | Histopathology

Histopathology of testis tissue was carried out as described previously (Zhang et al., 2019). Briefly, left testicular tissues from mice were removed and fixed in a 4% paraformaldehyde solution. The fixed tissues were dehydrated with graded concentrations of ethanol, embedded in paraffin, sectioned at 5 μm and then stained with hematoxylin and eosin (H&E). Photographs of tissue sections were taken and observed with a light microscope (Ts2R, Nikon).

2.5 | Sperm morphology

The entire epididymis was removed, weighed and minced in 1 ml normal saline for 15 min at 37°C to dissociate the spermatozoa, and then, the sperm straining was carried out using a Quick Sperm Stain Kit (Nanjing Jiancheng Bioengineering Institute, China). Hereafter, the stained spermatozoa were observed with a light microscope (Ts2R, Nikon), as previously reported by Wang et al. (2018).

2.6 | In vivo BTB integrity assay

Blood–testis barrier integrity was evaluated in mice by using a fluorescence tracing method (Ma et al., 2019). Briefly, 90 min after intravenous injection of 300 μl (1 mg/ml) FITC–dextran (Xi`an Ruixi Biological Technology Co., Ltd.) into the tails of mice, the right testes were removed and placed in a brown Eppendorf tube with Tissue-Tek OCT to prepare as frozen sections. Frozen sections were obtained using a cryostat microtome, and the fluorescently stained sections were detected with a fluorescence microscope (Ts2R, Nikon).

2.7 | Immunofluorescence staining assays

For immunofluorescence (IF) staining, testicular tissue slices were deparaffinised by xylene, rehydrated by gradient alcohol and then blocked using 10% goat serum for one hour, respectively. The sections were then incubated with primary antibodies against ZO-1 (1:400) overnight at 4°C, followed by the addition of fluorescent-labelled secondary antibodies at 37°C for one hour. Then, the sections were stained with DAPI for 3 min. The images were observed under a confocal microscope system (Zeiss LSM880, Carl Zeiss Jena, Germany).

2.8 | Western blot assay

After treatment, total proteins were lysed in ice-cold RIPA buffer. Protein concentrations were estimated by using a bicinchoninic acid (BCA) assay kit (Pierce). Then, 40 μg of protein lysates were separated with 10% or 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with TBS buffer containing 5% BSA for two h and then probed with the appropriate primary antibody at 4°C overnight. After cleaning with TBST, the PVDF membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies for 1.5 hr at 37°C. The protein bands were visualised using enhanced chemiluminescence (ECL) reagent (Millipore), and the intensities were quantified by a Tanon 4600SF Chemiluminescence Image Analysis System (Tanon, Shanghai, China) and normalised to GAPDH levels.

2.9 | Estimation of oxidative stress in the testis

The activities of superoxide dismutase (SOD), the levels of malondialdehyde (MDA) and the ratio of glutathione and total glutathione (GSH/T-GSH) in testicular samples were detected by using a classic colorimetric method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Protein concentrations were detected by a BCA protein assay kit. All the above indicators were normalised to protein concentration.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is a crucial biomarker of oxidative stress damage, and the estimation of 8-OHdG levels was similar to the method described in '2.7 Immunofluorescence assays'. The stained tissues were visualised by a fluorescence microscope (Ts2R, Nikon).

2.10 | Immunohistochemistry

The left testis was removed, fixed in a 4% paraformaldehyde solution and embedded in paraffin. The samples were sectioned at 5 μm, dewaxed with xylene and rehydrated with graded alcohol. Antigen retrieval was carried out in citrate buffer (pH 6.0) by heating in a microwave oven after the sections were treated with 3% hydrogen peroxide for 10 min. The primary antibody was added to the sections and incubated overnight at 4°C. And then, the sections were incubated with secondary polyclonal goat anti-rabbit antibody for 1 hr at 37°C. The sections were stained in DAB solution until the desired staining intensity was achieved after incubation with the SABC complex (Boster). Finally, cell nuclei were counterstained with hematoxylin, and the results were observed with a light microscope.

TABLE 2 Primer sequences for PCR amplification

Name	Primer sequences
ZO-1	Forward: 5′-TCATCCCAAATAAGAACAGAGC-3′
	Reverse: 5′-GAAGAACAACCCTTTCATAAGC-3′
HO-1	Forward: 5′-TGCAGGTGATGCTGACAGAGG-3′
	Reverse: 5′-GGGATGAGCTAGTGCTGATCTGG-3′
NQO1	Forward: 5′-CAGCCAATCAGCGTTCGGTA-3′
	Reverse: 5′-CTTCATGGCGTAGTTGAATGATGTC-3′
GAPDH	Forward: 5′-AATGGTGAAGGTCGGTGTGAACG-3′
	Reverse: 5′-TCGCTCCTGGAAGATGGTGATGG-3′

2.11 | Real-time quantitative PCR

Total RNA was extracted from testis samples by using Trizol reagent (Ta Ka Ra Biotechnology) according to the protocol. The purified RNA was used to synthesise single-strand cDNA with an All in One RT Master Mix Kit (Applied Biological Materials Inc.). Real-time quantitative RT-PCR was performed with Bright Green 2X qPCR Master Mix kit (Applied Biological Materilas Inc.) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The primer sequences are shown in Table 2. Relative gene expression was analysed by the $2^{-\Delta\Delta Ct}$ method using GAPDH as an endogenous reference.

2.12 | Statistical analysis

Data are presented as the mean ± standard deviation (*SD*). One-way analysis of variance (ANOVA) was used to evaluate the difference between groups, followed by Dunnett's *t* test. $p < .05$ was regarded as statistically significant. All analyses were performed using the SPSS program (version 18.0, Chicago, USA).

3 | RESULTS

3.1 | *Dioscorea zingiberensis* alleviates testis injury and spermatogenesis dysfunction without affecting blood glucose concentrations

A model of diabetic mice with testicular damage was established to evaluate the protective effect of DZ. Figure 2a shows that

there was no difference in the weight of the mice in each group. Figure 2b shows that the blood glucose levels of mice in the model group were significantly increased compared with that of the normal group, while there was no difference between model group and DZ groups. Figure 2c shows that the testicle index of mice in the model group was decreased significantly compared with that of mice in the normal group ($p < .01$), indicating that diabetes induced significant pathological atrophy in the testis of mice. *Dioscorea zingiberensis* (125 mg/kg) showed protective effects on the testicles of diabetic mice, increasing the testicle index, but DZ (125 mg/kg) did not statistically reverse the testis index ($p > .05$). The protective effect of DZ (250 mg/kg) on testis was more significant than that of model group. *Dioscorea zingiberensis* (250 mg/kg) obviously reversed the decrease in testis volume and testis index (Figure 2c,d). Testicular morphology and spermatogenesis were evaluated by H&E staining and sperm staining respectively. The pathological manifestations of the mice in the model group were observed including damaged seminiferous tubules, extensive gaps between germ cells and detached spermatogenic cells.

Unlike the model group, mice in the 125 and 250 mg/kg DZ groups showed normal testicular structure, regular seminiferous tubules and orderly germ cells arrangement (Figure 2e). The number of sperm counts in diabetic mice was decreased, and the structure of spermatozoa was also abnormal. In the mice treated with DZ, the number of spermatozoa was significantly increased, and the abnormal structures of spermatozoa, such as a folded tail, were improved (Figure 2f). In summary, the results showed that DZ improved testicular damage, especially morphological changes and spermatogenesis, without affecting body weight and blood glucose concentrations.

3.2 | DZ restores the BTB disruption induced by hyperglycaemia by upregulating ZO-1

The BTB, as a 'gatekeeper', protects developing germ cells. To observe whether DZ has a protective effect on the BTB, a fluorescence tracing method was carried out to evaluate the BTB integrity. After the induction of diabetes with STZ/HFD, significantly enhanced green FITC–dextran fluorescence was observed in the adluminal compartment and interstitial space of the testes, indicating that the permeability of the BTB was increased. In the STZ/HFD-induced diabetic model, DZ treatment significantly prevented green fluorescence from entering the intraluminal compartment (Figure 3a). To explore the mechanism by which DZ protected BTB integrity, we further evaluated the effect

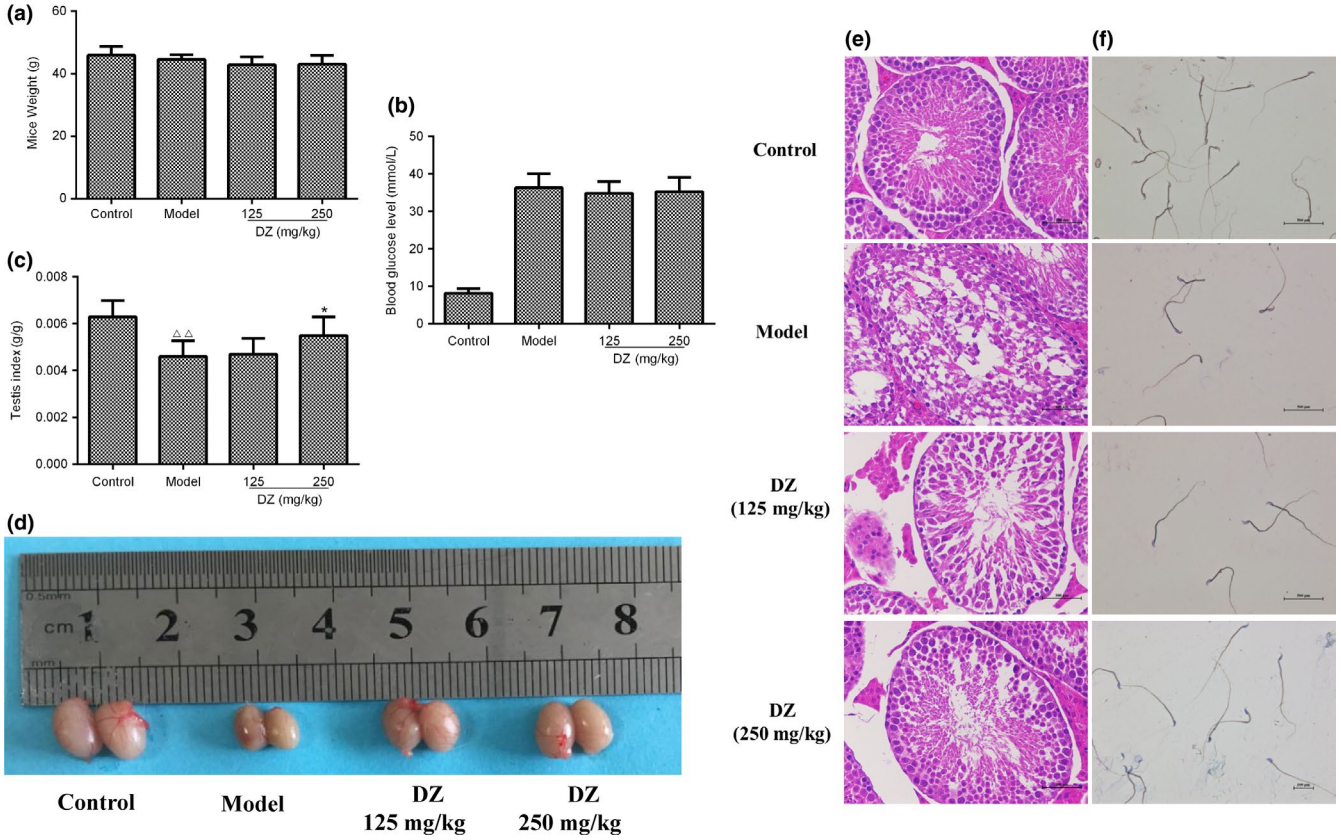


FIGURE 2 DZ alleviates testis injury and spermatogenesis dysfunction without affecting blood glucose concentrations. (a) Body weight, (b) blood glucose levels and (c) testicular weight index after DZ treatment for 10 weeks. (d) Representative testicular volume. (e) Testicular histological analysis in mice (stained with H&E, 400×). (f) Sperm staining (400×). The results are shown as the mean ± *SD* and are listed in a column chart. $^{\triangle}p < .01$ compared with the control group; $^{*}p < .05$ compared with the model group

of DZ on regulating the expression of junction proteins such as ZO-1. Western blot results indicated that the expression of ZO-1 was significantly decreased ($p < .01$) in STZ/HFD-induced diabetic mice compared with the control mice. After treatment with DZ, the expression of ZO-1 increased significantly ($p < .01$, Figure 3b,c). Real-time PCR showed that DZ significantly reversed the downregulation of ZO-1 in the testis induced by diabetes (Figure 3d). The protein expression of ZO-1 was also markedly higher in the DZ-treated group than that of ZO-1 in the model group ($p < .01$). The results were verified by IF analysis (Figure 3e). Taken together, the results indicated that DZ effectively restored the disruption of the BTB induced by hyperglycaemia by upregulating ZO-1.

3.3 | Effects of DZ on oxidative stress in STZ/HFD-induced diabetic mice

The testicle, as a vital organ that produces spermatozoa and secretes testosterone, contains many mitochondria and high levels

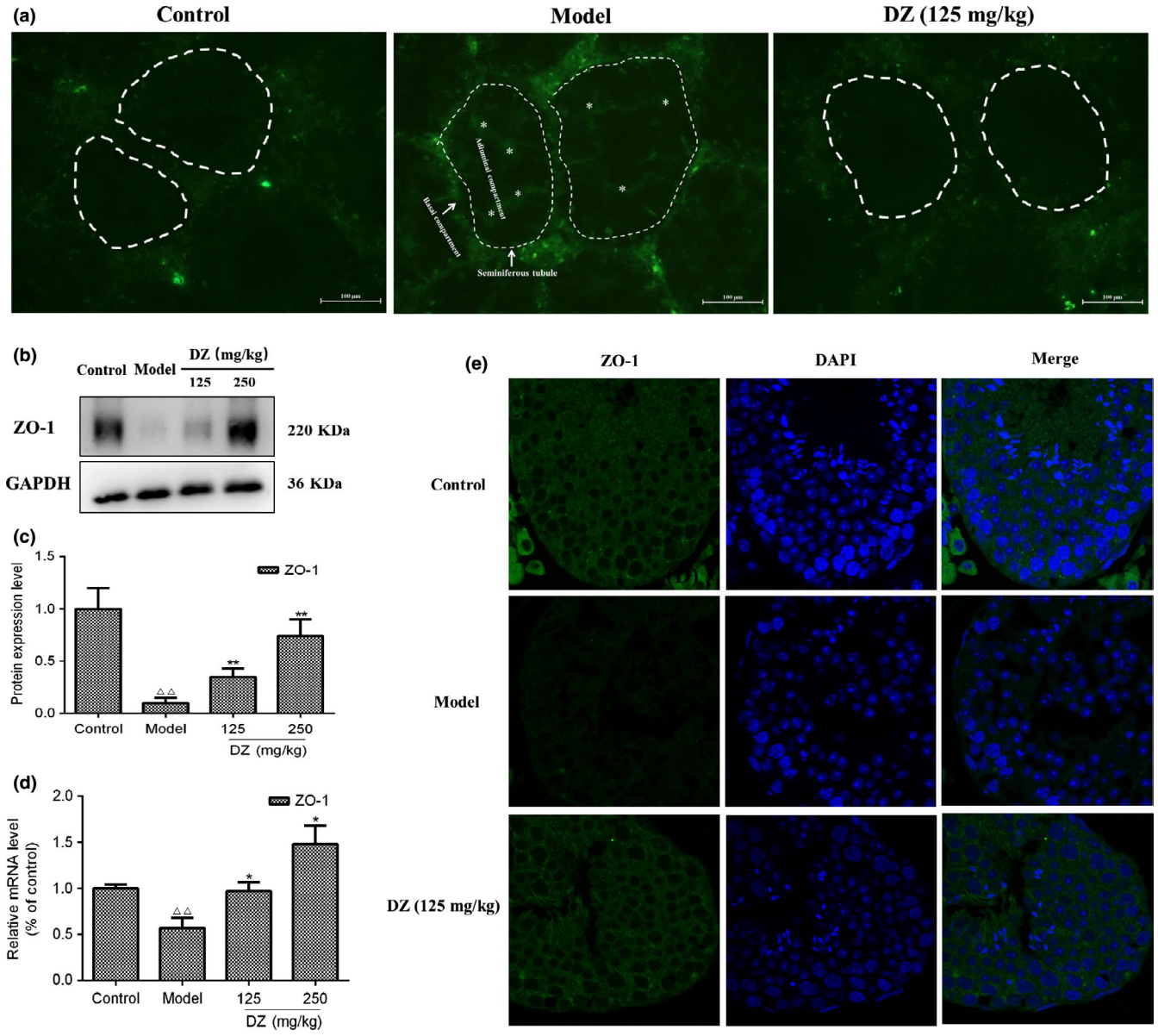


FIGURE 3 DZ restores the BTB disruption induced by hyperglycaemia by upregulating ZO-1. (a) BTB permeability was evaluated by a FITC fluorescence tracer assay in mice after DZ treatment for 10 weeks. (*) indicates that FITC entered the interstitial space from the adluminal compartment of seminiferous tubules in testis tissues. (b) The protein expression of ZO-1 in the testis was measured by Western blot analysis. (c) Results of greyscale analysis to Western blot was performed to determine the relative ratios of ZO-1. (d) The mRNA levels of ZO-1 were detected by real-time PCR. (e) The expression of ZO-1 was detected by IF (×1,000) with ZO-1 antibody (green) and DAPI (blue). The results are presented as the mean ± *SD* and shown in a column chart. $^{\triangle\triangle}p < .01$ compared with the control group; $^{*}p < .05$, $^{**}p < .01$ compared with the model group

of xanthine oxidase, which produce ROS and free radicals, and has a large amount of unsaturated fatty acids. These characteristics make the testicle tissue highly sensitive to oxidative stress-induced damage. Therefore, the protective effects of DZ on intratesticular oxidative stress damage caused by diabetes were investigated via detecting the MDA content, SOD activity and GSH/T-GSH levels in the testicle. Compared with the control group, the model group shows significantly reduced SOD activity and GSH/T-GSH level ($p < .01$) and significantly increased MDA content ($p < .01$). The high level of peroxidation in the testis may be one of the causes of testicle damage in mice. *Dioscorea zingiberensis* significantly reduced tissue MDA production and increased SOD activity (Figure 4a,b). However, DZ treatment ameliorated the ratio of GSH/T-GSH in the testis (Figure 4c). 8-OHdG is a principal marker of DNA damage, which can be induced by ROS. Figure 4d shows that the intensity of 8-OHdG fluorescence in the model group was significantly increased compared with that of the control group ($p < .01$), DZ (125 mg/kg) and DZ (250 mg/kg) significantly reduced the 8-OHdG fluorescence intensity ($p < .01$). These results showed that the antioxidant activity of DZ could protect against the intratesticular peroxidation caused by diabetes and restore the intratesticular oxidation–antioxidant balance in mice.

3.4 | DZ enhances the nuclear translocation of Nrf2 and activates Nrf2-dependent antioxidant enzymes

Nrf2 has been confirmed to have an antioxidant effect by regulating the expression of antioxidant enzymes, such as NQO1 and HO-1. To evaluate whether the antioxidant effects of DZ were

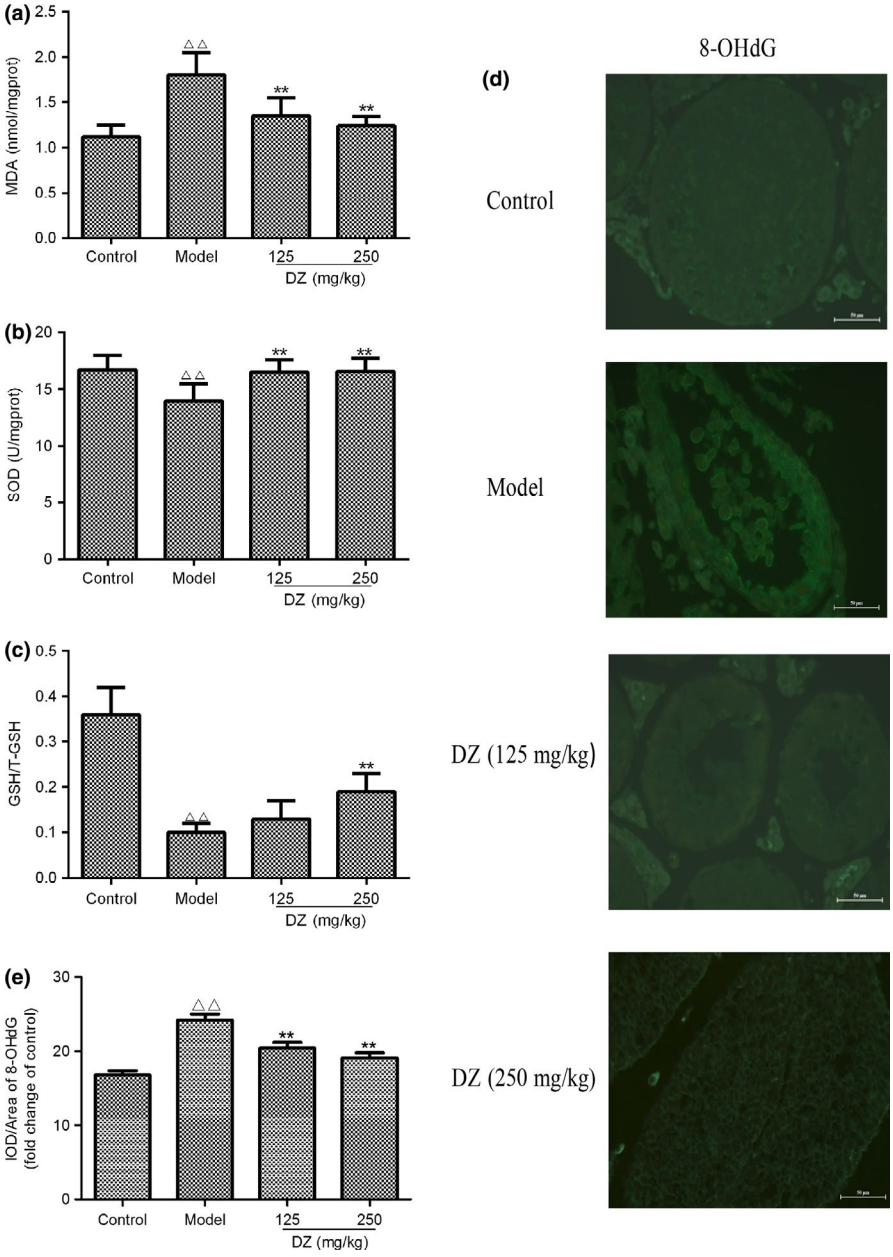


FIGURE 4 Effects of DZ on oxidative stress in STZ/HFD-induced diabetic mice. The levels of MDA (a), SOD (b) and GSH/T-GSH (c) in testicular tissues were detected by commercial assay kits after DZ treatment for 10 weeks. (d) The 8-OHdG expression in sections was determined by IF (×400) with 8-OHdG antibody (green), and a semi-quantitative analysis of 8-OHdG was determined (e). The results are presented as the mean ± *SD* and shown in a column chart. $^{\triangle\triangle}p < .01$ compared with the control group; $^{**}p < .01$ compared with the model group

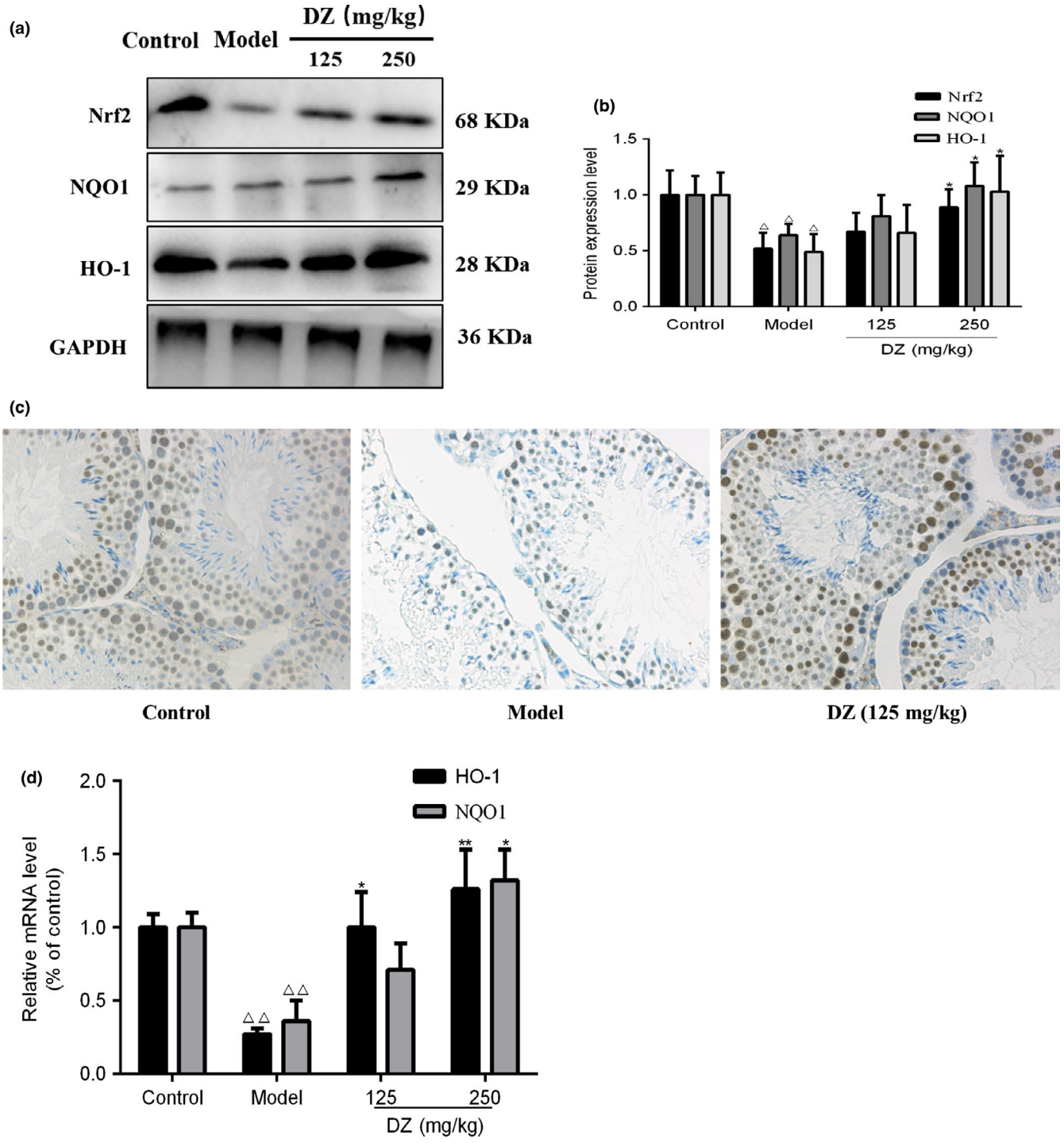


FIGURE 5 DZ enhances the nuclear translocation of Nrf2 and activates Nrf2-dependent antioxidant enzymes. (a) The expression of Nrf2, HO-1 and NQO1 was measured by Western blot analysis. GAPDH was used as the loading control. (b) The greyscale analysis was used to quantify the levels of Nrf2, HO-1 and NQO1. (c) Nrf2 protein expression was measured by immunohistochemistry. (d) NQO1 and HO-1 mRNA levels were detected by real-time PCR. The results are presented as the mean ± *SD* and shown in a column chart. $^{\triangle}p < .05$, $^{\triangle\triangle}p < .01$ compared with the control group; $^{*}p < .05$, $^{**}p < .01$ compared with the model group

related to the activation of Nrf2, the nuclear translocation of Nrf2 was investigated in testicular tissue. The results indicated that the expression of Nrf2, NQO1 and HO-1 was significantly decreased in STZ/HFD-induced diabetes mice. After DZ treatment, the protein expression of Nrf2, NQO1 and HO-1 increased significantly (Figure 5a,b). Moreover, DZ enhanced the nuclear translocation of Nrf2, as determined by immunohistochemistry (Figure 5c). Figure 5d shows that DZ significantly increased the mRNA levels of HO-1 and NQO1. It is suggested that DZ possesses potential antioxidant activity by activating the nuclear translocation of Nrf2 to promote the expression of antioxidant enzymes.

4 | DISCUSSION

In recent years, traditional Chinese medicines (TCM) have been utilised in diabetes and chronic diabetic complications because of its general beneficial effects as well as fewer side effects and toxic actions. Diabetes is closely implicated with male infertility, especially for people with type 2 diabetes. Therefore, the discovery of a highly effective medicine with low toxicity in the treatment of diabetic-induced male infertility is urgently needed. *Dioscorea zingiberensis*, as TCM, has a potent therapeutic action on male reproductive system disease. In this research, we evaluated the potential therapeutic effect and explored the functional mechanism in mice with HFD/STZ-induced diabetes. Our data indicated that DZ significantly reversed BTB disruption, testicular tissue injury and abnormal spermatogenesis in a mouse model of type 2 diabetes, as evidenced by the restoration of testicular function.

In general, the first step of testicular damage is the BTB disruption, which subsequently leads to meiotic arrest that negatively affects spermatogenesis and male fertility. Therefore, we speculated that the integrity of the BTB is compromised induced by diabetes. In our experiment, the permeability of the BTB in mice was significantly increased after long-standing diabetes mellitus, which occurred concurrently with the downregulation in the expression of ZO-1. This indicated that the abnormal alteration of ZO-1 was involved in the hyperglycaemia-induced disruption of the BTB. Some studies suggested the importance of TJs in the BTB, especially the ZO-1 protein, and that TJs could regulate cytoskeletal dynamics at cell junctions and affect male fertility (Fanning & Anderson, 2009; Li et al., 2019). Kuo et al. found that arecoline (a significant alkaloid in areca nut has a negative effect on male fertility) could reduce ZO-1 protein expression, leading to the disruption of the BTB, which represented a crucial route that mediates abnormal spermatogenesis, including low sperm quantity and quality (Kuo et al., 2014). Besides, ZO-1 is known to occupy a central position in the regulation of BTB dynamics as it could interact with claudins, occludin, cadherins, the actin cytoskeleton and GJs. Some studies suggested that the overexpression of ZO-1 can inhibit the incorporation of Cx43 (a vital protein in GJs) into GJs and the dissociation of the ZO-1/Cx43 interaction by phosphorylating c-Src on Tyr416, which lead to the internalisation and degradation of Cx43 (Sorgen et al., 2004). The androgen receptor plays a vital role in regulating the function of Sertoli cells. It is not only mediating androgen activities, but also Sertoli cell-specific deletion of the androgen receptor would reduce ZO-1 expression to further lead to a defective BTB (Wang et al., 2006; Willems et al., 2010). The reduced ZO-1 induced by diabetes is the critical role in the disruption of TJs in many chronic diabetic complications (Chehade, Haas, & Mooradian, 2002; Rincon-Choles et al., 2006). Besides, pre-clinical data suggested obesity could cause male infertility due to declines in the sperm function parameters. Meanwhile, damage of the blood–testis barrier (BTB) integrity by decreasing ZO-1 would be one of the critical underlying pathological mechanisms (Fan et al., 2015). Therefore, the results support the notion that the integrity of the BTB is sensitive to long-term high blood sugar and support the concept that the disruption of the BTB is crucial to diabetes-induced spermatogenesis dysfunction. In the present study, restoring the integrity of the BTB was a critical step in the treatment of diabetic testis injury and male infertility.

In the present study, we found that DZ exerts an antioxidant effect in testicular tissue and that this function may be the primary mechanism mitigating against testicular injury in mice with HFD/STZ-induced diabetes. Diabetes was related to a significant increase in ROS levels in testicular tissue, as shown by 8-OHdG staining and MDA assay. Under the normal physiological state, Nrf2 mediates the basal and inducible transcription of genes that encode enzymes, including NQO1 and HO-1, which are very important for protection against oxidative stress damage. Compared with wild-type males, the deletion of the Nrf2 gene leads to a 44% decrease in testicular sperm head counts, a 65% decrease in epididymal sperm counts and a 66% decrease in epididymal sperm motility (Nakamura et al., 2010). The decreased transcriptional activity of Nrf2 due to hyperglycaemia caused weakened antioxidant defence system and may represent an essential pathological mechanism in diabetic male infertility. Targeting and inducing Nrf2 have become a potential therapeutic strategy in diabetic male infertility (Ma et al., 2019; Wang et al., 2017; Zhao et al., 2018). In our research, we found that DZ could reduce the accumulation of ROS and enhance the expression of antioxidant enzymes in the testis tissue of mice by activating Nrf2.

Diosgenin, one of the aglycones of steroidal saponins extracted from DZ, is a main active ingredient that possesses a variety of therapeutic actions, including anti-inflammatory, immunoregulatory and antitumor effects. Diosgenin also has beneficial effects on the reproductive system in males. Wu et al. (2015) found that diosgenin could enhance the proliferation of TM4 cells and primary SCs by inducing SRC-ESR translocation and ERK/Akt-ESR transcriptional activity. Additionally, diosgenin was also used as a potential agent to protect testicular tissue against damage in STZ-induced diabetes in rats (Khosravi, Sedaghat, Baluchnejadmojarad, & Roghani, 2019). However, to date, there is limited data in the literature that evaluate the therapeutic mechanisms diosgenin (such as antioxidation, recovering BTB integrity and improving sperm quality) in testicular damage induced by diabetes. The present study will contribute to the theoretical basis for the further exploitation of diosgenin extract from DZ in the therapy of male infertility.

In conclusion, these results clearly showed that DZ, a traditional Chinese medicine, is a beneficial agent that attenuates testicular damage by ameliorating BTB disruption, testicular microstructure and spermatogenesis dysfunction by inducing the Nrf2/HO-1 antioxidant pathway and the TJ protein ZO-1 in HFD/STZ-induced diabetic mice. This study provides new insight into the mechanisms of the protective effect of DZ on diabetic-induced testicular damage and also provided theoretical support for its use in the treatment of male infertility. Further research will be carried out to investigate whether the upregulation of Nrf2 upregulates ZO-1 to reveal the correlation between Nrf2 and BTB integrity.

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CONFLICT OF INTEREST

The authors do not have any conflicts to disclose.

AUTHOR CONTRIBUTIONS

Bo Ma, Jie Zhou, Youli Xi, Jie Zhang, Xiaowei Zhou, Jiayi Chen and Chao Nie performed the experiments; Jie Zhou analysed the data; Bo Ma and Zhengbiao Zhu revised the manuscript; and Bo Ma, Jie Zhou and Youli Xi conceived the idea of the study and wrote the manuscript.

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