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Original Article

Effects of Sancai Lianmei Particle on autophagy and apoptosis in testes of diabetic mice via the Nrf2/HO-1 pathway

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Abstract: Objective: The aim of the current study was to evaluate the effects of Sancai Lianmei (SCLM) Particle on diabetic testicular dysfunction, exploring its mechanisms. Methods: A type 2 diabetic mouse model was induced with a high-fat diet and Streptozotocin (STZ). The mice were fed with 6 g/kg SCLM Particle daily. Protective effects of SCLM Particle on testicular function were assessed by sperm parameters, testis indexes, and H&E staining. Antioxidant capacity, testis malondialdehyde (MDA), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were determined using assay kits. Western blotting or qPCR was used to detect expression of anti-oxidative protein Nrf2/HO-1, autophagy relative protein P62/Beclin-1/LC-3, and apoptosis signal proteins Bcl-2 and Bax. Moreover, apoptotic cells were stained using TUNEL assay and autophagosomes were observed using transmission electron microscopy (TEM). Results: Administration of SCLM Particle considerably recovered testicular function and sperm quality, obviously improved testicular histopathologic structure, and significantly increased antioxidant enzyme activities. Apoptosis was reduced with the upregulation of Bcl-2 and downregulation of Bax. Autophagy was inhibited with decreased expression of P62, Beclin-1, and LC3. Autophagosomes were also reduced in germ cells under TEM. However, SCLM Particle significantly upregulated expression of Nrf2 and HO-1 in testes of diabetic mice. Conclusion: SCLM Particle alleviates testicular oxidative stress injuries and cell apoptosis in type 2 diabetic mice. Mechanisms may be associated with upregulation of Nrf2/HO-1 signal pathways and inhibition of excessive autophagy.

Keywords: Diabetic infertility, Sancai Lianmei (SCLM) particle, Nrf2/HO-1, autophagy, apoptosis

Introduction

Diabetes mellitus is one of the most common metabolic diseases. It has become a worldwide public health issue. An estimated 415 million adults have diabetes. By 2040, this is expected to rise to 642 million [1]. Recent data shows that the overall prevalence of diabetes mellitus in the Chinese adult population is estimated to be 11.6% (95% confidence interval [CI] 11.3-11.8%), far higher than the estimated worldwide prevalence [2, 3]. According to the survey, testicular dysfunction is one of the common complications of diabetes, mainly due to the loss of germ cells by apoptotic cell death. Impaired regulation of glucose and resultant hyperglycemia are major threats to the health of individuals in modern societies, given the rapidly rising prevalence affecting an increasing number of men in their reproductive years. Consequently, diabetes-induced hyperglycemia is likely to contribute to a decline in worldwide birth rates, especially in societies with a high diabetic prevalence [4]. Thus, diabetes mellitus has become a great obstacle in the fertility of humans. Current treatments are not satisfactory. It is necessary to find an effective and desirable therapeutic method to treat this disease. Traditional Chinese Medicine (TCM) plays an important role in the Chinese Health Care System, having certain advantages in preventing and treating DM, as well as its complications. Sancai Lianmei (SCLM) Particle has shown good effects regarding blood glucose reduction and antioxidant function in previous experiments [5-7]. In this study, type 2 diabetes

mellitus was constructed in a mouse model. The aim of the current study was to examine whether SCLM Particle could improve sperm qualities of diabetic mice through oxidative stress and apoptosis pathways.

Material and methods

Animal model and grouping

All animal protocols were approved by the Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine. Male C57BL/6J mice (Dashuo Experimental Animal Co. Ltd, Chengdu, China), aged 5-6 weeks, were acclimated at 20-26°C with a 12-hour light-dark cycle. They were fed with standard rodent chow and drank tap water for 1 week. Ten mice were randomly selected as the control group and fed with normal diet. The others were fed with a high-fat diet (fat energy supply was 60%), forming the model group. After 4 weeks, mice in the model group were injected intraperitoneally with 100 mg/kg of STZ (Sigma Aldrich, MO, USA) to induce T2D. Fasting blood glucose (FBG) was measured 7 days after injections with STZ, using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA, USA). Ultimately, mice with FBG levels of more than 11.1 mmol/L were considered diabetic and selected for experiments. Other animals with lower serum glucose levels were excluded. The mice were randomly allocated into four groups, including the control group (Ctrl, n = 10), diabetes mellitus group (DM, n = 10), DM with Sancai Lianmei Particle treatment group (DM/SCLM, n = 10), and DM with Metformin treatment group (DM/DMBG, n = 10). SCLM Particle (Hospital of Chengdu University of Traditional Chinese Medicine, China) was given by gavage at 6 g/kg every day for 4 weeks. This dosage has been confirmed as effective and safe [6]. DM/DMBG group mice were fed with 200 mg/kg metformin (Sino-us Shanghai Squibb Pharmaceutical Company, Shanghai, China). Control and DM group mice were administered the same amount of saline. Serum glucose and metabolic indexes were detected at the second and fourth week of administration.

Sample collection

The mice were sacrificed after 4 weeks of drug administration. Blood samples were collected from their eyes. The testis and epididymis were harvested for the following studies.

Determination of reproductive organ index

After weighing the body and testis, the index of testes in mice was calculated with the following formula: reproductive organ index (testis) = the weight of each rat reproductive organ (testis)/ body weight *1000‰.

Analysis of epididymal sperm parameters

Cauda epididymis was dissected and placed into 1 mL of prepared Ham's F10 medium for 30 minutes. A small tear was made for spermatozoa to swim out into the culture medium. Sperm density (10^6/mL), sperm vitality, and sperm survival rates were assessed using Weili Sperm Quality Automatic Detection System (WLJY-9000 type digital color, NanJing, China).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from frozen testes with RNA TRIzol (Invitrogen, America), according to manufacturer instructions. RNA concentrations and purities were quantified using a Nanodrop ND-1000 spectrophotometer. Complementary DNA (cDNA) was synthesized from total RNA using the RNA PCR kit (TaKaRa, DaLian, China), according to manufacturer protocol. TransStart Top Green qPCR SuperMix kit was used for qPCR analysis (initial template denaturation at 95°C for 5 seconds, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds). Primer sequences for genes included: β-actin forward (5'-gatgtgtttgggggaaggtct-3') and reverse (5'-tactcctgcttgctgatcca-3'); Nrf2 forward (5'-agcatagagcaggacatggagcaagt-3') and reverse (5'-ctggctggcatcatcagtggagagg-3'); HO-1 forward (5'-gctaagaccgccttcctgctcaacac-3') and reverse (5'-gcctctgacgaagtgacgccatctg-3'); LC3-II forward (5'-gccatgccgtccgagaagaccttca-3') and reverse (5'-atgctgtgcccattcaccaggagga-3'); Beclin-1 forward (5'-gcagtggcggctcctattccatcaa-3') and reverse (5'-gcagtggcggctcctattccatcaa-3'); P62 forward (5'-tggtgctactgcctcttctca-3') and reverse (5'-gggttactttggtccgcttt-3'). β-actin was used as the internal reference gene for normalization of gene expression levels. PCR was analyzed using the ΔΔcycle threshold method, as

described previously [8]. However, there was an adjustment for PCR reaction efficiencies, which was calculated from dilution series for each gene of interest.

Western blot analysis

Total testicular protein was extracted using lysate including 100 mM PMSF. Protein concentrations were measured using BCA protein assays (Beyotime, China). Regular Western blot protocol was performed, as described in previous studies [9]. Primary antibodies were as follows: Nrf2 (1:500), HO-1 (1:500), LC-3II/I (1:500), P62 (1:500), Beclin-1 (1:500), Bcl-2 (1:1000), Bax (1:2000), and β-actin (1:5000) (Affinity, America). Quantitative densitometry was performed on identified bands using Image Quantity one software.

Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay

Testis tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm thickness. Slides were stained for TUNEL using the ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Boster, Wuhan, China). Briefly, each slide was deparaffinized and rehydrated, then treated with proteinase K (20 mg/l) for 15 minutes. Endogenous peroxidase was inhibited using 3% hydrogen peroxide for 5 minutes, then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 hour. The TdT reaction was carried out in a humidified chamber at 37°C. Next, 3, 3-diaminobenzidine chromogen was added. Hematoxylin was used for counterstaining. For negative controls, TdT was omitted from the reaction mixture. Apoptotic cells exhibited a brown nuclear stain under the microscope. They were quantitatively counted manually. Results are presented as TUNEL-positive cells per microscope view.

Measurement of lipid peroxidation and antioxidant activity

Mouse homogenate supernatant of testes was used to assess oxidative products. Levels of malondialdehyde (MDA) and activities of superoxide dismutase (SOD) and 8-OHdG were determined using a microplate reader (1510, Thermo Fisher Scientific, Waltham, MA, USA). Protein content of the supernatant was determined using assay kits (Abcam, England). Objective protein activity was then measured at 450 nm against the blank.

Histopathology of testes

After anesthesia, testes were harvested and fixed by Bouin's fluid for 24 hours. After dehydrating in alcohol gradient and cleaning in xylene, testicular tissues were subjected to paraffin embedding. Afterward, tissue specimens were embedded in paraffin blocks. Sections measuring 5 μm were obtained for deparaffinization in xylene and rehydration in gradient ethanol from 100 to 70%. They were stained with hematoxylin and eosin. Testis tissues were examined, evaluating histopathological alterations under a standard light microscope (LeicaDM2500) with a digital camera (model-DFC-450C) (LeicaMicrosystems, Wetzlar, Germany). Finally, they were photographed.

Transmission electron microscopy (TEM)

For TEM observation, testes were fixed in ice-cold glutaraldehyde (2.5% in 0.1 mol/L cacodylate buffer, pH 7.4) for 24 hours, then fixed in osmium tetroxide. After dehydration with a graded series of acetone, the samples were embedded in Epon812. Semi-thin sections were obtained to locate the position using an optical microscope. These ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy. Electron micrographs were observed under a Hitachi H-600IV transmission electron microscope.

Statistical analysis

Quantitative data fitting a normal distribution are presented as mean ± SD, while ill-fitting data was analyzed using non-parametric tests. Comparisons were performed using one-way ANOVA for multi-groups. LSD testing was used for quantitative data fitting a normal distribution when there was equal variance. However, Tamhane's T2 test was used when unequal variance was present. Comparisons between two groups were analyzed with independent sample t-tests. Calculations were performed using the Statistical Package for Social Sciences, version 17.0, for Windows (SPSS, Chicago, IL). Differences are considered statistically significant at $P < 0.05$ and very significant at $P < 0.01$.

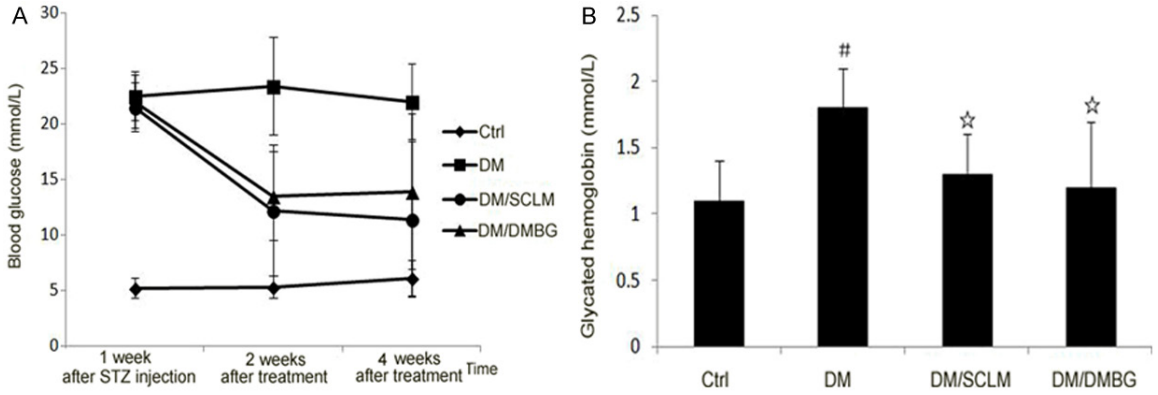

Figure 1. FPG and HbA1c changes in each group. A. FPG changes in each group. B. HbA1c changes in each group. Compared with Ctrl group: $^{\#}P < 0.05$; Compared with DM group: $^{*}P < 0.05$.

Results

Effects of SCLM particle on glucose control of T2DM

FPGs of DM, DM/DMBG, and DM/SCLM groups were 22.5 ± 2.2, 22.0 ± 2.4, and 21.5 ± 2.2 mmol/L, respectively after 1 week of STZ injections. They were significantly higher than those of the Ctrl group of 5.2 ± 0.9 mmol/L, consistent with the diagnosis of type 2 diabetes. After 2 weeks of administration, FPGs of mice in the DM/SCLM group and DM/DMBG group were significantly lower than those in the DM group (13.5 ± 4.0, 12.2 ± 5.9 *versus* 23.4 ± 4.4 mmol/L, $P < 0.05$). There were no differences between the DM/SCLM group and DM/DMBG group. FPGs showed similar changes in each group after 4 weeks of administration. Hemoglobin A1c (HbA1c) levels in the DM/SCLM group showed no statistical differences with the Ctrl group ($P > 0.05$), but levels were significantly lower than those in the DM group after 4 weeks of administration ($P < 0.05$), as shown in **Figure 1**.

Changes in reproductive organ indexes and sperm parameters

Reproductive organ indexes of the DM/SCLM group were lower than those of the Ctrl group ($P < 0.05$), but significantly higher those of the DM group ($P < 0.05$). Results indicate that SCLM Particle could increase testis weights. Additionally, compared with the DM group, sperm density, sperm vitality, and sperm survival rates were higher in the DM/SCLM group ($P < 0.05$). There was no statistical significance in the above sperm indexes between the DM/SCLM group and Ctrl group, except for sperm vitality. Sperm vitality in the DM/SCLM group was lower than that in the Ctrl group ($P < 0.05$). Results are shown in **Figure 2**.

Effects of SCLM Particle on testicular oxidative stress

Results of ELLISA showed that expression levels of SOD in the DM/SCLM group was close to that in the Ctrl group. Levels were significantly higher than those in the DM group (2.82 ± 0.34 *versus* 2.21 ± 0.31 ng/mL, $P < 0.01$). However, expression levels of MDA in the DM/SCLM group were significantly lower than those in the DM group (6.08 ± 1.00 *versus* 7.82 ± 0.87 nmol/mL, $P < 0.01$). Expression levels of 8-OHdG in the DM/SCLM group were also lower than those in the DM group (2.12 ± 0.38 *versus* 2.67 ± 0.23 ng/mL, $P < 0.05$), as shown in **Figure 3**.

Effects of SCLM particle on Nrf2/HO-1 signaling pathways

Relative mRNA expression of Nrf2 in the DM/SCLM group was significantly higher than that in the DM group (0.790 ± 0.156 *versus* 0.513 ± 0.136, $P < 0.01$). Relative protein expression of Nrf2 was also higher than that in the DM group (0.728 ± 0.087 vs 0.491 ± 0.093, $P < 0.05$). Compared with the DM group, relative mRNA and protein expression levels of HO-1 were both higher in the DM/SCLM group ($P < 0.01$ and $P < 0.05$ respectively), as shown in **Figure 4**.

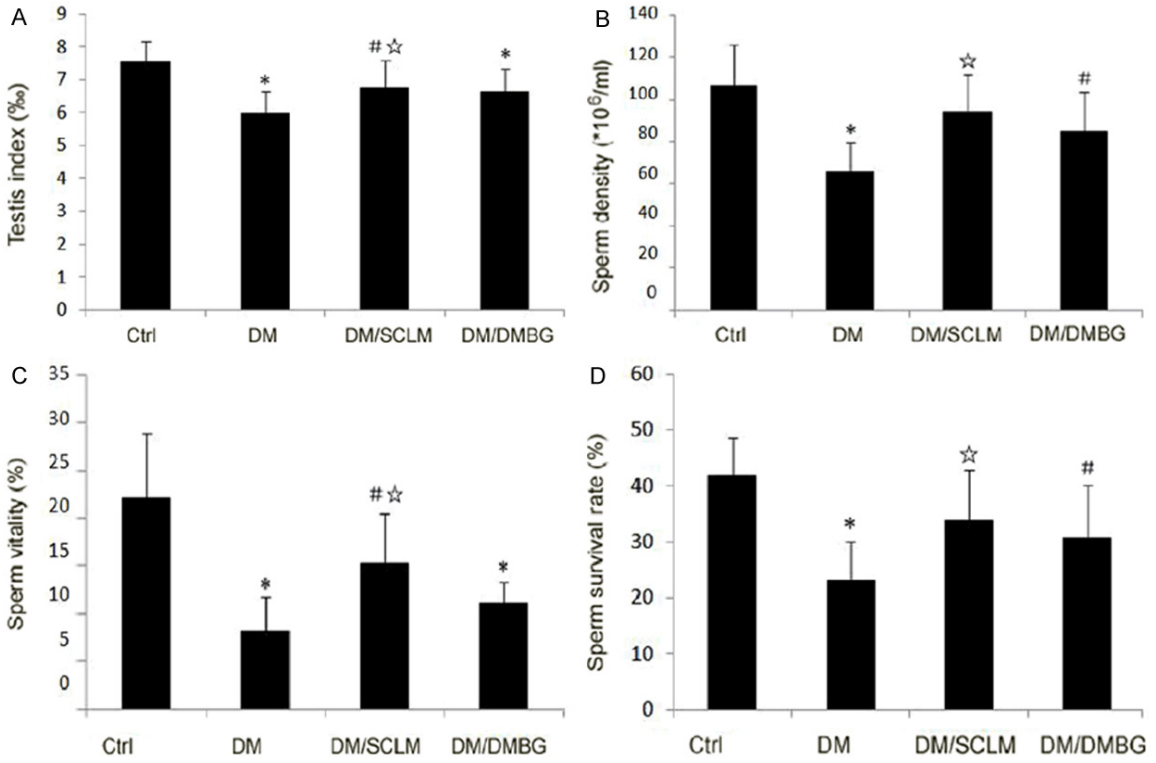

Figure 2. Effects of SCLM particle on testis index and sperm parameters. A. Reproductive organ index in each group, testis index = the weight of rat testis/body weight *1000‰. B. Sperm density in each group. C. Sperm vitality in each group. D. Sperm survival rate in each group. Compared with the Ctrl group: $^{*}P < 0.01$, $^{\#}P < 0.05$; Compared with DM group: $^{*}P < 0.05$.

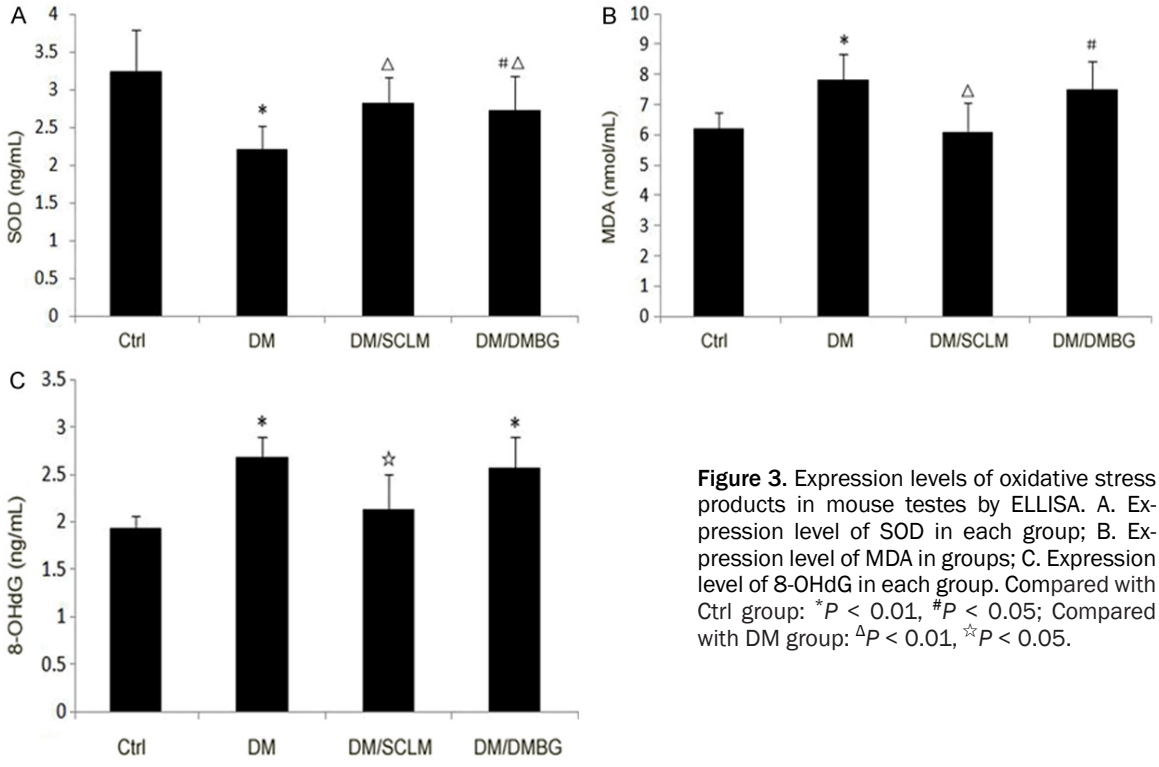

Figure 3. Expression levels of oxidative stress products in mouse testes by ELLISA. A. Expression level of SOD in each group; B. Expression level of MDA in groups; C. Expression level of 8-OHdG in each group. Compared with Ctrl group: $^{*}P < 0.01$, $^{\#}P < 0.05$; Compared with DM group: $^{\Delta}P < 0.01$, $^{\star}P < 0.05$.

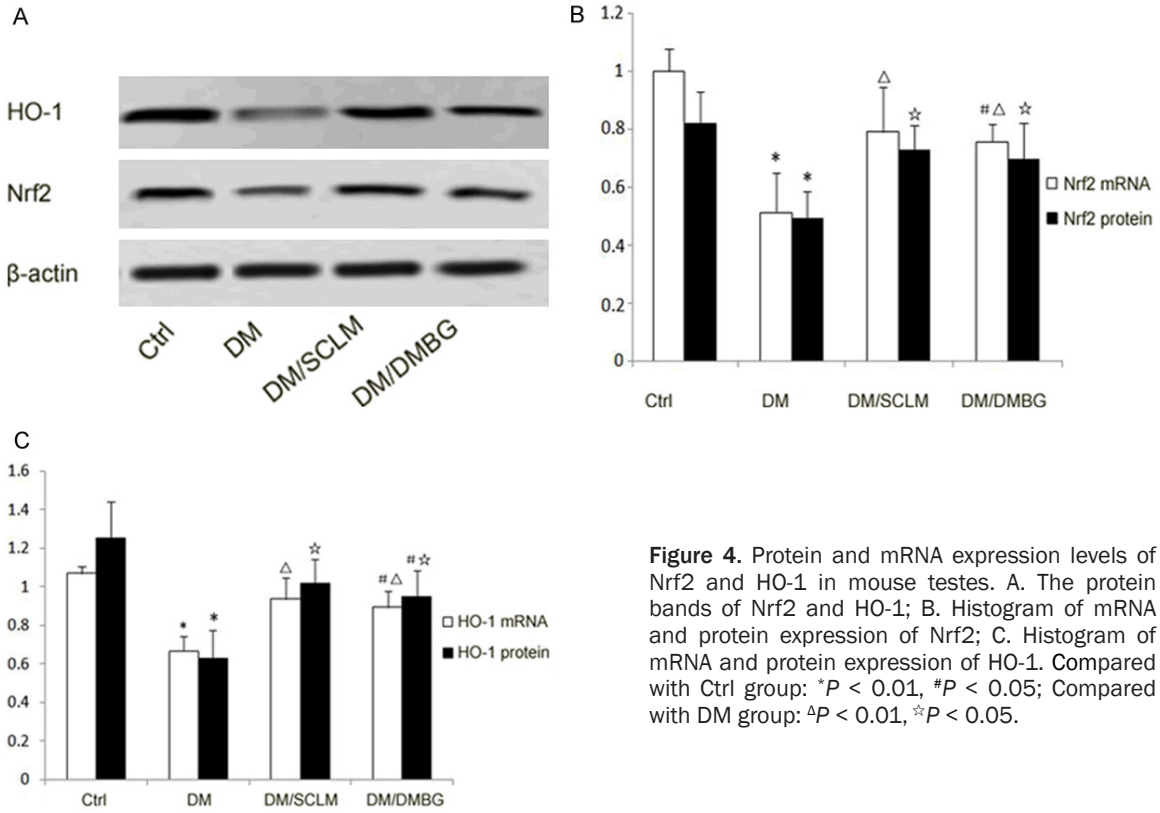

Figure 4. Protein and mRNA expression levels of Nrf2 and HO-1 in mouse testes. A. The protein bands of Nrf2 and HO-1; B. Histogram of mRNA and protein expression of Nrf2; C. Histogram of mRNA and protein expression of HO-1. Compared with Ctrl group: $^{*}P < 0.01$, $^{\#}P < 0.05$; Compared with DM group: $^{\Delta}P < 0.01$, $^{\star}P < 0.05$.

Effects of SCLM on autophagy in diabetic rat testes

Autophagy relative proteins of Beclin-1, LC-3 II/I, and P62 were detected using qPCR and Western blotting. These proteins in the DM group were significantly higher than in the Ctrl group. However, mRNA levels of Beclin-1, LC-3 II/I, and P62 in the DM/SCLM group were lower than those in the DM group ($P < 0.01$, $P < 0.05$, $P < 0.01$, respectively). Protein expression levels were also lower, compared with the DM group ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively), as shown in **Figure 5**. Moreover, autophagy was morphologically identified by TEM in spermatogonium, further confirming that it was excessively induced in the DM group, as shown in **Figure 7C**.

SCLM particle inhibits cell apoptosis in DM mouse testes

Testicular cell apoptosis was evaluated by detection of Bcl-2 and Bax protein expression with Western blotting and TUNEL assays. There were amounts of Bcl-2 expression in the Ctrl group, but expression levels in the DM group were significantly, decreased compared to the Ctrl group ($P < 0.01$). The trend was reversed in the DM/SCLM group, in which expression of Bcl-2 was increased, compared with the DM group ($P < 0.01$). Contrary to Bcl-2, expression levels of Bax in the DM group were increased, compared to the Ctrl group ($P < 0.01$). SCLM Particle attenuated expression of Bax, compared with the DM group ($P < 0.05$). TUNEL staining showed a significant increase in testicular apoptotic cell death in the DM group, compared with the Ctrl group. However, SCLM Particle was found to significantly, but not completely, prevent diabetes-induced cell apoptosis in testes. Results suggest that DM-induced testicular apoptotic cell death could be significantly attenuated by SCLM Particle treatment, as shown in **Figure 6**.

Effects of SCLM particle on pathological changes in diabetic mice testis

H&E staining of testes in the Ctrl group revealed normal histological images of seminiferous tubules. Germinal cells were filled in seminiferous tubules. The spermatogenic process was completed, including spermatogonium to sp-

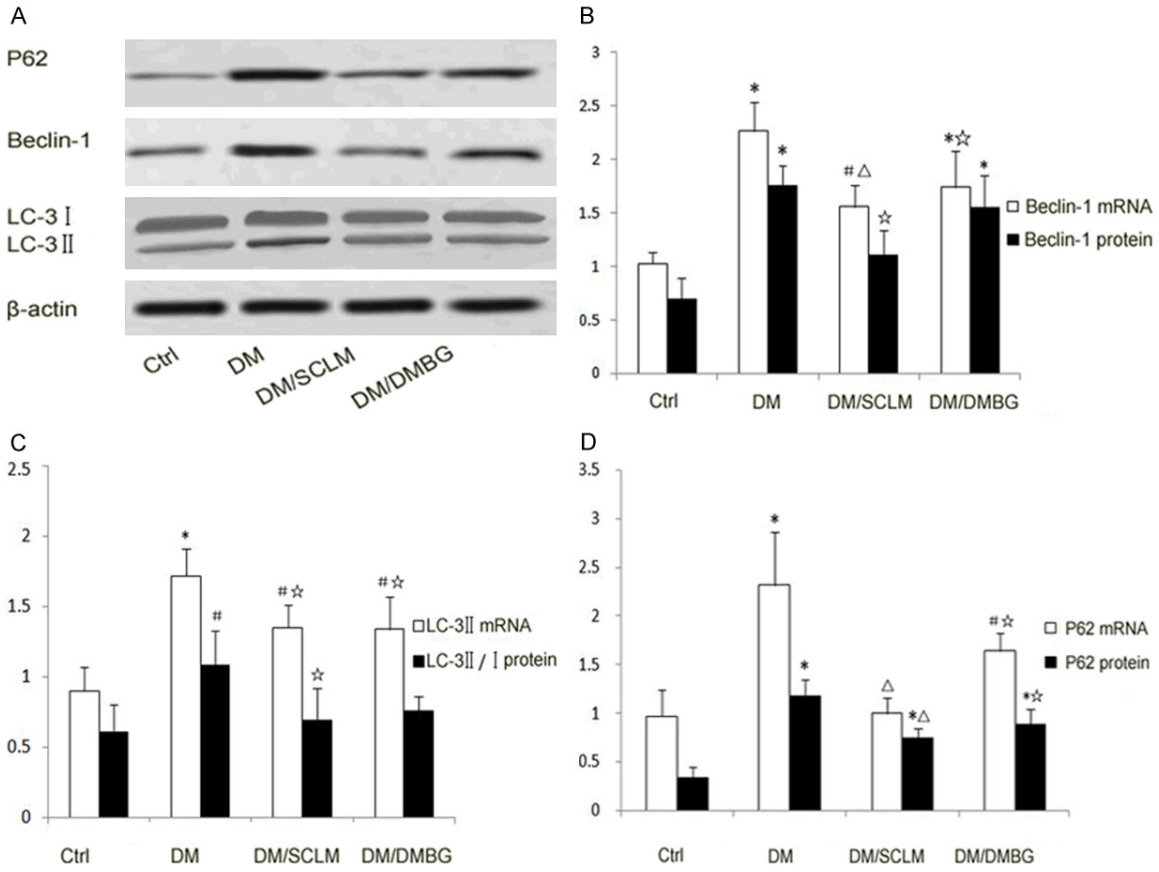

Figure 5. Autophagy relative proteins expression levels of Beclin-1, LC-3 II/I, and P62 in each group. A. The protein bands of Beclin-1, LC-3 II/I, and P62; B. Histogram of relative mRNA and protein expression of Beclin-1; C. Histogram of relative mRNA and protein expression of LC-3 II/I; D. Histogram of relative mRNA and protein expression of P62. Compared with Ctrl group: $^{*}P < 0.01$, $^{\#}P < 0.05$; Compared with DM group: $^{\Delta}P < 0.01$, $^{\star}P < 0.05$.

erm. Leydig cells were normal and well-developed. In the DM group, the seminiferous tubules became thinner and the lumen was hollowed. Spermatogenic cells developed incompletely. They were disarranged or lost, undergoing necrosis. Leydig cells were reduced or absent. However, SCLM Particle alleviated detrimental pathological changes. Almost normal spermatogenic cells and sperm could be observed in the DM/SCLM group. The tubule lumen was fatter than that in the DM group. There was also some normal Leydig cells detected in the DM/SCLM group. The ultrastructure of germ cells in testes was examined by TEM. In the Ctrl group, a well-developed spermatogonium with round nuclei was observed, with equally distributed chromatin, normal mitochondria, and ribosomes. In the DM group, cell nucleus damage could be detected, including degraded nuclei, nuclear membrane deformation, chromatin condensation, and margination. Moreover, damage in the cytoplasm could be detected, including reduced ribosomes, disappearing ridge, and swollen mitochondria. Autophagosomes were visible in the cytoplasm, as well. This was rare in the Ctrl group. However, pathological changes in the DM/SCLM group were much milder than those in the DM group, as shown in **Figure 7**.

Discussion

With increased morbidity and younger incidence, reproductive complications induced by diabetes have become an important cause of male infertility. Effective and safe approaches preventing diabetic testicular dysfunction have not been available [10]. However, aging societies and low birth rates have become a big obstacle for social and economic development in China. The state has implemented a second-child policy, aiming to increase birth rates and improve the population structure. Therefore, studying the pathogenesis and treatment of

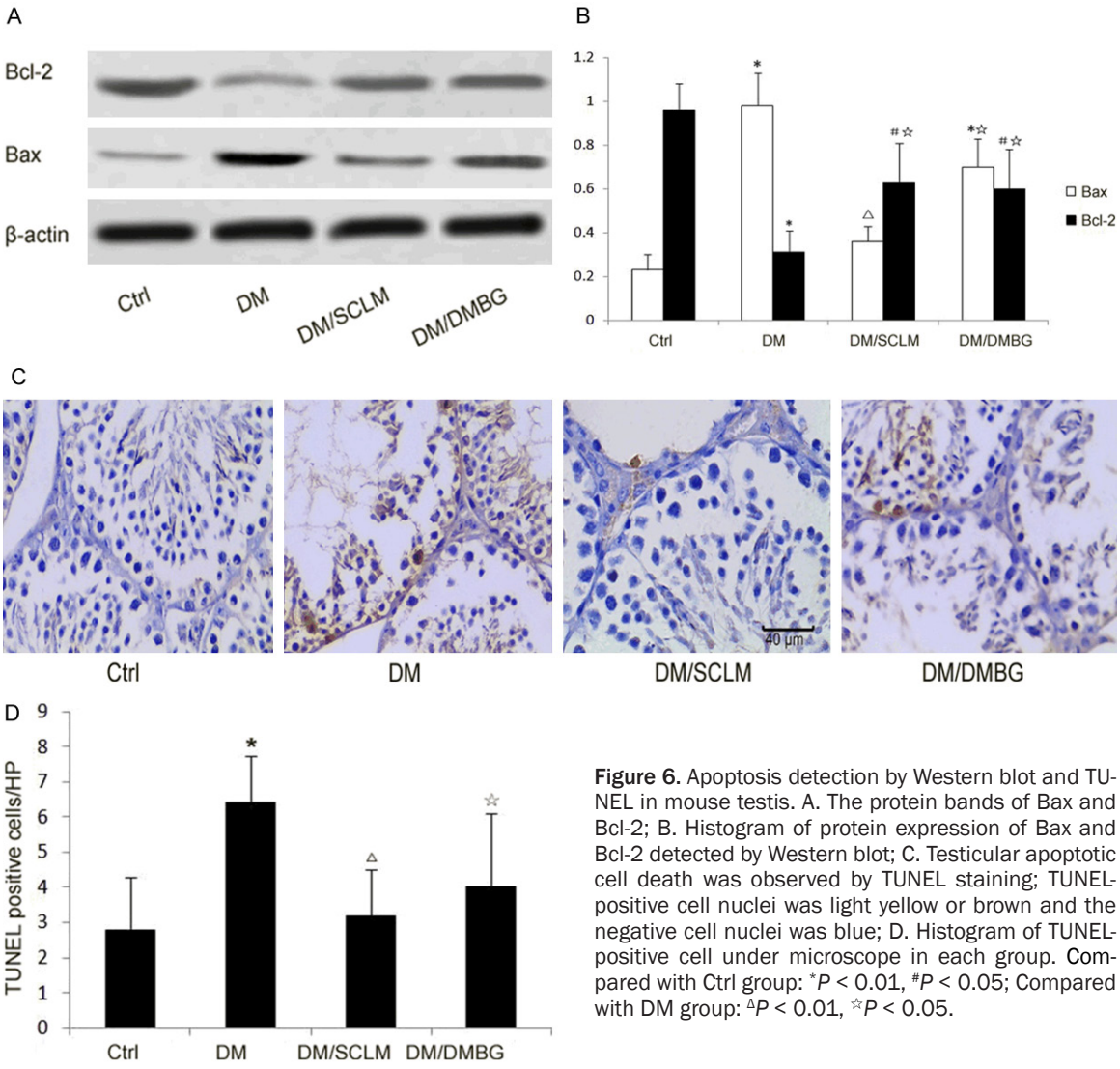

Figure 6. Apoptosis detection by Western blot and TUNEL in mouse testis. A. The protein bands of Bax and Bcl-2; B. Histogram of protein expression of Bax and Bcl-2 detected by Western blot; C. Testicular apoptotic cell death was observed by TUNEL staining; TUNEL-positive cell nuclei was light yellow or brown and the negative cell nuclei was blue; D. Histogram of TUNEL-positive cell under microscope in each group. Compared with Ctrl group: $^{*}P < 0.01$, $^{\#}P < 0.05$; Compared with DM group: $^{\Delta}P < 0.01$, $^{\star}P < 0.05$.

diabetic infertility has become an important issue in the treatment of male infertility. It is also meaningful for social development and family harmony.

In basic and clinical experiments, it has been confirmed that germ cell apoptosis is significantly increased in diabetic testes, compared to normal testes. Testicular apoptotic cell death occurs at low level during normal spermatogenesis. However, this process speeds up in some diseases, such as diabetes and other chronic diseases [11-13]. Increasing evidence has demonstrated that infertility or subfertility in diabetic individuals results from oxidative stress injury and destroyed oxidant-antioxidant defense of the male reproductive system [14]. Hyperglycemia and advanced glycation end products (AGEs) in diabetes lead to produce excessive oxidative stress products in germ cells, such as 4-HNE, MDA, and 8-OHdG. These products could cause germ cell mitochondria damage, leading to the release of cytochrome C. This activates apoptotic signaling pathways, such as caspase-3 [15]. The overproduction of oxygen free radicals has been highly correlated with abnormal cell autophagy and increased DNA fragments. This may be the mechanism of diabetic infertility [16]. The current study verified that excessive MDA and 8-OHdG were detected in DM mice testes. This causes abnormal autophagy in the mitochondria, Organelle damage, and nuclei degradation, eventually leading to cell death. Moreover, previous studies were checked, exploring the mechanisms of why excessive ROS is produced in diabetic indi-

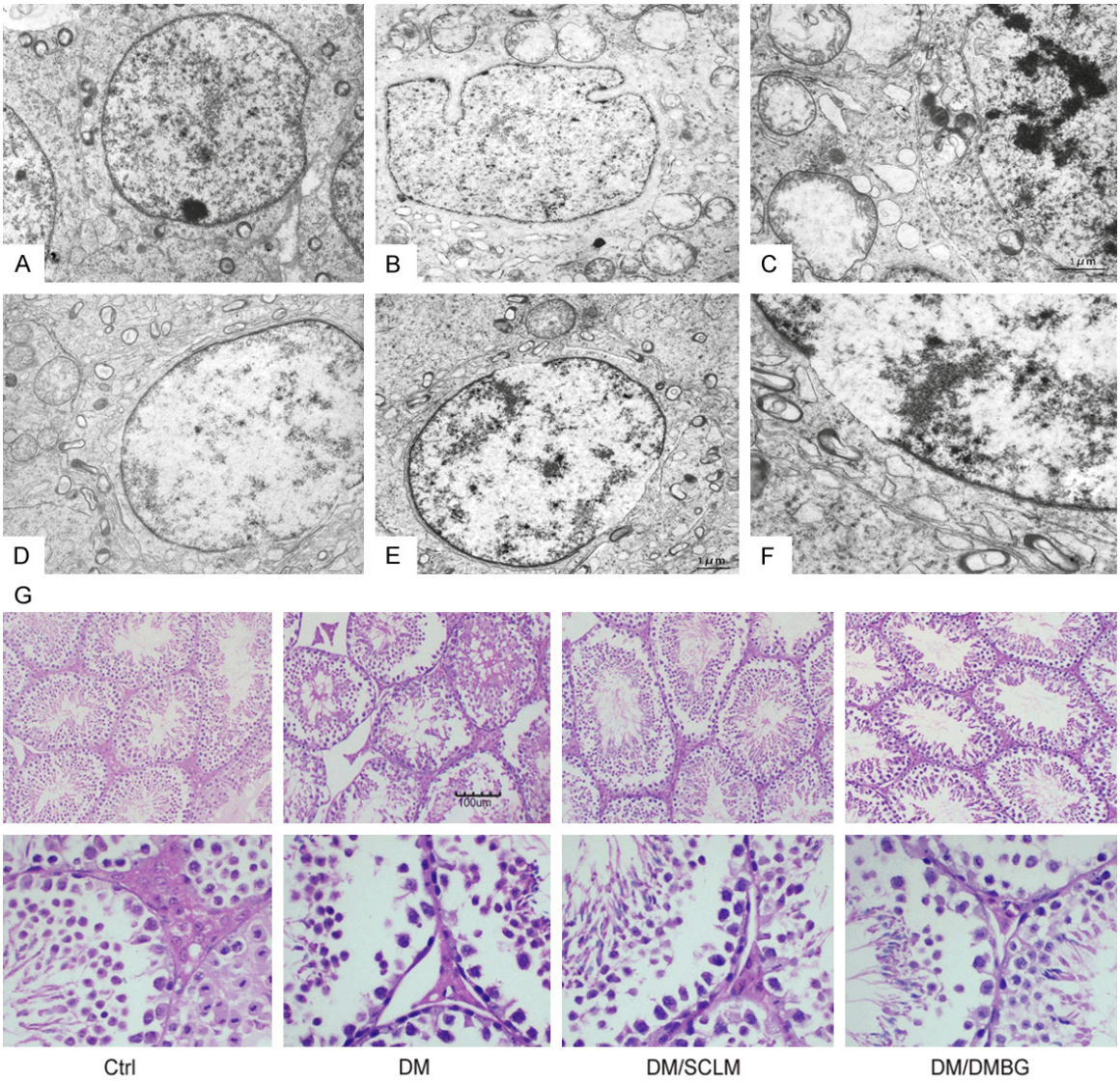

Figure 7. Pathological changes of mice testicular tissues and ultrastructure in germ cells. Ultrastructure in germ cells observed by TEM, (A) Ctrl group (×10000); (B) DM group (×10000), (C) DM group (×15000); Degraded nuclei, nuclear membrane deformation, chromatin condensation, disappeared ridge, swollen mitochondria, autophagosomes were shown in DM group; (D): DM/DMBG group (×10000); (E) DM/SCLM group (×10000); (F) DM/SCLM group (×15000), pathological changes became milder in this group. (G) Representative H&E staining in testes in each group (observed under 100 and 400 magnification). Reduction of spermatogenic cell layer, degraded germ cells, and reduced Leydig cells could be detected in the DM group.

viduals. Some scholars found that nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathways are a key signaling pathway in the process of oxidative stress injuries [17]. Nrf2 signaling pathways are inhibited in diabetic conditions. This aggravates oxidative stress injuries in diabetic patients [18]. Nrf2 regulates the transcription of antioxidant enzymes by interacting with antioxidant responsive elements (ARE). ARE sites include NAD(P)H (quinone oxidoreductase, NQO1), heme oxygenase-1 (HO-1) and glutamylcysteine ligase (GCL) [19]. HO-1 is one of the most important endogenous antioxidant protective factors in the body, playing an important regulatory role in many pathophysiological processes, especially protection against ischemia-reperfusion injuries and reduction of oxidative stress injuries [20]. Thus, enhancing expression of Nrf2 may be an effective therapeutic method improving spermatogenesis function. It was reported that sulforaphane (SFN) could increase expression

of Nrf2 and its downstream genes of HO-1, NQO1, and CAT in diabetic mice testis, leading to attenuate diabetes-induced oxidative stress damage and inflammation. It was also shown to preserve germ cell proliferation and cell viability [21]. Present results also showed that expression of Nrf2 was suppressed in the DM group, resulting in testicular cell damage and apoptosis, with increased oxidative stress damage and excessive autophagy. However, it is difficult to find desirable medicines to treat poor fertility induced by diabetes. Traditional Chinese Medicine (TCM) provides an alternative for improved testicular dysfunction in diabetes. Therefore, the current study was designed to evaluate the effects of herbal SCLM Particle against diabetic testicular dysfunction, further evaluating the relationship between these protective effects and Nrf2/HO-1 mediated anti-oxidative signaling pathways.

TCM plays an important role in the Chinese Health Care System, having certain advantages in preventing and treating DM, as well as its complications [22]. Diabetes was described as "Xiaoke" in Huang Di Nei Jing in TCM. The name was first found in Su wen·Qi bing lun, depicted as "spleen pyretic abundance...This person must eat amounts of sweet and fat food, in which fat makes person internal heat and sweet makes person full, helping the *Qi* overflow, turning to Xiaoke" [23]. Therefore, according to the theory of TCM, the pathogenesis of Xiaoke is the evil of dryness and heat. This consumes *Qi* and hurts *Yin,* leading to deficiencies of *Qi* and *Yin*. *Qi* deficiency causes insufficient fluid and *Yin* deficiency causes excessive internal heat. Thus, *Yin* fluid is not enough to raise the Jing, leading to impotence, fatigue, and infertility [5]. It is the reflection of the TCM theory of "*Yang* transformation into *Qi* and *Yin* transformation into shape". The treatment principle is to clear heat and fire, supplementing *Yin* and *Yang*. SCLM Particle, a variation of a classical Traditional Chinese prescription, was first mentioned in the book Wen Bing Tiao Bian in the Qing Dynasty [24]. It was created based on three ancient TCM formulas, "San Cai Tang", Jiao Tai Wan, and Fructus Mume. The particle consists of Renshen (Radix Ginseng), Tiandong (Radix Asparagi Cochinchinensis), Dihuang (Radix Rehmanniae), Wumei (Fructus Mume), Rougui (Cortex Cinnamomi Cassiae), and Huanglian (Rhizoma Coptidis). Main treatment effects have been described as a clearing of heat, moistening dryness, and a balancing of *Yin* and *Yang*. Many laboratory and clinical studies have confirmed the existence of effective components or the compound of SCLM, which were superior in improving insulin resistant, regulating blood glucose, and reducing oxidative stress damage and cell apoptosis [25]. Modern pharmacological research has found that ginsenosides Rb1, Rg1, Rg3, Rh2, Re, and IH-901 exert anti-diabetic and anti-oxidant effects [26]. Ginsenoside Rg1 could ameliorate testicular senescence changes in D-gal-induced aging mice via anti-inflammatory and anti-oxidative mechanisms and downregulate p19/p53/p21 signaling pathways [27]. Dihuang has been shown to have an inhibiting effect on advanced glycosylation end products (AGEs), which reacts on endothelial dysfunction. Mechanisms may be associated with the inhibition of endothelial cell oxidative stress injury, reduction of the generation of inflammation factors, and regulation of secretion from endothelial cells [28]. Rougui contains lots of cinnamaldehyde and cinnamic acid, which has been demonstrated to exhibit beneficial effects in attenuating disturbances of glucose and lipid metabolism [29]. Berberine extract from Huanglian may increase SOD activity and decrease MDA levels to reduce blood glucose in diabetic rats induced by alloxan and streptozotocin [30]. The basic and most important therapy for diabetic infertility is the lowering of blood glucose levels and prevention of complications. TCM has shown desirable therapeutic effects in treating this disease. In previous experiments, SCLM Particle was shown to reduce blood glucose levels and antioxidant function. SCLM Particle could significantly reduce hemoglobin A1c, FPG, and 2hPG levels, improving beta-cell function and insulin resistance of T2DM inadequately controlled with metformin. It has the advantages of decreasing blood lipid and improving the function of islet β cells with satisfactory safety and tolerance [6, 7]. It may also improve the release of nitric oxide and inhibit the secretion of endothelin-1 in type 2 diabetes patient plasma [5]. It reduces the production of MDA and increases SOD in serum, alleviating oxidative stress damage. However, results of *in vivo* and *vivo* animal toxicity tests have confirmed that SCLM Particle is safe and reliable, with no obvious toxicity [6].

The current study found that SCLM Particle significantly decreased FPG and HbA1c in DM mice, with effects equivalent to metformin. SCLM Particle may also attenuate oxidative stress injuries and inhibit testicular apoptosis in diabetic mice, leading to improved quality of sperm. Some indicators were better than metformin. Furthermore, exploring the mechanisms, the influence of SCLM Particle on autophagy and apoptosis was examined.

Autophagy is a stress response involved in clearance of long-lived proteins and digestion of damaged organelles, maintaining cellular dynamic balance and cell integrity. Moderate autophagy maintains homeostasis of organisms. It has been reported to play a protective role against testicular damages caused by hyperglycemia and hypoxia [31]. Hyperglycemia and oxidative stress in the testes of diabetic individuals cause hypoxia in the testis tissue. Amounts of ROS produced by the mitochondria under hypoxic conditions exerts a threat to cell survival. Mitochondrial autophagy is an adaptive metabolic response to the condition of hypoxia. Appropriate autophagy could reduce cell apoptosis, but abnormal autophagy increases incidence of cell apoptosis [32]. In this study, low levels of apoptosis could be observed in the Ctrl group, but abnormal autophagy occurred in DM mice testis, causing increased cell apoptosis. It could be attenuated by SCLM Particle. The p62 protein binds to the sites of autophagosome formation and can associate with both the autophagosome localizing protein LC3 and ubiquitinated proteins [33]. Therefore, p62 has been considered to act as a receptor for ubiquitinated proteins, organelles, and microbes, which sequesters into the autophagosome. The p62 protein interacts with the Nrf2-binding site of Keap1 and competitively inhibits Keap1-Nrf2 interaction. This is responsible for expression of a battery of gene-encoding antioxidant proteins [34]. Additionally, this study found that autophagy-relative proteins were significantly increased in DM testes, but SCLM Particle could decrease expression of P62, Beclin-1, and LC-3 II/I. TEM examination results showed that more autophagosomes were identified in diabetic testicular cells, compared to the SCLM treated group. Present results suggest that protective effects of SCLM on testicular damage may be regulated by levels of autophagy in diabetic testes.

Conclusion

The current study demonstrated that SCLM Particle could alleviate testicular dysfunction in diabetic mice. This compound significantly upregulates Nrf2/HO-1 signaling pathways, leading to a decrease of MDA and 8-OHdG and an increase of antioxidant enzyme SOD. Thus, it could attenuate oxidative stress damage. Moreover, SCLM Particle may also downregulate autophagy relative protein expression, leading to the inhibition of germ cell apoptosis. In summary, the current study demonstrated the protective effects of SCLM Particle on testicular dysfunction through upregulation of Nrf2/HO-1 signal pathways and inhibition of excessive autophagy in diabetic mice. Results from the present study support the possibility that SCLM Particle may be used as a potential agent for treatment of diabetic testicular dysfunction.

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Disclosure of conflict of interest

None.

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