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Studies on the mechanism of testicular dysfunction in the early stage of a streptozotocin induced diabetic rat model

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ABSTRACT

Streptozotocin (STZ) induced diabetic model has been widely used to study the effects of diabetes mellitus (DM) on male infertility, but it remains unclear whether the responses in this model are due to hyperglycemia or STZ per se. This study was designed to investigate the mechanism of STZ on testicular dysfunction. In the present study, sperm characteristics, serum testosterone, steroidogenic enzymes (StAR and 3β-HSD), and the vimentin apical extension of sertoli cells decreased significantly in the STZ group compared with those in the normal controls ($p < 0.05$), while Johnsen's score, testicular lipid peroxidation, spermatogenic cell apoptosis, and the expressions of NF-κB and Wnt4 significantly increased ($p < 0.05$). Insulin replacement mainly restored the decreased serum testosterone and steroidogenic enzymes, but not other parameters. The results indicated that spermatogenic dysfunction in the early stage of STZ-induced diabetic rats was due to direct STZ cytotoxicity to sertoli cells, which could be regulated by Wnt4 and NF-κB, while steroidogenic dysfunction might be a direct or indirect consequence of insulin deficiency. The results suggested that STZ-induced diabetic model, at least in the early stage, is not suitable to study the diabetes-related spermatogenic dysfunction.

1. Introduction

DM is one of the most prominent public health problems in modern societies and its incidence is rapidly increasing. The proposition that DM has adverse effects on male fertility has long been controversial [1]. However, recent studies have revealed that DM can affect spermatogenesis and steroidogenesis at various levels, which finally cause male infertility [2,3]. As expected, there are more studies with animal models than those with clinical data concerning DM-related male infertility. The STZ-induced diabetes rat is one of the most widely used models to study the effects of DM on male infertility.

STZ, an antibiotic isolated from *Streptomyces achromogenes*, is a well-known genotoxic agent and a potential source of oxidative stress [4]. Although STZ-induced diabetic rat in various animal studies has been demonstrated as a successful model for diabetes, it was reported that high doses of STZ may induce damage in tissues besides the pancreas [5]. STZ-induced diabetes significantly altered seminiferous tubules, body and reproductive organ weights, the level of serum testosterone, and sperm parameters [6,7]. However, it remains unclear whether these responses are due to hyperglycemia or STZ itself. Some studies showed that germ cell abnormalities in STZ induced diabetic epididymis do not correlate with blood glucose level [8], and insulin replacement was only able to prevent some adverse effects on certain epididymal regions [9].

In our preliminary experiment, we noticed that tight glycemic control (with insulin replacement) could not fully restore the testicular pathological changes and seminal parameters in the STZ-induced diabetes model. Hence we hypothesizes that the spermatogenic dysfunction in early-stage of STZ-induced rats might not be due to hyperglycemia. In this study, we aim to investigate the potential mechanism of STZ cytotoxicity on testicular reproductive dysfunction in the early stage of a diabetic rat model.

Abbreviations: Control, the Control group; STZ, streptozotocin or the STZ group; STZ + In, the Insulin group (STZ induced diabetic rats treated with insulin); DM, diabetes mellitus; WB, western blotting; MDA, malondialdehyde; TUNEL, terminal transferase-mediated dUTP-biotin nick end-labeling; NF-κB, nuclear factor-κB.

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2. Materials and methods

2.1. Study design

A total of 36 male 12-week-old Sprague–Dawley rats were obtained from the Animal Breeding Center at the Peking University Health Science Center. The experiments were approved by the institutional animal care and use subcommittee of our university. All animals were maintained in a clean environment on a 12 h light/12 h dark cycle. Rats were randomly divided into three equal groups: the Control group served as age-matched controls and received an intraperitoneal (i.p.) injection of citrate buffer; the STZ group received an i.p. injection of STZ in citrate buffer (55 mg/kg); the Insulin group received an i.p. injection of STZ and treated with intermediate-acting insulin twice daily (intracutaneous injection, 5–7 U/time). The rats were fasted for 16 h prior to injection. 4 weeks later, all rats were sacrificed and blood samples were taken for testing. Genital glands (epididymis and testes) were dissected out and spermatozoa were collected from the epididymis for analysis. Hematoxylin and eosin (HE) staining, immunohistochemistry (IHC), immunofluorescence (IF), western blot and terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay were conducted. Slides were photographed and recorded using a Leica DFC 425 C digital microscope camera system (Leica, Germany). Computerized histomorphometric analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

2.2. Body and reproductive organ weights

Body weights of all animals were weighed twice at the beginning and end of the study. Immediately after sacrifice, testes and epididymis were excised and their weights were recorded.

2.3. Plasma glucose and serum testosterone levels

Blood glucose levels were monitored at a regular interval throughout the study using a blood glucose analyzer (B. Braun, Melsungen, Germany). About 1 ml blood was drawn from the abdominal aorta and centrifuged at 2000 g for 10 min at 4 °C. Serum testosterone level was estimated using a commercial kit (Monobind 3725-300, USA) as per manufacturer's instructions. The optical density (OD) was measured at 450 nm with a reference wavelength of 620–630 nm using ELISA reader (ERBA Lisa 5, Transasia Biomedicals, India).

2.4. Evaluation of epididymal sperm density and motility

Sperm analysis was conducted as previous described [10]. The bilateral caudal epididymis was dissected out and spermatozoa were collected in 2 ml medium (Hams F10) containing 0.5% bovine serum albumin. After 5 min incubation at 37 °C, the epididymal sperm count was determined using the standard hemocytometric method and sperm motility was analyzed microscopically in 10 fields under a light microscope (Leica, Germany) using a 40× objective according to the World Health Organization recommended method. The epididymal sperm density was calculated by dividing the sperm count to caudal epididymis weight.

2.5. Measurement of malondialdehyde (MDA)

MDA concentration was measured with the thiobarbituric acid (TBA) reaction method. Briefly, the supernatant fraction was mixed with TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid (TCA) in 0.25 mM hydrochloric acid. The reaction mixtures were placed in boiling water, and then the absorbance of the supernatant was measured at 535 nm. The bicinchoninic acid (BCA) assay was used for protein quantitation. MDA levels were expressed as mmol/mg protein.

2.6. Histology analysis, immunohistochemistry (IHC) and immunofluorescence (IF)

The testicular tissues were harvested and fixed in Bouin's solution for a period of 6 h and then transferred to 70% ethanol until processing. The fixed tissues were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin in vertical direction. Sections of 4-μm thickness were cut using a rotor microtome. The paraffin sections were dewaxed in xylene for 20 min, rehydrated in serial graded ethanol solutions and then used for hematoxylin–eosin (HE) staining, IHC, IF and TUNEL assay.

For histological examination, sections were stained with HE staining. The histopathological changes in testicular tissue were evaluated by Johnsen's testicular score system. Thirty cross-sectioned tubules in each group were evaluated systematically, and a score between 1 (very poor) and 10 (excellent) was given to each tubule according to Johnsen's criteria [11].

Serial sections of tissues were then incubated for 10 min in 0.3% hydrogen peroxide to block endogenous peroxidase activity before IHC. After a wash with PBS, antigen retrieval was performed by boiling the tissue sections in 0.01 M citrate buffer (pH 6.0) for 10 min. The slices were then washed for 5 min in PBS and were incubated in 0.1% Triton X-100 and 5% goat serum for 30 min. The slides were subsequently incubated with primary antibodies overnight at 4 °C in a humidified chamber. Primary antibodies were mouse anti-vimentin (a sertoli cell marker, 1:50, Santa Cruz), rabbit anti-PCNA (1:200, Abcam), and rabbit anti-DDX4 (a germ cell marker, 1:400, Abcam). The sections for IHC were incubated with the MaxVision HRP-Polymer immunohistochemistry kit (Maxim, China) and were developed color with diaminobenzidine (DAB). The sections for IF were subsequently washed with PBS and were incubated at room temperature for 1.5 h with fluorescein conjugated secondary antibody. Sections were then counterstained with hematoxylin (IHC) and Hoechest 33342.

2.7. TUNEL assay

The level of DNA damage was detected via the TUNEL assay following the manufacturer's instructions (Beyotime, China). Briefly, 4 μm sections were treated with Proteinase K (20 mg/L) for 15 min (KeyGEN BioTECH, China). The sections were then treated with the reaction mixture containing TdT enzyme and biotin-11-dUTP for 60 min at 37 °C. The sections were washed with PBS, then were incubated with the streptavidin-linked tetramethylrhodamine (streptavidin-TRITC). Quantitative analysis of the apoptotic index was estimated as previously described with minor modification [12,13]. Briefly, the positive seminiferous tubules containing two or more TUNEL-positive cells were calculated in 30 randomly chosen fields in each group. The apoptosis index was calculated as the ratio of the positive seminiferous tubules of apoptosis to total number of seminiferous tubules in a cross section.

2.8. Western blotting (WB)

The cellular lysates from testicular tissue containing 20 μg protein were electrophoresed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, Massachusetts). Primary antibodies were rabbit anti-Wnt4 (1:200, Gene Tex), rabbit anti-NF-κB (1:200, Abcam), mouse anti-vimentin (sertoli cell marker, 1:50, Santa Cruz), anti-3β-hydroxysteroid dehydrogenase (3β-HSD,

1:50, Santa Cruz), and rabbit anti-steroidogenic acute regulatory (StAR, 1:50, Santa Cruz). After hybridization of secondary antibodies, the resulting images were analyzed with ChemiImager 4000 (Alpha Innotech Corporation, San Leandro, California).

2.9. Statistical analysis

Statistical analyses were performed with the SPSS 17.0 software. Data are expressed as mean ± SEM (the standard error of the mean). Multiple groups were compared using one-way analysis of variance followed by the Tukey HSD post hoc comparisons, and $p < 0.05$ was considered significantly.

3. Results

3.1. Blood glucose level, body weight and reproductive organ weight

The blood glucose level, body weight and reproductive organs weight (testes and epididymis) of the rats are presented in Table 1. Blood glucose levels in STZ-induced rats were significantly higher compared with those in the Control group ($p < 0.05$). In addition, the final body weight and reproductive organs weight of the rats in the STZ group were significantly lower than those in the Control group ($p < 0.05$). However, the rats in the Insulin group kept a normal blood glucose level, and had similar average body weight and reproductive organ weight as compared with the Control group ($p > 0.05$).

3.2. Epididymal sperm density and mobility

The caudal sperm density and mobility were significantly decreased in the STZ-induced diabetic rats compared to those of normal controls ($p < 0.05$, Table 1). Insulin replacement therapy on diabetic rats could not attenuate the abnormal sperm characteristics ($p > 0.05$). There was no significant difference in epididymal sperm density and motility between the STZ group and the Insulin group ($p > 0.05$). The results indicated that the damaged sperm characteristics in STZ induced rats did not correlate with blood glucose level.

3.3. Serum testosterone level

The total serum testosterone level was significantly lower in the STZ group than that in the Control group ($p < 0.05$). Conversely, diabetic rats treated with insulin replacement resulted in a full-recovery in the level of serum testosterone in comparison with untreated STZ-induced diabetic rats ($p < 0.05$). There was no significant difference in the level of serum testosterone between the Control group and the Insulin group ($p > 0.05$, Table 1).

3.4. Measurement of malondialdehyde (MDA)

STZ induced a significant increase in the level of MDA as compared to that in the Control group ($p < 0.05$, Table 1), suggesting a clear lipid peroxidation in the testicular tissue of STZ-induced diabetic rats. Insulin replacement partially but significantly attenuated the MDA levels in the testes of diabetic rats ($p < 0.05$). However, there was a significant difference in MDA levels between the Control group and the Insulin group ($p < 0.05$). These results indicated that the oxidative damage in the STZ-induced diabetic rats might be a combination effect of hyperglycemia and primary STZ cytotoxicity.

3.5. Pathological lesions in testis

The testes were histologically normal in the Control group, characterized by different stages of spermatogenesis in the multiple seminiferous tubules. However, the morphology of tubules in the STZ group and the Insulin group showed evident degeneration, varying from partially disorganised epithelium to totally disorganised epithelium with impaired organization of spermatogenesis stages (Fig. 1A). Insulin replacement could partially but significantly attenuate the pathological changes in the testes of diabetic rats as shown by Johnsen's score (Fig. 1B). There were also significant differences in pathological lesions between the Control group and the Insulin group ($p < 0.05$). Other typical changes including depletion of germ cells, declined layers of seminiferous epithelium, and cell debris in the lumen could also be seen both in the STZ group and the Insulin group. The deciduous cells in the lumen were further confirmed to be vimentin (a sertoli cell marker) positive cells and DDX4 (a germ cell marker) positive cells (Fig. 1C).

3.6. Disruption of sertoli cell vimentin filaments

The distribution and expression of sertoli cell vimentin filaments, one important element of cytoplasmic skeleton, were detected by IHC and WB, respectively. The vimentin filaments, surrounding the nucleus and radiating toward to the lumen, gave seminiferous tubule a characteristic "spoke-like" appearance in the Control group (Fig. 2A). IHC of vimentin in the STZ group revealed losing of their apical extension and distribution at their perinuclear localization. The distribution pattern of vimentin in the Insulin group was similar as that in the STZ group, characterized by dense vimentin staining around nucleus with the collapse of apical extension. Quantitative analysis by WB (Fig. 2B) showed

Table 1
Levels of blood glucose, body weight, reproductive organs weight, epididymal sperm parameters, serum testosterone and malondialdehyde.

Parameter	Control	STZ	STZ + In
Blood glucose (mg/dl)	107.14 ± 20.67*	421.42 ± 31.57	109.25 ± 33.23*
Initial body weight (g)	246.75 ± 4.1	248.25 ± 7.1	244.25 ± 6.3
Final body weight (g)	450.25 ± 12.71*	346.50 ± 74.59	446.25 ± 15.15*
Testis weight (g)	3.26 ± 0.21*	2.11 ± 0.15	3.20 ± 0.21*
Epididymis weight (g)	1.43 ± 0.17*	1.13 ± 0.11	1.45 ± 0.13*
Epididymal sperm density (10^8/g)	9.79 ± 1.98*	5.27 ± 2.27	5.46 ± 1.76#
Epididymal sperm mobility (%)	31.12 ± 6.65*	17.94 ± 4.01	16.96 ± 7.28#
Serum testosterone (ng/ml)	4.32 ± 0.39&	1.94 ± 0.78	4.27 ± 0.42&
Malondialdehyde (MDA) (nmol/mg)	4.62 ± 1.53*	11.76 ± 1.21	8.17 ± 1.35*,#

* $p < 0.05$ compared with the STZ group.
\# $p < 0.05$ compared with the Control group.
& $p < 0.01$ compared with the STZ group.

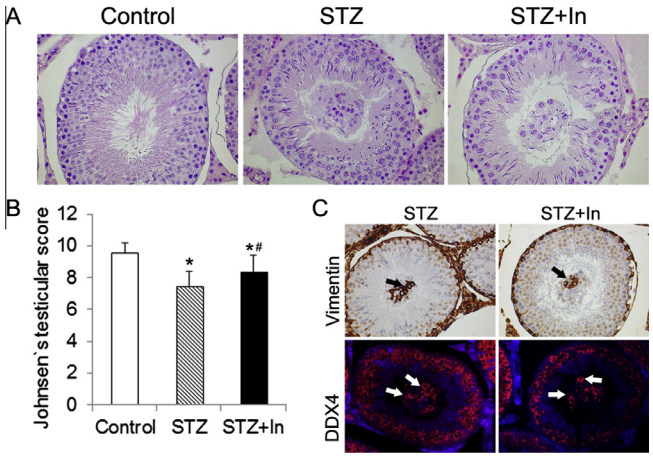

Fig. 1. Testicular histopathology. (A) Representative micrographs of hematoxylin–eosin-stained sections in the testes of rats, 400×. (B) Johnsen's testicular score. (C) Top: some deciduous cells in the lumen are confirmed to be vimentin (a sertoli marker) positive cell by using immunohistochemical staining (IHC), 400×. Bottom: there are also some deciduous cells in the lumen were DDX4 (a germ cell marker) positive cells by using immunofluorescent staining (IF), 400×. Black and white arrows indicate the deciduous cells in the lumen. Control, the Control group; STZ, the STZ group; STZ + In, the Insulin group (STZ induced diabetic rats treated with insulin). $^{*}p < 0.05$ versus the Control group, $^{\#}p < 0.05$ versus the STZ group.

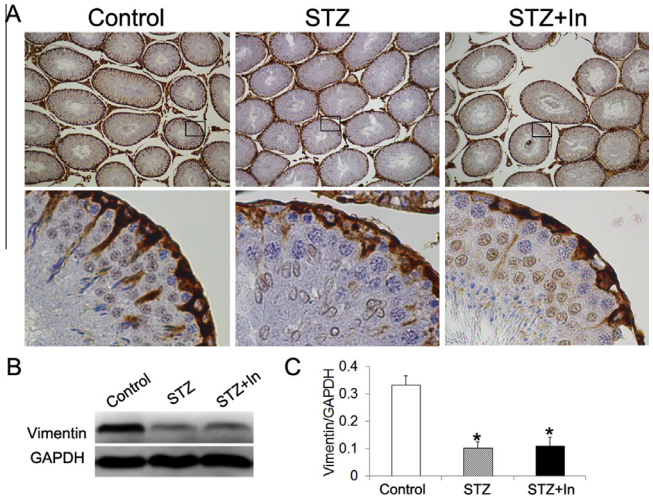

Fig. 2. The changes of sertoli cell vimentin filaments. (A) Immunohistochemical localization of sertoli cell vimentin filaments. The normal distribution of vimentin filaments in the Control group is observed radiating toward the lumen with a characteristic "spoke-like" appearance; The vimentin distribution in the Insulin group is similar as that in the STZ group, characterized by dense vimentin staining around nucleus with collapsed apical extension. Boxed areas in the 100× graphs are shown in the corresponding 1000× graphs. (B) The protein levels of vimentin in the testes are detected by using western blot (WB). (C) Quantitative analysis of vimentin protein expression in testes with WB. Data are shown as mean ± SEM. $^{*}p < 0.05$ versus the Control group.

the expressions of vimentin were significantly decreased both in the STZ group ($p < 0.05$) and the Insulin group ($p < 0.05$) compared with those in the Control group. There was no significant difference in vimentin expression between the STZ group and the Insulin group ($p > 0.05$) (Fig. 2C). These results indicated that sertoli cells might be the target of STZ in the testis.

3.7. Evaluation of TUNEL staining

We hypothesized the STZ-induced collapse of sertoli cell vimentin filaments impaired testicular physiology and ultimately led to spermatogenic cells apoptosis. In the present study, TUNEL-positive cells in the STZ group were significantly higher than those in the Control group. Most of the apoptotic cells observed were spermatogenic cells. Insulin replacement had no effect on the aspect of increased apoptosic index. The result was correlated well with the changes of sertoli cell vimentin filaments (Fig. 3).

3.8. Evaluation of NF-κB and Wnt4 expression

WB results showed that the expressions of NF-κB and Wnt4 were significantly higher in the STZ group than those in the Control group (Fig. 4A and B). Further, no significant expression changes of these two proteins were observed among the STZ-induced diabetic rats and the Insulin group. The results were also correlated well with the collapse of vimentin filaments. It indicated that the deleterious effects of STZ on sertoli cells might be regulated by Wnt4 and NF-κB.

3.9. Expression of steroidogenic enzymes

3β-HSD is steroidogenic enzyme in rat Leydig cells that catalyzes the oxidative conversion of δ5-ene-3-β-hydroxy steroid, and various ketosteroids. StAR, the transfer of cholesterol from the outer to the inner mitochondrial membrane, mediates the rate-limiting and acutely regulated step in steroidogenesis. In the present study, the expressions of 3β-HSD and StAR markedly decreased in the STZ group as compared with those in the Control group (Fig. 4C and D). Insulin replacement treatment resulted in a full recovery of the expressions of 3β-HSD and StAR as compared to the STZ group.

4. Discussion

STZ has been widely used to obtain models for the study of diabetes-related male infertility [3]. However, it remains unclear whether the testicular reproductive dysfunction in those models is due to hyperglycemia or the cytotoxicity of STZ itself. Previous studies suggested that oxidative stress was considered to act as causal links between elevated glucose and the complications of diabetes [14]. Elevated oxidative stress in the testicular milieu is demonstrated to have profound implications on testicular physiology and

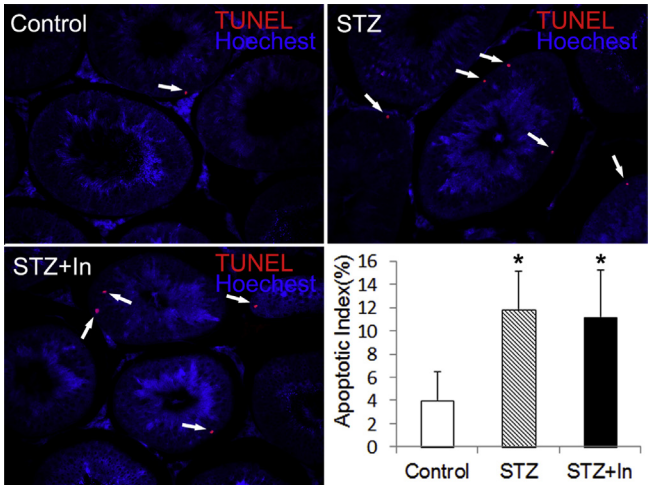

Fig. 3. Evaluation of TUNEL staining. The arrows indicate TUNEL-positive apoptotic cells emitting red signal in seminiferous tubules (200×). Bar graph shows quantitative analysis of the percentage of TUNEL-positive seminiferous tubules (containing two or more TUNEL-positive cells). Data are shown as mean ± SEM. $^{*}p < 0.05$ versus the Control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

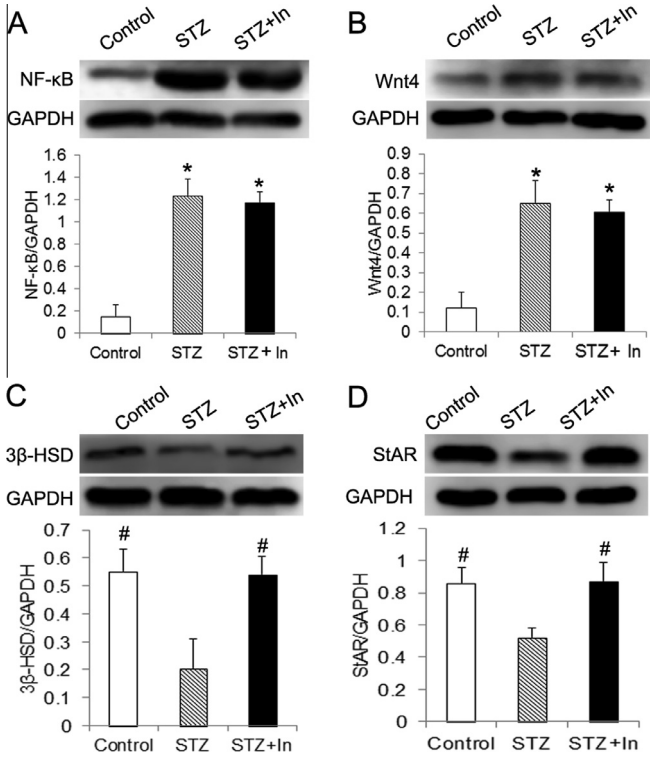

Fig. 4. Evaluation of protein expression by using WB. (A) Top: the expression of NF-κB; bottom: quantitative analysis of NF-κB protein expression. (B) Top: the expression of Wnt4; bottom: quantitative analysis of Wnt4 expression. (C) Top: the expression of 3β-HSD; bottom: quantitative analysis of 3β-HSD protein expression. (D) Top: the expression of StAR; bottom: quantitative analysis of StAR expression. Data are shown as mean ± SEM. $^{*}p < 0.05$ versus the Control group, $^{\#}p < 0.05$ versus the STZ group.

sperm function. Oxidative damage in the STZ-induced diabetic rats was ascertained in the present study. Lipid peroxidation was assessed in testicular lysate by measuring the MDA level. STZ treatment induced a significant increase in the level of MDA compared with that in the Control group. Insulin replacement could only partially attenuate lipid peroxidation in the testes of diabetic rats. These results indicated that the oxidative damage in the STZ-induced diabetic rats might be a combination effect caused both by both hyperglycemia and the primary STZ cytotoxicity.

Sertoli cells are the somatic cells in the seminiferous epithelium, providing physical support and environment milieu for germ cell development and maturation [15]. In a typical sertoli cell, vimentin filaments surround the nucleus, radiate out to the cell periphery, and terminate near Sertoli cells and adjacent germ cells [16]. Some researches have suggested that the vimentin filaments in sertoli cells served functions as diverse as positioning the sertoli cell nucleus, anchoring spermatogenic cells to the seminiferous epithelium or plasma membrane-nucleus communication [17]. On the contrary, collapse of vimentin filaments caused the loss of structural integrity of the seminiferous epithelium, along with germ cells apoptosis [18,19]. All these indicated that the vimentin filaments are necessary for testicular spermatogenesis.

In the present study, we found the sertoli cell vimentin filaments, characterized by loss of their apical extension and collapse at perinuclear localization, could not be recovered by insulin replacement. Further, we speculated that the collapsed vimentin induced by STZ might lead to precocious release of germ cells from underlying sertoli cells, and the deciduous cells may undergo apoptosis due to losing of support by the sertoli cells. As expected, we observed that the apoptotic index significantly increased in the STZ-induced diabetic rats as compared to that in the normal controls, and the increased apoptotic index could not be attenuated by insulin treatment. These results indicated that sertoli cells might be the direct target of STZ in the testis. However, to further determine the direct effects of STZ on sertoli cells, primary sertoli cell treated with and without STZ should be examined in future study.

Wnt4 is essential for normal male fetal reproductive tract development [20], and it is undetectable in normal adult testis [21]. A recent study has also shown that Wnt4 could reduced spermatogonial stem cells activity and increased germ cell apoptosis in vitro. Activated nuclear factor-κB (NF-κB) translocates into the nucleus and triggers the expression of proinflammatory cytokines, chemokines, and adhesion molecules [22]. In diabetes, NF-κB activation plays a critical role in the pathogenesis of inflammation [23]. WB analysis in this study showed that the expressions of NF-κB and Wnt4 in the STZ group were significantly higher than those in the Control group. In addition, the increased expressions of NF-κB and Wnt4 after STZ treatment could not be attenuated by insulin replacement. These results were completely coinciding with changes in sertoli cell vimentin filaments and spermatogenic cell apoptosis.

In insulin dependent diabetes, the testicular steroidogenesis diminished because of the absence of the stimulating effect of insulin on Leydig cells [24,25]. In the present study, the expression of 3β-HSD and StAR markedly decreased in the STZ-induced diabetic rat as compared to that in the normal controls. Interestingly, insulin replacement completely restored the expression of these two steroidogenic enzymes. The result provided supporting evidence as to why the levels of serum testosterone declined in the STZ group, while recovered to normal level when treated with insulin.

In conclusion, it could be reasonably postulated that spermatogenic dysfunction observed in this study was caused by STZ per se due to its cytotoxicity to sertoli cells, which was potentially regulated by Wnt4 and NF-κB. The difference was that steroidogenic dysfunction in the STZ-induced diabetic rats might be a direct or indirect consequence of insulin deficiency, which could be completely reversed by insulin replacement. Our results suggested that the STZ-induced diabetic model, at least in the early stage, is not suitable for studying the diabetes-related spermatogenic dysfunction. However, the conclusions were mainly based on the comparison among groups. Therefore, further confirmation of these findings with direct evidences, such as cytological experiments, should be conducted in future studies. In addition, the dynamic and long-term effects of STZ on testicular reproduction dysfunction should also be investigated.

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