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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,

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