Sirtuin (Sirt) 3 Overexpression Prevents Retinopathy in Streptozotocin-Induced Diabetic Rats

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Background: Sirtuin (Sirt) 3 could promote autophagy by downregulating the expression of genes related to neovascularization in retinal endothelial cells. In this study, we aimed to investigate the effect of Sirt3 overexpression on retinopathy in streptozotocin (STZ)-induced diabetic rats, and to assess its mechanisms.

Material/Methods: Ntraperitoneal injection of STZ in rats was used to produce a diabetic model. The study rats were divided into 4 groups (n=6 for each group): a control group; a model group; a model+scrambled adenovirus group; and a model+Sirt3 overexpression group. Hematoxylin and eosin (H&E) staining determined the pathological changes of retina tissues. Immunohistochemistry, fluorescence quantitative polymerase chain reaction, and western blotting were used to detect the expression of Sirt3, vascular endothelial growth factor (VEGF), and microtubule-associated protein 1A/1B-light chain 3 (LC3).

Results: In the model group, the inner limiting membrane was swollen, uneven and thickened, and the capillary endothelial cells occasionally protruded into the inner limiting membrane. These abnormalities were prevented by Sirt3 overexpression. Compared with the control group, the expression of Sirt3 at both mRNA and protein levels in the model group was significantly reduced, while the expression of VEGF was increased versus the control group (P<0.05). The expression of LC3 at both mRNA and protein levels was not different between the model group and the control group. Compared with the model group, the expression of Sirt3, LC3, and LC3-II was significantly increased, while the expression of VEGF was significantly decreased in the model+Sirt3 overexpression group (P<0.05).

Conclusions: Sirt3 overexpression has a preventive effect on diabetic retinopathy likely through promoting the expression of autophagy-related proteins and downregulating the expression of VEGF.

MeSH Keywords: Autophagy • Diabetes Mellitus, Type 2 • Diabetic Retinopathy

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Background

Diabetic retinopathy is one of the most common microvascular complications of diabetes mellitus. The main features of diabetic retinopathy are the occlusion and retinal hard exudates of retinal microvessels, which lead to blindness in severe cases [1]. Metabolic abnormalities caused by persistent hyperglycemia is the main pathogenesis of diabetic retinopathy, which can damage retinal microvascular system, including changes of capillary permeability, destruction of blood-retinal barrier, retinal leakage, macular edema, retinal vitreous hemorrhage, neovascularization, and retinal detachment [2].

Sirtuins, conservative nicotine adenine dinucleotide (NAD)-dependent deacetylases, consist of 7 subtypes [3]. Sirtuin (Sirt) 3 is an important member of sirtuins, which locates at the membrane of mitochondria. It functions in energy metabolism, biosynthesis, and antioxidant in the mitochondria by deacetylating target proteins [4]. Sirt3 mediates acetylation modification of autophagy-related proteins, affecting autophagy [5]. Importantly, endothelial Sirt3 is required for glycolysis and angiogenesis in coronary microvascular functions [6]. A recent study reported that the deletion of Sirt3 and Sirt5 was associated with inner retinal dysfunction in a mouse model of type 1 diabetes [7], which implicates Sirt3 as an important therapeutic target for diabetic retinopathy.

In a previous study, we reported that Sirt3 might promote autophagy by downregulating the expression of genes related to neovascularization in retinal endothelial cells [8]. That study not only provided a new insight into the mechanism of retinal neovascularization induced by human growth factor, but also proposed a candidate target for the treatment of neovascularization-related ophthalmopathy [8]. However, the exact function of Sirt3 overexpression in retinopathy has not been disclosed. In this present study, we proposed that Sirt3 contrasted retinal neovascularization by regulating the expression of autophagy and angiogenesis-related factors. In order to distinguish the function of Sirt3 in diabetic retinopathy, we constructed diabetic retinopathy in rats and evaluated whether Sirt3 overexpression promoted autophagy and downregulated the expression of angiogenesis-related genes. Our study would thus provide theoretical and experimental basis for treatment of diabetic retinopathy.

Material and Methods

Animals and modeling

Forty Sprague Dawley rats (male, 7–8 weeks old, 180–200 g) were obtained from Hunan Slake Jingda Laboratory Animal Co., Ltd. [SCXK (Hunan) 2016–0002]). All animal procedures were approved by Ethics Committee of the Second Affiliated Hospital of Nanchang University (No. YXS2017–24).

The animals were fasted for 12–14 hours before injection of streptozotocin (STZ) and 2 hours after intraperitoneal injection of STZ (55 mg/kg), the animals were administrated with food as previously described [9]. Three days after STZ injection, random blood glucose was measured, and blood glucose higher than 16.7 mmol/L was considered as a success of diabetes modeling. The experimental rats were then divided into 4 groups (n=6): a control group, a model group, a model+scrambled adenovirus group, and a model+Sirt3 overexpression group. The rats in the model group, the model+scrambled adenovirus group and the model+Sirt3 overexpression groups received treatment from the third day after STZ injection. In the model group, the rats received retinal injection of phosphate-buffered saline (PBS) (10 μL each eye) about 1 mm behind the sclera after deep anesthesia (5% isoflurane for induction and 2% for maintenance). The needle was kept in for 15 seconds after injection. The rats in the model+adenovirus control group (Vector: 20 μL adenovirus encoding vector, 10 μL per eye) and the model+Sirt3 overexpression adenovirus group (Sirt3: 20 μL adenovirus encoding Sirt3, 10 μL per eye) received 10 μL viruses (4.1×10^12 viral genome/mL) in each eye. At 8 weeks after viral injection, the rats in each group were anesthetized by inhalation of isoflurane. After decapitation, the eyeballs of each group were removed, and the retina was separated for biochemical analysis.

Hematoxylin and eosin (H&E) staining

The retinal tissues were fixed in paraformaldehyde (PFA, 4%) at 4°C overnight and dehydrated with 70%, 80%, and 90% ethanol and then mixture of alcohol and xylene (15 minutes), and xylene I (15 minutes) and xylene II (15 minutes). The tissues were placed in the mixture of xylene and paraffin for 15 minutes, then paraffin I and paraffin II for 50–60 minutes, respectively, then embedded with paraffin and sliced. After baking, dewaxing, and hydrating, the sections were dyed in H&E for 3 minutes, then differentiated by ethanol for 15 seconds, washed slightly, blued for 15 seconds. The images at 4 fields of each section were taken under a light microscope (BX51, Olympus, Japan).
Immunohistochemistry

The slides were baked in an oven at 65°C for 2 hours and placed in xylene for 10 minutes, replaced with xylene for 10 minutes; slices were placed in 100% ethanol, 95% ethanol, 80% ethanol and distilled water for 5 minutes, respectively. Slices were placed in citric acid buffer and moved into wet box and added with fresh hydrogen peroxide (3%) to remove the endogenous peroxidase (room temperature, 10 minutes). After that, slices were blocked in 5% bovine serum albumin at 37°C for 30 minutes and incubated with the primary antibody against Sirt3 (1:200) overnight at 4°C. After washing with PBS, the slices were incubated with the secondary antibody (goat anti-rabbit IgG, 1:100) at 37°C for 30 minutes. The staining was visualized with 3,3′-diaminobenzidine chromogen (DAB) and the images were taken under a light microscopy (BX51, Olympus, Japan).

Quantitative fluorescence polymerase chain reaction (PCR)

After extracting RNA using the TRIzol kit (CW0580S, CWBIO, China), the cDNA was synthesized according to the reverse transcription kit (Dalian Baosheng, Dalian, China). Using cDNA as template, the expression of Sirt3, VEGF (vascular endothelial growth factor), and LC3 (microtubule-associated protein 1A/1B-light chain 3) in each group was detected by reverse transcription quantitative polymerase chain reaction (PCR). The primers are listed as follows: LC3 F ACCGTGCTCACTACTTCTCTT; Sirt3 R CGTCAGCCGTATGTCCTC; VEGF F GAGTATCTTCAAAGCCGTTCCTG; VEGF R ATCTGCATAG GACGTTGCTCTC; β-actin F ACGTGAAGGATCCATAC; β-actin R TGGCAACAGGTCCATACC.

The reaction system included 9.5 μL RNase free dH₂O, 1 μL cDNA, 2 μL primers and 12.5 μL 2× qPCR mixture; PCR was conducted following the experimental protocol: denaturation 10 seconds at 95°C, annealing 30 seconds at 58°C, extension 60 seconds at 72°C (40 cycles). The expression of target genes was normalized to β-actin. The 2–ΔΔCq method was used to quantify the results as previously described [10].

Western blotting

The total protein was abstracted by adding the corresponding pyrolysis solution (4°C for 30 minutes) (28–9425–44, ReadyPrep; GE Healthcare Life Sciences). After centrifugation at 876×g for 10 minutes, the supernatant was collected. Protein concentration was determined using bicinechonic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were processed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and later transferred onto nitrocellulose membrane as previously described [11]. After blocking with 5% nonfat milk with Tween-20 at room temperature for 2 hours, the membrane were incubated with the primary antibodies against GAPDH (1:1000, TA-08, ZSbio, China), VEGF (1:1000, bs-1313R, Bioss), Sirt3 (1:1000, AF5135, Affinity), and LC3 (1:1000, ab48394, Abcam) overnight at 4°C. The secondary antibody (1:10 000, ab131368, Abcam, USA) was incubated with the membrane for 2 hours at room temperature. The expression of target proteins was normalized to GAPDH. At least 6 repeats were included in the western blotting.

Statistical analysis

All data were presented as mean ± standard deviation (SD) with 6 repeats. One-way ANOVA was used to assess the differences among groups using SPSS 17.0 statistical software, and significance was accepted when P<0.05.

Results

Sirt3 expression was decreased in retina tissue of diabetic rats

Compared with the control group, the fasting blood glucose of diabetic rats increased significantly 3 days after STZ injection (P<0.05) (Figure 1A) and the fasting blood glucose level in the model group was higher than 16.7 mmol/L, which ensured the successful modeling of diabetes. Eight weeks after diabetic modeling, the inner membrane of the retina was slightly swollen, uneven and thickened, and the endothelial cells of capillaries protruded from the inner membrane occasionally, the tissues of the retina were loose and edema, the arrangement of ganglion cells was loose and irregular, and the capillaries were dilated and new blood vessels were formed (Figure 1B). These morphological changes ensured the success of diabetic retinopathy.

Adenoviral-encoding Sirt3 prevents the pathological changes of retinal tissue in diabetic rats

Compared with the control group, Sirt3 at both mRNA and protein levels in the model group decreased significantly (P<0.05). By contrast, the decrease of Sirt3 expression in diabetic rats was prevented by Sirt3 overexpression compared to the model group (P<0.05) (Figure 1C–1E).

The inner membrane of the retina was stained by H&E to assess the morphological changes. In the model+Sirt3 overexpression group, the inner membrane of the retina was relatively intact, and no capillary endothelial cells were found to break through the inner membrane. Swollen, uneven and...
thickened inner membranes caused by diabetic modeling were prevented by Sirt3 overexpression (Figure 2). These data implicate that Sirt3 overexpression prevents morphological changes induced by retinopathy.

**Adenoviral-encoding Sirt3 reduces VEGF expression in diabetic rats**

VEGF level is low in the normal eyes, which is necessary to maintain the integrity of the ocular blood vessels [12]. Therefore, we detected the expression of VEGF in the retina. As shown in Figure 3, the expression of VEGF in the model group was obviously higher than that in the control group (P<0.05). By contrast, VEGF expression in model+Sirt3 overexpression group was significantly reduced compared to the model group (P<0.05) (Figure 3).

**Adenoviral-encoding Sirt3 increased LC3 expression in diabetic rats**

As a marker of autophagy, the expression of LC3 indicates the degree of autophagy [13]. The results of LC3 expression are shown in Figure 4A. LC3 expression at the mRNA level was comparable between the model group and the control group. By contrast, LC3 expression in the model+Sirt3 overexpression group was promoted in comparison with model group (P<0.05). As shown in Figure 4B, the expression of LC3-II at the protein level between the model group and the control group was comparable. Sirt3 overexpression promoted LC3-II expression in diabetic rats (P<0.05).

**Discussion**

Our study demonstrated that Sirt3 expression was downregulated in the retina of diabetic rats. Importantly, Sirt3 overexpression ameliorated the morphological changes of retina tissue after modeling diabetic retinopathy. Additionally,
Sirt3 overexpression promoted autophagy-related protein expression and prevented VEGF expression in diabetic retinopathy rat model. These results implicate therapeutic target of Sirt3 in diabetic retinopathy.

Diabetic retinopathy is one of the serious ocular complications of diabetes mellitus, featured by metabolic disorders and endocrine and blood system damage in the retina [14]. STZ-induced diabetic rat model has been widely used in the study of the pathogenesis, and prevention and treatment of microcirculation morphological changes [14]. The number of retinal capillaries and the basement membrane of capillaries are remarkably increased in STZ-induced diabetic rats [15]. In addition, changes in capillary endothelial cells and pericytes can alter capillary permeability as basement membrane plays an important role in maintaining the permeability of vascular wall [16]. Additionally, 2-month old diabetic rats can be used as diabetic retinopathy animal model [17]. Increased blood glucose is a marker of successful establishment of diabetic model [18]. In this present study, blood glucose level in the model group was greater than 16.7 mmol/L, which indicate that a diabetic rat model was successfully constructed. Diabetic retinopathy is characterized by the formation of neovascularization in the diabetic retina [19]. Results showed that the retina thickness of diabetic rats decreased and the number of pericapillary cells degenerated and apoptotic retina decreased 1 month after onset of diabetes mellitus [20]. Our results revealed typical pathological changes of retina tissue after modeling of diabetics.
Sirt3 is a histone deacetylase and locates on the mitochondrial membrane, which can regulate the acetylation level and activity of mitochondrial proteins, thus affecting a variety of biological processes [21,22]. Sirt3 overexpression can well contrast the hypertrophic stimuli, thereby protecting the myocardium against damage [23]. In addition, Sirt3 overexpression can increase the acetylation level and Parkin expression of FoxO3a, thereby activating autophagy and mitochondrial autophagy of cardiac myocytes, alleviating mitochondrial damage and mitochondria-dependent apoptosis, and playing an important protective role in diabetic cardiomyopathy [24]. Sirt3 levels are related to diabetic retinopathy, and negatively correlated with the duration of diabetic retinopathy [25]. Overexpression of Sirt3 has been shown to protect bovine retinal capillary endothelial cells and diabetic rat retinal capillary endothelial cells from hyperglycemia-induced injury [26]. By contrast, loss of Sirt3 expression under high glucose conditions is related to retinal dysfunction [27]. Sirt3 may play a role in regulating the related pathways of neovascularization [8]. Overexpression of Sirt3 inhibits retinal neovascularization induced by high glucose and insulin [8]. In our present study, we revealed that the expression of Sirt3 decreased remarkably after the construction of diabetic retinopathy. We applied a local injection of Sirt3 adenovirus. The pathological results showed that the retinopathy was prevented following Sirt3 overexpression. These results suggest that Sirt3 has a protective effect on retinopathy in diabetic model.

**Figure 3.** Adenoviral-encoding Sirt3 reduced VEGF expression in retinal tissue. (A) Representative immunohistochemical images of VEGF expression (upper panel) and quantification data of grey values of VEGF (down); triangle indicates Sirt3 expression. (B) mRNA expression of VEGF detected by real-time polymerase chain reaction. (C) Protein expression of VEGF detected by western blotting; (versus the control group, * P<0.05; versus the model group, * P<0.05). Sirt3 – sirtuin 3; VEGF – vascular endothelial growth factor; mRNA – messenger RNA.
VEGF expression is low in the normal eyes, which is necessary to maintain the integrity of the ocular blood vessels [12]. In diabetic retinopathy, the levels of VEGF in cells and body fluids were significantly higher than normal levels. The increase of VEGF level alters capillary permeability, which contributes to the hemorrhage and exudation of retinal blood vessels, increase of angiopoietin production, formation of retinal neovascularization and visual impairment. Blocking VEGF completely prevents retinal neovascularization [28]. Inhibition of vascular growth factor can also reduce the formation of new blood vessels [29]. Deletion of Sirt3 affects the expression of vascular endothelial progenitor cells and angiogenesis [30,31]. Our results showed that the expression of VEGF was increased in diabetic retinopathy model, which was prevented by overexpression of Sirt3. These results suggest that overexpression of Sirt3 may affect the formation of retinal neovascularization by regulating the expression of VEGF, ultimately performing protective role in retinal injury.

As a marker of autophagy, the expression of LC3 indicates the degree of autophagy [13]. The expression of Sirt3 can promote the expression of LC3-II [32]. Our previous results revealed that the expression of LC3 was not affected in human retinal endothelial cells treated with high glucose and insulin [8]. By contrast, LC3 may be upregulated in oxygen-induced neovascularization of rhesus monkey chorioretinal endothelial cells, and autophagy stimulates angiogenesis of aortic endothelial cells [33,34]. Overexpression of Sirt3 increased the expression of LC3 at mRNA and protein levels, suggesting that Sirt3 promoted autophagy of retinal neovascularization [8]. LC3 expression was not affected by diabetic retinopathy modelling, whereas overexpression of Sirt3 could promote the expression of LC3, thus promoting the autophagic activity of retina. These results suggest that Sirt3 possibly promotes autophagy to exhibit the preventive effect.

Our study demonstrated that Sirt3 overexpression prevented retinopathy in diabetic rats and also stimulated autophagy. In addition, literature supports autophagic degradation of VEGF as a critical protective mechanism of retinopathy [29]. Nevertheless, the exact mechanisms regarding the regulation of Sirt3 on LC3 were not elucidated in this present study. As has been reported, Sirt3 could function in the nucleus in the control of stress-related gene expression [35]. In this present study, LC3 expression at both mRNA and protein levels were regulated. It is probably that Sirt3 translocated into the nucleus to modulate LC3 expression. Additionally, the function of autophagy in retinopathy should be confirmed by genetic or pharmacological methods.

Figure 4. Adenoviral-encoding Sirt3 increased LC3 expression in retinal tissue. (A) mRNA expression of LC3 detected by real-time PCR. (B) Protein expression of LC-3 detected by western blotting; (versus the control group, * P<0.05; versus the model group, # P<0.05). Sirt3 – sirtuin 3; LC3 – microtubule-associated protein 1A/1B-light chain 3; PCR – polymerase chain reaction.
Sirt3 overexpression had a preventive effect on diabetic retinopathy likely promoting the expression of autophagy-related proteins and downregulating the expression of angiogenesis-related genes.

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