

Original Article

Inhibition of P66shc-induced oxidative stress protects against rat diabetic retinopathy

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Abstract: Diabetic retinopathy (DR) is one of the severest complications of diabetes. The role of oxidative stress response in onset and occurrence of DR has drawing our attention. As one key molecule in producing ROS, P66shc can be suppressed by SIRT1, whose up-regulation shows protective effects on DR. However, neither function nor mechanism of p66shc in DR is unclear yet. Intraperitoneal injection of STZ was performed to generate a diabetic model, on which blood samples were collected for measuring blood glucose, glycosylated hemoglobin, and lipid level. Serum indexes reflecting oxidative stress-related indexes such as MDA or SOD were quantified, along with tissue H₂O₂ releasing level. Gene expression level of SIRT1 and P66shc was measured by qPCR. Western blot was used to measure the protein expression of SIRT1, P66shc and apoptosis-related proteins Bcl-2, caspase 3 and caspase 9, in conjunction with TUNEL assay to measure retinal cell apoptosis. SIRT1 up-regulated and p66shc inhibited groups showed improved blood glucose, glycosylated hemoglobin and blood lipid levels, with decreased oxidation index MDA and higher anti-oxidation index SOD levels, plus decreased tissue H₂O₂ release, indicating significantly decreased oxidative stress level in rats with SIRT1 up-regulation or p66shc inhibition, which significantly suppressed retinal tissue apoptosis. SIRT1 up-regulation could suppress p66shc-induced oxidative stress and inhibited retinal tissue apoptosis. SIRT1 and p66shc can work as drug target for suppressing DR.

Keywords: p66shc, oxidative stress, diabetic retinopathy

Introduction

Diabetic retinopathy (DR) is one of severest complications of diabetes. The role of oxidative stress response in occurrence and progression of DR has drawn lots of research interests [1].

Mitochondria are the well-known source of reactive oxygen species (ROS), of which 95% come from mitochondria. Abundant amounts of ROS can damage large biomolecules including proteins, lipids and DNA. Adaptor protein P66shc is coded by oncogene Shc, and is the convergent point for mammalian metabolism, oxidative stress and aging, and is one key protein for cellular oxidative stress. Major functions of P66Shc include regulating signal transduction for cellular oxidative stress response and life cycle, and plays crucial roles in body mitochondrial oxidative metabolism and cellular oxidative injury [2]. P66shc produce large amounts of ROS to cause cellular oxidative stress and apoptosis via cytochrome C inside

mitochondria [3, 4]. Currently major researches for P66shc mainly focus on tumor proliferation/migration, and diabetes complicating with atherosclerosis [5]. Higher contents of P66shc in vascular endothelial cells in diabetic patients are believed to be the critical factor inducing oxidative stress in diabetic endometrium [6, 7]. Concurrent knockout P66shc gene can prevent endometrial cell dysfunction or oxidative stress induced by high glucose [8]. Therefore, P66shc is one important molecule in producing ROS. In recent years studies regarding the suppression of DR related hyperglycemia related stress memory by metformin, SIRT1 was found to be the possible target [9]. Other studies suggested that p66shc expression could be suppressed by SIRT1, which had protective effects on DR. However, the function or role of p66shc in DR is not conclusive yet.

This study utilized STZ-induced diabetic model, on which SIRT nucleotide sequence or anti-sense fragment of p66shc was injected into vit-

reous body, to up-regulate SIRT1 expression or to down-regulate p66shc expression level. Oxidative stress and cell apoptosis levels in retinal tissues were then separated measured to investigate possible roles and regulatory mechanisms of p66shc-involved oxidative stress in DR pathology.

Materials and methods

Major materials and reagents

Total protein extraction kit was purchased from Kaiji Biotech (China). Western blot lysis buffer and BCA protein quantification kit was provided by Beyotime (China). SIRT1 antibody and p66shc antibody were purchased from Proteintech (China). Horseradish peroxidase labelled goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L) antibody was purchased from Sanying Biotech (China). Test kits for SOD and MDA were purchased from Jiancheng (China). Blood glucose test paper was purchased from Roche (Swiss). Test kits for glycosylated hemoglobin, TG and TC were purchased from Huamei (China).

Experimental animal and grouping

Male SPF grade SD rats (6-8 weeks old) were provided by the Model Animal Center of Harbin Medical University. Animals were divided into four groups: normal control group, diabetic model group, diabetic plus SIRT1 up-regulation group and diabetic plus p66shc inhibition groups (N=10 each).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Harbin Medical University.

Generation and diabetic model and sample collection

SD rats (N=10 each) were fed with high-fat diet and received low dosage (30 mg/kg) of streptozotocin (STZ) via intraperitoneal injection to generate rat diabetic models following previous literatures [10, 11], with no animal death during model preparation. After 72 h, blood samples were collected from the tail vein. Successful model generation was defined when blood glucose level maintained above 16.7 mmol/L for more than one week. 17 weeks after successful model preparation, rats were continuously

fed. In diabetic plus SIRT1 up-regulation and diabetic plus p66shc inhibitor groups, weekly injection of SIRT1 nucleic acid fragments or anti-sense nucleic acid sequence of p66shc was performed into vitreous body at 100 nM concentration.

Seventeen weeks later, rats were weighted and opened the abdominal cavity after anesthesia. Blood samples were collected from abdominal aorta, and were centrifuged to obtain serum for further assays of oxidation-reduction and biochemical indexes. The eyeball was carefully extracted to separate the retina, which was kept at -80°C fridge for further use including Western blot and real-time PCR assay.

Assay for blood glucose, glycosylated hemoglobin and blood lipid levels

Serum samples were tested for ALT and AST activity following manual instruction of test kit. In principle, the amino-transfer product can react with 2, 4-dinitrophenylhydrazine to produce phenyl-hydrazone, which showed brown-reddish color in alkaline solution. Absorbance value was measured at 505 nm wave length to calculate activity in IU/L.

Serum MDA and SOD assay

MDA and SOD assays were performed using thiobarbituric acid (TBA) and xanthinoxidase approach, respectively, following manual instruction of test kits. Light intensity was measured by a microplate reader. Each index was measured in triplicates and was presented as mean \pm standard deviation (SD).

Western blot

Western blot was performed following previous literature [12]. Lysis buffer was firstly thawed in PMSF to reach final concentration at 1 mM. 100 mg fresh rat retinal tissues were collected and homogenized in lysis buffer, followed by 4°C lysis for 15 min. The mixture was centrifuged at 14000 rpm for 15 min, and the supernatant was collected to prepare total protein solution, which was quantified by BCA method to equalize concentration. Proteins were boiled for 5 min in denature, and was kept at -20°C. Proteins were separated in 8%-12% SDS-PAGE until separation of target proteins with adjacent molecular weights. Proteins

Retinal apoptosis and p66shc

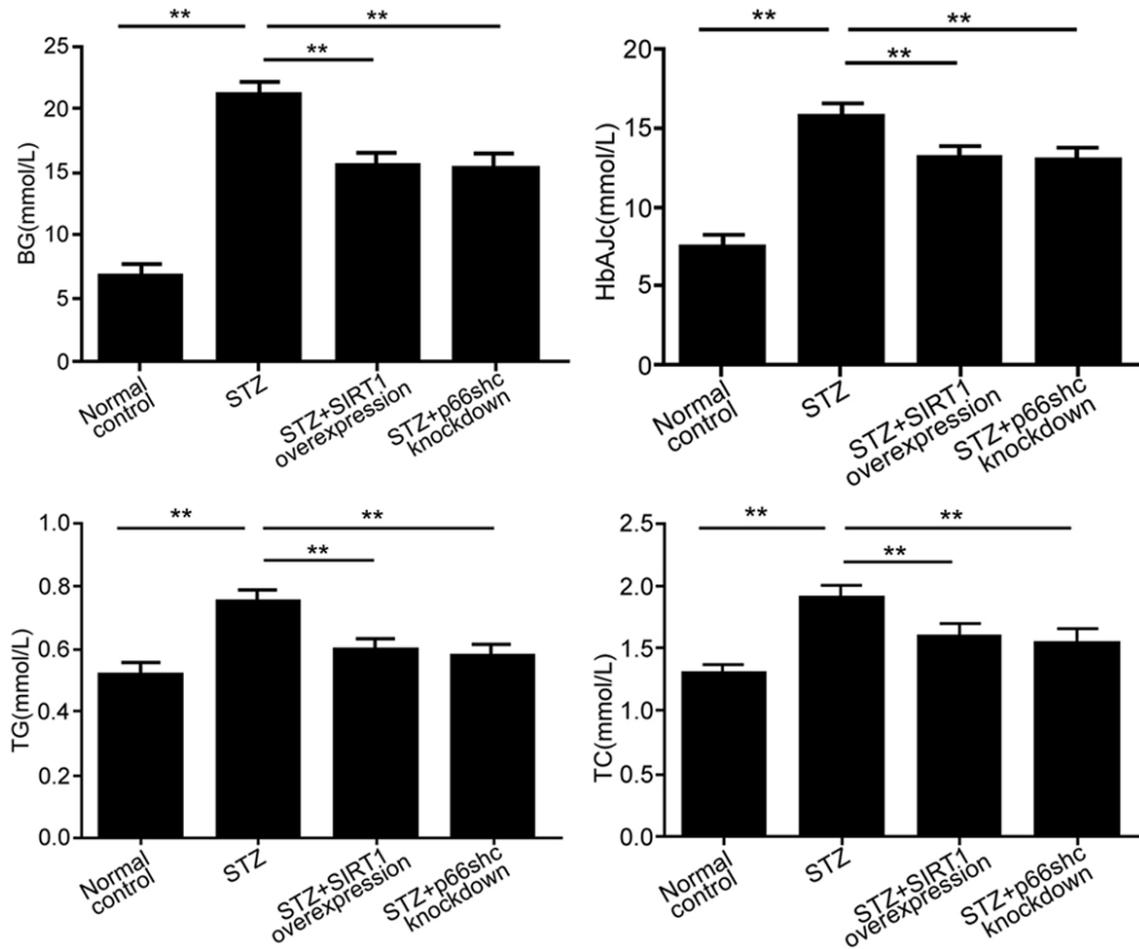


Figure 1. Effects of SIRT1 up-regulation and p66shc inhibition on blood glucose, glycosylated hemoglobin and blood lipid levels. **, P<0.01.

were transferred to PVDF membrane in 300 mA electric field. Primary antibody (1:1000) was added for 4°C overnight incubation. After rinsing in PBST for three times, secondary antibody (1:1000) was added for 37°C incubation for 2 h. The membrane was then developed.

Real-time quantitative PCR

100 mg rat retinal tissues were mixed with 1 mL Trizol for 5 min iced incubation. Tissues were homogenized on ice and were centrifuged for collecting the supernatant, which were saved and mixed with 200 µL chloroform for 15 s vigorous mixture. After 3 min room temperature incubation at 4°C, the mixture was centrifuged at 12000 g for 15 min. The upper aqueous phase was collected, and 1 mL ethanol was added for three times washing. The su-

pernatant was carefully discarded, and mRNA was re-suspended in 20 µL DEPC water.

SIRT1 primer was designed and synthesized by Sigma [13]. Primer sequences were: forward, 5'-CAACT TTGCT GTAAC CCTGT-3'; reverse, 5'-CAGCC ACTGA AGTTC TTTCA T-3'. P66shc primers were designed and synthesized by Sigma [14, 15]. Sequences were: forward, 5'-TACTT GGTTC GGGTG AGTGC-3'; reverse, 5'-GAGCA GGAAG TCCCG ACAA-3'. GAPDH was used as the internal reference (forward, 5'-TGACT TCAAC AGCGA CACCC A-3'; reverse, 5'-CACCC TGTTG CTGTA GCCAA A-3'). PCR amplification was performed in a 50 µL system following the manual instruction of test kit. Amplification conditions were: 50°C for 30 min, and 95°C for 5 min, followed by 40 cycles each containing 95°C 30 s, 55°C 30 s and 72°C 50 s, and ended with 72°C elonga-

Retinal apoptosis and p66shc

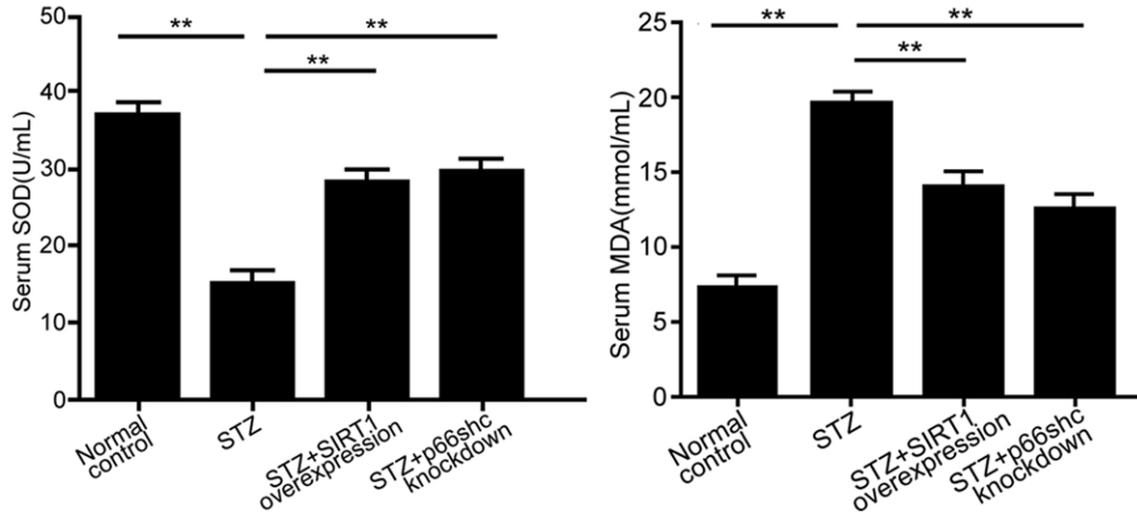


Figure 2. SIRT1 up-regulation or p66shc inhibition for rat serum SOD or MDA levels. **, P<0.01.

tion for 5 min. After reaction, real-time PCR amplification curve and melting curve were confirmed. Ct values of target gene were contrasted against those in internal reference gene. Gene expression was quantified by $2^{-\Delta\Delta Ct}$ method.

Tissue release of H_2O_2

Rat retinal tissues were prepared for homogenate in saline. Tissue release amount of H_2O_2 was measured by test kit following the manual instruction.

TUNEL for cell apoptosis

Rat retinal tissues were fixed in paraformaldehyde and prepared in paraffin sections. After immersing in 3% H_2O_2 -methanol solution for 10 min, slices were incubated in 0.2% Triton for 5 min, and were rinsed twice in PBS. Following the manual instruction, 50 μ L TUNEL reaction buffer was added for 37°C dark incubation for 1 h. With three times of PBS rinsing, a fluorescent microscope was used to capture the image.

Statistical methods

SPSS 19.0 software was used to process collected data. Measurement data were presented as mean \pm standard deviation for test of normality and equal variance. For those parameters with normal distribution, paired comparison was performed by student t-test. Com-

parison among multiple groups was performed in one-way analysis of variance. A significant difference was defined when P<0.05.

Results

General condition of rats

Compared to diabetic model rats, normal control group rats had shining furs and normal food intake. Diabetic rats had yellowish fur color, accompanied with significant body weight loss, polyuria and polydipsia. At early stage rats showed hyperactivity, followed by responsive retard, hyperhidrosis, horripilation, losing of fur and polyuria. In diabetic SIRT1 up-regulation group and diabetic p66shc inhibition group, rats showed improvement of various symptoms, but still worse than normal control group.

Biochemical indexes change

As shown in **Figure 1**, compared to normal control group, diabetic rats had elevated blood glucose, glycosylated hemoglobin and blood lipid levels, with statistical significance. Compared to diabetic rats, diabetic SIRT1 up-regulation group and diabetic p66shc inhibition group showed decrease of blood glucose, glycosylated hemoglobin and blood lipid levels, with statistical significance. These results suggested that SIRT1 up-regulation or p66shc inhibition could improve blood biochemical indexes caused by diabetes to certain extents.

Retinal apoptosis and p66shc

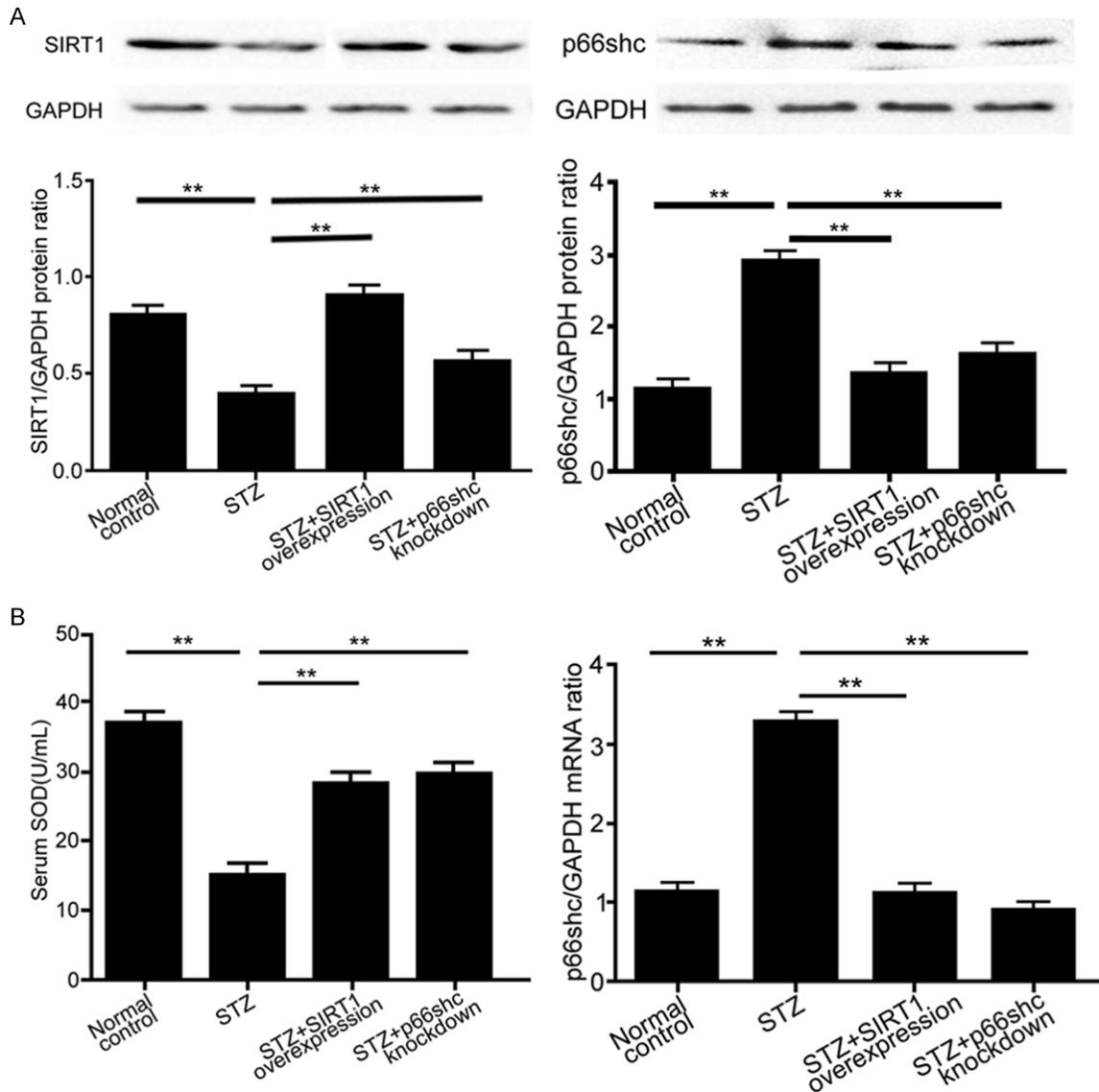


Figure 3. SIRT1 up-regulation or p66shc inhibition for rat retinal expression of SIRT1, p66shc and mRNA protein/mRNA. A. Rat retinal protein expression of SIRT1 and p66shc after SIRT1 up-regulation or p66shc inhibition. B. Rat retinal mRNA expression of SIRT1 and p66shc after SIRT1 up-regulation and p66shc inhibition. **, P<0.01.

Serum MDA and SOD levels

Oxidative stress level was measured by serum MDA and SOD assays. As shown in **Figure 2**, compared to normal control group, diabetic rats had significantly decreased serum SOD level. After SIRT1 up-regulation or p66shc inhibition, SOD level was significantly recovered compared to diabetic group, but still lower than control group. Comparing to normal control group, MDA level was significantly up-regulated in diabetic group, and showed weakened up-regulatory effects after SIRT1 up-regulation or

p66shc suppression. These results showed that SIRT1 up-regulation or p66shc inhibition could weaken oxidative stress level of rats.

Retinal SIRT1 and p66shc expression level

The expression levels of SIRT1 and p66shc in rat retinal tissues were shown as **Figure 3A** and **3B**. As compared to normal control group, diabetic model group showed significantly down-regulated and up-regulated protein or mRNA expression of SIRT1 and p66shc protein or mRNA, respectively. The treatment using

Retinal apoptosis and p66shc

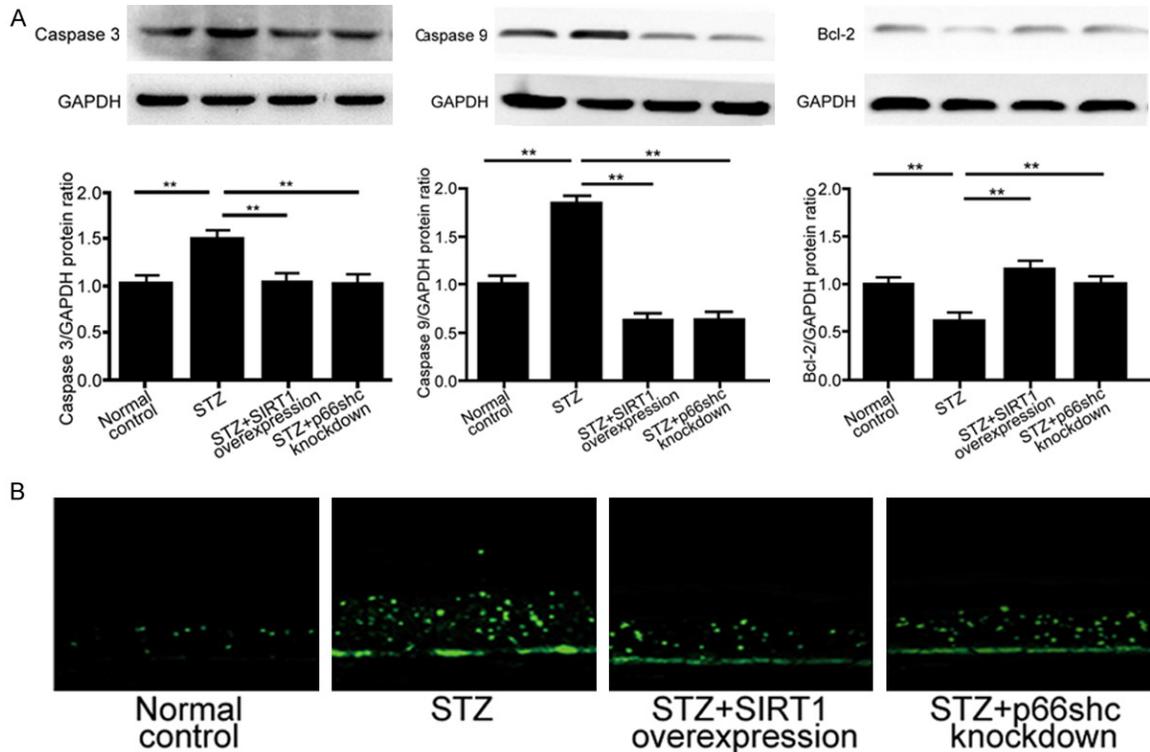


Figure 4. Effects of SIRT1 up-regulation or p66shc inhibition on rat retinal cell apoptosis. A. Protein expression of Caspase 3, Caspase 9 and Bcl-2 in rat retinal tissues after SIRT1 up-regulation or p66shc suppression. B. Effects of rat retinal cell apoptosis. **, P<0.01.

SIRT1 nucleotide sequence or anti-sense nucleic sequence of p66shc increased and decreased protein or mRNA expression level of SIRT1, p66shc, respectively. These results suggested the involvement of SIRT1 and p66shc in regulation of DR. moreover, SIRT1 can work as the upstream modulatory to suppress p66shc expression.

Retinal tissues apoptosis in all groups of rats

This study used Western blot to test expression of apoptosis and anti-apoptosis related proteins, and utilized TUNEL labelling to measure cell apoptotic level. Expression of apoptosis and anti-apoptosis related proteins was shown in **Figure 4A**. Compared to normal control group, diabetic model rats showed significantly elevated expression level of caspase 3 and caspase 9. Up-regulation of SIRT1 or down-regulation of p66shc significantly suppressed caspase-3 or caspase-9 expression level. In contrast, compared to normal group, diabetic model group had significantly lower Bcl-2 level. Treatment of SIRT1 up-regulation or p66-

shc down-regulation significantly increased Bcl-2 level. TUNEL staining results were shown in **Figure 4B**. In diabetic model group, the number of TUNEL positive cells was significantly increased. SIRT1 up-regulation or p66shc suppression significantly decreased the number of TUNEL positive cells. These results showed that SIRT1 up-regulation or p66shc down-regulation significantly inhibited apoptosis of retinal cells.

H₂O₂ release amount from rat retinal tissues

Test kit was used to quantify H₂O₂ level to reflect oxidative stress. As shown in **Figure 5**, compared to normal control group, diabetic rats showed elevated retinal H₂O₂ levels with statistically significant difference. After SIRT1 up-regulation and p66shc down-regulation, H₂O₂ level can be suppressed to certain extents. These results showed that SIRT1 up-regulation and p66shc down-regulation could protect diabetic induced oxidative stress to certain extents.

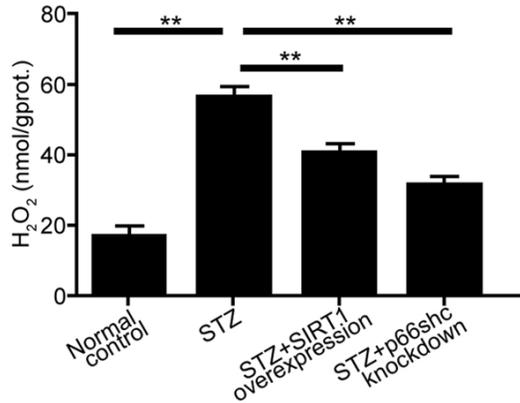


Figure 5. Rat retinal tissues H₂O₂ level after SIRT1 up-regulation or p66shc suppression. **, P<0.01.

Discussion

DR is one specific fundus change. As the most important sub-type of diabetic micro-vascular pathology, DR is one of major complications of diabetes [16]. Current opinions believed that elevated ROS production and release by oxidative stress is the major factor leading to DR [17, 18]. Early study has shown that, in lymphocytes from diabetic patients with DR, SOD activity was significantly suppressed [19] whilst MDA level was up-regulated [20], as consistent with our results. These indicated that during onset and occurrence of DR, oxidative stress may play important roles. Recent study also showed that certain compounds from Chinese medicine could improve diabetic with DR via suppressing oxidative stress [21]. Some studies showed that SIRT1 level was significantly suppressed in diabetes, and up-regulation of SIRT1 might be one functional target, although detailed mechanisms require further studies.

Abundant production of ROS is manifested as elevated production of free oxidative radicals but less clearance, leading to the imbalance of oxidation/anti-oxidation, to activate related signal pathways for cell apoptosis leading to apoptosis [22], thus forming one important step in retinal injury. Adaptor protein p66shc is encoded by oncogene Shc, and is the convergent point for metabolic, oxidative stress and aging response pathways in mammals. Major function of p66shc include the regulation of cellular oxidative stress response and life cycle signal transduction, whilst SIRT1 was believed to be the upstream regulatory factor of p66shc

[23]. Early study showed significant down-regulation and p66shc up-regulation in diabetes induced vascular oxidative stress and endothelial dysfunction, demonstrating the involvement of SIRT1-p66shc signal in diabetes induced vascular oxidative stress and vascular dysfunction regulation [23], as consistent with our initial hypothesis, which, however, has not been reported in DR. Other reports showed that p66shc could work as the marker for renal tubular oxidative injury in diabetic nephropathy [24]. Moreover, p66shc expressional change is correlated with longevity [25]. We thus believed that under SIRT1 regulation, p66shc-mediated oxidative stress participated in onset and progression of DR, although strong evidences are still lacking.

This study thus utilized a diabetic model induced by STZ. SIRT nucleic acid sequence or p66shc anti-sense nucleotide sequence was given by intra-vitreous injection to enhance SIRT1 expression or to suppress p66shc expression, respectively, in order to investigate the role of p66shc induced oxidative stress in DR pathology, along with regulatory mechanism of SIRT1. Our results showed that compared to diabetic model, SIRT1 up-regulation or p66shc down-regulation could improve serum biochemical index of diabetic rats to certain extents, suppress oxidative stress level, and inhibited retinal cell apoptosis, indicating the involvement of SIRT1 and p66shc in progression of DR. In assays for retinal protein expressions after SIRT1 up-regulation or p66shc down-regulation, we found that SIRT1 up-regulation was accompanied with p66shc suppression, whilst in rats with p66shc down-regulation, SIRT1 expression was not significantly altered. These results suggested that p66shc expression was under negative regulation by SIRT1, whilst p66shc could not affect SIRT1 expression. In summary, SIRT1 could negatively regulate p66shc to suppress oxidative stress response, and protect related apoptosis, thus alleviating DR conditions.

This study investigated the role of SIRT1 and p66shc in DR, and demonstrated regulatory role of SIRT1 on p66shc by Western blot. However, further regulatory mechanism on p66shc by SIRT1 requires further elucidation. In addition, this study investigated the protective role of the suppression on p66shc induced

oxidative stress against rat DR on model animals, but leaving detailed roles on retinal neurons or Muller cells to be further studied.

In diabetic model rats, up-regulation of SIRT1 or suppression of p66shc via intra-vitreous injection could suppress p66shc-induced oxidative stress and inhibit retinal tissues apoptosis. This study also proved regulatory effects of SIRT1 on p66shc. Therefore, SIRT1 and p66shc might work as the drug target for suppressing DR.

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Disclosure of conflict of interest

None.

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Retinal apoptosis and p66shc

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