Telomerase Reverse Transcriptase Promotes Proliferation, Reduces Apoptosis, and Enhances Autophagy in Cardiomyocytes During Myocardial Ischemia Reperfusion Injury

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Research article

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Abstract

Background: Telomerase reverse transcriptase (TERT) is reported to protect cells from ischemic injury in many diseases. However, the protective role of TERT in myocardial ischemia reperfusion injury (MIRI) remains unclear. The present study aimed to explore the protective roles of TERT in MIRI.

Methods: Rat myocardial H9c2 cells were exposed to oxygen and glucose deprivation (OGD) followed by reperfusion to mimic *in vivo*MIRI. A TERT overexpression model was constructed by adenovirus infection. Cell viability was detected using the CCK8 assay. Immunohistochemicalstaining of Ki67 was used to detect cell proliferation. Apoptosis was detected by TUNEL and flow cytometry analysis of Annexin V-FITC and propidium iodide. Apoptosis-related protein CC3 and autophagy related protein LC3 were detected by western blot analysis.

Results:TERT was increased in H9c2 cells after OGD. TERT overexpression promoted the proliferation, attenuated apoptosis, and enhanced autophagy in H9c2 cells after OGD.

Conclusions: This study indicates that TERT plays a protective role via promoting proliferation, suppressing apoptosis, and enhancing autophagy in myocardial cells after MIRI. Further study of TERT is expected to reveal new treatment targets for myocardial ischemia-reperfusion injury.

1. Introduction

Myocardial reperfusion after ischemic injury is believed to reduce ischemic injury and improve outcome; however, the process of reperfusion to the ischemic myocardium can induce further injury, called myocardial reperfusion injury[1]. This type of myocardial ischemia-reperfusion injury (MIRI) occurs after acute myocardial infarction as well as after some cardiovascular events including cardiovascular surgery such as coronary artery bypass grafting or cardiac transplantation and in patients surviving a cardiac arrest[2, 3]. The mechanisms of myocardial reperfusion injury include oxidative stress, intracellular Ca^{2+} overload, inflammation, and mitochondrial permeability transition pore opening which is a critical factor of cell injury[1].

Telomerase reverse transcriptase (TERT), an important component of telomerase, is the rate-limiting factor for telomerase activity and the key factor in regulating telomerase activity[4–6]. However, growing evidences have revealed that TERT also has other functions unrelated to telomere maintenance, including regulating gene expression, cell differentiation, apoptosis, and proliferation[6]. TERT is expressed in foetal mouse cardiomyocytes, but its expression is gradually decreased after birth, and there is almost no TERT expression in adult mouse cardiomyocytes; however, TERT expression increases after ischemic injury, suggesting that TERT may play a protective role in myocardial ischemic injury[7–9]. Therefore, in this study, we preliminarily explored the protective role of TERT in myocardial ischemia-reperfusion injury, to explore a new treatment target for myocardial ischemia-reperfusion injury.

2. Materials And Methods

2.1 Cell culture and myocardial ischemia-reperfusion injury

Rat myocardial H9c2 cell line (Procell) was used in our study. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) supplemented with 10% fetal bovine serum (FBS; Gibco) and then incubated at 37 °C in a humidified incubator containing 95% air and 5% CO_2. Myocardial cells were exposed to oxygen and glucose deprivation (OGD) for 6 h followed by reperfusion to mimic myocardial ischemia-reperfusion injury (MIRI) in vivo. To induce OGD treatment, the culture medium was replaced with DMEM without glucose, and then the cells were exposed to hypoxia in the airtight chamber with 95%N_2, 5%CO_2 at 37℃ for 6 h. After 6 h of OGD treatment, the culture medium was changed back to the normal DMEM with 5% FBS and then cultured in an incubator with normal condition at 37℃ to form reoxygenation.

2.2 Construction of adenovirus and infection

The TERT gene was Synthesized according to the CDS sequence of rat TERT (https://www.ncbi.nlm.nih.gov/nuccore/NM_053423.1), and constructed into pCDH-CMV-MCS-EF1-copGFP vector. And the TERT overexpression adenovirus plasmid was constructed. Viral particles were produced by transfection of TERT overexpression plasmid or empty vector plasmid along with packaging plasmid in human embryonic kidney (HEK) 293T cells. After amplification, the adenovirus was collected and purified and concentrated. And then filtered through a 0.2 μm filtration unit. The final adenovirus was mixed with storage liquid and preserved for the following experiment.

The packaged adenovirus was diluted to different ratios and added to H9c2 cells. The best MOI was 80 determined by western-blot after 48 hours. Then, the H9c2 cells were infected with TERT overexpression adenovirus and empty vector adenovirus. After 48 h, the cells were fed with fresh medium and exposed to OGD treatment. Experimental groups including: cells in normoxia as control (CON), cells treated by OGD (OGD), cells infected with empty vector adenovirus and treated by OGD (OGD + EMPTY), cells infected with TERT overexpression adenovirus and treated by OGD (OGD + TERT).

2.3 Detection of cell viability by CCK8

Cell Counting kit (CCK-8; Dojindo, Japan) was used to detect the cell viability. H9c2 cells in different groups were seeded in 96-well plates with the density of 1 × 104/well. At 48 h after OGD, a total of 10 μl of CCK-8 solution was added to each well and incubated for 3 h under standard cell-culture conditions (37 °C, 5% CO2). The absorbance was determined at 450 nm wavelength (A450 nm) with ELx808 microplate reader (BioTek Instruments, USA). The means of the optical density (OD) of 3 wells were used to calculate the percentage of cell viability as follows: Cell viability (%) = ($OD_{\text{treatment group}}/OD_{\text{control group}}$) × 100%. The experiments were repeated at least three times.

2.4 Immunohistochemical (IHC) staining of KI67

H9c2 cells in different groups were seeded on slides in 6-well plates. At 48 h after OGD, the cells on slides of different groups were treated with 3% hydrogen peroxide for 20 min, and then incubated with 1% bovine serum albumin and anti-KI67 (Abcam, UK, 1:100) for 2 h, and incubated with HRP-goat anti-Rat IgG for 60 min. Diaminobenzidine (DAB) was used as chromogen.

2.5 TUNEL assay

TUNEL assay was performed to detect the apoptosis of H9c2 cells according to the manufacturer's instructions (Keygen biotech, China). Briefly, cells were fixed with 4% paraformaldehyde at room temperature for 20 min and treated with 3% hydrogen peroxide at room temperature for 15 min, and then treated with 1% Triton X-100 for 5 min. Cells were then rinsed twice with PBS and incubated with biotinylated nucleotide and the terminal deoxynucleotidyl transferase, recombinant (rTdT) enzyme at 37 ℃ for 1 h in a humidified atmosphere in the dark. Samples were then rinsed with PBS for 3 times and were incubated with DAB. Alternate cells were processed in parallel without the rTdT enzyme as negative controls. Images were captured using a fluorescence microscope (Nikon, 80i).

2.6 Flow cytometry

Besides TUNNEL assay, flow cytometry was also used to detect the apoptosis of H9c2 cells. Cells were harvested with 0.25% trypsin and washed three times in PBS. Then, they were harvested and stained with Annexin V-FITC(AV) and propidium iodide (PI) (Dojindo, Japan) for 20 min at room temperature. After rinsed twice with PBS, cells were analyzed using flow cytometry (Millpore, guava easycyte). Cells stained with AV and PI or AV only were considered apoptosis. The percentage of apoptotic cells was calculated.

2.7 Western Blot

For western blot, cells were lysed in Radio Immunoprecipitation Assay (RIPA) with protease inhibitor cocktail (Beyotime, China), and then centrifuged at 14,000 rpm for 30 min at 4 ℃ to collect the supernatants.. Then, the protein concentration in the supernatant was determined by the BCA protein quantitation assay (Keygen biotech, China). Proteins were denatured in gel sample buffer by boiling for 5 min at 100 ℃. Equal amounts of protein samples (30ug) were electrophoresed on 12% Sodium dodecyl sulfate/Polyacrylamide gel electrophoresis (SDS/ PAGE) gels and electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche). Following blocking with Quickblock™ blocking buffer (Beyotime, China) for 1 h at room temperature, the membranes were incubated with TERT recombinant polyclonal antibody (Invitrogen), rabbit anti- LC3I/II antibody (Proteintech), and Cleaved-caspase-3 P17 (D175) polyclonal antibody CC3(4A BIOTECH), separately overnight at 4 ℃. The following day, membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20, and incubated with HRP-goat anti-Rat IgG secondary antibody (Santa Cruz Biotechnology, 1:5000) in blocking solution at room temperature for 1 h. Finally, immunoreactivity was visualized by the gel imaging system (Chemi-DOCTMXR+, BIORAD) and quantitatively analyzed. β-actin served as the loading control. The band intensity was measured, normalized by β-actin and calculated as the ratio of the optical density (OD). Three independent experiments were performed.

2.8 Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed by SPSS version 16.0 statistical analysis package. Student's t-test was used when comparison was made between two groups. Analysis of variance (ANOVA) with LSD post hoc test was used for multiple comparisons. $P < 0.05$ was defined as statistically significant.

3 Results

3.1 Expression of TERT expression in H9c2 cells after myocardial ischemia-reperfusion injury

Cultured myocardial cells (H9c2) were divided into four groups: cells in normoxia as control (CON), cells treated with oxygen and glucose deprivation (OGD), cells infected with empty vector adenovirus and treated with OGD (OGD + EMPTY), cells infected with TERT overexpression adenovirus and treated by OGD (OGD + TERT). Western blotting was conducted to determine the expression of TERT in different groups of myocardial cells at 48 h after OGD. As shown in Fig. 1, the expression level of TERT in OGD and OGD + EMPTY was significantly increased compared with that in the CON group ($P < 0.001$, Fig. 1), indicating that ischemia-reperfusion injury induced TERT expression in myocardial cells. Further, TERT expression in the OGD + TERT group was further increased significantly compared with that in the OGD and OGD + EMPTY groups ($P < 0.001$, Fig. 1). There was no significant difference in the TERT expression level between OGD and OGD + EMPTY groups ($P > 0.05$, Fig. 1). This demonstrated that the expression of TERT in myocardial cells was enhanced by infection with TERT overexpression adenovirus after OGD.

3.2 TERT promotes myocardial cell proliferation after myocardial ischemia-reperfusion injury

Cell counting kit 8 (CCK8) assay and immunohistochemical (IHC) staining of cell proliferation related protein Ki67 was used to determine the proliferation of myocardial cells in each group. At 48 hours after OGD, H9c2 cells in each group was collected for the following assays. As shown in Fig. 2A, the CCK8 staining revealed that the proliferation rate of H9c2 cells was decreased in the OGD and OGD + EMPTY group compared with that in the CON group ($P < 0.05$, Fig. 2A), whereas the proliferation rate of H9c2 cells in the OGD + TERT group was increased compared with that in the OGD and OGD + EMPTY group ($P < 0.05$, Fig. 2A). There was no significant difference in the proliferation rate between the OGD and OGD + EMPTY group ($P > 0.05$, Fig. 2A). Figure 2B shows the immunohistochemical staining of cell proliferation related protein Ki-67. The KI67 positive cells in the OGD + EMPTY group were decreased compared with the CON group, and the positive cells in the OGD + TERT group were increased compared with the OGD + EMPTY group. These results suggest that the proliferation of myocardial cells was reduced after myocardial ischemia-reperfusion injury, and that TERT can promote the proliferation of myocardial cells after myocardial ischemia-reperfusion injury.

3.3 TERT attenuates myocardial cell apoptosis after myocardial ischemia-reperfusion injury

To verify the effect of TERT on the apoptosis of myocardial cells after myocardial ischemia-reperfusion injury, flow cytometry and TUNEL assay were used to detect apoptosis in H9c2 cells. Cells in each group at 48 hours after OGD was collected for the following tests. Both flow cytometry (Fig. 3A) and TUNEL staining (Fig. 3B) showed that apoptosis of H9c2 cells in the OGD + EMPTY group was increased compared with that in the CON group. Further apoptosis in the OGD + TERT group was decreased compared with that in the OGD + EMPTY group. Western blot analysis to detect the apoptosis associated protein cleaved-caspase-3 (CC3) in H9c2 cells showed that the expression of CC3 in OGD + EMPTY was significantly increased compared with that in the CON group ($P < 0.001$, Fig. 3C, D). Further, CC3 expression in the OGD + TERT group was significantly decreased compared with that in the OGD + EMPTY group ($P < 0.001$, Fig. 3D). These results indicate that TERT can attenuate apoptosis of myocardial cells after myocardial ischemia-reperfusion injury.

3.4 TERT enhances autophagy of myocardial cells after myocardial ischemia-reperfusion injury

The expression levels of LC3-II/LC3-I were then analysed by western blot analysis to investigate the effect of TERT on cell autophagy activity after myocardial ischemia-reperfusion injury. As shown in Fig. 4, the expression rate of LC3-II/LC3-I was increased in the OGD + EMPTY group compared with that in the CON group ($P < 0.05$, Fig. 4). The expression of LC3-II/LC3-I in the OGD + TERT group was further increased compared with that in the OGD + EMPTY group ($P < 0.05$, Fig. 4). These results indicate that myocardial ischemia-reperfusion injury induced autophagy in myocardial cells, and that TERT enhanced this autophagy activity.

4 Discussion

In this report, we found that TERT was significantly upregulated after myocardial ischemia-reperfusion injury (MIRI). TERT can promote proliferation, attenuate apoptosis, and enhance autophagy in myocardial cells after MIRI.

The protective role of TERT in promoting cell proliferation and reducing apoptosis has also been reported in many other fields[6]. Our previous study showed that TERT can protect neurons from apoptosis induced by hypoxic-ischemic injury[10]. Oh reported that TERT overexpression can increase telomerase activity in cardiomyocytes and maintain telomere length, thus delaying irreversible cell cycle exit and promoting cell survival[7]. TERT can also promote proliferation, hypertrophy, and survival of cardiomyocytes, and both *in vivo* and *in vitro* experiments have shown that TERT can reduce apoptosis induced by ischemia injury in cardiac myocytes[7]. In a mouse model of myocardial infarction, TERT promoted the proliferation and survival of cardiomyocytes after myocardial infarction in mice, reduced the infarction area, increased the

metabolic activity in the infarcted area, and improved cardiac function. TERT also reduced scar formation by reducing fibre formation and increasing tissue remodelling and regeneration potential, thus ultimately reducing the mortality of heart failure after myocardial infarction[9]. Moreover, TERT did not enhance cardiac hypertrophy after myocardial infarction and thus, it did not alter heart morphology. The study also suggested that this mechanism may be related to the effect of TERT in maintaining telomere length and activating some signalling pathways associated with cardiac protection and regeneration. Thus, TERT may play a significant role in preventing heart failure after myocardial infarction[9]. However, whether TERT can play a role in promoting cell proliferation and anti-apoptosis in MIRI remains unclear. In this report we found that TERT expression was increased after MIRI, and that TERT overexpression promoted cardiomyocyte proliferation and reduced apoptosis after MIRI.

So far, the underlying mechanisms of TERT in cell protection upon myocardial ischemia-reperfusion injury remain to be explored. Acute myocardial reperfusion injury can occur due to complex mechanisms including reactive oxygen species(ROS) generation, Ca^{2+} overload, and mitochondrial permeability transition pore (mPTP) opening[11]. Yellon and Hausenloy concluded that the potential mediators during myocardial reperfusion injury include oxidative stresses, calcium paradox, inflammation, and mPTP[1]. Mitochondrial permeability transition pores may be a significant target for cardioprotection as they plays a critical role in injury[1]. The mitochondrial pathway mediating cell death (including apoptosis and necrosis) is critical in ischemic/reperfusion injury[12]. The key event in mitochondrial pathway-mediated apoptosis is mitochondrial outer membrane permeabilization resulting in the release of apoptogenic factors such as cytochrome C from the mitochondrial membrane, and activation of caspase, finally triggering apoptosis. Opening of the mPTP is a critical event in mitochondrial pathway-mediated necrosis. Ischemia results in intracellular acidosis, which leads to an increase in intracellular Ca^{2+}; Ca^{2+} overload promote mPTP opening during reperfusion and stimulates ROS production. These changes lead to the cessation of ATP synthesis and mitochondrial swelling, which may finally cause necrosis[12]. Nevertheless, there is an interconnection between the mitochondrial pathways of apoptosis and necrosis; it is also reported that mPTP opening may cause cytochrome C release and lead to apoptosis, and that apoptosis may lead to necrosis[12]. These changes significantly weaken cardiac function and increase heart infarction during reperfusion[3]. Therefore, these researches suggest that mitochondrial function plays an important role in the mechanisms underlying MIRI.

The effect of TERT on regulating mitochondrial function has been reported recently. TERT was believed to be located in the nucleus and cytoplasm and has active forms in the nucleus. However, studies have revealed that TERT can translocate into the mitochondria after injury, suggesting that TERT may also play a role in the mitochondrial injury pathways[3, 6]. Emerging evidences have shown the protective effect of TERT in mitochondrial function during stress; TERT can protect mitochondrial DNA (mDNA) damage by binding to mDNA, increasing the mitochondrial membrane potential, decreasing ROS production, and increasing respiratory chain activity[13, 14]. However, whether this function of TERT is also present in myocytes during MIRI remains unclear. In a rat model of TERT deficiency, lack of TERT was found to

increase mDNA damage and decreased the mitochondrial respiratory capacity during cardiac stress. These results confirmed a critical role of TERT in regulating mitochondrial functions in cardiac injury during stress, and suggested that TERT may regulate cardiac function during stress by regulating the mitochondrial Ca^{2+}, ROS production, and ATP production, which finally affect cell death[3]. Our previous research has shown that TERT may act as a neuroprotective agent via anti-apoptosis in neurons after hypoxic-ischemic injury. The underlying mechanisms may be associated with regulating the Bcl-2/Bax expression ratio, attenuating ROS generation, and increasing mitochondrial membrane potential[10]. Therefore, the effect of TERT on mitochondrial pathway-mediated cell death including apoptosis or necrosis during myocardial ischemic-reperfusion injury is the next focus of our research.

Autophagy is a process that delivers and degrades cytoplasmic materials in the lysosome. It is a recycling system that produces materials and energy for cell renovation[15]. However, the role of autophagy in cell injury is still controversial; generally, autophagy can promote cell survival, but some evidence suggests that autophagy can also induce cell death[12, 15]. In the heart, autophagy can promote adaption to hemodynamic stress[15]. It is already reported that ischemia-reperfusion injury can induce cardiac autophagy, but the role of autophagy during ischemia-reperfusion injury is still unclear. Increased autophagic capacity is suggested to protect myocytes against ischemia-reperfusion injury[16], whereas other reports have proposed that autophagy may be protective during ischemia, but may be harmful during reperfusion[17]. Microtubule-associated protein 1 light chain 3(LC3) is reported to be a reliable marker of autophagic activity[18]. In this study, the expression ratio of LC3-II/LC3-I was used to reflect autophagic activity. We found that the autophagic activity of myocardial cells was enhanced after myocardial ischemia-reperfusion injury; however, the exact role of autophagy during MIRI needs further research. Recently, a few studies have reported the role of TERT in regulating autophagy. In a model of nutrient deprivation, TERT was reported to activate autophagy activity by inhibiting mTORC1 kinase activity[19]. In mice with a specific genetic deletion of TERT, the autophagy activity was delayed after renal ischemia-reperfusion injury. The underlying mechanism may be mediated in part by increased mTOR signalling[20]. A recent study reported that TERT can promote autophagy in cancer cells under glucose deprivation through an HK2-mTOR pathway[21]. Our study also revealed that TERT can increase the autophagy activity of myocardial cells after myocardial ischemia-reperfusion injury. However, further research is needed to confirm the underlying mechanisms.

Further, telomere-independent effects of TERT, including regulation of gene expression, cell differentiation, and proliferation, are also reported. These effects of TERT might also be involved in the protection of myocardial ischemic injury[6]. TERT may participate in regulating the proliferation and differentiation of myocardial stem cells and endothelial progenitor cells, thus affecting myocardial regeneration and vascular regeneration after myocardial ischemic injury[6]. However, whether these mechanisms of TERT exist in MIRI and the underlying protection mechanisms of TERT in MIRI will be the focus of our next study.

5 Conclusion

Our findings demonstrate that TERT is significantly upregulated after myocardial ischemia-reperfusion injury (MIRI). Overexpression of TERT promotes proliferation, attenuates apoptosis, and enhances autophagy in myocardial cells after MIRI. Research on the regulating effects and mechanisms of TERT in MIRI is still a new field. Thus, in-depth studies on the protective role and mechanisms of TERT in myocardial injury are expected to explore new aspects of the protective effect of TERT on the myocardium, and provide a new theoretical basis and therapeutic target for attenuating injury and promoting repair during MIRI in future. But this research was based on Rat myocardial H9c2 cell line, in vivo studies are needed.

List Of Abbreviation

telomerase reverse transcriptase, TERT; myocardial ischemia reperfusion injury, MIRI; oxygen and glucose deprivation, OGD; human embryonic kidney, HEK; normoxia as control, CON; radio immunoprecipitation assay, RIPA; reactive oxygen species, ROS; cell counting kit 8, CCK8; mitochondrial permeability transition pore, mPTP; mitochondrial DNA, mDNA; microtubule-associated protein 1 light chain 3, LC3.

Declarations

Ethical Approval and Consent to participate The hospital ethics committeeapproval was granted of this study.

Consent for publication: All authors had agreed for publication

Availability of data and materialsAll data are available.

Authors' contributionsZhiFangand Jiao Li contributed to the whole study. Yingqiang Guo conceived of the study, contributed to the critical

review and study design. All authors read andapproved the final manuscript.

Competing interests The authors declare that they have no competing interests.

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Figures

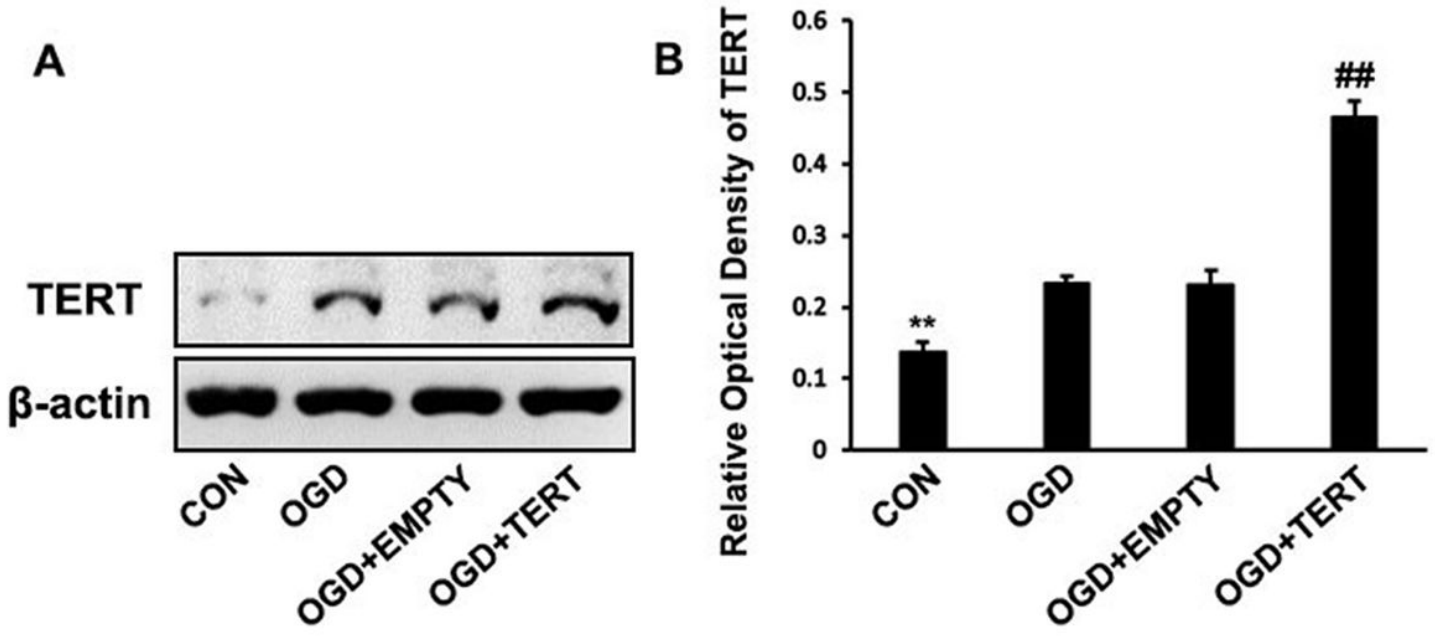

Figure 1

Expression of TERT in H9c2 cells. (A) Western blot showed that the expression level of TERT was significantly increased in OGD and OGD+EMPTY groups compared with CON group. And the expression level of TERT in OGD+TERT group was further increased compared with OGD and OGD+EMPTY groups. (B) Quantification of TERT expression. Data were obtained by densitometry and were normalized using β-actin as internal control. Values are expressed in relative optical density and are represented as mean ± SD. **P<0.001 versus OGD and OGD+EMPTY group, n=3. ## P<0.001 versus OGD and OGD+EMPTY group, n=3. (OGD, oxygen and glucose deprivation).

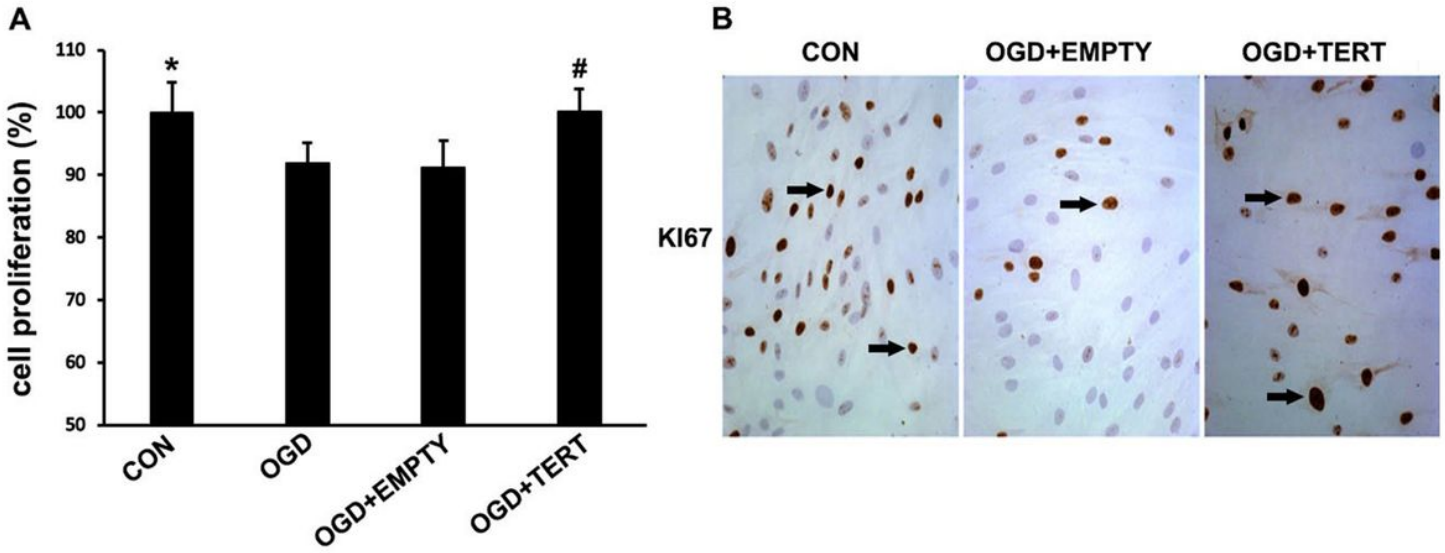

Figure 2

Proliferation of H9c2 cells. (A) CCK8 staining showed that the cell proliferation rate of H9c2 cells was decreased in OGD and OGD+EMPTY group compared with CON group. *P<0.05 versus OGD and OGD+EMPTY group, n=3. While the cell proliferation rate of H9c2 cells in OGD+TERT group was increased compared with OGD and OGD+EMPTY group. #P<0.05 versus OGD and OGD+EMPTY group, n=3. There was no significant difference about the proliferation rate between OGD and OGD+EMPTY group (P>0.05). (B) Immunohistochemical staining of KI67 showed that the KI67 positive cells in OGD+EMPTY group decreased compared with CON group, and the positive cells in OGD+TERT group increased compared with OGD+EMPTY group. (Black arrows showed the KI67 positive cells). (OGD, oxygen and glucose deprivation)

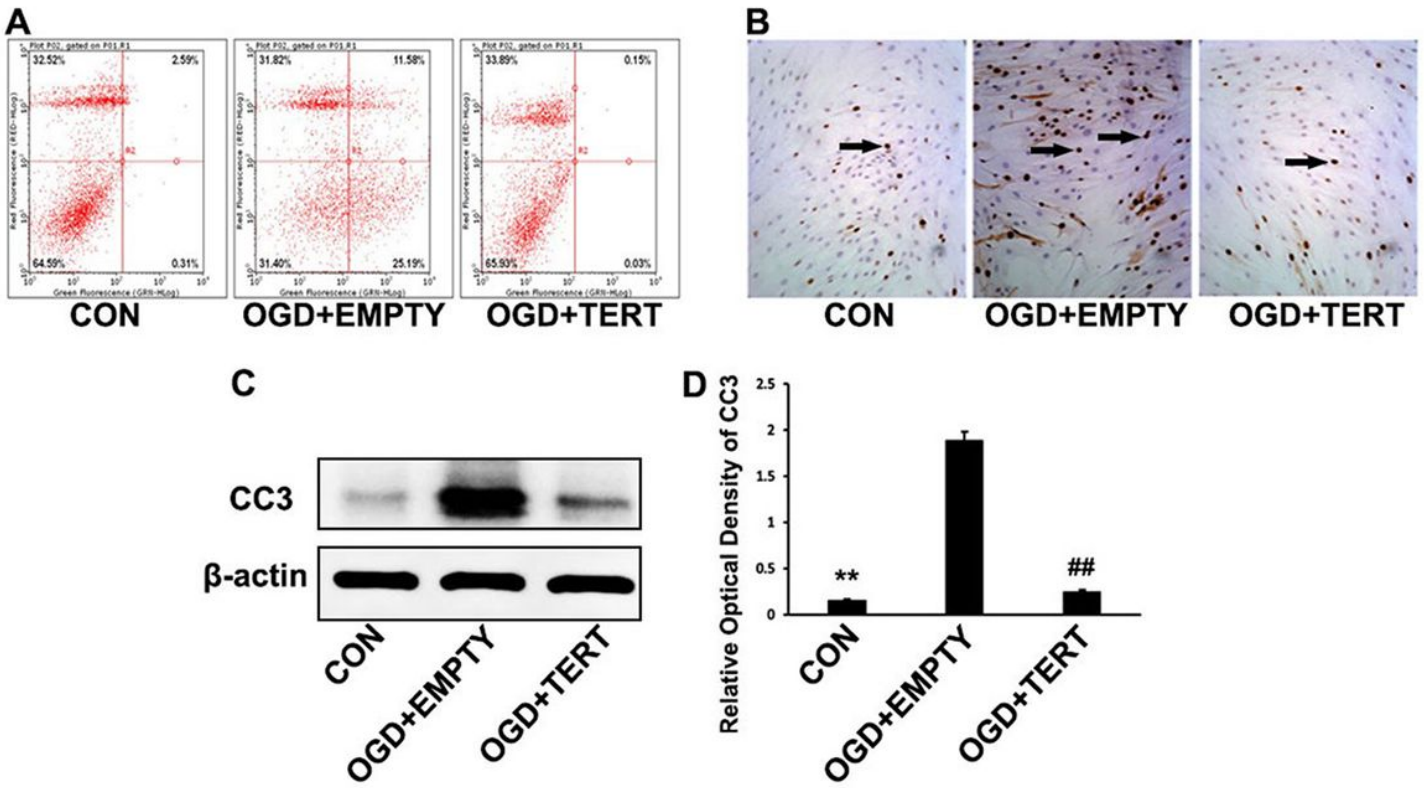

Figure 3

Apoptosis of H9c2 cells. (A) Flow cytometry and (B) TUNEL assay showed that apoptosis of H9c2 cells in OGD+EMPTY group increased compared with CON group. Cell apoptosis in OGD+TERT group decreased compared with OGD+EMPTY group. (Black arrows in Fig3.B showed the TUNEL positive cells). (C) Western blot showed that the expression level of CC3 was significantly increased in OGD+EMPTY group compared with CON group. And the expression level of CC3 in OGD+TERT group was decreased compared with OGD+EMPTY group. (D) Quantification of CC3 expression. Data were obtained by densitometry and were normalized using β-actin as internal control. Values are expressed in relative optical density and are represented as mean ± SD. **P<0.001 versus OGD+EMPTY group, n=3. ##P<0.001 versus OGD+EMPTY group, n=3. (OGD, oxygen and glucose deprivation).

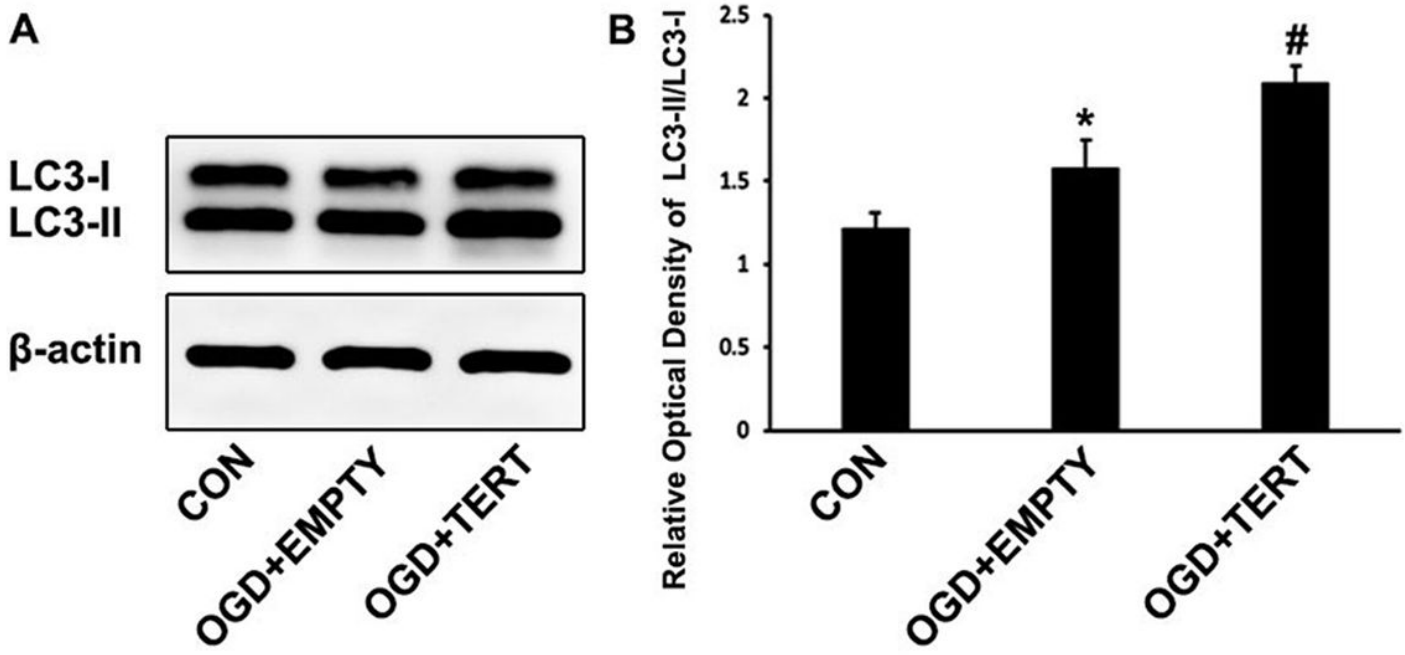

Figure 4

Autophagy of H9c2 cells. (A) Western blot showed that the expression rate of LC3-II/LC3-I was increased in OGD+EMPTY group compared with CON group. And the expression rate of LC3-II/LC3-I in OGD+TERT group was further increased compared with OGD+EMPTY group. (B) Quantification of rate of LC3-II/LC3-I. Data were obtained by densitometry. Values are expressed in relative optical density and are represented as mean ± SD. *P<0.05 versus CON group, n=3. #P<0.05 versus OGD+EMPTY group, n=3. (OGD, oxygen and glucose deprivation).