

Full-length article

Hypoxia reoxygenation induces premature senescence in neonatal SD rat cardiomyocytes¹

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Key words

cardiomyocytes; hypoxia reoxygenation; p16^{INK4a}; telomerase reverse transcriptase; β -galactosidase¹ Project supported by National Natural Science Foundation of China (No. 30671073).² These authors contributed equally to this study.³ Correspondence to Prof Ke-jiang CAO.
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Abstract

Aim: To investigate whether hypoxia reoxygenation induces premature senescence in neonatal Sprague-Dawley (SD) rat cardiomyocytes. **Methods:** Cardiomyocytes were isolated from neonatal SD rat heart and identified by immunohistochemistry. The control cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The hypoxic cultures were incubated in a modular incubator chamber filled with 1% O₂, 5% CO₂, and balance N₂ for 6 h. The reoxygenated cultures were subjected to 1% O₂ and 5% CO₂ for 6 h, then 21% oxygen for 4, 8, 12, 24, and 48 h, respectively. Cell proliferation was determined using bromodeoxyuridine labeling. The ultrastructure of cardiomyocytes was observed by using an electron microscope. β -Galactosidase activity was determined by using a senescence β -galactosidase Staining Kit. p16^{INK4a} and telomerase reverse transcriptase (TERT) mRNA levels were measured by real time quantitative PCR. TERT protein expression was determined by immunohistochemistry. Telomerase activities were assayed by using the TeloTAGGG Telomerase PCR ELISA^{plus} kit. **Results:** The initial cultures consisted of pure cardiomyocytes identified by immunohistochemistry. The proportion of BrdU positive cells was reduced significantly in the hypoxia reoxygenation-treated group ($P < 0.01$). Under the condition of hypoxia reoxygenation, mitochondrial dehydration appeared; p16^{INK4a} and TERT mRNA levels, β -galactosidase activity, TERT protein expression and telomerase activities were all significantly increased ($P < 0.01$ or $P < 0.05$). **Conclusion:** These data indicate that premature senescence could be induced in neonatal SD rat cardiomyocytes exposed to hypoxia reoxygenation. Although TERT significantly increased, it could not block senescence.

Introduction

Cellular senescence has been defined by Hayflick and Moorehead as the ultimate and irreversible loss of replicative capacity occurring in primary somatic cell culture^[1]. Senescence was generally studied in culture, different markers of the senescent phenotype, as mitochondrial dehydration^[2], senescence-associated β -gal activity^[3], and senescence-associated gene expression^[4,5]. Senescence has been identified in human fibroblasts^[6], keratinocytes^[7], endothelial cells^[8], smooth muscle cells^[9], tumor cells^[10], hematopoietic cells^[11], and cardiomyocytes^[12,13].

The mammalian heart is an obligate aerobic organ. Oxygen, beyond its indispensable role in cardiac energy metabolism, plays a central role in other biological processes that can be determinants of cardiac function, including the determination of cardiac gene expression patterns^[14]. The possibility that cardiac aging is an independent determinant of morbidity and mortality has faced opposition, and emphasis has been placed on age-associated changes, which increase the chances of cardiovascular events in the elderly. Treatment of cardiac diseases in elderly patients has resulted in a prolongation of the average lifespan. However, the maximum lifespan has not increased in the last 70 years^[15],

suggesting that cellular aging may play a more important role than generally expected.

Accumulating evidence suggests that telomerase plays a role in cellular senescence *in vitro* and *in vivo*^[16,17]. The enzyme telomerase, a ribonucleoprotein, is crucial for maintaining telomere length. Telomerase reverse transcriptase (TERT) is an important component of telomerase. It can counteract telomere shortening and prevent senescence^[18]. Senescence in human fibroblasts can be overcome by overexpression of TERT^[19]. Telomerase elongates short telomere, and the cells become immortalized.

Oxidative stress has been implicated in aging and numerous diseases^[20,21]. Nevertheless, whether premature senescence could be induced and blocked by TERT in neonatal Sprague-Dawley (SD) rat cardiomyocytes exposed to hypoxia reoxygenation remains unknown. In this study, we investigated the effect of hypoxia reoxygenation on neonatal SD rat cardiomyocytes.

Materials and methods

Experimental animals All experiments were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals of Nanjing Medical University, China.

Cardiomyocytes culture Neonatal SD rat hearts (1–2 d old) were removed under sterile conditions and washed 3 times in phosphate buffered saline (PBS) to remove excess blood cells. The ventricles were minced to small fragments and then agitated gently in a solution of 0.1% trypsin. The mixture was centrifuged at 1000×g for 15 min. The supernatant phase was discarded, and the cells were resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Isolated cells were cultured in a flask for 1 h at 37 °C in order to remove fibroblasts. The cultures were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Hypoxia reoxygenation The suspension of cells was diluted to 1.0×10⁶ cells/mL, and 1.5 mL was seeded in 6-well plastic plate and cultured for 72 h. Then, the control cultures were continuously incubated in 5% CO₂ and 95% air at 37 °C. The hypoxic cultures were placed within a modular incubator chamber (BioSpherix, Redfield, NY, USA), filled with 1% O₂, 5% CO₂, and balance N₂ for 6 h. The reoxygenated cultures were subjected to 1% O₂ and 5% CO₂ for 6 h, then 21% oxygen for 4, 8, 12, 24, and 48 h, respectively.

Electron microscopy For electron microscopy, cardio-

myocytes were digested with 0.1% trypsin, centrifuged at 1000×g for 10 min and fixed in 2.5% glutaraldehyde; they were postfixated in 1% osmium tetroxide in 0.1 mol/L phosphate buffer, dehydrated through a graded series of ethanol and propylene oxide, and then embedded in epoxy resin. Ultrathin sections of cardiomyocytes were stained with uranyl acetate and observed using an electron microscopy (JEM-1010, JEOL, Tokyo, Japan).

Cell proliferation assay Cell proliferation was determined using bromodeoxyuridine (BrdU) (Sigma, St Louis, MO, USA) labeling. Experimental cells were pretreated with 10 µmol/L BrdU for 30 min before hypoxia treatment and continued until time of hypoxia; normal cells were incubated with BrdU with an equivalent dose and time. The labeled cells were observed by immunocytochemistry using a mouse monoclonal antibody directed against BrdU 1:200 (Sigma, USA) and rabbit anti-mouse IgG as the secondary reagent. The labeled cells were viewed with the high-resolution Pathological Image Analysis System 1000 (High Resolution Pathological Image and Word Analysis System, Beijing, China). Stained BrdU and non-stained cells were counted in randomly selected areas on the sections to a total number of 500 cells per fish.

RNA isolation and real-time quantitative PCR Total RNA was isolated using the Trizol (Invitrogen, Life Technologies, Carlsbad, California, USA) method from the cells. RNA was treated with DNase to prevent contamination of genomic DNA. Total RNA (2 µg) was subjected to reverse transcription using reverse transcriptase (TaKaRa Biotechnology Co Ltd, Dalian, China) as described previously^[22]. Expression levels of p16^{INK4a} and TERT mRNA were quantified by real-time quantitative PCR, using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster city, CA, USA). With the use of standard curves, the amount of copy numbers of target genes in each sample was calculated and expressed as a ratio to the β-actin. Primers and probes of target genes were designed using Primer Express software (Applied Biosystems, USA) and they are listed in Table 1.

β-galactosidase staining Cell cultures were washed twice in PBS (pH 7.4) and fixed for 10 min at room temperature with 20% formaldehyde and 2% glutaraldehyde in PBS. After 3 washes in PBS, the cultures were incubated for 24 h at 37 °C in freshly prepared SA-β-galactosidase staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal; Sigma, USA (pH 6.0)] 400 mmol/L potassium ferrocyanide, 400 mmol/L potassium ferricyanide, and 200 mmol/L MgCl₂. The percent of SA-β-galactosidase positive cells was determined by counting the number of blue cells under bright field illumination, and the total number of

Table 1. Sequences of primers and probes for real-time quantitative PCR.

Gene	Forward primer	Reverse primer	Probe
p16 ^{INK4a}	5'-CCGAGAGGAAGGCGAACTC-3'	5'-GCTGCCCTGGCTAGTCTATCTG-3'	5'-CCATGCTGCTCCGGATCGCTCT-3'
TERT	5'-GGTCTTCCGCACGTTGGTT-3'	5'-CGCGCTCGCAAAGTTTCT-3'	5'-CTTCCACCAGGTGTCATCCCTGAAAGAG-3'
β -actin	5'-CCTGGCACCCAGCACAAT-3'	5'-GCTGATCCACATCTGCTGGAA-3'	5'-ATCGATCATTGCTCCTCCTGAGCGC-3'

cells in the same field under phase as a contrast.

Immunohistochemical procedures Slide chambers with cardiomyocytes were treated in 0.5% H₂O₂ for 5 min and washed in PBS. Non-specific binding was blocked with normal goat serum. Free-floating sections were incubated for 2 h at 37 °C in monoclonal antibodies myosin light chain antibody (Sigma, USA) and TERT (Acris, Hiddenhausen, Germany), all diluted at 1:100 in PBS. The sections were then washed in PBS and incubated for 30 min at 37 °C with the second antibody goat anti-mouse IgG. The protein contents were analyzed using the high-resolution Pathological Image Analysis System 1000.

Telomerase activities assay Cardiomyocytes were washed in PBS, and the pellet was lysed with 200 μ L lysis buffer for 30 min at 4 °C as previously described^[23]. The proteins were centrifuged for 20 min at 16000 \times g, and protein concentrations were determined in the supernatant using the Bradford assay. Telomerase activities were measured with 2 μ g protein by the Telo TAGGG Telomerase PCR ELISA^{plus} kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Statistical analysis Experiments were performed at least 3 times. Data were expressed as mean \pm SD. The *t* test and χ^2 were used to determine significant differences between 2 groups. *P* values less than 0.05 were considered to be significant.

Results

Identification of isolated cells as cardiomyocytes No spindle-shaped fibroblast could be seen. Immunocytochemical analysis revealed that most (>95%) cells in the isolated cultures were positive for sarcomeric myosin light chain (Figure 1).

Electron morphology of cardiomyocytes Cardiomyocytes showed normal morphology and equal nuclear chromatin distribution in the control group (Figure 2A). Most cells appeared to have cytoplasmic concentration, nuclear chromatin condensation and margination in the hypoxia group (Figure 2B). The cells approached mitochondrial dehydra-

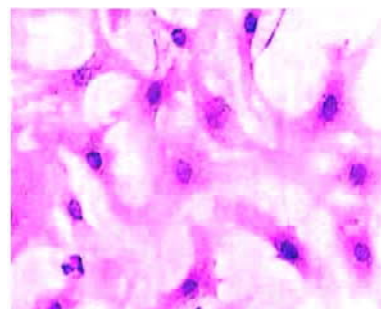


Figure 1. Identification of isolated neonatal SD rat heart cells as cardiomyocytes with monoclonal antibody sarcomeric myosin light chain, most (>95%) cells in the isolated cultures were positive for sarcomeric myosin light chain. Original magnification \times 100.

tion with enlarged, flattened nuclear morphology in the hypoxia reoxygenation group (Figure 2C).

Cell proliferation assay We examined cardiomyocyte proliferation in the hypoxia reoxygenation-treated group by measuring BrdU incorporation. The proportion of BrdU positive cells was 15.32% in the control group. It was reduced significantly in the hypoxia reoxygenation-treated group ($P<0.01$; Figure 3). These results indicated that cardiomyocyte proliferation could be blocked by hypoxia reoxygenation.

p16^{INK4a} and TERT mRNA expression We investigated whether hypoxia and reoxygenation treatment altered the expression of the p16^{INK4a} and TERT. With the use of real-time quantitative PCR, significant increases in p16^{INK4a} and TERT mRNA levels were observed in the hypoxia reoxygenation-treated group compared with the untreated group ($P<0.01$), respectively (Figures 4, 5).

β -galactosidase staining We investigated whether the hypoxia reoxygenation-treated cells were β -galactosidase-positive. With the use of a senescence β -galactosidase staining kit, a small part of the cells were positive for β -galactosidase in the control group, the hypoxia 6 h group, and the reoxygenation 4 and 8 h group, but β -galactosidase-positive cells significantly increased in the reoxygenation 12, 24, and 48 h group ($P<0.05$ and $P<0.01$; Figure 6A, 6B).

TERT protein In our study, TERT protein expression

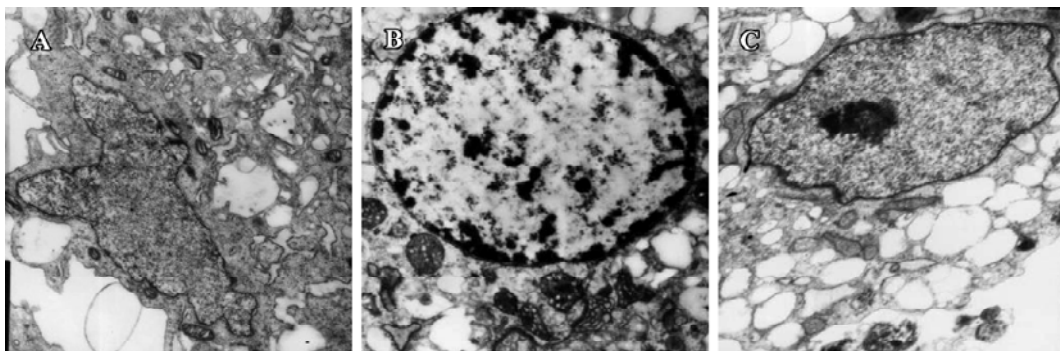


Figure 2. Electron micrographs of cardiomyocytes in the control group (A), hypoxia 6 h group (B), and reoxygenation 24 h group (C). Original magnification $\times 20\,000$.

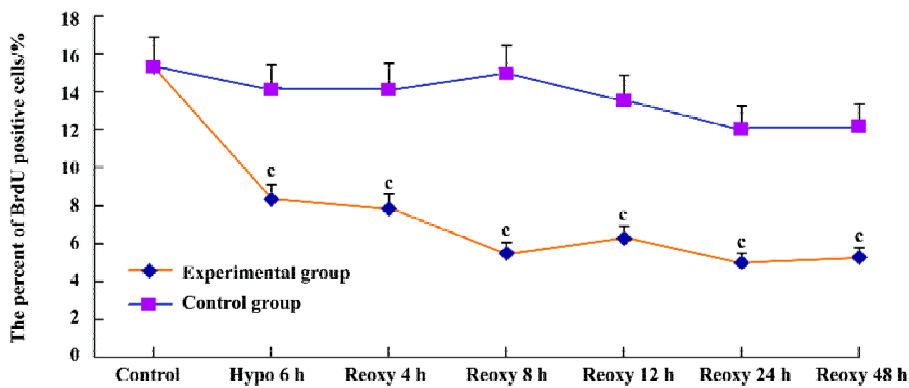


Figure 3. Effects of hypoxia and reoxygenation on cell proliferation in neonatal SD rat cardiomyocytes. $n=3$. Mean \pm SD. ^c $P<0.01$ vs control group.

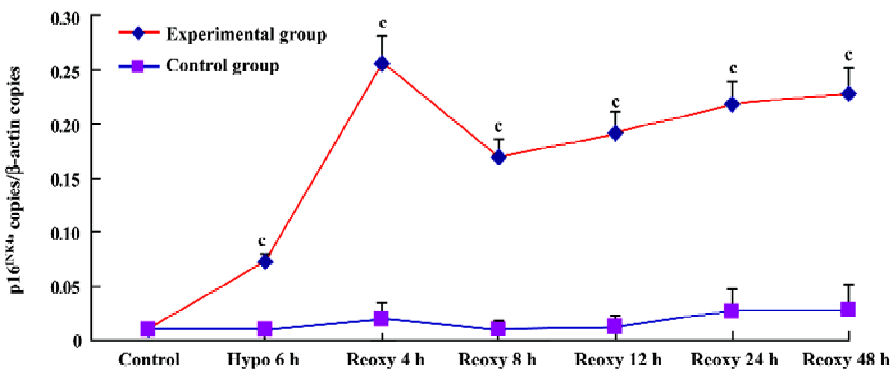


Figure 4. Effects of hypoxia and reoxygenation on p16^{INK4a} mRNA expression in neonatal SD rat cardiomyocytes. $n=3$. Mean \pm SD. ^c $P<0.01$ vs control group.

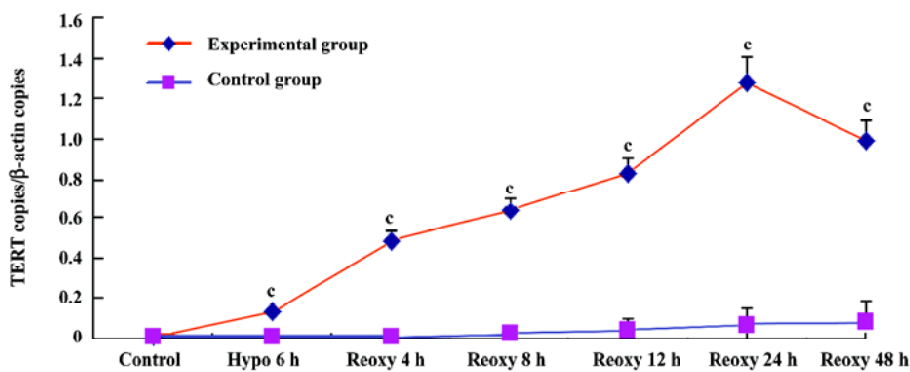


Figure 5. Effects of hypoxia and reoxygenation on TERT mRNA expression in neonatal SD rat cardiomyocytes. $n=3$. Mean \pm SD. ^c $P<0.01$ vs control group.

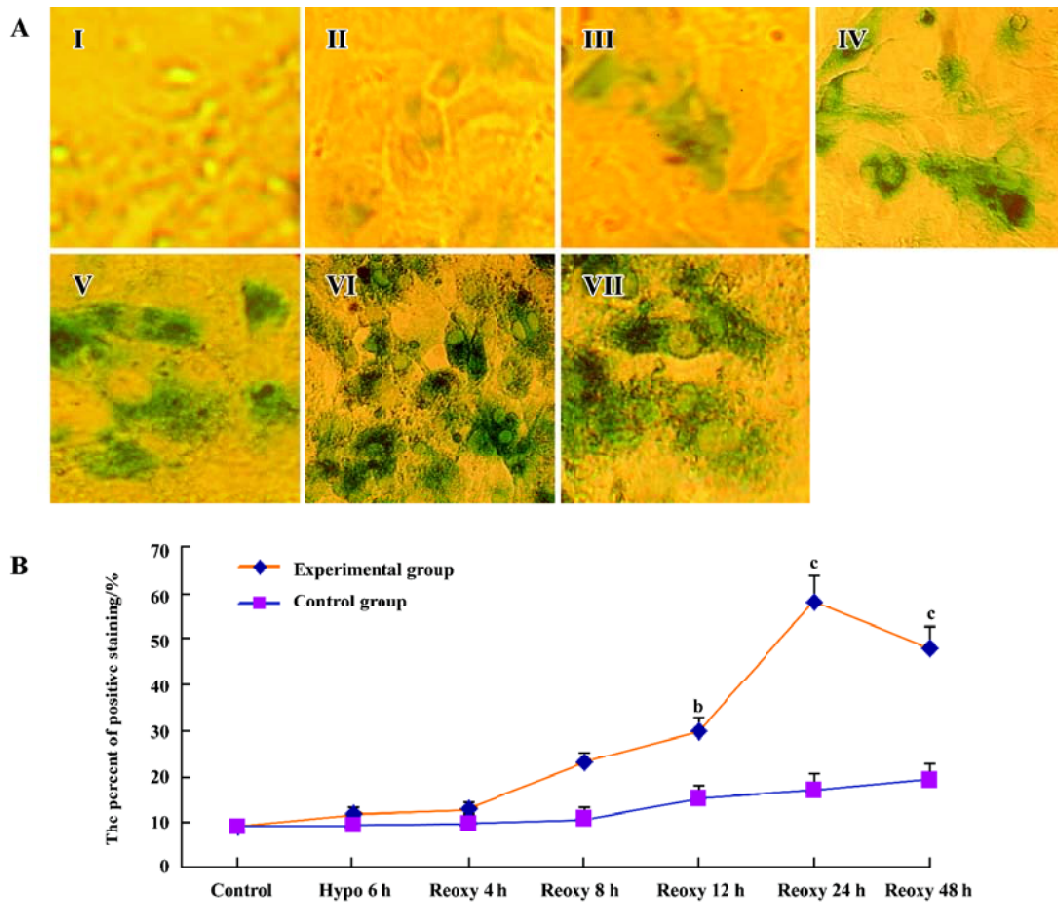


Figure 6. (A) Effects of hypoxia and reoxygenation on β -galactosidase activity in neonatal SD rat cardiomyocytes. (I) control group; (II) hypoxia 6 h group; (III) reoxygenation 4 h group; (IV) reoxygenation 8 h group; (V) reoxygenation 12 h group; (VI) reoxygenation 24 h group; and (VII) reoxygenation 48 h group. Original magnification $\times 400$. (B) Effects of hypoxia and reoxygenation on β -galactosidase activity in neonatal SD rat cardiomyocytes. $n=3$. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs control group.

was determined by immunohistochemistry. The immunohistochemical results showed that the positive cardiomyocytes were stained brown in the intranucleus and cytoplasm; the degree of color in each group was different. TERT protein expression in the group of hypoxia 6 h, reoxygenation 4, 8, 12, 24, and 48 h was significantly increased compared with control group ($P < 0.01$; Figure 7).

Telomerase activity assay With use of the Telo TAGGG Telomerase PCR ELISA^{plus} kit, relative telomerase activity increased significantly in the hypoxia reoxygenation-treated group compared with the control group ($P < 0.01$; Figure 8).

Discussion

In our study, most cardiomyocytes demonstrated cytoplasmic concentration, nuclear chromatin condensation and margination (Figure 1B), then chromatin gathered at the cen-

ter of cell nucleus in the hypoxia reoxygenation-treated group (Figure 1C). Mitochondrial dehydration, enlarged, flattened nuclear morphology could be observed in the reoxygenation-treated group (Figure 1C). The results were in accordance with a previously published study^[24].

Unlike other cells, the proliferation of cardiomyocytes is so limited after birth; the rate was only 15.32% in the control group. It was reduced significantly with hypoxia reoxygenation treatment (Figure 3). At or around birth, cardiomyocytes nearly lose their ability to divide. Cardiomyocyte DNA synthesis is associated with cell proliferation during fetal life, and a second DNA synthesis phase occurring after birth (up to approximately neonatal d 3) is associated only with binucleation^[25]. After birth, cardiac growth involves increasing the size of the myocytes without substantial increases in cell number.

p16^{INK4a} mRNA copies in the hypoxia reoxygenation-

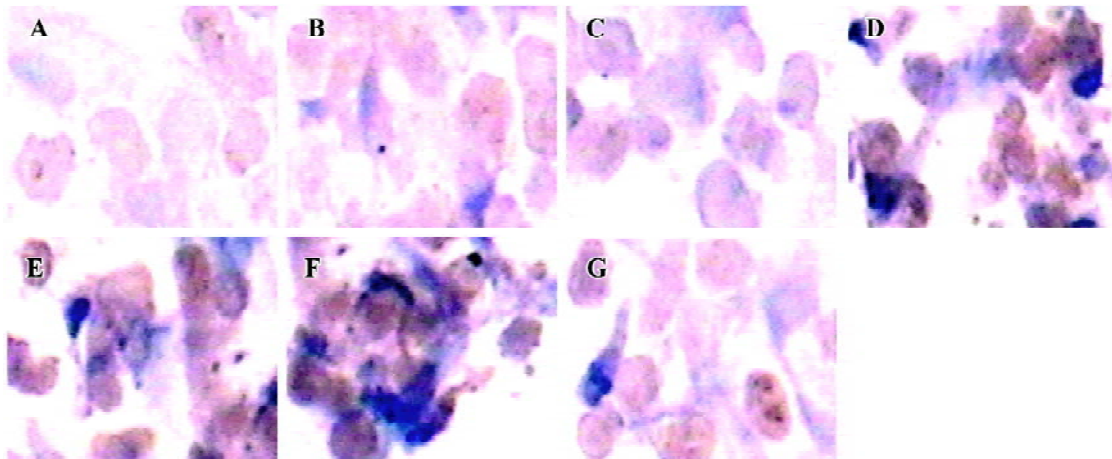


Figure 7. Effects of hypoxia and reoxygenation on TERT protein expression in neonatal SD rat cardiomyocytes. (A) Control group; (B) Hypoxia 6 h group; (C) Reoxygenation 4 h group; (D) Reoxygenation 8 h group; (E) Reoxygenation 12 h group; (F) Reoxygenation 24 h group; (G) Reoxygenation 48 h group. Original magnification $\times 100$.

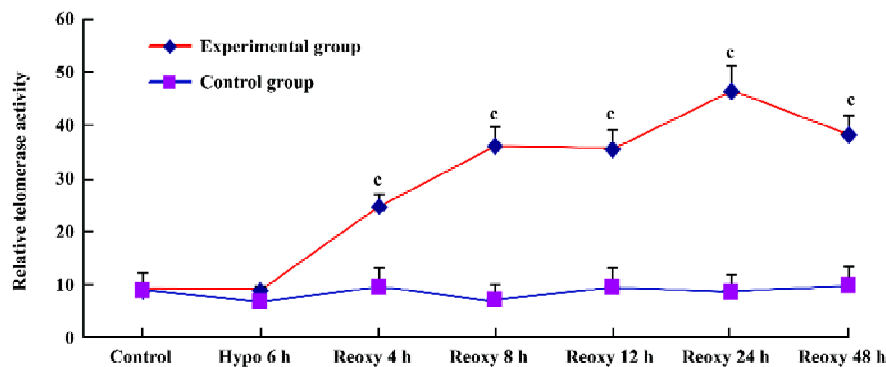


Figure 8. Effects of hypoxia and reoxygenation on relative telomerase activities in neonatal SD rat cardiomyocytes. $n=3$. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs control group.

treated cells were significantly increased compared with the untreated cells (Figure 4). p16^{INK4a}, an inhibitor of the cell cycle^[26], encoded potent tumor suppressor proteins^[27], and was recognized as the key regulator of premature senescence. Its expression levels were found to increase dramatically in premature senescent cells^[28] whose overexpression in mammalian cells could induce G₁ phase arrest^[29].

β -galactosidase activity has recently been used as a histochemical marker of premature senescence in human fibroblasts, keratinocytes, endothelial and smooth muscle cells^[30-32]. In our study, few cardiomyocytes were stained for β -galactosidase in the control group, the hypoxia 6 h group, and the reoxygenation 4 and 8 h group, but the staining intensity increased significantly in the reoxygenation 12, 24, and 48 h group (Figure 6A, 6B). Our findings are similar with a previous study on bone marrow cells^[21]. β -Galactosidase activity also correlated with replicative capacity in human mammary epithelial cells^[32]. It provided a simple assay

for chromosomes that induced senescence^[33]. β -Galactosidase activity increased in combination with the expression of senescence-associated proteins p53 and p21^{WAF1} as well^[34].

TERT mRNA and protein expression, relative telomerase activities, were all significantly increased in the hypoxia reoxygenation-treated cells compared with the untreated cells (Figure 5, 7, 8), but they could not inhibit senescence. It has been previously reported that the exposure of human cells to hypoxic conditions results in the induction of telomerase activity through human telomerase reverse transcriptase (hTERT) expression^[35,36]. TERT mRNA levels were reported to correlate with telomerase activity and to be implicated in the regulation of telomerase activity in cancer cells^[37]. Furthermore, telomerase activity in telomerase-negative cells can be restored by ectopic expression of TERT, suggesting that in certain cases, TERT is the only limiting factor for telomerase activation^[38]. The relationship between hypoxia and the induction of telomerase activity should be considered.

Hypoxic conditions could induce a DNA damage response by causing telomere damage. In response to this damage, hypoxia-inducible factor-1 α may induce telomerase in order to heal the damaged chromosome ends^[39].

The cells in the culture underwent senescence. This phenotype was attributable to unsuitable conditions (culture shock, oxidative stress) or to growth arrest mediated by p38 MAPK, and p19^{Arf}, not to telomere shortening *per se*^[40]. hTERT transduction also failed to prevent premature senescence in 21% O₂^[41]. Maybe the activation of p38 MAPK contributed to the onset of senescence induced by oxidative stress^[42]. p38 MAPK contributed to p53 accumulation in Ataxia Telangiectasia/TERT fibroblasts during oxidative stress-induced senescence^[43]. p38 MAPK phosphorylated p53 on Ser33 and Ser46 in the NH₂ terminal activation domain and thereby regulated the transactivation activity of p53^[43,44].

In summary, these data indicate that premature senescence could be induced in neonatal SD rat cardiomyocytes exposed to hypoxia reoxygenation. Although TERT significantly increased, it could not block senescence.

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