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Establishment of oxygen glucose deprivation reperfusion model of senescent SH-SY5Y cells

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ABSTRACT

Objective: To explore the establishment of an oxygen glucose deprivation/reperfusion model of senescent SH-SY5Y cells. **Methods:** SH-SY5Y cells were randomly divided into control (*D*-galactose 0 mmol/L group), *D*-galactose (25 mmol/L, 50 mmol/L, 100 mmol/L, 200 mmol/L, 400 mmol/L) groups, and treated with corresponding concentrations of *D*-galactose for 48 h. The changes of cell morphology, β-galactosidase, the cell morphology, β-galactosidase activity by microscopic observation, cell proliferation rate by EdU kit and cell survival rate by CCK-8 assay were used to determine the decaying concentration of *D*-galactose and to establish the senescence model. The senescent SH-SY5Y cells were randomly divided into control group (oxygen glucose deprivation without treatment group), oxygen glucose deprivation treatment (0.5 h, 1 h, 1.5 h, 2 h) group, followed by re-glucose reoxygenation for 24 h, and CCK-8 assay for the survival rate of senescent SH-SY5Y cells. **Results:** There were no significant changes in cell morphology and β-gal activity in the 25 mmol/L and 50 mmol/L groups compared with the control group ($P>0.05$), cytosolic hypertrophy was seen in the cells of the 100 mmol/L group, chromatin fixation in the cells of the 200 mmol/L group, and massive vacuolization in the cells of the 400 mmol/L group; the positive rate of β-galactosidase staining in the cells of the (100-400 mmol/L) group was significantly higher compared with the control group ($P<0.05$), with little difference between the 100 mmol/L and 200 mmol/L groups ($P>0.05$); the cell proliferation ability of the (100-400 mmol/L) group was significantly decreased in a concentration-dependent manner ($P<0.05$); the cell survival rate was decreased in a concentration-dependent manner ($P<0.05$), with IC_{50} between 100 mmol/L and 200 mmol/L. The survival of senescent SH-SY5Y cells showed a time-dependent decrease in oxygen-glucose deprivation ($P<0.05$), with an IC_{50} close to 1 h. **Conclusion:** *D*-gal concentration of 100 mmoL/L and 48 h of cell action could establish a survival rate of about 50% of senescent SH-SY5Y cells, and oxygen glucose deprivation of senescent SH-SY5Y cells for 1 h and reperfusion for 24 h could establish an oxygen glucose deprivation/reperfusion model of senescent SH-SY5Y cells with a survival rate close to 50%.

1. Introduction

Ischemic stroke is one of the diseases with high disability and mortality rates worldwide[1], often occurring in the elderly population[2]. The key to its treatment is to restore early blood reperfusion[3]. However, as reperfusion proceeds, secondary damage occurs in the original ischemic brain tissue, including increased apoptosis of neuronal cells and increased production of reactive oxygen species, which is called cerebral ischemia-reperfusion injury[4]. Cerebral ischemia-reperfusion injury can lead to serious brain diseases[3], and many research scholars have investigated the mechanism of action and effective neuroprotective drugs by establishing in vivo and ex vivo models[5−10], but few studies related to cerebral ischemia-reperfusion injury for aging have been reported. Compared with animal models, *in vitro* cellular models

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have the advantages of homogeneous study samples, exclusion of numerous interfering factors *in vivo*, facilitation of observation of single factors, and observation of regulatory mechanisms. Therefore, this study combined the popular *D*-galactose-decaying[11-13] model and the oxygen-glucose deprivation/reperfusion model[14-16] to jointly construct a model of oxygen-glucose deprivation-reperfusion injury in aging neuronal cells, laying a solid foundation for exploring the mechanisms of ischemia-reperfusion injury in the aging brain as well as new prevention and treatment methods.

2. Materials and methods

2.1 Materials

Human neuroblastoma cells (SH-SY5Y, Wuhan University Cell Bank). DMEM low sugar medium (Gibco, USA, item no. 11885084), fetal bovine serum (Gibco, USA, item no. 10100147). PBS buffer (Solarbio, China, item no. P1020), 0.25% EDTA trypsin (Solarbio, China, item no. T1300), cyanostatin (Solarbio, China, item No. P1400), *D*-galactose (Solarbio, China, item no. SG8010). Serum-free Freezing Solution (NCM Biotech, China, item No. C10400), CCK-8 kit (NCM Biotech, China, item no. C6050), β-galactosidase kit (Beyotime, China, item No. RG0039), BeyoClickTMEdU-594 kit (Beyotime, China, No. C0078S, including Hoechst 33342 (1000X) reagent), liquid nitrogen (Jinfeng Liquid Nitrogen Container Co., Ltd., China).

2.2 Cell culture, grouping of senescence models, and experimental treatments

SH-SY5Y cells were placed in DMEM low sugar complete medium with 10% fetal bovine serum and 1% penicillin for conventional culture passaging. Logarithmic growth phase SH-SY5Y cells were collected and inoculated in 6-well plates (cell density of 2×10^4 cells/mL), and after growing overnight, the complete medium with different *D*-galactose concentrations was replaced and the cells were divided into (1) control group: complete medium *D*-galactose concentration of 0 mmol/L according to the experimental design, referring to the study of Heidari *et al*[17]. (2) *D*-galactose 25 mmol/ L group: complete medium *D*-galactose concentration was 25 mmol/ L. (3) *D*-galactose 50 mmol/L group: complete medium *D*-galactose concentration was 50 mmol/L. (4) *D*-galactose 100 mmol/L group: complete medium *D*-galactose concentration was 100 mmol/L. (5) *D*-galactose 200 mmol/ L group: complete medium *D*-galactose concentration was 200 mmol/L. (6) *D*-galactose 400mmol/L group: complete medium *D*-galactose concentration was 400mmol/L, and incubation was continued in the normoxic incubator for 48 h for post-experimental index detection.

2.3 Morphology of SH-SY5Y cells in each group observed by ordinary light microscope

Logarithmic growth phase SH-SY5Y cells were collected and inoculated in 6-well plates (cell density of 2×10^4 cells/mL), and the cells were treated according to different groupings and subsequently placed under a light microscope to observe the changes in cell morphology and number.

2.4 Positive rate of β-galactosidase staining in each group of SH-SY5Y cells detected by β-galactosidase kit

Logarithmic growth phase SH-SY5Y cells were collected and inoculated in 6-well plates (cell density of 2×10^4 cells/mL), cells were treated according to different groupings, medium was aspirated, PBS washed once, staining fixative was added, fixed at room temperature for 15 min, fixative was aspirated, PBS washed 3 times, 1 mL of staining working solution was added, incubated overnight in a CO_2-free incubator protected from light, and β-galactosidase staining was observed by ordinary light microscopy.

2.5 EdU proliferation kit to detect the proliferation rate of SH-SY5Y cells in each group

SH-SY5Y cells at logarithmic growth stage were collected and inoculated in 6-well plates (cell density of 2×10^4 cells/mL), cells were treated in different groups, some of the medium was aspirated, EdU working solution pre-warmed at 37 ℃ was added and incubated for 6 h. Medium was aspirated, 1 mL of fixative was added to each well and incubated for 15 min at room temperature, fixative was aspirated, 1 mL of washing solution was added to each well and washed 3 times, 3-5 min each time. minutes each time. Aspirate the washing solution, add 1 mL of permeation solution and incubate for 15 min at room temperature; remove the permeation solution, add 1 mL of washing solution to each well and wash 1-2 times for 3-5 min each time, remove the washing solution, add 0.5 mL of Click reaction solution, shake the 6-well plate gently and mix well, incubate for 30 min at room temperature and avoid light, aspirate the reaction solution, add 1 mL of washing solution to each well and wash 3 times for 3-5 min each time. Aspirate the washing solution, add 1 mL of prepared 1XHoechst33342 solution to each well, incubate for 10 min at room temperature and avoid light, aspirate the 1XHoechst33342 solution, add 1 mL of washing solution to each well and wash 3 times, 3-5 min each time. The washing solution was aspirated and the nuclei and the staining of the value-added cells were observed under a fluorescent inverted microscope and photographed.

2.6 CCK-8 method to detect the survival rate of SH-SY5Y cells in each group

Logarithmic growth phase SH-SY5Y cells were collected and inoculated in 96-well plates (cell density of 10^4 cells/mL), and after treating the cells according to different groupings, the medium was aspirated and 100 μL of medium (containing 10 μL of CCK-8 reaction solution and 90 μL of sugar-free medium) was added to each well, incubated for 1 h at 37 ℃ and 5% CO_2 incubator, and the absorbance value of each well at 450 nm was detected by enzyme marker.

2.7 Establishment of senescent SH-SY5Y cell model

Based on the results of the above experiments, the optimal decay-causing concentration of *D*-galactose at 48 h was determined. The logarithmic growth phase SH-SY5Y cells were collected and inoculated in 6-well plates (cell density of 2×10^4 cells/mL), grown overnight, and then replaced with complete medium containing the optimum *D*-galactose concentration and continued to incubate in the normoxic incubator for 48 h.

2.8 Grouping of oxygen glucose deprivation/reperfusion models and experimental treatments

After establishing the senescent SH-SY5Y cell model, the senescent SH-SY5Y cells were divided into control group, oxygen-glucose deprivation 0.5 h group, oxygen-glucose deprivation 1 h group, oxygen-glucose deprivation 1.5 h group and oxygen-glucose deprivation 2h group. The experimental treatments were as follows: (1) Control group: senescent cells were placed in normal culture in normoxic incubator. (2) Oxygenose deprivation (0.5 h, 1 h, 1.5 h, 2 h) group: After establishing the senescent cell model, the old culture medium was aspirated and the pre-warmed sugar-free medium was added. The well plate was put into the homemade anoxic aeration box, the upper and lower holes were opened and 99.9% high purity nitrogen gas with a flow rate of 1 L/min was passed into them, and the upper and lower holes were closed after 5 minutes, followed by incubation in a 37 ℃, CO_2-free incubator for different time periods (0.5 h, 1 h, 1.5 h, 2 h), and after the incubation was completed, the well plate was removed from the anoxic box, the complete medium was replaced, and the incubation was continued in the normoxic incubator for 24 h .

2.9 CCK-8 assay to detect the survival rate of senescent SH-SY5Y cells

After establishing the senescent cell model, the senescent cells were treated with different groups of oxygen-glucose deprivation, re-glucose reoxygenation for 24 h. The old medium was aspirated, 100 μL of medium (containing 10 μL of CCK-8 reaction solution and 90 μL of sugar-free medium) was added to each well, incubated for 1 h at 37 ℃ and 5% CO_2 incubator, and the absorbance value of each well at 450 nm wavelength was detected by enzyme marker.

2.10 Statistical analysis

All data are expressed as ($\bar{x}\pm s$), and experimental data were analyzed using SPSS 23.0, and graphs were made using GraphPad Prism 8.0.1. One-way ANOVA was used between groups, and Tamhanes T2 test was selected for uneven variance; LSD test was selected for uneven variance.

3. Results

3.1 Morphology of SH-SY5Y cells in different D-galactose concentration groups observed by ordinary light microscopy

The results of ordinary light microscopy showed that there was no significant change in the number of cell morphology in the 25 mmol/L and 50 mmol/L groups after treatment with different concentrations of *D*-galactose for 48 h. The cells in the 100 mmol/L group were seen to be hypertrophied and flattened in shape; the number of cells in the 200 mmol/L group was significantly reduced, with chromatin fixation and cytosolic atrophy; cell fragmentation and vacuolation were widely seen in the 400 mmol/L group (Figure 1).

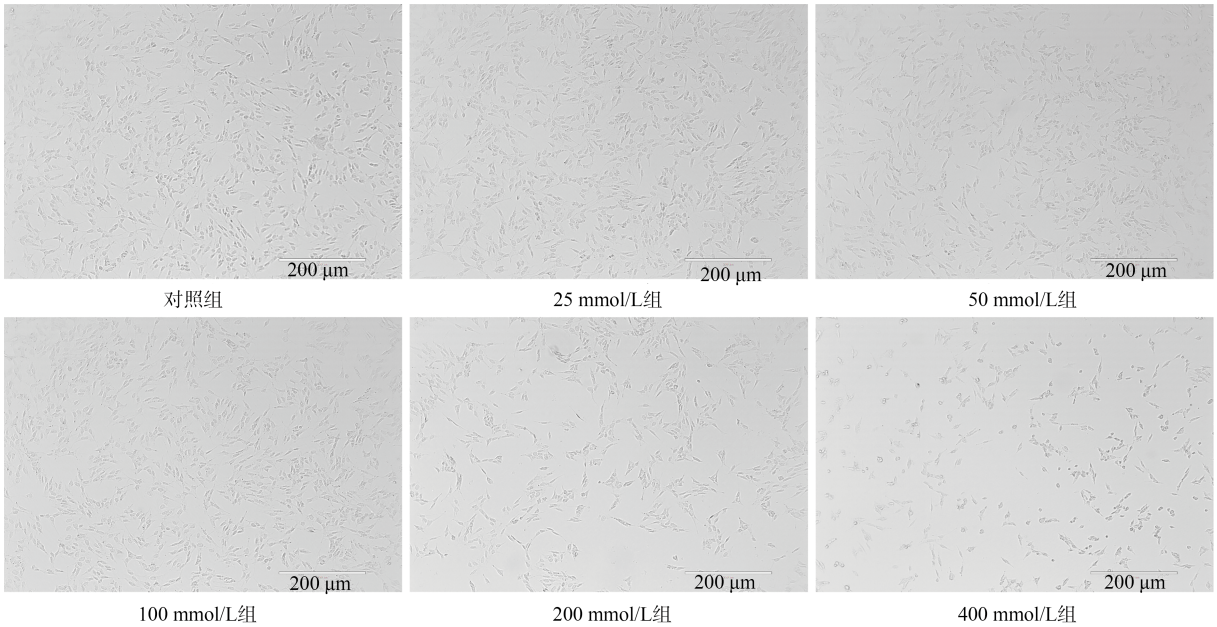


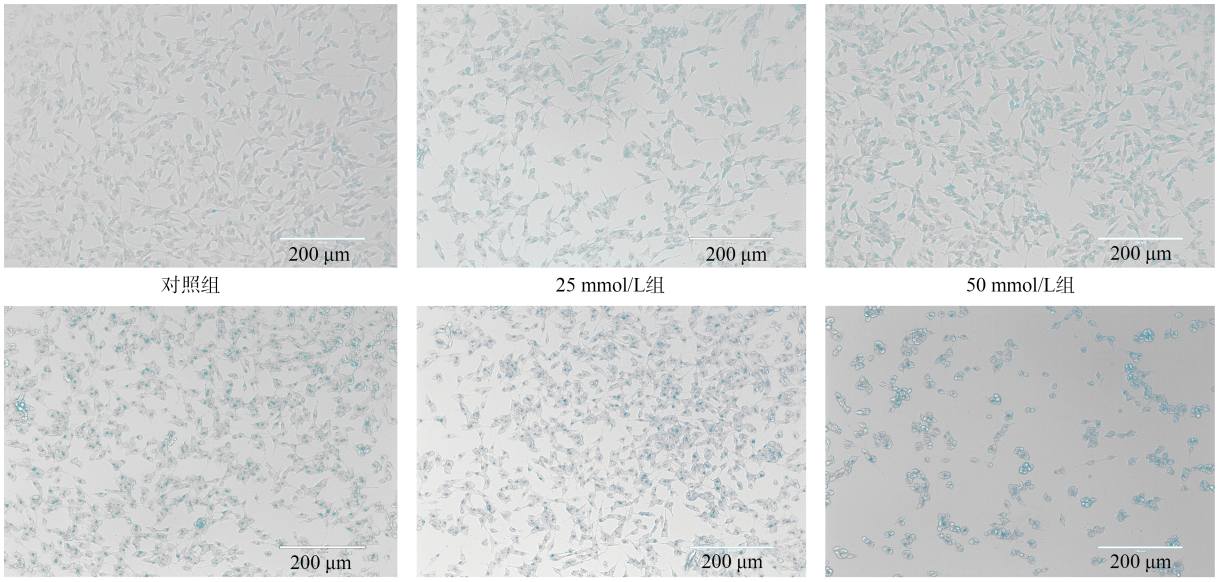
Fig 1 Changes in morphology and number of SH-SY5Y cells in each group (n=3, ×100)

3.2 Positive rate of β-galactosidase staining of SH-SY5Y cells in different D-galactose concentration groups detected by the β-galactosidase kit

The results of β-galactosidase kit showed that after 48 h of culture of SH-SY5Y cells in each group, the positive rate of β-galactosidase staining of cells in the (25 mmol/L to 400 mmol/L) group was significantly increased compared with the control group (P<0.05); the difference of positive rate of β-galactosidase staining between the 25 mmol/L and 50 mmol/L groups was not significant (P>0.05); the difference in the positive rate of β-galactosidase staining between the 100 mmol/L and 200 mmol/L groups was not significant (Figure 2 and 3).

3.3 Proliferation rate of SH-SY5Y cells in different D-galactose concentration groups detected by EdU proliferation kit

The results of the EdU kit showed that the cell proliferation rate was significantly lower in the 100 mmol/L, 200 mmol/L, and 400 mmol/L groups compared with the control group after 48 h of SH-SY5Y cell culture in each group (P<0.05); there was no significant difference in the cell proliferation rate between the 100 mmol/L and 200 mmol/L groups (P>0.05) (Figure 4 and 5).



Note: Compared with the control group, *P<0.05; between-group comparison, ns stands for P >0.05.

Fig 2 β-alactosidase staining of SH-Y5Y cells in each group (n=3, ×400)

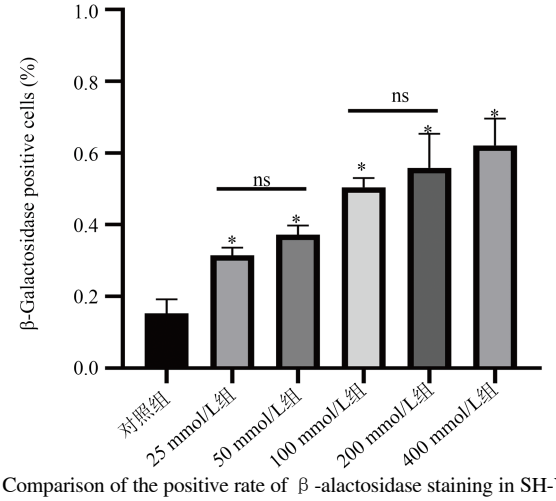


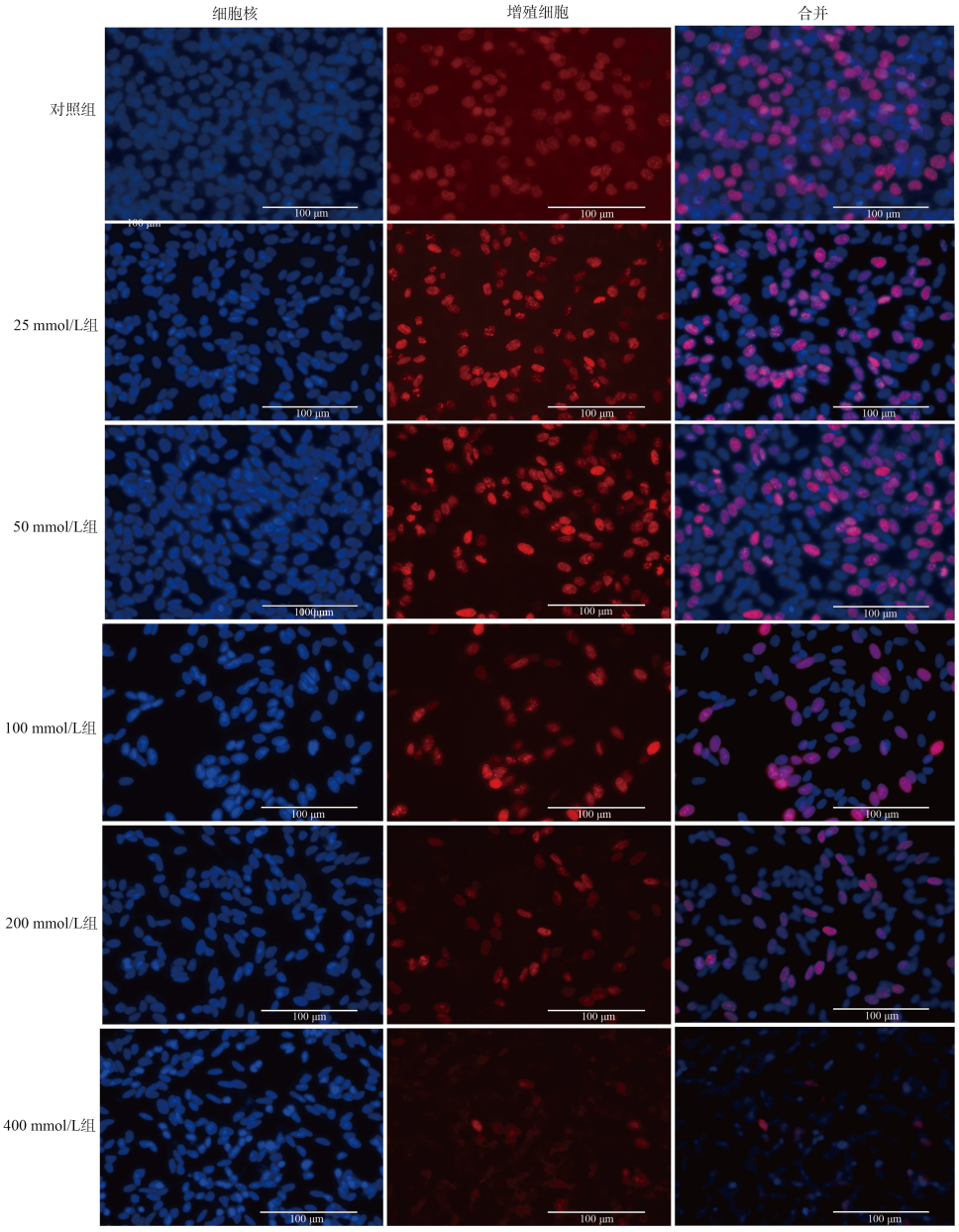
Fig 3 Comparison of the positive rate of β-alactosidase staining in SH-Y5Y cells in each group

3.4 CCK-8 method to detect the survival rate of SH-SY5Y cells in different D-galactose concentration groups

The results of CCK-8 assay showed that after 48 h of SH-SY5Y cell culture in each group, the survival rate of SH-SY5Y cells showed a D-galactose concentration-dependent decrease (P<0.05), and the cell survival rates in each group were (100.00 ± 0.00)%, (76.65 ± 0.08)%, (63.45 ± 0.09)%, (59.30 ± 0.11)%, (48.2 ± 0.11)%, (30.6 ± 0.62)% (Table 1).

3.5 CCK-8 method to detect the survival rate of aging SH-SY5Y cells at different times of oxygenose deprivation

The results of CCK-8 assay showed that the survival rate of senescent SH-SY5Y cells was found to decrease in a time-dependent manner (P<0.05) after different time periods of oxygen glucose deprivation, and the cell survival rates of each group were (100.00 ± 0.00)%, (94.12 ± 0.03)%, (53.85 ± 0..02)%, (41.88 ± 0.05)%, (23.58 ± 0.02)% (Table 2).



Note: Compared with the control group, $^{*}P<0.05$; compared with the 100 mM group, $^{\wedge}P<0.05$.

Fig 4 EdU fluorescence staining of SH-SY5Y cells in each group (n=3, ×400)

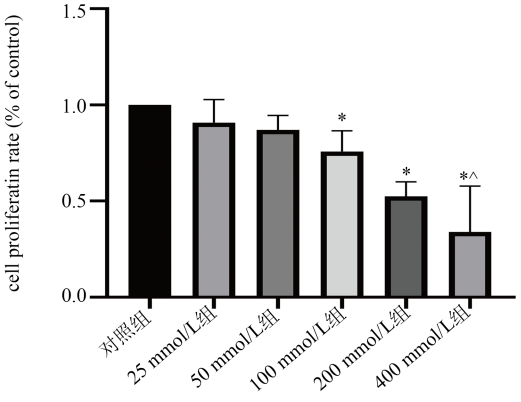


Fig 5 Comparison of proliferation rate of SH-SY5Y cells in each group (n=3)

Tab 1

Comparison of the survival rate of SH-SY5Y cells in each group (n=9, ($\bar{x}$±s))

Group	Survival rate (%)
Control	100.00±0.00
25 mmo/L	76.65±0.08*
50 mmo/L	63.45±0.09$^{*\&}$
100 mmo/L	59.30±0.05$^{*\&\$}$
200 mmo/L	48.29±0.11$^{*\&\$\%}$
400 mmo/L	30.61±0.62$^{*\&\$\%!}$
F	91.179
P	0.00

Note: Compared with the control group, $^{*}P<0.05$; compared with the 25 mmol/L group, $^{\&}P<0.05$; compared with the 50 mmol/L group, $^{\$}P<0.05$; compared with the 100 mmol/L group, $^{\%}P<0.05$; compared with the 200 mmol/L group, $^{!}P<0.05$.

Tab 2

Comparison of the survival rate of senescent SH-SY5Y cells in each group (n=6,$\bar{x}$±s)

Group	Survival rate (%)
Control	100.00±0.00
0.5 h	94.12±0.03*
1 h	53.85±0.02*#
1.5 h	41.88±0.05*#&
2 h	23.58±0.02*#&
F	977.711
P	0.00

Note: compared with the control group, *P<0.05; compared with the 0.5 h group, #P<0.05; compared with the 1 h group, &P<0.05; compared with the 1.5 h group, 'P<0.05.

4. Discussion

Compared to *in vivo* ischemia-reperfusion models of aging animals, *in vitro* oxygen glucose deprivation/reperfusion models of aging cells have the advantages of homogeneous study samples, facilitate the observation of single factors, and artificially controlled study conditions.

D-galactose-depletion is a commonly used model of accelerated aging[18]. SH-SY5Y cells are human neuroblastoma cells that are widely used in neuronal cell models because of their biochemical and functional properties of a variety of neurons[19]. In this study, SH-SY5Y cells were first denuded and then senescent cells were treated with oxygen glucose deprivation. In the morphological experiments of *D*-galactose-induced decay, we observed that cells in the 100 mmol/L group showed senescence manifestations such as cytosolic hypertrophy, cell flattening, and arbitrary orientation[20,21], and in the 200 mmol/L and 400 mmol/L groups, in addition to cytosolic hypertrophy, nuclear consolidation, cell vacuolization, and apoptotic or necrotic signs of cell fragmentation were also seen[22,23], which indicates that with the increase of *D* galactose concentration increased the degree of senescence in SH-SY5Y cells. pH 6, elevated β-galactosidase activity (SA-β-gal) is one of the senescence indicators[24], and this study found that overall SA-β-gal showed a concentration-dependent increase (P<0.05), but the difference between the 100 mmol/L and 200 mmol/L groups in SA-β-gal The difference was not significant.

The essence of senescence is the irreversible blockage of the cell cycle[25], and as detected by the EdU cell proliferation kit, we found that the cell proliferation rate appeared to decrease significantly (P<0.05) at *D*-galactose concentrations of 100 mmol/L or more. Based on the CCK-8 results, we found that the cell survival rate was (59.3 ± 0.05)% in the 100 mmol/L group and (48.29 ± 0.11)% in the 200 mmol/L group, with an IC_{50} between 100 mmol/L and 200 mmol/L/L. Based on the results of the above experiments, we decided to use 100 mmol/L as the optimal concentration of *D*-galactose for SH-SY5Y cell senescence.

In the D-galactose-induced senescence combined with oxygen glucose deprivation/reperfusion experiment, we found that the survival rate of SH-SY5Y cells showed a time-dependent decrease in oxygen glucose deprivation (P<0.05), and the cell survival rate was (53 ± 1.6)% at 1 h reoxygenation of SH-SY5Y cells for 24 h, which was close to 50%. Referring to the oxygen glucose deprivation model of non-aging SH-SY5Y cells established by previous groups[26], we found that the cell survival rate was (55.65±1.95)% at 2 h oxygen glucose deprivation, but in this experiment, we found that the cell viability at 2 h was only 23.5%, which shows that aging cells are more severely damaged after oxygen glucose deprivation/reoxygenation.

In summary, we first treated SH-SY5Y cells with different concentrations of D-galactose for 48 h, and detected the senescence by cell morphology, SA-β-gal, cell proliferation assay, and cell viability results, and determined the optimal senescence concentration as 100 mmol/L; subsequently, on the basis of the senescence, the senescent SH-SY5Y cells were oxygenose deprived for different times, and the senescence was detected by CCK-8 method. The cell survival rate was determined by CCK-8 method. Based on the IC_{50}, we finally determined that the model of senescent SH-SY5Y cells was constructed by using 100 mmol/L *D*-galactose for 48 h followed by 1 h of oxygen glucose deprivation and 24 h of re-glucose reoxygenation.

Authors' contributions

Zhang Qiao-tian, Jiang Chang-yue, Xie Lu: participated in the whole experiment, supervised the operation and wrote the paper; ZhuGe Xiang-zhen, Li Deli: cultured cells, tested the experiment-related indexes, analyzed the data statistically and participated in writing the paper; Hu Wan-xian: performed the EdU kit to detect the cell proliferation rate and the data statistically.

All authors declare that there is no conflict of interest.

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