

Optimal concentration and time window for proliferation and differentiation of neural stem cells from embryonic cerebral cortex: 5% oxygen preconditioning for 72 hours

Li-li Yuan^{1,2,*}, Ying-jun Guan^{2,*}, Deng-dian Ma³, Hong-mei Du²

1 Department of Histology and Embryology, Academy of Basic Medicine, Jining Medical University, Jining, Shandong Province, China

2 Department of Histology and Embryology, Academy of Basic Medicine, Weifang Medical University, Weifang, Shandong Province, China

3 Department of Otorhinolaryngology, Affiliated Hospital of Jining Medical University, Jining, Shandong Province, China

*Correspondence to:

Li-li Yuan or Ying-jun Guan, Ph.D.,
liliyuan06@126.com or
guanyj@wfmc.edu.cn.

orcid:

0000-0002-3689-7847 (Li-li Yuan)
0000-0002-9578-0497 (Ying-jun Guan)

doi: 10.4103/1673-5374.165526

<http://www.nrronline.org/>

Accepted: 2015-07-12

Abstract

Hypoxia promotes proliferation and differentiation of neural stem cells from embryonic day 12 rat brain tissue, but the concentration and time of hypoxic preconditioning are controversial. To address this, we cultured neural stem cells isolated from embryonic day 14 rat cerebral cortex in 5% and 10% oxygen *in vitro*. MTT assay, neurosphere number, and immunofluorescent staining found that 5% or 10% oxygen preconditioning for 72 hours improved neural stem cell viability and proliferation. With prolonged hypoxic duration (120 hours), the proportion of apoptotic cells increased. Thus, 5% oxygen preconditioning for 72 hours promotes neural stem cell proliferation and neuronal differentiation. Our findings indicate that the optimal concentration and duration of hypoxic preconditioning for promoting proliferation and differentiation of neural stem cells from the cerebral cortex are 5% oxygen for 72 hours.

Key Words: nerve regeneration; brain injury; neural stem cells; low oxygen; cerebral cortex; apoptosis; differentiation; microtubule-associated protein 2; glial fibrillary acidic protein; caspase-3; neural regeneration

Funding: This work was supported by the Science Foundation of Jining Science and Technology Bureau of China, No. 2012jnc07.

Yuan LL, Guan YJ, Ma DD, Du HM (2015) Optimal concentration and time window for proliferation and differentiation of neural stem cells from embryonic cerebral cortex: 5% oxygen preconditioning for 72 hours. *Neural Regen Res* 10(9):1516-1522.

Introduction

Stem cells in the central nervous system are defined by the characteristics of self-renewal and differentiation into neurons, astrocytes (Gage and Temple, 2013), and oligodendrocytes (Zawadzka et al., 2010). The fate of central nervous system stem cells to specific cell lineages can be changed by a single extrinsic factor (Guerout et al., 2014). Central nervous system stem cells that are used in the treatment of common degenerative and ischemic diseases have become a major research focus (Cheng et al., 2015).

Recently, the role of oxygen in proliferation and differentiation of neural stem cells (NSCs) has been examined (Xiong et al., 2013). Low oxygen condition promotes survival and proliferation of NSCs from the mouse ganglionic eminence (Horie et al., 2008). However, controversy remains surrounding the proper length of time and concentration of lowered oxygen preconditioning for improved NSC differentiation. There are many NSCs in the cerebral cortex (Borrell and Reillo, 2012), yet little research exists concerning the effect of low oxygen concentration *in vitro* on NSCs derived from the

cerebral cortex of embryonic rat. In this study, NSCs were isolated from the cerebral cortex of embryonic day 14 (E14) Sprague-Dawley rats, and cultured *in vitro*. Our aim was to examine the influence of low oxygen concentration and preconditioning time on cultured NSCs by comparing with normoxia, to provide a tool for NSC transplantation in the treatment of hypoxic-ischemic brain injury and degenerative diseases in the central nervous system.

Materials and Methods

Isolation and culture of NSCs

A total of 60 adult Sprague-Dawley rats, aged 3–6 months and weighing 220–250 g with a male to female ratio of 1:2, were housed and allowed free access to food and water under a 12-hour light/dark cycle, in accordance with the institutional guidelines on the care and handling of experimental animals (License No. SCXK (Lu) 20130001). Male and female rats were placed in the same cage at 8 p.m. E0 day was defined when sperm was found in the vagina of a female rat. E14 rat cerebral cortex was isolated for cell subculture. All

protocols were approved by the Institutional Animal Care and Use Committee of Jining Medical University, China and performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Proliferation and differentiation of NSCs

After incubation in trypsin, cells dissected from E14 rat cerebral cortices were mechanically dissociated, plated at a density of 5×10^5 /mL into 5 mL cell culture dishes in serum-free medium containing Dulbecco's modified Eagle's medium/F12 medium (Hyclone, Logan, UT, USA), 20 ng/mL of epidermal growth factor and basic fibroblast growth factor (Sigma, St. Louis, MO, USA), and 2% B27 (Gibco BRL, Grand Island, NY, USA), then placed into a saturated humidity incubator (Heraeus, Hanau, Germany) at 37°C with 5% CO₂. The medium was first changed after 24 hours, then every other day. After culturing for 7 days, cells were passaged by mechanical beating with a glass pipette, trypsin digested, and reseeded as single cells at 5×10^5 /mL in serum-free medium (first passage cells). Cells were passaged three times according to the above method. Third passage cells were processed for subsequent experiments.

Passage 3 neurosphere cell suspensions were centrifuged and the supernatant discarded. Next, 10% fetal bovine serum was added to plastic 6-well plates with poly-L-lysine-coated coverslips. Cells were treated with 50 mg/L 5-bromo-2'-deoxyuridine (BrdU) (Boster, Wuhan, China) and cultured in a saturated humidity incubator at 37°C with 5% CO₂ for 6 hours. Cells mounted on coverslips were fixed for 10 minutes using cold acetone, and nestin (Chemicon, Billerica, MA, USA) and BrdU (Accurate Chemical, Westbury, NY, USA) double immunofluorescence staining performed. The remaining slides were cultured for 7 days in medium containing serum, then fixed and prepared for immunofluorescence staining using microtubule associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP) (Chemicon). Slides were observed by inverted fluorescence microscopy (Leica, Wetzlar, Germany).

Hypoxic preconditioning

Third passage NSCs derived from E14 rat cerebral cortex were first cultured in serum-free medium for 7 days, including treatment with 5% O₂ for 72 or 120 hours then 20% O₂, or with 10% O₂ for 72 or 120 hours then 20% O₂. All groups were cultured in medium containing serum at 20% O₂. A normal control group (always 20% O₂) was also set up. Thus, NSCs were divided into groups of 5% O₂ 72-hours, 5% O₂ 120-hours, 10% O₂ 72-hours, 10% O₂ 120-hours, and a normal control, according to low oxygen concentration and intervention time. In low oxygen conditions, NSCs were housed in a tri-gas incubator (Heraeus,) at 37°C with 5% CO₂, with different oxygen concentrations set using nitrogen gas. In the normal control group, cells were placed in an incubator at normal humidity (*i.e.*, 37°C) with 5% CO₂. Immunofluorescent analysis of NSCs was performed using a laser scanning confocal microscope (Hi-

tachi, Tokyo, Japan).

Electron microscopy analysis of NSCs

After culturing for 7 days in serum-free medium, neurospheres from each group were collected separately and centrifuged at $1,500 \times g$ for 15 minutes. After supernatant removal, neurospheres were fixed in 3% glutaraldehyde and 1% osmium tetroxide, dehydrated, resin embedded, and then sliced into ultrathin sections. Ultrathin sections were double-stained using uranyl acetate and lead citrate, and imaged with a transmission electron microscope (Hitachi).

3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for NSC viability

Single cell suspensions of passage 3 NSCs were centrifuged, diluted to 1×10^5 /mL, and seeded into 96-well plates. In each plate, each group used four wells with a blank control group also. The wells were filled with 200 μ L serum-free suspension and the NSCs were observed every day. After 7 days, NSCs were divided into two portions, with one portion used for the MTT assay and the other for neurosphere counting. Optical density values of NSCs were measured by MTT assay. MTT (5 g/L, 20 μ L) was added to each well (of 96-well plates) and NSCs placed in an incubator of saturated humidity (37°C) at 5% CO₂ for 4 hours, then centrifuged and collected. Each well was treated with 150 μ L dimethyl sulfoxide and oscillated for 10 minutes. Finally, optical density values were automatically detected at 490 nm using a microplate reader (Thermo Electric, Shanghai, China).

Neurosphere counting

Neurospheres were observed using an inverted microscope at 200 \times magnification. The neurosphere number in each group was counted. Eight fields from each well (of 96-well plates) were randomly selected and four wells from each group were counted. The average number of neurospheres in each group was determined.

Apoptosis and differentiation of NSCs

In low-oxygen conditions, neurospheres were cultured in serum-free medium for 7 days, then centrifuged and the supernatant was removed. Next, neurospheres were resuspended in medium containing serum at a density of $0.5-1 \times 10^5$ /mL and cultured in 24-well plates with poly-L-lysine-coated coverslips. The differentiation and fixation methods were the same as before. After differentiation for 4 hours, four coverslips from each group were randomly selected and fixed for immunofluorescent staining of rabbit anti-caspase-3. Cell numbers of positive staining and Hoechst staining were counted using an inverted phase contrast microscope at 200 \times magnification. Five random fields from each coverslip were counted. After culturing for a further 7 days in medium containing serum, the remaining cells were used for immunofluorescent staining of MAP2. The observation index and MAP2 calculation method were the same as for caspase-3. All counts were performed by observers blinded to the experimental conditions.

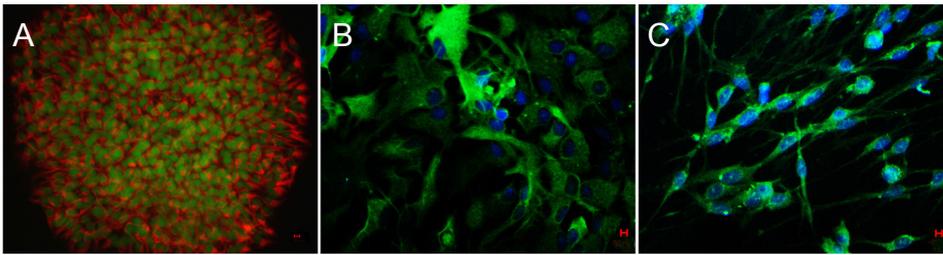


Figure 1 Expression of markers related to neural stem cell proliferation and differentiation (immunofluorescent staining, laser scanning confocal microscope).

(A) All neurosphere cells were positive for nestin (red) and BrdU (green). (B) After differentiation for 7 days, immunofluorescent staining showed that the cytoplasm of some cells was positive for GFAP (green) with Hoechst staining in the nucleus (blue). (C) MAP2 was expressed in the cytoplasm of some cells (green) with nuclear Hoechst expression (blue). BrdU: 5-Bromo-2'-deoxyuridine; GFAP: glial fibrillary acidic protein; MAP2: microtubule associated protein-2. Scale bar: 10 μ m.

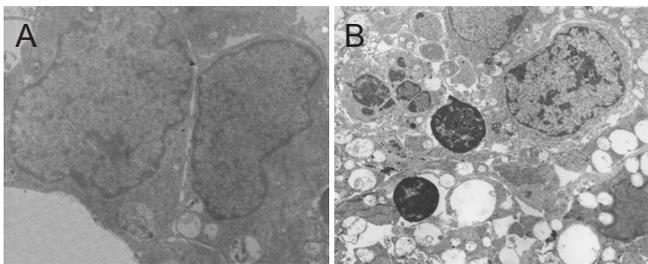


Figure 2 Influence of hypoxic preconditioning on the ultrastructure of neural stem cells from embryonic rat cerebral cortex (uranyl acetate and lead citrate double staining, transmission electron microscope).

After 7 days of culture in serum-free medium, neurospheres from each group were collected and treated for ultrathin sectioning. (A) In the normal control group (20% O₂, 7 days), only modest changes in the morphological signs of apoptosis were detected (\times 6,000). (B) Pyknosis and condensed chromatin near the nuclear membrane were detected in some neural stem cells in the 5% O₂ 120-hour group (\times 3,000).

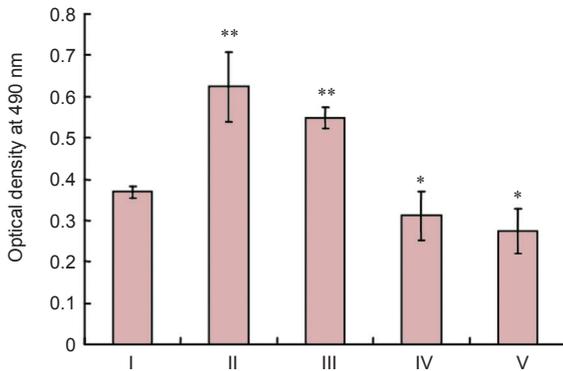


Figure 3 Effect of low oxygen on neural stem cell viability (MTT assay).

After culturing in growth medium for 7 days, neural stem cell viability was determined in each group using the MTT assay (optical density value). * $P < 0.05$, ** $P < 0.01$, vs. I (20% O₂, 7 days) (mean \pm SD, Student's *t*-test). All experiments were performed at least three times. I: Normal control group; II: 10% O₂ 72-hour group; III: 5% O₂ 72-hour group; IV: 10% O₂ 120-hour group; V: 5% O₂ 120-hour group. MTT: 3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

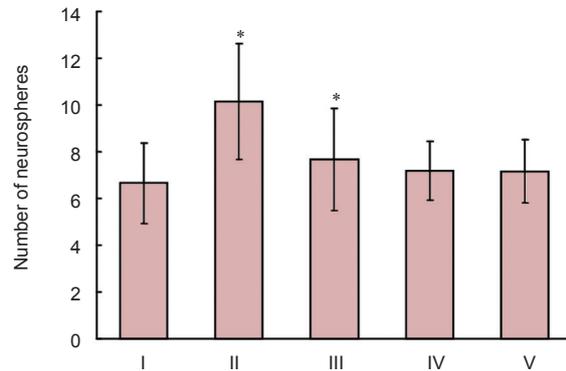


Figure 4 Distinct promotion of neural stem cell proliferation in the low oxygen 72-hour group as shown by neurosphere number.

The numbers of neurospheres in each group were counted from 96-well plates using an inverted microscope at 200 \times magnification. Eight fields of each well were randomly selected and four wells from each group were counted. The average neurosphere number in each group was obtained. * $P < 0.05$, vs. I (20% O₂, 7 days) (mean \pm SD, Student's *t*-test). All experiments were performed at least three times. I: Normal control group; II: 10% O₂ 72-hour group; III: 5% O₂ 72-hour group; IV: 10% O₂ 120-hour group; V: 5% O₂ 120-hour group.

Immunofluorescent staining

Fixed cells on coverslips were washed three times for 5 minutes in PBS containing 0.1% Triton X-100, followed by 2 M HCl incubation for 30 minutes at 37°C. After three PBS washes (this step was only used to detect BrdU), sheep serum was added to the coverslips for 30 minutes at room temperature. After serum removal, mixed antibodies composed of mouse anti-nestin monoclonal antibody (1:200; Chemicon) and mouse anti-BrdU polyclonal antibody (1:200; Accurate Chemical), or rabbit anti-caspase-3 monoclonal antibody

(1:100; Boster) were added to neurospheres on coverslips; or mouse anti-MAP2 polyclonal antibody (1:200; Chemicon) or mouse anti-GFAP polyclonal antibody (1:400; Chemicon) was added to differentiated cells on coverslips, then incubated overnight at 4°C. Coverslips were washed three times with PBS, and the cells were incubated with the corresponding fluorescent secondary antibody: Cy3 (sheep anti-mouse IgG; 1:80) or FITC (sheep anti-rat IgG; 1:200) (Sigma), or a mixture of both, for 60 minutes at 37°C in the dark. After three PBS washes, Hoechst 33258 (10 μ g/mL)

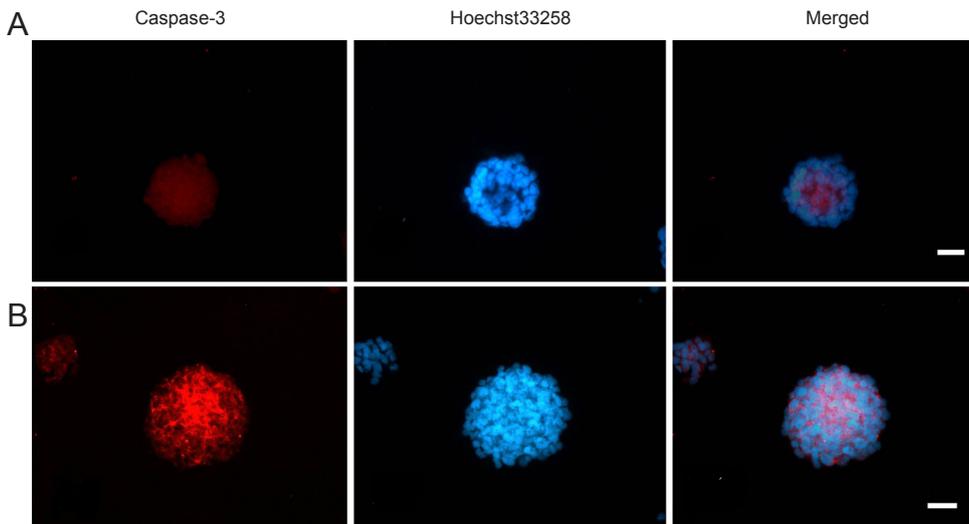


Figure 5 Effect of hypoxic preconditioning on neural stem cell apoptosis (immunofluorescent staining, fluorescence microscopy). After culturing for 4 hours in medium containing serum, apoptosis was detected by immunofluorescent staining of caspase-3 in neurospheres from the 10% O₂ 120-hour (A) and 5% O₂ 120-hour (B) groups. Caspase-3-positive cells (red) were mainly located in the center of neurospheres. Hoechst 33258 stained the nuclei of all cells (blue). Cell expression showed a significantly higher rate of apoptotic cells in the 5% O₂ 120-hour group than in the 10% O₂ 120-hour group. The rate of apoptotic cells was significantly higher in the 10% O₂ 120-hour and 5% O₂ 120-hour groups than in the normal control group (20% O₂, 7 days). Scale bars: 50 μm.

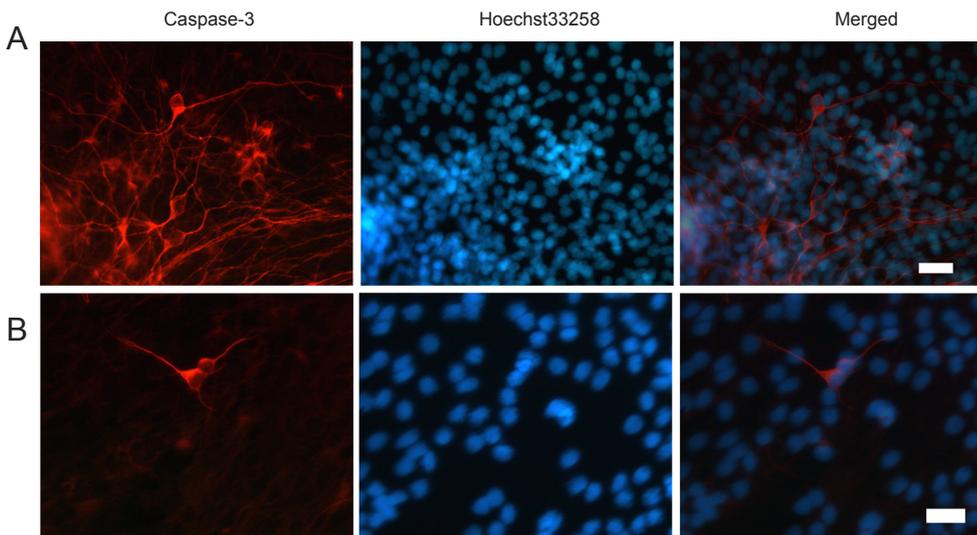


Figure 6 Influence of 5% O₂ preconditioning on neural stem cell differentiation (immunofluorescent staining, fluorescence microscopy). After culturing neurospheres for 7 days in medium containing serum, differentiation of neural stem cells to neurons was detected by immunofluorescent MAP-2 staining in the 5% O₂ 72-hour group (A) and 5% O₂ 120-hour group (B). MAP-2 was expressed in the cytoplasm of some cells (red), and Hoechst 33258 was detected in the nuclei of all cells (blue). A significantly higher proportion of neural stem cells differentiated to neurons in the 5% O₂ 72-hour and 120-hour groups than in the normal control group (20% O₂, 7 days). MAP-2: Microtubule-associated protein-2. Scale bars in A: 20 μm, B: 15 μm.

Table 1 Influence of hypoxic preconditioning on neural stem cell apoptosis as detected by immunofluorescent caspase-3 staining

Group	Number of caspase-3-labeled cells		Apoptotic rate (%)
	Positive	Negative	
Normal control (20%O ₂ , 7 days)	434	3,379	11.4
10% O ₂ 72-hour	559	4,206	11.7
5% O ₂ 72-hour	468	3,678	11.3
10% O ₂ 120-hour	859	2,791	23.5*
5% O ₂ 120-hour	1,163	2,273	33.8*
Sum	3,483	16,327	17.6

*P < 0.05, vs. normal control group (chi-square test). All experiments were performed at least three times.

Table 2 Effect of hypoxic preconditioning on neuronal differentiation of neural stem cells as detected by immunofluorescent MAP2 staining

Group	Number of MAP-2-labeled cells		Apoptotic rate (%)
	Positive	Negative	
Normal control (20% O ₂ , 7 days)	1,211	5,814	17.2
10% O ₂ 72-hour	1,341	6,366	17.4
10% O ₂ 120-hour	1,167	5,540	17.4
5% O ₂ 72-hour	1,471	6,231	19.1*
5% O ₂ 120-hour	1,113	4,736	19.0*
Sum	6,303	28,687	18.0

*P < 0.05, vs. normal control group (chi-square test). All experiments were performed at least three times. MAP-2: Microtubule-associated protein-2.

was added for 30 minutes at 37°C in the dark, followed by a further three PBS washes. Images were obtained and the number of positive cells was counted using a confocal laser scanning microscope (Zeiss, Jena, Germany) or fluorescence microscope (Leica, Solms, Germany). The calculation method was the same as before.

Statistical analysis

Statistical analysis was performed using SPSS 11.7 software (SPSS, Chicago, IL, USA). The measurement data results were expressed as the mean \pm SD. Comparisons of measurement data between groups were performed using Student's *t*-test. Numeration data were expressed as percentages. Comparison of numeration data between groups was performed by chi-square test ($\alpha = 0.05$).

Results

Characteristics of NSCs from embryonic rat cerebral cortex

Cells isolated from embryonic rat cerebral cortex were cultured *in vitro* and grown to neurospheres suspended in growth medium. Immunofluorescent staining (Figure 1A) showed that all cells within neurospheres were nestin/BrdU double positive cells. After culturing in medium containing serum for 7 days, neurosphere-derived cells were positive for GFAP or MAP2 (Figure 1B, C). These results indicate that cells derived from the cerebral cortex can be regarded as NSCs.

Effect of hypoxic preconditioning on the ultrastructure of NSCs derived from embryonic rat cerebral cortex

After culturing for 7 days in growth medium, neurospheres were collected from each group for electron microscopy. Pyknosis and condensed chromatin near the nuclear membrane were distinct in the 5% O₂ 120-hour group (Figure 2). In contrast, only modest changes in the morphological signs of apoptosis were detected in cells in the other low oxygen groups and normal control group.

Effect of hypoxic preconditioning on viability of NSCs from embryonic rat cerebral cortex

After culturing in growth medium for 7 days, NSC viability was determined by MTT assay. Significantly higher optical density values were observed in NSCs from the 10% O₂ 72-hour and 5% O₂ 72-hour groups compared with the normal control group (20% O₂, 7 days) ($P < 0.01$; Figure 3). In contrast, significantly lower optical density values were observed in the 10% O₂ 120-hour and 5% O₂ 120-hour groups compared with the normal control group ($P < 0.05$).

Influence of low oxygen on proliferation of NSCs from embryonic rat cerebral cortex

The average number of neurospheres in each group was calculated, and was significantly higher in the low O₂ 72-hour group compared with the normal control group ($P < 0.05$; Figure 4).

Low oxygen treatment for 120 hours promoted NSC apoptosis

After culturing for 4 hours in medium containing serum, neurospheres were fixed and caspase-3 immunofluorescent staining was performed. Caspase-3-immunoreactive cells were mainly located in neurosphere centers (Figure 5). Moreover, the rate of caspase-3-immunoreactive cells was significantly higher in the 10% O₂ 120-hour and 5% O₂ 120-hour groups compared with the normal control group ($P < 0.05$; Table 1).

5% O₂ treatment enhanced NSC differentiation into neurons

After culturing in medium containing serum for 7 days, immunofluorescent staining of NSCs was performed. A significantly higher proportion of MAP2-immunoreactive cells was observed in the 5% O₂ 72-hour and 120-hour groups compared with the normal control group ($P < 0.05$; Figure 6 and Table 2).

Discussion

Here, we have investigated the impact of lower oxygen levels than the 20% traditionally used to culture NSCs *in vitro*. Our major findings on NSCs derived from E14 rat cerebral cortex are: (1) hypoxic preconditioning for 72 hours enhances NSC viability and proliferation; (2) neuronal differentiation of NSCs is notably promoted with 5% O₂ preconditioning; and (3) after hypoxic preconditioning for 120 hours, the apoptotic rate of NSCs is strikingly elevated.

In previous studies addressing the effect of low oxygen on NSCs *in vitro*, the cells were isolated from the adult subependyma, E14 ganglionic eminence, and mesencephalon (Horie et al., 2008; Vernon et al., 2011). However, there are known NSCs in the cerebral cortex (Tao et al., 2006; Nakagomi et al., 2009), and the cells we used were derived from embryonic rat cerebral cortex (Shen and Zhang, 2003). Immunofluorescent staining for nestin, BrdU, MAP-2, and GFAP demonstrated that our *in vitro* cultured cells are NSCs.

Proliferation and differentiation of NSCs are a complicated process regulated by many internal and external factors (Yang et al., 2003; Zechel et al., 2007; Kim et al., 2015), and its exact control mechanisms are not clear. Change in oxygen concentration can affect biological characteristics of NSCs, including proliferation and differentiation (Horie et al., 2008). In previous studies, low oxygen concentration levels of mean tissue levels *in vivo* were examined (Hall et al., 1996). To follow a similar physiological environment, NSCs were cultured *in vitro* in decreased oxygen levels (5% oxygen), which are closer to physiological oxygen levels. The other oxygen level (10% O₂) was also used in the experimental groups, while 20% oxygen was used after hypoxic preconditioning.

Compared with the normal control group (20% O₂), in the low O₂ 72-hour group after culturing for 7 days in serum-free medium, the neurosphere number increased notably, and may be due to increased viability and proliferation. This is consistent with previous studies (Studer et al., 2000;

Clarke and van der Kooy, 2009). Proliferation of NSCs increased with hypoxic preconditioning in culture, partially by inhibition of caspase-dependent apoptosis. A previous study found that culture in low (4%) oxygen promotes survival of primitive NSCs by inhibiting apoptosis-inducing factor-dependent cell death, yet primitive NSCs undergo both apoptosis-inducing factor- and caspase-mediated cell death in 20% oxygen (Clarke and van der Kooy, 2009). In contrast, NSC survival in low oxygen is increased by inhibition of caspase-dependent cell death (Clarke and van der Kooy, 2009).

Moreover, we demonstrated that continually sustained lowered oxygen conditions (120 hours) are not beneficial during *in vitro* culture of embryonic rat cortical NSCs. Neurosphere number and NSC viability did not increase significantly between the low O₂ 120-hour and normal control groups. We also found visibly lower optical density values after low oxygen preconditioning for 120 hours compared with the normal control group. These results indicate that stem cell viability decreases with lowered oxygen preconditioning for 120 hours. Furthermore, in the low oxygen 120-hour group, the viability and number of neurospheres decreased, while in some neurosphere cells (after culturing NSCs in serum-free medium for 7 days including in 5% or 10% oxygen for 120 hours, then in 20% oxygen for 2 days), electron microscopy showed pyknosis and condensed chromatin near the nuclear membranes. Therefore, NSC apoptosis was detected by more than one method. Caspase-3 is important for apoptosis during cerebral ischemia and hypoxia (Lackner et al., 2007; Xu et al., 2007; Rodrigues et al., 2010). The high proportion of caspase-3-positive immunofluorescent cells in the 5% or 10% O₂ 120-hour groups suggests that NSC apoptosis is promoted by continual hypoxic preconditioning (120 hours). Our NSC culture procedure is not exactly the same as the previous study, which cultured NSCs in consistently low (4%) oxygen (or another low oxygen concentration) without 20% oxygen treatment. In our study, with culture of the low oxygen 120-hour group there are many possible mechanisms for inducing apoptosis: caspase-3 may act as an apoptotic agent and be more expressed; or erythropoietin may act as an anti-apoptotic agent or anti-oxidant and be less expressed, although we did not directly test the latter. Oxidative activity may be conducive to apoptosis in the low oxygen 120-hour group after restoring cultures to normal oxygen (20%) conditions. With low oxygen preconditioning for 120 hours, nitric oxide may cause NSC apoptosis by a p38 mitogen-activated protein kinase-dependent mechanism (Cheng et al., 2001; Yang et al., 2005; Song et al., 2013, 2014).

Low oxygen not only maintains self-renewal states of NSCs, but also affects cell-fate differentiation. Some studies have shown that low oxygen promotes neuronal differentiation of NSCs (Zhao et al., 2007; Bai et al., 2008). Our MAP2 immunofluorescent staining results show an increased proportion of neurons with 5% oxygen preconditioning during differentiation of NSCs derived from embryonic rat cortex. In our study, lowered oxygen preconditioning of NSCs was only present in the growth culture. Thus, the 5% oxygen conditions may enhance neuronal production by a mech-

anism that acts before neuronal differentiation. The Wnt signaling pathway, especially the Wnt/ β -catenin signaling pathway, plays an essential role in neuronal specification of NSCs (Haegele et al., 2003; Cui et al., 2011; Zhang et al., 2013; Bengoa-Vergniory et al., 2014). In the 5% O₂ group cultures, the Wnt signaling pathway may be activated and contribute to neuronal differentiation of NSCs, although we did not directly test this.

Altogether, hypoxic preconditioning with different oxygen concentrations and times has differential effects on the proliferation, apoptosis, and neuronal differentiation of *in vitro* NSCs derived from the cerebral cortex of E14 rats. Accordingly, 5% O₂ preconditioning for 72 hours not only promotes NSC proliferation but also contributes to their neuronal differentiation. With prolonged time (120 hours) of hypoxic preconditioning, NSCs distinctly become apoptotic. This study provides basic technology research for culturing NSCs *in vitro* before cell transplantation in treatment of central nervous system diseases.

Acknowledgments: We are very grateful to Professor You-hua Kong and Bao-hua Cheng from Jining Medical University in China for pre-reviewing this paper.

Author contributions: YJG and LLY designed the study. LLY and HMD performed experiments. DDM and LLY participated in the immunofluorescence measurements and statistical analysis. LLY wrote the paper. All authors approved the final version of the paper.

Conflicts of interest: None declared.

References

- Bai J, Luan Z, Zhou CL, Qu SQ, Jiang Y, Wang ZY (2008) Effect of hyperbaric oxygenation on the differentiation of implanted human neural stem cells into neurons in vivo. *Zhongguo Dang Dai Er Ke Za Zhi* 10:195-198.
- Bengoa-Vergniory N, Gorrone-Etxebarria I, Gonzalez-Salazar I, Kypta RM (2014) A switch from canonical to noncanonical Wnt signaling mediates early differentiation of human neural stem cells. *Stem Cells* 32:3196-3208.
- Borrell V, Reillo I (2012) Emerging roles of neural stem cells in cerebral cortex development and evolution. *Dev Neurol* 72:955-971.
- Cheng A, Chan SL, Milhavel O, Wang S, Mattson MP (2001) p38 MAP kinase mediates nitric oxide-induced apoptosis of neural progenitor cells. *J Biol Chem* 276:43320-43327.
- Cheng Y, Zhang J, Deng L, Johnson NR, Yu X, Zhang N, Lou T, Zhang Y, Wei X, Chen Z, He S, Li X, Xiao J (2015) Intravenously delivered neural stem cells migrate into ischemic brain, differentiate and improve functional recovery after transient ischemic stroke in adult rats. *Int J Clin Exp Pathol* 8:2928-2936.
- Clarke L, van der Kooy D (2009) Low oxygen enhances primitive and definitive neural stem cell colony formation by inhibiting distinct cell death pathways. *Stem Cells* 27:1879-1886.
- Cui XP, Xing Y, Chen JM, Dong SW, Ying DJ, Yew DT (2011) Wnt/ β -catenin is involved in the proliferation of hippocampal neural stem cells induced by hypoxia. *Ir J Med Sci* 180:387-393.
- Gage FH, Temple S (2013) Neural stem cells: generating and regenerating the brain. *Neuron* 80:588-601.
- Guerout N, Li X, Barnabe-Heider F (2014) Cell fate control in the developing central nervous system. *Exp Cell Res* 321:77-83.
- Haegele L, Ingold B, Naumann H, Tabatabai G, Ledermann B, Brandner S (2003) Wnt signalling inhibits neural differentiation of embryonic stem cells by controlling bone morphogenetic protein expression. *Mol Cell Neurosci* 24:696-708.

- Hall JE, Guyton AC, Brands MW (1996) Pressure-volume regulation in hypertension. *Kidney Int Suppl* 55:S35-41.
- Horie N, So K, Moriya T, Kitagawa N, Tsutsumi K, Nagata I, Shinohara K (2008) Effects of oxygen concentration on the proliferation and differentiation of mouse neural stem cells in vitro. *Cell Mol Neurobiol* 28:833-845.
- Kim BJ, Lee YA, Kim KJ, Kim YH, Jung MS, Ha SJ, Kang HG, Jung SE, Kim BG, Choi YR, Do JT, Ryu BY (2015) Effects of paracrine factors on CD24 expression and neural differentiation of male germline stem cells. *Int J Mol Med* 36:255-262.
- Lackner P, Burger C, Pfaller K, Heussler V, Helbok R, Morandell M, Broessner G, Tannich E, Schmutzhard E, Beer R (2007) Apoptosis in experimental cerebral malaria: spatial profile of cleaved caspase-3 and ultrastructural alterations in different disease stages. *Neuropathol Appl Neurobiol* 33:560-571.
- Nakagomi T, Taguchi A, Fujimori Y, Saino O, Nakano-Doi A, Kubo S, Gotoh A, Soma T, Yoshikawa H, Nishizaki T, Nakagomi N, Stern DM, Matsuyama T (2009) Isolation and characterization of neural stem/progenitor cells from post-stroke cerebral cortex in mice. *Eur J Neurosci* 29:1842-1852.
- Rodrigues CA, Diogo MM, da Silva CL, Cabral JM (2010) Hypoxia enhances proliferation of mouse embryonic stem cell-derived neural stem cells. *Biotechnol Bioeng* 106:260-270.
- Shen LH, Zhang JT (2003) Culture of neural stem cells from cerebral cortex of rat embryo and effects of drugs on the proliferation ability of stem cells. *Yao Xue Xue Bao* 38:735-738.
- Song J, Cho KJ, Cheon SY, Kim SH, Park KA, Lee WT, Lee JE (2013) Apoptosis signal-regulating kinase 1 (ASK1) is linked to neural stem cell differentiation after ischemic brain injury. *Exp Mol Med* 45:e69.
- Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, McKay R (2000) Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J Neurosci* 20:7377-7383.
- Tao K, Chen J, Wang G, Shu X (2006) Culture and identification of monoclonal neural stem cells derived from cerebral cortex. *J Huazhong Univ Sci Technolog Med Sci* 26:451-454.
- Vernon AC, Smith EJ, Stevanato L, Modo M (2011) Selective activation of metabotropic glutamate receptor 7 induces inhibition of cellular proliferation and promotes astrocyte differentiation of ventral mesencephalon human neural stem/progenitor cells. *Neurochem Int* 59:421-431.
- Xiong YJ, Yin B, Xiao LC, Wang Q, Gan L, Zhang YC, Zhang SM (2013) Proliferation and differentiation of neural stem cells co-cultured with cerebral microvascular endothelial cells after oxygen-glucose deprivation. *J Huazhong Univ Sci Technolog Med Sci* 33:63-68.
- Xu Q, Wang S, Jiang X, Zhao Y, Gao M, Zhang Y, Wang X, Tano K, Kanehara M, Zhang W, Ishida T (2007) Hypoxia-induced astrocytes promote the migration of neural progenitor cells via vascular endothelial factor, stem cell factor, stromal-derived factor-1alpha and monocyte chemoattractant protein-1 upregulation in vitro. *Clin Exp Pharmacol Physiol* 34:624-631.
- Yang M, Donaldson AE, Jiang Y, Iacovitti L (2003) Factors influencing the differentiation of dopaminergic traits in transplanted neural stem cells. *Cell Mol Neurobiol* 23:851-864.
- Yang SR, Cho SD, Ahn NS, Jung JW, Park JS, Jo EH, Hwang JW, Kim SH, Lee BH, Kang KS, Lee YS (2005) The role of p38 MAP kinase and c-Jun N-terminal protein kinase signaling in the differentiation and apoptosis of immortalized neural stem cells. *Mutat Res* 579:47-57.
- Zawadzka M, Rivers LE, Fancy SP, Zhao C, Tripathi R, Jamen F, Young K, Goncharevich A, Pohl H, Rizzi M, Rowitch DH, Kessaris N, Suter U, Richardson WD, Franklin RJ (2010) CNS-resident glial progenitor/stem cells produce Schwann cells as well as oligodendrocytes during repair of CNS demyelination. *Cell Stem Cell* 6:578-590.
- Zechel C, Moeckel S, Stoerchel P, Pawlak E, Tronnier V (2007) Influence of growth factors, neurotrophic factors and proteins of the extracellular matrix on expansion and differentiation of adult neural stem cells. *J Stem Cells Regen Med* 2:198-199.
- Zhang X, Chen L, Wang Y, Ding Y, Peng Z, Duan L, Ju G, Ren Y, Wang X (2013) Macrophage migration inhibitory factor promotes proliferation and neuronal differentiation of neural stem/precursor cells through Wnt/beta-catenin signal pathway. *Int J Biol Sci* 9:1108-1120.
- Zhao T, Zhang CP, Zhu LL, Jin B, Huang X, Fan M (2007) Hypoxia promotes the differentiation of neural stem cells into dopaminergic neurons. *Sheng Li Xue Bao* 59:273-277.

Copyedited by James R, Raye W, Wang J, Qiu Y, Li CH, Song LP, Zhao M