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Doxycycline can stimulate cytoprotection in neural stem cells with oxygen–glucose deprivation–reoxygenation injury: A potential approach to enhance effectiveness of cell transplantation therapy

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ABSTRACT

A substantial loss of transplanted neural stem cells is a major limitation to cell transplantation therapy of stroke. In this study, we provided *in vitro* evidence that doxycycline preconditioning of neural stem cells have resulted in decreased cell death and increased cell viability after oxygen–glucose deprivation–reoxygenation conditions that best mimics cerebral ischemia–reperfusion injury. Resistance to oxidative stress is one of the mechanisms of doxycycline-induced cytoprotection in neural stem cells as it significantly reduced the superoxide anion production. Moreover, doxycycline preconditioning also induced the expression of Nrf2 which is a basic transcription factor for a series of antioxidative and cytoprotective genes. Collectively, we suggested that doxycycline preconditioning of neural stem cells is a potential strategy to improve effectiveness of cell transplantation therapy.

1. Introduction

Recent experimental studies have highlighted the potential of stem cell transplantation as a novel therapeutic strategy for stroke [1]. A number of clinical trials have been performed and others are in process [2]. Transplantation of neural stem cells in the acute stage of stroke often reduces infarct size and reduces cell death in the penumbra area. This neuroprotective effect of grafted neural stem cells increases host cell survival and function [3]. However, hostile environment of ischemic brain reduces the efficiency of this approach [4]. Major challenges after cell transplantation is accelerated death of grafted cells which might be influenced by host inflammatory responses and production of reactive oxygen species after ischemia–reperfusion injury [5]. Several remedial approaches have been suggested to address this problem. Over expression of Bcl-2, a pro-survival factor, reduced the cell death and enhanced recovery after transplantation [6]. Alternatively, genetic modification of stem cells to over express paracrine factor can enhance neuroprotection in hostile microenvironment of host brain [7]. Beside better transplantation outcome of these *ex vivo* modifications of stem cells, safer and simpler strategies are still in demand.

Doxycycline is a tetracycline-derived antibiotic that also possesses potent anti-inflammatory and cytoprotective properties [8]. It is previously reported to exhibit neuroprotective properties in both focal and global brain ischemia in rats as well as in cell culture models of cell death [9,10]. At cellular level, doxycycline can prevent cell death by either reduction of microglial activation or blockade of apoptotic pathways [11]. Doxycycline has well characterized safety profile, clinical features and potential adverse effects [12]. In this study, we demonstrated that doxycycline preconditioning of NSC is a potential candidate approach to enhance efficiency of cell transplantation therapy in ischemic stroke.

2. Materials and methods

2.1. Antibodies

Anti-MAP2, anti-GFAP antibodies and anti-β actin were purchased from Sigma–Aldrich. Anti-Nrf2 was purchased from Santa Cruz Biotechnology. Anti-Nestin and Anti-O4 were purchased from Millipore.

2.2. Isolation and culturing of rat neural stem cells

The animals were handled in accordance with Northeast Normal University Guidelines for Care and Use of Laboratory Animals. All chemicals for cell culture were purchased from Invitrogen unless otherwise stated. Neural stem cells were isolated from bilateral subventricular zones of postnatal day 1 rat brains and resuspended in Neurobasal-A medium containing B-27 supplement, L-glutamine, 20 ng/ml fibroblast growth factor, and

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10 ng/ml epidermal growth factor. Cells were grown as monolayer cultures. For preconditioning, doxycycline (Sigma) was added to cell culture medium at final concentration of 8 μM for 24 h before harvesting the cells.

2.3. Oxygen–glucose deprivation (OGD) treatments

For OGD treatments, cells were cultured in a glucose-free balanced salt solution containing the following: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 14.7 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 10 mM HEPES; plates were then placed in an anaerobic chamber at 37 °C. After 8 h, the medium was replaced with the regular medium containing glucose and cultured in an incubator with 5% CO_2/95% air for reoxygenation.

2.4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.3% Triton X-100. Cells were incubated overnight with primary antibodies at 4 °C. Secondary antibody incubation was performed for 1 h at room temperature. Fluorescence was observed using confocal laser scanning microscopy (Olympus FV 1000).

2.5. Assessment of cell viability, cell death, and superoxide anion production

Cell viability was determined by using WST-1 assay kit (Roche). Lactate dehydrogenase (LDH) release was monitored by LDH-cytotoxicity detection kit (Roche). For TUNEL labeling, In situ cell death detection kit, TMR Red (Roche) was used and reaction was observed with confocal microscopy (OlympusFV1000). Superoxide anion production was detected by LumiMax™ superoxide anion-detection kit (Stratagene).

2.6. Real time PCR analysis

Real-time PCR was carried out with StepOnePlus™ Real-Time PCR System. Primers are as follows: Nrf2: 5′-TTCCCAGCCACGTTGAGAG-3′ and 5′–TCCTGCCAAACTTGCTCCAT-3′. GAPDH: 5′-CAGTGGCAAAGTGGAGATTG-3′ and 5′-AATTTGCCGTGAGTGGAGTC-3′. Relative mRNA expression was calculated by using $2^{-\Delta\Delta Ct}$ method and normalized to that of GAPDH.

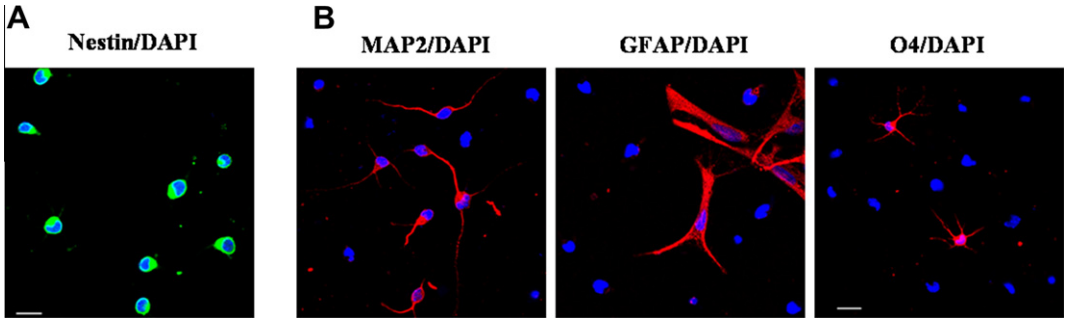

Fig. 1. Differentiation of neural stem cells. (A) Immunocytochemistry of neural stem cells monolayer culture stained with NSC marker Nestin. (B) After differentiation, the cells were stained with MAP2 for neurons, GFAP for glia and O4 for oligodendrocytes. DAPI is used to stain nuclei. Scale bar 20 μm.

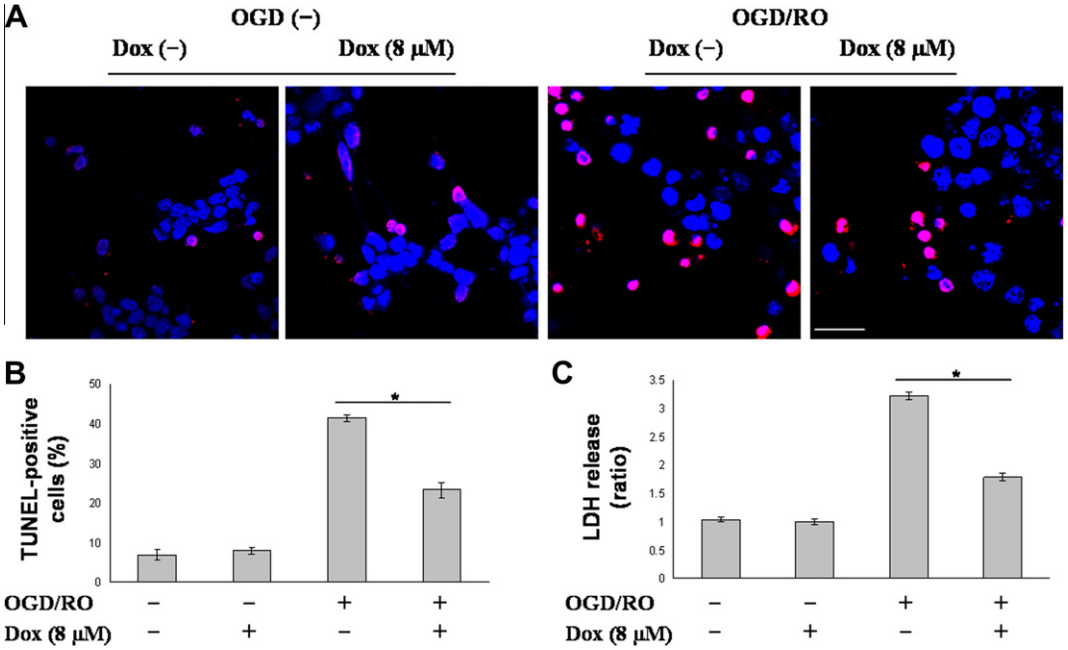

Fig. 2. Doxycycline preconditioning resulted in reduced cell death of NSCs after oxygen–glucose deprivation–reoxygenation injury. (A) Tunnel staining of doxycycline (DOX) preconditioned NSCs (^{PC}NSC) after 8 h of oxygen glucose deprivation (OGD) and 24 h of reoxygenation (RO). (B) Percentage of TUNEL positive cells. (C) Lactate dehydrogenase analysis (LDH) of ^{PC}NSC after 8 h of OGD and 24 h of RO.

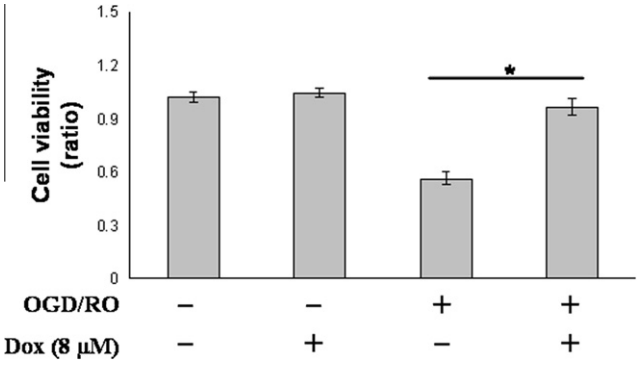

Fig. 3. Doxycycline preconditioning resulted in increased cell viability of NSCs after oxygen–glucose deprivation–reoxygenation injury. WST-1 assay of ^{PC}NSC after 8 h of OGD and 24 h of RO.

2.7. Western blot analysis

Total protein was isolated using radioimmunopreciptation lysis buffer supplemented with protease inhibitor cocktail (Roche). Equal protein quantity for every control and test sample was separated with 12% polyacrylamide gel electrophoresis. Resolved protein bands were electroblotted on polyvinylidene difluoride (PVDF) membrane (Millipore) and then blocked with 5% non-fat dry milk. After sequential incubation with primary and secondary antibodies, blots were visualized using electro-generated chemiluminescence (ECL, Amersham) reagent.

2.8. Statistical analysis

Data are always represented as the mean ± SEM for at least three independent experiments. Statistical analysis was performed using one way ANOVA to compare the means of groups, and p values < 0.05 indicate significant differences.

3. Results and discussion

For our study, we used oxygen–glucose deprivation (OGD) and reoxygenation, *in vitro* model that best mimics *in vivo* cerebral ischemia–reperfusion. Self renewing multipotent neural stem cells were isolated from postnatal day 1 rat brains and differentiated by using 0.5 μM retinoic acid (Fig. 1A and B). To elucidate the cytoprotective effect of doxycycline preconditioning on neural stem cells, LDH assay, tunnel assay and WST-1 assay were performed. LDH is rapidly released when cells are damaged by stress, injuries, chemicals or intercellular signals. LDH quantification revealed that, after 8 h of OGD and 24 h of reoxygenation, neural stem cells preconditioned with 8 μM doxycyline showed a significant reduction in cell death compared to non-preconditioned cells (Fig. 2C). Moreover, TUNNEL assay also showed significantly reduced cell apoptosis in preconditioned NSC after OGD/reoxygenation (Fig. 2A and B). A previous report showed that doxycycline reduces expression of cleaved caspase-3 and promotes neuronal survival in a rat model of neonatal hypoxia–ischemia [8]. This cytoprotective effect of doxycycline preconditioning was further supported by increase in viability of neural stem cells after OGD injury, measured by WST-1 assay (Fig. 3).

In ischemic stroke, ischemia–reperfusion and inflammation results in oxidative stress [13]. Neural stem cells subjected to 8 h of OGD and 1 h of reoxygenation showed a remarkable increase in superoxide anion production. The level of O_2^- was significantly reduced after doxycycline preconditioning of neural stem cells (Fig. 4A). This observation suggests resistance to oxidative stress is one of the mechanisms of doxycycline-induced cytoprotection in neural stem cells. Moreover, after doxycycline preconditioning of neural stem cells we investigated the expression of *Nrf2* gene. Nrf2 is a basic redox-sensitive transcription factor that regulates the basal and inducible expression of a battery of antioxidants and other cytoprotective genes [14]. Real time quantitative PCR analysis showed that doxycycline preconditioning significantly up-regulated the expression of Nrf2 mRNA under normal conditions (2-fold) as well as after OGD/reoxygenation (4.2-fold, Fig. 4B). These results were also supported by Western blot analysis that showed doxycycline preconditioning resulted in higher protein expression of Nrf2 under both normal and OGD/reoxygenation conditions (Fig. 4C). In conclusion, we provided *in vitro* evidence that doxycycline preconditioning reprograms NSCs to tolerate oxidative stress that results in reduced apoptosis and increased cell viability. Along with these beneficial effects,

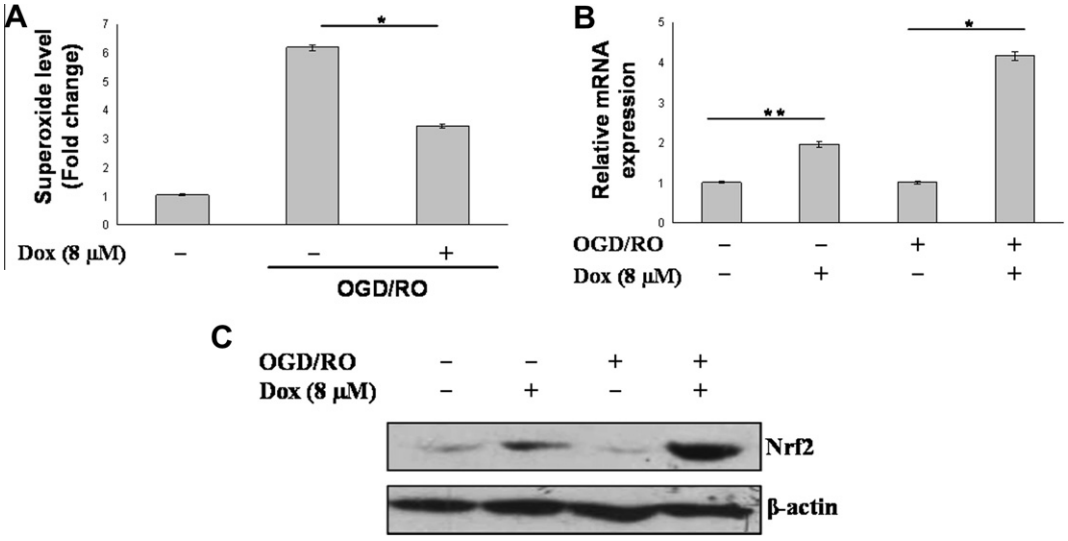

Fig. 4. Doxycycline preconditioning resulted in reduced superoxide anion production after oxygen–glucose deprivation–reoxygenation injury and upregulation of Nrf2, a transcription factor of antioxidative genes. (A) Preconditioning with doxycycline significantly reduced the elevated superoxide anion level after 8 h of OGD and 1 h of RO. (B) Real time quantitative analysis of *Nrf2* gene expression in DOX preconditioned NSC (^{PC}NSC) and DOX non-preconditioned NSC ($^{non\text{-}PC}$NSC) under normal conditions or after OGD/RO. GAPDH was used as endogenous control. (C) Western blot analysis of Nrf2 protein expression in ^{PC}NSC and $^{non\text{-}PC}$NSC under normal conditions or after 8 h of OGD and 24 h of RO. Error bars represent mean ± SEM. $^{*}p < 0.05$ versus $^{non\text{-}PC}$NSC after OGD/RO, $^{**}p < 0.05$ versus $^{non\text{-}PC}$NSC under normal conditions.

its simplicity, easy adoptability and minimal safety issues make doxycycline preconditioning a tempting approach to improve effectiveness of transplantation therapy in ischemic stroke.

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