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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.

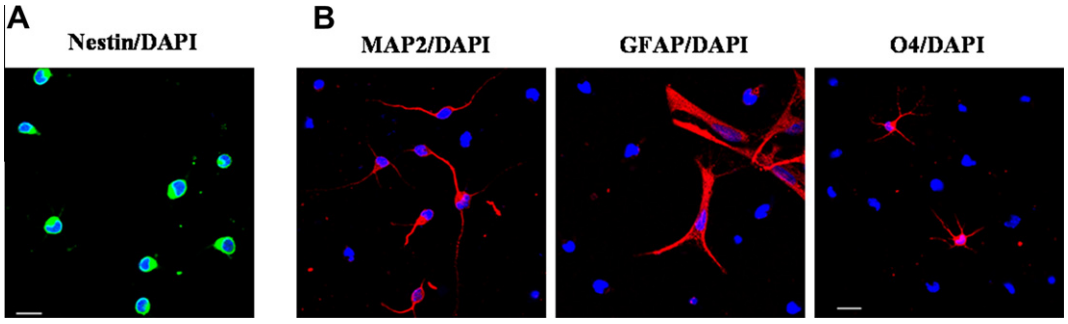

Fig. 1. Differentiation of neural stem cells. (A) Immunocytochemistry of neural stem cells monolayer culture stained with NSC marker Nestin. (B) After differentiation, the cells were stained with MAP2 for neurons, GFAP for glia and O4 for oligodendrocytes. DAPI is used to stain nuclei. Scale bar 20 μm.

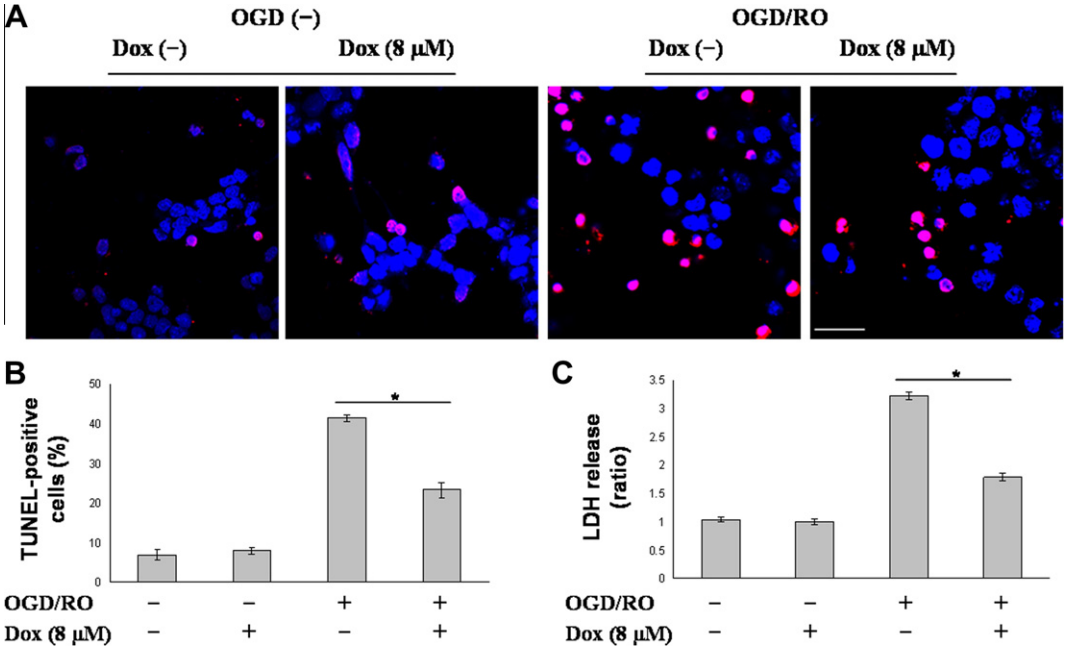

Fig. 2. Doxycycline preconditioning resulted in reduced cell death of NSCs after oxygen–glucose deprivation–reoxygenation injury. (A) Tunnel staining of doxycycline (DOX) preconditioned NSCs (^{PC}NSC) after 8 h of oxygen glucose deprivation (OGD) and 24 h of reoxygenation (RO). (B) Percentage of TUNEL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