Neurochemistry International 55 (2009) 710–716

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

# Simulated microgravity promotes cellular senescence via oxidant stress in rat PC12 cells

Jinghua Wang [a,1], Jifei Zhang [b,1], Shasha Bai [a], Guangyou Wang [a], Lili Mu [a], Bo Sun [a], Dandan Wang [a], Qingfei Kong [a], Yumei Liu [a], Xiuhua Yao [a], Ying Xu [c,*], Hulun Li [a,*]

[a] Department of Neurobiology, Heilongjiang Provincial Key Lab of Neurobiology, Harbin Medical University, 157# Baojian Road, Harbin, Heilongjiang 150081, China
[b] Department of Histology and Embryology, Mudanjiang Medical College, Mudanjiang, Heilongjiang 157011, China
[c] Department of Endocrinology, The first Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150001, China

ARTICLE INFO

*Article history:*
Received 27 April 2009
Received in revised form 23 June 2009
Accepted 7 July 2009
Available online 16 July 2009

*Keywords:*
Cell senescence
SA-β-gal
Simulated microgravity
Oxidant stress
PC12 cell

ABSTRACT

Microgravity has a unique effect on biological organisms. Organs exposed to microgravity display cellular senescence, a change that resembles the aging process. To directly investigate the influence of simulated microgravity on neuronal original rat PC12 cells, we used a rotary cell culture system that simulates the microgravity environment on the earth. We found that simulated microgravity induced partial G1 phase arrest, upregulated senescence-associated β-galactosidase (SA-β-gal) activity, and activated both p53 and p16 protein pathways linked to cell senescence. The amount of reactive oxygen species (ROS) was also increased. The activity of intracellular antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), was all significantly increased at 12 h after the microgravity onset, yet decreased at 96 h. Furthermore, concomitant block of ROS by the antioxidant N-acetylcysteine significantly inhibited the microgravity-induced upregulation of SA-β-gal activity. These results suggest that exposure to simulated microgravity induces cellular senescence in PC12 cells via an increased oxidant stress.



## 1. Introduction

Microgravity is a physical force that alters the function of cells and whole organisms (Norsk, 2005; Vunjak-Novakovic et al., 2002). Recent studies suggest that microgravity affects cellular functions such as proliferation (Dai et al., 2007), signal transduction (Vincent et al., 2005), gene expression (Clement et al., 2008), and oxidation (Stein, 2002). The molecular mechanism of microgravity on cellular system is still not well understood. Using DNA microarrays of T cells, it was shown recently that simulated microgravity revealed an alteration of NF-kB signaling and an inhibition of the early oncogenes *c-fos*, *c-myc* and *c-jun* expression (Boonyaratanakornkit et al., 2005). Simulated microgravity also induced fas, p53 and bax, whereas reduced bcl-2 in apoptosis induction of human thyroid carcinoma cells and osteoblastic cells (Kossmehl et al., 2002; Nakamura et al., 2003). Forty-eight hours exposure to simulated microgravity activated MAPK pathways in rat dermal fibroblasts (Loesberg et al., 2008). Simulated microgravity reduced RhoA activity and cofilin phosphorylation, decreased integrin signaling of human mesenchymal stem cells during the osteoblastogenesis (Saxena et al., 2007). In humans, exposure to microgravity caused aging-like changes, such as learning and memory disturbance, mild hypothyroidism, increased stress hormones, decreased sex steroids, insulin resistance, impaired anabolic response to food intake, anorexia, altered mitochondrial function, and systemic inflammatory response (Eddy et al., 1998; Biolo et al., 2003). Microgravity also induced aging symptoms in other biological systems, such as the fruit fly (Kim, 2000) and oat leaf segments (Miyamoto et al., 1995). In detailed mechanism, a recent study reported that microgravity caused changes in gene expression that direct fibroblast cells to premature senescence (Liu and Wang, 2008).

Cellular senescence can result from DNA damage, oncogene expression, supraphysiological mitogenic signals, and oxidative stress (Van Nguyen et al., 2007; Fridman and Tainsky, 2008; Furukawa et al., 2007). Senescent cells show specific characteristics, such as enlarged overall shape, shortened telomeres, high expressed senescence-associated β-galactosidase (SA-β-gal) at pH 6.0, and increased levels of the Cdk inhibitors p53 and p16 (Allsopp et al., 1992; Dimri et al., 1995; Nyunoya et al., 2006). The free radical theory of aging proposes that reactive oxygen species (ROS) produced in mitochondria cause damage to cellular macromolecules, and lead to cellular senescence and aging within an organism

* Corresponding authors. Tel.: +86 451 86662943; fax: +86 451 87502363.
*E-mail addresses:* lihulun@yahoo.com.cn (H. Li), xuyinglee@163.com (Y. Xu).
[1] These authors contributed equally to this work.

0197-0186/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2009.07.002

(Chen et al., 2004). Moreover, accumulation of senescent cells contributes to aging and age-related pathology (Campisi, 2005).

In this study, we examined the inductive effect of microgravity on senescence in neural crest-derived rat pheochromocytoma cells, PC12, which has been showed oxidation-sensitive to microgravity (Kwon et al., 2006), by simulating a microgravity environment with a rotary cell culture system (RCCS) bioreactor (Schwarz et al., 1992). The RCCS was developed by National Aeronautics and Space Administration (NASA) at the Johnson Space Center. It is a horizontally rotated, bubble free culture vessel with membrane diffusion gas exchange. The culture medium, cells and cell aggregate particles rotate with the vessel and do not collide with the vessel walls or any other damaging objects. Destructive shear forces are minimized because this system has no impellers, air lifts, bubbles, or agitators. This machine simulates microgravity conditions via rotating the vessel wall and medium containing cells at the same speed, producing a vector-averaged gravity comparable with that of continuous free fall condition on Earth (Bakos et al., 2002). We demonstrate that microgravity induces the cellular senescence of PC12 cells via the upregulation and release of ROS. These results characterize the effects of microgravity on cellular senescence in "reference" neural cells, which may further illuminate our understanding of native cells in the nervous system.

## 2. Materials and methods

### 2.1. Cell culture

Rat PC12 cells (Shanghai Cell Bank, China) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (HyClone, USA), supplemented with 10% horse serum (HyClone, USA), 5% fetal calf serum (FCS) (Gibco, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The 37 °C incubator circulated humid air containing 5% $CO_2$. Cells were subcultured every 3–4 days.

### 2.2. Microgravity exposure

Cells were seeded onto Cytodex-3 microcarriers (Sigma, USA), which were pretreated according to the manufacturer's instructions. Cells were cultured with serum-free DMEM for 24 h to synchronize the cell cycle, and then the medium was changed to DMEM with serum. Simulated microgravity exposure was carried out with an RCCS bioreactor (Synthecon, USA), in conjunction with a high aspect rotating-wall vessel (HARV) (Synthecon, USA). This HARV is able to reproduce a gravitational environment of $10^{-2}$g (Schwarz et al., 1992), which simulates microgravity conditions (MG). The culture vessel rotates at approximately 28–32 rpm, a previously established rate that maintains continuous cellular suspension (Lelkes et al., 1998). Normal gravity control PC12 cells (1G) were cultured on microcarriers without rotation (Wang and Good, 2001). The cultures were maintained for 6, 12, 24, 48, 72, and 96 h.

Cell culture media from 48 h of microgravity or normal gravity cultured PC12 cells were used to culture PC12 cells for another 48 h to evaluate the changes of the cell culture medium.

### 2.3. Senescence-associated β-galactosidase staining

At the end of culture, PC12 cells were washed with phosphate-buffered saline (PBS) and stained for SA-β-gal activity. First, cells were fixed in 3% formaldehyde for 10 min at room temperature. Cells were then washed three times with PBS, and incubated in 1 ml of X-gal solution (5 mmol/L of X-gal in 40 mmol/L of citric acid–sodium phosphate buffer, pH 6) at 37 °C for 12 h. After this incubation, a blue color developed in senescent cells, and proportions of senescent cells can be measured among the population.

All cells were counted by digital imaging, with Image Pro Plus software (Media Cybernetics, Silver Springs, MD). Approximately 600 total cells were counted for each group. The percentage of cells stained for SA-β-gal was calculated by the following formula: 100 × (number of cells stained with SA-β-gal/total number of cells).

### 2.4. Analysis of cell cycle by flow cytometry

PC12 cells were cultured under microgravity or normal gravity for 96 h, and then harvested by trypsinization and rinsed in PBS three times before fixation in 70% ethanol overnight at 4 °C. After two additional rinses with ice-cold PBS, cells were then incubated with RNase A (1 mg/ml) (Fermentas, Lithuania) in PBS at 37 °C for 30 min, then stained with 0.5 mg/ml propidium iodide (PI) (Sigma, USA) in PBS at room temperature for 15 min. Intracellular DNA was then labeled with PI, and the cell cycle distribution within each sample was determined by flow cytometry (Becton Dickinson, USA). Cells were detected at 488 nm excitation and analyzed with ModFit software.

### 2.5. Western blot analysis

Western blot analysis of proteins was performed with whole cell lysates. The Bradford method was used to ensure equal protein loading. Samples were fractionated on a 10% or 12% SDS-PAGE gel via electrophoresis. Proteins were transferred to 0.45/0.22 μm pore size PVDF membranes (0.22 μm for detect p16, 0.45 μm for detect other proteins) via semidry transfer. The membrane was blocked with 5 % non-fat milk for 2 h at RT, then incubated with anti-p16 (1:400 diluted, Santa Cruz Biotechnology, USA), anti-p53 (1:1000 diluted, Santa Cruz Biotechnology, USA) at 4 °C overnight. For detection, anti-rabbit or anti-mouse IgG AP-conjugated secondary antibody (1:1000 diluted, Promega, USA) was used, and β-actin (1:1000, Santa Cruz, USA) was used as a reference. All antibodies were diluted with TBST.

### 2.6. ROS activity assays

Intracellular ROS activity analysis of PC12 cells was performed after being stained with 10 μmol/L DCF-DA (Sigma, USA), as described previously (Shen et al., 2001). This compound is converted by intracellular esterases to 2′,7′-dichlorofluorescin, which can be oxidized by ROS to the highly fluorescent DCF. The fluorescence intensity was measured by flow cytometry (Becton Dickinson, USA) with excitation and emission settings of 488 and 530 nm, respectively.

$H_2O_2$ released into the culture medium was measured using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen, USA) (Sun et al., 2005), according to the manufacturer's recommendations. The $H_2O_2$ concentration was calculated based on a standard curve and the results were expressed as μmol/L.

For antioxidant treatment, PC12 cells were incubated with 0.05, 0.5, or 5 mmol/L of the antioxidant N-acetylcysteine (Sigma, USA) during 96 h microgravity culture.

### 2.7. Antioxidant enzyme activity assays

The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) in PC12 cell lysates was all determined with assay kits (Jian Cheng Biology Research Center, China). Estimation of all these biochemical parameters was determined according to the guidelines of the kits. SOD activity was measured through the inhibition of nitroblue tetrazolium (NBT) reduction by $O^{2-}$ generated by the xanthine/xanthine oxidase system. One unit of SOD activity was defined as the enzyme amount causing 50% inhibition in 1 ml reaction solution. GSH-Px activity was measured by using $H_2O_2$ and GSH as substrates. One unit of GSH-Px activity is defined as amount of enzyme required to degrade 1 μmol/L of GSH per min subtracting non-enzymatic reaction at 37 °C. CAT activity was detected using ammonium molybdate method by measuring the intensity of a yellow complex formed by molybdate and $H_2O_2$. One unit of CAT activity represents amount of enzyme required to decompose 1 μmol of $H_2O_2$ per second. Results were expressed as activity unit/mg protein (Ou et al., 2008).

### 2.8. Statistical analysis

Data are presented as the mean ± standard deviation, determined from three or more experiments per condition. Differences between pairs of groups were analyzed by a two-tailed Student's *t*-test. The level of significance was set as $P < 0.05$.

## 3. Results

### 3.1. Induction of senescence-like changes by microgravity

After 24 h culture on Cytodex-3 microcarriers (Fig. 1A), PC12 cells were attached and equally distributed throughout the microcarriers (Fig. 1B). However, after 96 h microgravity exposure, PC12 cells appeared to be flattened, enlarged senescent-liked cell morphology (Fig. 1C), compared with that cultured under normal gravity (Fig. 1D).

Senescent cells express high SA-β-gal activity at pH 6.0, which is widely used as a cellular senescence marker (Dimri et al., 1995). We thus tested whether microgravity could induce SA-β-gal activity in cultured PC12 cells. Increased SA-β-Gal staining relative to normal gravity control (1G) was observed at 24 h after onset of microgravity exposure (MG) (Fig. 2A). Microgravity also induced a time-dependent increase in the percentage of SA-β-gal positive PC12 cells. Significant increase in cell senescence was also present at the time points of 24, 48, 72 and 96 h, separately (Fig. 2B).