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Uric acid promotes neuronal differentiation of human placenta-derived mesenchymal stem cells in a time- and concentration-dependent manner★

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Abstract

Uric acid is an important, naturally occurring serum antioxidant. The present study investigates the use of uric acid for promoting proliferation and neuronal differentiation of mesenchymal stem cells derived from human placenta tissue. Human placenta-derived mesenchymal stem cells were pre-induced in the presence of either 0, 0.2, 0.4 or 0.8 mM uric acid in combination with 1 mM β-mercaptoethanol for 24 hours, followed by exposure to identical uric acid concentrations and 5 mM β-mercaptoethanol for 6 and 10 hours. Cells developed a neuronal-like morphology, with formation of interconnected process extensions, typical of neural cells. Immunocytochemistry and immunofluorescence staining showed neuron specific enolase positive cells were present in each group except the control group. A greater number of neuron specific enolase positive cells were observed in 0.8 mM uric acid in combination with 5 mM β-mercaptoethanol at 10 hours. After 24 hours of induction, Nissl bodies were detected in the cytoplasm of all differentiated cell groups except the control group and Nissl body numbers were greatest in human placenta-derived mesenchymal stem cells grown in the presence of 0.8 mM uric acid and 5 mM β-mercaptoethanol. These results suggest uric acid accelerates differentiation of human placenta-derived mesenchymal stem cells into neuronal-like cells in a time- and concentration-dependent manner.

Key Words: uric acid; human placenta-derived mesenchymal stem cells; differentiation; neural cells

Abbreviations: hPMSCs, human placenta-derived mesenchymal stem cells; BME, β-mercaptoethanol; NSE, neuron specific enolase

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INTRODUCTION

Human placenta-derived mesenchymal stem cells (hPMSCs) have the potential to differentiate *in vitro* and *in vivo*, into neural cells, therefore providing a novel, therapeutic stem cell source for the treatment of neurodegenerative diseases, including Parkinson's disease, brain ischemia and amyotrophic lateral sclerosis[1-3].

Uric acid is the end product of purine nucleotide metabolism. Experimental, clinical and epidemiologic evidence suggest that hyperuricemia plays a role in the pathogenesis of certain disease processes, including hypertension, insulin resistance, type II diabetes, cardiovascular and cerebrovascular evens[4-8]. Recent studies have shown uric acid to be an important serum antioxidant involved in the scavenging of single oxygen, peroxyl and hydroxyl radicals, thus protecting cells from oxidative damage[9-10] and delaying the onset of Alzheimer's disease and dementia[11-12]. Uric acid also stimulates proliferation of some cell types including T cells[13] and endothelial progenitor cells[14] and has been shown to protect dopaminergic neurons[15]. That is, uric acid has dual effects within the human body. Neuronal differentiation of mesenchymal stem cells (MSCs) is typically promoted using antioxidants including β-mercaptoethanol (BME), dimethyl sulfoxide and butylated hydroxyanisole[16]. In the present study, uric acid and BME were used to induce differentiation of hPMSCs into nerve cells in order to investigate the effects of uric acid during the differentiation process.

RESULTS

Morphology of isolated and cultured hPMSCs

Five days after initial hPMSCs seeding, phase-contrast microscopy revealed small, adherent cell colonies with fibroblast-like morphology (Figure 1A). Fibroblast-like cells continued to proliferate forming a homogeneous layer. hPMSCs reached 80–90% confluence 2 weeks later. Cell

passaging significantly accelerated cell growth and expansion, resulting in a more homogeneous culture with predominantly spindle-shaped morphology (Figure 1B).

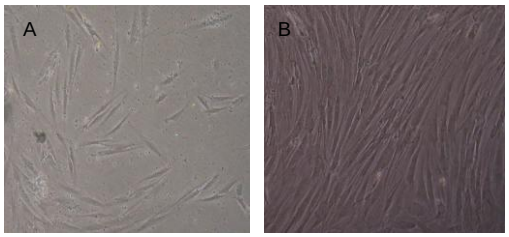

Figure 1 Morphology of human placenta-derived mesenchymal stem cells using phase-contrast inverted microscopy (× 100); 10 days after initial seeding (A) and after expansion to passage 3, showing homogeneous, spindle-shaped morphology (B).

Cell homogeneity was significantly increased after passage 3.

Phenotyping of cultured hPMSCs

Immunofluorescence analysis revealed that hPMSCs were positive for CD44, a marker for mesenchymal stem cells (Figure 2A) and negative for CD34, a marker for hematopoietic cells (Figure 2B), indicating that the isolated cells were hPMSCs.

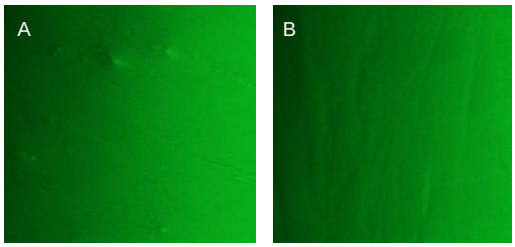

Figure 2 Immunophenotyping of human placenta-derived mesenchymal stem cells using immunofluorescence staining and immunofluorescence microscopy (× 100).

Cells were positive for CD44 (A) and negative for CD34 (B).

Effect of uric acid on hPMSCs proliferation

A methyl thiazolyl tetrazolium (MTT) assay indicated hPMSC proliferation was enhanced by uric acid in a dose-dependent manner (0.2, 0.4 and 0.8 mM). However, hPMSCs treated with 1.2 mM uric acid resulted in decreased proliferation. These results indicate that low and high concentrations of uric acid can accelerate and inhibit proliferation of hPMSCs respectively. The logarithmic growth phase of hPMSCs began at day 3 (Figure 3).

Uric acid induced differentiation of hPMSCs into neurons

Immunocytochemistry and immunofluorescence staining was used to detect neuron specific enolase (NSE) expression in differentiated hPMSCs. NSE expression was induced in the presence of 0.2, 0.4 or 0.8 mM uric acid in combination with 5 mM BME. NSE expression levels were greatest after exposure of hPMSCs to 0.8 mM uric acid for 10 hours (Figures 4, 5, Table 1).

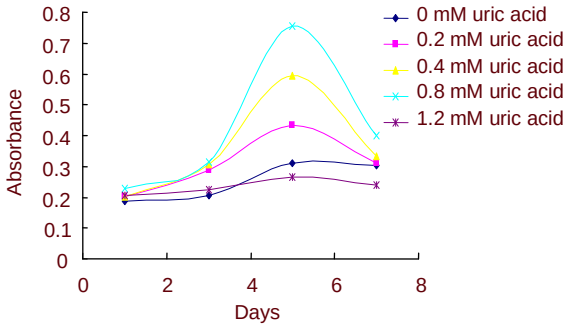

Figure 3 Effect of uric acid on the proliferation of human placenta-derived mesenchymal stem cells using a standard methyl thiazolyl tetrazolium assay.

Data are expressed from a mean of 12 wells for each group. 0.2, 0.4 and 0.8 mM uric acid enhanced cell proliferation in a dose-dependent manner.

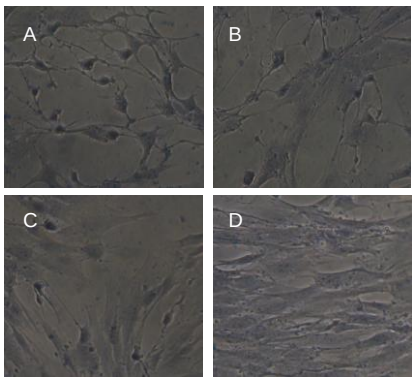

Figure 4 Neuron specific enolase expression in human placenta-derived mesenchymal stem cells induced with different concentrations of uric acid using diaminobenzidine immunocytochemical staining.

Light microscopy (× 100), 0.8 mM (A), 0.4 mM (B), 0.2 mM (C), 0 mM (D) uric acid groups.

Increased concentrations of uric acid resulted in increased diaminobenzidine staining (brown stain in cytoplasm), especially within the 0.8 mM uric acid group.

Uric acid induced Nissl bodies in hPMSCs

After neural cell induction of hPMSCs for 24 hours with 0, 0.2, 0.4 or 0.8 mM uric acid in combination with 5 mM BME, Giemsa staining was used to detect Nissl bodies in the cytoplasm of differentiated cells. Nissl bodies were detected in all groups except the control group (Figure 6). The quantity of Nissl bodies was most abundant in the 0.8 mM uric acid group.

DISCUSSION

MSCs can be induced to differentiate into various kinds of cells *in vitro*, including osteoblasts, adipocytes and nerve cells[17-20]. In addition, MSCs can be used to treat nervous system diseases and myocardial injury[21-23], promote the function of vascular cells and accelerate wound healing by promoting the formation of new blood vessel[23-25]. MSCs transplanted into animal models

display a protective effect towards damaged brain and spinal cord tissues[26-27].

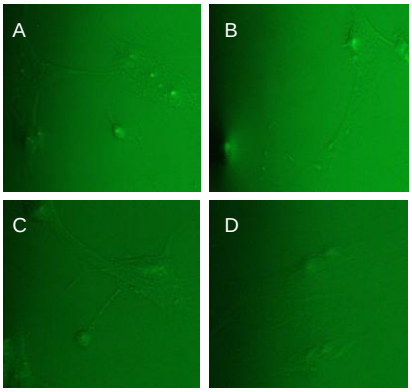

Figure 5 Neuron specific enolase expression in human placenta-derived mesenchymal stem cells induced with different concentrations of uric acid (immunofluorescence staining).

Immunofluorescence microscopy (× 100) images showing 0.8 mM (A), 0.4 mM (B), 0.2 mM (C), 0 mM (D) uric acid groups.

Increased concentrations of uric acid resulted in increased fluorescein isothiocyanate staining (green), especially within the 0.8 mM uric acid group.

Table 1 NSE positive cells (%)

Group	6-hour induction	10-hour induction
0.8 mM UA	62.9[a]	81.8[b]
0.4 mM UA	40.2[a]	53.6[b]
0.2 mM UA	25.0[a]	45.9[b]
0 mM UA	17.2	32.9

Ten fields of view were randomly selected and 100 cells were quantified using light microscopy (× 100).

The ratio of NSE positive cells in each group was calculated. [a]$P < 0.05$ *vs*. 0 mM UA group; [b]$P < 0.05$ *vs*. 6-hour induction (analysis of variance followed by *post hoc* analysis). NSE: Neuron specific enolase; UA: uric acid.

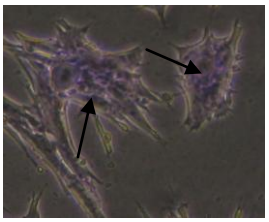

Figure 6 Nissl body formation within human placenta-derived mesenchymal stem cells following induction with 0.8 mM uric acid and 5 mM β-mercaptoethanol for 24 hours (Giemsa staining, light microscopy, × 200).

Nissl bodies stained dark blue in cytoplasm (arrows).

Data from this study indicate MSCs from placental tissue could act in an identical manner for similar therapeutic applications.

MSCs have been harvested from a number of tissues, including adipose, cord blood, bone marrow and embryonic[28-30]. Recent evidence shows that cells derived from human placenta tissue have unlimited self-renewal capabilities, coupled with an ability to differentiate into many cell types, arousing increased attention for their use in regenerative medicine[31]. However, human placenta tissue is reported to contain limited numbers of mononuclear cells. Although hPMSCs can be successfully induced into nerve cells, the quantity of induced cells is not sufficient, therefore limiting their clinical application. Consequently, there is a requirement for obtaining sufficient hPMSC numbers and ensuring their efficient induction into neural cells.

There is at present, no uniform standard for the identification of hPMSCs. hPMSCs are typically identified using cell morphology characteristics, cell surface marker expression and their ability to differentiate through various lineages[32-33]. In the present study, phase-contrast microscopy revealed hPMSCs with fibroblast-like morphology. Immunofluorescence analysis results showed that the cells were CD44 positive and CD34 negative, indicating a relatively pure hPMSCs population was obtained.

Both uric acid and BME are powerful antioxidants that exist naturally in the human body[34]. Using BME and dimethyl sulfoxide, Woodbury *et al* [35] reported that MSCs could be induced to differentiate into nerve cells. In the present study, we first investigated the influence of uric acid on promoting proliferation of hPMSCs. MTT assay data showed that uric acid used at low concentration (0.2, 0.4 and 0.8 mM) accelerates hPMSC proliferation, while at high concentration (1.2 mM), it inhibits hPMSC proliferation.

Interestingly, uric acid was found to accelerate differentiation of hPMSCs into neural cells in a time- and concentration-dependent manner, predominantly in cells treated with 0.8 mM uric acid.

In conclusion, uric acid accelerates proliferation of hPMSCs and their subsequent differentiation into neuronal-like cells in a time- and concentration-dependent manner. Using this method, we obtain greater numbers of hPMSCs and subsequent neuronal-like cells derived from hPMSCs. This may provide a new method for the scientific and clinical application of hPMSCs.

MATERIALS AND METHODS

Design

An *in vitro* comparative observation of repetitive measurements.

Time and setting

Experiments were performed in the Gout Laboratory of the Affiliated Hospital of Qingdiao University Medical College, China, from March to August 2011.

Materials

Human placentas were provided by the Department of Obstetrics and Gynecology of Qingdao Marie Hospital, China. The parturient had no severe or communicable

diseases. Informed consent was obtained from the parturient (including abortion and voluntarily donation).

Methods

Isolation and culture of hPMSCs

Harvested placentas were washed several times in phosphate buffered saline (PBS) to remove blood cells and subsequently sheared into 1 mm × 1 mm × 1 mm pieces using eye scissors. Tissue digestion was performed using 0.1% collagenase II (Sigma, St. Louis, MO, USA) solution at 37°C for 45 minutes and cell filtration was performed using a 200 mesh sieve. The resulting cell sediment was obtained by centrifugation at 1 000 r/min for 5 minutes at room temperature. The mononuclear cell fraction was separated by density gradient centrifugation and incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, HyClone, Logan, Utah, USA) at 37°C in a humidified atmosphere containing 5% CO_2 in a culture flask (T-175). Medium was replaced every 7 days thereafter. Non-adherent cells were progressively discarded by medium replacement. Cells were passaged to other culture flask (T-175) by trypsinization when MSCs reached 80% confluence. After passage, medium was replaced every 3 days. Cell morphology was observed using a phase-contrast inverted microscope (Olympus, Tokyo, Japan).

Immunofluorescence analysis of hPMSCs surface markers

hPMSCs (P3) were seeded into wells of a 6-well plate at a density of 5.0 × 10^4 cells/well. On reaching 50–60% confluence, expanded hPMSCs were washed three times with PBS, fixed with 4% paraformaldehyde (pH 7.4, 30 minutes) and rinsed with PBS (3 × 5 minutes). Cells were incubated for 1 hour with appropriate fluorochrome-conjugated antibody, either CD44-FITC or CD34-FITC (eBioscience, San Diego, CA, USA), and immediately analyzed by immunofluorescence (Olympus).

MTT detection for hPMSCs viability

hPMSCs (P4) were digested using 0.25% trypsin and seeded into four 96-well plates at a density of 1 000–10 000 cells per well. hPMSCs were starved by replacing the medium with serum-free DMEM/F12 for 24 hours to maintain synchronization at the same cell cycle phase. hPMSCs were treated with 0, 0.2, 0.4, 0.8, or 1.2 mM uric acid (Sigma). On day 2 of culture, 20 μL MTT (Sigma) was added to each well, the plate was placed in a box with volume fraction 5%CO_2 at 37°C to allow precipitation for 4 hours. 150 μL dimethyl sulfoxide (Sigma) was then added to each well. A shocking device was used for 10 minutes to lyse the cells and an enzyme-linked immune detector (Thermoelectron Corporation, Helsinki, Finland) was used to measure absorbance of each well at 485 nm. Absorbance was detected again at 3, 5 and 7 days to obtain a cell growth curve.

Grouping and intervention

hPMSCs (P4) were digested using 0.25% trypsin and seeded into three 24-well plates at a density 5.0 × 10^4 cells per cm^2. Experimentation was performed across five groups: untreated control, 0.8, 0.4, 0.2 and 0 mM uric acid groups.

On reaching 50–60% confluence, hPMSCs were pre-induced in serum-free culture medium containing 1 mM BME (Sigma) and uric acid (0, 0.2, 0.4 and 0.8 mM)[14] for 24 hours. This was replaced with 10% HI-FBS containing culture medium with 5 mM BME and identical respective concentrations of uric acid for 6 or 10 hours. Neuronal-like cells were identified using immunochemical and immunofluorescent staining. Giemsa staining was performed 24 hours after induction. Untreated group hPMSCs were cultured in DMEM/F12 medium supplemented with 10% HI-FBS in absence of BME or uric acid.

Immunocytochemical staining for NSE expression

hPMSCs were washed with PBS (3 × 10 minutes) and fixed with 4% paraformaldehyde (30 minutes/room temperature). After further PBS washes (3 × 10 minutess), cells were permeabilized with 0.1% Triton X-100 for 10 minutes prior to exposure to 3% bovine serum albumin (Sigma) for 15 minutes and incubation for 1 hour with primary rabbit anti-human NSE monoclonal antibody (1:100 dilution; Saierbio Corporation, Tianjin, China). Cells were washed three times in PBS and incubated for 30 minutes with secondary goat anti-rabbit antibody (1:200 dilution; BIOS Corporation, Sapulpa, OK, USA). Cells were then treated with avidin-biotin (BIOS Corporation) for 30 minutes, washed with PBS (2 × 5 minutes). 3,3'-diaminobenzidine tetrahydrochloride dehydrate (BIOS Corporation) staining substrate was mixed with the cells and left to react for 5 minutes. Addition of PBS for 5 minutes stopped the reaction and cells were washed twice with PBS. Finally, cells from each group were visualized by microscopy (Olympus) and photographed with a Nikon camera (magnification, × 200; Tokyo, Japan).

Immunofluorescence staining for NSE expression

hPMSCs were washed, fixed and permeabilized using the same methods as immunocytochemistry. The cells were incubated for 1 hour with the primary rabbit anti-human NSE monoclonal antibody (dilution at 1:100). After the reaction with the primary antibody, the cells were washed three times in PBS and incubated for 30 minutes with the secondary fluorescein isothiocyanate labeled goat anti-rabbit NSE antibody (dilution at 1:200; eBioscience). The cells were washed twice for 5 minutes with PBS, visualized using immunofluorescence microscopy (Olympus) and photographed with a Nikon camera (magnification, × 100).

Giemsa staining for Nissl body formation

hPMSCs were washed with PBS (3 × 10 minutes) and fixed with 4% paraformaldehyde (30 minutes/room temperature) prior to subsequent washing with PBS (3 × 10 minutes) and treatment with 1 mL/well Giemsa stain (Sigma). Cells were incubated in a box with 5% CO_2 at 37°C for 30 minutes, washed with PBS and visualized using light microscopy (Olympus). Photographs were

taken using a Nikon camera (magnification, × 400).

Statistical analysis

Data was expressed as a mean. Results were analyzed using analysis of variance followed by *post hoc* assessment using SPSS 17.0 software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Nailong Yang was responsible for the experimental concept, design, guidance and ethical approval. Jing Cui was responsible for conducting the experimental procedures, providing the images and writing the manuscript. Lili Xu and Peng Lin were responsible for image processing, data analysis and technical and informational support.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Qingdao Municipal Hospital Medical Ethics Committee and followed guidelines from the “Helsinki Declaration Requirements”.

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