Cobalt Chloride Enhances the Stemness of Human Dental Pulp Cells

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Abstract

Introduction: Hypoxia is a factor in controlling stem cell stemness. We investigated if cobalt chloride ($CoCl_2$), a chemical agent that mimics hypoxia *in vitro*, affected human dental pulp cell (hDPC) stemness by examining cell proliferation, stem cell marker expression, and osteogenic differentiation. **Methods:** hDPCs were cultured with or without 25 or 50 μmol/L $CoCl_2$. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine cell proliferation. The number of STRO-1^+ cells was determined by flow cytometry. The messenger RNA expression of the stem cell markers *REX1*, *OCT4*, *SOX2*, and *NANOG* and the osteogenic-associated genes *ALP*, *COLI*, and *RUNX2* were evaluated using reverse transcription polymerase chain reaction or real-time polymerase chain reaction. Osteogenic differentiation was assessed by alkaline phosphatase (ALP) activity and mineralization assays. **Results:** Although 25 and 50 μmol/L $CoCl_2$ suppressed hDPC proliferation, 50 μmol/L $CoCl_2$ increased the number of STRO-1^+ cells. Moreover, $CoCl_2$ dose dependently induced stem cell marker expression. Additionally, $CoCl_2$ treatment suppressed osteogenic-associated gene expression, ALP activity, and calcium deposition. The addition of apigenin, a hypoxia-inducible factor 1-alpha inhibitor, reversed the inhibitory effect of $CoCl_2$ on ALP activity. **Conclusions:** This study indicated that $CoCl_2$ may enhance hDPC stemness. *(J Endod 2017;■:1–6)*

Key Words

Cobalt chloride, human dental pulp cells, osteogenic differentiation, stemness

Significance

The use of $CoCl_2$ would be an advantage in the development of a method to increase the number of stem cells without the loss of their capability to differentiate, which is a major problem of cell-based approaches in current regenerative endodontics.

Human dental pulp stem cells (hDPSCs) are found in deciduous and permanent teeth and may be used in tissue regeneration because of their self-renewing and pluripotent ability (1–3). Dental pulp stem cells (DPSCs) are highly proliferative and formed dentin/pulplike complexes *in vitro* and *in vivo* (1, 2). However, the stem cells in the dental pulp represent <1% of the total cell population (4, 5). Therefore, stem cell amplification and stemness maintenance would benefit regenerative treatments.

Extracellular oxygen concentration influences stem cell characteristics, with low oxygen tension inducing cell division and maintaining stemness (6, 7). Tissue oxygen saturation is typically lower than that of air (20% oxygen) (ie, a hypoxic state). The oxygen tension in cat brain tissue and bone marrow ranges from 0.5%–7% and 0%–4%, respectively (8, 9). In the dental pulp, oxygen tension is 3% in mice and 4.5% in rabbits (10, 11). Therefore, these tissues possess a lower-than-atmosphere oxygen concentration (physiologic hypoxia) (12).

The oxygen saturation level may alter stem cell behavior. Low oxygen tension (hypoxia) up-regulated cell proliferation and stem cell marker messenger RNA (mRNA) expression while reducing the expression of osteoblastic markers in human bone marrow stromal cells (6). Moreover, mesenchymal stem cells (MSCs) had an increased proliferative life span and diminished adipogenic or osteogenic differentiation under hypoxia (7). Hypoxia also enhanced the proliferation and the number of STRO-1^+ cells while suppressing hDPC osteo/odontogenic differentiation (13, 14).

To simulate hypoxic conditions in the laboratory, several methods have been used. Physically creating hypoxia is achieved using a hypoxic chamber in which the oxygen level can be controlled. However, it is difficult to control and maintain steady oxygen tension using this method. Thus, adding a chemical substance, such as cobalt chloride ($CoCl_2$), into the culture media is an attractive alternative to physically creating hypoxia (15, 16).

$CoCl_2$ imitates hypoxia *in vitro* by preventing hypoxia-inducible factor-alpha (HIF-α) from being destroyed by oxygen (17). HIF-α regulates gene transcription in response to cellular oxygen reduction and maintains cell stemness (18). We previously found that $CoCl_2$ enhanced stem cell marker expression and inhibited osteogenic differentiation in human periodontal ligament cells (19). Our literature review revealed that investigation into the influence of $CoCl_2$ on hDPC stemness has not been reported. Therefore, this study evaluated the effect of $CoCl_2$ on hDPC cell proliferation, stem cell gene expression, and osteogenic differentiation.

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Materials and Methods

Tooth Collection

This study was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand, and informed consent was acquired from each participant. The teeth consisted of premolars and impacted third molars without caries or periapical lesions indicated for extraction.

Cell Isolation and Culture

After extraction, the teeth were placed in sterile tubes containing Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin G 100 U/mL (Invitrogen, Carlsbad, CA), 100 μg/mL streptomycin (Invitrogen), 5 μg/mL amphotericin B (Invitrogen), and 1% 200 mmol/L L-glutamine (Invitrogen) (culture media). The teeth were kept at 4°C, and pulp cell isolation was performed within 24 hours.

The dental pulp tissues were removed from the teeth and washed with sterile phosphate-buffered saline. The tissues were cut into approximately 1 × 1 mm pieces, digested with 3 mg/mL type I collagenase at 4°C for 1 hour, and centrifuged at 2000 rpm. The tissue was resuspended and cultured in 35-mm plates containing culture media. The explants were maintained in culture media at 37°C in a humidified 5% CO_2 atmosphere. When the cells from the explants reached confluence, they were subcultured at a 1:3 ratio. The culture media was changed every other day. Cells from passages 3 through 8 were used in this study. Cell lines from 3 donors were used.

To evaluate osteogenic differentiation, hDPCs were seeded in 24-well plates at a density of 1.25×10^4 cells/well in osteogenic media (OM) (culture media supplemented with 50 μg/mL ascorbic acid, 100 nmol/L dexamethasone, and 10 mmol/L β-glycerophosphate) with or without 25 μmol/L or 50 μmol/L $CoCl_2$. The media was changed every 48 hours. Cells cultured without $CoCl_2$ served as the control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide Assay

hDPCs were seeded into 24-well plates at a density of 1×10^4 cells/well and cultured in culture media with or without 25 or 50 μmol/L $CoCl_2$ (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 1, 3, and 6 days. The medium was changed every other day. After each culture period, the medium was replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (USB Corporation, Cleveland, OH) at 37°C for 30 minutes. The formazan product was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) solution and glycine buffer. Subsequently, the absorbance was evaluated using a microplate reader (Biotek Instruments, Winooski, VT) at 570 nm and converted into cell number using a standard curve.

STRO-1$^+$ Cell Quantification Using Flow Cytometry

hDPCs were cultured in 60-mm dishes at a density of 2×10^5 cells/dish in culture media with or without 50 μmol/L $CoCl_2$ for 6 days. The cells were harvested and incubated with a mouse STRO-1 primary antibody (Chemicon, Temecula, CA) and then incubated with a biotin-conjugated antimouse goat secondary antibody (Chemicon) on ice for 30 minutes. The cells were subsequently incubated with streptavidin–fluorescein isothiocyanate (Sigma-Aldrich) on ice for 30 minutes. The cells were resuspended twice in fluorescence-activated cell sorting buffer. The cells were fixed with 1% formaldehyde until analyzed using a flow cytometer (Beckman Coulter, Carlsbad, CA). Data analysis was performed using Cell Quest Software (BD Biosciences, Franklin Lakes, NJ).

Reverse Transcription Polymerase Chain Reaction and Real-time Polymerase Chain Reaction

hDPCs were seeded in 6-well plates at a density of 3×10^6 cells/well and incubated in culture media. The cells were treated with or without 25 or 50 μmol/L $CoCl_2$. hDPCs cultured without $CoCl_2$ served as the control. Total RNA was extracted using TRIzol reagent (Roche Diagnostics, Indianapolis, IN) per the manufacturer's protocol. One microgram of mRNA was converted into complementary DNA using the ImProm II Reverse Transcription System (Promega, Southampton, UK) and amplified by polymerase chain reaction (PCR) using Tag polymerase (Tag DNA Polymerase, Invitrogen) and primers for *REX1*, *OCT4*, *SOX2*, *NANOG*, *ALP*, *RUNX2*, or *COLI* in a DNA thermal cycler (Biometra GmH, Göttingen, Germany). The primer sequences are shown in Table 1. The amplified DNA was electrophoresed on a 1.8% agarose gel and visualized using ethidium bromide fluorostaining. The intensity of the specific bands was read by the Scion Image program (Scion Corporation, Fort Worth, TX). For real-time PCR, the reaction was performed using a LightCycler Nano (Roche Diagnostics) with a LightCycler_480 SYBR Green I Master kit (Roche Diagnostics).

Alkaline Phosphatase Assay

hDPCs were seeded in 24-well plates at a density of 2.5×10^4 cells/well and incubated in OM with or without 25 or 50 μmol/L $CoCl_2$ for 7 and 14 days. In some groups, cells were cultured in OM with 50 μmol/L $CoCl_2$ for 7 days before being replaced by OM without $CoCl_2$ for 7 days.

After the culture periods, the cells were treated with an alkaline lysis buffer. The supernatants were incubated at 37°C in an alkaline phosphatase (ALP) substrate solution containing 2 mg/mL p-nitrophenol phosphate (Invitrogen), 0.1 mol/L 2-amino-2-methyl-1-propanol (Sigma-Aldrich), and 2 mmol/L $MgCl_2$ for 30 minutes. The reaction was stopped using 50 mmol/L NaOH. The absorbance was measured at 410 nm with a microplate reader. Total cellular protein was measured using the BCA protein assay (Thermo Scientific, Waltham, MA) per the manufacturer's instructions. ALP activity was normalized to the total cellular protein.

Mineralization Assay

hDPCs were seeded at a density of 1.25×10^4 cells/well in 24-well plates. Osteogenic differentiation was performed similar to the ALP assay. After culturing for 28 days, the cells were fixed with absolute

TABLE 1. Reverse Transcription Polymerase Chain Reaction Primer Sequences

Gene	Forward primer Reverse primer
REX1	5′AGAATTCGCTTGAGTATTCTGA3′ 5′GGCTTTCAGGTTATTTGACTGA3′
OCT4	5′AGACCCAGCAGCCTCAAAATC3′ 5′GCAACCTGGAGAATTTGTTCCT3′
NANOG	5′GGAAGAGTAGAGGCTGGGGT3′ 5′TCTCTCCTCTTCCTTCTCCA3′
SOX2	5′ACCAGCTCGCAGACCTACAT3′ 5′ATGTGTGAGAGGGGCAGTGT3′
ALP	5′CGAGATACAAGCACTCCCACTTC3′ 5′CTGTTCAGCTCGTACTGCATGTC3′
RUNX2	5′CCCCACGACAACCGCACCAT3′ 5′CACTCCGGCCCACAAATC3′
COLI	5′ GTGCTAAAGGTGCCAATGGT 3′ 5′ ACCAGGTTCACCGCTGTTAC 3′
18S	5′GTGATGCCCTTAGATGTCC3′ 5′CCATCCAATCGGTAGTAGC3′