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Cobalt chloride supplementation induces stem-cell marker expression and inhibits osteoblastic differentiation in human periodontal ligament cells

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ABSTRACT

Objective: Low oxygen tension is one of the crucial factors of the stem-cell niche. However, the long-term hypoxic culture of stem cells is difficult and requires special equipment. In this study, we investigated whether mimicking hypoxia using cobalt chloride ($CoCl_2$) could maintain human periodontal ligament (HPDL) cell stemness.

Methods: HPDL cells were treated with either 50 or 100 μM $CoCl_2$. Cell proliferation was determined by an MTT assay. The mRNA expression of stem-cell marker and osteogenic associated genes were analyzed by RT-PCR and Real-time PCR. Osteogenic differentiation was determined by assaying alkaline phosphatase activity and *in vitro* mineralization.

Results: The results showed that the $CoCl_2$ supplementation had no effect on cell proliferation. $CoCl_2$ treatment increased the mRNA expression of the embryonic stem-cell markers *REX1* and *OCT4*. Culturing HDPL cells in osteogenic medium containing $CoCl_2$ resulted in a decrease in alkaline phosphatase activity, down-regulation of osteogenic associated gene expression, and suppression of mineralization. The use of Apigenin, an HIF-1α inhibitor, indicated that $CoCl_2$ might inhibit osteogenic differentiation through an HIF-1α- dependent mechanism.

Conclusion: This study shows that $CoCl_2$ treatment can induce stem-cell marker expression and inhibit the osteoblastic differentiation of HPDL cells. These findings suggest the potential application of $CoCl_2$ for maintaining the stem-cell state in the laboratory.

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1. Introduction

Stem-cell-based therapy is a promising approach for the treatment of various degenerative diseases. Embryonic and adult stem cells are the main cell types currently used in regenerative medicine. Because of the ethical concerns in obtaining embryonic stem cells,[1] adult stem cells have been intensively studied in the field of stem-cell research. Adult stem cells can be isolated from various tissues, including adipose tissue, bone marrow, and teeth.[2–4] Among adult tissues, the tooth is considered as one of the easily accessible sources of stem cells. Different types of healthy teeth such as exfoliated deciduous teeth, impacted teeth, and teeth extracted for orthodontic purposes can be easily obtained. Dental stem cells can be isolated from different parts of the tooth such as the dental pulp and periodontal ligament.[4,5]

The PDL contains cell populations capable of differentiating into cementoblasts or osteoblasts.[6,7] Periodontal ligament stem cells (PDLSCs) have been successfully isolated from the periodontal ligament of extracted human third molars.[4] PDLSCs are mesenchymal stem cells that are able to self-renew, with multilineage differentiation potential, including osteoblasts, adipocytes, chondrocytes, and neurons.[4,8–10] Human PDLSCs transplanted into immuno-compromised mice generated cementum-like tissue and dense type I collagen-positive PDL-like tissue.[4] The use of autologous PDLSCs, obtained from the extracted teeth of mini-pigs, could regenerate periodontal tissues in a porcine periodontitis model.[11] These data indicate the potential role of PDLSCs in stem-cell-based periodontal therapy. However, one of the limitations of adult stem cells is the limited and low amount of cells. Thus, finding laboratory methods to increase their proliferation, while maintaining the stem-cell state, is a challenge for the clinical use of stem cells.

Stem cells are resided in a special microenvironment known as the stem-cell niche. The niche is defined as an anatomical compartment, including cellular and acellular components, that provides signals controlling stem-cell behavior.[12,13] One of the critical components of the stem-cell niche is oxygen concentration.[13] Measuring the oxygen content of tissues known as stem-cell sources revealed a wide range of tissue oxygen concentrations that were lower than the inhaled oxygen tension of 21%. For example, hematopoietic stem cells were found to reside in a hypoxic microenvironment with an oxygen gradient between 1 and 6%.[14] The dental pulp, which contains dental pulp stem cells, has an oxygen profile of 3 and 4.5% in rats and rabbits, respectively.[15,16] However, cells are usually cultured under normoxic conditions, which might not be a suitable environment for the maintenance of the stem-cell state. These findings led to investigation into the effect of hypoxia on stem-cell behavior.

Hypoxia plays an important role in stem-cell proliferation and the maintenance of pluripotency. Exposing mouse bone marrow stromal cells to 3% O_2 enhanced cell proliferation and increased the expression of the embryonic stem-cell markers *OCT4* and *REX1*.[17] Similar studies on human dental pulp cells indicated that culturing with 3% O_2 promoted cell proliferation and increased the number of cells stained for the early mesenchymal stem-cell marker STRO-1.[18,19] Moreover, hypoxia was also found to maintain the stemness of bone marrow stromal cells and human dental pulp cells by suppressing their differentiation.[17,19]

To establish and maintain physical hypoxia, special equipment such as gas cylinders (containing a gas mixture of 95% nitrogen and 5% carbon dioxide), hypoxic chambers, and an oxygen analyzer are generally required. The use of this method to maintain a hypoxic environment in long-term cell culture, which usually requires media changes, faces the technical difficulty. The mimicking of hypoxia using a chemical reagent, so-called chemical hypoxia, is much more convenient and easier to establish. Cobalt chloride ($CoCl_2$) is a hypoxia-mimicking agent that is commonly used in hypoxic culture studies. $CoCl_2$ mimics the hypoxic response by inhibiting the activity of prolyl hydroxylase, a key enzyme in the oxygen sensing pathway.[20] In the present study, we tested whether $CoCl_2$ could maintain the stemness of human PDL (HPDL) cells as was found in physical hypoxic studies. The effect of $CoCl_2$ on cell proliferation, stem-cell marker expression, and osteogenic differentiation was examined.

2. Material and methods

2.1. Cell isolation and culture

The protocol for HPDL cell isolation was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University. HPDL cells were isolated and cultured as previously described.[21] The isolated cells were maintained in Dulbecco's modified Eagle's medium (Gibco, NY, USA) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 unit/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 5 μg/mL amphotericin B (Gibco) at 37 °C, in a humidified atmosphere containing 5% carbon dioxide. The medium was changed every 48 h. After reaching confluence, the cells were passaged at a 1:3 ratio. Cells from passages 3–6 were used in the experiments. When treating the cells with $CoCl_2$, $CoCl_2$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added to the culture medium at the designated concentration (50 or 100 μM).

To examine osteoblast differentiation, cells were seeded at a density of 2.5×10^4 cells/well in 24-well-plates and cultured in osteogenic medium (growth medium supplemented with 50 μg/mL ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate). The medium was changed every 48 h. Cells cultured in normal growth medium were used as the control.

2.2. Flow cytometry

Cells were harvested with trypsin-EDTA and resuspended in the wash buffer. For cell surface staining, cells were incubated with FITC-conjugated anti-CD44 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), FITC-conjugated anti-CD73 antibody (BD Biosciences Pharmingen), PerCP-CyTM5.5-conjugated anti-CD90 antibody (BD Biosciences Pharmingen), PE-conjugated anti-CD105 antibody (BD Biosciences Pharmingen), PerCP-conjugated anti-CD45 antibody (BD Biosciences