Expression and influence of BMP-4 in human dental pulp cells cultured *in vitro*

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Abstract. Effects of bone morphogenetic protein (BMP)-4 on proliferation and differentiation capacities of dental pulp cells through BMP-4 acting on human dental pulp cells cultured *in vitro* were investigated. Dental pulp tissues of lesion-free teeth extracted from patients due to orthodontics were taken, and human dental pulp cells were cultured *in vitro* using the tissue explant method. Immunocytochemical staining was used for the identification of vimentin and keratin. The dental pulp cells were divided into groups A and B. A total of 100 ng/ml BMP-4 was added into group A, while no inducer was added into group B as the control group. The cell growth curves at day 1, 2, 3, 5 and 7 after culture were drawn. At day 7, the cell count, alkaline phosphatase (ALP) activity, number of calcified nodules, and expression levels of dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1) and each gene related to dentinogenesis in each group were detected, respectively. Human dental pulp cells were conformed to the biological characteristics of dental pulp cells according to the identification of vimentin and keratin via immunocytochemical staining. With the prolongation of culture time, the number of cells in both groups was gradually increased, reaching the peak at day 5 and began to decline at day 7. The number of cells in group A was significantly greater than that in group B ($p<0.05$). According to the results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the relative messenger ribonucleic acid (mRNA) expression levels of ALP, DSPP and DMP-1 in group A were significantly higher than those in group B ($p<0.05$). BMP-4 can promote the growth of dental pulp cells and remarkably enhance the differentiation of dental pulp cells into odontoblasts.

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Introduction

With the development of society, people's living standard and quality have improved, and increasingly more people have begun to pay attention to oral problems. Moreover, the health problem of tooth, as an indispensable part of people's oral cavity, is also important (1). Dental disease is a common disease in clinic, and clinical transplantation and repair techniques play significant roles in the treatment of patients' pain and recovery of masticatory function (2,3). However, neither function nor sense of transplanted dentures can be comparable with natural teeth. Therefore, scholars all over the world are making efforts to find an effective method of tooth regeneration. In recent years, studies have shown that there is a certain correlation between tooth development and signaling pathway, and the combination with tissue engineering is an important key to tooth regeneration.

Dental pulp cells are regenerative cells with differentiation capacity, which, regulated and induced by several factors, can be differentiated into odontoblasts through continuous self-renewal capacity under appropriate conditions, repairing teeth (4). Bone morphogenetic protein (BMP) pathway plays an extremely important regulatory role in the interaction between dental epithelium and mesenchyme (5). Some studies indicate that (6) BMP signal can accurately regulate intracellular and extracellular factors, thus achieving intracellular and extracellular dynamic balance, which plays a crucial role in maintaining the normal tooth development. Once such balance is broken, the tooth development will be affected. The expression of BMP-4, as the first factor identified by human in the interaction between dental epithelium and mesenchyme, can well reflect the dental epithelium and mesenchyme-induced tooth potentiality, which can also induce some morphogenesis processes of dental germ, thus exerting a relative regulatory effect (7). In this study, therefore, human dental pulp cells were cultured *in vitro* under the influence of BMP-4 to observe the proliferation and differentiation capacities of dental pulp cells.

Materials and methods

Main reagents and instruments. High-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gibco; Thermo

Fisher Scientific, Inc., Waltham, MA, USA). BCIP/NBT alkaline phosphatase (ALP) staining kit was purchased from Sigma-Aldrich (Sigma-Aldrich: Merck KGaA, St. Louis, MO, USA). BMP-4 was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). TRIzol, reverse transcription kit, and fluorescence quantitative polymerase chain reaction (PCR) kit were from Invitrogen (Invitrogen: Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). ABI 7500 Fluorescence PCR amplification instrument was purchased from Applied Biosystems (Applied Biosystems: Thermo Fisher Scientific, Inc., Foster City, CA, USA).

Extraction and passage of dental pulp cells. Experimental samples in this study were taken from normal teeth extracted from patients aged <30 years old in China Medical University School and Hospital of Stomatology due to orthodontics. Before extraction, patients were informed and signed the informed consent. The study was approved by the Ethics Committee of School of Stomatology, China Medical University (Shenyang, China). After extraction, teeth were transferred to a sterile environment and dental crown was split to extract dental pulp tissues within 30 min. The extracted dental pulp tissues were cut into 1 mm^3 blocks and transferred into a culture flask. After 15% FBS and high-glucose DMEM were added, the culture flask was placed into a constant temperature incubator with 5% CO_2 for incubation at 37˚C, and the liquid was replaced once every 3 days. The culture medium was observed, and cells were digested with 0.25% trypsin when they covered >85% of the medium, followed by counting and passage. Finally, the cell growth curves at day 1, 2, 3, 5 and 7 were drawn.

Experimental grouping. In the experiment, the 3rd-5th generations of dental pulp cells in good growth conditions were selected and digested with 0.25% trypsin. The density was adjusted with high-glucose medium containing 10% FBS ($4.5x10^4$ cells/ml). Then cells were inoculated into a 48-well plate (500 μl/well), and cultured in the constant temperature incubator with 5% CO_2 at 37˚C. After cell adherence, they were divided into group A (100 ng/ml BMP-4) and group B (no treatment, experimental control group).

Immunocytochemical staining. The 3rd-5th generations of dental pulp cells in both groups were taken, inoculated into a 6-well plate at a density of $4.5x10^4$ cells/ml, and incubated in constant temperature incubator with 5% CO_2 at 37˚C for 3-5 days. When 85% cells were fused, they were rinsed with phosphate-buffered saline (PBS) 3 times (30-50 sec/time), fixed with 4% paraformaldehyde, rinsed again with PBS and air-dried. Then cells were soaked in Triton X-100 detergent for 5-10 min, rinsed with PBS, air-dried and soaked in 3% H_2O_2. After being rinsed again with PBS and air-dried, cells were sealed with FBS. Then, primary mouse monoclonal vimentin antibody (dilution, 1:200; cat. no. ab8978) and mouse monoclonal keratin antibody (dilution, 1:200; cat. no. ab169328) were added for incubation with ice at 4˚C overnight. The next day, the cells were taken, cultured in constant temperature incubator with 5% CO_2 at 37˚C, washed with PBS, air dried and added with secondary rabbit anti-mouse (HRP) IgG antibody (dilution, 1:1,000; cat. no. ab6728), followed by wet incubation at 37˚C. All the antibodies were purchased from Abcam (Cambridge, MA, USA). Finally, the secondary antibody was discarded. Then, horseradish peroxidase markers were added for labeling, and cells were washed with PBS and air-dried, followed by addition of developing solution for color development, hematoxylin-eosin staining, sealing and observation under a microscope (Olympus, Tokyo, Japan).

Table I. Primer sequences.

Genes		Primer sequences
ALP	F	5'-ACACCTTGACTGTGGTTACTGCTGA-3'
	R	5'-CCTTGTAGCCAGGCCCGTTA-3'
DSPP	F	5'-TTCTCCTACTCAGCCCATTTTA-3'
	R	5'-CCATCGTGACCGTATGTTTCTA-3'
DMP-1	F	5'-TGGGTTTGTTGTGATAGG-3'
	R	5'-GGAAGAGGTGGTGAGTGA-3'
U6	F	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'

ALP, alkaline phosphatase; DSPP, dentin sialophosphoprotein; DMP-1, dentin matrix protein-1; F, forward; R, reverse.

Reverse transcription-quantitative PCR (RT-qPCR). Total ribonucleic acid (RNA) was extracted using TRIzol solution from cells cultured at day 5 and 7 in strict accordance with the instructions. The concentration of total RNA extracted was detected using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan), and the purity of total RNA was detected via protein electrophoresis. Then, total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit in strict accordance with the manufacturer's instructions. The reaction system was prepared strictly according to the instructions of the fluorescence quantitative PCR kit. Expression of ALP, dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) messenger ribonucleic acid (mRNA) was detected. U6 was used as an internal control for amplification, and amplification primers were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The primer sequences are shown in Table I.

Cell staining. After culture for 12 days, cells in both groups were stained using the BCIP/NBT ALP kit according to the instructions of the BCIP/NBT ALP kit. The ALP-stained cells were observed under an inverted microscope, and the area of positive cells was recorded and analyzed using Image-Pro Plus software. The experiment was repeated 3 times.

Statistical analysis. In this study, Statistical Product and Service Solutions (SPSS) 22.0 software package (IBM Corp., Armonk, NY, USA) was used for the statistical analysis of data collected in this experiment. Enumeration data are presented as mean ± standard deviation (SD), analysis of variance was used for the intergroup comparison, and Student-Newman-Keuls test was performed for pairwise comparison. $P<0.05$ suggested that the difference was statistically significant.

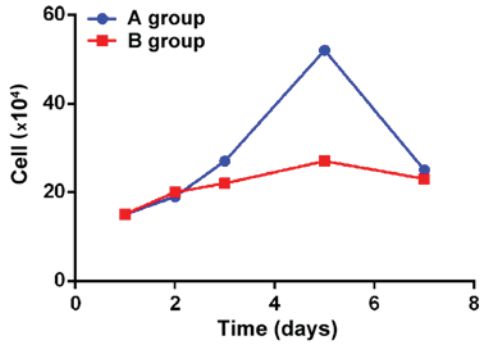

Figure 1. Cell growth curves. The comparison of cell growth curves between the two groups of cells shows that the cell growth rate in group A treated with BMP-4 is significantly higher than that in group B without any treatment, and there is a statistically significant difference between the two groups ($p<0.05$). BMP, bone morphogenetic protein.

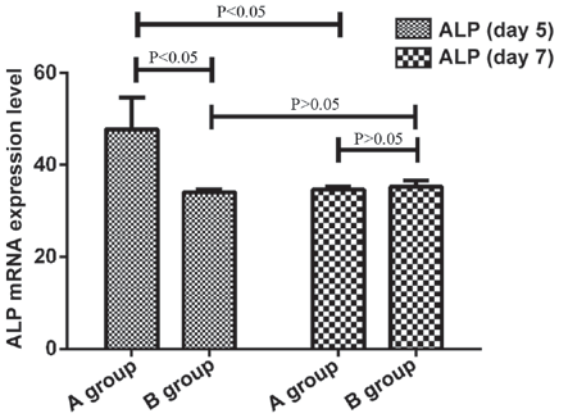

Figure 2. ALP expression level. Detection results of ALP expression levels in both groups of cells at day 5 and 7 via qRT-PCR reveal that the ALP expression level in group A at day 5 is higher than that in group B ($p<0.05$), and there is no significant difference at day 7 between the two groups of cells ($p>0.05$). Compared with that at day 7, the expression level in group A at day 5 is significantly decreased ($p<0.05$), while that in group B is slightly increased ($p>0.05$). ALP, alkaline phosphatase; RT-qPCR, reverse transcription-quantitative PCR.

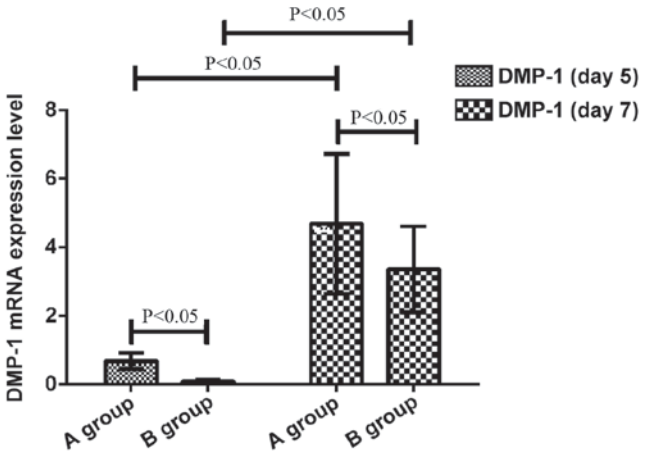

Figure 3. DMP-1 expression level. Detection results of DMP-1 expression levels in both groups of cells at day 5 and 7 via RT-qPCR reveal that the DMP-1 expression level in group A at day 5 is higher than that in group B ($p<0.05$), and there is a significant difference in the DMP-1 expression level at day 7 between the two groups of cells ($p<0.05$). Compared with those at day 7, the expression levels of DMP-1 in both groups of cells at day 5 are obviously increased ($p<0.05$). DMP-1, dentin matrix protein-1; RT-qPCR, reverse transcription-quantitative PCR.

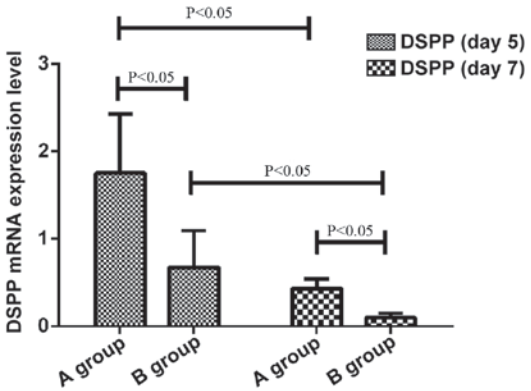

Figure 4. DSPP expression level. Detection results of DSPP expression levels in both groups of cells at day 5 and 7 via qRT-PCR reveal that the DSPP expression level in group A at day 5 is higher than that in group B ($p<0.05$), and there is a difference in the DSPP expression level at day 7 between the two groups of cells ($p<0.05$). Compared with those at day 7, the expression levels of DSPP in both groups of cells at day 5 are obviously decreased ($p<0.05$). DSPP, dentin sialophosphoprotein; RT-qPCR, reverse transcription-quantitative PCR.

Results

Cell culture results and morphological observation. Cells were cultured and observed. When dental pulp cells were cultured for 7 days, the difference in primary stem cells from other cells could be clearly seen, and cells were distributed in long fusiform type and stable after passage. It was found in the observation of vimentin and keratin via immunohistochemical staining that the vimentin staining was positive, while the keratin staining was negative, indicating that cells derived from the mesoderm and displayed the fibrous shape in this study, meeting the requirements of this experiment.

Analyses of cell growth curves in the two groups. In this study, the growth curves were drawn in both groups. It was manifested that the cell growth rate in group A added with BMP-4 was significantly higher than that in group B without treatment, and there was a statistically significant difference between the two groups ($p<0.05$). The cell growth rates in both groups were increased remarkably in an exponential manner at day 3 after culture, the cell proliferation reached the peak at day 5, and then slowed down (Fig. 1).

Detection of the expression of ALP, DSPP and DMP-1 in cells by PCR. At day 5 and 7 after culture, cells in both groups were taken to detect the expression of ALP, DSPP and DMP-1 via RT-qPCR. The expression levels of ALP, DSPP and DMP-1 in group A at day 5 after culture were significantly increased compared with those in group B, and there were statistically significant differences ($p<0.05$). Compared with those at day 5 after culture, the expression levels of DSPP in both groups of cells were significantly decreased at day 7 after culture ($p<0.05$), and the expression levels of DMP-1 in both groups of cells were significantly increased ($p<0.05$). Besides, compared with that at day 5 after culture, the expression level of ALP in

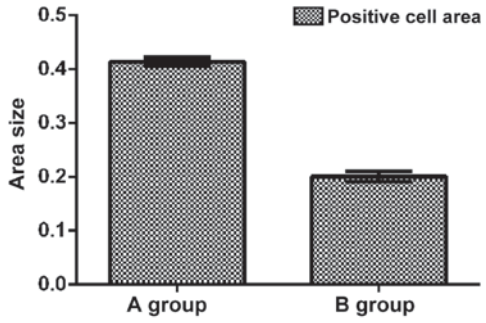

Figure 5. Detection of ALP activity. According to the results of ALP staining, the proportion of positive cells in group A (0.414±0.008) is significantly higher than that in group B (0.201±0.010) and there is a statistically significant difference between the two groups ($p<0.05$). ALP, alkaline phosphatase.

group A was decreased ($p<0.05$), but there was no significant difference in group B ($p>0.05$) (Figs. 2-4).

ALP staining results. At day 12 after culture, cells in both groups were stained. Staining results displayed that cells in both groups were stained successfully, and purple-black sediment could be observed in both groups, but it was significantly darker in group A than that in group B, showing strongly positive. Then, the proportion of positive area was determined using the Image-Pro Plus image software. Results showed that there was a significant difference in the proportion of positive cells in group B (0.201±0.010) compared with that in group A (0.414±0.008) ($p<0.05$) (Fig. 5).

Discussion

Dental pulp cells are predominantly distributed in adult dental pulp cells that possess multiple differentiation and self-renewal capacities, and the same characteristics as other tissue stem cells in the body (8). Studies have proved that (9) differentiation and self-renewal capacities of dental pulp cells are regulated by biological factors and signaling pathways. Recently it was demonstrated in a large number of studies that BMP, as an indispensable morphogenetic protein in tooth growth, can promote the growth and development of human teeth through the influence of multiple pathways (10-12).

As an extracellular signal molecule of transforming growth factor-β family, BMP-4 plays an important role in the tooth and bone growth and repair processes in the body, and also plays a regulatory role in the growth, apoptosis and differentiation of human dental pulp cells, which can maintain the renewal capacity of dental pulp cells through blocking the mitogen-activated protein kinase signaling pathway (13). In addition to its effects on the growth and development of teeth and bones, BMP-4 also exerts important effects on the embryonic development and differentiation in digestive, reproductive and nervous systems in the human body (14,15).

In this study, human dental pulp cells were cultured *in vitro* to observe the effect of BMP-4 on dental pulp cells. Through drawing the growth curves in both groups of cells, it was found that the cell growth rate in group A pre-treated with BMP-4 was significantly higher than that in group B, reaching the peak at day 5 after culture, and the cell proliferation was weakened after 5 days, possibly because the cell density was lower in the early stage of growth, and cells contacted less with BMP-4. With the cell growth, the secretion of BMP-4 was insufficient in both groups, but the content of BMP-4 in cells in group A was higher than that in group B due to the pre-treatment with BMP-4, thus increasing the cell growth rate.

It has been reported that (16) the inactivation of BMP-4 in mouse mandibular mesenchyme in animal model test leads to growth cease of mandibular molar in mouse, but the growth of maxillary molar is normal without significant differences from normal mouse. Moreover, Zhang *et al* (17) implanted the rhBMP4-attached agarose beads into the mouse tooth germ, and they found after culture *in vitro* for 24 h that the cell differentiation is significantly accelerated. It is visible through the above studies that BMP-4 plays an important role in the growth of human teeth, confirming the results of this experiment. ALP activity is an important index of cell mineralization ability, and the ALP expression level can directly reflect the degree of cell differentiation. Therefore, ALP can serve as a sign of dental pulp cell differentiation and formation (18). A number of scholars regard DSPP, one of the important components of dentin non-collagen protein, as the specific protein of dentin cells (19). Besides, DMP-1 can induce mesenchymal cells to form dentin cells, and promote the formation of mineralization (20). In this experiment, therefore, RT-qPCR was performed for both groups of cells cultured for 5 and 7 days, and the expression of ALP, DSPP and DMP-1 was detected. According to the results, the expression levels of ALP, DSPP and DMP-1 in group A at day 5 after culture were obviously increased with statistically significant differences compared with those in group B ($p<0.05$), exactly illustrating that differentiation markers of dental pulp cells pre-treated with BMP-4 are remarkably increased, and also well demonstrating that BMP-4 contributes to the growth of dental pulp cells. Finally, the proportion of positive cell area was detected via ALP staining, and results displayed that the positive area in group A was significantly larger than that in group B ($p<0.05$), indicating that pre-treatment with BMP-4 can increase the ALP activity and promote cell matrix calcification.

There were also some defects in this study. Its mechanism was not investigated deeply, and its regulatory mechanism and pathways involved in regulation were not fully understood. Therefore, its mechanism will be further studied in future experiments.

In conclusion, pre-treatment with BMP-4 can effectively promote the proliferation and differentiation of dental pulp cells, providing a new method for the differentiation of dental pulp cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NS designed this study, collected and analyzed the data, as well as wrote this manuscript. TJ and CW contributed to the extraction and passage of dental pulp cells. HS performed immunocytochemical staining. QZ conducted RT-qPCR. LL recorded and analyzed cell staining. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of School of Stomatology, China Medical University (Shenyang, China). Before extraction, patients were informed and signed the informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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