Effects of Naringin on the Proliferation and Osteogenic Differentiation of Canine Bone Marrow Stromal Cells In Vitro

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Abstract: The aim of the present study was to investigate the induction of osteogenic activity and osteogenic differentiation of canine bone marrow stromal cells (BMSCs) by naringin. BMSCs were separated and cultured in vitro and identified by flow cytometry. Then, different concentrations of naringin (1×10^4, 1×10^5, 1×10^6, and 1×10^7 mol/l) were added to BMSCs cultured in DMEM to induce their differentiation. The effects of naringin on BMSCs were evaluated by CCK-8 assay and by measuring the activity of alkaline phosphatase (ALP). Calcium nodules, a marker of osteogenesis, were detected by alizarin red staining. The results of cell-surface marker analysis showed that the cells were negative for CD34 and CD45 expression, with values of 0.126% and 0.075%, respectively, and positive for CD90 expression, a value of 95.4%. Naringin at a concentration of 10^6 mol/l obviously promoted cell proliferation, among the concentrations, this concentration achieved the best effects on proliferation and osteogenic differentiation. Calcium nodule staining was positive. The findings demonstrate that naringin can induce the differentiation of bone marrow stromal cells into osteoblasts and that naringin at a concentration of 10^6 mol/l can enhance the proliferation and osteogenic differentiation of BMSCs.

Key words: Naringin, BMSCs, Osteoinduction, Bone tissue engineering

Introduction

Tooth extraction, which can result from trauma, periodontal disease, periapical periodontitis and other phenomena, often causes alveolar bone absorption, which greatly challenges later denture repair and is not conducive to implant-supported restoration. In recent years, with the development and progress of bone tissue engineering, a solution to this problem has been developed. Traditional Chinese medicine is highly valued in our country and suggests that kidneys control bone formation. Therefore, bone-disease treatment from perspective of traditional Chinese medicine often starts with efforts to tonify the kidney, and Chinese kidney-tonifying drugs have the effect of promoting osteogenesis. Traditional Chinese medicine Rhizoma Drynariae has the effects of nourishing the kidneys, strengthening bones and relieving pain. Rhizoma Drynariae has been used as an effective medicine in orthopedics departments, and is commonly used for the clinical treatment of orthopedic diseases. In this study, we intended to investigate the effects of naringin, an active ingredient of Rhizoma Drynariae, on the proliferation and osteogenic differentiation of canine BMSCs in vitro.

Materials and Methods

Adult beagle dogs, both males and females, 1~2 years of age and weighing 10~15 kg, were purchased from the Ninth People’s Hospital, School of Medicine, Shanghai Jiao Tong University. Naringin was purchased from the Sigma-Aldrich. The other chemicals used in this study were of analytical reagent grade. The Ethics Committee of Yantai Stomatological Hospital approved the study (No. 2020-008).

Culture and passage of canine BMSCs

A total of 4 ml bone marrow was extracted from the beagle by iliac bone puncture, and the primary generation of cells was cultured by the whole bone marrow culture method. BMSCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, China) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 IU/ml penicillin/streptomycin (Gibco, USA) at 37°C under 5% CO₂. When cell confluence was close to 80%, cell passaging was performed. The culture method was based on the monograph of Situ Zhenqiang.

Flow cytometry to detect cell surface antigens

Third passage BMSCs cultured in vitro were washed with PBS buffer solution, digested with 0.25% trypsin (Gibco, USA) to form single-cell suspensions, placed in centrifuge tubes and centrifuged at 1,500 rpm for 15 min, and then resuspended in single-cell suspensions with PBS buffer solution containing 2% fetal bovine serum. The cells were incubated with CD34, CD45 and CD90 (BD, USA) monoclonal antibodies in the dark for 30 min at room temperature, washed with 3 ml PBS, resuspended in 400 ul PBS containing 2% FBS, and analyzed by flow cytometry. The software FlowJo 7.6.1 was used to calculate the positive rate of cell surface antigen, which was expressed as a percentage.
CCK-8 assay to detect drug toxicity

Third generation cells were digested with 0.25% trypsin, and the cell suspension was inoculated in 96-well plates at a density of $5 \times 10^4$/ml. A total of 200 µl of culture medium was added to each well. After 24 h of culture, the culture medium and unattached cells were discarded. Culture medium containing naringin of different concentrations was added to each group successively: the $1 \times 10^{-9}$ mol/l, $1 \times 10^{-8}$ mol/l, $1 \times 10^{-7}$ mol/l, $1 \times 10^{-6}$ mol/l, and blank control groups. Each group was cultured in an incubator at 37℃ under 5% CO2 and 100% humidity. On days 1, 2, 3, 4, 5 and 6, the optical density (at 450 nm) of each well was determined with a microplate reader. A cell growth curve was plotted with T on the horizontal axis and proliferation rate on the vertical axis. The proliferation rate was calculated as the ratio of the mean absorbance value of each well in the experimental group to the mean absorbance value of the control group.

Determination of alkaline phosphatase activity

Third generation cells were digested with 0.25% trypsin to obtain cell suspensions of $1 \times 10^4$/ml, which were seeded into four six-well plates at 2 ml/well, with six sub-plates each group. After 24 h of culture, the naringin was added to each six-well plate at $1 \times 10^{-9}$ mol/l, $1 \times 10^{-8}$ mol/l, $1 \times 10^{-7}$ mol/l, $1 \times 10^{-6}$ mol/l, or $1 \times 10^{-5}$ mol/l; a blank control plate was also established. The contents of total BCA protein and ALP in cells were detected on days 1, 3, 5 and 7 using kits (AnaSpec, USA) according to the manufacturer’s instructions, and the relative increment of ALP was calculated.

Calcium nodules assay

Third-generation cells were inoculated into 6-well plates at a density of $0.4 \times 10^5$/ml per well, treated with naringin, and cultured for 14 days, with the medium changed once every 3 days. The cells were then fixed in 4% paraformaldehyde for 30 min, rinsed 3 times with ddH2O, stained with alizarin red solution for 30 min and then rinsed repeatedly with ddH2O. The calcium nodules of the cells were observed under an inverted microscope.

Statistical analysis

SPSS20.0 software was used for statistical analysis, and the results are expressed as the mean ± standard deviation. One-way ANOVA was used to determine the significance, and $P<0.05$ was considered to indicate significance.

Results

Inverted phase-contrast microscopy observation

After the first replacement of culture medium on the 8th day of primary culture, some blood cells still exist in the petri dishes, as observed under low-power microscope. There were several adherent cell clusters in the culture dishes with large spaces between them. On the 12th day, the cells were adherent to the wall and had proliferated. The cell morphology was irregular, with the cells exhibiting a polygonal, spindle shape and protrusions. On the 14th day, the number of adherent cells had increased, and the cells had converged, accounting for 70–80% of the whole area of the culture dish. In addition, cell size had increased, and the cell morphology had changed to polygonal spindles. After the first passage, the P1-generation cells were well distributed as fibroblasts. Approximately 5 days after P1-cell culture, the dishes were covered, and P2 cells were subcultured. The cells showed monolayer growth and obvious orientation (Fig. 1).
Flow cytometry detection

Flow cytometry showed that cell homogeneity was high and that CD90 was expressed in more than 95% of the BMSCs. CD34 and CD45 expression was near zero, which ruled out the possibility that the cells composed a hematopoietic stem cell line and was consistent with the expression range of BMSC surface markers\(^4\) (Fig. 2).

Testing of naringin toxicity

Among the 5 experimental groups of canine BMSCs, those treated with naringin at concentrations of \(1\times10^{-7}\) mol/l and \(1\times10^{-6}\) mol/l exhibited significant increases in cell growth and proliferation with significantly greater growth and proliferation than those observed in the other groups at the same period of culture \((P < 0.05)\) (Fig. 3).

Quantitative detection of alkaline phosphatase (ALP)

Naringin at the concentration of \(1\times10^{-6}\) mol/l promoted ALP synthesis, with the group treated with this concentration having significantly greater ALP activity than the other groups with the same culture duration \((P < 0.05)\). There was no significant difference in ALP activity among the other experimental groups (Fig. 4).
two methods are the more commonly used methods: agglutination, flow cytometry and immunomagnetic bead separation. The first cells are: whole bone marrow adherent culture, density gradient centrifugation, under appropriate conditions\(^5,6\). Cells differentiate into osteoblasts, adipocytes, chondrocytes and other cell types, and calcium deposits gradually occur, and alizarin red staining showed the formation of calcium nodules (Fig. 5).

**Discussion**

After tooth extraction, alveolar bone resorption occurs, and the mass, structure and shape of alveolar bone changes, which affect later denture repair. Studies have shown that tooth extraction following immediate filling can prevent the absorption of alveolar bone and promote its regeneration. Filling materials include autogenous bone, allogeneic bone, heterogeneous bone and artificial synthetic material. In light of the advantages and disadvantages of various materials, tissue-engineered bone has become a research and application hotspot in recent years due to its advantages and disadvantages of various materials, tissue-engineered bone has become a research and application hotspot in recent years. In light of the advantages and disadvantages of various materials, tissue-engineered bone has become a research and application hotspot in recent years due to its unlimited source, non-antigenicity, predesigned shaping potential, and biological function. Bone marrow mesenchymal cells (BMSCs) are widely used as seed cells in bone tissue engineering. BMSCs can differentiate into osteoblasts, adipocytes, chondrocytes and other cell types under appropriate conditions\(^6\). In recent years, much research on traditional Chinese medicine extracts their active ingredients and materials or the combinations of Chinese and Western medicines has been conducted with the goal of promoting bone regeneration, and have revealed the therapeutic advantages of traditional Chinese medicine. Naringin, an active ingredient in bone fractals, is one of the materials of interest. Hu Qiyong et al.\(^7\) found that naringin at concentrations \(\geq 0.01\) mol/L could significantly promote the proliferation of human periodontal ligament cells and significantly increase their ALP activity. In the present experiment, naringin at a concentration of \(1 \times 10^{-6}\) mol/L was found to significantly promote the proliferation and differentiation of canine BMSCs, consistent with literature reports\(^8\).

At present, the main methods for separating bone marrow stromal cells are: whole bone marrow adherent culture, density gradient centrifugation, flow cytometry and immunomagnetic bead separation. The first two methods are the more commonly used methods\(^9\). In the present study, the whole bone marrow culture method was adopted. The bone marrow extracted for the experiment included a large number of blood cells and a small amount of stromal cells. After adherent culture, it was possible to remove most of the blood cells, fat precursor cells and fibroblasts and BMSCs with high purity were obtained after several fluid changes and 2 to 3 subcultures. To date, no specific monoclonal antibodies have been used to detect BMSC surface antigens. CD13, CD29, CD44, CD90 and CD105 are generally considered important markers on the surface of BMSCs\(^10\), but it is clear that BMSCs do not express hematopoietic stem cell surface antigens, such as CD14, CD34 and CD45\(^11\). In this study, CD90 was highly expressed in BMSCs obtained by adherent culture of whole bone marrow, whereas CD34 and CD45 expression was almost absent, consistent with domestic and foreign literature reports\(^11,12\). Relatively pure BMSCs can be obtained in the 3rd generation.

Changes in ALP activity and calcium content are important indicators of osteoblast proliferation, differentiation and osteogenic potential. They are often used as indicators for osteoblast identification and functional evaluation\(^12\). ALP is involved in the calcium and phosphorus metabolism of osteoblasts and promotes the transfer of extracellular organophosphorus components into cells; such transfer is an early indicator of osteoblast differentiation\(^13\). ALP releases inorganic phosphorus via the hydrolysis of organophosphates to promote calcium phosphate precipitation. Furthermore, it destroys the calcification inhibitor, initiates and continues the calcification process, and participates in the synthesis of inorganic calcium phosphate\(^13\). ALP is a specific enzyme in the process of bone formation\(^13\). Its activity largely reflects the functional status and differentiation degree of osteoblasts. Its expression indicates the beginning of bone differentiation and is positively correlated with the differentiation and maturation of osteoblasts. The higher the activity of ALP is, the more obvious the bone differentiation of osteogenic precursor cells is. In vitro culture, in this study, osteoblasts continuously deposited calcium salts, most of which were found in clone centers, forming unique calcium nodules. Macroscopic observation revealed that multiple white nodules had formed at the bottom of the petri dishes. Alizarin red staining confirmed that the treatment of BMSCs cultured in vitro with naringin at \(10^{-6}\) mol/L significantly promoted calcium deposition.

In conclusion, both the qualitative and quantitative aspects of the experiment confirmed that BMSCs in culture medium containing naringin, exhibited strong proliferative ability and osteogenic activity, that the strongest effects were observed at the concentration of \(1 \times 10^{-6}\) mol/L. In addition, the findings confirmed the feasibility of using traditional Chinese medicine and bone tissue engineering to promote bone regeneration. However, the stability and safety of naringin need to be determined...
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through *in vivo* animal experiments.

**Conflict of Interest**
The authors have declared that no conflict of interest exists.

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