

The effects of *Pongamia pinnata* on osteogenic differentiation and mineralization of human stem cells derived from the gingiva

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Objectives: The aim of the present study is to evaluate the effects of the extract of *Pongamia pinnata* on the morphology, viability, and differentiation potential of human stem cells derived from the gingiva.

Methods: Stem cells obtained from gingivae were cultured in an osteogenic medium in the presence of methanol extract of *Pongamia pinnata* (PPT) at concentrations ranging from 0.001 to 1%. Evaluations of cell morphology and cellular viability were done at Day 1. Alkaline phosphatase activity assays and Alizarin red S staining were performed to evaluate the osteogenic differentiation of stem cells.

Results: The morphology of stem cells in the presence of PPT at final concentrations of 0%, 0.001%, 0.01%, 0.1%, and 1% did not produce any noticeable changes when compared with the untreated control group. Application of PPT produced a significant increase in alkaline phosphatase activity when compared to the control group. The results of the Alizarin Red S staining showed a significant increase of absorbance with the 0.001% group.

Conclusions: Based on these findings, it was concluded that PPT could produce beneficial effects on mesenchymal stem cells with enhanced osteogenic differentiation.

Key Words : cell differentiation, herbal medicine, *Pongamia*, stem cells

Introduction

Stem cells have unique properties, including the capability of dividing and renewing themselves and the ability to give rise to specialized cell types.¹⁾ Stem cells have been applied in regenerative medicine.²⁾ Herbs have been used clinically for thousands of years, and these were suggested to be an effective source of therapeutics.³⁾ Moreover, in recent years, the effects of medicinal herbs on stem

cells have been tested.³⁻⁵⁾ The application of *Cimicifugae rhizoma* increased cell proliferation without morphologic changes.⁵⁾ Similarly, *Angelicae dahuricae* radix produced a marginal increase in stem cell proliferation.⁴⁾

The effect of differentiation of stem cells has been of interest. A previous report suggested that the therapeutic potential of stem cells has limitations due to cell survival and differentiation capabilities.⁶⁾ Thus, the effects of herbal extracts on differentiation

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of stem cells have been studied.^{7,8)} Tanshinone from the *Danshen* herb is shown to enhance osteogenic differentiation of bone marrow mesenchymal stem cells.⁸⁾ Maohuoside A from *Epimedium koreanum* Nakai, one of the most used herbs in East Asia for curing osteoporosis,^{9,10)} is shown to promote osteogenesis of mesenchymal stem cells via BMP and MAPK signaling pathways.⁷⁾ Moreover, the effects of herbal extracts have been tested on stem cells derived from the intraoral area. *Zanthoxylum schinifolium* herbal extract is reported to enhance the osteogenic potential of periodontal ligament stem cells.¹¹⁾ *Pongamia pinnata* (L.) Pierre has long been used in Oriental medicine to cure various diseases, including skin diseases and infectious diseases.¹²⁻¹⁵⁾ However, the effects of *Pongamia pinnata* on stem cells have rarely been tested. The aim of the present study is to evaluate the effects of the extract of *Pongamia pinnata* on the morphology, viability, and differentiation potential of human stem cells derived from the gingiva. Within the authors' knowledge, this investigation is the first to elucidate the effects of *Pongamia pinnata* on mesenchymal stem cells derived from gingiva.

Methods

1. Preparation of the materials

Pongamia pinnata (L.) Pierre was collected from Noakhali in Bangladesh by Md Salah Uddin. A voucher specimen recoded as PB022015 was deposited in the herbarium of the Korea Research Institute of Bioscience and Biotechnology. The dried seeds of *Pongamia pinnata* were ground. Five hundred mL of methanol was added to 58 g of the powder. The repercolation method was used for the extraction process at room temperature. The resultant product was filtered and concentrated under reduced pressure. Finally, 15.04 g of methanol extract of *Pongamia pinnata* (PPT) was obtained.

2. Isolation of stem cells from human tissue

The gingivae were obtained from healthy patients visiting the Department of Periodontics, Seoul St. Mary's Hospital, College of Medicine, the Catholic University of Korea. The Institutional Review Board reviewed and approved the study (KC11SISI0348). The participants signed informed consent forms. All the procedures were performed in accordance with the relevant guidelines and regulations. Isolation procedures were done based on the previous publication.¹⁶⁾ In short, the obtained gingiva was digested with dispase (Sigma-Aldrich Co., St. Louis, MO, USA) at 1 mg/mL and collagenase IV (Sigma-Aldrich Co) at a 2 mg/mL concentration.

3. Evaluation of cellular morphology

Stem cells were plated at a density of 2.0×10^3 cells/well in an osteogenic medium (alpha-minimal essential medium (α -MEM, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, Gibco), 200 mM L-glutamine (Sigma-Aldrich Co.), 10 mM of ascorbic acid 2-phosphate (Sigma-Aldrich Co.), 38 μ g/ml of dexamethasone, 2 mg/ml of glycerophosphate disodium salt hydrate, and 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich Co.)) The final concentrations of PPT were 0%, 0.001%, 0.01%, 0.1%, and 1%, respectively. The morphological evaluation was performed on Day 1 using inverted microscopy (CKX41SF, Olympus Corporation, Tokyo, Japan).

4. Evaluation of cellular viability

The cellular viability was evaluated on Day 1 based on the previous report using the Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) assay.¹⁷⁾ In short, cells were incubated with tetrazolium monosodium salt for 2 hours at 37 °C. Spectrophotometric absorbance at 450 nm was

measured using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

5. Alkaline phosphatase activity assays

Alkaline phosphatase activity assays were done on Days 3 and 7 using a commercially available kit (K412-500, BioVision, Inc., Milpitas, CA, USA). A microplate reader (BioTek Instruments Inc.) was used to measure the spectrophotometric absorbance of the samples.

6. Evaluation of Alizarin Red S staining

On Days 3 and 7, Alizarin Red S staining was performed. In short, the cells were washed, fixed, and stained with 2% Alizarin Red S Solution (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) and evaluated with a microscope (CKX41SF, Olympus Corporation). Ten percent cetylpyridinium chloride (Sigma-Aldrich Co.) was used to solubilize the bound dye, and spectrophotometric quantification was performed at 560 nm (BioTek Instruments Inc.).

7. Statistical analysis

The data are presented as means \pm standard deviations. A test of normality was performed and a one-way analysis of variance with a post hoc test was performed to determine the differences between the groups. The tests were performed using a commercially available program (SPSS 12 for Windows, SPSS Inc., Chicago, IL, USA), and the level of significance was 0.05.

Results

1. Evaluation of cell morphology

The morphology of stem cells treated with PPT at final concentrations of 0%, 0.001%, 0.01%, 0.1%, and 1% on Day 1 is shown in Figure 1. Stem cells in the control group showed fibroblast-like morphology on Day 1 (Figure 1A). The morphology

of stem cells in the presence of PPT at final concentrations of 0%, 0.001%, 0.01%, 0.1%, and 1% did not produce any noticeable changes when compared with the untreated control group (Figures 1B–1E).

2. Cellular viability

Results from the CCK8 assay revealed cellular viability on Day 1 and are shown in Figure 2. The relative values of CCK-8 at Day 1 for 0.001%, 0.01%, 0.1%, and 1% are 94.0 ± 5.4 , 94.3 ± 7.2 , 91.1 ± 4.9 , and 86.5 ± 5.2 , respectively, when the control (0%) group at Day 1 is considered 100% ($100.0 \pm 5.1\%$) ($P > 0.05$).

3. Alkaline phosphatase activity assays

The alkaline phosphatase activity treated with PPT at Days 3 and 7 is shown in Figure 3. The absorbance values of cells on Day 3 grown with 0%, 0.001%, 0.01%, 0.1%, and 1% PPT were 0.073 ± 0.000 , 0.075 ± 0.001 , 0.083 ± 0.000 , 0.078 ± 0.002 , 0.001 , and 0.081 ± 0.001 , respectively. The treatment of PPT (from 0.001 to 1% groups) showed a statistically significant increase in alkaline phosphatase activity when compared to the control group at Day 3 ($P < 0.05$). Application of PPT produced a statistically significant increase of alkaline phosphatase activity with the highest value at 0.01%. The absorbance values of cells on Day 7 grown with 0%, 0.001%, 0.01%, 0.1%, and 1% PPT were 0.104 ± 0.001 , 0.110 ± 0.000 , 0.122 ± 0.001 , 0.130 ± 0.001 , and 0.121 ± 0.000 , respectively ($P < 0.05$). Similarly, application of PPT produced a statistically significant increase of alkaline phosphatase activity and the highest value was seen at 0.1% group.

4. Mineralization assay

The results of the Alizarin Red S staining on Days 3 and 7 following treatment with different

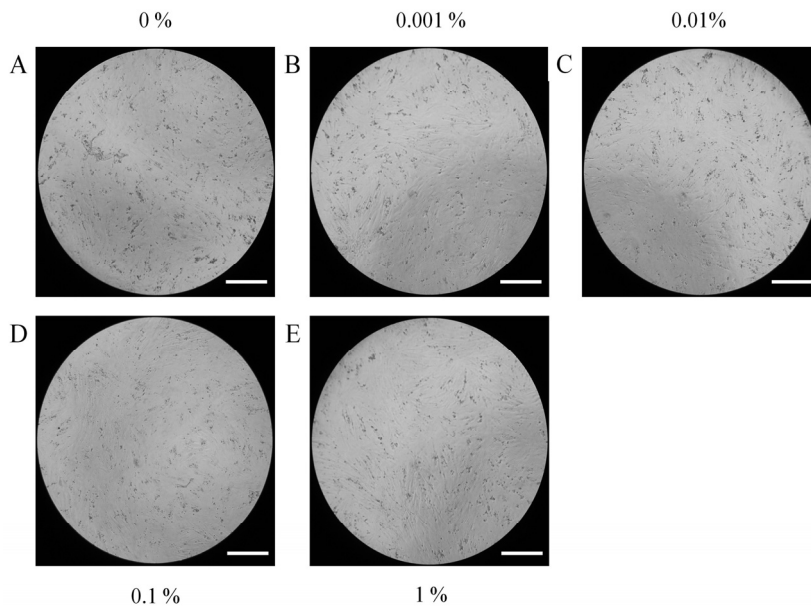


Fig. 1. Evaluation of cell morphology on Day 1 using inverted microscopy following treatment with different concentrations of PPT in osteogenic media.

- A. Control group (original magnification x 200)
 - B. 0.001% group (original magnification x 200)
 - C. 0.01% group (original magnification x 200)
 - D. 0.1% group (original magnification x 200)
 - E. 1% group (original magnification x 200)
- The bar indicates 200 μ m.

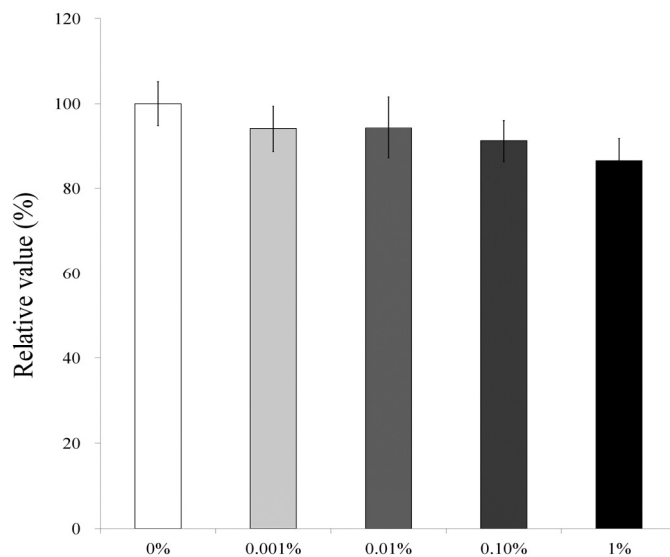


Fig. 2. The CCK-8 assay results at Day 1 cultured with osteogenic media. The data are presented as means \pm standard deviations.

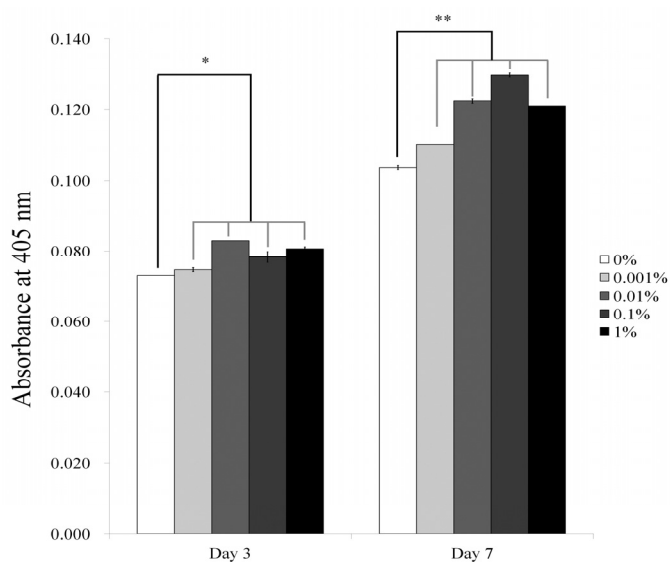


Fig. 3. Alkaline phosphatase activity on Days 3 and 7.

The data are presented as means \pm standard deviations.

* Statistically significant differences were noted when compared with the data from the control group at Day 3 ($P < 0.05$).

** Statistically significant differences were noted when compared with the data from the control group at Day 7 ($P < 0.05$).

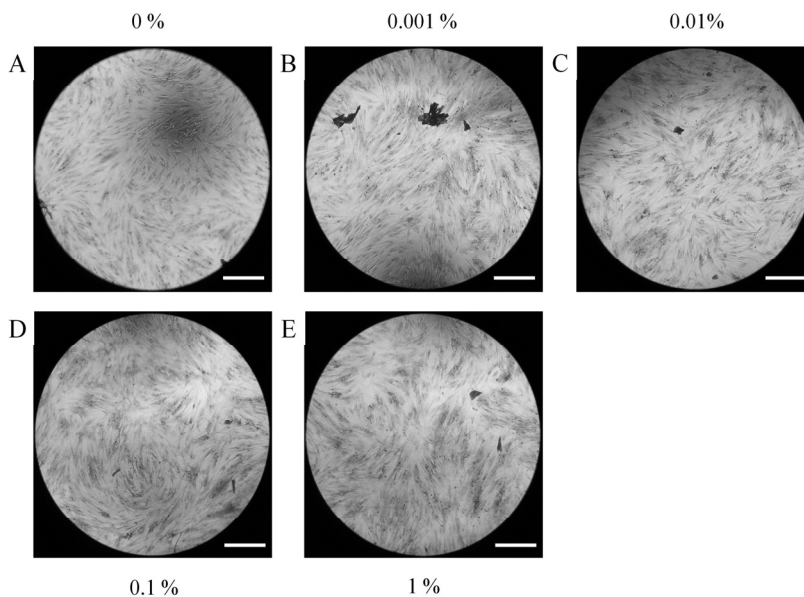


Fig. 4. Results of Alizarin Red S staining on Day 3 following treatment with different concentrations of PPT on osteogenic supplement.

A, Control group (original magnification $\times 100$)

B, 0.001% group (original magnification $\times 100$)

C, 0.01% group (original magnification $\times 100$)

D, 0.1% group (original magnification $\times 100$)

E, 1% group (original magnification $\times 100$)

The bar indicates $400 \mu\text{m}$.

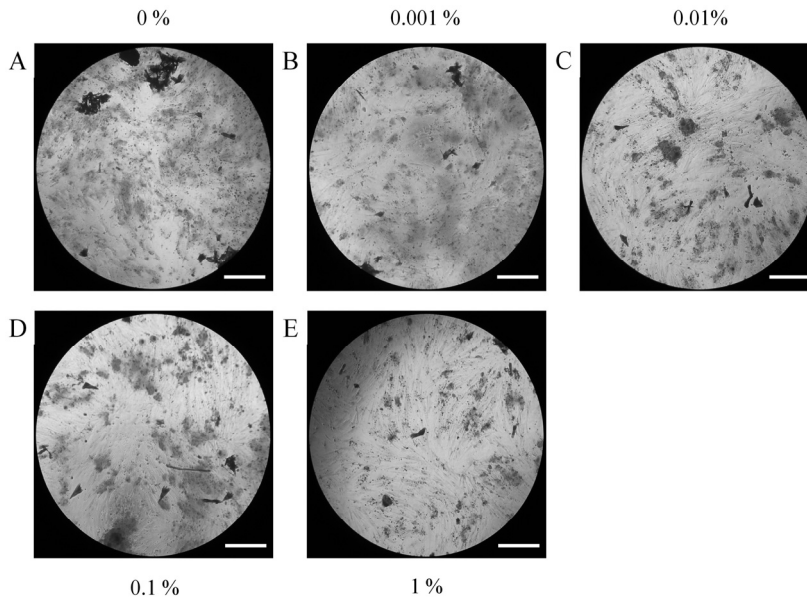


Fig. 5. Results of Alizarin Red S staining on Day 7 following treatment with different concentrations of PPT on osteogenic supplement.

- A. Control group (original magnification x 100)
- B. 0.001% group (original magnification x 100)
- C. 0.01% group (original magnification x 100)
- D. 0.1% group (original magnification x 100)
- E. 1% group (original magnification x 100)

The bar indicates 400 μ m.

concentrations of PPT in an osteogenic supplement are shown in Figures 4-5. The absorbance of cells on Day 3 cultured with 0%, 0.001%, 0.01%, 0.1%, and 1% PPT was 0.059 ± 0.002 , 0.044 ± 0.002 , 0.052 ± 0.005 , 0.049 ± 0.004 , and 0.063 ± 0.002 , respectively (Figure 6). The absorbance of cells on Day 7 cultured with 0%, 0.001%, 0.01%, 0.1%, and 1% PPT was 0.082 ± 0.002 , 0.102 ± 0.001 , 0.085 ± 0.002 , 0.084 ± 0.007 , and 0.080 ± 0.001 , respectively. A statistically significant increase of absorbance was noted with the 0.001% group on Day 7 ($P < 0.05$).

Discussion

This report discusses the effects of different concentrations of PPT on the osteogenic differentiation of stem cells derived from gingival

tissues. This study clearly showed that short-term application of PPT enhanced the proliferation of mesenchymal stem cells.

The application of a low dose of PPT enhanced alkaline phosphatase activity up to 25% and mineralization up to 24%. Alkaline phosphatase activity is used for the evaluation of early osteogenic differentiation.¹⁾ and Alizarin red S staining assay measures the presence of calcium in cellular deposits which is used for the evaluation of early matrix mineralization.¹⁷⁾ Alkaline phosphatase activities increased between 3 and 7 days and mineralization increased between 3 and 7 Days. The highest alkaline phosphatase activity was achieved with 0.01% PPT on Day 3 and 0.01% group on Day 7. A significant increase in mineralization was

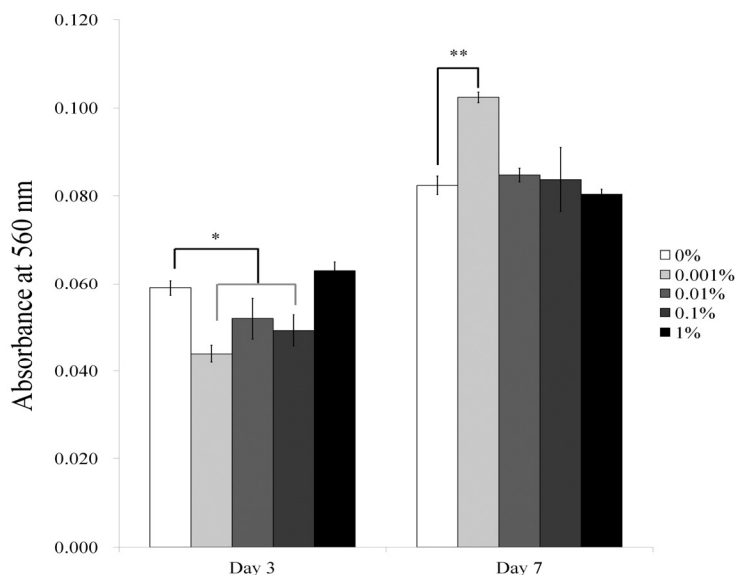


Fig. 6. Quantitative results of Alizarin Red S staining on Days 3 and 7.

The data are presented as means \pm standard deviations.

* Statistically significant differences were noted when compared with the data from the control group at Day 3 ($P < 0.05$).

** Statistically significant differences were noted when compared with the data from the control group at Day 7 ($P < 0.05$).

achieved in the 0.001% PPT group on Day 7. Different dosages may produce different effects on stem cells. A previous report showed that lower concentrations of a traditional herb enhanced the total metabolic activity and their alkaline phosphatase activity expression, but high concentrations significantly depressed the cell population.¹⁸⁾ It should be considered that there could be variations in doses to achieve the maximal effect, depending on the stage of cultured cell, the system, and the culture period.¹⁹⁾

In this study, methanol extracts of *Pongamia pinnata* were used. Various methods can be used for the extraction of herbs, including cold pressing, expeller pressing, and solvent extraction.^{3-5,11)} Different kinds of solvents including ethanol, methanol, butanol, hexane, methylene chloride, ethyl acetate, and water can be used for the extraction. A

relatively higher temperature is applied in expeller pressing when compared with cold pressing.²⁰⁾ Previous report showed that methanol extract showed higher functionality without compromising cellular viability and this study used methanol extract of *Pongamia pinnata* for the evaluation.²¹⁾ Different alcohol extracts including methanol, ethanol, and butanol may have different effects with different boiling points and solubility.^{22, 23)}

Human stem cells have been isolated and characterized from various tissues including bone marrow, periosteum, adipose tissue, and muscle.¹⁶⁾ Previous reports showed that mesenchymal stem cells derived from gingival tissue could be a novel source for stem cell-based therapy in bone reconstruction in clinical applications.²⁴⁾ It was shown that mesenchymal stem cells derived from human gingival tissue were superior to bone marrow-derived mesenchymal stem cells for cell therapy in

regenerative medicine.²⁵⁾ Similar to another source of stem cells, immunomodulatory properties were found with dental tissue-derived mesenchymal stem cells.²⁶⁾ It should be emphasized that stem cells derived from gingival tissue have several advantages, including ease of isolation, accessible tissue source, and rapid ex vivo expansion.²⁷⁾

Herbal extracts have been delivered with the use of scaffold materials including calcium silicate and tricalcium phosphate.^{28,29)} Calcium silicate has been applied with herbal extracts after seeding with human adipose-derived stem cells, and it was shown that the use of herbal extracts produced higher alkaline phosphatase activity, greater osteocalcin protein secretion, and clearly calcified tissue mineralization.²⁸⁾ Extracts from Epimedium were incorporated into poly lactic-co-glycolic acid/tricalcium phosphate using fine-spinning technology, and the supplement of scaffold materials with herbal medicine demonstrated enhanced bone repair with sustainable release.²⁹⁾

Based on these findings, it was concluded that PPT could produce beneficial effects on mesenchymal stem cells with enhanced osteogenic differentiation.

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Competing interests

The authors report no conflicts of interest related to this study. The author does not have any financial interest in the companies whose materials are included in the article.

Ethics statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consents were received from the participants.

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