

The Effects of *Moringa Oleifera* Lam. Leaves Extract on Osteogenic Differentiation of Porcine Bone Marrow Derived Mesenchymal Stem Cells

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Abstract— *Moringa oleifera* Lam., the Asian herbal plant, has various uses, such as anti-oxidation, and anti-inflammation. We have investigated the effect of *Moringa oleifera* Lam. leaves extract on osteogenic differentiation of porcine bone marrow derived mesenchymal stem cells. The potential of MSC for osteogenic differentiation has been well documented in many animal models especially on rodents. Despite the increasing importance of the pig as a large animal model. Previous studies have shown the benefits of *Moringa oleifera* leaves on anti-osteoporotic effect in osteoblastic cells. Therefore, this study aims to examine the effect of 100, 200, and 300ng/ml of ethanolic extract of *Moringa oleifera* Lam. leaves on osteogenic differentiation of porcine MSCs derived from bone marrow. The degree of osteogenic differentiation after *Moringa oleifera* Lam. leaves extract treatment was assessed by alkaline phosphatase (ALP) expression and ALP activity. The results showed that ethanolic extract of *Moringa oleifera* Lam. leaves enhanced osteogenic differentiation capacity of porcine bone marrow derived MSCs as demonstrated by increased ALP staining and ALP activity. The enhancement of osteogenic differentiation capacity of MSCs by those of *Moringa oleifera* Lam. leaves extract leads to the potential for using those for bone regeneration in pre-clinical models and bone alternative medicine.

Keywords—*Moringa oleifera* Lam. leaves, porcine bone marrow derived mesenchymal stem cells, osteogenic differentiation.

I. INTRODUCTION

Mesenchymal stem cells (MSC) are plastic adherent stromal cells found in special tissues and organs. With the capacity for self-renewal and multi-lineage differentiation, they are considered a promising cell source for tissue engineering, as they are easily accessible and not associated with ethical issues in relation to their use (1). It has been widely accepted that MSC based therapy has shown significant improvement of tissue regeneration in pre-clinical models and clinical trials. Despite the recent progress in MSC-based tissue regeneration in the last few decades, a major challenge remains how to restore new bone formation following disease or insult with the high quality and the bone volume that meet the needs of the body (2, 3).

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Purified and expanded MSC from various species have been shown to differentiate along osteogenic (4), chondrogenic (5), and adipogenic (6). Ringe and co-worker (7) established a protocol for the isolation of porcine MSC to compare their phenotype and their multilineage potential with human MSC. They report that the techniques used for osteogenic differentiation of porcine MSCs, which may serve the increasing demand for stem and progenitor cells in tissue engineering. As a result of the presented similarities between porcine and human MSC, porcine MSC are likely to be considered as a valuable model system for skeletal research.

After conducting several experiments on *Moringa oleifera* Lam. leaves (MOL) extract and understanding its osteoprotective effect, it was learned that this plant is having miraculous effect on bone integrity and it potentially improve osteogenesis. Various workers have indicated that this plant is having various phytochemicals, especially phytoestrogens which can have positive effect on bone. Recent work by Zhang and co-workers (8) has shown that flavonoids can stimulate osteoblastic proliferation and differentiation. In another study, Vali and his colleagues (9) also proved that Epigallocatechin-3-gallate like flavonoids induce bone mineralization and bone nodule formation. As MOL extract is rich in various flavonoids, one can speculate about osteoblastogenic potential of this plant (10, 11). In the present study, we examined whether MOL extract treated porcine bone marrow derived mesenchymal stem cells is capable of accelerating the osteogenic differentiation *in vitro*.

II. MATERIALS AND METHODS

A. Preparation of Extract

Leaves of *Moringa oleifera* Lam. were obtained in Phra Nakorn Sri Ayutthaya. Dried powder was prepared by drying *Moringa oleifera* Lam. in oven at 50° C. 100 gm dried powder of each component was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours. Methanolic extract was dried on water bath at 55° C. The leaves extract was freeze dried and stored at -70° C. Working solution was prepared by dissolving the extract in phosphate buffered saline (PBS).

B. Cell isolation and culture

Isolation and culture of porcine bone marrow derived mesenchymal stem cells (pBM-MSCs) from adult pig bone marrow. Bone marrow was aspirated from finished pig (n = 3; 5–6 months of age) collected at a local slaughter house. The marrow was drawn from femoral marrow cavity into syringes containing 1000 U heparin. Bone marrow samples were washed twice with PBS and twice with DMEM. Subsequently, the cells were washed twice with washing buffer and cultured in completed medium containing Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA), 2 mM L-glutamine (GibcoBRL, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was changed every 3-4 days. The plastic adherent fibroblast-like cells or so-called pBM-MSCs (about 80-90% confluence) were sub-cultured using 0.25% trypsin-EDTA (GibcoBRL, USA) and replated at density of 1×10^5 cell/cm² for further expansion.

C. Cell proliferation assay in vitro

The effect of 100, 200, 300, and 400 ng/ml ethanolic extract of MOL on pBM-MSCs proliferation was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay. *Ex vivo* expanded pBM-MSCs were seeded at passage three (1.0×10^4 cells/ well) in triplicate using a 96-well flat-bottom plate (Costar, Cambridge, MA, USA) and maintained in 100 µl medium with 100, 200, and 300 ng/ml ethanolic extract of MOL or standard culture medium for five days. Cells were treated with 5 mg/ml of MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 38 °C for 4 h. After cells were washed twice in PBS and treated with dimethyl sulfoxide, the absorbance in each well was measured at a wavelength of 490 nm using an automatic enzyme-linked immunosorbent assay (ELISA) reader (ELx800; BioTek Instruments Inc., Winooski, VT, USA).

D. Osteogenic differentiation

pBM-MSCs were plated into 35-mm tissue culture dishes and cultured in completed medium at 38°C for 24h. Subsequently, the cells were cultured in osteogenic differentiation medium containing DMEM supplemented with 10% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.1 µM dexamethasone (Sigma, USA) and 50 µg/ml ascorbic acid (Sigma, USA) in the 100, 200, and 300 ng/ml ethanolic extract of MOL. On day 7 of culture, 10 mM β-glycerophosphate (Sigma, USA) was added into each cultured dishes. Cells were harvested on 3, 7, and 14 of cultures for measuring alkaline phosphatase (ALP) activity using 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP; Sigma, USA) as a substrate. Cells cultured in completed medium served as a control and were treated in parallel to the experiment.

The quantitative alkaline phosphatase activity assay was measured using SensoLyte® pNPP alkaline phosphatase assay kit (AnaSpec, USA). Briefly, MSCs were cultured for 3, 7, and 14 days. ALP activity was measured by using microplate reader (BioTex, USA) using absorbance at 405 nm. The ALP

activity in each sample was calculated by comparing the measure OD values against a standard curve generated from 0 - 10 ng/ml of alkaline phosphatase standard solution. Each assay condition was done in triplicate and normalized with the concentrations of total cellular proteins using Bradford assay (Bio-Rad, USA).

E. Statistical analysis

Data analysis was carried out using SPSS10 statistical software. Data points are reported as the mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Statistical significance of (*) $p \leq 0.05$ was determined using the unpaired Student t-test or one-way analysis of variance (ANOVA). Statistical analysis was performed as described using at least three biological replicates unless otherwise stated.

III. RESULTS

A. Culture of pBM-MSCs

After primary culture, pBM-MSCs exhibited a morphologically homogeneous spindle-like population as shown in Figure 1. During the culture period, primary or passaged UC-MSCs displayed fibroblast-like morphological features, without visible morphologic alteration.

B. The effect of ethanolic extract of *Moringa oleifera* Lam. leaves on pBM-MSCs proliferation

pBM-MSCs were treated with ethanolic extract of MOL at various concentrations (50, 100, 200, 300 and 400 ng/ml) before the rate of cell proliferation was examined with the MTT assay. Ethanolic extract of MOL at 100 µg/ml was shown to enhance pBM-MSCs proliferation. Ethanolic extract of MOL at high concentrations (400 ng/ml) seemed to display an inhibitory effect on cell proliferation (Fig.2).

C. Ethanolic extract of *Moringa oleifera* Lam. leaves stimulates the osteogenesis of pBM-MSCs in vitro

To determine the effect of the ethanolic extract of MOL on the osteogenic differentiation of pBM-MSCs, the cells were cultured in osteogenic differentiation medium in the 100, 200, and 300 ng/ml ethanolic extract of MOL for 14 days (Fig. 3). After induction for 7 days, the expression of ALP was observed in pBM-MSCs cultured with osteogenic differentiation medium in the absence of ethanolic extract of MOL. Interestingly, treatment with 300 ng/ml ethanolic extract of MOL during osteogenic differentiation increased the ALP expression. In addition, pBM-MSCs treated with 300 ng/ml ethanolic extract of MOL for 14 days exhibited higher ALP expression (Fig. 3) as compared to pBM-MSCs treated with 300 ng/ml ethanolic extract of MOL for 7 days. The result of alkaline phosphatase staining also indicated the higher expression of alkaline phosphatase in pBM-MSCs treated with ethanolic extract of MOL for 14 days as compared to the 100, 200, and control groups respectively (Fig. 3).

D. ALP activity in pBM-MSCs

The activity of intracellular ALP in pBM-MSCs was also quantitatively assessed using colorimetric enzymatic assay at day 3, 7, and 14. The results demonstrated that the pBM-

MSCs cultured in osteogenic differentiation medium supplemented with 300 ng/ml ethanolic extract of MOL showed a clear superiority compared to the others (MSCs cultured in complete medium and MSCs cultured in osteogenic differentiation medium) (Fig. 4). As early as 14 days after ethanolic extract of MOL induction, ethanolic extract of MOL induced *p*BM-MSCs had about 2.5 folds increase in alkaline phosphatase activity. ($p < 0.05$).

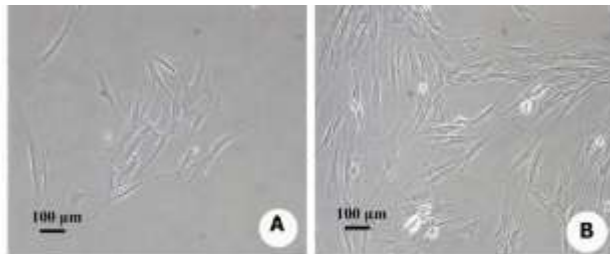


Fig 1. Morphology of primary cultured *p*BM-MSCs. A: Spherical-shaped cells at day 7 after the initial seeding; B: Adherent cells at day 3 of passage 3. Scale bar = 100 µm.

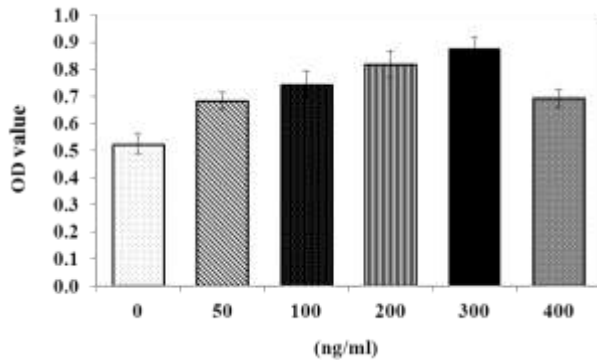


Fig. 2. Ethanolic extract of MOL at 50, 100, 200, and 300 ng/ml enhances *p*BM-MSCs proliferation, while ethanolic extract of *Moringa oleifera* Lam. leaves at high concentrations displayed an inhibitory effect on cell proliferation, shown by MTT assay.

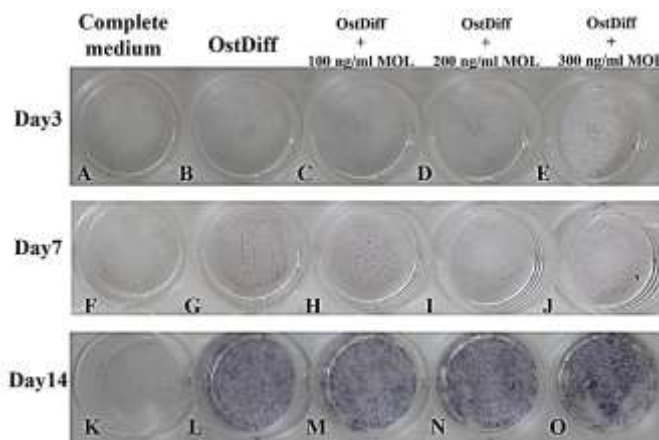


Fig. 3. The expression of alkaline phosphatase in *p*BM-MSCs cultured in osteogenic differentiation medium supplemented with 100, 200, and 300 ng/ml MOL for 3 (C, H, M), 7 (D, I, N), and 14 days (E, J, O, respectively) in comparison to those of MSCs cultured in osteogenic medium without MOL (B, G, L, respectively). *p*BM-MSCs cultured in DMEM supplemented with 10% FBS served as negative control (A, F, K, respectively).

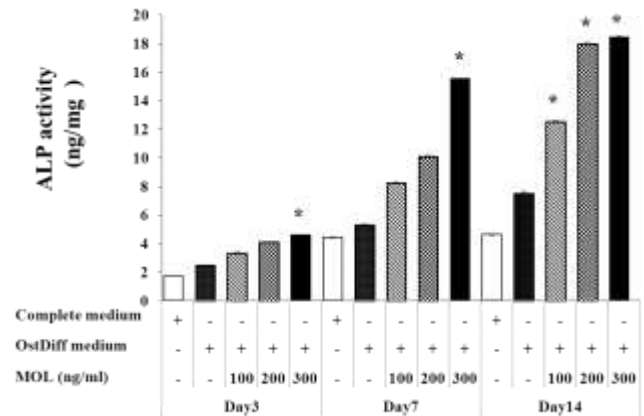


Fig 4.: Alkaline phosphatase activity of MOL induced osteogenic differentiation of *p*BM-MSCs. Data are expressed as mean \pm SEM. * $p < 0.05$: significant difference in comparison to MSCs cultured in osteogenic differentiation medium.

IV. DISCUSSION

The multi-lineage differentiation potential of MSCs has been extensively studied and culturing conditions for *in vitro* differentiation has been established. Although much progress has been made regarding the osteogenic differentiation process in the last decade, the restricted quantity and quality of functional osteocytes are the main obstacles for the therapeutic application. Exploratory for the new mediators that are able to enhance the osteogenic differentiation capacity of MSCs is thus necessary. This is the first study demonstrated that MOL treatment could enhance the osteogenic differentiation of *p*BM-MSCs as demonstrated by the increased expression of alkaline phosphatase activity and enhances ALP expression.

Treatment with MOL increased alkaline phosphatase activity as evidenced on day 7 and day 14 after osteogenic induction. This result is similar to the previous study which indicated that feeding of MOL to bilaterally ovariectomised rat can reduced bone loss (12). Moreover, methanolic extracts of MO components were found to have significant osteoblast stimulating property on osteoblastic cells (13). Due to flavonoid in ethanolic extract of MOL could induce *p*MSCs derived from bone marrow to undergo osteogenic differentiation (14).

As discussed by Ringe and co-worker (7), porcine MSC from bone marrow have been isolated routinely, and the mesenchymal differentiation pattern exhibits a reproducible and stable phenotype. MSC may be a source of easily accessible mesenchymal progenitor cells for the evolving field of tissue engineering to regenerate bone, cartilage, tendon, and adipose tissue, autologously. Due to the similarity between porcine and human MSC, swine may provide a useful animal model system to study and evaluate tissueengineered applications based on mesenchymal stem cells.

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