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Original Article

Effects of Baimai ointment medicated plasma on osteogenic differentiation of mesenchymal stem cells *in vitro*

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Abstract: The aim of the current study was to observe the effects of Baimai ointment (MPBM) medicated plasma for stimulation of osteogenesis in mesenchymal stem cells (MSCs) *in vitro.* This study tested both rat plasma and medicated plasma (BM to stimulate MSCs). CCK-8 assays were employed to detect proliferation levels of MSCs. This study also applied alkaline phosphatase (ALP) staining and Alizarin Red S staining to detect reactive osteogenic differentiation of MSCs. Moreover, mRNA levels of *ALP*, *OCN*, *Runx2*, *Col1a1*, *Col1a2*, *OPN*, and *OPG* were quantified using RT-qPCR. Protein levels of Akt and Wnt/β-catenin signaling pathways were measured using Western blotting. Compared with the control group, there were significant increases in proliferation of MSCs, as well as increased mRNA expression of *ALP, OCN, Runx2, Col1A1, ColA2, OPN*, and *OPG,* in the MPBM group ($P < 0.05$). Accordingly, phosphorylated Akt (Ser473, Thr308) and β-catenin signals were strongly activated in the MPBM group ($P < 0.05$). Results indicate that MPBM enhanced proliferation and osteoblastic differentiation of MSCs via Akt and Wnt/β-catenin signaling pathways, suggesting BM as a promising therapeutic drug for improved fracture healing.

Keywords: Baimai ointment (BM), rat, mesenchymal stem cell (MSC), osteogenic differentiation

Introduction

Bone fractures are typically caused by falls, trauma, and other secondary diseases, including diminished bone density and osteoporosis. Fractures are often accompanied by various degrees of injury to surrounding soft tissues. This results in compromised mobility, negatively impacting patient quality of life [1, 2]. Therefore, clinical management of fractures focuses on obtaining bone healing in the shortest possible time frame. The aim is to obtain the best possible functional recovery with the least number of complications. Some problems with bone healing can occur after bone trauma. Approximately 5% to 10% of bone healing cases are complicated by delayed union or non-union [3, 4].

MSCs, also known as multipotent mesenchymal stromal cells, are non-hematopoietic stromal stem cells. These cells mainly reside inside the bone marrow. They have been identified in a wide range of other tissues in the human body, including the brain, thymus, lungs, liver, spleen, and kidneys, among others [5-7]. MSCs are able to self-replicate and differentiate into a variety of cell types, including chondrocytes, osteocytes, and adipocytes [8, 9]. Osteogenic differentiation of MSCs is essential for maintenance of bone quality and quantity. Increased osteogenic potential of MSCs has been associated with increased bone formation and mechanical strength, translating to accelerated bone healing [10]. Cell therapy for delayed union and non-union after bone fractures using MSCs has been proposed [11, 12].

Baimai ointment (BM), a product manufactured by Tibet Cheezheng Tibetan Medicine, is a traditional Tibetan formula. Its anecdotal history spans thousands of years. The ointment is used

Table 1. Ingredients of Qingpeng ointment (Cheezheng®*)

Ingredients	Chinese name	Dosage
Curcumaelongae Rhizoma	Jiang Huang	36.3 g
Myristicae Semen	Rou Dou Kou	12.1 g
Nardostachyos Radix Et Rhizoma	Gan Song	19.4 g
Actinolite	Yang Qi Shi	12.1 g
licorice	Gan Cao	17.0 g
Moschus	She Xiang	0.17 g
Zingiberis Rhizoma	Gan Jiang	24.2 g
Carumcarvi	Zang Hui Xiang	31.5 g
Acorus calamus	Zang Chang Pu	17.0 g
Zanthoxyli Pericarpium	Hua Jiao	12.1 g
Alkali flowers	Jian Hua	18.2 g

*Standard number: Guo Jia Yao Pin Biao Zhun YBZ14322006-2012Z; License number: Guo Yao Zhun Zi Z20043178. Baimai ointment quality was examined according to Pharmacopoeia of The people's republic of China (Chinese Pharmacopoeia), edition 2010, Part I, appendix I R.

to relax tendons and activate collaterals. It is usually applied for treatment of traumatic injuries to meridians and tendons, tendon rigidity, clonus of the hands and feet, paralysis, hemiplegia, and limps. A previous clinical study provided evidence that topical application of BM can promote bone healing and nerve function recovery [13].

Some studies have demonstrated that complementary and alternative medicine can accelerate bone healing under specific conditions [14]. It would be beneficial to understand the active ingredients that promote bone healing [15]. Based on clinical observations, it was hypothesized that BM may promote bone fracture healing through stimulation of MSC osteogenic differentiation *in vitro*. The current study, therefore, investigated the effects of Baimai ointment (MPBM) medicated-plasma on osteogenic differentiation of MSCs *in vitro*.

Materials and methods

Cell culture

Rat primary MSCs were purchased from Cyagen (Cyagen Biosciences Inc. in Guangzhou, China). MSCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biowest, France), supplemented with 10% fetal bovine serum (FBS, Biowest, France) and 1% penicillin-streptomycin (T150731G001, Cyagen) in humidified 5% CO_2 at 37°C. Media were replaced every other day.

Preparation of BM

BM was obtained from Cheezheng Tibet Medicine using an herbal medicine license (Number Z20043178 Cheezheng Tibet Medicine Co. Ltd., Gansu, China). The ointment contained eleven traditional medicinal components (**Table 1**). Preparation of BM included the following steps. Except for moschus, the other 10 herbs were mixed and crushed into fine dust powder. This powder was added to a mixture of liquid paraffin, glycerol, polysorbate 80, methyl 4-hydroxybenzoate, and water. The mixture was stirred continuously at 80°C, then cooled. When the temperature dropped to 38°C, moschus was added. The mixture was stirred well until solidified. The quality of BM was tested and monitored by the China Food and Drug Administration (CFDA).

Preparation of rat medicated plasma

Rat medicated plasma was prepared according to published protocol [16, 17]. Twenty male Sprague-Dawley rats (Sippr BK Laboratory Animals Ltd, Shanghai, China), weighing approximately 200 grams, were randomly divided into control (n = 10) and MPBM groups (n = 10). They were housed in a 20°C-25°C air-conditioned room with a humidity level of 45% to 65%. They were housed under a 12-hour light-dark cycle and fed a standard diet with free access to tap water. Before preparation of the plasma, the rats fasted for 16 hours. The MPBM group was exposed to BM (0.09 g/kg body weight) by application to the dorsal skin of the mice, twice a day for three consecutive days. For the control group, only the vehicle solvent of BM was applied. One hour after the last administration, blood from the rats was collected and centrifuged (3,000 rpm/min for 20 minutes), obtaining the plasma. Finally, the plasma was sterilized by filtration and stored at -20°C prior to use.

Treatment of MSCs

MSCs were cultured with either 5% rat plasma in DMEM (control group) or 5% MPBM in DMEM (MPBM group), respectively. The groups were

Table 2. Primer sequences for qPCR

Gene name	GeneBank accession no.	mRNA sequences (5'-3')
ALP-F	NM_013059.1	TTCAACGGCACAGTCAAGG
ALP-R		CTCAGCACCAGCATCACC
OCN-F	M25490.1	GCAGGGATAACGGACTGAAG
OCN-R		GAGTAAAGTGGTCATAGTTCAGCTTG
Runx2-F	NM_001278483.1	ATCTTCAAGGCGCTGCAA
Runx2-R		CGGTGGACCCTGAGATTG
OSX-F	AY177399.1	AGCCATCCTGTTCACCAGAG
OSX-R		CATTCCCAGGGTGTCACAT
Col1a1-F	NM_053304.1	CATGTTCAGCTTTGTGGACCT
Col1a1-R		GCAGCTGACTTCAGGGATGT
Col1a2-F	NM_053356.1	CCTGGCTCTCGAGGTGAAC
Col1a2-R		CAATGCCCAGAGGACCAG
OPN-F	M14656.1	CCTCTGCATGAAGACGACATAA
OPN-R		GGTCAGGTTTAGAGCCACGA
OPG-F	U94330.1	AAGGAGCACAAACATGGCTG
OPG-R		TCTTAGGGTCTCGGAGGGAA
GAPDH-F	NM_031054.2	GTCGCCCATCATCAAGTTCC
GAPDH-R		GCATGGTCTCGATGGTGTTC

ALP = Alkaline phosphatase; *OCN* = Osteocalcin; *RUNX2* = runt-related transcription factor 2; *OSX* = Osterix; *Col1a1* = type I collagen, alpha 1 chain; *Col1a2* = type I collagen, alpha 2 chain; *OPN* = osteopontin; *OPG* = osteoprotegerin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

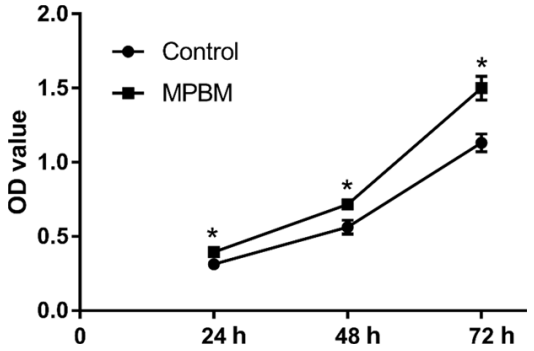

Figure 1. Comparison of proliferation abilities of MSCs between the groups. *P < 0.05, compared with the control group.

cultured for 24, 48, or 72 hours, then harvested for further analysis.

Cell counting kit (CCK-8) assay

Cell proliferation was measured using CCK8 assays (Dojindo, Japan). MSCs (3,000 cells/well) were incubated in 96-well plates. They were treated, as previously described, for 24, 48, and 72 hours. Following treatment, 10 μL of CCK-8 solution was added to each well, incubating for 0.5 hours at 37°C. Optical densities of each well were determined using a microplate reader (SYNERGY4, USA) at a wavelength of 450 nm. At least three independent experiments were performed.

Real-time quantitative PCR (RT-qPCR)

Total RNA was collected from cultured cells using TRIzol Reagent (Cat. No 15596-026, Invitrogen). First strand cDNA was synthesized using the RT Reagent Kit (Takara code DRR037A). Moreover, qPCR was performed with SYBR Premix Ex Taq™ (Cat. no. RR420R). The delta-delta Ct method was employed to analyze results. Specific primers for ALP, OCN, Runx2, OSX, Col1a1, Col1a2, OPN, and OPG are listed in (**Table 2**). These experiments were independently repeated three times.

Alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining

The cells were fixed in 4% paraformaldehyde at room temperature, then washed with PBS three times. Alkaline phosphatase (ALP) staining was performed using an ALP stain kit (Takara Bio Inc., USA), following manufacturer specifications. For detection of mineralized nodules, the cells were washed three times with PBS and fixed in 95% ethanol. Alizarin red S (Cat. No A5533; Sigma-Aldrich, Switzerland) was left on the plates overnight. The cells were then washed with water and examined under an upright microscope (Olympus BX53, Japan).

Western blotting

To examine protein expression, the cells were collected in lysis buffer (Beyotime, P0013B) containing PMSF. The lysates were centrifuged at 12,000×*g* at 4°C for 10 minutes. Protein concentrations were measured using a BCA Protein Assay Kit (Cat. No 23227, Pierce, USA). Proteins were separated using 15% SDS-PAGE and electroblotted to polyvinylidene fluoride (PVDF) membranes, using standard protocol. These membranes were incubated with primary antibodies and probed with corresponding secondary antibodies. Enhanced ch-

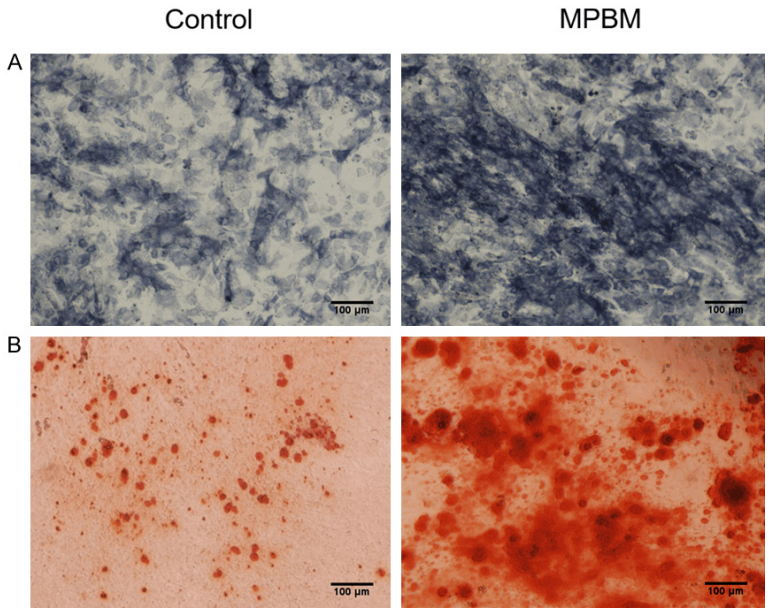

Figure 2. A. ALP histochemical staining of induced MSCs at day 7. Original magnification: 100×. B. Microscopic illustrations of induced MSCs stained by Alizarin Red S staining at day 21. Original magnification: 100×.

emiluminescence (Pierce Biotechnology, Rockford, USA) was used for protein visualization. The following antibodies were utilized: p-Akt Ser473 (1:1,000 dilution, CST, USA), p-Akt Thr308 (1:1,000 dilution, CST, USA), Akt (1:1,000 dilution, CST, USA), β-catenin (1:1,000 dilution, CST, USA), and GAPDH (1:2,000 dilution, CST, USA).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Additionally, Student's *t*-test was used to determine statistically significant differences between both groups. *P*-values ≤ 0.05 indicate statistical significance. Statistical analysis was conducted using SPSS 16.0 (SPSS Inc, Chicago, IL, USA) software.

Results

Effects of BM on MSCs proliferation

Proliferation of MSCs was measured via CCK-8 assays. CCK-8 results suggested a faster growth rate of MSCs in the MPBM group. OD490 values of the MPBM group were much higher than those of the control group at 24, 48, and 72 hours. Compared to the control group, the proliferation ability of treated MSCs was highest at 72 hours (**Figure 1**).

Effects of BM on MSCs ALP staining and Alizarin Red S staining

ALP activity is a marker of early-stage osteogenesis. MSCs were observed via ALP histochemical staining after osteogenic differentiation for 7 days (**Figure 2A**). Compared to the control group, higher ALP activity was observed in the MPBM group.

Mineralization of inducted MSCs was evaluated using Alizarin Red S staining on day 3 and day 7 of osteogenic differentiation (**Figure 2B**). The cells were enclosed by a mineralized matrix that was rich in calcium deposits, as evidenced by Alizarin Red S-positive regions. Compared to the control group, the MPBM group showed more Alizarin Red S-positive regions.

Effects of BM on MSC differentiation

Expression of osteogenic genes, including ALP, OCN, Runx2, OSX, Col1a1, Col1a2, OPN, and OPG, was determined via RT-qPCR after 7 days of induction. RT-qPCR analysis revealed that mRNA levels of genes associated with osteogenic differentiation, except for OSX, were significantly higher in the MPBM group than in the control group (**Figure 3**).

Effects of BM on MSCs Akt and β-catenin signaling activation

To determine the effects of MPBM on Akt and β-catenin signaling, Western blot analysis was conducted on the MSCs. There were significant differences in p-Akt (Ser473), p-Akt (Thr308), and β-catenin protein expression between the two groups. MPBM significantly increased p-Akt (Ser473), p-Akt (Thr308), and β-catenin protein expression, compared to the control group (**Figure 4**).

Discussion

Bone fracture healing is a complex process. MSCs have been shown to play a role in bone

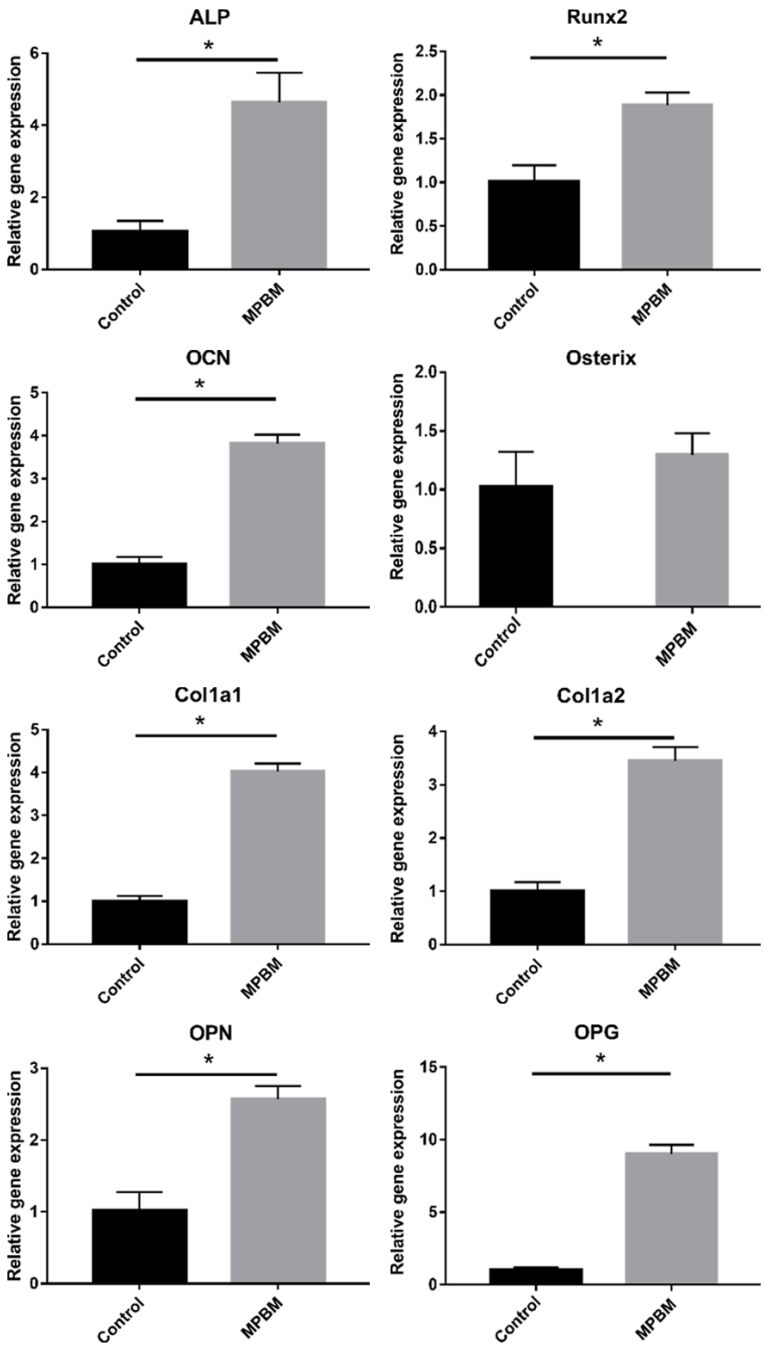

Figure 3. Osteogenic gene expression of induced MSCs at day 7. *P < 0.05 versus the control group.

remodeling and bone fracture healing [18]. The efficiency of MSCs, in terms of proliferation and differentiation, is crucial in the treatment of patients with bone non-unions or bone defects. Thus, exploring safe and effective treatments that regulate proliferation and osteogenic differentiation of MSCs is of vital significance [19].

The present study, using CCK-8 assays, showed that MPBM can enhance the proliferation of MSCs *in vitro*. Present findings suggest that BM could assist in bone fracture healing through the promotion of proliferation of MSCs. For example, BM was shown to stimulate BMSCs and promote osteogenic differentiation, as assessed by ALP and Alizarin Red S staining. ALP, a functional enzyme in osteoblasts, plays a major role in bone formation and bone mineralization. Therefore, the activity of ALP is often used to confirm the presence of osteoblasts and the degree of osteogenic differentiation [20, 21]. Alizarin Red S staining is generally employed to determine the degree of extracellular bone matrix nodules [22]. MPBM not only significantly upregulated expression of ALP, but also exhibited superior ability in stimulating MSCs to perform calcium deposition, compared with normal rat plasma.

The biological behavior of MSCs is closely connected with cell signaling pathways. Akt and Wnt/β-Catenin signaling pathways play important roles in proliferation and osteogenic differentiation of MSCs [23, 24]. Akt is a downstream serine-threonine kinase that transmits survival signals from growth factors [25]. β-Catenin, the core molecule of canonical Wnt/β-Catenin signaling, also plays an important role in MSC proliferation and differentiation, especially in osteoblast genesis and bone formation [26]. Compared to the untreated control group, the current study found that not only had β-Catenin increased expression, but expression levels of P-Akt (Th308 and Thr473) were also elevated following MPBM treatment. During the osteogenic differentiation process, MPBM promoted expression of osteogenic genes, including Wnt/β-catenin related genes, ALP, OCN, Runx2, Col1a1, and OPG [27, 28], as

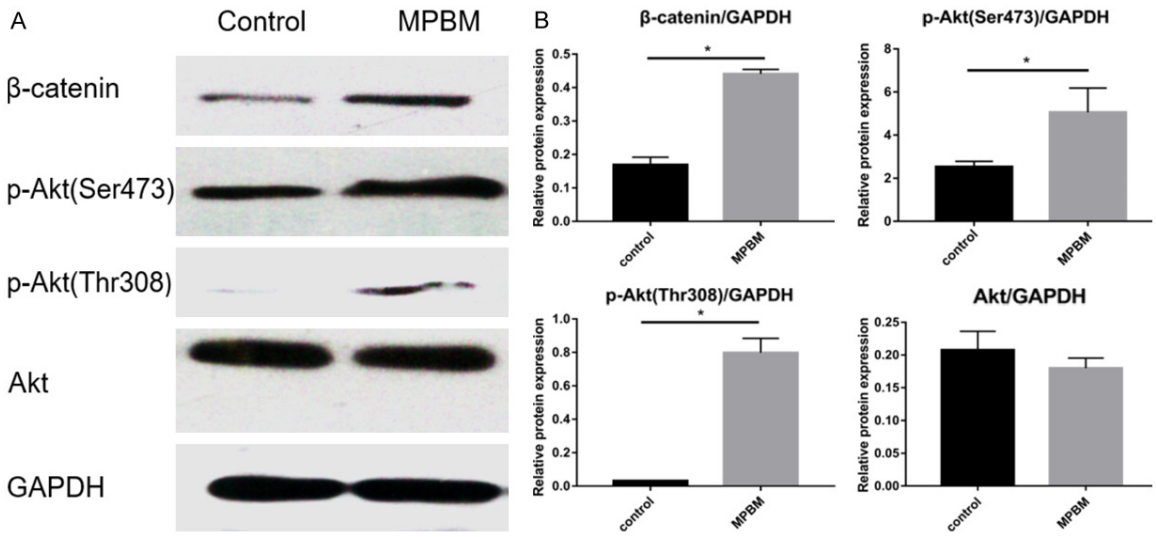

Figure 4. A. Expression levels of Akt and β-catenin signaling proteins detected via Western blot. B. Quantitative analysis. *P < 0.05 versus control group.

well as PI3K/Akt related genes, Col1a2, and OPN [29, 30]. They all play essential roles in the commitment of MSCs to osteoblastic lineage. The activity of OCN typically confirms the presence of osteoblasts, as well as the degree of osteogenic differentiation. Runx2 plays a vital role in the early stages of bone calcification. Col1 is the most abundant protein in the bone matrix. It is directly involved in mineralization and maturation of osteoblasts [31]. Expression of OPN has been linked to the mineralization front of the osteoid [32]. OPG can inhibit the differentiation and maturation of osteoclasts [33]. Current results indicate that MPBM could upregulate expression of these osteogenesis related genes, promoting the osteogenic differentiation of MSCs by modulation of Akt and Wnt/β-catenin signaling pathways.

To the best of our knowledge, this is the first study to systematically explore the effects of MPBM on osteogenic differentiation of MSCs *in vitro*. Current observations signified that a combination of Akt and β-catenin activation, in response to MPBM, could promote MSC growth and increase expression of osteogenic biomarkers. However, there were some limitations to the current study. As a result of methodological restrictions in herbal compounds and allowances for a more pharmacologically relevant assessment of mechanisms, this study applied medicated plasma instead of using the components of BM in the culture directly. As a result, the current study could not target which active ingredients of BM exert osteogenic effects.

Conclusion

In summary, the current study validated the suggestion that MPBM promotes proliferation and osteoblastic differentiation of MSCs via Akt and β-catenin signaling pathways. Current findings suggest that BM could be a promising alternative medicine for improved bone fracture healing.

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Disclosure of conflict of interest

None.

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