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Research article

# Feasibility of bone marrow mesenchymal stem cells harvesting from forearm bone

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ABSTRACT

*Introduction:* Mesenchymal stem cell is a promising therapeutic option in orthopedic filed and regenerative medicine. The feasibility of isolation method and characterization of Mesenchymal stem cell including growth kinetics, immunophenotypes and differentiation potency from small volume aspiration harvested from ulna and radius should be evaluated in order to utilize this cell in hand surgery.
*Materials and methods:* Mesenchymal stem cells were isolated and characterized from bone marrow of 12 patients who underwent internal fixation of fractures at radius or ulna. Population doubling time & clonogenic ability, immunophenotypes and trilineage differentiation potential of Mesenchymal stem cells were evaluated.
*Results:* Mesenchymal stem cells derived from bone marrow were attached to plastic flasks and became homogenous monolayer of fibroblast-like cells. They exhibited clonogenic ability and demonstrated positive markers which were shown by CD 73, CD 90, and CD 105 and negative markers which were shown by CD 34, CD 45. Mesenchymal stem cells derived from this source were capable of osteogenesis, chondrogenesis and adipogenesis.
*Discussion:* This study demonstrated the feasibility of bone marrow mesenchymal stem cells harvested from forearm bone marrow with small volume samples. This source should be useful in tissue engineering strategy or orthobiologic approach in orthopedic surgery.

## 1. Introduction

In hand surgery field, current tissue engineering approach involving cell-based therapy has potential role in hand surgery such as scaphoid nonunion, avascular necrosis, cartilage injury, osteoarthritis or reconstruction of bone deformity at wrist joint [1]. Mesenchymal stem cell (MSC) is a promising cell type in orthopedics and regenerative medicine [2]. Due to its differentiation potentials, it can be used as a progenitor cell for bone and cartilage regeneration. A conventional source of MSC is from bone marrow at the iliac crest or the femoral canal [3]. This cell type can be isolated from large volume aspiration with gradient-based technique [4]. However, this procedure is generally performed under general anesthesia. There are several reports on donor-site complications of the iliac crest procedure [5]. In order to minimize discomfort and potential harm of patients, the source of cells should be used from the same region rather than the remote site. Therefore, radius and ulna would be good candidate sources for MSC isolation.

To the best of our knowledge, reports on a technique for isolation and characterize bone marrow MSC from forearm bones which are radius and ulna are limited. Especially, bone marrow in these sites can be obtained with small volume aspiration. We would like to access for the feasibility of isolation method and to characterize of MSC from small amount aspiration samples harvested from ulna and radius. Thus, the objectives of this study were to determine [1] in vitro growth kinetics using population doubling time (PDT) and clonogenic ability [2], immunophenotypes and [3] differentiation potential of MSCs from radius and ulna obtaining with small amount aspiration.

## 2. Materials and methods

This study was designed as an in vitro experimental study. Twelve patients aged over 18 years diagnosed radius or ulna fractures which required operative treatment were included in this study for bone marrow harvesting and MSC isolation. The protocol of this study was approved by Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital (MURA2017/603). All participants provided informed consent before being enrolled to the study.

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### 2.1. Isolation technique

MSCs were isolated from the bone marrow ca nal through fracture sites of patients taken during internal fixation of fractures. Bone marrow was aspirated with 18 Gauge needle with 5 cc syringe and the sample was collected with EDTA tube. Red blood cell lysis buffer containing 154.4 mM $NH_4Cl$ (Sigma-Aldrich, USA), 10 mM $KHCO_3$ (Sigma-Aldrich, USA), and 97.3 μM EDTA (Fluka, Switzerland) was used to isolate MSCs. Briefly, RBC lysis buffer was added to bone marrow sample at a ratio of 9:1. The sample was mixed thoroughly and incubated for 10 min at room temperature prior to centrifuge at 160x g for 10 min at room temperature. Cell pellet was collected and resuspended with culture medium containing low-glucose DMEM (Gibco, USA) supplemented with 10 % FBS (Merck, Germany), 1 % GlutaMAX™ (Merck, Germany) and 1 % penicillin/streptomycin (Merck, Germany) and seeded onto 6-well culture plate. MSCs were maintained in 37 °C, 5 % $CO_2$ with humidified condition. After 3 days, non-adherent cells were removed and adherent cells were further cultured in fresh medium. The medium was changed every 3 days until the monolayer of adherent cells reached 80–90 % confluence. Cell passaging was performed using 0.25 % trypsin-EDTA (Invitrogen, USA).

### 2.2. Colony forming unit fibroblast assay

The $2^{nd}$ -$4^{th}$ passaged MSCs were evaluated for their clonogenic ability by using colony forming assays. Cells were seeded at low density in 6-well plate (200 and 300 cells/well) and cultured for 2 weeks in 37 °C, 5 % $CO_2$ with humidified condition. After two weeks, colonies were stained with crystal violet. Briefly, cells were washed with PBS and fixed in methanol (Honeywell, USA) for 5 min. Subsequently, the cells were incubated in 0.5 % crystal violet (Sigma Aldrich, USA) in 95 % ethanol (Merck, Germany) for 30 min at room temperature and washed twice with distilled water. Colonies consisting of over 50 cells were counted manually under a light microscope.

### 2.3. The population doubling time

Population doubling time (PDT) is the time taken to double the number of cell population. Cells were seeded in 6-well plate at the density of $5 \times 10^4$ and $1 \times 10^5$ cells/well. When reaching confluence, cells were trypsinized and manually counted using hemocytometer. PDT was calculated by the equation of PDT = culture time (CT)/population doubling number (PDN). CT was the duration of time from cell seeding to harvesting. PDN was calculated from PDN = log N/N0 × 3.31 where N was the cell number at the end of the cultivation period and N0 was the cell number at the initiation culture [6].

### 2.4. Flow cytometry analysis

Positive cell surface markers (CD73-PE/Cy7, CD90-APC, and CD105-PE; BD Biosciences, USA) and negative cell surface markers (CD34-PE and CD45-PerCP; BD Biosciences, USA) were evaluated by flow cytometry. Firstly, cells were trypsinized with 0.25 % trypsin-EDTA and washed twice with 1X PBS containing 2 % FBS and 1 mM EDTA by centrifugation at 160x g for 5 min. Next, cells were counted and adjusted for $2 \times 10^5$ cells per 50 μl. Appropriate concentration of each antibody was added and incubated at 4 °C for 30 min in dark. Unbound antibody was removed by washing with 1X PBS and discarded supernatant. Cell pellet was fixed with 300 μl of 1 % paraformaldehyde and analyzed with BD FACSCantoTM II Flow cytometry and FACSDIVA software version 6.1.3.

### 2.5. Differentiation assays

To demonstrate the differentiation potential of MSCs, passage 3–4 MSCs were cultured in vitro under (a) osteogenic, (b) chondrogenic and (c) adipogenic conditions. (a) Osteogenic potential: to induce osteogenesis, cells were cultured in basal medium until 70–80 % confluent before being changed to osteogenic differentiation medium containing basal medium supplemented of 100 nM dexamethasone (Sigma-Aldrich, USA), 10 mM β-glycerophosphate (Merck, Germany) and 50 μg/ml L-ascorbic acid (Sigma-Aldrich, USA). Osteogenic medium was refreshed every 3 days for 2 weeks. The cells were stained with 40 mM Alizarin Red S solution (Merck, Germany) to assess calcium deposition in the cells. (b) Chondrogenic potential: to induce chondrogenesis using the monolayer culture technique, cells were cultured in basal medium until 70–80 % confluent before being changed to chondrogenic differentiation medium containing 10 % FBS high-glucose DMEM supplemented with 10 nM dexamethasone (Sigma-Aldrich, USA), 1X Insulin-transferrin-selenium (Gibco, USA), 50 μg/mL L-ascorbic acid and 10 ng/mL transforming growth factor-β3 (TGF-β3). The medium was changed every 3 days. (c) Adipogenic potential: to induce adipogenesis, cells were cultured in basal medium until 60–70 % confluence and then changed to adipogenic differentiation medium containing basal medium supplemented with 1 μM dexamethasone (Sigma-Aldrich, USA), 10 μM insulin (Sigma-Aldrich, USA), 0.5 mM isobutyl-methylxanthine (IBMX) (Sigma-Aldrich, USA) and 0.2 mM indomethacin (Sigma-Aldrich, USA). Adipogenic medium was changed every three days for three weeks. Oil Red O (Merck, Germany) staining was used to assess lipid accumulation in the cells.

### 2.6. Gene expression analysis

Real time qRT-PCR was used to investigate the mRNA expression of osteogenic, chondrogenic and adipogenic differentiation markers. All primers in this study were well designed using Primer3 software following the guideline for primer designing [7]. Briefly, the primers were checked for their characteristics using OligoAnalyzer and OligoCalc programs. The primers lengths were 19–23 bp and contain 40–60 % G/C content. The melting temperatures of the primers were 52–60 °C. All primers contain less potential for secondary structure formation, including hairpin and primer-dimer. Each primer was specific to its target gene evaluated by using The Basic Local Alignment Search Tool (BLAST) software. In addition, each primer pair yieldd a single peak in the melting curve analysis and its product sizes were 140–220 bp. The gene specific primers were listed in Table 1. Total RNA was collected in Trizol® reagent (Thermo Fisher Scientific, USA) and extracted following the manufacturer's instructions. cDNA synthesis was performed from 1 μg of total RNA using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA). qRT-PCR was performed on C1000 Touch™ Thermal Cycler (Bio-Rad, USA) using SYBR® FAST qPCR Master Mix (Kapa biosystems/Roche holding AG, Switzerland). The PCR mixture consisted of 1 μl of template cDNA, 0.2 μl of each forward and reverse primers from 10 μM stocks, 3.6 μl of PCR-grade water, and 5 μl of SYBR master mix. PCR reaction was started with enzyme activation at 95 °C for 3 min followed by denaturation for 3 s at 95 °C, and annealing and extension at 60 °C for 20 s. Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method after normalization to the reference gene; GAPDH. Results were expressed as fold change over control (undifferentiated) cells.

### 2.7. Statistical analysis

The data from 12 MSCs samples were presented by descriptive analysis. The data comparison in for MSCs immunophenotypes, CFUs, and gene expression of MSCs was analyzed by t-test from the statistical package of GraphPad Prism. A p-value $< 0.05$ was considered to be statistically significant.

## 3. Results

Bone marrow samples were collected by sterile technique from 8 male and 4 female participants (n = 12) undergoing forearm fracture or distal end radius fracture. The average patient's age was 41.42 + 24.98 years (ranged from 18 to 90 years). The average bone marrow volume was 3.89 + 2.23 ml (range 0.5–8 ml).

**Table 1.** List of qRT-PCR primers for differentiation markers.

| Gene name | Primer | Sequence | GenBank Accession No. | Product size (bp) |
|---|---|---|---|---|
| RUNX2 | Forward | 5′-AACCCAGAAGGCACAGACAG-3′ | NM_001024630 | 192 |
| | Reverse | 5′-GCCTGGGGTCTGTAATCTGA-3′ | | |
| COL1A1 | Forward | 5′-GGGCAAGACAGTGATTGAATACA-3′ | NM_000088 | 143 |
| | Reverse | 5′-GGATGGAGGGAGTTTACAGGAA-3′ | | |
| SOX9 | Forward | 5′-GGTGCTCAAAGGCTACGACT-3′ | NM_000346 | 207 |
| | Reverse | 5′-TCGTTCAGAAGTCTCCAGAGC-3′ | | |
| COL2A1 | Forward | 5′-CCTGGTCTTGGTGGAAACTT-3′ | NM_001844 | 193 |
| | Reverse | 5′-CAGAGACACCAGGTTCACCA-3′ | | |
| PPAR-γ | Forward | 5′-TGAAGGATGCAAGGGTTTCT-3′ | NM_138711 | 200 |
| | Reverse | 5′-CCAACAGCTTCTCCTTCTCG-3′ | | |
| adiponectin | Forward | 5′- GCAGTCTGTGGTTCTGATTCC -3′ | NM_004797 | 221 |
| | Reverse | 5′-GTGCCATCTCTGCCATCAC-3′ | | |
| GAPDH | Forward | 5′-CAACTACATGGTTTACATGTTCC-3′ | NM_002046 | 206 |
| | Reverse | 5′-CAGCCTTCTCCATGGTGGT-3′ | | |

### 3.1. MSC morphology from forearm bones

The cells derived from bone marrow of forearm sources attached to plastic flasks and showed heterogeneous morphology at initial plating and then became a homogenous monolayer of fibroblast-like cells with passaging (Figure 1). Cells were expanded after 80–90 % confluent for 2 weeks.

### 3.2. MSC immunophenotypes

The cultured MSCs demonstrated the positive markers which were shown by CD73 (99.60 ± 0.30 %), CD90 (98 ± 1.76 %), and CD 105 (87.51 ± 6.92 %) and the negative markers which were shown by CD34 (2.09 ± 0.58 %) and CD 45 (0.07 ± 0.13 %) (Figure 2).

### 3.3. Clonogenic ability and in vitro growth kinetics

The clonogenic ability was evaluated using numbers of colony forming unit fibroblast (CFU–F). CFU-F of BM-MSCs after seeding number at 200 cells (16.59 ± 12.58 colonies) and 300 cells (21.00 ± 14.72 colonies) for 14 days was not significantly different. The population doubling time (PDT) of BM-MSCs was not different after seeding with the different density (PDT = 157.46 ± 59.47 h at 50,000 cells and 163.07 ± 56.78 h at 100,000 cells). The CFU-F number and PDT were shown in Figure 3.

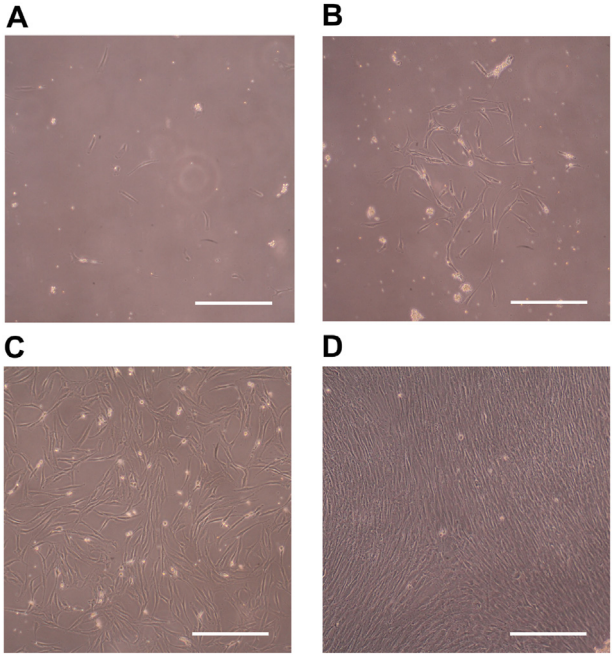


**Figure 1.** BM-MSC isolation and expansion of cells at day 3rd (A), 6th (B) 12th (C) and 15th (D) after isolation. Scale bar; 100 μM.

### 3.4. MSCs differentiation and gene expression

The morphology of MSCs after differentiation was assessed using light microscopy prior to induction. The MSC in control and differentiated condition was compared. Following differentiation cells were stained using appropriate specific staining solutions with positive staining seen following osteogenic, adipogenic, differentiation (Figures 4 and 5). The gene expression osteogenic marker of BM-MSCs was demonstrated by RUNX2 and COL1A1 gene (Figure 6). The gene expression of adipogenic marker of BM-MSCs was demonstrated by PPAR-γ and Adiponectin gene (Figure 7). The gene expression of chondrogenic marker of BM-MSCs was demonstrated by SOX9 and COL2A1 gene (Figure 8).

## 4. Discussion

MSC-based treatment is a promising strategy to improve outcomes in hand surgery via biological approach. MSC can be administrated as cell-based implantation at injured sites or as cellular component in tissue engineering-based approach [1]. MSCs are considered as the principal progenitors for bone regeneration. A conventional source of MSC is the iliac crest [3]. According to previous reports, MSCs from bone marrow source has a better osteogenesis differentiation compared to adipose tissue [8, 9]. These cells expressed higher ALP activity, calcium deposition and osteogenesis-related protein osteopontin which were bone forming markers [10]. Then, bone marrow should be a suitable source for bone regenerative applications. Conventionally, the density gradient-based method is required the high-volume aspiration of bone marrow (approximal 20–50 ml) [11]. However, in order to minimize discomfort and potential harm of patients, the regional source of bone marrow aspiration should be selected from same or adjacent regions. Therefore, we believe that radius and ulna should be optimal candidate sources for MSC isolation in hand surgery.

This study focused on the feasibility of MSC harvested from the forearm bones which should be utilized effectively in hand surgery. However, due to small-volume of bone marrow aspiration, the lysis buffer-based technique was modified in order to isolation MSC from these sources [11]. In this study, we demonstrated MSC characteristics which were obtained from bone marrow radius and ulna of patients who

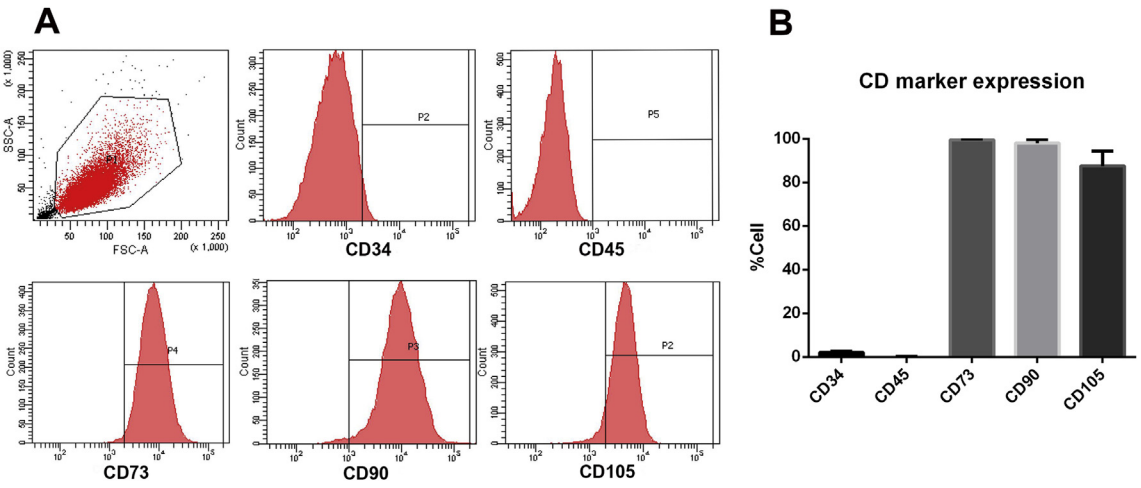


**Figure 2.** CD marker expression of MSCs using BD FACSCanto II flow cytometer. (A) Flow cytometry analysis demonstrated that BM-MSCs were positive for CD73, CD90, CD105 and negative for CD34, CD45. (B) Quantitative analysis of MSC marker expression was shown in bar chart. Data were shown as mean ± SD.

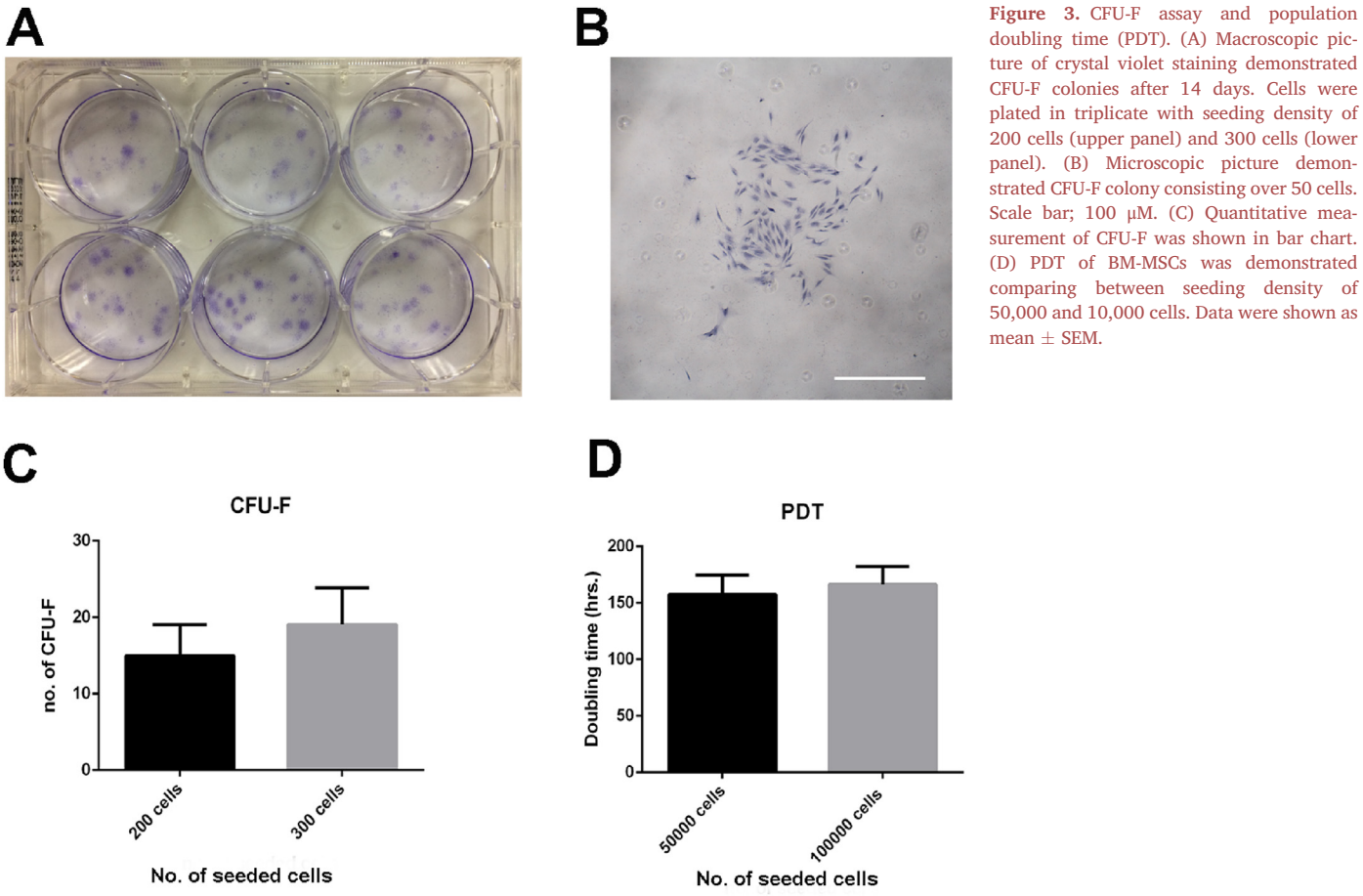


**Figure 3.** CFU-F assay and population doubling time (PDT). (A) Macroscopic picture of crystal violet staining demonstrated CFU-F colonies after 14 days. Cells were plated in triplicate with seeding density of 200 cells (upper panel) and 300 cells (lower panel). (B) Microscopic picture demonstrated CFU-F colony consisting over 50 cells. Scale bar; 100 μM. (C) Quantitative measurement of CFU-F was shown in bar chart. (D) PDT of BM-MSCs was demonstrated comparing between seeding density of 50,000 and 10,000 cells. Data were shown as mean ± SEM.

undergone fracture fixation procedures. Harvested cells were attached to a plastic surface with typical fibroblast-like shape and they demonstrated the clonogenic ability. These cells were able to differentiate into trilineage. They exhibited the markers of CD73, CD90 and CD105, meanwhile they did not express haematopoetic stem cell marker (CD45) or endothelial marker (CD34). This study demonstrated the property of MSCs from forearm source which were compiled to International Society for Cellular Therapy [12].

Conventionally, the iliac crest has long been used for bone marrow aspiration and this site is a common source of MSCs [13]. It has been reported that bone marrow from this area could be obtained up to 30–60 cc [4]. The relevant source for bone marrow harvesting should be closed to the region of treatment in orthopedic. Then, there are increasing evidences that these cells could be obtained from bone marrow from the proximal and distal femur, proximal and distal tibia, vertebral body, calcaneus, and humeral head in order to serve in each treatment region

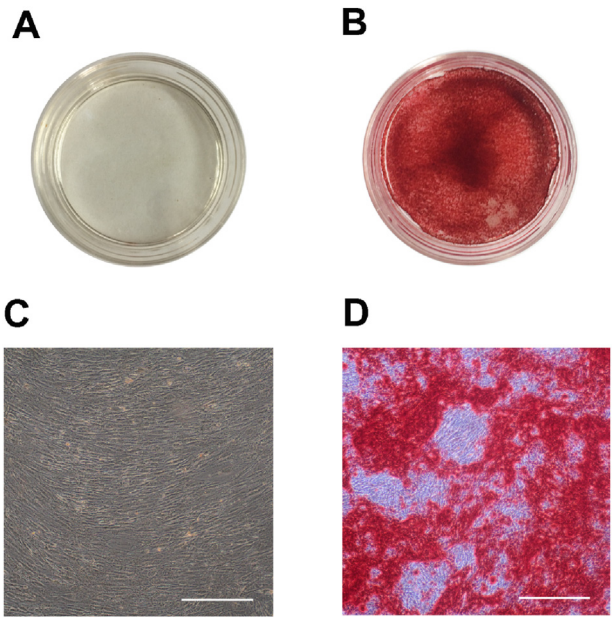


**Figure 4.** Alizarin Red S staining. Extracellular matrix staining using Alizarin Red S after 14 days of osteogenic differentiation. Macroscopic pictures showed control (A) and osteogenic induction of BM-MSCs after Alizarin Red S staining (B). Microscopic pictures demonstrated control (C) and osteogenic induction of BM-MSCs after Alizarin Red S staining (D). Scale bar; 100 μM.

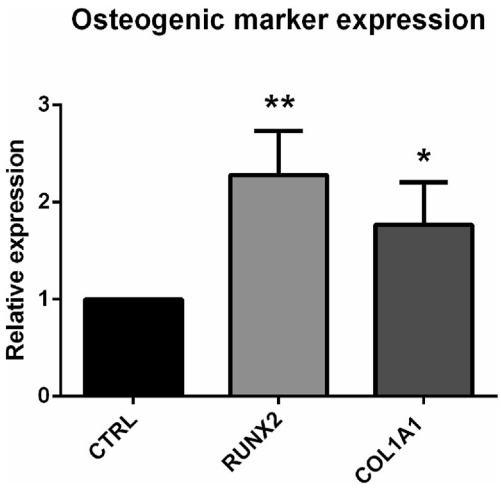


**Figure 6.** Osteogenic marker expression. RT-PCR result of osteogenic marker expression of BM-MSCs after 14 days of osteogenic differentiation. mRNA expressions of RUNX2 and COL1A1 weres significantly upregulated after osteogenic induction (*p = 0.0474 and **p = 0.0058, respectively). Data were shown as mean ± SEM. (CTRL = control expression, RUNX2 = Runt-related transcription factor 2 expression, COL1A1 = Collagen Type I Alpha 1 Chain expression).

[14]. A common technique used for MSC isolation from bone marrow is a density gradient based method which is required the high-volume aspiration of bone marrow [4]. In hand surgery field, the harvesting procedure from the bone marrow from forearm should be less invasive options for the surgical procedure. However, bone marrow from this bone including radius and ulna can be obtained with less amount of volume. The direct plating method modified by adding red cell lysis should be suitable for small-volume samples. Bone marrow sample was treated with red cell lysis buffer and treated cells were diluted with a basal medium and cultured without any disturbance at 37 °C for 3–4 days. The medium was replaced every 3–4 days and the contaminating other non-attaching cells were rinsed away. Since differences in methods of isolation, culture times and differentiation media used may result in very diverse outcomes [15]. So, in this study, source of cultured cells, isolation technique, and culture condition were consistently maintained for all experiments to obtain reproducible results. This developed protocol was ready to use in clinical setting. It was fast and convenient, especially, in small volume aspiration.

MSCs are capable of osteogenesis or osteogenic differentiation. Mesenchymal stem cell implantation has the potential role to manage non-union of hand and upper extremity bones. S. Giannotti et al., reported the encourage results one the treatment of pseudoarthrosis of the upper limb using expanded mesenchymal stem cells combined with autologous plasma gel. In this study, there were completely bone union in all treatment cases of pseudoarthrosis without any adverse events or complications [16]. Apart from osteogenesis effect, MSCs provided angiogenesis factors which were required to promote bone regeneration for example VEGF, in particular [17]. Therefore, a potential application in scaphoid non-union and kienbock's disease to promote healing process in these conditions.

Chondrogenesis ability of MSCs is desired in treatment of cartilage injury or osteoarthritis. It has been reported on the first pilot study to perform the intervention of cells injection on base of thumb arthritis. Matthew P. Murphy et al., demonstrated the effects bone marrow derived MSC treatment to the first carpometacarpal joint resulting in positive outcomes by improvement of symptoms and functions [18]. In addition,

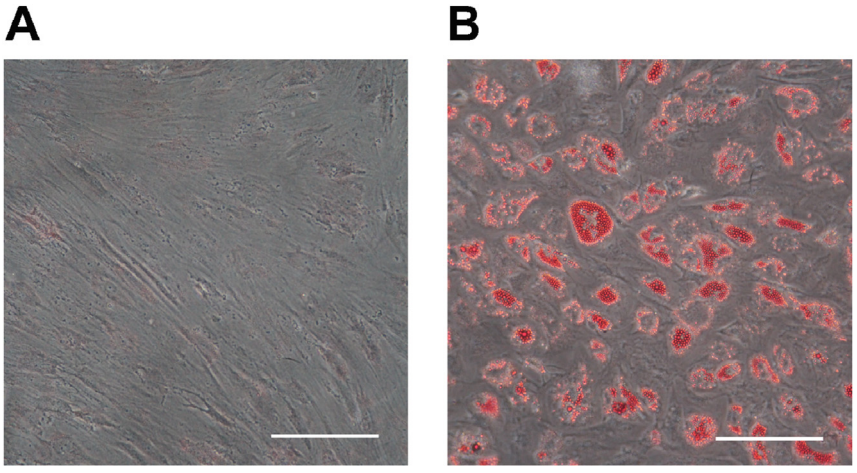


**Figure 5.** Oil Red O staining. Fat droplet staining using Oil Red O after 21 days of adipogenic differentiation. Microscopic pictures of control (A) and adipogenic induction of BM-MSCs after Oil Red O staining (B). Scale bar; 100 μM.

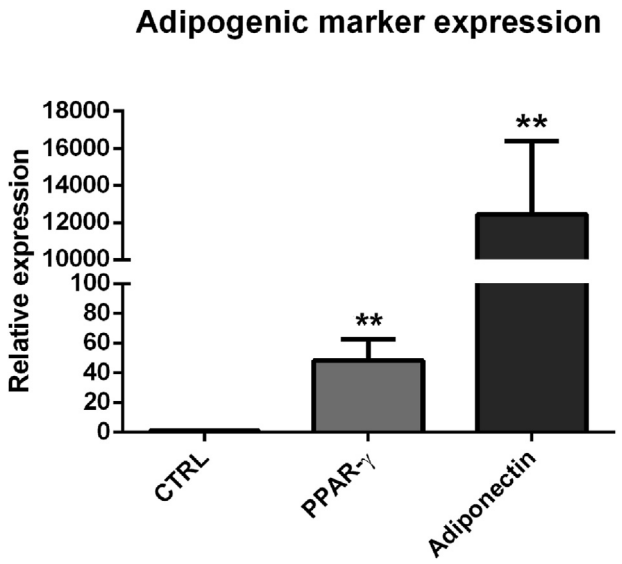


**Figure 7.** Adipogenic marker expression. RT-PCR result of adipogenic marker expression of BM-MSCs after 14 days of adipogenic differentiation. mRNA expressions of PPAR-γ and Adiponectin were significantly upregulated after adipogenic induction (**p = 0.0016 and **p = 0.0022, respectively). Data were shown as mean ± SEM. (CTRL = control expression, PPAR- γ = Peroxisome proliferator-activated receptor gamma expression).

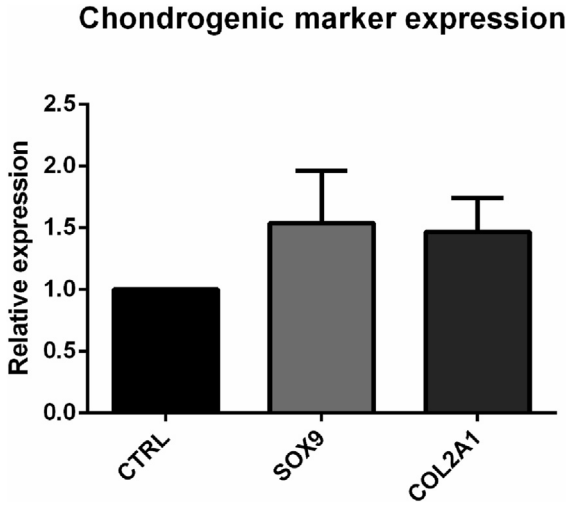


**Figure 8.** Chondrogenic marker expression. RT-PCR result of chondrogenic marker expression of BM-MSCs after 21 days of chondrogenic differentiation. mRNA expressions of SOX9 and COL2A1 were upregulated but not significantly difference (p = 0.1121 and p = 0.0547, respectively). Data were shown as mean ± SEM. (CTRL = control expression, SOX9 = SRY-Box Transcription Factor 9 expression, COL2A1 = Collagen Type II Alpha 1 Chain expression).

there is a long-term case report on osteochondritis dissecans of the elbow treated with bone marrow derived MSC [19]. It is increasing evidence on promising strategies to improve outcomes in hand and upper extremity conditions using biologics approach.

The authors acknowledge limitations to this present study. The study has a relatively small sample size. The potential factors including BMI and age which might affect the MSC were not evaluated or analyzed. In addition, the cases in this study were fractures from forearm in which may interfere the MSC characteristics and may not represent in MSC from healthy donor. However, we consistently obtained bone marrow in middle of bone marrow cannel adjacent from fracture sites and all technical steps were fully controlled in a standard laboratory condition.

In conclusion, this study demonstrated that the feasibility to obtain MSCs from forearm bone marrow with small volume aspiration. Isolated MSCs from these sources had properties according to International Society for Cellular Therapy criteria.

## Declarations

### *Author contribution statement*

Tulyapruek Tawonsawatruk: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Thepparat Kanchanathepsak: Contributed reagents, materials, analysis tools or data.

Thitinat Duangchan: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kuneerat Aswamenakul: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Aungkura Supokawej: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Data availability statement*

Data will be made available on request.

### *Declaration of interests statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

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