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Enhanced Bone Formation in Osteoporotic Mice by a Novel Transplant Combined with Adipose-derived Stem Cells and Platelet-rich Fibrin Releasates

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Abstract
Osteoporotic fracture is the main complication of osteoporosis (OP) and accounts for millions of injuries annually. Local intervention by intra-marrow injection has been a good option for preventing osteoporotic bone loss when the osteoporotic femoral fracture has been treated. In this study, tail vein transplantations were examined to evaluate the cell-based therapeutic approach for treating OP with adipose-derived stem cells (ADSCs) and platelet-rich fibrin releasates (PRFr) in an ovariectomized (OVX) mice model. Thirty-six 12-wk-old female ICR mice were randomly divided into six groups: untreated control; sham-operated; OVX-control; OVX-ADSCs; OVX-PRFr; and OVX-ADSCs+PRFr. Starting 8 wk after ovariectomy, the OVX mice received tail vein injections once each week for four consecutive weeks, then were evaluated radiographically and histopathologically 8 wk after the first injection. We also assessed changes to bone trabeculae in the proximal tibial growth plate. In OVX mice treated with ADSCs or PRFr alone, or with a combination of ADSCs and PRFr, the trabecular bone mineral density (BMD), bone volume ratios (BV/TV), and numbers (Tb.N) in the proximal tibia areas were significantly higher than that in the OVX-control group. Significant differences between OVX-treated mice and OVX controls were found for trabecular separation, but not for trabecular thickness. These results indicate that ADSCs or PRFr treatment enhances bone microarchitecture in OP. The treatment of bone loss of OVX mice with ADSCs+PRFr induced greater bone consolidation with bone tissue production ($P < 0.01$) when compared to the others. Thus, we conclude that the transplantation of ADSCs combined with PRFr might provide an alternative strategy for the treatment of various bone disorders in OP with an unlimited source of cells and releasates.

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Introduction

Osteoporosis (OP) is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitecture deterioration of bone tissue, leading to bone fragility and an increased risk of fracture[1]. Loss of estrogen seems to be the most important mechanism in the development of OP. The use of ovariectomized (OVX) rat or mice models is a well-established and reproducible method of simulating the postmenopausal condition and has been found effective in mimicking postmenopausal cancellous bone loss over a relatively short period of time[2]. During the initial period after ovariectomy, bone resorption exceeds bone formation causing a net loss of bone[3]. Statistically significant bone loss is detectable in the proximal tibial metaphysis after 14 d[3,4], in the lumbar vertebral body after 60 d[5].

Currently, bisphosphonates[6], hormone therapy with estrogen[7], selective estrogen receptor modulators/estrogen agonist-antagonists[8], denosumab[9], salmon calcitonin[10], and parathyroid hormone analogs[11] are the drugs of choice for OP[12,13] based on the goal of blunting the resorption component of bone homeostasis, a tightly coupled process of bone formation and bone resorption[14]. While professional organizations have compiled recommendations for the management of OP in various populations, a consensus has yet to develop as to which is the gold standard. These medications have yet to fully reduce the number of fracture cases and are associated with several side effects[15]. As the population continues to age and some types of medicines can cause bone loss, OP is likely to become an even more prevalent and serious health condition with economic burden for the society[16]. Therefore, a safer and more effective therapeutic strategy is urgently needed.

Bone remodeling is a complex process involving the close interactions between different progenitor cell lineages, mainly osteo- and adipo-progenitor cells[17]. It has been thoroughly proven that osteoblasts and adipocytes are both derived from mesenchymal stem cells within the bone marrow. Signaling pathways involved in these two processes could be modified to favor osteogenesis, resulting in the amelioration of OP[18]. Previous study has indicated that the increase in the number of adipocytes subsequently induced further apoptosis in osteoblasts and promoted the proliferation and differentiation of osteoclasts, resulting in an increase in bone resorption and overall bone loss[19]. Regenerative medicine approaches could be a better option in treating OP and/or osteoporotic damages compared with regular surgical techniques[20]. Therefore, bone marrow transplantation[21] and bone marrow cell intratibial injections[22] have been considered to enhance osteoporotic prevention or osteoporotic bone repair in animal models[23]. Thereby stem cell therapy for OP could potentially reduce the susceptibility of fractures and augment lost mineral density by either increasing the numbers or restoring the function of resident stem cells that can proliferate and differentiate into bone-forming cells[24]. Such OP therapies can be carried out by the exogenous introduction of mesenchymal stem cells (MSCs), typically procured from bone marrow, adipose, and umbilical cord blood tissues or through treatments with growth factors, drugs, or small molecules that recruit endogenous stem cells to osteoporotic sites[24].

Platelet concentrates for topical and infiltrative use—commonly termed platetet-rich plasma (PRP) or platelet-rich fibrin (PRF)—are commonly used or tested as surgical adjuvants or regenerative medicine preparations in most medical fields, particularly in orthopedic surgery, cardiovascular surgery, plastic/reconstructive surgery, sports medicine, dentistry, and oral implantology[25]. The concept is that within our blood there are growth factors, healing proteins, cell signals, and immune cells, which are part of the normal healing process that can be concentrated and applied or injected to a wound or surgical site to potentially enhance healing. Besides, a PRF clot that is squeezed to extract the releasate PRF (the serum exudate expressed from the clot) is called a PRF releasates (PRFr), and is rich in growth factors, leukocytes, lipids, and also proteins such as vitronectin and fibronectin[26]. This exudate may be used to hydrate graft materials, rinse the surgical site, and store autologous graft. In addition, it has been shown that the concentrations of growth factors such as platelet-derived growth factor (PDGF)-AB, transforming growth factor (TGF)-β1, epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), and P-selectin (a marker of platelet activation) were significantly higher ($P < 0.05$; < 0.05; < 0.001; < 0.05; < 0.001; < 0.01, respectively) in PRFr than in PRP[27]. In vitro study has demonstrated that autologous growth factors released by PRF gradually expressed stronger and more durable effect on proliferation and differentiation of rat osteoblasts than PRP[28].

As in previous reports, the observation that increased bone marrow fat content is closely associated with the development and/or consequence of OP[16] demonstrated that PRP treatment mainly exerted its action via promoting bone regeneration but also appeared to suppress adipogenesis within the marrow. Additional pertinent observation has demonstrated that PRP could stimulate the differentiation of embryonic fibroblasts into osteoblast-like cells and transplantation of these PRP-treated cells could significantly improve the bone architecture in OVX-SAMP8 osteoporotic mice[29].

Our previous study has provided an alternative method for functional reconstruction of rabbit osteochondral defects with an unlimited source of cells and blood-derived fibrin releasates[30]. We hypothesize that PRFr serve as a mixture of growth factors and act as a unique source/carrier of stem cells[27,31], which could induce osteogeneic differentiation and enhance osteogenesis, thereby favoring bone formation and ameliorate OP. The goal of this study was to develop a method to stimulate bone formation in order to restore proper bone remodeling process and explore the potential application of PRFr for promoting osteogenesis of

progenitor cells within the adipose-derived stem cells (ADSCs) using an osteoporotic mouse model.

Materials and Methods

Culture and Identification of Mouse ADSCs

A total of 5.0 g of subcutaneous adipose tissue was extracted from the ventral sides of 8-wk-old female ICR mice (also an albino strain, was named after the Institute of Cancer Research in the USA; BioLASCO Co., Ltd., Yilan, Taiwan) following CO_2 euthanasia. It was then immediately washed with phosphate-buffered saline (PBS) containing 1% penicillin/streptavidin (15140-122, Gibco, Santa Clara, CA, USA). Adipose tissue biopsies were chopped finely and adipocytes were isolated by collagenase digest (PBS solution, containing 0.2% collagenase type I, C0130, Sigma-Aldrich, St Louis, MO, USA) in a shaking water bath at 37°C for 30 min. After collagenase digest, the cell suspension was passed through a 100-μm nylon mesh and centrifuged at $500 \times g$ for 10 min. The supernatant was discarded and the cell pellet was resuspended in Minimum Essential Medium Eagle Alpha Modification (M0894, Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and added into 100-mm dishes for culture in a humidified 5% CO_2 incubator at 37°C. Culture medium was replaced 2 d later and once every 2 d thereafter. When the cells were 80% to 90% confluent, they were digested with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.05% trypsin, 0.5 mM EDTA, T4174, Sigma-Aldrich) and plated at a density of 3.3 × 105 cells per dish for further culture.

For molecular characterization, mouse ADSCs at passage 2 were harvested and resuspended in a culture medium at a density of 1×107 cells/ml. These cells were analyzed for expression of surface markers CD34, CD45, CD73, and CD90 by flow cytometry. Mouse ADSCs were stained with the rat anti-mouse CD34 (553731, BD Biosciences, San Jose, CA, USA) and CD73 (550738, BD); rabbit anti-mouse CD45 (ab10558, Abcam, Cambridge, MA, USA); and mouse anti-mouse CD90 (554895, BD). Cells (1 × 106) were incubated with the appropriate antibody in 0.1 ml PBS for 60 min at 4°C. Then, cells were washed and stained with phycoerythrin-conjugated goat anti-mouse immunoglobulin (Ig) (550589, BD), Alexa Fluor 647-conjugated goat anti-rat IgG (ab150159, Abcam), and DyLight 488-conjugated donkey anti-rabbit IgG (SA5-10038, Thermo, Waltham, MA, USA) secondary antibodies at 4°C for 30 min. Subsequently, cells were washed twice with PBS, centrifuged, then the final cell pellet was resuspended in fluorescence-activated cell sorting (FACS) sorting buffer (PBS, 1 mM EDTA, 25 mM HEPES, 1% FBS, pH 7.0), and filtered using a Falcon 35 μm cell strainer (352235, BD) before sorting. ADSCs were done on a BD FACS Aria cell sorter and the data were analyzed using BD FACSDiva software[32,33].

Xenogenic PRFr Preparation

A laboratory mouse has a circulating blood volume of about 1.5 to 2.5 ml (6% to 8% of the body weight)[34], making it difficult to get enough volume of mouse blood. Since rabbits are of the order lagomorphs and similar to rodents, large lot sizes available from pooled rabbit donors may be an alternative blood source[35]. Blood samples were collected from the experimental rabbits under general anesthesia. After adequate skin cleaning, disinfection, and sterilization, 6 ml of blood was drawn from the right jugular vein of each animal and collected in vacutainer tubes (367988, BD) with clot activator and gel. The vacutainer was rotated gently for 30 s and centrifuged at $3000 \times g$ for 10 min. The resultant jelly-like PRF needed to be separated from the gel clot without the red blood cells sinking to the bottom of the tube. Using forceps, the PRF clots were transferred into 15-ml sterile centrifuge tubes and the jelly-like PRF allowed to stand for at least 5 h[30]. Following centrifugation of the PRF at $5000 \times g$ for 10 min, the supernatant PRFr was collected in sterile tubes, frozen, and stored at –20°C until use. All preparation and delivery steps were carried out under standard disinfection procedures. One milliliter of whole blood yielded about 0.2 ml of PRFr in general.

Experimental Animals, Surgical Procedure, and ADSCs and/or PRFr Injection

All experiments were performed with the approval of the Institutional Animal Care and Use Committees of National Taiwan University (102-EL-82) and were carried out in accordance with the International Council for Laboratory Animal Science guidelines for the care and use of laboratory animals[36]. All efforts were made to minimize animal suffering and the number of animals used. After assessment of the anesthetic plane, back hair elimination, and disinfection, an approximately 0.5 cm linear incision (parallel to the spine) was made at 0.5 cm from the spine and 1 cm below the ribs. After the removal of bilateral ovaries, the abdominal muscles were then closed followed by the skin using an absorbable suture (Vicryl 3.0, ETHICON Inc., Somerville, NJ, USA). The same procedure was performed for the animals designed to the sham group but without ovary removal. Animals were then observed twice a day until healing of the incision had occurred.

OVX female mice (12 wk of age) were used for the experiments. Mice were then divided into six groups with six mice in each group: Control (Ctrl), Sham, OVX-Ctrl, OVX-ADSCs, OVX-PRFr, and OVX-ADSCs+PRFr. Serving as controls were unoperated animals and sham-operated animals who underwent the same manipulations as the OVX mice but without ovary removal. Injections consisting of ADSCs and/or PRFr were prepared in vitro under general sterile conditions and collected in a 1-ml syringe. Eight weeks after ovariectomy[37,38], the experimental mice received tail vein injections through a 26-gauge needle

once each week for four consecutive weeks (quadruple-injection). The OVX-ADSCs group received 3×10^5 ADSCs suspended in 0.6 ml of PBS and the OVX-PRFr group received 0.6 ml of PRFr, while the OVX-ADSCs+PRFr group received 3×10^5 ADSCs suspended in 0.6 ml of PRFr. Intravenous administration can be technically difficult, and the use of a restraining device is often required. It is common practice to stimulate dilation of the tail veins in mice by immersing them in warm ($40°C$) water for approximately 15 s[39]. Eight weeks after treatments, the animals were sacrificed and were subjected for micro-computed tomography (CT) analysis followed the protocol as described below.

Micro-CT Analysis

The methods used were in accordance with guidelines for the use of micro-CT in rodents[40]. The mice's left tibia were harvested 8 wk after the first injection, placed in a custom-fabricated jig with water, and scanned with a SkyScan 1176 MicroCT scanner (SkyScan, Kontich, Belgium) operating at 40 kV, 600 μA, 0.3 μm of rotation step, 0.5 mm Al filter, and 9 mm/pixel of scan resolution. The image slices were reconstructed using the NRecon (v.1.4.4, SkyScan) software system. The three-dimensional parameters of bone microarchitecture were calculated using CTAn (v.1.7.0.0, SkyScan) software. For trabecular bone, the proximal tibia was selected for analysis within a conforming volume of interest commencing at the growth plate and extending a further longitudinal distance of 0.5 to 1.5 mm in the proximal direction to assess site-specific responses to OVX and treatment. Microstructural measurements included total volume (TV, volume of region of interest, mm^3), bone volume (BV, volume of region segmented as bone, mm^3), bone volume fraction (BV/TV, %), BMD (g/cm^3), trabecular thickness (Tb.Th, mean trabecular thickness, mm), trabecular number (Tb.N, average number of trabeculae per unit length calculated as 1, mm^{-1}), and trabecular separation (Tb.Sp, distance between trabeculae, mm).

Histological Examination

To determine osteogenesis in the treated mice, tibia specimens of the mice were collected after 8 wk of treatment. Bone samples were fixed for 2 wk with 10% buffered formaldehyde, decalcified in a solution containing 20% sodium citrate and 50% formic acid in 1:1 dilution distilled water for 2 to 3 wk, dehydrated by an alcohol gradient and xylene soak, and placed on Baptist wax and embedded. Sections (5 μm) were cut sagittally along the tibial shaft axis and collected on glass slides before being deparaffinized and subjected to hematoxylin and eosin (H&E) staining using standard protocols. After mounting with coverslips, specimens were viewed and analyzed under a light microscope (Canon Inc., Tokyo, Japan). Relatively well-developed trabecular and cortical bone was observed in the tibia of untreated and sham control mice, and osteoporotic histological profiles were observed in OVX control mice as significant decreases in trabecular and cortical bone masses and increases in connective tissues.

Serum Biochemistry

At the end of the experimental period, the mice were euthanized by carbon dioxide inhalation and blood samples were collected by maximal terminal collection by cardiocentesis with a 1-ml tuberculin syringe with a 5/8-inch 25-gauge needle. Whole blood was clotted at room temperature for 2 h, and serum was separated by centrifugation at $700 \times g$ for 10 min and stored at $–80°C$ until analysis. Total calcium (Ca) and phosphorus (P) levels were quantified using commercial assays in the integrated biochemical analyzer Advia 1800 (Siemens Healthcare Diagnostics, Eschborn, Germany).

Statistical Analysis

All results were presented as mean $\pm$ standard derivation. The statistical significance of the tibia and biochemical effects was determined by one-way analysis of variance (ANOVA) using a Statistical Analysis System (2014, SAS/STAT User's Guide, ver. 13.2. SAS Institute, Inc., Cary, NC, USA), followed by ANOVA with Duncan's test. Differences with $P < 0.05$ and <0.01 between the test groups and the control group were considered to be statistically significant[41].

Results

Analysis of BMD in Tibial Bone Tissue

BMD analysis showed significantly different changes in proximal tibial trabecular BMD in the OVX groups ($P < 0.05$) (Fig. 1). Eight weeks after the first injection, the OVX-ADSCs (mean BMD, g/cm^3, 0.066 ± 0.008), OVX-PRFr (0.074 ± 0.009), and OVX-ADSCs+PRFr (0.077 ± 0.010) groups had a significant increase in tibial shaft BMD compared with the OVX-Ctrl (0.047 ± 0.006) group. In particular, the BMD in OVX-ADSCs+PRFr was higher than in OVX-ADSCs and OVX-PRFr.

Microstructure Analysis of Tibial Bone

Significant differences in bone mineral quantitative data were confirmed with the micro-CT analysis as shown in Fig. 1. Quantitative analysis of the tibia (Fig. 1) confirmed that the OVX-Ctrl group had the lowest values in BV/TV (4.89 ± 0.58) and morphological parameters such as Tb.N (0.57 ± 0.09). The sham-operated treatment showed minimal effects on the tibial bone mineral as well as in the Ctrl group. When the relative trabecular BV/TV was measured, it was clear that either ADSCs+PRFr-treated mice ($P <$
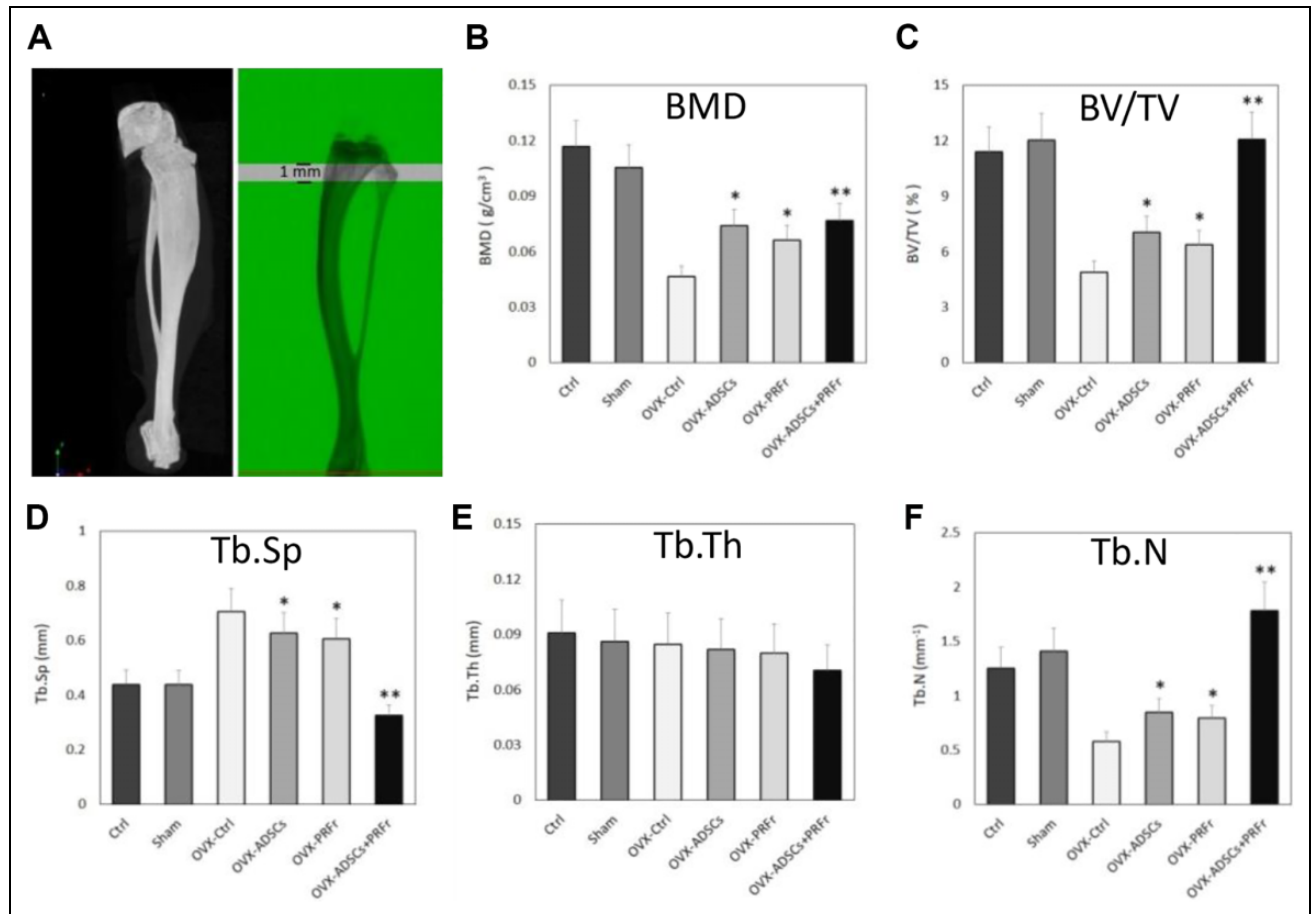
Fig. 1. A. Schematic representation of the trabecular bone area using micro-CT analysis. The proximal tibia was selected for analysis within a conforming volume of interest commencing at the growth plate and extending a further longitudinal distance of 0.5 to 1.5 mm in the proximal direction to assess site-specific responses to OVX and treatment. B–F. Micro-CT analysis of Ctrl, Sham, OVX-untreated, and OVX-treated tibia from ICR mice (six groups, $n = 6$) 8 wk post-transplantation. Illustration of BMD, BV/TV, Tb.Sp, Tb.th, and Tb.N from the entire group. The asterisks indicate significant differences as compared with the OVX-Ctrl group ($*P < 0.05$; $**P < 0.01$).
BMD: bone mineral density; BV: bone volume; CT: computed tomography; Ctrl: control; OVX: ovariectomized; Tb.N: trabecular number; Tb.Sp: trabecular separation; Tb.th: trabecular thickness; TV: total volume.

0.01) or ADSCs-/PRFr-treated alone ($P < 0.05$) demonstrated a significantly higher trabecular bone ratio. Otherwise, increased Tb.N and decreased Tb.Sp in OVX-injection groups were observed. Though the BV ($P < 0.05$) and Tb.N ($P < 0.05$) in tibial shaft showed a similar trabecular bone formation between OVX-ADSC and OVX-PRFr groups compared with the OVX-Ctrl group, it did show a progressive increase of trabecular bone mass in the ADSC or PRFr injections alone. However, injection with ADSCs+PRFr markedly increased the BV ($P < 0.01$) and bone parameters ($P < 0.01$) in the OVX groups except for Tb.Sp, which was significantly decreased ($P < 0.01$). Additionally, no significant changes in Tb.Th were observed in any of the treatment groups tested. Overall, the ADSCs+PRFr treatment did restore the BV and bone parameters to the normal levels as well as the Ctrl and Sham groups.

Histological Analysis of Tibial Bone

Figure 2 shows the H&E-stained diminished trabecular bone of the OVX mice. Decreased Tb.N and increased Tb.Sp in OVX-ADSCs/OVX-PRFr versus OVX-ADSCs+PRFr were observed. It can be obviously seen that more trabecular bone formation was found in the tibia while trabecular spacing was decreased in the OVX-ADSCs+PRFr group. Similarly, trabecular conjunction points were obviously increased in the OVX groups treated with ADSCs+PRFr when compared to the groups treated with ADSCs or PRFr alone.

Analysis of Mineral Elements in Serum

The quantitative analysis of Ca and P in serum at 8 wk post-transplantation is shown in Fig. 3. These data showed that the Ca had similar levels in the Ctrl and sham groups, while there were significantly higher concentrations of Ca in the
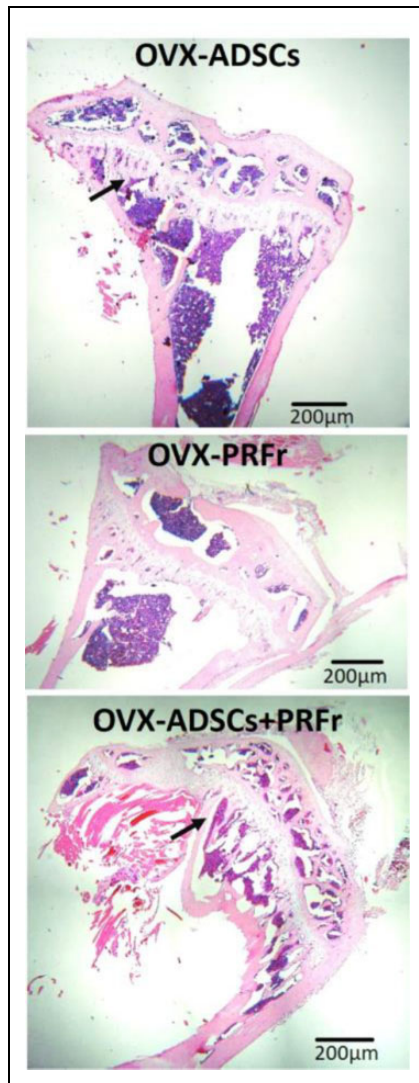
Fig. 2. Histological sections of osteoporotic mice proximal tibia 8 wk post-transplantation, of either ADSCs, PRFr, or in combination therapy (ADSCs+PRFr), stained using H&E staining methods. The black arrows indicate bony trabeculae. Scale bar—200 μm.
ADSC: adipose-derived stem cell; H&E: hematoxylin and eosin; OVX: ovariectomized; PRFr: platelet-rich fibrin releasate.

OVX-ADSCs, OVX-PRFr, and OVX-ADSCs+PRFr groups than in the OVX-Ctrl group. Figure 3 also showed similar P contents in all groups regardless of the unoperation/injection for the OVX models, except that the P content in the OVX-ADSCs+PRFr group was significantly higher than in the OVX-Ctrl group ($P < 0.05$), implying that the ADSCs+PRFr injection might be associated with positive bone parameters.

Discussion

OP is a skeletal disease characterized by decreased bone mass and microarchitectural deterioration of bone tissue, resulting in in an increased risk of fragility fractures. The pathophysiological involution OP of estrogen deficiency has been identified in both the early accelerated and late slow phases of bone loss in women following menopause. The accelerated phase in women is most apparent during the first decade after menopause and involves a disproportionate loss of cancellous bone. It is mediated mainly by the loss of the direct restraining effects of estrogen on bone cell function[42]. OVX mice are classically used as an animal model to simulate postmenopausal bone loss and patterns of bone loss after ovariectomy. In the present study, we intended to explore the feasibility of using ADSCs and PRFr as a cell-based therapeutic approach for treating OP in an OVX mice model by vein injection.

One of the key cellular players in bone physiology is the osteoblast, a bone-forming cell derived from bone marrow-resident mesenchymal stromal cells. Bone marrow-derived mesenchymal stem cells (BMSCs) are self-renewing in vivo. The shift in their differentiation from osteogenic to adipogenic lineage with increase in age can potentially affect osteoblast formation and bone remodeling, and thus be a factor in OP development and pathogenesis[43]. ADSCs have been shown to be pluripotent and explored for their usage in tissue engineering. We focused on ADSCs because of the easy isolation procedure and abundant supply and that fat-derived ADSCs are a more preferred source of autologous mesenchymal cells compared to BMSCs. Moreover, ADSCs proliferate at a higher rate than BMSCs[44], and have been found to secrete a variety of cytokines and growth factors with trophic paracrine effects that may mediate and stimulate tissue repair and regeneration[45]. In this study, flow cytometry was used to characterize ADSCs isolated from young female ICR mice. ADSCs were distinguished and they showed the highly positive expression of surface markers such as CD73 and CD90 and negative expression of CD34 and CD45.

Previous studies have shown that autologous growth factors released by PRF gradually expressed stronger and more durable effect on proliferation and differentiation of rat osteoblasts than those released by PRP[28]. A variety of tests suggested that PRF was superior to PRP in growth factor release, from the aspects of expression of alkaline phosphatase and induction of mineralization; therefore, the use of PRFr seems to be a more promising method to stimulate cellular activities related to healing responses in a way that is controllable and has a relatively long-term effect[28,46]. Although not yet conclusive, it appears that most research supports a positive role for platelet concentrates in bone regeneration in vivo[47,48]. A study further reported that PRP-treated osteo-progenitor cells underwent osteogenesis and they ameliorated OP in OVX-SAMP8 group model[14]. The stimulation of VEGF and MSCs combined with VEGF has been shown to enhance bone formation and healing. Another study has proven that the cross-talk between VEGF and bone morphogenetic protein (BMP) signaling pathways in MSCs promotes osteoblastic differentiation of MSCs, which aids in bone formation and fracture repair[49]. In
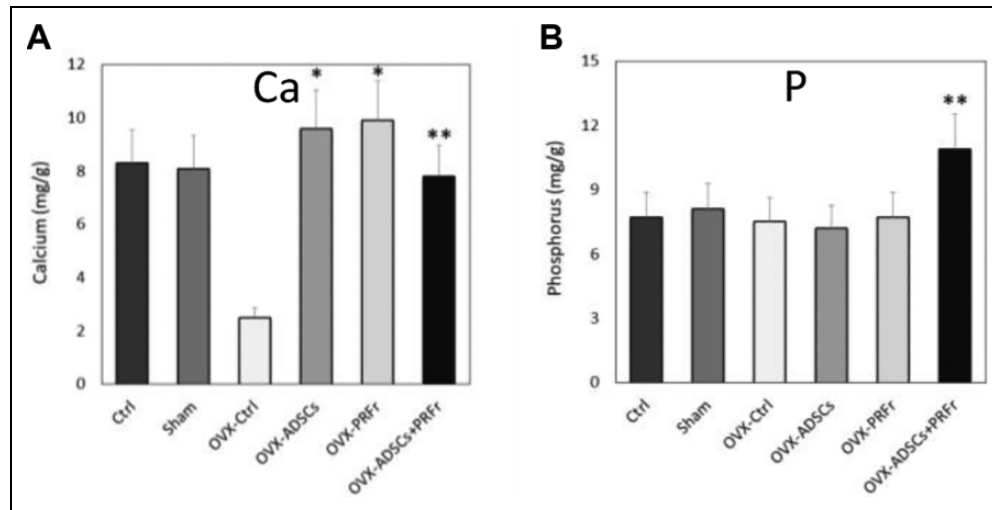
Fig. 3. Total calcium and phosphorous in serum (six groups, $n = 6$) 8 wk post-transplantation. The asterisks indicate significant differences as compared with the OVX-Ctrl group (*$P < 0.05$; **$P < 0.01$).
ADSC: adipose-derived stem cell; Ctrl: control; OVX: ovariectomized; PRFr: platelet-rich fibrin releasate.

assessing the efficacy of PRP and MSCs in the treatment of bone failure of OVX animals, a study using OVX-rat model showed good repair of osteoporotic bone defects (a 1.5-mm diameter tibial defect was made) treated with PRP and BMSCs[50], and also showed that the expression levels of osteogenesis-specific marker genes including RUNX2 (runt-related transcriptionfactor 2), OSX (osterix), and OPN (osteopontin) were significantly upregulated in the OVX-PRP/BMSCs rats[50]. However, PPARγ2 expression was suppressed particularly in the OVX-PRP/BMSCs group[50]. Similarly, a study demonstrated that xenogenic PRP (xenogeneic profiles of cytokines and growth factors) did not induce elevation of bone resorption[35]; and the treatment of bone failures in rabbits with secondary OP with allogeneic MSCs and xenogenic PRP could induce greater bone consolidation with mature and immature bone tissue production[35].

In the present study, by using a well-established osteoporotic mice model, we demonstrated that treatment with allogeneic ADSCs combined with xenogenic PRFr can be used to enhance bone formation. To our knowledge, this is the first experimental study of this kind to examine the efficacy of a combination of ADSCs and PRFr in OP treatment. Our results show a significant loss of BMD in the proximal tibia (40%) in mice 16 wk post bilateral OVX, as measured by micro-CT of the proximal tibia. Ultimately, ADSCs+PRFr transplants were shown to be effective in restoring tibial bone density, 8 wk post-transplantation. OVX mice that received ADSCs, PRFr, and ADSCs+PRFr transplants showed significantly higher bone regeneration (an average of 58%, 42%, and 64% of improved BMD, respectively, $P < 0.05$) over the OVX-Ctrl group. Quantification analysis also demonstrated that Tb.N clearly show significant losses (68%) in mice 16 wk after ovariectomy. The OVX group treated with ADSCs (27%; $P < 0.05$%),

PRFr (22%; $P < 0.05$%), and ADSCs+PRFr (120%; $P < 0.01$%) observed higher bone restoration when compared to OVX-Ctrl at 8 wk post-transplantation. During the late phase of bone healing, the newly formed bone in the proximal tibia was remodeled in the sham and the OVX-ADSCs+PRFr-treated mice, whereas remodeling was incomplete in the OVX-ADSCs, OVX-PRFr, and the OVX groups' mice, as observed in the micro-CT images. Notably, the significantly increased levels of BV and Tb.N of proximal tibia growth plates were observed in the OVX-ADSCs+PRFr ($P < 0.01$), OVX-ADSCs ($P < 0.05$), and OVX-PRFr ($P < 0.05$) groups' mice as compared with that in the OVX mice, indicating that the positive effect of ADSCs on bone formation might be enhanced in the presence of PRFr. As concentrated growth factors remarkably promoted the growth and osteogenic differentiation capacities of ADSCs in vitro[51], it is thus possible that PRFr may accelerate bone formation during skeletal repair by upregulating the proliferation and osteogenesis of ADSCs, which is consistent with that of an earlier report[50].

PRFr is rich in b-FGF, BMPs, EGF, PDGF, TGF, VEGF, insulin-like growth factor (IGF), nerve growth factor (NGF), and so on[27,52]. During the past few years, progress has been made toward the understanding of local regulation of bone remodeling especially in relation to OP. Cytokines have shown to be powerful regulators of bone resorption and formation, though under superior control from estrogen/testosterone, parathyroidhormone, and 1alpha,25(OH)$_2$D$_3$[53]. Collectively, BMPs are useful for improving fracture healing by both increasing osteogenesis and creating a favorable healing environment by altering cytokine release by endogenous cells[54,55]. To this end, the production of b-FGF as a result of BMP-2 exposure during the early phases of healing promotes the induction of BMSCs to become osteoblasts

while improving angiogenesis and mitogenesis simultaneously[55]. EGF protracts extracellular signal-regulated kinase and Akt phosphorylation and acts as an important mediator for osteogenic factor[56]. IGF-1 plays an important role in cell proliferation, differentiation, and apoptosis and is the major mediator of the effect of growth hormone on both bone growth and mineralization[57], via decreasing osteoblast apoptosis and promoting osteoblastogenesis through the phosphoinositide 3-kinase[58]. NGF promotes cartilage differentiation and increases osteoclast formation and healing of mice tibial fractures by increasing the levels of COL2A1 and SOX9 mRNA expression, and may be used for the prevention and treatment of OP[59]. Some of the cytokines primarily enhance osteoclastic bone resorption, e.g., interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor, while others, e.g., IGF, PDGF, and TGF-β, primarily stimulate bone formation[53]. Moreover, VEGF plays the role of a key upregulator of angiogenesis that is essential for bone development and regeneration, especially for large bone defects[60]. Therefore, PRFr may regulate and mediate the balance between osteogenesis and adipogenesis in vivo, which may also contribute to the positive effects of PRFr on bone formation in osteoporotic mice.

Our results suggest that ADSCs and PRFr may be useful in enhancing BV and treating pathological conditions of bone loss. Although mice are usually utilized for the purpose of initial evaluation and biological compatibility tests, these tests only provide limited information. Larger subjects and longer periods may provide more definitive and meaningful support for using this therapeutic approach. In conclusion, the present results indicated that ADSCs and PRFr promote new bone formation. Better results were obtained by combining the two into a single treatment for osteoporotic bone defects. Therefore, the combination therapy of ADSCs and PRFr may be a promising novel therapeutic strategy in osteoporotic bone regeneration.

Author Notes

Shinn-Zong Lin and Tzong-Fu Kuo contributed equally to this work.

Ethical Approval

Ethical approval to report this study was obtained from the Institutional Animal Care and Use Committees (IACUC) of National Taiwan University (NTU-102-EL-82).

Statement of Animal Rights

All experiments were carried out in accordance with the International Council for Laboratory Animal Science guidelines for the care and use of laboratory animals. This research was carried out in strict accordance with an approved IACUC protocol at National Taiwan University (NTU-102-EL-82).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, and/or publication of this article.

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References

1. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest. 2005;115(12):3318–3325.
2. Egermann M, Goldhahn J, Schneider E. Animal models for fracturetreatment in osteoporosis.Osteoporos. Int. 2005;16(2): S129–S138.
3. Wronski TJ, Cintrón M, Dann LM. Temporal relationship between bone loss and increased bone turnover in ovariectomized rats. Calcif Tissue Int. 1988;43(3):179–183.
4. Wronski TJ, Dann LM, Scott KS, Cintrón M. Long-term effects of ovariectomy and aging on the rat skeleton. Calcif Tissue Int. 1989;45(6):360–366.
5. Wronski TJ, Dann LM, Horner SL. Time course of vertebral osteopenia in ovariectomized rats. Bone. 1989;10(4):295–301.
6. Favus MJ. Bisphosphonates for osteoporosis. N Engl J Med. 2010;363(21):2027–2035.
7. Devine A, Dick IM, Dhaliwal SS, Naheed R, Beilby J, Prince RL. Prediction of incident osteoporotic fractures in elderly women using the free estradiol index. Osteoporos Int. 2005; 16(2):216–221.
8. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, Christiansen C, Delmas PD, Zanchetta JR, Stakkestad J, Glüer CC, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-yearrandomizedclinicaltrial Multiple outcomes of raloxifene evaluation (MORE) Investigators. JAMA. 1999;282(7):637–645.
9. Deeks ED.Denosumab: a review in postmenopausal osteoporosis. Drugs Aging. 2018;35(2):163–173.
10. Bandeira L, Lewiecki EM, Bilezikian JP. Pharmacodynamics and pharmacokinetics of oral salmon calcitonin in the treatment of osteoporosis. Expert Opin Drug Metab Toxicol. 2016;12(6):681–689.
11. Tabatabaei-Malazy O, Norani M, Heshmat R, Qorbani M, Vosoogh A, Afrashteh B, Kahkeshan F, Ajami A, Larijani B. Efficacy and safety of the biosimilar recombinant human parathyroid hormone Cinnopar® in postmenopausal osteoporotic women: a randomized double-blind clinical trial. Iran J Public Health. 2018;47(9):1336–1344.
12. Camacho PM, Petak SM, Binkley N, Clarke BL, Harris ST, Hurley DL, Kleerekoper M, Lewiecki EM, Miller PD, Narula HS, Pessah PR, et al. American association of clinical endocrinologists and American college of endocrinology: Clinical

practice guidelines for the diagnosis and treatment of postme-
nopausal osteoporosis−2016. Endocr Pract. 2016;22(suppl 4):
S1–S42.

13. Tu KN, Lie JD, Wan CKV, Cameron M, Austel AG, Nguyen
JK, Van K, Hyun D. Osteoporosis: a review of treatment
options. P T. 2018;43(2):92–104.

14. Fasipe OJ, Ibiyemi OB, Adelosoye AA, Idowu AA. Recent
advances and current trend in the pharmacotherapy of postme-
nopausal osteoporosis. J Health Res Rev. 2018;5(1):13–21.

15. Tella SH, Gallagher JC. Prevention and treatment of postme-
nopausal osteoporosis. J Steroid Biochem Mol Biol. 2014;142:
155–170.

16. Liu HY, Wu AT, Tsai CY, Chou KR, Zeng R, Wang MF,
Chang WC, Hwang SM, Su CH, Deng WP. The balance
between adipogenesis and osteogenesis in bone regeneration
by platelet-rich plasma for age-related osteoporosis. Biomater-
ials. 2011;32(28):6773–6780.

17. Duque G. Bone and fat connection in aging bone. Curr Opin
Rheumatol. 2008; 20(4):429–434.

18. Harada S, Rodan GA. Control of osteoblast function and reg-
ulation of bone mass. Nature. 2003;423(6937):349–355.

19. Wan Y, Chong LW, Evans RM. PPAR-gamma regulates osteo-
clastogenesis in mice. Nat Med. 2007;13(12):1496–1503.

20. Nguyen DT, Burg KJ. Bone tissue engineering and regenera-
tive medicine: targeting pathological fractures. J Biomed
Mater Res A. 2015;103(1):420–429.

21. Shen J, Tsai YT, Dimarco NM, Long MA, Sun X, Tang L.
Transplantation of mesenchymal stem cells from young donors
delays aging in mice. Sci Rep. 2011;1:67.

22. Mirsaidi A, Genelin K, Vetsch JR, Stanger S, Theiss F, Lindt-
ner RA, von Rechenberg B, Blauth M, Müller R, Kuhn GA,
Hofmann Boss S, et al. Therapeutic potential of adipose-
derived stromal cells in age-related osteoporosis. Biomaterials.
2014;35(26):7326–7335.

23. Hu L, Yin C, Zhao F, Ali A, Ma J, Qian A.Mesenchymal stem
cell: cell fate decision to osteoblast or adipocyte and applica-
tion in osteoporosis treatment. Int J Mol Sci. 2018;19(2):360.

24. Torres TM, Rubio M, Damia E, Cuervo B, Del Romero A,
Peláez P, Chicharro D, Miguel L, Sopena JJ. Adipose-
derived mesenchymal stem cells: A promising tool in the treat-
ment of musculoskeletal diseases. Int J Mol Sci. 2019;20(12):
3105.

25. Dohan Ehrenfest DM, Andia I, Zumstein MA, Zhang CQ,
Pinto NR, Bielecki T. Classification of platelet concentrates
(Platelet-Rich Plasma-PRP, Platelet-Rich Fibrin-PRF) for topi-
cal and infiltrative use in orthopedic and sports medicine: cur-
rent consensus, clinical implications and perspectives. Muscles
Ligaments Tendons J. 2014;4(1):3–9.

26. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mou-
hyi J, Gogly B. Platelet-rich fibrin (PRF): a second-generation
platelet concentrate. Part I: technological concepts and evolu-
tion. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;
101(3):e37–e44.

27. Burnouf T, Lee CY, Luo CW, Kuo YP, Chou ML, Wu YW,
Tseng YH, Su CY. Human blood-derived fibrin releasates:

composition and use for the culture of cell lines and human
primary cells. Biologicals. 2012;40(1):21–30.

28. He L, Lin Y, Hu X, Zhang Y, Wu H. A comparative study of
platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) on the
effect of proliferation and differentiation of rat osteoblasts in
vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod.
2009;108(5):707–713.

29. Lo WC, Chiou JF, Gelovani JG, Cheong ML, Lee CM, Liu HY,
Wu CH, Wang MF, Lin CT, Deng WP. Transplantation of
embryonic fibroblasts treated with platelet-rich plasma induces
osteogenesis in SAMP8 mice monitored by molecular imaging.
J Nucl Med. 2009;50(5):765–773.

30. Hsu YK, Sheu SY, Wang CY, Chuang MH, Chung PC, Luo
YS, Huang JJ, Ohashi F, Akiyoshi H, Kuo TF. The effect of
adipose-derived mesenchymal stem cells and chondrocytes
with platelet-rich fibrin releasates augmentation by intra-
articular injection onacute osteochondral defects in a rabbit
model. Knee. 2018;25(6):1181–1191.

31. Su CY, Kuo YP, Tseng YH, Su CH, Burnouf T. In vitro release
of growth factors from platelet-rich fibrin (PRF): a proposal to
optimize the clinical applications of PRF. Oral Surg Oral Med
Oral Pathol Oral Radiol Endod. 2009;108(1):56–61.

32. Arrigoni E, Lopa S, de Girolamo L, Stanco D, Brini AT. Iso-
lation, characterization and osteogenic differentiation of
adipose-derived stem cells: from small to large animal models.
Cell Tissue Res. 2009:338(3):401–411.

33. Liu G, Chen X. Isolating and characterizing adipose-derived
stem cells. Methods Mol Biol. 2018;1842:193–201.

34. Dutta S, Sengupta P. Men and mice: relating their ages. Life
Sci. 2016;152:244–248.

35. Rocha MAC, Silva LMC, Oliveira WA, Bezerra DO, Silva
GCD, Silva LDS, Medeiros BLDN, Baêta SAF, Carvalho
MAM, Argôlo NMN. Allogeneic mesenchymal stem cells and
xenogenic platelet rich plasma, associated or not, in the repair
of bone failures in rabbits with secondary osteoporosis. Acta
Cir Bras. 2017;32(9):767–780.

36. Committee for the Update of the Guide for the Care and Use of
Laboratory Animals. Guide for the care and use of laboratory
animals. 8th ed. Washington DC: Institute for Laboratory Ani-
mal Research, Division on Earth and Life Studies, National
Research Council. National Academies Press; 2011.

37. Kido HW, Bossini PS, Tim CR, Parizotto NA, da Cunha AF,
Malavazi I, Renno AC. Evaluation of the bone healing process
in an experimental tibial bone defect model in ovariectomized
rats. Aging Clin Exp Res. 2014;26(5):473–481.

38. Hoffmann DB, Sehmisch S, Hofmann AM, Eimer C, Komra-
kova M, Saul D, Wassmann M, Sturmer KM, Tezval M. Com-
parison of parathyroid hormone and strontium ranelate in
combination with whole-body vibration in a rat model of osteo-
porosis. J Bone Miner Metab. 2017;35(1):31–39.

39. Wang F, Nojima M, Inoue Y, Ohtomo K, Kiryu S. Assessment
of MRI contrast agent kinetics via retro-orbital injection in
mice: comparison with tail vein injection. PLoS One. 2015;
10(6):e0129326.

40. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jep-
sen KJ, Müller R. Guidelines for assessment of bone

microstructure in rodents using micro-computed tomography. J Bone Miner Res. 2010;25(7):1468–1486.

41. Snedecor GW, Cochron WG. Statistical methods, 8th ed. Ames, Iowa: Iowa State University Press; 1989.

42. Riggs BL, Khosla S, Melton LJ. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. J Bone Miner Res. 1998;13(5): 763–773.

43. Ganguly P, El-Jawhari JJ, Giannoudis PV, Burska AN, Ponchel F, Jones EA. Age-related changes in bone marrow mesenchymal stromal cells: A potential impact on osteoporosis and osteoarthritis development. Cell Transplant. 2017;26(9): 1520–1529.

44. Liao D, Gong P, Li X, Tan Z, Yuan Q. Co-culture with Schwann cells is an effective way for adipose-derived stem cells neural transdifferentiation. Arch Med Sci. 2010;6(2): 145–151.

45. Murphy MB, Moncivais K, Caplan AI. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Exp Mol Med. 2015;45(11):e54.

46. Dohan Ehrenfest DM, Bielecki T, Jimbo R, Barbé G, Del Corso M, Inchingolo F, Sammartino G. Do the fibrin architecture and leukocyte content influence the growth factor release of platelet concentrates? An evidence-based answer comparing a pure platelet-rich plasma (P-PRP) gel and a leukocyte- and platelet-rich fibrin (L-PRF). Curr Pharm Biotechnol. 2012; 13(7):1145–1152.

47. Cho AR, Kim HK, Kwon JY, Kim TK, Choi YM, Kim KH. The incorporation of platelet-rich plasma into calcium phosphate cement enhances bone regeneration in osteoporosis. Pain Physician. 2014;17(6): E737–745.

48. Oryan A, Alidadi S, Moshiri A. Platelet-rich plasma for bone healing and regeneration. Expert Opin Biol Ther. 2016;16(2): 213–232.

49. Cui Q, Dighe AS, Irvine JN Jr. Combined angiogenic and osteogenic factor delivery for bone regenerative engineering. Curr Pharm Des. 2013;19(19):3374–3383.

50. Wei B, Huang C, Zhao M, Li P, Gao X, Kong J, Niu Y, Huang R, Quan J, Wei J, Chu J. Effect of mesenchymal stem cells and platelet-rich plasma on the bone healing of ovariectomized rats. Stem Cells Int. 2016;2016:9458396.

51. Ma X, Ding L, Tang S, Li T, Pei J, Li Y. Effects of concentrated growth factors on proliferation and osteogenic differentiation in Beagle adipose-derived stem cells. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2018;43(1):1–6.

52. Kobayashi E, Flückiger L, Fujioka-Kobayashi M, Sawada K, Sculean A, Schaller B, Miron RJ. Comparative release of growth factors from PRP, PRF, and advanced-PRF. Clin Oral Investig. 2016;20(9):2353–2360.

53. Jørgensen NR. Cytokines and osteoporosis. Ugeskr Laeger. 1997;160(1):24–28.

54. Kessler S, Mayr-Wohlfart U, Ignatius A, Puhl W, Claes L, Günther KP. The impact of bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) on osseointegration, degradation and biomechanical properties of a synthetic bone substitute. Z Orthop Ihre Grenzgeb. 2003;141(4):472–480.

55. Rivera JC, Strohbach CA, Wenke JC, Rathbone CR. Beyond osteogenesis: an in vitro comparison of the potentials of six bone morphogenetic proteins. Front Pharmacol. 2013;4:125.

56. Iacob S, Cs-Szabo G. Biglycan regulates the expression of EGF receptors through EGF signaling pathways in human articular chondrocytes. Connect Tissue Res. 2010;51(5): 347–358.

57. Pass C, MacRae VE, Ahmed SF, Farquharson C. Inflammatory cytokines and the GH/IGF-I axis: novel actions on bone growth. Cell Biochem Funct. 2009;27(3):119–127.

58. Cao JJ, Kurimoto P, Boudignon B, Rosen C, Lima F, Halloran BP. Aging impairs IGF-I receptor activation and induces skeletal resistance to IGF-I. J Bone Miner Res. 2007;22(8): 1271–1279.

59. Sang XG, Wang ZY, Cheng L, Liu YH, Li YG, Qin T, Di K. Analysis of the mechanism by which nerve growth factor promotes callus formation in mice with tibial fracture. Exp Ther Med 2017;13(4):1376–1380.

60. Kempen DH, Lu L, Heijink A, Hefferan TE, Creemers LB, Maran A, Yaszemski MJ, Dhert WJ. Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration. Biomaterials. 2009;30(14):2816–2825.