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| Original Article |

Adipose Tissue-Derived Stem Cells in the Ovariectomy-Induced Postmenopausal Osteoporosis Rat Model

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Abstract : This study investigated the efficacy of adult adipose tissue-derived stem cells in restoring bone using an osteoporotic rat model. Thirty-six female Wistar rats (250 - 300 g, 12 weeks) were randomized into three equal groups: SHAM group (sham-operated), ovariectomy-induced (OVX) group and OVX with stem cell injection group (OVX with stem). Femur extraction and blood sampling were performed at 5, 6, 7 and 8 weeks. The proximal femoral metaphysis was scanned by micro-CT and evaluated for changes in various histomorphometric parameters. β-catenin expression was determined using H&E and immunohistochemistry. Bone metabolism was assessed using measurements of C-telopeptide of collagen type I (CTX), osteocalcin, and bone alkaline phosphatase (BALP). There was a trend for an increase in the bone mineral density (BMD), bone volume/trabecular bone volume (BV/TV) and trabecular number (Tb.N) in the OVX with stem group as compared with the OVX group, and decrease in the OVX with stem group as compared with SHAM group. The trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) of the OVX group were increased compared with those values in the SHAM and OVX with stem group. BALP levels were higher in the OVX group as compared with that in the other two groups and osteocalcin levels were highest in the SHAM group and slightly increased in the OVX with stem group as compared with the OVX group. This study may help us gain an understanding of the role of MSCs in the pathophysiology and treatment of osteoporosis.

Key words: *Ovariectomy, Osteoporosis, Animal model, Adipose tissue-derived stem cell*

1. Introduction

The World Health Organization (WHO) defines osteoporosis as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequential increase in fracture risk.[1-4] Osteoporosis may be caused, in part, by age-related decline in the number of osteoblast progenitors residing in the bone marrow [5] as well as the declining estrogen levels. Major therapeutic agents have been developed with the aim of preventing bone resorption. For most cases, osteoporotic patients are treated with antiresorptive drugs, such as bisphosphonates and selective estrogen receptor modulators.[6] While these anti-catabolic drugs are efficient in stabilizing bone mass, there is a need for anabolic drugs, which act alone or in conjunction with catabolic drugs, that target osteoblastic cells to increase bone formation and bone strength.[7] Recently, there have been introduced some anabolic drug such as parathyroid hormone (Fosteo, from Lilly) and several anti-sclerostin antibodies are in phase III clinical testing.

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, such as osteoblasts, chondroblasts and others. MSCs are often considered for cell and gene therapy strategies aimed at satisfying these requirements. These cells can be isolated from the bone marrow, adipose tissue and other sources, then cultured and

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expanded *in vitro* to increase the proportion of progenitor cells for use in therapy.[8,9] Indeed, adipose tissue-derived adult stem cells (ADMSCs) may have potential applications in the repair and regeneration of acutely or chronically damaged tissues. However, there are a few reports concerning the systemic effect and biological mechanism of MSC action in osteoporotic disease models. Therefore, the aim of this study was to investigate the effects of cultured ADMSCs in restoring an overall positive bone balance in the ovariectomy-induced postmenopausal osteoporosis rat model.

2. Materials and Methods

2.1 Animals and Experimental Design

For postmenopausal osteoporosis model, Animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Institute for Life Sciences, Seoul, Republic of Korea. Thirty-six female Wistar rats (250 - 300 g, aged 12 weeks) were divided into three groups of 12 rats: SHAM group (normal, sham-operated), ovariectomy (OVX)-induced osteoporosis group (OVX group) and OVX with stem cell injection group (OVX with stem). All rats in the OVX and OVX with stem groups were ovariectomized bilaterally and rats in the SHAM group were subjected to a sham surgery. Rats in the OVX with stem group received an injection of 2×10^6 ADMSCs into the lateral tail vein at 2 and 4 weeks after the surgery. Body weight was measured once per week for 8 weeks during the experimental period. Rats in all three groups were sacrificed at 5, 6, 7 and 8 weeks, followed by immediate blood sampling and the extraction of bilateral femurs.

2.2 Isolation and Culture of ADMSCs

Approximately 10 g of adipose tissue was harvested from the bilateral perirenal area of the female Wistar rats (250 - 300 g, aged 12 weeks). Harvested tissues were washed in saline and digested with 0.075% collagenase at 37°C for 1 h. The harvested adipose tissue was suspended in low-glucose Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA) containing fetal bovine serum (Gibco, Invitrogen) and 1% penicillin/streptomycin (Gibco, Invitrogen) (complete media), and then centrifuged for 10 min at 600 × g. The supernatant was discarded and the red blood cell content removed using 160 mM ammonium chloride for 3-5 min at room temperature. MSCs were isolated using a 70-μm nylon mesh filter and then centrifuged at 600 × g for 10 min. The pellet was resuspended in complete media and placed into a 175 cm^2 flask (Nunc, Invitrogen, Carlsbad, CA) as the primary culture in a humidified incubator at 37°C for 5 days under 5% CO_2. Non-adherent cells were removed by replenishing the media every 2-3 days with 10 mL of DMEM media. At 70-80% confluence, cells were harvested using 0.25% trypsin-EDTA and used for lateral tail vein injection.

2.3 Identification of ADMSCs

Fluorescence-activated cell sorting (FACS) was used to analyze the surface markers of rat ADMSCs. After 3 passages, the cells were trypsinized and resuspended in DMEM containing 10% fetal bovine serum (FBS). Samples were counted, centrifuged, and resuspended in PBS. The cells were placed into eppendorf tubes at 1×10^6 cells per 1.5 mL, washed twice with PBS, and incubated for 1 hour at room temperature with the following fluorescein isothiocyanate-conjugated antibodies (Peprotech Inc., USA) : anti-rat CD90 (1 : 200), anti-rat CD 29 (1 : 200), anti-rat CD45 (1 : 200), and anti-rat CD11b (1 : 200). In SHAM groups, the cells were incubated in PBS without antibodies. The samples were then washed twice with PBS and analyzed by FACS.

2.4 Blood sampling and Femur Extraction

Zoletil and Rompun were mixed in a 3:1 ratio, of which 200 μl was injected into each rat as intra-abdominal anesthesia. Blood (3 mL) was sampled from each rat by transcardiac puncture and transferred into a serum separator tube (SST tube, BD, Franklin Lakes, NJ). Samples were then centrifuged for 5 min in 1000 × g at 4°C. After centrifugation, the supernatant was collected and stored at -200°C.

Femurs were extracted from the rats, cleaned with normal saline, and wrapped with saline-soaked gauze prior to cryopreservation in -70°C.

2.5 Micro-computed Tomography (CT) Measurements

At first, the extracted femur samples were unfreezed, and then, they were scanned the proximal metaphysis by micro-CT (SKYSCAN 1172 high-resolution micro-CT, SKYSCAN, Kontich, Belgium). The micro-CT scans were acquired with rotation step of 0.4° at a tube voltage of 70 V, a tube current of 141 μA and pixel size of 18.81 μm. The image reconstruction and analysis were performed using SKYSCAN software (SKYSCAN N.V., SKYSCAN, Kontich, Belgium). The parameters evaluated included bone mineral density (BMD; g/cm^2), trabecular bone volume (BV/TV; %), trabecular number (Tb.N; mm), trabecular thickness (Tb.Th; mm) and trabecular separation (Tb.Sp; mm). Volume of Interest (VOI) was mean value of the maximal horizontal area of the cancellous bone region of the femoral head from upper most rudimentary growth plate to lower most cortical bone divided by 1.5 mm sliced 150 CT image in the analysis.

2.6 Pathological Evaluation

After 12weeks, all extracted and scanned femur specimens were fixed in 10% Buffered Neutral formalin for up to 7 days. Decalcification was performed with 8% Hydrochloric acid stock solution, 8% Formic acid solution, and ammonia solution. All specimens were saturated in fresh mixture solution every 3days. The decalcified femurs were embedded in paraffin and longitudinally cut into 4 μm-thick sections using a microtome (Leica Microsystems, Leica RM2255, Wetzlar, Germany). Sections were incubated in hematoxylin (Harris Hematoxylin-I, YD-Diagnostics.com, Yong In, South Korea) for 5 min and stained with eosin (Eosin Y solution, Sigma-Aldrich Co, St. Louis, MO) for 30 seconds. The H&E stained sections were observed by light microscopy.

2.7 Biochemical assay

All samples were centrifuged at 2000 rpm for 15 min at 4°C. Separated serum samples were measured with rat bone-specific alkaline phosphatase (BALP) rat ELISA kit (Kamiya Biomedical Company, Seattle, WA), rat osteocalcin enzyme-linked immunosorbent assay (ELISA) kit (Rat-MID™ Osteocalcin EIA, Immunodiagnostic System Inc., Boldon, UK), and rat C-telopeptide (Rat C-telopeptide of type I collagen (CTX-1) ELISA Kit (Cusabio Biotech Co., Ltd., Hubei, P.R. China) according to standard protocols and the manufacturers' instructions.

2.8 Immunohistochemistry for β-catenin

For β-catenin immunohistochemistry, paraffin-embedded slides were heated at 60°C for 1 h, deparaffinized with xylene and rehydrated through a series of graded alcohol solutions. For antigen retrieval, the tissue samples were treated with 0.25% Trypsin-EDTA (Gibco, Invitrogen, Carlsbad, CA) and incubated at 37°C for 5 min. We then followed a standard immunohistochemical method for the detection of β-catenin using rabbit monoclonal anti-β-catenin (Cell Signaling, Beverly, MA) at a 1:100 dilution, followed by Dako REAL™ EnVision™ HRP rabbit/mouse secondary antibody (Dako, DK-2600 Glostrup, Denmark). For negative control slides, the primary antibody step was removed. Antigen-antibody complexes were visualized using the DAB Substrate kit (Dako Liquid DAB+Substrate Chromogen System, K3468, Dako) and slides were counterstained with hematoxylin. β-catenin expression was evaluated by manual counting.

2.9 Statistical Analyses

The SAS (version 9.3, SAS Institute Inc, Cary, NC, USA) statistical package was utilized for statistical analyses. Data were represented as the mean ± standard deviation (SD). Two-way ANOVA with adjusting for time effector and Tukey's multiple comparison with post-hoc analysis were used to analyze the differences in weight, micro-CT data and biochemical assay measurements between the three groups (SHAM, OVX and OVX with stem group). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Culture of Isolated ADSCs in Monolayer and Identification of ADMSCs

ADMSCs cultured in monolayer had a fibroblast-like shape under an inverted phase-contrast microscope In FASC, characteristic expressions of stem cell related surface markers were confirmed. As control, cells were stained with isotype control IgG. ADMSCs expressed CD90 and CD29 which are adipogenic mesenchymal stem cell markers in 99% and 100%, respectively and were negative in CD11b and CD45 representing hematopoietic stem cell markers in 99.9% and 98%, respectively (supplementary material)

3.2 Body Weight

In all groups, the mean body weight increased over the 8 weeks after ovariectomy. Weight measurements for the rats in the OVX and OVX with stem groups was higher than that in the SHAM group, with body weight in the OVX with stem group slightly lower than that in the OVX group at 4, 6 and 7 weeks post-surgery. There was a significant difference in the total body weight between the SHAM and OVX or OVX with stem groups at 8 weeks post-surgery ($p < 0.05$) (Fig 1).

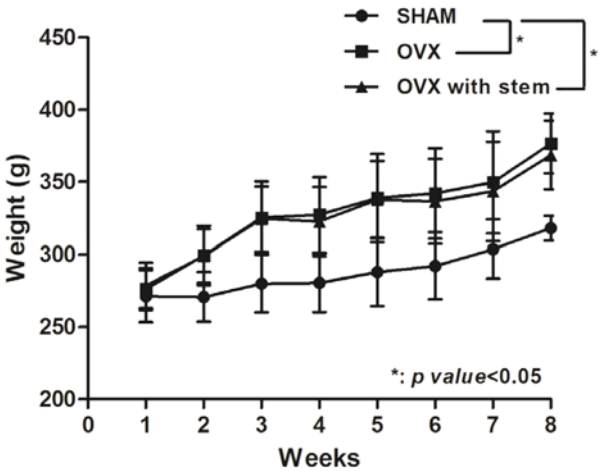

Figure 1. Mean body weight of the rats. The body weight in the OVX with stem group was slightly lower than that in the OVX group at 4, 6 and 7 week post operation (SHAM: sham operated group, OVX : ovariectomy group, OVX with stem : ovariectomy with stem cell injection group).

3.3 Micro-CT Measurements

There was a trend for an increase in the BMD, BV/TV and Tb.N in the OVX with stem group as compared with the OVX group, and a decrease in the OVX with stem group as compared with SHAM group. The Tb.Th and Tb.Sp of the OVX group were increased compared with those values in the SHAM and OVX with stem group. The difference between SHAM group and OVX or OVX with stem groups was statistically significant (Fig 2A~E).

3.4 Histology

Over the 5-8 weeks after surgery, histologic analyses of stained sections of the femur showed a thinning of the bony trabeculae and increased adipose tissue proportion at the bone marrow in the OVX and OVX with stem groups as compared with that of the SHAM group (Fig 3) These changes were more prominent in the OVX group than in the OVX with stem group.

3.5 Changes in the Serum Levels of Biochemical Markers

BALP levels were higher in the OVX group as compared with those in the other two groups, with the lowest level observed in the SHAM group ($p < 0.05$). Osteocalcin levels were highest in the SHAM group and slightly increased in the OVX with

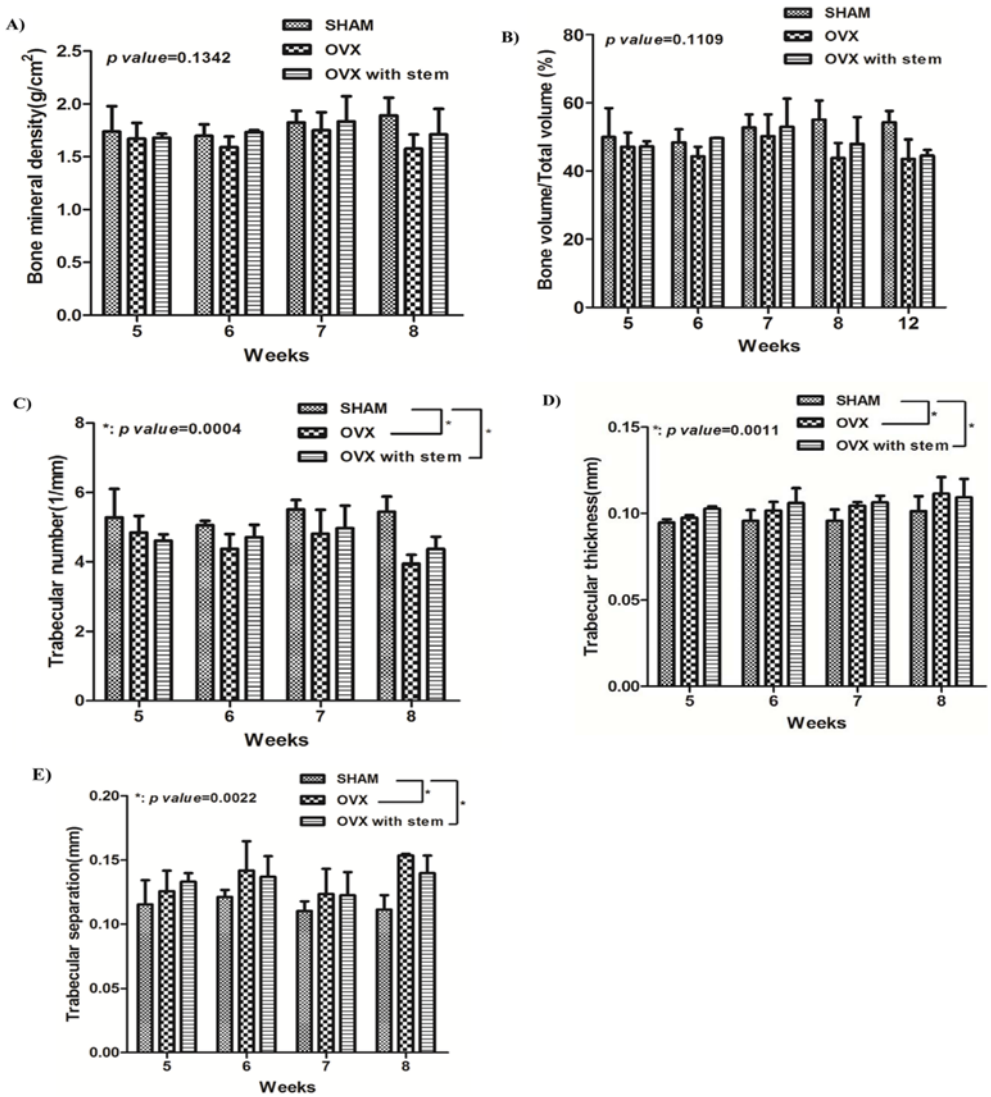

Figure 2. Micro-CT evaluation. There was a trend for an increase in the (A) bone mineral density (BMD), (B) trabecular bone volume (BV/TV; %) and (C) trabecular number (Tb.N) in the OVX with stem group as compared with the OVX group, and a decrease in the OVX with stem group as compared with SHAM group (D) and (E) trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) of the OVX group was increased compared with those values in the SHAM and OVX with stem group. (SHAM: sham operated group, OVX : ovariectomy group, OVX with stem : ovariectomy with stem cell injection group).

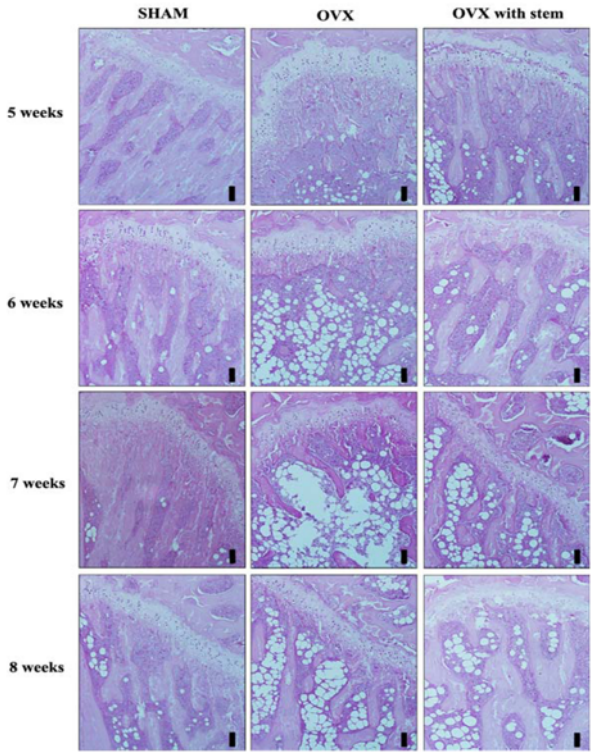

Figure 3. Hematoxylin-Eosin staining of the extracted femur. Sections of the femur were stained with hematoxylin and eosin. The results thinning of the bony trabeculae and increased adipose tissue proportion at the bone marrow for rats in the OVX group as compared with the SHAM and OVX with stem groups over the 5-8 weeks (Bars= x100, SHAM : sham operated group, OVX with stem group: ovariectomy with stem cell injection group).

stem group as compared with the OVX group (p <0.05). In contrast, CTX expression was highest in the OVX group as compared with the SHAM and OVX with stem groups (p > 0.05) (Fig 4A~C).

3.6 Immunohistochemistry for β-catenin

In the negative control samples, no β-catenin expression was observed in the growth plate or in the cortical bone, as expected, and positive β-catenin expression was observed in all three groups. The β-catenin expression was lowered in the OVX group as compared with that in the SHAM group. In contrast, the OVX with stem group showed increased positive β-catenin expression than that in the OVX group (p > 0.05) (Fig 5).

4. Discussion

There are two basic forms of osteoporosis that are based on whether the disease occurs as the primary disorder or secondary

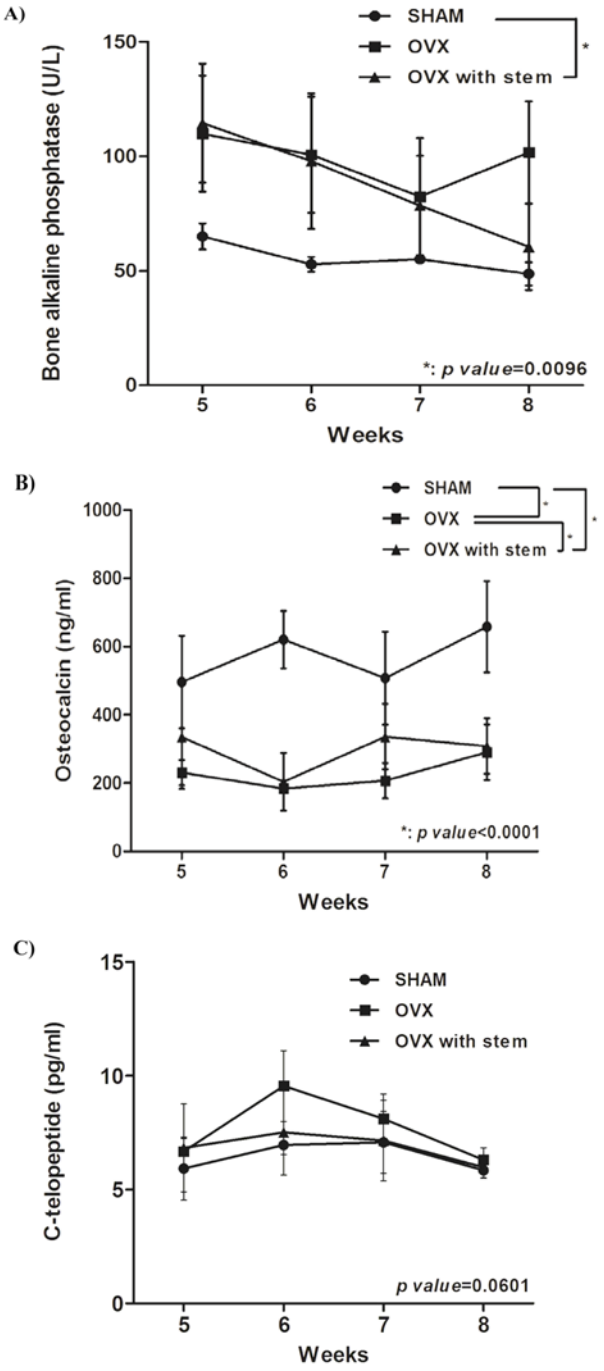

Figure 4. Changes in the levels of serum markers. A) The level of bone alkaline phosphatase (BALP) was higher in the OVX with stem group compare to the SHAM and OVX group. B) The level of osteocalcin was higher in the SHAM group compare to the OVX and OVX with stem group. And there was a slight increase in the OVX with stem group compare to the OVX group. C) The level of C-telopeptide of type I collagen (CTX) was highest in the OVX group compare to the SHAM and OVX with stem group. (SHAM: sham operated group, OVX : ovariectomy group, OVX with stem : ovariectomy with stem cell injection group).

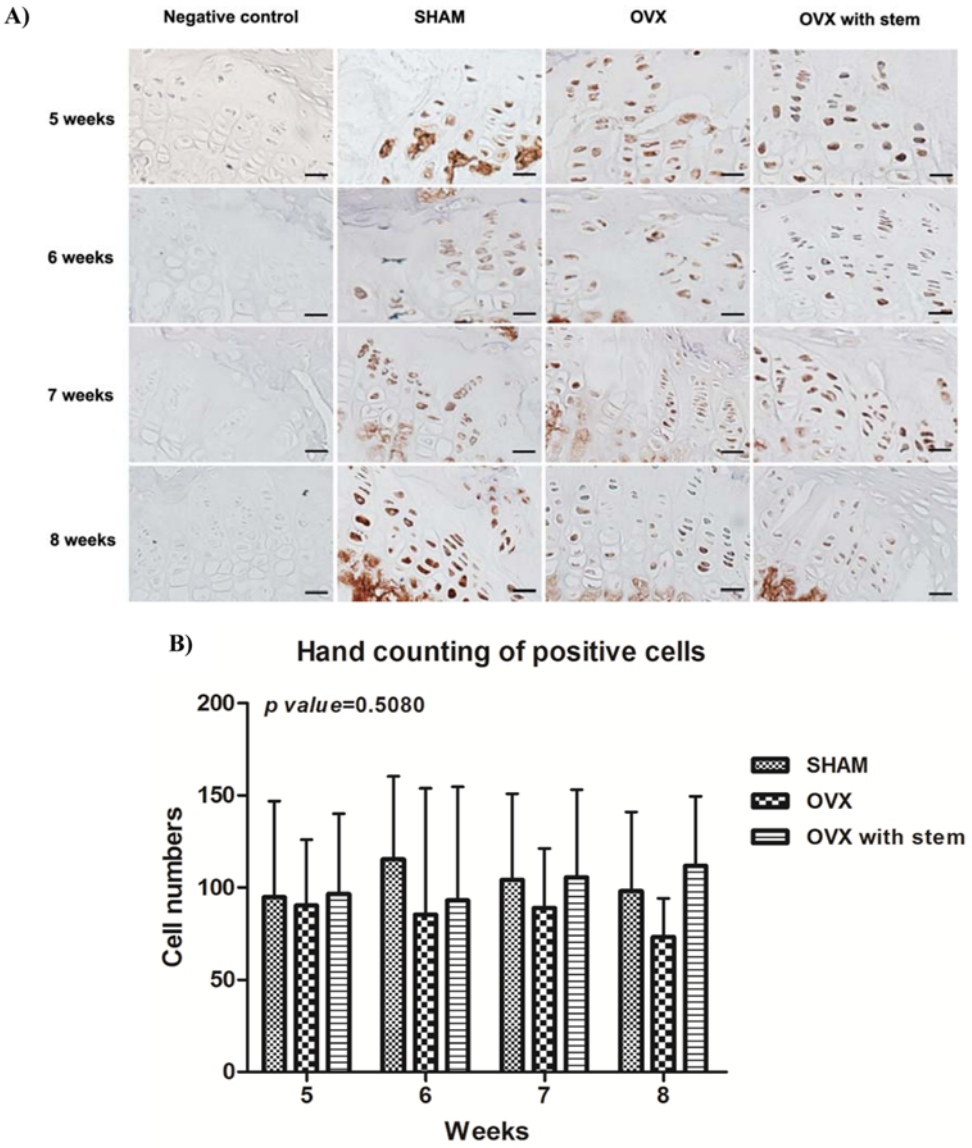

Figure 5. Immunohistochemistry for β-catenin. A) The β-catenin expression was lower in the OVX group compare to SHAM and OVX with stem group (SHAM: sham operated group, OVX : ovariectomy group, OVX with stem : ovariectomy with stem cell injection group) (Bars= x200).

to another identifiable medical condition or treatment. Primary osteoporosis is the result of the normal aging process.

It is particularly prominent in post-menopausal women in association with their decreased estrogen levels. Secondary osteoporosis, on the other hand, is caused by diseases of the endocrine system, the gastrointestinal system, the bone marrow. Treatment with anticonvulsants, heparin, and corticosteroids can also result in the development of secondary osteoporosis.[2,10-12] This model is suitable for postmenopausal osteoporosis, because the OVX is the most frequently used model for studying the events associated with postmenopausal osteopenia.[13] Furthermore, 12-weeks-aged- rat skeleton is more sensitive to the loss of ovarian hormones.[13-15]

There is still a clinical need for anabolic treatments that promote bone formation while simultaneously preventing bone deterioration. Previous reports about the stem cell in the treatment of osteoporosis are rare. One study shows that MSCs did not prevent estrogen-deficiency induced bone loss in rats after local or systemic administration to any significant degree.[16] In that study, it may be necessary to either combine MSCs with an osteoinductive carrier material, or provide an autologous stimulus by expressing a cell differentiation-inducing agent

such as BMP or TGF-β into the cells (MSC-based gene therapy) in order to achieve therapeutically significant bone regenerative effects because local 'catabolic' environment does not provide the required stimulus to the MSCs for their differentiation down the osteoblast lineage.[16] We think that compared to BMMSC, ADMSCs are more able to differentiate into lineages of mesodermal cells and tissues such as adipocytes, bone, cartilage, and skeletal muscle. For this reason, there are growing numbers of *in vivo* applications utilizing ADMSCs therapy in various diseases such as osteoarthritis, disc degeneration, liver regeneration, and vascular ischemia.[17] Therefore, in this study, we verified the potential for ADMSCs to restore a positive bone balance in osteoporotic rats using *in vitro* cultured and injected ADMSCs. Furthermore, we thought that ADMSCs, a prospective source of osteoblastic precursors, is proper source for bone regenerative medicine.[18]

ADMSCs express a range of chemokine receptors, which suggests that these cells may possess the potential to home to different tissues.[19] Intravenously administered ADMSCs have been shown to preferentially engraft into bone marrow tissue and bone.[20,21] Recent studies have attempted to provide evidence for the ability of circulating ADMSCs to migrate to a site of bone injury. In an interesting study that employed a parabiotic murine model, Muschler *et al*[22] showed that circulating osteogenic connective tissue progenitor cells are capable of homing to the fracture site and contributing to skeletal repair; this is in line with other suggestions of the homing potential of stem cells.[19] In addition, using an intravenous transplantation technique, another study showed that cells were immediately distributed over the entire body instead of directly engrafting into bone.[23] It may be that those cells specifically homing to the bone marrow compartment produce secretory factors to exert their therapeutic effects on bone. In theory, multiple injections of ADMSCs should provide an enhanced bone-forming effect as compared with a single injection.[18] For this reason, in this study, we made to plan of twice intravenous injections of ADMSCs. However, the effect of additional injections of ADMSCs into osteoporotic rats could be an avenue for exploration in future research.

There are other factors that affect bone strength, such as bony architecture. Trabecular bone is a highly metabolic tissue and is the most sensitive to estrogen withdrawal.[18,24] As such, trabecular bone loss is the most prominent pathophysiological change seen in post-menopausal osteoporotic humans and rodents.[18,25] In previous studies, these trabecular bone changes have been measured by micro-CT [26,27] histomorphometry, and serial histological sectioning of the tissue.[26,28] In this study, we also analyzed bone loss and trabecular architectural damage in rat femur by ex-vivo micro-CT. There was a trend for an increase in the BMD, BV/TV and Tb.N in the OVX with stem group as compared with the OVX group, and decrease in the OVX with stem group as compared with SHAM group. Furthermore, the Tb.Th and Tb.Sp of the OVX group ware increased compared with those values in the SHAM and OVX with stem group. We thought that these changes showed partial effects of injected ADMSCs in restoring an overall positive bone balance in our rat model.

Although bone density measurements are extremely important clinically, the technology is limited because it takes approximately 2-3 years before the efficacy of any intervention can be reliably determined.[2,29] Therefore, a more immediate measure of metabolic status is desirable. Biochemical markers of bone metabolism can provide a real-time assessment of bone resorption, formation, and turnover. Although bone biochemistry has been a subject of active research for many years, the clinical measurements of biochemical bone markers have been a relatively recent pursuit. For an effective treatment option, it will be first necessary to elucidate the roles of various markers in patients with bone disease.[2] Generally, osteocalcin and BALP are used as indices of bone formation and CTX as an index of bone resorption. In elderly women, serum levels of osteocalcin[30] and BALP [31] are increased[32], as are CTX levels.[32-34] Raised levels of these bone markers in the serum suggest an enhancement in bone turnover rates. In this study, the level of BALP was higher in the OVX group as compared with the OVX with stem group, and the SHAM group was the lowest of the three groups. This finding reflects results of previous reports[35], and shows that ADMSCs reduced the rebound increase in BALP expression in response to ovariectomy-induced osteoporosis. Osteocalcin levels were slightly increased in the OVX with stem group as compared with the OVX group, indicating a similar treatment effect of ADMSCs *($P<0.05$)*; this was also in line with previous studies.[18] CTX levels, on the other hand, were highest in the OVX group as compared with the other two groups, a finding that was also in agreement with a previous one.[36]

Cellular differentiation is determined by transcription factors, which are, in turn, regulated by extracellular signals including hormones and growth factors. The Wnt/β-catenin signaling pathway is a major physiological pathway involved in the bone healing process that induces the osteoblastic differentiation and maturation of MSCs.[37] In this study, we evaluated the expression of the β-catenin, and found a significantly lower expression of β-catenin in rats in the OVX group as compared with that in the SHAM group. By contrast, rats in the OVX with stem group showed increased positive β-catenin staining. This finding

suggests that the Wnt/β-catenin signaling pathway is involved in the ADMSC-induced increase in osteoblastogenesis to restore the osteoporotic effects of ovariectomy in rats.

However, our study has some limitations. First, because of use of the ex-vivo micro-CT, we did not get the continuous data from one subject. Second, this study has the insufficient number of animals therefore, failed to get a statistically significant result in all data. Third, we only evaluated the immunohistochemical staining of the bone, and then could not evaluate the precise intracellular physiologic mechanism of the Wnt/β-catenin signaling pathway. Fourth, we tried to the cellular distribution of the nano-particle labeled ADMSC by fluorescence microscopy. Because of the auto-fluorescence of the bone tissue, we could not check the migration of the ADMSC to the bone tissue.

5. Conclusion

Although there was non-continuous and statistical non-significant data in some results by some limitation of the study, this study may help us gain an understanding of the role of MSCs in the pathophysiology and treatment of osteoporosis. Furthermore, ADMSCs may be an attractive source of cells for the induction of the bone formation.

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Authors' contributions: All authors read and approved the final manuscript. JHJ and JRP designed and performed the experiments and wrote the manuscript. ESJ, DKK and SRJ participated in designing the experiments. JRP, and JKM provided assistance for data analysis, mouse-injection and extraction of the femur in the experiments, and ELISA assay, KHC participated in designing the experiments and drafting the manuscript.

Conflicts of Interest: The authors declare that they have no competing interests.

Ethical Statement: For postmenopausal osteoporosis model, Animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Institute for Life Sciences, Seoul, Republic of Korea.

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