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Efficacy of Human Adipose-Derived Stem Cells in Spinal Fusion in a Rat Ovariectomy Osteoporosis Model

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ABSTRACT

AIM: To evaluate the efficacy of human adipose-derived stem cells (h-ADSCs) in spinal fusion in an osteoporotic rat model.

MATERIAL and METHODS: Female Sprague-Dawley rats (n=40) underwent ovariectomy and were then randomly assigned into two groups: ovariectomy (OVX) (OVX + fusion) and h-ADSCs (OVX + fusion + h-ADSCs). Six weeks after OVX, we performed bilateral lumbar spinal fusion using the autologous iliac bone with or without administration of h-ADSCs. The efficacy of the spinal fusion was then assessed using manual palpation, lateral ending, morphogenic examinations, and histology six weeks the after fusion procedure.

RESULTS: Fusion bed volume was different between the two groups but not significantly. However, the fusion bed density was higher in the h-ADSC group than in the OVX group. Manual palpation (70% vs. 40%, p=0.112) and lateral bending (95% vs. 55%, p=0.011) produced higher fusion rates in the h-ADSC group than in the OVX group. Additionally, a histologic examination revealed new bone formation at the fusion bed between the lamina and implanted iliac crest bone in the h-ADSC group, whereas, in the OVX group, the fusion masses were composed of fibroblastic proliferation.

CONCLUSION: Our study demonstrates that the administration of h-ADSCs may have advantages in bone formation and consolidation but does not lead to bone overgrowth. These findings indicate that the administration of h-ADSCs is an alternative and efficient method for spinal fusion.

KEYWORDS: Adipose-derived stem cell, Spinal fusion, Osteoporosis, Ovariectomy

ABBREVIATIONS: h-ADSC: Human adipose-derived stem cell, **OVX:** Ovariectomy, **MSC:** Mesenchymal stem cell, **BMP:** Bone morphogenetic protein, **AIB:** Autologous iliac bone, **FBS:** Fetal bovine serum, **PBS:** Phosphate buffered saline, **DMEM:** Dulbecco`s modified eagle medium, **CT:** Computed tomography, **SMI:** Structure model index, **TGF:** Transforming growth factor

INTRODUCTION

Osteoporosis is one of the most common chronic systemic skeletal disorders characterized by less dense bone density, degeneration of bone microarchitecture, high bone fragility, and increased fracture risk (25). The prevalence of osteoporosis in patients undergoing spinal surgery is reportedly 50% in women and 16% in men aged > 50 years (3), with successful spinal fusion becoming increasingly difficult in elderly osteoporosis patients given that their bone

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strength is decreased (19). An important objective of spinal fusion surgery is to make solid arthrodesis of the spinal segment, and screw fixation is the most conventional method for providing immediate stability to the fusion site before osteogenesis occurs on the fusion bed (29). Although autologous bone grafting is considered the most common method for promoting spinal fusion, the amount of autologous bone is limited, and this procedure has caused severe complications such as donor-site bleeding, infection, and pain, with the rates of pseudoarthrosis ranging from 5 to 43% (10,35). Therefore, novel fusion enhancement techniques, including approaches with bone morphogenetic proteins (BMPs), are needed to overcome these limitations. However, treatment using BMPs has adverse side effects, such as pseudoarthrosis, bone overgrowth that causes neural tissue compression, heterotopic ossification, and increased financial burden due to the high cost of BMPs (1,31,34).

Therefore, the development of safe and effective therapies is critically needed for patients with spinal fusion. Regional gene therapy may represent an alternative treatment method for spinal fusion that bypasses the abovementioned complications. Recently, human adipose-derived stem cells (h-ADSCs) have become one of the most important sources of mesenchymal stem cells (MSCs), which can be differentiated into osteoblasts (22). Thus, this study was designed to evaluate the effectiveness of h-ADSCs for spinal fusion using a rat model of osteoporosis.

MATERIAL and METHODS

Animals

All animal care and laboratory procedures were performed in accordance with the guidelines and regulations approved by the Institutional Animal Care and Use Committee (IACUC) of the Clinical Research Institute, Hanyang University Hospital, Korea. The institutional review board (Hanyang University Hospital, Korea) approved this study.

Six-week-old female Sprague-Dawley rats (n=40), purchased from Orient Bio (Gyeonggi, Korea), were housed in pathogen-free ventilated cages with 12-hour day/night rotating shifts. Rats had free access to tap water and γ-irradiated food (LabDiet 5053, PMI, St Louis, MO) containing 0.81% calcium, 0.64% phosphate, and 2.3 IU/mg vitamin D.

Experimental Design and Surgery

The rats were assigned into the two following groups (20 rats each): ovariectomy (OVX) group (OVX + fusion operation + saline application) and h-ADSC group (OVX + fusion operation + h-ADSC application). After an adaptation period of 2 weeks, they underwent bilateral ovariectomy to induce osteoporosis (Figure 1A-D). After six weeks, posterolateral interlaminar fusion operation with autologous iliac bone (AIB) was performed bilaterally.

The spinal fusion surgery was performed under isoflurane anesthesia (2–4% isoflurane in oxygen continuously applied using a nose cone). The rats were placed on the operation table in the prone position and prepared using standard surgical methods. An incision was made over the spinous process of the lumbar spine (L2-L5) in the midline. L4 and L5 lamina was dissected bilaterally, and the level was checked by referencing from the iliac crest during surgery. Decortication of the L4-L5 lamina was performed using an electronic burr drill under the microscope (Figure 2A-C). About 0.2 g of autologous bone was harvested from the right iliac crest (Figure 3A-D). The bilateral iliac bone needed to be harvested from two rats as we could not harvest enough volume from a unilateral AIB. About 0.5 ml of saline (the OVX group) or h-ADSCs (the h-ADSC group) was administered between the decorticated transverse processes and AIB. After six weeks, all of the rats were euthanized with carbon dioxide (CO_2) in a sealed container. Various assessments for spinal fusion were then performed.

Isolation and Cultivation of h-ADSCs

Passage-3 h-ADSCs were provided by Hurim Biocell Co. (Seoul, Korea). With the prior consent of the volunteers, the h-ADSCs were obtained from lipo-aspirated fat tissues. In brief, blood and other components were washed away using phosphate-buffered saline (PBS). Then, these tissues were incubated at 37 °C for 30 minutes with 0.1% crude collagenase (Sigma Aldrich, St. Louis, Missouri, USA) (39). An equal volume of PBS with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, Utah, USA) was added to discontinue collagenase digestion. After centrifugation at 300 *g* for 5 minutes, we washed the pellets three times with PBS, and the viable cell number was assessed by trypan blue exclusion. Subsequently, cells were cultured in 15 mL of growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen, New York, USA) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in T75 culture flasks (Nunc, Rochester, Minnesota, USA). Cells were cultured at 37 °C in a humidified CO_2 incubator containing 5% CO_2 (Forma Scientific, Marietta, Ohio, USA) (4). The culture media was replaced every three to four days.

Identification of h-ADSCs

We used flow cytometry to evaluate cell surface antigens of cultured cells. The passage-3 cells were incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated cluster of differentiations (CD)31, CD34, CD45, CD90, CD105, or mouse IgG isotype control antibodies (BD Biosciences, Heidelberg, Germany) for 30 min at 4 °C in PBS. Cells were washed and analyzed with a FACS Calibur kit (BD Biosciences, Pharmingen, CA) (17).

Differentiation of h-ADSCs

Using standard methods, we qualitatively measured osteocyte and adipocyte differentiation. After trypsinization, h-ADSCs were placed on a 24-well culture plate and incubated for 24 h for cell attachment. Then, the medium was changed into a differentiation medium. All differentiation studies were performed using passage 3 cells (23).

Cells were cultured in a medium containing DMEM/10% FBS, 50 μmol/l dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich) for two weeks

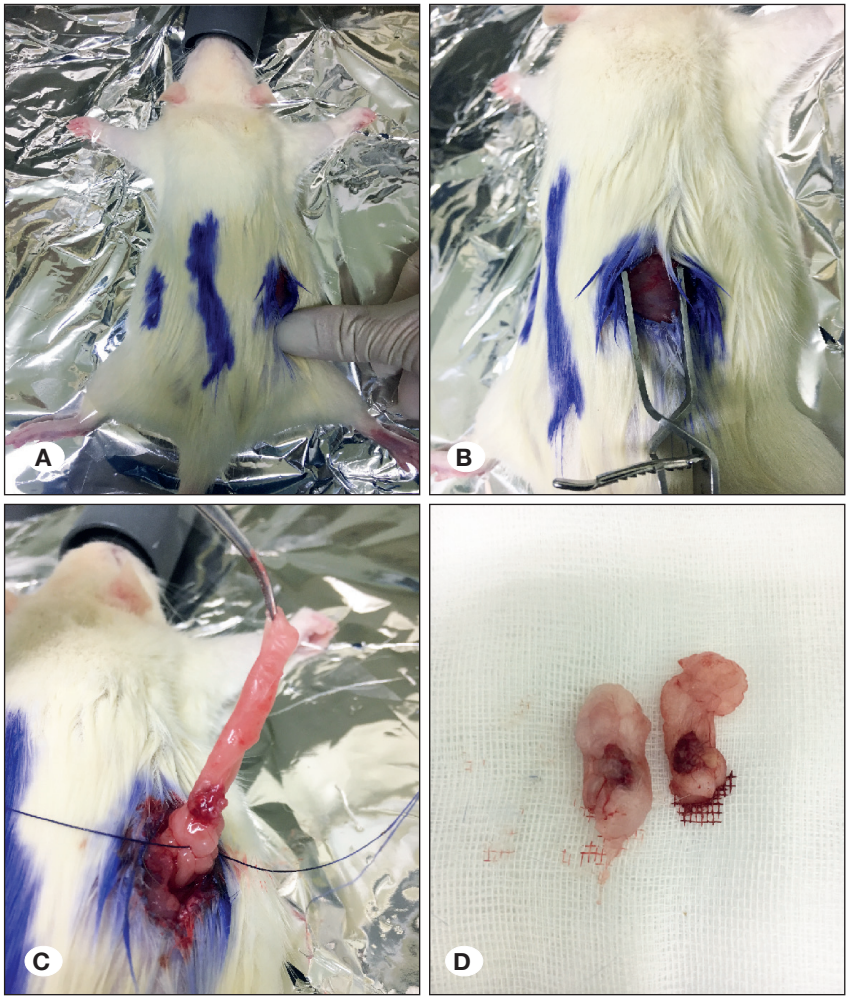

Figure 1: Bilateral ovariectomy to create rat model of osteoporosis.

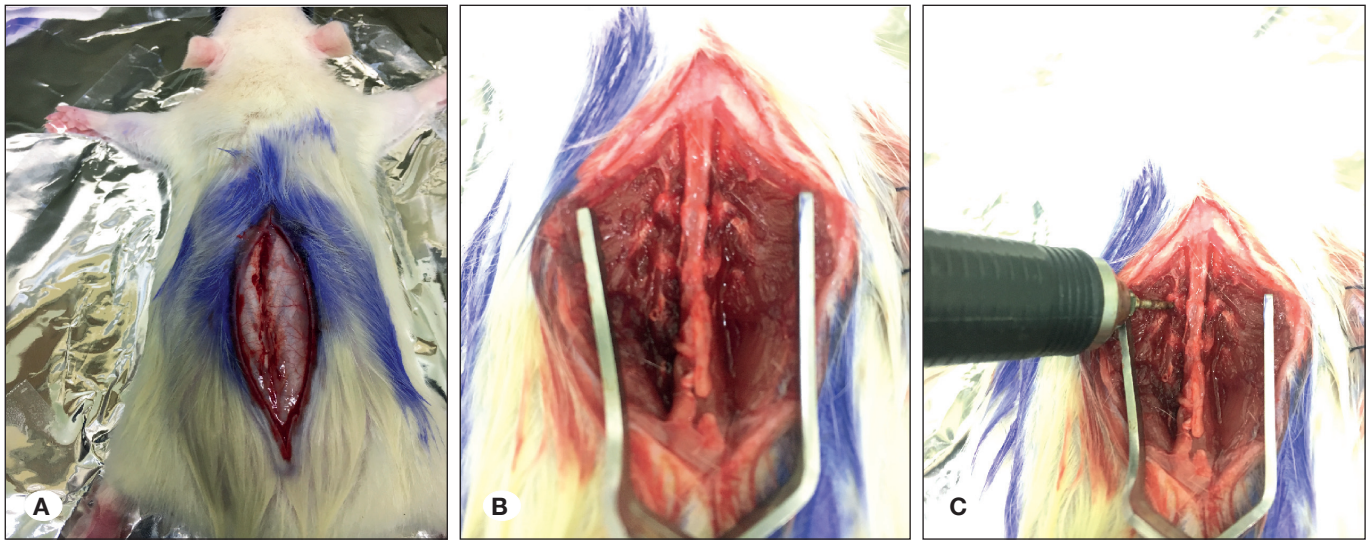

Figure 2: Posterolateral fusion; **A)** single midline incision, **B)** paramedian facial incisions, **C)** decortication to exposure cancellous bone.

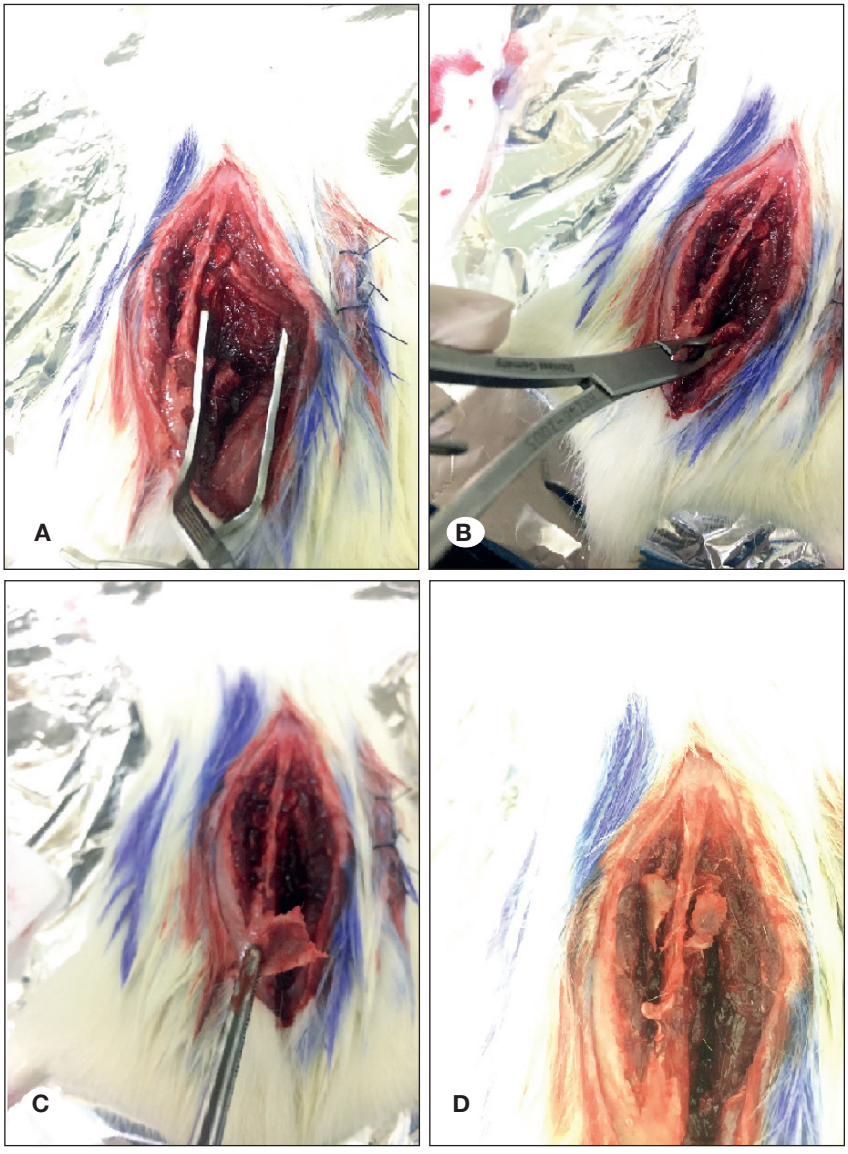

Figure 3: Harvesting of iliac bone graft.

to achieve adipocyte differentiation. Neutral triglycerides and lipids were assessed using Oil Red O staining with a conventional method (microscopic identification of Oil Red droplets to confirm adipose differentiation of MSCs) (23,38). Cells were cultured in an osteogenic differentiation medium consisting of L-DMEM, 10% FBS, 200 μM L-ascorbic acid-2-phosphate, 0.1 μM dexamethasone, and 10 mM β-glycerol phosphate (all from Sigma-Aldrich) for three weeks to achieve osteocyte differentiation (13). Alizarin Red S staining was used as a screening test to identify calcium deposits in MSCs. Differentiated MSCs with calcium deposits were seen with deep orange-red stains, while undifferentiated MSCs without calcium deposits were a paler pink under a light microscope (23).

Transplantation of h-ADSCs

The cultured h-ADSCs were harvested after trypsinization, suspended with 0.01 mL DMEM and 0.09 mL 1% sodium hyaluronate (LG Life Science, Seoul, Korea), and administered to the rats to induce bone formation (2.0×10^5 cells/rat; injection volume, 100 μL) (4).

Bone volume and Density Assessment

The formation of the bridging bone between bilateral laminas (fusion) was assessed using three-dimensional computed tomography (3D CT) (Siemens Somatome Sensation 64, München, Germany) 6 weeks after the spinal fusion procedure. The density of the bridging bone was examined using micro-computed tomography (Micro CT) (Sky-Scan 1172TM, Skyscan, Konetchy, Belgium). In addition, trabecular thickness, number, separation, and structure model index (SMI) were also measured.

Manual Assessment of Fusion

The Degree of fusion was evaluated by two examiners based on the reinforcement of the grafted bone and the formation

of bridging bone within the fusion bed in a blinded manner. The two examiners measured the lateral bending motion to classify the spine as fused or not fused. When any motion was observed from either side of the facets, it was defined as a fusion failure and declared not fused. Therefore, no bilateral bending motion was defined as a successful fusion, and the spine was classified as fused (28).

Histologic Evaluation

After the rats were sacrificed, each spine was dissected carefully to prevent the destruction of the fusion bed, and the specimens were fixed with 4% paraformaldehyde. After fixation, the specimens were decalcified in Calci-Clear Rapid for 9 h, rinsed with running tap water, and embedded in paraffin. Coronal sections around the lamina were obtained at the level of the fusion. The sections were then stained with Masson Trichrome and examined under a light microscope.

Statistical Analyses

The results are presented as mean ± standard deviation (SD). Differences in fusion status between groups were analyzed using the chi-square test. A p-value of < 0.05 was considered significant.

All statistical analyses were performed using R version 3.3.2 (http://www.r-project.org/).

RESULTS

Characteristics of h-ADSCs

ADSCs express MSC markers on their surface, such as CD 29, CD 44, CD 90, and CD 105, but do not express hematopoietic markers, such as CD 14, CD 34, and CD 45 (7). Our findings from the flow cytometry analysis indicated that the cultured cells were negative for hematopoietic markers (CD 31, CD 34, and CD 45) but positive for MSC markers (CD 90 and CD 105) (Figure 4).

Differentiation of h-ADSCs

We compared the levels of lipids and calcium deposits between differentiated h-ADSCs and control cultures using Alizarin Red S staining and Oil Red O staining. Differentiation of h-ADSCs into mesodermal cells, such as adipocyte and osteocytes, was determined by the presence of lipid droplets (Oil Red O staining) (Figure 5A) and calcium deposits (Alizarin Red S staining) (Figure 5B). These results were confirmed by comparing the differentiated samples with control samples that showed no biochemical and morphological changes.

Increased Spinal Fusion Rates in the h-ADSC Group

Using a manual assessment method, we found that the fusion rates at six weeks after the fusion procedure were 40% and 70% in the OVX and h-ADSC groups, respectively (p=0.112). In addition, the assessment of the success of the fusion using lateral bending revealed a significantly higher fusion rate in the h-ADSC group than in the OVX group six weeks after the fusion procedure (95% vs 55%, p=0.011) (Table I).

Increased density of the fusion bed and improved new bone formation in the h-ADSC group

There was no significant difference between the two groups in the volume of the fusion bed assessed using 3D CT images. However, the density of the fusion bed was higher in the h-ADSC group than in the OVX group (Figure 6). Micro CT scans revealed highly consolidated bone formation in the h-ADSC group (Figure 7). Histomorphometric analysis of the trabecular area indicated that the trabecular thickness (p=0.004), trabecular number (p=0.043), and SMI were higher in the h-ADSC group than in the OVX group six weeks after spinal fusion procedure (p=0.005). All microstructural indices, except for trabecular separation (p=0.723), differed significantly between the OVX and h-ADSC groups (Table II).

Furthermore, histologic evaluation revealed new bone formation at the fusion bed between the lamina and implanted

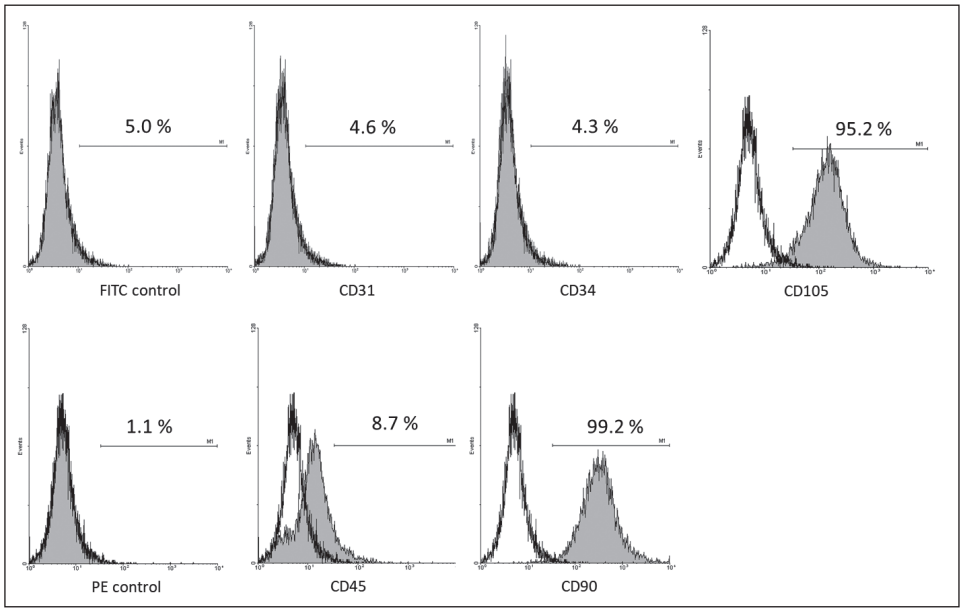

Figure 4: Flow-cytometry analysis of human adipose-derived stem cells (h-ADSCs) with mesenchymal and hematopoietic stem cell markers. The h-ADSCs were positive for mesenchymal markers (CD90 and CD105) but negative for hematopoietic markers (CD31, CD34, and CD45).

Table I: Manual Assessments of Spinal Fusion

	OVX (n=20)	ADSCs (n=20)	p
Manual palpation			
Fused, n (%)	8 (40.0)	14 (70.0)	0.112
Lateral bending			
Fused, n (%)	11 (55.0)	19 (95.0)	**0.011**

OVX: *Ovariectomy,* ***ADSCs:*** *Adipose tissue derived stem cells.*

Table II: Histomorphometric Analysis of the Vertebral Trabecular Area

	OVX (Mean ± SD)	ADSCs (Mean ± SD)	p
Trabecular thickness (mm)	0.13 ± 0.01	0.15 ± 0.01	**0.004**
Trabecular number (1/mm)	0.37 ± 0.07	0.46 ± 0.11	**0.043**
Trabecular separation (mm)	1.18 ± 0.20	1.14 ± 0.16	0.723
Structure Model Index	0.57 ± 0.14	0.81 ± 0.19	**0.005**

OVX: *Ovariectomy,* ***ADSCs:*** *Adipose tissue derived stem cells,* ***SD:*** *Standard deviation.*

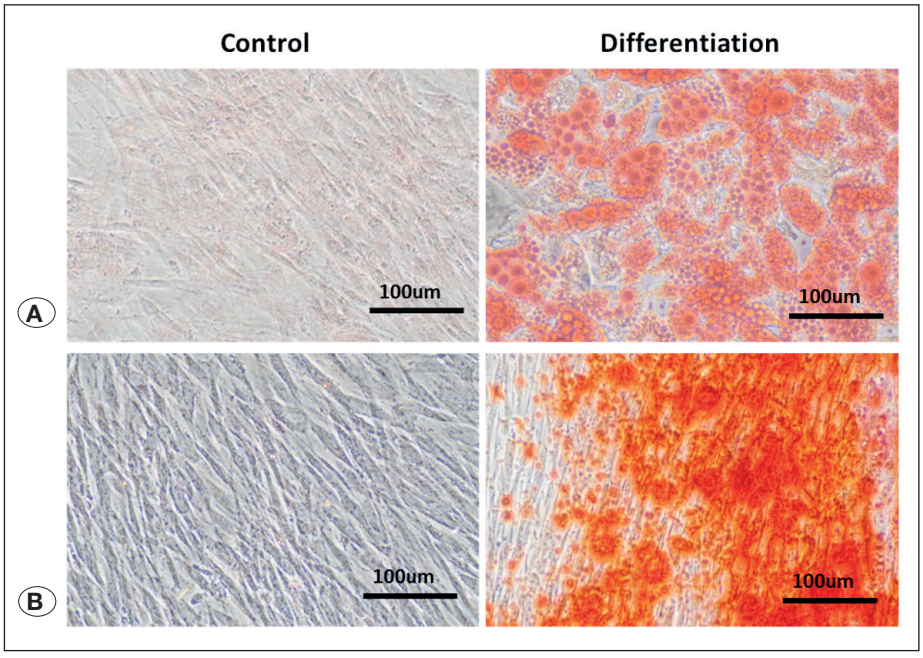

Figure 5: Adipogenic and osteogenic differentiation of h-ADSCs. **A)** Adipogenic differentiation was evidenced by the formation of lipid vacuoles (yellow) by Oil Red O staining at passage three in h-ADSCs. **B)** Osteogenic differentiation was evidenced by the formation of a mineralized matrix by Alizarin Red S staining at passage three in h-ADSCs.

iliac crest bone in the h-ADSC group six weeks after the fusion procedure (Figure 8A). In contrast, the fusion masses were only composed of proliferated fibroblasts, and the fusion process was delayed in the OVX group (Figure 8B).

DISCUSSION

We found that h-ADSCs increased fusion rate and bone formation but did not lead to a bone overgrowth in the osteoporotic rat spinal fusion model. As far as we know, this is a novel study demonstrating the therapeutic potential of h-ADSCs for spinal fusion. Lee et al. (16) presented the synergistic effect of human perivascular stem cells (hPSCs) and Nel-like protein-1 (NELL-1) in enhancing spinal fusion with osteoporotic conditions, revealing the possibility of establishing hPSCs based therapies for patients with osteoporosis. Our study has the advantage that hASDC alone was also effective in bone fusion in osteoporotic models.

Osteoporosis reduces osteoblast capacity, vascularity, and quality of bone marrow in the fusion bed. This is caused by

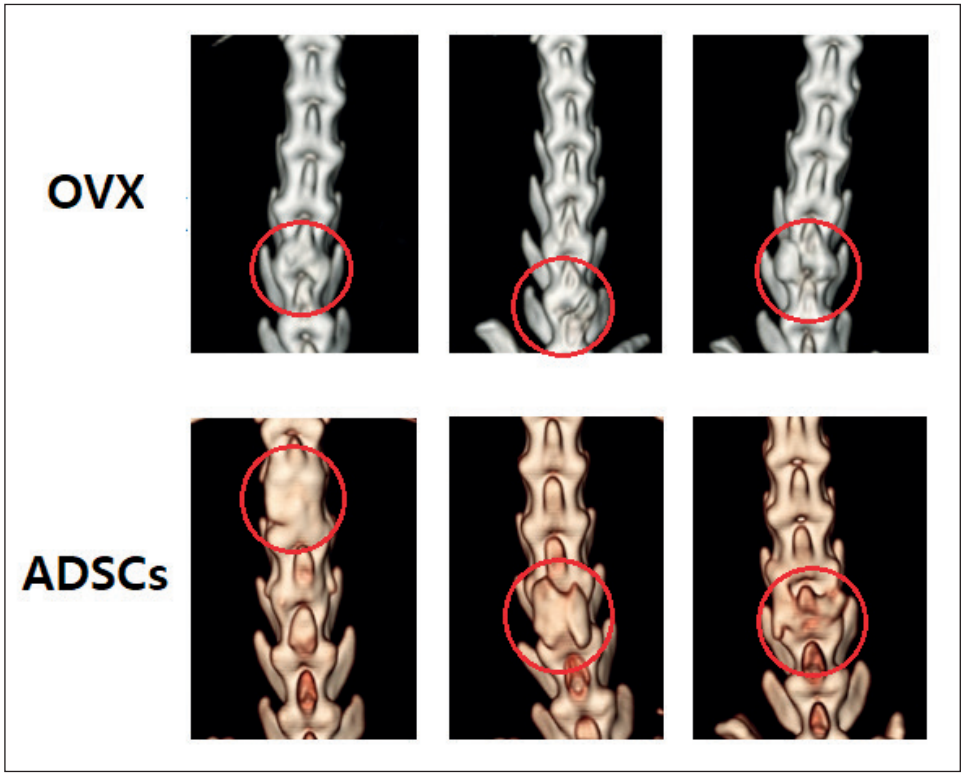

Figure 6: Three-dimensional (3D) reconstructed computed tomography scanning of fusion masses 6 weeks after surgery in the two groups. Grafted new bone was observed between the interlaminar spaces.

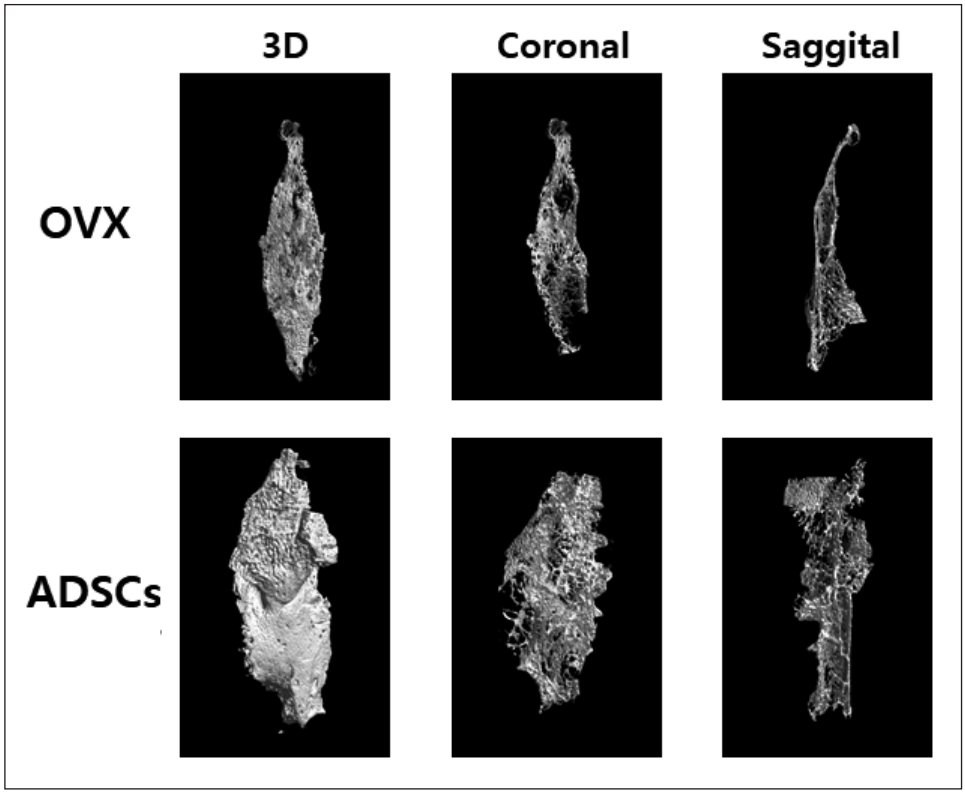

Figure 7: Microcomputed tomography of the fusion bed in the two groups. The fusion bed was larger and more highly consolidated in the h-ADSC group than in the OVX group.

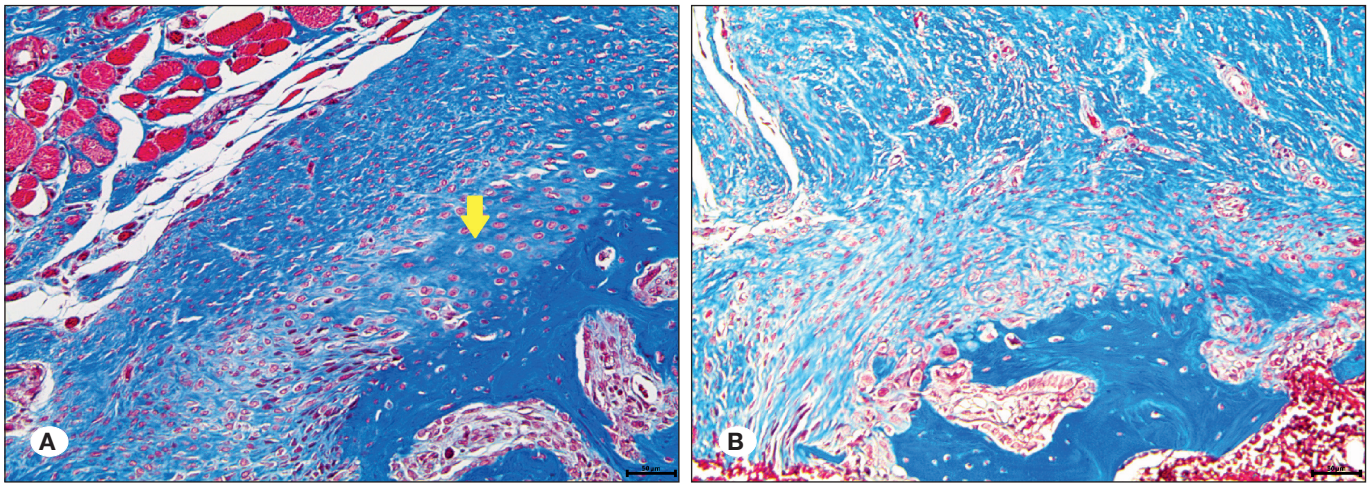

Figure 8: Histologic examination of the fusion bed (Masson-trichrome stain). New bone formation (yellow arrow) was found between the lamina and implanted iliac crest bone in the h-ADSC group **(A)**, but only fibroblastic proliferation was observed in the OVX group **(B)**.

cellular changes that cause estrogen shortage (including a decrease in osteoblastogenesis and an increase in osteoclastogenesis), reducing bone formation in the later stage in the OVX group (27). Previous studies found that the intramembranous bone formation around the decorticated transverse process was weaker in the OVX group than in the non-OVX group, and after spinal fusion surgery, osteoporosis had a negative effect on endochondral bone formation and maturation by adjusting bone remodeling (30). Therefore, spinal surgeons need to consider the types and number of implants in the graft layer, as well as the fusion surface, to increase the success rate of fusion in patients with osteoporosis requiring spinal fusion surgery (30).

Recently, regional gene therapy has emerged as an alternative treatment method for increasing the fusion rate. MSCs, which increase osteoinduction, can be transferred to a specific anatomical site using osteoinductive molecules (22) and have been confirmed to be effective in different tissues, including bone marrow (2), adipose tissues (40), muscles (11), neural tissues (8), and periosteum (26). Among these tissues, bone marrow is a well-known source of MSCs that can be differentiated into osteoblasts. However, bone marrow harvesting is challenging and associated with donor-site bleeding, infection, and pain (16).

Recently, adipose tissues have been suggested to be an alternative stem cell source for several reasons: (A) a sufficient number of MSCs can be collected with a relatively small volume of fat (6), (B) the number of MSCs in fat is maintained independent of the aging process (24), (C) human fat can be obtained by liposuction without complications, and (D) adipose-derived MSCs induce spinal fusion as efficiently as bone marrow-derived MSCs (22). In this study, h-ADSCs were isolated and differentiated into two lineages, indicating that h-ADSCs have osteogenic and adipogenic differentiation potentials. While it is largely believed that h-ADSCs are involved in a series of osteogenesis events, the process is not fully understood. Multiple signaling pathways, including transforming growth factor-β (TGF-β)/BMPs, Notch, Wnt/β-catenin, Hedgehog, and fibroblast growth factor signaling pathways, have been confirmed to participate in the differentiation of an osteoblast progenitor to a committed osteoblast (9). As a member of the TGF-β superfamily, BMP has many subtypes; among these subtypes, BMP-2 seems to play a significant role in the bone induction cascade, including mitosis, chemotaxis, and differentiation of MSCs during the bone remodeling process (20,32). Notably, previous studies (15,18,22) have suggested that BMP-2 can enhance ASC and heal large bone defects.

We performed manual palpation, a biomechanical bending test, and 3D CT and micro CT scans to evaluate the fusion status. Assessment of the fusion through lateral bending revealed a significant higher fusion rate in the h-ADSC group than in the OVX group (Table I). Additionally, histomorphometric indices were significantly different between the h-ADSC and OVX groups (Table II). In a two-dimensional analysis, newly formed bones in the h-ADSC group had more thicker trabeculae and tighter pacing than those in the OVX group. In a three-dimensional analysis, the newly formed bones were rod-like and plate-like in the h-ADSC group and the OVX group, respectively (5,14,36).

However, 3D CT scans showed that the volume of the fusion bed was not significantly different between the two groups (Figure 7). Based on our results, h-ADSCs likely increase the fusion rate by inducing osteogenesis and bone remodeling but do not lead to overgrowth of bone formation, which may be induced by BMP-2 (33,34,37).

Limitations

This study has several limitations. First, the OVX rat model used in the present study could not reproduce the human postmenopausal osteoporotic state completely (12,21). Second, we evaluated the fusion rates and bone overgrowth six weeks after surgery; thus, a longer time of assessment (more than six weeks) is needed to examine changes in the fusion segments. Third, no control (without ovariectomy) group was included in this study. Indeed, a previous study

has demonstrated a difference in fusion rates between normal and osteoporosis rat models (30). Fourth, we did not use immunocompromised rats that are less prone to reject xenograft tissue. However, there was no host transplant rejection during the experimental study period. Our findings are consistent with those of previous studies demonstrating that MSCs produce an immunosuppressive effect derived from various adult tissues, such as adipose and bone marrow (4,11). Fifth, we did not examine the signaling pathway involved in the effect of h-ADSCs on osteogenesis in spinal fusion as we only focused on the morphologic fusion effect of h-ADSCs in this study. Therefore, future studies are needed to identify the signaling pathways involved using real-time polymerase chain reaction and western blot analyses.

CONCLUSION

Our results indicate that the administration of h-ADSCs is advantageous in that h-ADSCs promote bone formation and consolidation without producing bone overgrowth. Since human fat is relatively easy and safe to obtain by liposuction, therapy with h-ADSCs represents an alternative and efficient method for spinal fusion. Future studies are needed to investigate the effects of h-ADSCs on the clinical outcomes of the spinal fusion procedure in osteoporosis patients.

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AUTHORSHIP CONTRIBUTION

Study conception and design: MKN, HJC

Data collection: MKN, ISB, BRD

Analysis and interpretation of results: ISB, HJY

Draft manuscript preparation: MKN, ISB

Critical revision of the article: KDK, HJC

All authors (MKN, ISB, HJY, BRD, KDK, HJC) reviewed the results and approved the final version of the manuscript.

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