Ovarian Tissue-Based Hormone Replacement Therapy Recovers Menopause-Related Signs in Mice

Dahyeon Yoo[1,2,3], Nanum Chung[3,4], Jungyoung Yoo[1,3], Chae Young Song[3,4], Chungmo Yang[3,5], Hye Won Youm[3], Kangwon Lee[6], Jin Hyun Jun[1,2,7], Jaewang Lee[1], and Jung Ryeol Lee[3,4]

[1]Department of Biomedical Laboratory Science, Eulji University, Seongnam;
[2]Department of Senior Healthcare, BK21 Plus Program, Graduate School of Eulji University, Seongnam;
[3]Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, Seongnam;
[4]Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul;
[5]Program in Nanoscience and Technology, Graduate School of Convergence Science and Technology, Seoul National University, Seoul;
[6]Department of Applied Bioengineering, Graduate School of Convergence Science and Technology, Seoul National University, Seoul;
[7]Eulji Medi-Bio Research Institute (EMBRI), Eulji University, Daejeon, Korea.

Purpose: In women, menopause manifests with a variety of symptoms related to sex-hormone deficiency. Supplementing steroid hormones with pharmacological drugs has been widely practiced. However, considering the possible complications associated with artificial hormone therapy, studies have been conducted to find an alternative to pharmacological hormone replacement therapy. Accordingly, this study aimed to evaluate the efficacy of tissue-based hormone replacement therapy (tHRT) for treating post-menopausal signs and symptoms.

Materials and Methods: CD-1 mice were ovariectomized, and the ovaries were cryopreserved. Following artificial induction of post-menopausal osteoporosis, cryopreserved ovaries were subcutaneously autografted, and indexes related to bone health were monitored for 12 weeks. Bone mineral density (BMD), bone mineral contents (BMC), total bone volume (BV), and body fat mass were measured by dual energy X-ray absorptiometry. Uterine atrophy was assessed histologically, and bone microstructures were imaged by micro-computed tomography analysis.

Results: Regardless of the number of grafted ovaries, the BMC, BMD, and BV values of mice that underwent ovary transplantation were better than those that did not undergo transplantation. The uteruses in these mice were thicker and heavier after auto-transplantation. Furthermore, the bone microstructure recovered after tHRT.

Conclusion: Recovery of menopause-related bone loss and uterine atrophy was achieved through tHRT. Ovarian tissue cryopreservation and transplantation may be applicable not only in patients wanting to preserve fertility but also in sex hormone-deficient post-menopausal women.

Key Words: Tissue-based hormone replacement therapy, menopause, symptoms, ovarian transplantation, bioimaging analysis

INTRODUCTION

One of the most important roles of the ovaries is the production of gametes and sex hormones.[1] Depletion of ovarian follicles and sex hormones leads to menopause, which can elicit numerous symptoms in women. Osteoporosis is the most common disease in post-menopausal women and is highly related to fracture-induced death in senile individuals. Sex-hormone deficiency also occurs due to vulvovaginal atrophy in women who have experienced menopause. Estrogen deficiency disturbs the homeostasis between bone formation and resorption as it significantly reduces bone density through excessive bone resorption. Over 1.5 million fractures per year are attrib-

uted to osteoporosis, including approximately 300000 hip and 700000 vertebral fractures.[2,3] Therefore, preventing menopause-induced osteoporosis is a socio-economic necessity in developed countries with an aging population.

Pharmacological hormone replacement therapy (pHRT) has been widely used to treat menopause symptoms. However, pHRT is associated with several side effects, including breast cancer, cardiovascular diseases, and venous thromboembolism.[4] Recently, bioengineering-mediated research has been considered to reduce pHRT-induced side effects.[5] No studies have investigated the optimization of biomaterials thus far.

Since 2004, ovarian tissue cryopreservation and auto-transplantation for fertility preservation have been clinically applied in women with cancer, premature ovarian insufficiency, and other gynecological conditions.[6,7] Notably, this technique is performed to restore the hypothalamus-anterior pituitary-ovary axis a few months after transplantation.[8-10] Expounding on this, we hypothesized that supplementing ovary-producing hormones by cryopreservation and auto-transplantation would improve menopause symptoms.

MATERIALS AND METHODS

Animals and experimental groups
A total of 160 eight-week-old female CD-1 mice was purchased from Orient Bio Inc. (Seongnam, Korea). The mice were housed in a room with a 12-h light/dark cycle. All experimental procedures were approved by the institutional animal care and use committee of the Seoul National University Bundang Hospital (BA-1910-282-082-01). Mice were randomly divided into the following four groups: sham (incision only), ovariectomy (OVX), ovarian tissue transplantation (OTPL) 1, and OTPL 2 groups. All surgeries were performed under anesthesia with zoletil and xylazine (30 mg/kg+10 mg/kg, intraperitoneal injection). Fig. 1 depicts the experimental scheme of this study.

Ovariectomy-induced osteoporosis and auto-transplantation of cryopreserved ovary
After 2 weeks of adaptation, 10-week-old female CD-1 mice were ovariectomized, and the ovaries were cryopreserved using a previously reported vitrification method.[11] The ovaries were successively exposed to the equilibration and vitrification solutions for 10 and 5 minutes, respectively. The ovaries were vitrified with liquid nitrogen and cryopreserved until transplantation. All surgical procedures in the other groups were performed identically to those in the sham group. Eight weeks after OVX, the cryopreserved ovaries were successively incubated in 1-M, 0.5-M, 0.25-M, and 0-M sucrose-based solutions for 5 minutes each and subcutaneously transplanted into each mouse in the OTPL 1 and OTPL 2 groups. A vitrified-warmed ovary was unilaterally grafted to mice in the OTPL 1 group, whereas mice in the OTPL 2 group underwent bilateral ovary transplantation. Mice were euthanized 0, 4, 8, and 12 weeks after auto-transplantation, and all tissues used in this study were collected and fixed in 4% paraformaldehyde.
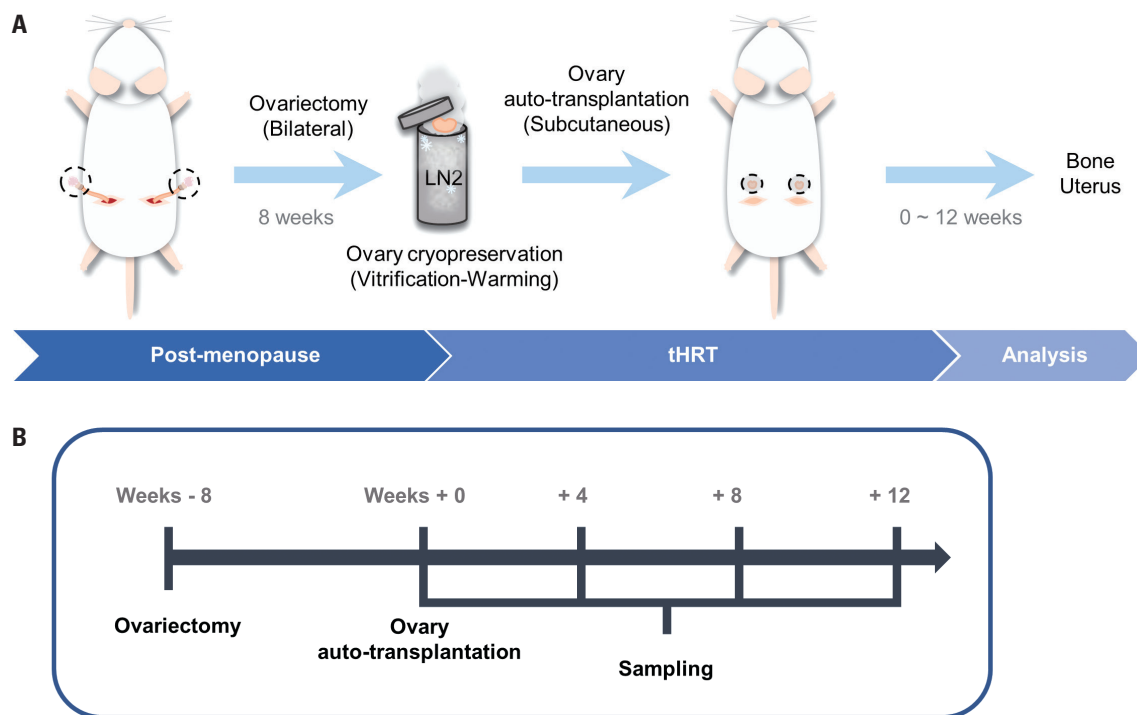

Fig. 1. An experimental scheme of this study. (A) Post-menopausal osteoporosis was induced by bilateral OVX. The ovaries were cryopreserved and subcutaneously transplanted 8 weeks after OVX. (B) Every 4 weeks after ovary transplantation, mice were euthanized for analysis. tHRT, tissue-based hormone replacement therapy; OVX, ovariectomy.

Bone mineral density, bone mineral contents, bone volume, and fat and body weight assessments using dual energy X-ray absorptiometry after OVX and OTPL

We demonstrated that bilateral OVX definitely decreased bone mineral density (BMD), bone mineral contents (BMC), and bone volume (BV) regardless of sites of measurement as shown in Fig. 2. Every second week after OVX or every fourth week after OTPL, the bone and body composition of 10 mice per group were analyzed using dual energy X-ray absorptiometry (DXA) (InAlyzer, Medikors, Seongnam, Korea), which is capable of scanning in vivo. BMD, BMC, and BV values of the body, femur, and lumbar vertebrae were calculated using InAlyzer software.

Eight weeks after OVX, all parameters were significantly lower in the OVX group than in the sham group. We either uni-

laterally or bilaterally transplanted ovaries into OVX mice to confirm the effectiveness of tissue-based hormone replacement therapy (tHRT). BMD, BMC, and BV after ovary transplantation were measured by DXA to investigate whether the grafts would reverse menopause-related bone loss. The indexes were measured following the region of interest (ROI) of the whole femoral bone and L4−5 vertebrae chosen by the researchers. Other characteristics, including body weight, fat, and fat in tissue weight, were evaluated using DXA analysis.

Micro-CT

Fixed femoral bones of two mice were scanned by micro-CT (Skyscan 1176) at 0, 4, 8, and 12 weeks post-transplantation to confirm the results of DXA analysis. The specimens were positioned horizontally for micro-CT scanning, and the systems were operated at 60 kV and 417 μA. Images were reconstructed
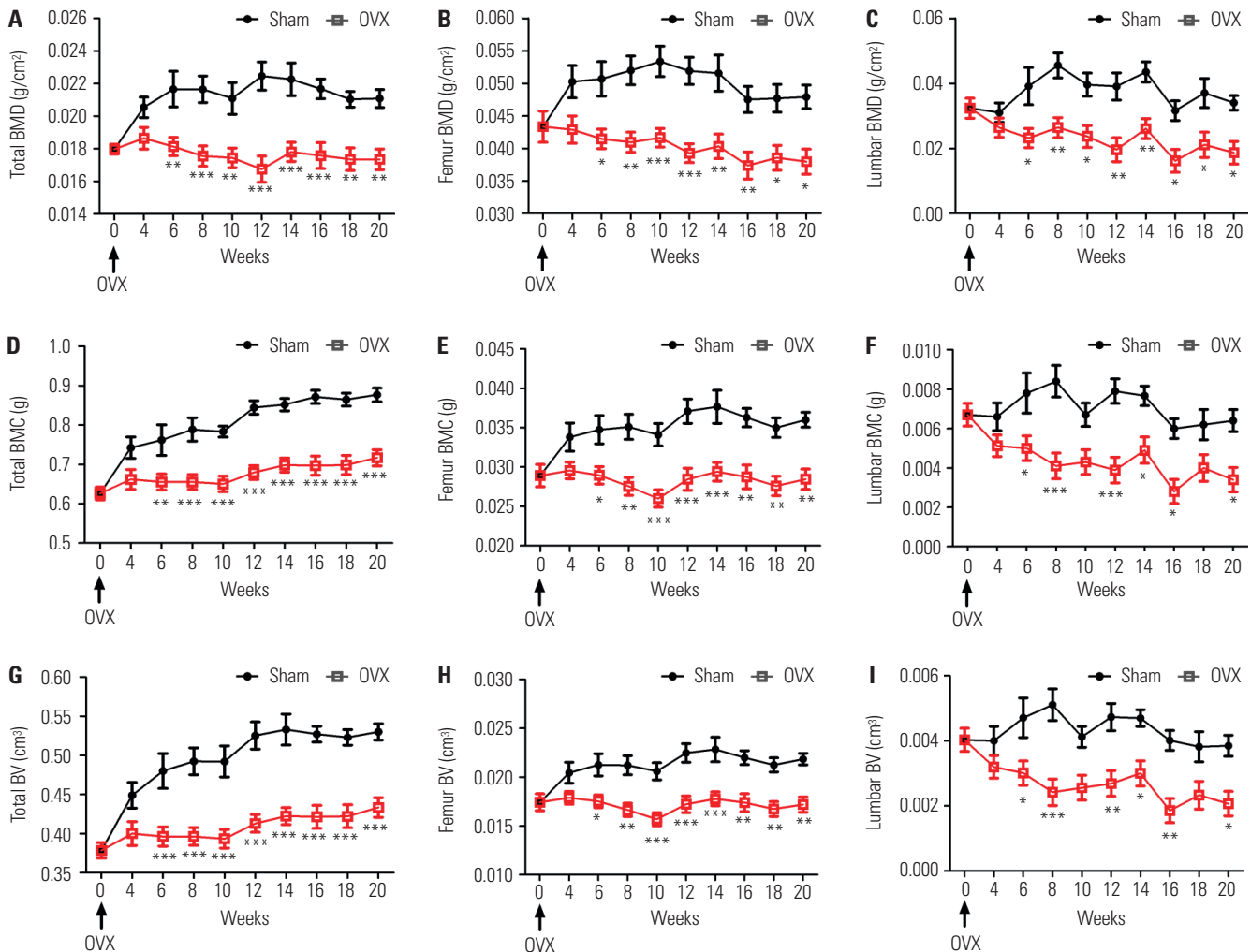

Fig. 2. Bioimaging analysis of the body, femur, and lumbar vertebrae measured DXA after OVX. (A-I) DXA was performed to evaluate OVX-induced osteoporosis in mouse models. BMD (A-C), BMC (D-F), and BV (G-I) of the body, femur, and lumbar vertebrae were measured until 20 weeks after OVX. Ten mice in each group were monitored every 2 weeks after OVX. Values of the femur and lumbar vertebral bones were calculated following the ROI. The mean of the OVX group (red empty squares) was compared with the of the sham group (black dots). Asterisks represent the p-value (*$p<0.05$, **$p<0.01$, ***$p<0.001$). BMD, bone mineral density; BMC, bone mineral contents; BV, bone volume; DXA, dual energy X-ray absorptiometry; OVX, ovariectomy; ROI, region of interest.

to be cross-sectional using NRecon software (Micro Photonics, Allentown, PA, USA). Tomograms were converted to 3D structures, which were sectioned transaxially using CTvox software (Version. 3.3.0.0, Bluescientific, Cambridge, UK).

Uterine weight, gross appearance, histology, and endometrial thickness

Although menopausal women experience vulvovaginal atrophy rather than uterine atrophy, we assessed uterine size, weight, and histology of ovariectomized mice in the present study, because it is difficult to evaluate vulvovaginal symptoms in mouse models. The uterus was dissected following euthanasia, and

the uterine weights of nine to 10 mice were measured. The uterine weight was divided into body weight. Gross examination was performed before fixation, and fixed uterine samples were embedded into paraffin blocks and sectioned into 4-μm layers. The slides were stained with hematoxylin and eosin according to general protocols. Endometrial thickness was manually measured using i-solution software (IMT i-solution Inc., Daejeon, Korea), and the average vertical and horizontal diameters were compared between groups.

Statistical analysis

All data are presented as a mean±standard error of the mean.
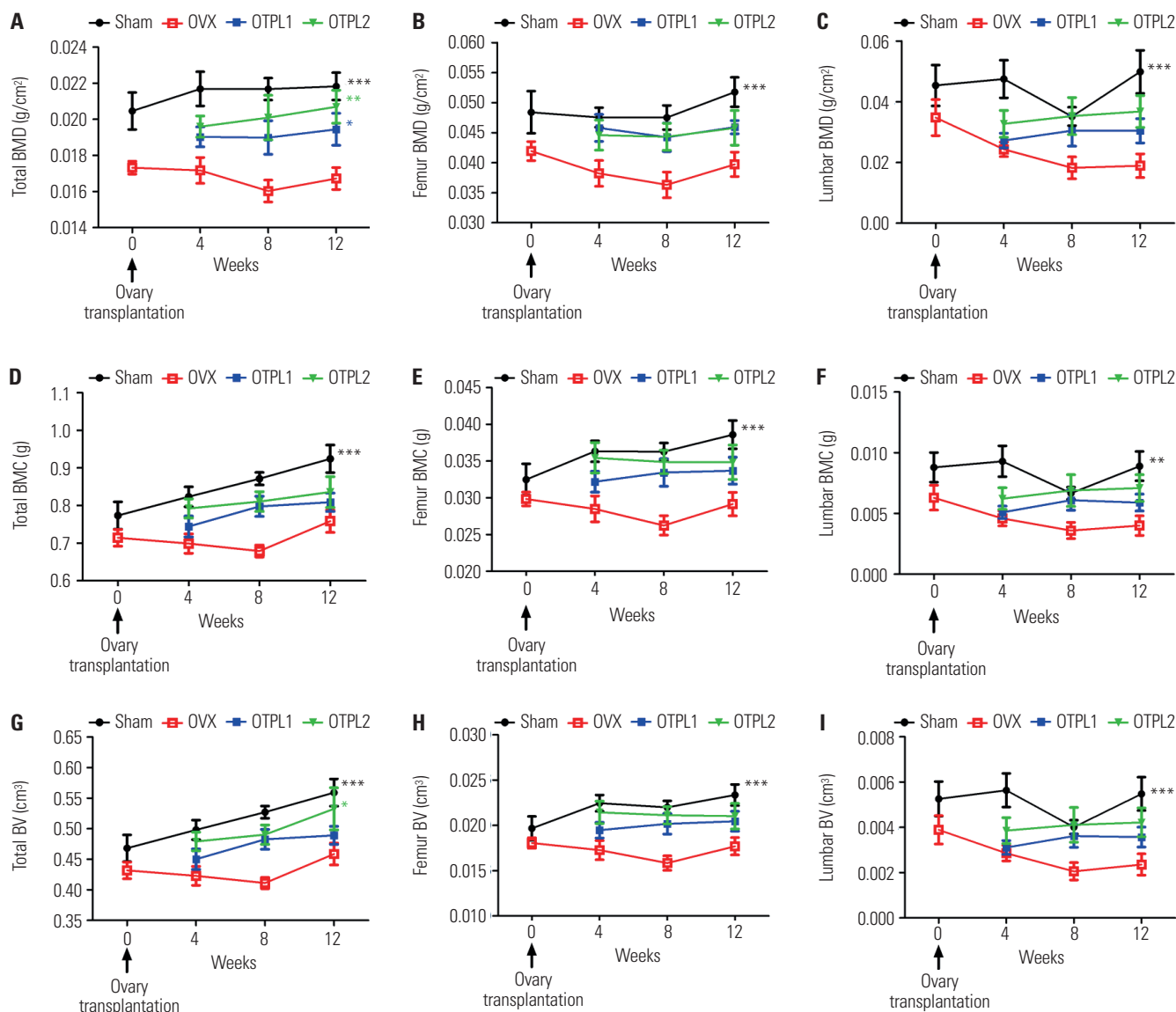

Fig. 3. Bioimaging analysis of the total body and femur and lumbar vertebrae measured by DXA after ovary transplantation. (A-I) Every 4 weeks after ovary transplantation, BMD (A-C), BMC (D-F), and BV (G-I) values of 9 or 10 mice per group were measured using DXA. Values of the femur and lumbar vertebral bones were calculated following the ROI. The means of the OVX groups (red empty squares) were compared with those of the sham (black dots), OTPL 1 (blue squares), and OTPL 2 (green triangles) groups. Asterisks represent the *p*-value at 12 weeks (*$p<0.05$, **$p<0.01$, ***$p<0.001$). DXA, dual energy X-ray absorptiometry; BMD, bone mineral density; BMC, bone mineral contents; BV, bone volume; OVX, ovariectomy; OTPL, ovarian tissue transplantation; ROI, region of interest.

To remove outliers (α=0.05), we performed Grubb's test using the outlier calculator that GraphPad offered (https://www.graphpad.com/quickcalcs/grubbs1/). Average (without outliers) values of mice per group were compared among groups using two-way ANOVA followed by Bonferroni posttests using GraphPad Prism 5 software (GraphPad Software Inc.).

RESULTS

Establishing a post-menopausal osteoporosis model and validating its efficacy

To establish a post-menopausal osteoporosis mouse model, the bilateral ovaries were surgically removed before OTPL. As show in Fig. 2, BMD, BMC, and BV gradually decreased regardless of the measured sites (body, femur, and lumbar vertebrae) 4 weeks after OVX. Based on our results, a period of 8 weeks after bilateral OVX was chosen for auto-transplantation of cryopreserved whole ovaries.

Recovery of bone health by auto-transplantation of vitrified-warmed ovarian tissue

BMD, BMC, and BV values in the body, femur, and lumbar vertebrae decreased in OVX-induced osteoporosis model animals. Four weeks after transplantation, all values increased; however, these values were still lower than those of the sham control group, as shown in Fig. 3. Fig. 3A-C show that the body BMD, femoral BMD, and lumbar vertebrae BMD values significantly decreased after removal of the ovaries; the body BMD recovered after ovary transplantation, regardless of the number of grafts. However, BMD values at the femoral and lumbar vertebrae were slightly higher than those after OVX. Regarding BMC, the OTPL 1 and OTPL 2 groups showed higher body, femoral, and lumbar vertebral values than those achieved with OVX, although there were no significant differences, as shown in Fig. 3D-F. Regarding BV, only the OTPL 2 group had a significantly increased body BV, compared with the OVX group, although there was no significant difference in the femoral and lumbar vertebral BV values (Fig. 3G-I). Therefore, we hypothesized that ovary-providing environments after transplantation might af-
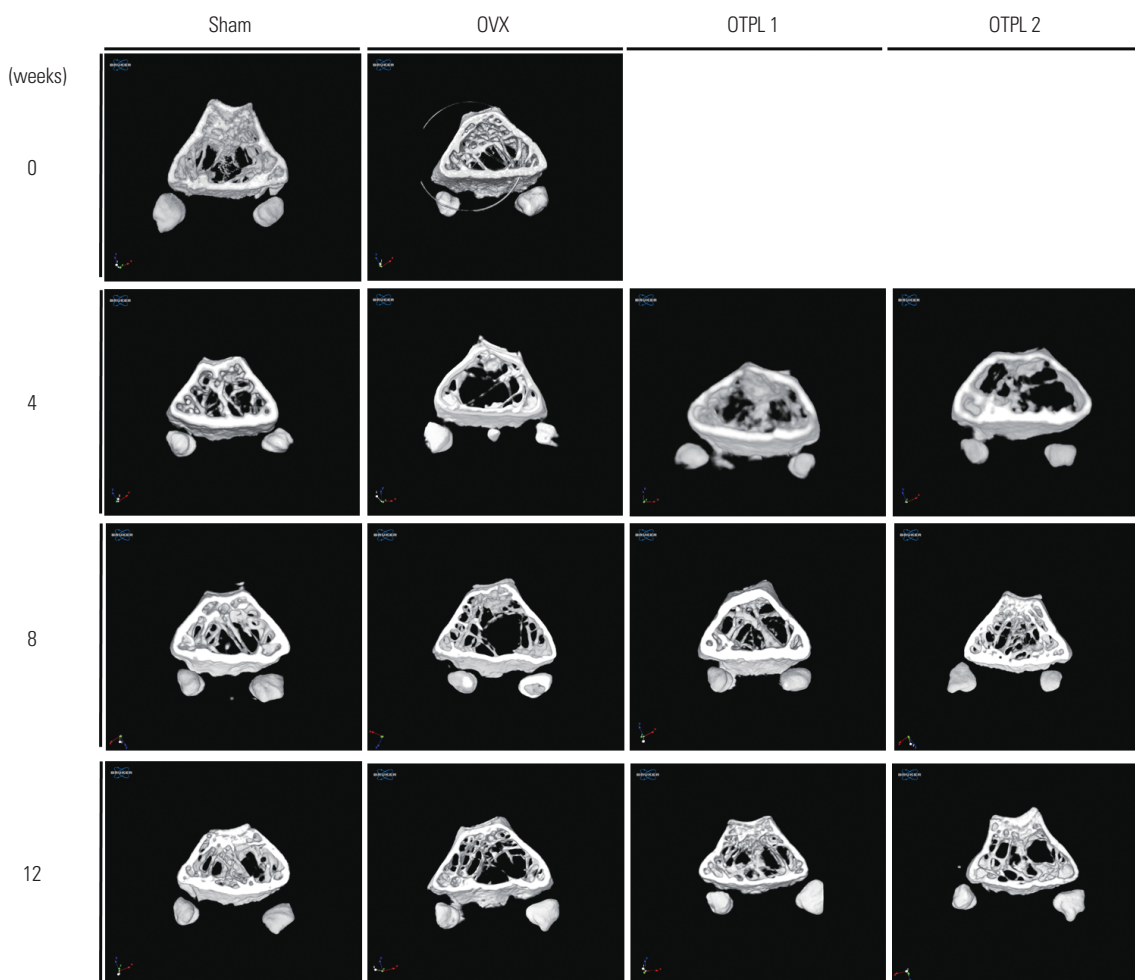

Fig. 4. Microstructure of the femoral bone imaged by micro-computed tomography. To assess bone recovery after ovary transplantation, fixed femurs of two mice were scanned using micro-computed tomography every 4 weeks after ovary transplantation. Tomograms were reconstructed to be cross-sectional images. OVX, ovariectomy; OTPL, ovarian tissue transplantation.

fect bone health in postmenopausal osteoporosis patients.

Changes in bone structure imaged by micro-CT

To investigate whether the auto-transplantation process may influence the microstructure of the femoral bone, a micro-CT analysis was performed. Micro-CT scans showed architectural differences between the OVX group and other groups (Fig. 4). Bilateral OVX strongly induced porosity in the femoral bone, while bone samples collected from the OTPL 1 and OTPL 2 groups had higher density than those from the other groups at 4, 8, and 12 weeks later.

Body weight and fat mass after auto-transplantation of ovarian tissue

In addition to bone health, we measured total body weight, in addition to absolute and relative fat mass, in model animals, which are commonly increased in postmenopausal women. Total body weight and fat mass values in the OVX group were significantly higher than those in the sham control group; however, these values were decreased in the OTPL 1 and OTPL 2 groups, as shown in Fig. 5.

Alterations in gross appearance, weight, histology, and thickness of the uterus

Uterine atrophy is common in post-menopausal women. Therefore, the uterus underwent gross examination and histological analysis. In Fig. 6A, the uteri in OVX mice were dramatically altered, whereas those in the OTPL 1 and 2 groups interestingly reverted to normal shape. These changes were confirmed when the uteri were weighed, as shown in Fig. 6B. Additionally, uterine histology showed severe atrophy and shrinkage in the OVX group, whereas these were surprisingly restored in both OTPL groups, regardless of the number of grafts (Fig. 6C and D).

DISCUSSION

In this study, we aimed to confirm the possibility of using tHRT

as an ideal therapeutic method to treat and prevent post-menopausal symptoms and effects, including osteoporosis, changes in body composition, and uterine atrophy. BMD, BMC, and BV values were increased in the OTPL 1 and OTPL 2 groups after auto-transplantation, regardless of the sites assessed (body, femur, and lumbar vertebrae). Interestingly, uterine characteristics, including gross appearance, weight, and endometrial thickness, were dramatically restored in both the OTPL 1 and OTPL 2 groups, indicating that auto-transplanted ovaries may restore endocrine function without any complications, which normally occurs with pHRT. Regimens with high doses of estrogen (50 µg $E_2 \pm P_4$) result in an abnormal uterus, which is consistent with concerns in human patients on estrogen therapy.[5]

In the present study, BMD, BMC, and BV values, as well as uterine status, were surprisingly comparable between the OTPL 1 and OTPL 2 groups, indicating that the number of ovaries transplanted into ovariectomized mice did not affect the recovery of bone health via the restoration of the endocrine system. These results suggest that treatment is not dependent on the dose of hormones nor amount of tissue transplanted, as the minimum dose and amount, respectively, are sufficient for treatment. In clinical practice, repeated transplantation with small amounts of tissue could be better as a long-term treatment option.

Silva, et al.[12] demonstrated that cryopreserved OTPL restored bone parameters, including median tibial cortical thickness and trabecular mean, in a rat model. In a previous study, ovaries in rats were bilaterally removed, vitrified, and reimplanted a week or month after OVX. This time period after OVX was apparently not sufficient to evaluate post-menopausal osteoporosis in rodent models. In our study, we observed that bone-related bioimaging indexes (BMD, BMC, and BV) gradually decreased and did not recover even after 20 weeks in ovariectomized mice. The OVX group showed a remarkable difference in BMD, BMC, and BV values, compared with the sham control, regardless of the time period. Therefore, we determined that a period of 8 weeks after OVX is suitable for investigating the effects of tHRT.
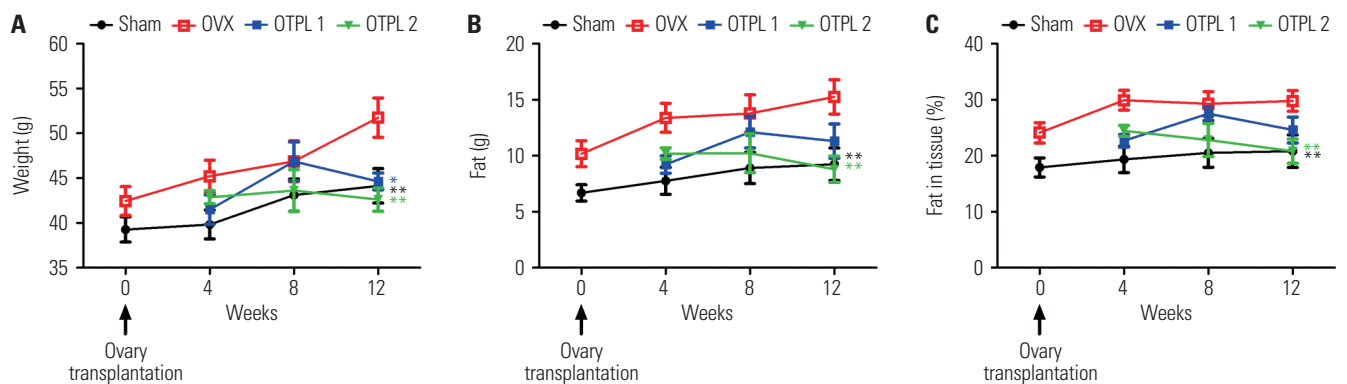

Fig. 5. Characteristics of animals used in the present study and body compositions after ovary transplantation measured by DXA. Body (A), fat (B), and fat in tissue (C) weights of 9 to 10 mice per group were assessed using DXA. The sham (black), OVX (red), OTPL 1 (blue), and OTPL 2 (green) groups are presented (*$p<0.05$, **$p<0.01$). DXA, dual energy X-ray absorptiometry; OVX, ovariectomy; OTPL, ovarian tissue transplantation.
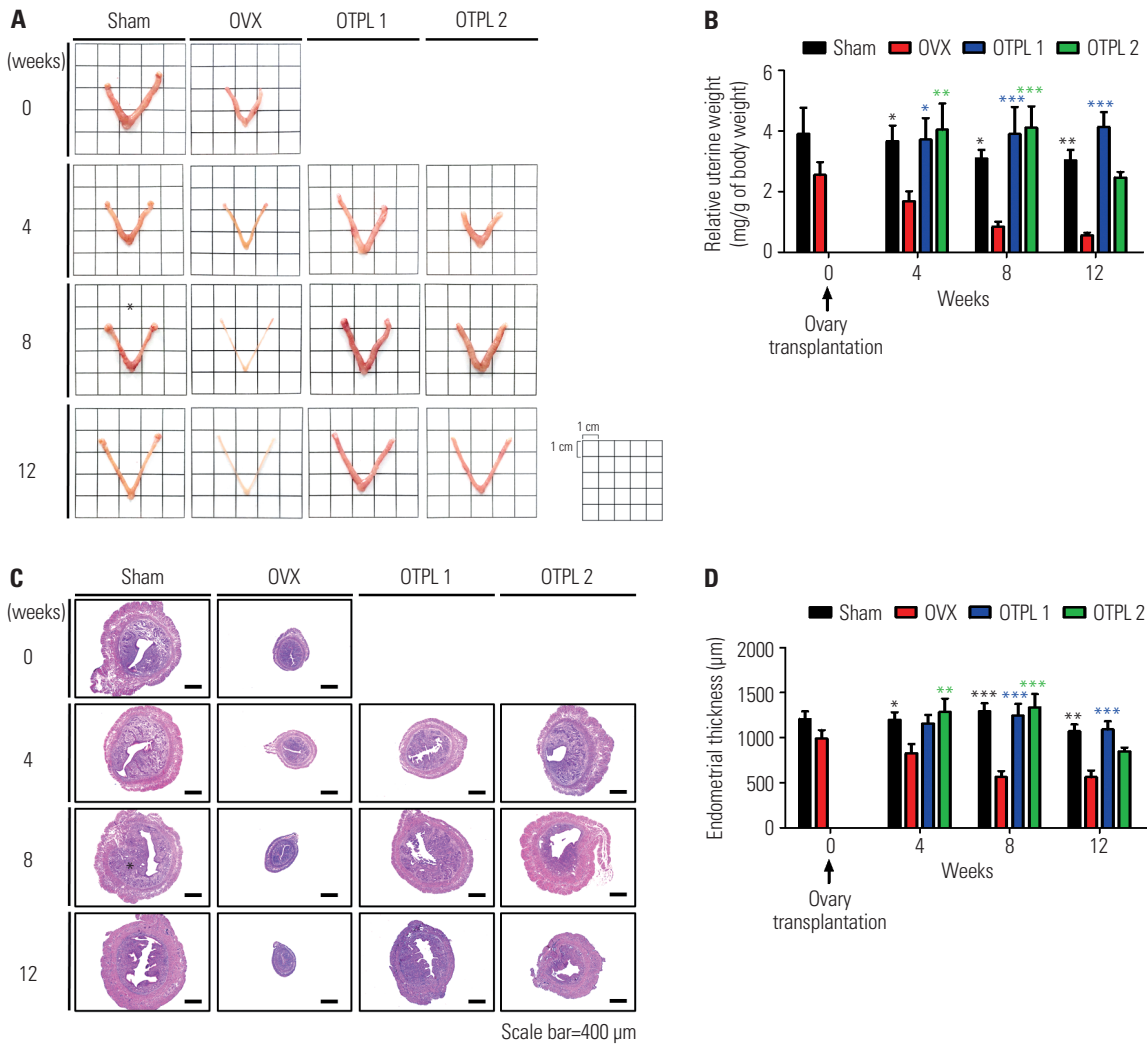
Fig. 6. Uterine recovery after ovary transplantation and evaluation every 4 weeks to check for recovery of hormones. (A) We performed a gross examination on the uterus. Grid size was about 1 cm×1 cm. (B) Uterine weight was measured and divided into body weight. (C) A fixed uterine sample was embedded in a paraffin block and sectioned into 4-μm layers. The slides were stained with hematoxylin and eosin. (D) Endometrial thickness was measured without the myometrium. The averages values of 9 to 10 mice per group were calculated. The means of the OVX groups (red) were compared with those of the sham group (black), OTPL 1 (blue), OTPL 2 (green) groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$). OVX, ovariectomy; OTPL, ovarian tissue transplantation.

In this study, we showed that the uterus and bone mass recovered to pre-OVX levels instead of ovary-related hormones [follicle stimulating hormone (FSH), luteinizing hormone, estrogen, and progesterone]. Our previous studies have demonstrated that serum FSH dramatically decreases after OTPL and induces the resumption of folliculogenesis in grafts.[13] We observed that estrogen is produced by in vitro folliculogenesis.[14] In addition to our study, many case studies have suggested that the transplanted ovary is the source of endogenous hormones as well as preserves fertility.[15-17] We did not measure serum estradiol levels in mice because we focused on the long-term effects of tHRT on not only hormone production but also on bone health. Based on our findings and those of previous studies, we assumed that the ovarian follicles in the graft may resume folliculogenesis and improve bone health via ovarian hormone production.

The most striking advantage of tHRT is graft longevity. Andersen, et al.[18] demonstrated that ovarian cortical strips, not whole tissue, have sustained function for 7 years in humans regardless of the presence or absence of cryopreservation. Kim[9] also showed that endocrine function after a second transplantation was sustained for 7 years. Jensen, et al.[19] demonstrated that 53 transplants maintained endocrine function up to 10 years. The cryopreservation of ovarian tissue can be performed to extend the longevity of grafts, resulting in an ideal postponed menopause in women.[20,21] As noted, the prevention of postmenopausal symptoms is vital in an aging society. In contrast, delayed menopause may result in unexpected symptoms, including estrogen-sensitive breast and uterine endometrial cancers; therefore, further studies related to tHRT are required.

tHRT has limitations, including safety issues, due to the high risk of ovarian cancer recurrence and other complications after

reimplantation, including leukemia, which can spread to the ovary.[22] Auto-transplantation of ovarian tissue for tHRT is inapplicable in patients with premature ovarian insufficiency because the ovary is non-functional. Particularly, *BRCA* mutation carriers will be at risk because there is still a lack of guidelines or expert consensus on OTPL in this population.[23,24] Although tHRT has several challenges regarding fertility preservation, it still merits consideration for endocrine restoration.

To replace pHRT, many regenerative medicine approaches with improved safety profiles have been published, including the use of cell-based encapsulations.[5] The ovarian follicle, a basic functional unit, has also been utilized to restore ovarian function, including sex steroid hormone production, in experimental animals.[25,26] Sittadjody, et al.[5] reported a novel cell-based HRT (cHRT) with fabricated constructs. They postulated that cHRT offers an attractive alternative to traditional pHRT. Similarly, Guo, et al.[27] and Liu, et al.[28] investigated the use of microencapsulated ovarian cells, which secreted estradiol and progesterone; these secretions may result in the prevention of osteoporosis in ovariectomized mice. Krotz, et al.[29] seeded three-dimensional complexes of granulosa cells and theca cells into micro-molded gels; this artificial ovary produced hormones in response to gonadotropins. Currently, mesenchymal stem cell-based therapy has been widely investigated considering its roles in osteoporosis, which include the genetic and transcriptional regulation involved in the pathogenesis of osteoporosis and regulation of signaling pathways associated with osteoporosis.[30] cHRT is thought to be in the experimental phase, not clinical.[31,32] In this respect, tHRT has already been clinically applied and demonstrated its restorative abilities in endocrine function.[33-35]

The present study has a few limitations. First, we did not assess serum estradiol levels after ovarian transplantation or the histology of the transplanted ovary, which can prove the direct recovery of ovarian function. However, we confirmed the hormonal effects of ovarian transplantation via increases in uterine weight and endometrial thickness, as well as confirming changes in body fat mass. Second, bone turnover markers and microarchitecture analysis data were missing, which could have helped us understand the changes in bone metabolism and strength. DXA data alone were utilized to check the recovery of bone density after ovarian transplantation because the purpose of this study was to demonstrate the possibility of tHRT as a therapeutic modality for post-menopausal osteoporosis. Third, we did not include a pHRT group in this study. Thus, we could not directly compare the efficacies of tHRT and pHRT. Fourth, we did not evaluate estrogen-related side effects of tHRT. Prior to clinical application, tHRT-associated safety issues, including breast cancer and thrombosis, should have been evaluated. Finally, this was an animal study, and further studies in human subjects are needed to confirm the role of ovarian transplantation as an alternative for pHRT.

Based on these findings, we suggest that tHRT could be an applicable therapeutic modality not only for fertility restoration but also for menopausal hormone therapy in mice. For tHRT application in humans, clinical studies with long-term follow-up are required to investigate the efficacy and safety of tHRT.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

Conceptualization: Dahyeon Yoo and Jaewang Lee. Data curation: Dahyeon Yoo, Jaewang Lee, and Jung Ryeol Lee. Formal analysis: Dahyeon Yoo, Nanum Chung, Jungyoung Yoo, Chae Young Song, and Chungmo Yang. Funding acquisition: Jaewang Lee and Jung Ryeol Lee. Investigation: Dahyeon Yoo, Nanum Chung, Jungyoung Yoo, Chae Young Song, and Chungmon Yang. Methodology: Dahyeon Yoo and Jaewang Lee. Project administration: Kangwon Lee, Jaewang Lee, and Jung Ryeol Lee. Resources: Kangwon Lee, Jin Hyun Jun, Jaewang Lee, and Jung Ryeol Lee. Software: Dahyeon Yoo. Supervision: Jin Hyun Jun, Jaewang Lee, and Jung Ryeol Lee. Validation: Hye Won Youm, Jaewang Lee, and Jung Ryeol Lee. Visualization: Kangwon Lee, Jin Hyun Jun, Jaewang Lee, and Jung Ryeol Lee. Writing—original draft: Dahyeon Yoo and Jaewang Lee. Writing—review & editing: Hye Won Youm, Jin Hyun Jun, Jaewang Lee, and Jung Ryeol Lee. Approval of final manuscript: all authors.

ORCID iDs

Dahyeon Yoo   https://orcid.org/0000-0002-4021-4498
Nanum Chung   https://orcid.org/0000-0001-9912-7615
Jungyoung Yoo   https://orcid.org/0000-0001-6357-7569
Chae Young Song   https://orcid.org/0000-0002-7566-7454
Chungmo Yang   https://orcid.org/0000-0002-4690-1061
Hye Won Youm   https://orcid.org/0000-0002-1735-7191
Kangwon Lee   https://orcid.org/0000-0001-5745-313X
Jin Hyun Jun   https://orcid.org/0000-0001-9898-4471
Jaewang Lee   https://orcid.org/0000-0001-6801-7149
Jung Ryeol Lee   https://orcid.org/0000-0003-3743-2934

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