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Basic Research

Beneficial Effects of Probiotics Derived from *Inonotus Obliquus* on Menopausal Symptoms in Ovariectomized Mice

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Physical debilitation and other health risks such as cardiovascular disease and osteoporosis often accompany decreased estrogen production in postmenopausal women. Phytoestrogens, natural compounds that functionally mimic estrogens, have emerged as a potential treatment method for hormonal dysregulation during menopause. Among these, chaga (*Inonotus obliquus*) extract has gained attention for its various health benefits, including anti-cancer and antioxidant properties. The aim of this study was to investigate the bone metabolic efficacy of lactic acid bacteria derived from *I. obliquus* in ovariectomized C57BL/6J female mice. The results demonstrated the estrogenic activity of probiotics derived from *I. obliquus* and its potential to alleviate osteoporosis symptoms by improving bone microstructure and density. Furthermore, probiotics derived from *I. obliquus* favored biochemical markers associated with a reduction in bone resorption. Additionally, effects on brain neurotransmitters and serum estradiol levels suggested its regulatory role in estrogen mimicry. These findings underscore the therapeutic potential of probiotics derived from *I. obliquus* in managing menopausal symptoms and thus warrant potential human investigation in improving bone health in postmenopausal women.

Keywords: Estrogen, *Inonotus obliquus*, Menopause, Osteoporosis, Ovariectomized mice

Abbreviations Used: Bone mineral density, BMD; Bone volume/total volume, BV/TV; C-terminal telopeptide, CTX; Estradiol, E2; Micro-computed tomography, Micro-CT; Norepinephrine, NE; Osteocalcin, OCC; Osteoprotegerin, OPG; Ovariectomy, OVX; Probiotics derived from *Inonotus obliquus*, pIO; Receptor activator of nuclear factors κB ligand, RANKL; Trabecular number, Tb. N.; Trabecular thickness, Tb. Th

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INTRODUCTION

Menopausal symptoms are linked to physical debilitation including the potential for cardiovascular disease and osteoporosis (Rymer and Morris, 2000). Osteoporosis in postmenopausal women heightens the risk of increased fracture due to a rapid decline in bone density and subsequent bone loss (Yong and Logan, 2021). Female sex hormones, estrogen and progesterone, are mainly produced in the ovaries. Estrogen, through its binding to estrogen receptors on cells throughout the body, plays a crucial role in metabolism and the transmission of biological signals (O'Neill and Eden, 2017). During menopause, there is a decline in ovarian function, resulting in reduced estrogen production and subsequent hormonal dysregulation (Woods et al., 2005). Therefore, treatment and prevention methods have been extensively researched, with one common approach involving the replacement of estrogen deficiency through the consumption of phytoestrogens (Krebs et al., 2004). Phytoestrogens are natural compounds derived from plants that have structure and function similar to estrogens with fewer side effects than synthetic estrogens (Patra et al., 2023).

Chaga (*Inonotus obliquus*) is a black birch fungus of the *Hymenochaetaceae* family that grows naturally in Russia (Kim et al., 2006). It is a pathogen that forms large, tuberous sclerotia on black birch trees and parasitizes on the stems, causing white rot (Zhong et al., 2009). In Russia and Finland, it is known as *I. obliquus* and is primarily found in the northern hemisphere of Russia and Japan. *I. obliquus* is primarily used as a functional and health food due to its anti-cancer (Ma et al., 2013), antioxidant (Cui et al., 2005), anti-diabetic (Wang et al., 2017), and anti-mutagenic (Ham et al., 2009) properties. Steroids or aromatic polyphenolic compounds found in *I. obliquus* mushrooms have demonstrated antioxidant, antitumor, and antiviral activities (Hwang et al., 2016).

Therefore, this study used ovariectomized C57BL/6J female mice to investigate the bone metabolic efficacy of lactic acid bacteria derived from *I. obliquus*. The study assessed bone mineral density (BMD), structural parameters of the proximal tibia, and various blood components influencing bone metabolism.

MATERIALS AND METHODS

Preparation of *I. Obliquus*-Derived Probiotics (pIO)

I. obliquus-derived probiotics (pIO) powders were obtained from Vitech Co., Ltd (Jeonbuk, Korea) and stored following the manufacturer's instructions until required. The samples were stored at 4°C until they were used for our experiments. Fresh probiotic was prepared weekly by dissolving the powders in water to the required concentrations of 1×10^9, 1×10^{10}, and 1×10^{11} cfu of pIO/kg of BW/day for oral administration to mice. To prepare fresh probiotics at the 10, 30, and 60 μg/mL necessary concentrations for treating cells in vitro, the powders were dissolved in 1x phosphate-buffered saline (PBS).

Estrogenic Activity Assay

Detailed procedures were described in a previous study (Lim et al., 2023). T47D-Kbluc cells, stably expressing estrogen response element (ERE) luciferase (Luc), were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 mg/mL streptomycin, and 0.2 U/mL bovine insulin. For the estrogenic activity assay, the culture medium was replaced with RPMI 1640 with 10 % charcoal-stripped FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin, and the cells were then incubated for 24 h. After incubation, the cells were seeded in 96-well culture plates and treated with pIO for 24 h. Luciferase activity was determined according to the manufacturer's instructions (Promega Corp., Madison, WI, USA).

MTT Assay for Cellular Viability

The MTT assay is a colorimetric measurement of cell metabolic activity, an indirect measure of cell growth (Cory et al., 1991). T47D-Kbluc cells were seeded in 96-well culture plates and treated with pIO (10, 30, and 60 μg/mL) for 24 h. After incubation, the cells were treated with 10 μL of thiazolyl blue tetrazolium bromide (5 mg/mL in PBS) for 3 h. MTT, a yellow tetrazole, is reduced to purple formazan and stains living cells. All the stained cells were then detached from the plate with 100 μL DMSO, and the OD_{540} was measured.

Animals

Detailed procedures were described in a previous study (Kim et al., 2020). A total of 36 female C57BL/6 mice (7 weeks old, weighing 15–20 g) were purchased from Raon Bio (Yongin, South Korea) and randomly allocated into six equal groups.

1. Control group (sham surgery group receiving oral saline).
2. Ovariectomy (OVX) group receiving oral saline.
3. Estradiol (E2) group (OVX mice receiving oral E2, 0.5 mg/kg of body weight/day).
4. OVX mice receiving oral pIO; 1×10^9 cfu of pIO/kg of BW/day.
5. OVX mice receiving oral pIO; 1×10^{10} cfu of pIO/kg of BW/day.
6. OVX mice receiving oral pIO; 1×10^{11} cfu of pIO/kg of BW/day.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience & Biotechnology (KRIBB-AEC-23276). Mice were provided ad libitum access to food and water. The room was maintained with a 12-h light–dark cycle (lights on at 09:00 and lights off at 21:00) and controlled temperature (21 ± 2°C).

Micro-Computed Tomography (CT) Analysis

Detailed procedures were described in a previous study (Lim et al., 2023). In brief, femurs were collected from the mice and fixed in 10% formalin for 24 h. Subsequently, the fixed femurs were stored in PBS at 4°C. Bone morphometric and microarchitectural data were obtained using a SkyScan 1276 micro-CT scanner (Bruker microcar, Kontich, Belgium). The femur was mounted on a sample chamber, and the sample was automatically rotated along its axis (angular step: 0.6°, reconstruction angular range: 360.00°). For 9 μm resolution images, a 0.5 mm Al filter, a 60-kV source voltage, and a 200 μA source current were used. Three-dimensional images and morphometric parameters were calculated using Nrecon, CTAn, and CTVol software (Bruker). The region of interest was defined between 0.5 and 2.5 mm below the growth plate of the proximal femur.

Biochemical Analysis of Serum and Brain

Detailed procedures were described in a previous study (Kim et al., 2020; Lim et al., 2023). Blood samples were collected from mice via cardiac puncture and subsequently centrifuged to obtain serum. The whole brain was homogenized and lysed using a lysis buffer. Total brain protein levels were quantified using a detergent-compatible (DC) protein assay kit (Bio-Rad, Hercules, CA, USA) and used for normalizing estradiol (E2) and norepinephrine levels. The serum levels of serotonin, C-terminal telopeptide (CTX), osteoprotegerin (OPG), and receptor activator of nuclear factors κB ligand (RANKL) osteocalcin (OCC), as well as the brain levels of E2 and norepinephrine were measured using commercially available ELISA kits following the manufacturer's instructions. ELISA kits were obtained from Abcam for OPG and

RANKL, Immunodiagnostic Systems (Boldon, UK) for CTX, Enzo Life Science (Farmingdale, NY, USA) for serotonin, Mybiosource (San Diego, CA, USA) for norepinephrine, and R&D systems (Minneapolis, MN, USA) for estradiol.

Statistical Analyses

The results are presented as the mean ± standard deviation (SD) of three or nine independent experiments. Statistical analysis was performed using Prism 5 software (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using either one-way ANOVA followed by Dunnett's test or unpaired Student's t-test, as appropriate.

RESULTS AND DISCUSSION

Estrogenic Activity of pIO in T47D-Kbluc Cells

Estrogenic activity refers to the ability of a chemical to mimic estrogen and exhibit estrogen-like functions by activating estrogen receptors (Nilsson et al., 2001). T47D-Kbluc cells were used to evaluate the cytotoxic and estrogenic activities of pIO. Notably, pIO demonstrated no cytotoxic effects at concentrations of 10, 30, and 60 μg/mL. Additionally, these cells stably expressed the ERE-Luc construct. The results indicated that treatment with pIO at a concentration of 60 μg/mL increased estrogen activity beyond normal levels (Figure 1).

Effect of pIO on Osteoporosis in OVX Mice

Bone metabolism is governed by osteoblastosis processes and bone resorption by osteoclasts (Teitelbaum, 2000). Estrogen deficiency promotes bone resorption by osteoclasts, leading to osteoporosis symptoms characterized by trabecular bone loss, simplification of bone microstructure, and reduction in bone mass (Lee et al., 2020). Micro-CT analysis of the femur was performed to investigate the effect of pIO on osteoporosis. Three-dimensional images of groups orally administered with pIO at doses of 1×10^9, 1×10^{10}, and 1×10^{11} cfu/kg showed increased and complex bone microstructure compared to the OVX group (Figure 2). BMD was significantly enhanced in the 1×10^{11} cfu/kg of pIO group compared to the OVX group. Moreover, bone volume/total volume (BV/TV) and trabecular thickness (Tb. Th.) values were significantly increased in both 1×10^{10} and 1×10^{11} cfu/kg of pIO groups. Additionally, trabecular number (Tb. N.) was significantly increased in both the 1×10^{10} and 1×10^{11} cfu/kg pIO groups (Figure 2). When compared with the E2 treatment group, the results suggest that pIO treatment may alleviate osteoporosis symptoms.

Effect of pIO on Biochemical Markers in OVX Mice

Various biochemical indicators are used in diagnosing osteoporosis (Lin et al., 2018). CTX is released from bone during bone resorption (Lee et al., 2020; Park et al., 2019), directly reflecting bone resorption and serving as a bone resorption index. RANKL and OPG are secreted by osteoblasts and regulate osteoclast genesis. OPG binds to RANKL and inhibits RANKL–RANK interaction (Zhao et al., 2019). These markers may describe bone metabolic status, and the effect of pIO on these markers was investigated. Oral administration of 1×10^{10} and 1×10^{11} cfu/kg of pIO groups significantly reduced serum CTX levels compared to the OVX group (Figure 3). Additionally, serum concentrations of OPG were restored. However, while the serum concentration of RANKL did not decrease, the RANKL/OPG ratio was significantly decreased by pIO treatment (Figure 3). These results suggest that pIO treatment may mitigate increased bone resorption caused by ovariectomy by inhibiting osteoclast differentiation.

Effects of pIO on Brain Neurotransmitters and Serum Estradiol Levels in OVX Mice

During the menopausal transition, hormonal changes occur as the secretion of ovarian follicular hormones decreases (Burger et al., 2008). These hormonal changes alter the hypothalamic–pituitary–ovarian axis and nervous system function (Yu et al., 2023). Menopausal depression is caused by these changes and is closely related to serotonin and norepinephrine (Kang et al., 2023). Therefore, we investigated the effects of pIO on brain estradiol, norepinephrine, and serum serotonin levels. As shown in Figure 4, oral administration of pIO at doses of 1×10^9, 1×10^{10}, and 1×10^{11} cfu/kg tended to increase estradiol and norepinephrine levels in the whole brain of the OVX group. Additionally, serum serotonin levels

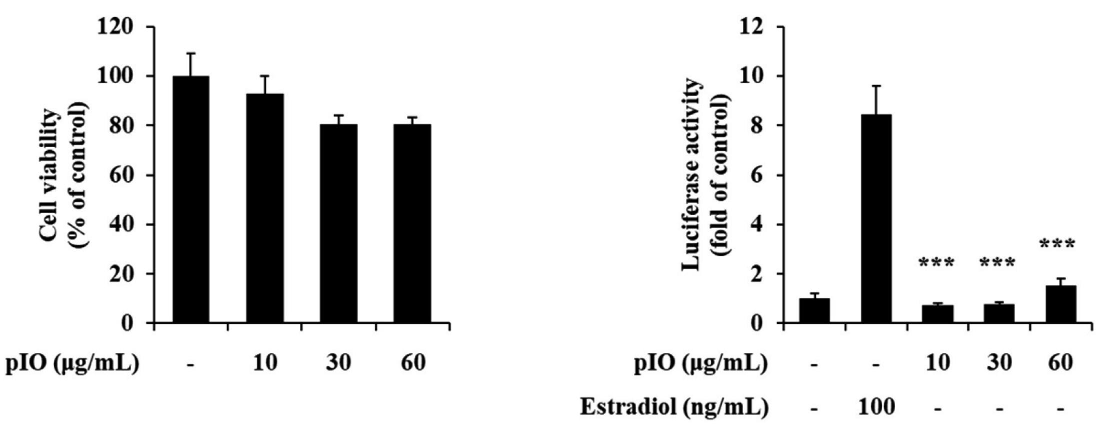

FIGURE 1 | Effect of pIO on the cytotoxicity and estrogenic activity of T47D-Kbluc cells. (A) cell viability; (B) the estrogenic activity on T47D-Kbluc cells. The results are expressed as the means ± standard deviations (SD). *P < 0.05 compared with the negative control group.

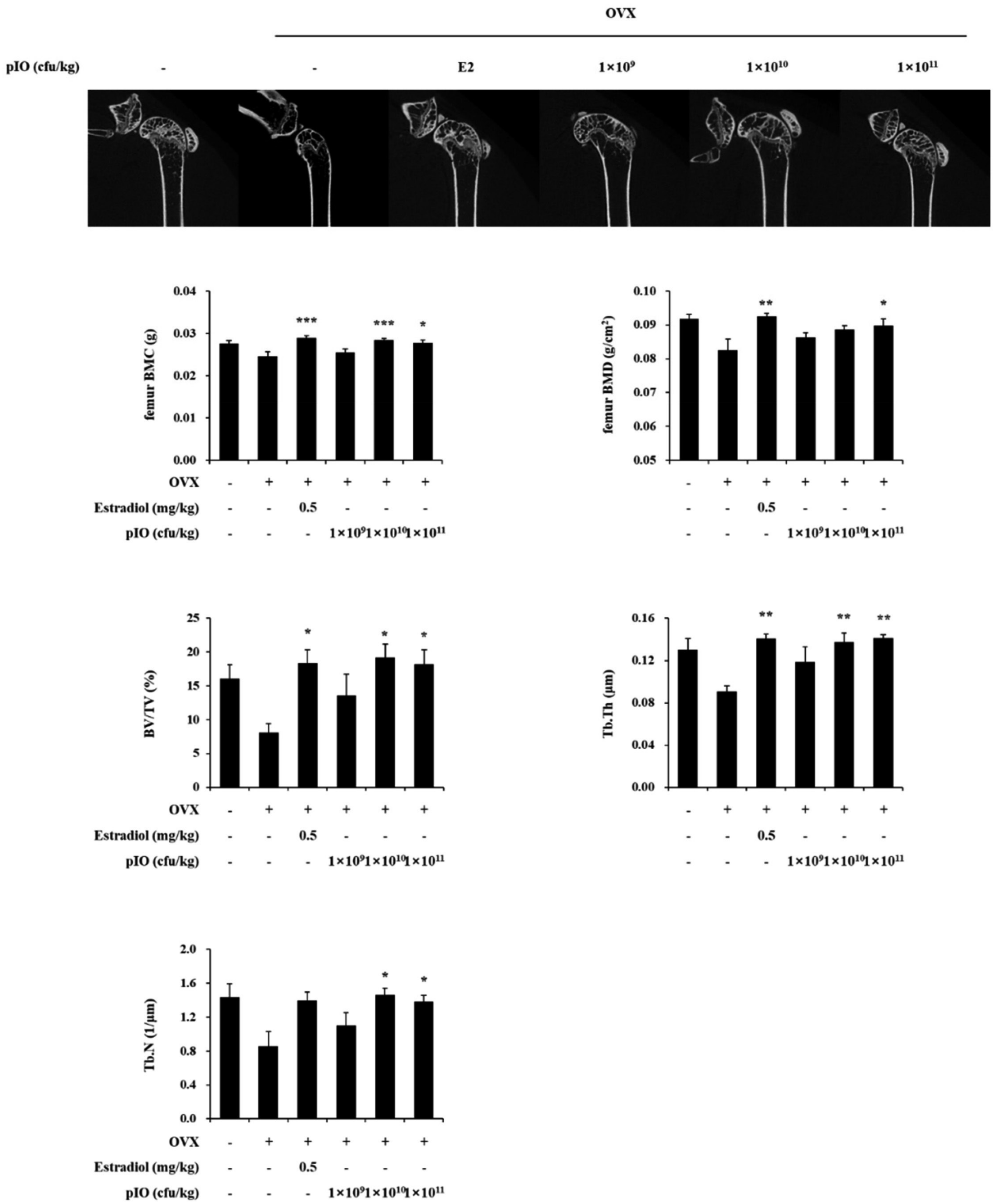

FIGURE 2 | Micro-CT analysis of the proximal femur of the OVX-induced bone loss in a mouse model. (A) Images of trabecular bone proximal femur of E2- or pIO-treated OVX mice. (B) Analysis of the trabecular bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular thickness (Tb. Th.), and trabecular number (Tb. N) of the femurs by micro-CT. The results are expressed as the means ± standard deviations (SD). *P < 0.05, **P < 0.01, and ***P < 0.005 indicate significant differences between the OVX group and the E2- or ECE-treated group.

tended to increase with pIO treatment. These results may explain how pIO exerts its regulatory effects through estrogen regulation and mimics estrogen.

In conclusion, this study investigated the potential bone metabolic efficacy of pIO using ovariectomized C57BL/6J female mice as a model of osteoporosis. First, pIO exhibited estrogenic activity devoid of cytotoxic effects, indicating its potential as a safe estrogen mimic. Second, the oral administration of pIO significantly improved bone microstructure parameters, including BMD, BV/TV, Tb. Th, and Tb. N, compared to the OVX group. These findings strongly suggest the effectiveness of pIO in mitigating osteoporosis symptoms. Furthermore, pIO treatment resulted in a decrease in serum CTX levels, a key indicator of bone resorption, and restored serum OPG concentrations while significantly reducing the RANKL/OPG ratio, indicating suppression of osteoclast differentiation. Finally, administration of pIO tended to increase brain estradiol and norepinephrine levels, as well as serum serotonin levels, suggesting that pIO has potential to regulate hormonal

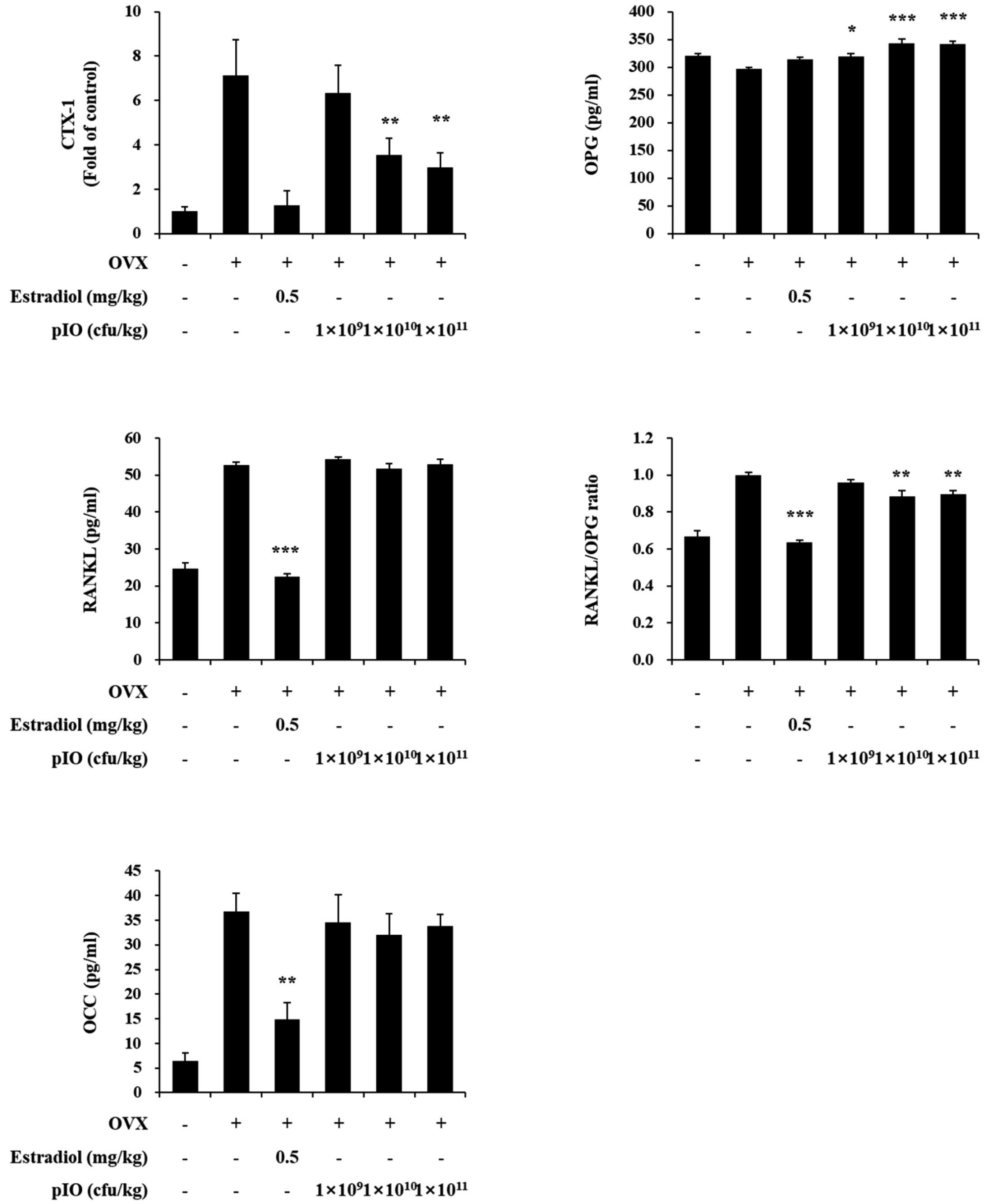

FIGURE 3 | Biochemical analysis of the serum markers of osteoporosis in estradiol- or ECE-treated OVX mice. The serum levels of CTX-1, RANKL, OPG, RANKL/OPG, and OCC were determined using ELISAs. The results are expressed as the means ± standard deviations (SD). *P < 0.05, **P < 0.01, and ***P < 0.005 indicate significant differences between the OVX group and the E2- or ECE-treated group.

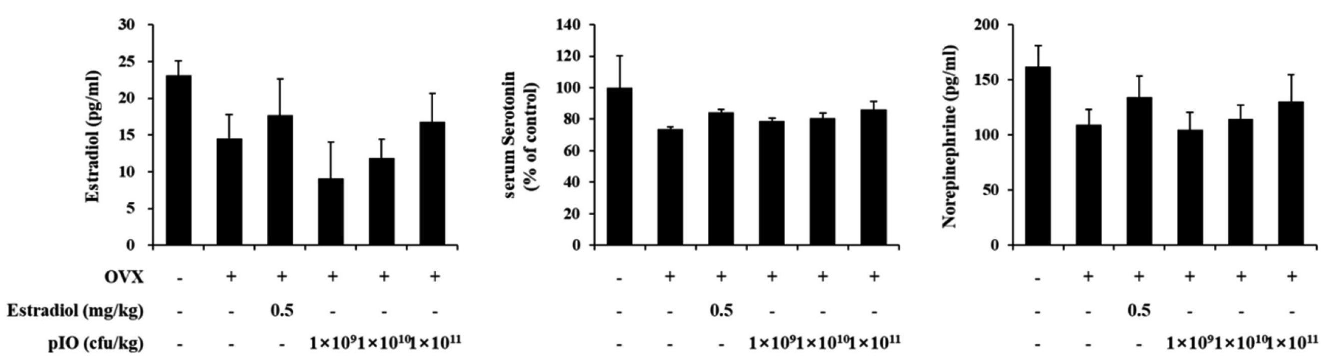

FIGURE 4 | Estradiol and norepinephrine levels in the brains and serotonin levels in the serum of E2- or pIO-treated OVX mice. Serum serotonin, estradiol, and norepinephrine levels were measured using ELISAs. The results are expressed as the means ± standard deviations (SD). *P < 0.05, **P < 0.01, and ***P < 0.005 are significantly different between the OVX group and the E2- or ECE-treated group.

changes associated with menopausal transition and influence neurotransmitter levels. These findings collectively demonstrate the potential of pIO as a therapeutic option for managing osteoporosis and associated menopausal symptoms. This highlights the need for further exploration and development of novel interventions to enhance the health of postmenopausal women.

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ASSURANCE OF THE ORIGINALITY OF DATA

The author(s) assure the readers and the publishers that all data presented here are original.

CONFLICT OF INTEREST DISCLOSURE

The authors state that there are no conflicts of interest to disclose.

AUTHORS' CONTRIBUTIONS

Seon Gyeong Bak and Nisansala Chandimali conducted formal analysis and writing—original draft. Hyung Jin Lim and Eun Hyun Park contributed to the formal analysis and investigation. Yeong Seon Won and Hyuck Se Kwon contributed to the investigation and methodology. Nayong Lee, Soon-Il Yun, and Hyunjeong Oh contributed to methodology. Sang-Ik Park and Seung-Jae Lee conceptualized the project and contributed to writing—review, editing, and project supervision.

DATA AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

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