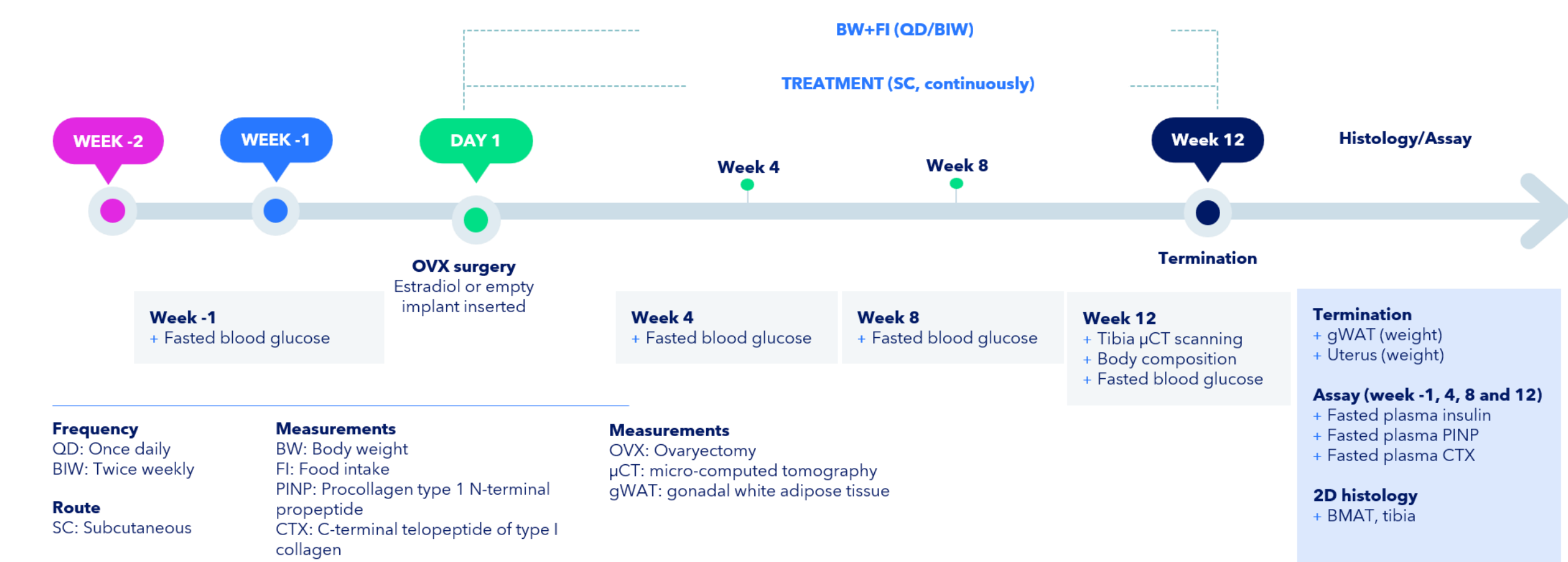


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Menopause is associated with significant health changes and a broad range of symptoms, including adverse changes in body composition and metabolism and an increased risk of osteoporosis. Estradiol (E2)-based hormone replacement therapy effectively alleviates several menopausal symptoms and protects against bone loss, but does not address all symptoms or health risks. These unmet needs, contraindications, individual benefit-risk profiles, and patient preferences highlight the need for developing additional treatments, including non-hormonal alternatives. The ovariectomized (OVX) rat is widely used to study metabolic and skeletal consequences of ovarian hormone depletion. This study aimed to characterize these changes and benchmark the response to continuous E2 treatment using metabolic assessments, micro-computed tomography (μ CT), histopathology, and bone-turnover biomarkers in the OVX rat model.

Ten-week-old female Sprague-Dawley rats underwent ovariectomy (OVX) or sham surgery (n=8-10 per group). OVX rats received a subcutaneous slow-release estradiol implant (E2; ~6.5 µg/day) or an empty control implant. All animals were maintained on a standard chow diet throughout the study. Metabolic assessments included body weight, food intake, MRI-based body composition, and 4-hours fasted blood glucose and plasma insulin. Blood samples were collected after 4 hours fasting at weeks -1, 4, 8, and 12 relative to surgery to measure bone turnover biomarkers. At 12 weeks after surgery, the proximal tibia was scanned using micro-computed tomography (µCT; SkyScan 1276) to assess bone architecture and mineral density. Animals were subsequently euthanized and tibial bone marrow adipose tissue (BMAT) was assessed histologically (Figure 5).



Group	Group name	Number of animals	Dosing Frequency	Dosing route	Dosing Concentration
1	Sham	10	Continuous	SC-implant	NA
2	OVX	10	Continuous	SC-implant	NA
3	OVX E2	8	Continuous	SC-implant	~6.5 µg/day

Figure 1. Study outline and group summary

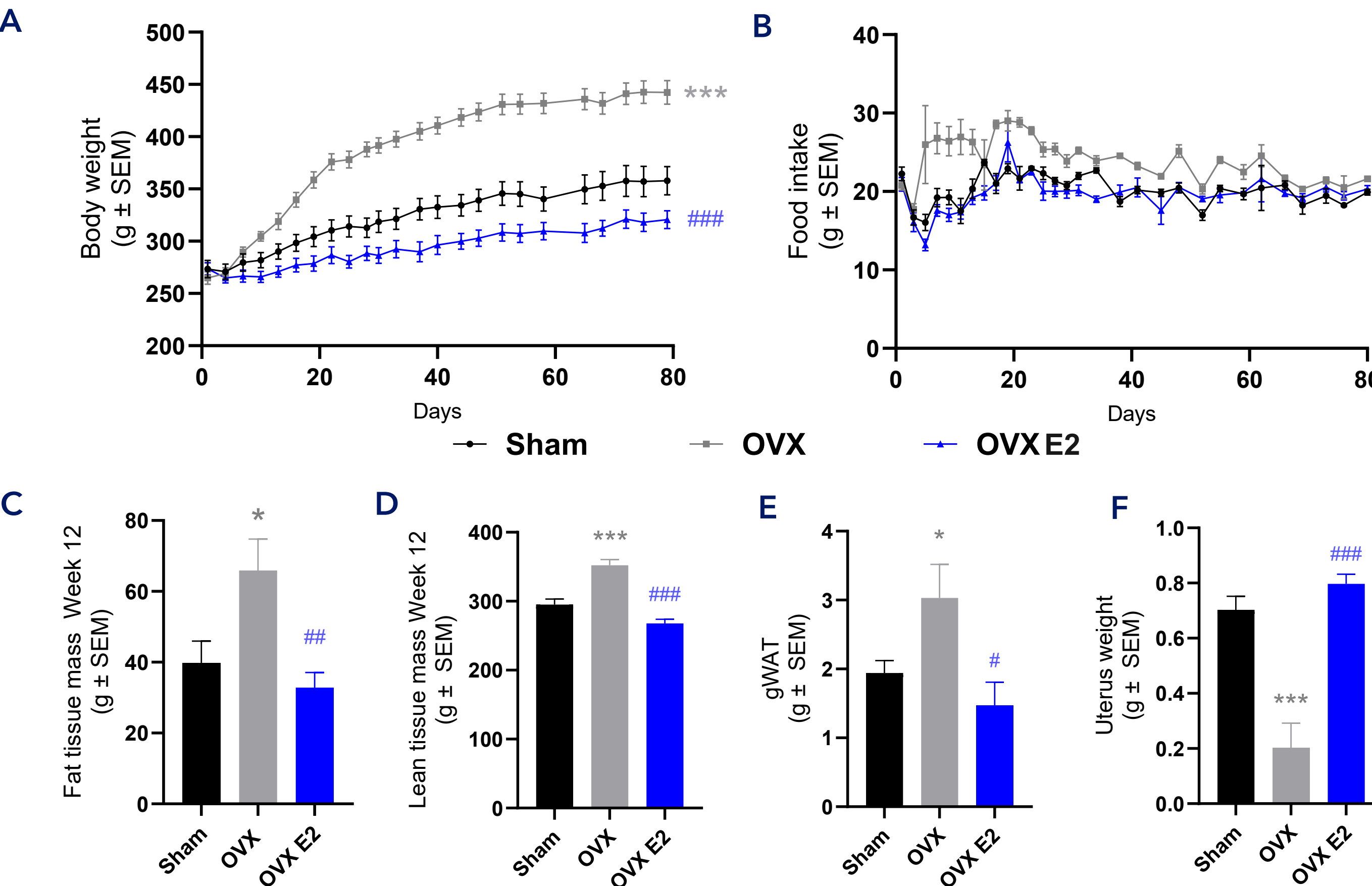


Figure 2. E2 prevented weight gain, fat tissue mass gain, and uterus atrophy in the OVX rats. (A) Body weight, (B) food intake (g), (C) absolute fat, and (D) lean tissue mass (g) at week 12. (E) Gonadal white adipose tissue (g) and (F) uterus weight (g) at termination. Data are shown as mean $\pm$ SEM, $n=8-10$. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared to Sham. # $p<0.05$ ## $p<0.01$ ### $p<0.001$ compared to OVX. Dunnett's test one-factor linear model. Only final body weight timepoint and day 29 food intake measurements were used in statistical comparison.

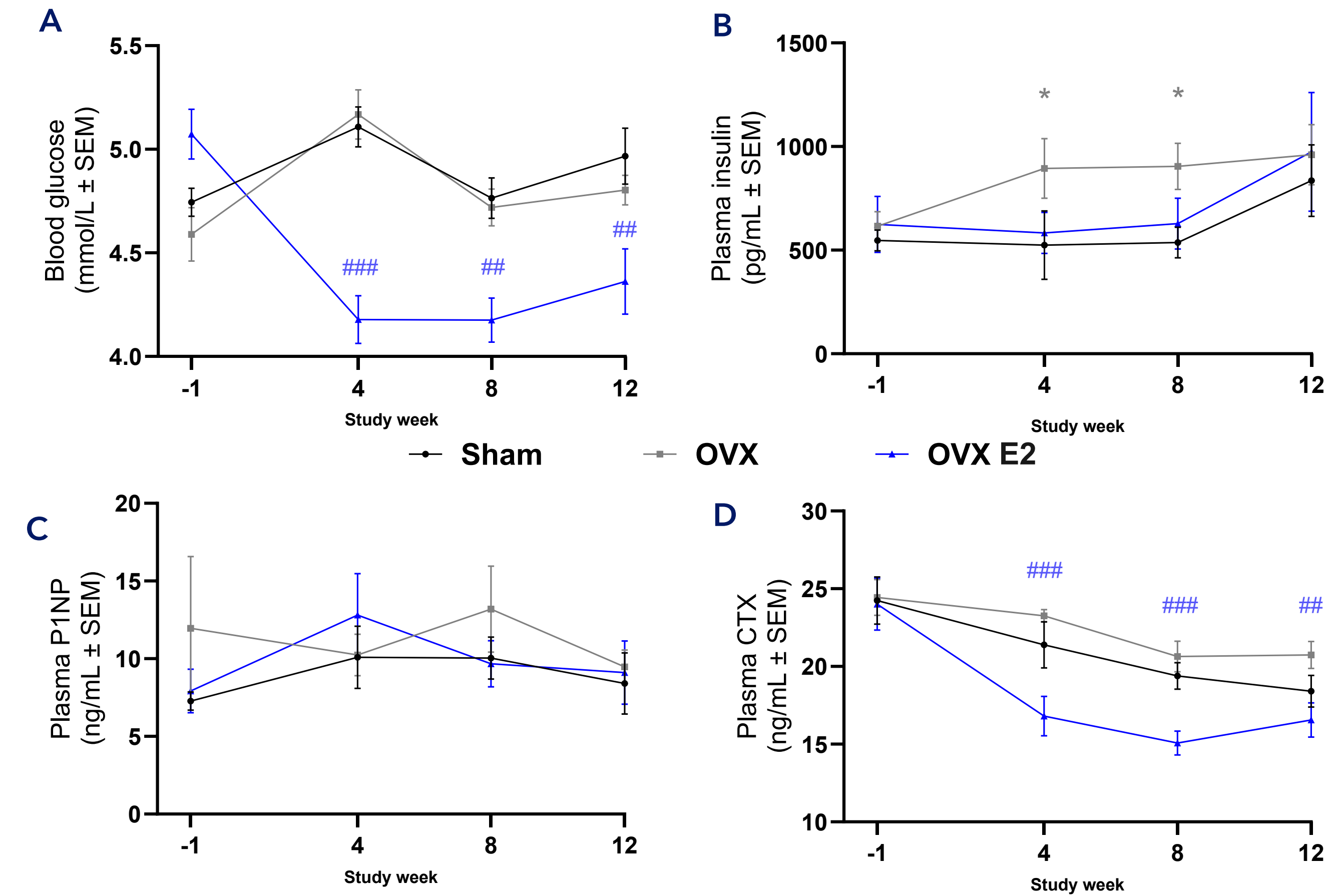


Figure 3. OVX induces hyperinsulinemia while E2 treatment reduces blood glucose and circulating CTX. (A) Fasting blood glucose, (B) fasting plasma insulin, (C) plasma procollagen Type I N-terminal Propeptide (P1NP, bone formation marker), and (D) plasma C-Terminal Telopeptide of Type I Collagen (CTX, bone resorption marker) measured from week -1 to week 12 after OVX surgery. Data shown as mean $\pm$ SEM, n=8-10. * $p<0.05$ compared to Sham. ## $p<0.01$, ### $p<0.001$ compared to OVX. Dunnett's test one-factor linear model.

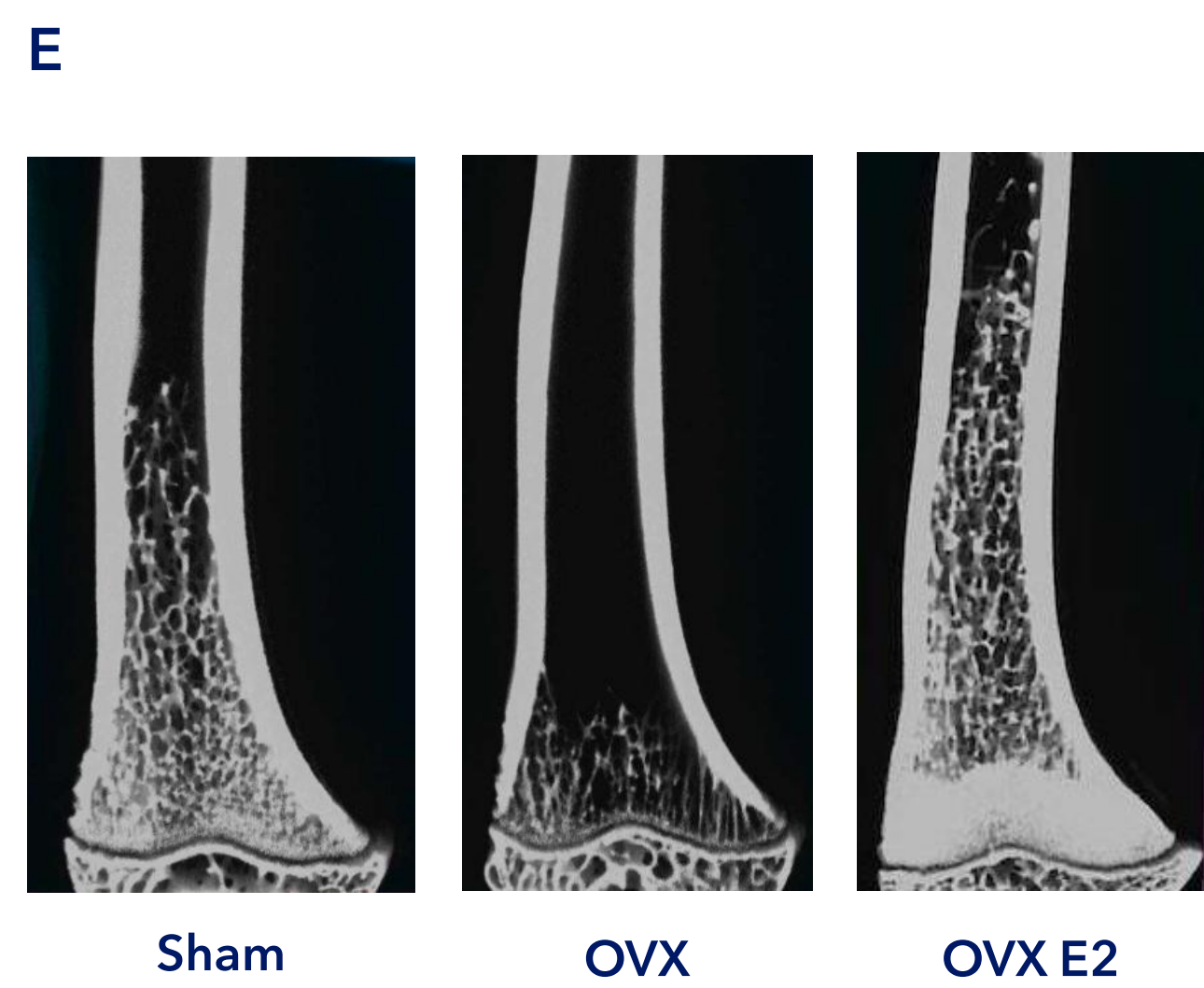
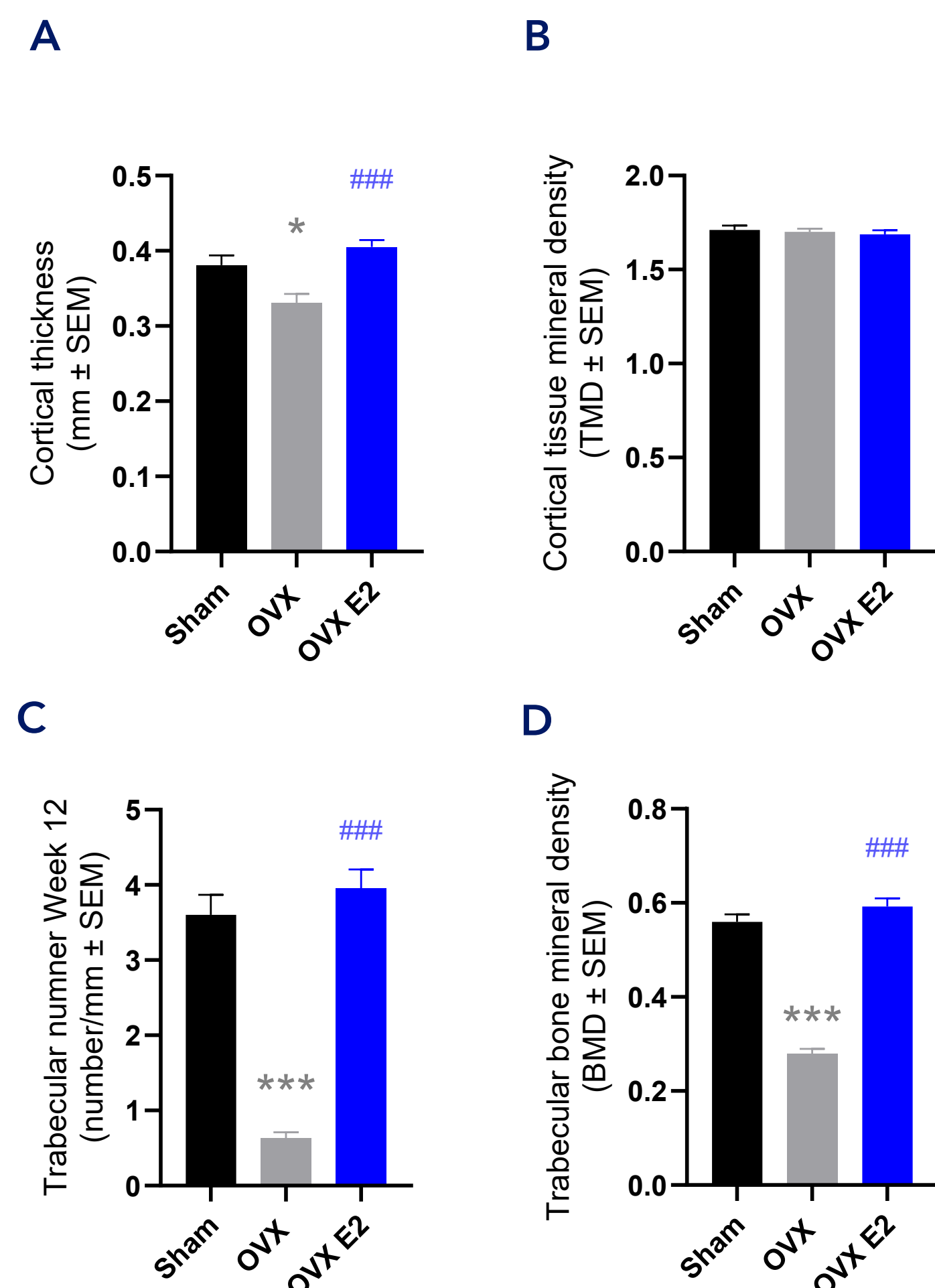


Figure 4. E2 prevented loss of cortical bone, trabecular bone and bone mineral density 12 weeks after OVX surgery. (A) Cortical thickness (mm), (B) cortical tissue mineral density (TMD), (C) number of trabeculae (number/mm), and (D) trabecular bone mineral density (BMD). (E) Representative images from sham, OVX, and OVX E2. Data shown as mean $\pm$ SEM, n=8-10. * $p<0.05$, *** $p<0.001$ compared to Sham. ### $p<0.001$ compared to OVX. Dunnett's test one-factor linear model.

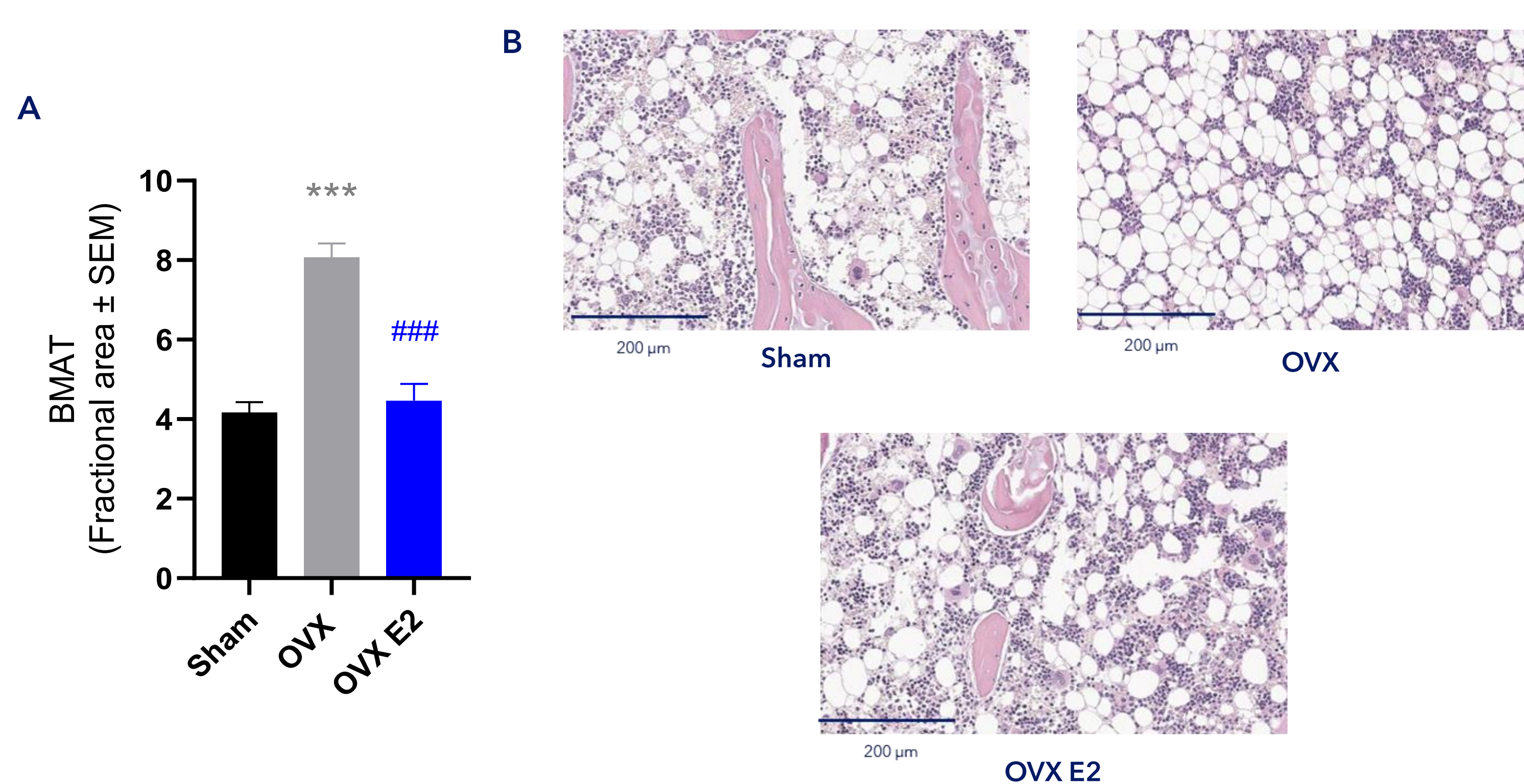


Figure 5. Treatment with E2 prevented build up of excess tibia bone marrow adipose tissue 12 weeks after OVX surgery. (A) Fractional area of adipocytes in bone marrow. **(B)** Representative images of H&E staining of tibia bone marrow from sham, OVX, and OVX E2 groups. Data shown as mean $\pm$ SEM, $n=8-10$. *** $p<0.001$ compared to Sham. ### $p<0.001$ compared to OVX. Dunnett's test one-factor linear model.

The OVX rat model demonstrates

- + Increased body weight, fat and lean mass, food intake, and plasma insulin
- + Reduced tibial cortical thickness, trabecular number, and bone mineral density
- + Increased bone marrow adiposity
- + Robust responses to estradiol (E2) treatment
- + E2 treatment prevented (1) weight and fat mass gain; (2) normalized glycemic control; (3) reduced plasma CTX levels; (4) preserved loss of cortical and trabecular bone; and (5) prevented BMAT expansion.

These findings support the OVX rat model as a translational animal model for testing novel therapies targeting menopause-associated metabolic changes and osteoporosis.