SYSTEMICALLY ADMINISTERED BONE MORPHOGENETIC PROTEIN-6 RESTORES BONE IN AGED OVX RATS BY INCREASING BONE FORMATION AND SUPPRESSING BONE RESORPTION*

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Running title: Systemic administration of BMP-6 in osteoporosis

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Although recombinant human bone morphogenetic proteins (BMPs) are used locally for treating bone defects in men, their systemic effect on bone augmentation has not been explored. We have previously demonstrated that demineralized bone (DB) from ovariectomized (OVX) rats can not induce bone formation when implanted ectopically at the subcutaneous site. Here we showed i*n vitro* that 17β-estradiol (E_2) specifically induced expression of BMP-6 mRNA in MC3T3-E1 preosteoblastic cells and that bone extracts from OVX rats lack BMPs. Next we demonstrated that ^{125}I-BMP-6 administrated systemically accumulated in the skeleton and also restored the osteoinductive capacity of ectopically implanted DB from OVX rats. BMP-6 applied systemically to aged OVX rats significantly increased the bone volume and mechanical characteristics of both the trabecular and cortical bone, the osteoblast surface, serum osteocalcin and osteoprotegerin levels, and decreased the osteoclast surface, serum C-telopeptide and IL-6. E_2 was significantly less effective, and was not synergistic with BMP-6. Animals that discontinued the BMP-6 therapy maintained the bone mineral density gains for another 12 weeks. BMP-6 increased *in vivo* the bone expression of ALK-2, ALK-6, Smad 5, alkaline phosphatase, collagen type I and decreased the expression of BMP-3 and BMP antagonists, chordin and cerberus. These results show, for the first time, that systemically administered BMP-6 restores the bone inductive capacity, microarchitecture and quality of the skeleton in osteoporotic rats.

Bone loss during aging and after menopause in women is known to result from an imbalance between bone formation and resorption leading to altered bone microarchitecture and excess bone fragility. Inferior bone strength and increased bone fracture rate of bone in patients with osteoporosis might be associated with decreased osteoinductive and thus self-regenerative bone capacity eventually due to lower content of growth and differentiation factors including bone morphogenetic proteins in the bone extracellular matrix (1-4).

Demineralized bone matrix (DBM) induces *de novo* bone formation when implanted into the rat muscle (5). On the contrary, DBM from OVX animals implanted into both normal and OVX rats induces only fibrous tissues suggesting that its decreased bone inducing activity is due to abnormal composition of bone from OVX rats and not to the 17β-estradiol (E_2) deficient microenvironment (1). Lack of specific signals needed for ectopic bone induction may, at least in part, explain diminished bone potency to heal fractures in osteoporotic patients (6,7). It has been demonstrated that fetal osteoblastic cell lines treated by E_2, specifically express BMP-6 mRNA, while gene transcripts of other members of the BMP family are unaffected (8). A functional relationship between E_2 and BMP-6 was further suggested by E_2 binding to BMP-6 gene promotor (9) and by increased BMP-6 immunostaining in bone marrow of mice treated with E_2 (10).

Although numerous studies have unequivocally demonstrated that BMPs induce new bone formation locally in animals and men, both ectopically and when implanted between bone ends in patients with delayed non-unions or acute fractures (11,12), so far it has not been shown that a systemically administered

recombinant BMP can effect the bone volume in OVX rats.

In these studies we tested the effect of systemically administered recombinant BMP-6 on the bone formation in aged OVX rats and showed for the first time that BMP-6 is a novel systemic bone anabolic agent and has a potential for treating bone loss in patients with osteoporosis.

Experimental Procedures

Demineralized bone matrix (DBM) - Bone matrix was prepared from 6 months old Sprague-Dawley rats, 3 months following OVX. After sacrifice, diaphyses of femurs and tibiae were removed and then powdered, sieved and demineralized as previously described (5). DBM from OVX rats was implanted subcutaneously (sc) in the pectoral region of normal and OVX rats, which were subsequently injected with 20, 50 and 100 μg/kg/day of BMP-6 intravenously (iv) from days 2 to 6 following implantation. Two weeks following implantation DBM, pellets were removed and embedded in paraffin, cut, stained with toluidin blue and examined for the presence of new cartilage and bone.

MC3T3-E1 preosteoblastic cell line and cell culture experiments - Stock cultures of non-confluent mouse-calvarial preosteoblasts (MC3T3-E1) were grown in alpha minimal essential medium (α-MEM), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. All culture reagents were from Life Technologies (Rockville, MD). MC3T3-E1 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were plated at 3×10^4 cells/cm^2 in 24-multiwell culture dishes (Costar) and cultured until reaching approximately 90% confluence. The growth medium was replaced by DMEM-F12 (without phenol red; Life Technologies) supplemented with ITS+culture supplement (Collaborative Biomedical Products, Bedford, MA) and L-ascorbic acid 2-sulfate (50 μg/ml; Sigma, St Louis, MO). Cells were stimulated with E_2, (Sigma, St. Louis, MO) at 10^{-7}–10^{-11} M for 12 and 72 h. At designated time points total cellular RNA was extracted and analyzed by semi-quantitative PCR.

Animals - Four, 6 or 7 months old Sprague-Dawley rats were subjected to OVX. Animals were anesthetized with an intraperitoneal injection of thiopenthal at doses of 4 mg/kg body weight. Thirty animals per experiment were subjected to sham surgery during which the ovaries were exteriorized but replaced intact. Bilateral ovariectomies were performed in the remaining rats from the dorsal approach and they were left untreated for a period of 6 to 18 months following surgery to await the development of osteopenia. BMP-6 at doses of 1, 10, 25 and 50 μg/kg was injected through the rat tail vein one or three times a week for 8 to 12, or 30 weeks (extended protocol). In total 390 sham and OVX rats were used in three protocols as follows:

a. (1) sham (n=30), (2) OVX (n=20), (3) OVX + BMP-6 (10 μg/kg intravenously (iv) 3xweek) (n=20), (4) OVX + BMP-6 (25 μg/kg iv 3xweek) (n=20), (5) OVX + BMP-6 (50 μg/kg iv 3xweek) (n=20), (6) OVX + E_2 (50 μg/kg intraperitoneally, ip 3xweek) (n=20) and (7) OVX + E_2 (50 μg/kg ip 3xweek) + BMP-6 (50 μg/kg iv 3xweek) (n=20). Therapy started 12 months following OVX and continued for 12 weeks (Figure 2).

b. (1) sham (n=30), (2) OVX (n=20), (3) OVX + BMP-6 (10 μg/kg iv 3xweek) (n=50) for 3 months and then group (3) OVX + BMP-6 was divided into: (4) OVX + BMP-6 (10 μg/kg iv 3xweek) (n=20), (5) OVX + E_2 (50 μg/kg ip 3xweek) (n=20) and (6) OVX + no therapy (n=10) for 4.5 months. Therapy started 6 months following OVX and continued for 7.5 months including first therapy (3 months) and second therapy (3.5 months), (Figure 4).

c. (1) sham (n=30), (2) OVX (n=20), (3) OVX + BMP-6 (1 μg/kg iv 3xweek) (n=20), (4) OVX + BMP-6 (10 μg/kg iv 3xweek) (n=20) and (5) OVX + BMP-6 (10 μg/kg iv 1xweek) (n=20). Therapy started 18 months following OVX and continued for 6 weeks (Figure 5).

All experiments and protocols were approved by the Institutional Animal Care Committee and the Ministry of Science and Technology (project 108295).

Recombinant BMP-6 - Human mature BMP-6 was produced in CHO cells as previously described (13, 14). Proteins were purified by using a Filtron Minisette (PallGelman) cross flow device. pH was adjusted to 5.5 and the material was dialyzed against 0.1 M Tris, 75 mM acetic acid, 0.2 M KH_2PO4, 5 mM EDTA, 6 M urea and then

filtered. Subsequently, ca. 150 mg protein was loaded onto a 5 ml HiTrapTM Heparin Sepharose HP (Amersham Biosciences) column that had been preequilibrated with 0.1 M Tris, 125 mM acetic acid, 5 mM EDTA and 6 M urea containing 0.3 M NaCl. Loading was performed at a flow rate of 4 ml/min. The column was loaded with maximal 30 mg of dialyzed protein. Dimeric BMP-6 species was eluted at a NaCl concentration of 0.7 M. Homogenous fractions were pooled, dialyzed against 10 mM NH_4-acetate, pH 4.0, lyophilized, and stored until used.

Biodistribution and pharmacokinetics of ^{125}I-labeled BMP-6 (^{125}I-BMP-6) - Highly purified BMP-6 (15.7 mg) was radioiodinated with 5 mCi of carrier-free Na ^{125}I using a modification of the lactoperoxidase method as described earlier (15, 16). Shortly, gel filtration on a Sephadex G-25 column was used to separate radioiodinated BMP-6 (^{125}I-BMP-6) from the free iodide. The column was eluted with 20 mM sodium acetate buffer, pH 4.5 containing 0.2 Tween-80 and 0.1% ovalbumin. The specific activity of the ^{125}I-BMP-6 preparation used in this study was 0.273 mCi/mg. Rats (n=50) received a single injection of ^{125}I-BMP-6 at a dose level of 10 µg/kg with the activity of 20 µCi. Injection volume was 500 µl. Animals were sacrificed 30 min, 1, 3, 6 and 24 h following injection. Tissues were removed, weighted and radioactivity was measured in a gamma counter. The relative uptake of ^{125}I-BMP-6 by tissues during time was expressed as nanograms of ^{125}I-BMP-6 per gram wet tissue weight.

Western blot analysis of bone extracts from sham and OVX rats - Femur and tibia from sham and OVX rats were excised, flash-frozen in liquid nitrogen, and crushed into a fine powder with o biopulverizer (Biospec Products, Bartlesville, OK, USA). Two hundred grams of bone powder were extracted at 4°C with 4 M guanidine-HCl and a mixture of protease inhibitors (5 mM benzamide, 0.1 M 6-aminohexanoic acid, 0.5 mM phenylmethylsulfonyl flouride, 5 mM N-ethylmaleimide). The protein extract was then ethanol-precipitated and lyophilized. The pellet was dissolved in 6M urea, 50 mM Tris, pH 7.0, containing 0.15 M NaCl and purified by a heparin-Sepharose column (Amersham Pharmacia Biotech). Bound proteins were eluted with 0.5 M, 1 M and 2 M NaCl in 10 mM sodium phosphate buffer (pH 7) at flow rate of 1 ml/min. The protein eluted by 0.5 M NaCl was collected and dialyzed extensively against distilled water and 30% acetonitrile, 0.1% TFA at 4°C. SDS-PAGE was run on a 10% gel according to the method of Laemmli (18). Samples were heated in boiling water for 3 min with or without dithiothreitol (100 mM) prior to electrophoresis. Following electrophoresis, the gel was blotted onto the nitrocellulose membrane and incubated with a goat polyclonal BMP-6 (Santa Cruz Biotechnology, CA), goat polyclonal BMP-2 (Santa Cruz Biotechnology, CA) and rabbit polyclonal BMP-7 primary antibodies (19). Alkaline phosphatase-conjugated anti-rabbit and anti-goat were used as a secondary antibody. The membranes were developed with the chromogenic substrate (Invitrogen Corporation, Carlsbad, CA).

In vivo and ex vivo bone mineral density (BMD) measurement by DXA - At 6 week intervals the animals were scanned for bone density measurements by dual-energy X-ray absorptiometry (DXA; Hologic QDR-4000, Hologic, Waltham, MA) (20). At the end of the experiment, animals were anesthetized, weighed and killed by cervical dislocation. The hind limbs and lumbar vertebrae were removed and fixed in 70% ethanol. The right femur and tibia, and the lumbar vertebrae (L1-5) were used for determination of the bone mineral content and BMD by DXA (21) equipped with a Regional High Resolution Scan software. The scan field size was 5.08 x 1.902 cm, resolution was 0.0254 x 0.0127 cm, and the speed was 7.25 mm/s. The scan images were analyzed and the bone area, bone mineral content and bone density of whole bones, proximal and distal metaphyses and the shaft of femurs and tibiae were determined.

PQCT - Isolated femurs were scanned by a peripheral quantitative computerized tomography (pQCT) X-ray machine (Stratec XCT Research M; Norland Medical Systems, Fort Atkinson, WI, USA) with software version 5.40. Volumetric content, density, and area of the total bone, trabecular, and cortical regions were determined as previously described (22, 23).

MicroCT - The microcomputerized tomography apparatus (µCT 40) and the analyzing software used in these experiments were obtained from SCANCO Medical AG (Bassersdorf, Switzerland) (24). The distal femur was scanned in 250 slices, each 13 µm thick in the dorsoventral direction

(25). Three-dimensional reconstruction of bone was performed using the triangulation algorithm. The trabecular bone volume (BV, mm^3), trabecular number (Tb. N, 1/mm), the trabecular thickness (Tb. Th, μm), and the trabecular separation (Tb. Sp, μm), were directly measured on 3D images using the method described by Hildebrand et al. (26). The trabecular bone pattern factor (TBPf) and the structure model index (SMI) were computed using software provided with the microCT machine (27,28).

Histology and histomorphometry - Animals were given a subcutaneous injection of the fluorochrome calcein at 10 mg/kg (Sigma Chemical, St. Louis, MO) at 14 and 4 days before death. The femurs were removed at death, prepared for histomorphometric analysis and quantified using a computer-aided image analysis system (Bioquant II, R and M Biometrics, Nashville, TN) as previously described (29-31). Statistical analyses were performed using StatView 4.0 packages (Abacus Concepts, Berkeley, CA). Organs were taken for histologic analyzes, embedded in paraffin, cut in 10-μm-thick sections, stained with hemalaun-eosin and toluidin blue to reveal potential therapeutic adverse effects.

Biomechanical testing - Using a materials testing system (Model 810, MTS Systems Corp., Minneapolis, MN), two types of mechanical testing were performed on the femur. Three-point bending test of the femoral shaft was used to determine the mechanical properties of the midshaft femur. The midshaft of the femur was subjected to three-point bending to failure at a displacement rate of 0.1 mm/sec, as described by Turner and Burr (32), using a 2.5 kN load cell (MTS Model 661, 14A-03). The maximal load and stiffness were calculated from the load-displacement curve. Indentation test of the distal femoral metaphysis was used to determine the mechanical properties of cancellous bone in the marrow cavity of the distal femoral metaphysis, as described previously (33,34).

Biochemical serum and urine parameters - Serum bone formation and resorption markers were measured by commercially available kits. Serum concentration of osteocalcin was measured by ELISA using rat osteocalcin EIA kits (Biomedical Technologies Inc., Stoughton, MA). Serum concentration of C-telopeptide (CTx) was measured by ELISA using RatLaps ELISA kits (Nordic Bioscience Diagnostics, Herlev, Denmark). Osteoprotegerin (OPG) level in serum was measured by ELISA using Biomedica rat OPG ELISA kit (Biomedica, Wien, Austria). Serum concentration of IL-6 was measured by ELISA using IL-6 Quantikine ELISA kit (R&D, Minneapolis, MN) following procedures provided by the manufacturer. The minimum detectable concentration of osteocalcin, CTx, OPG and IL-6 were 1ng/ml, 2 ng/ml, 0.73 pmol/l and 10 pg/ml, respectively.

RNA isolation and PCR analysis - RNA was extracted with TRIzol (Gibco BRL, Grand Island, NY, USA) and cDNA was synthesized from 4 μg of total RNA with Superscript II Rnase H-Reverse Transcriptase as indicated by the manufacturer (Gibco BRL). Samples were amplified with AmpliTaq DNA polymerase (Perkin Elmer Roche, New Jersey, USA). Sequences of primers are shown in Table 1. Reactions were performed in a GeneAmp 4800 thermal cycler (Perkin Elmer Cetus, Emeryville, USA) for 32-40 cycles. Results were visualized by gel electrophoresis in 1% agarose (Seakem GTG, Bioproducts, Rockland, USA) in TAE buffer (TRIS HCl, acetic acid, EDTA, pH 8.0) and stained with ethidium bromide (Sigma, St. Louis, MO).

Real time PCR - Two μg of purified total RNA from each rat bone were reverse transcribed with 200 U of MMLV reverse transcriptase RNase (BD Biosciences) using random hexamer primers (final concentration 20 pmol per sample) according to the manufacture's protocol. Gene expression of interest was measured using a commercial kit (LightCycler FastStart DNA Master SYBR Green, Roche Diagnostics, Mannheim, Germany) in the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described (35). Sequences of primers are shown in Table 1. Expression of four housekeeping genes was analyzed and geNorm software was used to identify the most suitable reference gene. GAPDH transcripts were used as a normaliser. Results are represented as fold change of the comparative expression level.

Statistical analysis - Distributions of quantitative data were tested with Kolmogorov-Smirnov test. Densitometric and histologic data measurement within the same time point were analyzed with One way ANOVA with one-sided Dunett-t post

hoc test against OVX and sham animals. Serum values of IL-6 were analyzed with paired t-test. Changes in gene expression were evaluated using One way ANOVA with one-sided Dunett-t post hoc test against OVX and sham animals.

RESULTS

Bone matrix from OVX rats is deficient in BMPs - Bone extracts from normal and OVX rats were analyzed by Western blot analysis following reduction with DTT (Fig 1A-C). Unlike bone extracts from normal rats, OVX animals had undetectable amounts of BMP-2, -6 and -7. (Fig 1A-C), suggesting that E_2 deficiency results with decreased amounts of BMPs in bone. This suggests that the lack of capacity of DBM from OVX rats to form new bone at an ectopic site in both normal and OVX rats may be at least in part due to BMP deficient DBM (1).

Intravenously administered BMP-6 induces ectopic bone formation by binding to sc implanted extracted DBM from OVX rats – Systemic effects of BMP-6 were tested by iv injections following sc implantation of DBM from OVX rats into both normal and OVX rats. New bone formation was found 2 weeks later in an amount similar to the one induced by implanting DBM from normal rats (Figs 1F and G). These results suggest that, following systemic administration of BMP-6, DBM from OVX rats, lacking new bone induction capacity, regained the osteogenic potential in both normal and OVX rats. A similar result was obtained when 8M urea extracted and thus non-osteogenic DBM from normal rats was implanted sc into male rats injected iv from day 2-5 with 100 μg/kg BMP-6 (data not shown). These results prompted us to test whether systemic administration of BMP-6 is effective in restoring bone volume in aged rats, 12-18 months following OVX.

Pharmacokinetics and biodistribution of systemically administered ^{125}I-BMP-6 - Prior to testing the efficacy of BMP-6 on the skeleton we tested its pharmacokinetic properties and biodistribution. High serum levels of ^{125}I -BMP-6 were achieved immediately after iv injection followed by a steady decline (Fig 1D). Approximately 60 ng BMP-6/ml was present in the circulation 1 min after injecting a dose of 10 μg BMP-6/kg. Biodistribution of ^{125}I-BMP-6 in the rat kidney increased during the first 4 h following injection, and decreased in the following 20 h, while the level of ^{125}I-BMP-6 in the liver increased immediately and decreased slowly throughout the experiment. The uptake of ^{125}I-BMP-6 in femur was 0.15% of the total applied dose (approximately 4.5 ng ^{125}I-BMP-6), reached the maximal value at 4 h and then declined slowly to 0.05% of the applied dose at 24 h following injection (Fig 1C). The maximal uptake of ^{125}I-BMP-6 in the tibia was 0.12% of the applied dose (approximately 3.6 ng ^{125}I-BMP-6) at 8 h following injection and declined slowly to 0.025% of the applied dose at 24 h following injection (Fig 1E). Autoradiographic analysis demonstrated that ^{125}I-BMP-6 remained intact in the serum, assayed at 4 h after administration (data not shown).

E_2 specifically induces expression of BMP-6 mRNA in MC3T3-E1 preosteoblastic cells in vitro - Prior to use of E_2 as a positive control in these studies we sought to confirm its specific effect on BMP-6 mRNA expression in osteoblastic cells *in vitro*. MC3T3-E1 pre-osteoblasts express BMP-2 and -4 during differentiation and *in vitro* bone formation (3). Addition of E_2 to cell cultures suppressed the expression of BMP-2 and BMP-4, but specifically induced the expression of BMP-6 transcripts in a dose-dependant manner (Fig 1H). The number of bone nodules on day 27 of culture were not affected (data not shown). E_2 induced BMP-6 was thus capable to replace the function of BMP-2 and BMP-4 in *in vitro* osteogenesis. Moreover, upon E_2 treatment, the expression profile of specific BMP receptors by MC3T3-E1 cells changed. Besides ALK-3 and ALK-6, MC3T3-E1 cells treated with E_2 expressed ALK-2 receptor, a specific BMP type I receptor reported to be preferentially used by BMP-6 in osteoblasts (data not shown).

BMP-6 restores bone volume in OVX rats - Twelve months following OVX rats were treated for three months with BMP-6 and the BMD was fully restored (Fig 2). Within 6 weeks of treatment rats regained the lost BMD and at 12 weeks following therapy they had higher hind limb (femur and tibia) BMD as compared to OVX, E_2 treated and sham animals (9%, 6% and 3% increase, respectively) (Fig 2). There were no significant differences between BMP-6 doses of 10, 25 and 50 μg/kg. Within the 12 week period hind limb BMD of sham treated rats decreased by

2%. BMD of the lumbar spine showed an increase of 9% at 12 weeks following BMP-6 treatment (data not shown).

Ex vivo BMD values of tibiae, femurs and vertebrae increased for 8-11% in rats treated with BMP-6, independently of a dose used. E_2 was about four times less effective than BMP-6 in restoring BMD (data not shown).

pQCT analyses of femurs showed that the total BMD was 13.8% higher and the total femoral bone mineral content (BMC) was about 18% higher in BMP-6 treated rats as compared to OVX rats. Cortical BMC was higher for 24%, cortical bone mineral area for 21% and the cortical thickness for 28% as compared to OVX rats (Table 2). Cortical bone parameters in femurs were preferentially enhanced over the trabecular bone by BMP-6 treatment (Table 2). Analyses of the tibiae showed a similar pattern, confirming a greater effect of BMP-6 on the cortical bone of the appendicular skeleton (data not shown).

MicroCT analyses of distal femurs showed that the bone volume (BV/TV) of rats treated with 10 μg BMP-6 was 78% increased compared to OVX control animals and 47% increased compared to rats receiving E_2 (Fig 3 and 4A). The trabecular number was higher 38% in BMP-6 and 19% in E_2 treated rats (Fig 4B). The trabecular thickness was increased 35% in BMP-6 treated as compared to OVX rats, and was 28% and 11% higher than in E_2 treated and sham animals respectively (Fig 4C). The trabecular separation was decreased 36% in BMP-6 treated rats (Fig 4D). Surprisingly, BMP-6 therapy increased the connectivity density for 33% (Fig 4E) and decreased the structure model index (SMI) for 42% (Fig 4F). Connectivity parameters were 38% better then in E_2 treated animals. However, none of the treatments were fully effective at restoring cancellous bone architecture and volume of sham rats. MicroCT analysis of the 5th lumbar vertebra showed 25% increased BV/TV, 8% increased trabecular number, 16% increased trabecular thickness and 29% decreased trabecular separation in BMP-6 treated rats (Table 3). This suggested that the trabecular bone of the lumbar vertebrae responded better, reaching almost the values of sham animals, to the systemic administration of BMP-6 as compared to long bones.

The three-point bending test was used to determine the mechanical properties of the midshaft femur. Maximal load and stiffness were 32% and 23% higher in BMP-6 treated animals as compared to OVX rats. Bones from BMP-6 treated animals absorbed 33% more energy than in sham animals (Table 4). Toughness was increased by 22% in BMP-6 treated rats as compared to sham rats (Table 4). The indentation test was used to determine the mechanical characteristics of trabeculae of the distal femoral metaphyses. Direct parameters: maximal load, stiffness and energy absorbed were increased about three to four fold in BMP-6 treated rats as compared to OVX control animals (Table 4). The ultimate strength showed the same trend. BMP-6 treatment improved the trabecular bone parameters as compared to OVX animals, but did not restore the mechanical properties to those exhibited by sham rats.

Histomorphometric analyses showed that the bone volume and the trabecular thickness of distal femurs were higher for 78% and 35%, respectively, in BMP-6 treated animals (Fig 3). Dynamic bone parameters showed increased mineral apposition rate for 48% and bone formation rate/bone volume for 18% in BMP-6 treated rats, while E_2 decreased the bone formation rate by 39% (Fig 5). Most importantly, BMP-6 both increased the osteoblast surface for 32% and decreased the osteoclast surface for 29%. E_2 decreased the osteoclast surface for 43% but did not have an effect on the osteoblast surface (Fig 5). Improved bone formation rates and mechanical properties of BMP-6 treated bones resulted from both increased number of osteoblasts and from potentially increased extracellular matrix production over E_2 therapy (Table 4, Fig 5 and 6).

The body weights in this experiment didn't change until day 375. Later, the body weight of BMP-6 treated rats was 22% higher then in those receiving E_2 and 11% higher then in sham animals, but was not different from OVX rats (data not shown).

Autopsy did not reveal any treatment-related gross pathology. Organ weights were normal, only rats treated with E_2 or E_2 and BMP-6 had increased uterine weights (data not shown). Histopathological findings of heart, lung, liver, spleen, kidney, uterus, pancreas, oesophagus, stomach, intestine, lymph nodes, bladder, brain, eyes, aorta, trachea, skeletal muscle, bone marrow (sternum) and femur were normal. Beyond

calcification of the tail at the site of BMP-6 injection no adverse effects were observed.

BMP-6 and E_2 do not act synergistically on increasing bone volume in OVX rats - Rats treated with E_2 at twelve weeks following therapy had similar femur BMD values as OVX control animals (Table 2). When both BMP-6 and E_2 were given to OVX rats BMD values at 12 weeks increased for 14%. However, BMD in rats treated with E_2 and BMP-6 was not different from rats treated with BMP-6 alone (Table 2). E_2 had no effect on cortical and trabecular thickness, while a combination of E_2 and BMP-6 increased the cortical thickness by 22% without an effect on the trabecular thickness (Table 2, Fig 4). These results suggest that E_2 alone did not have an effect on the bone in aged OVX rats unless combined with BMP-6.

Effects of BMP-6 on femoral gene expression - At three months following BMP-6 therapy the expression of ALK-2 and ALK-6, BMP receptors, as well as Smad 5, a downstream molecule in the BMP signaling pathway was increased (Fig 6). E_2 reduced the expression of ALK-2 and -6 and had no effect on Smad 5 (Fig 6). BMP-2 and BMP-3 expression was increased following OVX and was suppressed by both BMP-6 and E_2 therapy (Fig 6). On the other hand, OVX reduced the expression of BMP-6 in femurs, while E_2 increased the BMP-6 expression (Fig 6). BMP-4 expression was unchanged with both BMP-6 and E_2 treatment. Furthermore, BMP-6 increased the expression of alkaline phosphatase and collagen type I, reducing the expression of osteopontin (Fig 6). Expression of BMP antagonists, chordin and cerberus was decreased in rat femurs treated with BMP-6. The expression of twisted gastrulation was decreased following OVX and increased to normal levels following BMP-6 therapy (Fig 6). On the contrary, the expression of noggin was increased following OVX, BMP-6 therapy further increased the noggin expression, while E_2 had no effect (Fig 6).

Serum biochemical parameters- BMP-6 treatment at a dose of 10 μg/kg/3xweek increased the serum osteocalcin level, a bone formation marker, as compared to OVX animals, while E_2 did not have an effect (Fig 7A). Serum C-telopeptide values were lower in rats treated with BMP-6 indicating that BMP-6 suppressed the activity of osteoclasts (Fig 7B). Furthermore, BMP-6 increased the OPG serum levels as compared to OVX animals (Fig 7C), which may correspond to lower osteoclast numbers in BMP-6 treated rats (Fig 5). BMP-6 may, thus, increase the bone formation and suppress the bone resorption, which may in part explain its anabolic bone effect.

BMP-6 suppresses expression of IL-6 in spleen and IL-6 serum levels - BMP-6 decreased IL-6 serum levels 72 h following a single iv injection (Fig 8A). Three BMP-6 injections reduced the expression of IL-6 in the spleen to undetectable values as compared to OVX rats (Fig 8B) suggesting an additional mechanism by which BMP-6 might mediate the osteoclast production.

Maintenance of bone in OVX rats pre-treated by BMP-6 - To further explore whether the newly formed bone following BMP-6 therapy was maintained, BMP-6 was given to aged OVX rats for 12 weeks and was then discontinued. Animals were divided into three groups: untreated, treated with E_2 and those continuing the BMP-6 therapy. BMD was then monitored at 6, 12, 18, 24 and 30 weeks (Fig 9). BMP-6 therapy throughout the treatment period of 7.5 months increased the hind limb BMD values as compared to both OVX and sham rats which lost about 4% of BMD from the beginning of treatment (Fig 9). Hind limb BMD values in animals treated with BMP-6 were about 8% higher at 18 weeks than at 12 weeks, and about 14% higher as compared to OVX rats, while at 24 and 30 weeks BMD did not change any more (Fig 9). In rats treated with E_2 following the initial BMP-6 therapy BMD gains were maintained. Animals without any therapy maintained the BMD values until 24th week and then showed a decline to values still 10% higher than in OVX control rats (Fig 9). We suggest that animals that discontinued the BMP-6 therapy maintained the BMD gains for another 12 weeks while treatment with E_2 maintained the BMD until the termination of the study.

Ex vivo BMD values of the excised femur, tibia and lumbar vertebrae were similar to the *in vivo* results (data not shown).

Less frequently administered low BMP-6 dose is the most efficacious in increasing bone volume in OVX rats - In search for the most effective BMP-6 dose, aged OVX rats 18 months following OVX were administered BMP-6 less frequently at lower doses. Six weeks following the beginning of therapy BMP-6 treated animals showed higher BMD of hind limbs as compared to both OVX and

sham animals (Fig 10). Within 6 weeks 1 μg/kg/3xweek of BMP-6 increased the hind limb BMD for 11%. Doses of 10 μg/kg 1x/week and 3x/week increased the BMD for 9% and 8%, respectively (Fig 10). Surprisingly, aged rats gained the bone volume in an amount similar to young animals treated with BMP-6 (data not shown), although at the termination of the study the age difference was 2 years (Fig 10). This suggests a complex biology at the local bone tissue site perhaps requiring a recovery time needed to maximize the receptiveness of osteoblasts to the intermittent exposure of BMP-6.

DISCUSSION

In these studies we show that systemically administered BMP-6 in aged OVX rats has a pronounced anabolic effect significantly improving the bone quality. Both *in vivo* and *ex vivo* bone densitometric measurements revealed that BMP-6 restores trabecular bone of the axial skeleton and the cortical bone, while only a partial restoration of the trabecular bone occurs in long bones. The maximal load, stiffness and energy absorbed were increased about three to four fold in BMP-6 treated rats. Therapy with BMP-6 reduces serum C-telopeptide and IL-6, while increasing serum osteocalcin and osteoprotegerin levels, suggesting uncoupling of bone formation from bone resorption.

Not only hormones, like E_2 and PTH, have different effects on the peripheral and axial skeleton. For example, mutations of the cartilage-derived morphogenetic protein-1 (CDMP-1; GDF-5; BMP-14) cause Hunter-Thompson and Grebe chonrodysplasia (36,37). Both diseases are characterized by severe shortening of the limbs and multiple defects of limb joints and the normal axial skeleton (36,37). Grebe chondrodysplasia is caused by a point mutation in a Cdmp-1 gene, while in Hunter-Thompson chondrodysplasia there is an insertion in the mature domain leading to non-functional CDMP-1 protein.

So far the parathyroid hormone (PTH) is the only approved anabolic bone agent, which increases the trabecular bone volume, but may decrease the cortical bone mass (38) and produce cortical resorptive tunnels (39). PTH in aged OVX rats restores the bone volume by thickening the existing trabeculi without increasing the trabecular connectivity (40,41), although not influencing the trabecular mechanical strength (42). PTH also improves rigidity and stiffness of bone, but makes them more brittle, with less elasticity and less deformation before fracture (43). BMP-6 improves the trabecular microarchitecture in aged OVX rats by increasing the trabecular thickness, trabecular number and in particular the trabecular connectivity. BMP-6 also increases elasticity and energy absorbed. After discontinuation of the BMP-6 therapy animals maintained the BMD value for another 12 weeks. In comparative studies utilizing aged rats with similar post OVX time period, PTH withdrawal results in a loss of acquired BMD within 5 weeks (44). Unlike PTH, which circulates in serum, BMP-6 circulates when bone regenerates, like in patients with multiple bone fractures (Grgurevic et al, in preparation). This excludes other BMPs as potential circulating candidates in the regeneration of the skeleton including osteoporosis (Grgurevic et al, in preparation).

BMP-6 exerts its effects on bone via promoting bone formation and reducing bone resorption thereby providing a distinct means to accumulate the bone tissue mass. No known therapeutic agent achieves both effects by *in vivo* systemic administration. The currently available resorption inhibitors (E_2 and related compounds, bisphosphonates and calcitonin) have little effect on bone formation over a prolonged periods of time (45). It has been recently shown that leptin has a direct positive effect on the osteoblastic differentiation of stromal cells, and may also modulate bone remodeling by inhibiting the expression of receptor activator of nuclear factor κ B ligand (RANKL), the major downstream cytokine controlling the osteoclastogenesis in human stromal cells (46). However, the systemic effect of exogenously administered leptin on BMD has never been demonstrated. BMP-6 administration increases the bone formation, and in parallel increases OPG serum levels, uncoupling the osteoblast from osteoclast activity, leading to bone gain at both trabecular and cortical bone compartments.

IL-6 as a single molecule plays an important role in the pathophysiology of postmenopausal osteoporosis (47). We therefore studied its availability in BMP-6 treated rats and found that BMP-6 decreases the level of IL-6 in

serum and more importantly its expression in the spleen, as one of important organs for IL-6 synthesis. Recently, we and others have also shown that BMP-6 significantly reduces IL-6 production from marrow stroma and gastrointestinal system in rats (48,49). E_2 can also inhibit IL-6 expression via an E_2 receptor mediated transcriptional activity (49), which is supported by the fact that E_2 depletion results in IL-6 upregulation (51).

We and others (10) have suggested that E_2 induced osteogenesis is associated with increased levels of BMP-6 mRNA in mouse femurs, reflecting the emergence of clusters of BMP-6 positive stromal cells adjacent to active bone formation surface. We show that addition of E_2 to BMP-6 therapy has no a synergistic effect on BMD and the bone volume which may, at least in part, suggest a BMP-6 requirement for E_2 bone activity. On the other hand, E_2 maintained the gained BMD after discontinuation of BMP-6 prolonged therapy. It was suggested that E_2 specifically upregulates BMP-6 mRNA in osteoblastic cell lines (8). BMP-6 and E_2 crosstalk might be Wnt-mediated since BMPs are essential for Wnt-induced osteoblast differentiation (52), and Wnt signaling is also critical for the effects of E_2 on bone (53). Another example involves the transient upregulation of Cbfa-1 in response to BMP (54) and its' affection by E_2-OPG pathway (54). It has been also shown that E_2 prevents bone loss through a TGFβ-dependent mechanism, and that TGFβ signaling in T cells preserves bone homeostasis by blunting the T cell activation (56,57). However, a systemic effect of TGFβ on bone in animal models of osteopenia has not yet been studied.

Apart from BMPs, several other factors important for osteogenesis have been investigated in animal models of osteopenia. Basic FGF improves trabecular connectivity in aged OVX rats by increasing osteoid accumulation on trabecular surfaces and in between perforated trabecular rods (41). Like BMP-6, bFGF improves trabecular connectivity (58,59) but, as a general mitogen with serious adverse side-effects, its systemic administration is not practical (42,58). Human growth hormone (hGH) and insuline like growth factor I (IGF-I) in a rat model of osteopenia increase bone size and mineral content but decrease the BMD (59). Their effects on bone are currently investigated in clinical trials (59).

Numerous studies have demonstrated that BMPs are involved in local bone formation via stimulating proliferation and chemotaxis of bone progenitor cells, angiogenesis during development and bone repair, and endochondral osteogenesis by stimulating osteogenic compartment of bone stromal cells (2,3,60-63). It was never demonstrated that BMPs systemically targets bone marrow stem cells. Based on findings that BMP-6 successfully promotes restoration of bone in young and aged osteopenic rats, we suggest that it targets bone marrow stem cells which do not loose a self renewal and differentiation characteristics by ageing. However, it has been demonstrated that the amount of stem cells decreases by advanced age in both rats and men (64).

Apart from bone formation at the site of injection no side effects were observed by long standing systemic administration of BMP-6. Of particular interest is the fact that systemically administered BMP-6 influences bone formation at endosteal, periosteal, and trabecular bone compartment.

Among genes analyzed BMP-3 is of particular interest. Its upregulation following OVX and suppression by BMP-6 might be important since it has been suggested that BMP-3 is a negative regulator of *in vitro* and *in vivo* osteogenesis (65). BMP-3 inhibited alkaline phosphatase production induced by BMP-2 *in vitro* and BMP-3 knock-out mice had about twice the trabecular bone volume as wild-type controls (65). We have previously demonstrated that changes in BMP-3 and ALK-2 expression are reduced by mechanical loading which enables the induction of cartilage in a bone chamber (66). Treatment by BMP-6 might have inhibited BMP-3 expression and activity by formation of inactive BMP-3 heterodimers, but also inhibiting the effect of the BMP-3 homodimer.

Collectively, these results show, for the first time, that systemically administered BMP-6 restores bone volume and quality in osteoporotic rats making it a feasible candidate for treating osteoporosis in patients with pronounced bone loss.

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FOOTNOTES

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Conference presentations of these results received young investigator award at the 31st ECTS Symposium in Geneva (June 2005) and at the 27th ASBMR Annual Meeting in Nashville (September 2005).

FIGURE LEGENDS

Figure 1. A-C, Western blot analyses of bone extracts from normal rats show BMP-2, BMP-6 and BMP-7 at 35 kDa before and at 17 kDa after reduction with DTT. OVX animals had undetectable amounts of BMP-2, BMP-6 and BMP-7. Fractions following purification with the heparin sepharose chromatography were precipitated with SAS and subjected to SDS-PAGE and immunoblotting. Lanes 1 and 2 BMP-2, BMP-6 or BMP-7 protein standard (0.5 μg) -/+ DTT; lane 3 molecular mass marker; lanes 4 and 5 sham bone extract fraction of 0.5M NaCl -/+ DTT; lanes 6 and 7 OVX bone extract fraction of 0.5M NaCl -/+ DTT. Arrows indicate the mature BMP dimer and monomer of BMP of 35 (►) and 17 kDa (➔). Bands were visualized with specific BMP-2,-6,-7 antibodies. M: molecular mass marker. D, pharmacokinetic properties of iv injected ^{125}I-BMP-6 and E, biodistribution of ^{125}I-BMP-6 in femur and tibia; F, histology of OVX DBM implanted into a normal animal; G, histology of DBM from OVX rats implanted into normal rat and treated with BMP-6 showing new bone formation (toluidin blue staining, 25x magnification); H, RT-PCR analysis of BMP-6, -4 and -2 expression in MC3T3-E1 cells treated with E_2. Samples were normalized to GAPDH expression.

Figure 2. *In vivo* hind limb BMD in aged rats treated with BMP-6 (50 μg/kg) and E_2 (50 μg/kg). Within 6 weeks of treatment rats regained the lost BMD and at 12 weeks following therapy they had higher hind limb BMD as compared to OVX, E_2 treated and sham animals. O, significantly different from OVX and E, significantly different from animals treated with E_2 (by ANOVA Dunnett test, $P<0.05$); mo, months, TH, therapy.

Figure 3. μCT longitudinal and horizontal as well as histological analyses of distal femurs in aged rats treated with E_2 (50 μg/kg), BMP-6 (10 μg/kg) and E_2 (50 μg/kg) + BMP-6 (10 μg/kg) for 3 months

following 12 months of OVX. BMP-6 alone and in combination with E_2 increased the trabecular bone volume and connectivity. The standardized region of interest for μCT analyses began 4 mm above the intercondylar fossa and included 50 slices.

Figure 4. μCT analyses of the distal femur in aged OVX rats treated with E_2 (50 μg/kg), BMP-6 (10 μg/kg) and E_2 (50 μg/kg) + BMP-6 (10 μg/kg) for 3 months following 12 months of OVX. BMP-6 increased trabecular bone volume (BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th) and connectivity density (ConnD). BMP-6 decreased trabecular separation (Tb. Sp) and structural model index (0-plate to 3-rode) (SMI) as compared to control group (OVX). O, significantly different from OVX, E, significantly different from animals treated with E_2, and EB, significantly different from BMP-6 +E_2 treated animals (by ANOVA Dunnett test, $P<0.05$).

Figure 5. Dynamic histomorphometry of the distal femur in aged OVX rats treated with E_2 (50 μg/kg) and BMP-6 (10 μg/kg). Animals were treated for 3 months following 12 months of OVX (Figure 2). BMP-6 increased mineral apposition rate (MAR), bone formation rate/bone volume (BFR/BV) and osteoblast surface as a percentage of bone surface (Ob.S/BS) and decreased osteoclast surface as a percentage of bone surface (Oc.S/BS) compared to OVX group of rats. O, significantly different from OVX, and S, significantly different from sham gorup of animals (by ANOVA Dunnett test, $P<0.05$).

Figure 6. PCR analyses of rat femurs treated for three months with BMP-6 and E_2. BMP-6 decreased the expression of BMP-2, BMP-3, osteopontin (OPN), chordin and cerebrus; increased the expression of BMP-6, ALK-2, ALK-6 Smad 5, alkaline phosphatase (AP), collagen I (COL I), twisted gastrulation (Tsg) and noggin; and did not change the expression of BMP-4. E_2 (50 μg/kg) completely suppressed the expression of ALK-2 and -6 and had no effect on Smad 5 in aged OVX rats. OVX reduced endogenous expression of BMP-6, while E_2 increased BMP-6 expression. The fold change (FC) of gene expression obtained by real time PCR is shown below the gel images. Molecular marker (Mm) is shown in the first line of the gel and molecular weight of PCR products is indicated on the right side of the image (base pairs, bp). O, significantly different from OVX control rats ($p<0.05$, ANOVA, Dunnett test).

Figure 7. Serum values of osteocalcin (Oc), C-telopeptides (Ctx) and osteoprotegerin (OPG). Three months of BMP-6 (10 μg/kg) therapy increased the levels of Oc (*A*) and OPG (*B*) and decreased the levels of Ctx (*C*) in serum of aged rats 12 months following OVX; O, significantly different from OVX control rats ($p<0.05$, ANOVA, Dunnett test).

Figure 8. A, Decreased serum IL-6 levels 72 h following a single injection of BMP-6 (10 μg/kg); B, Semiquantitative RT-PCR analyses showed reduced IL-6 and β-actin expression in the spleen of rats treated for 7 days with BMP-6 (10 μg/kg/3xweek) as compared to OVX rats.

Figure 9. *In vivo* hind limb BMD in aged rats initially treated with BMP-6 (10 μg/kg) for 3 months and then subdivided into groups that continued BMP-6 (10 μg/kg) or E_2 (50 μg/kg) treatment for the following 4.5 months and the third group that discontinued the initial BMP-6 therapy. Rats that discontinued the BMP-6 therapy maintained the BMD gains until the 24^{th} week, while rats continuing the BMP-6 therapy increased BMD values for another 8%. Rats treated with E_2 after 12 weeks of initial therapy with BMP-6, maintained the gained BMD until the end of experiment at week 30. O, significantly different from OVX, and S, significantly different from sham gorup of animals (by ANOVA Dunnett test, $P<0.05$).

Figure 10. *In vivo* hind limb BMD of 26.5 months old rats treated with low doses of BMP-6 for 1.5 months, 18 months following OVX. Six weeks following the beginning of therapy BMP-6 treated animals showed higher BMD values of hind limbs as compared to both OVX and sham animals. BMP-6 at a dose of 1 μg/kg three times a week was most effective in restoring BMD. O, significantly different from OVX, gorup of animals (by ANOVA Dunnett test, $P<0.05$).

Table 1. Sequences of primers used for gene expression analysis.

Target gene	Semi-quantitative PCR	Real time PCR
IL-6	F CAAGAGACTTCCAGCCAGTTGC	
	R TTGCCGAGTAGACCTCATAGTGAC	
BMP-2	F GCAGAGCTCCAGATTTTTCG	
	R TTAAGACGCTTCCGCTGTTT	F TGAACACAGCTGGTCTCAGG
mouse	F GTTCCCTACAGGGAGAACACC	R TTAAGACGCTTCCGCTGTTT
	R GCCTGCGGTACAGATCTAGC	
BMP-3	F CACTCAGCTCTTACGGAAGGCC	F TGCTGTGGCTCTATGACAGG
	R CTGTTTCTTTTTGCTCCGGCTC	R TGGTGTTCACCAATTCTCCA
BMP-4	F TTCCTGGTAACCGAATGCT	
	R GGGGCTTCATAACCTCATAA	F TCTGGTCTCCGTCCCTAATG
mouse	F TTCCTGGTAACCGAATGCT	R AAACTTGCTGGAAAGGCTCA
	R GGGGCTTCATAACCTCATAA	
BMP-6	F AGGATGGGGTGTCAGAGGGAGA	
	R GTTGTGCTGCGGTGTCACCA	F TTCTTCAAGGTGAGCGAGGT
mouse	F CAGGAGCATCAGCACAGAGA	R TAGTTGGCAGCGTAGCCTTT
	R ATGTGTGCGTTGAGTGGGA	
ALK-2	F CCACCAACGTCGGAGATAGCA	F ACCACCAACGTCGGAGAT
	R TGCAGCACTGTCCGTTCTTCTT	R CCCCTCCACACTTCTCCATA
ALK-6	F CAGGTATAAAAGACAAGAAGCCAG	F CTCTGGGAGATTGCAAGGAG
	R TCTCTCATGTCCTCATAAGAAGGG	R TCATAAGCTTCCCCATTTGC
Smad 5	F TGATGAGGAAGAGAAATGGG	F GAATGCCACGTTTCCTGATT
	R GGAGGATAGGGGCTGTTAGG	R AGGGGTATCAGCTGGGAGTT
AP	F CGACACGGACAAGAAGCCCT	F CCTTGAAAAATGCCCTGAAA
	R AAGGTTGGCTCCAATGCAGG	R CTTGGAGAGAGCCACAAAGG
Osteopontin	F GTGAACTCGGATGAATCTGACG	F ATGGCTTTCATTGGAGTTGC
	R CTTGTCCTCATGGCTGTGAAAC	R GAGGAGAAGGCGCATTACAG
Collagen type I	F TATTGCTGGTGCTCCTGGCTTC	F TGCTGCCTTTTCTGTTCCTT
	R TCACCACGGGCTCCTCGTTT	R AAGGTGCTGGGTAGGGAAGT
Chordin	F GAACATCAAACCTCAGTGCCCC	F CCAGGGACAGCTACTTCGAG
	R TGTGAACCGAACCCTGCTGG	R AGAGGACGCTTTGAAGGACA
Cerberus	F TGGCATCGCTTCATGTTCAGA	F ACTGCTCACCCACCAAATTC
	R GTTCCGTCTTCACCATGCACTG	R AGCTGGGAGTCCAGAGATGA
Tsg	F TCCCTGATGTTCCTGATGTGCC	F TGTGAGCAAGTGCCTCATTC
	R CCGAGGGTTGCACATACCGA	R GGGAGGTGTGTCGCTGTAAT
noggin	F GAGCAAGAAGCTGAGGAGGA	F CCTGGCTTTCTGGTTCATGT
	R GTGGGGATCCATCAAGTGTC	R GCCGGGTAACTTTTGACGTA
GAPDH	F ACCACAGTCCATGCCATCAC	F AGACAGCCGCATCTTCTTGT
	R TCCACCACCCTGTTGCTGTA	R CTTGCCGTGGGTAGAGTCAT

All primers are for rat gene sequences; mouse primers were used for analyses of MC3T3 cell line in *in vitro* experiments.

Table 2. pQCT of the distal femur in aged OVX rats treated with BMP-6 and E_2

Groups	*Treatment*	*Total BMD (mg/mm^2)*	*Total BMC (mg)*	*Total area (mm^2)*	*Cortical BMD (mg/mm^2)*	*Cortical th. (mm)*
1.	Sham	743.7 ± 43.7 ^{O}	14.3 ± 3.2 ^{O}	19.2 ± 5.6	995.9 ± 87.2 ^{O}	0.80 ± 0.07 $^{O, E}$
2.	OVX	587.7 ± 38.3	11.3 ± 2.8	19.2 ± 3.2	945.5 ± 91.3	0.58 ± 0.04
3.	OVX + E_2	625.5 ± 41.4	12.6 ± 2.9	19.5 ± 4.2	974.3 ± 102.3	0.67 ± 0.04
4.	OVX + BMP-6	667.2 ± 50.8 ^{O}	13.3 ± 3.6 ^{O}	20.0 ± 4.9	1007.7 ± 106.5 ^{O}	0.74 ± 0.06 ^{O}
5.	OVX + BMP-6+E_2	670.8 ± 55.3 ^{O}	13.4 ± 3.8 ^{O}	19.9 ± 5.3	1014.4 ± 100.9 ^{O}	0.71 ± 0.05 ^{O}

Animals were treated for 3 months following 12 months of OVX (Figure 2); n=10 in all groups
Parameters analyzed include: Total BMD – bone mineral density, Total BMC – bone mineral content, Total area, Cortical BMD – bone mineral density and Cortical th. – thickness
Data are mean ± SEM, ^{O} significantly different from group 2 (by ANOVA Dunnett test, $P<0.05$)

Table 3. Bone volume and trabecular microarchitecture of 5th lumbar vertebrae in aged OVX rats treated with BMP-6 and E_2 by μCT

Groups	*Treatment*	*BV/TV (%)*	*Tr. Nm (1/mm)*	*Tr. Th (mm)*	*Tr. Sp (mm)*
1.	Sham	0.79 ± 0.07 ^{O}	2.56 ± 0.30	0.35 ± 0.08 ^{O}	0.18 ± 0.03 $^{O, E}$
2.	OVX	0.65 ± 0.08	2.51 ± 0.15	0.25 ± 0.04	0.21 ± 0.04
3.	OVX +E_2	0.75 ± 0.06	2.71 ± 0.08	0.28 ± 0.03	0.18 ± 0.02
4.	OVX + BMP-6	0.82 ± 0.07 ^{O}	2.81 ± 0.38 ^{O}	0.30 ± 0.04 ^{O}	0.14 ± 0.02 $^{O, E}$

Animals were treated for 3 months following 12 months of OVX (Figure 2); n=10 for all groups
Parameters analyzed include: BV/TV – trabecular bone volume, Tb. N – trabecular number, Tb. Th – trabecular thickness, Tb. Sp – trabecular separation
Data are mean ± SEM, ^{O} significantly different from group 2 and ^{E} significantly different from group 3 (by ANOVA Dunnett test, $P<0.05$)

Table 4. Biomechanical parameters of the femur from BMP-6 treated aged, OVX rats

Three Point Bending Test				*Indentation Test*			
Parameters	*Sham*	*OVX*	*OVX + BMP-6*	*Parameters*	*Sham*	*OVX*	*OVX + BMP-6*
F_u (N)	213.5 ± 9.2 ^{O}	168.9 ± 5.4	223.2 ± 5.9 ^{O}	F_u (N)	37.3 ± 7.9 ^{O}	3.3 ± 0.8	13.5 ± 3.9 $^{O, S}$
S (N/mm)	806.8 ± 46.5 ^{O}	646.7 ± 52.1	793.0 ± 38.1 ^{O}	S (N/mm)	177.0 ± 66.1 ^{O}	20.0 ± 4.1	83.3 ± 26.1 ^{O}
W (mJ)	49.2 ± 4.2 ^{O}	39.6 ± 3.5	65.6 ± 4.8 $^{O, S}$	W (mJ)	6.8 ± 1.2 ^{O}	0.6 ± 0.2	2.4 ± 0.8 $^{O, S}$
T (MJ/m^3)	4.3 ± 0.3 ^{O}	3.9 ± 0.4	5.3 ± 0.3 $^{O, S}$	σ (N/mm^2)	19.8 ± 4.2 ^{O}	1.8 ± 0.4	7.1 ± 2.1 $^{O, S}$

Animals were treated for 3 months following 12 months of OVX (Figure 2); n=10 in all groups; data are mean ± SEM
Femur diaphysis was subjected to three point bending to failure. Parameters analyzed include: maximum load (F_u), stiffness (S), energy absorbed (W) and toughness (T). Significant differences are indicated with respect to OVX control (O) and Sham (S) control rats ($P<0.05$ by ANOVA Dunnett test)
Indentation test provided data on mechanical properties of trabecular bone. Parameters analyzed include: maximum load (F_u), stiffness (S), energy absorbed (W) and ultimate strength (σ). Significant differences are indicated with respect to OVX control (O) and Sham (S) control rats ($P<0.05$ by ANOVA Dunnett test)

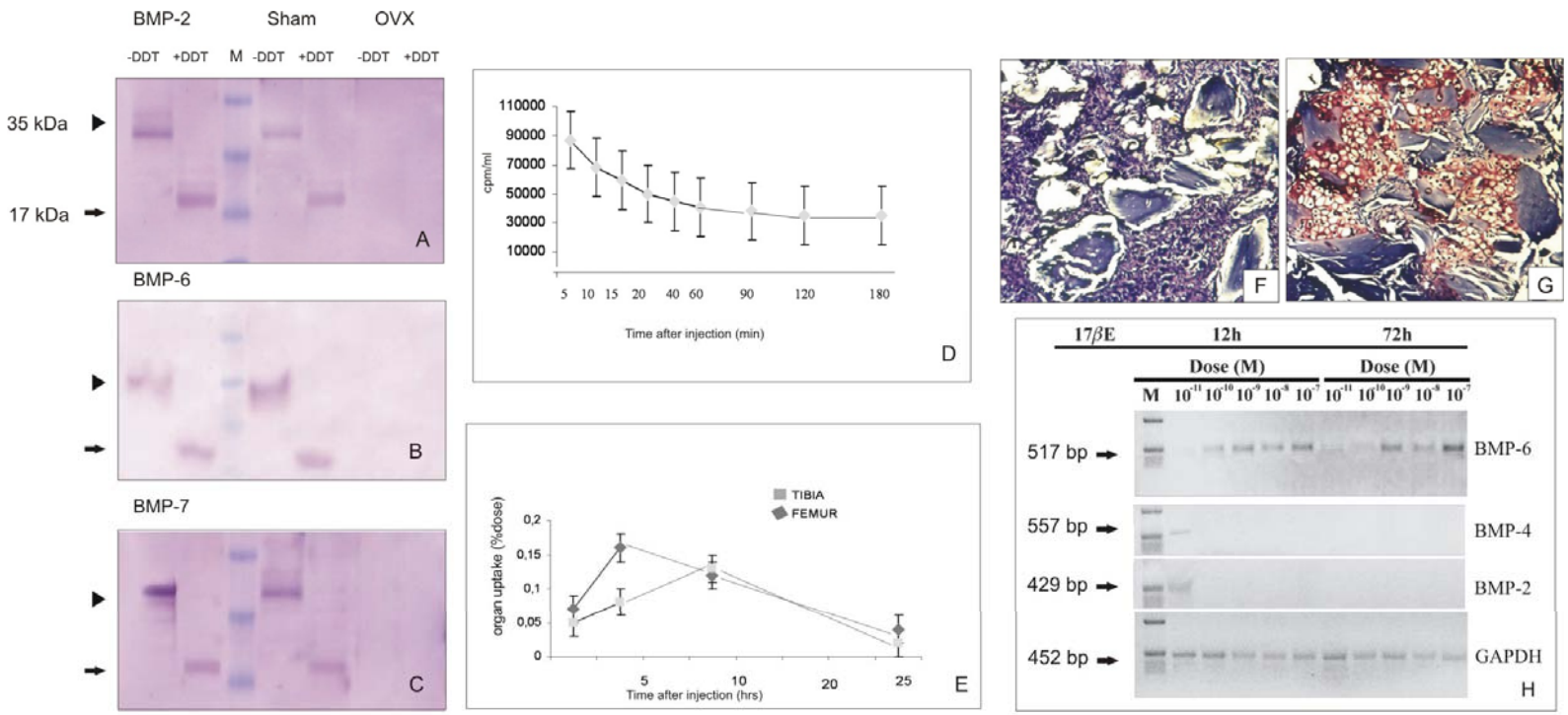
BMP-2
Sham
OVX
-DDT +DDT M -DDT +DDT -DDT +DDT
35 kDa
17 kDa
A
BMP-6
B
BMP-7
C
cpm/ml
110000
90000
70000
50000
30000
10000
5 10 15 20 40 60 90 120 180
Time after injection (min)
D
TIBIA
FEMUR
organ uptake (%dose)
0,2
0,15
0,1
0,05
0
5 10 20 25
Time after injection (hrs)
E
F
G
17βE
12h
72h
Dose (M)
Dose (M)
M 10-11 10-10 10-9 10-8 10-7 10-11 10-10 10-9 10-8 10-7
517 bp
BMP-6
557 bp
BMP-4
429 bp
BMP-2
452 bp
GAPDH
H

Figure 1.

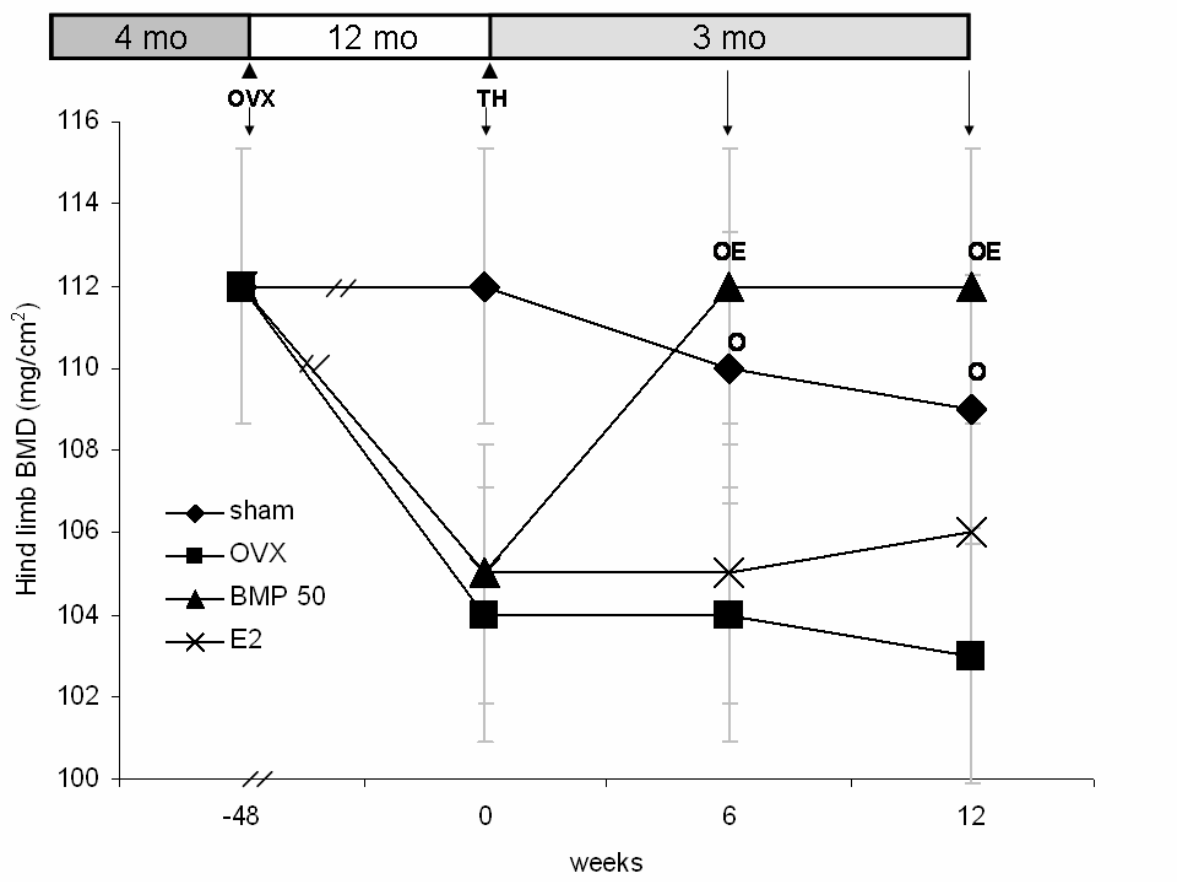
4 mo
12 mo
3 mo
OVX
TH
OE
OE
O
O
sham
OVX
BMP 50
E2
Hind limb BMD (mg/cm2)
116
114
112
110
108
106
104
102
100
-48
0
6
12
weeks

Figure 2.

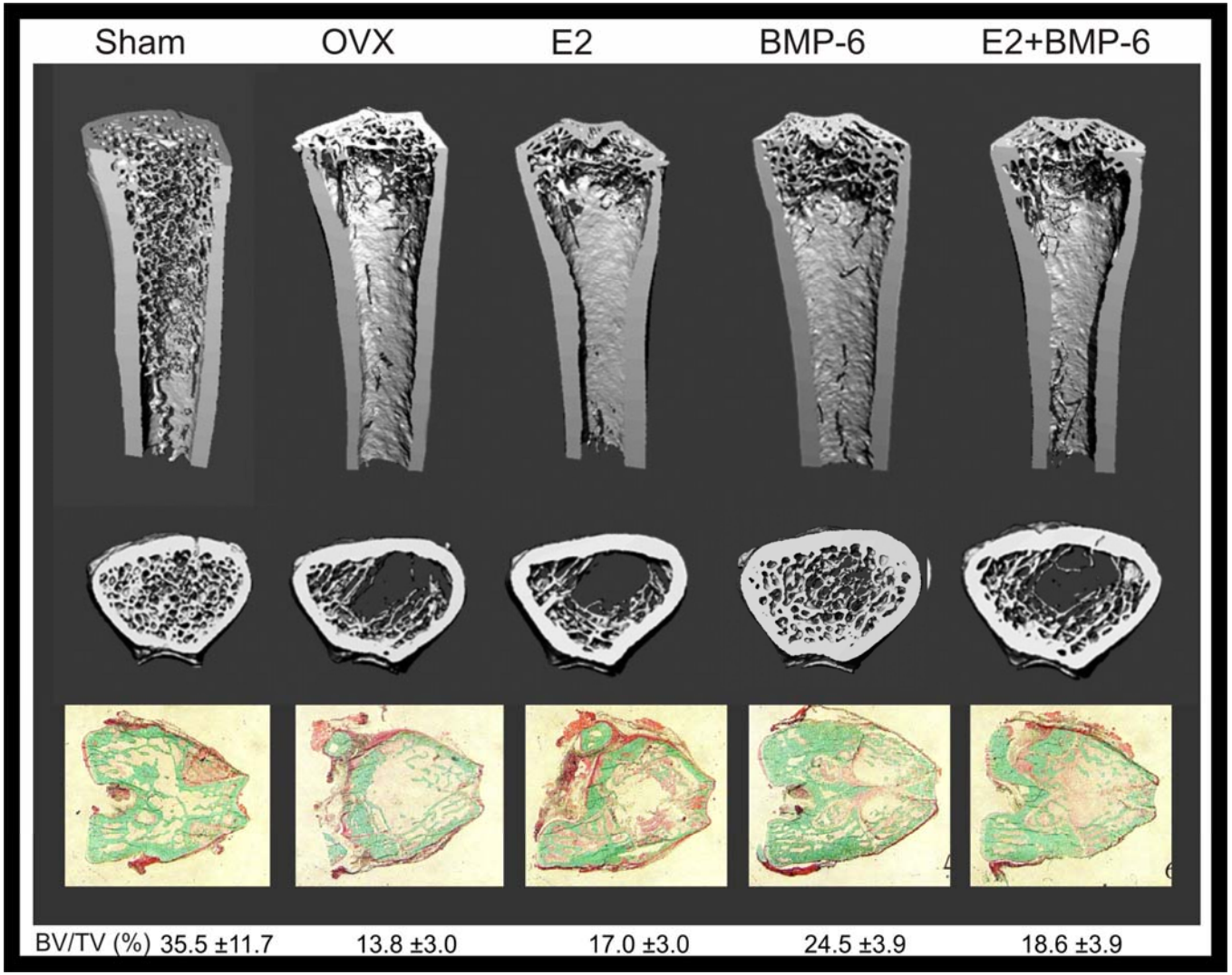

Figure 3.

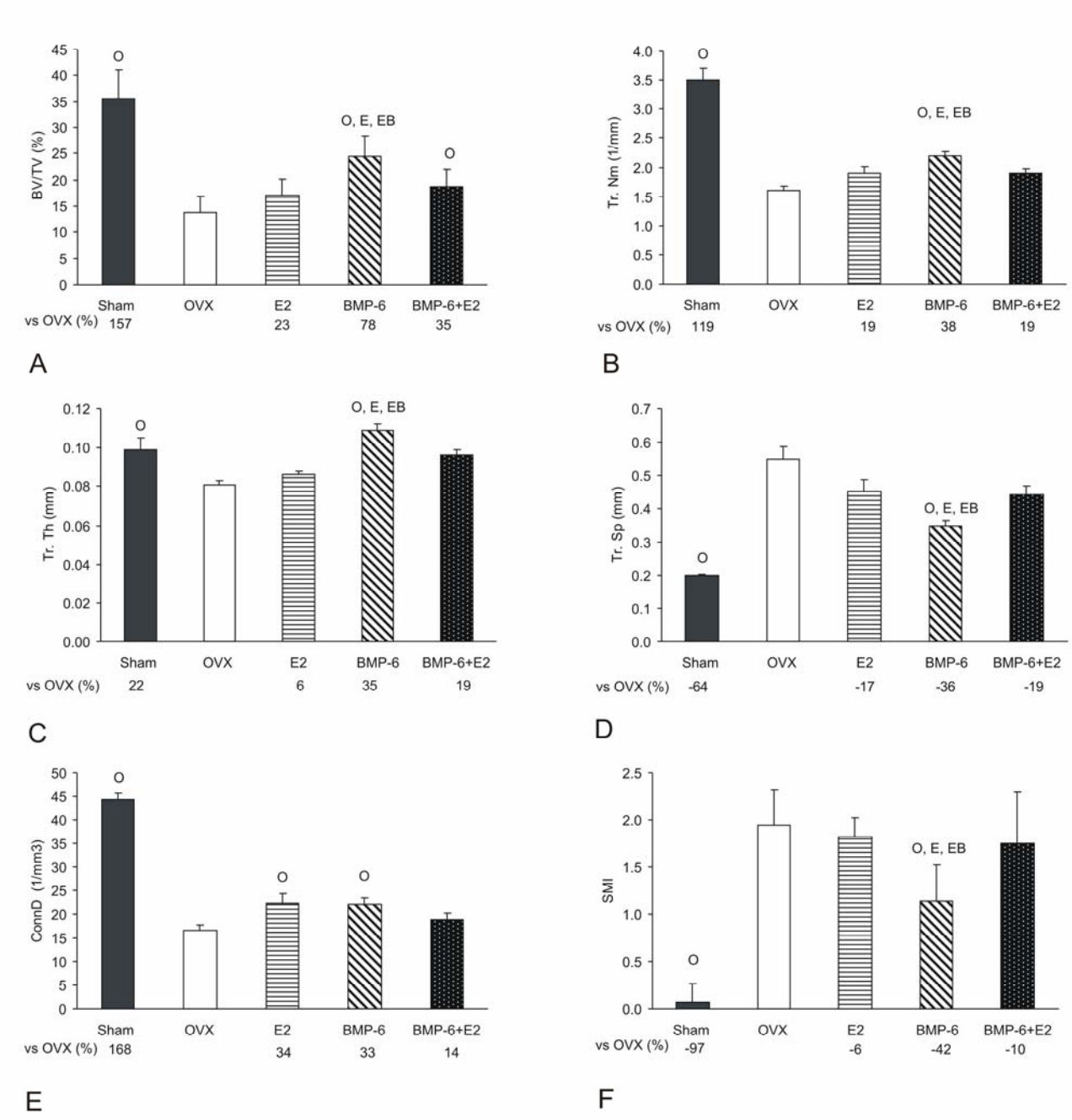
BV/TV (%)
O
O, E, EB
O
Sham
OVX
E2
BMP-6
BMP-6+E2
vs OVX (%) 157
23
78
35
A
Tr. Nm (1/mm)
O
O, E, EB
Sham
OVX
E2
BMP-6
BMP-6+E2
vs OVX (%) 119
19
38
19
B
Tr. Th (mm)
O
O, E, EB
Sham
OVX
E2
BMP-6
BMP-6+E2
vs OVX (%) 22
6
35
19
C
Tr. Sp (mm)
O
O, E, EB
Sham
OVX
E2
BMP-6
BMP-6+E2
vs OVX (%) -64
-17
-36
-19
D
ConnD (1/mm3)
O
O
O
Sham
OVX
E2
BMP-6
BMP-6+E2
vs OVX (%) 168
34
33
14
E
SMI
O
O, E, EB
Sham
OVX
E2
BMP-6
BMP-6+E2
vs OVX (%) -97
-6
-42
-10
F

Figure 4.

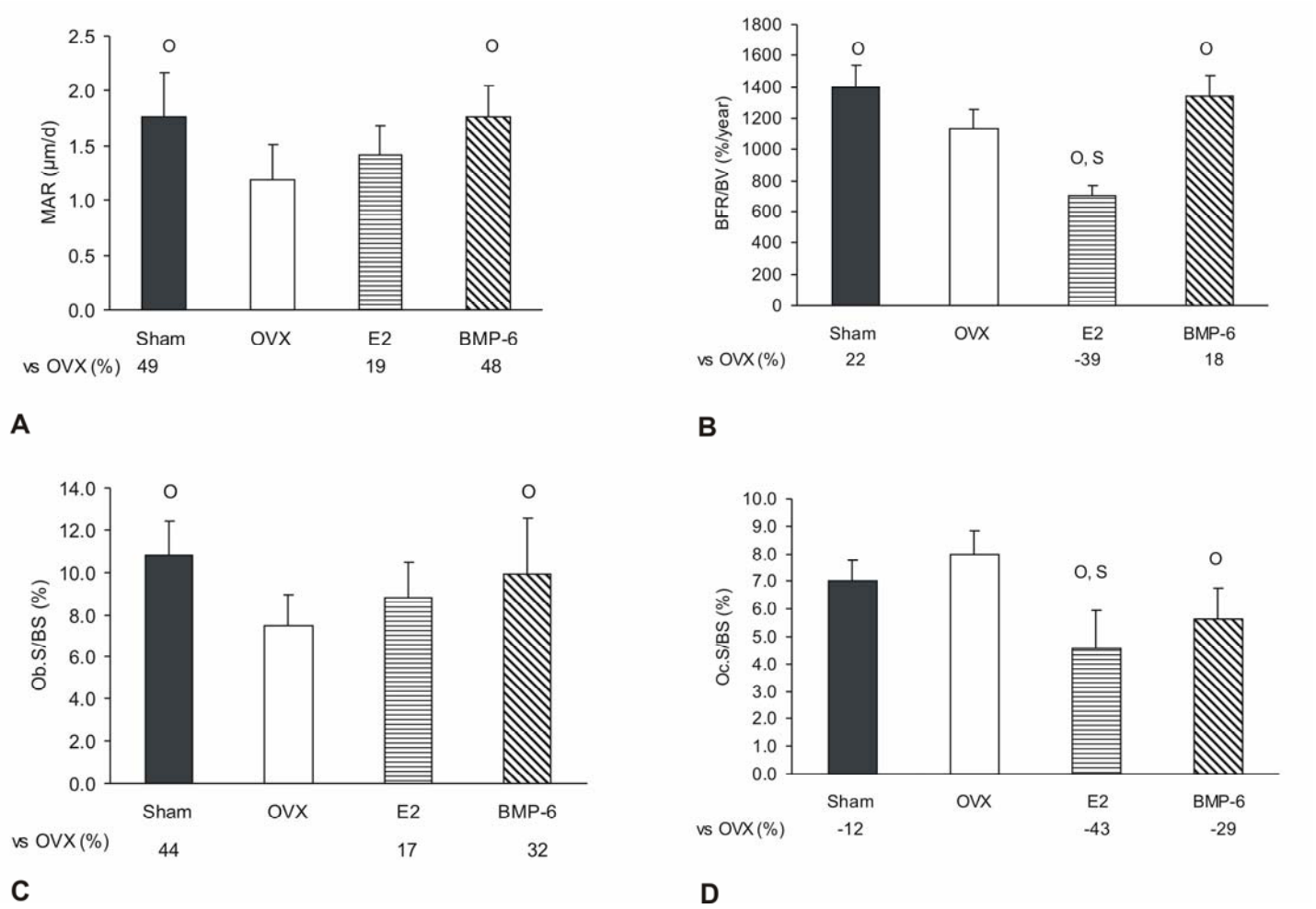
MAR (µm/d)
2.5
2.0
1.5
1.0
0.5
0.0
O
O
Sham
OVX
E2
BMP-6
vs OVX (%)
49
19
48
A
BFR/BV (%/year)
1800
1600
1400
1200
1000
800
600
400
200
0
O
O
O, S
Sham
OVX
E2
BMP-6
vs OVX (%)
22
-39
18
B
Ob.S/BS (%)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
O
O
Sham
OVX
E2
BMP-6
vs OVX (%)
44
17
32
C
Oc.S/BS (%)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
O, S
O
Sham
OVX
E2
BMP-6
vs OVX (%)
-12
-43
-29
D

Figure 5.

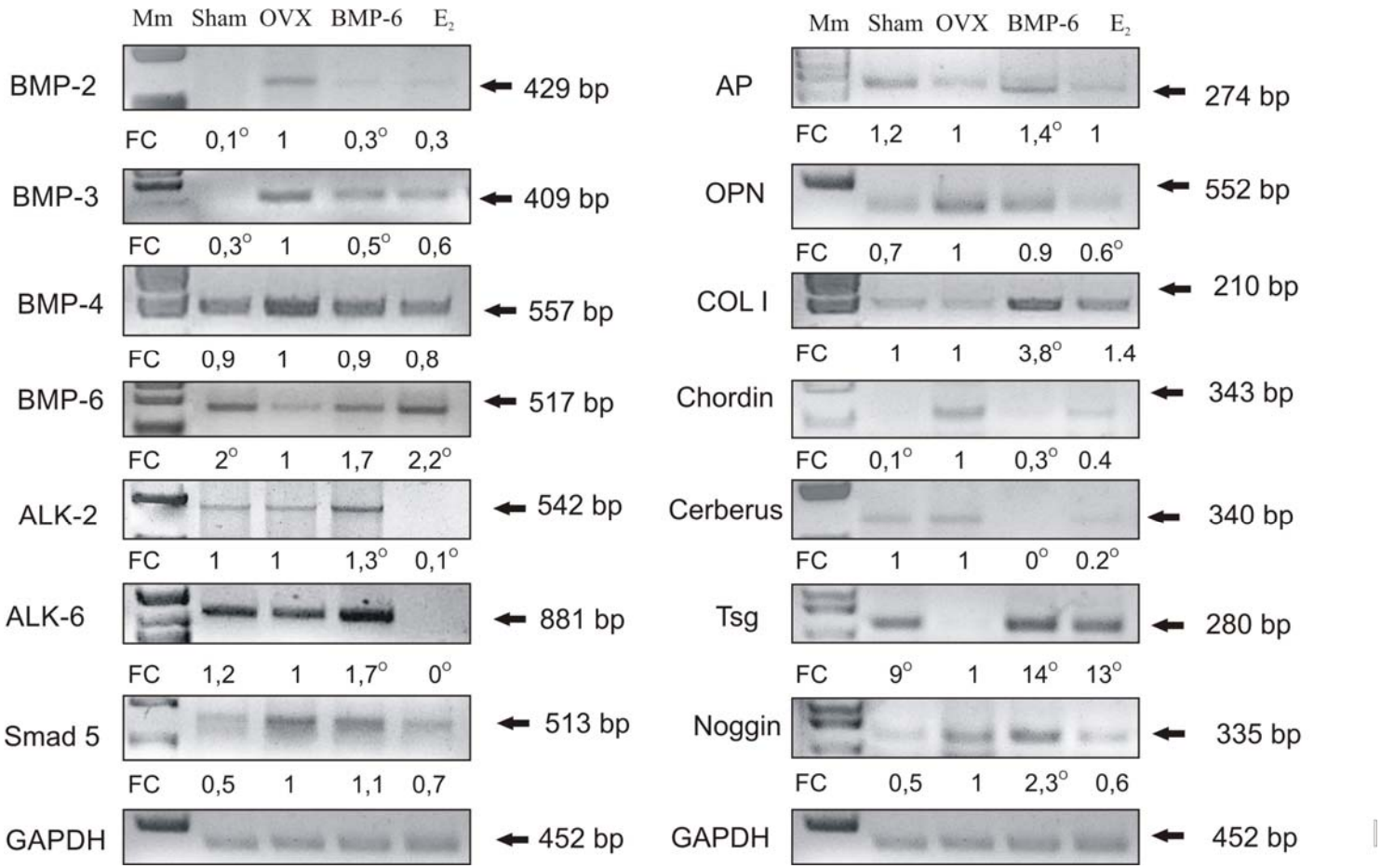
Mm Sham OVX BMP-6 E2
BMP-2
429 bp
FC 0,1° 1 0,3° 0,3
BMP-3
409 bp
FC 0,3° 1 0,5° 0,6
BMP-4
557 bp
FC 0,9 1 0,9 0,8
BMP-6
517 bp
FC 2° 1 1,7 2,2°
ALK-2
542 bp
FC 1 1 1,3° 0,1°
ALK-6
881 bp
FC 1,2 1 1,7° 0°
Smad 5
513 bp
FC 0,5 1 1,1 0,7
GAPDH
452 bp
Mm Sham OVX BMP-6 E2
AP
274 bp
FC 1,2 1 1,4° 1
OPN
552 bp
FC 0,7 1 0.9 0.6°
COL I
210 bp
FC 1 1 3,8° 1.4
Chordin
343 bp
FC 0,1° 1 0,3° 0.4
Cerberus
340 bp
FC 1 1 0° 0.2°
Tsg
280 bp
FC 9° 1 14° 13°
Noggin
335 bp
FC 0,5 1 2,3° 0,6
GAPDH
452 bp

Figure 6.

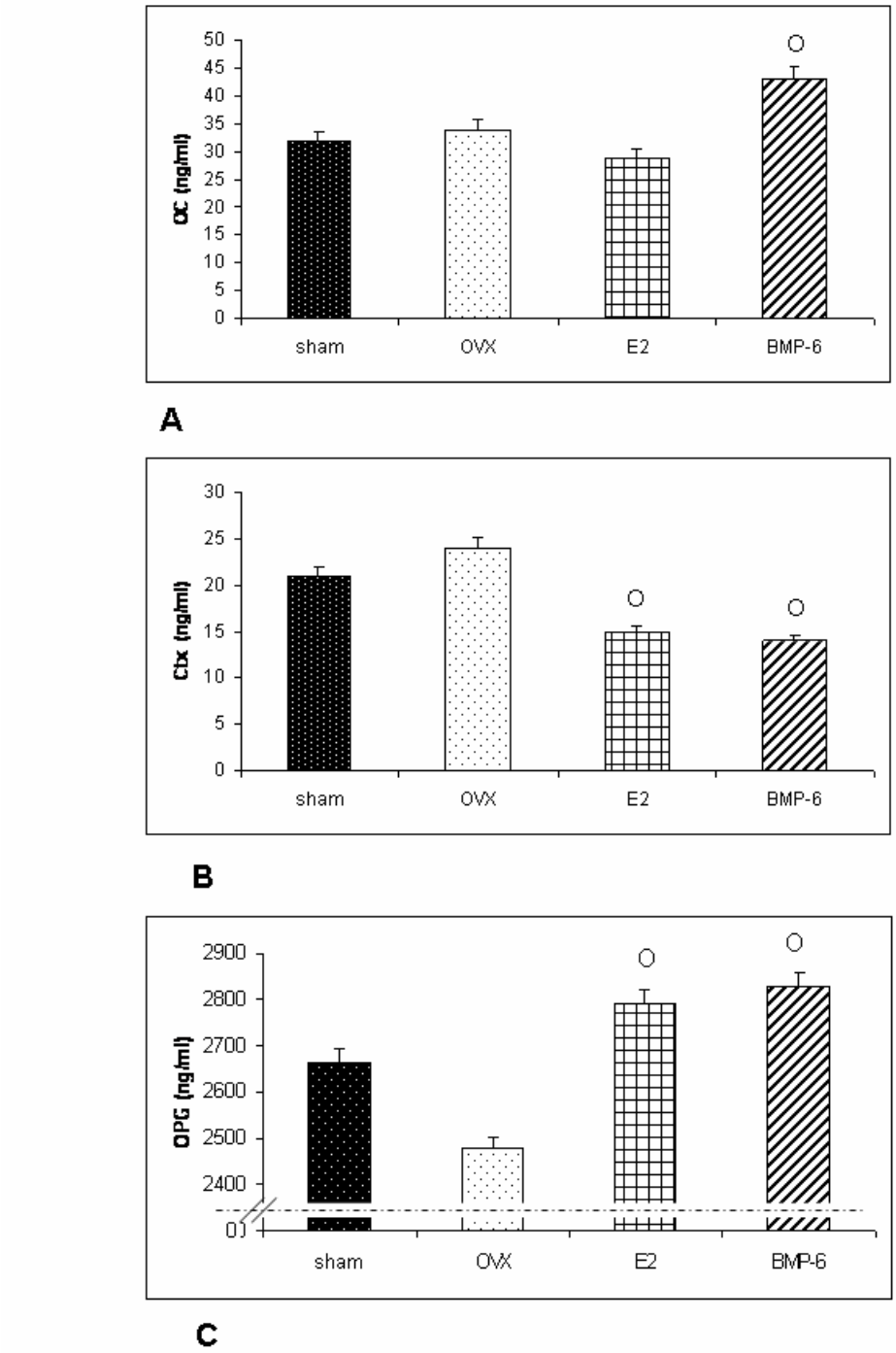
OC (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
sham
OVX
E2
BMP-6
A
Ctx (ng/ml)
30
25
20
15
10
5
0
sham
OVX
E2
BMP-6
B
OPG (ng/ml)
2900
2800
2700
2600
2500
2400
0
sham
OVX
E2
BMP-6
C

Figure 7.

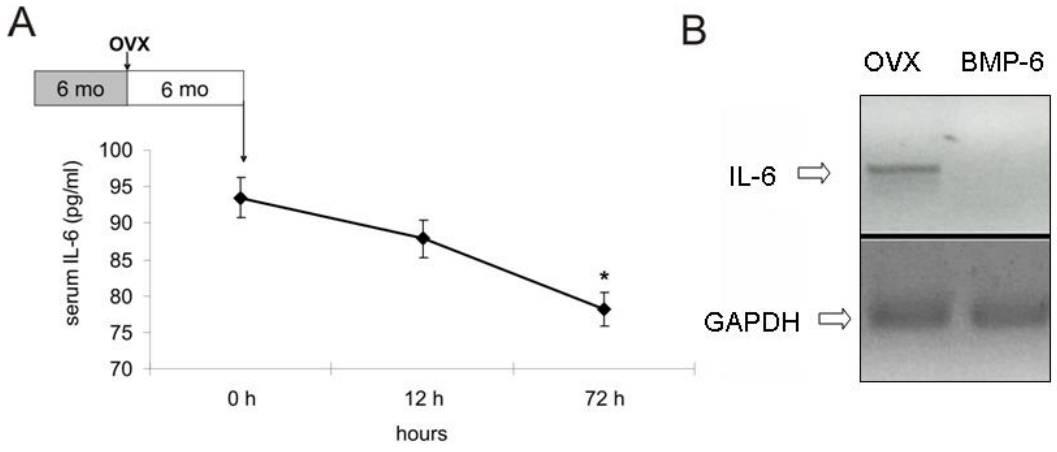
A
OVX
6 mo
6 mo
serum IL-6 (pg/ml)
100
95
90
85
80
75
70
*
0 h
12 h
72 h
hours
B
OVX
BMP-6
IL-6
GAPDH

Figure 8.

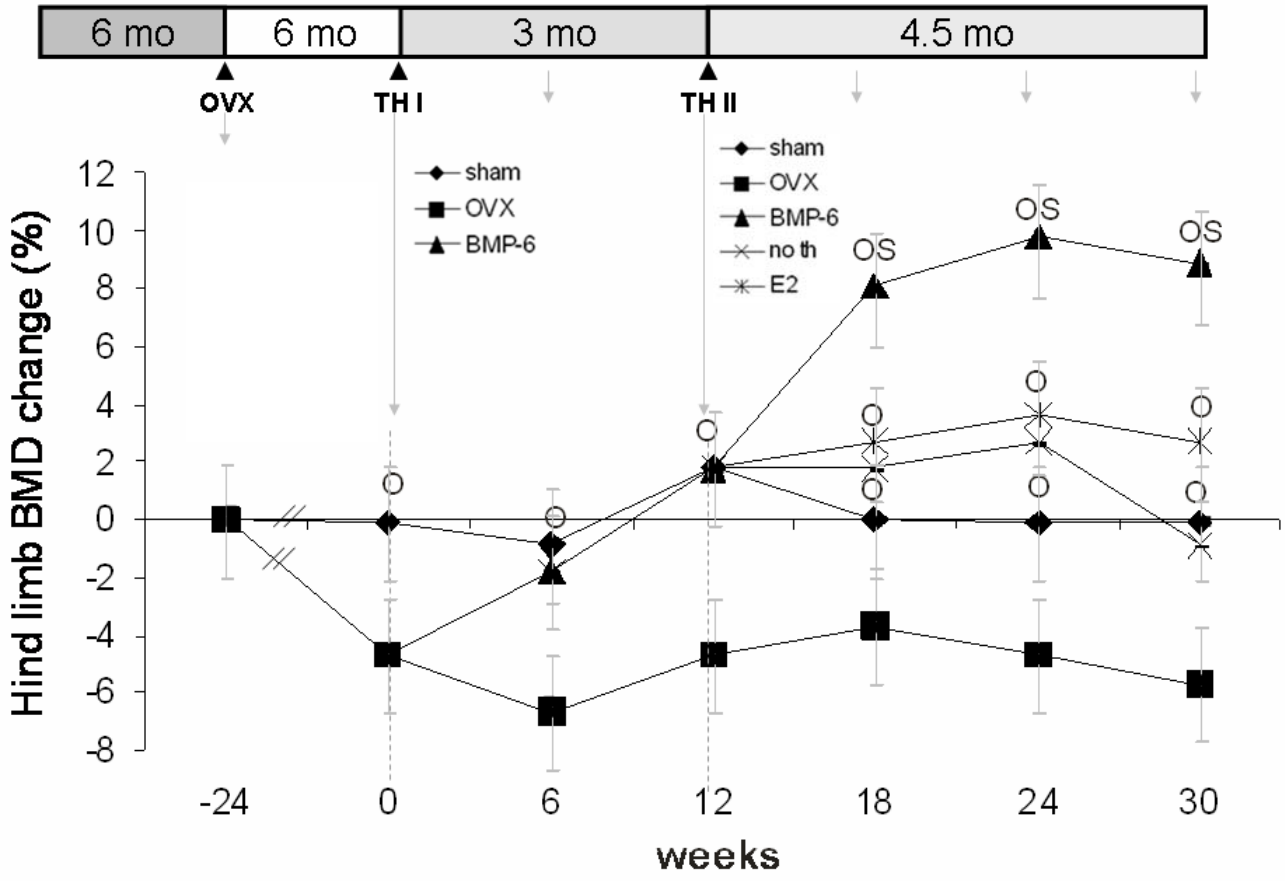
6 mo
6 mo
3 mo
4.5 mo
OVX
TH I
TH II
sham
OVX
BMP-6
no th
E2
Hind limb BMD change (%)
12
10
8
6
4
2
0
-2
-4
-6
-8
-24
0
6
12
18
24
30
weeks
O
OS

Figure 9.

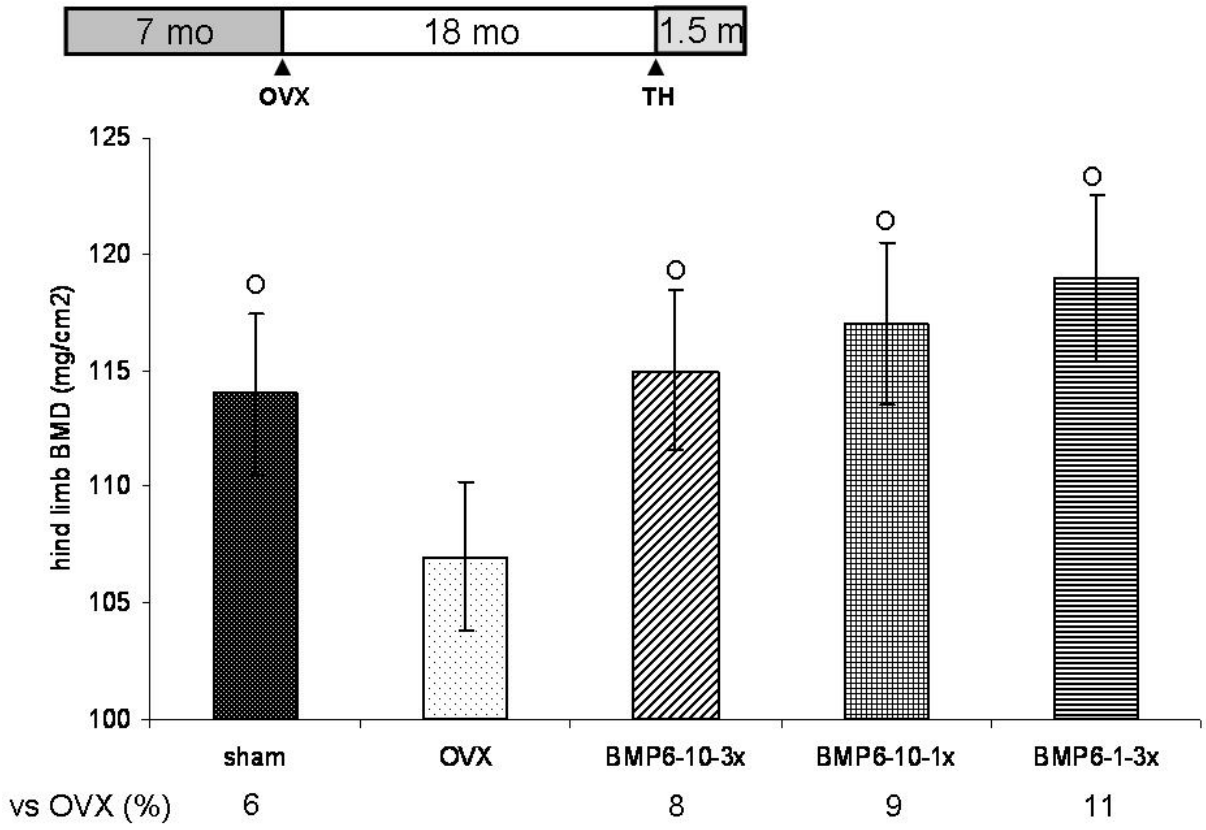
7 mo
18 mo
1.5 m
OVX
TH
125
120
115
110
105
100
hind limb BMD (mg/cm2)
sham
OVX
BMP6-10-3x
BMP6-10-1x
BMP6-1-3x
vs OVX (%)
6
8
9
11

Figure 10.