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Exogenous heparin binds and inhibits bone morphogenetic protein 6 (BMP6) biological activity

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Abbreviations:

BMP – bone morphogenetic protein

ALP – alkaline phosphatase

OC – osteocalcin

IU – international unit

HS – heparan sulfate

HSPG – heparan sulfate proteoglycans

OVX – ovariectomy

μCT - microcomputerized tomography

BMD - bone mineral density

DEXA – dual X-ray absorptiometry

DBM – demineralized bone matrix

ABSTRACT

Purpose. The purpose of this study was to explore the effect of heparin on bone morphogenetic protein 6 (BMP6) osteogenic activity.

Methods. Western blot analysis was used to confirm the binding of BMP6 to heparin and to observe its effect on BMP6 signaling in C2C12-BRE-Luc myoblasts. Real-time RT-PCR was performed for the expression analysis of alkaline phosphatase (ALP) and osteocalcin (OC) in C2C12 myoblasts treated with BMP6 and heparin for 72 hours. Rat ectopic bone formation assay was performed to explore the effect of heparin on BMP6 osteogenic activity. Two weeks following implantation the implants were analyzed morphologically and histologically. Mouse osteoporotic model was used to test the ability of BMP6 to improve the bone quality *in vivo* in the presence of heparin, followed by DEXA and μCT analyses. Blood coagulation was tested in rats priorly treated with BMP6.

Results. BMP6 specifically bound to heparin and induced Smad1/5/8 phosphorylation which was inhibited by heparin. After 48 and 72 hours of treatment, heparin inhibited BMP6-induced ALP and OC expression in C2C12 cells. Heparin dose dependently inhibited BMP6-induced new bone and cartilage formation in the rat ectopic bone formation assay, while in osteoporotic mice heparin inhibited the BMP6 potential to improve the bone quality as evidenced by decreased bone mineral density and trabecular bone parameters. Interestingly, BMP6 prevented the effect of heparin on the blood coagulation parameters.

Conclusion. The interaction of BMP6 with heparin might contribute to the heparin-induced osteoporosis and blood coagulation.

Keywords: heparin, osteoporosis, bone morphogenetic protein 6 (BMP6), C2C12 myoblasts, alkaline phosphatase (ALP)

1. INTRODUCTION

Osteoporosis is a rare, but potentially serious complication of long-term heparin therapy [1-5]. Although symptomatic fractures occur in less than 5% of patients receiving heparin [6], around one third of them have a reduction in bone density [7]. Long-term administration of standard heparin is not prescribed frequently, but is indicated for: the prevention of venous thromboembolism, treatment of pulmonary embolism and venous thrombosis, patients who undergo vascular surgery and coronary angioplasty, and selected patients with disrupted coagulation, like protein C deficiency [8].

Heparin is a highly sulfated glycosaminoglycan and the most negatively charged naturally occurring molecule. Structurally, it is similar to heparan sulfate, which is, in the form of heparan sulfate (HS) proteoglycans (PGs), found at the cell surface and in the extracellular matrix (ECM). Both heparin and HS consist of a core protein and highly sulfated glycosaminoglycan (GAG) chains. Although with different cellular localization, they share structural similarities [9]. GAGs are composed of disaccharide units of D-glucuronic acid-N-acetyl-D-glucosamine (GlcA-GlcNAc), modified by *N*-deacetylation/*N*-sulfation of GlcNAc, epimerization of GlcA to α-D-iduronic acid (IdoA), 2-*O*-sulfation of GlcA and IdoA and 6-*O*-sulfation of N-sulfoglucosamine [10]. Compared to heparan sulfate, in heparin N-acetyl groups correspond to less than 5% of all glucosamine units, but the degree of sulfation is higher than in heparan sulfates [9]. Despite differences, heparin derivatives are frequently used in exploring the HS role in binding and interaction with various molecules to initiate cell signaling [9].

The preferred routes of heparin administration are continuous iv infusion and sc injections [8]. In plasma, it binds to a number of different plasma proteins, which reduces its anticoagulant activity to about one third. Using ^{125}I-labeled heparin, it has been demonstrated that it accumulates and remains in bone long after stopping the treatment [11]. It induces bone resorption by increasing the number and activity of osteoclasts (OC), probably by inhibiting the expression of osteoprotegerin (OPG) [12]. In parallel, heparin reduces bone formation by decreasing the number and activity of osteoblasts (OB) [12]. However, the precise mechanism of heparin´s effect on bone remodelling still remains undetermined.

It has been reported that heparin and HS influence the activity of bone morphogenetic proteins (BMPs) [10, 13–15], which are members of the transforming growth factor-β (TGF-β) superfamily of proteins that regulate a variety of developmental processes including proliferation, differentiation, pattern formation and apoptosis [13, 14, 16 - 19]. In addition, several BMPs have confirmed osteogenic activity in different animal models, with BMP2 and -7 already in clinical use [20, 21]. BMPs were originally purified using heparin affinity chromatography [22] and subsequently cell surface and ECM heparan sulfate proteoglycans were shown to be critical for the biological activity of BMPs and their antagonists [10, 13, 23]. BMP2 and BMP4 have a heparin-binding domain at their N-terminus and strongly bind to heparin and HS via clustered basic residues in that domain [14]. Their binding to HSPGs could restrict the amount of BMPs available for signaling or limit their diffusion [14, 24]. Heparin/HS chains bind to BMP4 and repress BMP4-mediated expression patterns in *Xenopus* embryos [24]. Furthermore, combined deficiency of BMP4 and glypican-3, a cell surface proteoglycan, results in an abnormal skeletal development [25]. Syndecan-3, another member of cell surface HSPGs, interferes with the interaction of BMP2 and its receptor, thus inhibiting its activity during normal limb cartilage differentiation [14].

In addition, HSPGs could act as co-receptors and facilitate the interaction between BMPs and their receptors [26]. For example, Dally, a Drosophila homolog of the glypican family of cell surface HSPGs, acts as a co-receptor for Decapentaplegic (Dpp), a homolog of vertebrate BMPs, and regulates the sensitivity of cells to Dpp signaling [27]. Dally-like (Dlp), another member of the glypican family of cell surface HSPGs, interacts with Hadgehog (Hh) and acts as an Hh co-receptor, promoting Hh signaling strength in the Drosophila wing disc [28]. Compared to BMP2 and -4, BMP-5 to -8 have longer N-terminal sequences ahead of the first conserved cysteine and the allocation of basic residues within these sequences is quite different with the absence of clustered basic residues [29]. In spite of these differences BMP7 also binds to HS and heparin, which inhibits its activity *in vitro* [10, 30].

BMP6 has a major role in promoting OB differentiation and bone formation [31]. Hematopoietic stem cell (HSC)-derived BMP6 is responsible for enhanced OB differentiation and bone formation from bone marrow-derived stem cells (BMSCs) [32]. Also, exploring the role of BMP6 in the adult skeleton, it was found that it circulates in the plasma of healthy individuals [33], and when systemically applied to osteoporotic rats it restores the bone inductive capacity, microarchitecture and quality of the skeleton [34]. BMP6 expression is also strong in promoting the chondrocyte hypertrophy [35] and one study suggests its physiological role in maintaining growth plate function [36].

In the present study, we investigated the role of heparin in BMP6 signaling using C2C12-BRE-Luc mouse premyoblast cell line stably transfected with a reporter plasmid consisting of a BMP response element (BRE) from the *Id-1* promoter fused to a luciferase reporter gene [37]. Since BMP6 was shown to induce differentiation of C2C12 cells towards an OB phenotype [38], and is known to upregulate the *Id-1* gene expression [39 – 41], this cell line is a good experimental model for investigating the BMP6-mediated signaling *in vitro*. In a mouse model of osteoporosis, we explored the effect of the exogenous heparin on the BMP6-mediated osteogenic activity. We show that BMP6 specifically binds to heparin which inhibits the BMP6-mediated bone formation. In addition, we show that the removal or disruption of the cell-surface HS inhibits BMP6 signaling. Interestingly, BMP6 prevented the heparin's effect on the blood coagulation. Since BMP6 circulates in humans, its interaction with exogenous heparin might contribute to the heparin-induced osteoporosis and blood coagulation.

2. MATERIALS AND METHODS

2.1. Materials. C2C12-BRE-Luc BMP reporter cells, stably transfected with a reporter plasmid consisting of BMP response element (BRE) from the *Id-1* promoter fused to a luciferase reporter gene, were previously described [37]. DMEM/F-12, fetal bovine serum (FBS), antibiotic/antimicotic, phosphate buffer saline (PBS), NuPage 10% Bis-Tris gels, sample buffer, nitrocellulose membranes were from Invitrogen Life Technologies (Carlsbad, CA). Chondroitin sulfate, de-*O*-sulfated heparin, de-*N*-sulfated heparin, sodium chlorate, BCIP/NBT tablets, phosphatase inhibitor cocktail 2 and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Heparin for i.v. use (5000 IU/ml) was from Belupo (Croatia). Heparinase III (EC 4.2.2.8.) was from Iduron Ltd. (Manchester, UK). Recombinant human BMP6 and monoclonal anti-BMP6 antibody (7H2) were from Genera Research (Croatia). Anti-phospho-Smad1/5/8 antibody was from Cell Signaling Technology (Beverly, MA). Anti-β-actin antibody was from Millipore (Billerica, MA). Anti-Smad 1/5/8 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Protease and phosphatase inhibitor cocktails were from Roche (Basel, Switzerland). Luciferase Assay reagent and Reporter Lysis Buffer were from Promega (Madison, WI). Prestained SDS-PAGE marker and heparin-Sepharose beads were from BIO-RAD Laboratories Inc. (Hercules, CA). Sources of other materials are shown in the text.

2.2. Cell culture. C2C12-BRE-Luc cells were cultured in Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) containing 10 % FBS, 1% Antibiotic/Antimicotic at 37°C in a humidified atmosphere of 5% CO_2 in air. For sodium chlorate treatment cells were cultured in DMEM/F-12 supplemented with 5% dialyzed fetal bovine serum and 50 mM sodium chlorate for 4 days. For Heparinase III treatment cells were incubated with 25 mIU/ml for 3 hours in DMEM/F-12 at 37°C. All cell culture studies were performed with a cell passage less than 25.

2.3. BMP6 binding to heparin-Sepharose beads. 100 ng BMP6 was incubated with 50 µl heparin-Sepharose beads in 100 µl binding buffer (10 mM Na_2HPO_4, pH 7) with or without 2 mg intact heparin for 20 min on room temperature. After binding, the beads were washed twice with binding buffer. The bound protein was eluted with sample buffer and subjected to SDS-PAGE. BMP6 was detected by immunoblot using anti-BMP6 antibody. BMP6 (100 ng) was used as a positive control.

2.4. BCA protein assay. Protein concentration in lysates was determined by using BCA Protein Assay Kit (Thermo scientific) and the samples were normalized before immunoblot analysis.

2.5. Detection of Smad1/5/8 phosphorylation. C2C12-BRE-Luc cells were seeded in 10 cm Petri-dishes, $3x10^6$ cells per dish and cultured overnight in DMEM/F-12. The cells were then incubated with 50 ng/ml BMP6 and various experimental reagents for 30 min at 37°C. For BMP6 + heparin treatment, reagents were preincubated for 20 min at RT prior the treatment. After incubation, the cells were washed twice with ice cold PBS, scraped and centrifuged at 4°C for 10 min at 1100 rpm. Pellets were resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 1% TritonX-100, phosphatase and protease inhibitors) and lysis was done for 30 min on ice using an orbital shaker. The lysates were microcentrifuged for 10 min at 4°C and BCA was performed to normalize the samples.

2.6. Western immunoblotting. 45 µg of samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA and 0.05% Tween in TBS for 30 min at room temperature and then incubated overnight with primary antibodies at 4°C. Immunolabeling was detected using alkaline phosphatase-conjugated secondary antibodies and BCIP/NBT substrate (Sigma) according to the manufacturer's instructions. ImageJ software (NIH) was used for quantification of protein bend intensities.

2.7. Id-1 Reporter Gene Assay. C2C12-BRE-Luc cells were plated at a concentration of $3x10^4$ cells per well in 48-well plates containing DMEM/F-12 plus 10% FBS and allowed to attach overnight. Cells were washed with PBS and re-fed with 300 µl of DMEM/F-12 with 0.1% FBS for 7 hours. BMP6 and other reagents or their combinations were added to cells at appropriate concentrations for 15 hours, and then cells were then washed with PBS and lysed using 60 µl of the reporter lysis buffer. To measure the luciferase activity, 20 µl of cell lysate was added to 100 µl Luciferase Assay Reagent and luminescence was quantified using a Victor Wallack luminometer. The protein content of each lysate was analyzed using BCA Protein Assay Kit according to the manufacturer's instructions. Luciferase units were normalized to the protein content of each well. All experiments were repeated at least three times with three independent wells per condition.

2.8. RNA isolation and Real Time PCR. Total RNA was isolated from C2C12 cells cultivated in 10 cm Petri-dishes and treated with BMP6 (50 ng/ml) with or without heparin (10 µg/ml) for different time points using TRIzol (Invitrogen) according to manufacturer's instructions. The amount of RNA was determined by spectrophotometry. The cDNA was generated by reverse-transcription of 1 µg adjusted RNA using Super Script III First-Strand Synthesis System (Invitrogen) as indicated by the manufacturer's instructions. Gene expression of interest was measured by using a LightCycler FastStart DNA Master SYBR Green kit in a LightCycler instrument (Roche Diagnostics), as described [42, 43]. Briefly, 1µl of template was mixed with 9 µl of LightCycler FastStart DNA Master SYBR Green mix to which $MgCl_2$ and gene-specific forward and reverse PCR primers had been added to a final concentration of 2 µM for $MgCl_2$ and 0.5 µM for each primer. Results are represented as a fold change of the control expression level. *Gapdh* transcripts were used as a normalizer. The list of primers used is shown in Table 1.

2.9. Ectopic bone formation and morphometric analysis in rats. Bone matrix was prepared from 6-month-old Sprague-Dawley rats. After sacrifice, diaphyses of femurs and tibiae were removed and then powdered, sieved, and demineralized as previously described [44]. BMP6 (10 µg) with heparin (2, 20 or 200 IU) (international units) was added to demineralized bone matrix (DBM) to form pellets, which were then implanted subcutaneously in the pectoral region of normal rats. Pellets with acetate buffer only were used as a negative control and BMP6 (10 µg) alone as a positive control. Two pellets per group were analysed, altogether 10 rats. Two weeks following implantation of DBM, pellets were removed and embedded in paraffin, cut into 7- µm-thick sections, stained with toluidine blue, and examined for the presence of new cartilage and bone to reveal effects of the heparin on BMP6 osteogenic activity. Histomorphometric analysis is quantified using computer-aided image analysis system (SForm Image Analyzer software, Version 1.0). Data is shown as percentage of the Area of Interest in the implant tissue. All experiments involving animals were done in accordance and approved by Institutional Animal Research Committee.

2.10. Ovariectomized mice. Four-month-old CD-1 female mice were subjected to ovariectomy (OVX). Animals were anesthetized with an intraperitoneal injection of ketamine at doses of 100 mg/kg body weight. Six animals per experiment were subjected to sham surgery during which the ovaries were exteriorized but replaced intact. Bilateral ovariectomies were performed in the remaining mice from the dorsal approach and they were left untreated for a period of 3 weeks following surgery to await the development of osteopenia. BMP6 at dose of 10 µg/kg was injected through the tail vein three times a week for 7 weeks. Animals were divided into groups of six as follows: 1. sham; 2. OVX; 3. OVX (BMP6 10 µg/kg intravenously, 3 times/week); 4. OVX (heparin 0.5 IU/g, 3 times/week); 5. OVX (BMP6 10 µg/kg + heparin 0.5 IU/g intravenously, 3 times/week); 6. OVX (BMP6 10 µg/kg + heparin 1 IU/g intravenously, 3 times/week), altogether 36 animals.

2.11. Bone Mineral Density (BMD). Prior to ovariectomy and three and ten weeks after, the animals were anesthetized and hind limb were scanned for bone density by dual-energy x-ray absorptiometry (DEXA) (Hologic QDR-4000, Hologic, Waltham, MA) equipped with Regional High Resolution Scan software [45]. At the end of the experiment, animals were anesthetized and killed by cervical dislocation. The hind limbs were removed and fixed in 4% paraformaldehyde for further analysis by µCT. The right femur and tibia were also used for determination of BMD by dual-energy x-ray absorptiometry. The scan images were analyzed and bone mineral density of whole femurs and tibiae were determined.

2.12. µCT Analysis. The effect of heparin and BMP6 was tested on femur and tibia in OVX mice by µCT (µCT SkyScan 1076, Belgium). The distal femur and proximal tibia were scanned in 9 µm resolution with aluminum filter of 0,5mm in the dorsoventral direction. Three-dimensional reconstruction of bone was performed using the NRecon software (SkyScan, Belgium), and the volume of the trabecular bone (BV, U^3) was calculated. Trabecular parameters, including trabecular number (Tb. N, 1/U), trabecular thickness (Tb. Th, U), trabecular separation (Tb. Sp, U), and cortical parameters, including tissue volume (TV, U^3), bone volume (BV, U^3), and cortical thickness (Co. Th, U) were measured using CTAn software (Skyscan, Belgium) by using the method described previously [46].

2.13. BMP6 and heparin effect on rat blood coagulation. In the *ex vivo* experiment 1 ml of rat blood was collected in the citrate sterile tube using heparin-coated capillary tubes (n = 5) (Hirschmann Laborgeräte, Germany). One µg of BMP6 was added immediately after blood collection (n = 5), while control samples were treated with a vehicle (acetate buffer pH 4.0). *In vivo* rats were injected with 10 µg/kg of BMP6 (n = 5) or a vehicle (acetate buffer pH 4.0; n = 5). Ten minutes later blood was collected from the orbital plexus using heparin-coated capillary tubes. Coagulation parameters (PT, APTT, TT, fibrinogen and coagulation factor XI) were measured using standard laboratory procedures for human blood analysis. Altogether 25 animals were used in this experiment.

2.14. Statistics

Experimental values obtained by PCR analysis and DEXA are given as means ± SEM. Data obtained by µCT are presented as means ± STD. Data analyses were performed using Statistica 10 (StatSoft, USA) software. One way analysis of variance (ANOVA) with a Tuckey *post hoc* test was performed to determine the significance between the experimental groups. The results were considered significant if P value was lower than 0.05 ($P<0.05$).

3. RESULTS

3.1. BMP6 binds to heparin

To demonstrate that BMP6 binds to heparin, we incubated BMP6 with heparin sepharose beads and detected the bound BMP6 by Western blotting (Fig. 1). Coincubation with exogenous free heparin reduced the amount of the heparin sepharose bead bound fraction of BMP6 indicating that both immobilized and free heparin in solution bound BMP6.

3.2. Exogenous heparin inhibits BMP6 activity in vitro

Phospho-Smad1/5/8 were activated in BMP6-stimulated C2C12-BRE-Luc cells (Fig. 2a). To determine whether heparin interacts directly with BMP6 and influences its signaling, C2C12-BRE-Luc cells were treated at different time points with a combination of BMP6 (50 ng/ml) and heparin (10 μg/ml) (Fig. 2b). BMP6-induced Smad1/5/8 phosphorylation was reduced by exogenous heparin treatment. The same was observed in the Luciferase reporter assay, where heparin significantly reduced the BMP6-induced luciferase signal, while galactosaminoglycan chondroitin sulfate, another member of proteoglycan sugar chains, had no influence (Fig. 2c). These results suggested that BMP6 specifically bound to heparin/HS GAG chains and we assumed that free heparin competed with cell surface HSPGs for BMP6 binding. Because heparin and HS are modified by *N*- and *O*- sulfation, and these sulfated structures are responsible for the interaction with HS-binding proteins, we treated the cells with de-*O*- and de-*N*-sulfated heparins and BMP6 to test whether BMP6 requires specific sulfate groups for binding to heparin/HS. Neither de-*O*- nor de-*N*-sulfated heparin interfered with BMP6-induced luciferase signal (Fig. 2c). These results suggested that both *O*- and *N*-sulfation were essential for the BMP6 binding to HS chains.

3.3. Removal of HS from cell surface inhibits BMP6 activity

Heparan sulfate (HS) is ubiquitously expressed on cell surfaces and we determined whether HS expressed in C2C12 cells was involved in BMP6-mediated signaling. C2C12-BRE-Luc cells were pretreated with Heparinase III, HS lyase, to remove HS chains from the cell surface prior to stimulation with BMP6. As shown in Fig. 2d (lane 5), BMP6-induced Smad1/5/8 phosphorylation was inhibited in Heparinase III pretreated cells, suggesting that cell surface HS was important for BMP6 signaling. Similarly, the same treatment in the luciferase assay decreased the BMP6-induced activity by two fold (Fig. 2e).

Because sulfated structures of HS are responsible for its function and interaction with HS-binding proteins, these sulfate groups might be important for BMP6 signaling in C2C12-BRE-Luc cells. To explore if HS sulfation was involved in BMP6 signaling, C2C12-BRE-Luc cells were pretreated with sodium chlorate, an inhibitor of proteoglycan sulfation, and then BMP6-mediated Smad1/5/8 phosphorylation and luciferase induction were examined. The BMP6-induced Smad phosphorylation was inhibited (Fig. 2f, lane 5), as well as the luciferase induction, which was five fold decreased as compared to chlorate non-treated cells (Fig. 2g).

3.4. Cell-surface-anchored HS is important for BMP6 signaling

Proteoglycans (PGs) can be classified on the basis of their localization and type of the core protein. To determine the role of plasma membrane HS chains anchored through their core proteins in BMP6 signaling, we applied heparin exogenously and explored whether it substituted for the endogenous HS in heparitinase- and chlorate-treated C2C12-BRE-Luc cells. As shown in Fig. 2, exogenous heparin did not restore BMP6-mediated luciferase induction (Fig. 2e and 2g) nor Smad1/5/8 phosphorylation (Fig. 2d, lane 6 and 2f, lane 6) in either Heparinase III- or chlorate-pretreated cells. Moreover, exogenous heparin inhibited BMP6-induced Smad phosphorylation in normal C2C12-BRE-Luc cells (Fig. 2b, lanes 3-7). These results indicate that exogenous heparin could not rescue BMP6 signaling in HS-disrupted cells and that HS should be anchored on the plasma membrane for normal BMP6 signaling. Also, chondroitin-sulfate did not inhibit BMP6-mediated luciferase signal (Fig. 2c), suggesting that heparin-induced inhibition was a specific event not caused by the negative charges of the sulfate groups. Compared to de-*O*- and de-*N*-sulfated heparins, intact heparin showed the highest level of inhibition (Fig. 2c), suggesting that *N*- and *O*-sulfate groups of exogenous heparin were required for BMP6 signaling.

3.5. Heparin inhibits BMP6-mediated C2C12 differentiation

BMP6 is known to induce C2C12 myoblast differentiation towards the osteoblastic phenotype. However, at 24 hours following treatment (Graph 1a) heparin inhibited BMP6-induced alkaline phosphatase (*ALP*) expression, which was more pronounced after 48 and 72 hours. The same was observed for the osteocalcin (*OC*) gene expression (Graph 1b).

3.6. Heparin inhibits ectopic bone formation induced by BMP6 in vivo

BMP6 induces bone when implanted ectopically [47-49]. Heparin implanted with BMP6 on DBM, which was previously extracted with urea, dose dependently inhibited BMP6-induced ectopic bone with 200 IU heparin being the most effective (Fig. 3).

3.7. Heparin inhibits osteogenic activity of systemically administered BMP6 in OVX mice

We have previously shown that BMP6, given systemically, can restore bone in rats with osteoporosis [34]. When OVX mice were treated intravenously with BMP6 and two heparin doses (0.5 and 1 IU/g/day) for seven weeks, μCT analyses showed that heparin reduced the BMP6 osteogenic activity. In the femur, the higher dose reduced the BV/TV by 24% (Fig. 4a), the trabecular number by 29% (Fig. 4b) and increased the trabecular separation by 25% (Fig. 4c), as compared to BMP6 therapy alone. The lower dose showed a similar trend (Fig. 4). In tibiae, mice treated with heparin had the bone volume reduced by 35% (data not shown) and BV/TV by 30% (Fig. 5a). The trabecular number was reduced by 35% (Fig. 5b), while the trabecular separation was increased by 17% (Fig. 5c). Cortical bone parameters (bone volume, bone surface and cortical thickness) were not different (data not shown). The result was also supported by BMD values measured by DEXA, where both heparin treated animals showed lower BMD as compared to BMP6 treated mice. After 7 weeks BMD of femurs and tibiae in mice treated with BMP6 and heparin decreased dose dependently (Graph 2b and 2c).

3.8. BMP6 improves coagulation parameters

To further explore the specificity of BMP6 and heparin interaction, we used the blood coagulation pathway as an experimental model. When rat blood was collected in heparinized tubes the resulting activated partial thromboplastin time (APTT) and thrombin time (TT) were prolonged, while the coagulation factor XI value was undetectable (Table 2). Following addition of 1 μg of BMP6 to 1 ml of rat blood, the coagulation factor XI and the coagulation time were normalized (Table 2). To confirm these results *in vivo*, we administered BMP6 (10 μg/kg) intravenously and ten minutes later collected the blood using similar heparin-coated capillary tubes. The prothrombin time (PTT), activated partial thromboplastin time, thrombin time, fibrinogen (FBG) and coagulation factor XI were measurable and reached normal values (Table 2). These results confirmed that BMP6 interacts with the heparin activity *in vivo*. Surprisingly, in these experiments BMP6 had a similar effect as the enzyme hepzyme (Table 2.), a heparinase which specifically cleaves and inactivates heparin.

4. DISCUSSION

Long-term heparin treatment induces bone loss and increases the risk of fractures in patients [1-6] and in experimental animals [7, 11]. Here we explored the effect of exogenous heparin on BMP6 and found that BMP6 binds to heparin which, in contrast to endogenous heparan sulfate proteoglycans, blocks BMP6 signal transduction in C2C12 myoblasts and inhibits its osteogenic activity *in vivo* after local and systemic application.

Heparin belongs to highly sulfated glycosaminoglycans and is often used in exploring structurally similar heparan sulfate (HS) roles in binding and interaction with various molecules in cell signaling. It was revealed that BMPs are heparin/HS-binding molecules and their binding to specific HS GAG chains is necessary for their biological activity. GAGs have been found to have opposite effects on BMP activity in various *in vivo* and *in vitro* systems, enhancing it in some, while inhibiting their activity in other [10, 13, 30]. It may depend on multiple factors, like different BMPs examined [10, 13, 30, 50], the source, concentration and sulfation structure of specific GAGs, the availability or absence of cell surface GAGs, as well as BMP receptors and the assay used. Similarly, variable effects on the BMP efficacy were also observed in osteogenic assays [15, 30, 50]. In some studies it has been shown that heparin and HS enhance BMP2 activity in C2C12 cells [30, 50], while in others heparin had an opposite effect on the BMP signaling [15], inhibiting BMP2 [15] and BMP7 [10] binding to receptors and induction of Smad signaling. In contrast to BMP2 and BMP4, BMP6-induced alkaline phosphatase activity in C2C12 cells was inhibited by heparin [50]. BMP7 signaling was inhibited by heparin in rat osteosarcoma cells [10].

Here we showed that both *N*- and *O*-sulfate structures are needed for BMP6 binding and signaling in C2C12 cells. According to similarity in structure of mature domains of BMP6 and BMP7, we assume that BMP6 like BMP7 binds to negatively charged HS chains through basic lysine and arginine residues [10]. Alteration of the endogenous HS structure with chlorate treatment or its removal using heparinase III, inhibits BMP6-mediated signaling in C2C12-BRE-Luc cells, indicating that endogenous HS plays an important role in the BMP6 activity. Similarly, BMP7 activity was inhibited by removal/desulfation of cell surface GAGs [10]. We showed that exogenous heparin competitively inhibited BMP6 binding to endogenous HS, which disrupted the BMP6 signaling. Thus, for the proper BMP6 signaling, HS should be anchored on the plasma membrane. It is possible that HS binds BMP6, concentrating it on the plasma membrane, allowing the ligand-receptor interaction. On the other hand, it is interesting that exogenous heparin also binds to BMP6, probably preventing it to interact with the receptor complex.

It is known that BMPs support differentiation of C2C12 cells towards an osteoblastic phenotype [51, 52]. BMP6 in concentration of 50 ng/ml induced the expression of ALP by 180 fold and OC by 4 fold after 72 hours. The addition of heparin (10 μg/ml) attenuated the expression of both osteoblastic markers, which is also consistent with previous reports demonstrating that exogenous heparin can inhibit BMP2 and BMP7 binding to their receptors [10, 15].

We also showed that exogenous heparin dose dependently inhibited BMP6-mediated ectopic bone formation in rats which is in contrast to previous results showing that heparin potentiates BMP2 osteogenic activity [50, 53]. These differences are consistent with *in vitro* data, showing that heparin differently influences the activity of

various BMPs [15, 30, 50]. Using μCT analysis of femur and tibiae in a mice osteoporotic model, we demonstrated that exogenous heparin reduced the BMP6 osteogenic activity and its potential to improve the bone quality after 7 weeks of therapy. These results are consistent with previous studies showing that heparin induces bone loss leading to development of osteoporosis in rats [7].

The observation that BMP6 inhibited the heparin anticoagulant activity, probably by preventing its binding to coagulation factors, suggests that endogenous circulating BMP6 level might as well influence the coagulation process in patients.

We demonstrated that the addition of exogenous heparin to OVX mice significantly reduces the BMP6 osteogenic activity. These results support the clinical observations that heparin, when used as a thromboprophylactic agent after orthopedic surgeries, delays the fracture healing. Based on these results, we propose that exogenous heparin binds to BMP6 which influences the activity of mature osteoblasts as well as the differentiation of early osteoblasts and may be responsible for reduced bone formation leading to osteoporosis, a well-known side effect of the long term heparin therapy. Previous studies have shown that in pregnant women, almost one third of patients receiving heparin have a significant decrease of BMD, and that fractures occur in 2.2%-3.6% of all patients [6]. In non-pregnant women, the numbers are even higher. Our *in vivo* data support these observations.

BMP6 plays an important role in the iron metabolism by directly modulating the expression of hepcidin, a major regulator of iron homeostasis [54]. Endogenous BMP6 increases the hepcidin expression in the liver and reduces serum iron in mice [54 - 56]. In addition, loss of BMP6 leads to reduced hepatic hepcidin expression and the iron overload in *Bmp6* -/- mice which supports the involvement of BMP6 signaling pathway in the regulation of hepcidin [54, 57]. A recent publication showed that patients treated with low doses of heparin for prevention of a deep vein thrombosis showed a major decrease in serum hepcidin levels and increase in the serum iron [58]. This suppressed BMP6-mediated hepcidin expression may be due to a similar heparin mediated inhibition of BMP6 signaling as we describe here.

We propose that endogenous plasma membrane heparan sulfate stimulated BMP6 signaling, while exogenous heparin inhibited the BMP6 osteogenic activity.

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The authors declare that they have no conflict of interest.

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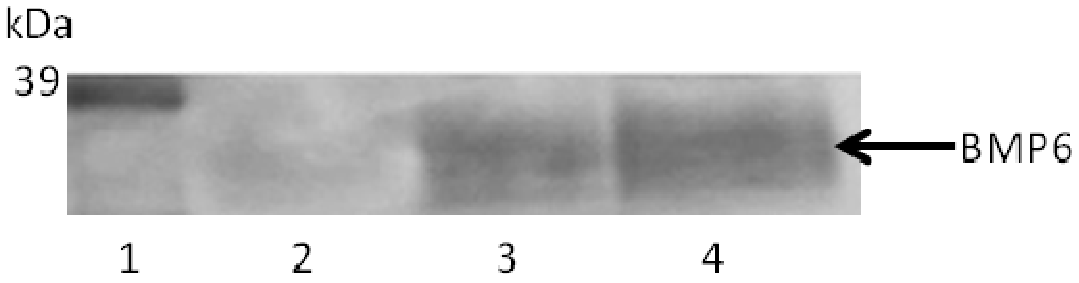
Fig. 1

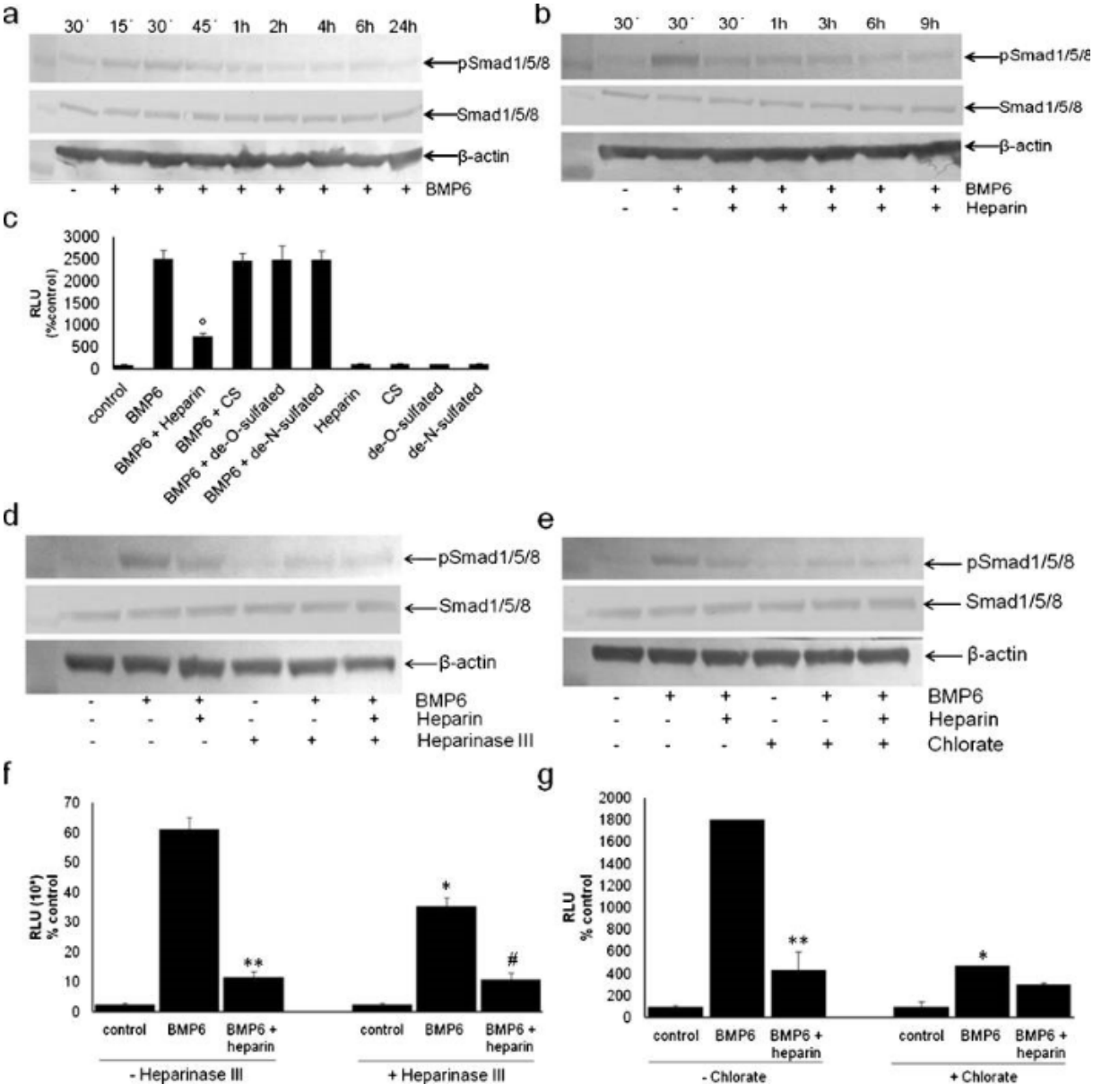
BMP6 binds to heparin-Sepharose. 100 ng BMP6 was incubated with heparin-Sepharose beads in the presence or absence of heparin (2 mg) for 20 min on room temperature. BMP6 bound to beads was eluted and subjected to SDS-PAGE and detected by immunoblot analysis with an anti-BMP6 antibody. Lane 1 – MWM (molecular weight marker); lane 2 – eluted BMP6 (100 ng) incubated with heparin beads and heparin; lane 3 – eluted BMP6 incubated with heparin beads; lane 4 - BMP6. Arrow indicates mature BMP6 (35 kDa). Intensity of the bends was quantified using ImageJ software and expressed as relative density compared to BMP6 standard.



Lane	Area	Percent (%)	Relative density
2	7396,27	11,92	0,241
3	23954,70	38,61	0,781
4	30688,03	49,47	1,000

Fig. 2

Exogenous heparin inhibits BMP6-mediated signaling. (a) BMP6 induces Smad1/5/8 phosphorylation. C2C12-BRE-Luc cells were stimulated with BMP6 (50 ng/ml) for indicated time points. (b) Cells were stimulated with BMP6 for 30 min or BMP6 and heparin (10 μg/ml) for various periods of time (30min-9h). (c) C2C12-BRE-Luc cells were stimulated with BMP6 alone or in combination with heparin, chondroitin sulfate and de-*O*- and de-*N*-sulfated heparin for 17h. BMP6-mediated signaling is inhibited by Heparinase III and chlorate treatment. (d, e) Heparinase III pretreatment interferes with BMP6-mediated signaling. C2C12-BRE-Luc cells were incubated in the presence or absence of Heparinase III (25mIU/ml) for 3 hours and then stimulated with BMP6 (50 ng/ml) for 30 min (d) or 10 ng/ml for 17h (e). (f, g) Chlorate treatment inhibits BMP6-mediated signaling. C2C12-BRE-Luc were incubated with or without sodium chlorate (50 mM) for 4 days and then stimulated with BMP6 (50 ng/m) for 30 min (f) or 10 ng/ml for 17h (g). (a, b) Cells were lysed and 45 μg aliquots were subjected to immunoblot analysis with an anti-phospho-Smad1/5/8 antibody as described in Materials and methods. β-Actin levels are shown as a loading control. Total Smad 1/5/8 levels showed no difference between samples. (c, f, g) The cells were lysed and the luciferase activity measured using Victor Wallack luminometer as described in Materials and methods. °$P<0.05$, comparison between BMP6 with heparin and BMP6 alone; *$P<0.05$, comparison between BMP6 with and without Heparinase III/chlorate; ** $P<0.05$, comparison between BMP6 without and with heparin; #$P<0.05$, comparison between BMP6 without and with heparin, all with Heparinase III.


a
30´ 15´ 30´ 45´ 1h 2h 4h 6h 24h
pSmad1/5/8
Smad1/5/8
β-actin
- + + + + + + + + BMP6
b
30´ 30´ 30´ 1h 3h 6h 9h
pSmad1/5/8
Smad1/5/8
β-actin
- + + + + + + BMP6
- - + + + + + Heparin
c
RLU (%control)
3000
2500
2000
1500
1000
500
0
control
BMP6
BMP6 + Heparin
BMP6 + CS
BMP6 + de-O-sulfated
BMP6 + de-N-sulfated
Heparin
CS
de-O-sulfated
de-N-sulfated
d
pSmad1/5/8
Smad1/5/8
β-actin
- + + - + + BMP6
- - + - - + Heparin
- - - + + + Heparinase III
e
pSmad1/5/8
Smad1/5/8
β-actin
- + + - + + BMP6
- - + - - + Heparin
- - - + + + Chlorate
f
RLU (10⁴) % control
70
60
50
40
30
20
10
0
control
BMP6
BMP6 + heparin
- Heparinase III
+ Heparinase III
g
RLU % control
2000
1800
1600
1400
1200
1000
800
600
400
200
0
control
BMP6
BMP6 + heparin
- Chlorate
+ Chlorate

Graph 1

Heparin inhibits BMP6-induced osteoblast differentiation. C2C12 cells were incubated with BMP6 (50 ng/ml) in the absence or presence of soluble heparin (10 µg/ml) for 24h, 48h and 72h. Expression of osteoblast markers alkaline phosphatase (ALP) (A) and osteocalcin (OC) (B) was measured by real-time RT-PCR. Data presented are fold change in gene expression from three experiments ± S.D. *P<0.05 vs control, #P<0.05 vs BMP6.

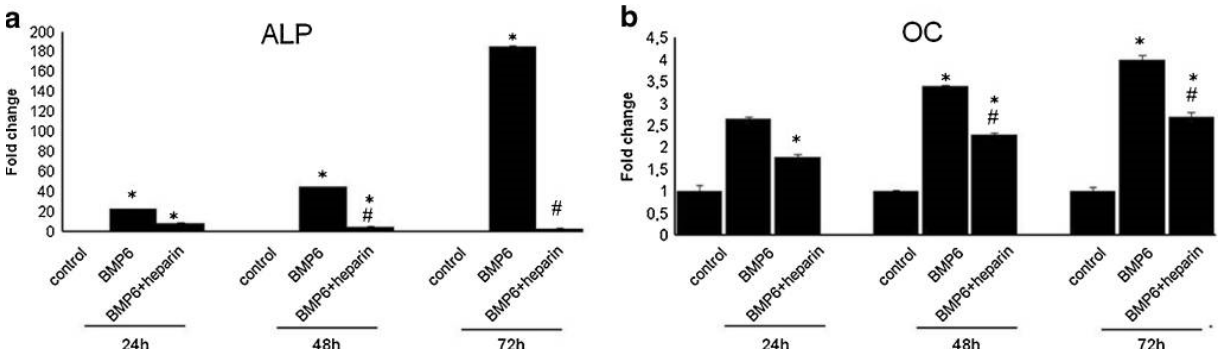


Fig. 3

Heparin inhibits the ectopic bone formation induced by BMP6 *in vivo*. BMP6 (10 µg) and 2, 20 or 200 IU (international units) of heparin were implanted subcutaneously to induce ectopic bone formation in rats. After 2 weeks implants were removed and examined histologically with toluidin blue staining. Morphometric analysis data is presented as BV/TV % of area of interest (newly formed bone) in the whole implant tissue. A – control (acetate buffer only); B – BMP6 (10 µg); C - BMP6 (10 µg) + heparin (20 IU); D - BMP6 (10 µg) + heparin (200 IU).

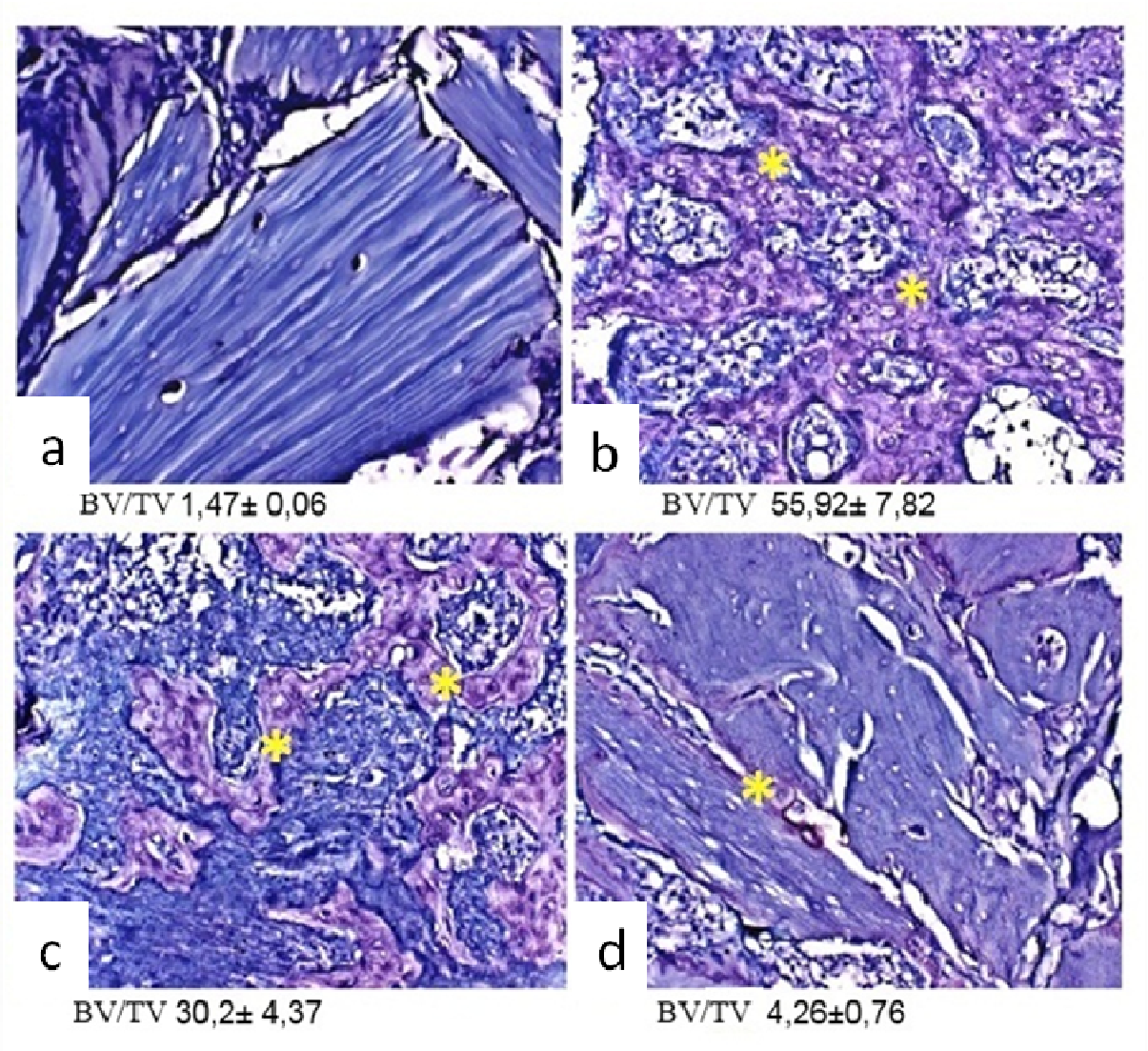


Fig. 4

μCT analysis of the distal femur of 4-month old OVX mice treated with BMP6 (10 μg/kg), heparin (0.5 IU/g), BMP6 (10 μg/kg) + heparin (0.5 IU/g) and BMP6 (10 μg/kg) + heparin (1 IU/g). 1 IU/g heparin with BMP6 significantly decreased the trabecular number (Tb.N.) (B) and increased the trabecular separation (Tb.Sp.) (C). Bone volume over tissue volume (BV/TV) values were also decreased (A). μCT images of femurs from all groups (D). Space encircled by the yellow line indicates the bone marrow cavity without trabeculi. *P<0.05 vs BMP6.

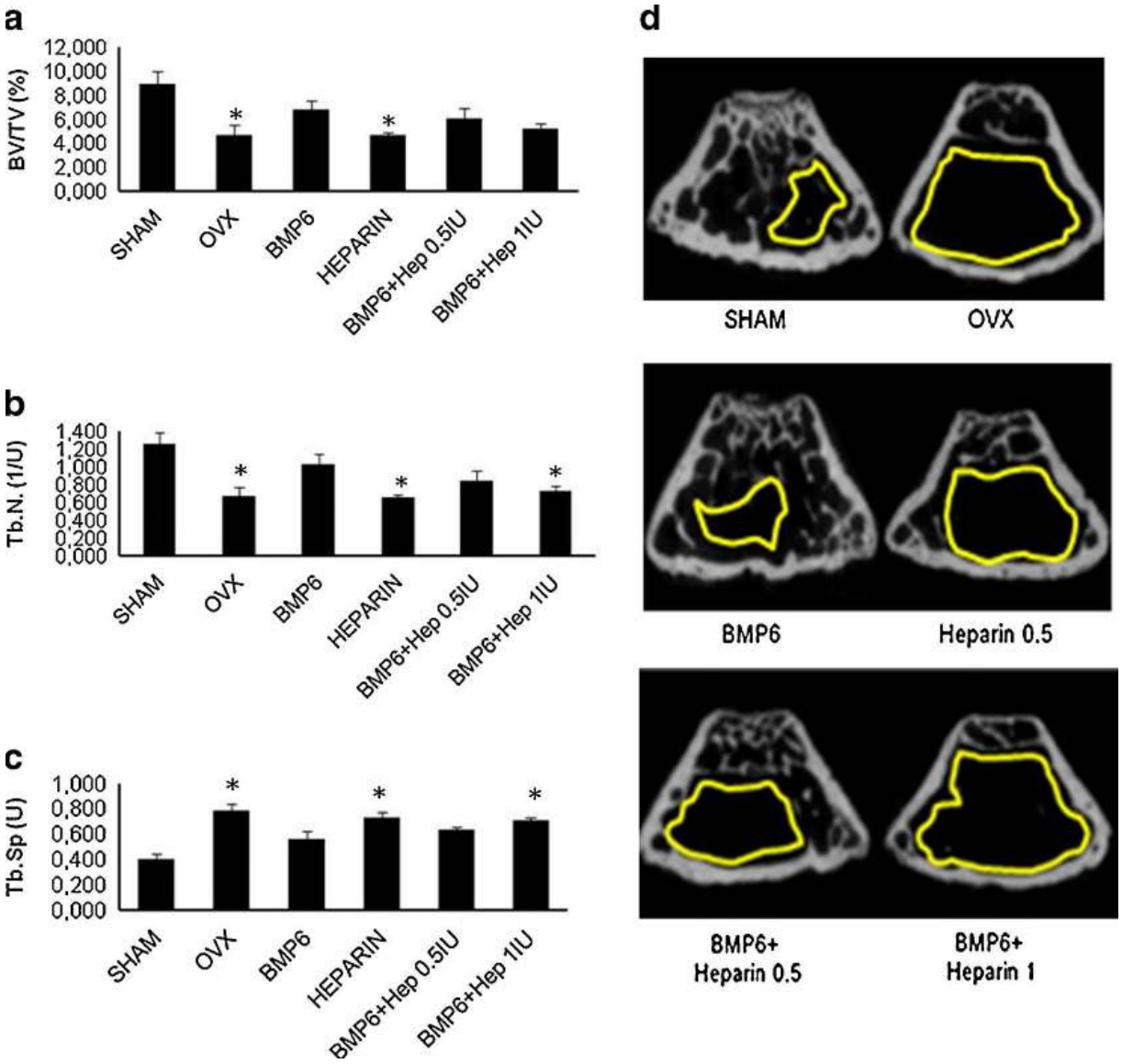
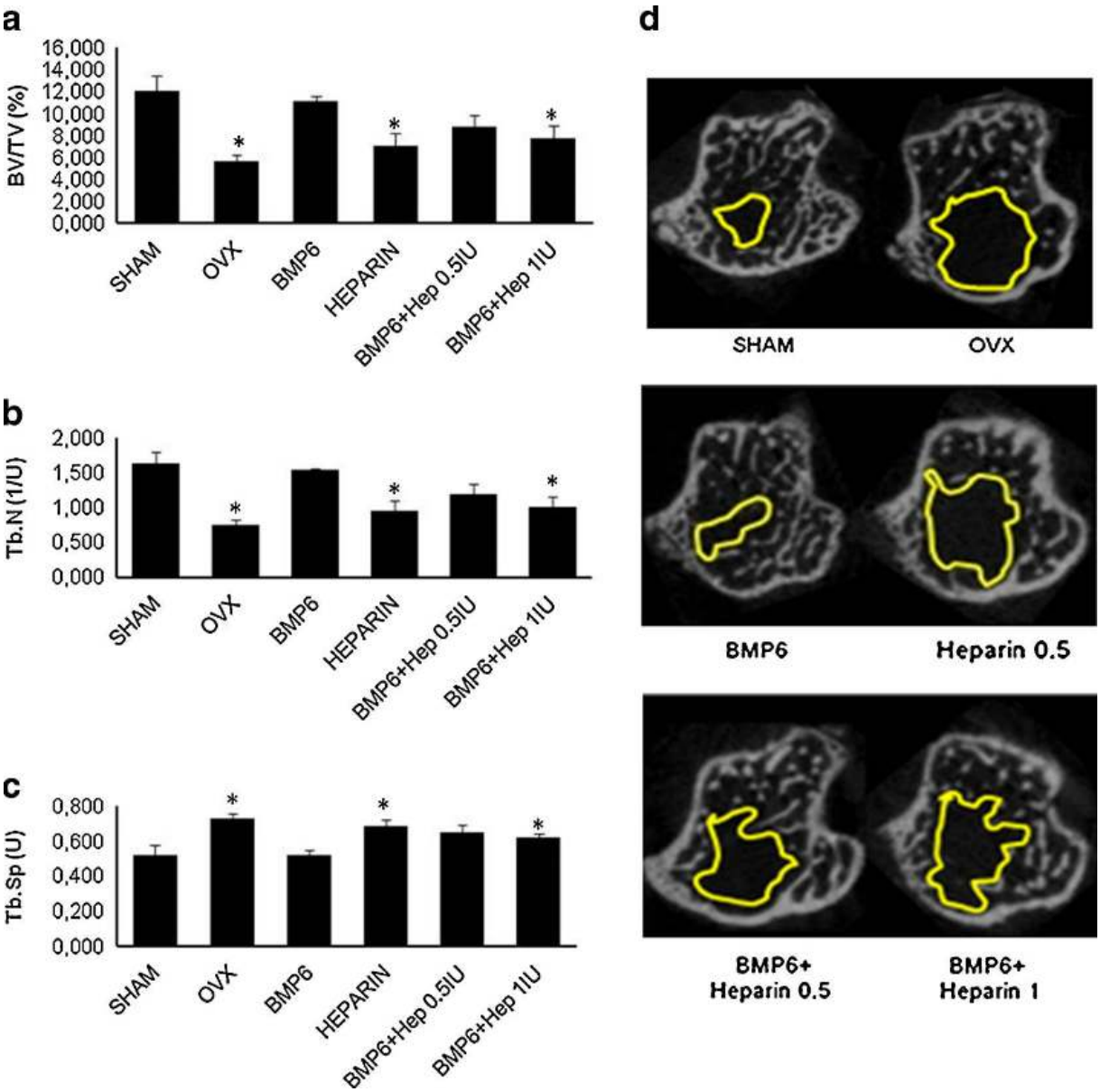


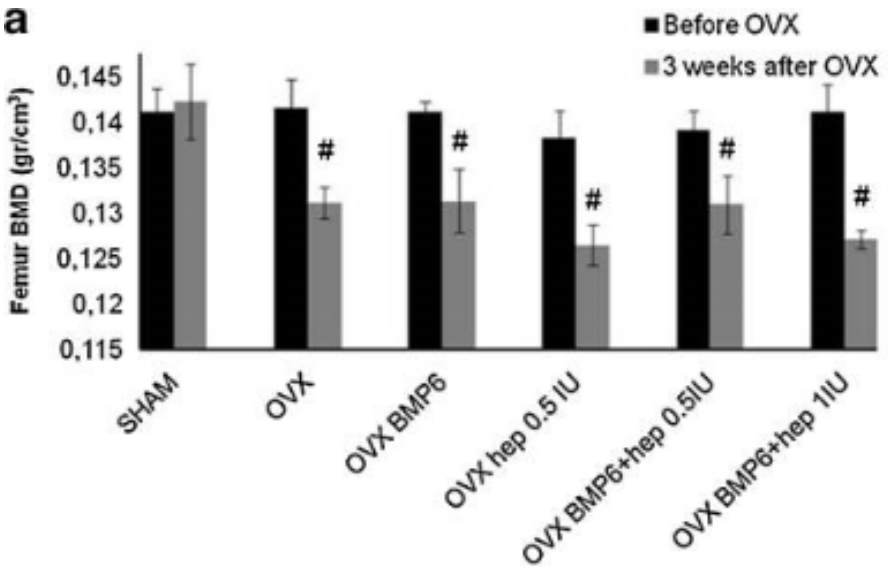
Fig. 5

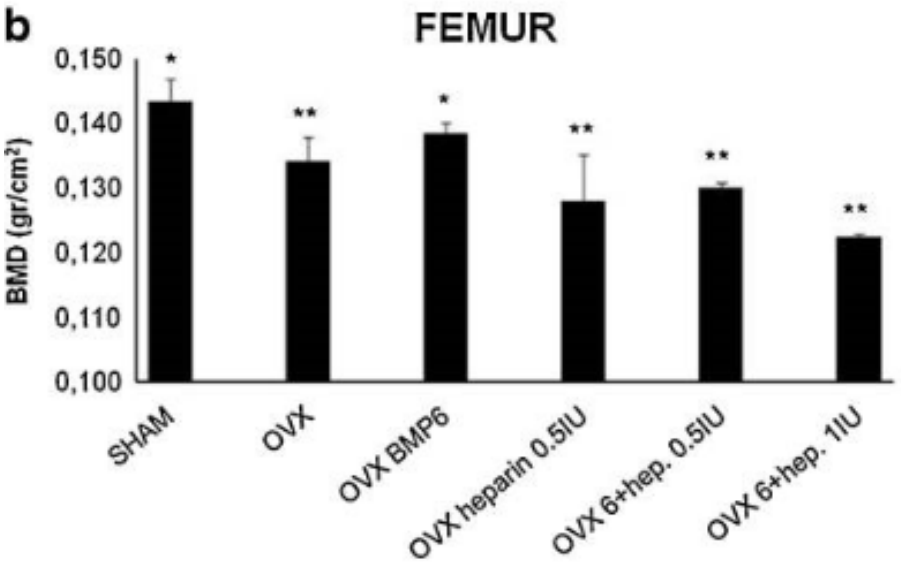
µCT analysis of the proximal tibiae of 4-month old OVX mice treated with BMP6 (10 µg/kg), heparin (0.5 IU/g), BMP6 (10 µg/kg) + heparin (0.5 IU/g) and BMP6 (10 µg/kg) + heparin (1 IU/g). One IU/g heparin with BMP6 significantly decreased the bone volume/tissue volume (BV/TV) (A) and the trabecular number (Tb.N.) (B) and increased the trabecular separation (Tb.Sp.) (C) compared to BMP6 treated mice. µCT images of tibiae from all groups of mice (D). Space encircled by the yellow line indicates the bone marrow cavity without trabeculi. *P<0.05 vs BMP6.



Graph 2

DEXA analysis of tibiae and femurs of 4-month old mice prior and 3 weeks after OVX (A), followed by 7 weeks of therapy (B, C). Mice were treated with BMP6 (10 μg/kg), heparin (0.5 IU/g), BMP6 (10 μg/kg) + heparin (0.5 IU/g) and BMP6 (10 μg/kg) + heparin (1 IU/g). One IU/g heparin with BMP6 significantly decreased BMD values in both femur (B) and tibiae (C) compared to BMP6 treated mice. Decreased BMD values were also seen in mice treated with lower dose of heparin (0.5 IU/g) and BMP6 (B). #P<0.05 vs before OVX, *P<0.05 vs OVX, **P<0.05 vs OVX BMP6.


a
Before OVX
3 weeks after OVX
Femur BMD (gr/cm²)
0,145
0,14
0,135
0,13
0,125
0,12
0,115
#
SHAM
OVX
OVX BMP6
OVX hep 0.5 IU
OVX BMP6+hep 0.5IU
OVX BMP6+hep 1IU


b
FEMUR
BMD (gr/cm²)
0,150
0,140
0,130
0,120
0,110
0,100
*
**
SHAM
OVX
OVX BMP6
OVX heparin 0.5IU
OVX 6+hep. 0.5IU
OVX 6+hep. 1IU

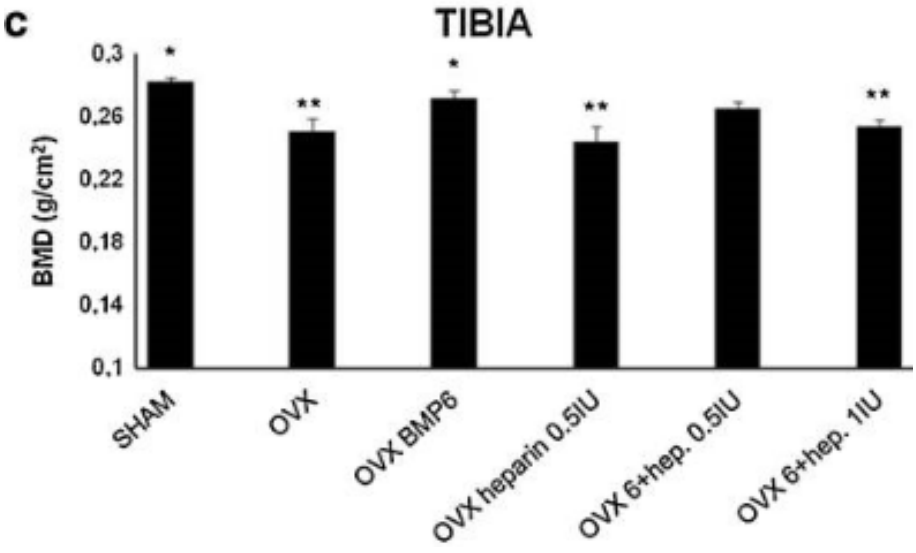

c
TIBIA
BMD (g/cm²)
0,3
0,26
0,22
0,18
0,14
0,1
*
**
SHAM
OVX
OVX BMP6
OVX heparin 0.5IU
OVX 6+hep. 0.5IU
OVX 6+hep. 1IU

Table 1

Sequences of primers used for gene expression analysis.

Target gene	Name	Forward 5′–3′	Reverse 3′–5′
ALP	Alkaline phosphatase	GATCATTCCCACGTTTTCAC	TGCGGGCTTGTGGGACCTGC
OC	Osteocalcin	CAAGTCCCACACAGCAGCTT	AAAGCCGAGCTGCCAGAGTT
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table 2

Ex vivo and *in vivo* correction of coagulation parameters after BMP6 treatment. Values are presented as a mean (n = 5 mice per group). s = second; mg/dl = milligram per deciliter; NM = not measurable; APTT = activated partial thromboplastin time; PT = Prothrombin time; TT = thrombin time; F XI = coagulation factor XI; FBG = fibrinogen.

Group	*n*	PT (%)	APTT (s)	TT (s)	FBG (mg/dl)	F XI
Ex vivo						
Control	5	84.6	NM	NM	85	NM
BMP6 1 µg	5	86.3	22.23	69.95	80	0.25
Hepzyme	5	80.0	21.60	78.30	80	0.21
In vivo						
Control	5	87.7	89.33	93.80	70	<0.025
BMP6 10 µg/kg	5	93.3	21.26	70.60	90	0.26