BMP-7 in Combination with Estrogen Enhances Bone Formation in a Fracture Callus Explant Culture

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In postmenopausal women, estrogen withdrawal results in decrease in bone density or osteoporosis. Osteoporosis leads to fracture and retards bone-healing response. Bone morphogenetic protein-7 (BMP-7), a member of the transforming-growth factor-β (TGF-β) superfamily, has been shown as a promising candidate that stimulates bone growth in its application to fracture healing. The purpose of this study was to determine whether BMP-7 could enhance bone formation in the absence of estrogen. Female rats underwent a controlled closed fracture at the midshaft of the right femur. The callus tissues were harvested from the fracture site eight days following the fracture, and were cultured in serum-free media. The explanted callus tissues were then treated with BMP-7, estrogen (E2) or both. We assessed bone formation by measuring alkaline phosphatase (AP) activity, expression of an osteogenic transcription factor, Runx-related transcription factor-2 (Runx2), production of nitric oxide (NO), and calcium mineralization. Supplementation of serum-free cultures with BMP-7 alone increased cell proliferation by twofold, caused a 6.5-fold increase in AP activity, and enhanced calcium mineralization after 48 h. Moreover, BMP-7 in combination with E2 caused a 8.2-fold increase in the AP activity. Runx2 protein expression was increased following stimulation with BMP-7 and E2. Interestingly, E2 induced the amount of NO production by twofold, whereas BMP-7 did not, either alone or with E2. Thus, BMP-7 could enhance early and late markers of bone fracture healing in callus explant cultures, except for NO. BMP-7 could be a promising growth factor in the treatment of fractures as a consequence of osteoporosis.

Keywords: osteoporosis; callus explant culture; BMP-7; estrogen; alkaline phosphatase; fracture healing

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Osteoporosis is an increasing health issue as global populations age, particularly in postmenopausal women where estrogen withdrawal results in further decreases in bone density. Osteoporotic bones are characterised by bone fragility with diminished bone density, micro-architectural deterioration, increased frequency of fracture and a retarded fracture healing process. A lack of estrogen has been shown to retard bone healing in several animal studies of spinal fusion and long bone fracture (Namkung-Matthai et al. 2001; Moazzaz et al. 2005). Hormone replacement therapy (HRT) could improve fracture healing, but because of potential side effects of HRT, alternatives to enhance osteoporotic fracture healing are eagerly sought.

Bone morphogenetic protein-7 (BMP-7), a member of the transforming growth factor-β (TGF-β) superfamily, is involved in proliferation, differentiation, and metabolism in a variety of tissues. Our work using BMP-7 in an osteoporotic rat spinal-fusion model has shown that recombinant human BMP-7 in a higher dose than normally need-
ed, administered locally at the site of spinal fusion, enhanced the bony fusion process (Lu et al. 2008). Further, BMP-7 application to vertebral bodies in an ovarectomised ovine model resulted in improved vertebral mechanical strength and histomorphometric parameters of osteopenic vertebra compared to controls (Phillips et al. 2006). BMP-7 may be a candidate for localized clinical use in cases of fracture in the absence of estrogen; however the molecular events involved in these models remain unknown. Complete understanding of the role BMP plays in bone formation will potentially lead to improved fracture healing care (Kloen et al. 2003).

Nitric oxide (NO) is a signalling molecule synthesized from L-arginine by nitric oxide synthases (NOSs) (Diwan et al. 2000) that is expressed during fracture healing. Riancho et al. (1995) showed that NO, which was produced constitutively by osteoblasts, may act as an autocrine stimulator of osteoblast growth. Koyama et al. (2000) demonstrated that slow release NO donors stimulate osteoblast growth and
differentiation. Interestingly, NOS inhibitors abolished the protective effect of oestrogen on bone in ovariectomised rats (Wimalawansa 2008b).

The callus explant model is an approach to study the effects of biochemical and morphological substances during the bone formation process, whilst keeping the number of animals to a minimum (Namkung-Matthai et al. 2000). An estrogen deficient explant callus culture was created using serum free cell culture media. Using this culture system we aimed to determine whether BMP-7 could enhance early bone formation markers Runx2 and alkaline phosphatase, as well as extracellular matrix mineralisation in serum free callus explant cultures. In addition, the presence or absence of fracture healing modulator molecule nitric oxide was evaluated.

Materials and Methods

Animal model

Eight female Sprague-Dawley rats weighing 300-350 g were used. The animal experiments were approved by the Animal Care and Ethics Committee (ACEC) of the University of New South Wales (UNSW) and are in accordance with guidelines of the National Health and Medical Research Council of Australia for animal research. Surgical procedures were performed under halothane inhalation anaesthesia. The right femur underwent a controlled closed fracture of the midshaft using a three point pressure “fracture creator” as described previously (Namkung-Matthai et al. 2001). There was no post-operative splinting. The rats were euthanased, and the callus was harvested from the fracture site eight days following the fracture.

Explant culture

The protocol of callus explant culture used was as previously described (Namkung-Matthai et al. 2001). Briefly, the freshly excised callus tissues from 8 rats were diced into small fragments of 10 mm, rinsed in PBS and approximately equal amounts of tissues were cultured in 48-well plates in phenol-red free α-MEM (Invitrogen, Mt Waverly, Australia) with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin for 24 h. Following equilibration with serum free medium (α-MEM + 1% penicillin/streptomycin), the explants were treated with either 17β-oestriadiol (oestrogen, E2; Sigma-Aldrich, St Louis, MO) at concentrations ranging from $10^{-5}$ to $10^{-11}$ M (dose response), or with single administrations of 100 ng/ml purified BMP-7 alone (Stryker-Biotech, Andover, MA), E2 at $10^{-7}$ M alone, or with E2 ($10^{-7}$ M) and BMP-7 together (100 ng/ml). Control cultures were with serum-free medium alone. Cultures were analysed at 0, 24 and 48 h.

Alkaline phosphatase activity

Alkaline phosphatase (AP) activity was determined in both conditioned media and in homogenized explant tissues using a modification of the method by Bradford (1976). Conditioned media and explant cultures were harvested at each time point. The tissue was homogenized with liquid nitrogen using a mortar and pestle, with added PBS buffer containing 0.1% Triton X-100. The homogenized tissues were then incubated for 30 minutes at 37°C, with the AP substrate p-nitrophenyl phosphate (p-NP, Sigma) at 2.5 µg/ml. Levels of the substrate metabolite p-nitrophenol (p-NP) produced were measured using a spectrophotometer (410 nm) and concentrations were determined by comparison with a p-NP standard curve. Protein concentrations in homogenized tissue extracts were estimated using BCA reagents (Pierce Biotechnology Inc., Rockford, IL). AP activity in explant and conditioned medium samples is expressed as nanomoles of p-NP generated per microgram of total cellular protein per minute.

Determination of DNA content

Total DNA content in homogenized explant tissues was estimated using a fluorescence method (Kim et al. 1988) following incubation with Hoechst 33258 dye (Sigma-Aldrich St. Louis, MO, USA). A standard curve was prepared from known concentrations of Herring Sperm DNA (Promega Corporation, Madison, WI). Sample fluorescence was determined using a HITACHI ModelF-4000 Spectrofluorometer (Hitachi Ltd., Tokyo, Japan) with emission at 475 nm and excitation at 350 nm. The DNA concentration from each sample was interpolated from the standard curve and then multiplied by the dilution factor.

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Fig. 1. Effect of BMP-7 and Estrogen on DNA concentration in rat callus explant cultures. Explant cultures stimulated with BMP-7 or estrogen (E2) and combination (BMP-7 + E2) for 48 h showing an increase in DNA content ($n = 4$, mean ± SEM, *$p < 0.05$, **$p < 0.01$).
**Histology**

At each time point, cultured explants were washed with PBS and fixed in 10% neutral buffered formalin for 2 hours. The tissues were then embedded in paraffin, cut for serial sectioning (4 µm) and mounted on Super Plus slides (Lomb Scientific, Australia). Tissue sections were stained with Hematoxylin-Eosin (H&E), Alcian blue and Masson’s Trichrome for general histological examination.

The number of cells in the cultured explant the cells were counted on 3 individual slides stained with H&E for each individual sample, using a 40x light microscope objective. For each slide the cells were counted in 6 randomly selected areas of the cultured tissue by three researchers independently.

**Alizarin Red S staining**

For alizarin red S (sodium alizarin sulphonate) staining, a 40 mM alizarin red S solution was prepared in distilled water and the pH was adjusted to 4.1-4.3 using 0.5% ammonium hydroxide. Tissue sections mounted on slides were deparaffinized and hydrated through a graded ethanol series. After washing in water, samples were stained with Alizarin red S solution at room temperature under moderate shaking for 10-15 min, rinsed in water, and washed in PBS for 15 min to remove unincorporated excess dye. Alizarin red S-positive staining represent mineralized matrix, and cells were identified as red colouration (Puchtler et al. 1969).

**Immunofluorescence staining of Runx2**

For immunohistochemical staining of Runt-related transcription...
factor-2 (Runx2), sections were deparaffinized and re-hydrated through a graded ethanol series and equilibrated in Tris-HCl buffer. Non-specific antigens were blocked with 5% normal goat serum for 30 minutes, following which slides were incubated with rabbit anti-Runx2 polyclonal antibody (1:100, Santa Cruz Biotechnology Inc., CA) at room temperature for 1 hour. After several washes with PBS, cells were incubated with Rhodamine-conjugated goat anti-rabbit immunoglobulins (1:200, Chemicon, Billerica, MA), for 1 h at room temperature in the dark. Negative controls were treated in a similar manner but with the omission of the primary antibody and were included in every experiment. Samples were counter-stained with 4′-6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich). Mounted with cover slips, the slides were visualized using a fluorescence microscope. All the staining procedures were repeated in three independent experiments.

**Nitrite concentration**

Nitrite, a stable end product of nitric oxide, was detected and quantitated in the culture media using the Greiss assay. Assays were performed on 100 µL of sample medium derived from a culture of explants. Once the Greiss reagent and sample were mixed, the absorbance was read 550 nm within 30 minutes. A standard curve consisting of nitrite (NaNO2) was used to quantify nitrite production. The results were normalized to the DNA concentration of explanted cultures (Namkung-Matthai et al. 2001).

**Statistics**

Data analysis among multiple experimental groups was performed with analysis of variance (ANOVA) and unpaired Student’s t test using SigmaStat software (SPSS Inc., San Rafael, CA). Differences were considered significant at \( p \leq 0.05 \).

**Results**

**BMP-7 Increases Cell Proliferation**

The DNA content of explant cultures at all time points was estimated. The treatment of estrogen-deficient callus explants with BMP-7 alone significantly increased DNA content after 48 h (\( p \leq 0.01 \), Fig. 1). DNA content following by E2 stimulation was less markedly increased at 48 h when compared with controls (\( p < 0.05 \)). The treatment of cultures with both BMP-7 and E2 resulted in the greatest (\( p < 0.01 \)) enhancement of DNA content after 48 h (Fig. 1).

Similar results were obtained from counting cells on H&E stained tissue sections (Fig. 2a), with slight increases at 24 h with E2 or BMP-7 treatment alone and more significant increases with combined BMP-7 and E2 compared to controls (\( p < 0.05 \), Fig. 2a). After 48 h, a 2-fold increase was detected with BMP-7 treatment either alone or with E2 when compared to control (\( p < 0.01 \)), while E2 alone increased cell numbers by approximately 48% (\( p \leq 0.05 \)). Examination of the H&E stained histological sections showed that the increased number of cells were osteoblast-like, present in the areas rich in capillary vessels within the callus explants (Fig. 2b). Under high magnification these osteoblast-like cells appeared to be large with a basophilic or granular appearing cytoplasm and a large spherical nucleus (Fig. 2b, B and D). This was most marked following treatment with BMP-7 alone, E2 alone (data not shown), or BMP-7 with E2 after 48 h (Fig. 2b, D).

**BMP-7 Increases Alkaline Phosphatase Activity in an Estrogen Deficient Environment**

The AP activity in control callus explant cultures at 48 h was low (<10 nmol p-NP/min/µg protein). Increasing concentrations of E2 caused a dose-dependent increase in AP activity at the 24 h time point between \( 10^{-11} \) and \( 10^{-5} \) M (Fig. 3a). A significant (6-fold) increase in AP activity was recorded in cultures stimulated with E2 at \( 10^{-11} \) M at 48 h, which reached a plateau for greater concentrations (Fig. 3a). A similar increase in AP activity was observed with BMP-7.
alone at 48 h (Fig. 3b). There were no significant differences between the BMP and E2 groups (Fig. 3b), indicating that the effects of E2 and BMP-7 were not additive, but that both agents could induce AP activity by up to 4-fold of control group.

**BMP-7 increases calcified mineralization of extracellular matrix**

Alizarin red S staining was performed to determine the pattern of mineralization within cultured explants under light microscopy. In 24-h cultures treated with either BMP-7 or E2 alone, well-mineralized matrix was observed only at the surface edge of the explant (Fig. 4, B and C); whereas a combination of both agents resulted in generalised, diffuse calcium deposition across the sections (Fig. 4, D). At 48 h Alizarin red S-positive stained tissue extended further into the explant with BMP-7 or E2 alone, but was more developed and extensive when both agents were combined (Fig. 4H). No Alizarin red S-positive staining was seen in control cultures (Fig. 4, A and E).

Masson’s Trichrome staining, which identifies newly formed bone tissue (red stain), showed a similar pattern. Untreated controls at either 24 h or 48 h showed almost no red staining (Fig. 5, A and E). Only a small amount of newly formed bone matrix was present within tissue sections stimulated with BMP-7 or E2 alone at 24 h (Fig. 5, B and C). However treatment with either E2 or BMP7 for 48 h (Fig. 5, F & G), and a combination of both BMP-7 and E2 at 24 h and 48 h (Fig. 5, D and H) led to large amounts of mineralized extracellular matrix deposition through the explant sections.

**Runx2 expression**

Immunostaining revealed that cells expressing Runx2
were present in all sections from both untreated and treated explant cultures, and that the protein was located in both the cell nucleus and cytoplasm (Fig. 6). The intensity and the number of Runx2 immunopositive stained cells revealed no observable differences in BMP-7, E2 or combination treated explants compared to untreated controls at 24 h (data not shown). However, the number of Runx2 immunopositive stained cells was increased in BMP-7-treated explants with (Fig. 6, H) or without (not shown) E2 at 48 h.

Nitric Oxide Production

Treatment of rat callus explant cultures with E2 at various concentrations resulted in a dose-dependent increase in nitrite production, indicating stimulation of NO synthase (Fig. 7a). The basal level of nitrite production in the explant cultures was not enhanced by stimulation with BMP-7 over a 48-h period (Fig. 7b). However addition of E2 to the cultures at $10^{-7}$M resulted in a 6-fold increase in the amount of NO, compared to controls at 24 h, which then decreased at the 48-h time point (Fig. 7a). Interestingly, treatment with BMP-7 and E2 together did not enhance NO production compared to cultures treated with either BMP-7 or E2 alone (Fig. 7b). Indeed the presence of BMP-7 appeared to counteract the nitrite production compared to that of cultures containing E2 alone.

Discussion

Osteoporosis is characterised by increased frequency of fractures and retarded bone healing. The proliferation and differentiation of osteogenic cells in the fracture callus plays an important role in bone re-union. This study utilises fracture callus explant cultures in serum free medium to produce estrogen-deficient conditions stimulating osteoporosis and investigate the potential for BMP-7 to influence osteogenic cell marker expression in an osteoporotic environment.

Treatment with BMP-7 induced the stimulation of cell proliferation and protein markers of new bone tissue in the absence of estrogen over a 48-h period. These data support the findings in an in vivo ovarectomised rat model where spinal fusion was accelerated following localised application of BMP-7 putty to the fusion site (Lu et al. 2008). BMP-7 was also effective in stimulating osteogenesis in a foetal rat calvaria cell (Yeh and Lee 1999) and enhanced osteogenic differentiation in mouse foetal bones in vitro (Haaijman et al. 1999).

Fracture healing is a complex process that involves a multitude of early events including nuclear proteins that regulate osteoblast differentiation and function, as well as later events organising calcified bony tissue. Here we have shown that the callus tissue treated with BMP-7 showed elevated AP activity and enhanced expression of Runx2, as well as evidence of extracellular matrix mineralisation characteristic of bone tissue. Alkaline phosphatase is one of the early markers of bone tissue growth (Eastell and Hannon 2008) and a number of BMP family members induce AP
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activity in vitro, including BMP-7 (Knippenberg et al. 2006). Runx2, a member of the Runt domain family of transcription factors, has been described as the master gene of osteogenic cell differentiation and bone matrix production (Karsenty 2008), possibly the earliest and most powerful molecular determinant of osteoblast differentiation. Activation of Runx2 increases osteocalcin and collagen I gene expression (Nakashima et al. 2002), which are also markers of bone formation.

The histological analysis in this study clearly showed that BMP-7 induced the formation of large amounts of new bony tissue in the absence of estrogen, which was mineralised throughout the callus after a 48-h period. The effect of BMP-7 was further enhanced when in combination with estrogen in the callus cultures, suggesting the two agents co-operated in promoting osteogenic growth. Whilst estrogen treatment alone could induce dose dependent stimulation of both AP and NO production, tissue mineralization occurred only on the periphery of the callus explant without BMP-7 and cell proliferation was not as elevated. A previous report has shown that estrogen administration improved fracture healing in an ovariectomised rat model, where new tissue resembled healthy (non osteoporotic) bone and investigations showed that the estrogen suppressed genes associated with osteoblast and osteoclast activity (Stuermer et al. 2010).

NO is expressed during fracture healing (Diwan et al. 2000), in which estrogen caused the dose-dependent stimulation of NO production. NO itself is a stimulator of AP activity, an early marker of bone formation (Namkung-Matthai et al. 2000). Estrogen plays a significant role in the regulation of bone function and fracture healing (Lorenzo 2003). It has been reported that circulating NO products are significantly lower in postmenopausal women, and estrogen supplementation restores their levels (Das 2002). NO donor therapy has been suggested as an attractive alternative to estrogen therapy to prevent and treat osteoporosis (Wimalawansa 2008a). Indeed, Armour et al showed that the anabolic effects of oestrogen on bone formation were blunted in eNOS-knockout mice (Armour et al. 2001). Interestingly, the results from the current study showed that NO concentrations were increased by stimulating with E2 but not by BMP-7. This is consistent with previous studies that estrogen stimulates NOS activity and mRNA expression levels in endothelial cells (Hayashi et al. 1995), and osteoblasts (Armour and Ralston 1998). The NO pathway may play a regulatory role in both the anti bone resorptive and anabolic effects of oestrogen on bone (van’t Hof and Ralston 2001). Further studies are needed to identify how and which specific signalling pathways affect the bone healing process in estrogen deficient environments.

Conclusion

This study demonstrated that in serum free callus explant cultures, markers of bone tissue formation were elevated by stimulating with BMP-7, suggesting its potential therapeutic application in promoting bone fracture healing in osteoporosis. Both early and late bone markers were further enhanced when BMP-7 was used in combination with estrogen. Estrogen alone was less effective in stimulating osteogenic markers in vitro, however its ability to stimulate fracture healing may be mediated by NO. Furthermore, BMP-7 stimulated the osteogenic pathway mediated by transcription factor Runx2, but not by NO. Further studies are needed to identify the underling mechanism, by which BMP-7 and estrogen counteract the effects of transcription factor Runx2 or NO, and the signaling pathways that are involved during the bone healing process.

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References


