Osteogenic actions of the osteogenic growth peptide on bovine marrow mesenchymal stromal cells in culture

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ABSTRACTS: The osteogenic growth peptide (OGP) regulates the differentiation of marrow mesenchymal stem cells derived from human and rodent cell lines into osteoblasts. Whether OGP directly regulates the bovine marrow mesenchymal stem cells differentiating into osteoblasts remains unknown. In this study, we evaluated the effects of OGP on the growth and differentiation of bovine marrow mesenchymal stem cells in culture. Our results showed that OGP promoted osteogenic differentiation of the bovine stem cells. OGP increased alkaline phosphatase (ALP) activity and mineralized nodule formation, and stimulated osteoblast-specific mRNA expression of Osteocalcin (BGP). On the other hand, OGP dose-dependently stimulated the expression of endothelial nitric oxide synthases. These results show for the first time a direct osteogenic effect of OGP on bovine marrow stromal cells in culture, which could be mediated by induction of endothelial nitric oxide synthases.

Keywords: osteogenic growth peptide; bovine; marrow stromal cells; eNOS

The osteogenic growth peptide (OGP) has attracted considerable clinical interest as a bone anabolic agent and a hematopoietic stimulator. It is present in mammalian serum in micromolar concentrations, increases bone formation and trabecular bone density, and stimulates fracture healing when administered to mice and rats (Bab and Chorev, 2002). In cultures of osteoblastic and other bone marrow mesenchymal stem cells (MSC), derived from human and rodents, OGP regulates proliferation, alkaline phosphatase (ALP) activity and matrix mineralization via an autocrine/paracrine mechanism (Spreafico et al., 2006).

MSC contribute to bone, cartilage, and fat tissue repair and regeneration, and have broader differentiation ability (Damoulis et al., 2007). MSC from different species show different biochemical characteristics. It has been demonstrated that OGP directly regulates rat MSC and favors differentiation into osteoblasts over adipocytes (Chen et al., 2007). Robinson D et al. have found that the main OGP activity in marrow systems derived from both rabbit and humans is a marked stimulation of ALP activity and matrix mineralization. However, in rabbit-derived cell culture this enhancement is accompanied by a reciprocal inhibition of proliferation. On the other hand, human cells show a concomitant increase in both parameters (Robinson et al., 1995). Considering this, it could be concluded that differentiation processes and mechanisms induced by OGP that are valid for MSC from one species may be not necessarily valid for others.

Cows are considered large-animal experimental models, and as such offer numerous advantages for making progress in the clinical application of OGP to medicine, especially in musculoskeletal health problems (Bosnakovski et al., 2004). The majority of research concerning OGP induced MSC differentiation has been done on rodents or humans (Jaquiery et al., 2005; Dai et al., 2007; Omae et al., 2007). However, research concerning ruminant animals such as cattle is rare.

Supported by the National Natural Sciences Foundation of China (No. 30771623), and National Supporting Project (No. 2006BAD04A05).

Additionally, various signal-transduction mechanisms have been shown to be particularly relevant to the growth and development of MSC (Damoulis et al., 2007). Nitric oxide (NO) generated by the nitric oxide synthase (NOS) is a signaling molecule constitutively produced in bone cells, which increases in response to several stimuli such as sex hormones, mechanical strain and proinflammatory cytokines (Armour et al., 2001). Endothelial NOS is the primary isoform expressed in bone cells (Bosnakovski et al., 2004; Jaquiery et al., 2005; Omae et al., 2007) and may have a significant role in trabecular bone growth and remodeling (Taylor et al., 2006). Damoulis et al. (2007) have demonstrated that the eNOS pathway plays a role in the osteogenic differentiation of MSC cultured in three-dimensional silk scaffolds. It is still unknown whether the eNOS pathway plays a role in the differentiation of bovine MSC regulated by OGP.

The objective of this study is to evaluate the effects of OGP on the growth and differentiation of bovine MSC in culture. In addition, we have also investigated possible mechanisms of action of OGP, such as the expression of eNOS.

MATERIAL AND METHODS

Harvest and isolation of bovine MSC

Bone marrow was obtained from the femurs of one day old Holstein Friesian calves. The marrow was drawn into 25-ml syringes containing 1 000 units of heparin after aseptic preparation of the harvesting fields and infiltration with local anesthetic.

Bovine MSC were isolated by modification of methods previously described (Mastrogiacomo et al., 2001; Holzer et al., 2002). Briefly, one volume of bone marrow sample was mixed with two volumes of phosphate-buffered saline (PBS), and the mixture was centrifuged at 1 000g for 4 minutes. The supernatant was discarded, and the pellet was washed two more times with Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Island, NY, USA). After determination of the cell viability and number using trypan blue staining, and after lysis of erythrocytes by the addition of 4% acetic acid, $5 \times 10^4/cm^2$ nucleated cells were plated in T-75 culture flasks in DMEM (low glucose) containing penicillin G 100 IU/ml, streptomycin 100 µg/ml, amphotericin B 0.25 µg/ml, HEPES 2.4 mg/ml, $NaHCO_3$ 3.7 mg/ml, and 10% fetal bovine serum (FCS, Lanhai Biological Technology Co. Ltd., China), hereafter termed standard growth medium. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After three days of culturing, the non-adherent cells were removed by changing the culture media. The adherent cells were grown to 90% confluence to obtain samples defined here as passage zero (P0) cells.

MSCs at P0 were washed with PBS and detached by incubation with 0.25% trypsin-EDTA for 2 to 3 min at 37°C. Complete medium was added to inactivate the trypsin. The cells were centrifuged at 1 000g for 3 min, the medium was removed, and the cells were resuspended in 2 to 5 ml of complete medium. The cells were counted using a hemacytometer and then plated as P1 in 75-cm^2 flasks at a density of 5×10^3 cells/cm^2. The medium was changed every 2 to 3 days, and the cells were passaged every 3 to 4 days. All cells used for the experiments were P3.

Semiquantitative RT-PCR

The total RNA of cells incubated in standard growth medium in the presence of 10^{-8}M dexamethasone (Dex, Sigma, USA), 10mM β-glycerophosphate (β-GP, Merck, USA) and 50 µg/ml ascorbic acid (Tianxin Co., China) or PBS for three weeks was extracted using Trizol reagent (Invitrogen, USA) following the recommendations of the manufacturer. The synthesis of complementary DNA (cDNA) and PCR amplification were carried out by a two-step method (Table 1). RT-PCR reagents were purchased from Sino-American Biotechnology Co. (China). According to the manufacturer's instructions, 10 µg of the isolated RNA was subjected to reverse transcription in 30 µl of reaction mixture containing 2 µl RT-PCR enzyme mix, 1.5 µl 20× buffer (Mg^{2+} free), 3 µl $MgCl_2$ (25mM), 1.2 µl dNTPs (2.5mM) and 20 pmol of each sense and antisense primers specific for the genes of interest and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All custom primers were also obtained from Sino-American Biotechnology Co. (China).

Methylthiazoletetrazolium (MTT) assay

The cells were plated at a density of 4×10^3 cells/well in 96-well plates in standard growth medium for 24 hours. They were then washed twice in PBS and

Table 1. Species primers for PCR application listed with expected fragments size and optimal annealing temperature

Gene	Primers	Annealing temperature (°C)	Fragment (bp)	GenBank No.
BGP	Ps: ATGAGAACCCCAATGCTGC	55	303	X53699
	Pa: CTAGACTGGGCCGTAGAAG			
GAPDH	Ps: CCTTCATTGACCTTCACTACATGGTCTA	60	127	U85042
	Pa: TGGAAGATGGTGATGGCCTTTCCATTG			

treated with OGP (10^{-11}, 10^{-9}, 10^{-7}M) or vehicle (PBS) for 72 hours. The measurement of cell viability was carried out using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. MTT (5 mg/ml, 20 µl/well, Sigma, USA) was then added to the cell cultures for a further four hours. The supernatant was discarded, followed by the addition of dimethyl sulfoxide (DMSO, 150 µl/well, Sigma, USA) and agitation for 10 min to dissolve the crystal completely. The OD values were measured at a wavelength of 490 nm on an ELISA reader (OG3022A, Nanjin, China).

ALP assay

The cells were plated at a density of 1×10^4 cells/well in 24-well plates in standard growth medium supplemented with OGP (10^{-11}, 10^{-9}, 10^{-7}M) or vehicle (PBS) for 14 days. The medium was changed every three or four days. ALP activity was assayed as previously described (Molinuevo et al., 2004). Briefly, the cell layer was washed with PBS and solubilized in 0.5 ml 0.1% Triton X-100. Aliquots of the total cell extract were used for protein determination by the Bradford (Bradford, 1976) technique. Measurement of ALP activity was carried out by spectrophotometric determination of initial rates of hydrolysis of para-nitrophenyl-phosphate to para-nitrophenol at 37°C for 10 min. The production of para-nitrophenol was determined by absorbance at 405 nm.

Mineralized bone matrix formation assay

The cells were plated at a density of 1×10^4 cells / well in 24-well plates in standard growth medium supplemented with OGP (10^{-11}, 10^{-9}, 10^{-7}M) or vehicle (PBS) for 21 days. The medium was changed every three or four days. Mineralized nodules were stained with Alizarin red S (Ueno, 2001). The dye taken up by extracellular calcium deposits was dissolved in 0.1N sodium hydroxide and quantified spectrophotometrically at 548 nm.

BGP protein assay

The cells were plated at a density of 1×10^4 cells/well in 24-well plates in standard growth medium supplemented with OGP (10^{-11}, 10^{-9}, 10^{-7}M) or vehicle (PBS) for 14 days. BGP ELISA kits were used to detect BGP protein levels. Briefly, cells were treated with various concentrations of OGP for the indicated times. The culture medium was then collected and measured for BGP. These samples were placed in 96-well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material with wash buffer (50mM Tris, 200mM NaCl, and 0.2% Tween 20), horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results are presented as the percentage of change in activity compared to the untreated control.

Western blot analysis of eNOS

Cells treated with OGP (10^{-11}, 10^{-9}, 10^{-7}M) or vehicle (PBS) for the indicated times were lysed and the protein concentrations determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). For western blotting, 50 mg of total cell lysates were subjected to SDS-polyacrylamide gel electro-

phoresis. The protein was transferred to polyvinylidene difluoride membranes using transfer buffer (50mM Tris, 190mM glycin, and 10% methanol) at 100 V for two hours. The membranes were incubated with blocking buffer (50mM Tris, 200mM NaCl, 0.2% Tween 20, and 3% bovine serum albumin) overnight at 4°C. After washing three times with wash buffer (blocking buffer without 3% bovine serum albumin) for 10 min each, the blot was incubated with polyclonal antibodies against eNOS for 2–15 h, followed by horseradish peroxidase-labeled secondary antibody for one hour. The membranes were washed again, and detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham, USA).

Statistical analysis

Data were analyzed with SPSS 10.0 statistical software (SPSS 10.0 for windows). The one-way-ANOVA and post hoc multiple comparison-LSD tests were performed. Each value represents mean ± standard error (SE) in all the figures. A significant level of 0.05 was defined for the differences between the groups.

RESULTS

Cell culture

Spindles bipolar to polygonal fibroblastic cells attached to the flask were observed after four days of culturing, at the first changing of the medium. After five to seven days, the cell culture reached confluence and, at the places where cells overlapped, exhibited more cuboidal morphology (Figure 1). Cells in the first or further passages had a uniform monolayer and needed approximately three days to reach confluence. Upon treatment with DEX after three weeks, mineralized nodules were formed and BGP mRNA was expressed (Figure 2).

The effect of OGP on the proliferation of bovine MSC

We first determined the effect of OGP on the cell proliferation of bovine MSC by the MTT assay. Our results showed that OGP showed a bell-shaped dose-response curve and was responsible for significant effects on bovine MSC proliferation at the concentrations of 10^{-9}M after 72 h treatment (Figure 3).

OGP regulates bovine MSC differentiation and mineralization

To examine the effect of OGP on bovine MSC differentiation, ALP activity and BGP protein was evaluated. Figure 4A shows that OGP significantly stimulated ALP in a bell shape dose-dependent manner, with a maximum at the concentration of 10^{-9}M (vs control, $P < 0.01$). In addition, OGP also dose-dependently stimulated the production of BGP protein (vs control, $P < 0.01$) (Figure 4B). Furthermore, the effect of OGP on the extent to which the bovine MSC mineralized the matrix was assessed after three weeks. Figure 4C show that OGP at 10^{-9}M (vs control, $P < 0.01$) significantly stimulated calcium deposition in mineralized nodules as assessed by Alizarin red S staining.

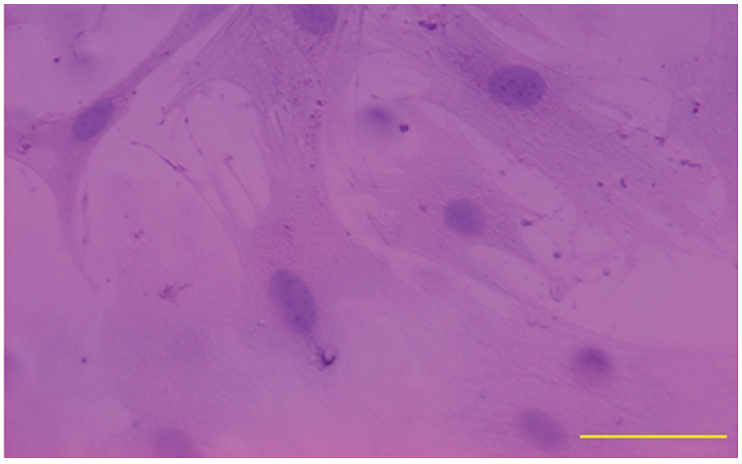

Figure 1. Morphology of bovine mesenchymal stem cells. Scale bar 100 μm

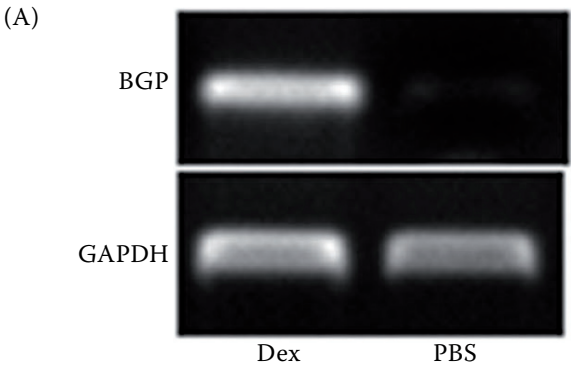

Figure 2. The effect of DEX on differentiation of bovine MSC. (**A**) RT-PCR analysis of the expression of an osteogenic specific gene, BGP, on day 21. Primers for GAPDH were used as a control. (**B**) Bovine MSC form calcificated nodules during treatment with DEX for three weeks. Scale bar 1 000 μm

eNOS expression is regulated by OGP

NO is known to be implicated in the metabolism of bones, especially as a mediator of cytokine effects on the remodeling of bone tissue. Thus, we investigated whether OGP had any effect on the expression of eNOS in bovine MSC, as evaluated by western immunoblot.

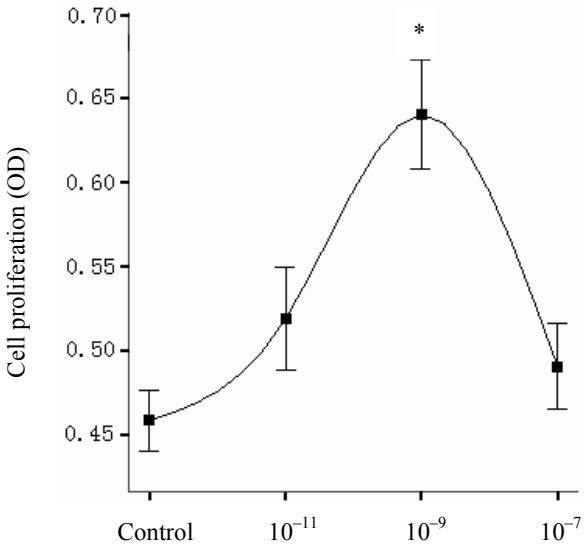

Figure 3. The effect of OGP on the cell proliferation of bovine MSC. Adhering cells that were incubated with different concentrations (M) of OGP for 72 h. Cell proliferation was determined by MTT. Each value is the mean ± SE of six independent experiments. The asterisk indicates a significant difference between control and OGP-treated groups (*$P < 0.01$)

The results indicated that OGP caused a significant increase in eNOS protein levels in bovine MSC (Figure 4D). To further examine the role of eNOS in OGP-induced cell differentiation, bovine MSC were pretreated with a eNOS inhibitor, 20mM L-NAME, for 4 h, then co-treated with 10^{-9} M OGP and the inhibitor for the indicated times. Addition of the inhibitor did not change ALP activity and BGP secretion, but prevented OGP-induced cell differentiation (Figure 5). Therefore, the differentiation of OGP-induced bovine MSC into osteogenic lineages may operate by an eNOS-dependent pathway.

DISCUSSION

MSC from different species exhibit different biochemical characteristics. The number of MSC found in canine bone marrow is fourfold higher than in humans. Canine MSC have a much faster doubling time, but lose osteogenic potential after the second passage. Furthermore, canine MSC do not form calcificated nodules during osteogenesis *in vitro*, which has been observed in human and rat MSC (Kadiyala, 1997). During osteogenesis induced by DEX, feline MSC was not observed to express ALP (Martin et al., 2002), and human MSC did not express BGP mRNA (Ringe et al., 2002). DEX is a potent inducer of BGP and ALP, which have been reported to be typical markers of osteogenic differentiation in other species (Kadiyala et al., 1997;

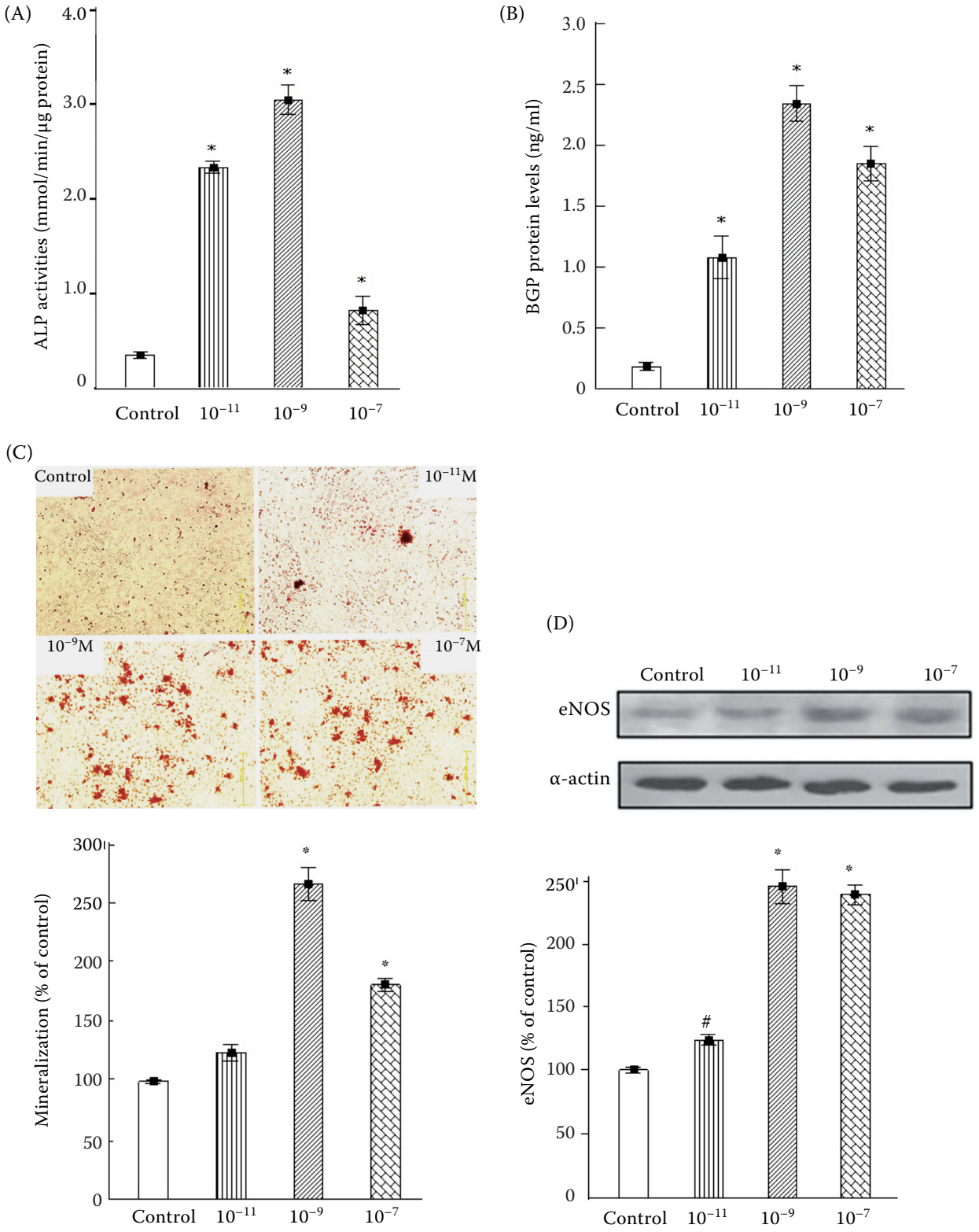

Figure 4. OGP increased ALP activity (**A**), BGP production (**B**), mineralization (**C**) and eNOS production (**D**). ALP activity was assessed by the conversion of *p*-nitrophenyl phosphate in 0.1M $NaHCO_3$-Na_2CO_3 buffer, pH 10, containing 2mM $MgSO_4$ and 0.1% Triton. The amount of BGP in culture medium was assessed by a BGP ELISA kit. The degree of mineralization was assayed by Alizarin Red S staining. The production of eNOS was determined by Western blot analysis. Each value is the mean ± SE of five independent experiments. The difference between control and OGP-treated groups ($^{\#}P < 0.05$, $^{*}P < 0.01$)

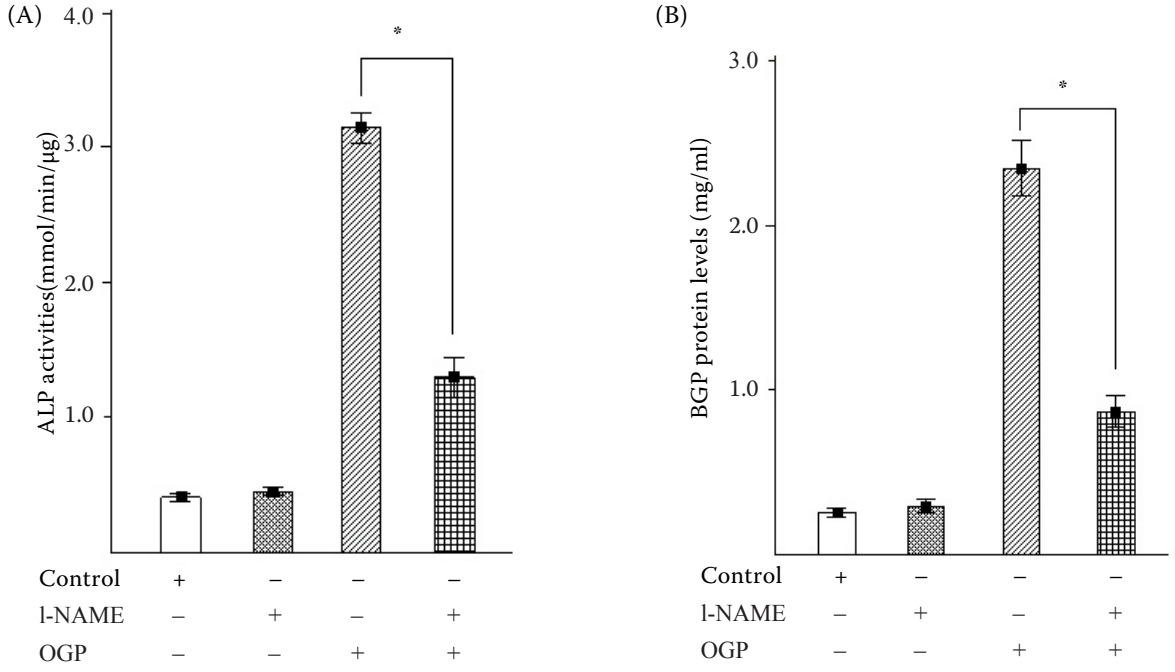

Figure 5. L-NAME inhibited the induction of ALP upregulation by OGP (**A**) and BGP stimulation (**B**). For A and B, cells were pretreated with or without L-NAME for 24 h, and then 10^{-9}M OGP was added for the indicated times. ALP activity (two week treatment) and BGP levels (three week treatment) were determined as described above. Each value is the mean ± SE of five independent experiments. The asterisk indicates a significant difference between the control and test groups (*$P < 0.01$)

Jaiswal et al., 1997; Worster et al., 2000; Martin et al., 2002). In this study we have found that bovine MSC possessed spindles, bipolar to polygonal fibrobiastic in shape, and exhibited a similar form to MSC isolated from other species (Haynesworth et al., 1992; Dennis and Caplan, 1993). Also, during osteogenesis induced by DEX, bovine MSC could form calcificated nodules and express BGP mRNA.

In *in vivo* rodent models, OGP enhances bone formation by increasing trabecular bone mass (Bab et al., 1992). Moreover, it induces a balanced increase in whole blood cell counts and overall bone marrow cells, and improves engraftment of bone marrow transplants (Gurevitch et al., 1996). Furthermore, OGP enhances, under unstable mechanical conditions only, the rate of fracture healing and accelerates callus formation in rabbits (Sun and Ashhurst, 1998).

OGP increases bone formation and trabecular bone density and regulates proliferation and osteogenic maturation of MSC (Robinson et al., 1995; Greenberg et al., 1997). It has been shown to be a mitogen for MSC and, furthermore, the simultaneous treatment of OGP and basic fibroblast growth factor (bFGF) results in a three-fold stimulatory effect as compared to OGP alone on MSC (Guido et al., 2002). It has been well documented that the stimulation of MSC to differentiate into osteoblasts is characterized mainly by increased expression of ALP and BGP (Rahnert et al., 2008). Chen et al have found that OGP promotes osteogenesis and inhibits adipogenesis as evidenced by a decrease in adipocyte numbers and an increase in ALP activity and mineralized nodule numbers. These effects appear to be at the level of commitment rather than at the level of maturation (Chen et al., 2007). Our results indicate that the presence of OGP (10^{-9}M) causes a significant increase in proliferation, AKP activity, BGP production and mineralization in bovine MSC.

NO released by the endothelial isoform of NOS (eNOS) could promote normal bone development, as mice lacking eNOS display defective in osteoblast maturation, resulting in delayed skeletal development (Aguirre et al., 2001). In the absence of eNOS, mice have a unique phenotype involving multiple organ systems. With regard to the skeleton, eNOS (–/–) animals have delayed skel-

etal maturation (Aguirre et al., 2001; Armour et al., 2001; Afzal et al., 2004). Recently, a biphasic mode of action of NO has been suggested, with lower doses supporting, and higher doses inhibiting, osteoclast and osteoblast function (Gyurkoa et al., 2005). Several groups have shown that eNOS is not only widely expressed on a constitutive basis in osteoblasts, osteocytes and osteoclasts, but also in bone marrow mesenchymal stem cells (Rob et al., 2001). Damoulis et al. have revealed that levels of eNOS increased significantly when MSC, microencapsulated in silk scaffolds, differentiated into osteoblasts (Damoulis et al., 2007).

In the present study, we demonstrate that OGP increases the expression of eNOS in a dose-dependent manner, with maximal stimulation at 10^{-9}M. This effect was found to closely correspond to the OGP-induced increase in MSC proliferation, AKP activity, and BGP protein levels, suggesting a link between NO production and bovine MSC growth or differentiation. Furthermore, OGP-mediated increase of ALP activity and BGP production is almost completely inhibited by L-NAME. These results support the hypothesis that eNOS plays an important role in OGP-mediated cell differentiation in bovine MSC.

In conclusion, these results show for the first time a direct osteogenic effect of OGP on bovine MSC in culture and suggest that this effect could be partly mediated by eNOS.

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Received: 2008–07–18
Accepted after corrections: 2008–09–15

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