Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Effects of intermittent negative pressure on osteogenesis in human bone marrow-derived stroma cells*

Zhi YANG[1], Miao LIU[1], Yin-gang ZHANG[†‡1], Xiong GUO[2], Peng XU[3]

([1]*Department of Orthopaedics, the First Affiliated Hospital, School of Medicine;* [2]*MOE Key Laboratory of Environment and Genes Related to Diseases, Institute of Environmental and Endemic Diseases;* [3]*School of Materials Science and Engineering, Xi'an Jiaotong University, Xi'an 710061, China*)

[†]E-mail: xgcgfd@126.com

Received Aug. 7, 2008; Revision accepted Oct. 11, 2008; Crosschecked Dec. 9, 2008

Abstract: Objective: We investigated the effects of intermittent negative pressure on osteogenesis in human bone marrow-derived stroma cells (BMSCs) in vitro. Methods: BMSCs were isolated from adult marrow donated by a hip osteoarthritis patient with prosthetic replacement and cultured in vitro. The third passage cells were divided into negative pressure treatment group and control group. The treatment group was induced by negative pressure intermittently (pressure: 50 kPa, 30 min/times, and twice daily). The control was cultured in conventional condition. The osteogenesis of BMSCs was examined by phase-contrast microscopy, the determination of alkaline phosphatase (ALP) activities, and the immunohistochemistry of collagen type I. The mRNA expressions of osteoprotegerin (OPG) and osteoprotegerin ligand (OPGL) in BMSCs were analyzed by real-time polymerase chain reaction (PCR). Results: BMSCs showed a typical appearance of osteoblast after 2 weeks of induction by intermittent negative pressure, the activity of ALP increased significantly, and the expression of collagen type I was positive. In the treatment group, the mRNA expression of OPG increased significantly (P<0.05) and the mRNA expression of OPGL decreased significantly (P<0.05) after 2 weeks, compared with the control. Conclusion: Intermittent negative pressure could promote osteogenesis in human BMSCs in vitro.

Key words: Negative pressure, Bone marrow-derived stroma cells (BMSCs), Osteogenesis
doi:10.1631/jzus.B0820240 **Document code:** A **CLC number:** R687

INTRODUCTION

Topical negative pressure (TNP) therapy that can stimulate angiogenesis and local blood flow is used routinely in clinical practice to treat and manage a variety of wounds (Heller *et al*., 2006; Kanakaris *et al*., 2007; Thompson and Marks, 2007; Loos *et al*., 2007), but very few reports have explored the effects of negative pressure on bone tissues. To date, only a few investigators have studied the responses of open fracture and lower-extremity wounds with exposed bone to negative pressure (Greer *et al*., 1998; DeFranzo *et al*., 2001). These studies provided evidence that their successes were due to reduction of tissue edema, thereby decreasing wound surface area and the stimulation of profuse granulation tissue formation, and covering bone. One of the major effects of negative pressure on the wound occurs to the mechanical stress mediated at the foam-wound interface. Mechanical stress has direct effects on angiogenesis (Ichioka *et al*., 1997). Angiogenesis in skeletogenesis, of which the importance was first recognized more than 50 years ago, plays an active role in bone formation and remodeling. In view of these characteristic features, we presume that negative pressure could potentially promote bone growth.

Bone marrow-derived stroma cells (BMSCs) are the main source of osteoblasts and they play a very

‡ Corresponding author
* Project (No. 20070421123) supported by the Postdoctoral Science Foundation of China

important role during the bone tissue repair process. In addition, they are the commonly used seed cells for bone tissue engineering. Shang *et al*.(2001) have successfully repaired bone defects by osteogenically induced BMSCs in a sheep model. BMSCs have a wide range of sources, a high degree of self-renewal capacity, and multiple differentiation potential, and they are also easier to be cultured in vitro. Therefore, as an initial step to better understand the effects of negative pressure on bone tissues, we examined the responsiveness of BMSCs to negative pressure and determined the mechanism of negative pressure on bone remodeling at the cellular and molecular levels.

MATERIALS AND METHODS

Cell culture

BMSCs were isolated from adult bone marrow donated by a hip osteoarthritis patient with prosthetic replacement, cultured in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose supplemented with 0.1 g/ml fetal bovine serum (FBS) and 0.01 g/ml penicillin-streptomycin, and maintained in a humidified incubator at 37 °C with 5% CO_2. Medium was replaced every 3~4 d. Passage 3 cells were used. Phase contrast light microscopy was used to observe cells morphology on Days 7 and 14.

Application of intermittent negative pressure

The CO_2 incubator (ASTEC, Japan) was technically customized to produce the intermittent negative pressure. A pressure sensor was installed in the incubator to precisely control and adjust the level of the negative pressure between −60 and 0 kPa. Passage 3 cells were plated in 6-well tissue culture plates at a density of 3×10^4 cells/well, and were divided into negative pressure treatment group and control group. After overnight attachment, in the treatment group, the medium was discarded, the cap of 6-well plates was opened, and negative pressure was intermittently administered for 2 weeks (pressure: 50 kPa, 30 min/times, and twice daily). Medium was replenished after each induction.

Cell growth assay

BMSCs were plated onto 60-mm dishes, in triplicate, in the presence of expansion medium, and at 1500 cells/cm^2. The cells were incubated at negative pressure as indicated above. At the end of assays on Days 1~7, the cells were rinsed with phosphate buffered saline (PBS), detached with trypsin-ethylene diamine tetraacetic acid (EDTA), and then counted with a hemacytometer.

Quantitative alkaline phosphatase (ALP) activity measurement

ALP activity was quantified using ALP reagent (RaiChem, CA, USA) on Day 14 and expressed as mU/ml, normalized to DNA concentration. The assay was evaluated using thawed-lysed samples. DNA content was determined using the fluorescent DNA quantification kit (Sigma, MO, USA) and expressed in μg DNA per ml of sample.

Immunocytochemical staining

Mouse monoclonal antibodies against human recombinant collagen types I (Sigma, MO, USA) were used in immunocytochemical staining. On Day 14, cells were fixed with 0.04 g/ml paraformaldehyde in PBS for 20 min and then rinsed with PBS. Thereafter, immunocytochemistry was performed using avidin-biotin-peroxidase method according to the manufacturer's recommendation of the kit (Sigma, MO, USA).

Real-time reverse-transcript polymerase chain reaction (RT-PCR)

Cells collected on Day 14 and at 3 different time points following cessation of negative pressure (1, 2, and 3 d) were lysed and total RNA was isolated using Tri-Reagent (Sigma, MO, USA). The ratio of absorbance at 260 nm to that at 280 nm was measured for verification of the purity of RNA. The total RNA was used for cDNA synthesis by reverse transcriptase with a cDNA synthesis kit (Sigma, MO, USA). Quantitative real-time RT-PCR was conducted to determine the mRNA levels of osteoprotegerin (OPG) and osteoprotegerin ligand (OPGL) with the following specific PCR primers: OPG, 5′-AATCAACTC AAAAATGTGGAATAGATGT-3′ (forward), 5′-GC GTAAACTTTGTAGGAACAGCAA-3′ (reverse); OPGL, 5′-GTGCAAAAGGAATTACAACATATC GT-3′ (forward), 5′-AACCATGAGCCATCCACC AT-3′ (reverse); β-actin, 5′-AGGCACCAGGGCG TGAT-3′ (forward), 5′-TCGTCCCAGTTGGTGA CGAT-3′ (reverse).

Statistical analysis

Statistical analyses of gene expression and ALP activity were performed using Student's *t*-test with significant difference at $P<0.05$.

RESULTS

The cell proliferation speed of the treatment group was lower than that of the control group (Fig.1). The cell morph changed from a shuttle shape to megagon with some prominences in the treatment group, but remained the shuttle shape in the control. On Day 14 of the experiment, positive immunostaining for collagen type I was observed in the treatment group, but not in the control group (Fig.2). The ALP activity of the treatment group [(14.56±1.83) mU/ml] increased by approximately 130% as compared to that of the control group [(6.26±1.29) mU/ml] ($P<0.01$).

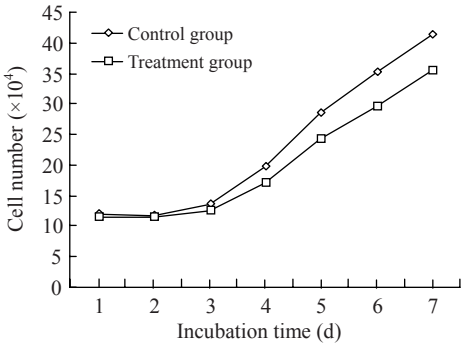

Fig.1 Negative pressure inhibit BMSC proliferation. BMSCs were plated in 60-mm dishes in expansion medium at 1500 cells/cm^2 and then allowed to proliferate on Days 1~7

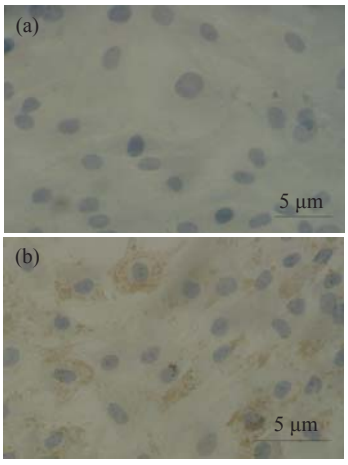

Fig.2 Immunohistochemical staining for collagen type I of BMSCs (steptavidin biotin complex method). (a) Control group; (b) Treatment group

The OPG mRNA level significantly increased after two weeks of application of negative pressure, but the OPGL mRNA level decreased. The OPGL/OPG mRNA ratio significantly deceased in the treatment group by approximately 70%, compared to the control group ($P<0.05$) (Fig.3).

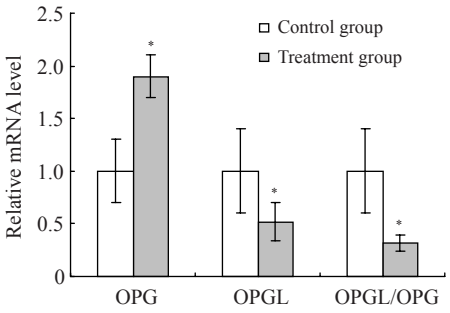

Fig.3 The change in OPG mRNA, OPGL mRNA, and OPGL/OPG mRNA ratio after 2 weeks of intermittent negative pressure exposure. RNA isolation is performed immediately after completion of negative pressure

n=6 for all groups; $^*P<0.05$, significant difference between the treatment group and the control group

One day after the exposure to 2-week negative pressure, the OPGL/OPG mRNA ratio was significantly deceased by approximately 60%, compared to the control ($P<0.05$) (Fig.4). However, two days after the exposure to 2-week negative pressure, the OPGL/OPG mRNA ratio was no longer significantly different from that of the control ($P>0.05$), indicating that negative pressure may contribute to the OPGL/OPG mRNA ratio difference.

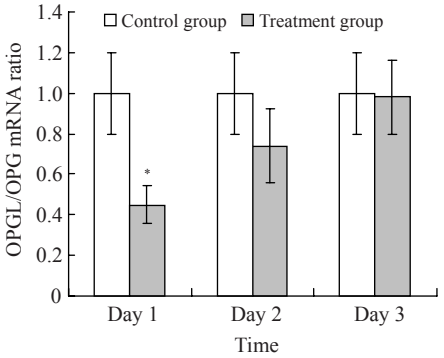

Fig.4 Time course study of OPGL/OPG mRNA ratio after 2 weeks of intermittent negative pressure exposure. RNA isolation is performed 1, 2, and 3 d after completion of negative pressure

n=6 for all groups; $^*P<0.05$, significant difference between the treatment group and the control group

DISCUSSION

The unique features of TNP therapy, which are thought to contribute to an optimized wound environment, include interstitial fluid flow and exudate management, oedema reduction, stimulation of angiogenesis and local blood flow, and effects on wound perfusion, growth factor, cytokine expression, and cellular activity, all leading to enhancing granulation tissue formation and improving wound-healing parameters (Petrie *et al*., 2003; Banwell and Musgrave, 2004; Potter *et al*., 2008). However, its effect on bone tissues is poorly understood. In this study, the effects of negative pressure on osteogenesis in human BMSCs were investigated. We examined the effects of negative pressure on ALP activity and the expression of collagen type I. Collagen type I is a major organic constituent of the extracellular matrix of bone. ALP, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in the calcification of bone. In the present study, ALP activity and the expression of collagen type I increased significantly in the presence of negative pressure through 14 d of culture.

Researchers speculate that the impacts of negative pressure to BMSCs were the results of joint mechanisms where the BMSCs were subjected to the stretch stress as well as hypoxia in short term. Various mechanical stimuli have been studied as in vitro model systems and have been shown to act through numerous signaling pathways to promote osteogenic differentiation of BMSCs and osteoblast activities (Mauney *et al*., 2004; Tang *et al*., 2006). Wiesmann *et al*.(2006) have reported that mechanical stimulation could promote the expression of collagen type I and osteonectin in MSCs. It has been shown that mechanical strain decreases osteoclastogenesis through OPGL and OPG signaling (Rubin *et al*., 2000; Kim *et al*., 2006). Rubin *et al*.(2000) have shown that substrate deformation resulted in a drop in OPGL/OPG mRNA ratio due to a significant change in OPGL expression but not OPG. Our results indicate that the decrease in OPGL/OPG mRNA ratio is due to both a decrease in OPGL mRNA level and an increase in OPG mRNA level. The drop in OPGL/OPG mRNA ratio was lost with time after cessation of negative pressure. This suggests that the effect of negative pressure on OPGL/OPG mRNA signaling is transient and may be cumulative. Lennon *et al*.(2001) have reported that cultures of rat MSCs at low oxygen tension (5% O_2) had a greater number of cells and generated more bone when implanted into rats compared with cultures grown at 21% O_2. Cell hypoxia in the negative pressure treatment group is believed to be a major stimulus for the expression of hypoxia-inducible factor-1 (HIF-1). HIF is an αβ-heterodimeric transcription factor that mediates the adaptation of many multicellular organisms to molecular oxygen, and is essential for the maintenance of cellular oxygen homeostasis. The expression of the α subunits is elevated during hypoxia and is maintained at low levels in most cells under normoxic conditions (Brown and Wilson, 2004; Lee *et al*., 2004). Towler (2007) has reported that strategies that promote HIF signaling in osteoblasts may augment bone formation and accelerate fracture repair. The HIF pathway activation promoted bone formation by enhancing angiogenesis (Wang *et al*., 2007). Osteogenesis and angiogenesis are tightly coupled during bone formation and repair. Activation of the HIF-pathway by negative pressure triggers hypoxia-responsive gene expression, such as vascular endothelial growth factor (VEGF), which plays a critical role in angiogenesis and promotes the osteogenesis in human BMSCs cultured in vitro by our experimental model system. In this study, the effects of negative pressure on OPGL and OPG were quantified at the mRNA level, and the expression of OPG in human BMSCs could be promoted by intermittent negative pressure, while the expression of OPGL could be inhibited in the same cells. We confirm that the change in OPGL/OPG mRNA ratio observed and the activation of the HIF-pathway in response to negative pressure directly affect the osteogenesis and osteoclastogenesis, which is consistent with previous studies. In conclusion, the effects of negative pressure on osteogenesis in human BMSCs were the results of joint mechanisms where the BMSCs were subjected to the stretch stress as well as hypoxia in short term.

In summary, the application of intermittent negative pressure induced a significant increase of OPG and collagen type I synthesis and a decrease of OPGL mRNA expression in cultured human BMSCs. This study has yielded important evidence on bone remodeling by our experimental model system, and the findings strongly suggest that intermittent negative

pressure could promote the osteogenesis in human BMSCs cultured in vitro.

This study revealed the mechanism of intermittent negative pressure to bone cells at the cellular and molecular levels. However, the expression levels of OPG mRNA and OPGL mRNA in BMSCs and what about the experimental model system applied to a scaffold fabricated by BMSCs and coral in bone tissue engineering need to be studied further, when BMSCs are induced at different intensity of negative pressure and different intermittent stimulating time. Future research could help to identify the optimal negative pressure intensity, frequency and the effect of stimulating bone regeneration in bone tissue engineering by the model system. That would lay a foundation to the research on the effect of negative pressure to bone tissues and bone tissue engineering.

References

Banwell, P.E., Musgrave, M., 2004. Topical negative pressure therapy: mechanisms and indications. *Int. Wound J.*, **1**(2):95-106. [doi:10.1111/j.1742-4801.2004.00031.x]

Brown, J.M., Wilson, W.R., 2004. Exploiting tumour hypoxia in cancer treatment. *Nat. Rev. Cancer*, **4**(6):437-447. [doi:10.1038/nrc1367]

DeFranzo, A.J., Argenta, L.C., Marks, M.W., Molnar, J.A., David, L.R., Webb, L.X., Ward, W.G., Teasdall, R.G., 2001. The use of vacuum-assisted closure therapy for the treatment of lower-extremity wounds with exposed bone. *Plast. Reconstr. Surg.*, **108**(5):1184-1191. [doi:10.1097/00006534-200110000-00013]

Greer, S., Kasabian, A., Thorne, C., Borud, L., Sims, C.D., Hsu, M., 1998. The use of a subatmospheric pressure dressing to salvage a Gustilo grade IIIB open tibial fracture with concomitant osteomyelitis to avert a free flap. *Ann. Plast. Surg.*, **41**(6):687. [doi:10.1097/00000637-199812000-00022]

Heller, L., Levin, S.L., Butler, C.E., 2006. Management of abdominal wound dehiscence using vacuum assisted closure in patients with compromised healing. *Am. J. Surg.*, **191**(2):165-172. [doi:10.1016/j.amjsurg.2005.09.003]

Ichioka, S., Shibata, M., Kosaki, K., Sato, Y., Harii, K., Kamiya, A., 1997. Effects of shear stress on wound-healing angiogenesis in the rabbit ear chamber. *J. Surg. Res.*, **72**(1):29-35. [doi:10.1006/jsre.1997.5170]

Kanakaris, N.K., Thanasas, C., Keramaris, N., Kontakis, G., Granick, M.S., Giannoudis, P.V., 2007. The efficacy of negative pressure wound therapy in the management of lower extremity trauma: review of clinical evidence. *Injury*, **38**(5):8-17. [doi:10.1016/j.injury.2007.10.029]

Kim, C.H., You, L., Yellowley, C.E., Jacobs, C.R., 2006. Oscillatory fluid flow-induced shear stress decreases osteoclastogenesis through RANKL and OPG signaling. *Bone*, **39**(5):1043-1047. [doi:10.1016/j.bone.2006.05.017]

Lee, J.W., Bae, S.H., Jeong, J.W., Kim, S.H., Kim, K.W., 2004. Hypoxia-inducible factor (HIF-1) alpha: its protein stability and biological functions. *Exp. Mol. Med.*, **36**(1):1-12.

Lennon, D.P., Edmison, J.M., Caplan, A.I., 2001. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J. Cell. Physiol.*, **187**(3):345-355. [doi:10.1002/jcp.1081]

Loos, B., Kopp, J., Hohenberger, W., Horch, R.E., 2007. Post-malignancy irradiation ulcers with exposed alloplastic materials can be salvaged with topical negative pressure therapy (TNP). *Eur. J. Surg. Oncol.*, **33**(7):920-925. [doi:10.1016/j.ejso.2006.12.018]

Mauney, J.R., Sjostorm, S., Blumberg, J., Horan, R., O′Leary, J.P., Vunjak-Novakovic, G., Volloch, V., Kaplan, D.L., 2004. Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. *Calcif. Tissue Int.*, **74**(5):458-468. [doi:10.1007/s00223-003-0104-7]

Petrie, N., Potter, M., Banwell, P., 2003. The management of lower extremity wounds using topical negative pressure. *Int. J. Lower Extrem. Wounds*, **2**(4):198-206. [doi:10.1177/1534734603261067]

Potter, M.J., Banwell, P., Baldwin, C., Clayton, E., Irvine, L., Linge, C., Grobbelaar, A.O., Sanders, R., Dye, J.F., 2008. In vitro optimisation of topical negative pressure regimens for angiogenesis into synthetic dermal replacements. *Burns*, **34**(2):164-174. [doi:10.1016/j.burns.2007.06.020]

Rubin, J., Murphy, T., Nanes, M.S., Fan, X., 2000. Mechanical strain inhibits expression of osteoclast differentiation factor by murine stromal cells. *Am. J. Physiol.: Cell Physiol.*, **278**(6):1126-1132.

Shang, Q., Wang, Z., Liu, W., Shi, Y., Cui, L., Cao, Y., 2001. Tissue-engineered bone repair of sheep cranial defects with autologous bone marrow stromal cells. *J. Craniofac. Surg.*, **12**(6):586-593. [doi:10.1097/00001665-200111000-00017]

Tang, L., Lin, Z., Li, Y.M., 2006. Effects of different magnitudes of mechanical strain on osteoblasts in vitro. *Biochem. Biophys. Res. Commun.*, **344**(1):122-128. [doi:10.1016/j.bbrc.2006.03.123]

Thompson, J.T., Marks, M.W., 2007. Negative pressure wound therapy. *Clin. Plast. Surg.*, **34**(4):673-684. [doi:10.1016/j.cps.2007.07.005]

Towler, D.A., 2007. Vascular biology and bone formation: hints from HIF. *J. Clin. Invest.*, **117**(6):1477-1480. [doi:10.1172/JCI32518]

Wang, Y., Wan, C., Gilbert, S.R., Clemens, T.L., 2007. Oxygen sensing and osteogenesis. *Ann. N. Y. Acad. Sci.*, **1117**(1):1-11. [doi:10.1196/annals.1402.049]

Wiesmann, A., Buhring, H.J., Mentrup, C., Wiesmann, H.P., 2006. Decreased CD90 expression in human mesenchymal stem cells by applying mechanical stimulation. *Head Face Med.*, **2**(1):8. [doi:10.1186/1746-160X-2-8]