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Original article

Effect of the same mechanical loading on osteogenesis and osteoclastogenesis *in vitro*

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ABSTRACT

Purpose: To investigate the influence of the same mechanical loading on osteogenesis and osteoclastogenesis *in vitro*.

Methods: Primary osteoblasts, bone marrow-derived mesenchymal stem cells (BMSCs, cultured in osteoinductive medium) and RAW264.7 cells cultured in osteoclast inductive medium were all subjected to a 1000 μstrain (με) at 1 Hz cyclic mechanical stretch for 30 min (twice a day).

Results: After mechanical stimulation, the alkaline phosphatase (ALP) activity, osteocalcin protein level of the osteoblasts and BMSCs were all enhanced, and the mRNA levels of ALP and collagen type I increased. Additionally, extracellular-deposited calcium of both osteoblasts and BMSCs increased. At the same time, the activity of secreted tartrate-resistant acid phosphatase, the number of tartrate-resistant acid phosphatase-positive multinucleated cells, matrix metalloproteinase-9 protein levels of RAW264.7 cells and the extracellular calcium solvency all decreased.

Conclusion: The results demonstrated that 1000 με cyclic mechanical loading enhanced osteoblasts activity, promoted osteoblastic differentiation of BMSCs and restrained osteoclastogenesis of RAW264.7 cells *in vitro*.

1. Introduction

Osteoclasts and osteoblasts are the key differentiated cell types that are responsible for bone resorption, and formation respectively.[1] Osteoclasts, a kind of large multinuclear cell, are derived from hematopoietic stem cells in bone marrow, blood and spleen.[2–5] Osteoblasts are progeny of resident bone marrow-derived mesenchymal stem cells (BMSCs) or bone marrow stromal cells.[1,6]

The balance between bone formation and bone resorption maintains adequate bone mass for each individual's habitual physical activity.[7] Mechanical loading has great influence on the skeleton because it can stimulate a series of intra- and intercellular events to inhibit bone resorption or promote bone formation.[8] Cyclic mechanical loading, at physiologically-relevant magnitudes, can enhance bone formation significantly.[9]

It is well-known that mechanical loading can affect or enhance osteoblastic differentiation of mesenchymal stem cells and osteoblasts' activity. For example, cyclic mechanical stretching (4000 με elongation at 1 Hz frequency) applied on BMSCs and C3H10T1/2 cells, resulted in more osteoblasts.[10] Cyclic-stretching at 500–1500 με promoted osteoblasts proliferation and increased collagen synthesis.[11] Some studies indicate that mechanical loading also influences bone marrow-derived pre-osteoclast-like cell activity, osteoclastogenesis and bone-resorption activity of osteoclasts.[12–15] *In vivo*, osteoblasts (pre-osteoblasts) and osteoclasts (pre-osteoclasts), in the area of bone formation or resorption, are stimulated by nearly the same mechanical loading. Therefore, stimulating osteoblasts (pre-osteoblasts), BMSCs and osteoclasts

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(pre-osteoclasts) with the same mechanical loading *in vitro* is an important means to study the effect of mechanical stimulation on bones.

However, the effect of the same mechanical loading on differentiation and activity of osteoblasts, BMSCs, and osteoclasts (pre-osteoclasts) *in vitro* is not fully understood. Few researchers have applied the same mechanical loading on these cells at the same time and investigated the effects of the same mechanical loading on these cells. In this study, in order to better understand how the same mechanical loading affects osteoblasts, BMSCs and pre-osteoclasts, we investigated the effects of the same mechanical loading on primary osteoblasts activity, osteoblastic differentiation of BMSCs and osteoclastic differentiation of RAW264.7 cells (pre-osteoclast-like cells).

2. Materials and methods

2.1. Animals, reagents and instruments

Mouse monocyte/macrophage cell line RAW264.7 cells were obtained from the cell culture center of Peking Union Medical College, China. C57BL/6 mouse and Wistar rats were obtained from the experimental animal center of Beijing. α-MEM medium, fetal calf serum, streptomycin, penicillin, collagenase I, trypsin were purchased from Invitrogen. Receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) were purchased from PeproTech Inc; tartrate-resistant acid phosphatase (TRAP) Staining Kit, ALP Activity Assay Kit, TRAP Activity Assay Kit, BCA Protein Assay Kit and Calcium Assay Kit were from Nanjing Jiancheng Biotechnology Co., Ltd, China; dexamethasone, β-glycerophosphate and ascorbic acid 2-phosphate were from Sigma–Aldrich; PVDF membranes were from Millipore; rabbit anti osteocalcin, goat anti matrix metalloproteinase-9 (MMP-9), RIPA lysis buffer and enhanced chemiluminescence detection kit were from Santa Cruz Biotechnology Co., Ltd; Quant Script RT Kit and SYBR Green I PCR Mix were from Beijing Cowin Biotechnology Co., Ltd, China.

2.2. Cells culture

Primary osteoblasts were isolated from neonatal C57BL/6 mice calvarial bone by a sequential enzyme digestion based on methods previously described.[16,17] Briefly, after the Wistar rats calvarial bone was minced with scissors and rinsed with PBS, the bone fragments were digested in 0.25% trypsin for 10 min one time, and in 0.1% collagenase I for 30 min 3 times. The first digestate was discarded, and the cell suspension was centrifuged twice. Then the collected cells were grown in α-MEM medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every three days and passage was conducted when the cells reached confluence, and the third passage cells were used for the following experiments.

BMSCs were isolated and cultured *in vitro* using the previously reported method.[10] Briefly, the femur and tibia of the two-month-old male Wistar rats were isolated. After rinsing three times with PBS, each end of femur and tibia was removed and douched with α-MEM medium. The flushed mixture was filtered through a 100 μm mesh, then centrifuged for 5 min at 800 g. The collected cells were re-suspended in medium and a 4-fold volume of red blood cell lysis buffer (0.15 mol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 10 μmol/L EDTA) was added. After 10-min incubation and 5 min centrifugation at 800 g, the cells were re-suspended in medium and centrifuged twice more. Then, the re-suspended cells were seeded to 25 cm^2 plastic flasks at a density of 2×10^5–3×10^5 cells per flask. Two weeks later, cell clones had formed, the cells were digested with 0.25% trypsin and passaged to new flasks. After the first passage, the medium was changed every three days and passage was performed when cells reached confluence, and BMSCs' third passage was used for the following experiments.

RAW264.7 cells can differentiate into osteoclast-like cells in the presence of 40 ng/mL RANKL and 40 ng/mL M-CSF, as was confirmed by bone resorption assay and TRAP staining in our lab. The cells were cultured in α-MEM medium mentioned above in flasks for the experiments.

2.3. Application of mechanical loading

The application of mechanical loading on the cells was conducted with a specially designed four-point bending device described previously.[18,19] In this instrument, the cells on the polystyrene cell carrier were subjected to homogeneously distributed uniaxial bending stimuli (Fig. 1).

Bending stimuli was measured in strain. Strain is the difference in length of the surface with and without application of bending stimuli divided by the length without the stimuli (1000 μstrain equals an elongation of 0.1%). The primary osteoblasts and the BMSCs cultured in osteoinductive medium (α-MEM medium mentioned above supplemented with 100 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 250 μmol/L ascorbic acid 2-phosphate), and the RAW264.7 cells cultured in osteoclast-inductive medium (α-MEM mentioned above supplemented with 40 ng/mL M-CSF and 40 ng/mL RANKL) were seeded to the cell carriers coated with osteoblasts extracellular matrix (ECM)

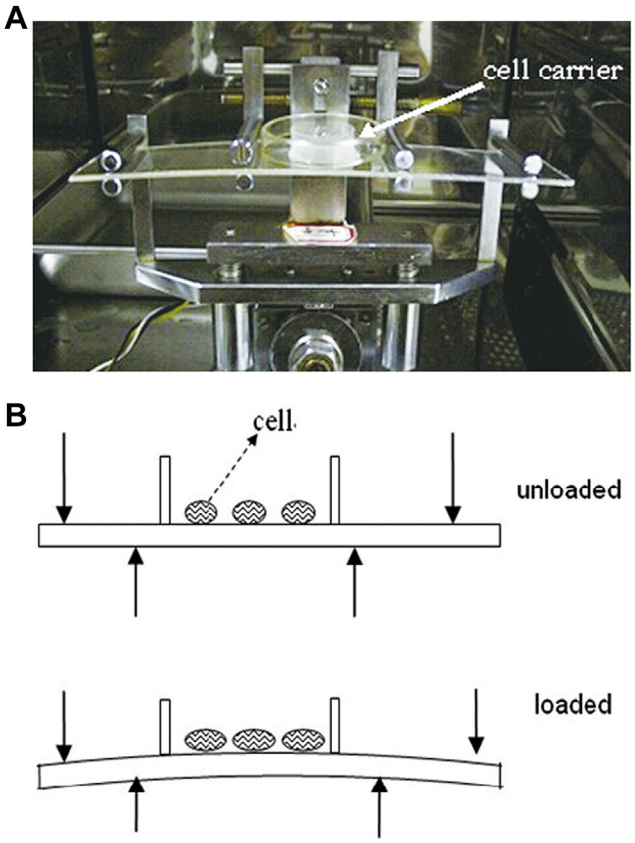

Fig. 1. A: Four-point bending device for application of mechanical loading on cells; B: Schematic chart of the device. When loaded, cells were stimulated by mechanical stretch.