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ORIGINAL ARTICLE

Mechanical Stimulus Inhibits the Growth of a Bone Tissue Model Cultured *In Vitro*[△]

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Objectives To construct the cancellous bone explant model and a method of culturing these bone tissues *in vitro*, and to investigate the effect of mechanical load on growth of cancellous bone tissue *in vitro*.

Methods Cancellous bone were extracted from rabbit femoral head and cut into 1-mm-thick and 8-mm-diameter slices under sterile conditions. HE staining and scanning electron microscopy were employed to identify the histomorphology of the model after being cultured with a new dynamic load and circulating perfusion bioreactor system for 0, 3, 5, and 7 days, respectively. We built a three-dimensional model using microCT and analyzed the loading effects using finite element analysis. The model was subjected to mechanical load of 1000, 2000, 3000, and 4000 μE respectively for 30 minutes per day. After 5 days of continuous stimuli, the activities of alkaline phosphatase (AKP) and tartrate-resistant acid phosphatase (TRAP) were detected. Apoptosis was analyzed by DNA ladder detection and caspase-3/8/9 activity detection.

Results After being cultured for 3, 5, and 7 days, the bone explant model grew well. HE staining showed the apparent nucleus in cells at the each indicated time, and electron microscope revealed the living cells in the bone tissue. The activities of AKP and TRAP in the bone explant model under mechanical load of 3000 and 4000 μE were significantly lower than those in the unstressed bone tissues (all $P < 0.05$). DNA ladders were seen in the bone tissue under 3000 and 4000 μE mechanical load. Moreover, there was significant enhancement in the activities of caspase-3/8/9 in the mechanical stress group of 3000 and 4000 μE (all $P < 0.05$).

Conclusions The cancellous bone explant model extracted from the rabbit femoral head could be alive at least for 7 days in the dynamic load and circulating perfusion bioreactor system, however, pathological mechanical load could affect the bone tissue growth by apoptosis *in vitro*. The differentiation of osteoblasts and osteoclasts might be inhibited after the model is stimulated by mechanical load of 3000 and 4000 μE .

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AS a composite material, formation and growth of the bone tissue is a complex process involving molecular, cellular and biochemical metabolic changes. But compared with other tissues *in vivo*, the structure of bone tissue is much denser, and stress environment and biological environment play important roles in the formation of its structure and function.¹ In the 19th century, Julius Wolff has suggested that external mechanical loading can affect the shape and structure of the bone.² The mechanical steady-state theory raised by Frost in 1987 had provided a better explanation to Wolff's law at the tissue level.³ Our previous researches have revealed that the mechanical strain can apparently regulate the MC3T3-E1 cells, a mouse monoclonal pre-osteoblastic cell line, to differentiate to the osteoblasts *in vitro* and regulate the bone reconstruction *in vivo*.⁴⁻¹¹ Bone growth *in vivo* is influenced by a combination of biochemical, mechanical, and other non-biological stimulations. When we want to illustrate how the stress affects bone growth *in vivo*, other factors or conditions may interfere with the results. Therefore, to investigate the roles of individual mechanical factor in bone growth, cancellous bone explant models *in vitro* can sweep away the interfering factors. Two studies have reported that a three-dimensional bioreactor system (Zetos™) could allow the controlled application of force and/or compression to three-dimensional explant models of cancellous bone maintained within specific chambers *ex vivo*.^{12, 13}

Unlike two-dimensional *in vitro* cell models, the explant models maintain the *in vivo* three-dimensional morphology and intercellular connections in the bone tissue. In this study, a rabbit cancellous bone explant culture model was constructed and used to investigate its response to physiological mechanical load. Our general assumption is that the physiological mechanical load can increase the reconstruction of rabbit cancellous bone explant culture model *in vitro*, and based on it, we will culture the tissue engineering model with mechanical load *in vitro*. The apoptosis in this bone model which was subjected to the pathological mechanical stress was specified for such hypothesis.

MATERIALS AND METHODS

Rabbits

Naturally mated 3-month old male New Zealand white rabbits were obtained from the Laboratory Animal Center of Academy of Military Medical Sciences. The care and use of animals were in accordance with the governmental guidelines for the care and use of laboratory animals.

Preparation of rabbit cancellous bone explant culture model

Under sterile conditions, rabbit femoral head were cut into 1-mm-thick cancellous bone tissue slices. After the cortical bone and adipose tissue were removed, these slices were made into about 8 mm in diameter and rinsed in sterile phosphate buffered saline (PBS) for 3 times. Thereafter, these bone slices were cultured with Dulbecco's Modified Eagle's medium (DMEM), containing 15% fetal bovine serum suitable for tissue living, in a new dynamic load and circulating perfusion bioreactor system which was independently developed by the Chinese Academy of Military Medical Sciences.¹⁴ Having been cultured for 0, 3, 5, and 7 days, the cancellous bone explant model was identified in morphology by hematoxylin-eosin (HE) staining and scanning electron microscopy (provided by Nankai University, Tianjin, China). DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, and fetal bovine serum was obtained from HyClone, USA.

MicroCT scanning and finite element analysis

At the same time, the cancellous bone explant culture model was multi-level scanned on a high-resolution microCT (Skyscan 1076 X-ray Microtomography, Belgium) in Beijing University of Aeronautics and Astronautics, China. Then the finite element models were input into AYSYS 12.0 software. During finite element analysis, a mechanical load of 1000, 2000, or 3000 µε was imposed onto the three-dimensional model by imitating the *in vivo* physiological stress range of bone.

Application of mechanical load on bone explant model

After cultured for 1 day, the bone explant model was stressed by dynamic load in the circulating perfusion bioreactor system which was made with piezoelectric ceramics, and controlled by installed computer software. Through the piezoelectric ceramic-loading rod transferring, these model samples were subjected to a sinusoidal stress loading of 1000, 2000, 3000, and 4000 µε respectively for 30 minutes per day for 5 days. The mechanical loading could lead to producing a compressive displacement in the bone tissue. Based on the compressive displacement (µm) which was displayed by the computer and the thickness of the bone tissue (m), the intensity of mechanical stress (µε) in the bone tissue could be calculated by the following formula: strengthen of mechanical stress (µε) = compressive displacement (µm) / thickness of the bone tissue (m).

Alkaline phosphatase (AKP) activity analysis

After mechanical load, the cancellous bone explant model

was rinsed for 3 times in PBS (unstressed bone tissues as the control group were incubated under the same conditions for the maximum period of mechanical loading application), then cut into about 1 mm³ sized cubes and tardily homogenized in RIPA lysis buffer (400 µl) containing protease and phosphatase inhibitors at 4°C. Thereafter, the extract of protein lysates was collected, and the total protein assay was performed using BCATM Protein Quantification kit (Nanjing Jiancheng Bioengineering Institute, China). According to manufacture's instructions of AKP Activity Assay kit (Nanjing Jiancheng Bioengineering Institute), the absorbance (OD) value of AKP in the total protein with equal dose was detected. We calculated the AKP activity with the following formula: $\text{AKP (U/gprot)} = (\text{S}_{\text{OD}}/\text{St}_{\text{OD}}) \times \text{St}_{\text{phonel quantity (0.003 mg)}/\text{S protein quantity (g)}$; where S_{OD} is sample OD value, St_{OD} is standard substance OD value, $\text{St}_{\text{phonel quantity}}$ is standard substance phonel quantity, S protein quantity is sample protein quantity.

Tartrate-resistant acid phosphatase (TRAP) activity analysis

The extraction and quantification of total protein in the bone explant model was performed as described above. OD values were measured at 530 nm ultraviolet spectra. Thereafter, we calculated the TRAP activity (U/gprot) basing on the manufacture's instructions of TRAP Assay kit (Nanjing Jiancheng Bioengineering Institute).

DNA Ladder detection

After mechanical load, the bone explant model was rinsed with ice cold PBS and cut into 1 cm³ in size, then homogenized on ice for 10 minutes. Thereafter, the DNA in the model samples was extracted using the DNA Ladder Assay kit (Nanjing Biobox Biotech Co., Ltd., China). The DNA fragments were visualized under ultraviolet light after being separated by electrophoresis on a 1.5% agarose gel by comparing with a 200-bp DNA ladder marker.

Caspase-3/8/9 activity detection

According to manufacturer's instructions of Caspase-3/8/9 Activity Detection kit (Nanjing Biobox Biotech Co., Ltd.), the protein in the bone explant model was extracted after these samples being subjected to mechanical loading for 5 days. Aliquots (50 µl) of the protein were removed and placed in a 96-well microplate containing caspase-3/8/9 reaction substrate, and the microplate was incubated at 37°C for 4 hours. The OD value was measured at 405 nm (TECAN, Switzerland), and the caspase-3/8/9 activity was determined through $\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}$.

Statistical analysis

All statistical analyses were performed using Statistics Package for the Social Sciences (SPSS) 11.0 (IBM SPSS, Inc., Chicago, IL, USA). The means and standard deviation were calculated for data from three separate experiments with triplicate samples. Statistical significance was determined with a two-tailed Student's *t* test. The difference was considered as statistically significant at $P < 0.05$.

RESULTS

Identification of rabbit cancellous bone explant model *in vitro*

The cancellous bone tissue slices can live for 7 days in the DMEM containing 15% fetal bovine serum under dynamic load in a circulating perfusion bioreactor system. HE staining showed that the histomorphology of the cancellous bone tissue slices cultured for 3, 5, and 7 days had no apparent difference compared with fresh slices. Osteocytes were trapped within the bone trabeculae with distinctly visible nucleus in cells (Fig. 1). Electron microscope scan also obviously detected living cells in the bone tissue cultured for 3, 5, and 7 days. However, there were not cells to be found on uncultured bone tissue because cells were covered by lots of adipose tissues (Fig. 2).

Results of MicroCT scanning and finite element analysis

Figure 3 shows the strain distribution in the cancellous bone explant model under 1000, 2000, and 3000 µε load, respectively. The strain at the nodes of the bone explant model with 1000, 2000, and 3000 µε load was mainly lower than 2000 µε. When the bone explant models received 3000 µε load, the strain of about 2000 nodes was higher than 4000 µε, however the range of physiological strain on bone *in vivo* is lower than 3000 µε. Therefore, we selected the mechanical load of 1000, 2000, 3000, and 4000 µε respectively to treat the explant culture model of the bone tissue *in vitro*.

AKP and TRAP activity

As displayed in the Table 1, mechanical load of 3000 and 4000 µε for 30 minutes per day for 5 days significantly reduced the activities of AKP and TRAP in the bone explant model compared with the control group (all $P < 0.05$). However, there was no significant difference in the activity of AKP or TRAP between 1000 µε group and 2000 µε group (all $P > 0.05$).

DNA ladder

As showed in Figure 4, DNA extracted from the cancellous bone explant model samples was fragmented into multiple fragments with lengths as integer multiples of approximately

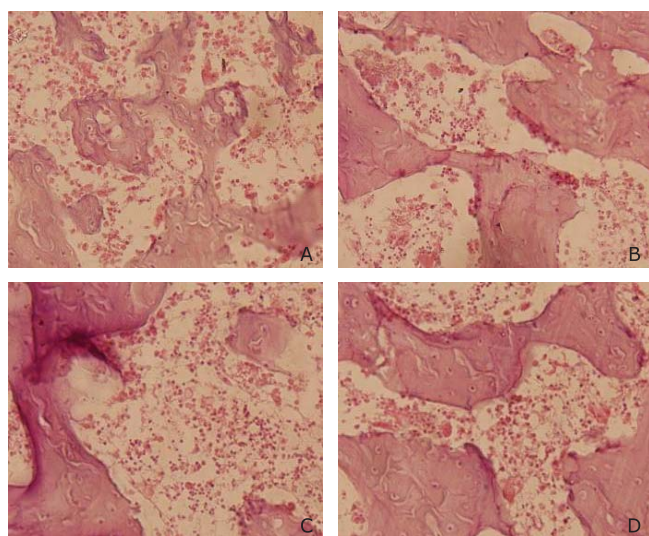


Figure 1. Photomicrographs of hematoxylin-eosin stained slices of rabbit cancellous bone cultured for 0 day (A), 3 (B), 5 (C), and 7 days (D) respectively *in vitro*. HE staining $\times 20$

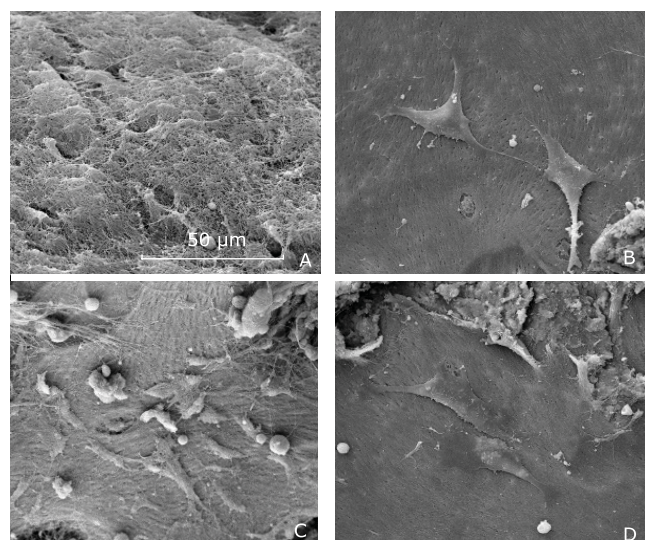


Figure 2. Results of electron microscopy scan of rabbit cancellous bone cultured for 0 day (A), 3 (B), 5 (C), and 7 days (D) respectively *in vitro*. $\times 1000$

Table 1. Comparisons of AKP and TRAP activities in the bone explant culture model receiving different mechanical loads for 5 days^S ($n=3$)

Group	AKP (U/gprot)	TRAP (U/gprot)
Control	27.350 $\pm$ 0.071	19.261 $\pm$ 0.103
1000 $\mu\epsilon$	26.309 $\pm$ 0.034	18.911 $\pm$ 0.081
2000 $\mu\epsilon$	28.121 $\pm$ 0.212	18.126 $\pm$ 0.134
3000 $\mu\epsilon$	13.365 $\pm$ 0.105*	15.961 $\pm$ 0.089*
4000 $\mu\epsilon$	10.161 $\pm$ 0.121*	16.023 $\pm$ 0.101*

^S: Plus-minus values are means $\pm$ SD.

AKP: alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase.

* $P < 0.05$ compared with the control group.

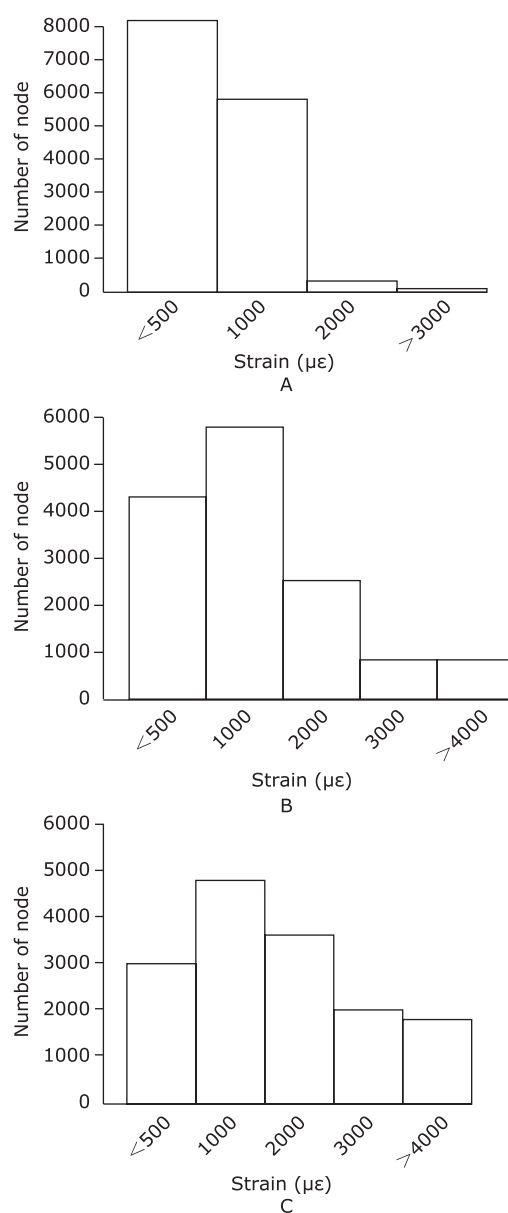


Figure 3. Distribution of strain in the cancellous bone explant model under 1000 (A), 2000 (B), and 3000 $\mu\epsilon$ load (C), respectively.

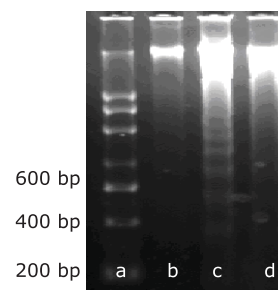


Figure 4. DNA ladder analysis result of apoptotic cells by agarose gel electrophoresis.

Lane a: 200-bp ladder marker; Lane b: control group; Lanes c and d: DNA extracted from the cancellous bone explant model stimulated by mechanical loading of 3000 and 4000 $\mu\epsilon$ forms characteristic 180-200 bp ladder.

180-200 base pairs. The DNA ladders were seen after mechanical load of 3000 and 4000 μe (Fig. 4, Lanes c and d). But no obvious DNA ladder patterns were observed in the control group during the entire experiment.

Caspase activity

The protein activity levels of caspase-3/8/9 in the mechanical load groups were significantly increased (all $P < 0.05$) compared with the control group (Table 2).

Table 2. Comparisons of caspase-3/8/9 activities in the bone explant culture model receiving 3000 and 4000 μe mechanical loads for 5 days[§] ($n=3$)

Group	Caspase-3	Caspase-8	Caspase-9
Control	1.422 $\pm$ 0.004	1.324 $\pm$ 0.043	1.328 $\pm$ 0.022
3000 μe	2.607 $\pm$ 0.038*	2.702 $\pm$ 0.012*	2.509 $\pm$ 0.004*
4000 μe	2.655 $\pm$ 0.017*	2.542 $\pm$ 0.025*	2.919 $\pm$ 0.073*

§: Plus-minus values are means $\pm$ SD.

* $P < 0.05$ compared with the control group.

DISCUSSION

The bone is an extremely complex organ composed of compact bone tissue containing cellular component and matrix component. It is difficult to culture bone tissue in simplified *in vitro* environment. We cultured the cancellous bone explant model in an environment suitable for tissue growth with dynamic load and circulating perfusion. This protocol provided the cancellous bone cells with O_2 , nutrient and mechanical load. The morphology of the model visualized by HE staining and scanning electron microscopy after 7 days of culture was normal.

Mechanical load is a key physiological regulatory mechanism for bone formation and is essential for the maintenance of skeletal architectural integrity and bone strength. According to the "mechanical steady-state theory" raised by Frost,³ the *in vivo* physiological mechanical stress of bone ranges from 1000 to 4000 μe . In order to test the effect of mechanical load on growth of the rabbit cancellous bone explant model *in vitro*, these bone tissue samples were subjected to mechanical stress of 1000, 2000, 3000, and 4000 μe , respectively. We found that the mechanical stress of 3000 and 4000 μe could simultaneously inhibit the activities of AKP and TRAP, reflecting proliferation and differentiation of osteoblasts and osteoclasts respectively.

As it is known that on the one hand osteoblasts and osteoclasts are the effector cells which are responsible for bone formation and bone resorption respectively,¹⁵⁻¹⁷ and mechanical stress can directly affect their developments *in vivo* or *in vitro*.^{18, 19} On the other hand, osteocytes, known

as the mechanosensors, have two key features of ability to detect mechanical stimuli and to send signals to other effector cells that regulate bone formation and resorption.²⁰⁻²⁴

Other experimental studies *in vitro* and *in vivo* also revealed the osteocytes network is the main sensor detecting strain in the bone tissue.²⁵ These cells, embedded in their lacunocanalicular system and largely distributed in bone, interconnecting by gap junctions between their cell processes, connecting with cells of the bone surface and of the bone marrow, are ideal place to sense bone loading and to direct remodelling according to strains.²⁶ Osteocytes are sensitive to biomechanical stress, particularly to fluid flow and shear stress induced by loading in the lacunocanalicular system.^{27, 28} You *et al*²⁹ indicated that osteocytes may function as mechanotransducers by regulating local osteoclastogenesis *via* soluble signals mechanically stimulated when co-cultured with osteoclasts. Likewise, some studies showed that sclerostin, an osteocytic protein belonging to the transforming growth factor/bone morphogenetic protein family which can inhibit Wnt signaling, is decreased by mechanical stimulation. Wnt has an essential role in osteoblast proliferation and differentiation, and the decrease of sclerostin could be a major signal to increase bone formation in response to loading.³⁰⁻³²

In this study, we detected apoptosis of bone cells in the cancellous bone explant model. Apoptotic cell death and subsequent removal of apoptotic cell debris by specialized phagocytic cells occur in numerous tissues during development³³⁻³⁶ and in response to injury.³⁷⁻⁴¹ Our experiment showed by DNA ladder assay that there were apparent apoptosis of cells in the bone tissue subjected to mechanical stress compared with the control group. The appearance of the DNA ladder bands further showed that mechanical stimulation led to the degradation of nuclear chromatin DNA to nucleosome fragments, which was characteristic of early apoptosis.⁴² Caspase-3, -8 and -9 are members of the caspase proteases family which has been known to play a major role in the execution phase of apoptosis. A series of members of caspase proteases family are all activated by some stimulating factors to participate in the apoptosis occurrence. It has been shown that the caspase-3 is the key protease among caspase family, because it is activated by a variety of apoptotic stimulating factors at the beginning of apoptosis, then the activated caspase-3 protease may act on some of the other caspase members to be activated in cascade, causing cell morphological changes, and to the end apoptosis to be completed.⁴³ Our experiment indicated that the mechanical stimulation of 3000 μe could activate the apoptosis program *via*

caspase-dependent route.

In summary, the cancellous bone explant models from the rabbit femoral head could be alive at least for 7 days in the dynamic load and circulating perfusion bioreactor system, and pathological mechanical loading (3000 and 4000 μE) could inhibit the proliferation or differentiation of bone cells, which might be a caspase-mediated process that lacks cell type specificity. Pathological mechanical load might affect bone tissue growth by cellular apoptosis *in vitro*.

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