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Research Article

Orbital fluid shear stress promotes osteoblast metabolism, proliferation and alkaline phosphates activity *in vitro*

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ABSTRACT

Prolonged disuse of the musculoskeletal system is associated with reduced mechanical loading and lack of anabolic stimulus. As a form of mechanical signal, the multidirectional orbital fluid shear stress transmits anabolic signal to bone forming cells in promoting cell differentiation, metabolism and proliferation. Signals are channeled through the cytoskeleton framework, directly modifying gene and protein expression. For that reason, we aimed to study the organization of Normal Human Osteoblast (NHOst) cytoskeleton with regards to orbital fluid shear (OFS) stress. Of special interest were the consequences of cytoskeletal reorganization on NHOst metabolism, proliferation, and osteogenic functional markers. Cells stimulated at 250 RPM in a shaking incubator resulted in the rearrangement of actin and tubulin fibers after 72 h. Orbital shear stress increased NHOst mitochondrial metabolism and proliferation, simultaneously preventing apoptosis. The ratio of RANKL/OPG was reduced, suggesting that orbital shear stress has the potential to inhibit osteoclastogenesis and osteoclast activity. Increase in ALP activity and OCN protein production suggests that stimulation retained osteoblast function. Shear stress possibly generated through actin seemed to hold an anabolic response as osteoblast metabolism and functional markers were enhanced. We hypothesize that by applying orbital shear stress with suitable magnitude and duration as a non-drug anabolic treatment can help improve bone regeneration in prolonged disuse cases.

1. Introduction

Bone forming osteoblast and osteocyte cells are highly responsive to mechanical loading. This mechanosensing cells response by converting the stress into anabolic signal, promoting cell differentiation and bone formation. Alternately, lack in mechanical loading has been associated with rapid bone loss especially in prolonged disuse such as bed rest [1], cast immobilization due to fracture [2], in injury of the spinal cord [3] and astronauts under microgravity environment [4]. Under physiological condition, bone is continuously renewed through the balanced activity of bone forming osteoblasts (anabolic activity) and bone resorbing osteoclasts (catabolic activity). Tight coupling of this activity with the influence of mechanical loading is essential for the repair of micro-damages and fractures on bone. It is believed that mechanical loading in a form of orbital shear stress [5], fluid shear stress [6], vibration [7] and cell stretching [8] is a potent anabolic stimulus to enhance osteoblast metabolism [9]. More notably, the mechanosensing property in osteoblast accelerates osteogenesis process, fundamentally required to maintain bone strength. Nevertheless, the biological signals involved during bone mechanotransduction leading to enhanced osteogenesis are not fully understood. Therefore, it is necessary to investigate the underlying mechanisms of mechanotransduction in order to uncover targets for bone fracture healing.

In vitro and *in vivo* models have shown that the mechanosensing properties of bone cells respond to loading by regulating cell differentiation and proliferation via biochemical activities. Cellular mechanosensing is initiated from the cell extracellular matrix (ECM) and into the cell interior. Mechanical load is transformed into biochemical signals which induce cell membrane protein conformational change. Alteration in the membrane protein structure modulates the cytoskeleton involved for cell metabolism,

Abbreviations: ALP, alkaline phosphatase; NHOst, Normal Human Osteoblasts; OCN, osteocalcin; ECM, extracellular matrix; RANKL, receptor activator of nuclear factor kappa-B ligand; OPG, osteoprotegerin; CLSM, Confocal Laser Scanning Microscope; OBM, osteoblast basal medium

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differentiation, proliferation, protein synthesis, and apoptosis [10]. The integrin glycoprotein found on cell membrane acts as mechanoreceptors which spans the cell membrane under loading. Mechanical signal is then channeled into the cell through the cytoskeleton framework, subsequently modifying gene and protein expression. Signal reorganizes the cytoskeleton components namely actin, tubulin, and intermediate filaments as a mean of mechanical adaption. Tubulin fibers responsible for cell division was seen depolymerized under mechanical loading [11]. On the contrary, increase polymerization of actin fibers has been reported under mechanical stimulation. Moreover, actin polymerization was shown to accelerate osteoblast differentiation from the mesenchymal lineage [12].

During osteoblastogenesis, osteoblast cells derived from mesenchymal lineage express specific bone markers. Numerous studies have shown that mechanical loading has an anabolic effect on the regulation of osteoblastogenesis while catabolic effect on osteoclastogenesis process [13–15]. The anabolic and catabolic effect in bone occurs through the OPG/RANK/RANKL pathway. The membrane bound protein; receptor activator of nuclear factor kappa-B ligand (RANKL) produced by osteoblast is a vital factor for osteoclastogenesis. The process is initiated when the receptor activator of nuclear factor kappa-B (RANK) on the surface of osteoclast progenitor binds to RANKL, differentiating into functional osteoclast. However osteoclastogenesis can be inhibited when osteoprotegerin (OPG) a decoy receptor for RANKL binds to RANKL. Inhibiting RANK/RANKL pathway by OPG stimulates osteoblast formation through the RANKL/OPG pathway. A 48 h study of continuous mechanical stimulation on osteoblast cells showed an increase in the production of OPG, consequently decreasing the RANKL/OPG ratio [14]. It has been generally established that the ratio of RANKL to OPG manages the balance between bone resorption and formation. Although mechanical stimulation leads to reduced RANKL/OPG ratio, nevertheless the stimulation magnitude plays a role [15]. Studies from Kim et al. showed that low frequency 1 Hz stimulation increased OPG mRNA while at higher frequency 10 Hz, RANKL mRNA was reduced, in which both stimulation magnitudes resulted in decreased RANKL/OPG ratio [15].

Once osteoblast cells are fully differentiated from mesenchymal cells, multiple bone matrix proteins are being expressed. Specific bone proteins are used to monitor osteoblastic phenotype during stages in osteoblast differentiation and formation [16–19]. The expression of type I collagen (COL-1) protein and alkaline phosphatase (ALP) activity is usually observed at early stages of osteoblast differentiation particularly during in immature stage. As osteoblast mature, bone maturation proteins; osteopontin (OPN) and osteocalcin (OCN) are expressed at maximal levels [18]. Numerous studies have shown that mechanical stimulation increases bone matrix protein ALP, COL-1, OCN and OPN, required for matrix synthesis [19–21]. Enhanced expression of bone matrix protein was seen under low frequency mechanical stimulation (1–3 Hz), resulting in peak bone mass [22]. These proteins contribute to matrix synthesis and ECM production, which in turn increases bone mass.

However, osteoblast response towards mechanical loading highly depends on the type of load, magnitude and duration [23]. Several *in vitro* studies have investigated the influence of mechanical loading on osteoblasts by applying load on the cell culture scaffold (collagen coated microcarrier) or directly onto the cells [24]. Kamkin could show that mechanical loading applied on osteoblast cells grown on collagen scaffolds increases osteoblast proliferation, ALP activity, OCN protein, and matrix production [25]. Given that osteoblast cells are highly responsive to mechanical signals, it is ultimately important to understand whether these anabolic signals are committed to the increase in osteogenesis function and can it be applied clinically. Therefore, we aimed to study continuous mechanical stimulation through orbital fluid shear stress on Normal Human Osteoblast (NHOst) cells. Of special interest were the effects of orbital fluid shear stress on the reorganization of the cytoskeleton towards NHOst metabolism, proliferation, and on osteogenic phenotypic markers.

2. Methods

2.1. Orbital fluid shear stimulation

A total of 2.5×10^5 NHOst cells/cm^2 were seeded into a T-25 Flask with full growth medium (OBM™). After pre-incubation for 24 h, OBM medium was replaced and 20 mM of HyQ HEPES was added. Culture flask was sealed with parafilm and transferred into a shaking incubator. NHOst cells were continuously shaken for 72 h at 250 RPM at 37 °C to induce orbital fluid shear stress [6]. Unstimulated NHOst cells grown at 37 °C with 5% CO_2 in a water-jacketed incubator with full growth medium for 3 days served as a control.

2.2. Change in NHOst cytoskeleton

NHOst cells were cultured on the 0.025 g Cytodex™ 3 microcarrier collagen beads (GE Healthcare) in a petri dish at density of 500,000 cells/ml. After 24 h of pre-incubation, NHOst cells attached onto Cytodex™ 3 were transferred to Lab-Tek II chamber slides (Thermo Fisher Scientific Inc., USA). Cells were exposed to orbital fluid shear stress at 24, 48 and 72 h. Unstimulated cells served as control. After each time points, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and stained according to our optimized method [26]. Fixed cells were incubated with DAPI (300 nM in PBS) for 20 min to stain the nucleus and then permeabilised with 0.1% Triton X-100 in PBS for 10 min. Alexa Fluor® 635 Phalloidin (6.6 μM in PBS) together with anti-α-tubulin conjugated with FITC diluted (1:50) in blocking solution (10% BSA, and 1% Triton X-100 in PBS) was added to the permeabilised cells and incubated for 1 h to stain actin and tubulin fibers. After 1 h, cells were washed twice with PBS and counterstained with DAPI (300 nM in PBS) for 30 min. Chambers were removed from the slides and cover slip was mounted with Prolong® Gold Antifade reagent to minimize photobleaching. Slides were stored at 4 °C in the dark until scanned with a Confocal Laser Scanning Microscope (CLSM) (Leica TCS SP5). Fluorescence intensity of nucleus (Excitation: 358 nm, Emission: 461 nm), actin (Excitation: 633 nm, Emission: 647 nm) and tubulin (Excitation: 494 nm, Emission: 518 nm) fibers was measured according to their wavelength.

2.3. Mitochondrial activity

NHOst mitochondrial activity was measured via MTS-based assay after 72 h of orbital fluid shear stress. A volume of 20 μl CellTiter 96® Aqueous One Solution (317 μg/ml) was added to the medium contained in each well (1:5). After incubation for 2 h, production of solubilised formazan was measured by absorbance at 490 nm. Mean optical density (OD) readings were converted into percentage (%) relative to control.

2.4. NHOst proliferation

Trypan Blue stain was used to measure the rate of NHOst cell proliferation. Both stimulated and control NHOst cells were harvested and resuspended in OBM. A volume of 20 μl of Trypan Blue