Cytotechnology **39:** 125–130, 2002.

Experimental model for stimulation of cultured human osteoblast-like cells by high frequency vibration

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Received 28 August 2001; accepted in revised form 5 May 2002

Key words: Cell culture, Mechanotransduction, Osteoblast like cells, Vibration

Abstract

Reliable and reproducible experimental methods for studying enhancement of osteoblast proliferation and metabolic activity *in vitro* provide invaluable tools for the research of biochemical processes involved in bone turnover *in vivo*. Some of the current methods used for this purpose are based on the ability of the osteoblasts to react metabolically to mechanical stimulation. These methods are based on the hypothesis that intracellular metabolic pathways could be influenced by the excitation of cytoskeletal components by mechanical cell deformation. Based on the same assumptions we developed a new experimental approach of biomechanical stimulation of cultured osteoblast-like cells by vibration. This method is based on the use of a specially designed vibration device that consists of an electric shaker with horizontally mounted well plate containing cell cultures. We used a first passage explant outgrowth of human osteoblast-like cell cultures, originating from samples of cancelous bone, collected from femoral necks of six donors during surgical arthroplasties of osteoarthritic hips. Well plates with replicates of cultured cells were exposed to a sine shaped vibration protocol in a frequency range of 20–60 Hz with displacement amplitude of 25 ($\pm$ 5) μm. We found that vibration at a distinct set of mechanical parameters of 20 Hz frequency and peak to peak acceleration of 0.5 ± 0.1 m/sec^2 is optimal for cell proliferation, and at 60 Hz frequency with peak to peak acceleration of 1.3 ± 0.1 m/sec^2 for metabolic activity. The presented easily reproducible experimental model should improve and simplify further research on the interactions between mechanical stimuli and intracellular biochemical pathways in osteoblasts.

Introduction

Mechanical stimulation is one of the important enhancing factors influencing osteoblast metabolism (Boppart et al. 1998; Duncan and Turner 1995; Neidlinger-Wilke et al. 1994). Current research methods in this field are based on the ability of cultured osteoblasts to react metabolically to mechanical stimulation. Stretching of cultured cells on elastic membranes and exposure of osteoblast cultures to a controlled fluid flow are the most popular models for this purpose (Neidlinger-Wilke et al. 1994; Banes 1999; Sejima 1999). Both of these methods are based on the hypothesis that intracellular metabolic pathways are influenced by the excitation of cytoskeletal components by mechanical cell deformation (Burger and Klein-Nulend 1998). Here we describe a different experimental approach to biomechanical stimulation of cultured osteoblast-like cells. The method is based on the application of external vibration directly to a cell culture plate. The vibration should cause mechanical deformation of the cells adherent to the culture plate surface. The deforming force should be uniformly transferred to all cultured cells. This relatively simple method of osteoblast stimulation *in vitro* can provide a reproducible experimental tool for research on cellular mechanotransduction pathways.

For determination of the optimal vibration parameters we have investigated the cellular response to vibration at infrasonic frequencies which are in the

range of a physiologic vibromyogram, e.g. about 20 Hz (Nigg 1998). Vibration peak to peak displacements of 20–30 μm, which are in the range of the osteoblast's cell diameter (Liu and Kalu 1990), were chosen empirically and have been confirmed by preliminary experiments (Levy et al. 1999). This set of vibration parameters has been chosen to investigate conditions that are equivalent to the upper limit of the normal vibration environment of bone cells *in vivo* (Nigg 1998). The impetus to investigate the response of osteoblasts to vibration in this specific range of mechanical parameters came from previous *in vivo* studies that suggested an existence of positive influence of 20 Hz vibration on the bone mass maintenance (Rubin et al. 1995).

Methods

Outline

- Culture of a confluent layer of osteoblast-like cells from cancellous bone samples were taken from femoral necks of donors during osteoarthritic hip replacements.
- Following a first passage, replicates of equal number of cultured cells were submitted to different vibration conditions using a specially designed vibration device.
- On completion of exposure to different controlled vibration protocols the total DNA content and alkaline phosphatase activity of all cultured cells replicates were measured and statistically compared to control replicates kept under static conditions.

Donors

Samples of cancellous bone were collected from the femoral necks of six donors during total hip replacements of osteoarthritic hips. The bone samples used are usually disposed after removal. The donors were in the age range of 55–77 years, one man and five women.

Cell cultures

Bone chips of cancellous bone, 2–3 grams in total, were incubated in DMEM [Dulbecco's Modified Eagles Medium] with heat-inactivated fetal calf serum (10%), 20 mM HEPES buffer, 2 mM L-glutamine, 100 mM ascorbate-2-phosphate, 10 nM dexametasone, 50 U ml-ml penicillin, 150 μ ml-ml streptomicin at 37 °C in humidified atmospheric environment of 95% air with 5% CO_2 [v:v] for 20–30 days. Human osteoblast-like cells grew out from the chips as adherent to the plastic tissue culture plates. The human bone cell cultures obtained by this method have been shown previously to express osteoblast-like characteristics (Gundle et al. 1998). These cells were passaged into 24 - well plates. Each well was seeded with 2×10^4 cells. Six replicate wells from each donor were prepared for each vibration condition. Following passage the cells were incubated in DMEM with 20 mM HEPES buffer, 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U ml-ml penicillin and 150 μ ml-ml streptomicin. Lower fetal calf concentrations were used at this stage in order to minimize its component's effect on cells.

The passaged cells were incubated for 24 h and subsequently exposed four times for periods of 120 s to the different vibration parameters at 24 h intervals. The exposure to vibration was done outside the cell culture incubator at ambient room conditions [25 °C]. The control replicates, kept in static conditions, were placed at the same distance from the vibrating shaker as the replicates exposed to the vibration protocol, and for the same time duration at the same time intervals to eliminate the possible influence of the electromagnetic fields and/or high frequency sound on the cultured osteoblasts. Twenty four h after the fourth exposure to vibration, all the replicates were washed by phosphate buffer solution and dissolved in Triton X − 100 (0.1 %) solution. All replicates were frozen at −20 °C and thawed twice for complete DNA recovery. Each replicate was then assayed for total DNA content and alkaline phoshatase activity.

Vibration device (Figures 1 and 2)

Well plates containing the cell cultures were connected to a horizontally oriented shaker [Mini-shaker Type 4810- Brüel & Kjaer, Denmark]. The amplitude, wave shape and frequency of the vibration provided by the shaker were controlled by an amplifier and pulse generator [Farnell, Function Generator – FG3]. Vibration peak-to-peak acceleration was measured with a piezoelectric accelerometer [4383 V- Brüel & Kjaer, Denmark] and displayed on a Vibration Measuring Amplifier [Type 2525 - Brüel & Kjaer, Denmark]. The displacement of vibration movement was measured by a LVDT [Linear Variable Displace-

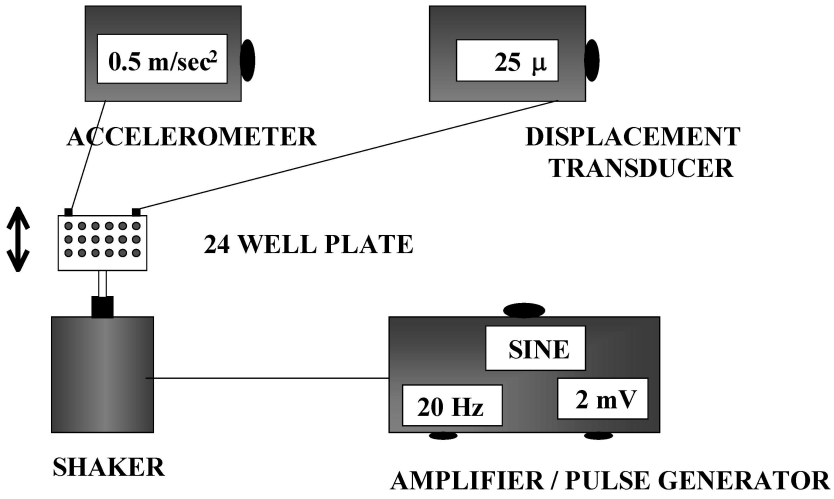

Figure 1. Experimental setup for vibration of cultured osteoblast−like cells−*Schematic presentation.*

ment Transducer - sensitivity 78.16 mV/V/mm, Solatron] and displayed on an oscilloscope.

Vibration protocol

Sine shaped vibration at 20, 30, 40 and 60 Hz was applied to well plates containing six replicates of cultured cells from each of the six donors. The vibration displacement in all experiments was kept in the range of 25 ± 5 μm. An additional six replicates of cell cultures from every donor were kept under static conditions and served as controls.

DNA assay

The total content of DNA in each replicate culture was measured fluorometrically using the Hoechst 33258 dye fluorescence enhancement method with

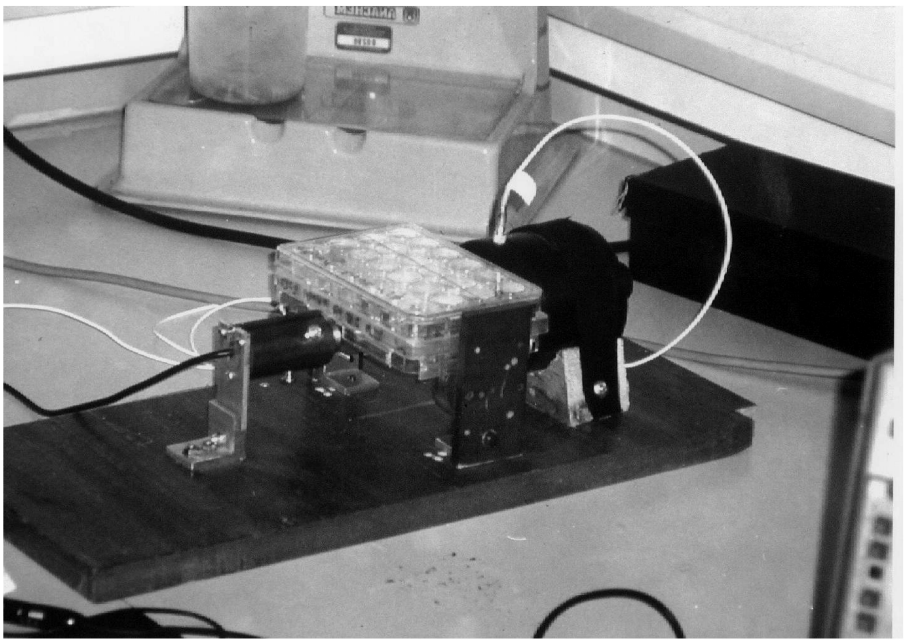

Figure 2. Photograph of the shaker with well plate ready for vibration experiment.

excitation at 355 nm and emission at 450 nm using a luminescence spectrometer [Model LS30 - Perkin Elmer] (Labarca and Paigen 1980). For calibration, standard solutions of salmon testes DNA in the range of 100–1000 ngr were used.

Alkaline phosphatase assay

Alkaline phosphatase activity was determined in dissolved cell culture replicates, after incubation with P-nitrophenyl phosphate substrate, by 410 nm wavelength spectrophotometry (Bessey et al. 1946).

Statistical analysis

The values of DNA content and Alkaline phosphatase activity per cell [alkaline phosphatase activity/DNA content] in replicates exposed to each vibration profile were compared to the values of the static controls by t test, after a normal distribution of values had been confirmed by the Kolmogorov-Smirnov test. If a normal distribution was not confirmed values were compared by the Mann-Whitney Rank Sum Test. P values of less than 0.05 indicated a significant difference between the compared groups. The results are expressed as means with standard deviation distribution.

Results

All the cultured cell replicates survived the exposure to the vibration protocol at the frequency range of 20–60 Hz. On microscopic examination of the cells after completion of the vibration protocol, normal morphological appearance of cells was verified in all replicates.

Exposure to vibration applied at 20 Hz frequency increased the DNA content significantly in cultures from five of six donors (Figure 3). Vibration at higher frequencies, from 30 to 60 Hz, showed either no change or decreased DNA content per well.

After exposure to vibration at 20 Hz frequency, alkaline phosphatase activity was significantly lower in 5 donors and without change in one additional donor when compared to static controls. Alkaline phosphatase activity gradually increased in cultures exposed to vibration with higher frequencies in the range from 30 to 60 Hz (Figure 4). At 60 Hz frequency cultured replicates from four donors showed a

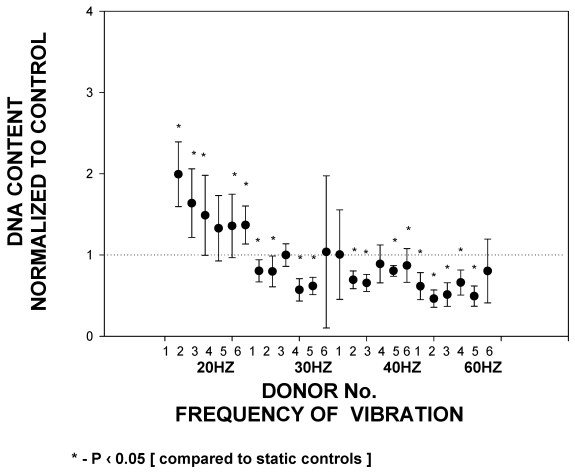

Figure 3. DNA contet of osteoblast−like cell cultures following vibration at frequencies of 20–60 Hz relative to static controls.

significantly higher alkaline phosphatase activity per cell comparing to controls.

Discussion

This study describes the development of a device to administer high frequency vibration to cultures of osteoblast-like cells. The rationale for this is that high frequency vibration has been shown to increase bone formation *in vivo* (Rubin et al. 1995) and that this type of mechanical stimulation is likely to be experienced

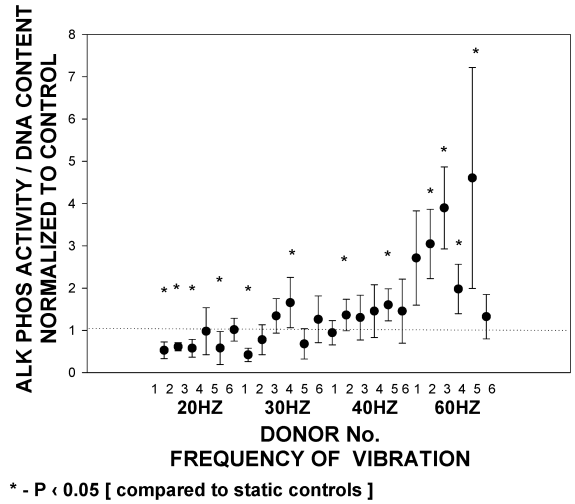

Figure 4. Alkaline phosphatase activity in cultured osteoblast−like cells following vibration at frequencies of 20–60 Hz relative to static controls.

in bone as a result of muscle vibration (Nigg 1998). The mechanism whereby osteoblasts respond to vibration *in vivo* is not known. We therefore propose to use this newly developed model to study in detail proliferation, differentiation and signaling responses in human osteoblasts.

During the last two decades there have been many studies demonstrating the responses of different cell types, especially bone-derived cells, to mechanical stimulation. However the large number of distinct experimental methods used has made it difficult to compare data. Most studies have used cyclic mechanical stimulation applied to stretchable membranes to which the cells were attached. This stimulation should be characterized by its displacement, frequency and acceleration [or wave shape of the applied force], but from most of these studies neither this information nor common optimal stimulation parameters for osteoblast-like cells activation are apparent (Table 1). Yet though these previous data do not provide a clear indication of the different mechanical factors required for osteoblast activation they do demonstrate that cellular deformation affects the intra-cellular cytoskeletal scaffold, which results in the activation of a cascade of secondary messengers that stimulate an active metabolic or proliferative response (Reich et al. 1997; Jacobs et al. 1998). These two responses appear not to be capable of being activated simultaneously (Stanford et al. 1995) and distinct intra-cellular messengers seem to mediate each response.

For these reasons when designing the current experimental method for mechanical activation, our intention was to build a device that provides a reproducible means of delivering a controlled cyclic movement, defined by its displacement, frequency and acceleration, in order to activate cultured osteoblast-like cells. This method was based on the assumption that cytoskeletal activation by cellular mechanical deformation is required. It was designed to overcome the technical difficulties of non uniform strains provided by the use of elastic membranes (Banes 1999) and the relative complicity of their maintenance. It was also designed to apply a uniform deforming force to a large number of cultured cell replicates. The use of a controlled vibration force applied to a cell culture dish meets these objectives. Thus a new technical design has been developed, adapted from that previously described for the investigation of the influence of vibration on cultured lymphocytes (Khalil and Qassem 1996).

We have chosen to investigate the *in vitro* influence of vibration parameters in the range otherwise known to be produced by the natural muscular vibration [in infrasonic range of frequencies] to which bone should be exposed *in vivo* (Nigg 1998). There appear to be no previous *in vitro* studies in this range, although the effectiveness of this range of vibration parameters has been already presented as effective for bone mass improvement in whole body vibration studies (Rubin et al. 1995; Smith and Gilligan 1996). Because our hypothesis is that a gradually changing acceleration should be less distractive to the cultured cells and therefore optimal for cellular response, we have used a sine wave acceleration pattern of the applied vibration.

The present results support our initial assumptions. First passage adult human osteoblast-like cells respond to vibration. Proliferation was optimally stimulated at a frequency of 20 Hz, displacement amplitude of 20–30 μm and peak to peak acceleration of 0.5 ($\pm$ 0.1) m/sec^2. Metabolism was more effective at 60 Hz frequency, displacement of 20–30 μm and peak to peak acceleration of 1.3 ($\pm$ 1) m/sec^2. Thus proliferation and metabolism are probably stimulated by distinct mechanical stimuli.

Table 1. Optimal parametric data of mechanical induction of proliferation of osteoblast like cells *in vitro* in different studies.

Reference	Frequency Hz	Displacement mm	Acceleration mm/sec^2	Time hours	Wave shape	Cells	Origin	Parameters investigated
Buckley et al. (1988)	0.05	0.009[a]	0.00089[b]	48	square	Osteoblasts	chicks	DNA,Cell No.
Tjandrawinata et al. (1997)	20–2000	NI	50000	0.12	sine	MC3T3E1	rat	mRNA:c-fos,c-myc
Neidlinger-Wilke et al. (1994)	1	0.0003[a]	0.0112[b]	1.2	NI	Osteoblasts	human	Cell No.
Jones et al. (1991)	1	0.0003[a]	0.0112[b]	0.07	NI	Osteoblasts	bullock	Cell No.
Stanford et al. (1995)	0.5	0.003[a]	0.03[b]	6	square	MC3T3E1	rat	DNA
Present study	20	0.025	450-550	0.13	sine	Osteoblasts	human	DNA

NI - not indicated

[a] average displacement estimated from strains applied to cells, assuming that the diameters of osteoblasts are in the range of 20–40 μm.

[b] calculated with approximation to sine shaped vibration.

There are several questions that cannot be verified as a result of this study. There is no information on the actual pattern of the mechanical deformation of cells and at what extent the media flow shearing forces and well plate movement contribute to it. There is also no information on the interactions between the cell cytoskeleton activation, by the presumed cell deformation, and the intracellular secondary messengers, that activate the observed proliferation and metabolic responses. These key questions will be addressed in future studies on the development of this experimental model.

To summarize:

- We described a device that delivers controlled vibration forces to bone derived cells adherent to the surface of a 24 - well plate. The values of displacement, frequency and acceleration can be controlled and recorded.
- External vibration caused stimulation of proliferation and metabolic activity of adult human osteoblast-like cells *in vitro*.
- Distinct and different vibration parameters were found to be optimal for either cell proliferation or metabolic activity.
- The spectrum of these optimal parameters is in the same range as the spontaneous muscle activity *in vivo*.
- The presented method of mechanical stimulation of human bone derived cells *in vitro* provides a relatively simple and easily reproducible tool for further research of cellular biomechanical pathways involved in maintaining bone mass and bone fracture repair *in vivo*.

These initial results should encourage further studies at microscopic, morphological and molecular levels for the better determination of the cellular and mechanical mechanisms involved in the described experimental model.

Acknowledgements

This work was supported by ''The Lord Nuffield research grant'' and by ''The Oxfordshire Health Authority research grant''.

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