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Effect of low-magnitude, high-frequency vibration on osteogenic differentiation of rat mesenchymal stromal cells

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Abstract

Whole body vibration (WBV), consisting of a low-magnitude, high-frequency (LMHF) signal, has been found to be anabolic to bone *in vivo*, which may act through alteration of the lineage commitment of mesenchymal stromal cells (MSC). Here, we investigated the effect of LMHF vibration on rat bone marrow-derived MSCs (rMSCs) in an *in vitro* system. We subjected rMSCs to repeated (six) bouts of 1-hour vibration at 0.3*g* and 60 Hz in the presence of osteogenic induction medium. The osteogenic differentiation of rMSCs under the loaded and non-loaded conditions was assessed by examining cell proliferation, alkaline phosphatase (ALP) activity, mRNA expression of various osteoblast-associated markers (ALP, Runx2, osterix, collagen type I alpha 1, bone sialoprotein, osteopontin, and osteocalcin), as well as matrix mineralization. We observed that LMHF vibration did not enhance the osteogenic differentiation of rMSCs. Surprisingly, we found that the mRNA level of osterix, a transcription factor necessary for osteoblast formation, was decreased, and matrix mineralization was inhibited. Our findings suggest that LMHF vibration may exert its anabolic effects *in vivo* via mechanosensing of a cell type different from MSCs.

Keywords

mesenchymal stromal cell; low-magnitude; high-frequency vibration; osteogenic differentiation; alkaline phosphatase; osterix

INTRODUCTION

Osteoporosis, a disease characterized by progressive deterioration of bone tissue due to an imbalance in the breakdown and rebuilding of bone, leads to increased bone fragility and susceptibility to fracture. Current measures for the prevention and treatment of osteoporosis

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are primarily drug-based, which delay disease progression but do not fully restore the balance in bone resorption and formation. Based on the premise that bone is a dynamic and self-regulating organ capable of adapting its mass and morphology according to its mechanical environment, some researchers have turned to a biomechanical approach to treating osteoporosis. Of recent interest is a mechanical signal known as low-magnitude, high-frequency (LMHF) vibrations that, when applied to the entire body of subjects (termed whole-body vibration, or WBV), produces anabolic responses in bone.[1,2] Such responses have been attributed to increased bone formation, which was observed in various animal models, including young mice,[3] ovariectomized rats,[4] and rats subjected to hind limb disuse by tail-suspension.[5]

The mechanism by which LMHF vibration induces anabolic responses at the cellular level remains largely unknown. Recent *in vivo* studies suggest that LMHF vibration directs the lineage commitment of bone marrow mesenchymal stromal cells (MSCs) in favor of osteogenesis over adipogenesis.[6,7] MSCs can form bone *in vitro* under appropriate chemical cues,[8,9] a process that has been shown to be modulated by mechanical signals. MSCs isolated from various sources, including bone marrow,[10–13] adipose tissue,[14] and calvariae,[15,16] have been tested for their response to a variety of mechanical stimuli *in vitro*, including tensile strain,[10–12] fluid flow-induced shear stress,[13–15] and pressure.[16,17] Several osteoblast-associated markers of differentiating MSCs were demonstrated to be upregulated by applied mechanical forces such as strain and fluid shear, possibly via activation of MAPK pathways.[10,12,18] However, the effect of LMHF vibration applied directly on MSCs has not been studied. Given the complex cellular heterogeneity of the bone marrow compartment, multiple cell type(s) including MSCs may respond to the LMHF signal and account for the observed anabolic responses *in vivo.* Hence, in this study, we aimed to delineate the effect of LMHF vibration on MSCs *in vitro*.

We hypothesize that LMHF vibration enhances the osteogenesis of MSCs *in vitro* in the presence of osteogenic factors, leading to increased expression of osteoblastic markers and matrix mineralization. To test our hypothesis, we subjected rat MSCs to repeated bouts of the mechanical stimulus at a magnitude of 0.3*g* and a frequency of 60 Hz to mimic the vibration conditions used in various animal and human studies[1,5,19]. We assessed and compared cell proliferation, alkaline phosphatase (ALP) activity, gene expression of certain transcription factors and matrix molecules, as well as their functional capacity to differentiate and form mineralized bone nodules between MSCs under LMHF stimulation and static controls.

METHODS

Bone marrow cell isolation and culture

Bone marrow cells were isolated from male Wistar rats (~4-week-old) as previously described[8] (see Supplementary Materials for details). After 6 days of primary culture, with medium changes every 2–3 days, cells of the first passage were seeded at a density of 5×10^3 cells/cm^2 for all experiments (with the exception of the proliferation study, where cells were seeded at 1×10^3 cells/cm^2 to prevent inhibition of growth due to cell contact) in multi-well plates. 48 hours later, cells were serum-starved overnight in α-MEM containing 10% antibiotics (AB) and 0.1% fetal bovine serum (FBS) to synchronize the cells. After the first bout of vibration, all cultures were maintained in osteogenic (OS) medium (α-MEM containing 10% FBS, 1x AB, 10 nM dexamethasone, 50 μg/ml ascorbic acid, and 5 mM beta-glycerophosphate).

Set-up of LMHF vibration system

A metal vibration plate was custom-made to contain two multi-well tissue culture plates as previously described.[20] The vibration plate was attached to a shaker (ET-127, Labworks Inc) that delivered vertical accelerations. The amplitude, waveform, and frequency of the vibration provided by the shaker were controlled with VibeLab computer program (Labworks Inc., Costa Mesa, USA). Peak-to-peak acceleration was measured at the centre of the vibration plate with a piezoelectric accelerometer (8632C5, Kistler, Amherst, USA), which output a voltage signal to the computer for feedback control between the desired and measured waveforms.

Vibration loading of cell cultures

After overnight serum-starvation to synchronize the cells[12], each culture well was completely filled with serum-free medium and tightly sealed with gas permeable sealing film (Excel Scientific, Victorville, USA) immediately prior to vibration. This minimized fluid perturbation (and thus fluid shear stress) within the wells when vibrations were applied. Plates were placed securely onto the vibration plate and subjected to 60 Hz of sinusoidal vibrations at 0.3*g* for 1 hour. Cells in the non-vibration group were placed on the same but stationary plate. After one hour, all cells (both vibrated and non-vibrated groups) received fresh OS medium. Cultures received five more vibration loading bouts over 6 days (Figure 1). Cell samples were collected immediately after the third consecutive bout of vibration (i.e. on days 2 and 6).

Proliferation assay

The amount of DNA in the cultured cell samples was measured using CyQUANT® Cell Proliferation Assays Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Briefly, cell layers were rinsed 2x with phosphate buffered saline (PBS) and stored at −80°C overnight. Cells were then lysed with the kit's lysis buffer containing a nucleic acid-binding fluorescence dye. Using a microplate reader with excitation at 485 nm and emission detection at 530 nm, fluorescence measurements were made and compared against a standard curve of known cell number.

mRNA quantification

Quantitative PCR was used to measure the mRNA levels of several early to late osteoblastic markers, including the osteogenic transcription factors runt-related transcription factor 2 Runx2 and osterix (Osx), ALP, and various bone matrix proteins, such as collagen type I alpha 1 (COL1A1), osteopontin (OPN), and bone sialoprotein (BSP). See Supplementary Material for detailed methods and rat-specific primer sequences (S-Table 1).

ALP assay

For quantitative analysis of ALP activity, ALP was extracted and detected with SensoLyte *p*-nitrophenyl phosphate (*p*NPP) ALP assay kit (Anaspec, Fremont, USA) according to the manufacturer's protocol. Briefly, cells were lysed using the kit's lysis buffer. Proteins were extracted by three rapid freeze-thaw cycles. Cell lysate was centrifuged for 15 minutes at 10,000*g* at 4°C. The supernatant was collected and combined with *p*NPP in a colorimetric reaction. Absorbance measurements at 405 nm were normalized to total protein content measured using BCA protein assay (Pierce, Rockford, USA). See Supplementary Material for ALP staining.

Quantification of matrix mineralization

Two weeks after the first bout of vibration (day 14), cell layers were rinsed 2x with calcium-free PBS. To solubilize calcium from the matrix, the samples were incubated overnight at

4°C in 0.6 N HCl. The supernatant was collected, centrifuged to remove cell debris, and reacted with *o*-cresolphthalein complexone, which produced colorimetric changes proportional to calcium content.[21] Absorbance measurements at 570 nm were compared against a standard curve of known calcium concentrations and normalized to DNA content. See Supplementary Material for von Kossa staining.

Statistical Analysis

A two-tailed *t*-test was used to compare means between two groups. A significance level of $p<0.05$ was employed. Experiments were repeated twice with cells pooled from 2 rats per experiment (n=6 for all assays). Data are reported as mean ± standard deviation.

RESULTS

MSC proliferation is not affected by LMHF vibration

We examined the effect of LMHF vibration on the proliferation of MSCs cultured in OS media. On day 0, cell samples were collected immediately post-vibration to verify that vibration did not promote detachment of cells from the culture plate. Both the vibrated and non-vibrated cultures proliferated over the course of 14 days (Figure 2a), and the proliferation rate did not differ significantly between both groups at all time points (Figure 2b).

ALP activity is not altered by LMHF vibration

The effect of LMHF on osteogenic differentiation was first assessed by quantifying ALP activity. The ALP levels expressed by rMSCs increased with time in both vibrated and non-vibrated groups in the presence of OS medium (Figure 3). However, at the time points studied, there was not a significant difference in ALP activity due to LMHF vibration.

mRNA level of Osx is inhibited by LMHF vibration

We further characterized the effect of LMHF vibration on osteogenic differentiation by examining the mRNA levels of several early to late bone markers. In both the vibrated and non-vibrated groups, the temporal expression patterns of the examined osteoblastic markers were comparable to those found in previous studies [22,23] (Figure 4A–F). The transcript levels of Osx, ALP, COL1A1, OPN, and BSP peaked at day 6, while that of Runx2 showed an increasing trend from day 2 to day 14. OCN was detectable at days 2 and 6, but could not be accurately quantified by qPCR due to its low expression levels. LMHF did not cause any significant change in the examined genes with the exception of Osx, which was decreased significantly on days 2 and 6 (Figure 4G).

Matrix mineralization is decreased by LMHF vibration

The effect of LMHF vibration on the functionality of the late-stage differentiated cultures was assessed by quantifying the amount of matrix calcium deposition. Cultures that received LMHF vibration loading contained a significantly lower amount of matrix mineralization normalized to the total number of cells (−24%) compared to the non-vibrated controls (Figure 5A). Matrix mineralization in both non-vibrated and vibrated cultures was confirmed by ALP/von Kossa staining (Figures 5B and 5C).

DISCUSSION

In this study, we investigated the potential osteogenic effect of LMHF vibration applied directly to MSCs. Contrary to our expectation, LMHF vibration did not enhance the osteogenic differentiation of rMSCs, as assessed by cell proliferation, ALP activity, mRNA

levels of various early to late markers of osteogenesis, and matrix mineralization. Specifically, we demonstrated that LMHF vibration inhibited the mRNA expression of Osx and decreased the amount of matrix mineralization. Taken together, the results suggest that the direct application of LMHF vibration to MSCs does not produce osteogenic effects.

LMHF vibration did not induce any changes in the proliferation of MSCs in our study. Various proliferation responses of MSCs under other mechanical stimuli were found in previous studies, from having no effect,[13] increasing proliferation,[24] to inhibiting cell growth.[12] Weyts *et al.* noted that the osteoblastic response to stretching depends on the stage of osteoblast maturation: stretching in early osteoblastic cultures caused apoptosis, while in more differentiated cultures proliferation was stimulated.[25] One possible explanation of our observation is that the mechanical stimulation was applied in the early stages of osteogenic differentiation (i.e. on days 0–6 of osteogenic induction).

Similarly, previous studies on the effect of mechanical stimulation on the level of ALP activity, an early osteoblastic differentiation marker, have yielded mixed results. We showed that the amount of ALP activity was not altered by LMHF vibration, which was consistent with certain studies that applied strain to human MSCs[12] or fluid flow to rMSCs[13] but differed from those that reported an upregulation[16,26] or downregulation.[27] The differential functional responses of MSCs to various forms of mechanical stimulation underscore the important concept that cellular responses in mechanotransduction are unique to the specific mechanical stimulus.

We continued to assess the effect of LMHF on osteogenic differentiation by measuring the mRNA levels of early to late osteoblast-associated markers. The time course mRNA expressions of the studied markers corresponded well with those reported by Malaval *et al.* in the same culture system, indicating that the manipulation of the cell cultures during our vibration experimental set-up did not affect the osteogenic differentiation in our *in vitro* model. We did not observe any differential expression between the vibrated and the non-vibrated groups in the majority of the genes studied. We did, however, observe a decreased mRNA level of Osx on days 2 and 6, which returned to control levels by day 14. Since Osx is crucial for osteoblast formation, the decrease in Osx expression may have contributed to the inhibited matrix mineralization observed in the vibrated cultures. Furthermore, as Osx is known to act downstream of Runx2 in the transcriptional control of bone formation,[28] we expected that Runx2 would also exhibit decreased expression under LMHF vibration. Although the observed unaltered level of Runx2 was unexpected, recent studies showed that Osx can be regulated via Runx-independent mechanisms,[29] and indicate an incomplete understanding of the molecular mechanisms underlying the action of Osx in osteogenic differentiation.

Our findings that LMHF vibration decreased Osx mRNA levels and matrix calcification but did not alter other indicators of osteoblast differentiation, including ALP activity, suggest that the intermediate signaling molecules mediating these observed effects may be ones other than those studied herein. Indeed, Simmons *et al.* have shown that increased matrix mineralization as a result of cyclic strain applied to differentiating human MSCs is mediated via extracellular signal-regulated kinase signaling but is independent of ALP stimulation. Furthermore, although widely used as an osteoblastic marker, ALP levels have been found to be independent of matrix calcification.[30]

Taken together, despite receiving repeated vibration stimulation, rMSCs have shown limited to no response from gene to protein expression levels of early to late osteogenic markers. This lack of response was unlikely due to a transient response that was not captured by the time points selected, as it has been shown that repetitive loading is able to amplify the

cellular response elicited by a single bout of loading.[13] In addition, it is improbable that potential differential effects between vibrated and non-vibrated groups have been masked by the presence of dexamethasone, a potent osteoinductive agent. We had chosen to supplement cultures with 10 nM dexamethasone beginning from after the first bout of vibration loading to the end of each time point such that the culture protocol is consistent with previous studies.[10,12,13] To confirm that dexamethasone had not suppressed any potential osteogenic effect of LMHF vibration on rMSCs, we repeated the same vibration protocol in the absence of any osteoinductive supplements. Quantification of proliferation and mRNA expression of early osteogenic markers showed no significant difference between vibrated and non-vibrated cells (results not shown), suggesting that LMHF vibration does not generate a baseline effect on the osteogenic differentiation of rMSCs even in culture medium deficient of a chemical osteoinductive agent.

The results from this study suggest that *in vivo*, cells may be responding to secondary mechanical stimuli induced by the vibration signal, such as shear stress due to bone marrow movement,[31] as opposed to vibration itself. Another possibility is that there may be alternative mechanisms by which WBV exerts its anabolic effect, possibly via a mechanosensor that is absent in the current *in vitro* model. Animal studies on the effect of mechanical loading and unloading suggest that alterations in the mechanical environment alter the cell fate of mesenchymal progenitors in the bone marrow.[6,7,32] However, the bone marrow is a heterogeneous compartment that houses hierarchical components of hematopoietic and mesenchymal cells. Thus, there may be heterotypic cellular interactions between the mechanosensor cell and the effector cells that are not immediately apparent in previous *in vivo* studies and in the current *in vitro* investigation.

A putative mechanosensor for detecting LMHF vibration is the osteocyte. Our laboratory has found that osteocytes under LMHF vibration release soluble factors that inhibit osteoclast formation.[20] Furthermore, osteocytes have been found to communicate with other cell types, such as MSCs,[33] through gap junctions[34] and soluble factors,[33] and such communication is mechanically regulated.[26,33,35] Future studies are needed to investigate the communication between osteocytes and MSCs and to elucidate the possible role of osteocytes in orchestrating MSC differentiation under vibration stimulation.

Our experimental set-up of LMHF vibration aimed to investigate whether the anabolic effect of LMHF vibration observed *in* vivo is a direct consequence of MSCs sensing and responding to the vibration signal. However, our system may not be replicating the *in vivo* environment as it lacks other cell types. Also, a vibration stimulus applied externally to the whole-body may translate to a different signal at the cellular level. However, the precise mechanical consequences of vibratory loading *in vivo* are currently unknown. Thus, as a first step to understand the cellular basis of vibration effects in bone, our *in vitro* system allows us to apply a controlled vibratory signal that is isolated from possible confounding cellular responses of other cell types and any secondary mechanical stimuli. Furthermore, although most *in vivo* studies have typically employed a loading duration of less than 20 minutes, we have chosen to use a 1-hour stimulation for two reasons. Firstly, in our preliminary tests, osteocyte-like MLO-Y4 cells did not respond to brief periods (e.g. 15 minutes) of LMHF vibration, which led us to establish a 1-hour stimulation protocol in our laboratory. Secondly, many *in vitro* studies testing the effects of other types of mechanical stimulation on bone cells usually employed periods of at least 1 hour,[26,36] and have shown that cellular response increased with increasing load duration from 0.5 to 2 hours.[36] Thus, in order to strike a balance between existing *in vitro* and *in vivo* mechanical loading regimes, which would allow us to compare our results with existing literature, we employed a 1-hour duration of vibration.

In conclusion, under our experimental conditions, we did not detect any osteogenic effect of vibration on rMSCs, but instead observed decreased mRNA level of Osx and inhibited matrix calcification, suggesting that other factors contribute to the anabolic effect of vibration on bone. We speculate that the osteogenic effect of LMHF is elicited through an alternative mechanism, where rMSCs may be activated by secondary mechanical stimuli induced by vibration, or indirectly activated via communication with osteocytes that are more mechanosensitive to LMHF vibration. Our results provide a better understanding of the cellular mechanism that underlies the bone's adaptive response to vibration. Such information is valuable in identifying the osteogenic components of the physical signal and facilitates the translation of the physical treatment of osteoporosis to clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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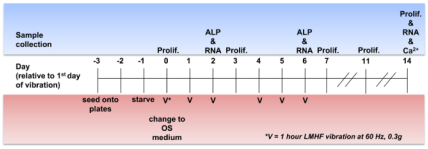

Figure 1. Vibration loading protocol

Cells were seeded at a density of 5,000 cells/cm^2 (or 1,000 cells/cm^2 for proliferation study). Two days later, they were serum-starved overnight, and subjected to 1 hour of LMHF vibration at 0.3*g* and 60 Hz on day 0. After vibration loading, the cultures received and were maintained in OS medium. The vibration loading was repeated on days 1–2 and 4–5. Samples were collected for proliferation study on days 0, 3, 7, 11, and 14; for ALP activity on days 2 and 6 (immediately after vibration); mRNA analysis on days 2, 6, and 14; and for mineralization assay on day 14.

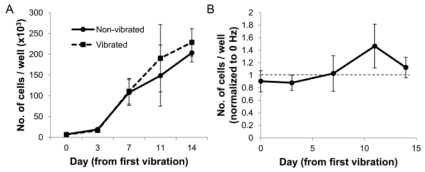

Figure 2. Proliferation of MSCs in OS medium

(A) Both vibrated and non-vibrated groups showed proliferation over the course of 14 days. (B) LMHF vibration did not affect proliferation rate (n=6).

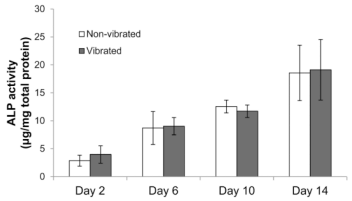

Figure 3. ALP activity

ALP activity increased from days 2 to 14. There were no significant differences in ALP activity levels between the vibrated and non-vibrated groups (n=6).

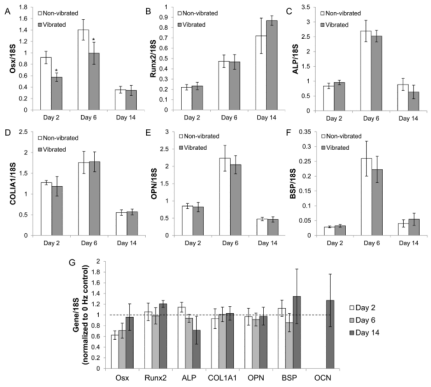

Figure 4. mRNA expression of early to late osteoblastic markers

Expression levels of Osx (A), ALP (C), COL1A1 (D), OPN (E), and BSP (F) were highest on day 6 while Runx2 expression increased over 14 days (B). (G) Vibrated cultures exhibited a lower mRNA level of Osx on days 2 (−38%) and 6 (−29%). *$p<0.01$ compared to the non-vibrated control (n=6).

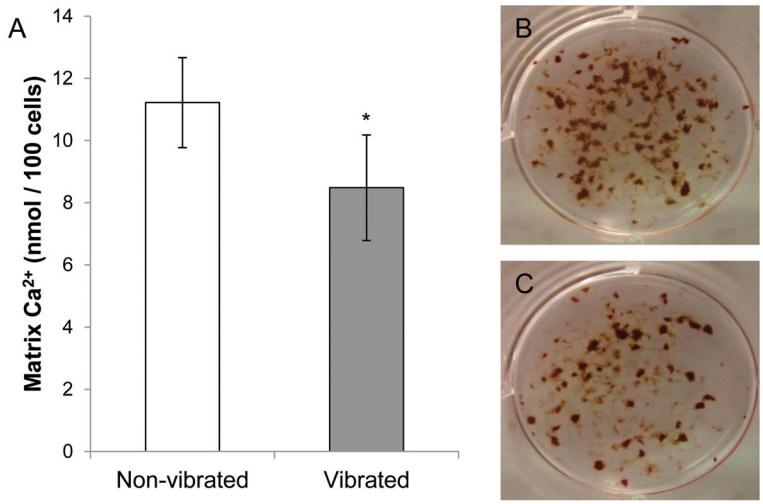

Figure 5. Matrix mineralization on day 14

(A) Cultures that were subjected to LMHF vibration showed 24% lower matrix calcium deposition.*$p<0.05$ compared to the non-vibrated control (n=6). Von Kossa/ALP staining of non-vibrated (B) and vibrated (C) cultures.