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Short communication

# Low-intensity vibrations accelerate proliferation and alter macrophage phenotype in vitro

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ABSTRACT

Macrophages are essential for the efficient healing of various tissues. Although many biochemical signaling pathways have been well characterized in macrophages, their sensitivity to mechanical signals is largely unexplored. Here, we applied low-intensity vibrations (LIV) to macrophages to determine whether macrophages could directly transduce LIV signals into changes in the expression of genes and proteins involved in tissue repair. Two different LIV signal frequencies (30 Hz or 100 Hz) were combined with two acceleration magnitudes (0.15*g* or 1*g*) to generate four distinct LIV signals that were applied to cultured murine macrophages. All four LIV signals significantly increased macrophage number after 3 days of stimulation with the combination of the smallest acceleration and the highest frequency (0.15*g* at 100 Hz) generating the largest response. Compared to non-LIV controls, gene expression of the pro-healing growth factors VEGF and TGF-β increased with all four LIV signals (Day 1). LIV also decreased protein levels of the pro-inflammatory cytokines IL-6, IFN-γ, and TNF-α (Days 1 and 3). These data demonstrate the sensitivity of macrophages to high-frequency oscillations applied at low intensities and may suggest that the benefit of LIV for tissue repair may be based on reducing inflammation and promoting a pro-healing macrophage phenotype.



## 1. Introduction

Macrophages play a critical role in the healing of various tissues (Duffield et al., 2005; Lucas et al., 2010; Mirza et al., 2009; Summan et al., 2006; van Amerongen et al., 2007). Tissue repair consists of overlapping phases of inflammation, proliferation, and remodeling, and macrophages are present in all phases. As tissue repair progresses, macrophages exhibit transitions in phenotype and function, although the precise factors regulating these transitions have not been fully elucidated (Novak and Koh, 2013a, b). During efficient healing, macrophages appear to orchestrate transition from one phase to the next, contributing to phagocytosis of necrotic tissue, cell proliferation, angiogenesis, collagen deposition and matrix remodeling. However, dysregulation of macrophage function can contribute to failure to heal or fibrosis in several pathological situations (Mirza and Koh, 2011; Mirza et al., 2013; Sindrilaru et al., 2011; Villalta et al., 2011). Thus, therapies that modulate macrophage phenotype and function may provide an avenue to promote tissue repair.

Accumulating evidence demonstrates that mechanical stimulation via low-intensity vibration (LIV; $\leq 1g$ acceleration magnitude of the oscillating plate) can be anabolic and/or anti-catabolic in musculoskeletal tissues including bone (Garman et al., 2007; Gilsanz et al., 2006) and enhance neuromuscular control (Fu et al., 2013). LIV has also been shown to accelerate bone regeneration (Hwang et al., 2009) and skin wound healing (Weinheimer-Haus et al., 2014) but the underlying mechanism(s) remains to be elucidated. While LIV has the potential to influence many cell types involved in tissue repair, macrophages are sensitive to mechanical signals as both cyclic and static stretch, albeit at much greater magnitudes than LIV, rapidly induced gene expression in monocyte/macrophages (Wehner et al., 2010; Yang et al., 2000). Thus, the aim of this study was to determine whether macrophages could directly transduce LIV signals into changes in the expression of genes and proteins involved in tissue repair. We also tested whether LIV frequency and magnitude may play a role in modulating macrophage response.

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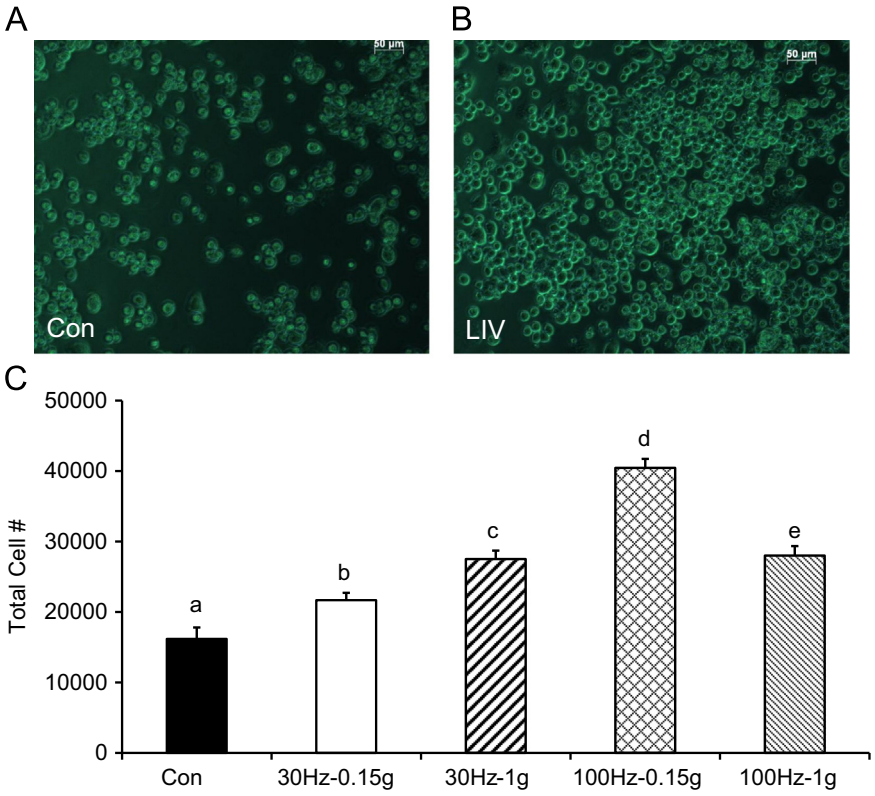


**Fig. 1.** (A and B) Representative images of cells double stained with calcein AM (green; live cells) and ethidium homodimer (red; dead cells) following exposure to (A) non-LIV sham or (B) LIV (100 Hz-0.15*g*) treatments. (C) Cells were exposed to one of four LIV signals or sham and cell number was determined after 3 consecutive days of stimulation. Labeled means without a common letter differ, $p \leq 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 2. Materials and methods

### 2.1. Cell culture

Murine macrophages (J774A.1, ATCC, Manassas, VA) were cultured in RPMI 1640 (Gibco, http://www.invitrogen.com) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS, Gibco 10082-147) and 1% penicillin/streptomycin and incubated at 37 °C, 5% $CO_2$.

### 2.2. Mechanical stimulation

Vibration was applied horizontally as described elsewhere (Uzer et al., 2012). Briefly, cells were exposed to either a non-LIV sham control, or one of the following four signal combinations of LIV frequency and acceleration: 30 Hz and 1*g*, 100 Hz and 1*g*, 30 Hz and 0.15*g*, and 100 Hz and 0.15*g*. Vibrations were applied for 20 min/session, 2 × /day; sessions were separated by a 2 h rest period. Non-LIV control cell culture plates were handled identically to experimental plates except that vibrations were not applied.

### 2.3. Cell number

Macrophages were seeded at a density of 7500 cells/cm$^2$. After allowing internal equilibrium for 24 h, cells were exposed to LIV or sham treatment ($n=9$ each) on each of three consecutive days. Cell number was determined via XTT cell proliferation assay (ATCC). On Day 3 of stimulation, immediately after LIV treatment, XTT reagent was added to the samples and incubated for 3 h prior to measuring absorbance. Cell number was calculated via comparison with a standard curve. Cell viability was checked with a live/dead cell assay (Invitrogen, NY) and visualized under a microscope (Zeiss, Germany).

### 2.4. Gene expression

To determine whether macrophages can respond within hours to the application of LIV with increased transcriptional levels of two pro-healing genes, cells were plated at a density of 18,000 cells/cm$^2$. 48 h after plating, cells were exposed to LIV or sham treatment ($n=9$ each). Immediately after the second LIV treatment, cells were lysed (TRIzol, Ambion, TX) and total RNA was isolated (RNeasy, Qiagen, CA). RNA concentration and quality were determined (NanoDropND-1000, Thermo Scientific, NY). cDNA was synthesized using standard protocols (High Capacity RNA to cDNA kit, Applied Biosystems, CA) and RT-PCR was performed (StepOne, Applied Biosystems) using Taqman primer (Applied Biosystems) probes for vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β. Relative gene expression was determined using the $2^{-\Delta\Delta_{Ct}}$ method with GAPDH as the endogenous control. Results were reported relative to non-vibrated control.

### 2.5. Flow cytometry

To test whether LIV induced changes in cell proliferation were accompanied by changes in protein levels of pro-inflammatory cytokines or whether LIV could decrease their production, flow cytometric analysis was performed for interleukin (IL)-6, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. Cells were plated at 18,000 cells/cm$^2$ 48 h prior to the start of the experiment and were then exposed to LIV at 0.15*g* and 100 Hz or sham treatment ($n=6$ each) for 3 days. The acceleration magnitude of 0.15*g* at a frequency of 100 Hz was selected for this experiment because this LIV combination was most effective at increasing cell number and altering VEGF expression. Immediately after the second experimental session on Day 1 or 3, cells were collected and fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton, and stained with fluorescently-labeled antibodies against IL-6, IFN-γ, or TNF-α (Abcam, Cambridge, UK). Live cells and dead cells were identified using calcein AM and ethidium homodimer (Invitrogen). Stained cells were analyzed by flow cytometry (FACScan, BD) and FlowJo software (Tree Star Inc. OR); percent cell populations were gated on the cells positive for these fluorescent antibodies.

### 2.6. Statistics

Data were presented as mean ± SD. All samples were run as triplicates and statistics were computed on averages of the triplicates, preserving the samples sizes described above. Differences between groups were identified by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests (SPSS 22.0, IBM, New York, NY). *p*-Values of less than 0.05 were considered significant.