RESEARCH ARTICLE
Primary cilia respond to intermittent low-magnitude, high-frequency vibration and mediate vibration-induced effects in osteoblasts

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Primary cilia respond to intermittent low-magnitude, high-frequency vibration and mediate vibration-induced effects in osteoblasts. Am J Physiol Cell Physiol 318: C73–C82, 2020. First published October 2, 2019; doi:10.1152/ajpcell.00273.2019.—Our objective was to investigate the role of primary cilia in low-magnitude, high-frequency vibration (LMHFV) treatment of MC3T3-E1 osteoblasts (OBs). We used chloral hydrate (CH), which has a well-characterized function in chemically removing primary cilia, to elucidate the role of primary cilia in LMHFV-induced OB osteogenic responses through cell viability assay, Western blot analysis, real-time quantitative RT-PCR, and histochemical staining methods. We observed a significant, 30% decrease in the number of MC3T3-E1 OBs with primary cilia (reduced from 64.3 ± 5%) and an approximately 50% reduction in length of primary cilia (reduced from 3 ± 0.8 μm) after LMHFV stimulation. LMHFV stimulation upregulated protein expression of the bone matrix markers collagen 1 (COL-1), osteopontin (OPN), and osteoclastin (OCN) in MC3T3-E1 OBs, indicating that LMHFV induces osteogenesis. High-concentration or long-duration CH exposure resulted in inhibition of MC3T3-E1 OB survival. In addition, Western blot analysis and RT-PCR revealed that CH treatment prevented LMHFV-induced osteogenesis. Furthermore, decreased alkaline phosphate activity, reduced OB differentiation, mineralization, and maturation were observed in CH-pretreated and LMHFV-treated OBs. We showed that LMHFV induces morphological changes in primary cilia that may fine-tune their mechanosensitivity. In addition, we demonstrated the significant inhibition by CH of LMHFV-induced OB mineralization, maturation, and differentiation, which might reveal the critical role of primary cilia in the process.

chloral hydrate; differentiation; high-frequency vibration; low-magnitude, mineralization; osteoblasts; primary cilia

INTRODUCTION
Osteoporosis, a chronic bone disease characterized by decreased bone mass and strength due to bone microstructure degeneration and susceptibility to fracture, is a common affliction among the elderly (35). Conventional osteoporosis treatment involves drug therapy. However, long treatment course, side effects, high cost, and poor patient compliance make drug treatment an unattractive option.

Low-magnitude, high-frequency vibration (LMHFV; frequency 10–100 Hz, intensity <0.5 g) is an effective and noninvasive treatment for osteoporosis (30, 35). LMHFV has been reported to upregulate osteogenic protein expression and improve the mechanical properties and density of long bones, microstructure, and cancellous bone, thus reducing the occurrence of fragility-induced bone fractures (5, 8, 31). Moreover, LMHFV treatment was found to upregulate factors involved in bone matrix regulation, such as cyclooxygenase 2 (COX-2), osteopontin (OPN), and prostaglandin-E2 (PGE2) in osteoblasts (OBs) (12, 13) and stimulate extracellular matrix (ECM) deposition (10a). Although LMHFV has been shown to regulate osteoblastic anabolic activity, its mechanism of action remains poorly understood.

Bone turnover and homeostasis are regulated by external mechanical stimuli, but the conversion of mechanical stimuli to biochemical signals in bone cells is poorly understood. Recent studies have shown that primary cilia, microtubule-based organelles that extend from the cell surface, may function as mechanosensors that regulate intracellular signal transduction and gene expression in bone cells (9, 16, 32). Primary cilia dysfunction can result in skeletal defects, such as nail and tooth dysplasia and shortening of limbs (14), as well as a variety of systemic diseases, such as polycystic kidney disease (39), Bardet-Biedl syndrome (2), and visual and hearing impairment (26). Moreover, primary cilia are implicated in OB differentiation and osteogenesis (9, 32). Removal of primary cilia in MC3T3-E1 OBs can significantly reduce osteogenesis in response to fluid flow (9).

Primary cilia may undergo changes in length in response to external mechanical stimuli to adjust their sensitivity (9, 11, 12). MLO-A5 OBs that were subjected to fluid shear stress for five consecutive days exhibited decreased length of primary cilia and number of OBs that express primary cilia (10a).

Thus, in this study, to explore the potential mechanisms of LMHFV-induced effect in the treatment of osteoporosis, we investigated the change of primary cilia subjected to LMHFV and the role of primary cilia in the biological effect of OBs’ response to LMHFV. We showed a significant decrease in the number of MC3T3-E1 OBs with primary cilia and an approximately 50% reduction in length of primary cilia after LMHFV stimulation. In addition, we provided evidence suggesting a critical role of primary cilia in LMHFV-induced OB mineralization, maturation, and differentiation through CH application.

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MATERIALS AND METHODS

Cell Culture

In this study, we used MC3T3-E1 cells (Zhongjiaoxinzhou Biotechnology, Shanghai, China), a physiologically relevant system for studying OBs (29). The MC3T3-E1 cells were cultured and passaged in α-minimum essential medium (α-MEM; Hyclone, Logan, UT) containing 10% fetal bovine serum (Clark Bioscience, Richmond, VA) and 1% penicillin-streptomycin (Hyclone) at 37°C in a 5% CO₂ humidified incubator (Fisher Scientific, Waltham, MA). To investigate osteogenic differentiation, MC3T3-E1 cells (2 × 10⁵ cells/well) were seeded in six-well plates containing 2 mL of α-MEM supplemented with 50 μg/mL ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO), 5 mM β-sodium glycerophosphate (Sigma-Aldrich), and dexamethasone (Xinhu Pharmaceutical, Shandong, China). For morphologic experiments, MC3T3-E1 cells (2 × 10⁵ cells/well) were seeded on glass slides.

Chloral Hydrate Treatment

MC3T3-E1 cells (2 × 10⁵ cells/well) were cultured in α-MEM medium containing chloral hydrate (CH; Sigma-Aldrich, Merck) at concentrations of 2, 4, or 8 mM for 24, 48, or 72 h to remove primary cilia from MC3T3-E1 cells. After CH pretreatment, cells were washed once with phosphate-buffered saline (PBS) and then recovered in fresh culture medium for 24, 48, or 72 h.

LMHFV Treatment

LMHFV was delivered at 35 Hz and a cell surface stress magnitude of 0.25 g (Cell Vibrator; designed by our Research Group, Jilin University, Changchun, China). MC3T3-E1 cells were cultured under static conditions and were stimulated with LMHFV for 20 min/day, 5 days/wk. Cells (2 × 10⁵ cells/well) were divided into six groups of at least three plates/group for subsequent experiments: control group, 12-day LMHFV group (cells were subjected to LMHFV for 12 days to study extracellular calcium deposition), 9-day LMHFV group (cells were LMHFV-induced for 9 days to study alkaline phosphatase differentiation), 7-day LMHFV group (cells were LMHFV induced for 7 days to study DNA content), 5-day LMHFV group (cells were LMHFV induced for 5 consecutive days), and 0-day LMHFV group (experiments were performed immediately after 20 min of LMHFV treatment).

Immunofluorescence

MC3T3-E1 cells (2 × 10⁵ cells/well) that were either pretreated with CH or subjected to 5 days of LMHFV treatment were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology, Shanghai, China) for 20 min at room temperature and were then washed and permeabilized with 0.2% TritonX-100 (BioFroxx, Guangzhou, China). Subsequently, the cells were incubated with mouse monoclonal α-acetylated tubulin primary antibody (ab24610; clone 6-11B-1; 1:1,000; Abcam, Cambridge, UK) at 4°C overnight and then were incubated with biotinylated goat anti-mouse secondary antibody (6-11B-1; 1:1,000; Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibodies (Abcam, Cambridge, UK) at 4°C overnight, and then stained with 10 g/mL FITC-conjugated streptavidin for 30 min at 37°C, and DNA was stained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The FITC- and DAPI-stained cells were covered in an anti-fade solution (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C. The excitation and emission wavelengths were used as specified by the manufacturer. Pictures were analyzed by three independent investigators using the ImageJ software (National Institutes of Health, Bethesda, MD) in a blinded fashion. Based on the microscopic pictures taken, cillum length was determined by the maximum intensity projection (MIP) method (10). The data for ≥100 cilia/treatment were obtained from at least three independent experiments, and the value is presented as means ± SD.

DNA Quantification

MC3T3-E1 cells (2 × 10⁵ cells/well) on day 7 of culture were first washed with PBS and then stored in carbonate dissolution buffer (Invitrogen Life Technologies, Paisley, UK) before harvesting and storage at 4°C for 24 h. Next, the cells were subjected to three rounds of freeze-thawing before resuspension in Tris-buffered EDTA solution. The Quant-IT PicoGreen reagent (Invitrogen Life Technologies, Paisley, UK) was then added and total DNA was measured at 485 nm excitation and 520 nm emission using an automated microplate reader (Bio-Rad Laboratories, Hercules, CA). Total DNA was calculated in reference to the standard curve.

Cell Viability Assay

MC3T3-E1 cells (5 × 10⁵ cells/well) plated in 96-well plates that were cultured for ≤7 days were incubated with 10 μL of CCK-8 (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C for 1 h and visualized using phase-contrast inverted microscopy (Olympus). The absorbance [optical density (OD)] at 570 nm was measured using an automated microplate reader (Bio-Rad Laboratories), and the cell viability ratio was calculated using the GraphPad Prism 5.0 software (La Jolla, CA).

Western Blot Analysis

MC3T3-E1 cells (2 × 10⁵ cells/well) subjected to 5 consecutive days of LMHFV stimulation were lysed in RIPA lysis buffer containing 0.1 mM phenylmethylsulfonyl fluoride protease inhibitor. Total cellular protein was quantified using the BCA assay (Beyotime Institute of Biotechnology, Shanghai, China) and then subjected to electrophoresis using 12% SDS-PAGE. The separated protein bands were transferred onto polyvinylidene fluoride (PVDF) membranes, which were probed with COL-1 (ab96723; 1:1,000), OCN (ab93876; 1:1,000), OPN (ab8448; 1:1,000), and histone H3 (ab1791; 1:2,000) polyclonal rabbit primary antibodies (Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies (A0208; Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at 37°C. The blots were detected using the enhanced chemiluminescence reagent (NCM Biotech, Suzhou, China), and the bands were quantified using the ImageJ software.

Real-Time Quantitative RT-PCR

Total RNA was extracted from the MC3T3-E1 cell (2 × 10⁵ cells/well) lysate using Tri-Reagent (Sigma-Aldrich, Merck) and then treated with DNase I (Invitrogen, Thermo Scientific) to remove DNA contaminant. Next, cDNA was obtained using the TaqMan reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was then performed, and the resulting mRNA expression levels were analyzed using an ABI Biosystems 7300 (Applied Biosystems, Singapore). The results were normalized with respect to the GAPDH gene. All reactions were performed in triplicate. The primers used for RT-PCR are as follows: COL-1, 5'-TTCCGGTGAAATCGTC-TC-3' (forward) and 5'-ACCTCGATTTCAATAGGACCAG-3' (reverse); Runx-2, 5'-TTCTGCAGGGTCTATCAGTT-3' (forward) and 5'-CTTCCATACGGCTAACCAC-3' (reverse); Cox2, 5'-GGTGGTGGCCGCTGGTGATG-3' (forward) and 5'-GTCCTTTCAAGGGAATGTGTCG-3' (reverse); GAPDH, 5'-GGCACAGTCGCAAGGATA-3' (forward) and 5'-ATGGTGTTGAGACGCTAGA-3' (reverse).

Alizarin Red Staining

MC3T3-E1 cells (2 × 10⁵ cells/well) exposed to LMHFV for 12 days (12-day LMHFV group) were first fixed with 4% paraformaldehyde and then washed with distilled water. The cells were then stained with 5 mg/mL Alizarin Red (AR) solution at pH 4.1 for 10 min to
were incubated with 5 ALP activity was then measured as previously described (17). Cells aldehyde for 20 min and then washed with PBS. Alkaline phosphatase stimulation (9-day LMHFV group) were fixed with 4% paraformaldehyde for 20 min and then washed with PBS. Alkaline phosphatase (ALP) activity was then measured as previously described (17). Cells were incubated with 5-bromo-4-chloro-3’-indolylphosphate (BCIP) and nitro-blue tetrazolium (NBT) staining solution (Alkaline Phosphatase Color Development Kit; Beyotime Institute of Biotechnology, Shanghai, China) for ≥5–30 min at room temperature in the dark until development of the desired blue-violet coloration, which in combination yield an intense black-purple color when reacted with alkaline phosphatase and were imaged using a scanner (Olympus Lifescience, Tokyo, Japan). Ten fields of view per sample were captured and percentage area of staining was quantified.

Statistical analysis

Data analysis was performed using SPSS for Windows (SPSS version 16.0; SPSS Inc., Chicago, IL), and data are expressed as means ± SD. Pairwise comparison was performed using one-way analysis of variance (ANOVA; a = 0.05), followed by Bonferroni post hoc tests. Data that did not follow a normal distribution or were heterogeneous were compared using the Mann-Whitney nonparametric comparison of means. A P value of <0.05 was considered statistically significant.

RESULTS

MC3T3-E1 Cells Express Primary Cilia

The presence of primary cilia (green) on MC3T3-E1 cells, which appeared as small, bright protrusions at the apical face of the cell membrane, was confirmed using laser confocal microscopy. Cell nuclei stained positively for DAPI (Fig. 1A). Both the primary cilia and cytoplasmic microtubule network (which appeared less bright than the stained primary cilia) were localized near the nucleus (Fig. 1, B and C).

**CH Damages and Removes Primary Cilia**

Cell morphology. OBs were treated with 0, 2, 4, and 8 mM CH for 24 h (Fig. 2A) or with 4 mM CH after 0, 24, 48, and 72 h (Fig. 2B) and recovered in fresh medium for 24 h (Fig. 3, A and B). In the absence of CH, the OBs appeared translucent and had a long fusiform shape. Dead cells were observed floating at the upper layer of the culture dish, and the adherent cells appeared shrunken for OBs treated with 4 mM CH for 48 h (Fig. 2B) and with 8 mM CH for 24 h (Fig. 2A). Similarly, treatment of OBs with 4 mM CH for 72 h resulted in a significant reduction in the number of cells, with only a minimal number of adherent cells (Fig. 2B). This indicates that prolonged exposure (>24 h) or a high concentration (8 mM) of CH causes cytotoxic effects in OBs.

Primary cilia regulate OB viability. The viability of CH-treated MC3T3-EL cells was assessed using the CCK-8 assay. CH treatment at high concentration or long duration reduced MC3T3-E1 cell viability compared with the control group (not treated with CH; Fig. 2, C and D). MC3T3-E1 cell viability was >95% after 24-h exposure to 4 mM CH (P > 0.05), but increases in CH concentration to 8 mM led to a significant decline in cell viability to 24.6% (P = 0.016).

We then studied the effect of CH on the prevalence and structure of primary cilia by assessing the CH concentration, exposure time, and rest time. Increasing the CH concentration to 4 mM for 24 h, followed by 24-h recovery or increasing the 4 mM CH treatment duration to 48 h, reduced the percentage of ciliated OBs from 64.3 ± 5.2 to 20.7 ± 3.5 and 10.3 ± 1.9%, respectively. We observed a nearly complete removal of primary cilia on cells treated with 8 mM CH for 24 h, followed by 24 h recovery or exposure to 4 mM CH for 72 h (Fig. 3, A, B, D, and E). Culturing in fresh medium allowed OBs to recover to their normal ciliated state; cells that were allowed the longest recovery time of 72 h showed almost 100% recovery of primary cilia (Fig. 3, C and F). OBs exposed to 4 mM CH for 24 h and then allowed a 24-h recovery in fresh culture medium showed disordered intracellular microtubule networks, which appeared as a strong but diffuse green staining near the nuclei (Fig. 3G). In addition, the average length of primary cilia was significantly reduced from 3 ± 0.8 μm in the...
control group to 1.03 ± 0.07 μm in the CH-treated group (Fig. 3H). The data of cilia length followed a normal distribution.

In conclusion, because of good deciliation together with good viability, we chose the optimal condition (4 mM CH for 24 h, followed by 24 h of recovery) and used it for the remaining experiments.

**LMHFV Causes Primary Cilia Morphological Alterations**

MC3T3-E1 cells stimulated with LMHFV for 5 consecutive days exhibited shorter, less well-defined, and stub-like primary cilia (Fig. 4A). In addition, we observed a ~30% reduction in the number of ciliated OBs in the LMHFV group compared with the static control group (Fig. 4B). Furthermore, the primary cilia of the LMHFV group showed an ~40% decrease (reduced from 3 ± 0.8 μm) in length compared with the static control group (Fig. 4C). There was no difference in the amount of total DNA in the static control and 7-day LMHFV groups (Fig. 4D).

**Primary Cilia Regulate LMHFV-Induced OB Differentiation, Mineralization, And Maturation**

Next, we investigated the effect of LMHFV on OB differentiation by measuring protein expression of the major bone matrix protein COL-1 and the two most common noncollagen bone matrix proteins OPN and OCN on day 5 after LMHFV treatment. Compared with the static control group, the LMHFV group had significantly higher COL-1, OPN, and OCN protein expression (Fig. 5, A–F). However, expression of these proteins was downregulated in response to CH pretreatment, indicating that CH almost negated the OB osteogenic effect of LMHFV. These results were confirmed with RT-PCR, which revealed that COL-1, COX-2, and RUNX-2 (transcription factor associated with OB differentiation) mRNA levels were downregulated in the LMHFV group in response to CH pretreatment, while LMHFV increased these measures, as shown in Fig. 5, G–I. These data indicate that the presence of primary cilia is necessary for LMHFV-induced osteogenic differentiation.

MC3T3-E1 cells that were either untreated or pretreated with 4 mM CH for 24 h followed by 24 h recovery in fresh medium were subjected to LMHFV stimulation. The cells were then stained with AR to assess the extent of matrix mineralization using phase contrast microscopy. A darker and larger stain area was observed in the LMHFV group compared with the control group (Fig. 6A). Moreover, CH pretreatment resulted in reduced calcium deposition compared with the CH-

Fig. 2. Effect of chloral hydrate (CH) on MC3T3-E1 cell viability. A–D: phase-contrast micrographs (A and B) and CCK-8 cell viability assay results (C and D) of osteoblasts (OBs) (5 × 10^4 cells/well) treated with 0, 2, 4, and 8 mM CH for 24 h, followed by 24 h of recovery in fresh medium (A and C) and OBs (5 × 10^5 per mL) treated with 4 mM CH after 0, 24, 48, and 72 h, followed by 24 h of recovery in fresh medium (B and D). Values are means ± SD (n = 3). *P < 0.05 or **P < 0.01 vs. control.
untreated groups (untreated and treated with LMHFV); the decrease in calcium deposition was significantly greater in the CH-treated LMHFV group (86.7% reduction) compared with the CH-treated group (not LMHFV-induced; 60.3% reduction), indicating the significant inhibition of CH in LMHFV-induced matrix mineralization (Fig. 6, A and C).

ALP staining of MC3T3-E1 OBs with BCIP/NBT after 9 days of LMHFV stimulation showed increased areas of ALP-positive colonies compared with the untreated control group and the LMHFV/H11001 CH-treated group (Fig. 6, B and D), indicating that CH prevented LMHFV-induced OB maturation.

**DISCUSSION**

We first sought to confirm the presence of primary cilia on MC3T3-E1 cells. A-F: immunofluorescence images (A–C) and semiquantitative profiles (D–F) of ciliated MC3T3-E1 cells (2 × 10^5 cells/well) pretreated with 0, 2, 4, or 8 mM CH for 24 h followed by 24 h of recovery in fresh medium (A and D), pretreated with 4 mM CH for 0, 24, 48, or 72 h followed by 24 h of recovery in fresh medium (B and E), and pretreated with 4 mM CH for 24 h followed by 0, 24, 48, or 72 h recovery in fresh medium (C and F). Representative primary cilia are indicated by yellow arrowheads. G and H: immunofluorescence images of primary cilia of MC3T3-E1 cells (G) and semiquantitative profile of the average length of primary cilia expressed on MC3T3-E1 cells (H) pretreated with 4 mM CH for 24 h followed by 24 h of recovery in fresh medium. Values are means ± SD (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

Fig. 3. Effect of chloral hydrate (CH) pretreatment on the number and length of primary cilia on MC3T3-E1 cells. A–F: immunofluorescence images (A–C) and semiquantitative profiles (D–F) of ciliated MC3T3-E1 cells (2 × 10^5 cells/well) pretreated with 0, 2, 4, or 8 mM CH for 24 h followed by 24 h of recovery in fresh medium (A and D), pretreated with 4 mM CH for 0, 24, 48, or 72 h followed by 24 h of recovery in fresh medium (B and E), and pretreated with 4 mM CH for 24 h followed by 0, 24, 48, or 72 h recovery in fresh medium (C and F). Representative primary cilia are indicated by yellow arrowheads. G and H: immunofluorescence images of primary cilia of MC3T3-E1 cells (G) and semiquantitative profile of the average length of primary cilia expressed on MC3T3-E1 cells (H) pretreated with 4 mM CH for 24 h followed by 24 h of recovery in fresh medium. Values are means ± SD (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.
significantly promote osteogenesis (21, 22, 33), but its mechanism of action remains unclear.

We hypothesized that LMHFV-induced vibrations stimulate tissue fluid flow, which in turn promotes bone formation. Since primary cilia were found to function as mechanosensors through their receptor tyrosine kinases (RTKs) and ion channels (6, 32) and also affect OB differentiation (39), we speculated that cilia may be involved in LMHFV-induced osteogenesis. Indeed, we observed a reduction in the density of ciliated OBs accompanied by structural distortion and shortening of primary cilia after LMHFV stimulation. Our findings are consistent with previous studies that reported similar reductions in the length of primary cilia upon exposure to mechanical stimuli (9, 25). The lack of change in total DNA in the static control and LMHFV groups indicates that the LMHFV-induced changes in cilia length and number are not due to increased OB proliferation. Taken together, these findings suggest that primary cilia can adjust their sensitivity to different mechanical stimuli by altering their morphology. Application of CH, which damages primary cilia, inhibited LMHFV-induced osteogenic responses (differentiation, mineralization, and maturation) in MC3T3-E1 OBs, indicating that LMHFV-induced osteogenic effects on OBs are predominantly mediated through primary cilia.

Based on the balance point model, cilia adjust their length through microtubule assembly and disassembly (24). Upon stress deprivation, the length of primary cilia in rat tail tendons was increased, and cilia length was restored to normal levels when subjected to cyclic loading (11). Mechanical and chemical stressors are additional stimuli that can affect cilia length and morphology. For instance, direct mechanical stress induces microtubule rupture, which disrupts the structural integrity of cilia, causing them to become distorted and shortened. The length of primary cilia is also affected by chemical changes in the microenvironment. For example, human adipose-derived stem cells (hASC) produced longer cilia when cultured in osteogenic differentiation medium than when cultured in expansion medium.
Cells that are actively dividing have a lower number of cilia compared with normal passive cells and tend to have shorter cilia, averaging 1–5 μm in length (3).

At present, primary cilia can be removed from cells via siRNA knockdown or CH treatment, with the latter being a more effective method that can negatively affect cellular architecture. Even 2 mM CH pretreatment is more effective at removing cilia than using the siRNA knockdown method (4). CH disrupts a cell’s ability to interact with the matrix (28), and high CH concentrations can affect mitosis (20), thereby reducing cell viability and promoting apoptosis. Thus, the use of CH to study the function of primary cilia must be properly controlled in terms of concentration and duration of treatment to maintain cell viability. We chose the CH concentration in our study based on the findings of Praetorius and Spring (28), who reported that 4 mM CH pretreatment for 24 h followed by 72 h of recovery restored the number of ciliated cells as well as returned the cilia morphology and structure to normal. We successfully replicated the observations of the Praetorius study and found that a 24-h recovery period after CH treatment is sufficient to return cells to the default condition. Using the CCK-8 assay, we confirmed that cell viability at 4 mM CH pretreatment was not affected and that CH concentrations exceeding 8 mM significantly reduced cell viability. We established the working concentration range of CH (< 8 mM) and showed that CH pretreatment at 4 mM for 24 h followed by 24 h of recovery is a safe and effective method to investigate primary cilia.

Although LMHFV stimulation could induce bone formation through primary cilia, other intracellular structures may similarly function as mechanosensors and also contribute to this physiological process. Potential mechanosensors may include glycocalyx (9), calcium channels (19, 23), integrins (27), adhesions (16), cytoskeleton (22a), and cell surface receptors such as bone morphogenetic protein-II (36), all of which have reported roles in cilia-mediated osteogenic responses. Clarification of the functional interplay between primary cilia and other mechanosensors is a potential area of focus in future studies.

In this study, we noted a significant reduction in bone matrix protein and mRNA levels (OCN, COL-1, COX-2, and RUNX-2) in OBs pretreated with CH despite LMHFV treatment. These protein and mRNA changes corresponded with inhibition of OB differentiation, mineralization, and maturation. This is consistent with a previous study that showed that disruption of the microtubule transporter proteins within cilia, such as PC1, PC2, and IFT88, inhibited hASC proliferation,
osteogenic gene expression, and calcium deposition and increased ALP activity (4).

Limitations of this study include the potential cytotoxicity of CH and the in vitro study design. Because CH-mediated destruction of microtubules can remove cilia as well as block mitotic spindle formation (20), we took special care to minimize the cytotoxic effects of CH and successfully identified an optimal treatment condition that does not negatively affect

Fig. 6. Chloral hydrate (CH) pretreatment inhibits low-magnitude, high-frequency vibration (LMHFV)-induced differentiation, mineralization, and maturation of MC3T3-E1 cells. Cells (2 × 10^5 cells/well) were pretreated with 4 mM CH for 24 h, followed by 24 h of recovery in fresh medium, and were subsequently divided into the static control group and LMHFV-treated group. A: images of Alizarin Red (AR) -stained cells on day 12. B: images of ALP-stained cells at day 9. C and D: comparison of AR-stained (C) and ALP-stained areas (D) among groups. Values are means ± SD (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. respective samples.
primary cilia morphology in the long term and number or cell viability. There are four types of bone cells in situ, but we used only a single cell type (the OB MC3T3-E1 cell line) in this study to simplify the study design. Moreover, our in vitro cell culture model may not adequately replicate the complex in vivo mechanical and biochemical environment. Thus, animal models should be used in future studies to provide deeper insight and confirmation of the findings of this study.

Conclusion

We demonstrated that primary cilia may respond to LMHFV through morphological alterations of microtubule cyclic loading sensitivity. In addition, our study demonstrated the significant inhibition by CH of LMHFV-induced OB mineralization, maturation, and differentiation, which might reveal the critical role of primary cilia in the process. Our findings provide novel insight into the underlying mechanism of vibration-induced osteogenesis and offer new perspectives in applying vibration therapy for preventing bone diseases that arise from dysfunctional cilia loading responses.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.S. and L.T. conceived and designed research; Y.-H.L., Z.C., Y.L., and J.S. performed experiments; D.Z. analyzed data; D.Z. interpreted results of experiments; Y.L. prepared figures; Y.-H.L. drafted manuscript; J.S. edited and revised manuscript; Y.-H.L., Z.C., Y.L., J.S., and L.T. approved final version of manuscript.

REFERENCES


27. Praetorius HA, Praetorius J, Nielsen S, Frokiaer J, Spring KR. 


