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Spontaneous cellular vibratory motions of osteocytes are regulated by ATP and spectrin network

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ABSTRACT

Vibration at high frequency has been demonstrated to be anabolic for bone and embedded osteocytes. The response of osteocytes to vibration is frequency-dependent, but the mechanism remains unclear. Our previous computational study using an osteocyte finite element model has predicted a resonance effect involving in the frequency-dependent response of osteocytes to vibration. However, the cellular spontaneous vibratory motion of osteocytes has not been confirmed. In the present study, the cellular vibratory motions (CVM) of osteocytes were recorded by a custom-built digital holographic microscopy and quantitatively analyzed. The roles of ATP and spectrin network in the CVM of osteocytes were studied. Results showed the MLO-Y4 osteocytes displayed dynamic vibratory motions with an amplitude of ~80 nm, which is relied both on the ATP content and spectrin network. Spectrum analysis showed several frequency peaks in CVM of MLO-Y4 osteocytes at 30 Hz, 39 Hz, 83 Hz and 89 Hz. These peak frequencies are close to the commonly used effective frequencies in animal training and in-vitro cell experiments, and show a correlation with the computational predictions of the osteocyte finite element model. These results implicate that osteocytes are dynamic and the cellular dynamic motion is involved in the cellular mechanotransduction of vibration.

1. Introduction

Low magnitude vibrations at high frequency (above 20 Hz) have been widely accepted to be beneficial for bone in both exercise and medical areas [1,2]. At the cellular level, vibrations are also demonstrated to be anabolic for osteocytes [3–5]. Our previous study shows that the response of osteocytes to vibration stimulation is frequency-dependent, but is not simply linear. Specifically, vibrations around 30 Hz have a positive effect on osteogenesis, while when the frequency reaches 90 Hz, the effect is opposite. A finite element model was built to seek explanations of the dynamic mechanical behaviors of osteocytes in our previous work. The harmonic response analysis showed the osteocyte finite-element model possessed several resonance frequencies points at 34 Hz, 86 Hz and 104 Hz etc. [6]. The computational result is inspiring that among these resonance frequencies, 34 Hz, is quite close to the effective vibration frequencies adopted in many rehabilitation trainings on animals [7–9]. So it is speculated that the frequency-dependent response of osteocytes to vibration may be related with the resonance motions of cells, which means a synergistic effect of the cellular spontaneous motions with external vibrations. However, the cellular spontaneous dynamic motions of osteocytes, especially at high frequencies, have not been confirmed.

The phenomenon of cell vibratory motions (CVM) or “flickering” was first observed in erythrocyte by Browicz T in 1890 [10]. Recently, CVM has been found in some kinds of eukaryotes [11,12] and thought to be closely related with specific cell functions [13]. For example, in neurons, CVM of 0.1–0.4 Hz is originated from the reorganization of the plasma membrane, and CVM around 1-3 Hz is related to changes of the membrane potential and/or spontaneous rhythmic activities.

Mechanism of erythrocyte flickering has been widely studied for

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decades, and both active and passive excitation mechanisms have been proposed [14]. In the active process, the role of adenosine 5′-triphosphate (ATP) in CVM is controversial. In some literatures, ATP is thought to be the energy resource of CVM [15,16]. The depletion of ATP causes decreased CVM amplitude [17]. While there are also reports demonstrate the independence of CVM on ATP [18,19]. On the other hand, the spectrin cytoskeleton underlying the cell membrane is thought to be the essential structural basis for CVM. The transient attachment/detachment of spectrin network to the cell membrane and the continuous remodeling of skeleton nodes result in the undulation of the cell membrane [20]. Besides, the dynamic spectrum of CVM is dominated by the spectrin-governed mechanical properties of cells, including the membrane tension and viscosity [21].

In osteocytes, the ATP molecule and spectrin network are both previously concerned for their critical roles in cellular mechanotransduction. The ATP molecule is acute mediator in osteocyte mechanotransduction [22]. It can not only mediates the P2 receptor pathway and Ca^{2+} influx in single osteocyte [23], but also propagates through junctional connections between osteocytes for cell-cell communications [24]. As for spectrin, our previous work has demonstrated the spectrin network is essential for maintaining the overall elasticity of osteocytes, and involved in several key signal pathways of mechanotransduction, including nitric oxide (NO) secretion, Cx43 connections and Ca^{2+} current pathway [25]. Although the contributions of ATP and spectrin to cellular mechanotransduction have been confirmed, little is known about their roles in the dynamic characteristic of osteocytes.

Hence, in this study, the CVM of MLO-Y4 osteocytes was recorded by DHM[1] and analyzed. The auto correlation function (ACF) and relaxation time of CVM were calculated as metrics of the dynamic characteristic of osteocytes. The contributions of ATP content and spectrin network to CVM of osteocytes were also studied to rationalize the frequency-dependent response of osteocytes to vibration.

2. Materials and methods

2.1. Cell culture

The murine long bone osteocyte Y4 (MLO-Y4) cell line was kindly provided by Dr. Lynda F Bonewald of University of Missouri-Kansas City [26]. The MLO-Y4 cells were maintained in Minimum Essential Medium (MEM) (Gibco, USA) supplemented with 5% (vol/vol) fetal bovine serum (FBS) (HyClone, South America) and 5% (vol/vol) calf serum (CS) (HyClone, New Zealand) at 37 °C, 5% CO_2. Cells were passaged on a thin glass-bottom culture dish (NEST, China) 24 h before DHM recording.

2.2. Drug treatments

2.2.1. Increase and reduce of intracellular ATP content

To explore the effect of ATP on CVM of osteocytes, four groups of cells with different intracellular ATP levels were set: Adenosine 5-triphosphate disodium salt ($ATPNa_2$) of 10 μM was added in culture medium to increase the exogenous ATP concentration (ATP + group) 1 h before DHM recording; To reduce the ATP concentration, cells were incubated in glucose and sodium pyruvate-free medium (11,966,025, Gibco, USA) for 2 h (ATP- group) or 4 h (ATP- group) before DHM recording. Also, a control group of cells with normal ATP level (CON) was set.

2.2.2. Disruption of spectrin network

To explore the effect of spectrin on CVM of osteocytes, diazene dicarboxylic acid bis [*N*,*N*-dimethylamide] (diamide, Sigma-Aldrich, MO, USA) of 500 μM was used to disrupt the spectrin cytoskeleton of MLO-Y4 osteocytes (DIA group) as described previously [25]. A DMSO control group was set and 0.1% DMSO was added in culture medium to exclude the effect of DMSO in the DIA solution.

To completely eliminate the ATP of MLO-Y4, antimycin A of 0.1 μM was added in culture medium for 30 min before the following treatment, and denoted as –ATP group. A control group of cells with normal level of ATP was denoted as + ATP group.

For all the drug-treated groups, MTT assay was conducted to exclude the cytotoxicity effect on cells.

2.3. ATP level measurement

The ATP concentration of MLO-Y4 osteocytes was measured by an ATP assay kit (Beyotime, China) based on bioluminescence. Briefly, cells were lysised by lysis buffer on ice. Excessive luciferin and firefly luciferase were added to cell lysates. The luciferin was excited by ATP in the presence of luciferase. The relative light unit (RLU) was measured by luminometer (Thermo Scientific, USA). The ATP concentration was proportional with RLU. For all groups, the ATP content was normalized with total protein amount for statistical analysis.

2.4. DHM recording of CVM on MLO-Y4 osteocytes

A customized DHM system was set up to record the holographic image of MLO-Y4 osteocytes as described in our previous study [27]. Briefly, holograms (512*512 pixels) of MLO-Y4 osteocytes were recorded at 180 Hz for 10 s in series. Therefore, 1800 holograms were acquired both before and after the drug treatments in all groups. For each sampling point on cell, 3*3 pixels were included.

2.5. Analysis of CVM on MLO-Y4 osteocytes

A decoupling procedure was adopted in the calculation of the vertical height of cell $z(t)$ from the holographic phase images [28]. The time-dependent membrane displacement is defined as the amplitude of CVM:

$$h(t) = \sqrt{(z(t) - z_0)^2} \tag{1}$$

where z_0 is the average height in 10s recording.

The autocorrelation function of the displacement $ACF \equiv \langle h(t)h(0) \rangle$, and the ACF is fitted with one-phase decay exponential function, and defined as ACF_f. The relaxing time $t_{relaxation}$ is defined as the time point when $ACF_f(t) = ACF_f(0)/e$ to compare the membrane tension and viscosity of cells [29].

For frequency analysis, $h(t)$ was Fourier-transformed to obtain the power spectrum of CVM ($Fh(t)$). A straight line was obtained by least square fit of Log_{10} scale of $Fh(t)$. The slope of the straight line is denoted as k_{PM}. The absolute value of k_{PM} was used as a characteristic metric for comparing the decay rate of CVM power spectrum [18].

According to Pelling's study [30], the Fourier transformed ACF (F_{ACF}), rather than $Fh(t)$, was used to analyze the non-random characteristic of CVM. As the random component of CVM has been filtered by ACF calculation according to *Wiener–Khinchin* theorem, the F_{ACF} is more powerful for the analysis of principle frequency of CVM.

A fixed-cell control group was set to exclude the coherent noise from the DHM system. Briefly, cells were fixed by 4% paraformaldehyde for 10 min and the same amount of culture medium was added before recording. For all data analysis, at least 15 sampling points were chosen randomly on each cell. > 15 cells from at least three repeated experiments were analyzed in all groups. For all the drug-treated groups, normalization to the untreated cells was conducted. The frequency spectrum was analyzed using purpose-written algorithms programmed in Matlab (version 2015).

[1] Digital holographic microscopy

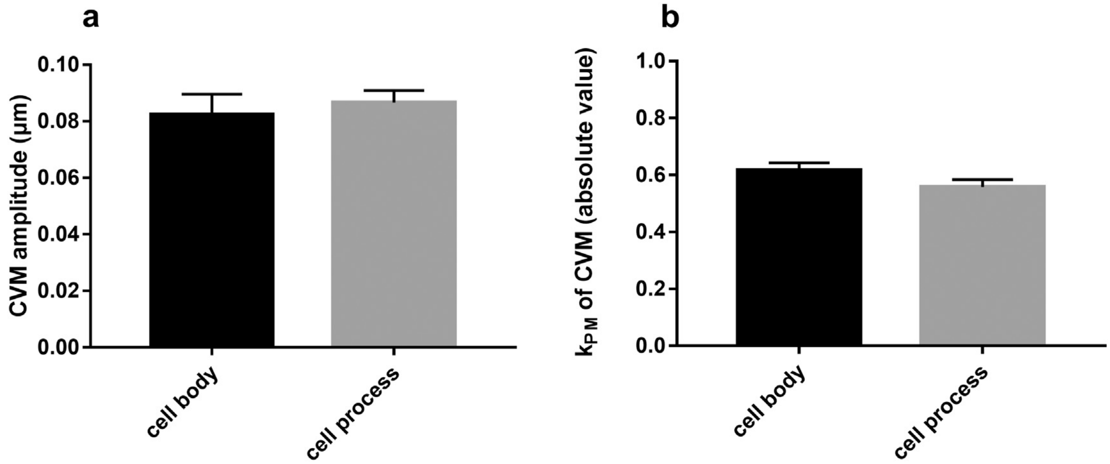

Fig. 1. The amplitude and k_{PM} of CVM on osteocyte. The average CVM amplitude of osteocyte was 0.08229 ± 0.007272 μm on the cell body and 0.08661 ± 0.004302 μm on the cell process. The absolute value of k_{PM} was 0.62 ± 0.027 on the cell body and 0.56 ± 0.026 on the cell process. No significant difference was observed between the cell process and cell body.

2.6. Statistical analysis

All values were expressed as mean ± standard error (SEM). Student's *t*-test was used to compare between two groups and One-way ANOVA was used to compare between > 3 groups followed by Turkey multiple comparison. Two-way ANOVA was used to analyze the effects of DIA with or without ATP and followed by multiple comparisons (GraphPad Prism 7). When *p*-value was under 0.05, the difference was thought to be significant.

3. Results

3.1. CVM of MLO-Y4 osteocytes

The CVM of MLO-Y4 osteocytes on both the cell body and cell process were measured by DHM. Results showed that, the average amplitude of CVM on the osteocyte cell body was 0.08229 ± 0.007272 μm ($n = 30$) and 0.08661 ± 0.004302 μm ($n = 25$) on the cell process (Fig. 1). No significant difference of the CVM amplitude can be observed between the cell body and cell process. The absolute value of k_{PM} of the cell body was 0.62 ± 0.027 on the cell body and 0.56 ± 0.026 on the cell process. Also, there is no significant difference between the cell body and cell process. Therefore, the sampling points on the phase image of cell were randomly selected in the following experiments.

3.2. Effect of ATP concentration on CVM

The intracellular ATP content of osteocytes was first confirmed (Fig. 2). The CVM amplitude of MLO-Y4 osteocytes with different ATP concentrations was analyzed. Results showed that when the ATP concentration was increased by $ATPNa_2$ (ATP+), the amplitude of CVM was also enhanced (Fig. 3a). The average amplitude of CVM was increased by 18.9% and the area under the recording curve (AUC) was increased by 19.1% compared with CON. On the contrary, the CVM was weakened with decreased ATP content (Fig. 3a). When the ATP content was consumed for 2 h (ATP-) in glucose and sodium pyruvate-free medium, the amplitude of CVM was decrease by 10.7%, and the AUC was decreased by 10.7%. When ATP was consumed for 4 h (ATP–) in glucose and sodium pyruvate-free medium, the amplitude of CVM was decreased by 10.4%, and the AUC was decreased by 10.4%. The duration of ATP consumption showed no obvious effect on either the CVM amplitude or AUC (Fig.3b).

The autocorrelation function (ACF) of CVM from osteocytes with

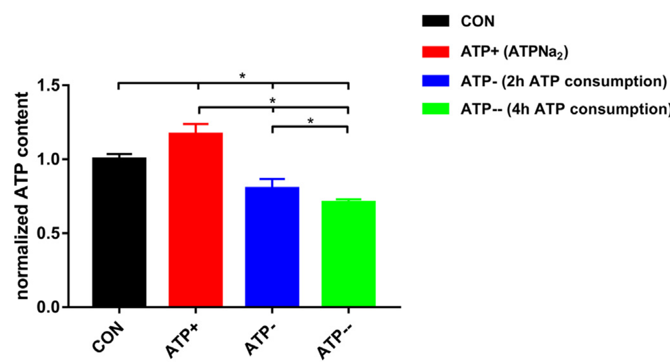

Fig. 2. Confirmation of intracellular ATP content of MLO-Y4 osteocytes. Exogenous $ATPNa_2$ was added and resulted in an increase of intracellular ATP content by 15.9% (ATP+). The consumption of ATP in glucose and sodium pyruvate-free medium for 2 h (ATP-) and 4 h (ATP–) decreased the ATP content of cell by 27.4% and 29.3% respectively. Significant difference of One-Way ANOVA was signed by * ($p < 0.05$).

different ATP levels was also compared. When the ATP level was elevated, the ACF curve decayed faster than the CON group. While when the ATP was consumed, the ACF curve decayed slowly (Fig. 4a). The relaxation time was calculated from ACF_f. The results showed the relaxation time of osteocytes of control group was 0.020 ± 0.0015 s, and the osteocytes with higher ATP content (ATP+) has a shorter relaxation time of 0.018 ± 0.0012 s. The ATP-consumed osteocyte (ATP-) has a longer relaxation time of 0.0354 ± 0.0023 s, and with more ATP being consumed, the relaxation time decreased to 0.030 ± 0.0021 s (Fig. 4b).

3.3. Effect of spectrin disruption on CVM amplitude of osteocytes

MLO-Y4 osteocytes were treated by DIA to disrupt the spectrin cytoskeleton for 20 min and CVM was measured both before and after drug treatment. The CVM amplitude was normalized to the CVM amplitude before drug treatment. The heat map of the normalized CVM amplitude of a single osteocyte from CON (Fig. 5a) and DIA (Fig. 5b) is respectively represented. It was obviously that the CVM amplitude of osteocytes was enhanced by the spectrin disruption (Fig. 5c). Meanwhile, the ATP content was also increased after the DIA treatment (Fig. 5d).

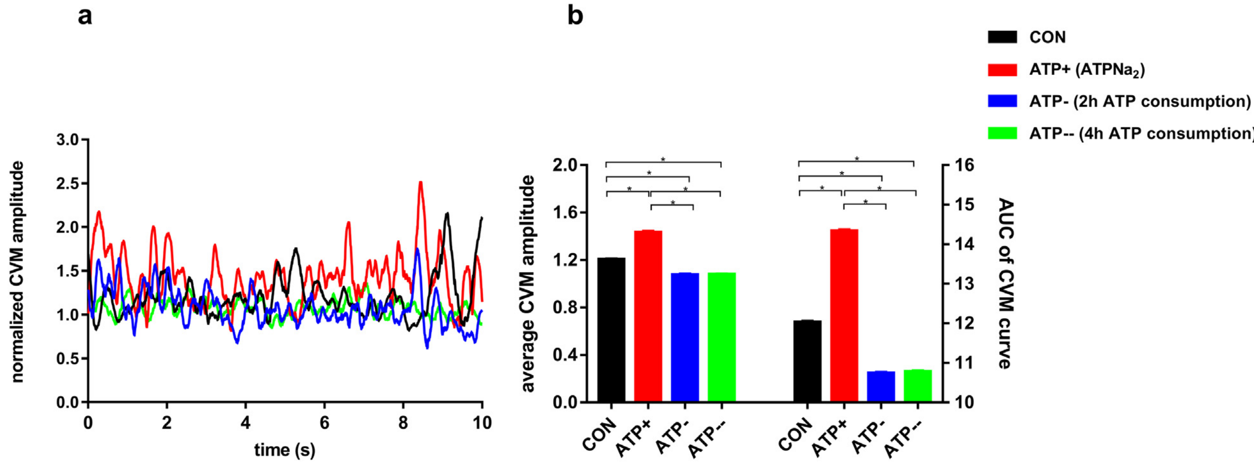

Fig. 3. The CVM of MLO-Y4 osteocytes under different ATP levels. (a) The CVM curve of MLO-Y4 osteocytes in 10s. Osteocytes of control group showed a dynamic vibration motions with irregular peaks and troughs (black curve). The increase of ATP content caused a more intense vibration motion of osteocytes with higher peaks (ATP +, red curve) and consumption of ATP in glucose and sodium pyruvate-free medium for 2 h/4 h caused a alleviated vibration motion of osteocytes (ATP-/ATP–,blue/green curve). (b) Quantification of the CVM motions with the average amplitude (left Y- axis) and area under the CVM curve (right Y-axis). Significant difference of One-Way ANOVA followed by Turkey *post*-test was signed by * ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

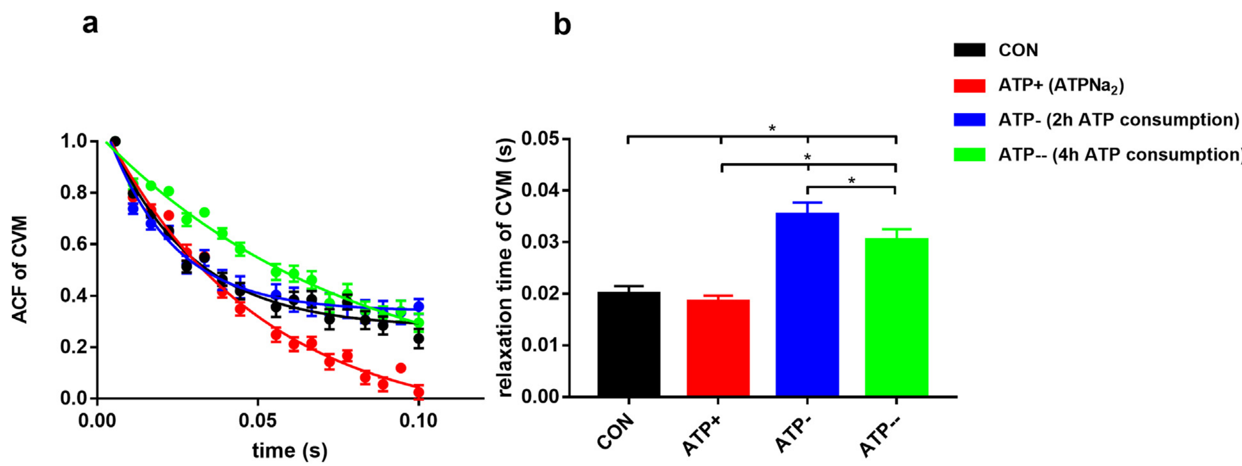

Fig. 4. The ACF analysis of CVM from MLO-Y4 osteocytes. (a) The AFC curve of MLO-Y4 osteocytes under different ATP levels. The ACF curve decayed faster when exogenous ATP was added (ATP +) and decayed slowly when ATP was consumed in glucose and sodium pyruvate-free medium for 2 h or 4 h (ATP-/ATP–). (b) Correspondingly, the $t_{relaxation}$ of ATP + ($ATPNa_2$) was shorter than the $t_{relaxation}$ of CON, and the $t_{relaxation}$ of ATP- (2 h ATP consumption) was longer. Although the $t_{relaxation}$ of ATP-- (4 h ATP consumption) was still longer than the $t_{relaxation}$ of CON, it was shorter than the $t_{relaxation}$ of ATP- (2 h ATP consumption). Significant difference of One-way ANOVA followed by Turkey post-test was signed by * ($p < 0.05$).

3.4. Effect of spectrin disruption on CVM is partly relied on ATP

The treatment of DIA enhanced the CVM of MLO-Y4 osteocytes, and meanwhile introduced an increase of ATP content, which could also increase the CVM. To further elucidate the mechanism of CVM enhancement caused by DIA (the spectrin disruption or the increase of ATP?), the effect of DIA treatment on CVM with (+ATP) or without ATP (-ATP) was studied. Results showed that, the treatment of DIA caused an increase of CVM amplitude in normal ATP environment, and when ATP was depleted, the treatment of DIA also increased the CVM amplitude of osteocytes, but to a lesser extent (Fig. 6a and Table 1). From the ACF analysis, it is observed that the MLO-Y4 osteocytes relaxed much more slowly in the absence of ATP, and the treatment of DIA showed an obvious recovery effect (Fig. 6b and Table 1). For the absolute value of k_{PM}, the treatment of DIA also displayed a similar promoting effect in both +ATP and –ATP environment (Fig. 6c and Table 1). Two-way analysis shows an interaction effect of ATP and DIA on both the CVM amplitude and relaxation time, but not on k_{PM} of CVM (Table 2).

3.5. Spectrum analysis of CVM of MLO-Y4 osteocytes

To ascertain whether a resonance effect involves in the frequency-dependent response of MLO-Y4 osteocytes to vibration, the spectrum of CVM was analyzed by F_{ACF}. The F_{ACF} of paraformaldehyde-fixed cells showed three system frequency peaks at about 23 Hz, 45 Hz and 68 Hz (Fig. 7). To eliminate the influence of the coherent noise of the recording system, frequencies bands of 25-40 Hz, 47-62 Hz and 75-90 Hz were chosen for analysis.

It was observed that, the MLO-Y4 osteocytes showed four frequency peaks at around 30 Hz, 39 Hz, 83 Hz and 89 Hz (blue curve, Fig. 8). After the DIA treatment, the CVM of osteocytes showed four frequency peaks at around 51 Hz, 55 Hz, 80 Hz, and 86 Hz (yellow curve, Fig. 8). When ATP was depleted, frequency peaks at around 81 Hz and 89 Hz were observed (green curve, Fig. 8), and the DIA treatment did not affect the frequency peaks in the absence of ATP (red curve, Fig. 8).

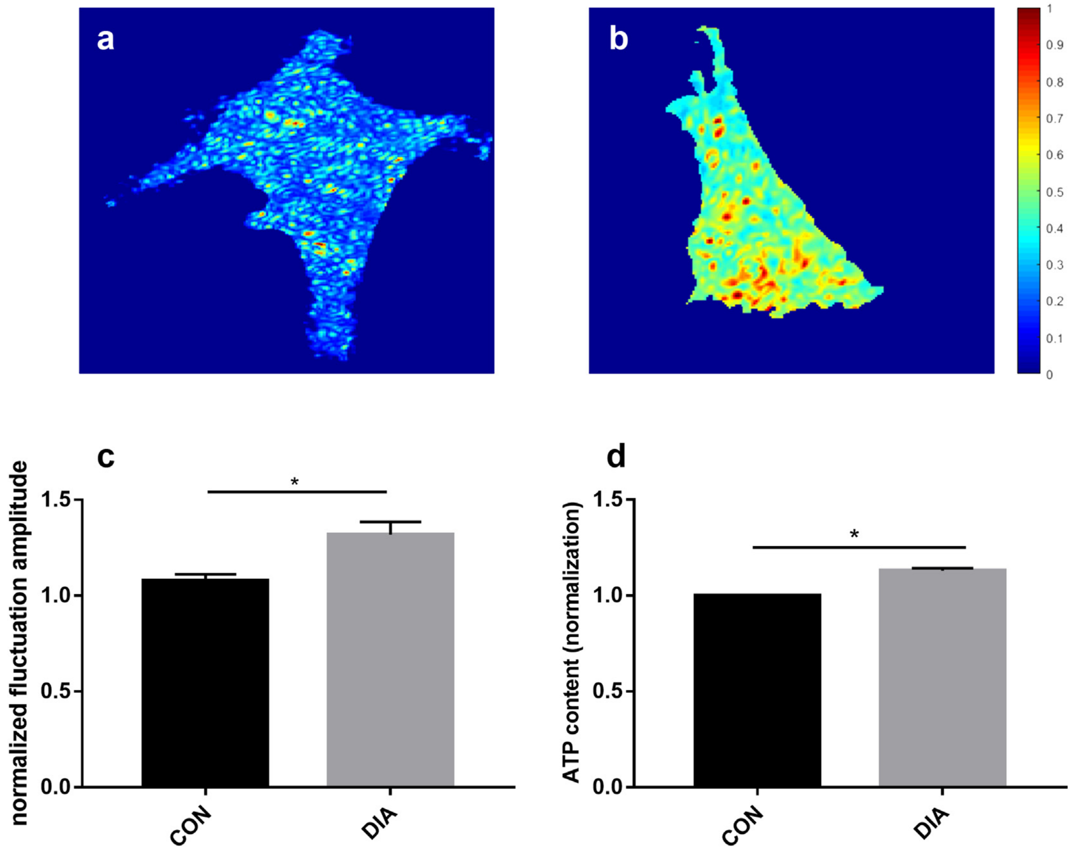

Fig. 5. The effects of DIA treatment on CVM amplitude and ATP content of MLO-Y4 osteocytes. (a) Heat map of CVM amplitude of a representative control osteocyte. (b) Heap map of CVM amplitude shows increased CVM amplitude of DIA-treated osteocytes. (c) Quantification of CVM amplitude shows an increase of 16.6% after DIA treatment accompanied by a 13.1% increase of ATP content (d). Significant difference of t-test was signed by * ($p < 0.05$).

4. Discussion

Osteocytes, although previously thought to be inactive embedded in bone tissue, are demonstrated to be quite dynamic in the present study. The MLO-Y4 osteocytes display spontaneous vibratory motions regulated by ATP content and spectrin network. In the present study, the CVM amplitude of osteocytes observed by DHM is around 80 nm. It is on the same order of magnitude with the “flickering” amplitude of erythocyte, which varied from 32 nm to 400 nm in different studies [21,31,32]. While unlike the anuclear erythocyte, the plasma membrane of osteocytes is not only confined by the submembrane spectrin network, but also connected with the F-actin and anchored by the focal adhesions to the substrate [33]. However, the CVM of osteocytes is still obvious. We speculate that, the confinement of the cytoskeleton and focal adhesion to the cell membrane is not global, but only concentrated to some connection nodes. In Szabo's study on fibroblast [12], the atomic force microscope (AFM) detected a pulsation of 1-2 nm on the membrane, which is relatively mild compared with CVM of osteocytes measured in our study. During the AFM detection, an indenting force, although very low, was applied on cells. So it is speculated that the fluctuation sensed by the AFM tip was mainly induced by the contraction of the stress fibers rather than the spontaneous membrane

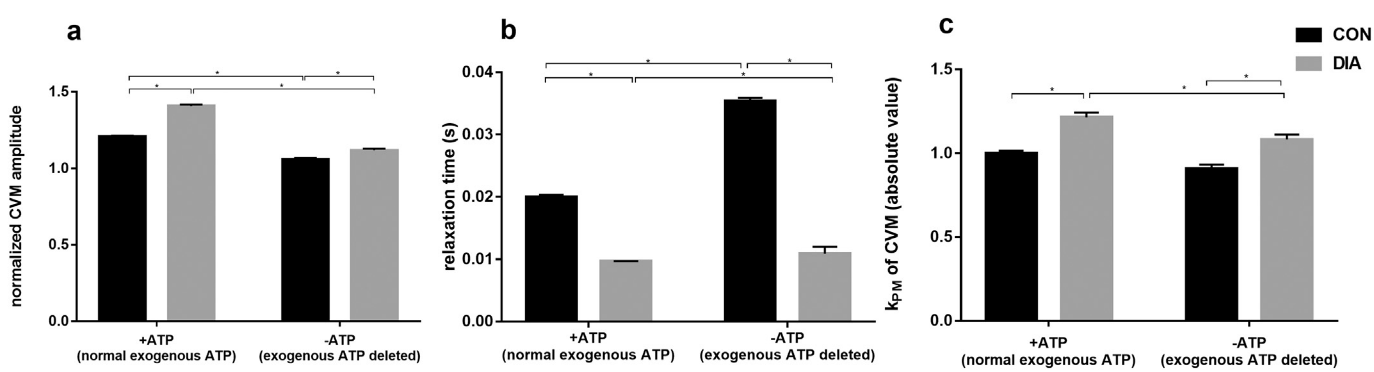

Fig. 6. Effect of DIA on osteocytes CVM in the presence or absence of ATP. (a) The DIA treatment increased the CVM amplitude by 16.6% in the presence of ATP, while only caused an increase of 5.7% when ATP was depleted by antimycin A. (b) The DIA treatment significantly decreased the $t_{relaxation}$ in the presence of ATP. The depletion of ATP by antimycin A caused an obvious increase of $t_{relaxation}$, and the DIA treatment recovered the $t_{relaxation}$. (c) The DIA treatment caused a similar extent of increase of the absolute value of k_{PM} in the presence and absence of ATP. Significant difference of multiple t-test followed Two-way ANOVA was signed by * ($p < 0.05$).

Table 1
Effect of DIA on osteocyte CVM in the presence/absence of ATP.

	DIA effect on CVM amplitude	DIA effect on $t_{relaxation}$	DIA effect on k_{PM}
+ ATP (normal exogenous ATP)	+ 16.6%	− 55%	+ 21.6%
− ATP (exogenous ATP deleted)	+ 5.7%	− 69.2%	+ 19.3%

Table 2
Interaction effect analysis of DIA/ATP effect on CVM of MLO-Y4 osteocyte. * stands for the statistical significance.

	CVM amplitude	$t_{relaxation}$	k_{PM}
DIA	$p < 0.0001$ ***	$p < 0.0001$ ***	$p < 0.0001$ ***
ATP	$p < 0.0001$ ***	$p < 0.0001$ ***	$p = 0.0001$ ***
Interaction effect	$p < 0.0001$ ***	$p < 0.0001$ ***	$p = 0.4847$

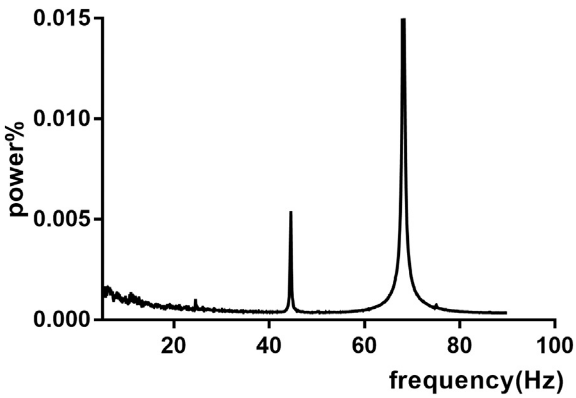

Fig. 7. Spectrum analysis of paraformaldehyde-fixed cells shows three main coherent frequency peaks of the recording system. Three frequency peaks around 23 Hz, 45 Hz and 68 Hz were observed.

undulation. Besides, as it was demonstrated by Adachi [34] that the cell process of osteocyte is more sensitive to the monotonous deformation than the cell body, the CVM of the cell process and cell body was compared in our study, and no difference was found. It demonstrates that the CVM of osteocytes, at least in the situation of monotonous stimulation, does not account for the different mechanosensitivity of different parts on osteocytes and vice versa.

The ATP concentration is thought to be a governing factor of erythocyte flickering [15,18,35]. In the model proposed by Dubus and Fournier [36], the spectrin network is regarded as a triangular network attached to a fluid-bilayer membrane. The coupling strength between the spectrin and cell membrane is weakened by ATP through phosphorylation, thus guarantees the flickering characteristic of red blood cell [37,38]. In the present study, the CVM of osteocytes is increased with ATP concentration and decreased when ATP is consumed. It indicates that, the ATP is also the energy resource of the CVM in MLO-Y4 osteocytes. On the other hand, in the absence of ATP, the DIA-induced spectrin disruption also enhanced the CVM of osteocytes, but in a lesser extent. We speculate the DIA-induced cell softening [25] is contributing to the enhancement of CVM, which is independent of ATP. The mechanical property of cell is regarded as another important factor in cell membrane fluctuations [19]. Theoretical relations between mechanical properties, including bending modulus [39–41] and tension modulus [42] with the mean-square CVM amplitude have been built. These relations indicate a negative correlation between the stiffness and fluctuation amplitude in red blood cells. Although these theoretical descriptions are derived from the relative simple red blood cell structure, similar phenomenon is also found in MLO-Y4 osteocytes. A fluctuated membrane can be regarded as a non-equilibrium system, and the relaxation time is related to the bending elasticity, tension and bulk liquid viscosity of a membrane [43,44]. Specifically, the relaxation time of a tense membrane is longer than the relaxation time of a deflated membrane under the same condition [29]. In correspondence, the relaxation time of osteocytes decreases after DIA treatment both in the presence and absence of ATP in the present study, which implies an increased rheological property of the osteocytes.

Inspired by the results from harmonic analysis of finite element calculation in our previous study, spectrum analysis of CVM was performed in the present study. The absolute value of PM slope increased after the DIA treatment, indicating a more rapid decay of power with frequency and more non-random components in CVM. Frequency peaks can be observed around 30 Hz, 39 Hz, 83 Hz and 89 Hz, which are quite close to the main frequencies from the computational prediction (34 Hz, 86 Hz) by finite element model of osteocyte [6]. In our previous animal study using local vibration as a countermeasure against the bone loss of tail-suspended rats, vibration at 35 Hz was demonstrated to be the most effective [45]. So it is speculated that, vibration around 35 Hz is special. Studies of Tanaka [46] and Zhao [47] demonstrate that bone formation of mice tibia and ulnae is enhanced by a resonance effect. So it is speculated that when vibrations around the peak frequencies are applied, a resonance phenomenon should be invoked, thus activate the downstream mechanotransduction of osteocytes. Further, as the most abundant cells in bone, the resonance of osteocytes may unitedly contribute to the resonance response of bone tissue to vibration.

In addition, it is a limitation of this study that the MLO-Y4 cell line, instead of primary osteocytes from bone was utilized for CVM measurement. Future studies of CVM on primary osteocytes, as well as other bone cells, such as osteoblasts and osteoclasts would further elucidate the response of bone to vibration stimulations.

5. Conclusions

In conclusion, we have found the MLO-Y4 osteocytes display spontaneous cellular vibratory motions. Frequency peaks of the vibratory motions can be found and are possibly involved in the resonance response of osteocytes to vibration stimulation. The dynamic motions of cells are determined by both the ATP content and spectrin structure. As the ATP and spectrin structure are also involved in the mechanotransduction of osteocytes, the CVM may be a novel evaluation gauge for the mechanosensitivity of osteocytes. Besides, establishment of theoretical equation between CVM and mechanical properties for osteocytes would offer a new way for the measurement of cells' bending modules and shear modules.

Author contribution

XT.W., LW.S., F.P.,W.X. and YB.F. conceptualized the study. XT.W., RY.C., and X.Y. collected and assembled data. XT.W. and LW.S. drafted the manuscript. W.X. and YB.F. revised the manuscript. YB.F. and LW.S. provided financial support. All authors reviewed manuscript for final approval.

Declaration of Competing Interest

The authors declare that they have no competing financial interests.

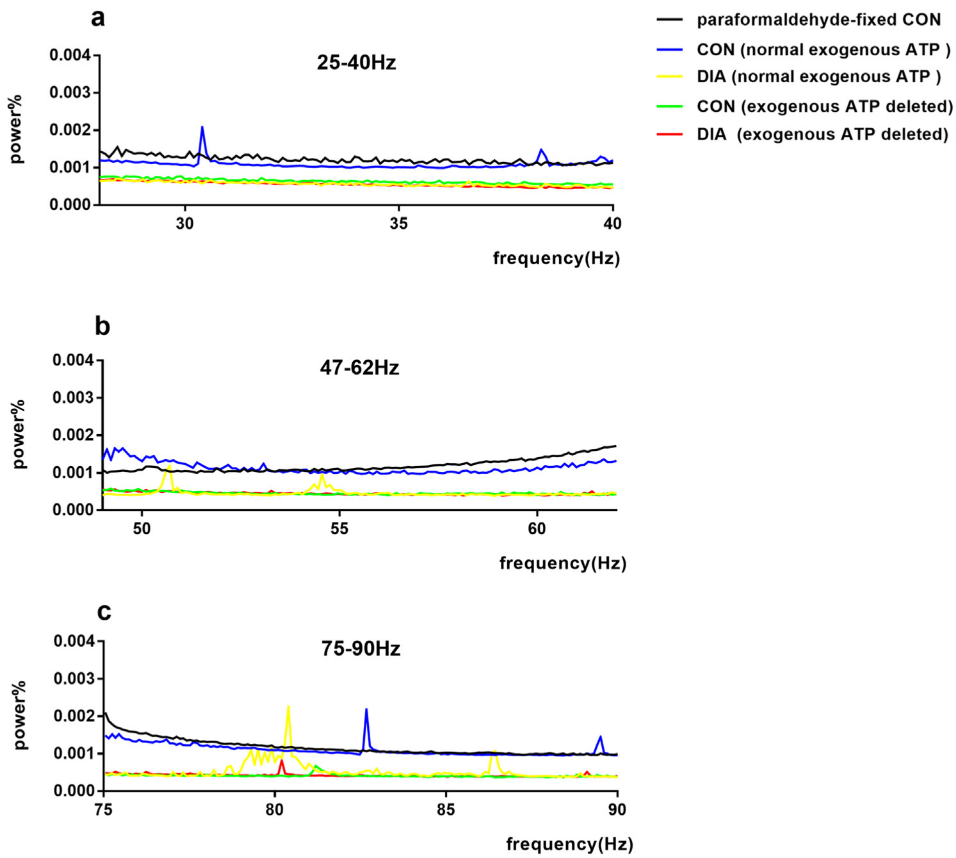

Fig. 8. Spectrum analysis of osteocytes CVM. (a)-(c) Four frequency peaks at around 30 Hz, 39 Hz, 83 Hz and 89 Hz (blue curve) of osteocytes of CON were observed, and the treatment of DIA caused a shift of the frequency peaks to 51 Hz, 55 Hz, 80 Hz, and 86 Hz (yellow curve). After the deleption of ATP by antimycin A, only two small peaks at around 80 Hz and 89 Hz can be observed (green and red curves). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

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