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Technical Note

Dynamic hydraulic fluid stimulation regulated intramedullary pressure☆

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ABSTRACT

Physical signals within the bone, *i.e.* generated from mechanical loading, have the potential to initiate skeletal adaptation. Strong evidence has pointed to bone fluid flow (BFF) as a media between an external load and the bone cells, in which altered velocity and pressure can ultimately initiate the mechanotransduction and the remodeling process within the bone. Load-induced BFF can be altered by factors such as intramedullary pressure (ImP) and/or bone matrix strain, mediating bone adaptation. Previous studies have shown that BFF induced by ImP alone, with minimum bone strain, can initiate bone remodeling. However, identifying induced ImP dynamics and bone strain factor *in vivo* using a non-invasive method still remains challenging. To apply ImP as a means for alteration of BFF, it was hypothesized that non-invasive dynamic hydraulic stimulation (DHS) can induce local ImP with minimal bone strain to potentially elicit osteogenic adaptive responses *via* bone–muscle coupling. The goal of this study was to evaluate the immediate effects on local and distant ImP and strain in response to a range of loading frequencies using DHS. Simultaneous femoral and tibial ImP and bone strain values were measured in three 15-month-old female Sprague Dawley rats during DHS loading on the tibia with frequencies of 1 Hz to 10 Hz. DHS showed noticeable effects on ImP induction in the stimulated tibia in a nonlinear fashion in response to DHS over the range of loading frequencies, where they peaked at 2 Hz. DHS at various loading frequencies generated minimal bone strain in the tibiae. Maximal bone strain measured at all loading frequencies was less than 8 με. No detectable induction of ImP or bone strain was observed in the femur. This study suggested that oscillatory DHS may regulate the local fluid dynamics with minimal mechanical strain in the bone, which serves critically in bone adaptation. These results clearly implied DHS's potential as an effective, non-invasive intervention for osteopenia and osteoporosis treatments.

Introduction

Aging or functional disuse of the bone can subsequently create a number of physiological or pathophysiological changes in the skeleton of the affected subjects (*e.g.* elderly, long-term bed-rest patients, and astronauts who participate in long-duration spaceflight missions), leading to conditions such as osteopenia [1]. Studies of mechanobiology and novel modalities of mechanical loading have demonstrated their abilities in regulating bone strength [2–10]. However, the underlying mechanotransductive mechanisms, namely, how mechanical signals are delivered to bone cells and how the bone cells respond to such signals, remain unclear.

As a potent regulator in bone adaptation, bone fluid flow (BFF) with altered velocity or pressure acts as a communication media between an external load and the bone cells, which then regulate bone remodeling [11–16]. In converse, discontinuous BFF can initiate bone turnover and result in osteopenia [17–20]. Physical signals such as intramedullary fluid pressure (ImP) have been suggested to initiate BFF and to influence the osteogenic signals within the bone [18]. A few studies using surgical methods on *in vivo* animal models have shown that BFF can be altered by ImP without bone deformation, and that ImP alone is sufficient to induce potent adaptive responses in the bone. Qin et al applied a direct alteration of ImP to an isolated turkey ulna without deforming the bone tissue and found increased bone formation in response to the applied pressure [18]. Similarly, a more recent study introduced a novel microfluidic system for generating dynamic ImP and BFF within the femurs of alert mice to induce osteogenic responses [21].

Therefore, ImP-induced BFF provides a great potential in developing novel mechanical stimuli as countermeasures for disuse bone loss. Previous *in vivo* study using oscillatory electrical muscle stimulation (MS) in a hindlimb suspension (HLS) functional disuse rat model has demonstrated that oscillatory MS-induced muscle contraction can generate ImP and bone strain to mitigate disuse osteopenia [9,22,23]. A potential

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mechanism of the interrelationship between vasculature adaptation and applied ImP alteration has been suggested in a later study [24]. Induced ImP possibly triggers the transformation of the bone nutrient vasculature, leading to the ultimate alteration in blood supply to the bone. However, to non-invasively isolate the ImP factor from the bone strain factor in an *in vivo* setting still remains challenging. Furthermore, in order to establish the translational potential of ImP, a novel and non-invasive method that directly couples an external load and internal BFF would be an attractive intervention. To reach this goal, our group has recently developed a novel, non-invasive dynamic hydraulic stimulation (DHS). Its promising effects on mitigating disuse bone loss have been shown *via* a 4-week rat HLS study followed by μCT and histomorphometry analyses [25]. The results indicated a great potential of DHS to become a novel countermeasure for clinical osteoporosis/osteopenia treatments.

Identifying stimulation parameters within an optimal loading regimen, *e.g.* frequency, is important to maximize the effectiveness of the stimulation and to generate beneficial adaptive responses in the bone. As an important determinant of bone adaptation to mechanical loading, loading frequency has been shown with a positive correlation to the improved bone quality in various studies [26–28]. Interestingly, effective loading frequencies seem to differ among various loading modalities [8,26,27,29]. Whole-body vibrations are known to be more effective at high frequencies (>30 Hz). On the other hand, an ulna axial loading study in mice reported a higher effect at lower loading frequencies (5 Hz and 10 Hz) as opposed to higher frequencies (20 Hz or 30 Hz) [29]. Related to ImP and BFF loading theory, relatively lower frequencies (5 Hz and 10 Hz) were reported to be more effective in bone adaptation in the femur [21] and tibia [8], respectively.

To test the hypothesis that non-invasive DHS can induce local ImP with minimal strain to potentially elicit osteogenic adaptive responses, the objective of this study was to evaluate the immediate effects on femoral and tibial ImP and bone strain induced by DHS over the tibia within a broad range of loading frequencies.

Materials and methods

Animals

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University. Surgical experiments were performed on three 15-month-old female Sprague Dawley virgin rats (Charles River, MA; body mass 426 ± 36 g) to measure the ImP and bone strain simultaneously during DHS loading.

ImP measurements

Each animal was anesthetized through standard isoflurane inhalation. An approximately 1 cm incision was made at the anterior knee region of the animal's right leg to expose the distal femur and proximal tibia. From the distal end of the femur and the proximal end of the tibia, a 1 mm hole was carefully drilled into each of the right femoral and tibial marrow cavities, respectively. Guided by a 16-gauge catheter, a micro-cardiovascular pressure transducer (Millar Instruments, SPR-524, Houston, TX) was inserted into each of the femoral and tibial marrow cavities (Fig. 1). The catheter and the pressure transducer apparatus were sealed tightly within the drilled holes.

Bone strain measurements

Similar to the above surgical procedure, a 2 cm incision was made at the anterior side of the right tibia and the lateral side of the right femur. A linear single element strain gauge (120 Ω, factor 2.06, Kenkyojo Co., Tokyo) was firmly attached to the flat surface of each of the same tibia and femur within the mid-diaphyseal regions (Fig. 1). The exposed muscles underwent minimal disruption, and the open skin was sutured before applying DHS.

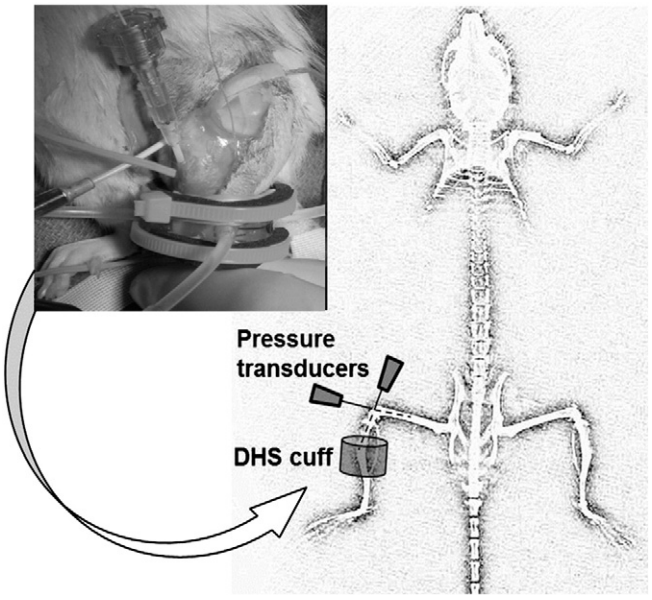

Fig. 1. Surgical experiment setup. For the ImP measurements, a micro-cardiovascular pressure transducer was inserted into each of the marrow cavities of both tibia and femur. For bone strain measurements, a single element strain gauge was firmly attached to the flat surface of each of the same tibia and femur at the mid-diaphyseal region. Simultaneous ImP and bone strain were then measured in both tibia and femur during DHS on the tibia.

DHS loading

DHS was achieved through a custom-made inflatable cuff placed around the right tibia, with the similar setup in our recently published study [25]. Briefly, the inflation and deflation of the cuff were driven by a syringe pump with the loading magnitudes and frequencies delivered by a programmable waveform/signal generator (HP33120A, Hewlett-Packard, Palo Alto, CA). A pressure sensor was included to monitor the stimulation amplitude throughout the entire stimulation process. The pressure stimulation was achieved by 40 mmHg static pressure + the peak-to-peak dynamic pressures given by the function generator that was set at 1.5 V constant voltage. To start the experiment, DHS was applied to the operated tibia by placing the stimulation cuff around the mid-tibia region and loaded at frequencies of 0.5 Hz, 1 Hz, 1.5 Hz, 2 Hz, 2.5 Hz, 3 Hz, 3.5 Hz, 4 Hz, 5 Hz, 6 Hz, 7 Hz, 8 Hz, 9 Hz, and 10 Hz. Measurements of ImP and bone strain of the stimulated right tibia and un-stimulated right femur were recorded simultaneously using a strain gauge amplifier (SCXI-1000, National Instruments, Austin, TX). For each animal, the entire frequency spectrum was repeated for at least three times.

Fast Fourier Transform (FFT) analysis

Each run of data recording was put through a Fast Fourier Transform using MatLab. The random noise was removed by zeroing any values in each power spectrum that were below a threshold. This threshold was defined as the middle point between the lowest peak of the signal and the average of the random noise. Once the noise was removed, each frequency step was analyzed individually. Each step was divided into twenty intervals. The difference between the maximum and minimum values was calculated and averaged over all intervals for that particular frequency step. This was taken as the peak-to-peak value for the corresponding loading frequency (Fig. 2).

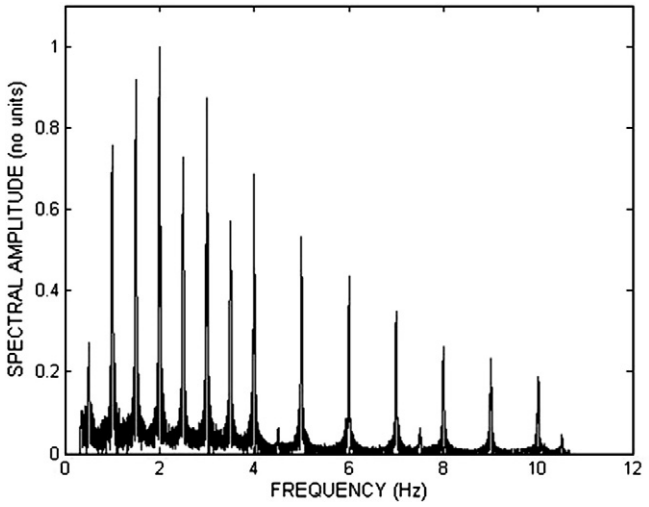

Fig. 2. Representative traces of ImP measurements from DHS loading at various frequencies over time.

Statistical analysis

The values of ImP measurements were reported as mean ± SD. The effects of treatments were evaluated using a Kruskal–Wallis one-way ANOVA on ranks and Dunn's pairwise comparison *post-hoc* test using GraphPad Prism 3.0 Software (GraphPad Software InC., La Jolla, CA). Power analysis was performed using the Statistical Power Calculator of Researcher's Toolkit provided by DSS Research (Fort worth, TX and Arlington, VA) to calculate the statistical power of each ImP measurement value at each frequency point compared to the baseline value with 95% confidence interval.

Results

Tibial and femoral ImP induced by DHS

Approximately 1 mmHg tibial ImP and 5 mmHg femoral ImP were generated by normal heart beat within the marrow cavities. Oscillatory DHS over the tibial region of each rat's right hindlimb, loaded at various frequencies, generated additional fluid pressures in the tibial marrow cavity but not in the femoral marrow cavity. Tibial ImP generated by DHS at each frequency was plotted and shown in Fig. 3. The observed responding trend of the ImP (peak-to-peak) values against frequency was induced in a nonlinear fashion during DHS loading. The induced ImP values (peak-to-peak) were in the order of 1.98 ±1.57 mmHg at 0.5 Hz ($p > 0.05$), 9.20 ± 4.58 mmHg at 1 Hz ($p > 0.05$), 13.98 ± 3.23 mmHg at 1.5 Hz ($p < 0.01$), 14.48 ±3.10 mmHg at 2 Hz ($p < 0.01$), 13.99 ± 2.58 mmHg at 2.5 Hz ($p < 0.01$), 13.18 ± 2.07 mmHg at 3 Hz ($p < 0.01$), 12.52 ± 2.08 mmHg at 3.5 Hz ($p < 0.01$), 12.09 ± 2.18 mmHg at 4 Hz ($p < 0.05$), 11.52 ± 2.48 mmHg at 5 Hz ($p < 0.05$), 10.56 ± 2.67 mm Hg at 6 Hz ($p > 0.05$), 9.59 ± 3.21 mmHg at 7 Hz ($p > 0.05$), 8.55 ± 2.97 mmHg at 8 Hz ($p > 0.05$), 7.76 ± 3.70 mmHg at 9 Hz ($p > 0.05$), and 7.28 ± 4.85 mmHg at 10 Hz ($p > 0.05$). The ImP reached the peak at 2 Hz.

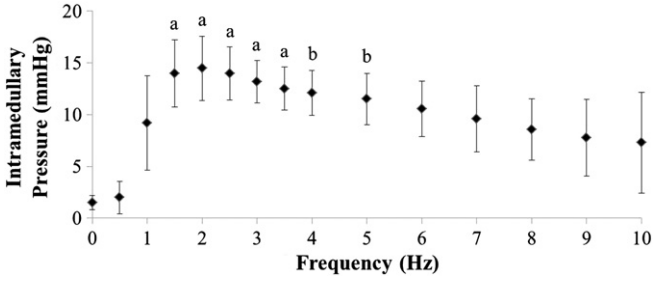

Fig. 3. Graph shows mean ± SD values of the ImP measurements. ImP in the tibia increased significantly with DHS loading frequency at 1.5 Hz, 2 Hz, 2.5 Hz, 3 Hz, 3.5 Hz, 4 Hz, and 5 Hz. In the loading frequency spectrum from 0.5 Hz to 10 Hz, a maximum ImP of 14.48 ± 3.10 mmHg was observed at 2 Hz, which was around 7 folds higher than at 0.5 Hz (1.98 ± 1.57 mmHg). [a]$p < 0.01$ *vs.* baseline ImP; [b]$p < 0.05$ *vs.* baseline ImP.

Statistical power analysis

The power analysis showed sufficient statistical power of each ImP measurement value compared to the baseline value with 95% confidence interval in the following frequencies: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 Hz. The strong statistical power indicates that our current sample size is sufficient to reject the null hypothesis when false. The effect of DHS on ImP induction can well be determined over the frequency range of 1 Hz to 10 Hz.

Effect of DHS on tibial and femoral bone strain

Oscillatory DHS over the tibial region of the rats' hindlimbs at various loading frequencies generated minimal bone strain within the tibia. Maximal bone strain measured at all loading frequencies was smaller than 8 με. Similar to the femoral ImP measurements, no detectable induction of bone strain was observed in the un-stimulated femur.

Discussion

For the first time, this study demonstrated a non-invasive method that can be applied to an *in vivo* rodent model to isolate ImP and bone deformation, which are the two key determinants for BFF. The promising results from this study indicated that DHS can generate local fluid pressure in bone with simultaneous minimal bone strain. DHS over the rat tibia induced a peak of tibial ImP at 2 Hz. While the observed tibial bone strain over the range of loading frequencies was smaller than 8 με, relatively high ImP values were observed as a function of loading frequency. Tibial ImP measurements between the loaded 1.5 Hz and 5 Hz showed significant increases compared to the baseline level. These results suggested that DHS can potentially produce high fluid pressure gradients within the local marrow cavity. As a key determinant of BFF, loading generated fluid pressure in bone may have strong potentials in attenuating disuse bone loss, if loaded at proper frequencies.

The results from this study coincided with one of our recently published *in vivo* experiment, in which DHS loading at frequency of 2 Hz was shown to have mitigation effect on disuse trabecular bone loss in a rat HLS functional disuse model [25]. For example, trabecular bone volume fraction in the stimulated tibia was increased by 83% when a DHS protocol at 2 Hz was applied for four weeks in conjunction with HLS. The high surface area of the trabecular network allows it to expose to the rapid change in fluid pressure, leading to the pronounced effects of DHS observed in the trabecular bone. The DHS stimulation cuff was designed as dynamic circular compressions around the diaphysis region of the loaded bone, which does not provide direct physical contact over the metaphyseal region. However, the anabolic/anti-catabolic effects of DHS on disuse trabecular bone strongly support the ImP-induced BFF mechanism. According to the beneficial role of mechanotransduction in triggering bone remodeling [30,31], strong evidence has suggested that interstitial fluid flow in the bone can be altered by external muscular activities through various mechanisms [32,33]. Based on the muscle pump theory, it is thought that muscle contraction compresses the blood vessels in the muscle, which generates an arteriovenous pressure gradient that further increases the hydraulic pressure in the skeletal nutrient vessels and amplifies the capillary filtration in bone [19,20,34]. Increased vessel pressure can directly increase the ImP that further drives BFF [35]. Direct circular compressions provided by DHS over the surrounding muscles have demonstrated its ability on ImP inductions and potential in attenuating disused bone loss [25].

The observed ImP inductions have indicated a nonlinear response in the DHS loading spectrum between 0.5 Hz and 10 Hz, in which they peaked at 2 Hz. The nonlinear relationship between the DHS loading frequencies and the corresponding ImP inductions is presented in Fig. 3. The frequencies and ImP values are positively correlated, however, it is highly frequency-dependent and they do not correlate into a linear relationship. This response to the loading frequency range is different from our previous observation using oscillatory muscle stimulation (MS), in which oscillatory MS induced maximal ImP at 20 Hz. Oscillatory MS was achieved *via* two disposable needle-sized electrodes inserted into the quadriceps of the stimulated rats. The electrodes were then connected to the waveform generator with 2 V supplies to induce muscle contraction [9,22]. DHS provides an external stimulus that increases dynamic compressions on the surrounding muscles over the tibia, as opposed to direct muscle stimulation provided by oscillatory MS. Based on the characteristics of the tissue material, *e.g.* the viscoelastic nature of the surrounding muscle tissue, the loads at high frequencies could be quickly damped. Due to the different physical orientations of how oscillatory MS and DHS contact the loaded tissue, as well as the different material densities and viscosities within hard and soft tissues, maximal DHS-induced ImP may result at relatively lower frequency compared to MS. This also suggests that direct hydraulic coupling may influence bone adaptation in a more physiological frequency range, where the normal heart rate of the rat is within the average range of 360 times per minute [39]. Furthermore, the mechanotransductive sequence through different connective tissues during DHS may attenuate the high frequency response in bone, *e.g. via* the connective pathway from the contacted muscle *vs.* tendon to bone, resulting in peak ImP at a different frequency. To further reveal this complex mechanism, future investigation may need to focus on muscle kinetics and BFF generation.

Previous studies from our group have shown that BFF can be altered by ImP with minimal bone strain, and that ImP alone is sufficient to induce bone adaptation. A loss of cortical bone by 5.7% was resulted in a disuse avian ulna [18]. An increase in cortical bone mass by 18% was shown with a direct fluid loading into the ulna at 20 Hz for 4 weeks. Moreover, the study also observed a strong correlation between the transcortical fluid pressure gradient and total bone formation. Similarly, generation of dynamic ImP and BFF within the femurs of HLS mice using a novel microfluidic system significantly reduced the disuse bone mass loss in both trabecular bone and cortical bone [21]. Our present study demonstrated DHS's ability in ImP induction, which strongly suggests its potential in attenuating disuse bone loss. This hypothesis is further supported by the mitigation effect of DHS at 2 Hz, indicated in our recent publication [25]. The response of bone cells to dynamic fluid flow mechanical stimulation was further demonstrated by a longitudinal *in vivo* study, which indicated that the MSC population was positively influenced by the DHS-derived mechanical signals. Changes of MSC number in response to DHS bias their differentiation towards osteoblastogenesis, leading to bone formation even under disuse conditions [36]. Further study on the underlying mechanism in response to mechanical signals, *e.g.* mechanobiological modulation of cytoskeleton and calcium influx into osteoblastic cells [37], can provide insights for the future developments of novel and effective osteoporosis treatments. Interesting, DHS on the tibia only affected the tibial ImP but not the femoral ImP, indicating the local effect of DHS. This is suggesting that the function of DHS may be site specific. More detailed *in vivo* experiments are needed to validate this observation. Moreover, while increased bone fluid pressure and BFF regulation are strongly correlated, our current results of DHS-driven ImP induction suggest a possible mechanism that the induced ImP may subsequently enhance BFF. Future studies may extend our current experimental focus and incorporate further experiments of tracer transport determinations to verify this hypothesized mechanism [31,38].

In summary, oscillatory DHS can generate local ImP as a function of stimulation frequency with minimal strain, where the induced dynamic ImP may subsequently enhance BFF. DHS, if applied at an optimal frequency, has strong potential in preventing and attenuating bone loss under disuse osteopenia condition. Results from this study provide evidence that may facilitate the development of a biomechanical based intervention for osteoporosis prevention and treatment, which provide great insights for future clinical applications.

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