Published in final edited form as:

Bone. 2010 April ; 46(4): 1075–1081. doi:10.1016/j.bone.2010.01.371.

In situ permeability measurement of the mammalian lacunar-canalicular system

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Abstract

Bone is capable of adapting its mass and structure under mechanical cues. Bone cells respond to various mechanical stimuli including substrate strain, fluid pressure, and fluid flow (shear stress) *in vitro*. Although tissue-level strains are well documented experimentally, microfluidic parameters around bone cells are quantified mainly through theoretical modeling. A key model parameter, the Darcy permeability of the bone lacunar-canalicular system (LCS), is difficult to measure using traditional methods due to the co-existence of the larger vascular and smaller LCS porosities. In this paper, we developed a novel method to measure the LCS permeability by rapid compaction of intact mammalian bones and recording the intramedullary pressure (IMP). Six canine metacarpals were subjected to three step-compression tests with peak loads of 50, 100, or 200 lbs, while the IMP was simultaneously recorded using a catheter pressure transducer. The loading ramp time was chosen to be ~2 ms, which was long enough to allow pressure equilibrium to be established between the marrow cavity and the vascular pores, but short enough to observe the LCS fluid flowing into and out of the vascular pores. This loading scheme permitted us to differentiate the contribution of the two intermingled porosities to the IMP responses. The time constant of the IMP pressurization and relaxation due to the LCS was found to be 8.1 ± 3.6 sec ($n = 18$). The mid-shaft cortex of the metacarpals mainly consisted of osteons with an average radial thickness of 65 ± 27 μm , which served as the characteristic distance for the LCS fluid to relax. The LCS permeability was obtained via poroelastic analysis to be $2.8 \pm 1.8 \times 10^{-23}$ m^2 , which was smaller than previous theoretical predictions (order of 10^{-19} to 10^{-22} m^2), but within the range of previous experimentally based estimations (order of 10^{-22} to 10^{-25} m^2). Our results also show that osteoblasts and osteocytes experience hydraulic pressures that differ by three orders of magnitude under physiological compressive strains. These estimates of the *in vivo* mechanical environments may be used to design *in vitro* models for elucidating the cellular and molecular mechanisms of bone adaptation and pathological bone loss.

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Keywords

Darcy permeability; Intramedullary pressure; Fluid pressure; Rapid compaction; Poroelasticity; Osteocytes; Osteoblasts

Introduction

Mechanical environment of bone provides various forms of stimuli that drive bone to adapt its mass and structure to meet its mechanical demands [1]. Quantification of the mechanical stimuli has been performed at both tissue and cellular levels [2-5]. *In vivo* tissue-level strains, which have been well-established using strain gages, range from 600 $\mu\epsilon$ during walking up to 2000 $\mu\epsilon$ during vigorous activities [2,3]. Strains at the cellular levels have been mapped using finite element analysis or imaging correlation strain quantifications [4,5], but are thought to be too small to directly elicit cellular response [6,7]. Studies suggest that load-induced interstitial fluid flow (IFF) through the lacunar-canalicular system (LCS) [8] and its secondary effects such as shear stress and/or drag force acting on the cell membranes may be the primary mechanical stimuli that bone cells respond to (see a recent review [9]). *In vitro* studies have demonstrated that bone cells are more sensitive to IFF than substrate strain [6,7]. Besides IFF, bone cells are also found to respond to hydraulic pressure [10-12]. We have recently shown that both IFF and fluid pressure induce anabolic responses in osteoblasts, but alter different cytoskeletal components [12]. Despite the importance of IFF and fluid pressure in bone cell functions, quantitative measurements of the microfluidic environment are lacking due to the difficulty of probing mineralized bone tissues.

The intraosseous pressure was typically determined using a pressure transducer inserted into the larger marrow cavity [13-16]. In the absence of applied loading, the range of intramedullary pressure (IMP) reported in literature is often below the systemic blood pressure (see a recent review [17]). Under mechanical loading, IMP has been shown to reach 6.8 kPa (51 mmHg) in turkey ulnae during a step compression of 600 $\mu\epsilon$ [14]. The IMP of mammalian long bone can vary between 10 and 120 mmHg depending on the applied loading conditions [15,17]. Electrical stimulation of rat quadriceps at 20 Hz increased the IMP to 45 mmHg and attenuated the trabecular bone loss induced by hind limb suspension [16]. Anabolic responses to altered IMP have been well documented [13,15,16], and measurements of IMP have provided quantitative data on the pressure environment subjected to osteoblasts lining the marrow cavity and connecting vascular pores.

Quantifying the fluid pressure and flow velocity around osteocytes (the presumed “mechanosensors” [18]), however, remains quite a challenge due to the presence of hierarchical and multi-scaled pores, including the vascular pores (on the order of 10 μm) and the LCS pores (on the order of 0.1-1 μm) that surround osteocytes [9,19]. Although the dimensions of osteocytic lacunae and canaliculi are on the order of 10 and 1 μm , respectively, the effective pore sizes are much smaller (6-12 nm) due to the presence of the cell body, cell process, and the pericellular matrix filling in the extracellular fluid space in the LCS [9]. Poroelastic modeling has been the primary means to quantify the microfluidic stimuli around osteocytes [8,20-24]. The key parameter in these models is the Darcy permeability at the LCS level. Traditional permeability experiments were performed by subjecting bone samples to applied pressure gradients and measuring the resulting flux [25-27]. The Darcy permeability obtained in these studies (on the order of 10^{-11} to 10^{-15} m^2) was a lumped permeability of the intermingled vascular and LCS pores, which was dominated by the larger vascular pores [19]. Weinbaum and colleagues first developed an ultrastructural model where the effective Darcy permeability of the LCS was estimated based on the anatomical features of the LCS and the assumed pericellular matrix organization [8]. This model, widely adopted in later studies,

provides a valuable tool to estimate the LCS permeability that ranges from 10^{-19} to 10^{-22} m^2 [20,21,23,28]. Recently, experimentally based estimations of LCS permeability were reported [19,24,29]. Smit et al. (2002) [29] reported the LCS permeability to be on the order of 10^{-22} m^2 by fitting a finite element analysis of fluid flow to the streaming potential data [30]. Oyen (2008) estimated the LCS permeability to be on the order of 10^{-24} m^2 using a nanoindentation technique along with poroelastic analysis [24]. Cowin and colleagues recently found the LCS permeability to be on the order 10^{-24} to 10^{-25} m^2 based on un-confined compression tests of single osteons [31]. Overall, these experimentally based estimations (10^{-25} m^2 to 10^{-22} m^2) are several orders of magnitude smaller than theoretical estimations (10^{-19} m^2 to 10^{-22} m^2) [20,21,23,28]. Further investigation of the LCS permeability is highly needed in order to more accurately predict the micro-mechanical environment experienced by osteocytes within bone.

In this paper, we developed an *in situ* method to measure the LCS permeability by monitoring the IMP of intact mammalian bone under rapid compression. The present study extended the pioneering work of Qin who had pressurized tubular bones under step compression [14]. The novelty of our method was the selection of the ramping time (2 ms), which was longer than the fluid relaxation time of the vascular pores (on the order of 10 μsec [20]), but shorter than the fluid relaxation time of the LCS pores (on the order of seconds). By exploiting their dramatic difference in permeability and relaxation time, we successfully differentiated the contributions of the physically intermingled vascular and LCS pores on the IMP recordings, from which the LCS permeability was estimated using poroelastic analysis. The data presented herein provide a more accurate quantification of the *in vivo* fluid pore pressure and fluid flow, the physical signals that modulate the adaptation processes of osteoblasts and osteocytes to external loading. These data may be used to design more realistic cell culture studies to decipher the cellular mechanisms of bone adaptation and pathological bone loss due to disuse.

Methods

Sample preparations

A total of 6 metacarpal specimens from beagle dogs (3 to 4 years in age) were used in this study. The frozen bone samples were kindly provided by Dr. Mitchell Schaffler (City College of New York). Each frozen sample was thawed at room temperature. After cleaning off the surrounding soft tissues, the second or third metacarpals were isolated from the limbs, and the two ends of the bone were capped in aluminum fixtures of 1cm in height, width and depth, filled with epoxy resin (D805, 3M, Minnesota, USA). The mid-shafts of the bone samples were aligned with the centerline of the fixtures to minimize bending during the step loading (detailed below). The whole preparation was wrapped in wet paper towels with phosphate buffered solution (PBS) at 4 °C until the resin was hardened.

To record load-induced IMP, a 0.5mm diameter hole was bored through the mid-shaft of the bone. A PBS-filled catheter was inserted into the marrow cavity through the hole and sealed with epoxy resin. Leakage was checked by injecting PBS via the catheter. A Mikro-Tip catheter pressure transducer (Millar Instruments, Houston, Texas) was then placed and sealed inside the catheter (Fig 1). The signal from the pressure transducer in the catheter was amplified through a controller (PCU 2000, Millar Instruments) and recorded to a computer at 250Hz through a NI-USB-6221 A/D board using the LabView software (National Instruments, Texas). The pressure transducer was calibrated according to the manufacture's instruction prior to testing each of the samples.

Loading Conditions

The specimen was fixed vertically on the platform of a hydraulic MTS testing machine (MTS858, Bionix Test System, Eden Prairie, MN). A small compression force (5 lbs) was applied for 10 minutes prior to testing in order to eliminate any slippage in the system. The baseline pressure reading was recorded during this time. Each specimen was then subjected to a series of step loadings with the peak load increasing from 50, 100, to 200 lbs. The resulting strains were estimated to be approximately 900, 1800, and 3600 $\mu\epsilon$, based on the cross-sectional bone area in the mid-shaft (obtained after mechanical tests) and the elastic modulus of cortical bone (11.5 GPa) [32]. Please note that these strains were higher than the physiological strains associated with normal gaits, but were necessary to obtain an adequate signal-to-noise ratio of the IMP recordings. In the load-control mode, the MTS machine was tuned to ramp from the preload (5 lbs) to peak load within 2 ms, and then hold the peak load steady for ~60 sec. During the dwell time, the IMP peaked and maintained at a steady-state level. The load was then released at the same rate (Fig 2A). However, the load recordings revealed some fluctuations during the dwell time, resulting in fluctuations of the IMP recordings.

Analysis of IMP responses

In response to the rapid compression, two types of behaviors were observed in the IMP recordings during both the onset and removal of loading: i) the initial instantaneous jump or fall, and ii) the subsequent exponential rise to peak pressure or return to baseline pressure. Given the relatively high permeability of the vascular regions, the IMP was assumed to equilibrate throughout the marrow cavity and the surrounding Haversian canals and Volkmann's canals [20], resulting in the instantaneous responses of the IMP. The subsequent exponential rise and decay of the measured IMP were attributed to LCS fluid moving into vascular pores (pressurization) and out of the vascular pores back into the LCS pores (relaxation). To determine the time constant during pressurization (τ_1) and relaxation (τ_2) for each loading condition (50, 100, and 200 lbs peak compression), exponential functions were used to fit the IMP recording ($P(t)$) similarly to Qin et al [14] using the following equations (1 and 2), respectively:

$$\frac{P(t) - P(0^+)}{P_{peak} - P(0^+)} = (1 - e^{-t/\tau_1}) \quad (1)$$

$$\frac{P(t) - P(T^+)}{P(T^-) - P(T^+)} = e^{-(t-T)/\tau_2}, \quad (2)$$

where the time point $t = 0$ corresponds to the onset of loading, $t = 0^+$ indicates the time when the peak load is reached (2 ms), and the corresponding IMP pressure is termed as $P(0^+)$; T is the dwell time of the peak load and P_{peak} is the peak pressure obtained during the dwell time. To fit the pressure relaxation period (Eq. 2), $P(T^-)$ and $P(T^+)$ are the pressures prior to and right after releasing the load, respectively. The difference between both time constants was evaluated using a paired t-test, where significance was defined by a p-value < 0.05 .

Osteonal thickness

Previous studies of load-induced streaming potentials and fluid flow simulations have shown that the characteristic distance for the fluid to relax in cortical bone is the spacing between the vascular pores (*i.e.*, the osteonal cortex thickness) [21,33,34]. To determine the osteonal thickness in dog metacarpals, three transverse sections of 500 μm thick were cut at the mid-

shafts of two samples with a 0.3 mm thick diamond blade (Buehler, IL, USA). Each section was then polished down to a final thickness between 50 and 100 μm using 1200, 2400 and 4000 grit abrasive paper (Buehler). Each section was then mounted on a cover glass with an aqueous mounting media, and then imaged with a $5\times$ lens (EC-Plan Neofluar Lens, Zeiss). The average osteonal thickness, defined by the outer radius minus the inner radius, was determined for 50 randomly selected osteons.

Poroeelastic estimation of LCS permeability

The measured pressurization and relaxation time constants were used to determine the pressure diffusion coefficient (c) and permeability (k) of a given osteon. Based on the poroelastic model described by Zeng et al. (1994) [8], the pore pressure (p) within an osteon under dynamic stress $\sigma(t)$ is defined by the following:

$$\frac{\partial^2 p}{\partial r^2} + \frac{1}{r} \frac{\partial p}{\partial r} + \frac{1}{r^2} \frac{\partial^2 p}{\partial \theta^2} - \frac{1}{c} \frac{\partial p}{\partial t} = \frac{B}{3c} \cdot \frac{d\sigma(t)}{dt}, \quad (3)$$

where r and θ are the cylindrical coordinates with the origin located at the center of the osteon, B (estimated below) is the Skempton factor that describes the relationship between changes in total stress to changes in pore fluid pressure [8,35]. The pressure diffusion coefficient c is proportional to the square of the characteristic length l (osteonal thickness) and inversely related to the measured characteristic time constant τ as shown below:

$$c = \frac{l^2}{\tau}. \quad (4)$$

The LCS permeability was then determined from the pressure diffusion coefficient (Eq. 5) as described in detail in Weinbaum et al. (1994):

$$k = c\mu \left[\frac{(1-2\nu)}{2G(1-\nu)} \right] \left[\frac{9(1-\nu_u)(\nu_u-\nu)}{B^2(1+\nu_u)^2(1-2\nu)} \right], \quad (5a)$$

$$B = \frac{\frac{1}{K} - \frac{1}{K_s}}{\frac{\nu_0}{K_f} + \frac{1}{K} - \frac{(1+\nu_0)}{K_s}}, \quad (5b)$$

where μ is the viscosity of body fluid, which is assumed to be that of seawater (1.06×10^{-3} kg/ms), the drained shear modulus of bone (G) is 5 GPa, the drained Poisson's ratio of bone (ν) is 0.325, and the un-drained Poisson's ratio (ν_u) is set to 0.33 [8,35]. To be consistent with previous studies, the drained bulk modulus (K) is 15 GPa, the bulk modulus of the solid phase (K_s) is 17.6 GPa, the bulk modulus of the fluid phase (K_f) is the same as seawater, 2.3 GPa, and lastly the fluid volume fraction for the lacunar-canalicular extracellular space (ν_0) is on the order of 0.05 [35]. As a result, B is calculated to be 0.35 and equation 5a is reduced to equation 6 to calculate the LCS permeability:

$$k = c\mu / 18.6 \text{ GPa} \quad (6)$$

Results

IMP responses and characteristic time constants

Two behaviors were observed in the IMP recordings for both the onset and removal of the step loading (Fig. 2A). Upon the onset of compressive loading, IMP instantaneously increased to 55-60 % of the peak pressure (Figs. 2B and 2D), followed by an exponential rise and eventual plateau of the IMP (Figs. 2B and 2D). Once the load was released, the IMP instantaneously dropped by 55-60 % of the peak pressure, followed by a similar exponential decay before the IMP returned to baseline (Figs. 2B and 2D). The magnitude of the peak IMP was between 5 and 20 mmHg, and was found to be linearly proportional to the applied load [P_{peak} (mmHg) = $0.0042 \times \text{Strain}$ ($\mu\epsilon$), $R^2 = 0.96$] (Fig. 2C).

The time constants during both pressurization and relaxation were found to be independent of the loading magnitude and were 7.2 ± 3.2 sec and 9.1 ± 3.8 sec, respectively. Since there was no significant difference between these two constants (p-value of 0.06), we used the average value (8.1 ± 3.6 sec) in later calculations of the LCS permeability (Table 1). The exponential functions defined by each time constant fit the measured IMP responses very well with high correlation coefficients (0.98 ± 0.01 , Fig. 2D).

Osteonal thickness

Light microscopy examination demonstrated that the metacarpal bone was predominately osteonal bone (> 80% of the total volume), except for the lamellar bone near the periosteum and endosteum (Fig. 3). The randomly selected osteons ($N = 50$) were measured to be 81 ± 24 μm at the outer radius and 16 ± 5 μm at the inner radius, leading to an average osteonal thickness of 65 ± 27 μm (Table 1). This thickness was assumed to be the characteristic distance for pressurization and relaxation of the LCS fluid into and out of the vascular pores, which was responsible for the observed exponential rise/decay in the IMP recordings during the step-compression tests.

Estimation of the LCS permeability

Based on the average time constant of the IMP exponential response and the measured osteonal thickness, the pressure diffusion coefficient (c) and LCS permeability (k) were derived to be $5.3 \pm 3.2 \times 10^{-10}$ m^2/s (using equation 4) and $2.8 \pm 1.8 \times 10^{-23}$ m^2 (using equation 6), respectively. The calculations are summarized in Table 1.

Discussion

The purpose of this study was to determine the Darcy permeability of intact mammalian bone at the lacunar-canalicular system (LCS) level. The LCS permeability, a key parameter used to quantify the microfluidic environment around osteocytes, has been difficult to measure due to the co-existence of the larger vascular pores and the smaller LCS porosity [9,19]. To overcome this challenge, we developed a novel method based on the pressurization of the intact osteonal mammalian bone using a rapid compression test. The ramping time of the loading was carefully chosen to lie between the characteristic relaxation times of the vascular porosity and the LCS porosity. This method ensured the separation of the IMP responses due to the two porosities and the estimation of LCS permeability using poroelastic analysis. The experimentally based LCS permeability obtained in the present study allows more accurate estimations of the cellular mechanical environments (e.g., fluid pore pressure and fluid flow), which may be used to design

physiologically relevant *in vitro* models to study the cellular and molecular mechanisms of bone adaptation and pathological bone loss.

Our experimentally based estimations of LCS permeability ($2.8 \pm 1.8 \times 10^{-23} \text{ m}^2$) are in reasonable agreement with results from other studies (10^{-25} m^2 to 10^{-22} m^2) [19,24,29], and at least one order of magnitude smaller than theoretical estimations that have been widely used [8,20,21,23,28]. Most importantly, the present study demonstrates two distinct behaviors of the intramedullary pressure of mammalian bone under rapid compression (Fig. 2). By choosing a loading speed between the characteristic relaxation times of the larger vascular pores and smaller LCS pores, we successfully differentiated the pressurization/relaxation responses attributed to these two porosities in a time sequence. The initial, nearly instantaneous rise and fall in IMP during the onset and release of loading was due to the fast fluid relaxation in the vascular porosity (on the order of $\sim \mu\text{s}$), and the subsequent exponential rise or decay in IMP during the onset or release of loading was due to a much slower fluid relaxation in the LCS porosity (on the order of $\sim 8 \text{ sec}$) (Fig. 2B). As predicted in previous studies [36], the characteristic time for the pressure generation and relaxation in a consolidating porous medium is proportional to the fluid viscosity and the loading area, and inversely proportional to the applied loading pressure and the Darcy permeability. In the current application the average pressure in the two porosities resulting from the step loading differed by three orders of magnitude (detailed below), the permeability of the smaller LCS and larger vascular pores differed by at least eight orders of magnitude (Table 2). This suggests that the permeability of the porosity is the dominating factor in determining the characteristic relaxation time. The relatively small permeability of the LCS indicates a longer time for fluid to drain into and out of from the vascular pores and marrow cavities, which leads to the longer exponential rise or decay in IMP (Fig. 2). The same mechanism applies to the larger porosities that have a much higher permeability and thus much shorter pressurization and relaxation time. We assume that fluid exchanges freely among LCS pores, vascular pores and marrow cavity, because the lining cells are unlikely to serve as a fluid transport barrier.[37] The relative contribution of the vascular and LCS pores to the overall IMP response appeared to be on the same order under the current experimental setting, although the IMP response associated with the vascular pores appeared to be larger (Fig. 2D).

The two distinct behaviors of the canine IMP measured in this study were consistent with previous studies of avian bones [14,30]. Turkey ulnae under bending and compressive loads displayed two distinct characteristic relaxation time constants (150 ms and 10 sec) in IMP, which were attributed to the vascular and lacunar-canalicular porosities, respectively [14]. The LCS relaxation times are comparable (8 sec vs. 10 sec) for canine and turkey bones. The characteristic relaxation time of the vascular pores in avian bone (150 ms) appeared to be longer than that of canine cortical bone ($< 2 \text{ ms}$), which was probably due to the difference in vascular organizations of the two species. Canine bone mostly consists of axial osteonal canals and radial Volkmann's canals (Fig. 3). In contrast, the avian bone contains many parallel, circumferential vascular channels with fewer frequent radial connections to the marrow in the cortex (see Figure 2 of Mi et al. [38]).

We derived the LCS permeability based on the measured time constant ($\sim 8 \text{ sec}$) of the IMP exponential responses, assuming that the LCS fluid relaxed into the Haversian canals of the osteons (Fig. 3). Previous studies of streaming potential measurements and bone fluid flow simulation support this assumption. Otter et al. (1994) found that the characteristic relaxation time of the streaming potential across cortical bone samples remained the same when the sample thickness was reduced from 4 mm to 1 mm [34]. In addition, the first detailed streaming potential measurements across bone by Starkebaum et al. (1979), showed that the potential gradients near Haversian canals were 30 times higher than the overall gradient across the sample's entire thickness during pure bending [33]. Furthermore, computational simulations

of fluid flow across osteonal bone replicated the findings of Starkebaum and confirmed that Haversian canals serve as a low-pressure reservoir for LCS fluid to relax [21]. In our canine samples, the average osteonal thickness was found to be 65 μm , which was within the reported range of $63 \pm 13 \mu\text{m}$ [39]. Using the poroelasticity theory developed by Cowin and colleagues, we found a LCS permeability on the order of $2.8 \times 10^{-23} \text{ m}^2$ would result in a characteristic relaxation time constant that matched our experimental measurement (8 sec). Given the variability of osteonal thickness (40-150 μm) and the IMP relaxation time constant (4-12 sec), we found the LCS permeability in the range of $2.8 \pm 1.8 \times 10^{-23} \text{ m}^2$ (Table 1). As shown in the poroelastic analysis (Eq. 5a), the estimated permeability k was inversely proportional to the square of the Skempton factor B . If the Skempton factor B of the cortical bone is reduced from 0.35 (the value used in the present study) to 0.20 as suggested in a recent study [40], the estimation of the LCS permeability is expected to be 3-fold higher ($8.4 \pm 5.4 \times 10^{-23} \text{ m}^2$), but remains to be on the same order (10^{-23} m^2).

The LCS permeability estimated in the present study was at least one order of magnitude smaller than theory-based estimations, but within the range of experimentally based estimations (Table 2). In particular, Beno et al (2006) estimated the LCS permeability for canine bone to be between 1×10^{-22} and $1 \times 10^{-19} \text{ m}^2$, based on images of individual lacunae and the total number of canaliculi emanating from each lacuna. However, several morphological characteristics of the LCS were not measured directly in that study, but based on electronic microscopic data of murine LCS, such as dimensions of the cell process, canalicular wall, and effective fiber spacing of the pericellular matrix. Besides, three other studies provide experiment-based estimations (Table 2). Smit et al (2002) reported the LCS permeability to be on the order of 10^{-22} m^2 , based on streaming potential data reported by Otter et al (1992) [30]. Oyen (2008) reported the LCS permeability on the order of 10^{-24} m^2 , based on nanoindentation tests on hydrated bone [24]. Cowin and colleagues reported the LCS permeability on the order of 10^{-24} to 10^{-25} m^2 , based on stress relaxation tests on single osteons [19]. The large variation among these experiment-based estimates may be due to different methodologies and the poroelastic properties of bone used in the analyses. Further investigations need to address these issues. Overall, the experiment-based studies suggest that the LCS has a much smaller permeability than the vascular pores, and the existing theoretical models tend to over-estimate the LCS permeability. Selective permeability studies on cortical bone are summarized in Table 2.

The IMP measurements and LCS permeability obtained in the present study allow more accurate estimations of the mechanical stimuli experienced by osteocytes and osteoblasts. Based on our measured LCS permeability, the hydraulic pressure within the LCS is predicted to reach a peak value of 5 MPa at the cement line when the osteon is compressed up to 1000 μe at 1 Hz using the analytical solution given by Zeng et al. (1994)[8]. Meanwhile, our IMP measurements never exceeded 30 mmHg, even under the sub-failure load ($\sim 4000 \mu\text{e}$ compression) *ex vivo* (Fig. 2C). Due to presence of the systemic blood pressure (17 to 33 mmHg) [17], the IMP in a living, mechanically loaded bone is expected to be on the order 60 mmHg (8 kPa). Therefore, the magnitude of fluid pressure that osteoblasts experience in bone ($\sim 8 \text{ kPa}$) is approximately three orders of magnitude smaller than the pore pressure experienced by osteocytes (up to 5 MPa). The implications of such large difference in pressure stimulation on the cellular responses of the two cell types are unclear. Osteoblasts have been shown to respond to hydraulic pressures *in vitro* [10,12], but the applied pressure ($\sim 500 \text{ mmHg}$) was much higher than the values reported here (60 mmHg). In contrast, there have been few studies of osteocytes subjected to hydraulic pressures on the order of MPa [11] and most of *in vitro* studies using flow chambers used very low pressures (at the kilopascal level). It would be more physiologically relevant if the osteocytes are studied under substantially higher pore pressures (around megapascal level) in future mechanotransduction studies. The above analysis focuses on fluid pressurization as bone experiences mostly compressive loading *in vivo*. However,

curved bones such as tibia and femoral head may experience local tensile strains due to the combination of bending and compression. Large tensile strains are recently proposed to induce negative gauge pressures that may lead to cavitation [41]. The mechanisms by which the body avoids cavitation and the cellular responses to negative pressures are unclear and thus need further investigations.

There are several limitations in the present study. First, the study was performed on *ex vivo* bone samples. Despite taking extensive precautions to maintain hydration of the samples, some alterations of the tissue during preparation were unavoidable. For example, the two ends of the bone were capped in aluminum fixtures with an epoxy resin in order to securely fix the samples on the testing machine. Thus, the surrounding soft tissues needed to be removed, which exposed and possibly dehydrated the periosteum, resulting in the collapse of associated blood vessels. In addition, we did not immerse the bone in solution during testing, but were only able to apply PBS on the bone surfaces to keep it moist. Although the overall testing period was relatively short for each sample (10 min), dehydration may have occurred to some degree while the samples were subjected to three sequential tests. However, these alterations may have a beneficial effect of reducing the leakage from the periosteum. The relatively steady plateau of the peak pressure (Fig. 2B) suggests that the leakage at the periosteum of our samples was minimal. A leaky periosteum would provide an alternative relaxation route besides the Haversian canals, which would complicate our analysis. Another limitation of this study was the use of an average osteonal thickness as the characteristic relaxation length for the LCS fluid draining into the marrow cavity and vascular pores. Although the majority of canine cortical bone consisted of osteons, there was ~20% of lamellar bone around the periosteum and endosteum (Fig. 3). The thickness of the lamellar region was on the order of 100-300 μm (Fig. 3). In addition, the trabecular bone at the two ends of the metacarpals may have contributed to the IMP response. Since the typical dimension of trabeculi and lamellar cortical bone does not dramatically differ from the diameter of osteons, the fluid relaxation distance used in the present analysis is expected to be a reasonable approximation for the current setting. Lastly, there was a concern about the use of frozen samples because possible damage to the cellular components and alteration of the LCS permeability may occur during the thawing process. Our recent studies demonstrate that transport of small molecules (like sodium fluorescein) is not altered in live bones versus that in bones two hours postmortem, and the mechanical loading-enhanced transport is the same for both freshly sacrificed and frozen bones (Liyun Wang, unpublished data). These data suggest that freezing and thawing is unlikely to alter the LCS Darcy permeability significantly. However, testing of live bones *in vivo* will be needed to address this concern.

Despite these limitations, we were able to measure the LCS permeability and intramedullary pressure by using rapid compression of the entire bone. By choosing the loading rate between the characteristic relaxation times of the vascular and LCS porosities, we successfully differentiated the responses in the intramedullary pressure by these two intertwined porosities. The characteristic relaxation time of mammalian osteonal bone was found to be on the order of 8 sec, and the LCS permeability was estimated to be on the order of 10^{-23} m^2 . Using this experimentally validated LCS permeability, more accurate evaluation of the microfluidic environments for osteocytes during physiological loading conditions was readily obtained using well-established poroelastic models. Our results reveal that under physiological compression of 1000 $\mu\text{ε}$ at 1 Hz, osteocytes experience a 1000-fold higher hydraulic pressure than osteoblasts. The results of the present study provide quantitative data on bone permeability at the lacunar-canalicular level and the mechanical environments of bone cells, which may be used to design more physiologically relevant *in vitro* models to investigate the mechanisms of bone adaptation and mechanotransduction.

Acknowledgments

This study was supported by NIH (AR054385, AR043222 and P20RR016458), University of Delaware Research Foundation and the office of Naval Research grant N00024-07-C-4212. The authors thank Dr. Mitchell Schaffler for providing the bone samples and Dr. Stephen Cowin for commenting on the manuscript.

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Figure 1.

A schematic shows that the canine metacarpal sample was capped at both ends within epoxy resin and aluminum fixtures and positioned vertically on an MTS machine. The bone was subjected to rapid compression, while the intramedullary pressure (IMP) was recorded simultaneously, using a small pressure transducer sealed within a catheter that was inserted into the bone marrow cavity via a 0.5mm hole bored on the bone cortex.

Figure 2.

The IMP response to rapid compaction of the intact canine metacarpals. **[A]** The loading waveforms of three representative tests show that the samples were subjected to a rapid compression with a short ramp time (2ms) to reach the defined peak loads (50, 100, or 200 lbs). **[B]** Two distinct behaviors were observed in the IMP responses: the instantaneous jump/fall during the onset and release of the load due to the contribution of the vascular pores (including marrow cavity), and the subsequent slower exponential rise and decay, due to the smaller LCS pores. The relatively steady IMP during load dwell time suggested minimal leakage at the periosteum. **[C]** The mean IMP that built up during compaction increased linearly with increasing loading magnitude [$P_{peak} \text{ (mmHg)} = 0.0042 \times \text{Strain } (\mu\epsilon)$, $R^2 = 0.96$]. **[D]** The average time constants for the IMP exponential rise/decay during the onset and removal of the load were determined to be 7.2 ± 3.2 sec and 9.1 ± 3.8 sec with an average of 8.1 ± 3.6 sec by fitting IMP recordings with exponential functions using equations 1 and 2. Good fitting between the measured IMP recordings (solid line) and the exponential functions (broken line) was achieved with correlation coefficients of 0.98 ± 0.01 . The time constants and variability in IMP pressure (gray area) were found to be independent upon the magnitude of applied load.

Figure 3.

Representative photographs of [A] the entire cross-section and [B] the blow-up of the boxed area of a transverse section of the mid-shaft of a canine metacarpal. The dominant features of the bone included: i) osteons with an average outer radius of $81 \pm 24 \mu\text{m}$ and inner radius of $16 \pm 5 \mu\text{m}$, which were well distributed in the majority of the cortex area ($\sim 80\%$); and ii) the Haversian and Volkmann's canals, which were well-connected with the marrow cavity. The lamellar bone along the periosteum and endosteum comprised $\sim 20\%$ of the total area with a thickness varying from 100-300 μm . Based on previous experimental and simulation studies [21,34], the thickness of the osteons (indicated by l in panel B, $l = 65 \pm 27 \mu\text{m}$) was assumed to be the characteristic distance for the fluid in the lacunar-canalicular system to drain into the marrow cavity and the vascular pores (canaliculi are not shown at the current scale).

Table 1
Summary of Calculation of the LCS permeability

Parameters (units)	Source	Mean (std)
LCS fluid relaxation time constant (τ , sec)	Measured from IMP	8.1 (3.6)
Osteonal thickness (l , μm)	Measured from images	65.5 (27.2)
Pressure diffusion coefficient (c , m^2/s)	Equation 4	5.3×10^{-10} (3.2×10^{-10})
LCS Darcy permeability (k , m^2)	Equation 6	2.8×10^{-23} (1.8×10^{-23})

Table 2
Summary of selective studies on the Darcy permeability of cortical bone

	Permeability (m ²)	Methods / Species or / Transport Direction	Study
VP	3.00×10 ⁻¹³	Transport Chamber / Bovine / Longitudinal	Rouhana et al. (1981) [26]
	5.00×10 ⁻¹⁵	Transport chamber / Dog / Radial	Li et al. (1987) [25]
	1.00×10 ⁻¹¹ or 1.00×10 ⁻¹³	Theory / Rat / Longitudinal or Radial	Dillaman et al. (1991) [42]
	6.35×10 ⁻¹³ or 1.90×10 ⁻¹⁵	Theory / - / Longitudinal or Radial	Zhang et al. (1998) [20]
	1.10×10 ⁻¹³	Transport Chamber / Bovine / Radial	Malachanne et al. (2008) [27]
LCS	1.47×10 ⁻²⁰	Theory / - /	Zhang et al. (1998) [20]
	1.70×10 ⁻²⁰	Biot Theory / Bovine	Wang et al. (1999) [21]
	2.20×10 ⁻²²	Finite element analysis, fitting streaming potential data / Dog / Radial	Smit et al. (2002) [29]
	7.50×10 ⁻²⁰ or 6.70×10 ⁻²¹	Biot Theory / - / Longitudinal or Radial	Gururaja et al. (2005) [43]
	1.50×10 ⁻¹⁹ to 1.00×10 ⁻²²	Biot Theory / - / Dog	Beno et al. (2006) [28]
	3.79×10 ⁻²¹	Biot Theory / Mouse	Zhou et al. (2008) [23]
	4.14×10 ⁻²⁴	Nanoindentation / Human	Oyen (2008) [24]
	1.00×10 ⁻²⁴ or 1.00×10 ⁻²⁵	Stress relaxation of single osteons / Bovine	Galilani et al. (2009) [31]
	2.8×10 ⁻²³	Step loading of intact bone / Dog	Present Study

Notes: VP = vascular porosity; LCS = lacunar-canalicular system. Theoretical and experimentally based estimations are indicated in the column of Methods. The transport directions (radial or longitudinal) are listed when applicable, especially for the VP measurements.