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MORPHOLOGY, LOCALIZATION AND ACCUMULATION OF IN VIVO MICRODAMAGE IN HUMAN CORTICAL BONE

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Abstract

In vivo, microdamage occurs in the form of linear microcracks and diffuse damage. However, it is unknown whether the age-related changes in bone quality predispose bone to form one type of damage morphology over the other during in vivo loading. In this study, histological and histomorphometrical analyses were conducted on transverse cross sections, obtained from the tibiae of aging human bone (age 19 to 89), to investigate the in vivo accumulation and localization of damage morphologies. The results demonstrate that old donor bone (83 ± 3 years) contains more linear microcracks than younger donor bone in the cortices predominantly subjected to compressive ($p < 0.01$) and tensile loading ($p < 0.01$). In contrast, young donor bone (40 ± 10 years) contains more diffuse damage than older donor bone in the cortex predominantly subjected to tensile loading ($p < 0.01$). The formation of damage morphology showed no correlation with bone geometry parameters and exhibited distinct preferences with bone microstructure. Linear microcracks formed in the interstitial bone ($p < 0.01$) and were either trapped or arrested by the microstructural interfaces (cement line and lamellar interface) ($p < 0.05$). Areas of diffuse damage, however, were preferentially associated with secondary osteonal bone ($p < 0.01$) and had no relationship with the microstructural interfaces ($p < 0.01$). Based upon these findings, we conclude that age-related changes in bone microstructure, but not bone geometry, play a key role in the propensity of old donors to form linear microcrack over diffuse damage under in vivo loading conditions.

Keywords

Aging; Microcracks; Diffuse Damage; In vivo Loading; Bone Quality

INTRODUCTION

It is well known that both cancellous and cortical tissue contribute to the fracture resistance of bone [1,2]. Quantitative or qualitative changes in cancellous bone and in cortical bone are therefore expected to affect bone fragility. Historically, only the quantitative changes, measured by bone mass, have been considered to be a significant predictor of fracture risk [3]. However, recent evidence shows that not all the variability in bone fracture can be explained by bone mass alone [4–9]. For example, Kanis [2005] noted that even though the changes in BMD (bone mineral density) between the ages of fifty and eighty years predict a

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four-fold increase in hip fracture risk, the risk in reality increased thirty-fold. Thus, age–related changes in bone quality could affect the mechanical competence of bone including the formation of microdamage.

In vivo microdamage occurs in the form of linear microcracks [10–13] and diffuse damage [14,15]. Previous studies provide evidence that different damage morphologies (linear microcracks and diffuse damage) have different effects on the mechanical properties of bone [16–19]. For example, Burr *et al*. [1998] demonstrated that stiffness loss has a linear relationship with diffuse damage and a quadratic relationship with linear microcracks. They also found that tensile damage accumulates more easily but crack growth is greater in compression [16]. We showed that cortical bone compartmentalizes the damage morphologies in different regions and the sequence of damage production in different phases of cyclic loading to dissipate energy and resist a catastrophic fracture [18]. More significantly, the propensity of older human bone to form linear microcracks over diffuse damage has been suggested to play a critical role in bone fragility [19].

Using an in vitro model, damage morphology has shown to be a combined function of local strain and tissue properties [20,16,17,18,19]. Linear microcracks form under compressive loading [20,16,17,18,19] in the interstitial bone and either stop at the cement lines (i.e the osteonal boundaries) [20] or develop along the lamellar interfaces (i.e. the interlamellar boundaries) [19]. In contrast, diffuse damage forms under tensile loading [20,17,18] as submicroscopic cracks [20] and has no relationship with the lamellar arrangement of bone [19]. In addition to the tissue properties, bone geometry could also affect damage morphology. Specifically, narrower tibias have been shown to fail in a brittle manner [21] suggesting that bone width may play a role in the development of damage morphology. The interaction of strain mode (tensile vs. compressive) with bone microstructure and geometry may therefore alter the mode and magnitude of damage formation with aging under in vivo loading conditions.

Thus, the aim of this study was to determine the in vivo occurrence and accumulation of linear microcracks and diffuse damage in human cortical bone and to investigate their relationship with bone microstructure and geometry.

MATERIALS AND METHODS

Transverse sections were obtained from twenty-four human cadaveric tibiae (Age range 19 to 89 years; Sex: males and females) who died suddenly showing no apparent bone disease. These sections were separated into two groups (Young, mean age = 40 ± 10 years, N = 11, N_{male} = 6, N_{female} = 5; and Old, mean age = 83 ± 3 years, N = 13, N_{male} = 7, N_{female} = 6). The preparation of sections included cutting a 1 cm long segment from the distal diaphysis followed by en bloc staining in 1% basic fuchsin [22]. After staining, the segments were embedded in polymethyl methacrylate (PMMA) and serial transverse sections (100 μm thickness) were obtained. The center of each section was located 3.5 cm below the middle of the diaphysis. This technique of staining and sectioning is known to mark only the damage present in bone at the time of the donor's death [23]. Transverse sections, and not longitudinal sections, were used because the distinction between the interstitial and secondary osteonal bone is often difficult in longitudinal sections [24,19].

Histological analyses of microdamage

Microdamage was measured in the anterior and posterior cortices at 200× magnification using bright-field microscopy (IX81, Olympus, Melville, NY 11747). The anterior and posterior cortices were chosen because under in vivo loading they are subjected primarily to tensile and compressive loading, respectively [Peterman *et al*. 2001] [25]. In basic fuchsin stained sections, linear microcracks appear as a sharply defined line (Figure 1) and diffuse damage appears as

an area of pooled staining (Figure 1) [20,15,18,19]. Selective areas containing diffuse damage or a linear microcrack were examined under a laser confocal microscope (Zeiss, Thornwood, NY 10594; Excitation = 543 nm; Emission = 560 nm) to verify the presence of damage (Figure 1). Following the histological assessment of microdamage, the bone sections were subjected to geometric and microstructural analyses.

Geometric analyses

The transverse sections were scanned and converted to 8 bit BMP digital images using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA, 95110). In conjunction with Image J 1.36 [http://rsb.info.nih.gov/ij/], a macro (computer program), available for the estimation of cross sectional moments [http://www.hopkinsmedicine.org/FAE/mmacro.htm], was used to calculate the principal moments of inertia (Imax, Imin), polar moment of inertia (J), anterior cortex width (Aw), posterior cortex width (Pw), tibia width in the anterior-posterior direction (APw), section modulus in the anterior-posterior direction (Sm.AP), polar moment of inertia normalized to tibia length (J/L), and cortical area (Ct. Ar) of the bone cross sections [21,26]. The macro provides a better estimate of the principal moments of inertia than the two concentric ellipse method [27,28]. To avoid any operator bias in the calculation of cortex width, the anterior cortex width (Aw) and the posterior cortex width (Pw) were measured where the axis, representing the maximum bending rigidity, crossed the anterior and posterior cortices, respectively.

Microstructural analyses

Because of donor-to-donor variability in areas of interstitial and osteonal bone, linear microcracks and diffuse damage were normalized to interstitial/osteonal areas. The interstitial and secondary osteonal bone areas were determined by taking images from the anterior and posterior cortices under brightfield microscopy at 125 × magnification. Average measurements were taken over four fields to reduce any measurement errors [29,30]. The quantities measured from each selected field included the total area of the field, total area of secondary osteons including haversian canals, total area of the haversian canals, and total area of all pores (> 200 μm^2) including the haversian canals [31,30,26]. The osteon fragments that had a distinct haversian canal and a clear cement line were included in the quantification of the secondary osteonal area [26]. The microstructural parameters of interest were calculated as follows [31, 30,26]:

$$\text{Secondary osteonal area}(\%) = \frac{\text{Total osteonal area excluding canals}}{\text{Selected field area}} \times 100 \quad (1)$$

$$\text{Porosity}(\%) = \frac{\text{Area of pores and canals}}{\text{Selected field area}} \times 100 \quad (2)$$

$$\text{Interstitial area}(\%) = 100 - (\text{Secondary osteonal area}(\%) + \text{Porosity}(\%)) \quad (3)$$

To analyze the role of the microstructural interfaces in the development of damage morphology, linear microcracks and diffuse damage were counted based on the following classification (Figure 2): LM_1: linear microcrack that either formed at the lamellar interface or was stopped at the cement line; LM_2: linear microcrack that neither formed at the lamellar interface nor stopped at the cement line; DD_1: diffuse damage patch that was either limited to one lamella or stopped at the cement line; and DD_2: diffuse damage patch that was neither limited to one lamella nor stopped at the cement line. The lamellar interface, defined as the boundary between adjacent lamellae, was visualized using a bright-field microscopy with a polarizing filter [19, 32].

Statistical analyses

Following a check for normality, the differences in microdamage accumulation [linear microcrack density (#/mm^2), diffuse damage density (mm^2/mm^2)] first between males and females and then between the young and old groups were compared by an unpaired *t*-test. For those variables failing the normality test, a Mann-Whitney test was used for comparison. The association between damage morphology and bone microstructure was determined by comparing the number of linear microcracks associated with interstitial bone area (#/mm^2) vs. osteonal bone area (#/mm^2) and the diffuse damage areas associated with interstitial bone area (mm^2/mm^2) vs. osteonal bone area (mm^2/mm^2). The effects of the microstructural interfaces on damage morphology were determined by comparing the number of LM_1 vs. number of LM_2 and the number of DD_1 areas vs. the number of DD_2 areas. Either a paired t-test or a Wilcoxon signed-rank test was used for all the above comparisons. Linear regression analysis was conducted to examine the relationship between the geometrical parameters and damage morphology. SigmaStat 2.0 (SYSTAT Software Inc, Chicago, IL 60606) was used for all the statistical analyses.

RESULTS

Because no gender-related differences in morphology or accumulation of microdamage were found ($p > 0.05$) for both the young and old groups, data from males and female donors were pooled for microdamage comparisons. Damage accumulation and morphology were significantly different between the young and old groups. Compared to young donors, cortical bone cross sections obtained from old donors contained 3.5-fold more linear microcracks in the cortex subjected to compressive loading (posterior cortex) ($p < 0.01$, Figure 3a) and nearly 3-fold more linear microcracks in the cortex subjected to tensile loading (anterior cortex) ($p < 0.01$, Figure 3a). In contrast, bones obtained from young donors contained 25-fold more diffuse damage than bones obtained from old donors in the cortex subjected to tensile loading (anterior cortex) ($p < 0.01$, Figure 3b). Diffuse damage accumulation in the cortex subjected to compressive loading (posterior cortex) was minor and not statistically significant between the young and old groups ($p = 0.662$, Figure 3b).

The differences in the development of microdamage between the young and old donors could not be fully explained by bone geometry. Both morphologies of damage had weak correlations with bone geometrical parameters (Table 1). Damage morphology, however, exhibited different preferences with bone microstructure. Twenty-two-fold more linear microcracks formed within interstitial bone than secondary osteonal bone ($p < 0.01$, Figure 4a) while nearly 4-fold more diffuse damage developed in secondary osteonal bone than interstitial bone for both young and old donors ($p < 0.01$, Figure 4b).

Linear microcracks and diffuse damage showed distinct relationships with the microstructural interfaces for both young and old donors. A post-hoc analysis revealed that 88% of the linear microcracks either developed along the lamellar interface or were stopped at the cement line ($p < 0.05$, Figures 2 and 5a). In contrast, 79% of the diffuse damage patches were neither limited to one lamella nor were stopped at the cement line ($p < 0.01$, Figures 2 and 5b).

DISCUSSION

The differences in linear microcrack formation between the young and old donors found in this investigation are consistent with previous studies that showed an age-related increase in linear microcracks density [11–13]. Characterization of age-related changes in in vivo damage morphology has not been reported previously. To our knowledge this is the first study to report the effects of age on damage morphology development and to identify the relationship between damage morphology and the microstructural interfaces in vivo.

Here, we investigated the incidence and accumulation of linear microcracks and diffuse damage in the anterior and posterior cortices of the tibia. The anterior and posterior cortices were chosen because dynamic gait simulation of human cadaveric tibia shows that the primary loading on the tibia is bending and that the maximum tensile and compressive strains occur in the anterior and posterior cortices, respectively [25]. Unlike experiments done on human subjects, where strain gages were placed either on the antero-medial or on the medial aspect of the tibia [33, 34,35], the human cadaveric model allows strains to be recorded from all the four cortices and therefore provides a more comprehensive information about the tibia loading milieu [25].

In a previous investigation, we reported that diffuse damage forms at an early stage in the fatigue life while the formation of linear microcracks occurs towards the end of the fatigue life [18]. The ability of cortical bone to compartmentalize damage morphology production in different stages of cyclic loading, therefore, plays an important role in resisting a catastrophic fracture [18]. Results of this study show that, under in vivo loading, bones obtained from young donors have a higher propensity than bones obtained from old donors to form diffuse damage that is known to dissipates more energy and is self-limiting (does not grow further) [36,16, 18]. More importantly here we show that in addition to forming significantly less diffuse damage in the tensile cortex, the aging human bone forms significantly more linear microcracks than young human bone in both the tensile and compressive cortices. Linear microcracks are traditionally associated with compressive loading and the terminal phase of bone fracture characterized by rapid crack propagation and catastrophic fracture [16,18]. These results suggest that other modes of loading including mixed mode loading may combine with changes associated with advancing age including the decline in bone tissue quality and consequently dominate the formation of linear microcracks in both the tensile and compressive cortices [37].

In vivo microdamage accumulation depends on the imbalance between the rate at which the remodeling process repairs damage and the rate at which microdamage is initiated [38]. The accumulation of in vivo damage reported in this study could therefore be a result of several factors including bone quality, applied load, or/and a decline in the remodeling rate. In a previous study [19], we have used an in vitro model to examine the tissue level mechanical properties (bone quality) and found that specimens obtained from old donors form more linear microcracks than specimens obtained from young donors while young donors form more diffuse damage than old donors under similar loading conditions [19]. Thus, bone quality plays a role in damage morphology development. However, a decline in the remodeling rate will exacerbate the effects of bone quality and result in the accumulation of more microdamage.

A recent study by Tommasini *et al*. [2005] have demonstrated that in young males bone fragility can also be predicted by bone geometry. Bones obtained from narrower tibias were more brittle and accumulated more damage compared to bones obtained from wider tibias [21]. Here, we show that only the formation of diffuse damage in the posterior cortex has a significant correlation with cortex width ($r^2 = 0.271$). However, the magnitude of diffuse damage accumulation in the posterior cortex was very limited and showed no difference between the young and old groups (Figure 3b). Therefore, bone geometry may be a good predictor for stress fracture risk in young adults, but it does not predict the age-related changes in in vivo damage morphology where other factors including bone turnover-mediated changes in bone quality may play a role in microdamage development.

Specifically in contrast to bone geometry, the changes in the turnover-based bone quality parameters including the interstitial and secondary osteonal content of bone can provide a better understanding of factors that predispose bone to form one type of damage morphology over the other, as shown by the localization of linear microcracks and diffuse damage with interstitial and secondary osteonal bone, respectively (Figures 4a and 4b). Interstitial and secondary

osteonal bone are reported to have different chemical composition [39,40] and mechanical properties [41,39]. Interstitial bone is characterized as an older tissue that is more highly mineralized [39,40] and stiffer [41,39] than secondary osteonal bone. Consequently, interstitial bone is more likely to dissipate energy in a brittle manner [42] by forming linear microcracks [43,44] over diffuse damage, as was found here.

The relationship between different damage morphologies and microstructural interfaces was also distinct. Eighty-eight percent of the linear microcracks either followed the lamellar interface or stopped at the cement line (Figures 2 and 5a). However, only 21% of the diffuse damage patches were related to the microstructural interfaces (Figures 2 and 5b). Thus, the lamellar interface and the cement line in bone are associated with the arrest and trapping of linear microcracks but not diffuse damage.

Previous studies show that the lamellar interface [40,19] and the cement line [45,11,12,20, 46,47] affect bone toughness differently. Jepsen *et al*. [1999] and Diab *et al*. [2006] demonstrated that the lamellar interface has an intimate relationship with linear microcracks suggesting that the orientation of the lamellae may play a role in resisting crack propagation [19]. The preferential orientation of the lamellae parallel to the longitudinal axis of bone [48] could provide bone with toughness [49,50]. Additionally the lamellar interface also keeps the cracks physically isolated from each other which in turn delays microcrack coalescence [40]. On the other hand, the cement line is known to contribute to bone toughness by acting as a barrier to crack propagation or by deflecting crack growth [45,11,12,20,46,47]. Moshin *et al*. [2006] found that microcracks less than 100 micron in length stopped at the cement line while microcracks in the range of 100–300 micron were deflected as they encountered the osteonal boundaries. Therefore, despite the reduced propensity of older donor bone to form diffuse damage, the microstructural interfaces can still act to provide the bone with some toughening mechanisms to resist linear microcrack propagation (Figure 5a).

The disassociation between diffuse damage and the microstructural interfaces (Figure 5b) also suggest that age-related changes at the ultrastructural level may have a greater affect on the formation of diffuse damage than linear microcracks. However, the age-related variations investigated in this study are limited to the effects of bone microstructure on damage morphology development, and no attempt has been made to identify the ultrastructural features associated with these changes. For example, it is known that aging bone is characterized by an increase in the fraction of highly mineralized bone [51] and the modification of collagen by denaturation [52] or non-enzymatic glycation [53,54]. All the above factors may make bone more prone to form linear microcracks over diffuse damage and should be investigated further.

A limitation of this work is that linear microcracks and diffuse damage were observed only in the transverse sections. In a previous study [19] we examined longitudinal sections and found that cortical bone displays comparable trends in damage morphology development to what was reported here. Hence, it is reasonable to consider that the orientation of the sections will not affect the morphology and accumulation of microdamage although the actual length of microcracks may be longer in the longitudinal sections [55].

In conclusion, the results of this investigation demonstrate that age-related changes associated with bone quality (as measured by bone microstructure) affect the propensity of bone to form one mode of damage morphology over the other. Following in vivo loading, old donor bone contained more linear microcracks than young donors in the cortex subjected to compressive loading and the cortex subjected to tensile loading. In contrast, young donors formed more diffuse damage than old donors in the cortex subjected to tensile loading. The formation of damage morphology had distinct relationships with bone microstructure. Linear microcracks and diffuse damage colocalized with interstitial and secondary osteonal bone, respectively.

Furthermore, linear microcracks were more often arrested by the microstructural (lamellar and cement line) interfaces than was diffuse damage. A better understanding of the relationship between bone quality and damage morphology can provide a better insight into the age-related increase in bone fragility.

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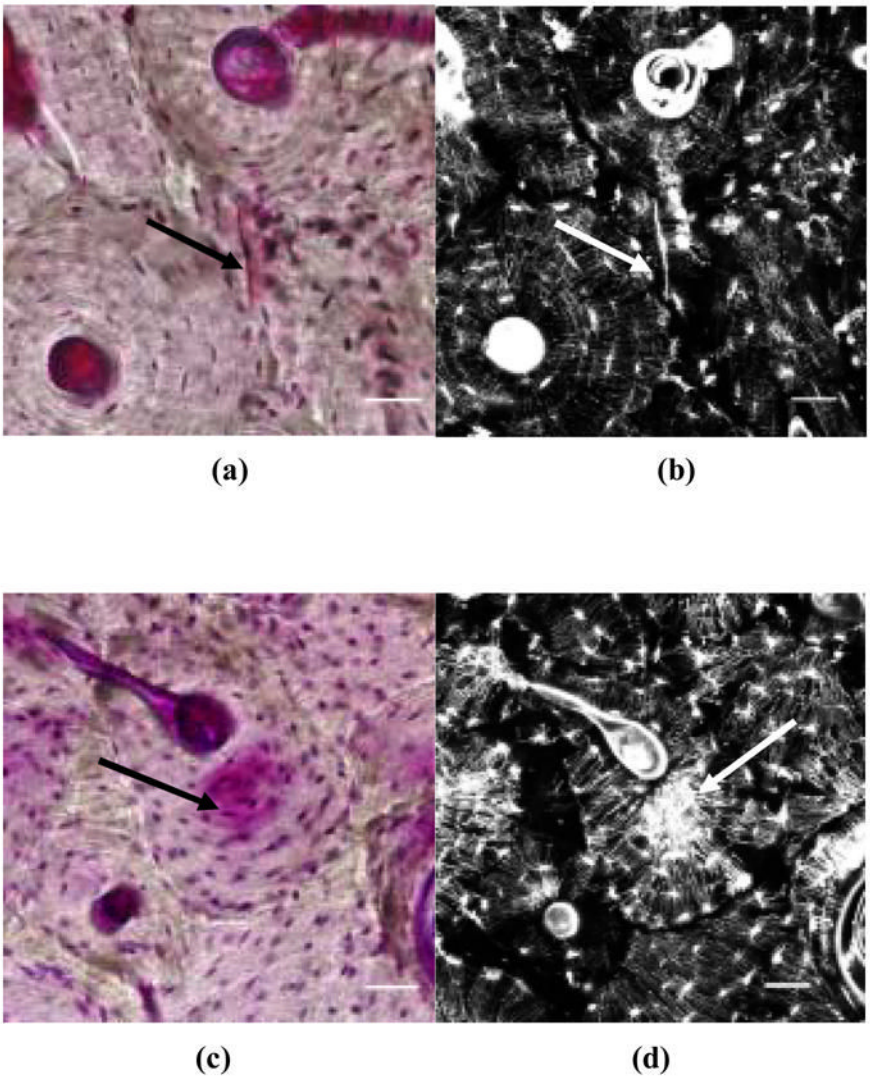


Figure 1.
In vivo linear microcracks (a: under bright-field microscopy; b: under a laser confocal microscope) and diffuse damage (c: under bright-field microscopy; d: under a laser confocal microscope) in human cortical bone. Scale bars = 50 μm.

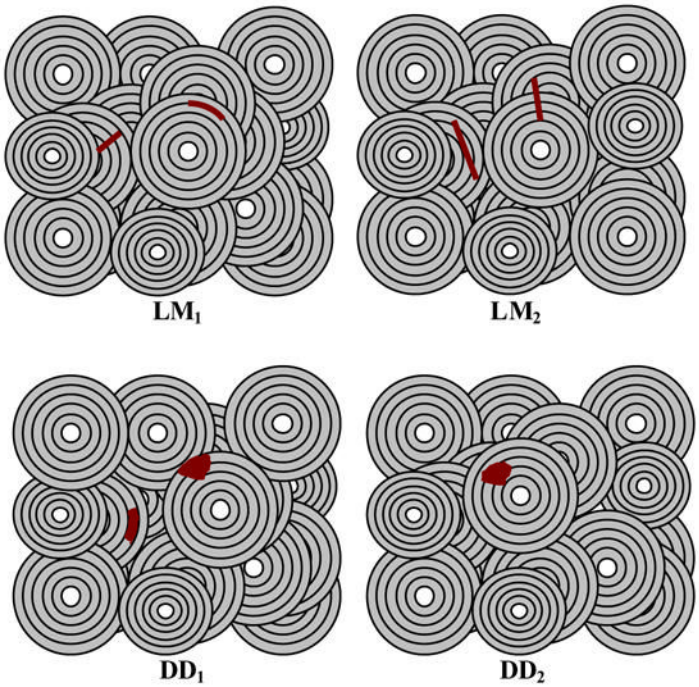
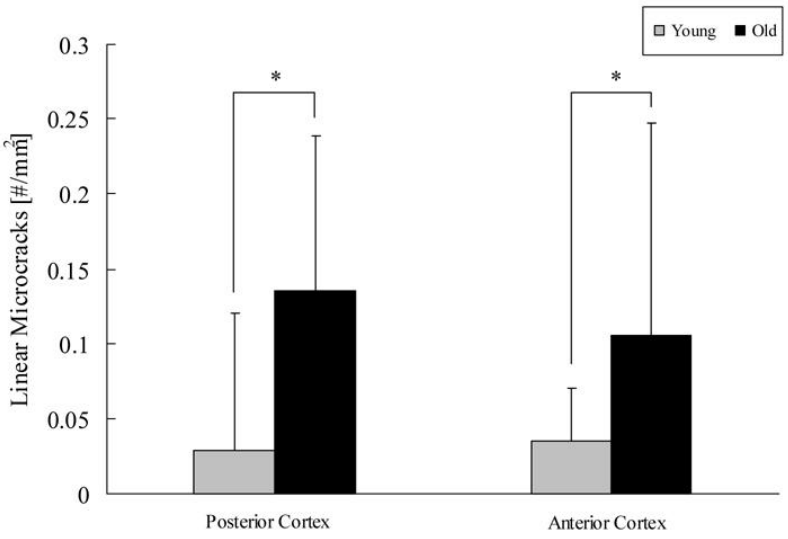
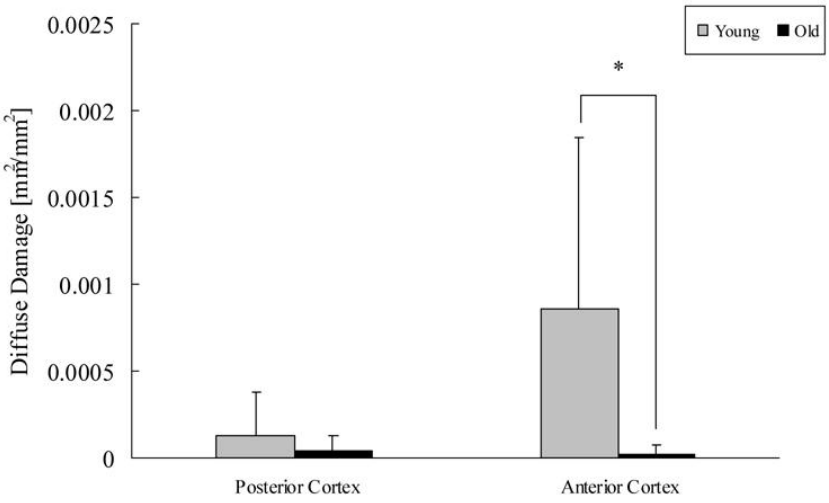


Figure 2.
Schematic representation of microdamage morphologies and their relationship with the microstructural interfaces. LM_1: linear microcrack that either formed at the lamellar interface or was stopped at the cement line. LM_2: linear microcrack that neither formed at the lamellar interface nor was stopped at the cement line. DD_1: diffuse damage patch that was either limited to one lamella or was stopped at the cement line. DD_2: diffuse damage patch that was neither limited to one lamella nor was stopped at the cement line.



(a)



(b)

Figure 3.

Bones obtained from old donors contained more linear microcracks than the bones from young donors in both the anterior and posterior cortices (a). In contrast, bones obtained young donors contained more diffuse damage than bones from old donors in the anterior cortex (b). * indicates $p < 0.05$.

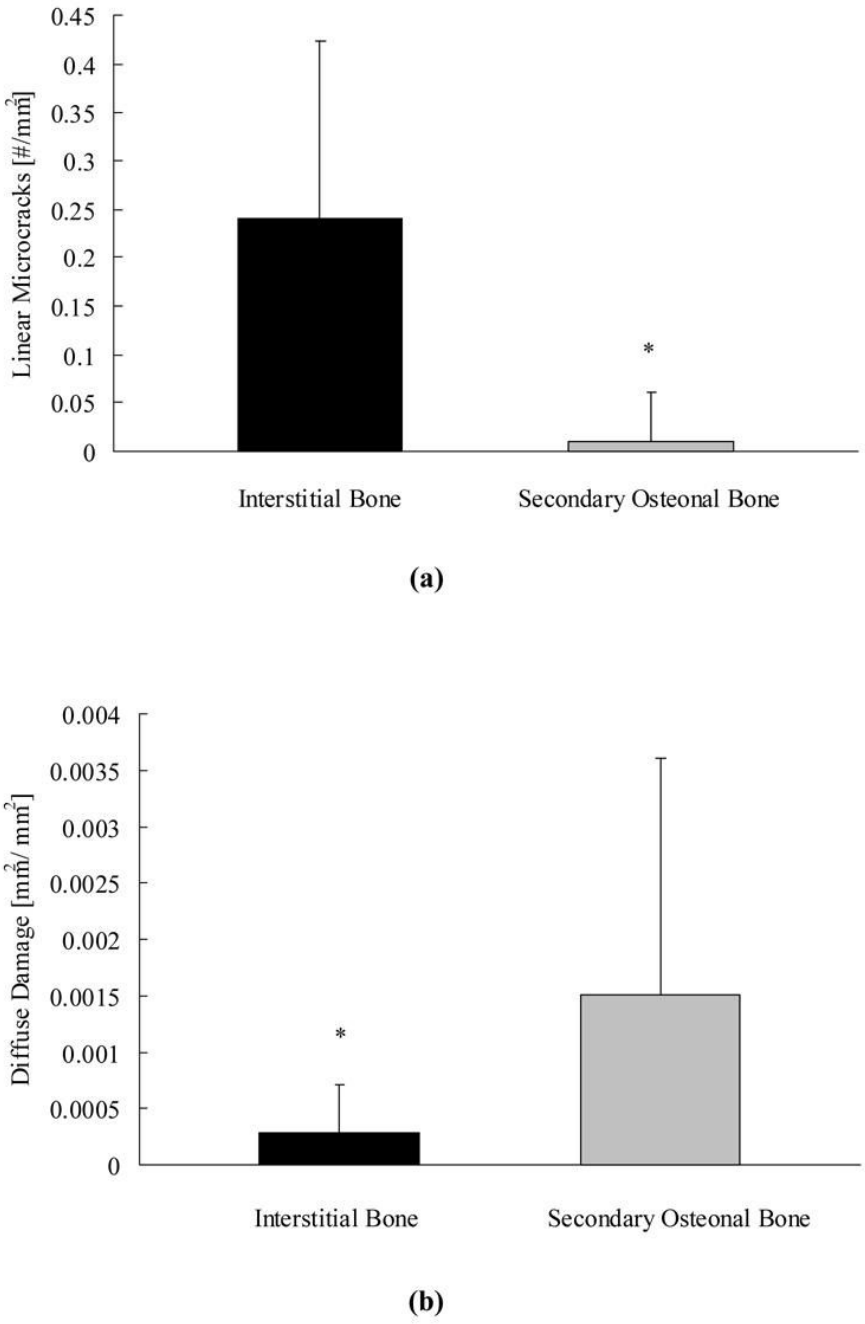


Figure 4.
More linear microcracks formed in interstitial bone than secondary osteonal bone (a), while more diffuse damage developed in secondary osteonal bone than interstitial bone (b). * indicates $p < 0.05$.

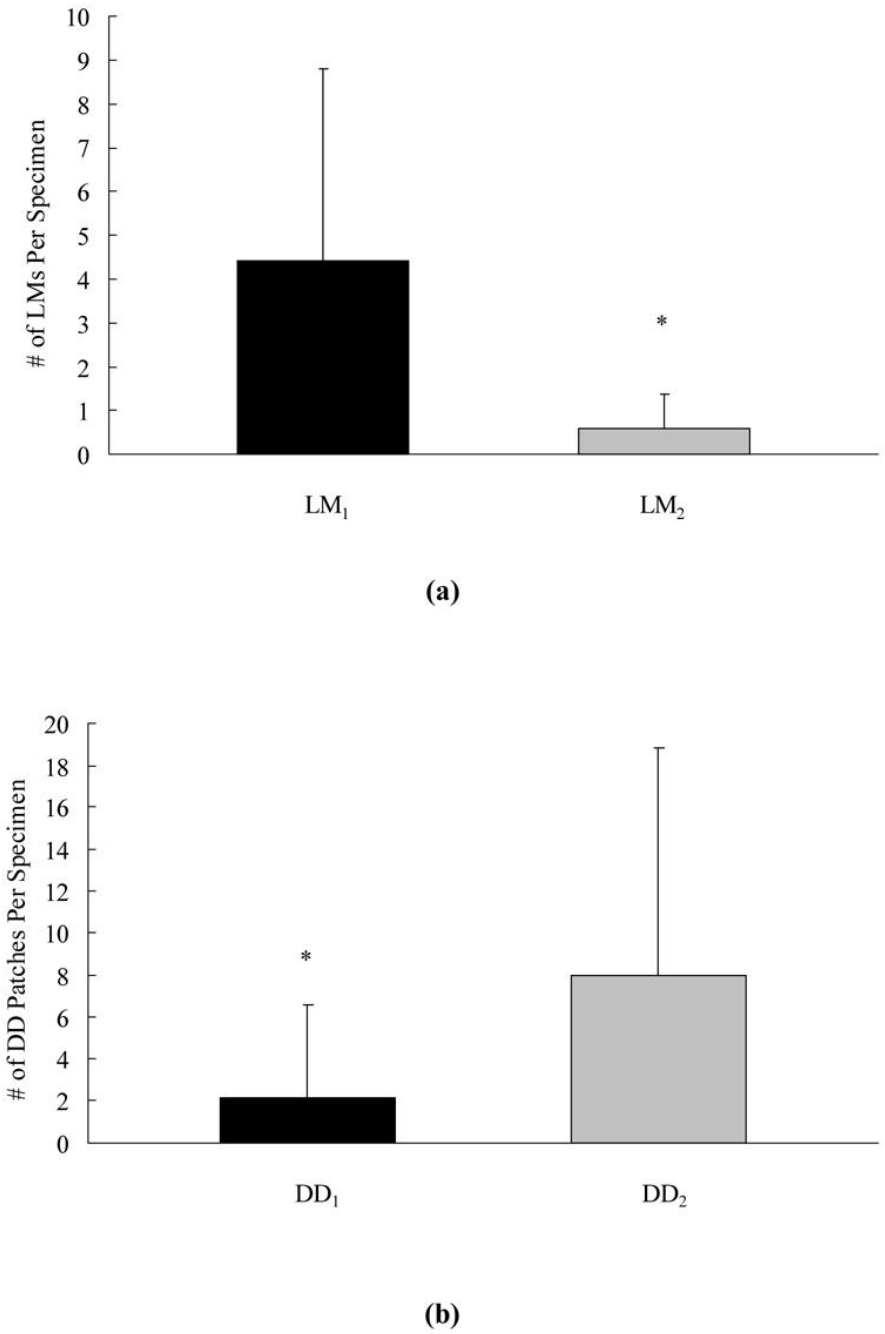


Figure 5.

More linear microcracks either formed at the lamellar interface or were stopped at the cement line (LM_1) (a). In contrast, diffuse damage patches were neither limited to one lamella nor were stopped at the cement line (DD_2) (b). * indicates $p < 0.05$.

Table 1

Correlation between damage morphology and the geometric parameters. The first and second rows correspond to the r^2 and p values, respectively. Significant correlations are shown in bold.

	Imax	Imin	J	Aw	Pw	APw	Sm. AP	J/L	Ct.Ar
LM $_{\text{Posterior Cortex}}$	0.011	0.007	0.010	N/A	0.003	0.003	0.015	0.017	0.001
	0.633	0.690	0.645		0.801	0.787	0.565	0.547	0.907
LM $_{\text{Anterior Cortex}}$	0.003	0.001	0.001	0.011	N/A	0.030	0.000	0.003	0.005
	0.802	0.904	0.898	0.631		0.419	0.998	0.787	0.737
DD $_{\text{Posterior Cortex}}$	0.026	0.001	0.015	N/A	**0.271**	0.039	0.008	0.007	0.068
	0.453	0.858	0.570		**0.009**	0.358	0.676	0.691	0.220
DD $_{\text{Anterior Cortex}}$	0.084	0.038	0.069	0.089	N/A	0.057	0.040	0.035	0.095
	0.171	0.361	0.214	0.157		0.261	0.351	0.380	0.143