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COLLAGEN MUTATION CAUSES CHANGES OF THE MICRODAMAGE MORPHOLOGY IN BONE OF AN OI MOUSE MODEL

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Abstract

Previous studies have postulated that ultrastructural changes may alter the pattern and capacity of microdamage accumulation in bone. Using an osteogenesis imperfecta (OI) mouse model, this study was performed to investigate the correlation of collagen mutation with the microdamage morphology and the associated brittleness of bone. In this study, femurs from mild OI and wild type mice were fatigued under four-point bending to create microdamage in the specimens. Then, the microdamage morphology of these specimens was examined using the bulk-staining technique with basic fuchsin. Similar with the results of previous studies, it was observed that linear microcracks were formed more easily in compression, whereas diffuse damage was induced more readily in tension for both wild-type and mild-type mice. However, less diffuse damage was found in the tensile side of mild OI mouse femurs (collagen mutation) compared with those of wild type mice, showing that the microdamage morphology is correlated to the brittleness of bone. The results of this study provide direct evidence that supports the prediction made by the previous numerical simulation studies, suggesting that microdamage morphology in bone is significantly correlated with the integrity of the collagen phase.

Keywords

Linear microcrack; diffuse damage; collagen mutation; osteogenesis imperfecta; fatigue

1. INTRODUCTION

Fragility fractures among the post-menopausal women and elderly are a major concern in the health care of our society [1,2]. Such fractures are mainly due to bone mass loss and tissue quality deterioration. Tissue quality deterioration in bone may be manifested in the architecture and the accumulation of microdamage. Micro-architecture in trabecular bone can be clinically assessed using noninvasive imaging techniques such as in vivo peripheral quantitative

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computed tomography (pQCT) [3,4] and micro-magnetic resonance imaging (microMRI) [5, 6]. However, detection of microdamage in living human individuals may be technically challenging and relies currently on invasive sampling by biopsies [7-9]. In addition, previous in vitro studies have shown that microdamage accumulation significantly relates to the toughness of bone [10-13]. Since microdamage accumulation contributes to bone fragility, understanding its underlying mechanism becomes indispensable.

Numerous studies [10,14-37] have indicated that bone could be considered as a fiber-reinforced composite material at the ultrastructural level, in which mineral crystals are responsible for its stiffness and collagen fibrils offer its toughness. Using a probabilistic failure model of the composite, numerical simulations have predicted that the morphology of microdamage in bone is significantly related to collagen fibril properties such as the diameter, modulus, and nonlinearity of collagen fibrils [38]. To our knowledge, however, no experimental evidence is available to support the prediction. Animal models of osteogenesis imperfecta (OI), in which defective collagen phase is present due to genetic mutation of collagen, are usually used to study the underlying mechanism of the disease. Existing mouse models for OI include osteogenesis imperfecta murine (oim) [39], Mov-13 mouse [40], Brittle IV mouse [41], and Crtap mouse [42]. In this study, osteogenesis imperfecta murine (oim) was chosen to determine the correlation of the defective collagen phase with the morphology of microdamage induced by fatigue in bone.

2. MATERIALS AND METHODS

2.1 Specimen Preparation

Two groups of mice (oim/+ and +/+) were used in this study (N=10 per group). The oim/+ mouse had a so-called mild type of osteogenesis imperfecta, whereas the +/+ mouse were wild-type controls of the same strain (C57BL/6J). All mice were obtained from Jackson Laboratories and housed in the Animal Core of University of Texas Health Science Center at San Antonio. At the age of 5 months, animals were sacrificed by CO₂ suffocation. Their femurs and tibias were dissected, cleaned of soft tissue and stored in PBS-wrapped gauze at -20°C until testing. All animal procedures were approved by IACUC at both the University of Texas Health Science Center at San Antonio and the University of Texas at San Antonio.

2.2 Mechanical Testing

Monotonic testing was first conducted until failure on each mouse tibia using a four-point bending fixture on an electromagnetic mechanical testing system (Bose ELF 3300, Bose Corporation, Minnetonka, MN). The four-point bending fixture had a span length of 3.0 mm between loading points and 6.5 mm between supporting points. The anterior side of mouse tibias was loaded in compression and the posterior side in tension, respectively. The monotonic test was conducted under the displacement control with a rate of 0.005 mm/s at room temperature. Bone samples remained hydrated with a dripping PBS solution during the testing.

Fatigue tests were then performed on the mouse femurs in the same four-point bending configuration at room temperature. Mouse femurs were fatigued under load control using a sinusoid waveform with a frequency of 2 Hz. The peak load of the fatigue waveform was set as 70% of predetermined yield load of mouse femurs and the minimum load as 20% of the yield load. The tests were stopped when reaching 40% degradation of the original stiffness [43]. The choice of this stopping point had ensured a considerable amount of microdamage generation without creating an event of catastrophic failure. All samples were tested under wet conditions with a dripping irrigation system. After the tests, the mid-diaphyseal portion of the femurs was placed in 70% ethanol alcohol for later microdamage morphology analysis.

2.3 Histological Analysis

The mid-diaphysis of mouse femurs were bulk stained with basic fuchsin following an established staining protocol [44]. After staining, bone specimens are re-hydrated to facilitate the sectioning procedure since the hydrated bone samples are less brittle than in alcohol [45, 46]. Then, the bone sections were embedded, cut perpendicularly to the long axis of bones and polished to 100 μ m thick slices. Fluorescence images of the bone slices were taken under a fluorescence microscope with a maximum excitation wavelength of 560nm and a peak emission wavelength of 610nm. The linear crack density (Cr.Dn: total number of cracks per unit bone area), microcrack length density (Cr. Ln: total length of cracks per unit bone area) and diffuse damage density (Df.Dm.Ar: total diffuse damage area per unit bone area) were measured using a public domain program ImageJ (National Institutes of Health) on both tension and compression sites of the specimens.

2.4 Geometrical Analysis

Geometrical dimensions of wild-type and mild-type mouse bones were quantified and compared in this study. Both femoral and tibial lengths were measured using a digital caliper. The cross-section images of diaphyseal femurs from the two animal groups were examined using a public domain program ImageJ to detect any geometric differences between the wild-type and mild-type mice, such as the cortical thickness, periosteal diameter, and the moment of inertia along the medial-lateral plane, which was calculated with a custom MATLAB (MathWorks, Natick, MA) script.

2.4 Statistical Analysis

Student-*t* tests were performed to compare the differences in linear crack and diffuse damage densities between mild-type and wild-type mice to assess significant differences in terms of microdamage morphology induced by fatigue in bone. The differences in geometrical dimensions between the wild-type and mild-type mouse bones were also evaluated using student-*t* tests. The significance level was set at *p*-values less than 0.05.

3. RESULTS

3.1 Mechanical testing

Monotonic testing indicated that the toughness of bone (tibias) was significantly lower for mild-type mice than for wild-type mice (Figure 1a). From the load-displacement curve, the work-to-failure of bone from mild-type mice was about half the value of the wild-type controls due to the significantly reduced ultimate load and failure displacement (Figure 1b). The ultimate load of mild-type mouse tibias (21.8 $\pm$ 7.5 N) was significantly (*p* = 0.003) less than that of wild-type mouse tibias (32.2 $\pm$ 5.4 N). The failure displacement of mild-type mouse tibias (0.211 $\pm$ 0.067 mm) was significantly (*p* = 0.04) lower than that of wild-type mouse tibias (0.293 $\pm$ 0.095 mm).

The fatigue tests demonstrated that the femurs from mild-type mice had a significantly shorter fatigue life than that of wild-type mice (Figure 2a). It took the femurs of the mild-type mice 7,990 cycles to reach ~40% stiffness degradation, whereas the femurs from wild-type mice reached the same percentage of stiffness degradation by 19,170 cycles (Figure 2b).

3.2 Bone geometry

No significant differences were observed in the overall size and geometry of the femur from both wild-type and mild-type mice. Both femur and tibia lengths were not significantly different (*p*>0.80) between wild-type and mild-type mice (Table 1). The optical photographs of the mid-diaphyseal cross-section also showed a similar result (Figure 3). No significant differences

($p > 0.12$) were noted in the geometrical parameters, such as periosteal diameters and cortical thickness between wild-type and mild-type mouse femurs (Table 1). In addition, no significant difference ($p > 0.50$) was detected in the moment of inertia along the medial-lateral plane, which contributed to the mechanical properties of bones tested under the bending mode (Table 1). These results suggest that the genetic mutation in collagen in the mild-type mice did not significantly affect the size and shape of bone.

3.3 Microdamage morphology

Both linear microcrack and diffuse damage were observed in femurs of both mild-type and wild-type mice (Figure 4). Linear microcracks were defined as damage that was stained as individual lines, whereas diffuse damage was defined as areas of patches with augmented basic fuchsin staining. The histomorphometry analysis indicated that more linear cracks were formed in the compression region than in the tension regions of the specimens irrespective of mice groups ($p < 0.01$). However, there was no significant difference in the linear crack density between the two animal groups ($p = 0.72$) (Tables 2 and 3). On the other hand, less diffuse damage ($p = 0.005$) was observed in the tensile region of the mild-type control specimens compared with the wild-type ones (Tables 2 and 3).

4. DISCUSSION

It has been reported that the size and mechanical properties of collagen fibrils may have a significant effect on the microdamage progression in bone. The numerical simulation of bone mineral-collagen composite in the previous study predicts that the smaller diameter and weaker strength of collagen fibrils would inhibit diffuse damage formation in bone [38]. To verify the prediction, osteogenesis imperfecta (OI) mouse model was used in this study. The OI mutation is a single nucleotide deletion in *pro α 2(I)* collagen gene, COL1A2, resulting in a frame-shift altering the last 48 amino acids of the carboxyl terminal propeptide in *pro α 2(I)* collagen [39, 47]. Consequently, OI mice do not synthesize functional *pro α 2(I)* collagen chains. This defect not only causes deleterious changes in collagen fibrillar structure [48], but also leads to a smaller fibril diameter [49] and the reduced collagen content (by 20%) in bone [50].

The results of this study indicate that the mild OI mouse bone demonstrates distinct microdamage morphology in tension compared with that of controls. The mild-type mice mainly produce linear microcracks in bone with limited diffuse damage formed in the tensile side, whereas the wild-type controls exhibit a significantly higher capacity of producing diffuse damage in addition to a similar amount of linear microcracks compared to that of mild-type ones. Since the mild-type mice possess defective collagen fibrils compared with the wild-type controls, the above experimental observations verify the previous prediction by the probabilistic failure model of bone mineral-collagen composite that reduced amount/size and weakened collagen fibrils facilitate the formation of linear microcracks and inhibit the formation of diffuse damages. It is noteworthy that the only difference in microdamage morphology between the wild-type and mild type mice bones is that the wild-type mouse bone form more diffuse damage (Tables 2 and 3), which coincides with the higher toughness of the wild-type mouse bone. This result suggests that diffuse damage can dissipate more energy than linear microcracks.

OI may also result in other ultrastructural changes that may contribute to bone brittleness, including enhanced local mineralization, disorganized collagen fibrils and mineral crystals, altered crystal size, Ca-P ratio and crystallinity of minerals [49-51,52]. For instance, enhanced degree of mineralization has been observed in oim mouse bones using Fourier transform infrared spectroscopy (FTIR) and quantitative backscattered electron image (qBEI) techniques [52,53]. Higher mineralization would also inhibit diffuse damage formation but facilitate formation of linear microcracks based on the numerical prediction using the aforementioned

probabilistic model. In addition, disorganization of collagen fibrils and mineral crystals in oim mouse bone may prohibit the collagen/apatite debonding along a preferential orientation [54, 55], thus suppressing the formation of diffuse damage in bone.

It has been reported that there are almost no visual differences between mild-type and wild-type mice [56], but the quality and biomechanical properties of bone in mild-type mice is significantly reduced [50,56,57]. The results of this study have again confirmed the observation.

It is also noted from the results of this study that diffuse damage are most likely induced by tension, whereas the formation of linear microcracks are favored more in compression regions. This is in good agreement with those reported in the previous studies [12,58]. By testing human cortical bone, Ebacher et al. reported that diffuse damage generally oriented transversely to the longitudinal tensile stress, whereas crosshatching cracks are long and straight but oblique to the loading axis [59]. Several other investigators also reported similar observations [11, 12].

There are some limitations for this study. First, power analysis indicated that the sample size of 10 mice per group yielded a power of 0.71 when quantifying the diffuse damage at the tensile region of mouse femurs. However, additional specimens (N=17 per group) may be needed in the future study to achieve a power of 0.90, resulting in more reasonable conclusions. Second, reduced thickness of collagen fibrils is not the only changes in the ultrastructural components of osteogenesis imperfecta mice. Additional changes include collagen and calcification disorganization, and reduced calcified needles. Consequently, the prediction of microdamage dependence on ultrastructural properties from the previous study [38] is insufficient for the OI model and may be extended to account for these additional ultrastructural changes.

5. CONCLUSIONS

Adverse alterations in collagen fibrils (reduced thickness and disorganization) and mineral crystals (reduced calcified needles and calcification disorganization) observed in osteogenesis imperfecta mice may result in the reduced capability to form diffuse damage in bone, thereby leading to more brittle behavior of the tissue.

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(a)

(b)

Figure 1.

(a) Comparison of work-to-failure of monotonic tests between tibial bones of mild-type of osteogenesis imperfecta and wild-type mice; (b) Typical load-displacement curves of four-point bending tests for mild-type and wild-type mice tibias.

Figure 2. (a) Comparison of fatigue life between femurs of mild-type and wild-type mice; (b) Typical modulus degradation curves of femoral bones of mild-type and wild-type mice.

Figure 3.
Comparison of cross sections of mice femurs. (a) wild type; (b) mild type.

Figure 4. Microdamage morphology of mice femoral bone from fatigue tests. (a) diffuse damage; (b) linear microcrack.

Table 1

Comparison of geometrical parameters of wild-type and mild-type mouse bones.

	Wild-type (n=10)	Mild-type (n=10)	p-value
ML diameter (mm)	1.26 ± 0.13	1.27 ± 0.09	0.80
AP diameter (mm)	1.27 ± 0.09	0.92 ± 0.04	0.63
Cortical thickness (mm)	0.19 ± 0.04	0.17 ± 0.02	0.12
Moment of inertia (mm ⁴)	0.039 ± 0.009	0.036 ± 0.007	0.50
Femur Length (mm)	16.28 ± 0.74	15.78 ± 0.96	0.25
Tibia Length (mm)	18.05 ± 1.21	17.99 ± 1.08	0.91

Table 2

Microdamage quantification at the tensile region of mouse femurs

Mice	Df.Dm.Ar	Cr. Dn	Cr. Ln	B.Ar
Mild type	0.012 ± 0.027	8.61 ± 4.94	0.39±0.29	0.22±0.032
Wild type	0.077 ± 0.084*	9.38 ± 9.84	0.44±0.40	0.24±0.030

Df.Dm.Ar is diffuse damage area per unit bone area (mm^2/mm^2); Cr.Dn. is the number of microcracks per unit bone area ($\#/\text{mm}^2$); Cr. Ln is the length of microcracks per unit bone area mm/mm^2 ; B.Ar is the bone area under consideration (mm^2).

* significant differences ($p<0.05$) between mild-type and wild-type

Table 3

Microdamage quantification at the compression side of mouse femurs

Mice	Df. Dm. Ar	Cr. Dn	Cr. Ln	B.Ar
Mild type	0.017 ± 0.027	18.61 ± 11.96	1.38 ± 0.97	0.25 ± 0.029
Wild type	0.034 ± 0.038	18.06 ± 10.38	1.04 ± 0.76	0.25 ± 0.031

Df.Dm.Ar is diffuse damage area per unit bone area (mm^2/mm^2); Cr.Dn. is the number of microcracks per unit bone area ($\#/\text{mm}^2$); Cr. Ln is the length of microcracks per unit bone area (mm/mm^2); B.Ar is the bone area under consideration (mm^2).