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The effect of low fluoride concentrations on microdamage accumulation in mouse tibias under impact loading

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Abstract Microdamage accumulation in bone is one of the mechanisms for energy dissipation during the fracture process. Changes in the ultrastructure and composition of bone constituents due to aging or diseases could affect microdamage accumulation. Low concentration (1 mM) of sodium fluoride (NaF) has been used in this study to investigate the effect of ultrastructural changes on microdamage accumulation in mouse tibias following free-fall impact loadings. Twenty-two tibias were divided randomly into control and NaF-treated groups. Free-fall impact loading was conducted twice on each tibia to produce microdamage. The elastic modulus of NaF-treated tibias decreased significantly after the impact loadings, while there was no significant difference in the modulus of untreated samples between pre- and post-damage loadings. Microdamage morphology analysis showed that less and shorter microcracks existed in NaF-treated tibias compared with control bones. Meanwhile, more and longer microcracks were observed in tensile regions in untreated samples compared with that in compressive regions, whereas no significant difference was observed between tensile and compressive regions in NaF-treated bones. The results of this study indicate that more energy is required to generate microcracks in NaF-treated bone than in normal bone. A low concentration of fluoride treatment may increase the toughness of bone under impact loading.

1 Introduction

Bone is a natural hierarchical composite comprised of mineral (hydroxyapatite, HA), proteins (mainly type I collagen), and water [1]. The ultrastructural and compositional changes of bone due to aging or diseases can affect the mechanical behavior or the quality of the tissue [2,3]. According to a National Institutes of Health (NIH) consensus [4], bone quality is determined by multiple factors, such as turnover, mineralization, and microdamage accumulation. Among them, microdamage accumulation serves as a mechanism for energy dissipation in bone [5–9]. Two major types of microdamage have been observed in human bone: linear microcrack and diffuse damage [10–12]. Several previous studies have investigated the contribution of ultrastructural and material properties of bone constituents to microdamage accumulation in bone [13–16]. For example, the mineralization level of bone as well as the mutation of collagen can alter the pattern and capacity of microdamage accumulation in bone [15,16]. However, very limited information is available regarding the contribution of mineral-collagen interaction to microdamage accumulation in bone [17].

Fluoride treatment ex vivo has been proposed to change the interfacial bonding between mineral and collagen in bone [18–20]. Soaking tibial cortical bone in sodium fluoride (NaF, ~1 M), lessens microcracks as compared to normal bone after compression testing [21]. However, experimental studies

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have demonstrated that higher levels of fluoride concentration ($\geqslant 0.145\,M$) cause partial dissolution of bone mineral and reprecipitation of calcium fluoride (CaF_2) and some fluorapatite (FAp)/fluorahydroxyapatite (FHAp) type material [22,23], while lower concentrations ($\leqslant 0.001\,M$) induce an exchange of fluoride with hydroxyl ion in bone mineral surfaces without dissolution of the apatite [24,25]. Therefore, fluoride treatment at a low concentration offers a better tool to study the effect of changes at mineral-collagen interface in bone.

So far, microdamage accumulation in bone after bending or torsion fatigue tests has been well studied [10,26]. But microdamage accumulation in bone induced by impact loadings is still poorly understood. Gymnasts, basketball players, and soldiers who experience more impact loads are prone to stress fracture rather than fatigue fracture. Besides, more than 90 % of lower limb fractures are associated with falls [27], and one of the risk factors for fracture following a fall is the history of past falls [28]. Therefore, the goal of this work is to study the effect of fluoride treatment ex vivo at a low concentration on microdamage accumulation in bone after impact loading.

2 Materials and methods

2.1 Sample preparation

Twenty-two unpaired tibias were obtained from 16-week old female C57BL/6H (B6) mice (Peking University Health Science Center, Beijing, China). The procedures in this study were approved by the Peking University Institutional Animal Care and Use Committee. Bone samples were wrapped in 0.9 % NaCl soaked gauze and kept frozen at $-20\,°C$ until

further treatment. All tibias were divided randomly into a control group ($n=11$) and an experimental group ($n=11$). Mechanical tests were performed immediately after thawing of the tibias from the control group. The samples from the experimental group were treated with 1 mM non-buffered NaF using the protocol proposed by Walsh and Guzelsu [18]. The protocol involved an initial 24-h treatment with a detergent solution (0.1 % Nonidet P40 (NP-40)) to remove organic barriers for ion exchange, followed by 72 h in the fluoride solution. Each tibia was placed in a 2 ml vial filled with the treatment solutions under constant shaking at room temperature. After the treatment, mechanical testing was conducted immediately.

2.2 Mechanical testing

A drop-tower system was designed for the free-fall impact experiment (Fig. 1). An aluminum cylinder (2.76 g of weight) was used as the impact mass for free-fall. A plastic pipe (40 cm of height) was set up as the channel of falling. The loading tap was made of polymethylmethacrylate (PMMA), which had an elastic modulus of 18.6 GPa. The proximal end of tibia was glued to a steel plate, and the distal end was contacted with the loading tap. Each sample was conducted impact loading twice to produce enough microdamage while not breaking the bone.

The force-displacement curve was acquired before and after impact loadings by an electronic universal testing machine system (Shimadzu Autograph AGS-X 1 kN) under compression load control with 20 mN/s of loading rate and 8 N of maximum load. Assuming the tibia was a cantilever beam of "s" shape suffering off-axial compression (Fig. 2), the equation for elastic modulus was
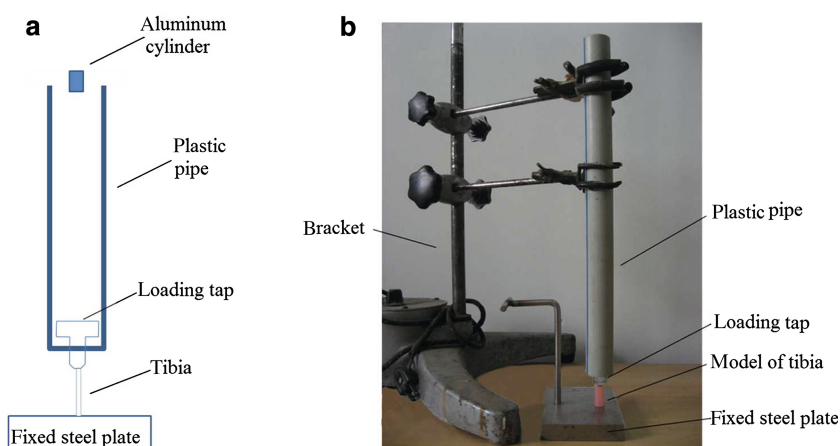

Fig. 1 The self-designed drop-tower system. An aluminum cylinder, which was used as the impact mass, fell along the plastic pipe. The impact loading was applied to the distal end of the tibia through a loading tap. The proximal end of the tibia was fixed on a steel plate
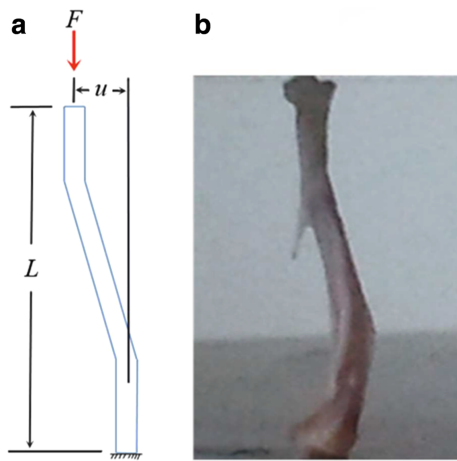
Fig. 2 A simplified model **a** was used to calculate the elastic modulus of mouse tibia **b**. Assuming the tibia was a cantilever beam of "s" shape suffering off-axial compression, the elastic modulus can be calculated using Eq. (1)

$$E^2 = (F^2/d)[u^2 L^3/(6I^2)]. \tag{1}$$

Here, F is the loading force; d is the displacement; u is the off-axial distance; L is the length of the tibia; I is the moment of inertia.

For simplicity, the cross-sectional shape of each tibia was assumed to be the same along its long axis. Then, the moment of inertia for each tibia was measured by point counting techniques [29] using the cross-sectional image obtained in the microdamage morphology analysis. By regression of the force-displacement data, the elastic modulus of each tibia was then determined.

All samples were tested under wet conditions with an irrigation system. After mechanical tests, the mid-diaphyseal portion of the tibia was then preserved in 70 % ethanol for at least 48 h before microdamage morphology analysis.

2.3 Microdamage morphology analysis

The tested tibias were bulk-stained with 1 % basic fuchsin (J.T. Baker, Phillipsburg, NJ, USA) in a graded series of alcohols (80 %, 90 % and 100 %) under a 20-psi vacuum [30]. Samples were stained for 2 h in each step, followed by rinsing in 100 % alcohol to remove the excessive stain. After that, the stained samples were then embedded in PMMA, and five cross-sections with 0.5 mm apart were cut from the middle portion of each sample using a low-speed diamond saw (Buehler LTD, Evanston, IL, USA). The obtained slices were then ground and polished by hand to 120 μm.

The polished slice was observed and photographed using a fully automated upright microscope system (Leica DM6000B, Leica Microsystems Ltd., Beijing, China) at 200 magnification under green incident light (the range of wave-

length was 515 and 560 nm). To screen out partially-stained artefactual cracks, the same slice was also photographed under ultraviolet (UV) incident light (the range of wave-length was 355 and 425 nm) [31]. The crack density (Cr.Dn = total number of microcracks/bone cross-sectional area, #/mm^2), crack surface density (Cr.S.Dn = total length of microcracks/bone cross-sectional area, mm/mm^2), and delamination density (Dl.Dn = total number of inter-lamellar cracks/bone cross-sectional area, #/mm^2) were measured using ImageJ software package. The regions of diffusely basic fuchsin staining were identified as diffuse damage areas [10]. The number of linear microcracks was obtained by counting the microcracks with a length less than 200 μm, while the amount of delamination was acquired by counting the cracks with a length greater than 200 μm [16]. The data for the five slices obtained from the same sample were averaged for each tibia. As impact loading was conducted on the whole tibia, the tibia of curved shape suffered an off-axis load, leading to tensile and compressive regions in the cross-section [32]. The crack density, crack surface density, and delamination density were also calculated in both tensile and compressive regions.

2.4 Statistical analysis

F tests were employed firstly to compare the variances between the control group and the NaF-treated group. Then, differences in microdamage morphology and elastic modulus between the two groups were evaluated using Student t tests. Paired t tests were employed to compare the differences of elastic modulus in each group measured before and after impact loadings. A significant difference was considered when the p value was less than 0.05.

3 Results

Two untreated bones were broken in the second free-fall impact loading while none of the NaF-treated tibias were broken. The broken bones had been excluded from the control group before further analysis. Therefore, the sample number of the control group for statistical analysis was nine, while the number of NaF-treated group was still 11.

The typical load versus displacement curves for the two groups before and after impact loadings were shown in Fig. 3. The elastic modulus was calculated by regression of the whole data using Eq. (1). Statistical analysis revealed that elastic modulus of bone in the control group before impact was not different from that in the control group after impact loadings ($p = 0.383$). However, after free-fall impact loadings, the experimental group had significantly lower values of elastic modulus than before ($p = 0.022$). There were no significant differences observed between the control group
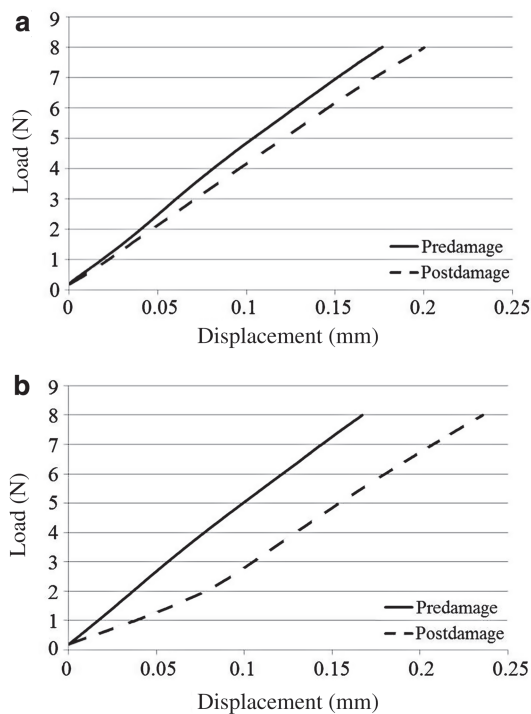
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Fig. 3 The typical load versus displacement curves from the control group **a** and the NaF-treated group **b** before (*solid lines*) and after (*dashed lines*) impact loadings

Fig. 4 Elastic moduli in the control group and the NaF-treated group before (*black columns*) and after (*gray columns*) free-fall impact loadings. Star denotes there exists a significant difference (<0.05). (*Error bars* represent standard deviations)

icantly higher than those from the NaF-treated group, while inter-lamellar cracks were not significantly different between the two groups (Table 1).

and the NaF-treated group either before ($p = 0.136$) or after ($p = 0.767$) impact loadings (Fig. 4).

Diffuse damage, linear microcracks, and inter-lamellar cracks were detected in the cross-sections of tibias in this study (Figs. 5, 6). Diffuse damage was defined as a cluster of feather-like patches perpendicular to the periosteal surface. Linear microcracks were defined as damage that was stained as individual lines. Inter-lamellar cracks were long delaminating cracks parallel with the endosteal surface (Figs. 5, 6). The partially-stained artefactual cracks can be screened out under UV incident light (Fig. 6b, c). As diffuse damage was observed only in a few slices, the histomorphometry analysis of diffuse damage was excluded from this study.

The tensile and compressive regions in the cross-section of each tibia were determined according to the loading mode and simulation results [32]. The crack density, crack surface density, and delamination density were compared between the two groups among both tensile and compressive regions. In the control group, the crack density and crack surface density in compressive regions were significantly different with those in tensile regions, whereas the delamination density was not significantly different between the two loading regions. However, the number and the length of linear microcracks were not significantly different between tensile and compressive regions in the NaF-treated group (Table 1). On the other hand, the crack density and crack surface density in the whole cross-section from the control group were signif-
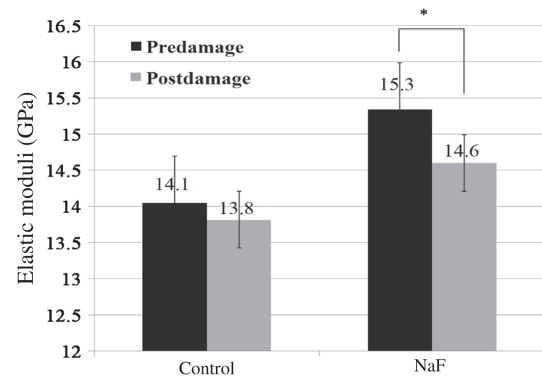
4 Discussion

The effects of low concentrations of NaF treatment ex vivo on microdamage accumulation in mouse tibias under impact loadings have been investigated in this study. Fluoride treatment has been frequently used to investigate the influence of ultrastructural changes on the mechanical properties of bone [18–22,33,34]. Numerous studies have demonstrated that fluoride ions interact with HA in different ways depending on the concentration and other factors [23,25]. At the concentration level of 1 mM, fluoride ions can exchange with hydroxyl groups to generate a monolayer of FHAp or FAp without dissolution of the mineral phase [24,25]. In addition, the treatment protocol used in this study has been shown not to denature the collagen phase [18]. Therefore, NaF treatment with low concentration is an ideal model to study the contribution of mineral-collagen interaction to microdamage accumulation in bone.

Microdamage can be accumulated in bone even in normal daily activities. However, the load conditions in daily activities are different from impact loading. The time span for an impact loading in this study is less than 0.1 ms, which is much less than any qusi-static or fatigue loading. As the collagen component of bone is a visco-elastic material, its response to impact loading should differ from that to other loadings. In addition, the average impact force in this study was 96.0 N, which was far beyond the breaking force of mouse tibia under the monocompressive test (26.8 N). Therefore, it is important to study microdamage accumulation in bone induced by falls.
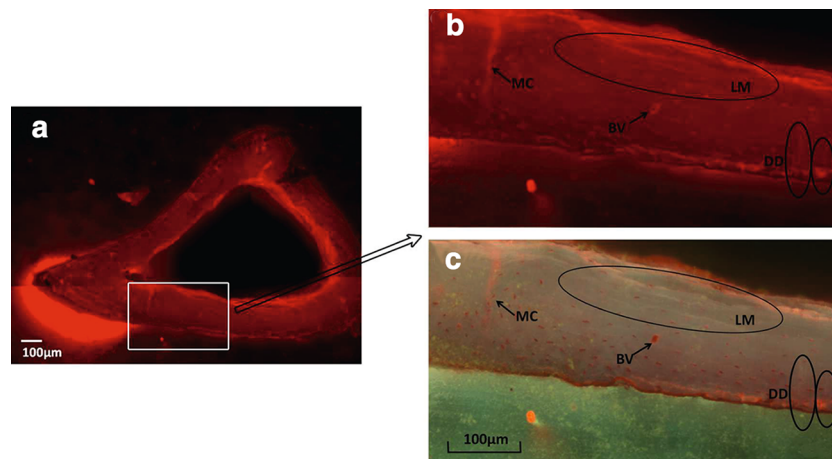
Fig. 5 a Bulk-stained cross-section of tibia in the control group viewed under green incident light. **b** Microdamage accumulation was observed under green incident light, and **c** UV incident light at 200 magnification. DD: diffuse damage; MC: microcrack; LM: inter-lamellar cracks; BV: blood vessel
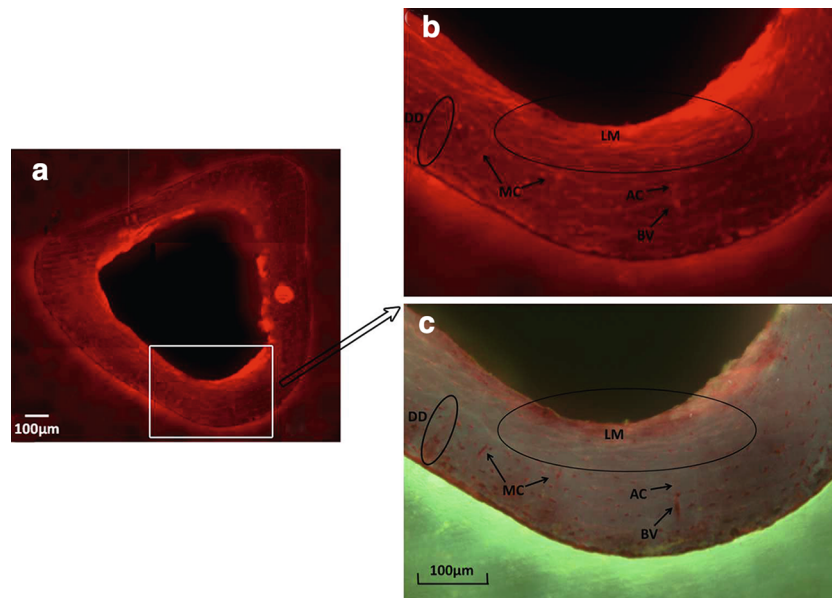

Fig. 6 a Bulk-stained cross-section of tibia in the NaF-treated group viewed under green incident light. **b** Microdamage accumulation was observed under green incident light, and **c** UV incident light at 200 magnification. Partially-stained artefactual crack was screened out under UV incident light (c). DD: diffuse damage; MC: microcrack; LM: inter-lamellar cracks; BV: blood vessel

Table 1 Microdamage quantification of mouse tibial bones mean (standard deviation)

		Cr.Dn (#/mm^2)	Cr.Ln (mm/mm^2)	Dl.Dn (#/mm^2)
Compression	Control	5.82 (1.30)[a]	0.275 (0.037)[a,c]	9.61 (4.38)
	NaF	4.99 (2.06)	0.197 (0.090)[a]	11.44 (4.57)[a]
Tension	Control	8.84 (2.42)[a,b]	0.422 (0.126)[b,c]	9.57 (5.15)
	NaF	5.03 (1.93)[b]	0.209 (0.083)[b]	6.58 (1.59)[a]
Sum (C+T)	Control	14.7 (3.33)[c]	0.697 (0.150)[d]	19.2 (6.19)
	NaF	10.1 (3.41)[c]	0.406 (0.161)[d]	18.0 (4.97)

Cr.Dn is the total number of linear microcracks per unit cross-sectional area (#/mm^2). Cr.Ln is the total length of linear microcracks per unit of cross-sectional area (mm/mm^2). Dl.Dn is the total number of inter-lamellar cracks per unit of cross-sectional area (#/mm^2). Groups with the same superscript letter are significantly different ($p < 0.05$)

Before free-fall impact loadings, the elastic modulus of NaF-treated tibias is not significantly different from that of normal tibias (Fig. 4), which is not consistent with other observations [21,22,34]. This discrepancy can be attributed to differences in the fluoride concentrations. The level of fluoride concentrations used in other studies was high enough to change the structurally effective mineral content of bone by partial dissolution of bone mineral and reprecipitation into calcium fluoride (CaF_2) [22,34]. However, it has been demonstrated that at the concentration level of this study, the mineral phase in bone was not dissolved [24,25]. As the mineral phase imparts stiffness to bone, it is reasonable that the modulus of NaF-treated tibias in this study is not changed significantly. A similar result has been observed in a previous study in which the modulus of femurs did not increase significantly following 1 mM fluoride treatment [17]. Therefore, the elastic modulus of cortical bone is supposed not to increase after fluoride treatment with low concentrations.

On the other hand, the responses of NaF-treated tibias to impact loadings are distinct from those of untreated tibias. The elastic modulus of treated tibias after impact loadings is significantly lower than before impact loadings, whereas the modulus of control samples does not change significantly before and after free-fall impact loadings (Fig. 4). This may be ascribed to the changes of mineral-collagen interaction in bone after NaF treatment. Fluoride-induced mineral-collagen detachment has been observed in another study from the fracture surface [33]. The weakened mineral-collagen interaction may be accounted for by the increase of modulus loss. This may imply that fluoride-treated bone is more sensitive to microdamage compared with normal bone. However, the underlying mechanisms need more study.

Microdamage morphology analysis has shown that less and shorter linear microcracks in the whole cross-section have been observed in NaF-treated tibias than in control samples (Table 1). These observations are consistent with others, which reported that bone treated with 1 M buffered NaF had less microcracks than the normal bone under compression via scanning electron microscopy (SEM) [21]. It has been well accepted that during the post-yielding of bone, microdamage accumulation serves as a pathway for energy dissipation. In this study, the degrees of impact energy imposed on the control group and the NaF-treated group were the same. Therefore, less microdamage in a NaF-treated tibia implies that more energy is required to generate microdamage in NaF-treated bone than in normal bone. In addition, the toughness of bone appears to have an inverse correlation with the level of microdamage existing in bone [6,9]. Less microdamage in NaF-treated tibia suggests that more energy is needed to break NaF-treated bone compared to normal bone after the two impact loadings. It could be confirmed by our experimental observations in which two untreated bones were broken

in the second free-fall impact loading while none of the NaF-treated tibias was broken. The results of this study suggest that low concentrations of NaF treatment will increase the toughness of bone under impact loading conditions. A similar conclusion has been obtained in another study in which a significant increase in toughness has also been observed at higher concentrations of fluoride (2.0 M) under tensile testing [22].

At the nanostructural level, a model for bone material failure has been proposed: interfacial sliding between two components is followed by rupture of collagen crosslinks [35]. In addition to microdefects formation, interfacial sliding is also responsible for energy dissipation during the damage process. Compared with HA, the monolayer of FHAp or FAp induced by fluoride treatment in this study has been proved to have an enhanced affinity for anions (carried by collagen) due to the increased surface charge density [36,37]. Therefore, it is reasonable to speculate that interfacial sliding in NaF-treated bone can dissipate more energy than in normal bone. The collagen phase was not denatured by the treatment in this study [18]. The energy for rupture of collagen crosslinks or breakage of collagen fibils in NaF-treated bone is then considered to be similar with that in normal bone. Accordingly, it can be assumed that more energy is required to generate detectable microdefects in NaF-treated bone than in normal bone.

The differences of microdamage accumulation in tensile and compressive regions in each group indicate that the mechanisms of microcracking are also different between treated and untreated bones. In NaF-treated tibias, the number and the length of linear microcracks are not significantly different between tensile regions and compressive regions. But the inter-lamellar crack density in tensile regions of NaF-treated bones is significantly lower than that in compressive regions. On the other hand, more and longer linear microcracks are observed in tensile regions compared with compressive regions within the control group. Moreover, statistically less and shorter linear microcracks are found in NaF-treated tibias than in untreated tibias in tensile regions. It has been well established that linear microcracks are commonly observed in compressive regions rather than in tensile regions [11,13,16,38,39]. More linear microcracks in tensile regions would result in a greater toughness loss [38]. There has been a demonstration by Reilly and Currey that experimentally more compression damage (i.e., linear microcracks in this study) in tensile regions will reduce impact strength of tibias [39]. Therefore, based on the observations in this study, it can be speculated that fluoride treatment with low concentrations could improve the bones' energy-absorbing ability on impact by inhibiting the compression damage forming in the tensile region.

Unlike normal daily activities, the stress in bone during impact loading is larger than the yield stress. It has been

demonstrated that diffuse damage occurs at low stresses while linear microcracks first form at higher stresses [10]. This may be an explanation for the observation in this study that diffuse damage only existed in a few slices. Once the linear microcracks formed, they have a tendency to grow into long cracks that are likely to lead to fracture of bone [10]. Thereby, the strategy for enhancing bone toughness of gymnasts, basketball players, and soldiers, who are prone to stress fracture due to multiple impact loads, is to inhibit the propagation of microcracks. The observations of this study imply that low-does fluoride therapy may be an effective method.

One limitation of this study is that there is no data in this work to prove the changes at mineral-collagen interface after fluoride treatment. Experimental evidence in other studies has demonstrated that the exchange of fluoride with hydroxyl ions of apatite gives rise to an FHAp type material at lower concentrations of NaF (<1 mM). But other factors, such as pH and time of immersion, can affect the ultrastructural response. However, the experimental design in this study has been tried in order to meet the conditions in other studies except for the fluoride concentrations. The second limitation is that the process of microcracking has not been monitored during the twice free-fall impact loadings. Using different fluorescent agents can differentiate microdamage initiation and propagation in each loading step [40,41]. Knowing the microdamage before and after impact loading will give us a better idea of how fluoride affects microdamage formation and propagation under impact loading. Lastly, more comparisons of mechanical properties, such as the deformation during impact process, work to failure, and ultimate strain, would enhance the speculations in this study. This would be for future work.

5 Conclusion

The results of this study have indicated that more energy is required to generate microdamage in NaF-treated bone than in normal bone. Low concentration of fluoride treatment is supposed to increase the toughness of bone under impact loading conditions. Less linear microcracks (compression damage) in tensile regions of NaF-treated bone suggest that fluoride treatment with low concentrations could improve the bones' energy-absorbing ability to impact by inhibiting the compression damage forming in the tensile region. However, NaF-treated bone is more sensitive to microdamage compared with normal bone potentially due to the weakened mineral-collagen interface in bone.

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