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Effects of Collagen Unwinding and Cleavage on the Mechanical Integrity of the Collagen Network in Bone

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Abstract. The objective of this study was to investigate how molecular level changes in the collagen network affect its mechanical integrity. Our hypothesis is that the cleavage and unwinding of triple helices of collagen molecules significantly reduce the mechanical integrity of the collagen network in bone, whereas collagen crosslinks play a major role in sustaining the structural integrity of the collagen network. To test this hypothesis, the collagen molecular structure was altered in demineralized human cadaveric bone samples in the following two ways: heat induced unwinding and pancreas elastase induced cleavage of collagen molecules. Along with control specimens, the treated specimens were mechanically tested in tension to determine their strength, elastic modulus, toughness, and strain to failure. Also, the percentage of denatured collagen molecules and amounts of two major collagen crosslinks (hydroxylysylpyridinoline and lysylpyridinoline) were determined using high-performance liquid chromatography techniques. It was found that unwinding of collagen molecules may cause more reduction in stiffness (E) but less strain to failure (ϵ_f) than cleavage. Both collagen denaturation types cause similar changes in the strength (σ_s) and work to fracture (W_f) of the collagen network with no significant changes in hydroxylysylpyridinoline and lysylpyridinoline crosslinks. The results of this study indicate that the integrity of collagen molecules significantly affect the mechanical properties of the collagen network in bone, and that collagen crosslinks may play an important role in maintaining the mechanical integrity of the collagen network after collagen denaturation occurs.

Key words: Bone — Collagen — Denaturation — Tensile properties — Demineralized bone

Bone is an integral living tissue comprising minerals, proteins, and water [1]. Previous studies have shown that mineral content contributes predominantly to the stiffness of bone [2, 3] whereas the quality of the collagen matrix plays a major role in sustaining the toughness of bone [4–11].

The collagen network in bone has a highly hierarchical structure [12]. Triple helical collagen molecules (such as collagen type I), stabilized by numerous hydrogen bonds, are aggregated together by stable trivalent bonds (mature crosslinks) to form microfibrils [13–15]. They are subsequently constructed into fibrils, super-fibrils, then finally a complex collagen network in bone [16]. In addition to causing abnormal metabolism in bone [17], changes in collagen molecules and crosslinks may affect the mechanical integrity of the collagen network, and subsequently lead to the altered bone strength and toughness [4, 9, 18]. For example, genetic defects in collagen molecules may lead to osteogenesis imperfecta, a brittle bone disease [19–21]. Heat-induced unraveling of collagen triple helices may significantly reduce bone mechanical integrity [22]. Also, collagen denaturation has been reported to relate significantly to the age-dependent decrease in bone toughness [10]. In addition, the enzymatic cleavage of amide links between amino acids in collagen chains may cause a set of changes in mechanical parameters of bone [23–26]. Moreover, a reduction in the concentrations of collagen hydroxypyridinium crosslinks has been reported to be associated with reduced bone strength [17, 27]. However, these studies provided merely qualitative information about the correlation between collagen and bone mechanical properties. To elucidate the mechanisms of collagen-related changes in bone quality it is necessary to understand how collagen molecules affect the mechanical integrity of the collagen network.

The objective of the present study was to investigate how cleavage and unwinding of collagen molecules affect the mechanical integrity of the collagen network in bone. Our hypothesis is that the cleavage and unwinding of triple helices of collagen molecules significantly reduce the mechanical integrity of the collagen network in bone, and collagen crosslinks play an important role

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in sustaining the structural integrity of the denatured collagen network. To test this hypothesis, the collagen molecular structure was altered in demineralized human cadaveric bone samples in the following two ways: heat induced unwinding and pancreas elastase induced cleavage of collagen molecules. Then, effects of such changes on the mechanical integrity of the collagen network were investigated.

Materials and Methods

Reagents

Guanidinium chloride, Tris, Brij 35, iodoacetamide, NaN_3, α-chymotrypsin, pepstatin-A, homoarginine, pyridoxine, and pancreas elastase (EC3.4.21.36) were purchased from Sigma (St. Louis, MO, USA); EDTA, ribose, and heptafluorobutyric acid were purchased from Aldrich (Milwaukee, WI, USA); Acetonitrile and methanol were purchased from Burdick & Jackson (Muskegen, MI, USA); AccQ-Fluor Reagent Kit was purchased from Waters Corp. (Milford, MA, USA).

Sample Preparation

Ten human cadaveric femurs were collected from six individuals with a mean age of 41 ± 15 years through the Musculoskeletal Transplant Foundation. All these donors were carefully screened for any abnormal pathologies of bone. From each femur, a 100 mm-long section of the mid-diaphysis was used for specimen preparation. A total of 12 specimens were extracted from the medial aspect of the same femur. Four of them (30 mm × 4 mm × 2 mm) for tensile test, four (7 mm × 4 mm × 2 mm) for collagen denaturation analysis, and the remaining four (7 mm × 4 mm × 2 mm) were used to measure the concentrations of collagen crosslinks. All specimens were immersed in a buffered 0.5 M EDTA solution, (0.1 M Tris, 0.02% NaN_3, PH7.4) at 4°C for 6–10 weeks until total removal of minerals. The EDTA solutions were changed every 4 days. The calcium content of EDTA extracts was detected using a flame colorimetry to ensure the completion of demineralization. After demineralization, all specimens were washed three times for 10 min in PBS, and then stored in a storing buffer (50 mM Tris, 0.2 M NaCl, 10 Mm $CaCl_2$, 0.05% Brij 35, and 0.02% NaN_3) at 4°C. The specimens were divided into three groups based on the pretest treatments: controls, heated treatment, and enzyme (pancreas elastase) treatment. The control specimens were stored in the storing buffer at 4°C without any treatment. Heat treatments were performed by incubating the specimens in the storing buffer at 70°C for 1 hour to induce unraveling of triple helices of collagen molecules [28, 29]. The enzyme treatment was conducted by immersing demineralized specimen in a solution containing 0.074 U pancreas elastase per mg demineralized specimens at 30°C in a water bath for 4 days. Then the specimens were washed in phosphorus-buffered saline solution (PBS) three times for 1 hour each and stored in the storing buffer at 4°C. Pancreas elastase is an endopeptidase that hydrolyzes peptide bonds on the C-terminal side of amino acids bearing uncharged non-aromatic side chains [30]. In fact, after the cleavage of peptide bonds of type I collagen, the triple helix may be sensitive to unwinding at 37°C. Since the incubations for enzymatic cleavage treatment were performed at 30°C in this study, the triple helical formation of cleaved molecules can still remain intact at this temperature. Therefore, such a treatment can specifically cleave certain amide links in the backbone chains of collagen molecules without interrupting their triple helical structures.

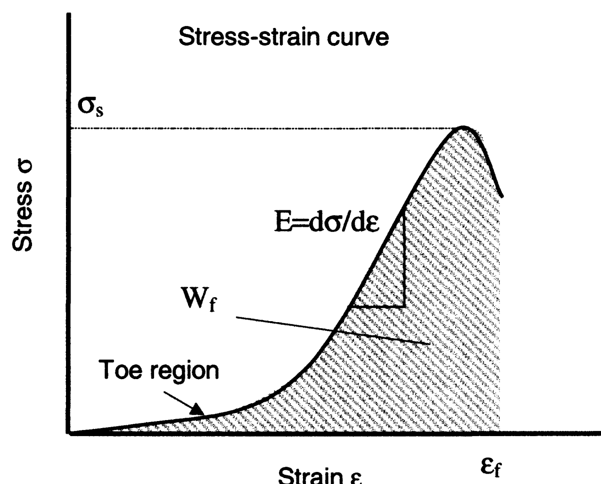

Fig. 1. Determination of mechanical properties from the stress-strain curve of demineralized bone specimens. E is the elastic modulus, $_f$ is the strain at failure, W_f is the work to fracture, and σ_s is the tensile strength of the specimen.

Tensile Test

Flat dumbbell-shaped specimens were prepared using a diamond saw and a bench top milling machine. Before demineralization, a 5 mm portion at both ends of the specimens were embedded in a plastic block to facilitate gripping of specimens. The overall length of the specimen was 30 mm. The gage length, width, and thickness were 10 mm, 24 mm, and 2 mm, respectively. The test specimens were mounted in an Instron mechanical test machine (Model 1011) with a loading rate of 10 mm/min until failure. The load-deformation curve was recorded using a computer-aided data acquisition system. Based on the curve, the elastic modulus (E), ultimate strength (σ_s), work to fracture (W_f), and strain to failure (ϵ_f) were calculated as shown in Figure 1.

Denatured Collagen Analysis

A selective digestion technique was used to determine the amount of denatured collagen molecules (as percentage of the total amount of collagen) [31]. The demineralized bone samples were digested at 37°C in a 500 μl incubation buffer (0.1 M Tris HCl, 1 mM Iodoacetamide, 1 mM EDTA, and 10 μg/ml pepstatin-A) containing 1 mg/ml α-chymotrypsin. The denatured and intact collagen can be separated by removing the supernatant (containing the denatured collagen) from the remaining insoluble matrix (containing the intact collagen). Then, the amounts of the denatured collagen (in the supernatant) and the intact collagen (in residue) were determined by measuring hydroxyproline in each of the two fractions using a AccQ · Tag Amino Acid Analysis Column, which is packed with 4 μm silica base bonded with C18 in a high performance liquid chromatography (HPLC) system (Waters Corp.).

Collagen Crosslinks Measurement

A simplified HPLC assay [32] was used to quantify the pyridinium crosslinks in the collagen network. Briefly, demineralized bone samples were hydrolyzed at 110°C furnace for 20 hours, then vacuum dried. The hydrolysate was dissolved in water containing 10 nmol pyridoxine/ml and 2.4 μmol homoarginine/ml as internal standards. After diluted fivefold in dilution solution (0.5% (v/v) heptafluorobutyric acid in 10% (v/v) acetonitrile), the sample was injected into the

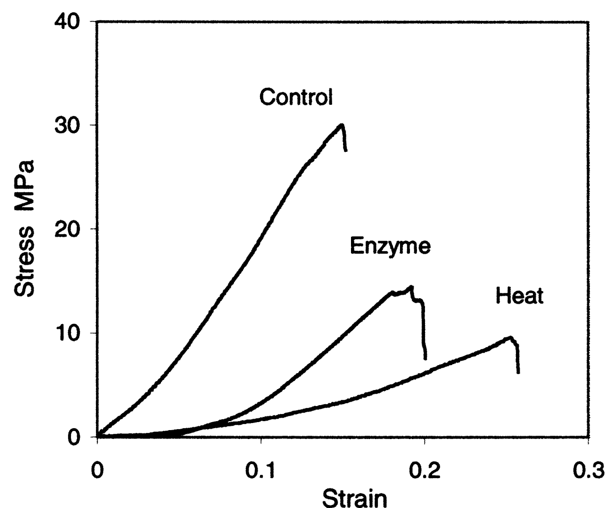

Fig. 2. Typical stress-strain curves of the collagen network with and without treatments. Collagen denaturation induced by heating and enzymatic cleavage makes the collagen network weaker, more compliant, and less tough. Compared with heat-treated specimens, the enzyme-treated one indicates a greater stiffness and less strain of failure.

HPLC system, and a two isocratic-step chromatography was run together with programmed fluorimeter. Finally, the concentrations of hydroxylysylpyridinoline and lysylpyridinoline crosslinks were normalized by the total amount of collagen as moles per mole collagen. Waters AccQ · Tag Amino Acid Analysis method was used to determine the amount of hydroxyproline (300 mol of hydroxyproline per mol of collagen molecules). The column for collagen crosslinks analysis was Waters S5 ODS2 Column, 4.6 mm × 150 mm, packed with 5 μm spherical silica particles with 80 Å pores.

Statistical Analysis

All experiment data were organized as mean ± standard deviation. Repeated measures analysis of variance (ANOVA) was performed to detect the effects of the different treatments on both the mechanical properties and biochemical properties of the collagen network. A post hoc test (Fisher's Protected Least Significant Difference) was conducted to compare results between the groups. Moreover, regression analyses were performed to explore the relationships between the molecular integrity and the mechanical properties of the collagen network. In all analyses, significance was considered only when $P < 0.05$.

Results

Significant differences were found in mechanical and biochemical parameters measured in this study between the treated groups and the control group. Figure 2 shows the typical stress-strain curves of the enzyme-treated, heat-treated, and control specimens. The stress-strain responses were significantly different between these specimens, showing that the heat-treated specimens had the longest toe region and least stiffness, followed by the enzyme-treated specimens, and finally the control specimens. In addition, the heat-treated group demonstrated greater strain to fracture but less stiffness than the enzyme-treated group.

Compared with controls, heat-treated specimens exhibited an almost 5× increase in the percentage of denatured collagen (%DC) as shown in Table 1. Correspondingly, significant changes in all mechanical properties were observed for the heat-treated specimens ($P < 0.05$): nearly threefold reduction in strength (σ_s), an approximate sevenfold reduction in modulus of elasticity (E), about a twofold reduction in work to fracture (W_f), and an almost 2.5-fold increase in the strain to failure (ϵ_f). However, no significant changes were observed in the two collagen crosslinks measured: hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).

The enzyme (pancreas elastase)-treated specimens showed similar changes in mechanical properties compared with the heat-treated specimens. Again, no significant changes were found in HP and LP collagen crosslinks compared with controls, indicating that pancreas elastase does not affect the collagen crosslinks. The *post hoc* multiple comparisons showed no significant differences in σ_s, E, W_f, %DC, and the two types of the collagen crosslinks (HP and LP) between the enzyme-treated and heat-treated specimens ($P > 0.05$). However, the enzyme-treated specimens exhibited less increment in the strain to fracture ($_f$) compared with the heat-treated specimens ($P < 0.05$).

The regression analyses demonstrated that the mechanical properties of the collagen network related significantly to the percentage of denatured collagen molecules (including both unwinding and cleavage), as shown in Figure 3. The tensile strength and elastic modulus of the collagen network dropped sharply (almost 3×) at about 20% collagen denaturation, but exhibited no significant changes thereafter. Also, the collagen network maintained structurally intact irrespective of collagen denaturation. However, the work to fracture decreased gradually with increasing collagen denaturation. Different from the other parameters, the strain to fracture of the collagen network increased with increasing collagen molecule denaturation.

Discussion

The results of this study support our hypothesis that cleavage and unwinding of triple helices of collagen molecules significantly change the mechanical integrity of the collagen network in bone, and it was observed that the collagen network can sustain its structural integrity even when large amounts of collagen molecules denaturation (unwinding or cleavage) occurs, suggesting that collagen crosslinks play an important role in keeping the collagen network structurally intact.

Table 1. Experimental data and ANOVA analyses ($n = 10$)

	σ_s (MPa)	E (MPa)	W_f (N/mm)	ϵ_f (%)	%DC	HP	LP
Control	29.4 ± 4.91	405 ± 129	1.13 ± 0.363	9.78 ± 1.25	7.86 ± 1.71	0.094 ± 0.012	0.052 ± 0.014
Heated	7.37 ± 2.10[a]	55.6 ± 19.8[a]	0.627 ± 0.170[a]	22.4 ± 3.29[a]	39.3 ± 5.08[a]	0.095 ± 0.014	0.052 ± 0.013
Enzyme	7.98 ± 3.58[a]	74.6 ± 32.7[a,b]	0.640 ± 0.336[a]	16.8 ± 4.26[a,b]	33.2 ± 13.0[a]	0.089 ± 0.018	0.047 ± 0.011
ANOVA	$P < 0.0001$	$P < 0.0001$	$P < 0.004$	$P < 0.0001$	$P < 0.0001$	$P > 0.05$	$P > 0.05$

σ_s = ultimate strength; E = elastic modulus; W_f = work to fracture; ϵ_f = strain to fracture; %DC = percentage of denatured collagen molecules; HP = hydroxylysylpyridinoline moles per mole collagen molecules; LP = lysylpyridinoline moles per mole collagen molecules

[a] Significantly different from controls

[b] Significantly different from the heated specimens

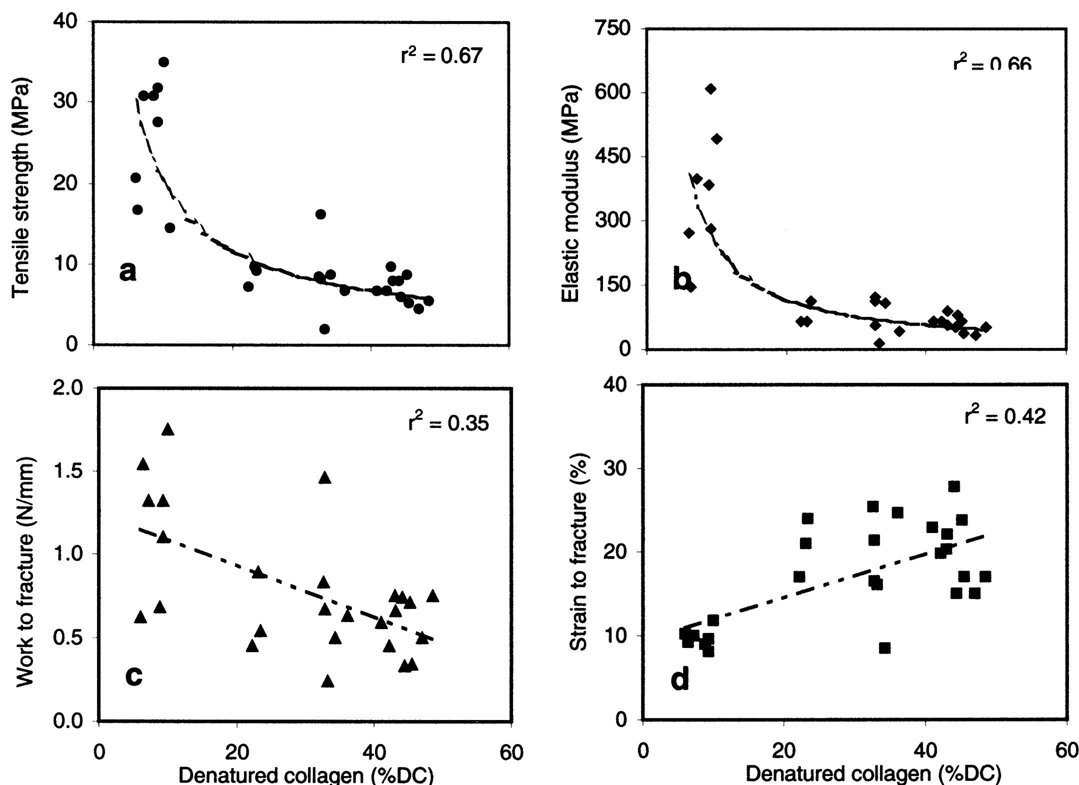

Fig. 3. Relationships of collagen denaturation with the mechanical properties of the collagen network: (**a**) tensile strength; (**b**) elastic modulus, (**c**) work to fracture, and (**d**) strain to fracture.

There are several limitations to the present study. First, only longitudinal mechanical properties of the collagen network were measured. Since bone is an anisotropic material with collagen fibrils being oriented in a slight angle with respect to the long axis of bone [34], changes in the mechanical integrity of the collagen network induced by the treatments performed in this study might be different in other orientations [35]. Secondly, due to the limited space between collagen molecules within the fibril, elastase may not be able to penetrate into the intrafibrillar water compartment. Thus, degradation of the fibrils can only start from the surface. Most likely, a significant part of the collagen molecules in the center of the fibrils remain unaffected. Both limitations may result in non-uniformity of material properties within the test specimen. In this study, such non-uniformity is not considered important, because only relative comparisons were performed between control and treated specimens.

The mechanical properties of the control specimens tested match with the results reported in the literature. Catanese et al. [36, 37] measured tensile properties of demineralized human cadaveric bones (femur, tibia, and humerus). They reported that the elastic modulus is between 275 ± 94 MPa and 450 ± 50 MPa, the ultimate stress varies between 15 ± 4.2 to 26 ± 4.7 MPa, and the strain to failure falls in the range of 8.4 ± 1.6%. In this study, the ultimate stress, tensile elastic modulus, and strain to fracture of demineralized human femurs were determined to be 29.4 ± 4.9 MPa, 405 ± 129 MPa, and 9.78 ± 1.25%, respectively, comparable with the data obtained by Catanese et al. However, using

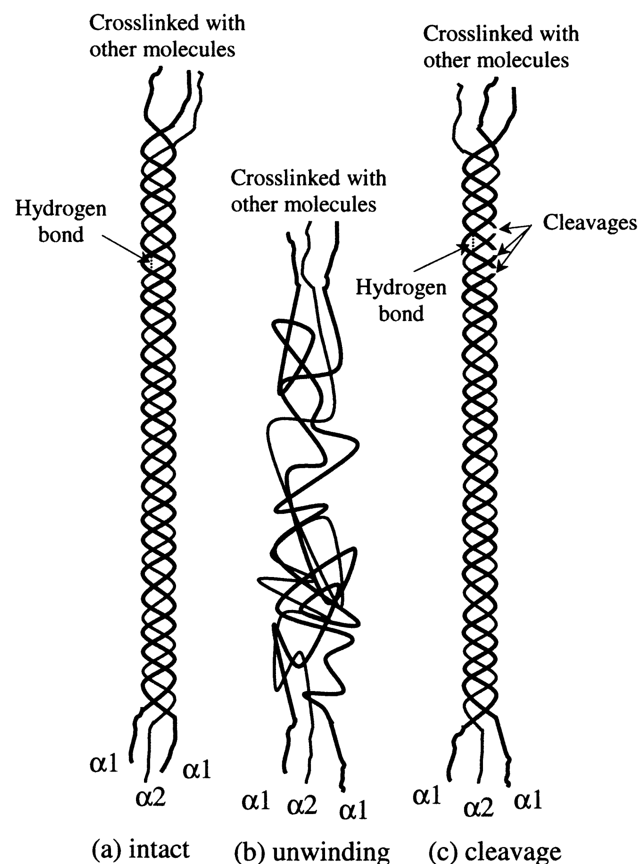

Fig. 4. Schematic representation of structural changes in collagen molecules under different treatments: (**a**) intact collagen molecule, (**b**) heating-induced unraveling, and (**c**) enzymatic cleavage of collagen chains.

bovine bone samples, Bowman et al. reported that the ultimate stress, average modulus, and ultimate strain of demineralized bone are 61.5 ± 13.1 MPa, 613 ± 113 MPa, and 12.3 ± 0.5%, respectively. These values are much greater than those of humans, indicating that the mechanical properties of the organic matrix of bovine bone may be different from those of human bone. In fact, a recent study by Catanese et al. [33] has demonstrated that the monotonic mechanical properties of demineralized bone may vary significantly due to the heterogeneity of bone tissues.

In this study, heating was used to induce unwinding of collagen molecules by breaking hydrogen bonds in the triple helices of collagen molecules [29, 31]. It has been known that type I collagen molecules have a triple helical structure formed by three α chains (Fig. 4a). If triple helices of collagen molecules are unraveled by breaking the hydrogen bonds between the chains, these molecules lose their rigidity to resist deformation (Fig. 4b). Such damages to collagen molecules consequently lead to the weakening of the collagen network, and finally the deterioration of bone quality [5, 6]. One of the manifestations of such a change in the collagen network is reflected in the elongated toe region of the stress-strain curve for heat treated specimens (Figure 2). Moreover, the mechanical properties of the collagen network significantly decrease with collagen denaturation, suggesting that the molecular integrity plays a predominant role in determining the strength; stiffness, and toughness of the collagen network. However, it is noteworthy that after heat treatment the specimens still maintain intact, suggesting that the unwinding of collagen molecules did not lead to a total disintegration of the collagen network. Thus, it is presumable that collagen crosslinks play a significant role in maintaining the collagen network intact by holding unraveled molecules together. In fact, these intermolecular collagen crosslinks are a major structural component of the collagen network and contribute to the mechanical integrity of bone [14].

Pancreas elastase is an enzyme that cleaves certain amide links in the polypeptide chains of collagen molecules. A recent study has reported that the elastase is able to cleave the triple helix of collagen molecule in fragments similar in size to those produced by mammalian collagenase, and that the clipping of collagen molecules by the elastase is limited to alanine sites in the triple helix of collagen [38]. Although the enzymatic cleavage can essentially cut chains of collagen molecules, the cleaved molecules may still remain the triple helical formation at 30°C, a incubation temperature used in this study (Fig. 4c). Thus, structural changes in collagen molecules induced by the enzymatic cleavage are obviously different from those by heating. However, similar changes in mechanical properties were observed for the pancreas elastase-treated specimens compared with the heat-treated ones. The significant differences between the two groups was the distinct length of toe region and the extent of strain to failure (ϵ_f), showing that ϵ_f increased about 100% in the heat-treated specimens, but only 50% in the pancreas elastase-treated specimens with respect to the controls (Table 1). Since the elastase cleavage of collagen molecules does not interrupt the triple helical structure of the collagen molecules, the hydrogen bonds present in the cleaved collagen molecule may still function in sustaining the stiffness of the collagen network. This may explain the differences in the length of the toe region in the stress-strain curve and the amount of strain-to-failure between the two groups.

The results of the present study indicate that the mechanical properties of the collagen network relate significantly with the extent of collagen denaturation for both unwinding and cleavage (Fig. 3). The tensile strength (σ_s) and elastic modulus (E) of the collagen network are very sensitive to the collagen denaturation, showing significant decreases as the amount of denatured collagen increases up to about 20%. However, it is noteworthy that no considerable changes are observed thereafter. This may suggest that collagen crosslinks begin to play a predominant role in withholding the remaining strength and stiffness of the collagen network

after collagen denaturation. However, the work-to-fracture (W_f) and strain-to-fracture (ϵ_f) of the collagen network do not show such a threshold as a function of collagen denaturation (Fig. 3). Considering the stress-strain behavior of the three types of demineralized bone specimens (Fig. 2), W_f and ϵ_f are most likely determined by the compliance of collagen molecules in the network, which is reflected by the elongated toe region in the stress-strain curve.

In summary, this study indicates that irrespective of differences in collagen denaturation caused by unwinding and cleavage, both mechanisms make the collagen network significantly weaker, more compliant, and less tough. Furthermore, the study demonstrates that collagen crosslinks may play an important role in sustaining the mechanical integrity of the collagen network when collagen denaturation reaches a certain extent.

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