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Original article

Effects of abnormal collagen crosslinks on hypersonic longitudinal wave velocity in bovine cortical bone

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Abstract

Abnormal collagen crosslinks such as advanced glycation end products (AGEs) in bone are known to reduce mechanical strength. Here, we have investigated the effects of glycation-induced AGE crosslinks on longitudinal wave velocity through cortical bone using a micro-Brillouin light-scattering technique (μ-BR). Given that this technique requires transparent specimens, extremely thin (< 100 μm in thickness) cortical bone specimens were fabricated from a bovine femur and used as reference and AGE specimens after incubation. Hypersonic longitudinal wave velocities in the GHz range decreased in all AGE specimens. The rates of velocity decrease were more than 5% in the bone axis direction and approximately 5% in tangential direction due to the incubation for two weeks. Because the specimens used in this study were extremely thin, we should next examine the velocity changes in thick *in vivo* bone.

KEY WORDS: Micro-Brillouin scattering, Advanced glycation end products, Collagen crosslinks, Cortical bone, Collagen film

Introduction

Bone has a complicated hierarchal structure, being primarily composed of collagen and minerals such as hydroxyapatite (HAp). The structure results in anisotropic and heterogeneous properties. The National Institutes of Health (NIH) consensus development panel has noted that the bone strength depends on not only bone mineral destiny (BMD) but also bone quality [1)] such as micro and macroscopic structure and material property which finally affects bone elasticity. Although reduction of BMD and deterioration of bone structure are known to lead to bone weakness and fracture, the diabetic disease and adynamic bone disease can actually weaken bones with no effect on bone structure or BMD stay normal [2,3)]. Such effects result in part from deterioration in the quality of collagen, which occupies 50% of the total volume of bone. One example is the abnormal collagen crosslinks, known as advanced glycation end product (AGE) crosslinks, are nonenzymatic crosslinks [4,5)]. Nonenzymatic crosslinks have detrimental effects on the mechanical and biological functions of bone. A typical AGE crosslink is pentosidine, which is formed by nonenzymatic glycation or oxidation reactions resulting in tissue maturation [6)]. The accumulation of pentosidine in bone is considered to exacerbate brittleness of collagen fibers and deteriorates the mechanical properties of bone, particularly post-yield properties and toughness [7,8)]. However, while accumulation of AGE crosslinks has been associated with a decrease in the mechanical properties of cortical and trabecular bone [5,9,10)], the effects of AGE crosslinks on ultrasonic and hypersonic wave velocity in bone have not yet been investigated as the complicated heterogeneity and anisotropic structure of bone hamper measurement.

Here, we investigated the effects of the glycation-induced AGEs crosslinks on the hypersonic longitudinal wave velocity in cortical bone using a micro-Brillouin light-scattering technique [11)]. This optical technique enables non-destructive high-resolution measurement of wave velocity in the GHz range. Owing to the high resolution measurements, we have already reported the local anisotropy of wave velocity in the bovine cortical bone and the single trabecula [12,13)]. The wave velocity measurement also enables evaluation of newly formed bone which is softer than the matured bone [14)].

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Materials

Given that the micro-Brillouin light-scattering technique requires transparent specimens, we fabricated super-thin bovine cortical bone specimens and artificial collagen films for scattering measurements. Details are described below.

Bone specimens

Fig. 1 illustrates the procedure for specimen preparation. A ring-shaped cortical bone specimen was obtained from the mid-shaft of the right femur of a 32-month-old bovine. In the plane of the bone axis and tangential direction, 8 plate specimens (10 mm×10 mm) at the anterior, posterior, medial, and lateral parts were sliced out and ground to a thickness of approximately 70 μm using a lapping machine (ML-521-d; Maruto, Tokyo, Japan) to ensure sufficient optical transparency.

Collagen film specimens

Fig. 2 shows the artificial thin collagen gel membrane (Collagen Type-I; Asahi Techno Glass Corporation, Tokyo, Japan). The membrane is supported with a nylon membrane ring (outer diameter: 33 mm, inner diameter: 24 mm) and has a thickness of approximately 20 μm. Half of the membrane was used as a reference specimen, while the other half was used as an AGE specimen.

Incubation for glycation

To induce glycation, the specimens were incubated in a mixture of Phosphate buffered saline (PBS; 166-23555; Wako, Osaka, Japan), D-(-)-Ribose (R9629; SIGUMA-ALDRICH, St. Louis, USA), Protease Inhibitor Cocktail Set III (without EDTA; 539134-; Calbiochem, San Diego, USA) and Penicillin-Streptomycin (15140; gibco, Carlsbad, USA) [14]. Reference specimens were put in a mixture of PBS and penicillin-streptomycin. All specimens were kept in an incubator at 37 °C for 14 days.

Determination of AGEs in bone

Following incubation, all specimens were hydrolyzed in 6 N HCl at 110 °C for 24 hours. Hydrolysates were analyzed for cross-links and hydroxyproline levels using a high-performance liquid chromatograph (HPLC) (LC9; Shimadu, Kyoto, Japan) with fluorescence detection fitted with a cation exchange column (0.9 × 10 cm, Aa pack-Na; JASCO, Ltd., Tokyo, Japan) [15]. It was assumed that collagen weighed 7.5 times the measured weight of hydroxyproline, with a molecular weight of 300,000 Da [15]. The obtained data were used to calculate AGE values as mol/mol of collagen. AGE levels were determined using a fluorescence reader (JASCO FP6200) at wavelengths of 370 nm excitation and 440 nm emission and normalized to a quinine sulfate standard [16].

Micro-Brillouin scattering technique

Brillouin scattering measurement was performed using a six-pass tandem Fabry-Pérot interferometer (JRS Scientific Instruments, Zurich, Switzerland) [17]. The system uses a solid state laser with a wavelength of 532 nm, and includes an optical microscope system near the specimen. The actual spot diameter of the focused laser beam on the specimen was approximately 10 μm [18], enabling evaluation of wave

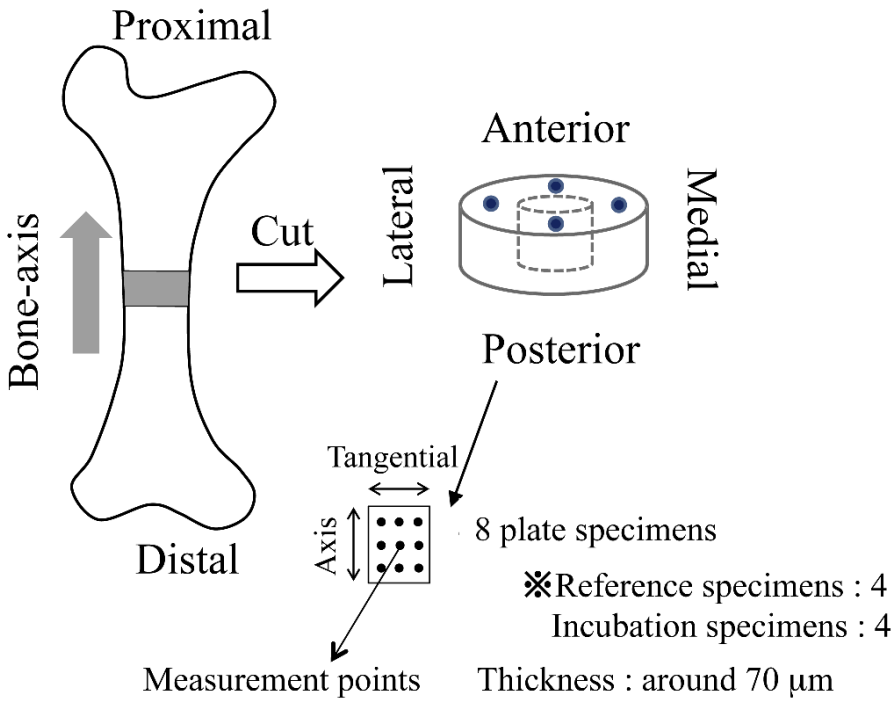

Fig 1. **Bone specimen preparation.**

properties in a small area. The laser power near the specimen was approximately 11 mW, which was sufficiently low to avoid any degradation of the specimen. The scattered light was received by a photomultiplier (Hamamatsu, R464s, Shizuoka, Japan) and averaged by a photon counter before analogue-to-digital conversion. After a number of interferometer scans, the averaged spectrum was recorded on a computer.

The wavelength of the observed phonons was determined based on the scattering geometry, which specifies the directions of incident and scattered light. The reflection-induced ΘA (RIΘA) scattering geometry [19] is shown in *Fig. 3*. This geometry enables the simultaneous observation of phonons propagating in each direction of wave vector of $q^{\Theta A}$ and q^{180} in one measurement (the frequency is in the GHz range). The wave velocity of $q^{\Theta A}$ is measured in the area where the incident and reflected light interfere. From the observed spectrum, we obtain the frequency shift $f^{\Theta A}$, which gives us the wave velocities as follows

$$v^{\Theta A} = f^{\Theta A} \frac{\lambda_0}{2 \sin(\Theta/2)}$$

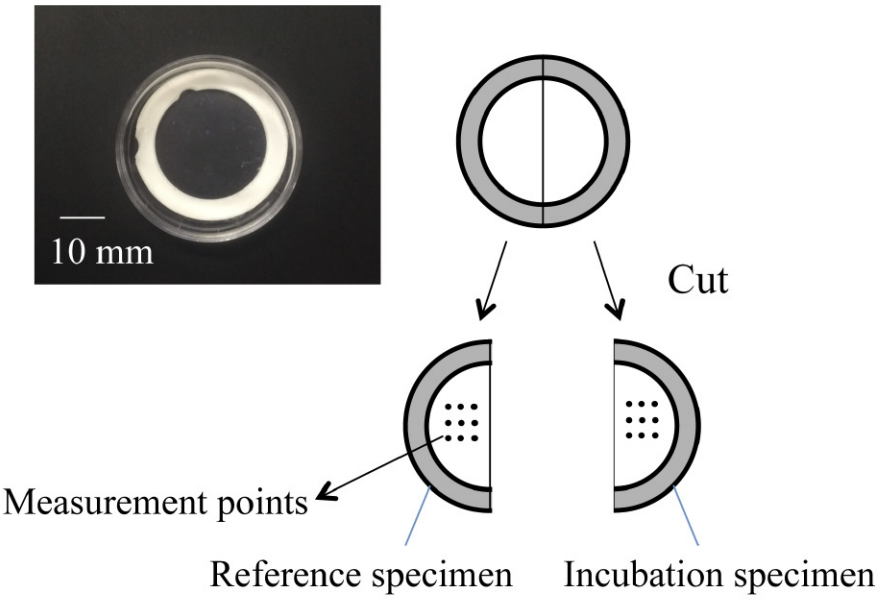

Fig 2. **Thin collagen gel membrane specimen preparation.**

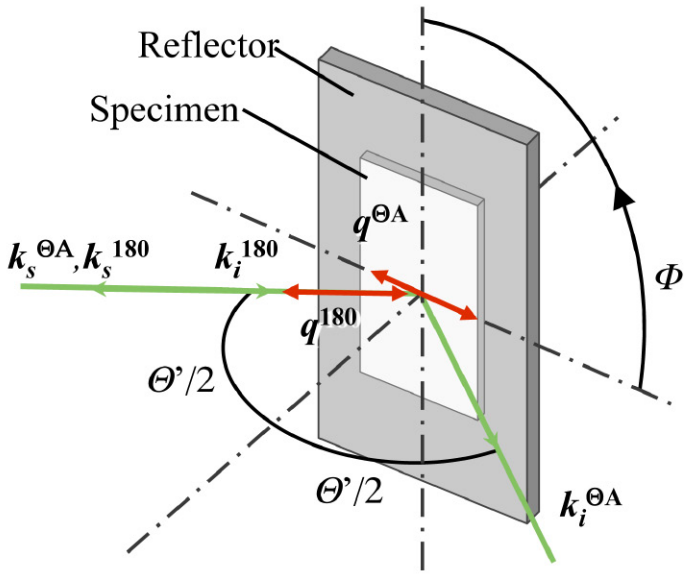

Fig 3. **RIΘA scattering geometry.**

k_i , the wave vector of the incident light; k_s, the wave vector of the scattered light; q, the wave vector of the sound wave; $\Theta/2$, the angle between the incident laser beam and the normal line of the specimen surface; Φ, the rotation angle in the plane.

Here, v is the acoustic velocity, λ_0 is the wavelength of the incident laser, and $\Theta/2$ is the angle between the incident laser beam and the line normal to the specimen surface. This measurement gives us the longitudinal wave velocity in the GHz range, which is much higher than the frequency range of ultrasonic diagnostic systems. Of note, the good spatial resolution of the measurements reduces the effects of microstructure on the wave velocity. In addition, we can select the direction of wave propagation by rotating the specimen, which easily provides anisotropic information.

Results

Table 1 shows the amount of collagen crosslinks before and after incubation (14 days) using a HPLC system. As we can see, after incubation, the amount of AGE crosslinks increased in all specimens [7,15]. The increases of crosslinks in the reference specimens were much smaller than in incubated specimens.

Table 1. Amounts of collagen cross-links.

(mg quinine / mg collagen)

	Anterior	Posterior	Medial	Lateral
Before incubation	0.0833	0.0511	0.0492	0.0537
After incubation	0.1921	0.1817	0.1592	0.1656

Fig. 4 shows the wave velocity in the bone axis direction before and after incubation. In bone axis direction, the wave velocities decreased by 6.1% at lowest and 7.4% at highest in all specimens after incubation (paired t-test, $p < 0.01$, $n = 9$). In the tangential direction, it decreased by 4.6% at lowest and 5.5% at highest as shown *Fig. 5* (paired t-test, $p < 0.01$, $n = 9$). The decreases in wave velocity in reference specimens were all within 0.5%, values which were much smaller than average decreases in the incubated specimens (*Fig. 6*).

Next, to investigate the relation between velocity and crosslinking in the specimens, we measured the velocity changes as a function of time. One measurement specimen was from the posterior part which has haversian structure and the other was from the lateral part which has plexform structure. The results are shown in *Fig. 7*.

To confirm the effect of AGE crosslinks on wave velocity, we also investigated the effects of AGEs on wave velocity in the thin collagen gel membrane (Collagen Type-I; *Fig. 8*). After incubation of 14 days, wave velocity decreased by 8.0 % in the incubated specimens (paired t-test, $p < 0.01$, $n = 9$), but by less than 1% in reference specimens. *Fig. 9* shows the velocity change in collagen as a function of time.

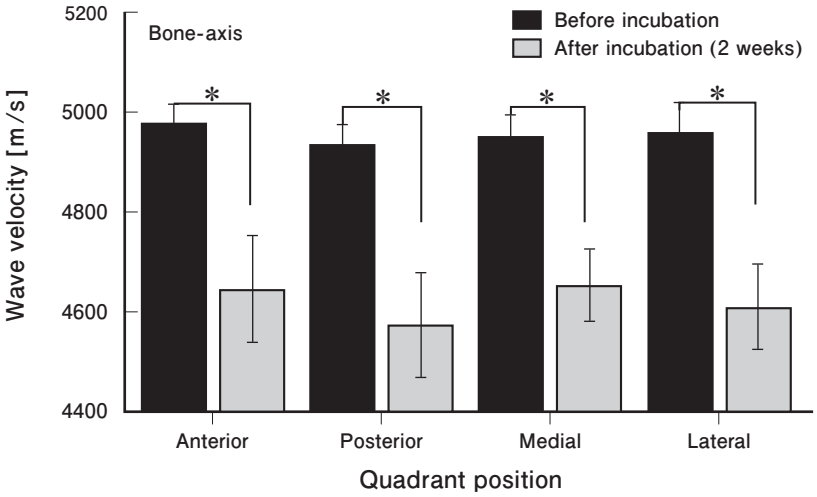

***Fig 4.* Variation of velocities in bone axis direction.**
The effects of glycation are shown.

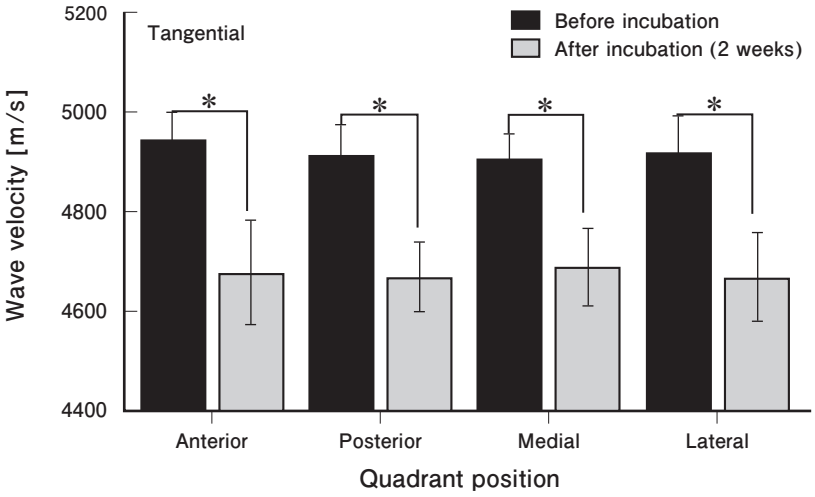

***Fig 5.* Variation of velocities in tangential direction.**
The effects of glycation are shown.

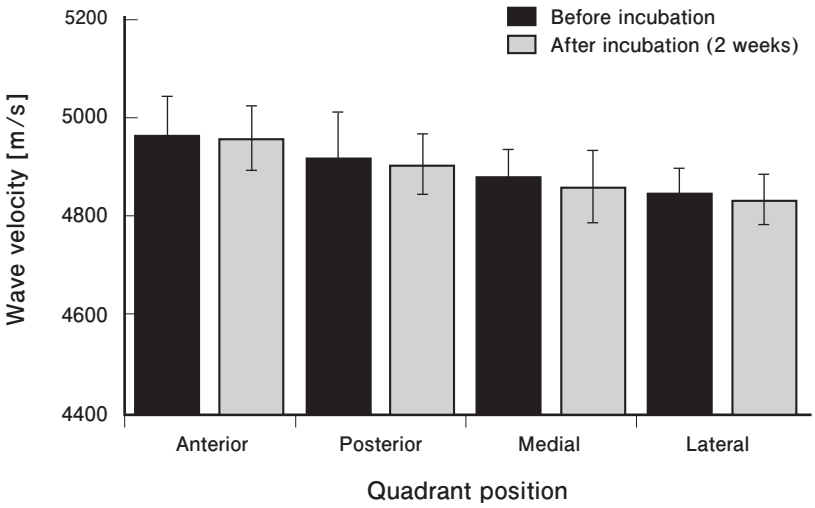

Fig 6. **Variation of velocities in bone reference specimens in the axis direction.**

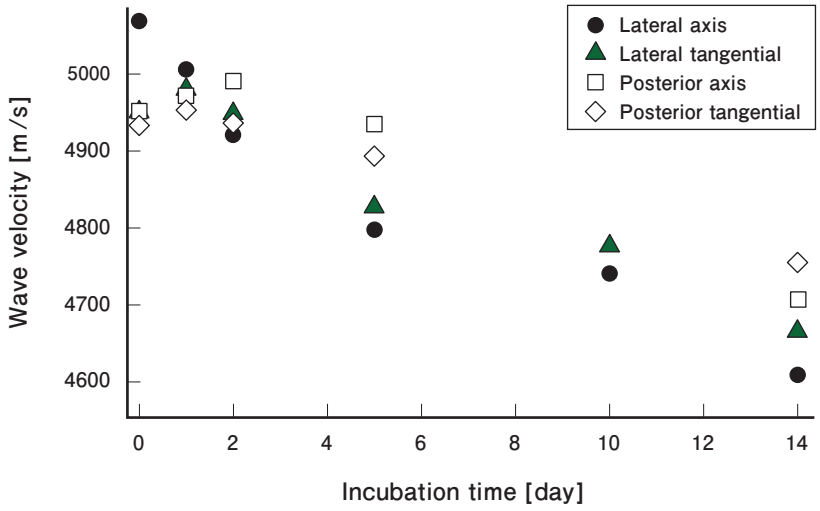

Fig 7. **Variation of velocity in bone due to incubation time.**

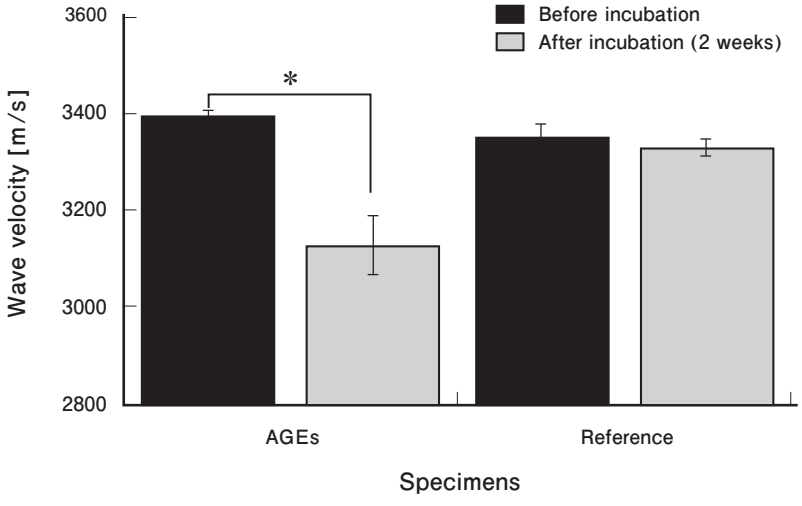

Fig 8. **Variations of velocities in collagen membrane specimens.**

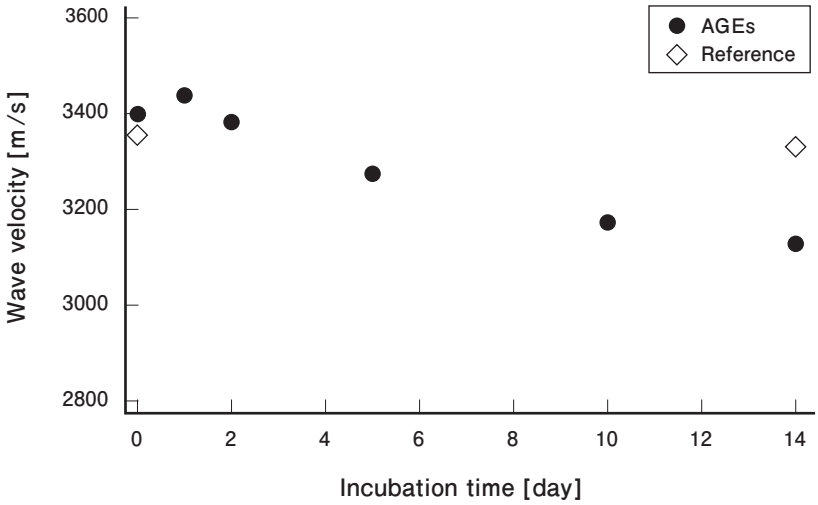

Fig 9. **Variation of velocity in collagen membrane specimens due to incubation time.**

Discussions

The decreases of wave velocity in bone axis direction are bigger than in tangential direction as shown in ***Figs. 5, 6*** and ***7***. This showed AGE crosslinks reduce the elastic property in bone axis direction more than in tangential direction. Wave velocity increased slightly at almost bone specimens in the first three to four days but decreased over time (***Fig. 7***). The initial increase of wave velocity seems to indicate an increase in the elastic property of bone due to the progress of glycation. Given that the specimens measured by a micro-Brillouin scattering method were extremely small and thin compared with the cortical bone in the midshaft of the femur, we suspect that glycation proceeded much more rapidly in our specimens than it might *in vivo*.

As mentioned above, changes in wave velocity reflect changes in elastic properties of bone, with decrease in velocity subsequently indicating decreased bone elasticity. The initial increase of wave velocity is understandable, considering the crosslinking process of common polymeric materials [20]. However, reasons for the observed subsequent decrease in wave velocity should be discussed carefully. Volume contraction due to rapid crosslinking in thin specimen may have caused physical defects in specimens. Because Brillouin scattering measurements provide the averaged wave velocity in the measured area (spot diameter 10 μm), the minute defects reduce wave velocity and elasticity. Discussion should therefore be conducted with the microscopic morphological data for specimens before and after incubation.

On examining velocity change as a function of time, the same tendency was noted, in the incubated collagen specimen (***Fig. 9***). These results suggest that the decrease of wave velocity due to AGEs in bone derives from the velocity decrease in collagen. The precise comparison of the bone and collagen data seems difficult because the composition and the initial wave velocities are different, however, the final velocity decreases after 14 days incubation were both less than 10%.

Conclusion

With the progressive formation of AGE crosslinks, hypersonic wave velocity in cortical bone specimens initially increased slightly and then decreased. This decrease appears to be due to elasticity changes in collagen of bone. However, of note: the method used on the present study required extremely thin specimens for transparency during measurements. As such, glycation may have progressed unnaturally rapidly. Future studies should investigate whether or not a similar decrease in velocity occurs in large cortical bones during the normal glycation process *in vivo*.

Acknowledgments

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Reference

1) NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy. Osteoporosis prevention, diagnosis, and therapy. JAMA. 2001; 285: 785-795.
2) Inada M, Terada M, Nishizawa Y, et al. Protective effect of an aldose reductase inhibitor against bone loss in galactose-fed rats: Possible involvement of the polyol pathway in bone metabolism. Metabolism. 1999; 148: 904-909.
3) Atsumi K, Kushida K, Shimizu S, et al. Risk factors for vertebral fractures in renal osteodystrophy. Am J Kidney Dis. 1999; 33: 289-293.
4) Saito M, Fujii K, Mori Y, et al. Role of collagen enzymatic and glycation induced cross-links as a determinant of bone quality in spontaneously diabetic WBN/Kob rats. Osteoporos Int. 2006; 17: 1514-1523.
5) Vashishth D, Gibson GJ, Khoury JI. Influence of nonenzymatic glycation on biomechanical properties of cortical bone. Bone. 2001; 28: 195-201.
6) Saito M, Fujii K, Marumo K. Degree of mineralization-related collagen crosslinking in the femoral neck cancellous bone in cases of hip fracture and controls. Calcif Tissue Int. 2006; 79: 160-168.
7) Saito M, Soshi S, Tanaka T, et al. Intensity-related differences in collagen post-translational modification in MC3T3-E1 osteoblasts after exposure to low-and high-intensity. Bone. 2004; 35: 644-655.
8) Saito M, Marumo K. Collagen cross-links as a determinant of bone quality: A possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. Osteoporos Int. 2010; 21: 195-214.
9) Hernandez CJ, Tang SY, Baumbach BM, et al. Trabecular microfracture and the influence of pyridinium and non-enzymatic glycation-mediated collagen cross-links. Bone. 2005; 37: 825-832.
10) Wang X, Shen X, Li X, et al. Age-related changes in the collagen network and toughness of bone. Bone. 2002; 31: 1-7.
11) Sakamoto M, Kawabe M, Matsukawa M, et al. Measurement of wave velocity in bovine bone tissue by micro-Brillouin scattering. Japanese Journal of Applied Physics. 2008; 47: 4205-4208.
12) Mathieu V, Fukui K, Matsukawa M, et al. Micro-Brillouin scattering measurements in mature and newly formed bone tissue surrounding an implant. J Biomech Eng. 2011 Feb; 133(2): 021006. doi: 10.1115/1.4003131.
13) Tsubota R, Fukui K, Matsukawa M. Local ultrasonic wave velocities in trabeculae measured by micro-Brillouin scattering. The Journal of the Acoustic Society of America. 2014 Feb; 135(2): EL109-14. doi: 10.1121/1.4862883.
14) Viguet-Carrin S, Farla D, Bala Y, et al. An *in vitro* model to test the contribution of advanced glycation end productsto bone biomechanical properties. Bone. 2008; 42: 139-149.
15) Saito M, Marumo K, Fujii K, et al. Single column high-performance liquid chromatographic- fluorescence detection of immature, mature and senescent cross-links of collagen. Anal Biochem. 1997; 253: 26-32.
16) Saito M, Grynpas MD, Burr DB et al. Treatment with eldecalcitol positively affects mineralization, microdamage, and collagen crosslinks in primate bone. Bone. 2015; 73: 8-15.
17) Sandercock JR. Trends in Brillouin scattering: Studies of opaque materials, supported films, and central modes. In: Light Scattering in Solids III, Springer, Berlin, Heidelberg, 1982; 173-206.
18) Matsukawa M, Shintani K, Tomohiro S, et al. Application of Brillouin scattering to the local anisotropy and birefringence measurements of thin layers. Ultrasonics. 2006 Dec 22; 44 Suppl 1: e1555-9.
19) Krüger JK, Embs J, Brierley J, et al. A new Brillouin scattering techniques for the investigation of acoustic and opto-acoustic properties: Application to polymers. Journal of Physics D: Applied Physics. 1998; 31: 1913-1917.
20) Matsukawa M, Nagai I. Ultrasonic characterization of a polymerizing epoxy resin with imbalanced stoichiometry. The Journal of the Acoustic Society of America. 1996; 99: 2110-2115.