Determination of the relationship between collagen cross-links and the bone–tissue stiffness in the porcine mandibular condyle

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**Abstract**

Although bone–tissue stiffness is closely related to the degree to which bone has been mineralized, other determinants are yet to be identified. We, therefore, examined the extent to which the mineralization degree, collagen, and its cross-links are related to bone–tissue stiffness.

A total of 50 cancellous and cortical bone samples were derived from the right mandibular condyles of five young and five adult female pigs. The degree of mineralization of bone (DMB) was assessed using micro-computed tomography. Using high-performance liquid chromatography, we quantified the collagen content and the number of cross-links per collagen molecule of two enzymatic cross-links: hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), and one non-enzymatic cross-link: pentosidine (Pen). Nanoindentation was used to assess bone–tissue stiffness in three directions, and multiple linear regressions were used to calculate the correlation between collagen properties and bone–tissue stiffness, with the DMB as first predictor.

Whereas the bone–tissue stiffness of cancellous bone did not differ between the three directions of nanoindentation, or between the two age groups, cortical bone–tissue stiffness was higher in the adult tissue. After correction for DMB, the cross-links studied did not increase the explained variance. In the young group, however, LP significantly improved the explained variance in bone–tissue stiffness. Approximately half of the variation in bone–tissue stiffness in cancellous and cortical bone was explained by the DMB and the LP cross-links and thus they cannot be considered the sole determinants of the bone–tissue stiffness.

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1. Introduction

Bone tissue consists of mineralized matrix, mainly hydroxyapatite (HA), and non-mineralized matrix, predominantly fibers. Both components are important determinants of the mechanical properties of bone (Borah et al., 2000; Burr, 2002; Follet et al., 2004; Turner et al., 1990).

The compressive strength of bone is determined largely by the degree of mineralization of bone (DMB) that increases bone stiffness (Van der Linden et al., 2001; Van Ruijven et al., 2007). However, an invaluable role in the bone’s ductility and flexibility (Hernandez et al., 2005; Oxland et al., 1995) – in other words, in its toughness (Garnero et al., 2006; Wang et al., 2001) and fracture resistance (Zioupos et al., 1999) – may be played by the presence of collagen fibers. So far, however, contradictory results are published on the relationship between collagen content and the mechanical properties of bone (Ding et al., 2002; Nafei et al., 2000).

Collagen molecules consist of three polypeptide strands connected by cross-links. Although it has been suggested that the individual collagen structure is ‘stiffened’ by an increase in the...
number of cross-links (Avery and Bailey, 2006), which thus increases overall bone–tissue stiffness, the literature on this topic is inconsistent. Some studies found that the number of cross-links was correlated with bone stiffness (Banse et al., 2002; Rath et al., 1999); others found that it was not (Vashishth et al., 2001; Ziopos et al., 1999). Two frequently studied enzymatic cross-links in bone are hydroxyllysylpyridinoline (HP) and lysylpyridinoline (LP), also referred to as pyridinium and deoxypyridinoline (Knot and Bailey, 1998). Non-enzymatic cross-linking occurs by advanced glycation end products (AGEs), although pentosidine (Pen) is just one of the many AGEs. Pen measurement is a common quantification method in bone as it can be precisely quantified (Saito et al., 1997) and as it is considered as a marker for the accumulation of other non-enzymatic glycation products (Bank et al., 1998).

Each of the above-mentioned studies reporting on the relationship between collagen cross-links and bone mechanical properties used mechanical tests at macroscopic or structural level. Their results depended greatly on the bone-density of the samples, which made it difficult to test the hypothesis that bone–tissue stiffness is influenced by the presence of collagen cross-links. In contrast, results from nanoindentation are independent of the bone density (Silva et al., 2004), enabling the examination of the correlations between collagen cross-links and bone–tissue stiffness without bone density-related interference.

The aims of this study were therefore to examine the tissue stiffness of cancellous and cortical bone using nanoindentation and to assess the relative contribution to the bone–tissue stiffness of the DMB, collagen, and the HP, LP, and Pen cross-links. It was hypothesized that the bone–tissue stiffness is correlated with the collagen content and the number of cross-links studied. To increase the variation in the collagen parameters, we used cancellous and cortical bone-samples of two different ages.

2. Materials and methods

The mandibular condyles of ten female domestic pigs (Sus scrofa) were used. All animals originated from the same lineage had been raised under identical, standardized conditions at a pig farm associated with the Faculty of Animal Sciences at Wageningen University in the Netherlands, and showed no signs of disease. They were divided into two groups of five animals: a young group (average age of 9.4 ± 0.4 weeks) and an adult group (average age of 10.26 ± 5.0 weeks). Power calculations were performed in PASS software based on data from previous work (Willems et al., 2010). These calculations resolved that total sample sizes of 7 and 9 were needed to test the reported differences and variations in DMB and the number of HP, LP, and Pentosidine cross-links, respectively, on the basis of a power (1–β) of 0.9.

Approval was obtained from the Committee for Animal Experimentation at the Faculty of Animal Sciences of Wageningen University. The pigs' heads were obtained and stored at −20 °C within 4 h postmortem before further processing. Fifty bone-samples were prepared in a way similar to that described in our earlier procedure (Willems et al., 2010). In brief, using a water-cooled micro-saw, we cut a horizontal bone-slice approximately 5 mm thick at the level of the medial and lateral poles. One cubic-shaped cancellous bone-sample was derived from the center of each condyle and one cortical bone-sample was derived from each lateral pole. Each of these samples was prepared for nanoindentation by dehydration by dehydration in 70%, 90%, and 100% ethanol, and embedded in polymethylmethacrylate. To expose the bone microstructure on the superior, anterior, and lateral sides, the embedded samples were then cut across three planes. Finally, under deionized water, the bone microstructure on the superior, anterior, and lateral sides, the embedded samples were prepared for nanoindentation by dehydration, and polished using a micro-cloth and an aluminum polishing paper.

The bone-sample (particle size 0.05 m) was indented at its center (Fig. 1). In total, 300 indents were made (10 animals × 2 bone-samples per animal × 3 directions per bone-sample × 5 indents per direction). The load protocol comprised two conditioning steps. At the start of the third unloading, the Oliver and Pharr method was used to assess the tissue stiffness (Oliver and Pharr, 1992). The maximum load was 8 mN, before final unloading at a rate of 0.08 mN/s. The corresponding maximum indentation depth was approximately one micron. The contact stiffness S, which is the slope of the initial portion of the final unloading curve, and the contact area A, were used to determine the reduced modulus (E_r) of the sample-indenter combination. Poisson's ratio of the Berkovich diamond indenter was 0.07; its Young's modulus was 1140 GPa. Poisson's ratio of bone was assumed to be 0.3.

The DMB was determined using a micro-computed tomography system (μCT 40, Scanco Medical AG, Brütiselen, Switzerland) as previously described (Willems et al., 2007). The bone-samples were scanned at a resolution of 10 μm and a peak voltage of 45 kV (effective energy: 24 kV). Four projections, each with an integration time of 250 ms, were performed for each scan angle in each slice. To improve the signal-to-noise ratio, the projections were then averaged.

Volumes of interest as large as the complete sample were defined in order to analyze the DMB at the same level as the collagen properties. Bone was distinguished and separated from background using the same threshold for samples from the same age group. The computed X-ray attenuation in each voxel was represented by a gray value in the three-dimensional reconstruction, which was converted into a DMB value using reference measurements of a calibration phantom (QB M GmbH, Möhrendorf, Germany) containing hydroxyapatite grains in concentrations of 0, 100, 200, 400, and 800 mg/cm³.

The collagen content and the numbers of the HP, LP, and Pen cross-links were assayed using high-performance liquid chromatography as described in detail elsewhere (Bank et al., 1996). Briefly, the complete bone-samples were hydrolyzed at 110 °C in HCl and vacuum dried. The hydrolysate was then dissolved in 10 nmol pyridoxine/ml water and 2.4 mmol homoaarginine/ml water as internal standards.

Reversed-phase chromatography was performed. The collagen content was expressed as milligrams of collagen per milligram of dry bone tissue. The numbers of HP, LP, and Pen cross-links were determined and divided by the absolute amount of collagen, resulting in the average numbers of HP, LP, or Pen cross-links per triple helix of collagen. Statistical analysis was performed as follows: preliminary testing by one-way ANOVA showed no statistically significant difference in tissue stiffness between the three directions of indentation, which were therefore pooled and averaged. We also pooled and averaged data on the collagen parameters from the two samples of cancellous bone. Because of aberrations in the HPLC peak of hydroxyproline, the collagen content data from one young cancellous sample and from one adult cortical bone-sample were excluded from further analysis.

Independent sample t-tests were performed in order to establish whether there were significant differences between the two age groups. The differences between the cancellous and cortical bone-samples were tested for statistical significance using paired sample t-tests. Correlations between bone–tissue stiffness and the DMB and collagen parameters were calculated using Pearson's correlation coefficients (r). The coefficient of determination (r²) between bone–tissue stiffness and the collagen parameters was calculated using multiple regression analysis with DMB as the first predictor. All tests were carried out using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA); p-values of less than 0.05 were considered statistically significant.

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Fig. 1. A frontal view of one of the porcine condyles from which samples of cancellous bone and cortical bone were obtained. Asterisks: samples used to determine the DMB and the bone–tissue stiffness (shown in detail). The other samples were used for collagen assays. Gray values: increasing DMB from light towards dark.
3. Results

Fig. 2 shows the values of Young's modulus reflecting bone–tissue stiffness. In the samples derived from the adult group, the tissue stiffness of both the cancellous and the cortical bone-samples was higher than it was in the samples obtained from the young group. However, this age-related difference was statistically significant only in the cortical bone (p < 0.05). There were no statistically significant differences in bone–tissue stiffness between the cancellous and cortical bone within either age group.

In cancellous bone, the average DMB in the adult group was 1004 ± 41 mg HA/cm³, which was higher (p < 0.001) than the DMB in the young group, (533 ± 46 mg HA/cm³). In addition, in cortical bone, the bone was more highly mineralized (p < 0.001) in the adult group (925 ± 35 mg HA/cm³) than in the young group (593 ± 37 mg HA/cm³). In the adult group, the cancellous bone was more highly mineralized (p < 0.05) than the cortical bone.

Table 1 shows a summary of the collagen content and the number of cross-links. The samples from the young group, the number of Pen cross-links was higher in both bone types (p < 0.05). The number of HP and LP cross-links did not differ significantly either between the young and adult animals or between cancellous and cortical bone-samples. Comparison of cancellous and cortical bone from the adult animals showed that the number of Pen cross-links was higher in cortical bone (p < 0.05).

Correlation analyses showed that the DMB was positively correlated with bone–tissue stiffness (r = 0.72, p < 0.01, n = 10). However, when correlation analyses were carried out separately for the two age groups, there was no significant correlation in the young group (r = 0.51, NS, n = 5) or in the adult group (r = 0.00, NS, n = 5), (Fig. 3A). The collagen content, HP, and Pen cross-links were not statistically significantly correlated with bone–tissue stiffness. In contrast, the number of LP cross-links was negatively correlated with bone–tissue stiffness (r = −0.68, p < 0.01, n = 10).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Cancellous bone</th>
<th>Cortical bone</th>
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<tbody>
<tr>
<td></td>
<td>Young (SD)</td>
<td>Adult (SD)</td>
</tr>
<tr>
<td>Collagen content (mg/ mg of dry bone)</td>
<td>0.15 (0.03)</td>
<td>0.26 (0.10)</td>
</tr>
<tr>
<td>HP (nr. of cross-links/triple helix)</td>
<td>0.09 (0.05)</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>LP (nr. of cross-links/triple helix)</td>
<td>0.03 (0.02)</td>
<td>0.01 (0.00)</td>
</tr>
<tr>
<td>Pen (nr. of cross-links/triple helix)</td>
<td>0.39 (0.25)*</td>
<td>0.04 (0.02)**</td>
</tr>
</tbody>
</table>

Significant differences (p < 0.05) between “young and adult animals and between “cancellous and cortical bone. HP = Hydroxylysylpyridinoline, LP = Lysylpyridinoline, Pen = Pentosidine.

Fig. 2. Bone–tissue stiffness (mean ± SD) measured using nanoindentation at the cores of trabecular elements. Asterisk: higher cortical bone–tissue stiffness in the adult group than in the young group (p < 0.05).

Fig. 3. In the young and the adult group separately, no significant relationships were detected between mean bone–tissue stiffness and (A) DMB and (B) the number of LP cross-links. HA = hydroxyapatite.
correlated with bone–tissue stiffness \( r = -0.480, p < 0.05, n = 10 \). However, when correlation analyses were carried out for both age groups separately, no significant correlation was found in the young \( r = -0.11 \) nor in the adult \( r = 0.08 \) group (Fig. 3B).

For the DMB alone, the coefficient of determination of bone–tissue stiffness was 51%. After correction for DMB, the cross-links studied did not increase the explained variance. In the young group, however, LP significantly improved the explained variance in bone–tissue stiffness; the coefficient of determination increased by 40% in the young group \( p < 0.05 \), while in the adult group no significant change (7%) was found.

4. Discussion

This study had two aims: to examine the tissue stiffness of cancellous and cortical bone and to assess the relative contribution that the DMB, the collagen, and the HP, LP, and Pen cross-links make to bone–tissue stiffness. Nanoindentation was used as a test method because its results could not be affected by the density of bone-samples.

As dehydrated and embedded bone may have a higher Young’s modulus than wet bone–tissue has (Hengsberger et al., 2002), any results obtained using nanoindentation should be interpreted with caution (Donnelly et al., 2006). Because the stiffness of dehydrated and embedded bone is linearly related to that of wet bone, and as it can be expected that all dehydrated and embedded samples underwent this rise in bone–tissue stiffness, it is nevertheless safe to correlate bone–tissue stiffness with collagen parameters (Mitra et al., 2006).

In this study, it was chosen to indent trabecular elements of 110 \( \mu \)m in width only in order to make a clear comparison in tissue stiffness between bone from young and adult specimens, between the three indentation directions, and between cancellous and cortical bone. Possibly, this choice narrowed the DMB range. But as bone from two age groups was used, a relatively large range in DMB values was obtained. Large age-related changes in DMB and collagen values in comparable, healthy, porcine mandibular condyles have previously been discussed (Willems et al., 2010). Therefore, it cannot be expected that this approach did affect the determination of the relative contribution of the DMB, collagen and the HP, LP, and Pen cross-links to the bone–tissue stiffness, which was the objective of this study.

It should be noted that the current values of trabecular elements of the same size are not representative for the bone–tissue stiffness in the porcine mandibular condyle. Inclusion of a wide range of trabecular widths would have resulted in representative Young’s moduli for the porcine mandibular condyle. But, as the mandibular condyle differs from other bone structures with regard to factors such as the loading and the rate of bone remodeling, it still would not have been possible to compare the bone–tissue stiffness with that in other bone structures.

Our finding that the tissue stiffness of cortical bone tissue was higher in bone-samples from adult animals than from young ones is in agreement with Mulder et al. (2007), stating that bone–tissue stiffness may increase with age.

As bone–tissue stiffness has been reported to differ between these bone types (Zysset et al., 1999), it is remarkable that bone–tissue stiffness did not differ in this study, either according to age group or between cancellous and cortical bone. Generally, cancellous bone is known to remodel faster than cortical bone (Jee, 1999), resulting in a relatively low DMB in cancellous bone. As mean DMB is known to be an important determinant of bone–tissue stiffness, one would expect the DMB and bone–tissue stiffness both to be lower in cancellous bone. In our study, however, the DMB in the adult group was somewhat higher in cancellous bone than in cortical bone. This suggests that there is a continuously high rate of remodeling not only in the cancellous part of the bone in the mandibular condyle, but also in the cortical part. As our indentation protocol comprised the selection of trabecular elements with a width of 110 \( \mu \)m, it is also possible that our test method masked the differences between cancellous and cortical bone. If so, the differences between cancellous and cortical bone that have been reported in literature may have been due to differences in the thickness of the trabecular elements studied.

Because bone–tissue stiffness did not differ significantly between the three directions of indentation, our data were averaged for further analysis. The lack of elastic anisotropy revealed by these data may have been caused by the multiple loading directions of the mandibular condyle, which might produced a more isotropic bone structure than in other bones. Another cause might be differences in bone–tissue stiffness between or within single trabecular elements which, irrespective of the direction of indentations, are known to be caused by the heterogeneous intratrabecular distribution of bone mineral (Zysset et al., 1999).

At \( r = 0.72, R^2 = 0.51, \) half of the variance in bone–tissue stiffness was explained by the DMB alone, a finding that is consistent with Zysset et al. (1999). However, bone–tissue stiffness was measured locally at various sites, whereas the mean DMB values were obtained from the complete bone-sample. Correlation of two variables originally assessed at different levels might lead to an underestimation of this correlation. On the other hand, as Mulder et al. (2007) have shown that, if the local mineral distribution is disregarded, as it was in our study, the correlation between the DMB and bone–tissue stiffness might be over-estimated.

In our study, collagen content was not statistically significantly correlated with bone–tissue stiffness. This is consistent with the findings of Žiggut-Carrin et al. (2008) in a study involving young bovine femurs. Similarly, our finding of a negative correlation between the number of LP cross-links and bone–tissue stiffness confirms earlier reports by Banse et al. (2002). However, as the number of LP cross-links is strongly negatively correlated with the DMB (Wassen et al., 2000), the negative correlation between the number of LP cross-links and bone–tissue stiffness might be the result of co-variation.

The addition of the number of cross-links to the multiple regression analysis did not significantly increase the coefficient of determination of bone–tissue stiffness of 51.5%. In young bone, the coefficient of determination increased by 40% in the young bone after the addition of the parameter LP to the multiple regression analysis, whereas such a significant increase was not found in the adult bone. This suggests that possible differences in bone–tissue stiffness as a result of changes in collagen properties can be best studied in bone-samples with a relatively low DMB or in demineralized bone-samples. However, in order to meet the second aim of this study, we did not demineralize the bone-samples: demineralization of all bone-samples might have led to a higher coefficient of determination between the collagen parameters and bone–tissue stiffness. Lastly, it should be mentioned that the cross-links studied are just some of the many collagen cross-links in bone–tissue. For instance, the pyrroles are situated on the same location as the LP cross-links (Hanson and Eyre, 1996) and are characterized as major types of trivalent cross-links (Saito and Marumo, 2010).

In conclusion, under the experimental conditions of our study, a relatively low percentage of the variance in bone–tissue stiffness was explained by both the DMB and the cross-links studied, suggesting that these parameters are not the sole determinants of this mechanical property of bone tissue.
Conflict of interest statement

None declared.

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