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Higher number of pentosidine cross-links induced by ribose does not alter tissue stiffness of cancellous bone

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ABSTRACT

The role of mature collagen cross-links, pentosidine (Pen) cross-links in particular, in the micromechanical properties of cancellous bone is unknown. The aim of this study was to examine nonenzymatic glycation effects on tissue stiffness of demineralized and non-demineralized cancellous bone.
A total of 60 bone samples were derived from mandibular condyles of six pigs, and assigned to either control or experimental groups. Experimental handling included incubation in phosphate buffered saline alone or with 0.2 M ribose at 37 °C for 15 days and, in some of the samples, subsequent complete demineralization of the sample surface using 8% EDTA. Before and after experimental handling, bone microarchitecture and tissue mineral density were examined by means of microcomputed tomography. After experimental handling, the collagen content and the number of Pen, hydroxylysylpyridinoline (HP), and lysylpyridinoline (LP) cross-links were estimated using HPLC, and tissue stiffness was assessed by means of nanoindentation.
Ribose treatment caused an up to 300-fold increase in the number of Pen cross-links compared to nonribose-incubated controls, but did not affect the number of HP and LP cross-links. This increase in the number of Pen cross-links had no influence on tissue stiffness of both demineralized and nondemineralized bone samples. These findings suggest that Pen cross-links do not play a significant role in bone tissue stiffness.

1. Introduction

Bone tissue is, apart from water and lipids, composed of mineral, mainly hydroxyapatite, and extracellular matrix, containing predominantly collagen fibers which consist of three polypeptide chains [1] connected by cross-links. An important mature cross-link is pentosidine (Pen) which is an advanced glycation end product (AGE) formed as a result of non-enzymatic glycation [2]. Other important mature collagen cross-links in bone extracellular matrix are hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), which originate post-translationally from immature types of cross-links during primary mineralization [3]. Both mineral and collagen change their content and architecture as the tissue develops and matures. Bone density increases [4–6] and trabeculae thicken, decrease in number, and become mineralized to a higher degree [7–9]. Changes with age, such as an increase [10–12] or a decrease [13] in the number of Pen cross-links, have been reported. Besides, age-related changes in the number of HP [14,15] and LP cross-links [11,13,15,16] have been shown.

Local differences in mineralization degree and the number of collagen cross-links can occur even within single trabeculae because of differences in "tissue age" [17–20]. A high "tissue age" results in cross-link alterations, which might affect bone mechanical properties.

For instance, patients suffering from diabetes have high levels of Pen cross-links in their bones [21]. The causal relationship between these high levels of cross-links and the patient's impaired mechanical properties of bone tissue, such as a decrease in toughness, has however not been elucidated [21,22]. Bone mechanical properties can be assessed at different levels. At the macroscopic level, mechanical tests are performed on complete bone trabeculae or on larger structural units such as cubically or cylindrically shaped bone samples. Results from this kind of tests depend on the size of the bone samples used [23]. Tests at the microscopic level aim to examine material or tissue properties of bone, and such micromechanical tests are executed very locally. Bone mineral, bone collagen, and bone mechanics-related variables can all vary with age or with bone remodeling rate and have often been correlated. This has been done mainly by assessing mechanical

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variables at the macroscopic level, such as stiffness [18,19,24–29], ductility, toughness, and fracture resistance [10,29–31] or at the microscopic level, such as tissue stiffness or microhardness [32–38].

However, the above studies are mainly descriptive and lack experimental approach. An experimental study design offers the opportunity to avoid and control potential co-variation. Experimental studies show, for instance, that bone demineralization affects the mechanical properties both at the macroscopic [39–41] and the microscopic level [42,43]. It has been suggested that collagen might influence bone tissue stiffness, albeit to a limited extent [44]. An experimental verification of this influence is, however, lacking. In contrast to HP and LP cross-links, the number of Pen cross-links can reliably be changed *in vitro*. Non-enzymatic glycation of bone by ribose incubation results in an increase in the number of AGEs. This increase in the number of AGEs results in 'stiffening' of the collagen structure without a concomitant increase in cortical bone stiffness at the macroscopic level [45]. An increasing number of Pen cross-links, which is not accompanied by changes in the mineralization degree, does not change cortical bone stiffness [46] but does result in inferior post-yield mechanical properties [47]. Experimental manipulation causing an up to threefold increase in the number of AGEs changes fracture resistance but not cancellous bone stiffness [48,49]. Taken together, data from this limited number of experimental studies are consistent with the concept that toughness, tensile strength or post-yield properties of bone are influenced by collagen constituents whereas the stiffness of bone tissue depends mainly on its mineralized compartment.

In these experimental investigations, however, the mechanical properties of bone have been characterized at the macroscopic level, and at that scale, the stiffness of bone is affected by the bone's architectural features such as its density or its anisotropic nature as well [24,50–52]. Conversely, measurements from a micromechanical loading device, because of its local nature, are independent of bone density [53]. Nanoindentation is a micromechanical testing modality which might increase the likelihood of revealing the potential mechanical effects of changes in the number of Pen cross-links in bone tissue. Hitherto only two experimental studies addressed the correlation between the presence of collagen cross-links and the micromechanical properties of the bone which contains them [54,55]. Viguet-Carrin et al. [54] found in non-demineralized cortical bone no influence of a change in the number of Pen cross-links on the micromechanical bone properties. More recently, Paschalis et al. [55] reported that decreases in the number of immature collagen cross-links and in the number of HP cross-links result in lower bone tissue stiffness. It is currently unknown whether the number of Pen cross-links affects the micromechanical properties of cancellous bone.

The aim of this study was, therefore, to examine the effect of non-enzymatic glycation on the tissue stiffness of demineralized and non-demineralized cancellous bone. We hypothesize, on the basis of the abovementioned work of Paschalis et al., that an increase in the number of Pen cross-links might result in higher bone tissue stiffness. We further hypothesize that this effect might be more distinct in demineralized than in non-demineralized cancellous bone, assuming that demineralization impairs the influence of the mineralized part of the bone on the bone tissue stiffness, which might elucidate the micromechanical role of Pen cross-links, if present at all.

2. Materials and methods

2.1. Bone samples

Mandibular condyles of six adult (age: 61.7 ± 20.7 weeks) female domestic pigs (*Sus scrofa*) were used. All animals originated from the same breeding lineage were raised under standardized conditions at a farm associated with the Faculty of Animal Sciences at Wageningen University in the Netherlands, and showed no signs of disease. 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 post mortem before further processing.

Sixty cancellous bone samples (Fig. 1) were prepared as previously described [13]. In brief, a horizontal bone slice approximately 5 mm thick was cut at the level of the medial and lateral poles of the mandibular condyles using a deionized water-cooled microsaw (MBS 240 E, Proxxon GmbH, Föhren, Germany). Except for the period of time during preparation and testing, all bone samples were stored at −20 °C as a lower temperature is likely to cause microcracks in the (cancellous, not cortical) bone, whereas a higher temperature might result in tissue degradation [56,57]. At no stage were the samples exposed to direct sun light or temperatures above 40 °C.

2.2. Study design

The study design is shown schematically in Fig. 2. Samples in group A were kept untreated whereas all other samples were incubated. Group B was incubated with PBS alone, acting as a control group. To each group, two originally neighboring bone samples were assigned from each condyle, of which the first sample was used for collagen determination and the second one for micromechanical testing.

2.3. Experimental modifications

All samples in groups B–E were incubated at 37 °C, pH 7.4 for 15 days as follows. Incubation solutions were refreshed five times per week and 0.02% sodium azide (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and protease inhibitors (Roche Diagnostics Nederland B.V., Almere, the Netherlands) were added to prevent degradation. Samples in groups B were incubated in 0.1 M phosphate buffered saline (PBS), whereas the incubation solution of group C consisted of 0.1 M PBS and 0.2 M ribose to increase the number of Pen cross-links by non-enzymatic glycation.

Groups D and E received the same incubations as groups B and C, respectively, which was followed by superficial demineralization, or full demineralization of the bone sample surface, in 8% ethylenediaminetetraacetic acid (EDTA) for 2 h at 37 °C.

2.4. Microcomputed tomography

All bone samples were scanned using a microcomputed tomography system (μCT 40, Scanco Medical AG, Brüttisellen, Switzerland) before and after the experimental handling in order to quantify the microarchitecture, as expressed by bone volume fraction, trabecular thickness, trabecular separation, and degree of anisotropy. Scanning was performed at a resolution of 10 μm and a peak voltage of 45 kV (effective energy 24 keV). Four projections, each with an integration time of 250 ms, were performed for each scan angle in each slice.

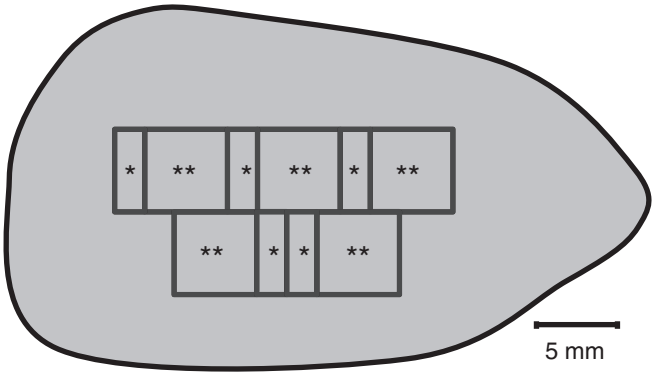

Fig. 1. Superior view on the condylar bone slice from which ten cancellous bone samples were obtained per animal. *: used for collagen assessment, **: used for nanoindentation.