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Mechanical stiffness of TMJ condylar cartilage increases after artificial aging by ribose

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ABSTRACT

Objective: Aging is accompanied by a series of changes in mature tissues that influence their properties and functions. Collagen, as one of the main extracellular components of cartilage, becomes highly crosslinked during aging. In this study, the aim was to examine whether a correlation exists between collagen crosslinking induced by artificial aging and mechanical properties of the temporomandibular joint (TMJ) condyle. To evaluate this hypothesis, collagen crosslinks were induced using ribose incubation.

Methods: Porcine TMJ condyles were incubated for 7 days with different concentrations of ribose. The compressive modulus and stiffness ratio (incubated versus control) was determined after loading. Glycosaminoglycan and collagen content, and the number of crosslinks were analyzed. Tissue structure was visualized by microscopy using different staining methods.

Results: Concomitant with an increasing concentration of ribose, an increase of collagen crosslinks was found. The number of crosslinks increased almost 50 fold after incubation with the highest concentration of ribose. Simultaneously, the stiffness ratio of the samples showed a significant increase after incubation with the ribose. Pearson correlation analyses showed a significant positive correlation between the overall stiffness ratio and the crosslink level; the higher the number of crosslinks the higher the stiffness.

Conclusion: The present model, in which ribose was used to mimic certain aspects of age-related changes, can be employed as an *in vitro* model to study age-related mechanical changes in the TMJ condyle.

1. Introduction

Collagen is the main component of the extracellular matrix (ECM) of connective tissues like bone and cartilage. Its molecules have helical structures which pack together to form collagen fibrils in fiber-type collagens (Ghazanfari, Khademhosseini, & Smit, 2016; Lodish et al., 2000; Panwar et al., 2015). These fibrils progressively rearrange and crosslink at the inter-fibrillar level to provide structural integrity needed by a tissue for proper remodeling and load-bearing capacity (Wilson et al., 2014). Every change in molecular or structural organization of collagen caused by development, pathological conditions, or

aging, has an impact on collagen's contributions to tissue function (Odetti et al., 2000; Panwar et al., 2015; Scharf et al., 2013; Wilson et al., 2014).

Aging is accompanied by a series of cellular, molecular, and structural changes in the mature tissues that ultimately influence their properties and functions (Panwar et al., 2015). Non-reversible collagen crosslinking is a prominent change in the ECM during aging. By altering the mechanical properties of collagen, it could result in an age-related degenerative effect on tissue maintenance (Ait-Belkacem et al., 2012; Bai, Phua, Hardt, Cernadas, & Brodsky, 1992; Brüel & Oxlund, 1996; Tang, Zeenath, & Vashishth, 2007). Such age-related changes are

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thought to play a key role in the etiology of osteoarthritis (OA) which is characterized by progressive destruction of cartilage (Bank, Bayliss, Labefer, Maroudas, & Tekoppele, 1998; Basser, Schneiderman, Bank, Wachtel, & Maroudas, 1998). Mechanical loading is considered a dominant factor for the onset or progression of cartilage damage. It has been shown that the age-related increase of collagen crosslinking increased tissue stiffness. This was considered to make the tissue more susceptible to mechanically-induced damage (Bourne, Lippell, & Torzilli, 2014).

Collagen crosslinking often occurs due to enzymatic and/or non-enzymatic reactions. Enzymatic crosslinking mainly occurs during development and maturation. Non-enzymatic crosslinking reactions can take place due to aging-associated modifications and certain diseases (Wilson et al., 2014). These reactions, called glycation, happen in the presence of a reducing sugar and lead to the production of advanced glycation end-products (AGEs) in the tissue (Brüel & Oxlund, 1996; DeGroot et al., 1999; Panwar et al., 2015). When AGEs are produced, they remain in the tissue until it is renewed. Cartilaginous collagen found in the joints has an extremely slow turnover rate and remains in the tissue after maturation without renewing (Verzijl et al., 2000). As a result, long-term accumulation of AGEs in the joints happens at older age (Bank et al., 1998; DeGroot et al., 1999; DeGroot et al., 2001b). Such an accumulation of AGEs would also happen in young adults due to the presence of extra sugar in the body in diabetes (Illien-Junger et al., 2013; Kohn, Cerami, & Monnier, 1984; Odetti et al., 2000). Resembling the same condition, the excessive exposure of tissue to sugar has been used *in vivo* and *ex vivo*, as a method for the induction of artificial aging (DeGroot et al., 2001a, 2001b; Verzijl et al., 2002; Vos et al., 2012a). Pentosidine, a crosslink between arginine and lysine residues in collagen molecules, is a well-characterized and reliable measure of AGEs (Bank et al., 1998; Sell & Monnier, 1989).

The effect of this artificial aging has been studied specifically on mechanical properties of ECM in different types of tissues including bone (Panwar et al., 2015; Willems et al., 2014), hyaline cartilage (Bank et al., 1998; Chen et al., 2002; Verzijl et al., 2002), and tendon (Reddy, 2004; Reddy, Stehno-Bittel, & Enwemeka, 2002). It has also been utilized to study aging effects as the main risk factor for OA (Verzijl et al., 2002; Vos et al., 2012a; Willett, Kandel, De Croos, Avery, & Grynepas, 2012). There is, however, no study in the literature on the effect of such a crosslinking induction on cartilage of the temporomandibular joint (TMJ).

In the present study, the aim was to examine whether a correlation exists between collagen crosslinking induced by artificial aging and mechanical properties of the TMJ condyle. Evaluation of TMJ cartilage is of particular interest because the TMJ condyle is covered with fibrocartilage rather than hyaline cartilage which cover other articular joints (Athanasίου, Almarza, Detamore, & Kalpakci, 2009; Koolstra, 2002). It has a different cartilaginous structure and composition as compared to articular cartilage. Interestingly, in the regeneration of damaged hyaline cartilage, it is replaced by fibrocartilage containing collagen type I. This is generally considered a material of inferior quality at these locations, while in the TMJ it performs well under various loading conditions. The TMJ condyle of the porcine was selected on the basis of its similarities to the human jaw joint, especially in its mechanical characterization (Herring, 2003).

2. Materials and methods

2.1. Sample preparation

The heads of young pigs (aged 6–8 months old, gender not known) were collected from a local slaughterhouse. The left and right TMJ condyles were dissected within 12 h after sacrifice, and stored at -20°C . The dissected condyles were inspected visually to exclude the ones with gross abnormality, such as vascularization and fibrillation of the surface. In total, 24 condyles were used for this study.

2.2. Crosslinking induction

The condyles were divided into four groups: three groups for incubation with different concentrations of ribose and one control group. For each incubation group, five condyles were used (all from the right side of the mandibles); for the control group, nine condyles (all from the left side of the mandibles). For crosslinking induction, intact condyles were incubated in phosphate buffered saline (PBS) containing 125, 250, or 500 mM ribose, a protease-inhibitor (PI) cocktail (2 mM Ethylenediaminetetraacetic acid; 5 mM benzamidine; 10 mM N-ethylmaleimide; 1 mM phenylmethylsulfonyl fluoride (Kim, Wong, Helfrick, Thomas, & Athanasίου, 2003)), and sodium azide 0.02% for 7 days at 37°C . These groups were labeled as *R125*, *R250*, and *R500*, respectively. The control samples were incubated in the same condition but without ribose. PI was added to minimize tissue degradation and sodium azide to prevent bacterial growth during the incubation time. For the next steps of the study, five regions per condyle were examined, i.e., central, medial, lateral, posterior, and anterior.

2.3. Mechanical characterization

For the mechanical loading, a compression loading test was performed by using a custom-made instrument equipped with a 25 N load-cell (Fazaeli, Ghazanfari, Everts, Smit, & Koolstra, 2016). A rigid cylindrical indenter with a diameter of 4 mm was used for loading. This apparatus produces cyclic displacements of an indenter and simultaneously measures the compressive reaction force. The average thickness of each region was measured prior to loading using micro-CT scanning as described in a previous study (Mirahmadi, Koolstra, Lobbezoo, van Lenthe, & Everts, 2017). To locate each region of interest perpendicular to the cylindrical indenter, the condyles were fixed in a container with adjustable tilting capability. The container was filled with PBS containing PI during the test. A tare load of 0.2 N was introduced to each region to maintain proper loading plate contact, followed by 5 min of relaxation time. Afterwards, 60 cycles of 1% strain at a frequency of 1 Hz were applied as preconditioning, followed by 5 min relaxation time. Thereafter, the condyle was cyclically loaded at a strain level of 5% for 20 cycles at 1 Hz. It has been shown that 10 cycles of loading would be enough for TMJ disc to reach a steady state (Tanaka et al., 2002). Force-displacement curves were used to calculate stress-strain values using the initial thickness of each region and the cross-sectional area of the indenter. Instantaneous modulus (E_{Ins}) was calculated from the peak stress of the first cycle of loading. Steady state modulus (E_{St}) was similarly calculated from the average peak stresses of the last five cycles. Representative stress-strain curves from different groups are shown in Fig. 1.

2.4. Biochemical analyses

To examine the effect of crosslinking induction on biochemical properties, a plug of 4 mm was punched out from each mechanically-loaded region of the condyles. All plugs were weighed and lyophilized overnight; the dry weight (DW) was measured afterward to calculate water content.

To measure the amount of GAG, about 2 mg of the dried samples were incubated in papain digestion buffer at 60°C overnight and used for colorimetric quantification after reaction with dimethyl methylene blue (DMMB) (Ghazanfari et al., 2015). The GAG values were normalized to the tissue dry weight.

One portion of the digested sample was hydrolyzed with 6 M HCl at 95°C for 20 h, dried overnight, and used for the quantification of hydroxyproline (Hyp) and pentosidine (Pen) as measures of collagen content and of the number of collagen crosslinks, respectively. The amount of Hyp was measured following neutralization and a reaction with chloramine-T and dimethyl amino-benzaldehyde (Paul et al., 2013). To determine the collagen content, it was assumed that Hyp

Fig 1. Representative stress-strain curves of control and treated groups from the posterior region.

comprises approximately 10% of the collagen weight (Reddy et al., 2002).

For the determination of the Pen level, high-performance liquid chromatography (HPLC) was used as described in detail elsewhere (Bank, Jansen, Beekman, & te Koppele, 1996). The number of Pen crosslinks were determined and expressed as mole per mole collagen, assuming 300 Hyp residues per triple-helical of collagen molecule and a molecular weight of 300,000 g/mol (DeGroot et al., 1999; Vos et al., 2012b).

2.5. Microscopy

The plugs adjacent to those used for the biochemical analysis from all regions were fixed in 4% formalin and used for histology. Cryosections with the thickness of 10 μ m were stained with SafraninO/Fast green and Picrosirius red. To eliminate the possible effect of sectioning and processing on the structure of collagen fibers, two pieces with a thickness of approximately 1 mm from the central region of one sample incubated with the highest concentration of ribose (R500) and another sample from the control group were stained with collagen type I and II antibodies (see Supplementary material for more detail). Confocal microscopy was used to make the images from thick slices.

2.6. Statistical analyses

To analyze the effect of ribose incubation and region on mechanical and biochemical properties, two-way analyses of variance (ANOVA) with LSD post-hoc test with the assumption of normal distribution of data were performed. Linear regression with Pearson correlation analysis was conducted to assess the correlation between the ribose concentration and the quantified parameters as well as the correlation between pentosidine and stiffness. All values are reported as mean $\pm$ standard deviation. The statistical tests were carried out using SPSS 23 for Windows (SPSS Inc., Chicago, IL, USA), a $p < .05$ was considered statistically significant.

3. Results

3.1. Crosslinking induction

Collagen crosslinking increased with the amount of ribose added to the samples. The condyles incubated with a high concentration of ribose, i.e., R250 and R500 showed a significant increase in the pentosidine (Pen) level (Fig. 2B); being almost 50-fold after incubation with R500. Linear regression analysis demonstrated a significant positive correlation between ribose concentration and Pen level. The increasing trend of Pen with ribose concentration was similar for all regions (Fig. 2A).

3.2. Mechanical properties

When ribose concentration increased, there was an increasing trend in stiffness as defined by instantaneous and steady state moduli (E_{Ins} and E_{St}). This was apparent for all regions (Fig. 3A, D). Statistical analyses revealed the absence of a statistically significant interaction between concentration and region on compressive moduli ($p = 0.954$ for instantaneous and $p = .708$ for steady state moduli). The anterior regions showed the lowest stiffness for both incubated and control samples; posterior and central regions were the stiffest regions (Fig. 3A, D). A general stiffness ratio was calculated by dividing the stiffness of the incubated group with the average stiffness of the control group. Linear regression revealed significant correlations between general stiffness ratios and the ribose concentration (Fig. 3B, E). Linear regression also showed significant correlations between general stiffness ratios and Pen increase ($R = 0.308$ for general E_{Ins} ratio and $R = 0.265$ for general E_{St} ratio, both $p < .01$; Fig. 3C, F). An almost 50-fold increase in cartilage Pen levels increased the general E_{Ins} ratio by about 1.5-fold from the control to R500.

3.3. Biochemical analyses

GAG content results demonstrated no differences among the groups with respect to regions (Fig. 4A). However, when all regions for each condyle were grouped on the basis of the ribose incubation (Fig. 4B), GAG concentration negatively correlated with ribose. This reduction was significant for condyles incubated with the highest ribose concentration, i.e., from $8.6 \pm 1.0 \mu\text{g}/\text{mg DW}$ for the R500 to $7.2 \pm 0.4 \mu\text{g}/\text{mg DW}$ for the control group.

With respect to the amount of collagen, no significant difference was found between the various regions (Fig. 5A). The results also showed that less collagen was measured when the concentration of ribose increased (Fig. 5B). The reduction of collagen was significant for the R500 group when compared to the control group.

3.4. Microscopy

Histology showed that the TMJ condylar cartilage was covered with a dense collagenous layer (Fig. 6A–D). When the concentration of ribose increased, spaces appeared within the collagenous matrix (arrows in Figs. 6 and 7). In the control group, collagen fibers in the superficial layer were aligned parallel to the surface (Fig. 6E). However, this parallel orientation changed following the incubation with ribose. As it is shown in Fig. 6 (panels E–H), collagen fibers were oriented in different directions when the concentration of ribose was increased, being most pronounced in the R500 group (Fig. 6H). This was confirmed with immunostaining when thicker sections of the central region from the control group and the R500 group were stained with collagen type I and

Fig. 2. Pentosidine (Pen) amount of different regions and ribose concentrations. Regional (A) and overall (B) Pen amount for different ribose concentrations. Pen level increased significantly after ribose treatment in all region; # shows significant differences between R500 and other groups with one-way ANOVA). There was also a significant positive correlation between overall Pen content and ribose concentration (Pearson correlation analyses). ** $p < .01$, *** $p < .001$ significantly different from the control group with one-way ANOVA.

Fig. 3. Instantaneous (A) and steady state (D) moduli (E_{Ins} and E_{St}) of different regions showed that the anterior region has the lowest elastic moduli of all regions (# $p < .01$). General ratio of elastic moduli for incubated samples versus control (B) and (E) showed a significant positive association between increased ribose concentration and elastic modulus with Pearson correlation analyses. R500 and R250 groups had significantly higher general E_{Ins} ratios than the control and R125 groups (one-way ANOVA, * $p < .05$). There were also significant positive correlations between Pen amount and both general ratios with Pearson correlation analyses (C and F).

Fig. 4. GAG amount of different regions. Regional (A) and overall (B) GAG content for different ribose concentrations. There was a significant negative correlation between overall GAG content and ribose concentration (Pearson correlation analysis). GAG content decreased significantly after incubation with R500 (one-way ANOVA, ** $p < .01$).

Fig. 5. Collagen content of different regions. Regional (A) and overall (B) collagen content for different ribose concentrations. There was a significant negative correlation between overall collagen content and ribose concentration (Pearson correlation analysis). Samples incubated with R500 showed significantly lower collagen content (one-way ANOVA, *** p < .001).

II antibodies (Fig. 7A, B). In line with the Picrosirius red stained sections (see Fig. 6) confocal microscopy revealed a disorganized orientation of the collagen fibers. Confocal microscopy images also confirmed that the disorganization in collagen fibers observed in histological sections was not due to tissue processing.

4. Discussion

The present study investigated the aging-like effect of ribose-induced crosslinking on the crosslink level of collagen and the concomitant effect on biomechanical characteristics of the TMJ condylar cartilage. Ribose incubation significantly correlated with the number of collagen crosslinks, approximated with Pen, and the compressive stiffness. The level of Pen as a measure of AGEs showed a significant positive correlation with the increasing ribose concentrations. The rise in Pen was in line with those reported in previous studies (Bank et al., 1998; DeGroot et al., 1999; DeGroot et al., 2001b; Vos et al., 2012a;

Willett et al., 2012). Bank et al. measured about 50 times more Pen in human articular cartilage after its incubation with 600 mM ribose for 86 h; the increase in Pen level measured in our study was similar to the increase they obtained in a shorter time with a comparable ribose concentration. A possible explanation for this different level of Pen could be the difference in tissue types. We used TMJ condylar cartilage; a cartilage quite different from articular cartilage: fibrocartilage versus hyaline cartilage. In other words, it contains both collagen type I and type II. In contrast to hyaline cartilage, collagen type I can be detected throughout the TMJ condylar cartilage (Almaraz & Athanasiou, 2004). Although both collagen type I and II are fibrillar collagens, Antipova and Orgel (2010) have shown that collagen type II has more possible interfibrillar crosslinking sites than collagen type I. Collagen type II has similar peptide chains in its triple helical structure with three lysine groups for interfibrillar crosslinking, whereas collagen type I has different peptide chains of which only two can provide potential crosslinking sites (Antipova & Orgel, 2010). We speculate that these

Fig. 6. Micrographs of histologic sections showing a sagittal view of the central region of control and incubated samples. A–D show sections stained with SafraninO/Fast green. The superficial fibrous layer stained in blue shows the presence of collagen fibers in this layer. This area is shown at a higher magnification in the inserted figure (40X). The micrographs show that spaces within the matrix of the cartilage increased gradually when the concentration of ribose increased. E–H represent the collagenous structure of the sections under polarized light microscope specifically in the superficial layer. Sections were stained with Picrosirius red. As the concentration of ribose increased, spaces between the collagen fibers also increased. The arrangement of collagen fibers, which was parallel to surface in the control group, changed after incubation. Arrows show the spaces appeared within the matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Confocal microscopy of the central region from the control group (A), and the central region of the R500 group (B). Thick samples, that stained for collagen type I (green), II (red), and nuclei (blue) showed disorganization in the collagen fibers specifically in the superficial layer in the incubated sample in comparison to the control. Arrows show the spaces appeared within the matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differences in collagen molecular structure may create the different results in the crosslinking with ribose. Another reason could be the difference in the tissue's exposure to sugar. As Bank and coworkers have used small cartilage plugs rather than the intact joint that was used here, the tissue plugs were exposed to ribose solution from different directions, whereas the intact condyle was exposed to the solution only from the surface. The method used in the present study is more mimicking the *in vivo* situation than the method in which tissue plugs are used.

The results of stiffness measurement depicted that E_{Ins} and E_{St} were region-dependent; a finding in line with previous studies (Lamela, Pelayo, Ramos, Fernández-Canteli, & Tanaka, 2013; Lu, Mow, & Guo, 2009; Tanaka et al., 2014). The stiffness in compressive loading is thought to be due to the presence of the negatively charged proteoglycans, which are able to trap water and thus contribute to the cartilage viscosity (Kuroda et al., 2009). A recent study has also shown that the compressive properties of the tissue are determined by the Pen amount for both artificially induced and naturally occurring crosslinks (Julkunen et al., 2009). Moreover, after ribose incubation, roughly 10% of the detected crosslinks belonged to proteoglycans (Bank et al., 1998). These findings suggested that compressive properties of articular cartilage during maturation and aging were controlled not only by proteoglycan content but also by Pen. Our results confirm that there is also such a correlation between Pen and compressive stiffness in fibrocartilaginous TMJ condyle.

During AGE formation, crosslinks are created particularly between lysine and arginine residues of collagen molecules (Sell & Monnier, 1989). It has been assumed that collagen crosslinking makes the whole collagen network stiffer; however, it has been recently indicated that it is not the collagen network but sliding capacity in the fiber at the fibril level is influenced. The fibril sliding in the fiber of tendon collagen was reduced remarkably after getting more crosslinks (Li, Fessel, Georgiadis, & Snedeker, 2013). Fessel et al. also showed that the induction of more crosslinks does not influence the stiffness of the collagen fibers in tendon, but instead alters fiber-fiber and fibril-fibril sliding which consequently influences the mechanical response (Fessel et al., 2014; Gautieri et al., 2017). The same explanation could be extrapolated to the results of TMJ condylar cartilage, in which the dominant collagen is type I, specifically in the superficial fibrous layer. This layer of the TMJ condylar cartilage plays an important role in its compressive and friction properties (Lu et al., 2009; Ruggiero et al., 2015).

The above-mentioned effects of crosslinking on fibrils are also in good agreement with our microscopic observations, which showed a dramatic change in the fibrous layer of condylar cartilage after incubation with ribose. It has been observed that inter-fibrillar crosslinks

were created due to diabetes or following incubation with a sugar, and that those crosslinks caused closer packing of several fibrils (Bai et al., 1992; Odetti et al., 2000). We therefore assumed that, due to the packing of fibrils in some areas, more Pen can result in the shrinkage in some areas and the formation of spaces in neighboring areas (arrows in Figs. 5 and 6).

It is known that in OA, the collagen network is damaged at early stages; this damage can further lead to proteoglycan loss. Therefore, structural changes of collagen related to the increase in Pen level as shown here may result in the onset or progression of OA as it has been shown by De Groot et al. (DeGroot et al., 2004). In contrast, at the late stage of OA, the amount of AGEs was inversely correlated with cartilage damage, most probably due to an altered collagen and proteoglycan turnover rate in OA (Vos et al., 2012b).

Surprisingly, when Pen increased, a reduction in the amount of GAG as an indicator of the level of proteoglycans was observed. However, this reduction in GAG content did not reflect the aging-associated reduction in GAG which has been previously shown (Kuroda et al., 2009). Firstly, the water content which has a direct correlation with the amount of proteoglycans did not change (see Supplementary material). Secondly, it has been shown that after the creation of crosslinks in cartilaginous matrix, about 10% of detected Pen in the tissue was derived from proteoglycans (Bank et al., 1998). This means that proteoglycans were also involved in crosslinking reactions which could result in a lower digestibility. Therefore, we assume that the decrease in the amount of GAG was due to differences in the digestibility of crosslinked proteoglycans. The same reasoning as mentioned above for GAG reduction can apply to the observed reduction in collagen because the molecular solubility of collagen decreases by an accumulation of AGE (Wilson et al., 2014). Kulmala et al. have also reported a reduction in the collagen content after incubation of the articular cartilage with threose (Kulmala et al., 2013). Crosslinking has also been shown to decrease the susceptibility of articular cartilage to enzymatic digestion (DeGroot et al., 2001b; Ghazanfari, Driessen-Mol, Bouten, & Baaijens, 2016).

It has been demonstrated that compressive properties of the superficial layer in the TMJ condyle play an important role in interstitial fluid pressurization (IFP). IFP provides around 90% of tissue support under applied load (Ruggiero et al., 2015). AGE production reactions modify free amino groups of the collagen molecules and other proteins in the tissue matrix (Sell & Monnier, 1989). These modifications change the fixed charge density and its distribution within the matrix, thereby influencing the tertiary structure of collagen molecules, the interactions of collagen with other proteins and water, and the fluid pressure resulting from fixed charge density in the matrix (DeGroot et al., 1999). Our results demonstrated a clear increase in compressive stiffness,

concomitant with a rise in Pen level as well as structural changes in the fibrous layer of the condyles. The potential correlation between our finding in this study and the fixed charge density of proteoglycans, permeability, IFP, and loading distribution within the condylar cartilage provides more insights into the mechanism behind the aging-like effect of crosslinking on tissue function.

Although our study provided insights into biomechanical and biochemical consequences of AGE formation in the TMJ condylar cartilage, it had some limitations that should be considered. First, ribose used in this study can induce only an effect of collagen crosslinking. Although this method has been used widely, there are several other parameters which can influence the mechanical responses in the cartilaginous matrix. Moreover, there are some contradictory results which noted that crosslinking had a different effect than aging on collagen structure. Belkacem et al. have found that ribose incubation increased collagen fibril diameter, while aged samples have shown an overall reduction in their collagen fibril diameter (Ait-Belkacem et al., 2012).

Second, the condyles used in the present study were from young pigs in maturation stage, which undergo enzymatic crosslinking. Even though the enzymatic and non-enzymatic reactions have different routes, it might have an influence on crosslinking of the collagen molecules. The obtained results were not compared with results from naturally aged samples. Such a comparison can give a better insight into the differences and the similarities of the aging model in this study on biomechanical responses.

Thirdly, the increase in the collagen crosslinking correlated significantly with moduli ratios in our study. However, the individual regions did not show a significant increase in modulus both for steady state and instantaneous. Since the moduli of the R500 samples were always higher than for the other groups, it can be the small number of samples used for the experiments which did not result in significant differences.

Finally, some assumptions were made in mechanical characterization which can influence the results, e.g., the average of the last five reaction force cycles was used to calculate the steady state modulus. In addition, the edge effect of indenter on measured stiffness was neglected assuming that the effect is the same in all experiments.

In conclusion, an *ex vivo* crosslinking method was used to stimulate AGE formation and to examine the effect of crosslinking on biomechanical properties of the TMJ condylar cartilage without other age-related alterations. Incubation with ribose correlated significantly with the amount of Pen and stiffness. The accumulation of AGEs not only influenced the compressive properties of the tissue but also the structure of the tissue itself. Although our approach does not mimic all aspects of aging, it can be considered as a practical model for simulating age-related (mechanical) changes in the TMJ condyle. Our findings are important since TMJ condyle has a unique structure among other load-bearing articular joints in which tissue matrix contains collagen type I throughout the cartilage.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.archoralbio.2017.12.010>.

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