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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 (Aït-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, Lafeber, 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, & Grynpas, 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 (Athanasiou, 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, & Athanasiou, 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