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Aging does not change the compressive stiffness of mandibular condylar cartilage in horses

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Running headline: Aging does not stiffen fibrocartilage

Abstract

Objective: Aging can cause an increase in the stiffness of hyaline cartilage as a consequence of increased protein crosslinks. By induction of crosslinking, a reduction in the diffusion of solutions into the hyaline cartilage has been observed. However, there is a lack of knowledge about the effects of aging on the biophysical and biochemical properties of the temporomandibular joint (TMJ) cartilage. Hence, the aim of this study was to examine the biophysical properties (thickness, stiffness, and diffusion) of the TMJ condylar cartilage of horses of different ages and their correlation with biochemical parameters.

Materials and methods: We measured the compressive stiffness of the condyles, after which the diffusion of two contrast agents into cartilage was measured using Contrast Enhanced Computed Tomography technique. Furthermore, the content of water, collagen, GAG, and pentosidine was analyzed.

Results: Contrary to our expectations, the stiffness of the cartilage did not change with age (modulus remained around 0.7 MPa). The diffusion of the negatively charged contrast agent (Hexabrix) also did not alter. However, the diffusion of the uncharged contrast agent (Visipaque) decreased with aging. The flux was negatively correlated with the amount of collagen and crosslink level which increased with aging. Pentosidine, collagen, and GAG were positively correlated with age whereas thickness and water content showed negative correlations.

Conclusion: Our data demonstrated that aging was not necessarily reflected in the biophysical properties of TMJ condylar cartilage. The combination of the changes happening due to aging resulted in different diffusive properties, depending on the nature of the solution.

Key words: Cartilage, stiffness, diffusion, aging, temporomandibular joint

Introduction

The temporomandibular joint (TMJ) of the jaw is composed of the temporal fossa, mandibular condyle, and articular disc. It is a synovial, bilateral joint with a unique morphology and function. Unlike hyaline cartilage in other articular joints with collagen type II as the dominant collagen, condylar cartilage in the TMJ is a fibrocartilaginous tissue which contains both collagen type I and type II. It has a dense superficial layer of fibrillar collagen type I. Condylar cartilage absorbs and distributes shear, tensile, and compressive forces during rotational and translational jaw movement. This cartilage is a secondary cartilage and the center of growth in the mandible [1, 2]. During aging, gradual structural and compositional changes occur in TMJ cartilaginous extracellular matrix (ECM) [3-5]. ECM changes have been reflected in the biophysical and mechanical performance of hyaline cartilage [5, 6].

Similar to other articular cartilage, TMJ condylar cartilage consists of three main constituents, i.e., collagen, proteoglycans (PGs), and water which together create the functionality of the matrix. Each of these components changes due to aging. The bulk of the dry weight of the cartilage is *collagen* [7]. The prominent functional feature of collagen is maintaining tissue integrity and stability under tensile loading [8, 9]. Collagen fibers are oriented parallel to the cartilage surface at the superficial layer of TMJ condyle [10]. The superficial layer controls the mechanical response of hyaline and fibrocartilage [11, 12] due to its influence on the interstitial fluid pressurization. Cartilaginous collagen has an extremely slow turnover rate; as a consequence, non-enzymatic crosslinks between collagen molecules result in accumulation of advanced glycation end products (AGEs) in cartilage with increasing age [13, 14]. One of these AGEs, pentosidine, may serve as a well-characterized and reliable measure of AGEs; it has been reported abundantly present in articular cartilage. The accumulation of AGEs leads to stiffening of hyaline cartilage with aging [9, 14]. *Proteoglycans* (PGs), the second abundant component of cartilage, are

considered to contribute to the compressive stiffness of cartilage due to their negatively charged glycosaminoglycan (GAG) chains [15, 16]. The resultant highly negative fixed-charge density (FCD) also provides a hydrophilic environment with a water content of up to 80% which reduces with increasing age [8]; however, the total amount of PG does not change remarkably during aging [3]. The changes happen in the composition, structure, and hydrodynamic size of PGs during aging [15, 17, 18]. Next to the structural role of PGs, they also contribute to nutrient and solute transport in cartilage due to their highly hydrated nature [18].

Articular cartilage is an avascular tissue in which the transport of metabolites depends mainly on diffusion [19, 20]. PGs and water content strongly contribute to the permeability and diffusion [20]. Yet, little is known about the effect of aging-associated changes on the diffusive properties of cartilage. This knowledge is considered also to provide valuable information for treatment and regeneration strategies. It has been shown that when the number of crosslinks increases, the hyaline cartilage not only becomes stiffer, but it becomes also less diffusive to anionic contrast agents [21, 22]. However, there are other compositional and structural changes in aging cartilage beside crosslinking that can influence diffusion. To the best of our knowledge, there is no study on the alteration of cartilage diffusion with natural aging in TMJ condylar cartilage. Therefore, the aim of this study was to investigate the effect of aging-associated changes on tissue biomechanics and diffusion, and their correlation with biochemical composition of mandibular condylar cartilage. We hypothesized that when the TMJ condylar cartilage becomes older, it gets more crosslinks, becomes stiffer and less diffusible. In the present study, we collected equine samples from different ages. Horse is one of the large animal models which has been widely used in orthopedic research [23, 24]. Different ages of equine samples are easily accessible, and the thickness of their cartilage is close to that in humans [23, 25]. To evaluate our hypothesis, we determined

the mechanical properties of the cartilage under compression. Next, we measured the diffusion of a negatively charged (Hexabrix) and an uncharged (Visipaque) contrast agent into TMJ cartilage of horses in different ages, using contrast-enhanced computed tomography (CECT). . Finally, the biochemical and structural changes of the tissue matrix were assessed. The correlations between age and biophysical and biochemical properties of the tissue were then examined.

Materials and methods

Sample preparation

Ten equine heads from different ages were obtained from the faculty of veterinary medicine of the University of Utrecht, the Netherlands (gender not specified). The animals had been sacrificed to serve as educational specimens in dissection lectures, hence, for a reason other than the present study. Their use was according to the ethical standards of the University of Utrecht. The age of the animal, as estimated from their teeth, ranged between 2.5 and 18 years old. Right and left condyles were dissected from the temporomandibular joints. The quantitative measurements of both condyles at each age were averaged since the aging effect was the main concern of this study. The thickness of the cartilage in the central region of condyles was measured using μCT scanning of the cartilage covered with 20% barium sulfate in agarose gel, as described in a previous study [26]. Knowing the thickness, strain-control cyclic compression was applied to the central region of condyles for calculating the cartilage stiffness. Afterward, for the diffusion tests, cylindrical osteochondral plugs (diameter 6 mm) were drilled out from frozen condyles. Sagittal tissue slices with a thickness of about 3-5 mm were cut from the adjacent area for histological staining using a band saw. Surrounding tissue of the cartilage plugs was collected for

biochemical analysis as shown in Figure 1A. The plugs were stored at -20˚C in PBS enriched with a protease inhibitor cocktail (PBS-PI [27]) and sodium azide 0.02% prior to diffusion test.

Suggested spot for Figur 1

Mechanical testing

To determine the stiffness of the central region of the intact condyle, cyclic unconfined compression loading tests were performed by using a custom-made instrument as described previously [28]. Briefly, a solid cylindrical indenter (diameter 4mm) applied strain-control cyclic displacements of the cartilage and simultaneously the compressive reaction force was recorded. A tare load of 0.2 N was introduced to the region of interest. Then, 60 cycles of 1% strain at a frequency of 1Hz were applied as preconditioning; after 5 min relaxation time, a strain level of 5% was applied for 20 cycles at 1Hz. Instantaneous modulus (E_{Ins}) was calculated from the peak force of the first cycle of loading. Under cyclic loading, a steady-state response is usually reached within 10 cycles [29]. Therefore, steady state modulus (E_{St}) was calculated from the peak force average of the last five loading cycles. As compressive stiffness is different topographically across the condyle [28], only the central region was tested from all the condyles. The stiffness of right and left condyles of each head were averaged and reported for each age.

Contrast enhanced computed tomography (CECT)

For diffusion measurements, each plug was thawed in PBS-PI at room temperature. The lateral sides of the plug were sealed using a thin layer of cyanoacrylate (Histoacryl, Braun Surgical S.A., Rubi, Spain),

followed by wrapping in parafilm to allow diffusion only from the surface (Figure 1B). Diffusion of two contrast agent with different electric charge (negative and uncharged) was evaluated for each plug. The order of contrast agents' diffusion was chosen randomly and was kept identical for all the samples. Hexabrix solution containing ioxaglate (Hexabrix, 1269 g/mol, charge = -1, GE Healthcare, Netherlands) was prepared with a concentration of 0.08M in PBS-PI. After the diffusion test with Hexabrix, the plug was washed out for 48h with PBS-PI at 4˚C with several changes of solution. Thereafter, the plug was used for the diffusion of Visipaque, i.e., a neutral contrast agent. Visipaque solutions that contained iodixanol solutes (Visipaque, 1550.191 g/mol, charge=0, GE Healthcare, Netherlands) were used without any dilution with a concentration of 0.42M. Images were acquired using a µCT apparatus (Bruker SkyScan 1272, SkyScan, Kontich, Belgium) with an isotropic voxel size of 10µm with 66 kV tube voltage and 166 µA current at 18 time points. Average gray index for the whole cartilage thickness was calculated at each time point using µCT software (CTAn version1.16.4.1, SkyScan). A volume of interest from the reconstructed image was chosen with the same software as to include the whole thickness of the cartilage (Figure 1C). Gray values of cartilage at time zero were taken as zero concentration of the contrast agent. Contrast agent concentration within the cartilage was normalized to the bath concentration which was taken as 100%. The concentration gradient of the bulk at different time points was used to define the flux through the cartilage surface with the following formula:

$$J = - h \, \partial C / \partial t$$

where J is the flux, h is the cartilage thickness, C is the bulk concentration within the cartilage, and t is time.

Biochemical analyses

To examine the effect of aging on the tissue constituents, the tissue that surrounded the plugs was weighted and subsequently freeze-dried for 24h. Wet and dry weights were used for calculating water content. Dried tissue samples were digested with papain and the amount of GAG was measured with a colorimetric assay using dimethyl methylene blue. Hydroxyproline (Hyp) amount, for the assessment of collagen content, and pentosidine (Pen) amount, as a measure of the number of collagen crosslinks, were assessed after hydrolyzing the digested solution with 6M HCl. The amount of Hyp was measured following neutralization and a reaction with chloramine-T and dimethyl amino-benzaldehyde [30]. The collagen content was determined assuming that Hyp comprises approximately 10% of the collagen weight [31]. For the determination of the Pen level, high-performance liquid chromatography was used as described in detail elsewhere [32-34].

Microscopy

The sagittal slices of tissue from the central region were fixed in 4% formalin and decalcified with neutral EDTA for 6 weeks. Sections with a thickness of 10 μm were stained with SafraninO/Fast green (SafO/FG) and Picrosirius red (Picro). The structure of the cartilage was visualized using light microscopy after SafO/FG staining and collagen fibers spatial alignment was visualized using polarized light microscopy.

Statistical analyses

Non-parametric Spearman rank correlation was used to analyze correlations between aging and other quantified parameters. Quantified parameters are presented as a mean value of right and left condyle for each horse. The statistical tests were carried out using SPSS 23 for Windows (SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

Results

Mechanical testing

The thickness of cartilage in the central region of the condyle for each age (pooled right and left) is presented in Figure 2. The thickness decreased from 0.8 mm for the youngest sample (2.5 years old) to 0.27 mm for the oldest one (18 years old). Correlation analyses showed a statistically significant negative correlation between age and the thickness.

Suggestedspot for Figure 2

The compressive stiffness of the TMJ condylar cartilage in the central region was measured under cyclic indentation. It appeared that the stiffness did not change substantially with age. No statistically significant correlation was observed between age and both E_{Ins} and E_{st} (Figure 3).

Suggested spot for Figure 3

Contrast enhanced computed tomography (CECT)

Figure 4 shows the normalized concentration and flux of Visipaque with the time of diffusion for samples from horses of 3 and 18 years old. As can be seen in Figure 4 A, normalized concentration increased sharply in the early time points, and reached a plateau after around 10 h and equilibrated afterward. Flux, which showed the concentration changes over time in the thickness of the diffusing substrate, showed a remarkable decline in the first hour of diffusion (Figure 4 B).

Suggested spot for Figure 4

Normalized concentration shows the accumulation of diffusing solution into the cartilage which was then compared after equilibrium was reached at 48h for both contrast agents (Figure 5 A, B). Flux defines the rate of the solution diffusing which is an indication of the slope of normalized concentration curves, most importantly at the early time after 15 min of diffusion (Figure 5 C, D). The normalized concentration of Visipaque in younger samples (2.5, 3, 4 years old) was slightly higher than that in other ages; however, no statistically significant correlation was found between normalized concentration and age for either contrast agents. The correlation analyses showed a statistically significant negative correlation between Visipaque flux and age (Figure5 D). In contrast, no significant correlation was found between Hexabrix flux and age (Figure 3B).

Suggested spot for Figure 5

The correlation analyses between age and diffusion parameters when controlling for biochemical factors are shown in Table 1. The correlation between Visipaque flux and age disappeared with a dramatic decline whilst we control for the influence of collagen content. Such an effect with a minor decline was also observed when we control for the effect of Pen form the correlation analyses between Visipaque flux and age. The correlation between Visipaque flux and age was not affected with GAG and water content as the partial correlations did show noticeable change. The correlation coefficient of -0.855 turned to -0.935 whilst controlling for GAG and to -0.802 whilst controlling for water content.

Suggested spot for Table 1

Biochemical analyses

The results of the biochemical analyses of the samples of different ages are presented in Figure 6. When the horse TMJ condyle became older, collagen content significantly increased (Figure 6 A, rho= -0.952, $p<0.001$). The collagen content increased from around 67% in the young samples to 94% in the oldest one. The amount of GAG increased with advancing age, as shown with the positive correlation coefficient (Figure 6 B). A similar trend was also observed for Pen, while water content decreased with age Figure 6 C, D). Collagen crosslinks measured with Pen increased around 15-fold with age.

Suggested spot for Figure 6

Microscopy

As shown in Figure 7A, the cartilage became thinner with increasing age. The amount and the distribution of collagen and GAG in the SafO/FG stained sections demonstrated a series of age-related alterations. For instance, the young samples stained more intensely red, thus indicating a high level of GAG content; the young adult and middle-aged samples had less red staining and therefore likely contained less GAGs. In the young samples, the distribution of the GAGs was more uniform, and throughout the cartilage except for the superficial zone. With aging, chondrocyte clusters increased within the cartilage, and loss of GAG staining in the 1/3 upper part of cartilage was observed. During the preparation of the samples for histology, it was observed that the superficial layer of the cartilage easily detached from the underlying part in the samples of young ages, particularly in those of 2.5 years old. This phenomenon can be observed in Figure 7A, indicated by black arrows. The structure and the thickness of the fibrous superficial layer also varied notably by age. The waviness of collagen fibers decreased by advancing age, while the fibers became thicker and more packed (Figure 7B).

Suggested spot for Figure 7

Discussion

This study investigated the effect of natural aging on biophysical properties of equine TMJ condylar cartilage and their correlation with their biochemical components. Our results showed that when the condylar cartilage became older, the stiffness of the TMJ condyle did not increase significantly, whereas the number of collagen crosslinks remarkably increased. However, the diffusion of the neutral contrast

agent reduced with aging. The correlation between diffusion and age for Visipaque was strongly influenced by collagen content and crosslink level.

Age-dependent alteration of the TMJ condylar cartilage included the change in its thickness**;** it decreased significantly with aging. Our results are in accordance with a reduced thickness observed in hyaline cartilage [6, 35, 36] and also in the mandibular cartilage [37, 38]. Rodriguez et al. have reported a larger thickness for immature hyaline cartilage in horses than that of mature animals [36]. Moriyama et al. suggested that the tidemark advancement that happened by aging lead to eventual thinning of cartilage [35]. An apparent decrease in the cellularity of the cartilage as seen in the histological staining can also result in the cartilage thickness reduction due to the lack of proliferation capacity of chondrocytes [18]. Our results proved that there was a decline in the diffusion of Visipaque with aging. Thereby, the lack of enough metabolite transport from the cartilage surface might result in blood vessel invasion and subchondral bone advancement [39].

One of the most prominent age-associated changes in cartilage is the increase in the number of collagen crosslinks [9, 14]. As expected, we measured a significantly higher Pen level in the samples of older horses as compared to that of younger ones, but our finding in this study did not show any statistically significant increase in the stiffness with aging (Figure 3). We previously showed that incubation of porcine TMJ condyles with ribose as an aging-effect simulation increased Pen level and consequently the stiffness of the cartilage [28]. Positive correlations between Pen increase on stiffening of cartilage has been also shown in natural aging both in hyaline cartilage and in fibrocartilage of TMJ disc form different species [6, 14, 29].

The origin of the seemingly contradictory findings could be related to several parameters that have changed with aging, e.g. Pen level, GAG, and the region of the cartilage. Firstly, in a previous study, we measured a 50 fold increase in Pen levels leading to a 1.5 fold increase in stiffness [28]. The crosslink level of naturally aged samples in this study was only 15-fold higher than the young samples. It is likely that the Pen increase in the older equine condyle of our study could not result in significant changes in the contribution of collagen network under compression. Secondly, it is well known that the compressive stiffness of the cartilage depends not only on GAG content [18, 40] but also on the amount of sulfation and ratios of different GAGs [15, 18, 41]. With advancing age, the amount of keratan sulfate (KS) increased and chondroitin sulfate (CS) decreased. Older aggrecan becomes weaker under compression as they have a lower proportion of CS/KS than younger ones [17]. The results of our biochemical analyses for GAG content showed no significant difference with increasing age, which is in line with previous studies on the equine [3, 42] and human [43]articular cartilage. However, our microscopy images clearly showed changes in the distribution and intensity of stained GAG within the matrix. Therefore, we can conclude from the above-mentioned reasons that the changes in GAGs have been likely compensated with Pen increase with aging which resulted in the same mechanical response. And finally, TMJ cartilaginous matrix dynamically changes to adapt to loading with aging [4]. TMJ condylar cartilage undergoes diverse loading patterns in different regions which results in regional-dependent stiffness [16, 28]. Each region might change differently with aging. However, it was shown in equine articular cartilage that the stiffness of one region increased significantly while the stiffness in another region showed minor changes with aging [44].

The diffusive properties of cartilage depend on the size of the diffusing molecule and the biochemical and physical structure of the cartilage matrix [45]. Together, these contribute to the diffusion alterations

through two main properties i.e., steric hindrance and/or electrostatic effects [21, 22, 46, 47]. In this study, Pen, Collagen, and GAG showed positive correlations with age concomitant with a negative correlation between water with age (Figure 6). These changes are all in favor of reducing the diffusion. Visipaque showed a reduced diffusion with an increased aging as we expected. In contrast, Hexabrix diffusion did not change notably with aging. Since Hexabrix has a similar molecular weight as Visipaque, the most important factor in creating the difference between these two contrast agents was the electrostatic interactions [22]. Visipaque is a neutral contrast agent of which diffusion is mainly controlled by steric hindrance. As correlation analyses also revealed, collagen content strongly influenced the correlation between age and diffusion (Table 1). Thereby, the young samples with higher water content and lower collagen and Pen level showed a higher flux and normalized concentration. On the other hand, electrostatic interactions within cartilage matrix are controlled through the GAGs molecules as well as Pen level [21, 22, 48]. Age-related changes in collagen crosslinking have been shown to lead to increased fix charge density while it reduces sulfation of GAGs and the total of their negative charge [15, 18, 41, 48]. Our microscopy images also confirmed that the overall distribution of GAGs changed remarkably during aging. Although the biochemically assessed GAG content showed a slight increase, the upper 2/3 of cartilage showed hardly any GAG.

We studied several aspects of aging in equine TMJ condylar cartilage. Yet, there is some limitation related to our study. First, we studied only one region of the condyle. The TMJ condyle experiences different loading pattern in each region. Second, we only applied the compression testing while the condyle also experiences shear and tensile in which tissue can differently respond with aging. And third, we did not measure the charge density and the changes in ECM sub-molecules. For instance, hyaluronan, which extensively changes with aging [49], has shown an important role in diffusion [50]. Further

investigation of distribution and content of different collagen types as well as GAG sub-chains with aging can reveal valuable information about the contribution of these components in the biophysical response of cartilage.

In conclusion, we measured a reduction in water content and in thickness of equine TMJ condylar cartilage with aging, whereas collagen content and Pen level increased. This combination of biochemical alterations resulted in a similar response under compressive loading and diffusion of Hexabrix into the cartilage in all ages. These findings demonstrated that the changes happening in aging mandibular condylar cartilage are not similar to that of hyaline cartilage. Our hypothesis of a reduction of diffusion with aging was confirmed using an uncharged contrast agent implying that the neutral nutrition exchange might be more affected with aging. For future work, investigating such effects in diffusion of different agents (negative, neutral, and positive) into pathological situations, and in hyaline cartilage by aging can provide relevant insight into clinically relevant topics.

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Author contributions

F.M., J.H.K., G.H.L., F.L. and V.E., contributed in the conception and design of the study; F.M. conducted the experiments, analyzed the data, and wrote the manuscript text. J.S. and R.S. contributed in biochemical measurements and interpretation. F.M., J.H.K., S.F., G.H.L., F.L., and V.E. contributed in the interpretation of the data. H.W. and V.A. contributed in the interpretation of diffusion data. All authors contributed in critical revision of the article and final approval of the version to be published. J.H.K. takes the responsibility for the integrity of data.

Conflict of interest

There is no conflict of interest related to this study.

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Figure 1: Tissue preparation steps. (A) An osteochondral plug (Ø = 6mm) was drilled from the central region of the condyles for diffusion test, a sagittal slice was taken for histology, and the surrounding tissue was used for biochemical tests. (B) Sagittal view of a plug in µCT holder for diffusion test. (C) Pseudo-colored image of the contrast enhanced computed tomography of an osteochondral plug at zero hours and 48h. The attenuation of cartilage increased after diffusing of the contrast agent.

Figure 2: Cartilage thickness of the central region of condyles in relation to age. Each value represents the mean thickness of right and left condyle per horse. The thickness of the cartilage declined sharply from the younger to the older ones. A significant negative association was found between age and thickness (rho: Spearman's rank correlation coefficient).

Figure 3: Stiffness of condyles versus age, expressed as (A) the instantaneous (E_{Ins}) and (B) steady state (E_{St}) modulus. Spearman's rank correlation analyses showed no significant changes in the stiffness of the samples with age.

Figure 4: Representative graphs of diffusion measurements within 48 h. (A) Normalized concentration and (B) diffusive flux of Visipaque by time from samples at age of 3 and 18 years old. The concentration of the solution in the cartilage increased rapidly in the first time points and it reached equilibrium thereafter.

Figure 5: Diffusion measurements for Hexabrix and Visipaque. (A) Normalized concentration of Hexabrix and (B) Visipaque at the equilibrium after 48 h of diffusion. Normalized concentration of Hexabirx did not change with age. In case of Visipaque, a decreasing trend by advancing age was observed, although it was not statistically significant. Diffusive flux of (C) Hexabrix and (D) Visipaque after 15 min of diffusion. Visipaques flux showed a sharp decline with age.

Figure 6: Biochemical composition versus age and collagen content (A), GAG content (B), water content (C), and pentosidine (D) level of samples. Collagen, GAG, and Pen level showed significant positive correlations with advanced aging while water content was negatively correlated.

Figure 7: Histology of equine condylar cartilage of different age (staining with SafO/FG). In the young samples (upper row), a more uniform distribution of GAGs was apparent. There is an increasing amount of blue-stained matrix (primarily collagen and non-GAG components) with age. The intensity and distribution of GAG (red) varied considerably among the samples, with the highest amount found in the young samples. The structure becomes more compact with age; particularly in the superficial collagen

layer. Black arrows indicate the detachment of the superficial layer during sample preparation. (black scale bar: 300 µm, labels in white boxes indicate the age of the horses).

(B) Orientation and thickness of the collagen fibers in relation to age (staining with Picro staining). Polarized microscopy revealed orientation and thickness of collagen fibers in the superficial layer became more packed and oriented in parallel to the surface when the condyles get older (white scale bar 200µm).

Table 1: Correlation coefficients between diffusion parameters and age, and its partial correlations when controlling for biochemical contents. Spearman's rank correlation coefficients are presented for normalized concentration (Nor. Conc.) at the late time point (t=48 h) and flux at early time point (t=15min). Significant association was found between Visipaque flux and age. P values are presented in brackets for each correlation. (Degree of freedom equals 7 for all partial correlations).

	Hexabrix		**Visipaque**	
	Flux (t=15min)	Nor. Conc. (t= 48h)	Flux (t=0.25 h)	Nor. Conc. (t= 48h)
Age	-0.624 (0.054)	0.212 (0.556)	**-0.855**** (0.002)	-0.539 (0.108)
Controlling for:				
Collagen	-0.217 (0.575)	0.072 (0.853)	-0.195 (0.615)	0.177 (0.649)
GAG content	-0.480 (0.191)	-0.379 (0.315)	-0.934*** (0.000)	-0.751* (0.020)
Water content	-0.318 (0.404)	0.162 (0.678)	-0.802** (0.009)	-0.139 (0.722)
Pen	-0.356 (0.346)	0.746* (0.021)	-0.644 (0.061)	-0.068 (0.861)

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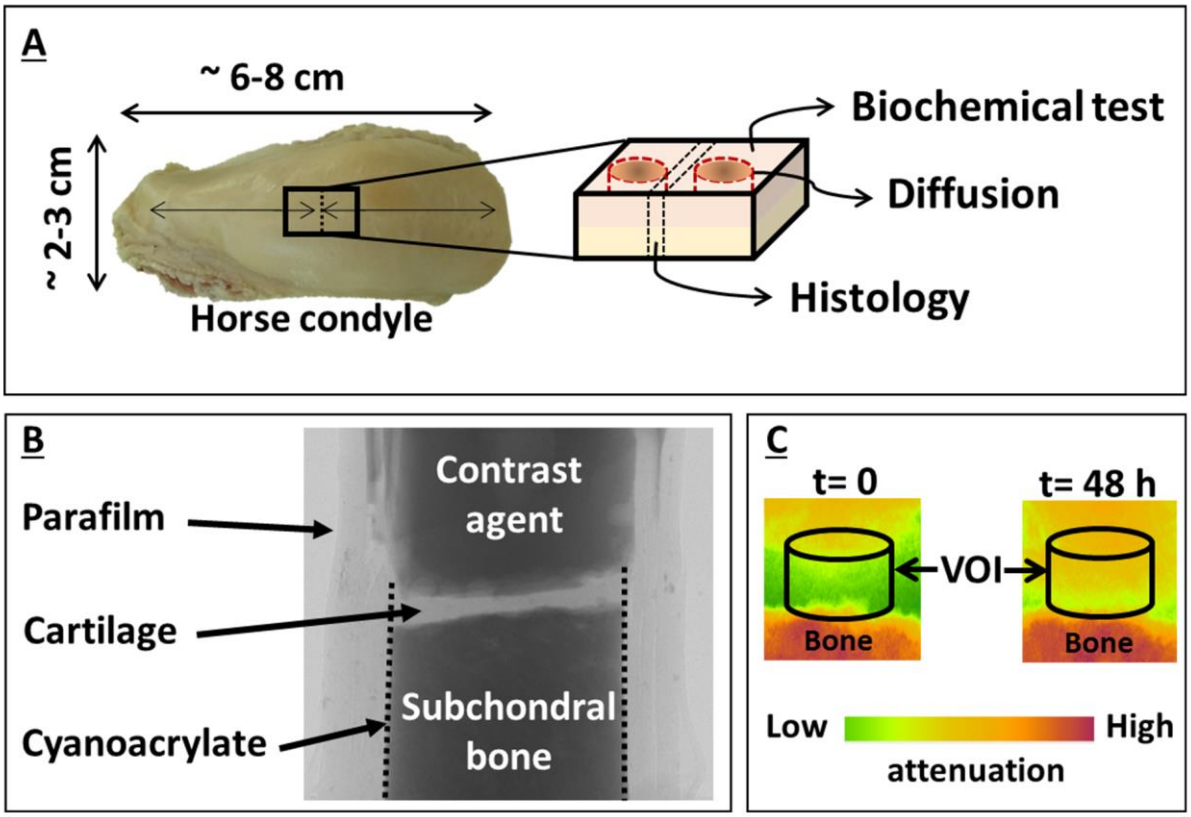

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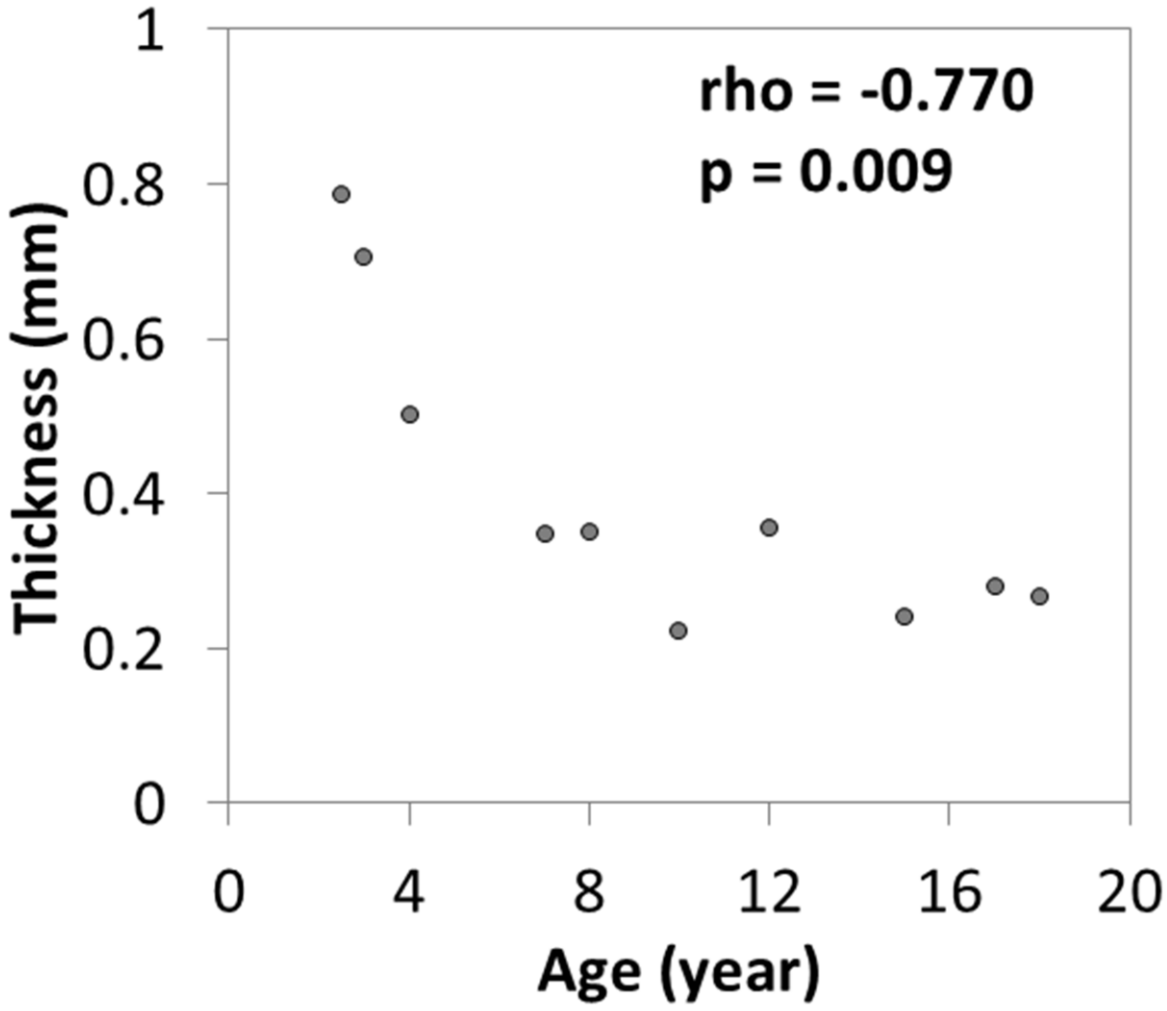

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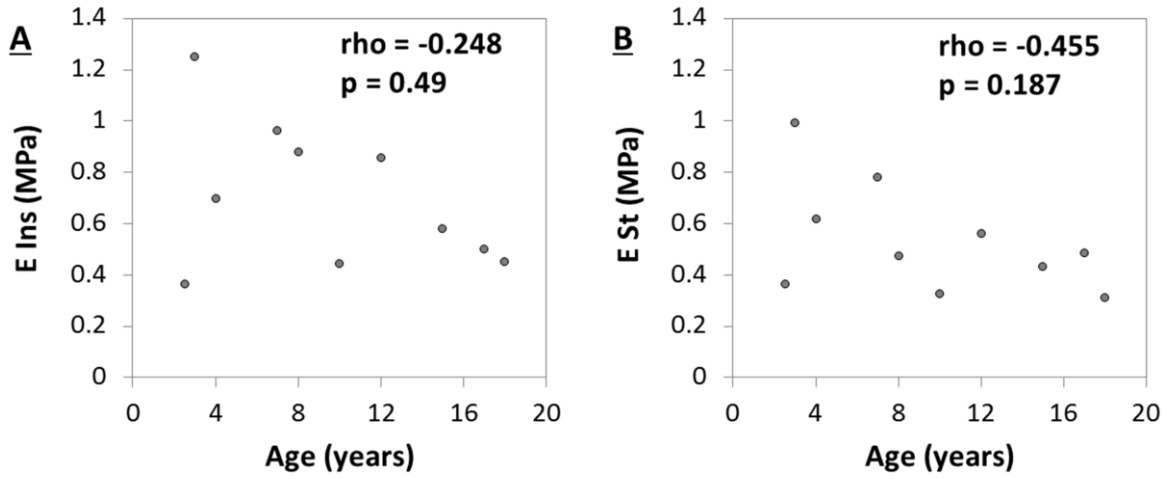

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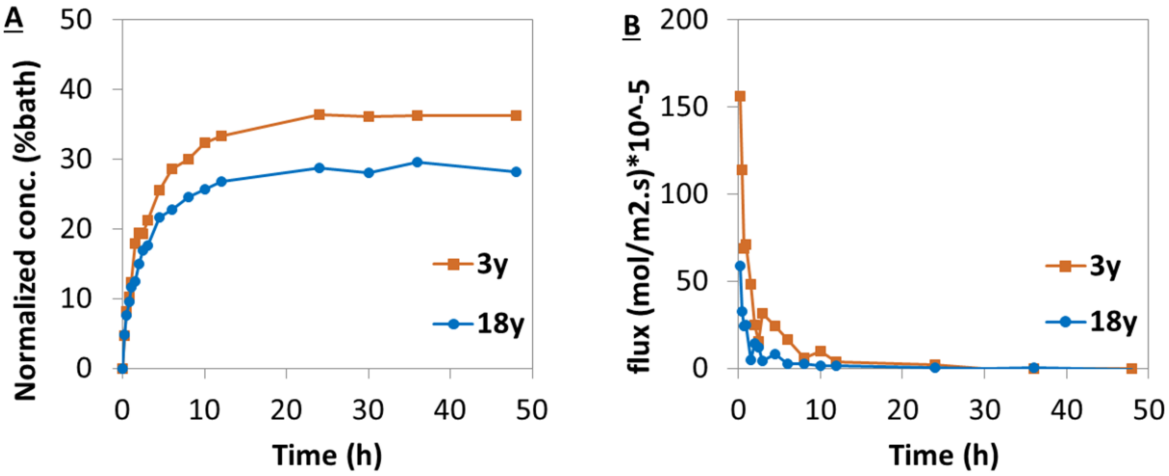

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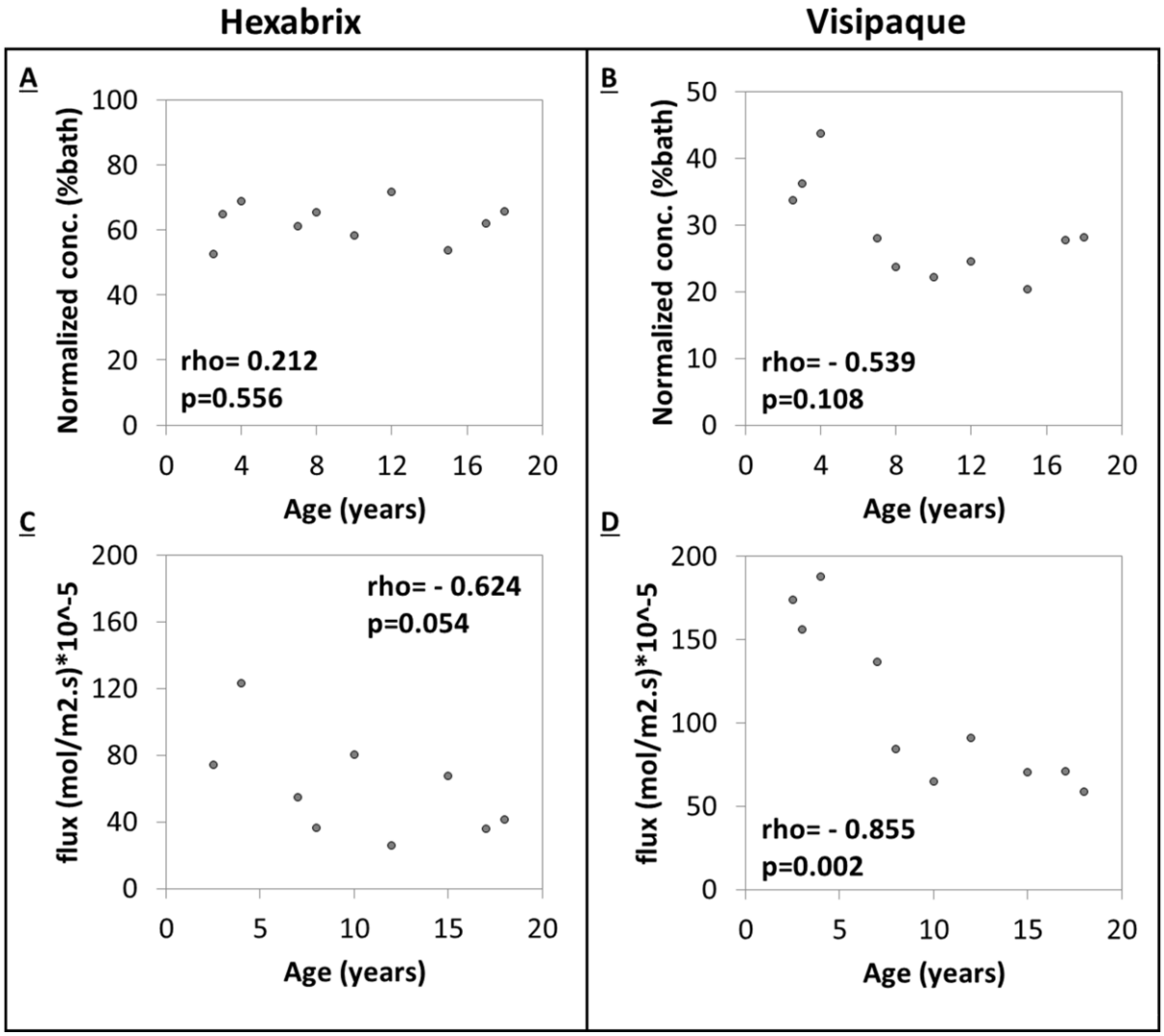

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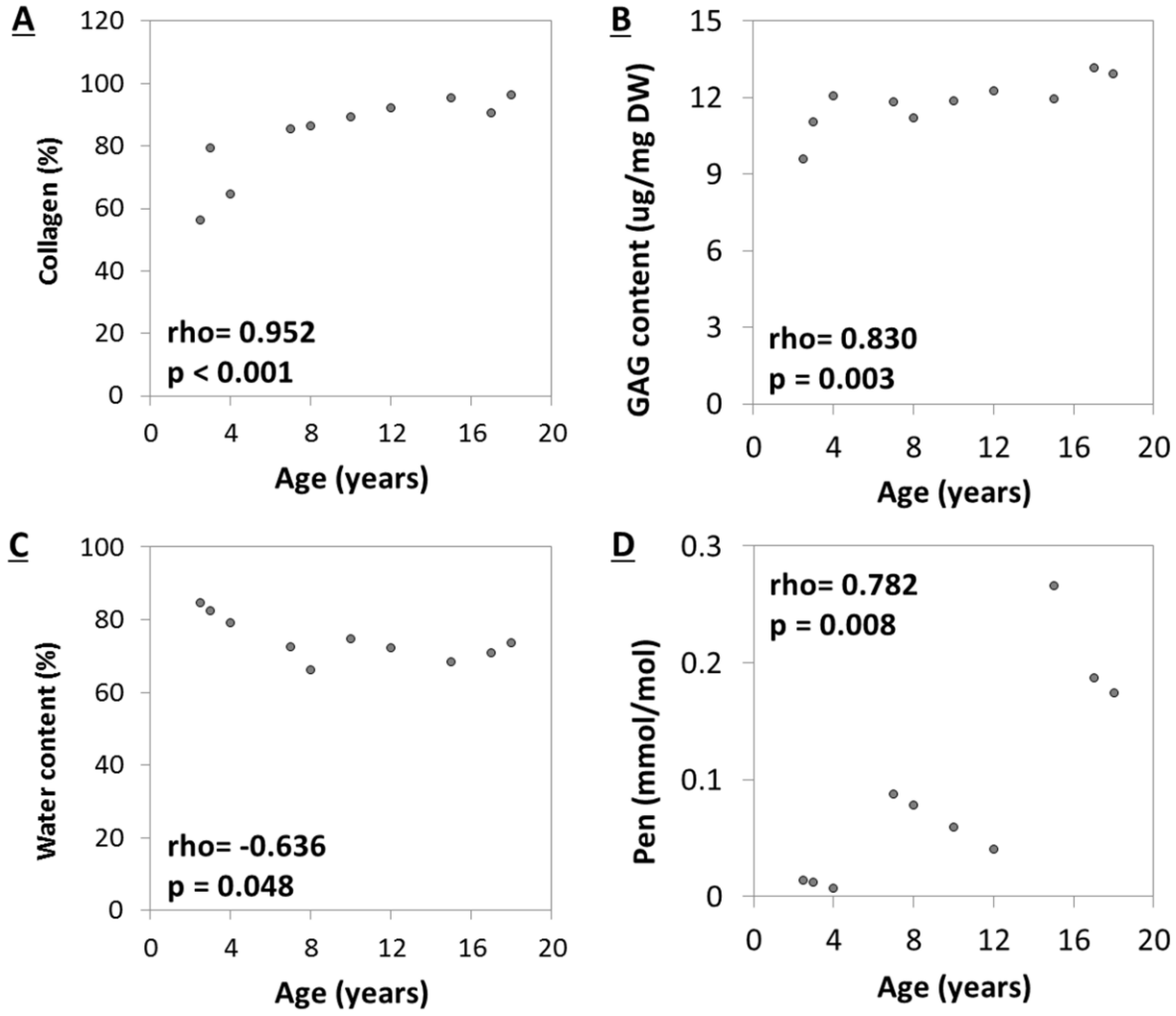

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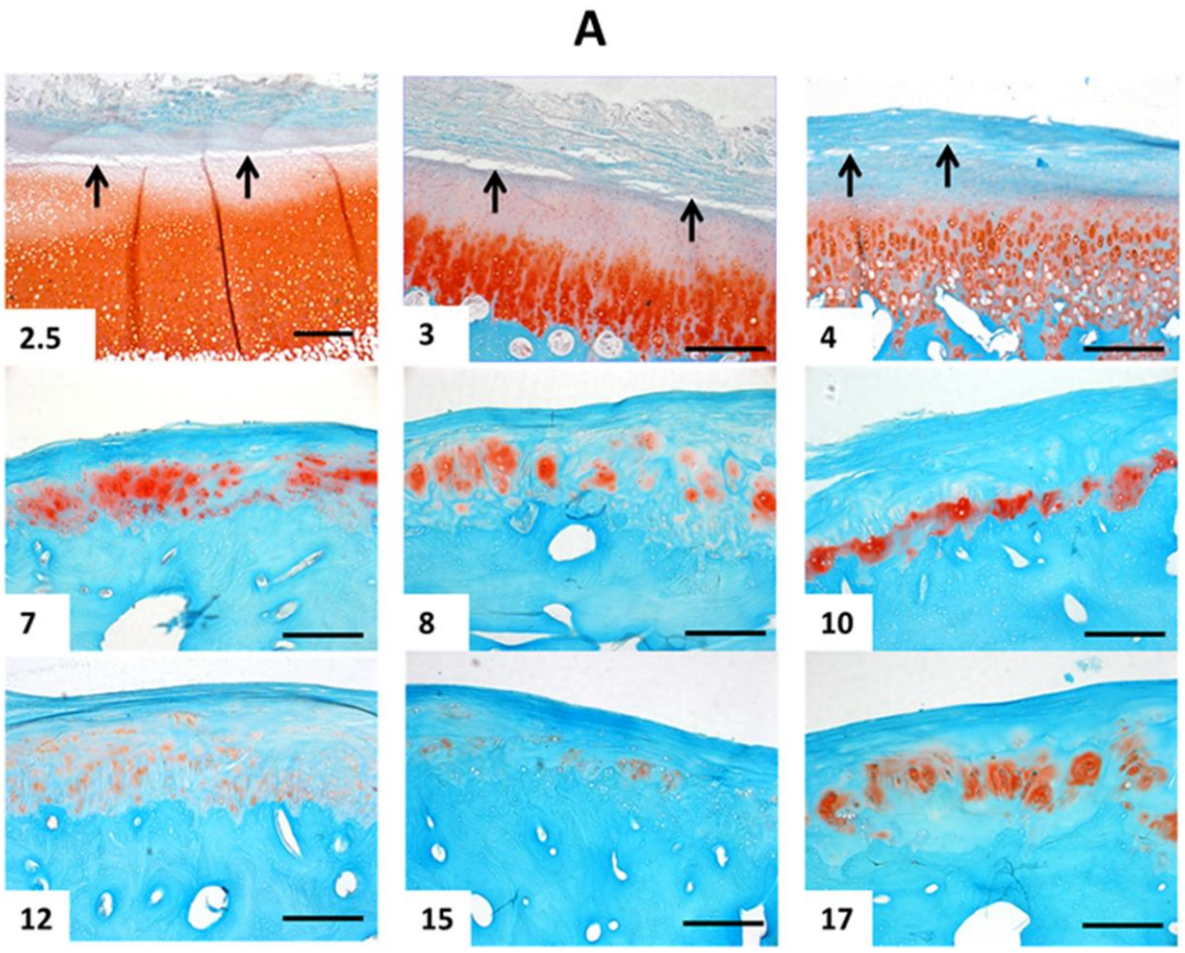
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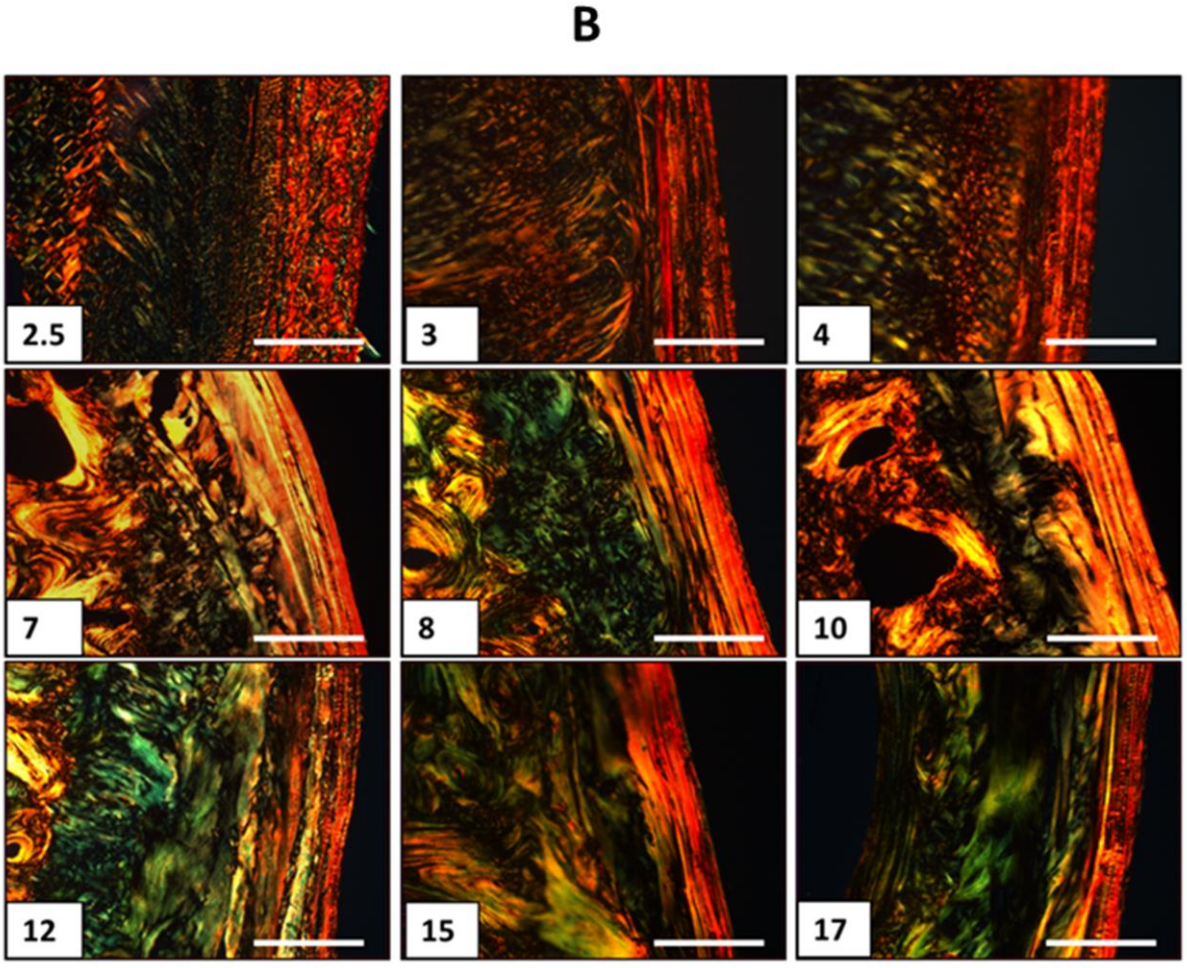
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