A microscopic and macroscopic study of aging collagen on its molecular structure, mechanical properties, and cellular response

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Non-standard abbreviations:

AGEs Advanced glycation end-products

HPLC High-performance liquid chromatography

ELISA Enzyme-linked immunosorbent assay

PFTE Polytetrafluoroethlene (Teflon)

MMPs Matrix metalloproteinases

ABSTRACT

Collagen is the most important component of the extracellular matrix. During aging, collagen structure changes, detrimentally affecting biophysical and biomechanical properties due to accumulation of advanced glycation end-products (AGEs). In this study we conducted both microscopic and macroscopic studies of different aged collagens to investigate their influence on fibrillogenesis, mechanical and contractile properties of reconstituted hydrogel constructs from these collagens seeded with corneal stromal fibroblasts. A high magnetic field was applied during the fibrillogenesis of the different aged collagens to reveal the capacity of collagen fibril alignment. A non-destructive indentation technique and optical coherence tomography were used to determine the elastic modulus and dimensional changes respectively with and without cell presence. Our data revealed that older collagens exhibit higher viscosity and faster gelation rates than younger specimens. Young collagens were able to form highly aligned fibrils in the resulting hydrogels under magnetic fields, while older collagens did not. The collagen constructs from the youngest animals demonstrated a higher elastic modulus and increased contraction in comparison to the older collagen. These results provide clear evidence that aging resulted in the alteration of collagen molecular structure, which may be linked to AGEs accumulation. The results from this systematic study of collagen aging may enhance our understanding of age-associated pathology.

KEYWORDS: Alignment, contraction, fibrillogenesis, modulus.

1. Introduction

Collagen is a key life-long structural protein that is the most prevalent protein in the body (1-5) constituting to approximately one-third of the total body mass (6). There are more than 20 types of collagen in animal tissues which vary in the length (2), with collagen type I being the most abundant form (5) and most commonly utilized for research and commercial applications (7). Collagens are made up of 3 polypeptide subunits referred to as α-chains that assemble into a triple helix structure (2, 8, 9). The triple helices form microfibrils which pack together to form larger fibrils or fibers in tissues (9). Following fiber formation the collagen chains are progressively joined by cross-links (10) which increases the structural and biochemical stability of the tissue (9). This self-assembly into large-scale structures occurs under physiological conditions. Networks of type I collagen can also be formed *in vitro* (3). Such structures often have high tensile strength (2, 11) although they can have little strength in flexion or torsion (11). Tissue specific architecture is often pivotal to tissue function and the ability of a tissue to sustain an applied load (11). The primary role of the collagen networks is to provide a supportive extracellular framework for cells, allowing for adhesion, aggregation, cell attachment, morphogenesis and development (4). Detrimental changes to biochemical and biomechanical interactions all result, amongst other causes, from the aging process (12) and consequently collagen loses flexibility, enzymatic digestibility (13) and becomes less deformable (14, 15). *In vivo* this manifests as a decline in anatomical integrity and function often affecting multiple organ systems and resulting in increased pathology, disease, and a higher risk of death (16). Tissues that are most vulnerable to such changes include the kidney, capillary basement membranes, cardiovascular and pulmonary systems (17). In fibrotic and degenerative disorders, altered regulation of collagen synthesis and degradation are likely to be of importance (4). Collagen molecules have active reactive groups on their side chains and any chemical reactions with these amino groups will have a direct effect on the triple helix properties and so on tissue and organ function (13). Such changes are often associated with inter- and intra-molecular cross-linking of the peptides. Cross-linking can occur enzymatically (lysyloxidase mediated (14)) or non-enzymatically. Enzymatic processes usually occur during developmental and maturation stages of the collagen; whereas non-enzymatic changes occur due to the aging process, which damages proteins and associated biomolecules (13). Frequently non-enzymatic changes are a result of the glycation process, which subsequently induce the accumulation of advanced glycation end-products (AGEs) (18).

AGEs are a complex and heterogeneous group of compounds whose presence have been shown to increase in tissues with chronological, normal and physiological aging (15, 16). AGEs formation causes proteins to cross-link through a reaction of the reducing oxo-group of sugars with a free ε-amino group of the protein (amino group of lysine or arginine) (14) which involves a series of reactions to form a Schiff base (16). The initial Schiff base and resulting Amadori products undergo an irreversible series of reactions leading to cross-linked structures (13). The phenomenon was first described by Lois Camille Maillard in 1912 in relation to food studies (16). However, it was much later that these Maillard reactions were shown to occur slowly *in vivo* during glycation and that they controlled tissue modifications during aging (16).

Advanced glycation occurs over a period of weeks (15) thus affecting long-life proteins such as collagen, disrupting collagen fibril arrangement and spatial architecture during cross-linking. Cross-linking interferes in cell-matrix interactions affecting adhesion and cell spreading, impeding function and decreasing tissue remodeling capabilities (15). AGEs are formed continually in the human body (16) although they are accelerated by some physiological situations such as aging and pathophysiological conditions such as diabetes (15, 19). The process of advanced glycation itself is not harmful, only its products are (15). The assessment of non-enzymatic changes is difficult (13) and unfortunately AGEs measurement varies widely between studies. Many different methods exist for measuring AGEs and as of now there is no gold standard method for detecting AGEs. The methods most commonly used include high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (15). In this study we aim to reveal the change in molecular structure of collagen due to aging *via* a simple magnetic alignment technique and investigate the effect of collagen structural changes on their mechanical properties and cellular capacity of matrix remodeling in reconstituted collagen hydrogel constructs by a non-destructive indentation technique (20-22) and imaging modality. We hypothesize that inter- and intra-molecular cross-linking of collagen due to aging interferes greatly with the fibrillogenesis process. Consequently, the mechanical properties of the hydrogel construct are affected, which in turn influences the cell's ability to contract and remodel the collagen constructs.

2. Materials and Methods

2.1. Extraction and characterization of aging collagen

Native type I collagen was extracted from rat tail tendons of different-age animals, a few days (newborns), 2 months (young adults), 6 months (adults) and 2 years (old adults), as previously described (23). Briefly, acid-soluble type I collagen was obtained from rat tail tendons by acetic acid extraction then purified by dialysis against distilled water and lyophilized. The lyophilized, purified, and non-pepsined collagen was stored at -80°C until further use. For preparing 3D gels, lyophilized collagens were sterilized in ethanol, dried, and then dissolved in sterile 18 mM acetic acid at a final concentration of 5 mg/ml. Commercially available rat tail collagen (BD Biosciences Mountain Science, UK) was used for comparison.

A semi-micro viscometer (size 75, Cannon-Ubbelohde, Cannon Instruments, USA) was used to measure the kinematic viscosity of the different collagen solutions according to the manufacturer's instructions. All collagen samples were diluted using 0.1% acetic acid (*v/v*; Sigma-Aldrich, UK, diluted in phosphate buffered saline, PBS) to working concentrations of 0.5 mg/ml to allow for viscosity measurements to be recorded. BD collagen was also diluted using the same procedure. Briefly, 2 ml of each collagen sample were added to the lower reservoir of the viscometer. The viscometer was clamped into the holder and inserted into a water tank that remained at a constant temperature of 20 ºC. The viscometer, sample, and the holder were left for approximately 15 minutes to allow for the sample to stabilize to the bath temperature. A vacuum pump (FB70155, Fisherbrand, Loughborough, UK) was used to apply suction through the capillary tube and collect the sample into a collecting bulb. The efflux time was measured by allowing the collagen

solution to flow freely between two measuring marks on the apparatus. The time for the collagen meniscus to pass between two defined measuring markers was measured in seconds. The kinematic viscosity of the sample was calculated by multiplying the efflux time by the viscometer constant which was calibrated to 0.007813 mm^2/s^2 (Poulten Selfe and Lee, PSL, Essex, UK) for the viscometer used.

2.2. Corneal stromal cell culture

Human corneal tissue remaining from corneal transplantation was used for the isolation of stromal cells. This research has received approval from Birmingham NHS Health Authority Local Research Ethics Committee, and only tissues from donors which have been consented for research were used in this study. The endothelial and epithelial layers were physically stripped using sharp-point forceps. The remaining stromal layer was cut into smaller pieces and cultured in cell culture flasks containing Dulbecco's modified eagle medium (DMEM; Biowest, France) supplemented with 10% fetal calf serum (FCS, Biowest, France), 1% antibiotic and antimitotic solution (A+A, Sigma-Aldrich, UK) and 2 mM L-Glutamine (Sigma-Aldrich, UK) at 37˚C, 5% CO_2, allowing stromal cells to migrate out from the tissue. Media was changed every 2-3 days and the cells were passaged at confluence. Third passage corneal stromal cells were used for experiments.

2.3. Fabrication of acellular and cell-containing hydrogels

Collagen hydrogels from both extracted and commercially available BD rat tail collagen were investigated and manufactured. To neutralize the collagen solution and to fabricate extracted collagen hydrogel constructs, NaOH and other cell-supporting additives were added to the given collagen solution. For making 1 ml of neutralized collagen gel solution, 100 µl MEM (10X concentration), 100 µl $NaHCO_3$ (22g/l), 90 µl 0.1M NaOH, 10 µl L-Glutamine (100X concentration), 100 µl H_2O, 100 µl FCS and 500 µl collagen at the concentration of 5 mg/ml were mixed to give constructs of final collagen concentrations of 2.5 mg/ml. Commercially available rat tail collagen (BD) hydrogels was prepared according to the manufacturer's instructions as previously described (21), using 10X DMEM in place of PBS. The collagen mixture (500 µl at 2.5 mg/ml) was cast into a filter paper ring (internal diameter 20 mm) on non-adherent PTFE plates. Cellular constructs were produced following the same protocols except the stromal cells were suspended throughout the both extracted and BD hydrogel solutions and prior to casting (cell density; 1 x 10^6 cells/ml). The cell-collagen mixture was cast for each construct. Gelation was achieved by incubation at 37˚C, 5% CO_2 for 35-60 mins.

2.4. Characterization of collagen

2.4.1. Magnetic alignment of collagen

Acellular collagen hydrogel samples were prepared as described previously (see section 2.3) at collagen concentration 2.5 mg/ml. 150 µL of gel was pipetted into a Millicell EZ 4 well glass slide chamber (Merk Millipore, Oxford, UK) and allowed to set. The collagens were aligned under 12 Tesla magnetic fields (CRETA, Grenoble) during fibrillogenesis. The magnetically aligned hydrogels were viewed using polarized light microscopy (SP-60P, Brunel, UK) and the collagen fiber organization images were captured using digital photography (D5000, Nikon, Japan). Collagen stromal cells were seeded at density 20,000 cells per well in 100 µl media. The cells were allowed 2 hrs to adhere before the media was topped up to 500 µl per well. The cells were cultured for 3 days before their orientation was viewed using fluorescent microscopy (Eclipse T*i*-S, Nikon, Japan).

2.4.2. Collagen contraction

Hydrogels cast into filter paper rings were effectively confined to the dimension of the ring. This permitted analysis of confined contraction in terms of a change of thickness of the hydrogel construct via optical coherence tomography (OCT). Acellular collagen hydrogels were produced as a control. An in-house built OCT was used to measure the change in thickness of constructs daily. The detailed description of the instrument can be found elsewhere (24). The acquired images were analyzed using ImageJ (NIH, USA). Recorded data was averaged and represented as a mean value ± the calculated standard deviation. Acellular control data was relatively constant for the duration of the experiment so the data was accumulated and displayed as an average of 7 days and represented as a mean value ± the calculated standard deviation.

2.4.3. Modulus measurement

The mechanical properties of the constructs were measured daily using a non-destructive spherical indentation technique (20, 22, 25) which permitted repeated analysis of constructs over time. The in-house built instrumentation consists of a sample holder with a spherical indenter and an image acquisition system (22, 25, 26). Hydrogel constructs were circumferentially clamped. A PTFE sphere of known weight and radius (0.0711 g and 2 mm radius) was placed centrally on the construct, causing uniform deformation of the hydrogel. The extent of the deformation was recorded and applied to a theoretical model (27) to calculate the elastic modulus of the constructs.

2.4.4. Cell viability and morphology

Cell viability was observed at day 7 using a live-dead fluorescent double staining kit (Fluka, Switzerland) able to simultaneously stain viable cells green, and dead cells red; it was used according to the manufacturer's instructions. Stained hydrogel constructs were washed in PBS and examined using fluorescent microscopy (Eclipse T*i*-S, Nikon, Japan).

Cell morphology was observed at day 7 in the hydrogel constructs and day 3 in the magnetically aligned samples using phalloidin tetramethylrhodamine-B-isothiocyanate (Sigma-Aldrich, UK) to fluorescently stain actin filaments of the cells which effectively displays directionality. Briefly, each sample was fixed in 500 µL 10% neutral buffered formalin solution (Sigma-Aldrich, UK) for 60 mins, before washing 3 times in PBS. 500 µL of a 10 µg/ml phalloidin tetramethylrhodamine-B-isothiocyanate solution was applied to each sample and incubated at room temperature, in the dark for 40 mins. All immunostained samples were further counterstained with DAPI (1:500; prepared in PBS; Sigma-Aldrich, UK) in order to visualize cell nuclei and viewed using fluorescent microscopy (Eclipse Ti-S, Nikon, Japan).

2.5. Statistics

All data was analyzed using AbiPrism statistical software. The data was subjected to a normality (Kolmogorov-Smirnoff) test. The data was normally distributed, so comparisons were performed using either T-tests for pairs of data, or one-way analysis of variance (one-way ANOVA) followed by a Bonferroni post-test. Significance was indicated to determine if the effects of time, collagen age and collagen concentration were statistically significant at three levels: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Three specimens per group have been tested. Recorded data was averaged and represented as a mean value ± the calculated standard deviation.

3. Results

3. 1. Collagen quality

Native type I collagen was obtained by acetic acid extraction from rat tail tendons of different ages: a few days (referred to as “newborn”), 2 months, 6 months and 2 years. Collagens of all ages were in a homogenous solution in acetic acid and colorless in appearance. Commercially available rat tail collagen (BD Biosciences, Mountain Science, UK) was used as a comparison. Differences in viscosity were observed between the collagen groups. The oldest collagen solution (2 years) was visibly more viscous than the younger collagens (newborn and 2 months). Although all collagens formed homogenous hydrogels, the gelation time for the younger collagen was significantly longer than that of the older collagens. For example, the newborn collagen took up to an hour to gelate while the 2-year old collagen formed a gel within 10 minutes. Viscosity studies were performed using a semi-micro viscometer and the kinematic viscosity was calculated (Fig. 1). ANOVA tests revealed that the age of the collagen had a statistically significant effect on the viscosity of the collagen solution. The newborn collagen has a significantly lower viscosity than all other samples ($p \leq 0.001$ inclusive). There was no significant difference between the viscosity of the 2-month and 6-month old samples. The 2-month collagen has a significantly lower viscosity than the 2-year ($p \leq 0.001$) and BD samples ($p \leq 0.05$). The 6-month collagen had a significantly lower viscosity than the 2-year old sample ($p \leq 0.001$), but was not significantly different from the commercially available BD sample. The

viscosity of the 2-year collagen was significantly greater ($p \leq 0.001$) than the BD sample, and was almost twice as great as the youngest (newborn) collagen.

3.2. The alignment degree of fibrillogenesis under a magnetic field

Type I Collagens from different-aged rats (newborn, 2-month, 6-month and 2-year) were prepared at a final collagen concentration of 2.5 mg/ml. Following exposure to a 12 Tesla magnetic field during fibrillogenesis with a step heating programme, collagen fibers in the newborn and 2-month samples aligned perpendicularly to the applied magnetic field, whereas fibers in the 6-month and 2-year specimens remained randomly oriented (Fig. 2E-H). Following seeding with corneal fibroblasts and culture for 3 days it was observed that the cell orientation mirrored the topography of the aligned or disorganized collagen surface. The newborn and 2-month collagen surfaces caused the cells to align and grow in the defined direction (Fig. 2A and B) whereas the cells on the 6-month and 2-year collagen surfaces failed to align and continued to grow in a random orientation (Fig. 2C and D).

3.3. Collagen contraction

Collagen hydrogel constructs of different-age collagens were prepared at a final collagen concentration of 2.5 mg/ml. Corneal fibroblasts seeded in the hydrogel constructs prior to gelation caused contraction during the culture. Additional acellular constructs were prepared as a control. The contraction of the constructs was limited to a change in thickness since the presence of a filter paper ring support prevented the hydrogels from contracting horizontally. The change in thickness of all constructs was measured daily for 7 days using OCT. The thickness of all acellular constructs remained constant and showed no significant differences between groups throughout the duration of the culture period (Fig. 3A). All cellular constructs contracted thus making the constructs thinner. Highest contraction occurred within the first 3 days. ANOVA tests revealed that the age of the collagen had a statistically significant effect on the change in thickness of the construct.

The cells in the youngest collagen constructs (newborn) caused the greatest degree of contraction and by the end of the experiment was less than 20% of its original thickness (Fig. 3B). The newborn collagen decreased more significantly in thickness than all other collagens used ($p \leq 0.001$ days 1-7 inclusive for age 2-month, 6-month, 2-year and BD) with the exception of the 2-month collagen (where $p \leq 0.001$ days 1-4, $p \leq 0.05$ days 2 and 4 and $p \leq 0.05$ on day 3). Similar trends were observed when comparing 2-month collagen to 6-month, 2-year, and BD collagens ($p \leq 0.001$ days 1-7 inclusive). The 6-month collagen also decreased in thickness more significantly than 2-year and BD collagens ($p \leq 0.05$ day 2-4; $p \leq 0.01$ day 3-5; $p \leq 0.001$ day 6-7). The 2-year old collagen decreased more significantly than the BD collagen on day 1 only ($p \leq 0.001$).

3.4. Collagen modulus measurement

The elastic modulus of the different aged collagen constructs in both acellular and cellular constructs was measured daily for 7 days. All acellular control hydrogels retained a constant modulus with a visible trend showing that the younger the collagen, the higher the modulus (Fig. 3C) and so were accumulated into a bar graph for easier comparisons to be drawn (Fig. 4). The modulus of the acellular newborn constructs was significantly greater than 2-month, 6-month, 2-year and BD constructs for the duration of the experiment ($p \leq 0.001$ inclusive). Acellular 8 week constructs were significantly greater in modulus than the 6-month, 2-year and BD ($p \leq 0.001$) constructs; 6-month constructs were significantly higher in modulus than the 2-year and BD constructs. There was no significant difference between the 2-year and acellular BD constructs. The modulus of the cellular constructs (Fig. 3D) manufactured using newborn collagen was significantly greater than the 6-month, 2-year and BD cellular collagen constructs for the duration of the experiment ($p \leq 0.001$ inclusive). However, the newborn collagen only showed a significantly greater modulus when compared to the 2-month collagen at day 5 ($p \leq 0.05$) and day 6 ($p \leq 0.05$). Similar trends were observed when comparing the modulus of the 2-month collagen to the 6-month and 2-year collagens ($p \leq 0.001$ day 2-7, with the exception of 6-month collagen at day 5 where $p \leq 0.05$). The 6-month collagen also had a significantly higher modulus when compared to 2-year collagen ($p \leq 0.05$ day 2 and 4, $p \leq 0.01$ day 3 and 5, $p \leq 0.001$ day 6-7). There was no significant difference in the modulus of the 2-year collagen when compared to the BD collagen.

3.5. *Cell viability and morphology*

All collagen constructs exhibited good cell viability following 7 days culture (Fig. 5F-J) with no obvious differences in cell viability when comparing the different-age collagens or commercially available collagen (BD). The morphology of all the cells was characteristically fibroblastic in appearance (Fig. 5A-E). However, it was observed that in the specimens constructed from the younger collagens (newborn and 2-month) (Fig. 5A and B), cell populations appeared compared to the other samples. This denser appearance was likely to be due to the high contraction occurring, thus causing the surrounding collagen matrix to be denser. All older samples appeared to have disorganized actin arrangements that were less dense in appearance (Fig. 5C-E).

4. Discussion

It is well known from clinical pathology and normal daily life that aging affects connective tissues considerably, such as wrinkle formation and reduced skin elasticity as humans become older. However to date, there is no systematic report of the molecular changes of aging collagen and its influence on the mechanical properties of reconstructed collagen hydrogels and the cellular response when fibroblast-like cells are seeded into the hydrogel. To our knowledge, the present report is the first study conducted whereby the molecular and functional changes of aging collagen have been investigated in parallel using microscopic and macroscopic modalities. It is revealed that with aging, there are systematic changes in molecular

structure of type I collagen which triggered macroscopic changes of the reconstructed collagen hydrogels in an age-dependent manner. In our study, through measurement of the macroscopic parameters including kinematic viscosity, fibril alignment, elastic modulus and the contraction capability (thickness change), we have obtained further evidence and new insights that aging changes the collagen monomer structure. The changes at the monomeric level considerably affect the fibrillogenesis process, which alters the architecture of collagen fibers and the global network in the hydrogel.

Type I collagen is a fiber-forming protein and an important scaffolding component of the ECM. The source, age, extraction method, and post-processing, all result in a collagen with a defined structure regarding the nature of the fibrils and the extent of cross-linking. As all the different collagens (excluding the BD samples) were sourced, extracted, and processed using the same protocols it can be assumed that the collagen age will be the only contributing factor to the observed differences in collagen structure and behavior. We hypotheses that the quality and quantity of collagen monomers (tropocollagen) extracted from connective tissue, such as tendons in our study, will bear a mark of aging. In this study, we used an acid extraction method. Unlike enzymatic extraction methods, the extracted products consist primarily of monomeric tropocollagen with intact telopeptides (28). It is believed that telopeptides are essential for the end-to-end aggregation of tropocollagen molecules, which is a crucial step in fibrillogenesis (29). It has been shown that the removal of telopeptides dramatically alters the *in vitro* self-assembly process of collagen, and the resulting fibril packing order is detrimentally affected (7). Further studies have demonstrated that in collagen, the N-terminal telopeptide regions of α-chains are responsible for intra-molecular covalent cross-linking of α-chains through lysine residues (30). Our results confirm that the older tissues (even at 2-year) still contained a substantial portion of acid-soluble tropocollagen. However, the viscosity of the older collagens was higher than the younger collagen counterparts; this implies that the older solubilized tropocollagen solution contained a substantial number of trimeric, polymeric triple-helices, or AGEs aggregates. The variation in alignment capacity during fibrillogenesis of the different-age collagens when exposed to a high magnetic field provided further evidence that structural alterations to the telopeptide region had occurred in the older samples with reduced end-to-end cross-linking of tropocollagen which prevented the formation of homogenous and denser packaged fibers.

Biological polymers such as collagen and fibrin are diamagnetically anisotropic. The diamagnetic anisotropy of such proteins largely depends on the relative orientations of the peptide bonds and aromatic residues present in their structure (31). As collagen contains few aromatic groups its diamagnetic anisotropy is entirely due to the regular arrangement of the peptide bonds that form the collagen triple helix. Worcester (31) has shown that the diamagnetic anisotropy of a single peptide bond in the collagen triple helix conformation is $-0.25|\Delta\chi_p|$, where $\Delta\chi_p$ is the diamagnetic anisotropy of a single peptide bond. The diamagnetic anisotropy of collagen is negative and consequently molecules tend to orient perpendicular to an applied field. As diamagnetic anisotropy is approximately additive; the anisotropy of a single collagen

molecule consisting of about 2730 peptide bonds is $-680|\Delta\chi_p|$. When N molecules are aligned parallel, the diamagnetic anisotropy of the ensemble, $\Delta\chi$, is N times greater following the equation [1]:

$$\Delta\chi=-680\times N\times|\Delta\chi_p| \qquad [1]$$

The orienting effect of the magnetic torque is countered by randomizing Brownian motion so the degree of alignment in a magnetic field, H, depends on the ratio in equation [2]

$$|\Delta\chi|H^2/kT \qquad [2]$$

where k is Boltzmann's constant and T the absolute temperature. In order to reach a high degree of orientation, the condition, $|\Delta\chi|H^2>20kT$, has to be satisfied (31). For collagen fibers this means that $680\times N\times|\Delta\chi_p|H^2>20kT$. The experiments reported here were carried out at near room temperature in a field of 12 Tesla (i.e. 1.2×10^5 G (Gauss)). By using the lowest value, $|\Delta\chi_p|=8.9\times10^{-30}$ erg G^{-2} (28), we calculate that a high degree of magnetic orientation requires a freely rotating group of approximately 10,000 near mutually parallel collagen molecules. The number of collagen molecules within fibrils easily exceeds this number. When acid solubilized collagen is neutralized the solution becomes viscous then gelates as self-assembly into fibrils and fibers occurs. In the presence of a strong magnetic field a gel of highly aligned fibers results, provided assembly is not so fast that gelation occurs before orientation has maximized. For orientation to work, the initial solution must be largely composed of intact single molecules, or small parallel aggregates thereof, which come together in a parallel fashion giving rise to a high magnetic anisotropy. However, if the solution contains a significant proportion of disorganized aggregates, in which the constituent molecules are not parallel, or the molecules are not freely rotated, the effective diamagnetic anisotropy of these groups is strongly reduced or eliminated making magnetic orientation virtually impossible.

Our experiments demonstrate that solutions of collagen extracted from young rats readily formed aligned gels, whilst gels produced from older animals showed a random morphology. This suggests that the collagen solutions derived from young rats consist largely of monomeric tropocollagen molecules, which come together in a parallel fashion and rotate as a group to attain high orientation before gelation takes place. In contrast, the collagen solutions extracted from older rats oriented poorly, probably because they contained a high proportion of weakly anisotropic aggregates, for example, AGEs aggregates and polymeric tropocollagens as schematically shown in Fig. 6. Hence, the fibrillogenesis under a magnetic field provided a simple technique to reveal molecular alterations of collagen due to aging.

When corneal fibroblasts were seeded onto the aligned and non-aligned collagen surfaces, the orientation of the cell populations simply replicated the alignment architecture underneath, manifesting as parallel densely packed, aligned cell cytoskeletons in the younger collagen samples, whilst the cells seeded on the older collagen specimens were randomly orientated in morphology and less densely packed. In fact, the aligned collagen fibers acted as contact guidance for the cells. The different molecular structure of extracted collagens due to aging can be used to explain the variation in the modulus of the acellular collagen samples.

The presence of trimeric or polymeric tropocollagen or AGEs accumulation in the older collagen specimens caused difficulties regarding molecular rotation and movement during fibrillogenesis. As a result, the formed hydrogel had a poor quantity and quality of fiber packing resulting in a hydrogel construct with an amorphous-like ultrastructure in comparison to densely packed fibrous structure in younger specimens. Hence, at the given collagen concentration, the younger collagen hydrogels (newborn and 2-month) with highly organized fibrous structure resulted in a higher construct modulus compared to the randomly and loosely packaged (older) specimens (6-month and 2-year), which is consistent to mechanical properties appearing in non-biological engineering materials.

The variation of elastic moduli in cell-containing constructs from different-age collagens involved a slightly different mechanism, which was the cellular contraction capability. We and other groups have already observed that fibroblasts can “sense” the mechanical strength of their surrounding environment. They contract soft substrates through actin-myosin attachment mechanisms that are controlled by complex intracellular signaling pathways (32) and the contraction ceases once they have reached a harder environment. It has been proven that under the given fibrous collagen hydrogel, higher seeding cell numbers and lower collagen concentrations trigger the largest contraction rates (26). In the present study, the used corneal stromal cells have a proven contraction capacity (25). Although the same cell seeding density and collagen concentration were used for different-age collagen constructs, the microstructures of the formed collagen hydrogel were quite different. The younger collagen hydrogel specimens consist of organized fibrous ultra-structures with less inter-molecular cross-linking; whilst the older collagen hydrogels are randomly packed fibrous structures with a highly cross-linked network. Thus, the individual fibroblasts encountered different local mechanical environments in younger and older collagen hydrogels as shown in Fig 7. Although the modulus of acellular collagen in the younger hydrogels was higher than in the older collagen, the cells applied strain that pulled and contracted the fibers and fiber bundles, not the entire constructs, thus remodeling it. The cells in older collagens experienced a cross-linked aggregated collagen network. It is difficult for a single cell to contract this network environment even though the older acellular collagen hydrogels had the lowest modulus. The local environment was already too great for an individual cell to overcome the strain of the matrix. The possible explanation why the modulus in youngest collagen specimens was highest is due to the additive effect of cell contraction and high acellular modulus. The well packed acellular fibrous structure in the younger collagen specimen guided cell organization. Upon contraction, the younger, denser collagen hydrogel constructs, adopted a highly organized 3D structure, which has been confirmed by our actin staining images (Fig. 5A and B). These experiments imply that we can predict hydrogel microstructure by fibroblast contractibility.

The acellular and cellular contraction experiments support our hypothesis that aged collagen is not really “stiffer” or “stronger”. Instead, it is weak and less deformable. Our multiple measurements can be used to predict the microstructural changes in collagen during the aging process through macroscopic parameter differences. Fibroblasts seeded into collagen hydrogels have previously been shown to influence the

structural characteristics of the matrix (26, 33). Guidry and Grinnel (34) have previously investigated the contraction of collagen gels using human skin fibroblasts seeded on the top of collagen constructs that were attached to an underlying solid support. They determined that gel reorganization was not due to collagen degradation and that the protein content within the collagen gels was not destroyed. Thus, the reorganization and contraction of the gels occurred *via* a physical rearrangement of pre-existing collagen fibrils, rather than a replacement of the pre-existing fibrils via newly synthesized matrix (34). We expect that a similar scenario is occurring in our 3D experiments. In addition, the regular media changes in our investigation would have prevented the potential accumulation of matrix metalloproteinases (MMPs) that may degrade the collagen matrix.

It has been frequently documented that age-dependent intra- and inter-molecular cross-linking results in changes in the biophysical characteristics of purified soluble collagen (6). Formation of AGEs might be the main process for the cross-linked structure. AGEs affect virtually every tissue in the human body (16) and accumulation of AGEs in collagenous tissues negatively affects their functions (35) as it affects cellular signaling and activation of gene expression. ECM composition, matrix modeling proteinases, and integrins are all important factors modulating cell behavior, in terms of migration, proliferation, spatial organization, and differentiation *in vitro* and *in vivo (6)*. Our findings are in agreement with previous studies (6, 14) in that increasing age causes structural changes in collagen, which triggers downstream reactions. In studies on intramuscular connective tissue (35) it was suggested that differences in AGEs concentration in young and old tissues *in vivo* was related to the chronological component of aging in that the longer the protein residues are exposed to glucose, the greater the risk of AGEs formation (35). The rate by which glycation occurs may be influenced by the location of glycation sites (36), which differs with age, thus causing increased differences in the collagen structure. Age-related differences in the degree of collagen cross-linking certainly lead to changes in rigidity of 3D structure of the reconstructed collagen hydrogels (6).

5. Conclusion

Our study has demonstrated that increasing age of collagen aging has a direct impact on the biomechanical properties of 3D collagen constructs. A combination of non-destructive monitoring techniques is able to demonstrate the impact of the collagen matrix on the cell's ability to manipulate and remodel its surrounding environment. We are able to predict microscopic differences in the collagen monomer and hydrogel constructs by measuring macroscopic parameters, which provide a good platform to further investigate the mechanisms of aging and the screening of new agents for slowing the aging process.

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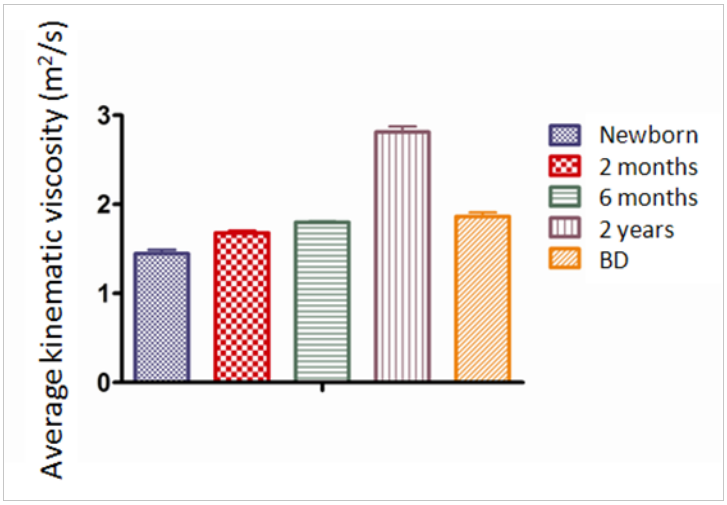

Fig 1: The average kinematic viscosity of 0.5 mg/ml collagen from different aged rats and commercially available (BD) collagen; n=3.

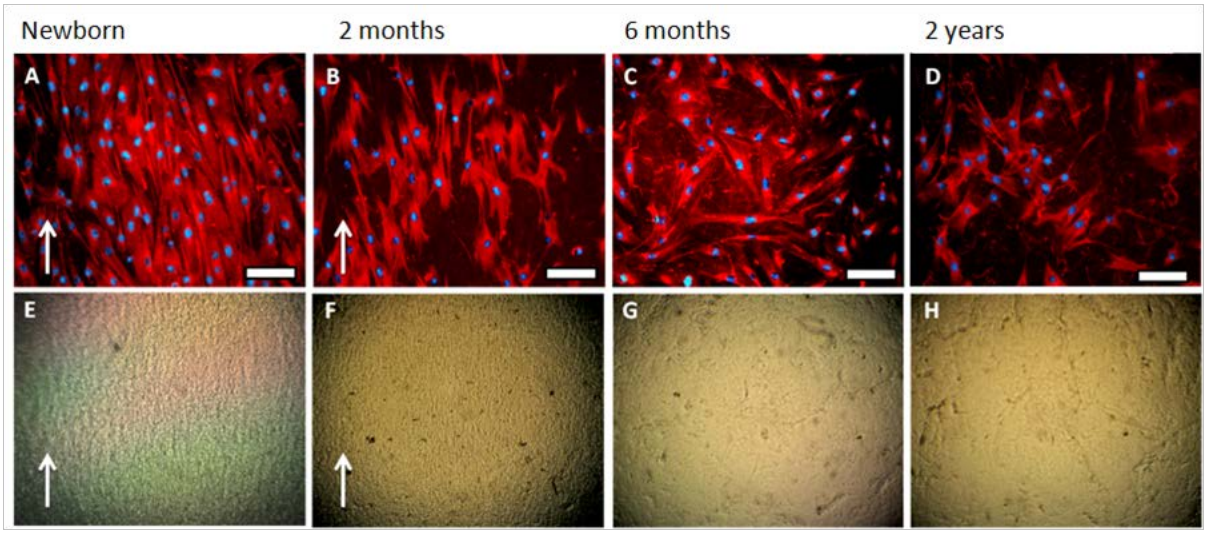

Fig 2: Representative fluorescent phalloidin tetramethylrhodamine-B-isothiocyanate (red) and DAPI (blue) stained corneal stromal cells (**A**-**D**) cultured for 3 days on different aged collagens fibrillogenesed under a 12 T magnetic field at concentration 2.5 mg/ml, scale bar = 100 μm. The corresponding polarized light microscope images (**E**-**H**) of different aged collagen samples under 12 T magnetic field, the newborn (**E**) and 2 month (**F**) collagen solutions aligned, whereas the 6 month (**G**) and 2 year old (**H**) specimens did not. The white arrows indicate the directionality of the collagen fibers.

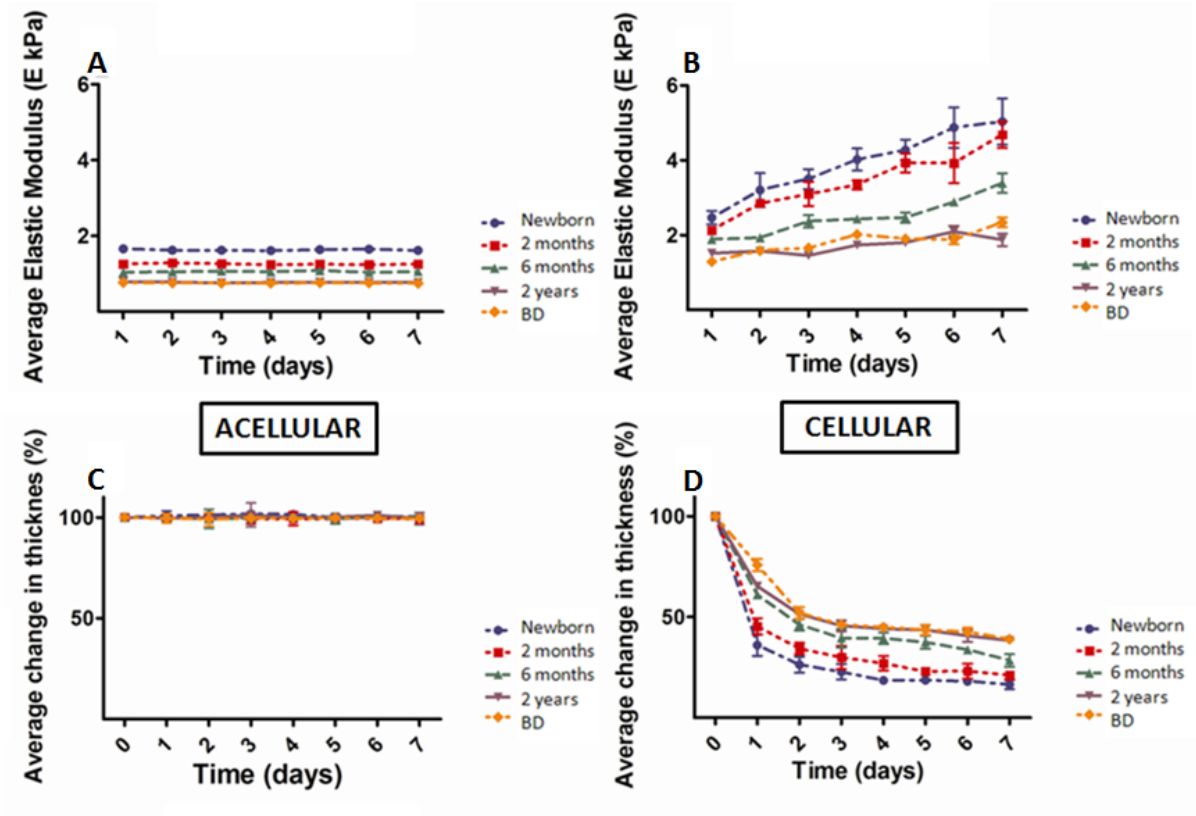

Fig 3: The average elastic modulus, measured using a spherical indentation technique, of 2.5 mg/ml collagen concentration acellular (**A**) and cellular (**B**) constructs using collagen extracted from different aged rats cultured for 7 days; the average change in thickness of the corresponding acellular (**C**) and cellular (**D**) constructs, measured using optical coherence tomography; n=3

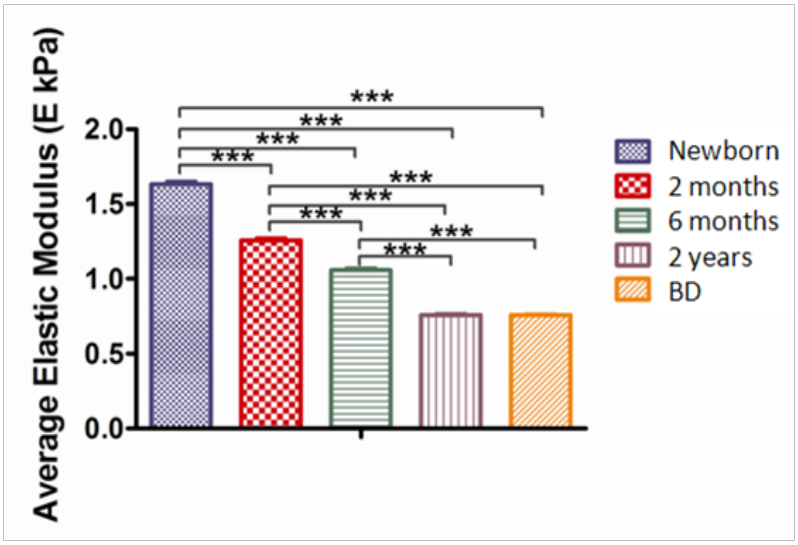

Fig 4 Accumulated elastic modulus results of collagen extracted from different aged rats (and BD collagen following 7 days culture; n=7.

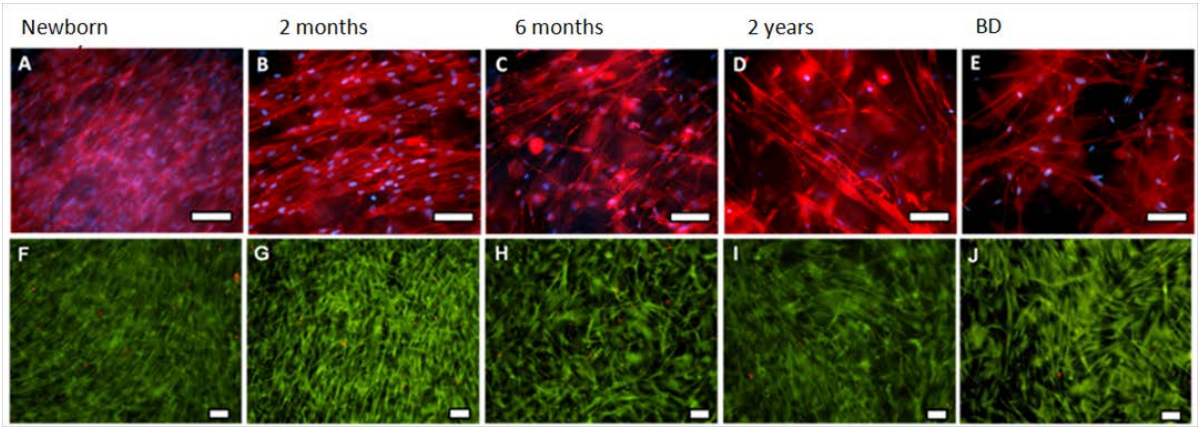

Fig 5: Representative fluorescent phalloidin tetramethylrhodamine-B-isothiocyanate (red) and DAPI (blue) stained corneal stromal cells (**A-E**); and representative live-dead fluorescent images (**F-J**) of cells cultured in different aged 3D collagen hydrogel constructs and commercially available BD collagen constructs following 7 days culture. Green represents live cells, red represents dead cells' scale bar = 100 μm.

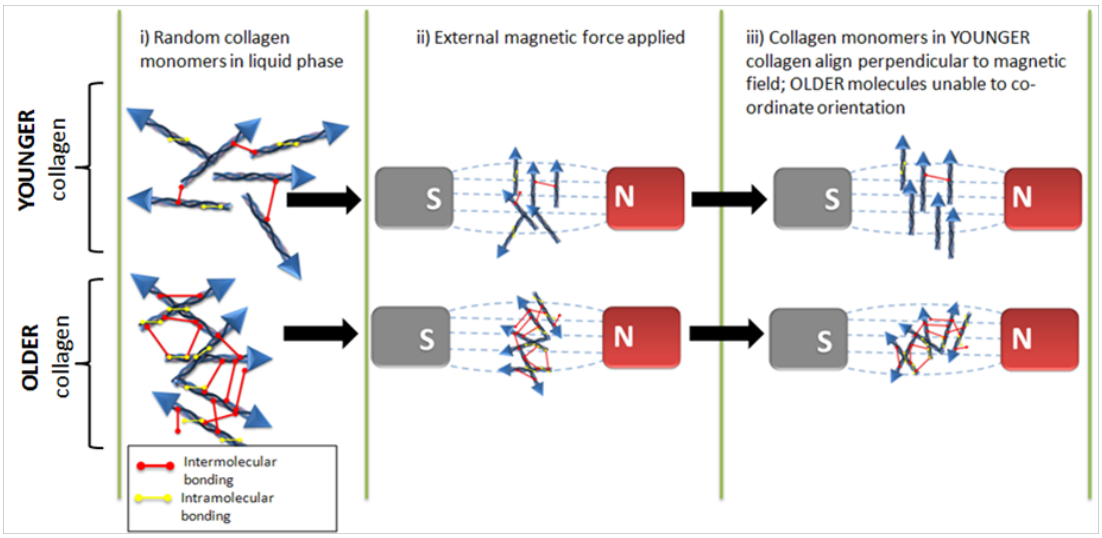

Fig 6: Schematic representation of the mechanisms of collagen alignment in young and aged collagen under external magnetic field (12 T). The younger collagen contains a greater number of monomeric tropocollagen molecules which have large diamagnetic anisotropy and so can overcome thermo randomisation and align; older collagen molecules contain a high proportion of weakly anisotropic aggregates and are unable to overcome thermo randomisation and thus remain random in their orientation.

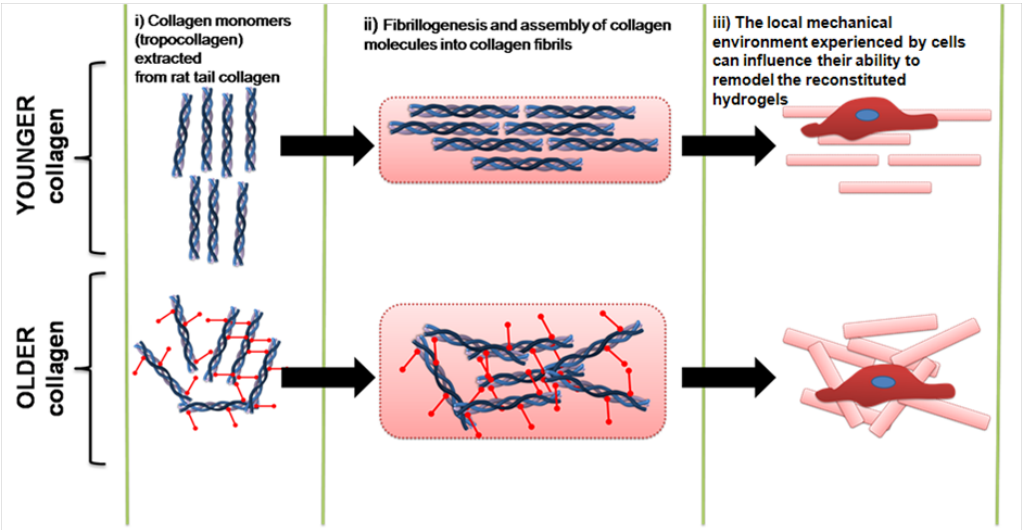

Fig 7: Schematic representation of the mechanisms of cellular collagen contraction in young and aged collagen. The cells in the younger collagens experienced densely packed collagen fibers or fiber bundles and were able to contract them; whilst cells encounter highly cross-linked collagen constructs in older collagen specimens and were unable to contract the specimens.