Cartilage Matrix Formation by Bovine Mesenchymal Stem Cells in Three-dimensional Culture Is Age-dependent

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Abstract

Background Cartilage degeneration is common in the aged, and aged chondrocytes are inferior to juvenile chondrocytes in producing cartilage-specific extracellular matrix. Mesenchymal stem cells (MSCs) are an alternative cell type that can differentiate toward the chondrocyte phenotype. Aging may influence MSC chondrogenesis but remains less well studied, particularly in the bovine system. **Questions/purposes** The objectives of this study were (1) to confirm age-related changes in bovine articular cartilage, establish how age affects chondrogenesis in cultured pellets for (2) chondrocytes and (3) MSCs, and (4) determine age-related changes in the biochemical and biomechanical development of clinically relevant MSC-seeded hydrogels. **Methods** Native bovine articular cartilage from fetal ($n = 3$ donors), juvenile ($n = 3$ donors), and adult

($n = 3$ donors) animals was analyzed for mechanical and biochemical properties ($n = 3$ –5 per donor). Chondrocyte and MSC pellets ($n = 3$ donors per age) were cultured for 6 weeks before analysis of biochemical content ($n = 3$ per donor). Bone marrow-derived MSCs of each age were also cultured within hyaluronic acid hydrogels for 3 weeks and analyzed for matrix deposition and mechanical properties ($n = 4$ per age).

Results Articular cartilage mechanical properties and collagen content increased with age. We observed robust matrix accumulation in three-dimensional pellet culture by fetal chondrocytes with diminished collagen-forming capacity in adult chondrocytes. Chondrogenic induction of MSCs was greater in fetal and juvenile cell pellets. Likewise, fetal and juvenile MSCs in hydrogels imparted greater matrix and mechanical properties.

Conclusions Donor age and biochemical microenvironment were major determinants of both bovine chondrocyte and MSC functional capacity.

Clinical Relevance In vitro model systems should be evaluated in the context of age-related changes and should be benchmarked against human MSC data.

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Introduction

Articular cartilage is a specialized tissue that distributes loads during normal joint movements [3]. Cartilage undergoes remarkable alterations in composition, organization, and mechanical properties with aging [11, 36, 52]. A major portion of the population will have cartilage pathology, including osteoarthritis [20]. Short of total joint arthroplasty, current treatments for traumatic cartilage injury and disease such as microfracture or osteochondral autografting offer satisfactory short-term solutions without

evidence of long-term function [15, 27, 44]. Autologous chondrocyte implantation (ACI) uses *in vitro* expanded chondrocytes for implantation into a defect, but also fails to produce functional, integrated repairs [28, 34, 35].

One limitation of ACI is the age of donor chondrocytes. The literature suggests lower proliferation, extracellular matrix (ECM) forming potential, and more senescence in aged human chondrocytes [5, 21, 31]. Similarly, aged bovine chondrocytes produce less cartilage ECM in three-dimensional culture [50], and adult canine chondrocytes generate functional grafts only when expanded in specialized media [39]. Adkisson and coworkers noted that immature human chondrocytes in a scaffold-free system produced cartilage-like ECM superior to adult chondrocytes [1].

This evidence suggests that donor age limits the clinical potential of autologous chondrocytes and has motivated many groups to investigate mesenchymal stem cells (MSCs). MSCs are a multipotent cell type found in bone marrow that can differentiate along osteogenic, chondrogenic, and adipogenic lineages [4]. Like chondrocytes, however, MSC properties also change with age; MSC density in bone marrow decreases and aged MSCs are slower to proliferate [47]. Regardless, aged MSCs can produce functional repair tissue. Rabbit tendon injuries repaired with autologous MSCs from young or aged animals produced repair tissue with equivalent material properties [16]. The literature is conflicting whether osteogenic and adipogenic MSC differentiation is age-dependent, with some studies suggesting it is independent of age [42, 46, 49] and others dependent on age [14, 30]. For human MSC chondrogenesis, both age-dependent and age-independent findings have also been noted [37, 41, 43]. Recent findings showed a decreased chondrogenic potential in aged human male MSCs but no decline in female MSCs [41]. Another recent report on fetal and adult human MSCs showed similar adipogenic and osteogenic differentiation, but age caused diminished cartilage ECM formation [8].

Using an equine model, Kopesky and coworkers reported adult MSCs in hydrogels form superior engineered tissue compared with juvenile MSCs and adult chondrocytes [29]. In contrast, we found juvenile bovine MSCs are inferior in terms of functional ECM production to donor-matched chondrocytes in various hydrogels [17, 23, 25, 33] but have not considered MSC age in our hyaluronic acid (HA) hydrogel system. Thus, although the literature demonstrates aging affects MSC and chondrocyte function, the relative effects of aging of bovine chondrocytes compared with MSCs is unknown.

The objective of this study was to confirm age-related changes in native cartilage and determine the effects of aging on bovine MSCs and chondrocytes in three-dimensional pellet and hydrogel culture. Specifically, we sought to (1) confirm age-related changes in bovine articular cartilage, establish how age affects chondrogenesis in cultured pellets for (2) chondrocytes and (3) MSCs, and (4) determine age-related changes in the biochemical and biomechanical development of clinically relevant MSC-seeded hydrogels.

Materials and Methods

To analyze developmental differences in bovine cartilage with age, trochlear groove cartilage from fetal ($n = 3$ donors), juvenile ($n = 3$ donors), and skeletally mature (adult) ($n = 3$ donors) stifle joints was analyzed for biochemical content, biomechanical properties ($n = 3$ –5 per donor), and histology (Fig. 1A). Cartilage samples were sectioned with a freezing stage microtome to obtain 1-mm thick $\times$ 4-mm diameter samples for mechanical testing. After testing, samples were analyzed for DNA, sulfated glycosaminoglycan (GAG), and collagen content. Histologic staining for proteoglycans and collagens was performed and split-line directions evaluated across each joint and at each age.

Fig. 1A–C Experimental groups for analysis of fetal, juvenile, and adult native cartilage (A), pellet study of chondrocytes (CHs) and mesenchymal stem cells (MSCs) of fetal, juvenile, and adult origin

cultured for 6 weeks in chondrogenic medium with (CM+) and without (CM-) TGF- β 3 (B), and the investigation of MSCs within a three-dimensional hyaluronic acid (HA) hydrogel context (C).

Fetal (second or third trimester; JBS, Souderton, PA), juvenile (3–6 months; Research 87, Boylston, MA), and adult bovine limbs (2–3 years; Animal Technologies, Tyler, TX) were acquired within 24 hours of slaughter. MSCs from three donors of each age were isolated from tibial or femoral bone marrow extractions by plastic adherence [33] and maintained separately in growth medium (DMEM, 10% fetal bovine serum, and 1% penicillin-streptomycin-fungizone; Invitrogen) through Passage 2 or 3. Diced, full-thickness articular cartilage from three donors of each age group was enzymatically digested to release the chondrocytes, which were used without passaging [33].

Chondrocytes (primary) and bone marrow-derived MSCs (expanded) from fetal, juvenile, and adult bovine donors (three donors per age) were isolated, formed into cell-rich pellets (200,000 per pellet), and cultured for 6 weeks in medium with (CM+) or without (CM–) the chondrogenic induction factor transforming growth factor- β 3 (TGF- β 3) [33]. Biochemical assays for DNA, sulfated GAG, and collagen content in each pellet were performed ($n = 3$ per donor) and proteoglycan and collagen distribution was assessed by histology (Fig. 1B).

Bone marrow-derived MSCs from fetal, juvenile, and adult bovine donors (three donors per age) were also encapsulated in photocrosslinked HA hydrogels [10] and cultured for 3 weeks in chondrogenic medium with TGF- β 3. Cell viability was analyzed (LIVE/DEAD staining kit; Invitrogen, Carlsbad, CA) [17], and mechanical testing ($n = 4$) was performed followed by biochemical assays ($n = 4$) for DNA, GAG, and collagen content and histologic analysis of proteoglycan and collagens (Fig. 1C).

Hyaluronic acid (approximately 65 kDa; Lifecore, Chaska, MN) was methacrylated to form the photocrosslinkable HA macromer as in Burdick et al. [10]. HA gel solution was prepared at 1% (mass/volume) in phosphate-buffered saline (PBS) with 0.05% w/v of the photoinitiator I2959 (2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone; Ciba-Geigy, Tarrytown, NY) and MSCs were suspended at 20 million cells/mL before being ultraviolet-polymerized between glass plates separated by a 2.25-mm spacer [17]. Cylindrical constructs were removed from gel slabs using a sterile 4-mm-diameter biopsy punch (Miltex, York, PA).

The equilibrium and dynamic moduli of native cartilage and MSC-seeded hydrogel constructs were determined in unconfined compression using a custom device in a PBS bath [32]. The equilibrium modulus was derived from stress relaxation (10% strain; approximately 1200-second relaxation) data and the dynamic modulus from five cycles of sinusoidal deformation (1% strain amplitude) performed immediately after stress relaxation [40].

Cartilage, pellet, and hydrogel samples were papain-digested and assayed for DNA, GAG, and collagen content

using the Picogreen (Molecular Probes, Eugene, OR), 1,9-dimethylmethylene blue [19], and orthohydroxyproline assays [38, 45], respectively, as described in Erickson et al. [17] and Huang et al. [25].

Split-line direction was evaluated across fetal and juvenile stifle joints. A round 1.25-mm-diameter needle was dipped in India ink and inserted perpendicular to the cartilage surface to the level of the subchondral bone. India ink was drawn into the formed gaps, creating a clearly visible line. This process was repeated in a grid with 5-mm intervals across the joint surface.

Histologic analysis of cartilage, pellets, and hydrogels was performed. All samples were fixed in 4% paraformaldehyde. Cartilage and hydrogels were embedded in paraffin and sectioned to 8 μ m. Pellets were cryosectioned to 12 μ m. Sections were stained for proteoglycans with Alcian blue (pH 1.0) and for collagen by picrosirius red [17].

Cartilage and pellet data are reported as the mean $\pm$ SD of results for three donors of each age group ($n = 3$ –4 samples per donor per assay). Hydrogel data are reported as the mean $\pm$ SD of four samples from each MSC donor age. We determined differences in biochemical content and mechanical properties among fetal, juvenile, and adult native cartilage using one-way analysis of variance (ANOVA). We assessed differences in biochemical content between cell pellets with age (fetal, juvenile, and adult) and media supplementation (with and without TGF- β 3) using a two-way ANOVA. We used SYSTAT 13 (Systat Software, Chicago, IL) for all analyses, including the Fisher's least significant difference post hoc testing of pairwise comparisons.

Results

Cell density, collagen content, organization, and equilibrium modulus of native cartilage depended on donor age. Fetal cartilage DNA content was two- and fourfold greater than juvenile and adult cartilage, respectively (Fig. 2A). GAG content (per wet weight) ranged between 5% and 6% regardless of cartilage age (Fig. 2B). Adult cartilage collagen content (10.3%) was approximately two- and fourfold greater than juvenile ($p = 0.005$) or fetal cartilage ($p < 0.001$; Fig. 2C). The compressive modulus of juvenile (0.73 MPa) and adult (0.64 MPa) cartilage was 50% to 75% higher than fetal (0.41 MPa) cartilage (Fig. 2D). Histologic staining confirmed the level of biochemical constituents (Fig. 3) and split-line analysis showed marked differences between fetal and juvenile cartilage with clearly demarcated split-line patterns in juvenile specimens, whereas fetal specimens lacked organization and directionality (Fig. 4).

Fig. 2A–E DNA content (A) decreased as the donor age of bovine cartilage increased (F = fetal; J = juvenile; A = adult). Glycosaminoglycan (GAG) content (B) did not change with age, but collagen content (C) increased significantly. Cartilage equilibrium compressive modulus (D) increased slightly with age, whereas the dynamic modulus (E) was independent of age (three donors; $n = 3$ –4 per donor).

The biochemical content of chondrocyte pellets depended on donor age and TGF- β 3 supplementation. The DNA content in adult chondrocyte pellets cultured in CM+ for

Fig. 3 Histologic staining of proteoglycans (top) and collagens (bottom) show age-related changes in glycosaminoglycan (GAG) and collagen content and localization while providing a visual confirmation of decreasing cellularity with age. Depth-dependent collagen organization increased with donor age (Stain, Alcian blue and picrosirius red; original magnification, $\times 100$; 50- μm scale bar).

6 weeks was approximately two- and threefold greater than in juvenile ($p < 0.001$) or fetal ($p < 0.001$) chondrocyte pellets (Fig. 5A). The GAG levels in fetal chondrocyte pellets in CM+ were at least 50% less than either juvenile ($p = 0.016$) or adult ($p = 0.361$) pellets (Fig. 5B). The collagen content in fetal chondrocyte pellets was greater than juvenile pellets in CM+ and greater than both juvenile and adult pellets ($p < 0.001$) in CM– (Fig. 5C). Interestingly, CM+ decreased GAG ($p = 0.002$) and collagen ($p < 0.001$) content of fetal chondrocyte pellets (Fig. 5B–C).

When MSCs were formed into pellets, the DNA content increased with age, whereas ECM levels decreased. The DNA content was generally higher in juvenile and adult

Fig. 4 Split-line analysis revealed prominent alignment of collagen fibers in juvenile articular cartilage (right). The star-shaped splitting pattern observed in fetal samples (left) indicated collagen in this immature cartilage is less organized.

pellets than in fetal pellets in CM– or CM+. For adult MSC pellets, CM+ did not alter DNA content ($p = 0.942$), whereas CM+ increased fetal ($p = 0.402$) and juvenile ($p < 0.001$) MSC pellet DNA by approximately threefold. In CM–, MSCs produced very little GAG regardless of age. In CM+, MSCs from all age groups increased in GAG content with fetal MSCs accumulating two- and 15-fold higher levels than juvenile ($p < 0.001$) or adult ($p < 0.001$) MSCs, respectively (Figs. 5B, 6). CM+ increased collagen content in MSC pellets for each age group with the greatest collagen accumulation in CM+ fetal pellets.

MSC chondrogenesis in three-dimensional hydrogels was likewise dependent on donor age. After 3 weeks in CM+, DNA content in fetal MSC-seeded gels increased by 48% ($p < 0.001$), juvenile DNA changed very little (-15% ; $p = 0.377$), and adult DNA decreased (-35% ; $p < 0.001$; Fig. 7C). The GAG content of both fetal and juvenile MSC-seeded constructs reached approximately 3%, a level approximately 15-fold higher than adult MSC-laden gels ($p < 0.001$; Figs. 8A, 9). Collagen content reached 0.20% in fetal and 0.28% in juvenile MSC-seeded constructs on Day 21, whereas adult MSC-seeded hydrogels contained approximately 10 times less collagen

Fig. 5A–C DNA (A), glycosaminoglycan (GAG) (B), and collagen (C) content of mesenchymal stem cell and chondrocyte (CH) pellets from fetal (F), juvenile (J), and adult (A) bovine donors cultured in chondrogenic medium with (CM+) and without TGF- β 3 (CM-). Data represent the mean $\pm$ SD for three donors per age and three pellet analyses per donor.

(0.03%; $p < 0.001$; Figs. 8B, 9). The equilibrium and dynamic moduli of fetal and juvenile MSC hydrogels reached approximately 90 kPa and approximately 800 kPa, respectively (Fig. 8C–D). The modulus of HA gels seeded with adult MSCs remained at acellular levels after 3 weeks, 15-fold less than fetal or juvenile MSC gels ($p < 0.001$; Fig. 8D).

Discussion

Donor cell age may be an important determinant of the success of autologous tissue engineering; however, the current literature presents contradicting evidence in a variety of model systems and culture contexts for MSCs. Our first objective was to confirm age-related changes in bovine articular cartilage. Second, we sought to establish how age modulates chondrogenesis of chondrocyte pellets and, third, MSC pellets. Lastly, we investigated age-related differences in biochemical and biomechanical potential of MSCs in hydrogels.

This work was not without limitations. First, the hydrogel study used only MSCs, which was motivated by previous work indicating chondrocyte function is limited in this HA hydrogel formulation [17]. Second, only TGF- β 3 was used, in which additional growth factors may have elicited different results related to donor age. TGF- β 3 is consistently used in tissue engineering to elicit a chondrogenic response; however, it remains possible our age-related observations are the result of changing responsiveness to TGF- β 3, which was not studied. Third, we did not evaluate hypertrophic markers; however, we have previously demonstrated bovine MSCs in agarose hydrogels do not deposit appreciable amounts of mineral or collagen Type X [24]. Finally, this work was carried out in vitro, and the performance of these cells may be altered in vivo (eg, within the synovial joint).

Consistent with previous studies [11, 51, 52], our findings demonstrate that as bovine cartilage matures, mechanical properties and collagen content increase, GAG

Fig. 6 Proteoglycan staining of fetal, juvenile, and adult mesenchymal stem cell (MSC) pellets cultured in chondrogenic medium with TGF- β 3 (CM+) for 6 weeks. Fetal MSC pellets accrued more

proteoglycan than juvenile pellets; adult MSCs formed the smallest pellets with the least amount of proteoglycan (Stain, Alcian blue; original magnification, $\times 50$; 500- μ m scale bar).

Fig. 7A–C Calcein AM labeling of viable MSCs in HA hydrogels (**A**) on Day 21 showed more cells in fetal MSC gels and a dramatic decline in viable cells for adult MSCs. Ethidium labeling (**B**) indicated a greater number of adult MSCs were nonviable compared with gels seeded with fetal or juvenile MSCs (Stain, Calcein AM and Ethidium Homodimer; original magnification, $\times 100$; 250- μm scale bar). DNA content (**C**) on Day 21, normalized to initial DNA levels, showed fetal MSCs increased in number while adult MSC numbers declined significantly ($n = 4$; dashed line represents Day 0 levels).

content remains stable, and cellularity declines. In human articular cartilage, Temple and colleagues showed no age-related biochemical changes and a decrease in equilibrium modulus for only the 60 + age group; however, the youngest (21–39 years) age group was already skeletally mature [48]. Studying younger donors, Kempson found increasing tensile properties of human articular cartilage until the third decade and suggested refinement of the collagenous network for 30 years [26]. We also observed a marked change in the superficial collagen staining intensity in juvenile and adult bovine samples, consistent with previous studies of fetal to juvenile cartilage [2]. In fully formed and specialized adult cartilage tissue, prevailing collagen orientation in this surface zone defines a “split-line” direction [9, 13] that is remarkably consistent among all human subjects [7]. In bovine joints, we observed similar split-line patterns in juvenile femoral condyles, trochlear grooves, and patellar cartilage surfaces. Notably, these patterns were entirely absent or poorly defined in fetal cartilage surfaces. This suggests

that coincident with load-bearing use, cartilage undergoes a rapid alteration in not just the amount of biochemical constituents, but also in the structure and functional assembly of these molecules.

Along with changes in cartilage structure and function, chondrocytes extracted from bovine cartilage of differing ages showed differences in biosynthetic activities in a three-dimensional pellet system. TGF- $\beta 3$ increased DNA content at each age, and most in adult pellets, suggesting a switch from differentiated to proliferative activities. GAG and collagen deposition in fetal and juvenile bovine chondrocyte pellets was generally higher than adult chondrocyte pellets. Interestingly, fetal and juvenile chondrocyte pellets in chemically defined medium with TGF- $\beta 3$ accumulated less GAG and collagen (fetal only) than those cultured without TGF- $\beta 3$, a result that has not been previously reported. In contrast, TGF- $\beta 3$ improved both GAG content and mechanical properties for immature chondrocytes in the context of three-dimensional agarose hydrogels [33]. This may indicate a microenvironmental

Fig. 8A–D Biochemical content of MSC-seeded HA constructs after 21 days in culture showed an age-dependent accumulation of GAG (A) and collagen (B). The equilibrium compressive modulus (C) and dynamic compressive modulus (D) of MSC constructs likewise developed in an age-dependent fashion after 21 days ($n = 4$ constructs per age).

influence (such as cell-to-cell contact) in the interpretation of this soluble factor.

Unlike chondrocytes, pellets formed from bovine MSCs of different ages were age-dependent. TGF- β 3 initiated robust chondrogenesis, consistent with the literature [6]. Aged MSC pellets with TGF- β 3 accumulated less GAG

and collagen than immature MSCs. This decline in MSC potential has been observed in both murine [30] and male (but not female) human [41] MSCs in pellet format, although donors were skeletally mature. Another study suggests a small decline in matrix production from adult MSCs compared with fetal cells [5]. However, these reported deficiencies in aged human pellets were not as marked as observed in this study with bovine cells.

Bovine MSC chondrogenic capacity in a three-dimensional HA hydrogel environment was also evaluated. These gels support both human and bovine MSC chondrogenesis [12, 17]. In this study, we used a 1% w/vol HA formulation that maximizes matrix formation by juvenile bovine MSCs [18]. Similar to pellets, bovine MSCs in this three-dimensional context were highly age-dependent with fetal and juvenile MSCs producing robust samples with compressive properties reaching approximately 20% of native tissue values within 3 weeks. Conversely, adult MSCs produced little ECM and only minor changes in mechanical properties. Tran-Khanh and coworkers, using bovine chondrocytes, reported a similar age-related decrease in biochemical and biomechanical properties in agarose hydrogels [50]. In contrast to these findings, Kopesky and coworkers found adult equine MSCs in a self-assembling peptide hydrogel generated constructs with greater mechanical properties than either juvenile chondrocytes or MSCs, although only dynamic properties were reported [29].

We have studied aging in bovine cartilage, three-dimensional cell pellets, and in three-dimensional hydrogels intended for cartilage tissue engineering. Our observations confirm that age is an important modulator of cartilage properties and of the MSC and chondrocyte response to TGF- β 3 in pellet culture. Most notably, bovine chondrocytes decrease in matrix-forming capacity in pellet culture with advancing age, but these decreases are smaller than those seen in human chondrocytes [1]. Likewise, bovine MSCs show a sharp decrease with age in cartilage matrix-forming capacity that is more severe than reported for human MSCs in this same format [5]. Overall, at each age, and under ideal conditions (absence of TGF for chondrocytes, presence of TGF for MSCs), bovine chondrocytes in pellet culture produce more GAG and collagen than MSCs, consistent with our previous findings [17, 22, 33]. Taken together, when considering an autologous cell-based tissue engineering strategy for cartilage repair, age must be an important consideration. Bovine cells are and remain a valuable tool for optimizing new material formulations, but care must be taken to ascertain the similarity in response of cells from this source in comparison to human cells.

Fig. 9 Picrosirius red staining of collagens (top) and Alcian blue staining of proteoglycans (bottom) supported the quantitative biochemical measures (Stain, picrosirius red and Alcian blue; original magnification, $\times 50$; 250- μ m scale bar).

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