

アルジネートマイクロスフェアを用いた猫関節軟骨細胞の3次元培養

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Three-Dimensional Culture of Feline Articular Chondrocytes in Alginate Microspheres

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ABSTRACT. Chondrocytes isolated from proximal femoral articular cartilage from 3 adult cat cadavers were expanded in monolayer culture and subsequently cultured in alginate microspheres for 24 days. Cell proliferation and production of proteoglycans in alginate microspheres were observed during day 18 and 24. Quantification of chondroitin sulfates (CS) by capillary electrophoresis revealed that cultured chondrocytes synthesized CS6 but not CS4. Three-dimensional culture using alginate microspheres is a useful *in vitro* technique to study proliferation and metabolism of chondrocytes; however, further modifications are needed to apply the technique to feline articular chondrocytes.

KEY WORDS: alginate microsphere, articular chondrocyte, chondroitin sulfate.

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Degenerative joint disease (DJD) is a common problem in veterinary practice, particularly in dogs and horses. Because of the high prevalence of DJD in these species in depth studies on chondrocyte biology have been undertaken. In contrast, DJD in cats has not been studied in detail due to a lack of severe clinical symptoms. This view has begun to change after a study reporting a relatively high incidence of DJD in geriatric cats based on a radiographic survey [9]. We have also conducted a radiographic and pathologic study on DJD in cat hip joints and found that 14.1% and 35.3% of the cats had radiographic and gross pathologic signs of DJD, respectively (submitted). Despite the surprisingly high incidence of DJD in cats, little is known regarding the underlying pathoetiology thus far. Investigations on metabolic and proliferative activities of chondrocytes are of particular importance since they are known to change during processes of aging and DJD. The metabolic activity of chondrocytes has been often studied by investigating constituents of proteoglycans, particularly chondroitin 4-sulfates (CS4) and chondroitin 6-sulfates (CS6) which are the two predominant glycosaminoglycans in articular cartilage (AC). For example in people, AC from neonates has a CS6:CS4 ratio of approximately 1:1, while the ratio progressively increases with increasing age [1, 4]. A similar age-related change in the ratio of CS6 and CS4 was observed in horses [3]. In people with DJD, the ratio of CS6 and CS4 was lower in patients with DJD than those without DJD [18]. The increased synthesis of CS4 was thought to be associated with chondrocyte proliferation in a repair process of damaged cartilaginous tissues. These reports suggested

that changes in the amounts of CS6 and CS4 in the newly synthesized cartilage matrix could serve as an indicator of metabolic activities of articular chondrocytes. These biochemical changes in AC have not been reported in cats.

In vitro culture systems hold significant advantages in facilitating studies on chondrocyte metabolism under various conditions. Three-dimensional culture using alginate has been successfully used in studying chondrocyte metabolisms in various species including humans [6, 7, 13], rabbits [12], and dogs [15, 16, 19]. Alginate microspheres allow chondrocytes to produce and retain proteoglycans around themselves as seen in AC. Quantification of CS in canine intervertebral disk chondrocytes cultured in alginate microspheres has been performed by use of capillary electrophoresis [15]. By using this technique, temporal changes in patterns of CS synthesis by canine articular chondrocytes in alginate microspheres have been demonstrated, validating this culture system as a valuable tool to study dynamic metabolic activities of chondrocytes *in vitro* [16]. We speculate that feline articular chondrocytes may also undergo age-related biochemical changes as reported in other species, leading to the development of DJD. The objective of this study was to evaluate proliferative and metabolic activities of feline articular chondrocytes in alginate microspheres in an attempt to provide information necessary for the development of a suitable culture system for feline articular chondrocytes.

Isolation of articular chondrocytes: Three adult domestic cat cadavers were collected from a local animal shelter immediately after euthanasia. All cats had radiographically normal hip joints. Femoral and acetabular AC were grossly examined to confirm that the joints have no signs of degenerative changes. The use of these cadavers was approved by the Institutional Animal Care & Use Committee at the University of Florida. AC tissues were aseptically excised from

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both proximal femoral heads and placed in Hank's balanced salt solution (HBSS, Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.). Excised cartilage was diced and digested in Ham's F-12 medium (Sigma Chemical Co., Ltd.) containing 5% fetal bovine serum (FBS, Sigma Chemical Co., Ltd.), 0.4% pronase (Sigma Chemical Co., Ltd.), and 0.004% deoxyribonuclease (DNase, Sigma Chemical Co., Ltd.) for 90 min at 37°C in humidified atmosphere (5% CO₂/95% air). The digested tissues were centrifuged for 5 min at 1,500 rpm and washed three times with Dulbecco's phosphate buffered saline (DPBS, Sigma Chemical Co., Ltd.). After washing, the tissues were further digested in Ham's F-12 medium containing 5% FBS, 0.012% collagenase (Sigma Chemical Co., Ltd.), and 0.004% DNase for 18 hr at 37°C in humidified atmosphere. The digested cells were washed three times with HBSS and strained through a 70 µm filter (Cell Strainer, Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) to eliminate debris. Subsequently, the isolated chondrocytes were counted using a hemacytometer and the viability of the cells was measured by Trypan blue exclusion technique. The chondrocytes were then suspended in Ham's F-12 medium containing 10% DMSO and stored in liquid nitrogen until used for monolayer culture.

Three-dimensional culture of chondrocytes in alginate microspheres: Frozen cells were thawed in a water bath at 37°C for 1 min and washed three times with HBSS. Thawed cells were counted and the viability was measured as described. Based on cell counting chondrocytes were seeded in a culture flask at a concentration of approximately 600 cells/cm² (9×10^4 cells/150 cm²) in Ham's F-12 medium containing 10% FBS, 2 mM L-glutamine, and 1% antimycotic/antibiotic solution (termed culture medium) at 37°C in humidified atmosphere. The culture medium was changed every third day. The culture was continued until approximately 80% of the surface was covered by the proliferated chondrocytes at which point the proliferated cells were freed by trypsinization, using a 0.05% trypsin solution containing 0.02% EDTA (Sigma Chemical Co., Ltd.). Freed cells were washed three times with HBSS and the number and viability of the cells were measured as described. The cells were resuspended in DPBS containing 1.2% of alginate (Kelton LV[®], Kelco Company, Chicago, IL, U.S.A.) at a cell concentration of 5×10^5 /ml [7]. The alginate suspension containing chondrocytes was dropped into a 102 mM CaCl₂ (Sigma Chemical Co., Ltd.) solution, 1 to 2 cm away from the surface through a 22-gauge needle. The microspheres were hardened in 102 mM CaCl₂ solution for 10 min, washed three times with HBSS and one time with culture medium, and cultured in culture medium at 37°C in humidified atmosphere for 24 days.

Quantification of DNA Contents: Twenty microspheres were collected from each sample on day 0, 12, 18, and 24. Quantification of DNA contents in microspheres was carried out by fluorometric assay using Hoechst 33258 dye solution (Sigma Chemical Co., Ltd.) as previously described [11]. Fluorometric enhancement was measured by spectro-

fluorometry (TKO 120 Mini Fluorometer, Hoefer Scientific, San Francisco, CA, U.S.A.) at an emission range of 400 to 550 nm and an excitation wavelength of 365 nm immediately after the reaction. A standard curve was made by measuring known concentrations of highly polymerized calf thymus DNA (Sigma Chemical Co., Ltd.) [11]. The DNA contents of 20 microspheres were calculated based on the standard curve.

Alcian blue staining of proteoglycans: To confirm production of proteoglycans by cultured chondrocytes on day 12, 18 and 24, alginate microspheres were hardened in 55 mM CaCl₂ for 45 min at room temperature and fixed in 2.5% glutaraldehyde in 0.1 M PBS for 4 hr at room temperature. Fixed microspheres were stained in a 0.1% alcian blue solution containing 0.4 M MgCl₂ and 2.5% glutaraldehyde in 25 mM sodium acetate (pH 5.6) at 4°C. Stained microspheres were observed under a phase contrast microscope.

Quantification of CS by capillary electrophoresis: One hundred microspheres were randomly selected on day 18 and 24. The microspheres were washed with HBSS and placed in 10 ml of 4 M GuHCl solution containing 50 mM Tris-HCl, 10 mM N-ethylmaleimid, 0.36 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C for overnight [20]. The extracted solution was strained through a 70 µm filter (Cell Strainer, Becton Dickinson Labware). To eliminate other proteins, samples were subjected to equilibrium density centrifugation after extraction. A CsCl (Fisher Scientific, Pittsburg, PA, U.S.A.) solution was added to the samples at a concentration of 0.55 g/g of sample solution. The samples were centrifuged for 48 hr at 40,000 rpm, 8°C, using a fixed angle rotor (Type T70.1, Beckman Instrument, Inc., Palo Alto, CA) in a centrifuge tube (Ultra-Clear Centrifuge tube 344322, Beckman Instrument, Inc., Palo Alto, CA). Newly synthesized proteoglycans were collected from the deepest layer in the tube [17]. The collected samples were run through a PD-10 column. Then elution was performed with 0.02 M Tris-HCl. Obtained elute was stored in -20°C until following digestion. Extracted samples (275 µl) were digested with 5 mU of chondroitinase ABC (Seikagaku America, Inc., Ijamsville, MD, U.S.A.) containing 80 µl of 100 mM Tris-HCl (Sigma Chemical Co., Ltd.) and 40 µl of cinnamic acid (CA, Sigma Chemical Co., Ltd.) for 3 hr at 37°C. After 3 hr of digestion, the digestion was terminated by heating the samples in boiling water for 1 min [10]. Capillary electrophoresis (BioFocus[®]3000, Bio-Rad, Hercules, CA) was carried out to quantify the proportion of CS4 and CS6 in the digested samples as previously described [15]. Fused silica capillary column (50 µm i.d., 375 µm o.d., 50 cm length, Bio-Rad) was used. The digested samples were loaded under vacuum and electrophoresed for 15 min at 23°C, 15 kV in 40 mM phosphate (Sigma Chemical Co., Ltd.), 40 mM SDS (Sigma Chemical Co., Ltd.) and 10 mM sodium borate (Sigma Chemical Co., Ltd.) at pH 9.0. The eluant was monitored at 232 nm. Peak areas for both CS4 and CS6 were standardized by that of the standard marker (CA).

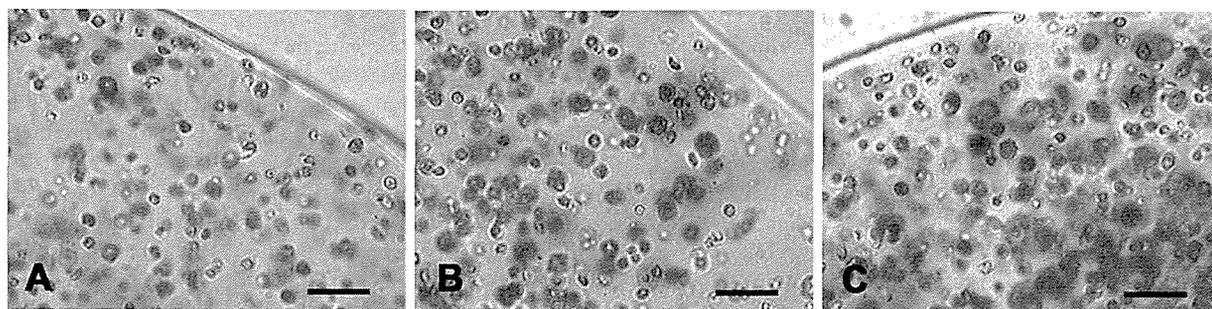


Fig. 1. Alcian blue staining of feline articular chondrocytes encapsulated in alginate microspheres. Images were taken under a phase contrast microscope on day 12 (A), 18 (B), and 24 (C). Alcian blue stained proteoglycans that have been synthesized by and retained around chondrocytes similar to proteoglycan deposition in the extracellular matrix in articular cartilage. The amount of proteoglycans progressively increased from day 12 to day 24. bar=100 μ m.

Absolute amounts of CS4 and CS6 were calculated from the standard curve which had been established by measuring known concentrations of serially diluted disaccharide samples (Seikagaku America, Inc.) as previously described [15].

Cell isolation and viability: The mean number of isolated chondrocytes from both proximal femoral AC was 6.38×10^5 , ranging from 6.15×10^5 to 6.6×10^5 . The mean viability of isolated cells was 100%. After thawing the cells, the mean cell viability dropped to 79.0%. The chondrocytes were recovered from the monolayer culture after 10 days. The mean number of cells recovered was 7.06×10^6 , ranging from 6.67×10^6 to 7.75×10^6 . At this point, the mean cell viability was 88.1%, ranging from 78.0% to 97.4%.

Proliferation of feline chondrocytes in alginate microspheres: In all samples, the DNA contents in 20 microspheres progressively decreased from day 0 to day 18 and increased from day 18 to day 24 (Table 1). Direct observation of cell aggregates in the microspheres under a phase contrast microscope seemed to correlate well with the DNA measurements where the formation of cell aggregates was pronounced after day 18 (data not shown).

Production of proteoglycans: Production of proteoglycans by chondrocytes in alginate microspheres was confirmed by alcian blue staining. Positively stained substances were already seen around some of the chondrocytes in alginate microspheres on day 12 (Fig. 1). The proportion of cell aggregates with alcian blue positive substances considerably increased on day 18 and 24, and this seemed to correspond to the increased rate of colony formation by proliferated chondrocytes (Fig. 1).

Quantification of CSs: At day 18 and 24, amounts of CSs were quantified by capillary electrophoresis. In all samples, only one peak following a peak of the standard marker (CA) was detected on electropherograms. The peak was identified as CS6 from the time at which the peak appeared and by adding 1 μ g of CS4 and CS6 to the samples. In 2 samples, the amount of CS6 increased from day 18 to day 24 while it was decreased in 1 sample (Table 1).

In the present study, we investigated the usefulness of three-dimensional culture using alginate microspheres in evaluating proliferative and metabolic activities of feline

Table 1. Proliferation and production of chondroitin 6-sulfates (CS6) by feline articular chondrocytes in alginate microspheres

Cat number		Day			
		0	12	18	24
Cat 1	DNA	554.4	530.6	488.9	524.6
	CS6	NA	NA	1.510	1.545
Cat 2	DNA	792.9	661.8	637.9	661.8
	CS6	NA	NA	1.926	1.729
Cat 3	DNA	590.2	357.7	280.2	316.0
	CS6	NA	NA	1.673	1.742

DNA values are presented as ng per 20 microspheres. CS6 values are presented as μ g per 100 microspheres. CS6=chondroitin 6-sulfate, NA=not applicable.

articular chondrocytes. We observed that DNA contents decreased from day 0 to day 18 and increased from day 18 to day 24 in all samples. The results suggested that compared to chondrocytes from other species feline chondrocytes may require a longer culture period to proliferate when alginate microspheres are used. In a previous report, when primary canine articular chondrocytes (without preceding monolayer culture) were encapsulated in alginate microspheres, chondrocytes steadily proliferated over a period of 20 days [14]. Primary canine intervertebral disk chondrocytes were also reported to proliferate in alginate microspheres over a period of 14 days [19]. In our study, we expanded feline chondrocytes to approximately 10-fold in monolayer culture before they were encapsulated in alginate microspheres. Because of the limited number of chondrocytes that could be harvested from feline proximal femoral AC (approximately 6.0×10^5 cells) expansion of chondrocytes in monolayer culture was necessary to carry out all experiments in our study. The use of monolayer culture was also based on a report that 6.4×10^5 chondrocytes were required to successfully quantify CS with capillary electrophoresis [15].

We think that the expansion step in monolayer culture did not affect metabolic activities of feline chondrocytes as proteoglycan synthesis was clearly observed by alcian blue

staining of the microspheres. However, the effects of monolayer culture on the metabolic activity of feline chondrocytes encapsulated in alginate microspheres need to be further examined to ensure that feline chondrocytes metabolically "re-differentiate" in microspheres. Such considerations were highlighted in alginate microsphere culture of rabbit chondrocytes where re-expression of aggrecan and type II collagen genes required 4 days and suppression of type I and III collagen synthesis required 2 weeks of culture in microspheres after monolayer culture [12]. With immunocytochemical techniques, expression of type I and II collagen can be evaluated in feline chondrocytes cultured in alginate microspheres which will provide important insights into the metabolic status of encapsulated chondrocytes in alginate microspheres. It is also important to investigate the effects of various supplements in the culture medium on proliferation and re-differentiation of feline chondrocytes. Among various reagents used, transforming growth factor β -2 (TGF β -2) and ascorbic acid are known to stimulate chondrocyte proliferation in monolayer culture [2, 5, 21]. Addition of these growth stimulatory reagents in feline chondrocyte alginate culture may enhance proliferation of chondrocytes thereby promote production of cartilage-specific type II collagen and the active form of CS such as CS4.

As mentioned, it was evident by alcian blue stain that feline articular chondrocytes produced proteoglycans in alginate microspheres with progressively increased amounts over the culture period. Similar to the previous observation in canine intervertebral disk chondrocytes [19], newly synthesized proteoglycans were retained around the chondrocytes as well-demarcated alcian blue positive substances. Quantification of CS revealed that newly synthesized CS was mainly, if not all, CS6. It has been known that abundant CS4 are present in AC where chondrocytes actively proliferate such as in endochondral ossification centers [8]. For that reason, it was plausible that a small number of actively proliferating chondrocytes in alginate microspheres did not synthesize enough quantity of CS4 that could be detected by capillary electrophoresis in our study. On the other hand, detected CS6 was likely to be those accumulated mostly during the early stage of the culture period. It has also been known that CS is not detected by capillary electrophoresis if the total DNA contents are lower than 748 ng/20 microspheres [14]. In our study, all but one of the samples did not have DNA contents greater than 748 ng/20 microspheres at any time points during the study. This again suggested that the detected CSs were mainly CS6 that have been synthesized and accumulated over the culture period.

The present study provided initial considerations necessary for the use of alginate microspheres for investigations on proliferation and metabolism of feline articular chondrocytes. It would be ideal for researchers to be able to examine dynamic changes of metabolic activities of feline

chondrocytes during both actively proliferating and senescent phases within the same culture system. Further optimizations on the culture length, cell density, and methodology for CS quantification should be considered in feline chondrocyte culture using alginate microspheres in future investigations.

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