Effect of Ascorbate and Two Different Media on Canine Chondrocytes in Three-Dimensional Culture*

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ABSTRACT

The objective of this study was to determine the effects of ascorbate and two different culture media on cell morphology and extracellular matrix formation of canine chondrocytes grown in a three-dimensional (3-D) culture system. Articular cartilage harvested from the humeral head of three adult canine cadavers was used to obtain chondrocytes for primary culture. Subcultured chondrocytes were seeded in a 3-D medium of RPMI-1640 (R), RPMI-1640 with 50 µg/mL ascorbate (RA), Ham’s F-12 (F), or Ham’s F-12 with 50 µg/mL ascorbate (FA) in agarose. Samples were harvested at 5, 10, 15, and 20 days of 3-D culture and analyzed for histologic appearance and proteoglycan staining, electron microscopic appearance, dimethylmethylene blue assay for glycosaminoglycan (GAG) content, and immunohistochemical staining for collagen type II production. Chondrocytes in all four groups maintained appropriate morphology and produced matrix over the entire study period. Chondrocytes from groups R and RA produced more GAG and collagen type II than did those from groups F and FA on days 10 ($P = .00791$) and 15 ($P = .0173$). Chondrocytes from group RA produced more GAG on days 5 ($P = .0154$) and 20 ($P = .0044$) than did those in groups R, F, and FA. With respect to matrix production, RPMI-1640 is superior to Ham’s F-12 for 3-D culture of canine chondrocytes. The addition of ascorbate at 50 µg/mL to RPMI-1640 did have a positive effect on the production of GAG but had minimal effect on type II collagen production. Determining the most ideal in vitro microenvironment for canine chondrocytes grown in a 3-D culture system has important implica-
tions to the in vivo application of this tech-

n INTRODUCTION

Physeal and articular cartilage are unique tissue defined by their extracellular matrix (ECM). Cartilage-specific glycosaminoglycans (GAG) and collagen type II are the major ECM components in determining appropriate tissue architecture and function. The ECM is synthesized and maintained by chondrocytes and must remain ordered and intact to maintain tissue function. In vitro models of physeal and articular cartilage in health and disease have been developed. Monolayer culture systems do not allow for appropriate maintenance of chondrocyte phenotype and cartilage-specific ECM production. Chondrocytes in explant culture systems (culture in which tissue is harvested in a sterile fashion and placed directly into culture medium) maintain their differentiated phenotype, have low mitotic activity, are not exposed to exogenous proteolytic activity, and their ECM is similar to that observed in vivo. However, explant cultures do not allow for appropriate study of cartilage in a state of development or repair. Cook et al have described a three-dimensional (3-D) chondrocyte culture system that maintains cell phenotype and cartilage-specific ECM synthesis; provides a model of cartilage in development and repair; and allows for study of cellular, ECM, and synovial fluid events. Although GAG, proteoglycan, and type II collagen production have occurred without the addition of ascorbate to this 3-D culture system, specific evaluations of the effects of the presence or absence of ascorbate have not been reported. Furthermore, most studies evaluating chondrocytes in culture have used Ham’s F-12 for the culture medium.

The purpose of this study was to determine the effects of ascorbate on canine chondrocytes in 3-D culture and to compare Ham’s F-12 and RPMI-1640 in order to determine the optimal medium for proliferation, differentiation, and matrix synthesis. GAG production, type II collagen, and morphology were of particular interest. Greater GAG production, type II collagen secretion, and normal cellular morphology suggest more normal chondrocyte function and health. Such information will be beneficial in the formation of the ideal growth medium for chondrocytes in 3-D culture. Knowledge of the influence of components of a medium on cells in vitro is important for understanding factors that may contribute to the ideal environment for chondrocytes and articular cartilage in an in vivo application.

n MATERIALS AND METHODS

Chondrocyte Isolation

Canine chondrocytes were harvested, cultured, and subcultured as previously described. All aspects of the experimental protocol were performed simultaneously in samples from all dogs. Cells from each dog were treated separately and not mixed. Within 1 hour of euthanasia, full thickness articular cartilage slices were aseptically obtained from the caudolateral surface of the humeral head of three adult canine cadavers, 2 to 4 years of age, using a No. 10 scalpel blade. The slices were placed in Hank’s balanced salt solution containing penicillin (100 IU/mL)/streptomycin (100 µg/mL)/amphotericin (2.5 µg/mL) (HBSS-p/s/a) and transported to a laminar flow hood. The cartilage slices were diced with a No. 10 scalpel blade into 2-mm cubes and rinsed twice with 10 mL of HBSS-p/s/a while placed on sterile gauze. The cartilage slices were placed in Hank’s balanced salt solution containing penicillin (100 IU/mL)/streptomycin (100 µg/mL)/amphotericin (2.5 µg/mL) (HBSS-p/s/a) and transported to a laminar flow hood. The cartilage slices were diced with a No. 10 scalpel blade into 2-mm cubes and rinsed twice with 10 mL of HBSS-p/s/a while placed on sterile gauze. The cartilage from each dog was placed in 30 mL of clostridial collagenase (0.5 mg/mL; 324 U/mg) in HBSS-p/s/a and incubated at 37°C with 5% CO₂ with slow stirring (60 revolutions per minute) for 15 hours.

The collagenase solution containing chon-
drocytes from each dog was filtered through sterile gauze to remove cartilage fragments. The gauze was rinsed with 10 mL of HBSS-p/s/a. The suspension was centrifuged at 400 g for 10 minutes; the supernatant was discarded and the cells were resuspended in 5 mL of RPMI-1640 medium with 2 mM L-glutamine containing penicillin (100 IU/mL)/streptomycin (100 µg/mL)/ampicillin (2.5 µg/mL) plus 10% fetal bovine serum (RPMI-p/s/a + FBS). Chondrocytes from each dog were plated in 25-cm² culture flasks and incubated at 37°C with 5% CO₂ and 95% humidity. The medium was changed every 2 to 3 days.

When cells were confluent (10 days), the medium was discarded and the flasks were rinsed with 10 mL of HBSS-p/s/a. Chondrocytes were detached from the flask by adding 1 mL of 0.05% trypsin + 0.53 mM EDTA in HBSS and incubating at 37°C for 5 minutes. Eight milliliters of RPMI-p/s/a + FBS were added to the suspension. Chondrocytes from each dog were split 1 to 9 in three 75-cm² flasks and incubated at 37°C with 5% CO₂ and 95% humidity. The medium was changed every 2 to 3 days.

Three-Dimensional Culture

When cells were confluent (15 days), the medium was discarded, the flasks were rinsed with 10 mL of HBSS-p/s/a. Chondrocytes were detached from the flask by adding 1 mL of 0.05% trypsin + 0.53 mM EDTA in HBSS and incubating at 37°C for 5 minutes. Eight milliliters of RPMI-p/s/a + FBS were added to the suspension. Chondrocytes from each dog were split 1 to 9 in three 75-cm² flasks and incubated at 37°C with 5% CO₂ and 95% humidity. The medium was changed every 2 to 3 days.

Sample Collection

The contents of one well from each dog in groups R, RA, F, and FA were collected at days 5, 10, 15, and 20 of 3-D culture. GAG assay controls were collected on day 20. The contents of each well were divided into three portions. One portion was placed in 10% buffered formalin for histologic processing. The second portion was weighed and placed in 1 mL of distilled, deionized water and stored at -80°C for subsequent GAG assay. The third was prepared for electron microscopy.

Histologic Examination

Five µm-thick sections were cut from each sample after histologic processing in the routine manner. Sections were stained with hematoxylin and eosin and toluidine blue. Sections were examined by a pathologist who was blinded to the treatment groups. Sections were evaluated for cell numbers and morphology and toluidine blue staining of proteoglycans.

Glycosaminoglycan Assay

Total sulfated GAG content was quantitated
by dimethylmethylene blue assay. Frozen samples were thawed and sections digested in 1.5 mL of 0.5 mg/mL papain (14 U/mg) in distilled, deionized water at 65°C for 4 hours. A 100-µL aliquot of the digest solution was assayed for total GAG concentration by addition of 2.5 mL of dimethylmethylene blue solution and spectrophotometric determination of absorbency at 525 nm. Known concentrations of bovine trachea chondroitin sulfate A were used to construct the standard curve. Results were standardized to correct for differences in sample weights. Total GAG content for these samples was reported in µg/mL/g.

Transmission Electron Microscopy
A portion of the contents of each well of a 24-well culture plate was minced into approximately 1-mm cubes and fixed in 2.5% glutaraldehyde in cacodylate buffer. The cubes were embedded in epon resin, processed for electron microscopy, and stained with lead citrate/uranyl acetate. Sections were viewed under a transmission electron microscope and evaluated for cell morphology and matrix production by a pathologist who was blinded to the treatment groups.

Immunohistochemistry
Unstained sections were deparaffinized. Endogenous peroxidase quenching was performed using 3% H₂O₂ in water. A 30-minute pepsin digestion was conducted. Immunohistochemical staining was performed using a commercially available avidin–biotin–peroxidase kit. A serum block was performed and the primary antibody (rabbit antibovine type II collagen) applied at a 1:1000 dilution for 16 hours at 4°C. Hematoxylin counter stain was used. Positive (canine trachea and meniscus) and negative (isotype matched primary antibody against human cytomegalovirus) controls were stained concurrently. Sections were examined by a pathologist who was blinded to the treatment groups.

Analysis
All statistical analyses were performed using a computer software program. Data from the three dogs in each group were combined and means ± standard errors were determined. A Friedman repeated measures analysis of variance was performed to determine differences within a group over time. When significant differences were obtained, an all pairwise multiple comparison (Student-Newman-Keuls) was performed to determine where differences occurred. Differences among groups were analyzed in the same manner. Significance was set at P < .05.

Results
Histologic Evaluation
Chondrocytes appeared round to oval with round, basophilic nuclei on hematoxylin– and eosin–stained sections. The cells were found primarily as individuals but were commonly seen in small groups or clusters. Evidence of proteoglycan production was shown with toluidine blue staining of pericellular and territorial matrix. All four groups had histologic evidence of both pericellular and territorial matrix by day 5. Although there were minimal differences between groups, groups R and RA appeared subjectively to have more cells with territorial matrix staining and more intense matrix staining. Marked increases in matrix staining were noted in groups R and RA on day 10 compared with their day-5 appearance, whereas groups F and FA showed only minimal change. Staining of the territorial matrix was subjectively more intense in groups R and RA than in groups F and FA on days 15 and 20 (Figure 1).

Immunohistochemical Staining
There was minimal pericellular staining of
type II collagen in groups R and RA by day 10. Pericellular and territorial staining were observed in groups R and RA on day 15. Subjectively, staining was more intense and widespread for group RA than for group R. The pericellular and territorial staining of collagen type II in groups R and RA had increased in intensity and amount on day 20 relative to day 15. Groups F and FA had no to minimal type II collagen staining on days 15 and 20 (Figure 2).

Transmission Electron Microscopy

Cells in all four groups that were evaluated appeared to be viable in that they contained dilated endoplasmic reticulum, intracytoplasmic lipid vacuoles, and membrane extensions. A notable difference between the RPMI-1640 and Ham’s F-12 groups was that groups R and RA contained a pericellular fine fibrillar network by day 10. No network was noted in groups F and FA on any day (Figure 3).

Glycosaminoglycan Production

Canine chondrocytes in groups R and RA produced GAG in 3-D culture as early as day 5 and throughout the study period. Measurable GAG production was not seen in groups F and FA until day 10 and remained less than 15 µg/mL/g for the duration of the study. Mean values for GAG production by group R were 2.32, 71.07, 116.78, and 33.92 µg/mL/g on
days 5, 10, 15, and 20, respectively. Mean values for GAG production by group RA were 8.65, 70.84, 112.63, and 75.17 µg/mL/g, on days 5, 10, 15, and 20, respectively. Mean values for GAG production by group F were 0.00, 6.03, 10.78, and 4.17 µg/mL/g on days 5, 10, 15, and 20, respectively. Mean values for GAG production by group FA were 0.00, 12.01, 5.55, and 4.99 µg/mL/g on days 5, 10, 15, and 20, respectively. Chondrocytes in group RA had significantly more GAG production than did groups F and FA on days 5, 10, 15, and 20 (P < .05). Chondrocytes in group RA had significantly more GAG production than did group R on day 20 (P < .05). Chondrocytes in groups R and RA had highest measured GAG levels by day 15 and then declined by day 20 (Figure 4).

DISCUSSION

The objective of this study was to determine if the addition of ascorbic acid at a previously published concentration to two different commonly used tissue culture media would influence matrix synthesis and morphology of canine chondrocytes in a 3-D culture model. Based on previous studies in the authors’ laboratory without the addition of ascorbic acid, the authors’ hypothesis was that the addition of ascorbic acid at 50 µg/mL would not have measurable effects on chondrocytic morphology or biosynthetic capabilities. Such information is important for understanding normal articular cartilage physiology and for formulating the optimal 3-D culture model.

Although dogs are capable of synthesizing adequate amounts of ascorbate, dog owners often opt for providing exogenous ascorbate as a means of potentially optimizing skeletal development. Humans cannot synthesize adequate amounts of ascorbate and may have disorders of physeal and articular cartilage with hypovitaminosis C because ascorbate is a co-factor for the lysyl- and prolyl-hydroxylases. Hydroxylation of proline to hydroxyproline stabilizes the collagen triple helix and is necessary for normal collagen secretion. Because of the influence ascorbate has on the formation of normal collagen, it is commonly added to cell culture media.

The results of the study reported here demonstrated that successful culture of canine chondrocytes in 3-D was more dependent on using RPMI-1640 versus Ham’s F-12 than on the addition of ascorbate at 50 µg/mL. This was shown by the more normal morphology and greater ECM production in groups R and RA than in groups F and FA over time.

Numerous differences exist between RPMI-1640 and Ham’s F-12 in terms of components and their concentrations. RPMI-1640, unlike Ham’s F-12, contains hydroxyproline, which is necessary for formation of a normal collagen triple helix. This may be the explanation for the greater collagen type II secretion by the cells grown in RPMI-1640 that was demon-
strated immunohistochemically. These immunohistochemical findings were paralleled by the electron microscopic appearance of a pericellular fine fibrillar network that was consistent with the previously reported appearance of type II collagen.\textsuperscript{17}

Another notable difference between RPMI-1640 and Ham's F-12 is the pH. The published pH for RPMI-1640 is 8.2, whereas that for Ham's F-12 is 7.0. Researchers have suggested that a more acidic environment may be detrimental to chondrocytes grown in 3-D culture.\textsuperscript{7} Based on these findings, further evaluation of the effect of pH on chondrocytes in 3-D culture is necessary.

The significantly greater GAG production by chondrocytes in group RA versus those in group R on days 15 and 20 may have been attributable to a positive effect of ascorbic acid alone or a synergism between ascorbate and one or more of the constituents of RPMI-1640. This is in contrast to previous reports that the addition of ascorbate to culture media had no influence on proteoglycan synthesis by human\textsuperscript{18} and rabbit\textsuperscript{19} chondrocytes in 3-D and monolayer culture, respectively. Those studies, however, were evaluating proteoglycans and not solely GAG. Techniques that measure the amounts of proteoglycans of differing molecular weights would be necessary to determine the effect of ascorbate on proteoglycans and the quality of the ECM.\textsuperscript{20}

Glycosaminoglycan levels decreased in all groups from days 15 to 20, which is consistent with previous studies in the authors' laboratory and secondary to imbibition of water over time by the agarose plugs.\textsuperscript{7,8} Thus it is a relative decrease in GAG production.

The concentration of ascorbate in this study was set at 50 µg/mL based on previous studies of chondrocytes in culture.\textsuperscript{21–23} Comparing the effects of differing concentrations of ascorbate, ranging from 25 µg/mL to 400 µg/mL, on chondrocytes in 3-D culture would allow assessment of the effects of varying concentrations of ascorbate.\textsuperscript{24} Although large-dose dietary supplementation of ascorbic acid has been reported to suppress the development of experimentally induced arthritis in rats\textsuperscript{25,26} and guinea pigs,\textsuperscript{27} dogs normally synthesize enough ascorbic acid to make it a nonessential nutrient.\textsuperscript{12} Thus varying concentrations of ascorbate may have little effect on the biosynthetic capabilities of canine chondrocytes. Nevertheless, such investigations are warranted to augment understanding of cartilage physiology and optimize cell culture techniques.

**CONCLUSION**

As has been shown in previous studies,\textsuperscript{7,8} this 3-D model does allow viability and differentiation of canine chondrocytes. It also permits the production of the components of normal cartilage matrix. The use of RPMI-1640 versus Ham's F-12 appeared to be more significant than did the addition of ascorbate at a concentration of 50 µg/mL with regard to viability and matrix production of canine chondrocytes.

**REFERENCES**