

Synovial fluid does not inhibit collagen synthesis

Bovine cruciate ligament studied in vitro

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An organ culture model of four bovine anterior cruciate ligaments was used to compare synovial fluid with bovine serum, lactated Ringer's solution, and Basal Medium Eagle's (BME) to determine their relative abilities to support in vitro conversion of proline into hydroxyproline. There was no difference between serum and synovial fluid in their ability to support collagen synthesis. These results suggest that bovine synovial fluid is not inhibitory in that it is similar to bovine serum in supporting ligament collagen synthesis under the conditions described.

There is little known about ligament healing or ligament cell metabolism in a synovial environment. Synovial fluid has been implicated as one of the potential reasons for variable results after primary anterior cruciate ligament repair or reconstruction (O'Donoghue 1950, 1955, O'Donoghue et al. 1966, 1971, MacIntosh and Tregonning 1976, Warren and Marshall 1978, Andrish and Holmes 1979, Clancy et al. 1981, Odensten et al. 1985) despite the fact that it has received very little scientific attention in this context.

Our purpose in this investigation was to study the influence of synovial fluid on ligament metabolism under controlled circumstances, comparing bovine synovial fluid with bovine serum and other control media in terms of their support of bovine ligament cell-collagen synthesis in vitro.

Materials and methods

Tissue preparation and distribution

After a series of pilot investigations designed to optimize methods of ligament organ culture and to minimize variability, four fresh, young adult, intact, bovine stifle (knee) joints were obtained from a local slaughterhouse. Under sterile conditions, the anterior cruciate ligament was removed from each joint, placed into a sterile Petri dish, and kept moist with a few drops of Ringer's solution.

Ringer's was chosen as a nonnutrient, isotonic salt solution, which was chosen arbitrarily as a metabolic baseline. The synovial membrane was removed from all the ligament surfaces and discarded. Each ligament was then further dissected along its longitudinal axis into five sections of approximately equal size. One of the five fragments from each of the four ligaments was randomly assigned to one of five experimental culture media: bovine synovial fluid, bovine serum (Sigma), lactated Ringer's (Travenol), Basal Medium Eagle's (Gibco), and a "zero" control pretreated with cyclohexamide (40 µg/mL), washed twice, and then resuspended in Basal Medium Eagle's.

Media

Sterile synovial fluid was obtained by needle aspiration of 18 normal, young adult, bovine radiocarpal

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joints. This synovial fluid specimen was pooled and centrifuged at 200 g for 20 min to remove all the cells. The clear synovial fluid supernatant was then aliquoted and stored at -70°C until the day of culture.

Bovine serum was chosen as a control for synovial fluid, because serum is a well-known culture medium, and is presumed to be an essential stimulator of healing. As noted above, based on previous experiments in our laboratory, lactated Ringer's was chosen to provide a baseline control for collagen metabolism, and Basal Medium Eagle's was chosen as a standard nutrient medium that does not contain proline or hydroxyproline.

Small aliquots of the acellular synovial fluid and the bovine serum were analyzed for proline and hydroxyproline content by calibrated amino acid analysis. Subsequently, all the media had their cold proline concentrations adjusted to equal that of bovine serum (found in this case to have the highest natural proline concentration) to provide equal amounts of cold precursor within each medium. Ascorbic acid, a cofactor of collagen synthesis, was added to all the media to a final concentration of 50 $\mu\text{g}/\text{mL}$. To ensure sterility, penicillin and streptomycin were also added to final concentrations of 100 U/mL and 100 $\mu\text{g}/\text{mL}$, respectively. All the media were buffered with 0.02 M Hepes. Osmolalities of all the culture media were measured by freezing-point depression, and were found to be within 10 milliosmoles of each other, thus decreasing the possibility of osmotic differences in nutrient absorption. To all the media, ^3H -proline (New England Nuclear #NET-323, specific activity 35.2) was added and mixed to make a final fluid concentration of 20 $\mu\text{Ci}/\text{mL}$.

With the exception of proline manipulations, ascorbic acid, and antibiotics, each of the metabolic media was therefore undiluted.

Culture conditions

Prior to culture, each of the five fragments from each ligament was blotted twice aseptically and the wet weight was recorded. Using sterile 15 mL polystyrene culture tubes, one fragment from each ligament was placed in one of five 5 mL culture volumes for treatment with one of the test solutions. Cultures were incubated for 44 hours in a humidified incubator at 37°C with 5 percent CO_2 . This interval of culture was demonstrated in pilot experiments (Figure 1) to represent a period with measurable levels of collagen synthesis with minimum

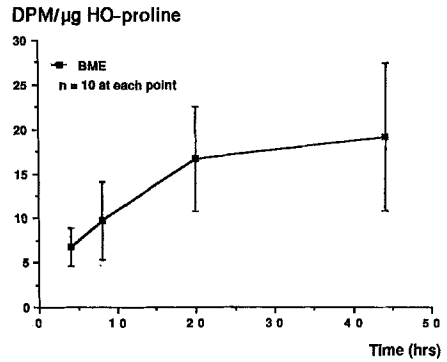


Figure 1. Graph of a pilot experiment showing mean specific activity (\pm SD) of 10 ligament fragments incubated in BME. Autoradiographs confirmed cellular activity. Note that absolute specific activity is a function of the activity of the proline precursor and that the ligament specimen studied was thus different in this pilot experiment than in the series reported.

risks of evaporation and no contamination. Autoradiographs in those pilot experiments also confirmed cellular, rather than nonspecific, metabolic activity at the 44-hour culture interval.

A 100- μL aliquot of each radiolabeled culture medium was assayed to quantify residual radioisotope after 44-hour incubations to ensure an excess of isotope and to measure isotope depletion.

After incubation, each ligament fragment was blotted twice, and a wet weight was recorded. The fragments were then washed extensively for 4 hours at 4°C in distilled water to remove nonincorporated label according to a previously described protocol (Frank et al. 1988).

Each lyophilized fragment was hydrolyzed completely and analyzed for hydroxyproline content and for newly synthesized collagen by methods described elsewhere (Frank et al. 1988). Data from all four ligaments were combined to compare means for each experimental medium.

Results

Water content and swelling

The initial preculture water content of all four ligaments was 73.7 ± 2.23 percent. No individual ligament had a water content different from the mean of the 20 fragments.

Table 1. Percentage swelling (after 44 hours) of bovine cruciate ligament incubated in different culture media, as a measure of potential proline and nutrient delivery in each test solution. Mean *SD*

Medium	Percent increase in wet weight	
Cyclohexamide/BME	59	13
BME	69	17
Ringer's	69	13
Bovine serum	50	18
Synovial fluid	54	14

All the fragments swelled between 50 and 70 percent in all the culture media, with fragments in BME and Ringer's swelling slightly more than those in bovine serum or synovial fluid. However, no statistical differences could be determined among the culture media for this parameter, suggesting similar delivery by diffusion of the proline precursor to the ligament cells in each case (Table 1).

Collagen synthesis

Cyclohexamide pretreatment completely eliminated collagen synthesis in our system, confirming that no synthesis could occur in intact enzyme systems in the absence of cell viability. This data was not included in the statistical analysis. All the other culture media supported the conversion of ^3H -proline into ^3H -hydroxyproline in each of the four ligaments (Table 2). The data can be analyzed as a two-way factorial design with no replicates. That is, there are two factors (the cow and the medium), and all possible combinations of the two factors were tested once. The "cow" can be thought of as a blocking factor—hence, a factor expected to introduce variability into the measurements of the medium

effect that we wish to correct for. Initially, a two-way ANOVA was performed; however, a check of the residuals (Box et al. 1978) suggested the possibility of an interaction between the two factors. Therefore, the logarithms of the collagen synthesis measurements were analyzed in place of the raw data. In this analysis the two-way ANOVA found, as expected, that there was a difference in measured collagen synthesis between ligaments ($P = 0.00001$), and there was also a difference when comparing the collagen synthesis measured in the different media ($P = 0.0007$). In this analysis a check of the residuals (Box et al. 1978) indicated that the possible interaction had been corrected by the logarithmic transformation of the raw data.

Tukey's multiple comparison test was then performed to compare the average values of collagen synthesis in the four media. It showed that BME gave more collagen synthesis than any other medium ($P = 0.05$). Ringer's gave less collagen synthesis than any other medium. Tukey's test did not reveal any difference between synovial fluid and serum.

Further analysis of the data using nonparametric methods, including the Friedman test and the Wilcoxon signed rank tests, did not provide any additional insights (Lehmann and D'Abrera 1975).

Discussion

The majority of previous investigations on speculated synovial effects have looked at tendons or ligaments in a so-called synovial environment. Lundborg and Rank (1978, 1980), for example, examined gap healing of sutured tendon fragments within a joint (Matthews 1976) and suggested that cells were supported in a manner that they termed tissue culture in situ. This speculation was supported by evidence from Amiel et al. (1986), Whiteside and

Table 2. Specific activities (DPM/ μg hydroxyproline) of the four anterior cruciate ligaments in various culture media. Numbers in italics are logarithmic transformations for statistical analysis

Medium	Anterior cruciate ligaments								
	1		2		3		4	Average	
Cyclohexamide/BME	0		0		0		0	0	
BME	12.4	<i>1.093</i>	14.7	<i>1.167</i>	9.8	<i>0.991</i>	5.1	<i>0.708</i>	10.5
Ringer's	7.4	<i>0.869</i>	6.5	<i>0.813</i>	6.0	<i>0.778</i>	1.8	<i>0.255</i>	5.4
Bovine serum	9.3	<i>0.968</i>	8.0	<i>0.903</i>	8.1	<i>0.908</i>	3.9	<i>0.591</i>	7.3
Synovial fluid	7.5	<i>0.875</i>	9.4	<i>0.973</i>	6.5	<i>0.813</i>	2.5	<i>0.398</i>	6.5

Sweeney (1980), and some of Lundborg's earlier investigations (Eiken et al. 1975), which collectively suggested a possible nutrient role for synovial fluid in the joint. Others, however, have noted the presence of confounding variables in such experiments, including the unknown contributions of synovial cells to measures of ligament metabolism (Amiel et al. 1986) and to tendon healing (Potenza and Herte 1982, Menon et al. 1985). In fact, the only in vitro experiment in which synovial fluid alone was tested on the proliferation and plating efficiency of ligament cells themselves (Andrish and Holmes 1979) demonstrated an apparent inhibitory effect.

As opposed to those findings, our organ culture model has demonstrated that bovine synovial fluid and bovine serum are comparable culture media for collagen synthesis by bovine ligament tissue. This similarity between serum and synovial fluid rules out any marked inhibitory effect of synovial fluid on collagen metabolism.

One possibility for the apparent superiority of an artificial culture medium to other solutions and the surprising lack of superiority of serum over simple Ringer's lactate is that normal cruciate ligament cells are relatively dormant, explaining the lack of substantial stimulation of collagen metabolism by serum. None of these solutions was very stimulatory of collagen synthesis; possibly a limitation of the culture system or perhaps an important intrinsic limitation of the ligament itself. On the other hand, cells were, nonetheless, performing some baseline metabolic activity and were synthesizing some collagen in all the test solutions, as shown by the complete inhibition of collagen synthesis by the inhibitor of protein synthesis—cyclohexamide. The culture conditions used were therefore, at a minimum, supportive of some collagen synthesis.

Another possible explanation of the lack of stimulation by nonculture media is that some essential nutrient or nutrients that we have not measured may have been present in lower concentrations in synovial fluid, bovine serum, and Ringer's (as compared with BME), and may have been exhausted before the completion of the experiment. We minimized this possibility by attempting to ensure that an excess of medium was present: by having a relatively large fluid volume per piece of ligament tested in every case. Other important factors (ascorbic acid, osmolarities, proline) were also controlled and were comparable in order to provide consistent and favorable baselines for some of the factors that could influence the results. An excess of all the nutrients could not be proven, however, because quantities of all the essential factors for ACL cells are unknown.

A third possibility that may explain apparent increases in metabolism of ACLs in BME is that cells may have preferentially proliferated in BME, resulting in apparent increases in collagen synthesis, rather than true increases per cell. This seems unlikely, because we have failed to culture bovine cruciate ligament cells in any of these media in other experiments.

Finally, we should comment on differences between our results and those of Andrish and Holmes (1979). Whereas synovial fluid may be inhibitory to cell proliferation in vitro for a variety of reasons, including potential inhibition of cell attachment, cell motility, or cell division itself, we would suggest that cell collagen metabolism is probably not affected. Inhibition of cell proliferation by synovial fluid is therefore probably not due to an inhibition of matrix production.

Instead, synovial fluid appears to be similar to serum in at least supporting collagen synthesis in fibroblasts; and one might speculate that, similar to others (Eiken et al. 1975, Matthews 1976, Lundborg and Rank 1978, 1980, Rank et al. 1980, Whiteside and Sweeney 1980, Amiel et al. 1986), cruciate ligament cells may be specifically adapted to metabolize collagen in a synovial environment.

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