The Evaluation of Glucosamine/Chondroitin-Sulfate and Hyaluronate on Cartilage Healing – A Rabbit Animal Injury Model

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ABSTRACT

Background: Alternative therapies (glucosamine-chondroitin-sulfate-manganese) (GS/CS) and injectable hyaluronate (H) are used for arthritic conditions of the knee. There is clinical evidence of the effectiveness of GS/CS in humans and domestic animals, and of H in humans. This is the first study to evaluate the effect of combined therapy on the healing of cartilage. **Methods:** This rabbit cartilage injury model study used four groups of ten rabbits; Group I (control group), II (oral GS/CS/Manganese), III (H intra-articular) and IV (both therapies). **Results:** A pathologist (blinded) graded all specimens by two cartilage-healing scales. None of the controls healed the defect with hyaline cartilage while two in each of the single therapy groups and four in the combined therapy group healed with hyaline cartilage. Cellular morphology and matrix staining indicative of hyaline cartilage healing evaluated separately were statistically significant. Trends were noted in the overall scores and healing of cartilage adjacent to the defect was better in the control group. **Conclusions:** Hyaline cartilage is superior to fibrocartilage and was found only in treated groups. **Clinical significance:** Arthritic conditions are prevalent and the use of GS/CS and H are in common use. This study is the first to evaluate the effect of these on healing cartilage.

INTRODUCTION

As the population ages and arthritis becomes more prevalent, the search for "new" and "alternative" therapies will increase. Glucosamine-HCL and Chondroitin-Sulfate compounds are readily available and in common use. Scientific data regarding these therapies is limited and not definitive. Hyaluronate is FDA approved and in use for the treatment of the arthritic knee; nevertheless, little is published as to its efficacy in the healing of articular damage. We have found no literature that looks at the effect of combined therapies. However, we believe that patients use both Glucosamine and Chondroitin compounds and receive Hyaluronate injections in the course of treatment of the arthritic knee. Our rabbit model study was designed to evaluate the effect of these modalities singularly and in combination.

Glucosamine/Chondroitin compounds are advertised widely in lay publications. These compounds are marketed as "chondroprotective agents,"[18] and their availability in drug stores, health food stores, and the Internet is widespread. In that they are readily available, patients may use these compounds for indications such as chronic arthritis and acute injury. In a case report, an osteochondral impaction injury in a basketball player treated with a Glucosamine/Chondroitin-Sulfate formula showed significant improvement of MRI changes to normal appearing cartilage[40].

Much of the reported research is on the clinical use in veterinarian medicine. Glucosamine-HCL-Chondroitin-Sulfate compound is commonly used as an additive to the diet of athletic and pleasure horses to reduce the incidence of musculoskeletal lameness[21]. Synergism has been reported when Glucosamine and Chondroitin-Sulfate are given together [8, 20, 23].

Clinical studies have shown Glucosamine and Chondroitin-Sulfate to be effective in the treatment of osteoarthritis[1,3-5,7,9,12,13,16,17,22,33,36,38]. When used in combination with Manganese

Ascorbate, a reduction in the symptoms of DJD of the knee was noted in the U.S. Navy diving and special warfare group when taken for 16 weeks[26].

Despite human and equine clinical success, the mechanism of action of these compounds is not clear. Glucosamine and Chondroitin are components of the glycosaminoglycans (GAGs), which are components of the repeating disaccharide units of endogenous polysaccharides[11]. No convincing evidence proves that orally administered compound results in increased levels of GAGs in the cartilage or synovial fluid.

Hyaluronan is produced by synoviocytes and chondrocytes, and is found in synovial fluid and articular cartilage[2,3]. Hyaluronan is a high molecular weight and viscosity substance. In osteoarthritis, however, Hyaluronan becomes depolymerized resulting in a decrease in molecular weight and viscosity. Like Glucosamine/Chondroitin-Sulfate, Hyaluronan has also been used in the management of thoroughbred racehorses. Injected intra-articularly, it is frequently used in the treatment of DJD of the human knee. Altman found Hyaluronan administered intra-articular for five consecutive weeks reduced pain and improved function and was at least as effective as naproxen with fewer side effects[2].

In that all of these substances are commonly used in the treatment of osteoarthritis (physician and self-prescribed), we sought to study the effect of these agents singularly and in combination on a rabbit injury model. The injury would be of the type that would be expected to generate an arthritic condition. Glucosamine-Chondroitin compounds form the building blocks of synovial fluid and articular cartilage, and they have been shown clinically to benefit injured cartilage. We hypothesized that the oral administration of Glucosamine-Chondroitin or the intra-articular addition of Hyaluronan or both may result in improved cartilage healing.

MATERIALS AND METHODS

Forty adult male New Zealand White rabbits weighing 3-5 kg. were obtained. The study protocol was approved by the Animal Welfare Committee at the University of Texas - Health Science Center in Houston. Licensed veterinarians supervised all phases of the study, and all personnel involved in the study completed the required institutional rabbit methodology course. The animals were anesthetized using Ketamine I.M. at a dose of 18 mg/kg and Xylazine I.M. at 4 mg/kg. Isoflourane was available for inhalation and was titrated to effect. Cefazolin (20 mg/kg I.M.) was administered. The prepatellar area of the right knee was shaved and the entire right lower extremity sterilely prepped using a betadyne solution. A midline incision was made through the skin and a medial parapatellar incision made into the joint. Using a 2mm-drill point, a 6mm deep defect was created in the weight-bearing portion of the medial femoral condyle. The area was then irrigated with approximately 10 cc of normal saline and the medial joint capsule repaired with 4-0 Vicryl interrupted sutures. The skin was closed with 3-0 Vicryl interrupted sutures. Marcaine 0.25% (1mg/kg) was infiltrated in the skin about the wound. The animals were closely monitored for evidence of pain as evidenced by poor feeding, limited ambulation, and general appearance. Buprenorphine (0.02-0.05 mg/kg S.Q. q. 8 hrs) was administered as needed. The wound was inspected three-times/day for evidence of infection.

The animals were randomly divided into four groups of ten rabbits each. The number of rabbits was determined using power analysis data from published repair studies on rabbit cartilage[30]. Group I (control) was fed a daily diet of standard rabbit food (Teklad®). Group II (Gluc/Chond) was fed a diet of Teklad® rabbit food that had been manufactured with the addition of Glucosamine-HCL, Chondroitin-Sulfate and Manganese (Cosamin DS,Nutramax

Laboratories Inc, Edgewood,MD)*. The formula resulted in the intake of 17mg/kg/day of Chondroitin-Sulfate and 21mg/kg/day of Glucosamine. The Glucosamine/Chondroitin was also available to be administered by oral gavage should the rabbits fail to consume their diet, although this did not occur. All animals consumed the entire daily diet. Group III (Hyal) received the standard rabbit diet (Teklad®). In addition, an injection of 0.5 ml of hyaluronan (Hylan G-F, Synvisc® Wyeth,Madison NJ) was placed in the right knee of each rabbit at weeks 4,5, and 6 post injury. The animals were sedated with Ketamine (20 mg/kg IM) and Acepromazine (1 mg/kg IM) prior to each injection. Group IV (Hyal/Gluc/Chond) received combination therapy including the diet supplemented with Glucosamine, Chondroitin-Sulfate, and Manganese and the three injections of Hyaluronan at weeks 4, 5, and 6.

At 12 weeks, the rabbits were euthanized using Pentobarbital IV (50-60 mg/kg). The distal femur was removed and sent to pathology for microsectioning using a standard paraffin technique and stained with Safarin-O. The slides were numbered and logged. An independent pathologist with no knowledge of the nature of the study evaluated the defect based on two scales[25,35]. As the two scales require extensive evaluation of each slide and the pathologist must be accustomed to the use of these scales, only one pathologist evaluated the slides. In that this pathologist graded all slides with absolutely no knowledge of the nature of the study we felt this sufficient.

STATISTICAL METHODS

Mann-Whitney tests were performed (SPSS Version 10.0.5, SPSS, Inc., Chicago, Illinois) to compare the study groups to the control groups for each of the categories in both histological scales. Significance levels were taken to be 0.05.

* Cosamin DS contains TRH122 low molecular weight Chondroitin-Sulfate and FCH649 Glucosamine-hydrochloride and Manganese.

RESULTS

Histology evaluation with the Pineda scale (Table I) is grouped into four categories: filling of the defect, reconstitution of the osteochondral junction, matrix staining, and cell morphology in the defect. In this grading scale, a lower number indicates a better outcome. The results are shown in Figure 1. A Mann-Whitney test was performed in each of the four categories to compare the study groups to the control group, and a significance level of less than 0.05 was used. There were no significant differences detected with respect to any of the four categories, although near-significant p values were found in the cell morphology category.

The Moran scale (Table II) grades each histology section in nine categories: cell morphology, matrix staining, surface regularity, structural integrity, thickness, bonding to adjacent cartilage, hypocellularity, chondrocyte clustering, and degenerative changes in adjacent cartilage. In this grading scale, a higher score indicates a better outcome. The results are represented in Figure 2. Again, Mann-Whitney tests were used to compare the study groups to the control group. No significant differences were noted when surface regularity, structural integrity, thickness, bonding to the adjacent cartilage, hypocellularity, or chondrocyte clustering were studied. The Hyaluronan group had a significantly better score than the control group when cell morphology was studied. The group treated with both Hyaluronan and Glucosamine/Chondroitin scored significantly better than the control when matrix staining was studied. Figure 3 illustrates the stained sections (Safranin-O) with two examples from each group. The upper photomicrograph represents the section in that group receiving the highest overall grade, while the lower photo is that of the lowest overall score in that group.

DISCUSSION

Many new dietary supplements have become available, some of which are marketed as "chondroprotective" and many of which are marketed to those with already existent osteoarthritis. Hyaluronan has been approved by the FDA for viscosupplementation of the arthritic knee. It is unclear from numerous in vitro and in vivo studies as to the efficacy of these agents. No prior study has evaluated the use of these agents, independently or in combination, for the treatment of acute osteochondral damage, which would be expected to lead to osteoarthritis.

Glucosamine, Chondroitin-Sulfate, and Hyaluronan are found in natural cartilage, and Hyaluronan occurs naturally in the synovial fluid. Glucosamine is the primary component of glycosaminoglycans (GAGs), which make up the proteoglycans found in the matrix of cartilage. It is these GAGs that are hydrophilic and allow cartilage to imbibe fluid. Glucosamine is also a component of Hyaluronan. Glucosamine has been shown to increase the production of GAGs in the body and to promote proteoglycan synthesis by fibroblasts and chondroblasts[25,33]. Glucosamine has been shown to have an anti-inflammatory effect, which is independent of prostaglandin[32]. This effect is 50-300 times slower, but 1000-4000 times less toxic than the indomethicin to which it was compared[15]. Multiple other studies have confirmed that although the onset of action is slow, the effectiveness is at least equal to and, in some cases, superior to non-steroidals [12,13,15,32]. In a clinical, controlled study, Glucosamine had significantly better pain relief than placebo, and the improved symptoms were thought to be due to anti-inflammatory and analgesic effects[27]. Glucosamine alone and in combination with Chondroitin-Sulfate have been shown in multiple trials to be effective in reducing pain and disability [14-16,18,19,29,33].

Chondroitin-Sulfate is the prominent glycosaminoglycan (GAG) found in articular cartilage. Multiple randomized placebo controlled studies have been performed in which the Chondroitin-Sulfate was administered orally, intra-articularly, and intramuscularly[10,24,28,34,37,39]. One clinical study compared Chondroitin-Sulfate to diclofenac Na followed by placebo in the treatment of osteoarthritis of the knee[31]. Chondroitin-Sulfate was found to be more effective although the mode of action was slower. Blanco reports an increased production of NO by chondrocytes of osteoarthritic joints and found Chondroitin-Sulfate to reduce NO formation[6].

Manganese is found in combination with Glucosamine and Chondroitin-Sulfate in the commercial preparation that we used. A Manganese deficiency results in abnormal formation of cartilage and bone[41]. There are no specific studies indicating a benefit of exogenous Manganese in the treatment of osteoarthritis[26].

There are numerous preparations available for Glucosamine and Chondroitin-Sulfate. Some are available in singular and some in combination. These over- the-counter products are not required to comply with FDA standards of labeling and there may be variability in the quantities of ingredient in the product. We chose to use a product which is available only by prescription as it contained Glucosamine, Chondroitin-Sulfate and Manganese in quantities meeting FDA manufacturing standards. In addition, because the product was mixed with the Teklad® diet, the diet was assayed to assure uniformity. The dose was based on the equivalent dose which would be taken by a 72 kg adult taking the recommended dose of three commercially available capsules per day (Glucosamine 21mg/kg/day and Chondroitin-Sulfate, 17mg/kg/day).

Our data demonstrate that although all defects filled in at least 75% during the study period, all of the control animals had fibrocartilage only in the defect. In both the Glucosamine/Chondroitin and the Hyaluronan groups, there were two animals with exclusively

hyaline cartilage in the defect, and the group treated with both Glucosamine/Chondroitin and Hyaluronan there were four animals with exclusively hyaline cartilage in the defect. Studies have demonstrated that fibrocartilage will break down under the high loads seen in the knee joint during normal activities, and that hyaline cartilage is necessary for a healthy joint. Very broad based grading scales showed only strong statistical trends among our groups with the treated groups performing better than controls (combined therapy best). Statistical significance was noted in hyaline cartilage healing, suggesting that treatment with either Hyaluronate or Glucosamine-HCL-Chondroitin-Sulfate-Manganese compound, or both will increase the chances of hyaline cartilage healing of a defect injury in the knee.

Minor degenerative changes were found in areas adjacent to the defect in some of the study specimens. These changes were not noted in the control group. There is no logical explanation for this finding, but since statistical significance was noted, it does require mention and should be evaluated in future studies.

Non-steroidal anti-inflammatory drugs have been the primary treatment for osteoarthritis. This class of drug is known to have a high rate of intolerance ranging from gastric upset to major bleeding and death. Recently, COX-2 specific drugs have been developed in an effort to decrease these risks. In addition, intra-articular viscosupplementation with Hyaluronan has become available as another treatment modality. Clearly, there is a significant need for alternate therapies, and our growing and aging population is seeking therapies outside of contemporary medicine. The shelves of pharmacies and health food stores are replete with compounds promising to relieve arthritis symptoms. Clinical and equestrian studies support the use of Glucosamine and Chondroitin-Sulfate. The action of these substances is not profound and the delay of action is long. The Office of Alternative Medicine of the National Institute of Health

felt that these substances deserved further study and they are now funding a multicenter placebo controlled clinical study.

Hyaluronan is available for intra-articular injection and is classified by the FDA as a device. It is felt that it functions as a mechanical lubricant to the knee as opposed to having a direct pharmacological or biochemical effect. The normal dose of Hyaluronan is 3cc per injection. We used 0.5cc per injection in the study animal. Per kilogram, this is greater than the adult human dose, but the quantity of 0.5cc was so small, that anything less would result in most of the product remaining in the needle. Thus, we felt this to be the minimum possible dose of this very viscous material.

The efficacy of these agents in relieving the symptoms of osteoarthritis is well established. We felt that, individually or in combination, these compounds may improve the quality and quantity of cartilage repair thus reducing the risk of later osteoarthritis. This pilot study, utilizing an acute injury animal model (rabbit), was performed to determine if there was a benefit to either oral supplementation with Glucosamine-HCL and Chondroitin-Sulfate or intra-articular administration of Hyaluronan or both to cartilage healing. We used two grading scales, which are very broad based and include the quality of cartilage (hyaline vs. fibrocartilage), as only one aspect. When our overall results were examined, strong statistical trends in the overall scores were noted. The combined therapy of Glucosamine-HCL-Chondroitin-Sulfate compound and Hyaluronan injections were superior to the control group but only to a near-significant p-value. All of the control groups healed the defect with fibrocartilage, while two in the Glucosamine-Chondroitin group and two in the Hyaluronate group healed entirely with hyaline cartilage. In the combined therapy group, four healed exclusively with hyaline cartilage.

Thus, in all, we found that all controls healed with fibrocartilage, while eight of thirty treated animals healed entirely with hyaline cartilage. Cell morphology was superior to a near-significant p-value in the treated animals (indicative of hyaline cartilage), but when the overall scores were evaluated, only strong statistical trends were noted. We believe that these findings are supportive of a role of Glucosamine-Chondroitin-Manganese, Hyaluronate injections, or both in the healing of damaged cartilage. However, larger numbers of animals are required if statistical significance is to be achieved. The comprehensive nature of the Moran and Pineda scale may be such that the clinically relevant healing with hyaline cartilage is overshadowed. The majority of prior studies of cartilage healing use only cell morphology and matrix staining, which are indicative of hyaline cartilage, as the only parameter to judge outcomes. We used the Moran and the Pineda Scales as more recent studies have used these more all-inclusive scales. However, we would predict that cell morphology and matrix staining, which predict the formation of superior Hyaline cartilage, would be of greatest significance.

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Table I. The Pineda Scale for the grading of healing of articular cartilage including subcatagories and scores. Note that in this scale, smaller numbers indicate better results.

I	Filling of defect		scale
		125%	-1
		100%	0
		75%	1
		50%	2
		25%	3
		0%	4
II	Reconstitution of osteochondral junction		
	Yes		0
	Almost		1
	Not Close		2
III	Matrix Staining		
	Normal staining		0
	Reduced staining		1
	Significantly reduced staining		2
	Faint staining		3
	No stain		4
IV	Cell Morphology		
	Normal		0
	Mostly hyaline and fibrocartilage		1
	Mostly fibrocartilage		2
	Some fibrocartilage, but mostly nonchondrocytic cells		3
	Nonchondrocytic Cells Only		4

Table II	Moran Scale; Histologic grading system for articular cartilage healing.	
	Note; larger numbers indicate a better result.	
Category		
Nature of the repair tissue		Scores
	Hyaline Cartilage	4
	Mostly Hyaline Cartilage	3
	Mostly fibrocartilage	2
	Mostly non-cartilage	1
	Non-cartilage only	0
Matrix Staining (Safranin-O)		
	Normal Staining	3
	Moderate	2
	Slight	1
	None	0
Structural Integrity		
	Normal structure	2
	Slight disruption	1
	Severe disruption	0
Surface Regularity		
	Smooth and intact	2
	Slight disruption	1
	Severe disruption	0
Filling of the defect		
	100%	2
	>50%<100%,or >100%	1
	<50%	0
Bonding to host tissue		
	Bonded	2
	Partially bonded	1
	Not bonded	0
Degenerative changes of the repair tissue		
	Normal cellularity and cell morphology	3
	Mild hypocellularity and cell cluster	2
	Moderate hypocellularity and cell degeneration	1
	Severe hypocellularity and cell degeneration	0
Degenerative changes of the adjacent cartilage		
	Normal cellularity, cell morphology, and matrix staining	3
	Mild hypocellularity and cell clustering, moderate matrix staining	2
	Moderate hypocellularity and cell degeneration,decreased matrix staining	1
	Severe hypocellularity and cell degeneration, poor or no matrix staining	0

Figure Key

Figure 1. The Pineda scale is represented. Note that a smaller number indicates a better score. In the area of cell morphology which grades for the healing with hyaline vs. fibrocartilage, the study groups did better than the controls where in defect filling, the controls were superior.

Figure 2. The Moran scale is represented. Only statistically significant catagories are illustrated. Note that in this scale, a larger number represents better results. In these catagories, the study groups were better than the controls in cell morphology and matrix staining which were indicative of the quality of healing in the defect area, but the appearance of the cartilage in areas adjacent to the defect was better in the control group.

Figure 3. These photomicrographs represent two example sections (Safranin-O stain) from each of the four groups. The worst and best of each group by the two grading systems are illustrated with the better healing on the top.