Effect of Glucosamine Sulfate on Osteoarthritis in the Cruciate-Deficient Canine Model of Osteoarthritis

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Abstract

Objective. Osteoarthritis (OA) is a major cause of musculoskeletal pain and disability worldwide. The investigation of disease-modifying treatment options for OA has become an important aspect of orthopedic care. To assess the effect of intra-articular and oral glucosamine sulfate (GS) versus placebo on osteoarthritis in a canine model. **Materials.** In this randomized, placebo-controlled, double-blinded study, OA was induced by anterior cruciate ligament transection (ACLT) according to the Pond-Nuki model in 32 canines. All canines were allocated into 4 treatment subgroups with treatment administered for 8 weeks: GS (400 mg) intra-articular, placebo intra-articular, GS (200 mg/kg body weight) oral, and placebo oral. The contralateral nonoperated stifle (knee) served as control. After 8 weeks, the medial and lateral femoral condyles, the medial and lateral tibial plateau and patella were histologically examined and anatomic changes quantified by light microscopy using the modified Mankin score. **Results.** After 8 weeks, mean Mankin score values significantly ($P < 0.002$) decreased in the intra-articular GS group (8.1; range 7.9–8.8) compared with the intra-articular placebo group (13.9; range 11.6–15.9) and again significantly ($P < 0.002$) in the oral GS group (12.1; range 9.9–12.7) compared with the oral placebo group (15.1; range 12.5–17.0). Mean Mankin score values were significantly ($P < 0.002$) lower in the intra-articular GS group compared with the oral GS group. **Conclusion.** Both, intra-articular and oral administered GS significantly reduced histological signs of OA in the Pond-Nuki model, with the intra-articular application being more effective compared to oral administration.

Keywords

experimental osteoarthritis, articular cartilage, glucosamine sulfate, canine, drug therapy, knee joint

Introduction

Osteoarthritis (OA) is a common and major cause of morbidity and disability.^{1,2} The pathophysiology of OA involves complex interaction of mechanical stress, oxidative damage, and inflammatory mediators and the catabolic-anabolic balance of the joint, synovia, matrix, and chondrocytes.^{3,4}

The main objectives in the management of OA are to reduce symptoms, minimize functional disability and limit progression.³ Currently available medical therapies address the treatment of joint pain in patients suffering from OA.¹ Analgesics as well as traditional and cyclooxygenase-2-selective nonsteroidal anti-inflammatory drugs (NSAIDs) have suboptimal effectiveness,^{1,5} and there is some question about their safety, especially in light of recent reports of increased cardiovascular risk.^{1,5}

Glucosamine is an aminomonosaccharide occurring naturally in almost all human tissues, largely in proteoglycans of articular cartilage. The normal source of glucosamine is endogenous biosynthesis from glucose. Exogenous

glucosamine sulfate (GS) is the preferential source for proteoglycan biosynthesis. GS is consequently incorporated into proteoglycan molecules⁶ and shows a special tropism for cartilaginous tissues.⁷

Two long-term clinical studies suggested structure-modifying effects of GS by radiographic preservation of joint space and improved WOMAC (Western Ontario and McMaster Universities Arthritis Index) score values compared with placebo-treated patients.^{8–10} However, the use of GS remains a matter of debate,^{1,2} and recent studies comparing glucosamine

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with placebo for OA found mixed results regarding efficacy of glucosamine for pain relief and physical function.¹¹⁻¹³

In vitro and *in vivo* experiments with GS indicated anabolic effects by stimulating the synthesis of proteoglycans and hyaluronic acid and anticatabolic effects by inhibiting metalloproteinases.^{3,4,9,10}

The aim of this prospective, randomized, double-blinded study was to assess the efficacy of intra-articular and oral administered GS on OA in dogs for the treatment of osteoarthritis.

Methods

A total of 32 skeletally matured (closed epiphyses) beagle canines with a preoperative median age of 15 months (range 13-18 months) and a mean body weight of 13.25 ± 1.59 kg were included in this study. Body weight was monitored and documented once per week during the weeks (8 weeks). Final mean body weights calculations at the time of death/sacrifice (57th postoperative day) did not differ significantly from the preoperative measurements ($P = 0.78$).

Osteoarthritis was induced using the standard operating procedures of the approved Pond-Nuki model.¹⁴ According to this model the left stifle (knee) of all 32 canines was destabilized in general anesthesia by anterior cruciate ligament transection (ACLT) using a standard parapatellar mini-arthrotomy with consecutive development of OA with defined morphological and biochemical characteristics.¹⁴ The nonoperated contralateral knee of all 32 canines was dissected at the end of the study and served as control.

All 32 canines were randomized and allocated into 4 groups (#1 i.a., #2 i.a., #1 p.o., #2 p.o., where i.a. = intra-articular and p.o. = oral [per os]). Injectable solutions of GS and placebo, as well as the capsules for oral GS and placebo administration were provided by Rotta Research Laboratorium, Monza, Italy. Having the identical appearance and viscosity GS and placebo were blinded to the investigators knowing only the use of substance #1 or #2 until study data acquisition was accomplished. Canines randomized for intra-articular applications were injected into the operated left knee, only. The right knee of all 32 canines served as control and remained untouched for the entire study. Only the left operated knee was injected according to the randomized group allocation with either substance #1 or #2.

Contents, Dosages, and Administration Intervals

Intra-articular Applications. Two ampules of a preassembled set (ampule A, containing either 400 mg GS and 2.0 mL water for injection q.s. [#1 i.a.] or 2.0 mL water for injection q.s., only [#2 i.a., blinded to the administrator]; ampoule B, containing either 2.0 mg sodium hydroxide and 1.0 mL water for injection q.s. [#1 i.a.] or 1.0 mL water for injection

Figure 1. Dissection and preparation.

(A) Cartilage specimen of a dissected knee joint: The knee joint was dissected in toto and 5 specimens taken: lateral and medial femoral condyles, patella, lateral and medial tibial plateau. (B) Cartilage specimen of the medial femoral condyle cut into 3 equal pieces. The middle piece was fixed and embedded in paraffin for histological examination. (C) A (visible) cartilage thickness decrease was mainly localized in weightbearing areas (lateral and medial plateau of the tibia).

q.s., only [#2 i.a., blinded to the administrator]) were drawn up in one 5-mL syringe. Injections were started on the day of the operation and were repeated once weekly over a period of 5 weeks (6 injections overall).

Oral Applications. Orally administered capsule presented an identical appearance and were therefore blinded to the administrator. GS was orally administered in a daily medication of 200 mg/kg body-weight for a period of 8 weeks by mixing capsules into the food. Complete takings were controlled. Placebo was orally administered on a daily basis for a period of 8 weeks by mixing capsules in the food. Complete takings were controlled.

Sample Processing and Statistical Analysis Patterns

All canines were sacrificed after 8 weeks (57th postoperative day). Both knee joints were dissected and results of a macroscopic evaluation documented. Osteochondral samples of both, the operated and nonoperated (contralateral) knee joint were harvested from 5 defined anatomical sites: lateral and medial femoral condyles, patella, lateral and medial tibial plateau (Fig. 1). The harvested samples were cut in 3 equal pieces (Fig. 1) and the middle piece fixed in 2.5% glutaraldehyde in 0.1 molar phosphate buffer (pH 7.4) and finally embedded in paraffin for

Table 1. Histological Mankin Score.^a

I.	Structure		III.	Stainability of the cartilaginous matrix with safranin O	
	Normal	0		Normal	0
	Slight surface irregularities	1		Slight reduction	1
	Moderate surface irregularities	2		Moderate reduction	2
	Severe surface irregularities	3		Severe reduction	3
	Cleft in transitional zone	4		No dye noted	4
	Cleft in radial zone	5			
	Cleft in calcified zone	6			
	Loss of transitional zone	7			
	Loss of radial zone	8			
	Loss of calcified zone	9			
	Complete disorganization	10			
II.	Cell		IV.	Tidemark zone	
	1. Tangential zone			Intact	0
	Normal	0		Multilayered	1
	Swelling of cells	1		Indistinct	2
	Disappearance of cells	2		Crossed by blood vessels	3
	2. Transitional and radial zone		V.	Formation of pannus tissue on the cartilaginous surface	
	Normal	0			
	Slight hypercellularity	1		Normal	0
	Moderate hypercellularity	2		Slight	1
	Severe hypercellularity	3		Moderate	2
	Slight cloning	4		Marked	3
	Moderate cloning	5			
	Severe cloning	6			
	Slight hypocellularity	7			
	Moderate hypocellularity	8			
	Severe hypocellularity	9			
	Disappearance of cells	10			

^aModification by Sakakibara et al.¹⁵ Score values range from 0 to 32.

histological examination. Serial sections of 2 µm were stained with safranin-O for light microscopic evaluation.

The Mankin score (**Table 1**) in the modification of Sakakibara et al.¹⁵ was used to quantify findings. The score includes histological and histochemical assessment parameters of degenerative changes in hyaline articular cartilage and ranges from 0 (no lesions) to 32 (severe lesions). All 5 sites were examined separately with each specimen scored twice by 3 different raters.

Final score values were calculated as mean deriving from single score values.

Intrarater reliability was calculated using the intraclass correlation coefficient (ICC) of the first and second assessment of each rater. Mean score values of the rater providing the highest ICC were used for further statistical analysis. The Mann-Whitney *U* test was used to analyze Mankin score values between both medications (#1 or #2) and administration (i.a. or oral) groups. *P* values were adjusted in order to maintain the error level of *P* = 0.05 according to Bonferroni-Holm. All tests were 2-tailed and a *P* value of

0.05 or less was considered to be significant. Data analysis was performed with SPSS for Windows 10.0 (SPSS Inc, Chicago, IL, USA).

Results

Knee joints of all 32 canines were available for analysis. No complication in terms of death (prior to the end of the study), illnesses, significant weight loss, postoperative infections, hemarthrosis occurred during the study. Compared with the nonoperated contralateral side, histological analysis revealed significant higher Mankin score values in the operated knee joints treated with either GS or placebo (*P* < 0.002). Both, the intra-articular as well as the oral GS administration group presented significantly lower mean Mankin score values compared with the placebo-treated counterpart groups (*P* < 0.002). Intra-articular GS-treated canines demonstrated significantly lower mean Mankin score values compared with canines treated using the oral application mode (*P* < 0.002). Mean score values

Table 2. Application Mode–Associated Mankin Score Values.

		P Value
GS i.a.	Placebo i.a.	
8.1 (7.9-8.8)	13.9 (11.6-15.9)	0.002
GS p.o.	Placebo p.o.	
12.1 (9.9-12.7)	15.1 (12.5-17.0)	0.002
GS i.a.	GS p.o.	
8.1 (7.9-8.8)	12.1 (9.9-12.7)	0.002
Placebo i.a.	Placebo p.o.	
13.9 (11.6-15.9)	15.1 (12.5-17.0)	0.172

GS, glucosamine sulfate; i.a., intra-articular drug application; p.o., oral drug application (per os).

Figure 2. Boxplot results. Boxplots of the Mankin-score values of rater 1. The Mankin scores were significantly ($P < 0.002$) lower in the glucosamine sulfate (GS) groups than in the placebo groups.

between intra-articular and oral placebo-treated canines did not differ significantly ($P = 0.172$) (Table 2, Fig. 2).

Visual Macroscopic Analysis

Eight weeks postoperative, all 32 canines presented macroscopic cartilage pathology on the operated knee. The cartilage revealed a bluish discoloration compared to the glossy white surface of the nonoperated contralateral joint. Macroscopic findings were found to vary between animals allocated to the GS-treated groups compared with the placebo-treated groups: Placebo-treated joints presented a more roughened and irregular articular surface compared with the GS-treated joints, with the cartilage thickness being visibly more decreased in the placebo-treated joints. The major cartilage thickness loss was localized at the weightbearing areas of the lateral and medial tibial plateau (Fig. 1).

Visual Microscopic Analysis

The nonoperated contralateral knee presented physiological cartilage histology results in all animals: Regular, full-thickness hyaline articular cartilage of uniform structure. Cartilage thickness as well as safranin-O staining intensity were superior compared with the operated contralateral knee joints. Cartilage lesions were found in all operated joints.

Compared with all other operated stifles, knee joints treated with intra-articular GS presented least loss of cartilage thickness and safranin-O staining, whereas oral placebo-treated knees presented the highest incidence of cartilages thickness and safranin-O staining loss (Fig. 3).

Discussion

In this study, canines treated with either intra-articular or oral GS demonstrated less osteoarthritic changes after ACL transection compared with those treated with placebo. Compared with oral administered GS, canines receiving intra-articular GS presented less signs of OA. This is the first placebo-controlled study to assess the efficacy of intra-articular versus orally administered GS on OA in a canine model.

The precise mechanism of action of glucosamine has not been fully elucidated yet.¹⁰ Glucosamine is an aminosaccharide, acting as a preferred substrate for the biosynthesis of glycosaminoglycan chains and, subsequently, for the production of aggrecan and other proteoglycans of cartilage.^{9,10} Because of this essential role that aggrecans play in giving the cartilage its hydrophilicity, compounds enhancing synthesis of aggrecans may be beneficial in cases of OA, a disorder characterized by an increase in matrix structural protein turnover, with catabolism being predominant over synthesis.^{9,10} Cartilage unrelated effects, such as the inhibition of superoxide-radical generation or the inhibition of superoxide-radical generation have been suggested to explain the fast onset of action on symptoms noted in short-term clinical trials.¹⁰ The anabolic effect of GS was primarily thought to be attributable to its capacity for providing building blocks for the synthesis of glycosaminoglycans by chondrocytes.^{6,16}

However, the use of GS in OA is a matter of controversy. In a recent analysis from Wandel *et al.*¹³ reviewing 10 trials with an average of at least 100 included patients, the authors concluded, that glucosamine produced no clinical relevant effect on pain or structure of OA-affected joints. A different recent meta-analysis concentrated on the structure-modifying effect of glucosamine.¹⁷ Their conclusion regarding joint space narrowing and odds ratios revealed that GS was not effective at producing structural modifications after 1 year of treatment, but GS produced a small to moderate effect on medial joint space narrowing after 3 years. In contrast, 2 recent pivotal (3 years) studies revealed GS to have structure (disease) modifying properties by inhibiting joint space narrowing in knee OA.^{8,10,17} Normally, GS is not a rate-limiting component but in cases of injury or rapid repair, the amount of

Figure 3. Histological results. **(A)** Cartilage of the lateral femoral condyle of an *operated knee* after intra-articular glucosamine sulfate (GS) application. Intensely stained matrix with a slight loss of safranin-O staining in zone I and II and uniform structure of hyaline articular cartilage (safranin-O, 100 $\times$). **(B)** Cartilage of the lateral femoral condyle of an *operated knee* after oral GS application: advanced loss of safranin-O staining, hypocellular tangential zone (safranin-O, 100 $\times$). **(C)** Cartilage of the lateral femoral condyle of an *operated knee* from after intra-articular placebo treatment showing a lack of safranin-O staining and irregular chondrocytes formation in zone I and II: chondrocyte multiplication or migration, to form clones (chondrones) and moderate hypercellularity (safranin-O, 100 $\times$). **(D)** Cartilage of the medial femoral condyle of an *operated knee* after oral placebo treatment showing surface irregularities and inappropriately ovoid and round superficial chondrocytes. Typical pathological lesions, including disorganization and loss of cartilage (safranin-O, 100 $\times$).

GS can be limiting. This was shown in a model of repairing cartilage in young rabbit joints injured with chymopapain.¹⁸

Glucosamine increased the proteoglycan content of repairing young rabbit cartilage, but had no effect on the healthy articular cartilage. GS offers stressed chondrocytes an equilibrium.⁶ Gouze *et al.*¹⁸ investigated the influence of glucosamine on changes in cartilage matrix metabolism, which are regulated by mechanical compression. They observed a decrease of proteoglycan synthesis and aggrecan mRNA expression linked to static compression reversed by glucosamine. Glucosamine reversed in addition the increase of MMP-3 mRNA expression caused by loading. Sandy *et al.*¹⁹ showed that glucosamine inhibited the aggrecanase response to IL-1 in rat chondrosarcoma cells. Therefore, Gouze *et al.*¹⁸ concluded that GS appears to have a constitutional effect on IL-1 or static compression-mediated changes in enzyme expression involved either in catabolism or anabolism of proteoglycans. In further experiments, Gouze *et al.*¹⁸ studied the influence of glucosamine on IL-1 β mediated effects in rat chondrocytes. IL-1 β causes a marked decrease of the expression and activity of galactosyl- β -1, 3-Glucuronosyltransferase I, a key enzyme involved in glycosaminoglycan biosynthesis, reflected in a decrease of proteoglycan content. Glucosamine fully prevented the inhibitory effects of IL-1 β . Shirazi *et al.*²⁰ presented similar findings in studies with GS in cartilage explants obtained from patients with osteoarthritis undergoing total knee replacement. Shirazi *et al.*²⁰ concluded that GS has a protective effect on OA articular cartilage by inhibiting local NO and MMP-3 production. Furthermore, GS reversed the IL-1 β inhibition in proteoglycan synthesis. In this study, both intra-articular and oral administered GS significantly reduced histological signs of OA in the Pond-Nuki model, with the intra-articular application being more effective compared with oral administration.

The structure modifying of glucosamine of OA joints remains controversial. The systematic review that is the basis for OARS recommendations for the management of hip and knee OA was recently updated using more recent publications in glucosamine²¹. The analysis gathered the results of 19 randomized controlled trials from a total of 20 retrieved placebo-controlled trials. Sixteen of them used GS (13 oral, 2 intra-muscular, and 1 intra-articular) and 3 of them used glucosamine hydrochloride (GH). The review revealed a decrease of effect size for pain evaluated without discrimination between GS and GH, corresponding to a moderate symptomatic efficacy.²¹ The same analysis revealed a small but significant effect size for GS for reduction of joint space loss in the medial compartment of knee OA, but a nonsignificant effect on joint space narrowing in hip OA patients after a 24-month treatment. An identical conclusion was reached by the Glucosamine/Chondroitin Arthritis Intervention Trial (GAIT).¹ Results of another clinical trial were in line with the previously mentioned analysis and reported no significant difference in the WOMAC pain or function score with GS compared with placebo.²² However, in a recent clinical trial by Petersen *et al.*²³ serum level of cartilage oligomeric matrix

protein, a marker of cartilage degradation, was studied. Study results showed that combined with exercise, GS treatment reduced the level of serum cartilage oligomeric matrix protein. Again, in this study, an OA structure modifying effect of OA was seen canines treated with either oral or intra-articular GS.

GH and GS are identical from a chemical and structural point of view.¹⁶ The addition of salt does not justify the difference in efficacy or biological effects observed in different studies.¹⁶ Indeed, both GS and GH dissociate in the acidic milieu of the stomach, resulting in the release of glucosamine itself.²⁴ GH was also tested in the rat after transection of the anterior cruciate ligament.²⁵ GH administered orally at a dose of 1,000 mg/kg/d produced a chondroprotective effect and reduced the serum level of the collagen degradation marker CTX-II.²⁵ GS used in this study led to a significant decrease of osteoarthritic structural changes in the intra-articular as well as the oral administration modes compared with placebo. This findings are in line with previously reported results.²¹

The benefits of the use of GS for OA have long been greeted with skepticism due to the lack of reliable information regarding their absorption, pharmacokinetics, and mechanism of action.²⁶ Pharmacokinetic studies on GS in dogs using ¹⁴C-glucosamine and ³⁵S-labeled chondroitin sulfate found that 87% of an orally administered dose of radio-labeled GS and 70% of the labeled chondroitin sulfate were absorbed.²⁶ Other studies reported that GS is bioavailable after oral dosing and is reported to have a tropism for articular cartilage.²⁵ GS is classically administered orally to human subjects at a dose of 1,500 mg/d.¹⁶ In clinical trials involving OA patients, it was shown to reduce pain and provide functional improvement in addition to structure-modifying effects.^{11,16} It is important to point out that many *in vitro* studies were performed in different culture systems, with various formulations and concentrations of glucosamine. Concentration used in the *in vitro* studies were “super physiological”—in some cases up to 2,000 times higher than the maximal concentration that can realistically be achieved in plasma (10 μ M) after oral administration of 1,500 mg of GS in human subjects.¹⁶ Furthermore, some of these studies compared the effects of 2 formulations of glucosamine in order to provide evidence for the superiority of one or another.^{27,28} For example, GS has shown to be a stronger inhibitor of gene expression than GH.²⁸ In conclusion, extrapolation of the *in vitro* data to the *in vivo* situation should be done with great caution.¹⁶ In this study, dogs received an oral dose of 200 mg/kg body weight, which is 10 times higher compared with the standard dose recommendation of 1,500 mg/d for an adult with a body weight of 75 kg. This might have sharpened the results, as significant lesser OA changes were seen in the orally treated GS group compared with the placebo group. However, in this study best results were achieved with intra-articular injection of GS.

The Pond-Nuki model has been reported to be a reliable procedure for induction of experimental OA.¹² In this study, histological analysis revealed significant higher Mankin score values in the operated knee joints treated with either GS or placebo compared with the nonoperated contralateral knee. These results characterize the Pond-Nuki model used in this study to be an effective and reliable procedure to reproducibly induce experimental OA in canines.

Limitations

The researchers operating the canines, administering drugs and performing the histological evaluation were identical. Although blinded to the administered substances until completion of the analysis, there might be a certain study bias.

Conclusion

In this study both, intra-articular and oral administered GS reduced histological signs of osteoarthritis in the Pond-Nuki model, with the intra-articular application being more effective compared to oral administration.

Animal Welfare

The study was conducted in the approved facilities of the Institute for Animal Experiments of the Ruprecht-Karls University Heidelberg, Germany (Institut für Versuchstierkunde, Ruprecht-Karls-Universität Heidelberg).

Ethical Approval

The protocol of the study was submitted and approved by the Institutional Ethical Review Board (#A103C-041999; equivalent to US Department of Agriculture and/or AAALAC standards in the United States).

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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