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The Fate of Oral Glucosamine Traced by ^{13}C Labeling in the Dog

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Abstract

Objective: It has remained ambiguous as to whether oral dosing of glucosamine (GlcN) would make its way to the joint and affect changes in the cartilage, particularly the integrity of cartilage and chondrocyte function. The objective of this study was to trace the fate of orally dosed GlcN and determine definitively if GlcN was incorporated into cartilage proteoglycans. *Design:* Two dogs were treated with ^{13}C-GlcN-HCl by oral dosing (500 mg/dog/d for 2 weeks and 250 mg/dog/d for 3 weeks). Cartilage was harvested from the tibial plateau and femoral condyles along with tissue specimens from the liver, spleen, heart, kidney, skin, skeletal muscle, lung, and costal cartilage. Percentages of ^{13}C and ^{13}C-GlcN present in each tissue sample were determined by inductively coupled plasma mass spectroscopy (ICP-MS) and nuclear magnetic resonance spectroscopy, respectively. *Results:* In the case of dog 1 (2-week treatment), there was an increase of 2.3% of ^{13}C present in the articular cartilage compared to the control and an increase of 1.6% of ^{13}C in dog 2 compared to control. As to be expected, the highest percentage of ^{13}C in the other tissues tested was found in the liver, and the remaining tissues had percentages of ^{13}C less than that of articular cartilage. *Conclusion:* The results are definitive and for the first time provide conclusive evidence that orally given GlcN can make its way through the digestive tract and be used by chondrocytes in joint cartilage, thereby potentially having an effect on the available GlcN for proteoglycan biosynthesis.

Keywords

animal models, osteoarthritis disease modification, articular cartilage, tissue, drug delivery

Osteoarthritis (OA) is the most prevalent type of arthritis and negatively affects millions of adults in the United States alone.[1] OA presents in patients as swelling, stiffness, and pain in the joints, particularity the knee and hip, leaving the patient affected with limited mobility.[2] OA is characterized by matrix loss in articular cartilage due to an imbalance in matrix production and degradation and local inflammation. Proteoglycans, along with collagen fibers, are the major constituent of cartilage and are responsible for the positive pressure in cartilage, allowing the joint to withstand loads. Frequently used pharmacological therapies in the treatment of OA are analgesics or anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs may alleviate the pain but have little to no disease-modifying properties and come with negative side effects, especially in the gastrointestinal (GI) tract.[3,4] Agents that limit disease progression and favorably change the joint structure, or structure-modifying drugs, would be a more suitable therapeutic approach. Among the most notable agents is glucosamine (GlcN), which is available as a neutraceutical and widely used by OA patients. GlcN is formed by intracellular reactions using glucose as a substrate and is needed to generate glycosaminoglycan chains, which are an essential component of proteoglycans. Along with galactosamine, GlcN makes up 30% to 50% of the proteoglycan volume in articular cartilage.[5,6] The rationale for using GlcN in OA patients is that it is a crucial component of cartilage and thought to add to the biosynthesis of glycosaminoglycans and ultimately increase functional proteoglycans.[6] The precise mechanism of how oral doses of GlcN affect the tissues of the joint is not known, and it remains a controversial therapeutic approach.

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There is a large amount of attention placed on exploring the effects of GlcN on joint cartilage, chondrocyte function, and inflammation, as well as many clinical studies, many of which were recently reviewed.[7,8] There are examples of positive and negative outcomes with GlcN in animal models[9-14] such as in a rabbit OA model, where lesions in the cartilage along with synovial inflammation were decreased upon treatment with GlcN-SO_4.[15] Among the many clinical studies that have been reported, numerous have shown varying results and run the gamut of research approaches, including those that are based on patient-reported effects to those with clinically measurable outcomes and in the form of large clinical trials. There is noted variability in the outcome of reported clinical trials, and this in part may be linked to the glucosamine salt used and study design. Of the two typical forms, glucosamine SO_4 has shown more efficacy,[3,16-18] whereas other studies have shown less convincing data of efficacy such as in a double-blind study with 1583 OA patients in whom no significant reduction in joint pain with oral dosing of GlcN-HCl was found compared to placebo except in a subgroup of more severe patients.[19] However, in a certain subset of milder patients, benefit was shown as measured by disease-modifying parameters in the study prolongation for up to 2 years of treatment.[20] Very recently, another study found that treatment with GlcN-SO_4 for 12 weeks gave a significant lessening of pain and increased function in the knee compared to a placebo.[21] In other studies, it was also found that once-a-day oral treatment with 1500 mg GlcN-SO_4 made GlcN bioavailable systemically in the plasma and in the synovial fluid of the joint, in concentrations that are in the 10-μM range.[22] Different meta-analyses of GlcN have also encountered varying results. The latest update of a Cochrane review[17] showed in an analysis of 25 randomized controlled trials that on the Lequesne index, GlcN was better for reducing pain and increasing function compared to a placebo. Another meta-analysis found such heterogeneity among its 15 trials that no conclusion could be elicited,[23] but it has been suggested that such heterogeneity may be caused by differences in glucosamine formulations and study designs.[24]

In vitro studies have shown that GlcN's efficacy in part can be attributed to its inhibition of interleukin (IL)–1 induction by blocking the NF-κB pathway and preventing COX-2 expression in chondrocytes.[25] Byron *et al*.[26] found that prostaglandin E2 production was reduced significantly compared to control with a 1-μM concentration of GlcN when equine chondrocytes were stimulated with IL-1.[8] In addition, it has also been found that aggrecan core protein increases in human OA chondrocytes, whereas MMP-3 activity decreases upon treatment with GlcN-SO_4 at clinically relevant concentrations.[27]

Despite the variation in these study results and regardless of which GlcN salt form is used, the perplexing question has remained as to the fate of orally ingested GlcN. All the studies showing benefit or not are predicated on the concept that GlcN taken orally has a fate that is related either directly to its trafficking to the joint or indirectly through affecting systemic pathways and eventually chondrocytes and other cells within the joint tissues. This ambiguity stems from the fact that substances containing large quantities of GlcN are not digested to a large extent.[5] Orally administered GlcN is absorbed almost up to 90% in the GI tract.[28] The GlcN is then metabolized in the liver, with much of it being eliminated in the urine.[6] A study looking at the clearance of radioactivity from radiolabeled GlcN-SO_4 has shown that the radioactivity is mostly removed as carbon dioxide (50%) during respiration and in the form of GlcN from the kidneys (~35%) and in fecal waste (~2%).[29]

In our present study, we set out to test the presence of carbon 13 in tissue specimens and to do so by definitive methods such as inductively coupled plasma mass spectroscopy (ICP-MS). We took this approach in this study because ^{12}C to ^{13}C ratios are naturally very stable in tissues and plants. One notable 1-specimen study was performed using carbon and nitrogen tracing to characterize dietary intake characteristics of ancient populations ("ice men") since the carbon isotope ratios (C13:C12) are so stable within specific plant types and among aquatic and terrestrial sources.[30] ICP-MS was introduced in the early 1980s and unites high-temperature ICP technology with the low limits of detection of mass spectrometry for elemental determinations and isotopic analysis of test samples. The atoms present in the sample are converted to ions by argon plasma in the ICP source, and these ions are then analyzed in the attached mass spectrometer by their mass-to-charge ratios.[31] This combination allows separation of different atomic species of individual elements. Additional benefits of ICP-MS include the vast dynamic range and detection limits in the parts per trillion for a large amount of elements.[32]

In this study, we built on these clinical findings and our previous *in vitro* experiments[33] showing that when cartilage explants are cultured in medium containing ^{13}C-GlcN, the labeled carbon is detected and metabolized into the galactosamine moieties of chondroitin sulfate. We hypothesize that, when taken orally, GlcN can be available in the joint and incorporated into the proteoglycans of the articular cartilage. Since our objective was not to measure GlcN's effect on cartilage metabolism but its fate when ingested, we used an available ^{13}C-GlcN-HCl formulation. Our findings would predictably relate to either the HCl or SO_4 forms. Our study objective was straightforward: By using sophisticated analysis tools, we wanted to follow the fate of ^{13}C-GlcN and support whether orally ingested GlcN can make its way to the articular cartilage, where putative changes may occur in proteoglycan biosynthesis and ultimately cartilage function.

Materials and Methods

Animal Model

The study was conducted with approval of the Institutional Animal Use and Care Committee at the University of Pennsylvania. Three dogs were part of this study. Two dogs (~10-kg Beagles) were treated with ^{13}C-GlcN-HCl (Sigma-Aldrich, St. Louis, MO) by oral dosing (combined with food). To parallel a clinically relevant dose in this study, we used a glucosamine equivalent dose based on the human dose that is clinically used (GlcN-SO_4).[3,17,18] One dog was untreated, 1 (dog 1) was given 500 mg/dog/d (~50 mg/kg/d) for 2 weeks, and 1 (dog 2) was given 250 mg/dog/d for 3 weeks: After allometric conversion, the former is roughly equivalent to a 1500-mg daily dose in an adult human (~20 mg/kg/d). The dogs were sacrificed at the end of the noted dosing periods approximately 24 hours after the final dose, and cartilage was harvested (dog 2) from the tibial plateau and femoral condyles along with tissue specimens from the liver, spleen, heart, kidney, skin, skeletal muscle, lung, and costal cartilage. Tissues were also taken from an untreated dog. All tissues were snap frozen and stored at –80°C until tested.

Magnetic Resonance Spectroscopy Measurements

As background for this study experiments were performed using bovine cartilage explants and cultured with 13C -labeled GlcN for 13 days followed by 13carbon MRS analysis. The methods and characterization of the metabolic products determined from this MRS data was based on standards and previously reported.[33]

All experiments were performed on a Bruker AM-500 spectrometer (Bruker Optics, Billerica, MA). Samples were coarsely chopped and placed in a 5-mm-diameter nuclear magnetic resonance tube with 0.5 ml phosphate-buffered saline (PBS). Broadband, proton-decoupled ^{13}C magnetic resonance spectroscopy (MRS) measurements were obtained at 125.8 MHz using a composite pulse decoupling with a WALTZ-16 sequence and a flip angle (90° flip angle 16 μs) with 32K points, sweep width of 38 kHz, and a repetition delay of 400 ms. In total, 25,000 acquisitions were made for each specimen. After the data acquisition, the free induction decays were line broadened with 20 Hz of exponential weighting, Fourier transformed to the frequency domain, and manually phased. Peaks were referenced relative to the carboxyl/carbonyl peak at 177.5 ppm.

Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

Tissues were weighed into Oak Ridge Teflon centrifuge tubes (Nalge Nunc International, Rochester, NY) to 0.01 mg on an M-220D analytical balance (Denver Instruments, Denver, CO). The tissue samples were digested in trace metal–grade nitric acid (Fisher Scientific, Waltham, MA) at 90°C for 2 hours on a Select Heat block (VWR Scientific, Radnor, PA). The sample digests were then diluted with 18.3 megaohm water to achieve a 5% nitric acid test solution. The test solutions were maintained in Elkay mineral-free 15-ml conical centrifuge tubes (Sherwood Services AG, Schaffhausen, Switzerland).

Test solution analysis was performed on a PE-SCIEX ELAN 6000 ICP mass spectrometer (PerkinElmer) that was equipped with a cross-flow nebulizer. Total elemental detection counts for carbon 12 and carbon 13 were measured. Instrument validation for the C12:C13 ratio was performed on control canine cartilage obtained from the diagnostic laboratory necropsy floor. Repetitive analyses of the validation sample had total count coefficients of variation of less than 1% for both C12 and C13. In addition, all repetitive analyses had C12:C13 ratios of 98.73%:1.27%. The research samples were analyzed in triplicate. From the total counts, C12:C13 ratios were mathematically derived for each of the triplicates.

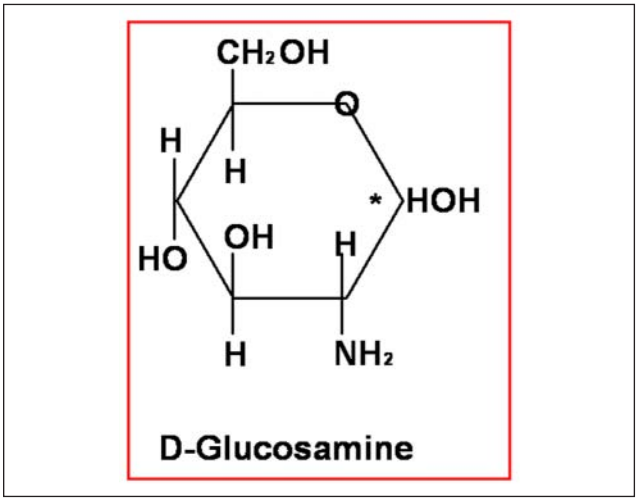

Figure 1. Chemical structure of D-glucosamine and labeled carbon (*).

Results

Figure 1 shows the chemical structure of D-GlcN and labeled carbon (^{13}C) at position C1. This C1 carbon position was chosen to be labeled because its integrity is maintained throughout the formation of glycosaminoglycans,[34] and therefore the labeled C1 will be detectable in the production of cartilage proteoglycans. Previously, we showed that after a period of time, specimens cultured in media containing labeled GlcN can increase the intensity of the signal at the anomeric region on MRS spectra. **Figure 2** shows stacked spectra of cartilage incubated in labeled GlcN for 3, 6, 10, and 13 days along with a plot of the control at day 6.[33]

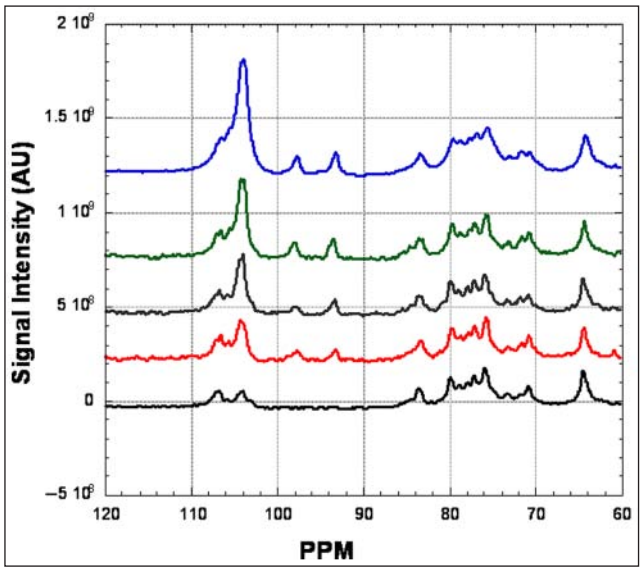

Figure 2. Kinetics of glucosamine (GlcN) uptake and metabolization. ^{13}C magnetic resonance spectroscopy (MRS) spectra from specimens grown in labeled GlcN, illustrating the increase in signal intensity in the anomeric region over the course of the culture period, from days 0, 3, 6, 10, and 13 (from bottom to top).

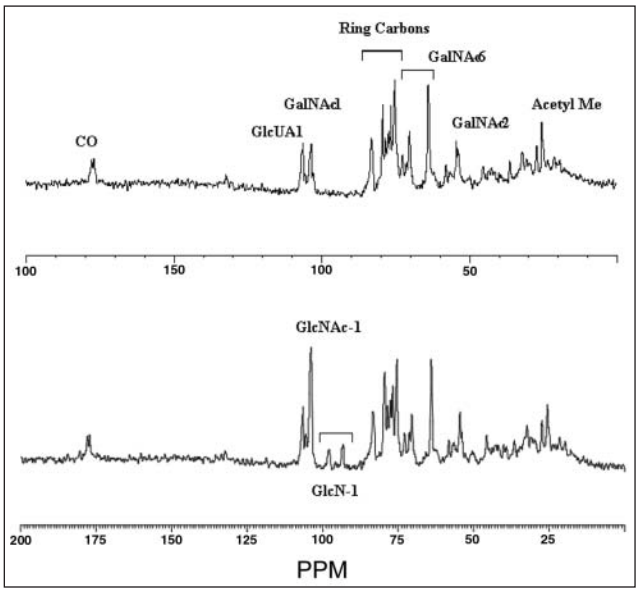

Figure 3. ^{13}C spectra of (**A**) control and (**B**) treated cartilage explants cultured with or without ^{13}C-GlcN for 13 days. LFC: left femoral condyle

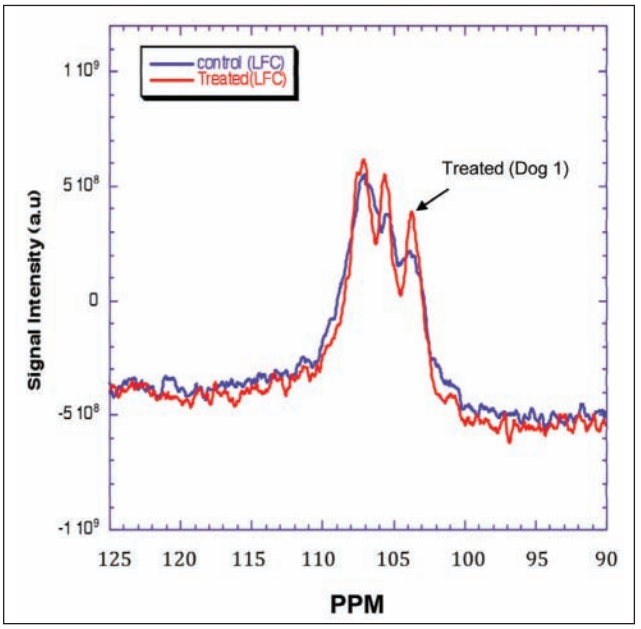

Figure 4. ^{13}C spectra of canine cartilage from dog 1 treated and control. Treatment was 500 mg/d for 2 weeks. LFC: left femoral condyle

Figure 3 shows representative ^{13}C spectra for unlabeled cartilage and cartilage tissue maintained in GlcN enriched to 99% ^{13}C at the C1 carbon position. All samples were incubated in Dulbecco's modified Eagle's medium (DMEM) with the appropriate labeled supplement for 13 days prior to MRS analysis.

In these spectra, aliphatic resonances appear in the region of 0 to 50 ppm. The C3 carbon of lactate at 19.5 ppm is not apparent in the control spectrum. Resonances from the C2 to C5 carbons of ring structures appear in the region of 50 to 90 ppm, including a sharply resolved peak at 63.5 ppm that corresponds to the C6 carbon of GalNAc in cartilage. The outliers at 177.2 ppm and 177.8 ppm correspond to the carboxyl and acetyl carbonyl (CO) resonances, respectively. The most important region of interest lies at 90 to 120 ppm, where the anomeric (C1) carbons resonate. In the control spectrum, 2 broad, complex peaks in the region of 100 to 110 ppm correspond to the expected resonance regions for the C1 carbons of the glycosaminoglycan (GAG) chains. Two new peaks became apparent in the treated cartilage spectrum, corresponding to the α and β anomers of unbound GlcN at 93 ppm and 97.5 ppm, respectively (Figure 3B). The ^{13}C positions have been assigned for all of the carbons of the major GAG subunits in isolated, purified form.

Figure 4 shows the expanded ^{13}C spectral region between 100 and 110 ppm from cartilage of both GlcN-treated and untreated dogs. Although unresolved, there are 5 peaks in this region, and based on the phantom results, these resonance peaks were assigned as follows: 107.1 ppm, GlcUA of Ch-6S; 106.6 ppm, GlcUA of Ch-4S; 105.7 ppm, GlcUA and 2-acetoamidoglucosamine (GlcNAc) of keratan sulfate (KS); 104.3 ppm, GalNAc of Ch-6S; and 103.7 ppm, GalNAc of Ch-4S.

Although all 5 resonances are elevated in treated tissue when compared to control, the resonances of GalNAc of

Table 1. Inductively Coupled Plasma Mass Spectroscopy Data: Articular Cartilage

Sample	Carbon^{12}, %	Carbon^{13}, %	Ratio
Control	98.73	1.27	0.0129
Dog 1	98.68	1.32	0.0134
Control	98.73	1.27	0.0129
Dog 2	98.71	1.29	0.0131

Table 2. Inductively Coupled Plasma Mass Spectroscopy Data: Other Tissues (Dog 2)

Sample	Carbon^{12}, %	Carbon^{13}, %	Ratio
Liver	98.71	1.29	0.0131
Kidney	98.72	1.28	0.0130
Skin	98.73	1.27	0.0129
Spleen	98.73	1.27	0.0129
Lung	98.72	1.28	0.0130
Heart	98.72	1.28	0.0130
Costal cartilage	98.72	1.28	0.0130
Skeletal muscle	98.72	1.28	0.0130

Ch-6S at 104.3 ppm and GalNAc of Ch-4S at 103.7 ppm have higher elevation, which is consistent with the results from bovine tissue.[33]

Femoral condyle cartilage from dog 1 (2-week treatment of 500 mg/d) had 98.68% of the carbon present in the form of ^{12}C and 1.32% present as ^{13}C, whereas the carbon present in the control femoral condyle cartilage was 98.71% ^{12}C and 1.29% ^{13}C (triplicate data points had identical ratios; **Table 1**). For dog 1, this corresponded to an increase of 2.3% of ^{13}C in the treated dog's cartilage compared to the control dog. Dog 2, which was treated with a lower daily dose of labeled GlcN (250 mg/d) for a longer time course of 3 weeks, had 98.71% of the carbon in the femoral condyle cartilage in the form of ^{12}C and 1.29% in the form of ^{13}C. This corresponds to an increase of 1.6% of ^{13}C present in the femoral condyle of the treated dog when compared to control (**Table 1**). These data were confirmed by nuclear magnetic resonance (NMR) spectroscopy where all 5 peaks of the GlcN spectrum were elevated in both treated dogs as compared to controls. We analyzed the ^{12}C to ^{13}C ratio in dog 1, dog 2, and the control dog (0.0134, 0.0131, and 0.0129, respectively). The measurements were made 3 times for each animal and resulted in the exact same values. Hence, the ratios for the treated dogs were significantly higher than that for the control dog.

We analyzed the percentages of ^{12}C and ^{13}C in various tissues from dog 2 to determine the distribution of ^{13}C-GlcN by ICP-MS (**Table 2**). The amount of ^{13}C in normal tissues within an animal was very consistent. Other tissues analyzed showed predictable levels of ^{13}C content similar to that determined in control cartilage. As expected, the highest percentage of ^{13}C in all the other tissues tested was found in the liver, and the remaining were all less than that of the articular cartilage. We analyzed the ^{12}C to ^{13}C ratios in the other tissues, and these were also similar to the control's ratios, again except for the liver, which had a ratio consistent with dog 2.

Discussion

Considering that tissue ^{12}C to ^{13}C ratios are naturally very stable, as can be seen by the consistency in the spleen, heart, kidney, skin, skeletal muscle, lung, and costal cartilage and the fact that ICP-MS is a method of exceptional sensitivity, the increases noted in the percentage of ^{13}C in femoral condyle cartilage are very impressive. For dog 1, the increase of 2.3% is well within an expected range if the labeled GlcN is incorporated into cartilage proteoglycans. These findings were supported by NMR spectroscopy, where all 5 peaks of the GlcN spectrum were elevated in both treated dogs as compared to controls. The results are definitive and for the first time provide conclusive evidence that orally given GlcN can make its way through the digestive tract and be used by chondrocytes in joint cartilage, thereby potentially having an effect on the available GlcN for proteoglycan biosynthesis.

These results support the findings by Persiani *et al*.,[22] who found that GlcN was made bioavailable in the joints of OA patients upon oral dosing of GlcN-SO_4, as well as several large animal studies that showed GlcN's presence in the synovial fluid of joints.[35,36] Our findings on levels in tissues are in accordance with many human studies that established an increase of GlcN level in serum.[37,38] Although our study uses a limited number of animals, the indication that the lower dose resulted in less (~30%) ^{13}C-GlcN in joint cartilage when compared to the higher dose is suggestive of a dose responsiveness and parallels other studies showing variation in plasma levels with different doses and peak plasma levels of glucosamine sulfate in patients with OA at the 1500-mg/kg dosage.[39] The inclusion of plasma analysis and earlier time points is planned for a future study.

There remains controversy over the effects of GlcN and the specific benefit in joint disease and, moreover, which salt form is linked with these outcomes. Although our study was performed with GlcN-HCl, the results would predictably be the same regardless of whether it was the HCl or SO_4 salt, given these both result in free glucosamine in solution. In fact, in light of the higher oral bioavailability of glucosamine sulfate compared to glucosamine hydrochloride that has been observed in animals and humans,[39,40] it could be speculated that if ^{13}C-labeled glucosamine sulfate had been used in the present study, higher amounts of ^{13}C glucosamine might have been detected in the cartilage, but further comparative studies are warranted. Our present study has its limitations both in study size and the fact it

was not designed as a pharmacokinetic study. Rather, this unique initial study was to answer a straightforward question about the fate of oral glucosamine and to provide a rational basis for assuming that GlcN can arrive at a target site. Additional studies should be made to determine what the elimination half-life of glucosamine is in the dog and what changes in absorption and distribution would there be if tissues were examined at different time points. It is possible that if the tissues were collected earlier after the last dose, the level of incorporation in the cartilage could have been even higher than that determined, as has been shown in plasma.[39] In addition, pharmacokinetic studies showed that this is true also in humans when the different dose regimens used in clinical trials have been directly compared in a review.[7] Although a number of studies continue to be performed to elucidate the effects and pathways that may be affected by GlcN and follow-up is needed with additional clinical trials, this study clearly determined the fate of ^{13}C-GlcN and, by conclusive methodology, that GlcN taken orally makes its way to the articular cartilage where putative changes may occur in chondrocyte function and proteoglycan biosynthesis and post-translation modifications.

Conclusion/Summary

Using this analytical approach, we have demonstrated that orally ingested GlcN can be traced to relevant joint tissues. The percentage of ^{13}C in cartilage in both dosing regimens was higher than in any other tissue tested except the liver, where it would be expected to be high. Considering that tissue ^{12}C to ^{13}C ratios are naturally very stable and the fact that ICP-MS is a method of exceptional sensitivity, the increase of 2.3% is sizable and well within an expected range if the labeled GlcN was incorporated into cartilage proteoglycans.

The results are definitive and for the first time provide conclusive evidence that orally given GlcN can make its way through the digestive tract and can be used by chondrocytes in joint cartilage. Although clearly it was possible to affect tissue levels of GlcN in our study, it may remain that the disparate results obtained in many studies may still be due to a number of other reasons, including the different GlcN salts used at different dose regimens with consequent lower systemic and possibly cartilage bioavailability of GlcN, but also a different study design, stage of joint disease when treated, and individual patient variations and predispositions. It remains intriguing that GlcN taken orally can potentially have an effect on the available GlcN for proteoglycan biosynthesis in clinically relevant tissues.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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