This study was conducted to study absorption of glucosamine (GlucN) and chondroitin sulfate (CS) in horses immediately after feeding. Six mature mares were used in a replicated 3 x 3 Latin-square-designed experiment. The experiment consisted of three 15-day periods, which included 10 days of diet adaptation followed by a 5-day sampling period. Blood was drawn after feeding on one day during each sampling period. Horses were fed a control diet (40% hay, 60% concentrate) balanced to meet NRC requirements for maintenance of mature horses (NRC, Nutrient requirements of horses, 1989). In one experimental diet, 2.0 g CS and 5.5 g GlucN were added to the basal ration at each feeding. In the other experimental diet, 3.5 g CS and 8.5 g GlucN were added to the basal ration at each feeding. After collections, blood was centrifuged and plasma was harvested and stored until analyzed for the presence of each compound. Analyses for plasma GlucN were performed using high-performance liquid chromatography. CS in the plasma was analyzed using a color reagent, dimethylmethene blue, followed by ultraviolet spectrophotometry. There were no significant differences (P < .05) in the concentration of either CS or GlucN in plasma, when comparing the three different diets. This leads to a conclusion that these compounds were not absorbed intact through the intestinal wall into the bloodstream. This poses a question as to whether oral forms of these compounds are absorbed and are able to migrate to joints through the blood to improve joint function.

1. Introduction

Degenerative joint disease and its associated joint pathology contribute significantly to musculoskeletal lameness and loss of function in performance and pleasure horses [1]. Numerous dietary supplements containing glucosamine (GlucN) and/or chondroitin sulfate (CS) are marketed as a way to help support, improve, or restore the health of horse’s joints [2]. The main goal of the medical therapy in treatment of degenerative joint disease is to restore and maintain normal joint function by alleviating pain, decreasing joint inflammation, and protecting the cartilage from further injury [3]. GlucN and CS are macromolecules endogenous to cartilage and have been used in veterinary medicine for several years. Elucidating their modes of action at the cellular level is becoming an important area of study in arthritis research [4]. The first step in this line of research is to determine the absorption of these products from the gastrointestinal (GI) tract in the equine.

Articular cartilage is composed of chondrocytes, which synthesize and deposit around themselves a watery matrix composed of collagen and proteoglycans [1]. Glycosaminoglycans (GAGs) are highly negatively charged long chains of repeating disaccharides that attach to a core protein [5]. GAGs aggregate with hyaluronic acid to form proteoglycan macromolecules [6]. Any insult to cartilage can cause a loss of GAG content and, therefore, a loss of elasticity and ability to bear and transmit forces efficiently, resulting in a
cascading cycle of more cartilage insults. Conditions such as this result in a need to supply the raw materials (nutrients) to the cartilage, so that the cartilage may replenish itself [1]. CS is the most abundant GAG in the body [7]. The major site of metabolism for circulating CS is the liver, where the GAG, depending on the animal species, may be partly degraded to oligosaccharides that subsequently lose their sulfate groups as inorganic sulfate [8].

The bioavailability of oral CS in animals is a subject debated in the literature. Andermann and Dietz investigated the absorption of CS in rabbits after oral administration and found neither absorption nor release of a characteristic clearing factor into the bloodstream [9]. One factor that may affect the absorption of CS is the chain length of the molecule. The mammalian intestinal epithelium is a highly effective barrier, which hinders the diffusion of a wide variety of compounds, especially those that are charged and/or have a high molecular mass [8]. Absorption of CS has been proven in studies in which radiolabeled compounds were used. Palmieri et al. demonstrated that >70% of the radioactivity administered orally to rats and dogs is absorbed [10]. The labeling methods used by the authors suggest that each CS molecule bears a single label at its reducing end [8]. The in vivo enzymatic degradation of the polymer proceeds step-by-step from the nonreducing end toward the reducing end [11]. This sort of labeling is not representative of the whole molecule. Conte et al. reported on the plasma concentration of CS given orally to man and concluded that the absolute bioavailability of the GAG was 13.2% of the administered dose [12]. Conte et al. reported that when 3H-CS was fed to rats and dogs, >70% of radioactivity was absorbed [11]. They found low-molecular-weight CS in synovial fluid of these animals; however, to date, there are no enzymes known to catalyze reoxidation of the monosaccharides of GAG chains [11].

GlucN, an amino sugar synthesized by chondrocytes from glucose and glutamine, is an important intermediate for the formation of numerous compounds, including GAGs [6]. GlucN absorption from the GI tract has been studied on many occasions in many animals. In a study of intestinal absorption of GlucN in rats, it was reported that GlucN is easily absorbed through a simple carrier-mediated transport across the intestinal wall. GlucN was found to be absorbed without modification to its molecular structure [13]. The liver is the main organ responsible for the metabolism and biotransformation of exogenous GlucN [14]. In a study using uniformly labeled GlucN, it was found that GlucN is rapidly and well absorbed from the GI tract of dogs. It was reported that the absorption in dogs was 87% of the administered dose [15]. In another study using labeled GlucN fed to rats, it was reported that the radioactivity found was not due to GlucN, but instead was from chemical entities, which changed over time [14].

The aim of this study was to improve the understanding of the absorption of GlucN and CS after oral administration in horses. Given the abundance of commercially available products containing these products, it is imperative that members of the equine industry understand how GlucN and CS work as chondroprotective agents. Ultimately, an increase in the knowledge of these products will lead to improvement in the prevention or management of cartilage degeneration in athletic and performance horses.

2. Materials and Methods

Six mature horses were used in a replicated 3 × 3 Latin-square-designed experiment. The ages and weights of the horses ranged from 3 to 10 years and from 472 to 557 kg, respectively. The horses were maintained at the Texas A&M University Horse Center using protocols approved by the Institutional Agricultural Animal Care and Use Committee. Before the experiment, all horses were dewormed and vaccinated, and some of them, when deemed necessary, had their teeth floated.

The horses were randomly assigned to one of three treatment groups, with two horses per group. The control diet was fed at 1.5% of body weight and was balanced to meet or exceed NRC requirements for maintenance of mature horses. It contained 40% hay and 60% concentrate [16]. The horses on the level 1 diet were administered a dose of 2.0 g CS and 5.5 g GlucN, top-dressed on the control diet twice a day. The horses on the level 2 diet were receiving 3.5 g CS and 8.5 g GlucN, fed at the same time and in the same manner. CS and GlucN dosages were determined by review of literature in which these compounds were administered in an oral form [8,11,17,18].

Horses were kept in individual stalls at feeding time, fed, and allowed 2 hours to consume their meals, after which, any refusals were weighed back and discarded. Horses had free access to water. The GlucN hydrochloride used was obtained from Sigma Chemical Co., St. Louis, MO. The chondroitin-4-sulfate used was obtained from CarboMer, Inc., Westborough, MA.

In each treatment period, horses were managed in drylot pens during a 10-day diet adaptation period with ad libitum access to water. The diet adaptation period was followed by a 5-day sampling period involving total collection of urine and feces (for other studies), for which, horses were confined to tie stalls and wore collection harnesses for 23 hours a day. Horses were walked for 1 hour each day during the collection period to alleviate any discomfort due to confinement.

Blood samples were taken through venipuncture with indwelling jugular catheters. The blood was collected into anticoagulant tubes and after harvesting, it was stored at −20°C until later analyses. Blood samples were taken around feeding on one day during each collection period at −30 minutes, 30 minutes, 60 minutes, 90 minutes, 2 hours, 2.5 hours, 3 hours, 3.5 hours, 4 hours, 4.5 hours, 5 hours, 5.5 hours, 6 hours, 7 hours, and 8 hours.

2.1. CS Analyses

CS in the plasma was analyzed using a color reagent, dimethylmethylene blue (DMMB), followed by ultraviolet (UV) spectrophotometry [8]. The DMMB assay for sulfated GAGs has found wide acceptance as a quick and simple method of measuring the sulfated GAG content of tissues and fluids [19].

After centrifugation of noncoagulated blood, plasma was retrieved and frozen at −20°C until analyses. One milliliter of plasma was mixed with 0.94 mL of 20 mM sodium phosphate buffer and 60 μL of papain solution (10 mg/mL) in the same buffer to eliminate proteins. The sodium buffer contained 2 mM ethylenediaminetetraacetic acid. 
and 2 mM dithiothreitol at pH 6.8. The samples were incubated in a shaking water bath for 30 minutes at 45°C, followed by 30 minutes at 55°C, and finally at 62°C for 120 minutes. Cloudy samples were incubated further to eliminate as much protein as possible. After centrifugation for 30 minutes at 3,000 g, the supernatants were analyzed the same day using UV spectrophotometry [8].

To detect CS in the plasma, a DMMB reagent was used, same as the one described previously. Two milliliters of the DMMB reagent was pipetted into a disposable polystyrene cuvette and absorbance at 525 nm was read. One hundred microliters of sample was added, and the absorbance at 525 nm was read again 15 seconds after mixing [8]. The reliability and accuracy of the method were tested using plasma samples spiked with known amounts of CS.

2.2. GlucN Analysis

Plasma was obtained by centrifugation of non-coagulated blood and was kept frozen at −20°C until analyses. Plasma was treated with acetonitrile to precipitate most of the proteins. Approximately 500 μL of the plasma/acetonitrile mixture was submitted to the Texas A&M Protein Chemistry Laboratory (PCL) for analyses.

GlucN standards ranging from 2 μg/mL to 1,000 μg/mL were prepared in water. Norvaline was used as the internal standard for the assay. Free amino acid standards were obtained from Sigma Chemical Co. (St. Louis, MO) and Agilent Santa Clara, CA.

A duplicate set of GlucN standards ranging between 2 and 8 μg/mL was prepared for every assay. Five nanomoles of 24 amino acids found in serum was added to 200 μL of GlucN standard to further simulate the conditions found in the serum. Additionally, 5 nmol of internal standard was added to all samples and standards.

Samples were then spun in a centrifuge to further remove any precipitate before analysis, and a 200-μL aliquot was taken per sample.

All samples and standards were dried in a Savant Speed-Vac with radiant cover and then reconstituted in 50 μL of 0.4M borate buffer. One microliter of resuspended sample was injected after automated pre-column derivatization with ortho-phthalaldehyde in the presence of 3-mercaptopropionic acid to produce the isoindole derivative of the amino acids. Using a UV detector, this derivative is detected at 338 nm. The amino acids and GlucN were separated on a Hypersil C-18 column by gradient elution using sodium acetate buffer. Chromatographic conditions to be used were identical to those used for routine amino acid analysis in the Texas A&M PCL, except that the eluants contained a 3/4 strength sodium acetate concentration to allow separation in the middle of the chromatogram, where alanine, the first anomer of GlucN, and arginine elute (Eluant A: 14.5 mM sodium acetate, 0.05 mM ethylenediaminetetra-acetic acid with 180 μL triethylamine and 3 mL tetrahydrofuran per liter. Eluant B: 14.5 mM sodium acetate, 40% acetonitrile, 40% methanol, 20% water). Derivatized amino acids were separated on a Hypersil AA column from Agilent.

2.3. Statistical Analysis

Resulting data were analyzed by analysis of variance appropriate for the Latin square design, using STATA statistical software (Stata Corp, 2001 College Station, TX). When necessary, means were further separated using a Fisher–Hays means comparison test. Differences were considered significant at P < .05.

3. Results and Discussion

3.1. Glucosamine

GlucN elutes as a broad peak. The first and last peaks represent the α and β conformers (as shown by the arrows in Fig. 1), and the area between these two peaks represent the linear form of the molecule. Although it is not possible to integrate the whole area under the curve because of the elution of arginine and taurine (Fig. 1), it is possible to quantify GlucN using one or both (simultaneously) of the anomeric peaks with linear results. In a blind study performed by the Texas A&M PCL, spiked plasma samples in the range of 2-15 μg/mL were submitted for analysis. These samples were analyzed using both the α and β peaks, averaging the results. Recovery of the GlucN in the spiked samples averaged at 98% (95%-100%; Fig. 1). This analysis resulted in very accurate results. Quantitation threshold was determined to be 1 μg/mL, eight times the signal-to-noise ratio for these conditions.

GlucN was not detected in the horse plasma for any of the 250 samples (Fig. 2). As shown in Figure 3, if GlucN were present in the plasma samples, it would elute as two peaks. Previous studies on absorption of GlucN have reported higher values than the current study. In one such
study, an oral dose of 125 mg/kg body weight was administered to horses, and the bioavailability was found to be 2.5% [20]. Unfortunately, this oral dose is approximately 5- to 10-fold higher than typical administration levels in horses, limiting the validity of the results.

It is not clear whether the GlucN molecule is absorbed in its entirety or is degraded before absorption [21]. In the current study, only whole GlucN was analyzed in the blood. Previously, it has been shown that GlucN from exogenous sources is incorporated into the metabolic pathway of GAG synthesis [22]. After entering the cells, GlucN may be phosphorylated to give glucosamine-1-phosphate and subsequently may be N-acetylated, yielding acetylglucosamine-1-phosphate. N-acetylglucosamine is one of the intermediates in this GAG synthesis pathway. These events could possibly account for the fact that no free GlucN was detected in the blood (L. Roden, personal communication, 2004). In an experiment in which D-glucosamine was given to rats through the stomach tube, it seemed likely that GlucN is phosphorylated, converted to fructose-6-phosphate, and metabolized [23]. Also, in previous studies, the bioavailability of GlucN has been studied in rats, dogs, and man using radiolabeled GlucN [13, 15]. In a study performed by Setnikar et al. using labeled GlucN fed to rats, it was reported that the radioactivity found was not due to GlucN, but instead was from chemical entities, which changed over time [14]. Another study evaluating serum levels of GlucN after oral treatment at clinically relevant doses reported a bioavailability of approximately 5% after nasogastric administration [24]. This level was found to be at least 500 times lower than those reported to be efficacious in having an effect on modification of chondrocytes in vitro. The authors concluded that a large proportion of GlucN taken orally is eliminated due to the poor absorption in the gut or, on absorption, is metabolized by cells in the gut lining and liver and/or cleared by the kidney [24].

3.2. Chondroitin Sulfate

There were no significant differences between treatments in the amount of CS found in the blood based on diet (Table 1). To better visualize and further examine the change in CS over time, the data points were normalized to baseline values. When the blood samples were taken after feeding, there was no significant (P < .05) change in the blood concentration due to feeding CS. At no point was there a significant increase in plasma CS levels over the baseline value during the 8-hour sampling period after feeding (Fig. 4).

Figure 4 illustrates that there was no significant post-prandial response to feeding CS in diets 2 and 3. If CS was present in the plasma after the horses were fed, there should have been an increase in concentration of CS when horses were fed diets 2 and 3 compared with diet 1. The results obtained here seem logical when looking at the chemical makeup of CS. Mass of CS can range from 14,000 Da to 30,000 Da. Its weight would suggest that absorption across the GI mucosa, which contains a variety of GAG-degrading enzymes, is low [25]. In a review of previously reported findings on CS absorption, authors of one study found that a lower-molecular-weight CS had a higher bioavailability, although not statistically significant from the higher-molecular-weight CS, when given orally. The authors of that particular study also indicated that the CS used was experimentally prepared and was not available commercially [26].

In the current study, there was no effect on plasma CS concentrations due to oral administration of CS to horses. Previous studies have used radiolabeled CS, and absorption results were based on recovery of radioactivity. One research group reported when CS is fed to rats and dogs, >70% of label is absorbed. Total radioactivity in that study was based on the sum of exogenous CS and of several labeled molecular species derived from its depolymerization and catabolism [11]. This study and one performed by Palmieri et al. used a labeling method that produces a CS molecule with a single label at its reducing end [10]. This sort of labeling is not representative of the whole molecule, and measuring the radioactivity of any absorbed material

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Chondroitin Sulfate</th>
<th>SE</th>
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<tbody>
<tr>
<td>Control</td>
<td>62.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Level 1 diet</td>
<td>79.9</td>
<td>7.4</td>
</tr>
<tr>
<td>Level 2 diet</td>
<td>75.1</td>
<td>7.3</td>
</tr>
<tr>
<td>Total</td>
<td>72.2</td>
<td>4.1</td>
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</tbody>
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![Fig. 3. Sample of horse plasma overlaid with 10 μg/mL standard.](image)

![Fig. 4. Postprandial curve: Chondroitin sulfate levels in plasma around feeding. Mean change in plasma CS concentrations ± SEM after an oral dose.](image)
actually means following the metabolic fate of the very last residue in the polymer backbone [8].

4. Conclusions

This study was conducted to further elucidate unanswered questions surrounding bioavailability of two compounds commonly found in equine joint supplements, GlucN and CS. Six mature horses were fed diets with pure GlucN and CS, top-dressed on their feed. Blood samples were taken and appropriate analyses were run to determine whether whole GlucN or whole CS was present or increased in the blood due to oral dosing. When feeding pure GlucN to mature horses at two different amounts, there was no detectable GlucN measured in the plasma. These data are obviously different from previous reports, but a lot of questions about the exact fate of exogenous GlucN in the equine GI tract remain to be answered. CS is a molecule known to be endogenous to horses. When horses were fed an exogenous source of CS, plasma samples showed no significant increase in amounts of CS to produce a postprandial curve. It is well documented in the literature that the mammalian GI tract is not suited for allowing whole CS to be absorbed. Due to the lack of significant amounts of either GlucN or CS in the blood, this study does not support the theory that oral doses of GlucN and CS are readily available to the joints through the bloodstream. With the enormous impact that “nutraceuticals” are having on the equine industry, it is important to know whether resources are being used efficiently and effectively. The ability to lessen the effects of degenerative joint disease and osteoarthritis is important. However, more research is needed to determine whether oral products containing GlucN and CS are actually efficacious in that regard.

References