

Oral glucosamine and chondroitin sulfate on synovial fluid biomarkers from osteoarthritic equine joints

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ABSTRACT: Several studies, mainly *in vitro*, have shown that chondroitin sulfate (CS) and glucosamine (GlcN) do have chondro protective and anti-inflammatory actions. The aim of the present study was to investigate whether oral CS/GlcN supplementation has effects on the CS, hyaluronic acid (HA) and prostaglandin E₂ (PGE₂) concentrations on synovial fluid of equine osteoarthritic joints. Horses with mild osteoarthritis (OA) in tibiotarsal joint received daily PO doses of CS and GlcN (2.8/3.1 g) for 25 days. Synovial fluid (SF) and urine samples were collected before treatment (day 0), and every 7 days, until day 55 (30 days after the end of treatment). Urinary CS increased upon oral treatment, indicating that this compound was systemically distributed. Concerning the SF, CS concentration increased after the end of the treatment and returning to baseline afterwards, while HA and PGE₂ concentrations did not change. Despite the systemic distribution, oral supplementation of CS/GlcN for 25 days was insufficient as an anti-inflammatory support. However, it is possible to infer that there was an anabolic effect upon cartilage matrix.

Key words: chondroitin sulfate, horse, glucosamine, osteoarthritis, synovial fluid.

Efeitos da glucosamina e condroitim sulfato oral sobre biomarcadores do líquido sinovial de articulações osteoartríticas de equinos

RESUMO: Vários estudos, principalmente *in vitro*, têm mostrado que o condroitim sulfato (CS) e a glucosamina (GlcN) possuem ação condroprotetora e anti-inflamatória. O objetivo deste trabalho foi investigar se a administração oral de CS/GlcN possui efeito sobre as concentrações de CS, ácido hialurônico (AH) e prostaglandina E₂ (PGE₂) do líquido sinovial (LS) de articulações equinas com osteoartrite (OA). Cavalos diagnosticados com OA tibiotársica grau leve receberam por via oral doses diárias de CS e GlcN (2,8g/3,1g) por 25 dias. Amostras de LS e urina foram coletadas antes do tratamento (dia 0) e a cada 7 dias até o dia 55 (30 dias após o fim do tratamento). Houve aumento do CS urinário, indicando a distribuição sistêmica desse composto. No LS, a concentração de CS aumentou após o final do tratamento e retornou aos valores basais em seguida, enquanto as concentrações de HA e PGE₂ não apresentaram alterações. Apesar da distribuição sistêmica, a suplementação oral de CS/GlcN por 25 dias foi insuficiente como medida anti-inflamatória. Contudo, pode-se inferir que houve efeito anabólico sobre a matriz cartilagínea.

Palavras-chave: cavalo, condroitim sulfato, glucosamina, líquido sinovia, osteoartrite.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) remain the treatment of choice for most cases of equine lameness, because of the undisputable analgesic effect of many of these drugs. In cases of chronic pain, such as osteoarthritis (OA), wherein the equine patient requires ongoing, daily pharmaceutical support to maintain functionality and quality of life, the use of NSAIDs is limited by its potential side effects, which may include gastric ulceration

and renal functions changes (REED et al., 2006; RAIDAL et al., 2014). This led to the development of “alternative” or “complementary” treatments for chronic cases.

Currently, there are no disease-modifying therapies with strong evidence of efficacy in equine OA; therefore, its management is based on relieving symptoms and improving functions. Common therapies for joint diseases are intra-articular (IA) injections of corticosteroid, with or without hyaluronic acid (HA); polyacrylamide hydrogel

(PAAG); blood-derived products such as platelet-rich plasma (PRP), autologous conditioned serum (ACS), autologous processed plasma; and mesenchymal stem cell (MSC). Corticosteroid provides better pain relief and functionality in the short- and medium-term, but over again alternatives are needed for older horses and horses with chronic OA. The IAHA alone is often not enough to decrease lameness substantially; although, its greatest benefit may be its long-term disease-modifying effects (CONTINO, 2018). The PAAG is used with promising results, which studies showed alleviates lameness and joint effusion in osteoarthritic horse joints (TNIBAR et al., 2015); however, this treatment requires further investigation.

Most equine clinical studies have shown that PRP has positive effects when used to treat joint injuries; however, these studies have a high risk of bias and PRP products vary greatly in composition, making it difficult to compare their efficacies (BROSSI et al., 2015). The most common ACS used on horses is called IRAP, and it is superior to corticosteroids in treating joint disease with a concurrent soft tissue injury (CONTINO, 2018). Several studies have shown that intra-articular mesenchymal stem cell (MSC) injection helps to reduce synovial inflammation and results in better cartilage repair, when compared to other currently available therapies and surgical techniques; however, production of hyaline tissue have yet to be achieved (ORTVED; NIXON, 2016).

Finally, systemic therapy with HA, polysulfated glycosaminoglycans (PSGAG), pentosanpolysulfate sodium(PPS)and bisphosphonates as well as other therapies such as shockwave and physical therapy are indicated in the management and treatment of joint disease (CONTINO, 2018).

One of the main components of the cartilage extracellular matrix, responsible for many of its properties, is the proteoglycan aggrecan, which is composed by a ~200 kDa protein core, glycosylated with chondroitin sulfate (CS, ~100) and keratan sulfate (KS, ~30) side chains. Thus, CS is a prominent component of the cartilage matrix (WATANABE, 1998).

Under normal conditions, rates of proteoglycan synthesis and degradation are in equilibrium, while in OA the degradative processes take prevalence, and it was believed that CS and/or glucosamine (GlcN) could provide substrates to increase the proteoglycan synthesis and cartilage regeneration. Thus, in the 1990's, CS and GlcN were introduced as "nutraceutical agents" for treatment of OA. Their efficacy in OA were demonstrated in

a number of clinical trials (McALINDONet al., 2000; VANGNESS et al., 2009), and several *in vitro* studies have shown that CS and GlcN have chondroprotective and anti-inflammatory actions (BASSLEER et al., 1998; CHAN et al., 2005; VALLIÈRES & DU SOUICH, 2010; HARLAN et al., 2012).

However, beneficial effects of CS/GlcN oral administration in horses are still controversial. When CS is administered orally, presumably, the intact CS molecules are degraded by gastrointestinal enzymes and subjected to a large first-pass effect in the liver, reaching the systemic circulation at lower concentrations than *in vitro* studies, compromising the efficacy of its chondro protective and anti-inflammatory properties (WELCH et al., 2012; HIGLER et al., 2014).

CS and GlcN are currently recommended for humans as a "SYSADOA" – Symptomatic Slow-Acting Drug for the treatment of OA. It means that they provide pain relief and increased joint mobility only after a relatively long period of regular administration (1-2 months), and their effects last long after the end of treatment (6-9 months) (MORITA et al., 2018).

Studies in horses have shown that CS and GlcN are more effective in improving cartilage metabolism when in association (LAVERTY et al., 2005; RODGERS, 2006; BACCARIN et al., 2012). Nowadays, numerous oral supplements containing GlcN and CS, together with other nutrients, are commercially available (RAMEY et al., 2002). These preparations are known as Disease-Modifying Osteo Arthritis Drugs (DMOADs).

The aim of the present study was to investigate the effects of oral administration of CS and GlcN on synovial fluid CS, HA and prostaglandin E₂ (PGE₂) concentrations in horses with mild OA. The hypothesis is that these compounds have anti-inflammatory and chondro protective activities that would be useful in the management of joint diseases in horses.

MATERIALS AND METHODS

This study was approved by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo – CEUA/USP (949/2006).

Animals

Horses were submitted to clinical and radiographic evaluation for inclusion criteria: lameness due to OA in the tibiotarsal joint (grade 1-5) with or without joint effusion; radiographic lesions compatible

with OA type 1 or 2, according to McILWRAITH (2002); no prior or current treatment with CS, glucosamine (GlcN), and/or anti-inflammatory drugs.

Six horses, aged 6-10 years old, weighing 350-450 kg, were diagnosed as mild osteoarthritis (OA) in one of the tibiotarsal joint and were selected to be included in the study. At that time, they were subjected to similar exercise and nutritional managements (pellets, coast cross hay and water *ad libitum*).

Treatment

Osteoarthritic horses received daily 30 g-doses, PO, of CS/GlcN supplement for 25 days. The oral CS/GlcN supplement used in the study provided active ingredients of 2800 mg CS and 3100 mg GlcN per dose (30 g scoop) (HIGLER et al., 2014). The supplement also contained amino acids (L-methionine, Met; L-lysine, Lys), and minerals (sulfur, selenium, manganese, copper, and zinc). Before use, this preparation was analyzed for CS and GlcN (DIETRICH & DIETRICH, 1976; GIUSTI et al., 1988), which have shown that the concentrations stated by the manufacturer were correct. No other intramuscular, intravenous or topical therapies were administered during this time.

Urine and SF samples were collected the day before initiation of treatment (day 0), and on days 7, 14, 21 of treatment, as well as on days 28, 35, 42, 49 and 55 post-treatment (up to 30 days after the end of treatment). All urine samples were collected via urethral catheterization or natural urination. The arthrocentesis was performed medially to the saphenous vein, just below the medial malleolus of the tibia, in the dorsomedial face of the tarsus (STASHAK, 2002). All SF and urine samples were centrifuged to remove debris ($2000 \times g$, 15 min, 4 °C), and the supernatants were aliquoted and stored at -80 °C.

Urinary Glycosaminoglycans

Urinary glycosaminoglycans were isolated from urine samples by ionexchange chromatography as previously described (VIEIRA et al., 2005). These compounds were identified by agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9 (PDA) buffer (DIETRICH & DIETRICH, 1976), and quantified by densitometry of the gel slabs. Urinary creatinine was measured by the alkaline picrate method in automatic biochemical analyzer (HEINEGÅRD & TIDERSTRÖM, 1973).

Synovial Fluid Glycosaminoglycans and PGE₂

HA and CS concentrations were determined as previously described (MACHADO et

al., 2012). In brief, synovial fluid (SF) samples (50 µL) were submitted to proteolysis with maxatase solution (4 mg/mL in 0.05 mol/L Tris-HCl, pH 8.0, 100 µL). After overnight incubation at 50 °C, maxatase was heat inactivated (10 min, 100 °C), and debris were removed by centrifugation (3000 × g for 15 min at 24 °C). The supernatant was freeze dried, and re-suspended in distilled water (25 µL). Aliquots (5 µL) were submitted to agarose gel electrophoresis in PDA buffer, and stained with 0.1% Toluidine Blue in 50% ethanol:1% acetic acid (for sulfated glycosaminoglycans), and then in 0.05 M sodium acetate buffer, pH 5 (for HA). Compounds were quantified by densitometry of the agarose gel slabs.

PGE₂ was quantified by ELISA, using a commercial PGE₂ EIA kit (Monoclonal Antibody, Cayman Chemical Company, USA) (PRADELLES et al., 1985), and adapted to horse SF by BACCARIN et al. (2014). To check for possible dilution of the SF samples due to effusion, urea concentrations were measured by urease-glutamate dehydrogenase method, using an automatic biochemical analyzer. PGE₂, HA and CS are given as concentrations.

Statistical analysis

Data were evaluated for normality using the Kolmogorov-Smirnov test. Afterwards, the unpaired t tests and ANOVA followed by Tukey-Kramer test were used to compare time points. Graph Pad Instat 3 software was used to perform the statistical analysis. Statistical significance was set at a p<0.05.

RESULTS

All six horses, initially, presented, lameness (scores 1-3). Two of these animals (#2 and #4) also presented joint effusion. Upon treatment with CS + GlcN, the lameness of five animals improved; although, the differences were not statistically significant. One horse maintained its initial lameness score (#3), and one (#2) maintained joint effusion. Others clinical signs of osteoarthritis were not observed at the beginning of the trial and the physical exams did not change after CS+GlcN administration.

The CS increased in the urine compared to baseline values during the treatment (days 7 and 14, P<0.05), possibly due to the excretion of exogenous CS (Figure 1). This finding showed the systemic distribution of the CS given PO.

Concerning the SF (Figure 2), CS concentration increased after the end of treatment (days 28; P<0.01 and 35; P<0.05), returning to baseline afterwards. In contrast, the SF HA concentrations did

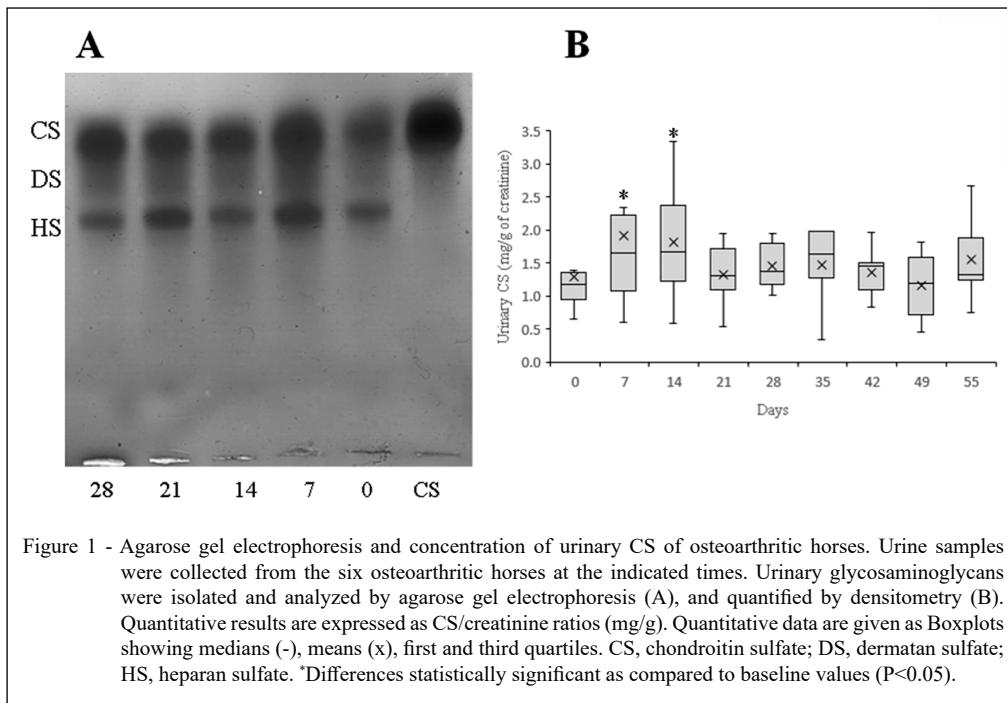


Figure 1 - Agarose gel electrophoresis and concentration of urinary CS of osteoarthritic horses. Urine samples were collected from the six osteoarthritic horses at the indicated times. Urinary glycosaminoglycans were isolated and analyzed by agarose gel electrophoresis (A), and quantified by densitometry (B). Quantitative results are expressed as CS/creatinine ratios (mg/g). Quantitative data are given as Boxplots showing medians (-), means (x), first and third quartiles. CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate. *Differences statistically significant as compared to baseline values ($P<0.05$).

not vary during all the experimental period (treatment and post-treatment).

Concerning PGE₂ concentrations, no variations were observed (Figure 3) ($P>0.05$). In all figures the data points are presented relative to time 0.

DISCUSSION

VIEIRA et al. (2005) reported that urinary CS and KS reflect the turnover rates of cartilage proteoglycans, and BACCARIN et al. (2012) have shown that treatment with CS and GlcN (given every 5 days by IM route, 25 days) led to a long lasting increase in the urinary CS and KS, and significantly improved the OA clinical signs. In the present study, CS and GlcN were given PO every day, per 25 days.

Despite the higher doses of CS and GlcN (PO: 2.85 g CS and 3.15 g GlcN/day; IM: 0.75 g CS and 0.75 g GlcN every 5 days), the urinary CS did not reach the levels obtained upon IM administration (13 mg/g of creatinine). This suggested that less intact CS reached the urine. It is not surprising that the bioavailability of CS given PO is lower (22-32%) (DU et al., 2004; LAVERTY et al., 2005; MEULYZER et al., 2008), but it is also possible that the CS degradation products are effective as chondro protectors and/or anti-inflammatory agents (WELCH

et al., 2012). In dogs, the bioavailability of oral CS is 5%; however, it reaches 200-278% when multiple doses are offered (ADEBOWALE et al., 2002). RODGERS (2006) reported that oral administration of CS and GlcN (1.2 g/3.9 g twice daily) during 8 years in OA horses dramatically decreased the frequency and number of intra-articular infiltrations of HA and/or corticosteroids. Additionally, according RODGERS (2006) sustained oral dosing of CS may result in significantly higher measurable joint levels that would correlate with the beneficial effects on cartilage seen in the *in vitro* studies.

Results of others studies using oral CS+GlcN supplement have varied; no beneficial effects were observed in stride length of aged horses after three months supplementation (HIGLER et al., 2014), in contrast stride characteristics (joint motion, stride length and swing duration) were improved in veteran horses after 12 weeks of oral CS+GlcN supplementation (FORSYTH et al., 2006).

As already mentioned, the transitory increase in urinary CS upon treatment is possibly due to exogenous CS, but the SF CS is not, since it increased after the end of treatment. The CS and GlcN have been shown *in vitro* to increase the proteoglycans and glycosaminoglycans(GAGs) synthesis (BASSLEER et al., 1998; LEGENDRE

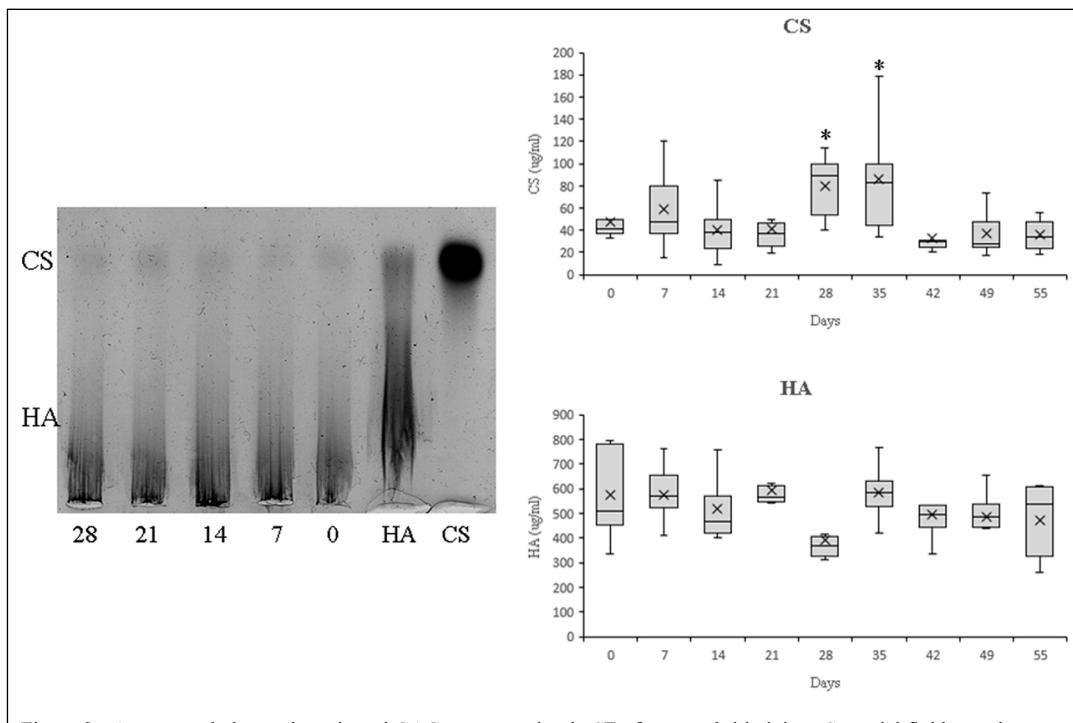


Figure 2 - Agarose gel electrophoresis and GAG concentration in SF of osteoarthritic joints. Synovial fluid samples were submitted to proteolysis, the glycosaminoglycans were precipitated by methanol, re-suspended in water and analyzed by agarose gel electrophoresis as described in Methods. The CS and HA were quantified by densitometry. Quantitative results are given as Boxplots showing medians (-), means (x), first and third quartiles. *Differences statistically significant as compared to baseline values ($P<0.05$). HA, hyaluronic acid; CS, chondroitin sulfate.

et al., 2008). Therefore, it is possible that oral CS/GlcN administration for 25 days has stimulated the synthesis of CS by the cartilage matrix, suggesting anabolic effects.

Repeated arthrocentesis was reported as a potential confounding factor for the use of synovial PGE₂ and GAG concentrations as markers of joint disease. However, based on VAN DEN BOOM et

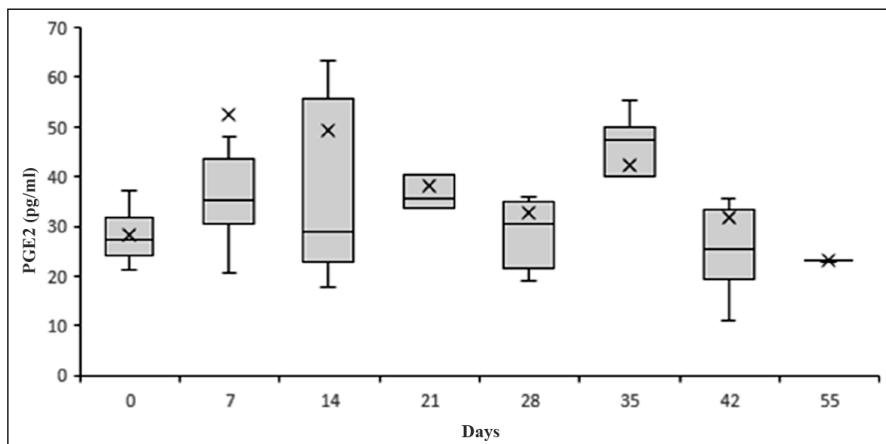


Figure 3 - PGE2 in SF of osteoarthritic joints. PGE2 was measured in the synovial fluid of osteoarthritic horses before (day 0), during (days 7, 14, 21), and after 25 days of oral supplement treatment (day 55). Quantitative results are given as Boxplots showing medians (-), means (x), first and third quartiles. *Differences statistically significant as compared to baseline values ($P<0.05$).

al. (2005) study, such a confounding effect can be avoided if one week or more separates punctures. The SF HA and PGE₂ concentration did not vary during all the experimental period; although, *in vitro* studies have shown that GlcN alone or in combination with CS has the potential to reduce PGE₂ levels (CHAN et al., 2005). In addition, it has been shown that CS and GlcN increase the concentration and improve the composition of hyaluronic acid, which may play a role in its anti-inflammatory effects (TRUMBLE, 2005).

The oral supplement used in this study was a CS/GlcN combination that delivered 2.8 g CS/3.1 g GlcN (active ingredients) per dose. This gives a total daily dose of 5.9 g of active CS/GlcN for a typical 450-kg horse for 25 days. Our results suggested that long-term use of a PO CS/GlcN supplement is necessary to decrease PGE₂ concentrations in SF.

CONCLUSION

Oral supplementation with CS increased urinary CS in horses with mild OA, indicating that this compound was systemically distributed. Although, the oral supplement containing CS + GlcN (25 days) has led to synovial fluid CS concentration transient increase, suggesting anabolic effects, no anti-inflammatory effects were observed.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

Protocol nº 949/2006.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

RYAB, JMC and YMM conceived and designed experiments. JMC, TSLM, APLM performed the experiments. RYAB, JMC and YMM carried out the lab analyses. JJM and RYAB prepared the draft of the manuscript. JJM, RYAB and YMM critically revised the manuscript. All authors approved of the final version.

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