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Communication

[*Comunicação*]

Clinical study of chondroitin sulfate urinary excretion following intramuscular application of the chondroitin sulfate and glucosamine association in horses

[*Estudo da excreção urinária de condroitim sulfato após administração intramuscular da associação de condroitim sulfato e glucosamina em cavalos*]

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Lameness in horses is often associated to a certain degree of osteoarthritis (OA), a chronic disorder characterized by metabolic and structural changes in the hyaline cartilage, leading to degradation of the extracellular matrix (ECM) contents, such as collagen and proteoglycans. Accordingly, pain and loss of joint mobility are common causes of decreased performance and early retirement in sports horses. The usual therapeutic protocols– such as non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular steroids – are routinely employed to suppress pain and improve joint function. However, their potential adverse effects can allow the progression of cartilage degeneration. In such context, osteoarthritis modifying drugs have presented considerable evidence of chondroprotection effect, favoring the chondrocyte anabolism, and modulating the inflammatory cascade (Moreira *et al.*, 2019). These drugs act as adjuvants and can be employed for long-term periods, allowing the decreasing of dose or administration frequency of other drugs that may have a deleterious potential (Trumble, 2005).

Several *in vitro* studies have demonstrated the anabolic, anticatabolic, anti-inflammatory, antiapoptotic and antioxidant effects of the chondroitin sulfate (CS), manly when associated to glucosamine (GlcN) (Henrotin *et al.*, 2010). However, the oral administration of the combination of CS and GlcN can result in lower bioavailability of these glycosaminoglycans (GAGs), once they are degraded by the gastrointestinal tract enzymes and subjected to the hepatic first pass effect, reaching the systemic circulation in lower concentrations (Moreira *et al.*, 2019). Therefore, the intramuscular administration of this association seems to be more beneficial and promising.

The CS is the main excreted GAG in the urine of mammals and, although its exact mechanism is not completely understood, some evidence suggests a systemic origin, followed by the chondral metabolism, hepatic catabolism, and renal filtration. The increase of excretion may, therefore, be related to a higher turnover rate of the ECM in horses, due to a high metabolic chondral activity (Vieira *et al.*, 2005). In this regard, as a systemic biomarker, identifying and quantifying CS in urine offers a method to evaluate and monitor OA, besides contributing to investigations of new drugs and clinical trials (Baccarin *et al.*, 2012).

The present study aimed to evaluate the urinary excretion of CS followed by the intramuscular application of the CS and GlcN association in healthy horses to identify its excretion peak and decline, characterizing the systemic distribution of exogenous CS, up to its return to baseline values. This research project was approved by the Ethics Committee on Animals Use of the School of Veterinary Medicine and Animal

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Science (CEUA/FMVZ), protocol 1096020221. Six healthy, male, mixed Mangalarga Paulista breed horses, weighted between 300-400kg were included. Horses were not athletes and were free of laboratory (hemogram, renal and hepatic profiles) and physical, radiographic and ultrasonographic orthopedic changes. Subjects were acclimatized and kept in stalls, receiving coast-cross hay, mineral supplementation, and water *ad libitum.*

The urine samples were always collected by mornings, at the same time, for seven consecutive days: D-1, D0, D1, D2, D3, D4, D5, D6 and D7 through urethral catheterization or spontaneous urination and, after collection, samples were stored at -20°C until analysis. Compound injection was performed on D0, immediately after urine collection. All horses received a single intramuscular administration of a commercial solution based on chondroitin sulfate A (750mg/mL) and glucosamine sulfate (750mg/mL), at a dose of 2mL/100kg of body weight.

The urinary CS was extracted by ion exchange chromatography, adapted from Baccarin *et al.* (2012), in Q-Sepharose Fast Flow columns (Cytiva), previously balanced. The samples were thawed, centrifuged at 2000 rpm for 10 minutes (Legend RT; Sorvall) and a 2mL aliquot of the supernatant was separated for creatinine measurement. Each sample (10mL) was diluted in 30mL of distilled water and Q-Sepharose was added, followed by the homogenization step (BHS-300 homogenizer; Benfer) at low speed for one hour and then applied to the column. Subsequently, elution was carried out with 3 ml of 0.3 M NaCl, followed by 5mL of 2 M NaCl, and each fraction was collected in a test tube, precipitated under agitation and slow addition of methanol. After 24 hours at -20°C, the formed precipitates were collected by centrifugation at 330 G for 15 minutes, vacuum dried for one hour and resuspended in 50 µL of distilled water. The products were stored in Eppendorf tubes at -40°C and subsequently submitted to agarose gel electrophoresis (0.5%) in 1,3-diaminopropane-aceto (PDA) buffer at 0.05 M and pH 9 (Dietrich *et al.*, 1977). The CS was fixed to the gel with cetavlon and for its identification, the slides were submerged in 0.1% toluidine blue dye, and the excessive dye was removed with 1% acetic acid and 50% ethanol. The measurement of CS was performed by densitometry (QuickScan Touch Plus, Helena Laboratories) and urinary creatinine by reaction with acid picrate (alkaline medium) and determined by an automatic biochemical analyzer (Labmax240, Labtest), as described by Lustgarten and Wenk (1972). The CS/creatinine ratio (mg/g) excreted was used as it is more reliable for interpretation of results, since this relationship is independent of the time of urine collection and removes the bias of hydration degree of the horses, as described by Michelacci *et al*. (1989).

The resulted data were subjected to descriptive analysis with estimated mean, median, standard deviation, 25% and 75% percentiles of the CS (mg/L), creatinine (g/L) and CS/creatinine ratio (mg/g) variables. To assess the differences between the moments, normal distribution was tested with the Shapiro-Wilk normality test to determine the parametric and non-parametric approach. For variables with normal distribution, the difference between the moments was verified with the ANOVA test of repeated measures followed by the Bonferroni correction as post-hoc. For variables without normal distribution, the difference between the moments was analyzed using the Friedman test followed by Nemenyi's test with Bonferroni correction as post-hoc. All tests were considered significant when $p<0.05$ and all analyzes were performed in the R 4.0.4 environment (R Core Team, 2021). To evaluate and compare the variations of excreted CS during the seven consecutive days after intramuscular injection of a solution based on CS and GlcN, the urine samples from D-1 and D0 were established as the baseline parameter.

Results showed that there was a statistically significant ($p<0.0097$) increase in CS excretion (mg/L) on D1 (24 hours) compared to baseline (D0). Then, there was a statistically significant decrease in D7 ($p<0.05$), compared to D1 (Figure 1). Regarding the CS/creatinine ratio, there was a statistically significant increase ($p<0.012$) on D1 (24 hours) compared to baseline, besides a statistically significant decrease in levels on D5 ($p<0.015$), when compared to D1 and to D2 ($p<0.002$) (Figure 2). There was no significant difference ($p<0.05$) in creatinine (g/L) levels at the evaluated moments.

In the present study, a single application of the injectable solution was able to promote a

significant increase in the urinary excretion of CS (expressed as a CS/creatinine ratio, $p<0.012$) 24 hours after administration, indicating that the exogenous molecule was distributed systemically. Baccarin *et al.* (2012) found similar results, with a notable increase in urinary CS concentration 24 hours after intramuscular administration in horses with mild OA, returning to its baseline levels 24 to 48 hours after the peak. On the other hand, results obtained in this study showed a significant decline in CS levels only five days after treatment.

GlcN is a precursor and structural component of CS, and after oral administration it is rapidly absorbed by glucose transporters (GLUTs), demonstrating a wide tissue distribution and a tropism for articular cartilage (Trumble, 2005; Neil *et al.*, 2005). In horses, the currently recommended doses for oral administration result in incipient plasma concentrations, requiring doses 5 to 10 times higher to increase their bioavailability (Neil *et al.*, 2005).

In turn, CS is the predominant GAG in connective tissues and the exogenous molecule can influence the metabolism of healthy and diseased cartilages. Its metabolic destination after oral administration is variable according to its size and molecular weight, as well as intestinal degradation and hepatic biotransformation (Neil *et al.*, 2005; Trumble, 2005; Du *et al.*, 2004). Although it demonstrates rapid absorption and tropism for cartilage and synovial fluid, when administered orally, it is unknown whether the therapeutic effect is due to the intact or fragmented molecule or to disaccharides. Furthermore, in vitro projects suggest that intact administered CS, not exposed to enzymatic degradation, stimulates chondrocytes, and protects them against degradation (Lippiello *et al.*, 2000). In this sense, the intramuscular treatment of the injectable combination of CS and GlcN seems to be more advantageous.

CS disaccharides are capable of accumulating in plasma, promoting a substantial residual effect (Neil *et al.*, 2005), therefore, although there is a need for a prolonged treatment for the onset of effects, their benefits persist for long periods even after the treatment discontinuation (Bottegoni *et al.*, 2014; Baccarin *et al.*, 2012). Considering the slow action and the residual effect of the drug, it is imperative to determine the interval between administrations. In the present study, the period between drug application and the moment when the CS urinary concentration declines provide relevant information that supports the use of this interval. Therefore, according to the results presented, it is suggested that the application interval of the injectable combination of CS and GlcN at the aforementioned concentrations, at a dose of 2mL/100kg, should be five days.

Most studies evaluating the effects of GAGs on equine OA extrapolate doses used in previous studies or follow the recommendations of manufacturers of commercial products and, according to Pearson and Lindinger (2009), treatment outcomes are vulnerable to the employed underdoses. The authors consider the lack of testing of other doses, including higher than recommended ones, as a limitation of the study, although employed posology was sufficient to raise the urinary levels of the CS concentration.

In short, the studies demonstrate encouraging trends and evidence of the effectiveness of chondroprotectors, so there is a growing interest from the veterinary pharmaceutical industry to investigate and invest in research on the efficacy and safety of these drugs, to prove their benefits in the prevention and therapy of OA in horses. Intramuscular administration of the CS and GlcN-based solution for injection provides superior potential in bioavailability and may be a drug presentation with surpassing results. In conclusion, a single administration of CS and GlcN by intramuscular route increases the urinary levels of CS in 24 hours in healthy horses, which declined only from the fifth day after injection. This interval must be respected when using CS-based chondroprotectors in the management of OA in horses.

Keywords: glycosaminoglycans, chondroprotectors, osteoarthritis, horses

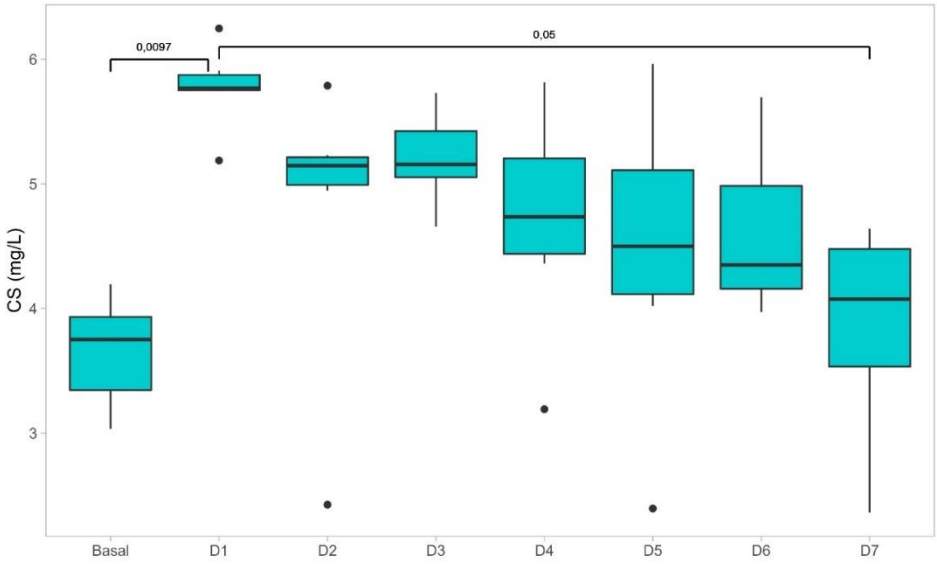

Figure 1. Inside line of boxes presents the median (50%) of data in each moment. The inferior limit of boxes corresponds to the first quartile (25%) and the superior limit, to the third quartile (75%). Lines' extremities correspond to the superior and inferior limits of distribution and the black dots represent the outliers. After administration of intramuscular CS and GlcN, a statistically significant increase of urinary levels of CS (mg/L) was found on D1 in relation to the baseline (p<0,0097). There was a statistically significant decrease on D7 (p<0,05) when compared to D1. The brackets represent the statistically significant differences between evaluated moments.

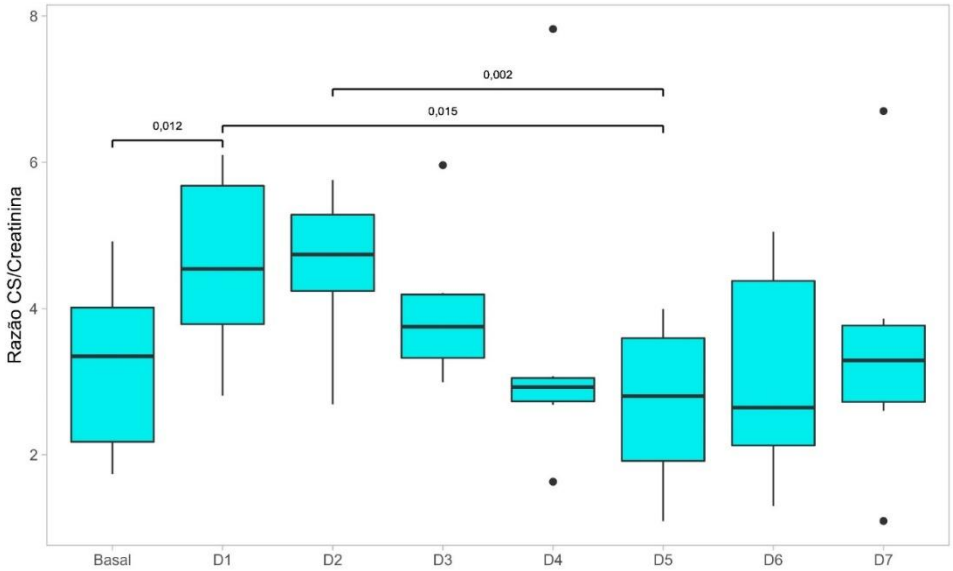

Figure 2. Inside line of boxes presents the median (50%) of data in each moment. The inferior limit of boxes corresponds to the first quartile (25%) and the superior limit, to the third quartile (75%). Lines' extremities correspond to the superior and inferior limits of distribution and the black dots represent the outliers. After administration of intramuscular CS and GlcN, there was a statistically significant increase of urinary levels of the CS/creatinine (mg/g) ratio on D1 in relation to the baseline (p<0,012), and a statistically significant decrease on D5 when compared to D1 (p<0,015) and D2 (p<0,002). The brackets represent the statistically significant differences between evaluated moments.

RESUMO

A osteoartrite é uma importante afecção do aparelho locomotor de equinos, e os condroprotetores têm obtido papel importante no manejo da dor e como adjuvantes na redução de doses e na frequência de aplicação de outros fármacos que apresentam efeitos deletérios. O condroitim sulfato (CS) e a glucosamina (GlcN) são condroprotetores com potenciais efeitos anti-inflamatórios e anticatabólicos, cuja administração oral é a via mais frequente e controversa por resultar em baixa biodisponibilidade. Por essa razão, a administração intramuscular pode ser mais vantajosa. O objetivo do presente estudo foi avaliar os períodos de tempo para se atingir o pico de excreção urinária de CS e o início de declínio, após injeção intramuscular de produto à base de CS e de GlcN, em seis cavalos hígidos, na dose de 2 mL/100kg de condroitim sulfato A (750 mg/mL) e sulfato de glucosamina (750 mg/mL). Amostras de urina foram coletadas, no mesmo horário, no dia anterior (D-1), no dia da administração (D0), as quais constituíram o valor basal, e por mais sete dias (D1 a D7). O CS urinário foi extraído por cromatografia de troca iônica em coluna de Q-Sepharose Fast Flow, identificado por eletroforese em gel de agarose e mensurado por meio de densitometria, enquanto a creatinina urinária foi mensurada por analisador bioquímico automático. Os níveis de CS excretado (mg/L) aumentaram (P<0,0097) no D1 (24 horas) em relação ao momento basal (D0) e diminuíram no D7 (P<0,05) em relação ao D1, além dos níveis da relação CS/creatinina (mg/g) se elevarem significativamente (P<0,012) no D1 (24 horas) em relação ao basal e diminuírem no D5 (P<0,015), se comparados ao D1 e ao D2 (P<0,002). Não houve diferença significativa (P<0,05) da creatinina (g/L) nos momentos avaliados. Uma única aplicação de CS e de GlcN injetável foi capaz de aumentar consideravelmente as concentrações urinárias de CS 24 horas após, decaindo a partir do quinto dia. Esse período fundamenta o intervalo de cinco dias entre tratamentos injetáveis desses condroprotetores na OA em equinos.

Palavras-chave: glicosaminoglicanos, condroprotetores, osteoartrite, equinos

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