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Urinary glycosaminoglycans in horse osteoarthritis. Effects of chondroitin sulfate and glucosamine

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ABSTRACT

Our objectives were to characterize the urinary excretion of glycosaminoglycans (GAGs) in horse osteoarthritis, and to investigate the effects of chondroitin sulfate (CS) and glucosamine (GlcN) upon the disease. Urinary GAGs were measured in 47 athletic horses, 20 healthy and 27 with osteoarthritis. The effects of CS and GlcN were investigated in mild osteoarthritis. In comparison to normal, urinary GAGs were increased in osteoarthritis, including mild osteoarthritis affecting only one joint. Treatment with CS + GlcN led to a long lasting increase in the urinary CS and keratan sulfate (KS), and significant improvement in flexion test of tarsocrural and metacarpophalangeal joints was observed. In conclusion, urinary CS and KS seems to reflect the turnover rates of cartilage matrix proteoglycans, and the measurement of these compounds could provide objective means of evaluating and monitoring joint diseases.

1. Introduction

Osteoarthritis (OA) is a chronic condition characterized by progressive degeneration of articular cartilage accompanied by sclerosis of the subchondral bone (Reginster, 2002). Clinical features include joint effusion, pain, instability, limitation of motion and functional impairment. OA results in the destruction and breakdown of articular cartilage, and its presence and severity is usually documented by X-ray imaging. Breakdown of the cartilage components – collagen, proteoglycans and other proteins – results in the generation of fragments of these macromolecules, which can eventually be detected in the blood, synovial fluid, or urine. Systemic biomarkers (serum or urine) offer a potential method for quantifying the disease status, and these indicators in serum or urine would provide objective means of evaluating and monitoring OA. Furthermore, the identification of biomarkers is one way to accelerate drug discovery and trials.

Cartilage is the mammalian tissue that contains the highest concentration of glycosaminoglycans (GAGs)[1], especially chondroitin sulfate (CS) and keratan sulfate (KS), which occur in the tissue as proteoglycans. There are evidences indicating that most of the urinary GAGs are of systemic origin: CS is the main urinary and plasmatic GAG in many mammalian species, but it is not present either in the urinary tract (Pereira et al., 2004; de Lima et al., 2005) or kidney (Cadaval et al., 2000); CS given to rats was rapidly excreted in the urine, part as polymeric CS and part as degradation products (Michelacci et al., 1992); cartilage and cornea are the only tissues that contain significant amounts of KS, and trace amounts of KS are present in mammalian urine (Pereira et al., 2004; Vieira et al., 2005). Consequently, these compounds could be systemic biochemical markers of joint diseases.

We have previously shown that when growth and calcification processes take place in human cartilages, changes occur in the structure and composition of cartilage proteoglycans, especially aggrecan. These changes were observed with age, in osteoarthritis (Michelacci et al., 1979), and in cartilage tumors, both chondrosarcoma (Mourão et al., 1979) and enchondromatosis (Michelacci et al., 1981).

Because horses have naturally occurring OA (Richardson and Loinaz, 2007), which is similar to that of humans, the horse was chosen as a species to investigate a possible correlation between urinary excretion of GAGs and OA. The main urinary GAGs in horses are CS, dermatan sulfate (DS) and heparan sulfate (HS), with small amounts of KS. A marked decrease in urinary GAGs occurred with age for healthy horses, both sedentary and athletes, and the concentration of KS increased with age. Athletic horses excreted

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[1] Abbreviations: CS, chondroitin sulfate; GAG, glycosaminoglycan; GlcN, glucosamine; GLUT, glucose transporter protein; HAS, hyaluronan synthase; i.p., intraperitoneal; i.m., intramuscular; KS, keratan sulfate; p.o., *per oral*.

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less GAGs in the urine than age-matched sedentary horses (including KS) (Vieira et al., 2005).

Horses are also a good model to investigate the potential of "nutraceutical" agents, such as glucosamine (GlcN) and CS, for the treatment of OA. From the treatment standpoint, they are easy to administer and generally received as benign (Trumble, 2005), and both the dosing and the patients' adherence are easy to control.

The molecular mechanisms mediating anti-arthritic activities of GlcN and CS are not completely understood. The transport of GlcN through plasma membrane is facilitated by glucose transporter proteins (GLUTs) (Uldry et al., 2002). Chondrocytes express several GLUTs and actively import and metabolize GlcN, but not N-acetylglucosamine. GlcN inhibits glucose transport in a non-competitive fashion (Shikhman et al., 2009), and in equine chondrocytes, GlcN reduced the interleukin-1β-induced expression of inflammatory reaction-related enzymes (Neil et al., 2005b).

Concerning CS, many authors have shown anti-inflammatory activities for CS in cultured chondrocytes or cartilage explants from different mammalian species (reviews in du Souich et al., 2009; Iovu et al., 2008). Also, there are evidences suggesting that the combination of GlcN and CS may be more effective in preventing cartilage degradation (Dechant et al., 2005). However, most of the above cited studies were performed in cultured cells or cartilage explants. There are few in vivo studies concerning the biochemical effects of CS and/or GlcN in horses (Forsyth et al., 2006; Neil et al., 2005a). So, the objectives of the present study were to characterize the urinary excretion of GAGs in athletic horses, both healthy and with OA, and to investigate the effects of CS and GlcN upon clinical and biochemical markers of OA in these animals.

2. Materials and methods

2.1. Materials

Standard chondroitin 4-sulfate (from whale cartilage), chondroitin 6-sulfate (from shark cartilage) and DS (from hog skin) were purchased from Sigma Chemical Co. HS (from bovine pancreas) and chondroitinases AC and B (from *Flavobacterium heparinum*) were prepared by methods previously described (Aguiar et al., 2003; Dietrich and Nader, 1974). Agarose (standard, low M_r) was purchased from Bio-Rad Laboratories. Q-Sepharose Fast Flow was purchased from Amersham Pharmacia Biotech do Brasil Ltda. MST1 anti-KS monoclonal antibody was obtained as previously described (Alves et al., 1994). Two commercial preparations of CS and GlcN from Vetnil Ind. e Com. de Produtos Veterinários Ltda. were used: (A) an isotonic solution of CS (75 g/l) and GlcN (75 g/l), administered by intramuscular route (i.m.), (10 ml = 750 mg CS + 750 mg GlcN); and (B), a mixture of GlcN (800 mg), selenium (4 mg), vitamine E (600 mg), and amino acids (L-glutamine, 800 mg, L-arginine, 200 mg, proline, 400 mg, DL-methionine, 800 mg, L-lysine, 800 mg), given by oral route (p.o.). Before use, these preparations were submitted to analysis for CS and GlcN (Dietrich et al., 1977; Giusti et al., 1988), which have shown that the concentrations stated by the manufacturer were correct.

2.2. Animals and urine samples

This work was approved by the *Ethical Committees of Universidade Federal de São Paulo – UNIFESP* (CEP 0800/07) and *Universidade de São Paulo – USP* (946/2006), and was carried out in accordance with *UNIFESP* and *USP guidelines*, and also in accordance with EC Directive 86/609/EEC for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

All urine samples were collected via urethral catheterization always at 10:00 a.m. The samples were stored at −20 °C until use.

To evaluate the variations in the concentration of urinary GAGs in horses, urine samples were collected from three healthy athletic horses for six consecutive days.

For the determination of horse urinary excretion pattern and to identify possible changes that occur in OA, urine samples were collected from 47 warmblood athletic horses, 20 healthy and 27 with OA.

To evaluate the urinary excretion of exogenous CS, three athletic horses with mild OA were selected by physical and radiographic criteria. Urine samples were collected on days 1 and 2, at 10:00 a.m., and soon after the urine sample collection of day 2, CS + GlcN was i.m. administered. Urine samples were collected on the subsequent five days, always at 10:00 a.m. On day 7, CS + GlcN was again i.m. administered soon after the urine sample collection, and again urine samples were collected on the subsequent five days. Creatinine and GAG concentrations were measured.

2.3. Treatment

The effects of CS and GlcN administration were investigated in six athletic horses with mild OA affecting only one joint, selected by radiographic criteria. These animals were dressage and jumping warmblood horses, 8–12 years old. The inclusion criteria were:

1. Lameness due to either metacarpophalangeal or tarsocrural joints for more than one month, increased articular volume or history of intermittent joint lameness;
2. Radiographic image of either metacarpophalangeal or tarsocrural joints compatible with osteoarthritis types 1 or 2, according to McIlwraith (2002);
3. Absence of treatment with GAGs and/or anti-inflammatory drugs.

After the first urine sample collection, these animals were treated every five days, for 25 days, with 10 ml of i.m. CS + GlcN (preparation (A), Section 2.1). Urine samples were collected immediately before each dosing on days 1, 6, 11, 16, and 21. Subsequently, urine samples were collected every seven days, up to the 60th day. Two month later (day 115), the same animals received daily p.o. GlcN doses (800 mg/day, preparation (B), Section 2.1) for 25 days, and again urine samples were collected every five days during the treatment, and every seven days after the end of treatment, up to the 184th day.

2.4. Glycosaminoglycan identification and quantification

Urinary GAGs were analyzed by two different methods: (1) Dialysis: Urine samples (500 μl) were dialyzed against pure water, freeze-dried in Speed Vac (VR-1 Heto Lab), and resuspended in 20 μl of water; (2) Ion-exchange chromatography: Urine samples (10–40 ml) were diluted with three volumes of water, and then applied to an ion-exchange column of Q-Sepharose Fast Flow (5 ml bed volume, 1 × 6.5 cm), previously equilibrated with water. The column was eluted in a stepwise fashion with 0.3 M NaCl (20 ml) and 2 M NaCl (20 ml).Three ml fractions were collected. Three volumes of methanol were added to each fraction, slowly and under agitation. After standing for 24 h at −20 °C, the precipitates formed were collected by centrifugation, vacuum dried and resuspended in 100 μl of water.

Aliquots of these preparations (5 μl) were submitted to agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9 (PDA) (Dietrich et al., 1977). After fixation with cetyltrimethylammonium bromide and Toluidine Blue staining, the urinary GAGs were quantified by densitometry of the gel slabs (Scanner