

Serum concentrations of keratan sulfate, osteocalcin, and pyridinoline crosslinks after oral administration of glucosamine to Standardbred horses during race training

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Objective—To determine the effects of orally administered glucosamine on concentrations of markers of bone and cartilage metabolism in Standardbred horses during race training.

Animals—Twenty 16- to 20-month-old Standardbreds beginning race training.

Procedure—Horses were randomly assigned to 2 groups. One group received glucosamine hydrochloride (4 g, PO, q 12 h), and the second (control) group received glucose (4 g, PO, q 12 h). Serum samples were obtained prior to onset of the study (baseline) and at regular intervals for 48 weeks for determination of concentrations of keratan sulfate (KS), osteocalcin (OC), and pyridinoline crosslinks (PYD).

Results—Osteocalcin concentrations changed significantly with time; mean serum concentrations were significantly higher than baseline values for samples obtained at 24 to 48 weeks after onset of the study. Although a significant effect of time was observed for mean concentration of KS, concentrations did not differ significantly from baseline values at any time during the study when groups were analyzed separately. However, pooled analysis revealed significant increases of mean serum KS concentration at weeks 24 and 30. Significant changes in serum PYD concentrations were not detected. Oral administration of glucosamine did not significantly affect serum concentrations of any of the markers.

Conclusions and Clinical Relevance—Increased serum OC in clinically normal Standardbreds during race training may reflect bone formation that accompanies adaptive remodeling of the appendicular skeleton. For these experimental conditions, glucosamine did not appear to exert a detectable influence on serum concentrations of these 3 markers of connective tissue metabolism. (*Am J Vet Res* 2002;63:1106–1110)

Interest in the use of biological markers for animals with osteoarthritis (OA) has been prompted by the recognition that pathophysiologic processes in articular tissues are advanced by the time routine radi-

ographic alterations become detectable. Identification and monitoring of metabolic changes in cartilage and bone that precede radiographically detectable changes would be of considerable value. Potential utility of such markers is not limited to early diagnosis; they could also be used to provide prognostic information, elucidate disease mechanisms, and facilitate investigation of the metabolic effects of medications intended to prevent or treat joint damage in its incipient stages.

A number of molecules found in serum and synovial fluid have been investigated for their use in identifying initial changes in cartilage and bone in animals with OA. Keratan sulfate (KS), a glycosaminoglycan unique to articular cartilage, is 1 such marker that is released into the general circulation during episodes of cartilage breakdown.¹ The potential for use of KS as a marker of early cartilage damage was originally recognized because of the fact that there are substantial increases in KS concentration prior to the onset of macroscopic cartilage damage in dogs subjected to transection of a cranial cruciate ligament.² Subsequently, serum and synovial fluid concentrations of KS have been used to monitor the development of cartilage lesions in a number of studies^{3–7} that involved experimentally induced and naturally occurring joint disease. Although findings have not been consistent, increased blood concentrations of KS have been observed in horses with joint diseases.^{4,6}

Markers of OA are not limited to those reflecting aberrations of cartilage matrix. Growing recognition of the role of subchondral bone in the progression of OA has resulted in an increasing frequency for the use of markers of bone metabolism as indices of disease progression. Two such bone-derived molecules are osteocalcin (OC) and pyridinoline crosslinks of type-I collagen (PYD), both of which are used as markers of OA.^{8–11} Osteocalcin is a product of osteoblasts. It is a noncollagenous protein, and its concentration in the circulation is regarded as a sensitive indicator of bone formation. Moreover, OC expression is increased in subchondral bone of osteoarthritic joints, and OC mRNA can be detected in chondrocytes from osteoarthritic but not normal joints.¹² Pyridinoline crosslinks are found in mature collagen of bone and other tissues. Increased concentrations of PYD in blood or urine are most commonly used as an indicator of bone resorption.⁹ Humans with OA have increased concentrations of this marker in their urine, which decrease after intra-articular administration of corticosteroids into affected joints.¹³

Glucosamine is a precursor of the disaccharide

Received Dec 26, 2001.

Accepted Apr 2, 2002.

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subunits of cartilage proteoglycans. It is a putative antiarthritic compound that holds promise for use in the treatment of animals with OA. It is purported that glucosamine possesses anti-inflammatory and disease-modifying (chondroprotective) effects. Because of its relative safety, glucosamine could have great use for treatment of joint disease even if it is only modestly effective.^{14,15} Few appropriately designed and controlled clinical trials have not been conducted; however, the results of studies¹⁶⁻¹⁹ suggest that glucosamine administration can be used to reduce pain and improve joint mobility in humans and horses with OA.

Analysis of *in vitro* studies²⁰⁻²³ indicates that glucosamine sulfate increases proteoglycan synthesis by chondrocytes and may have a number of anti-inflammatory properties. Our laboratory group conducted studies^{24,25} that revealed glucosamine is a potent inhibitor of proteoglycan loss and matrix metalloproteinase synthesis or activity, as well as a potent inhibitor of nitric oxide production, in equine cartilage explants. Potential metabolic consequences of glucosamine administration, as manifested by serum concentrations of metabolic markers, have not been studied. Therefore, the objective of the study reported here was to determine the potential influence of oral administration of glucosamine on serum concentrations of OC, KS, and PYD in young Standardbreds during race training.

Materials and Methods

Horses—Twenty Standardbreds that were 16 to 20 months old and just beginning race training were randomly assigned to 1 of 2 groups. Horses in the treatment group received glucosamine hydrochloride^a at a dose and frequency corresponding to the maintenance amount (4.0 g, PO, q 12 h) recommended by the manufacturer of a commercially available glucosamine-containing nutraceutical preparation^b throughout the entire study. Horses in the control group received a similar amount of glucose (4 g, PO, q 12 h).

Collection of samples—Blood samples were obtained from an external jugular vein. Samples were centrifuged (300 × g for 10 minutes), and sera were harvested, apportioned into 1.5-ml centrifuge tubes,^c and stored at -80 C until analysis. Samples were obtained before the start of the study (baseline samples; week 0) and at 2-week intervals until conclusion of the study 48 weeks later. All blood samples were collected between 10 AM and noon.

Training schedule—For the first 12 weeks, training consisted of slow jogging for a distance of 1.6 km on 5 days each week. Subsequently, jogging distance and intensity of exercise were gradually increased; by 20 weeks, horses were jogging 4.8 km 5 d/wk and also performing brief intervals of more intense speed (ie, running a distance of 400 m at a speed of 8.89 m/s) as part of each workout. From weeks 20 to 24, horses exercised 5 d/wk. Four of these workouts consisted of jogging for a distance of 4.8 km, and the fifth workout consisted of running a distance of 1.6 km at a speed of 8.20 to 8.89 m/s.

During the subsequent 2 months (weeks 25 to 32), horses exercised 6 d/wk. Four of those exercise periods consisted of jogging a distance of 5.6 km. The other 2 days each week were devoted to exercise of increasing intensity. At week 25, horses started by running a distance of 1.6 km at a speed of 8.89 m/s. Speed was increased by 0.14 m/s every other workout until horses reached a speed of 11.43 m/s. After that, increases in speed were in increments of 0.08 m/s until week 32 to 34, at which time horses were entered in competitive races. In general, horses raced once weekly, had a brief respite

from training on the day after each race, and exercised in the form of jogging for the next 4 days. This schedule was maintained until the study was completed on week 48.

Measurement of KS concentrations—Serum KS concentration was quantified by use of an ELISA that included an inhibition step.^{26,27} Incubation of serum with the anti-KS monoclonal antibody^d was performed at pH 5.3. The detection process required incubation with the secondary antibody (goat anti-mouse IgG conjugated to horse radish peroxidase^e), and color development was initiated by use of *o*-phenylenediamine.^f All concentrations of KS were reported as equivalent units of an international standard of KS.⁸

Measurement of OC and PYD concentrations—Concentrations of OC and PYD in serum samples were quantified by use of commercially available ELISA^{h,i} that were performed in accordance with the manufacturer's instructions. Detailed descriptions of the use of the 2 assays for samples obtained from horses have been provided elsewhere.²⁸

Statistical analysis—Data were analyzed as a split-plot ANOVA by use of the model $y = \mu + \text{group (glucosamine)} + \text{error}_1 + \text{time} + (\text{group} \times \text{time}) + \text{error}_2$ where *y* was the response variable (ie, concentration of OC, KS, or PYD). When we detected a significant time effect, mean values for each sample period were compared with baseline values by use of the Bonferroni *t* test for multiple comparisons. Data were reported as mean ± SEM, and a value of $P < 0.05$ was considered significant.

Results

Sixteen of 20 horses completed the entire 48 weeks of the study. Four horses were removed from training prior to the conclusion of the study and were excluded from the analysis. Therefore, there were 7 horses in the control group and 9 horses in the glucosamine group. Three of these 4 horses were withdrawn from training by the stable manager because of lack of athletic ability or immaturity at week 24 (1 horse in the control group) and 28 (2 horses, 1 from the control group and 1 from the glucosamine group). Lameness was not observed in those 3 horses. A fourth horse (control group) was removed at week 30 because of suboptimal performance. The stable manager attributed the problem in that horse to lameness in the carpal region referable to skeletal immaturity; however, a complete examination was not conducted.

Although samples were obtained at intervals of 2 weeks, economic considerations restricted our analysis to fewer samples. Alterations in concentrations of markers were modest during the early portion of the study, and analyses were weighted for sample times corresponding to more rigorous training conducted later during the study. Specifically, samples that were analyzed were obtained prior to the onset of treatment and training (ie, baseline) and at weeks 12, 20, 24, 30, 36, 40, 44, and 48.

Baseline value for mean ± SEM serum OC concentration was 30.48 ± 3.35 ng/ml. Serum OC concentration was not significantly ($P = 0.53$) affected by glucosamine treatment, and there was not a significant ($P = 0.32$) interaction of treatment × time. Osteocalcin concentrations changed significantly ($P < 0.001$) with time, and the highest mean value (61.49 ng/ml) was recorded at week 30 (Fig 1). For the control group, serum OC concentration was significantly higher than the baseline value for weeks 24, 30, 36, 40, 44, and 48. Mean OC concentration in serum for glucosamine-

treated horses was significantly different from the baseline value at weeks 12, 24, 30, 36, 40, 44, and 48.

Baseline value for mean KS concentration was 182.55 ± 9.20 ng/ml, and it did not vary widely over the course of the study. Concentrations of KS were not significantly ($P = 0.96$) affected by glucosamine admin-

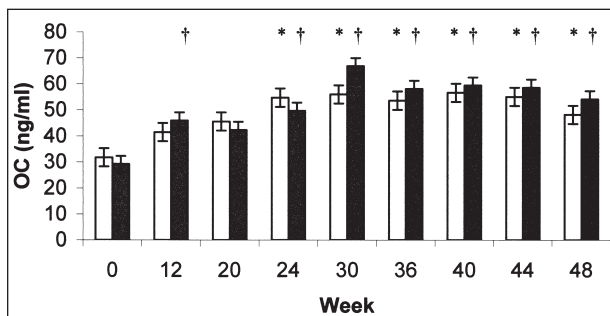


Figure 1—Mean \pm SEM serum concentrations of osteocalcin (OC) in Standardbred horses that were in race training and that were administered glucose (control group; $n = 7$; open bars) or glucosamine hydrochloride (4 g, PO, q 12 h; $n = 9$; solid bars). *Serum OC concentration of the control group was significantly ($P < 0.001$) higher than the baseline value (week 0) for that group. †Serum OC concentration of the glucosamine-treated group was significantly ($P < 0.001$) higher than the baseline value for that group. Values did not differ significantly ($P = 0.53$) between glucosamine-treated and control groups.

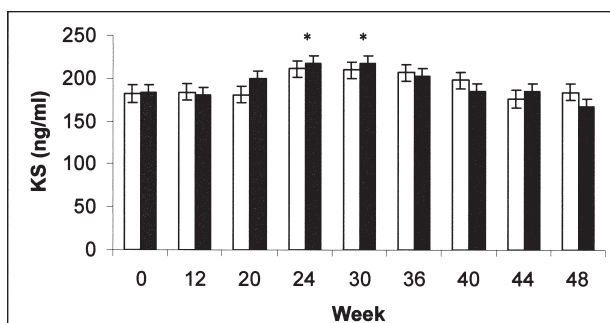


Figure 2—Mean \pm SEM serum concentrations of keratan sulfate (KS) in Standardbred horses that were in race training and that were administered glucose (open bars) or glucosamine (solid bars). Concentrations of KS differed significantly ($P < 0.001$) over time. *Pooled values for all horses of both groups were significantly ($P < 0.001$) higher than the baseline value (week 0). Values did not differ significantly ($P = 0.96$) between glucosamine-treated and control groups.

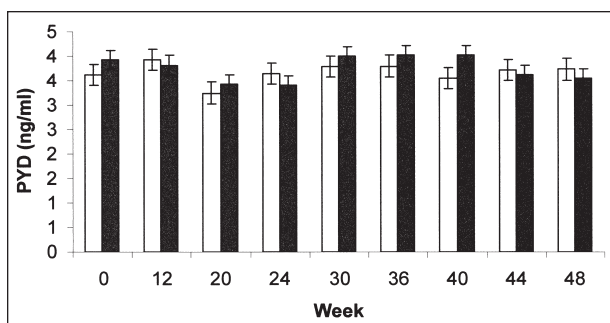


Figure 3—Mean \pm SEM serum concentrations of pyridinoline crosslinks of type-I collagen (PYD) in Standardbred horses that were in race training and that were administered glucose (open bars) or glucosamine (solid bars). Values did not differ significantly over time ($P = 0.11$) or between glucosamine-treated and control groups ($P = 0.45$). Week 0 = Baseline values determined prior to the start of the study.

istration, and there was not a significant ($P = 0.63$) interaction of treatment \times time. There was a significant ($P < 0.001$) effect of time; however, when glucosamine-treated and control horses were analyzed separately, none of the time points had a value that was significantly different from the baseline value (Fig 2). Because we did not detect a significant difference between the glucosamine and control groups, a subsequent pooled analysis was conducted, which revealed that KS concentrations at weeks 24 and 30 were significantly higher than the baseline value.

Analysis of mean serum PYD concentrations revealed a baseline concentration of 3.77 ± 2.05 ng/ml, and it did not change significantly over the course of the study. Specifically, there was not a significant effect of glucosamine treatment ($P = 0.45$) or time ($P = 0.11$), and there was not a significant ($P = 0.67$) interaction of treatment \times time (Fig 3).

Discussion

Physical activity is among the most important of the numerous factors that influence skeletal structure. It has been clearly established that bone density is higher in athletes than in sedentary individuals.²⁹⁻³¹ Speculation exists that augmented bone density in certain locations may enhance loads experienced by the overlying articular cartilage and contribute to OA lesions.^{29,32,33} The study reported here was prompted by observations of potential cartilage-sparing actions of glucosamine in vitro and the documented use of the selected molecules as markers of incipient arthritis.

Contrary to a number of studies of exercising horses in which investigators used the same markers we used as well as other markers as indices of cartilage and bone turnover, we did not include a control group of sedentary, age-matched horses. Inclusion of such a group would be required to specifically address the effects of exercise on serum concentrations of these markers. Given the central focus of the potential modifying influence of glucosamine on serum concentrations of the markers, it was considered appropriate to conduct the study without this control group.

The dose and frequency of glucosamine administration corresponded to that recommended by the manufacturer of a popular nutraceutical product. Although testing a range of doses would have been of interest, our pool of similarly aged horses that were trained in a similar manner by the same group of individuals and that did not receive other nonsteroidal anti-inflammatory drugs or other antiarthritis preparations was limited. For this reason, we elected to administer glucosamine at a dosing rate and frequency equivalent to that currently recommended. Although we cannot rule out the possibility that glucose and glucosamine may have similarly influenced concentrations of markers, it would appear to be unlikely. We are not aware of any reports indicating that oral administration of modest amounts of glucose (4 g, PO, q 12 h) would influence bone or cartilage metabolism. Moreover, unlike glucosamine, glucose does not appear to exert an effect on cartilage metabolism in vitro.²⁵

We observed a significant and sustained increase in the mean serum concentration of OC during train-

ing, compared with the mean baseline OC concentration. Temporally, serum concentrations were unchanged during the early training period, but they increased significantly between 12 and 24 weeks and remained increased for the duration of the study. Serum OC concentration is influenced by a number of factors. In addition to increases that accompany skeletal growth, weight-bearing exercise induces changes in serum concentrations of OC,³⁴ probably reflecting exercise-induced bone deposition; however, the precise nature of these responses is incompletely characterized. For example, humans undergoing aerobic training have transient reductions of blood OC concentrations, which are followed by a return to baseline concentrations. This is in contrast to subjects who perform anaerobic exercise or resistance training in which rapid augmentation of serum OC occurs prior to detectable changes in bone density.^{35,36} These data suggest that, among a multiplicity of other factors, the type of exercise has an impact on the osseous response. Thus, a likely explanation for the increased mean OC concentrations observed in our groups of horses is bone deposition that accompanies the adaptive remodeling response of osseous tissues to training. This hypothesis is supported by the observation of increased concentrations of bone alkaline phosphatase and carboxy-terminal propeptide of type-I procollagen (markers of bone formation) that have been documented in treadmill-exercised Thoroughbreds.³⁷ In contrast to a study²⁷ in which OC concentrations gradually decreased in 2-year-old horses, we observed a persistent increase in serum OC concentration. A longer period for collection of samples would have been required to determine whether there would have been a gradual return to baseline concentrations.

We cannot rule out the possibility that increases in serum OC concentrations were reflecting metabolic changes in bone that accompany incipient OA; however, similar responses were characteristic of the entire group of horses, and we did not detect concomitant increases in concentrations of KS or PYD. Lack of parallel increases in the serum concentrations of these degradative markers of cartilage and bone discourages a conclusion that OC concentrations indicate the development of osteoarthritic lesions in subchondral bone.

We did not detect a significant effect of glucosamine on the observed increases in serum OC concentrations, similar to results obtained after prolonged exercise in yearling Quarter Horses.²⁷ Changes induced by training that may have led to osteoarthritic changes in bone, which were mirrored by OC concentrations and influenced by glucosamine administration, were undetected. We are not aware of any other data addressing this issue, and the effects, if any, of glucosamine administration on bone deposition remain to be determined.

Many investigators have expressed interest in the use of KS as an index of cartilage breakdown, and numerous studies have been conducted concerning its use as a marker of cartilage damage. We found that when horses from both treatment groups were considered collectively, there was a transient increase in serum KS concentration at weeks 24 and 30. This could indicate a temporary increase in proteoglycan

turnover in cartilage in response to increased training intensity or may have been a nonspecific response related to growth. The latter possibility seems less likely than the former because other reports^{4,38} indicate a peak of this marker early in life. Data from *in vitro* studies²³⁻²⁵ support a cartilage-sparing role of glucosamine, and it was believed that training-induced matrix turnover in cartilage could be detected and, furthermore, that glucosamine could play an inhibitory role in that process. Because KS concentrations were not markedly influenced by the intensity of training used in our study, this question remains unanswered.

Little information is available on the effects of exercise on serum KS concentrations in horses. In a study³⁹ in which investigators correlated changes in serum concentrations of metabolic markers with various types of running-type exercises in humans, the concentration of KS was transiently increased in conjunction with exercise. This exercise-induced increase in KS concentrations was attributed to an effect of mechanical loading in combination with a possible underlying high turnover rate of cartilage matrix in the athletes during training. Lacking a control group of sedentary, age-matched horses, we cannot draw conclusions on the effects of exercise on KS concentrations in horses trained in the manner described in our study.

Significant changes were not observed in serum concentrations of PYD in the horses of the study reported here, which suggested that gradually increasing the exercise intensity did not result in detectable breakdown of type-I collagen. The effects of exercise performed on a treadmill on another marker of collagen I breakdown (ie, pyridinoline cross-linked telopeptide of type-I collagen) in 2-year-old Thoroughbreds have been reported.³⁵ In that study, concentrations of pyridinoline cross-linked telopeptide of type-I collagen in exercised horses were higher than those of sedentary control horses early and late during the 12-month study, but significant differences were not detected at 4 and 6 months. For Thoroughbreds in that study, concentrations decreased significantly during the course of the study, which is contrary to the consistent concentrations of PYD detected in our horses during the course of the 48-week study reported here. It remains to be determined whether the lack of gradually decreasing concentrations of PYD among our horses reflects training-induced collagen turnover or is indicative of biological variation.

Glucosamine did not appear to influence serum concentrations of PYD in the horses of our study. This suggested that the training regimen was not associated with collagen breakdown. Another possibility is that glucosamine had little influence on the processes involved in collagen turnover for horses maintained in these conditions.

Four horses were withdrawn from training prior to conclusion of the study. Of these horses, only 1 was lame. A diagnostic evaluation was not conducted; however, the stable manager attributed the poor performance of that horse to lameness in the carpal region related to skeletal immaturity. Training was successfully resumed in the horse the next season without a recurrence of lameness. The remaining horses were

withdrawn for reasons of immaturity (2 horses) and lack of inherent ability (1). Because these horses were not included during the later stages of the study when more vigorous training would have been most likely to have a measurable influence on turnover of connective tissue, samples from these 4 horses were not analyzed. Because of the lack of specific data, it cannot be known with certainty that exclusion of these horses influenced the results; however, given the fact that withdrawal of 3 of the 4 horses was for reasons other than lameness, it seems an unlikely source of bias.

^aGlucosamine HCl, Sigma Chemical Co, St Louis, Mo.

^bCosequin, Nutramax Laboratories, Edgewood, Md.

^cMicro tube, Sarstedt, Nümbrecht, Germany.

^dAnti-keratan sulfate monoclonal antibody, ICN Biomedicals Inc, Aurora, Ohio.

^eAnti-mouse IgG (Goat), HRP conjugate, ICN Biomedicals Inc, Aurora, Ohio.

^fo-phenylenediamine, Sigma Chemical Co, St Louis, Mo.

^gProvided by Dr. Eugene Thonar, Department of Biochemistry, Rush Presbyterian, St Lukes Medical Center, Chicago, Ill.

^hNovocalcin, Quidel Corp, Mountain View, Calif.

ⁱSerum Pyd, Quidel Corp, Mountain View, Calif.

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