Comparison of the antioxidant effects of synovial fluid from equine metacarpophalangeal joints with those of hyaluronic acid and chondroitin sulfate

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Objective—To evaluate the antioxidant effects of synovial fluid (SF) pooled from metacarpophalangeal joints of healthy horses or horses with various pathological conditions, and to compare them with the antioxidant effects of hyaluronic acid (HA) and chondroitin sulfate (CS).

Sample Population—SF from 1 metacarpophalangeal joint was obtained from 42 horses immediately after humane slaughter. Samples were classified into 3 groups on the basis of origin: healthy joints or joints with chronically damaged cartilage or vascularly congested synovial membranes as detected via macroscopic evaluation.

Procedures—Antioxidant effects were evaluated by use of rat liver microsomal fractions treated with Fe³⁺-ascorbate as a free radical generator system leading to oxidative stress. Amounts of thiobarbituric-reactive substances and glutathione transferase (GSH-T) conjugation activity were measured.

Results—SF from healthy and chronically damaged joints inhibited microsomal lipid peroxidation, whereas SF from joints with congested synovial membranes had only a slight effect. Hyaluronic acid and CS did not inhibit microsomal lipid peroxidation. Moreover, GSH-T activity was detected in all SF samples, which had similar activity regardless of disease status. All SF samples as well as HA and CS protected rat microsomal GSH-T activity against oxidative damage. Only SF samples from joints with congested synovial membranes protected microsomal thiols against oxidation, an effect also evident with HA and CS.

Conclusions and Clinical Relevance—The antioxidant mechanisms associated with the response to metacarpophalangeal joint damage in horses appeared to act on different targets, depending on whether the damage was acute or chronic. (Am J Vet Res 2010;71:399–404)
tal liver cytosolic proteins and 3% of total liver microsomal proteins.10–11

Virtually all pathological conditions are associated with disturbances in the redox balance of a system. This is particularly relevant in degenerative joint disease because oxidative stress is a marker for the disease. Studies12–13 have been conducted to evaluate the antioxidant ability of several enzymes (ie, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and GSH-T) in human joints. Because GSH-T activity appears to have antioxidant ability in human joints,12,13 this activity may be present in synovial fluid from equine joints. It has been proposed that glycosaminoglycans, such as HA and CS, may act as antioxidants in joint tissues.14–19 Nevertheless, the concentration of HA in synovial fluid decreases in chronically damaged metacarpophalangeal joints.20 In inflammatory joint conditions in horses, an increase in the amount of reactive oxygen species in diseased joints can occur, accompanied by an increase in total antioxidant capacity.2

The antioxidant ability within joints is far from understood in horses. The purpose of the study reported here was to evaluate the antioxidant effects of synovial fluid pooled from metacarpophalangeal joints of healthy horses or horses with various pathological conditions and to compare them with the antioxidant effects of HA and CS.

Materials and Methods

Synovial fluid—Samples of synovial fluid (3 to 4 mL) were obtained by aseptic puncture of 1 metacarpophalangeal joint at the level of the upper synovial sac in each of 42 mixed-breed horses immediately after slaughter at a local slaughterhouse by use of a captive bolt. Aspirated synovial fluid containing visible blood was discarded. Afterward, joints were dissected and inspected macroscopically for abnormalities in the cartilage and synovial membrane. Joints were subsequently classified by use of the following categories: healthy = bright and pearly white cartilage with a smooth cartilage surface, non–vascularly congested synovial membrane, and yellow, transparent synovial fluid with no evidence of blood; chronically damaged = cartilage with yellowish, opaque coloration and erosion or wear lines; and vascularly congested = abnormal coloration of the synovial membrane, and yellow, translucent synovial fluid with no evidence of blood. All samples were centrifuged at 4,000 g for 15 minutes at 4°C. Protein concentration was measured in the supernatant of each sample as described elsewhere.21 Samples with similar protein concentrations were pooled before freezing to avoid artifacts when performing concentration-dependent assays. The supernatants were stored at –80°C until processed.

Liver microsomal fraction—We decided to use rat liver microsomes in the study because GSH-T activity in such microsomes is inhibited by oxidative damage.23 Adult male Sprague-Dawley rats (body weight, 200 to 250 g) maintained at the vivarium of the School of Chemical and Pharmaceutical Sciences, Universidad de Chile, were used. Rats were allowed to consume pelleted food and were housed in a controlled environment (22°C, with lights on from 7:00 AM to 7:00 PM). All procedures were performed in accordance with protocols approved by an institutional ethics committee and with the guidelines for laboratory animals of the US National Research Council.

Microsomal fractions were prepared by differential centrifugation as described elsewhere.24 In brief, food was withheld from rats for 15 hours but free access to water was provided prior to euthanasia via decapitation. Livers were accessed via celiotomy, perfused in situ with 4 volumes of 25 mL of saline (0.9% NaCl) solution, excised, and placed on ice. Liver tissue (9 to 11 g of wet wt), devoid of connective and vascular tissue, was homogenized at 4°C with 5 volumes of 0.15M KCl, with 8 strokes in a homogenizer. Homogenates were then fractionated via centrifugation at 4°C with 1 of 2 devices at 9,000 X g for 15 minutes; sediments were discarded. Supernatants were then centrifuged at 105,000 X g for 60 minutes. Pellets (microsomes) were stored at –80°C until used.

Preincubation of liver microsomes with test media—Microsomes (1.0 mg of protein) were incubated (final volume, 1 mL) with various volumes of synovial fluid, CS3 (0.31 or 0.62 mg), or HA† (0.31 or 0.62 mg) for 15 minutes at 37°C with constant agitation. The amount of CS or HA in the incubations was selected on the basis of the physiologic concentration of HA in joint synovial fluid of horses (approx 0.5 mg/mL).24

Oxidative conditions—A mixture of 50µM FeCl3 and 1mM ascorbate was used as a generator of oxygen-free radicals to induce oxidative stress. This system has been routinely used by our research group.23

Microsomal lipoperoxidation assay—To evaluate the antioxidant capacity of the various synovial fluid samples collected, rat microsomal membranes, with or without preincubation with synovial fluid, were exposed to the Fe2+-ascorbate mixture. Synovial fluid antioxidant capacity is reflected by the inhibition of the lipoperoxidation of these microsomal membranes. The extent of microsomal lipid peroxidation after preincubation of microsomal membranes with Fe2+-ascorbate was estimated by determining the rate of TBARS generated.25 In brief, microsomes (1 mg of protein/mL) were incubated with the Fe2+-ascorbate mixture in 50mM phosphate buffer (pH, 7.4) for 20 minutes at 37°C with constant agitation. Control samples contained all of the reagents but microsomal protein. Following incubation, protein was precipitated with 10% trichloroacetic acid (final concentration) and separated by centrifugation at 9,000 X g for 10 minutes at 4°C. Supernatants were then incubated with 0.5% thiobarbituric acid (final concentration) for 1 hour at 37°C with constant agitation to generate TBARS. Absorbance values at 532 nm were established for the generated TBARS, which were assayed in conditions in which their formation was dependent on time and protein concentration. Nanomoles of TBARS of microsomal protein was calculated by use of the extinction coefficient of the conjugate (ε532 = 156mM–1cm–1).

GSH-T activity—Glutathione transferase conjugation activity was assayed as described elsewhere.24 In
brief, 100 µL of synovial fluid was incubated with 1mM 1-chloro-2,4-dinitrobenzene (substrate) and 4mM glutathione (cofactor) in 100mM phosphate buffer (pH, 6.5). Control samples without glutathione were assayed. Appearance of the conjugated product was continuously recorded for 3 minutes at 37°C in a spectrophotometer. To calculate GSH-T activity, an ε<sub>410</sub> value of 9.6mM<sup>-1</sup>cm<sup>-1</sup> for the conjugate was used. When indicated, synovial fluid samples were supplemented with 0.1 mg of microsomal protein/µL. To test whether synovial fluid samples protected GSH-T activity from damage induced by the Fe<sup>3+</sup>-ascorbate mixture, microsomal protein (0.1 mg/mL) in the presence or absence of synovial fluid (100 µL) was preincubated with Fe<sup>3+</sup>-ascorbate for 15 minutes at 37°C with constant agitation prior to assaying GSH-T activity. Results are expressed in nanomoles of conjugate per minute per milligram of microsomal protein.

**Microsomal thiol concentration**—An assay was performed to determine any antioxidant effects of all types of synovial fluid, HA, or CS in preventing a decrease in thiol concentration induced by Fe<sup>3+</sup>-ascorbate. Microsomal proteins contain thiols groups, which can be oxidized as a result of oxidative stress. This may lead to the gain or loss of function of microsomal enzymes, as has been described for GSH-T<sup>23</sup> and uridine diphosphate–glucuronyl transferases.<sup>23</sup>

Cytosolic thiol groups were titrated as described elsewhere.<sup>25</sup> In brief, microsomes (1 mg of protein/mL) were preincubated in the presence or absence of the Fe<sup>3+</sup>-ascorbate mixture in 50mM phosphate buffer (pH, 7.4). Control samples contained all reagents but microsomal protein. Control and microsomal samples were incubated for 20 minutes at 37°C with constant agitation. Afterward, microsomal thiol concentration was titrated with 0.6mM 5,5-dithiobis(2-nitrobenzoic acid)<sup>32</sup> for 1 hour at 37°C under constant agitation; following incubation, samples were centrifuged at 9,000 × g for 10 minutes at 4°C. Absorbance of the supernatants at 410 nm was then measured. Thiol concentration of the samples was estimated on the basis of the equimolar appearance of 5-thio-2-nitrobenzoic acid (ε<sub>410</sub> = 13,600mM<sup>-1</sup>cm<sup>-1</sup>) in the supernatants, expressed as nanomoles of thiols per minute per milligram of microsomal protein.

**Statistical analysis**—Data are presented as mean ± SD of at least 4 independent experiments (ie, experiments performed on different days). Differences found between the synovial fluid from healthy, chronically damaged, or vascularly congested joints were evaluated with respect to lipid peroxidation, thiol oxidation, and GSH-T activity. Groups of test data were compared by use of 1-way ANOVA with a Dunnett test for multiple comparisons. Values were considered significantly different at P < 0.05. All analyses were performed with commercially available software.<sup>1</sup>

**Results**

**Synovial fluid**—Synovial fluid samples were obtained from 18 healthy metacarpophalangeal joints, 18 chronically damaged joints, and 6 joints with vascularly congested synovial membranes. Mean ± SD protein concentrations in synovial fluid were 8.96 ± 0.6 mg/mL for healthy joints (18 pooled samples), 10.06 ± 0.5 mg/mL for chronically damaged joints (18 pooled samples), and 8.5 ± 1.9 mg/mL for joints with congested synovial membranes (6 pooled samples).

**Effect of SF on lipid peroxidation**—Synovial fluid from healthy and chronically damaged joints inhibited lipid peroxidation in rat liver microsomes by 40%, compared with the value for untreated membranes (1.95 ± 0.25 nmol of TBARS/min/mg of microsomal protein). This value remained unchanged at all volumes of synovial fluid tested (10 to 100 µL; Figure 1). On the other hand, synovial fluid from joints with congested synovial membranes inhibited microsomal lipid peroxi-
Effect of HA and CS on lipid peroxidation—Preincubation of microsomes with HA or CS failed to inhibit microsomal lipid peroxidation induced by Fe³⁺-ascorbate. Nevertheless, the combination of equal concentrations of HA and CS (0.312 mg/mL each) inhibited lipoperoxidation by 17%, compared with the amount of peroxidation in untreated microsomal membranes (Figure 2).

GSH-T activity—Activity of GSH-T was evident in all synovial fluid samples, which had similar values regardless of their classification as healthy or diseased (approx 10 nmol of conjugate/min/mg of protein; Figure 3). These values were significantly lower than the GSH-T activity of rat liver microsomal membranes (used as a positive control specimen) and close to the sensitivity limit of the technique used for these experiments. Therefore, we evaluated the GSH-T activity of a mixture of microsomes and synovial fluid. Results indicated that the GSH-T activity associated with the various categories of synovial fluid, although low, significantly (P < 0.001) differed from the values for the mixtures of synovial fluids with microsomes.

Effect of synovial fluid on microsomal GSH-T activity—Incubation of microsomes with various types of synovial fluid partially prevented the Fe³⁺-ascorbate—in-
duced inhibition of microsomal GSH-T activity (Figure 4). Residual GSH-T activities in the presence of Fe

ascorbate for microsomes alone and for microsomes preincubated with synovial fluid from healthy, chronically damaged joints and joints with vascularly congested synovial membranes were 59.3%, 68.7%, 74.6%, and 82.8%, respectively.

Effect of HA and CS on inhibition of microsomal GSH-T activity—Both HA and CS were able to prevent inhibition of GSH-T activity by Fe

ascorbate by approximately 40% (Figure 5).

Effect of synovial fluid, HA, and CS on oxidation of microsomal thiols—Treatment of microsomes with the Fe

ascorbate mixture reduced the microsomal thiol concentration by 61.8%, compared with the concentration in untreated membranes (45.3 ± 11.4 nmol of thiol/mg of protein). Preincubation of microsomes with synovial fluid from vascularly congested joints, HA, and CS reduced the damage from 61.8% to 45.8% (Figure 6). However, preincubation with synovial fluid from healthy and chronically damaged joints failed to prevent Fe

ascorbate–induced damage to microsomal thiols.

Discussion

Oxidative damage of biomolecules in joint tissues is related to an imbalance between oxidant and antioxidant processes. In the study reported here, synovial fluid from metacarpophalangeal joints of equine cadavers inhibited lipid peroxidation in rat liver microsomes as induced by Fe

ascorbate, regardless of the physiologic or pathological condition of the joint. The extent of this antioxidant capacity, however, was different for each type of sample, with synovial fluid from joints with vascularly congested synovial membranes having a lower capacity than the other types of synovial fluid. In synovial fluid from healthy and chronically damaged joints, this protection was independent of synovial fluid volume. It is possible that at this range, saturation of the protective effect had been reached for these 2 types of synovial fluid. Our findings were suggestive of a loss of the antioxidant ability of synovial fluid during an acute inflammatory process (joints with congested synovial membranes) of the equine metacarpophalangeal joint, which is overcome in chronic conditions, whatever the cause of the inflammation (chronically damaged joints).

In contrast to those findings, only synovial fluid samples from joints with congested synovial membranes prevented, although only partially, the microsomal thiol concentration induced by Fe

ascorbate. We decided to use the Fe

ascorbate system for inducing oxidative damage in rat liver microsomes because this is a widely accepted superoxide anion generator and has been routinely used by our research group to promote oxidative damage in biomolecules. In fact, in the present study, treatment of microsomes with Fe

ascorbate reduced microsomal GSH-T activity in our system by 40% in comparison with the amount of GSH-T activity in untreated membranes. In an earlier study, we found that oxidative damage to microsomal GSH-T leads to a gain of function in this enzyme. Joints with vascularly congested synovial membranes are affected by an acute inflammatory process; thus, synovial fluid samples from these joints are likely to have the highest degree of oxidative damage and, presumably, the highest GSH-T activity. Our results indicated that GSH-T activities from all synovial fluid samples were direct inhibitors. Two possibilities could account for these findings. First, minute changes in GSH-T activity might yield considerably different antioxidant capacities of synovial fluid, and these changes could not be detected in our study because of the pooled sample used (samples from 6 joints). Second, an antioxidant biomolecule other than GSH-T might undergo a gain of function during oxidative stress.

Preincubation of microsomes with HA and CS (or a combination of both) failed to have an antilipoperoxidative effect, even though these compounds existed in synovial fluid. As has been reported, the content of HA in the synovial fluid decreases in joint disease. Similar to HA, CS is another disaccharide found in synovial fluid. If the antioxidant effect of synovial fluid is attributable to the presence of such compounds, then preincubation with either or both compounds in combination should mimic the effect of preincubation with synovial fluid. For that reason, the antioxidant effect of HA and CS was also tested in identical conditions used for synovial fluid. Most likely, the high polarity of these compounds prevented them from interacting with microsomal membrane lipids, which might explain the lack of antilipoperoxidative effect detected. In contrast, HA and CS protected microsomal GSH-T activity and microsomal thiol oxidation from inhibition by use of Fe

ascorbate treatment. Again, the high polarity of HA and CS would allow them to directly interact, presumably, with thiol groups through hydrogen bridges, partially protecting these thiol groups from oxidation.

Results of the study reported here suggested that the antioxidant effect of synovial fluid from the metacarpophalangeal joints of horses is attributable, at least in part, to the effects of HA and CS in this fluid (protecting cysteinyl residues from oxidation) and the presence of GSH-T activity (antilipoperoxidative effect). This activity may be important for protecting against oxidative stress in horses with inflammatory conditions of the joints. In human joints, GSH-T activity in synovial fluid has also been detected. This activity can have several sources, including destruction and subsequent release of GSH-T by chondrocytes and synoviocytes in synovial fluid, necrosis of synovial membrane cells during inflammation and release of GSH-T, or protection of the joint through specific release of GSH-T by chondrocytes and synoviocytes in synovial fluid, necrosis of synovial membrane cells during inflammation and release of GSH-T. It has been reported that GSH-T is secreted into particular extracellular fluids such as plasma, CSF, and vitreous humor. Because GSH-T activity was not increased in the synovial fluid from joints with congested synovial membranes in our study, an increase of this activity during the inflammatory process is unlikely in horses. Therefore, the presence of GSH-T in equine synovial fluid may represent a native antioxidant defense rather than a response to joint injury. Potentiating this activity by pharmacological induction of GSH-T or direct infiltration of joints with GSH-T may be useful for preventing and treating joint damage in horses.
References


