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ORIGINAL ARTICLE

Antioxidant capacity and oxidative damage determination in synovial fluid of chronically damaged equine metacarpophalangeal joint

Alejandro Villasante · Oscar F. Araneda · Claus Behn · Marco Galleguillos · Hector Adarmes

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Abstract In order to determine oxidative stress in equine joints with degenerative processes, we analyzed synovial fluid (SF) antioxidant capacity and the concentration of oxidative damage biomarkers in healthy and chronically damaged metacarpophalangeal joints. SF samples were collected from joints of thirty 2–5 year-old crossbreed male equine, macroscopically classified at *post mortem* inspection and later histologically confirmed. The antioxidant capacity was determined measuring uric acid and the concentration of sulfhydryl groups and the total radical trapping antioxidant potential (TRAP). The oxidative damage was determined by assessing malondialdehyde (MDA) and carbonyl protein concentration. TRAP was significantly higher ($p<0.05$) in the group with chronic damage (CD). The sulfhydryl groups and concentration of uric acid did not show significant difference between the groups ($p>0.05$). Although carbonyl concentration did not show significant difference between groups, it was slightly higher in the group with CD ($p=0.05009$). Concentration of MDA did not show significant difference ($p>0.05$) between groups. The observed significant increase in TRAP in the group with CD could be related to the participation of components other than protein, sulfhydryl groups, or uric acid coming from degenerating joint tissues. These findings could be helpful for a better understanding of the oxidative stress role in equine joints with chronic degenerative process.

Keywords Synovial fluid · Oxidative stress · Free radicals · Antioxidant capacity

Introduction

Increasing evidence of cellular process signaling and pathologic cellular pathways being regulated by reactive oxygen species (ROS) and nitrogen oxidative species (NOS) has been

A. Villasante (✉) · M. Galleguillos · H. Adarmes
Department of Biological Animal Sciences, Faculty of Veterinary Medicine, University of Chile, Santa Rosa 11.735, Santiago, Chile
e-mail: vill0378@vandals.uidaho.edu

O. F. Araneda · C. Behn
ICBM, Faculty of Medicine, University of Chile, Santiago, Independencia 1027, Santiago, Chile

reported over the last few years (Orun et al. 2007; Virgili and Marino 2008; Emerling et al. 2009; Ma et al. 2009; Diehn et al. 2009). ROS are generated from cell oxygen metabolism and can be divided into free radicals (high reactive molecules), such as superoxide anion ($O_2^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$), and non radicals (low reactive molecules), such as hydrogen peroxide (H_2O_2), hipochlorous acid (HClO), and ozone (O_3) (Dimock et al. 2000; Diehn et al. 2009).

In order to protect tissues against oxidative damage caused by prooxidants, there are several lines of antioxidant defenses at the intra- and extracellular level (Finkel and Holbrook 2000; Sureda et al. 2004; Orun et al. 2007; Hanen et al. 2009; Venkatesh et al. 2009).

Living systems synthesize prooxidants as a consequence of their normal homeostasis (Gutiérrez 2002; Valdez et al. 2000). Nevertheless, the net increase of prooxidants is caused by an imbalance between the generation and neutralization rates, which lead to oxidative stress. This condition has been associated with the aging process of living systems. One of the premises of the "Free Radical" theory of aging states, that over time free radicals induce an unrepaired oxidative damage to biological macromolecules that get accumulate during life (Beckman and Ames 1998; Muller et al. 2007). Oxidative stress can also disrupt cell homeostasis and progressively the entire tissue, affecting the nature of its structure-function and leading to the genesis of pathologies such as osteoarthritis (Gutiérrez 2002; Henrotin et al. 2003). Osteoarthritis is the most prevalent joint disease in equines and humans, progressing in both species similarly (Frisbie et al. 2002; van der Harst et al. 2005; Frisbie et al. 2007).

Within the joint, trauma can cause an increase in prooxidants production, cited as the main osteoarthritic etiological factor (Kehrer 1993; Kidd et al. 2001). The post traumatic inflammatory episode leads to the activation of phagocytes by proimflammatory cytokines (IL-1, IL-6 and TNFα). This activation sets up the respiratory burst (NADH oxidase, superoxidodismutase and mieloperoxidase), which leads to the sequenced production of anion superoxide, hydrogen peroxide and hypochlorous acid (Henrotin et al. 2003; Murphy et al. 2008). The increased ROS and NOS production from joint cellular components causes the oxidative damage of the chondrocyte mitochondrial genome, contributing to the chondrodegenerative processes. This degenerative process disrupts mitochondrial respiration, promoting aging, cell death, functional failure, and degeneration of chondrocytes and other joint cellular components (Grishko et al. 2009). Also, oxidative damage can affect the cartilage homeostasis through the oxidation of joint molecular components that play a role in the integrity of the cartilage tissue such as collagen, proteoglycans and hyaluronan (Greenwald and Moy 1979; Monboisse et al. 1984; Bates et al. 1985; Henrotin et al. 2003). The Nitric oxide ($NO^{\cdot}$) produced by chondrocytes in osteoarthritic joints can inhibit the synthesis of proteoglycans and the chondrocyte response to insulin growth factor (IGF-1) (Sandell and Aigner 2001; Henrotin et al. 2003). Hence, the self repair capacity of the cartilage is decreased under oxidative stress because of the disturbance of both the chondrocyte's energetic metabolism and the capacity to synthesize matrix components. This causes apoptosis of chondrocytes and the loss of matrix cellular components, leading to the characteristic histological changes of the joint cartilage under degeneration process (Sandell and Aigner 2001; Yudoh et al. 2005; Grishko et al. 2009).

Increased production of ROS is involved in the pathogenesis of acute equine joint diseases (Dimock et al. 2000). From this background, it is likely that ROS also play a role in the pathology of non-inflammatory chronic equine joint. Therefore, we hypothesize that in equine joints with non-inflammatory chronic damage, antioxidant capacity should be decreased due to an accumulative exhaustion of the antioxidant defenses, causing higher levels of oxidative damage biomarkers and a decrease in the total antioxidant capacity.

The main goal of this study was to determine in the SF from both a healthy and a group of joints with non-inflammatory chronic damage the following: TRAP, uric acid, and sulfhydryl concentration as antioxidant potential parameters and MDA and protein carbonyl concentration as oxidative damage estimators.

Materials and methods

Animals and biological samples

Synovial fluid samples (4 ml) were collected from the metacarpophalangeal joints from a total of 30 crossbred male equines immediately after slaughter. The age range was 2–5 years old. Age was determined by denture chronology. A healthy group and a group with CD were established, both made up of 15 equines. The inclusion/exclusion criteria was similar to Adarmes et al. (2003), based upon a macroscopic classification done at the *post mortem* inspection. Healthy joints showed a mother of pearl coloration with a smooth cartilage surface (Fig. 1a), in addition to a non-congestive synovial membrane and yellow/transparent SF. The chronically damaged joints showed cartilage with a dull, grossly yellowed color and a pitted surface with remarkable rub lines (Fig. 1b), in addition to a non-congestive synovial membrane. We also discarded SF with blood traces due to its antioxidants, ionic metals (such as Fe^{+2} and Fe^{+3}), and free radical components, all of which could affect either the determination of the antioxidant potential or the concentration of oxidative stress biomarkers. To confirm the conformation of both groups, a cartilage sample of 5 mm^2 was taken to quantify the degeneration level of the articular cartilage using a cartilage degeneration index (CDI) similar to Brommer et al. 2003a and Brommer et al. 2003b. This procedure was performed for the entire joint to provide a general CDI value. To confirm the non- inflammatory conditions, SF samples (2 ml) were taken from each joint to perform a WBC count and differential quantification. WBC concentrations were determined using an automated cell counter. Smears of SF were examined cytologically to determine the differential WBC count. Both the CDI and the non-inflammatory criteria, allowed us to exclude joints with acute damage and to confirm the healthy group.

For the extraction of the SF the skin of the metacarpophalangeal joint area was removed, followed by an aseptic puncture at the synovial sacs level using a 10 ml sterilized syringe. Subsequently the syringe was kept in ice until treatment.

Sample treatment

Samples were centrifuged at 3,000×g for 15 minutes at 4°C. Then the supernatants were aliquoted in labeled tubes to be stored at −86ºC until analysis.

Protein determination

Protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin as the standard. Proteins were quantified spectrophotometrically at 750 nm.

Total antioxidant capacity determination

Synovial fluid antioxidant capacity was surveyed by the "Total trapping antioxidant potential" (TRAP) according to Lissi et al. (1992). This is a chemo-luminescence technique

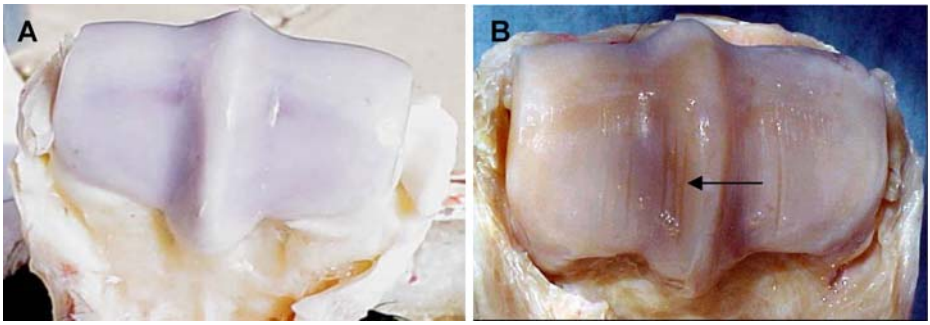

Fig. 1 Macroscopical view of both a healthy (**a**) and a chronically damaged (**b**) metacarpophalangeal cartilage. Arrow indicates rub lines of a cartilage pitted surface

that allows the non specific assessment of antioxidant molecules in the sample. The 2,2′-azo-bis (2, amidinopropane) was used as a thermolitic system to generate peroxil radicals. The results were expressed in mg/dl of uric acid equivalents and standardized by protein concentration.

Sulfhydryl groups determination

Reduced sulfhydryl groups were used in this study as antioxidant markers since they have been cited as both protein (ceruloplasmin and albumins) and peptide (glutathione) functional groups with antioxidant properties (Gutteridge 1986; Ojano-Dirain et al. 2005). We proceeded according to Hu (1994) using a Tris-EDTA solution and the Ellman's reagent (5, 5′-Dithio-bis -2-nitrobenzoic acid or DTNB). Sulfhydryl groups were quantified spectrophotometrically at 412 nm and later standardized by the protein concentration.

Uric acid determination

Uric acid concentration was surveyed as antioxidant estimator since it has been cited as an antioxidant molecule (Becker et al. 1989; Stienfelt et al. 2005). We used a Valtek Diagnostics commercial Kit (Santiago, Chile) with an enzymatic mixture of Uricasa-Peroxidasa as reagent. The uric acid was quantified spectrophotometrically at 520 nm.

Carbonyl proteins determination

Carbonyl proteins were used as protein oxidative damage estimator since they have been cited as protein oxidative biomarkers (Craig et al. 2007; Oikawa et al. 2009; Dalle-Donne et al. 2009 and Wong et al. 2009). We proceeded according to Reznick and Packer (1994), upon the use of 2, 4- dinitrophenylhydrazine (DNPH) as reagent. The long wave used to quantify the protein concentration was 280 nm; for the carbonyl concentration, it was 370 nm.

Malondialdehyde (MDA) determination

We used MDA as lipoperoxidation estimator due to it has been cited as a biomarker to be assessed in determining lipoperoxidation (Janero 1990; Meagher and Fitzgerald 2000; Moselhy and Demerdash 2004 and Koklu et al. 2007). We proceeded according to Cadenas

et al. (1996), using the TBARS assay. The MDA was quantified spectrophotometrically at 535 nm.

Statistic analysis

Each variable was analyzed with both a normality test (Kolmogorov-Smirnov test) and an equality of variance test (Levene's Test). All variables showed a normal distribution and equal variances. A T- test was applied for detection of significant differences between the means of both groups using a significance value of $p<0.05$. The results are expressed with the mean±S.D. Statistical analysis was carried out with Statistical Package for Social Sciences Software (SPSS 12.0 for Windows; SPSS, USA).

Results

The results for protein concentration, antioxidant estimators and oxidative damage estimators are summarized in Table 1. Protein concentration did not show a significant difference ($p=0.44$) between groups. For the healthy group, the protein concentration was 10.92±5.34 mg/ml; for the group with CD, it was 9.67±3.28 mg/ml. Regarding the antioxidant estimators, TRAP was significantly higher ($p=0.034$) in the group with CD. The total concentration of antioxidants for the healthy group was 0.68±0.40 μg/mg of protein; for the group with CD, it was 1.07±0.56 μg/mg of protein. Concentration of Sulfhydryl groups did not show a significant difference ($p=0.89$) between groups. For the healthy group, sulfhydryl group concentration was 5.96±3.15 nmoles/mg; for the group with CD, it was 5.95±2.98 nmoles/mg of protein. The uric acid concentration did not show a significant difference ($p=0.12$) between groups. For the healthy group, the uric acid concentration was 0.80±0.69 mg/dl; for the group with CD, it was 0.51±0.48 mg/dl. Regarding the oxidative damage estimators, the concentration of carbonyl proteins did not show a significant difference ($p=0.05009$) between groups. Nevertheless, the group with CD was remarkably close to significance. For the healthy group, the concentration of carbonyl proteins was 1.40±1.18 nmoles/mg of protein; for the group with CD, it was 2.55±1.82 nmoles /mg of protein. The MDA concentration was not significantly different ($p=0.469$) between groups. For the healthy group, it was 3.58±1.98 nmoles/ml; for the group with CD, it was 2.98±2.46 nmoles/ml.

Discussion

We have chosen the equine as a model to work with because degenerative forms of arthritis significantly affect the potential of the equine to perform successfully. Our model was non-inflammatory since the most prevalent of these joint pathologies, osteoarthritis, is classically considered an inherently non-inflammatory disorder. However recent debate has strongly questioned the non-inflammatory character of the disease. Even when there is no doubt regarding the changes that this disorder cause on cartilage and underlying bone in the later stages, there is less consensus about the role of an inflammatory condition in the initial phase of the disease (van der Harst et al. 2005).

Equine has also been used as a model to study human osteoarthritis, since it proceeds similarly in both species (Frisbie et al. 2002; Frisbie et al. 2007). Nevertheless, equine cannot be considered an absolute model for all type of human joint disorders because of the existence of interspecific differences. This is the case, for instance, of autoimmune joint

Table 1 Concentration of proteins, antioxidant parameters and oxidative damage parameters in the metacarpophalangeal joint synovial fluid

	Healthy group (n=15)	Chronic damage group (n=15)
Protein determination (mg/ml)	10.92±5.34[a]	9.67±3.28[a]
Antioxidant estimators		
TRAP (μg/mg of protein)	0.68±0.4[a]	1.07±0.56[b]
Sulfhydryl groups (nmoles/mg of protein)	5.96±3.15[a]	5.95±2.98[a]
Uric acid (mg/dl)	0.8±0.69[a]	0.51±0.48[a]
Oxidative damage estimators		
Carbonyl proteins (nmoles /mg of protein)	1.4±1.18[a]	2.55±1.82[a]
MDA (nmoles/ml)	3.58±1.98[a]	2.98±2.46[a]

Data are presented as mean±SD; n=number of equines. Values superscripted by the same letter are not significantly different ($P > 0.05$)

disorders, which generate intra-articular immune complexes characterized by auto antibodies anti-collagen type I and II of the cartilage. The role of these immune complexes in autoimmune joint disorders has been well established in human, rat and dog, but it is less clear for the case of the equine (Osborne et al. 1995).

Oxidative stress, which is caused by an imbalance between the antioxidant defenses and the prooxidant generation systems, has been associated with the pathogenesis of several inflammatory and non-inflammatory pathologies.

In joints with chronic damage such as osteoarthritis, cells and joint biomolecules become targets of free radicals that disrupt joint homeostasis, which in consequence induces the loss of the biomechanical function of the joint (Yudoh et al. 2005 and Grishko et al. 2009).

The aim of this study was to determine the total antioxidant capacity and level of oxidative damage in the SF of equine metacarpophalangeal joints with chronic damage. To perform the study, we established two equine metacarpophalangeal joint groups (healthy and with chronic damage).

To our knowledge, in previous studies TRAP has not been determined within the equine joint with degenerative conditions. Contrary to our hypothesis, TRAP level in the SF of the group with CD was significantly higher than in the healthy group. We suggest that this increase is a consequence of an accelerated turnover rate of matrix biocomponents with antioxidant properties (i.e., glycosaminoglycans, hyaluronic acid and proteoglycans) due to a higher activity of matrix degradative enzymes (metalloproteinases and aggrecanases) that has been described in osteoarthritic joints (Dimock et al. 2000; Sandell and Aigner 2001; Campo et al. 2003). The increased activity of theses enzymes is triggered by proinflamatory cytokines such as IL-1, 17, 18 and TNFα which inhibit the synthesis of tissue inhibitor of metalloproteinases (TIMPs) (Caron 1992; Sandell and Aigner 2001; Kidd et al. 2001; Goodrich and Nixon 2006; Grishko et al. 2009).

With the aim to evaluate the antioxidant role in the SF of some specific molecules, we quantified the concentration of reduced sulfhydryl groups and uric acid. These molecules were used as antioxidant biomarkers since both have been cited as effective antioxidant components in biological systems. There is evidence of higher oxidate sulfhydryl levels and lower reduced sulfhydryl levels in the plasma of human patients with rheumatoid arthritis (Hu 1994; Yardim-Akaydin et al. 2003; Giustarini et al. 2005). Uric acid is another antioxidant molecule which has been described both as a cardiovascular protector due to its

capacity to neutralize hydroxyl radicals (Becker et al. 1989) and as the most important bird plasmatic antioxidant (Stienfelt et al. 2005).

Nevertheless, our outcomes indicate that neither proteins, sulfhydryl groups, nor uric acid were the cause of the increased TRAP level within the group with CD.

Regarding oxidative damage estimators, although carbonyl concentration did not show a significant difference ($p=0.05009$) the carbonyl concentration within the group with CD tended to increase. This increase suggests that proteins were the target of oxidative damage, which was also detected by Dimock et al. (2000) where higher SF protein carbonyl concentration ($P<0.01$) was observed in the disease joints group. Previous studies that have done in other species, have also described an increase in the concentration of protein carbonyl in several tissues, as consequence of an oxidative damage of proteins (Wong et al. 2009; Dalle-Donne et al. 2009 and Oikawa et al. 2009).

Lipid peroxidation products are measured as an estimator of oxidative damage on lipids molecules. Malondialdehyde is one such product generated in tissues by free radical action. In our study MDA concentrations did not display significant difference between groups. We suggest to reasons for this lack of difference: (1) MDA is a highly soluble molecule that can diffuse rapidly from the joint to the bloodstream and (2) biomolecules such as GAGs and proteoglycans can reduce lipoperoxidation within the joint (Campo et al. 2003). GAGs and proteoglycans can be released from the cartilage matrix since an increase in the biosynthetic activity, as a trade off mechanism in order to repair the damaged cartilage and because of the turnover of matrix biocomponents mentioned before (Sandell and Aigner 2001; Campo et al. 2003).

Therefore, we suggest that the increase in the antioxidant capacity detected in the SF in equine joints under degenerative process could be due to a compensatory respond of chondrocytes to the cartilage chronic damage and because of the loss of matrix components with antioxidant properties in the SF.

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