Measurement of Metalloproteinase-3 levels in synovial fluid from horses as biomarker of joint disease - Medición de los niveles de Metalloproteinasa-3 en líquido sinovial como biomarcador de enfermedad articular

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Summary

Synovial fluid Metalloproteinase 3 (MMP-3) levels were measured in 27 horses, whose underwent arthroscopy of fetlock / tarsal joint for lameness diagnosis or removal of osteochondral fragments. The degree of cartilage damage in each joint was scored by a modified scale (Brommer et al., 2004) and correlated with clinical, radiological, arthroscopic findings as well as MMP-3 levels in synovial fluid from samples collected aseptically from each joint affected after induction of general anaesthesia prior to arthroscopy according to the law terms of ethics committee and animal welfare (2010/63/EU). MMP-3 levels, were analysed using a commercially available equine ELISA kit (No. E0101Eq of USCN Life sciences Inc). Obtained values were evaluated by non-parametric Mann-Whitney test to establish differences between joints, age and groups of severity cartilage damage. Furthermore, correlations were estimated by Spearman rank’s correlation. MMP-3 levels in synovial fluid were significantly higher in joints with severe cartilage lesions than those with healthier joints in mature horses (p=0,012). These findings suggest that MMP-3 levels in synovial fluid likely reflect an increase in cartilage metabolic activity as result of their remodelling and it may therefore be, a useful indicator of degeneration however, it requires deeper investigation in the horse.

Key words: Inflammatory biomarkers, cartilage damage, diagnosis, osteoarthritis, equine
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Resumen
Los niveles de Metaloproteinasa -3 (MMP-3) fueron medidos en el líquido sinovial de 27 caballos, sometidos a artroscopia de la articulación del menudillo/tarso para diagnóstico de cojeras o extirpación de fragmento osteocondral. El grado de daño del cartílago fue calificado en cada articulación mediante una escala modificada (Brommer et al., 2004) y fue correlacionado con los hallazgos clínicos, radiológicos, artroscópicos y los niveles de MMP-3 de las muestras de líquido sinovial colectadas asépticamente de cada una de las articulaciones afectadas, después de la inducción anestésica y antes de la artroscopia de acuerdo con los términos del comité de ética y bienestar animal (2010/63/UE). Los niveles de MMP-3 fueron analizados utilizando un kit de Elisa disponible comercialmente para equinos (No. E0101Eq of USCN Life sciences Inc). Los valores obtenidos se evaluaron mediante la prueba no paramétrica de Mann- Whitney, para establecer diferencias entre las articulaciones, edad, y grupos de severidad de daño del cartílago. Así mismo, se calcularon correlaciones mediante el coeficiente de correlación de Spearman. Los niveles de MMP-3 en el líquido sinovial fueron significativamente más altos en las articulaciones con lesiones graves del cartílago que aquellos con articulaciones más sanas en los caballos maduros (p=0,012). Estos hallazgos sugieren que los niveles de MMP-3 en el líquido sinovial probablemente reflejan un aumento en la actividad metabólica del cartílago como resultado de su remodelación, y por lo tanto pueden ser un indicador útil de su degeneración, sin embargo, esto requiere una investigación más profunda en el caballo.

Palabras clave: biomarcadores de inflamación, daño del cartiláge, diagnóstico, osteoartritis, equino.

INTRODUCTION
Equine osteoarthritis OA or degenerative joint disease accounts for approximately 60% of all cases of lameness in athletic and pleasure horses [McIlwraith et al., 2010]. Is characterized by variable degrees of articular cartilage degeneration, subchondral bone sclerosis, and marginal osteophyte formation [McIlwraith et al., 2010]. This common form of arthritis can occur in humans, horses, dogs and cats [Mobasheri & Henrotin, 2010], and its early diagnosis has become a major challenge, in both human like veterinary medicine [Zrimsek et al., 2007]. The traditional diagnosis is based on clinical, radiographic, and arthroscopic observations but it is often complicated. Since OA is a disease clinically silent for a long period, radiography changes are visible only in advanced stages, once the disease has become established [Van Weeren & Firth, 2008; McIlwraith, 2011; Chiaradia et al., 2012]. Therefore, a poor correlation with clinical signs and marked articular cartilage degeneration can be present despite normal radiographic appearance of the joint [Van den Boom et al., 2004]. Only arthroscopy, although more invasive,
allows direct visualization of cartilage and has become the gold standard for assessing cartilage lesions in vivo [Fuller et al., 2001; Brommer et al., 2004; Van den Boom et al., 2005]. Although, there are another diagnostic noninvasive or minimally invasive methods used for the evaluation of progressive joint disease (scintigraphy, magnetic resonance imaging MRI, computed tomography CT, etc.), which may give us useful information, there are still severe limitations in the detection of early articular cartilage damage [Brommer et al., 2004]. Recently, researchers have focused to identify molecules that can be useful as early markers and of great interest is the option that concentrations of these biomarkers can be analyzed through methods minimally invasive, using body fluids such as synovial fluid (SF), blood and urine [Frisbie et al., 2008]. Within them, several equine studies have investigated presence of MMP activities in naturally occurring joint disease. In such studies, increased synovial fluid MMP-3 [Brama et al., 2000], and MMP-2 and MMP-9 levels [Clegg et al., 1997; Frisbie et al., 2008] have been detected in OA joints suggesting a role for these enzymes in matrix degradation. MMP-3 is believed to play a significant role in cartilage matrix destruction because it functions to degrade a number of cartilage components and is critical for the activation of other MMPs [Fujita et al., 2006]. Furthermore, general MMP activity in joints with moderate to severe cartilage damage has been related to the severity of cartilage changes [Van den Boom et al., 2005]. It is therefore the aim of this study, to analyse levels of MMP-3 in equine SF to correlate them with cartilage lesion and clinical findings in horses with natural occurring joint disease and determine their usefulness in the diagnosis of cartilage damage.

1. MATERIALS AND METHODS

1.1 Animals and samples

Synovial fluid samples were obtained of 44 joints from twenty-seven horses undergoing arthroscopy at the University of Veterinary medicine Vienna and the equine clinic Bargteheide. The horses underwent arthroscopy due to OCD lesions, intra articular fractures or aseptic (traumatic) arthritis of fetlock (metacarpophalangeal) or hock joints (tarsocrural). A complete physical and lameness examination was performed on all cases. The median age of the horses was 4.8 years (range 1-13 years) and them were assigned to two groups: young (1 to 3 years) and mature (4-13 years). Those horses, who had received intra articular anaesthesia prior to arthroscopy were excluded from the study to avoid biased results due to synovial fluid dilution. Join surfaces were evaluated by arthroscopy, affected and intact joint surfaces were compared. All samples were performed under appropriate ethical guidelines (2010/63/EU). Synovial fluid samples were collected aseptically using plain collection tubes (VACUETTE®) by use of needle arthrocentesis (without lavage) into at the time of surgery, while the horses were under anaesthesia. Samples were centrifuged (gravitations for 10 minutes), decanted and stored at -80°C until them were assayed.
1.2 Radiographic and arthroscopic scores

Radiographic and arthroscopic images of each one of the injured horses were evaluated and then a score was assigned for each affected joint. Briefly, standard radiographic views of the affected joint were assessed to determine; joint space narrowing, subchondral bone changes (sclerosis and lucency), periarticular remodelling and/ or exostosis. For each radiographic variable were assigned a score from 0 to 3, with 0 indicating non diseased, 1: mild 2: moderate, and 3: severe changes. The maximal radiographic score was 12. Arthroscopy was performed following premedication and induction of general anaesthesia. Joints were examined visually by using a 30 Karl Storz, standard arthroscope, during continuous intra-articular infusion of acetated Ringer’s solution. Arthroscopic video recordings were reviewed, and an arthroscopic score was assigned by an orthopaedic surgeon (JE) according a modified scale used [Brommer et al., 2004]. Briefly, cartilage lesions were evaluated according to the type and size. By type of lesions were categorized from 0 to 4 as 0= normal cartilage, 1 = swelling and softening of the cartilage, 2 = superficial fibrillation, 3 = deep fibrillation down to subchondral bone, and 4 = exposure of subchondral bone. The size of these lesions was estimated as a percentage of the cartilage surface (0-100%) that could be seen. The score of cartilage damage for each joint was calculated using a percentage of lesion size multiplied and the category of cartilage lesion (% size of lesion x category lesion). Then these values were assigned on a scale points created by the author to create a score from 0 to 4, where score 0 = healthy cartilage, score 1: mildly affected; score 2 = moderately affected; score 3 = severely affected; score 4 = destruction of cartilage.

1.3 MMP-3 Measurements

A sandwich enzyme immunoassay was used to measure MMP-3 according to the manufacturer's instructions (No. E90101 Eq of USCN Life sciences Inc.). Briefly, microwells of microplate are precoated with an antibody specific to MMP-3. Standards (3.12, 6.25, 12.5, 25, 50, 100, 200 ng/ml) , serum and synovial samples were diluted 1:5, added into each well in duplicate and incubated during 2 hours at 37°C. Then, a biotin-conjugated antibody preparation specific for MMP-3 was added and again incubated. After washing, Avidin conjugated to Horseradish Peroxidase (HRP) was added into each well and following a further incubation period, the excess enzyme conjugated was removed by washing. Finally, TMB (3, 3’, 5, 5’-tetramethylbenzidine) substrate solution was added, which gives a blue coloration; the intensity of colour was proportional to the concentration of MMP-3 in the sample. The enzyme substrate reaction was terminated by addition of sulphuric acid solution and the colour changed to yellow, which was read at 450 nm. The minimum detectable dose of equine MMP-3 is typically less than 1.37 ng/ml according to the sensitivity reported by manufacture. The concentration of MMP-3 in the samples was determined by comparing the optical density (O.D.) of the samples to the standard curve and expressed in ng/ml.
1.4 Statistical analysis

Statistical evaluation was performed by the use of statistical software (SPSS 19.0 for Windows; Inc, Chicago). Obtained values of synovial fluid MMP-3 did not show a normal distribution. Therefore, differences of these factors between fetlock and TC joints, between age groups (1-3 and 4-13 years) and between groups of severity for cartilage damage were evaluated by use of the nonparametric Mann-Whitney test. Furthermore, correlations between MMP-3 in synovial fluid with clinical examination (lameness, swelling, and flexion test), radiographic score, and severity of cartilage changes were estimated by calculating Spearman rank’s correlation. A value of P <0.05 was considered statistically significant for all analyses.

2. RESULTS

2.1 Clinical examination

44 affected joints were identified from 27 horses, including 16 fetlocks and 28 TC joints of hind and forelimbs. Lameness was only observed in 12 horses in at least one limb (1-2/5). Swelling was evident in 27 joints from 18 horses. The pain flexion test (37 joints) from 23 horses, showed mild and moderate positive response in both joints but severe positive response was present in three TC joints. The most joints (38 joints) were diagnosed with osteochondritis OC, followed by aseptic arthritis (4 joints), and intra-articular fracture (2 joints).

2.2 Radiographic changes

In fetlock joint evaluation, mild to moderate changes were observed like as subchondral bone plate irregularities of the third proximal (10 joints), third distal at the MC/MTIII, pointed joint margins, mild subchondral bone sclerosis of P1 (7 joints), and mild joint space narrowing (2 joints). No changes were detected in 4 joints. Moreover, radiographic examination of tarsocrural joint revealed only mild changes in 15 joints (34,1%), such as periarticular remodelation (11 joints), lucencies (5 joints) and sclerosis of subchondral bone (1 joint). No changes were evident in 13 joints (29, 6%).

2.3 Arthroscopic changes

Score grade 0: Cartilage articular surface intact in approximately 100 %, was evident in 6 joints (4 fetlocks and 2 TC joints).

Score grade 1: Softening and swelling were observed affecting all joints with a percentage approximated between 10 - 60 %. Superficial fibrillation was observed in 2 joints (10%), deep fibrillation in 2 joints (10%) and exposure of subchondral bone in only one joint (5%). Total evaluated joints were 16 (8 fetlocks and 8 TC).
Score 2: Softening and swelling (25 %), superficial fibrillation (15 %), deep fibrillation (10-15 %) and exposure of subchondral bone (2 %) were observed and in 2 joints. Total evaluated joints were 8 (1 fetlock, 7 TC).

Score 3: Of these joints, 4 joints presented fibrillation superficial (30%), deep fibrillation (30%), exposure of subchondral bone (10%), and deep fibrillation (15%). Only one had deep fibrillation (50%) affecting its surface. Total evaluated joints were 7 (2 fetlocks and 5 TC). Softening and swelling (30-100%) were evident in the most joints (6).

Score 4: Normal articular surface was no visible. Softening and swelling (30-50%) were visualized in 2 joints. One of these joints was affected simultaneously with superficial (35 %) and deep (35 %) fibrillation, while the other, included deep fibrillation (50%) and exposure of subchondral bone (30%). In the remaining 4 joints, was noted with superficial fibrillation (50-100%) and deep fibrillation (20-50%) with exposure of subcondral bone (20%). Total evaluated joints were 6 (1 fetlock, 5 TC).

The most commonly affected sites for each joint were identified at level of dorsal surface of the condyles of the third metacarpal/metatarsal (MC/MT III) bone, followed by the aspect dorsoproximal of proximal phalanx (P1) in fetlock joint. Simultaneously in TC joint, at intermediate ridge of the distal tibia (IRDRT), followed also for lateral trochlea ridge of talus (LTRT), medial malleolus (MM) and medial trochlea ridge of talus (MTRT).

2.4 ELISA Measurements: MMP-3 measurement was evaluated in 44 synovial fluid samples but only was found in 32. These levels ranged between 3,6 ng/ml to 78,1 ng/ml (mean ±SD; 32,7 ± 26), as shown in figure 1.

![Figure 1. MMP-3 levels in synovial fluid](http://www.veterinaria.org/revistas/redvet/n101017/101709.pdf)
2.5 Differences and correlations:

2.5.1 Differences between fetlock and tarsocrural joints: There was a significant difference for swelling in tarsocrural joint compared with fetlock joint (p=0.005), such as radiographic score and cartilage score findings (p= 0.017) and (0.032 respectively) being tarsocrural joint more affected. No more significant differences were observed.

2.5.2 Differences between groups of age: A significant difference was viewed between synovial fluid levels of MMP-3 for both groups: young and mature horses being synovial fluid higher in mature horses than younger (p=0.022).

2.5.3 Differences between severity groups of cartilage score: A significant difference was observed between the groups of score 0 and 2 related to lameness (0.033) and swelling (0.018). Both groups of score 3 and 4 showed significant differences in the levels of MMP-3 in synovial fluid (0.039 and 0.012, respectively) compared with group score 0 (Figure 2).

![Figure 2](http://www.veterinaria.org/revistas/redvet/n101017/101709.png)

**Figure 2.** Levels of synovia fluid MMP-3 in the different degrees of cartilage score.

2.6 CORRELATIONS

2.6.1 Correlations (Spearman rank): Statistical analysis revealed strong correlations among MMP-3 levels in synovia fluid with cartilage score lesions (0.001). Additionally, there was also a strong correlation between lameness with swelling (0.001) and flexion test (0.008). In the same form, swelling was correlated, with flexion test (0.006) as well as with cartilage score (0.05). However, there were no significant correlations between other parameters.
Discussion

In a normal cartilage, an equilibrium exists between anabolic (chondrocyte synthetic activity) and catabolic (enzymatic matrix degradation) activities that maintains the structural and functional integrity of extracellular matrix (ECM) [Kidd et al., 2001; Goldring & Otero, 2011]. This degradation of ECM is effectuated by several proteinases, of which metalloproteinase are the most important. The MMP family is a group of zinc-dependent endopeptidases that are structurally and functionally related to OA process. The degradation of extracellular cartilage matrix by MMPs depends on an increase in the biologically active forms [Fujita et al., 2006]. When MMPs are activated, these proteolytic enzymes degrade the extracellular matrix (e.g. collagens and proteoglycans). In adult articular cartilage, metabolism tends to be extremely slow, whereas in young individuals, it will be maintained at a substantially higher level to allow for growth and remodeling [Brama et al., 1998].

Our results showed that mean levels of MMP-3 in synovial fluid was significantly higher in joints with cartilage damage in mature horses. It suggests that these changes related to OA may be a natural occurring age-related process in the horses and, it also would explain, an increase in the metabolic activity accompanying cartilage remodeling. Therefore, our results indicate that MMP-3 levels may be a useful tool in assessing the amount of damage in horses suspected of OA. MMP-3 or stromelysin is considered the most important proteinase responsible for cartilage degradation [Hegemann et al., 2002]. Since it degrades a number of cartilage components such as aggrecan, fibronectin, and collagens, and is critical for the activation of other MMPs [Fujita et al., 2006].

Several equine studies have investigated presence of MMPs in naturally occurring joint disease. In such studies, increased synovial fluid MMP-3 levels [Brama et al., 1998; Brama et al., 2000] have been detected in OA joints. However, only a few studies have investigated presence of equine synovial fluid MMPs related to cartilage damage [Jouglin et al., 2000; Van de Boom et al., 2005]. In this study, we found a significant difference in the levels of MMP-3 between joints without cartilage damage (score 0) and joints with severe cartilage damage (score 4). Levels of MMP-3 in synovial fluid tended to increase significantly with severity score of cartilage lesions, which could be a novel finding in horses and demonstrates a link between changes in articular cartilage.

On the other hand, although radiographic changes were more evident in fetlock than tarsocrural joint, oppositely, swelling, pain flexion test and cartilage lesions were more significant in tarsocural joint. As mentioned before, radiographic changes only can be detected once OA is completely established or when the appreciable changes of subchondral bone are greater to 30-40%. In this studio, cartilage damage with exposure of subchondral bone assessed by arthroscopy was not greater to 30%. Therefore, it is likely that the radiographic changes were not correlated with cartilage injury,
demonstrating once again that radiography is an insensitive method to detect cartilage lesions.

We have used the Société Française d’Arthroscopie (French Arthroscopic Society, SFA) grading system previously reported [Ayral et al., 1996; Brommer et al., 2004]. This scoring system has been used in human arthroscopy evaluating five categories and is widely applied and accepted in the diagnosis of joint changes in both, humans and horses, however, no validated scoring system exists today in equine arthroscopy [Fuller et al., 2001; Gangl et al., 2007]. An increase in the percentage of size of these lesions affecting a joint, suggests a chronic inflammation process, on the contrary, a small lesion suggests an acute process, maybe associated to trauma. Softening and swelling of cartilage visualized in a great number of joints, may suggests early changes in the articular cartilage by inflammation, taking into account that most of these horses had a lesser or greater degree of synovitis. A major presence of superficial and deep fibrillation in combination with exposure of subchondral bone in the cartilage score groups 3 and 4 were noted. All these lesions in a joint are consistent with early changes in OA and they are indicative of a chronic inflammatory process. Moreover, a complicating factor in chronic disorders with an intermittent clinical course, such as OA, is that the activity of the disease process may vary at the time. Therefore, marker levels may not to accurate reflect the overall disease stage [Van Weeren et al., 2005].

One limitation of our study was the interpretation of reliable values from diseased horses without the use of healthy controls. In addition, the evaluation of horses with spontaneous joint disease derives in other limitations such as, preexistent disease conditions, additional affected joint, previous treatments and exercise. Further comparisons between healthy horses, different joints and other joint diseases require to be investigated.

In the future, monitoring of several biomarkers and evaluating repeated measurements, might improve the interpretation of results. Additionally, the support of more advanced imaging tools such ultrasonography, MRI, CT, when these are available, may be useful for understanding and diagnosis of early cartilage damage.

Conclusions

Because OA is common in horses, future equine studies should focus on to get a better and earlier diagnosis that may provide new means of evaluating. Consequently, learning about the physiology and cartilage repair mechanism could be critical for understanding OA process and achieve an optimal treatment. At present this study concludes, a positive correlation between SF MMP-3 with the grade of cartilage damage and these levels were significantly different according to age, which could be associated significantly to chronic age-changes. Therefore, MMP-3 may be a potential candidate for assessment of cartilage damage however; it needs more research in the horse.
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