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Prospective Biochemical Markers for Osteoarthritis in Horses

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Abstract: A central feature of the osteoarthritis (OA) involves erosive destruction of the articular cartilage extracellular matrix (ECM) on the surfaces of joints. The resultant loss of joint function makes studies on mechanisms underlying ECM degradation critical for treatment of the disease and prevention of disability. In the present study synovial and serum samples were collected from normal (n=20), Early OA (n=10) and Late OA (n=20) adult male horses attended from surgery clinic, faculty of veterinary medicine Cairo University. The activities of different types of matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs) in synovial fluid were estimated by Substrate and Reverse zymography respectively and the expression of MMP-13 was determined by Western blot. The level of procollagen type IIC-propeptide (PIICP) in serum and synovial fluid was measured by ELISA. The activity of pro and active form of MMP-9 and level of PIICP was significantly increased in early stage of OA. While the activity of both proMMP-2 and active MMP-13 as well as level of expression of the later one were increased significantly in Late OA. In addition, the activity of TIMP-1 was significantly decreased in different stages of OA. Such biomarkers could be used to predict and monitor osteoarthritis pathogenesis.

Key words: ECM · MMps · TIMPs · PIICP · ELISA

INTRODUCTION

Equine species consider as the most important species in Egypt economy especially horses and donkeys. The ability of the horses to do these hard works refers to healthy fore and hind limbs especially joints. Horse's joints give the skeleton flexibility and allow him to walk, trot, run, jump and move his head and neck.

Osteoarthritis (OA) is degenerative joint disease, common in athletic horses, occurring as a result of trauma or excessive use of the joint during performance and training. OA is characterised by destruction and loss of articular cartilage, poor cartilage repair, changes to the subchondral bone plate, synovitis and capsulitis [1].

Early diagnosis of OA is a major problem, in both human and veterinary medicine. Visible lesions of articular cartilage can be detected using arthroscopy, which is a successful but invasive method. The use of markers from serum or synovial fluid give a chance to diagnosis OA at an earlier stage, monitor pathological changes of the disease and the effects of treatment [2].

Chondrocytes play an important role in both the physiological metabolism in development and growth of cartilage matrix and in the pathological degradation in joints resulting from sever mechanical strain [3]. The degradative activity of chondrocytes is greatly stimulated by cytokines such as interleukin-1 beta (IL-1β) or tumour necrosis factor alpha (TNF-α), are the predominant mediators of inflammation [4]. These cytokines are capable of inducing Matrix metalloproteinases (MMPs) and reducing synthesis of tissue inhibitor metalloproteinases (TIMP) [5].

MMPs are responsible for cleavage of extracellular matrix molecules of cartilage [6]. They are responsible for degradation of different substrates, according to their specific substrates; MMPs are classified into six groups [7].

These groups include Collagenases (MMP-1, MMP-8 and MMP-13), Gelatinases (MMP-2 and MMP-9), Stromelysins (MMP-3 and MMP-10), Matrilysins (MMP-7 and MMP-26), Membrane-typematrix metalloproteinases (MMP-14, MMP-15, MMP-16,

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MMP-17,MMP-24 and MMP-25) and others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28) [8, 9].

In normal tissues, there are a balance between MMPs and TIMP by binding non-covalently in a 1:1 ratio with high affinity to both pro and active MMP catalytic sites resulting in either prevention or loss of MMPs activity [10]. During OA disease, disruption of the balance between MMPs and TIMPs is occurred by increasing the concentration of MMPs [11].

In OA development changes occur not only ~~in~~ the degradation of matrix molecules, but also in the rate of their synthesis. Type II collagen is synthesized as procollagen containing three identical α chains: a central triple helical domain is flanked by non-helical N-terminal and C-terminal propeptides. These propeptides are removed by specific proteinases in a form of Procollagen type II C-propeptide (PIICP) before the incorporation of type II collagen into collagen fibrils [12].

The present study investigated the molecular and biochemical events of OA for monitoring and predicting consequences of the disease for prospective prognosis and treatment.

MATERIAL AND METHODS

Horses: Fifty adult male normal and osteoarthritic horses of 6-10 years age were used in the present study. The animals were examined clinically and by x-rays for diagnosis and grading of osteoarthritis at surgery clinic - surgery department, Faculty of veterinary medicine -Cairo University.

Serum Samples: Blood samples were collected from the jagular vien for serum preparation.

Synovial Fluid Samples: Synovial fluid samples were collected from healthy and affected joints (Distal interphalangeal or carpal or tarsal or fetlock joints). The samples were centrifuged at 10.000 x*g* for 20 min at + 4°C. The Supernatants were aliquoted and stored at - 20°C [13]. Synovial samples were diluted before use in a ratio of 1:5 by PBS pH (7) to reduce their viscosity [14].

Substrate Zymographic Analysis

***Gelatin zymography*:** The activity of MMP-2 and MMP-9 were detected in gelatin zymography [15]. Briefly, synovial fluid samples were separated by SDS-PAGE on 7.5% (W/V) gels, containing 1 mg/ml gelatin under non reducing conditions. Then washed twice for 15 minutes each in 2.5% (V/V) Triton X-100 and then incubated in development buffer (0.05 *M* Tris-HCl pH 8.8, 5 m*M* CaCl2, 0.02% NaN3) for overnight incubation. Gels were stained with 0.1% coomassie brilliant blue R250 in methanol:acetic acid:water (4.5:1:4.5, v:v:v).

Casein Zymography: Stromelysins (MMP-3,10), MMP-1, MMP-7, MMP-12 and MMP-13 activities were detected on 12% Casein zymography by incorporation of 50mg/ml (W/V) casein in the gel [16].

Heparin-Enhanced Substrate Zymography: The activity of MMP-1 and MMP-13 were detected in 7.5% gelatin zymogrphy by loading 10µl heparin (0.3mg /ml in 1x sample buffer without SDS) to the lanes of regular and zymogram gels within 20-30 minutes after electrophoresis began [17].

Western Blotting: Detection of MMP-13 by Western blotting was carried out [18]. Briefly, synovial fluid were separated by 10% SDS-PAGE followed by electro-blotting by tank transfer technique to polyvinylidene fluoride (PVDF). The primary antibodies used was MMP-13 polyclonal antibody.HRP-conjugate Goat anti-Rabbit IgG secondary antibody were diluted 1:1000 before use, these being developed using the DAB Substrate chromogen Kit.

Reverse Zymographical Analysis: TIMPs were estimated by reverse zymography [15].15% regular and reverse zymogram containing 1 mg/ml gelatin, BHK conditioned media with 1% SDS were used.

Zymogram gels and membrane of western blot were digitally scanned in true color.The images and membrane were then processed using commercially available software (GelQuant.NET), after conserved to gray scale.

Measurement of Piicp Concentration: The concentration of PIICP in the synovial fluid and serum was measured by commercial ELISA kit (*WKEA MED SUPPLIES CORP*).

Statistical Analysis: Results are presented as mean ±SD. A statistical analysis of different groups was performed using ANOVA. $P<0.05$ was considered significant for all analysis [19].

RESULTS

Substrate Zymography: The gelatinolytic activity of ProMMP-9 and ProMMP-2 were represented in all synovial samples. The ProMMP-9 were significantly increased and displayed as active form (86KDa) in early

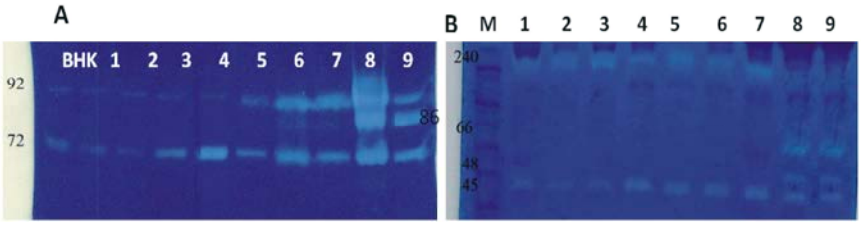

Fig. 1: Substrate zymography. (A) 7.5% Gelatin Zymogram of synovial fluid. The baby hamster kidney (BHK)marker is shown at the left with molecular weight(92 &72 KDa).the pro-MMP-9 & pro-MMP-2 with genolytic activity are shown at 92 and 72KDa respectively. Lanes 1-3 correspond to Normal synovial fluid, Lanes 4-7correspond to Late OA and Lanes 8,9 correspond to Early OA.Gelatinolytic activity at 86KDa present in Early OA.(B) 12% casein Zymogram of synovial fluid: Lane M. Blue Eye Prestained protein marker(10-245 KDa) is shown at the left. 1st band at 240KDa and 2nd band at 45KDa. Lanes 1-3 correspond to Normal synovial fluid, Lanes 4-7correspond to Late OA and Lanes 8,9 correspond to Early OA.66 and 48 KDa represent caseinolytic activity in Early OA.

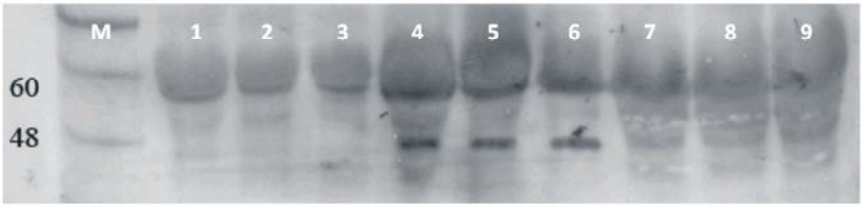

Fig. 2: Western blot. Lane M.Blue Eye Prestained protein marker(10-245 KDa) is shown at the left. ProMMP-13 are represented in all synovial fluid samples(60KDa) & active MMP-13 are represent in osteoarthritic samples(48KDa). Lanes 1-3 correspond to Normal synovial fluid, Lanes4-6 correspond to Late OA and Lanes 7-9 correspond to Early OA.

Fig. 3: 15% Reverse Zymogram of synovial fluid: Lane M.Blue Eye Prestained protein marker(10-245 KDa) is shown at the right. TIMP-1 are represented in all synovial fluid samples. Lanes 1-3 correspond to Normal synovial fluid, Lanes 4-7 correspond to Late OA and Lanes 8,9 correspond to Early OA.

OA(Figure 1A), while proMMP-2 were significantly increased in late OA (Table 1). Caseinolytic activity of MMP-complex (240 KDa) and MMP-1 or MMP-3 (45 KDa) are expressed in all synovial samples (Figure 1B). MMP- complex significantly decreased in orthopaedic synovial fluid samples, while MMP activity at 45 KDa significantly increased in late OA (Table 1). Early OA synovial fluid displayed bands at 66KDa and 48KDa (Figure1B) that represent MMP-2 and MMP-13 respectively.

Table 1: Activities of MMPs in substrate zymography.

Animal groups	Normal	Early OA	Late OA
Gelatin zymography			
ProMMP-9	$0.39^{b}\pm0.02$	$0.65^{a}\pm0.04$	$0.43^{b}\pm0.04$
ProMMP-2	$0.47^{b}\pm0.04$	$0.48^{b}\pm0.03$	$0.67^{a}\pm0.02$
Casein zymography			
1st band	$0.61^{a}\pm0.02$	$0.33^{c}\pm0.00$	$0.42^{b}\pm0.03$
2nd band	$0.46^{b}\pm0.04$	$0.41^{b}\pm0.01$	$0.66^{a}\pm0.03$
Heparin-enhanced zymography	$0.52^{b}\pm0.060$	$0.54^{b}\pm0.003$	$0.74^{a}\pm0.040$

MMP-13 (48KDa) activity in heparin enhanced zymography was displayed in all synovial samples, with significantly increased in late OA (Table 1).

Western Blotting: The expression of ProMMP-13 (60KDa) was detected by western blot in all samples (Fig. 2), with increasing in Late OA. While the active form (48KDa) was not detected in normal synovial fluid and its expression is elevated with OA especially in Late OA (Table2).

Reverse Zymography: The bands of TIMP-1 (30KDa) were represented in all samples (Fig. 3), with significantly decrease in osteoarthritic samples especially at Late OA (Table3).

Table 2: Expression of MMP-13 by Western blotting

Treatments	MMP-13 (Pro-form)	MMP-13 (active-form)
Normal	$0.37^{b}\pm0.042$	
Early OA	$0.48^{ab}\pm0.025$	$0.21^{b}\pm0.008$
Late OA	$0.53^{a}\pm0.042$	$0.79^{a}\pm0.008$

Table 3: Activities of TIMPs in Reverse Zymography

Treatments	Reverse zymography
Normal	$0.521^{a}\pm0.021$
Early OA	$0.225^{b}\pm0.028$
Late OA	$0.168^{b}\pm0.034$

Table 4: Concentration of PIICP in serum(S) and in synovial fluid (SF)

Treatments	PIICPS (µg/L)	PIICPSF (µg/L)
Normal	$0.69^{b}\pm0.001$	$0.69^{b}\pm0.001$
Early OA	$5.99^{a}\pm1.542$	$19.93^{a}\pm3.084$
Late OA	$0.68^{b}\pm0.001$	$0.69^{b}\pm0.000$

Concentration of PIICP: The concentrations of PIICP µg/L in serum and synovial fluid was significantly increased in serum and synovial fluid of Early OA (Table 4).

DISCUSSION

Osteoarthritis is a disease with multifactorial etiologies and affects all adjacent tissues in joints. Morphological, biochemical, structural and biomechanical changes of the extracellular matrix and cells are observed in cases of OA which lead to the degeneration of articular cartilage. The degeneration is characterized by softening, fibrillation, ulceration and loss of cartilage tissues [6].

In OA cartilage, the balance between anabolic and catabolic equilibrium of chondrocytes metabolism is controlled by pro-inflammatory cytokines via activation of catabolic pathway by inducing MMPs and reducing TIMPs [5].

MMPs is one of the predominant proteinases belong to a zinc-dependent proteases family, they are responsible for the characteristic matrix degradation in OA [20].

MMPs are secreted as inactive zymogens then activated by losing 8 to 10 kDa N-terminal propeptide, this step is proposed as a fundamental step in articular cartilage degradation [21]. The result in the present study was matching this hypothesis as gelatinolytic activity of proforms MMP-2 and MMP-9 (Table1) are significantly increased in different stages of osteoarthritis. In this study the significant increase of proMMP-9 activity at Early OA, result in MMP-9 active form (86KDa) was displayed after its activation (Fig.1A).

These results were in agreement with Zrimsek *et al*. [13] who stated that, Gelatin zymograms of synovial fluid from normal and osteoarthritic joints show proenzymes and active forms of MMP-2 and MMP-9 in osteoarthritc joints. Elevated gelatinolytic activity of MMP-2 and MMP-9 have also been observed in horses joints [22], in canine osteoarthritic and rheumatoid synovial fluid samples [23], In cows with septic and aseptic arthritis [24]. In Addition, significant increase in activity of proMMP-2 of Late OA (Fig. 1A) supporting the previous observation of Aigner *et al*. [25], who reported that MMP-2 was up-regulated in late stage of osteoarthritic Knee cartilage.

In healthy, resting tissues some MMPs such as MMP-7, MMP-19, MMP-24, MMP-25 and MMP-26 are expressed at low levels and many of the other, such as MMP-1, MMP-3, MMP-9, MMP-10, MMP-11 and MMP-13 are marginally expressed [26].

In the current study the caseinolytic activity of these MMP-complex (240KDa) were significantly decreased (Fig.1B) in orthopaedic synovial fluid in accordance with Patricia *et al*. [7], in which MMP complex of different molecular weight at 240KDa and 130KDa were represented.

In early stages of OA, there is an imbalance of MMP regulation towards enhanced activity under the effect of pro-inflammatory cytokines, resulting in a loss of matrix, in particular at the cartilage surface [27]. The appearance of proteinolytic activity of MMPs at 48 and 66KDa in Early OA samples (Fig. 1B) were expected to be MMP-13 and MMP-2 respectively to ensure that MMPs regulated toward enhanced activity. MMP-13 (Collagenase 3) is a potent proteolytic enzyme that plays a major role in the degradation of type II collagen the main collagen component of cartilage [1].While, gelatin the denatured form of collagen easily to be digested by gelatinases mainly MMP-2 [7].

The caseinolytic activity at 45KDa in the present study (fig.1A) represented either MMP-1 or MMP-3 that responsible for degradation of Col I, II, III, VII, VIII, X, gelatin and Col II, IV, IX, X, XI, gelatin respectively [8]. These casinolytic activity were significantly increased in Late OA samples(Table1) in accordance with Blaine [28] who recorded that the bands presents at 45-50 KDa indicated the presence of MMP-3 in canine stifles of osteoarthritc synovial fluid. The increment of MMP-1 activity have also been observed in synovial fluid of osteoarthritc horses [29], confirming the potential of MMP-1 to serve as a biochemical marker for joint disease.

MMP-13 is one of collagenases that responsible for degradation of collagen II and other substrates such as Col I,III,IV,IX,X,XIV,gelatin [8]. In the current study, Proteinolytic activity of MMP-13(48 KDa) (Table1) was elevated in late OA samples in comparison to others. These data in the converses of Aigner *et al*. [25], who showed that, MMP-13 was up-regulated in late stage of osteoarthritic Knee cartilage. Many different in vivo studies have shown the importance of MMP-13 in osteoarthritis. Administration of specific MMP-13 inhibitors to animal models of osteoarthritis has shown a significant reduction in the severity of OA [30-32].

In the current study, the increment of MMP-13 activity in Late OA samples was confirmed through western blotting (Fig. 2) by using specific antibody (anti-MMP-13).These results supporting the previous observation of Ryu *et al*. [14] and Lynne *et al*. [33] who reported that MMP-13 expression was elevated in osteoarthritic synovial fluid.

The observed increment of different types of MMPs in the current study indicates the role of MMPs in the progression of osteoarthritis. In early stages and during OA progression there is an imbalance of MMP regulation towards enhanced activity [20]. The overexpression of matrix-degrading enzymes resulting in a loss of matrix, in particular at the cartilage surface. Subsequently, there is an increase of water content in the matrix, a decrease of proteoglycans and cleavage of collagen type II. Due to damages in the structure of the collagen network, there is also a loss of tensile strength in the cartilage and, thereby, altered biomechanical properties of cartilage with a reduced stiffness [27]. The progressive structural changes in articular cartilage followed by subchondral bone thickening, deformation of the articular surface, osteophyte formation. Advanced progression result in synovial intima cell hyperplasia and synovial fibrosis in subchondral bone, the synovial membrane and the synovial fluid respectively [34].

Once MMPs are released, tissue inhibitors of matrix metalloproteinases (TIMPs) regulate their proteolytic and biologic activity by covalent binding and blocking MMP activity in a ratio 1:1 favoring a balance of ECM homeostasis [35]. In the current study the activity of TIMPs that represented at 30 KDa (Fig.3) indicate the present of TIMP-1 [36].TIMP-1 activity was reduced in the osteoarthritic synovial fluid in comparison to the normal. The decrement of TIMP-1 concentration have also been observed in osteoarthritic human joints [37], in posttraumatic osteoarthritis, primary osteoarthritis, or pyrophosphate arthritis [38, 39]. The disturbance in the MMP-TIMP balance is shifted towards MMP result in an excess of activated MMPs leading to cartilage degradation [1]. Chondrocytes try to compensate cartilage degradation during osteoarthritis by enhanced proliferation and synthesis of collagen type II (COL2AI) that not able to sustain mechanical and environmental factors [40]. The release of noncollagenous carboxypeptide extension of type II procollagen molecules in the synovial fluid; procollagen II- propeptide(PIICP) used as an index of the synthesis and degradation of type II collagen due to its relatively short half life(14-16 hours) [41].

In the current study the concentration of PIICP was significantly increased (Table 4) in Early OA in both serum and synovial fluid. These results have also been observed in human synovial fluid [4, 12].

In early osteoarthritis, increased PIICP levels show accelerated synthesis and degradation of matrix collagen and indicate progressive cartilage loss accompanied by joint space narrowing result in minimal change on plain radiographs [12].

In Conclusion, MMP-9 and PIICP could be used as predictors for early stage of OA and both proMMP-2, active MMP-13 and decrement of TIMP-1 activity were used as predictors for Late OA. These biomarkers are useful in discriminating between different stages of OA and monitor OA Pathogenesis.

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