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14-3-3 η Protein is Detectable in Blood Serum and Fetlock Joint Synovial Fluid of High Performance Horses

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Abstract

Objective: To investigate whether 14-3-3 eta protein is detectable in synovial fluid and blood serum of high performance horses and to study whether any significant correlation between this and matrix metalloproteinase 1 and 3 in horse synovial fluid.
Design: Clinical study.
Animals: Eleven standard breed horses (seven high performances and four controls).
Procedures: Blood serum and fetlock synovial fluid of seven high performance and four control horses were subjected to western blot for detection of 14-3-3 eta, gama, matrix metalloproteinase 1 and 3 proteins.
Results: High level of 14-3-3 eta protein was detected in synovial fluid of high performance horses compare to those of control ($P<0.05$). 14-3-3 gama protein was detectable in synovial fluid and significant differences was found between high performance and control horses ($P<0.05$). The level of matrix metalloproteinase 1 and 3 were significantly increased in samples of high performance horses relative to of that control ($p<0.05$). The results showed that 14-3-3 eta protein was significantly increased in serum samples of high performance horses relative to of that control ($P<0.05$).
Conclusions and Clinical Relevance: 14-3-3 eta isoform can be readily detected in synovial fluid and blood serum of high performance horses and presence of this protein has significant effect on the levels of matrix metalloproteinase 1 and 3 in synovial fluid.
As the high performance horses are always prone to joint diseases, this protein might be use as an early diagnostic tool in some joint inflammatory conditions.

Key words: horse, 14-3-3 eta, matrix metalloproteinase, joint, arthritis.

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Introduction

Joint diseases are the most important cause of lameness and diminution of athletic function and wastage in high performance horses.[1,2] Repeated traumatic injury, joint instability, infection and osteochondrosis are the most important causes.[2] Joint problems should be timely diagnosed and treated, if not an irreversible degeneration process of the cartilage is produced and the result is osteoarthritis (OA).[2] Between equine skeleton joints, fore fetlock has the largest number of site-specific traumatic and degenerative lesions.[3] The susceptibility to injury of the fetlock joint is probably related to large range of motion and transmittance of full body weight to the ground during one phase of the stride.[3]

Articular cartilage is an anural, avascular, alymphatic hyaline cartilage and characterized by abundant extracellular matrix (ECM). OA is the result of imbalance of some degradiating peptides and remodeling of ECM components within the joint.[4] In horses with excessive exercise load a net catabolic response by chondrocytes and fibroblast-like synoviocytes (FLS) characterized by increased expression of matrix metalloproteinase 1 (MMP), MMP-3, MMP-9, interleukin 1 β, tumor necrosis factor α and cycloxygenase-2 which can affect ECM.[5]

MMPs are a family of structurally related zinc-binding endopeptidases and participate in the normal physiology of connective tissue during development, morphogenesis, bone remodeling and wound healing.[6] There are over 20 MMPs [7,8] and are known to play a vital role in cleavage and degradation collagens and aggrecans of ECM.[9,10] MMPs especiallyMMP-1, MMP-3 and MMP-13 have an integral role in connective tissue turnover and they regulate collagen degradation.[11,12] Though the exact mechanism is not completely understood, in normal articular cartilage the level of MMPs is tightly regulated to basal levels for matrix turnover and remodeling,[13] and is accomplished by tissue inhibitors of metalloproteinase (TIMPs).[14] In OA this balance is lost resulting cartilage degradation and failure.[14] An imbalance in the activation of MMPs and inhibition can be a key event in shifting the MMP cascade from physiologic to pathologic conditions.[15] MMPs can accumulate in the tissues and fluid and play a prominent action in tendon, ligament and articular cartilage degradation.[15,16] The concentration of MMPs has been shown to be increased in cartilage, synovial membrane and synovial fluid of patient with arthritis/osteoarthritis.[17,18]

The 14-3-3 η protein is a member of abundant family of acidic dimeric molecules that have a wide range of functions. They are known to be involved in numerous cellular events, including regulation of the cell cycle, cell growth, differentiation and apoptosis.[19] They are composed of seven isoforms in mammals (β, γ, ε, η, σ, τ,ξ). Since the discovery of the first 14-3-3 protein in 1967, the member of this family have been repeatedly rediscovered based on their new biological activities, primarily in signal transduction pathways.[20] Although these proteins are primarily intracellular, there have been some reports indicating the presence of these proteins extracellulary.[21] This family proteins influence divers biological activities by regulating the subcellular localization of target proteins[19]. Recently, releasable form of 14-3-3 σ was found to have a potent MMP-1 stimulatory effect on dermal fibroblasts.[22] This finding was the first indication of a relevant extracellular biological function for this important family of proteins. In another study, high levels of two specific isoforms of 14-3-3 proteins (η and γ) in synovial fluid of human patient with joint inflammation have been shown.[23] These findings suggest that the presence of these proteins in sera and synovial fluid can be used as biomarkers in inflamed joints.

It is generally accepted that marked articular cartilage degeneration can be present despite normal radiographic appearance of the joint.[24] Therefore, biochemical markers of early

articular cartilage degeneration would provide clinicians with a useful tool to assess the current status of the cartilage.
In the present study, we asked whether 14-3-3 η proteins is detectable in synovial fluid and blood serum from high performance horses. The second question was to determine whether this protein play any role in joint damage process at the same horses.

Materials and Methods

Horse and experimental set-up

Fetlock joint synovial fluid and blood serum were collected from seven high performance standard breed horses. The age of horses was between 6 to 8 years old. Control samples were collected from four horses using only for pleasure riding without any inflammatory joint history or any other abnormalities based on medical records. The age of control horses were between 4 to 5 years old. After informed consent synovial fluid and blood serum were collected using a syringe gage number 18. Synovial fluid samples and blood serums were clarified by centrifugation at 1500 xg for 15 min and stored in aliquots at -80ºC for subsequent analysis. Samples were analyzed for 14-3-3 η, γ, MMP-1 and MMP-3 proteins using western blot. The University of British Columbia Ethic committee has approved this study.

Antibodies

Antibodies raised in rabbit against human 14-3-3 η, and γ were generously provided by Dr. Aitken (School of Biomedical and Clinical Sciences, University of Edinburgh, Scotland) and monoclonal anti-human MMP-1 and MMP-3 antibodies were purchased from R&D systems (Minneapolis, MN). Horseradish peroxidase conjugated secondary antibodies against mouse and rabbit IgG were obtained from Bio Rad Lab. (Hercules, CA).
To verify if anti-human 14-3-3 η, γ, MMP-1 and MMP-3 antibodies were able to detect these proteins in horses, we tested three horse synovial fluids along with three different human samples. The human samples were: keratinocyte lysates as positive control for 14-3-3 proteins; human dermal fibroblast lysate as positive control for MMP-1 protein and human fibroblasts conditioned medium as positive control for MMP-3 protein. Each experiment was done in triplicates using three different cell strains.

Western blot analysis

For western blot analysis, 5µl of either synovial fluid or blood serum from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% (wt/vol) acrylamide gel for MMPs and 12% (wt/vol) acrylamide gel for 14-3-3 proteins. Separated proteins were electrotransfered onto PVDF membranes (Millipore, Bedford, MA, USA). Nonspecific proteins on membranes were blocked in 5% skim milk powder in phosphate buffered saline (PBS) with 0.05% Tween20 overnight at 4ºC. Immunoblotting was performed using rabbit anti-14-3-3 η, γ (1:1000 dilution), MMP-1 and MMP-3 (1:250 dilution) monoclonal antibodies. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,500 dilution). Immunoreactive proteins were then visualized using western blotting luminal reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Ponceau-S staining was used for all membranes to ensure that loading control was even (images not shown).

Statistics

The levels of 14-3-3 and MMP proteins in test and control samples were quantified using densitometry. Statistical analysis was performed using the SPSS 11 program for Windows (SPSS Inc. Chicago IL, USA). The means of the groups were compared using student t-test. Differences were considered statistically significant when $P<0.05$.

Results

Verification of human antibodies to detect horse 14-3-3 and MMP proteins

In order to detect the presence of horse 14-3-3 and MMP proteins using human antibodies, three different horse synovial fluid samples (randomly chosen) loaded on a SDS-PAGE along with three different strains of human keratinocyte lysates, human fibroblasts or human fibroblast conditioned media samples (Fig. 1). Using western blot analysis we were able to detect the presence of all four proteins tested (14-3-3 η, γ, MMP-1 and MMP-3). This result showed that human 14-3-3 and MMP antibodies are able to detect horse 14-3-3 and MMP proteins as well and they can be used for the subsequent experiments.

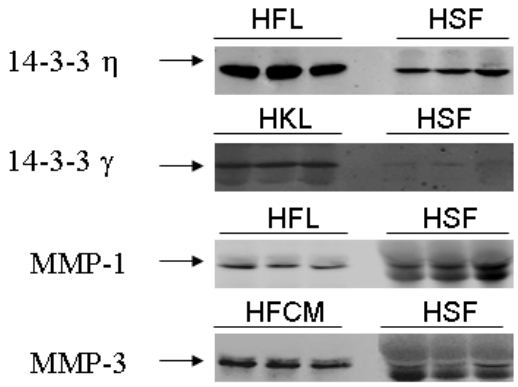

Figure 1. Human antibodies detect horse 14-3-3 and MMP proteins. Three randomly chosen synovial fluid of horses were used to verify that human antibodies are able to detect 14-3-3 and MMP proteins. Human keratinocyte lysates were used as positive control for 14-3-3 η and γ, human fibroblasts lysates were used as positive control for MMP-1 and human fibroblast conditioned media were used as positive control for MMP-3.
HFL; Human Fibroblast Lysate, HSF; Horse Synovial fluid, HKL; Human Keratinocyte Lysate, HFY; Human Fibroblast Lysate, HFCM; Human Fibroblast Condition Media.

Presence of 14-3-3 η and γ in synovial fluid

To examine whether there were differences in the presence of 14-3-3 η and γ protein in synovial fluid of high performance horses, 5µl of synovial fluid from control samples and from high performance horses were loaded on a SDS-PAGE (Fig. 2). The results showed that 14-3-3 η was present only in synovial fluid samples from high performance horses compared to those of controls (n=7 and n= 4 respectively, P<0.05) (Fig. 2A). 14-3-3 γprotein was significantly increased in synovial fluid samples of high performance horses compared to those of controls (n=7 and n= 4 respectively, P<0.05) (Fig. 2B).

Presence of MMP-1 and MMP-3 in synovial fluid

Due to previous studies [23, 25] showing that some 14-3-3 isoforms stimulate MMP-1 and MMP-3 expressions, the presence of these proteins were also analyzed using western blotting. To examine this, 5µl of synovial fluid from control samples and from high performance horses were loaded on a SDS-PAGE (Fig. 3). The results showed that MMP-1 was significantly increased in samples of high performance horses relative to of that control (n=7 and n= 4 respectively, P<0.05) (Fig. 3A). Very similar results were observed with respect to MMP-3, indications a significant increase in expression of MMP-3 in samples of high performance horses compared to those of controls (n=7 and n= 4 respectively, P<0.05) (Fig. 3B).

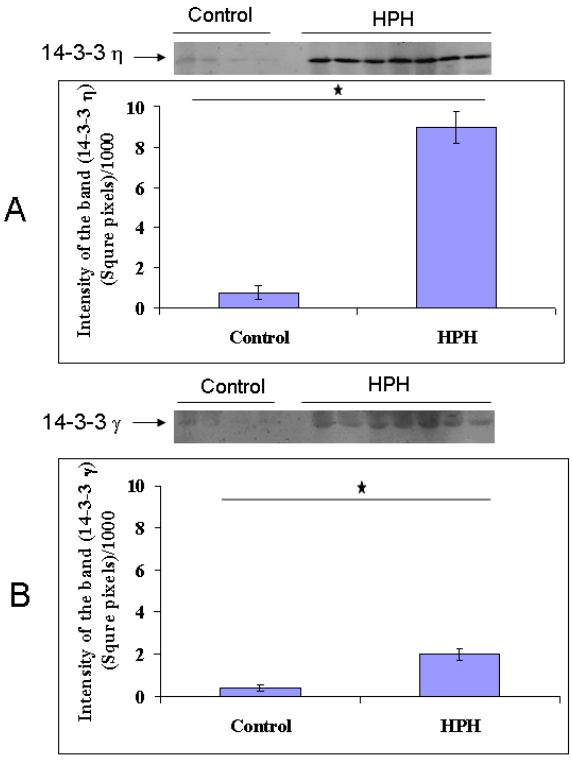

Figure 2. Presence of 14-3-3 η and γ in synovial fluid of high performance horses. The intensity of the bands for 14-3-3 η (Figure 2A) and 14-3-3 γ (Figure 2B) were quantified by densitometry.
HPH; High Performance Horses

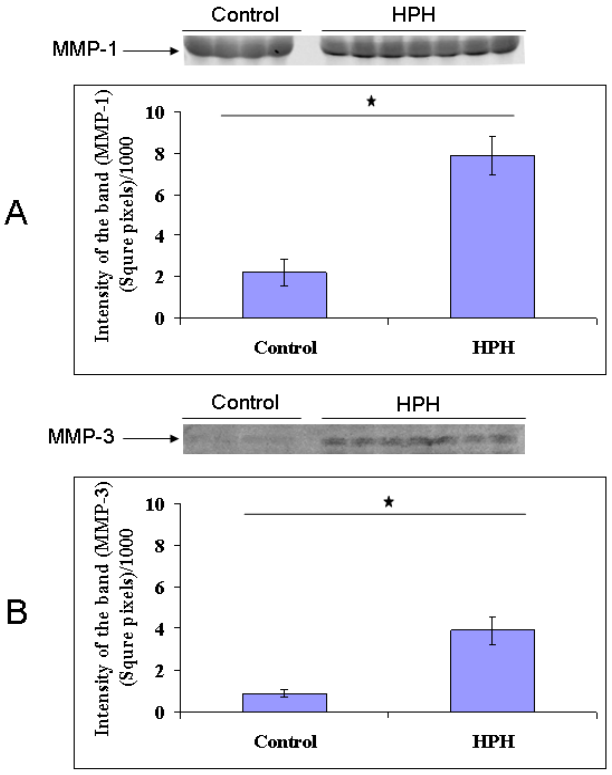

Figure 3. Presence of MMP-1 and MMP-3 in synovial fluid of high performance horses. The intensity of the bands for MMP-1 (Figure 3A) and MMP-3 (Figure 3B) were quantified by densitometry.
HPH; High Performance Horses

Detection level of 14-3-3η in serum samples of high performance horses

Due to the fact that 14-3-3 η protein might be used as a joint damage biomarker in either synovial fluid or serum sample, we wanted to see whether the level of 14-3-3 η protein in synovial fluid reflects to that in serum from the same horses. To achieve this, serum sample from high performance horses and those of control were subjected to western blot. The results showed that 14-3-3 η protein was significantly increased in serum samples of high performance horses relative to of that control (n=7 and n= 4 respectively, $P<0.05$) (Fig. 4).

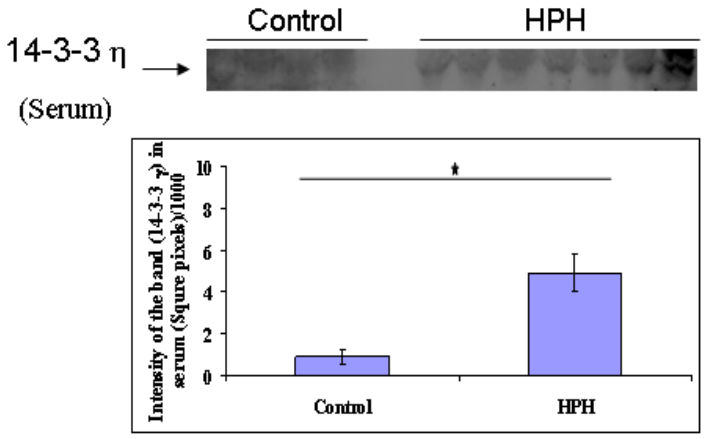

Figure 4. Detection of 14-3-3η in serum samples of high performance horses. The intensity of the bands for 14-3-3 η was quantified by densitometry. HPH; High Performance Horses

Discussion

Osteoarthritis is a common and biggest problems affecting the horse industry and too much cost regarding treatment, loss of use and prolonged disability.[26] The main object of the present study was to detect 14-3-3 η protein as a potent MMP-1 and MMP-3 stimulator in synovial fluid of high performance horses. Our previous results in human and dog with joint inflammation, demonstrated that levels of these proteins had significant correlation with the level of 14-3-3 η in synovial fluid.[23] Early diagnosis of OA is a major problem in human and veterinary medicine. As the high performance horses are prone to joint damage, we asked question,whether14-3-3 η in synovial fluid has any effect to develop joint problem in horses. We also wanted to see whether this protein could be detectable in blood serum of the same horses.

Like previous study in dog, we conducted some experiments to confirm that antibodies against human 14-3-3 and MMP proteins are able to detect these proteins in horse. As shown in Fig.1, the size of 14-3-3 and MMP bands detected in horse samples was identical to these of human. These finding indicate that human antibodies can be used to address question raised in this study.

Articular cartilage is a dynamic organ in the body. Any change in the composition of this organ either due to increased degradation or decreased production will change the mechanical

properties of cartilage.[27] During arthritis or OA, joint tissue promotes to express IL-1 and TNF-α which up-regulate the gene of some degradative enzymes especially MMP proteins.[28,29,30] In the other words OA is a complex interactive, degradative and repair process which involves cartilage, bone and synovium and is characterized by progressive and irreversible degradation of articular cartilage ECM.[31, 32] Most part of this complex interactive is mediated by MMPs. Some 14-3-3 protein isoforms (β, σ and η), have potent MMP-1 and MMP-3 stimulatory effect in fibroblasts.[23, 25] Based on the results from human and dog, increasing level of MMP-1 and MMP-3 in synovial fluid of high performance horse might be due to 14-3-3 η protein.

Present study is supporting previous results showing high levels of 14-3-3 η and γ isoforms in synovial fluid from human and dog with joint inflammation. However, it is the first study showing high level of 14-3-3 η in synovial fluid and blood serum of high performance horses. 14-3-3(s) are primarily intracellular proteins, but under certain pathological conditions these proteins can be found in tissue fluid.[33,34] Present studies showed high level of 14-3-3 η protein is detectable in synovial fluid of high performance horses. Unlike to the study in human and dog we have not found high level of 14-3-3 γ isoform at the same synovial fluid. The mechanism by which these proteins are released into synovial fluid is not completely understood. However, it is known that keratinocytes released 14-3-3 proteins via exosomes.[34, 21] It is not clear why synovial fluid of high performance horses contain such high level of only 14-3-3 η but not other isoforms such as γ.[23, 35] It is possible that this reflects differential expression of 14-3-3 isoforms by cells within the synovium.

One of the major step in etiopathogenesis of OA is breakdown of the ECM by proteolytic enzymes like MMPs.[36,37] The MMPs stimulatory effects of the 14-3-3 η isoform might induce FLS and chondrocytes to produce high level of these enzymes, [38] especially MMP-1 and MMP-3 which can damage ECM.

Present study demonstrated that 14-3-3 η isoform can be readily detected in synovial fluid and blood serum of high performance horses. As the high performance horses are always prone to joint diseases, this protein might be use as an early diagnostic tool in some joint inflammatory conditions. This protein might also be used as one of the predisposing factor for joint damage in high performance horses.

Acknowledgements

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چکیده

رد یابی پروتئین ۳-۳-۱۴ اتا در مایع مفصل قلمی بند انگشتی و سرم خون اسب های با عملکرد ورزشی بالا

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هدف- تحقیق حاضر به جهت مشخص کردن اینکه آیا ایزوفرم اتا پروتئین ۳-۳-۱۴ در مایع مفصلی و سرم خون اسبان ورزشی قابل رد یابی می باشد انجام شده است. همچنین در این تحقیق تاثیر این پروتئین در فعالیت آنزیمهای ماتریکس متالوپروتئیناز ۱ و ۳ در مایع مفصلی نیز بررسی شده است.

طرح- مطالعه درمانگاهی.

حیوانات- یازده راس اسب استاندارد برد شامل ۷ اسب ورزشی با عملکرد بالا و ۴ اسب گروه کنترل.

روش کار: مایع مفصلی و سرم خون از تمام اسبان اخذ شد و با استفاده از روش وسترن بلات پروتیئن ۳-۳-۱۴ اتا و گاما و ماتریکس متالوپروتئیناز های ۱ و۳ اندازه گیری شدند.

نتایج- مقادیر بسیار بالائی از پروتئین ۳-۳-۱۴ اتا در مایع مفصلی اسبهای ورزشی یافت شد که بطور معنی داری نسبت به گروه کنترل بالا بود ($P<0.05$). ایزوفرم گاما پروتئین ۳-۳-۱۴ نیز به میزان معنی داری در اسبان ورزشی نسبت به گروه کنترل بالا بود ($P<0.05$). میزان فعالیت آنزیمهای ماتریکس متالوپروتئیناز های ۱ و ۳ نیز در اسبان ورزشی به میزان معنی داری نسبت به گروه کنترل بالا بود ($P<0.05$). نتایج تحقیق حاضر نشان داد ایزوفرم اتا در سرم خون اسبان ورزشی قابل رد یابی بوده و بطور کاملا معنی دار نسبت به گروه کنترل بیشتر است ($P<0.05$).

نتیجه گیری- تحقیق حاضر نشان داد پروتئین ۳-۳-۱۴ اتا در سرم خون و مایع مفصلی اسبان قابل رد یابی بوده و افزایش این پروتئین تاثیر معنی داری در افزایش فعالیت آنزیمهای ماتریکس متالوپروتئیناز ۱ و ۳ در مایع مفصلی دارد. چون اسبان ورزشی همواره مستعد بیماری های مفاصل هستند این پروتئین می تواند بعنوان یکی از مارکر های تشخیص زود هنگام التهاب و بیماری مفاصل در اسب باشد.

کلید واژه ها- اسب، ۳-۳-۱۴ اتا، ماتریکس متالوپروتئیناز، مفصل، آرتریت.