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SOME NEW IMMUNOLOGICAL TESTS USED FOR EARLY DETECTION OF FOALS ARTHRITIS

By

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ABSTRACT

New immunological bioactive parameters are needed for the early detection of arthritis in foals. A total number of 90 serum and synovial fluid samples were collected from clinically normal foals (20), suspected foals (43) and foals showing signs of clinical arthritis (27) for the measurement of haptoglobin (Hp), serum amyloid A(SAA) and Cyclooxygenase-2 (COX2) concentrations .The level of Synovial fluid Hp recorded a significant elevation ($P<0.05$) in foals showing signs of clinical arthritis. Meanwhile its elevation wasn't significant in the suspected foals. At the same time, the serum Hp levels did not record a significant elevation for both groups. A significant increase in the levels of serum and synovial fluid SAA concentration for both suspected foals and foals showing signs of clinical arthritis. The levels of COX2 displayed a significant increase in serum and synovial fluid of both suspected foals and foals showing signs of clinical arthritis. Additionally, a strong positive correlation between COX2 concentration in synovial fluid and its concentration in blood at. 0.985 On the similar ground a positive correlation at 0.983 was found between SAA concentration in synovial fluid and its concentration in blood. It was concluded thatCOX2 and SAA have a potential usefulness as inflammatory markers in horses, showing joint inflammation, when their concentrations are measured either in synovial fluid or in blood serum.

INTRODUCTION

Horses are an important part of many people's lives and a big industry in many regions. In training and competition they may risk inflammation of joints (arthritis). An early discovery of inflammation may exclude the use of drugs and a shortened period of rest. **(Hanfried *et al.*, 2012)**. Arthritis is an unwelcome diagnosis for any horse; it is one of the most common conditions that affect performance of horses. It is progressive and permanent deterioration of articular cartilage, the specific type of cartilage that lines the ends of bones where they come together to form a joint It can occur in horses of any age but is more

commonly found in older horses. In fact, it is believed to be responsible for up to 60% of all lameness. The joints most often affected by arthritis include the knee, fetlock, coffin, hock, and pastern (where it is often referred to as "ringbone"). The key to keeping arthritis under control is early detection and quick action to decrease damaging inflammation. **(Meszoly and Joanne, 2010).** Arthritis horses is also called Degenerative Joint Disease (DJD),including osteoarthritis, remains a leading cause of lameness and decreased quality of life among horses. Methods currently used to assess the overall health of a joint include physical and lameness exams and radiology. So far, there are no specific tests that can be performed on synovial fluid to facilitate a diagnosis of joint inflammation and disease **(Kentucky Equine Research Staff, 2016)**. The immediate detection of an inflammatory response and the monitoring of its clinical course are primary challenges for veterinary medicine. The search for early inflammation markers has therefore been an important focus to the veterinary medical research. Special attention has been on the identification of biochemical parameters that have the specificity and sensitivity to both signal the presence and evaluate the intensity of an inflammatory response. Acute Phase Proteins (APP) is a group of biological markers that have a direct response to an acute inflammation and a fast decrease in concentrations upon removal of inflammation. **(Prato *et al.*, 2011).** Haptoglobin is a so called positive acute phase protein, i.e. it increases when the inflammatory system is stimulated. As examples an increased equine serum concentrations of haptoglobin can be seen in e.g.: non-infectious arthritis. **(Carolyn and Belgrave, 2014).** Serum amyloid A (SAA) is the major protein in horses and presents the following characteristics: present at very low or undetectable levels in the serum of healthy animals but increase rapidly from 10 to 1000 times during the acute phase response (APR). This level of response is related to the size of the damaged tissue and can be expressed in a wide dynamic range and decrease rapidly in response to treatment. **(Casella *et al.*, 2012).** The prostaglandin G/H synthase enzymes, commonly termed COX-1 and COX-2, differ markedly in their responses to regulatory stimuli and their tissue expression patterns. COX-1 is the dominant source of "housekeeping" prostaglandins, whereas COX-2 synthesizes prostaglandins of relevance to pain, inflammation, and mitogenesis. Prostaglandin H synthase (PGHS) two isoforms, (COX-1 and COX-2), catalyze the oxygenation of arachidonic acid (AA) to PGH2, a key intermediate in the biosynthesis of all prostanoids. The discovery of COX-2 15 years ago infused new life and vigorous research into the prostaglandin field and yielded significant new insights into the basis of inflammation,

pain, and fever. **(William *et al.*, 2001) and Rimon *et al.*, 2010).** The goal of the current study is to provide updated information regarding these biomarkers (haptoglobin (Hp),serum amyloid A (SAA) and Cyclooxygenase-2 (COX2) by using a commonly available automated assay.

MATERIAL AND METHODS

Experimental design:

(90) Foals up to one year old were separated into three groups based on the signs. The first group consisted of (20) foals showing no symptoms and considered as clinically normal foals. The second group comprised of (43) suspected foals, that were showing early warning signs, such as changes in behavior or performance, changes in the way that, the horse moves, as shortening of stride, hollowing of the back, or raising of the head, Unwillingness to perform tasks that came easy in the past, Stiffness that goes away as the horse warms up, and also warmth or pain in the area of the joint. And the third group consisted of (27) foals showing signs of clinical arthritis such as swelling around the joint or lameness, as shown in Fig.(1). Foals were excluded from the study if there were clinical evidence of concurrent diseases.

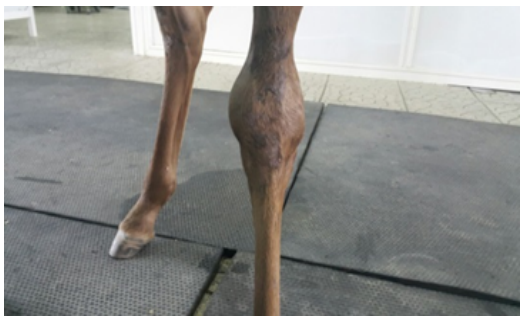


Fig. (1): A foul showing swelling in his knee joint

Sampling:

After clinical examination of the animals, blood samples were collected via jugular vein puncture, then the blood samples were allowed to clot by leaving it undisturbed at room temperature for 15-30 minutes, and the clot was removed by centrifugation at 1,000-2,000 xg for 10 minutes. Synovial fluid samples were collected as described by **(McIlwraith *et al.*, 2001)** using sterile needles and syringes. All sites were prepared aseptically. Synovial fluid was transferred to plain vacutainer tubes; excess negative pressure on the syringe was avoided to prevent haemorrhage. Fig. (2)

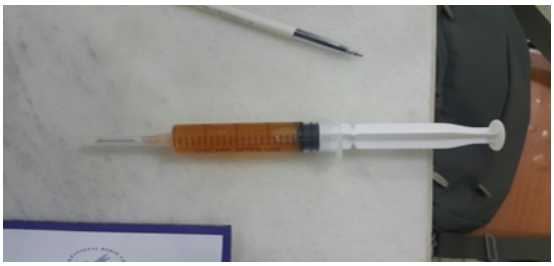
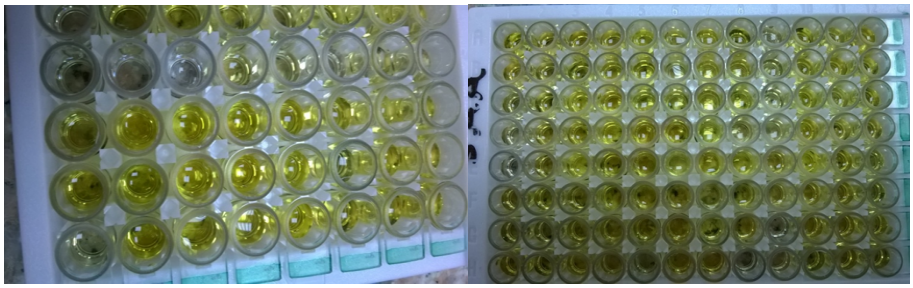


Fig. (2): Synovial fluid sample.

Determination of haptoglobin and serum amyloid a concentrations:

The samples under this study were subjected to measurement of Hp and SAA using sandwich ELISA. Ready coated anti-horse 96-well microtiter ELISA plates were applied (Sunredbio Co., Shangahi, China). The procedures were followed according to the instructions provided with the kits; Fig. (3 and 4).



A **B**

Fig. (3 A and b): showing the results of Hp (A) and SAA determination (B).

Determination of COX2 concentration:

It was carried out according to **Doré (2010).**

Statistical analysis:

The results were analysed statistically according to **Petrie and Watson (1999)** for determination of means, standard deviation and correlation.

RESULTS

Table (1): Determination of Haptoglobin (Hp) concentration (mg/dl) in blood serum and synovial fluid samples.

Type of sample	Clinically normal foals N (20)	suspected foals N(43)	Foals showing signs of clinical arthritis N (27)
Serum	0.10 ^{A} ± 0.01	0.11 ^{A} ± 0.01	0.14 ^{A} ± 0.02
Synovial fluid	0.28 ^{A}± 0.08	0.37^{AB} ± 0.02	0.51^{B}*± 0.09

*** Significantly different from control at P<0.05**

In comparison with the control group in (Table1), the level of Synovial fluid Hp recorded a significant elevation (P<0.05) in foals showing signs of clinical arthritis. Meanwhile its elevation wasn't significant in the suspected foals. At the same time, the serum Hp levels did not record a significant elevation for both groups.

Table (2): Determination of SAA concentration (mg/L) in serum and synovial fluid samples.

Type of sample	Clinically normal foals N (20)	suspected foals N(43)	Foals showing signs of clinical arthritis N (27)
Serum	1.83 ^{A}± 0.18	2.81^{B*} ± 0.12	3.62^{c*} ± 0.2
Synovial fluid	6.8 ^{A}± 0.88	44.22 ^{B*} ± 0.88	75.3 ^{c*}± 1.48

*** Significantly different from control at P<0.05.**

(Table 2) showed a significant increase in the levels of serum and synovial fluid SAA concentration for both suspected foals and foals showing signs of clinical arthritis than the control.

Table (3): Determination of Cox2 (U/L) in serum and synovial fluid samples.

Type of sample	Clinically normal foals N (20)	suspected foals N(43)	Foals showing signs of clinical arthritis N (27)
Serum	33.58 ^{A} ± 1.32	63.11 ^{B*} ± 1.40	92.44 ^{c*}± 2.14
Synovial fluid	20.38 ^{A} ± 1.1	33.18 ^{B*} ± 1.44	42.1 ^{c*}± 1.18

*** Significantly different from control at P<0.05.**

The levels of COX2 displayed a significant increase in serum and synovial fluid of both suspected foals and foals showing signs of clinical arthritis than the control', as shown in (Table 3).

Table(4): Correlation between Hp, SAAandCOX2 levels in Serum and Synovial fluid samples.

Correlations						
Studied parameters	Serum Hp	Synovial fluid Hp	Serum SAA	Synovial fluid SAA	Serum COX2	Synovial fluid COX2
Serum Hp		0.762**	0.735**	0.752**	0.692**	0.721**
Synovial fluid Hp			0.973**	0.978**	0.969**	0.926**
Serum SAA				0.983**	0.941**	0.963**
Synovial fluid SAA					0.981**	0.941**
Serum COX2						0.985**
Synovial fluid COX2						

**** Correlation is significant at the 0.01 level (2-tailed).**

(Table 4) illustrated a strong positive correlation between COX2 concentration in synovial fluid and its concentration in serum at 0.985. On the similar ground a positive correlation at 0.983 was found between SAA concentration in synovial fluid and its concentration in serum.

DISCUSSION

In veterinary medicine, horses are considered to be both pets and athletes; unsoundness due to inflammatory joint disease or joint infection can be career limiting or life ending for a horse. Thus, an area of clinical significance for veterinarians is the diagnosis and treatment of joint infections, meanwhile diagnosis of septic arthritis early in the disease process allows for immediate treatment, **(Ludwig, 2016).** Following treatment of septic arthritis, 56 to 81% of horses return to their original function, **(Schneider *et al.,* 1992) and Wright *et al.,* 2003)** therefore rapid diagnosis is critical for early treatment and a better prognosis **(Gibson *et al.,* 1989)**. Successful treatment of septic arthritis involves several goals: prompt and accurateрecognition of the condition, thorough diagnostic examinations, complete elimination of infection, timely resolution of inflammation and pain, and a speedy return to function. **(Morton, 2005).** Here in this study a trial to investigate rapid, accurate, and applicable methods for the early and rapid detection of equine arthritis was achieved to make it easier to eliminate the causes with the minimal losses as well as preventing joint problems and arthritis in horses. The acute phase response is a complex systemic early-defense system activated by trauma, infection, stress, neoplasia, and inflammation. Although nonspecific, it serves as a core of the innate immune response involving physical and molecular barriers and responses that serve to prevent infection, clear potential pathogens, initiate inflammatory processes, and contribute to resolution and the healing process. Acute phase proteins, an integral part of the acute phase response, have been a focus of many applications in human diagnostic medicine and recently have been identified in common animal species. Potential applications to diagnosis, prognosis, assessment of animal health, and laboratory animal welfare are readily apparent.**(Carolyn *et al.,*2009).** Equine haptoglobin (Hp) is a positive acute phase reactant, the levels of which increase significantly in horses in response to inflammation, tissue injury or disease. The present study showed that, the levels of synovial fluid Hp recorded a significant elevation ($P<0.05$) in foals showing signs of clinical arthritis. Meanwhile its elevation wasn't significant in the suspected foals. At the same time, the serum Hp levels did not record a significant elevation for both groups. These findings were supported by **(Barrachinaa *et al.,* 2016)** who reported that haptoglobin was a potential useful marker of joint inflammation in

horses. Moreover, **(Rosenkranz *et al.*, 2010)** found that levels of Hp correlate with clinical disease activity and that, the presence of Hp in the inflamed tissue suggested the role Hp plays in the progression and pathology of the disease and can also be used as a biomarker of disease activity. Regarding the synovial fluid Hp levels, it was evident in this study that they recorded a significant elevation for groups, suspected foals and foals showing signs of clinical arthritis. On the same ground, **(Carolyn and Belgrave, 2014)** measured serum and synovial haptoglobinpre - and post-arthritis induction, they found that haptoglobin increase in horses is significant in both serum and synovial fluid, but this increase was higher in synovial fluid than in serum. Every few years a new buzzword begins circulating in the horse health industry. And recently, a biomarker called SAA has become such a word, garnering attention from the equine veterinary community for its ability to indicate inflammation **(Lu, 2014).** Serum amyloid A (SAA) is one of the major acute-phase proteins predominantly produced by the liver **(Moutsopoulos and Madianos, 2006)**. The production of acute-phase SAA (A-SAA) is stimulated by pro inflammatory cytokines, such as interleukin-6 (IL-6), Il-1, tumor necrosis factor (TNF), interferon-γ, and transforming growth factor- (TGF-) **(Migita *et al.*, 2011)** .These proteins have several roles, including the transport of cholesterol to the liver for secretion into the bile, the recruitment of immune cells to inflammatory sites, and the induction of enzymes that degrade extracellular matrix. **(Zhang, 2005).** SAA also acts as a cytokine, influencing cell adhesion, migration, proliferation and aggregation **(Targońska-Stepniak, 2010)**.The current study examined the SAA concentration in blood serum and synovial fluid samples. The results showed a significant increase in the levels of serum and synovial fluid SAA concentration for both suspected foals and foals showing signs of clinical arthritis. These results seems to be in agreement with **(Jacobsen *et al.*, 2006) and (Nakamura, 2011)**, who studied acute phase protein expression for synovial fluid and serum samples and found high SAA levels in animals with suspected bacterial infection, infectious arthritis, and other high inflammation joint problems and that, the circulating concentration of SAA protein is increased by 1000-fold within 24 to 48 h following infection/inflammation from a basal level of 5-8 μg/mL.Lately, **Katy (2013)** stated that SAA is the major protein in horses and presents the following characteristics: they are present at very low or undetectable levels in the serum of healthy animals but increase rapidly from 10 to 1000 times during the acute phase response (APR). This level of response is related to the size of the damaged tissue and can be expressed in a wide dynamic range and decrease rapidly in response to

treatment, although not totally reduced in the absence of recovery. The present results (Table 2) showed that, measurements of SAA should make a significant contribution to diagnosis and management of arthritis in horses due to its significant elevation in the synovial fluid samples of both suspected foals and foals showing signs of clinical arthritis, Similarly, **(Jacobsen *et al.,* 2006)** suggested that, synovial fluid SAA concentration was a good marker of infectious arthritis and appeared to reflect changes in inflammatory activity. Finally, they recommended that further studies of the SAA response in osteoarthritic joints to assess its usefulness in diagnosis and monitoring of osteoarthritis is warranted. Moreover, **(Belgrave, 2013)** results indicated that assessment of SAA concentration can provide valuable information regarding the clinical state of horses and may be more useful for patient monitoring and as a prognostic indicator than are traditional markers of inflammation. **(Larson, 2014)** also stated that SAA is extremely useful in determining the presence of infection at a very early stage. It would allow not only earlier treatment but can monitor whether the treatment is working and when the proper time to end treatment would be. Inflammation is the immune system's response to infection and injury and has been implicated in the pathogeneses of diseases such as arthritis, cancer, atherosclerosis, stroke and epilepsy. The two isoforms of cyclooxygenase, COX-1 and COX-2, have been identified. Whereas constitutively expressed COX-1 is thought to mediate 'housekeeping' functions, and thus is responsible for the production of prostaglandins that are required for normal physiological activities, inducible COX-2 expressed in immune cells is a key player in initiating the inflammatory response by converting arachidonic acid, an polyunsaturated fatty acid (PUFA), into proinflammatory prostaglandins (mainly PGE2) and triggering production of other proinflammatory chemokines and cytokines. Because of this, a therapeutic strategy for inflammatory diseases has involved inhibition of COX-2, though this is now challenged by the finding that COX-2–derived oxidative metabolites of PUFAs in activated macrophages possess anti-inflammatory and antioxidant properties. Thus, COX-2 has a dual role in the initiation and resolution of inflammation by generating PUFA - derived pro - and anti-inflammatory mediators, respectively. **(Chen, 2010) and Liang Dong *et al.,* 2011).** For more understanding,Cox-2: Cyclooxygenase-2, is an enzyme primarily of immune-mediating cells - eg, macrophages, PMNs, and synoviocytes that acts to speed up the production of certain chemical messengers, called prostaglandins that play a key role in promoting inflammation. When cox-2 activity is blocked, inflammation is reduced. Unlike

cox-1, cox-2 is active only at the site of inflammation, not in the stomach. **(Wilson *et al.*, 2004) and Medical Dictionary 2016).**This means that, the stimulation of COX-1 enzyme is done on a continuous basis by the body but COX-2 enzyme didn't present at normal condition and produced only at the time of need. **(Khezar, 2013).** Since the previous studies proved that COX2 played an important role in promoting inflammation ,so our study was established to evaluate the levels of COX2 in blood serum and synovial fluid samples of suspected foals and foals showing signs of clinical arthritis. It was clear that by increasing the severity of arthritis, the levels of COX2 displayed a significant risein serum and synovial fluid of both groups. (Table 3), these results came in parallel with the results obtained by **(Kurumbail *et al.*, 2001)**, who concerned that, COX-2 is unexpressed under normal conditions in most cells, but elevated levels are found during inflammation. Furthermore, **(Chen *et al*., 2008)** revealed that prostaglandins generated under inflammatory conditions reflect the activity of the highly inducible COX-2 isoform. Identically, **(Foye and Lemke, 2008)** noticed that COX-2 enzymes play an important role in inflammation and pyrexia. Moreover, **(Sobolewski *et al.*, 2010)** results showed that, the inducible COX-2 isoform, was upregulated during both inflammation and cancer. Recently, it was stated in the **(Medical dictionary 2016)** that, unlike cox-1, cox-2 is active only at the site of inflammation, not in the stomach, and that when cox-2 activity is blocked, inflammation is reduced. **(Chen, 2010)** explained the significant rise in the levels of COX2 during subclinical and clinical arthritis by the fact that inducible COX-2 expressed in immune cells is a key player in initiating the inflammatory response by converting arachidonic acid (AA, C20:4), a polyunsaturated fatty acid (PUFA), into proinflammatory prostaglandins (mainly PGE2) and triggering production of other proinflammatory chemokines and cytokines. Therefore, prostaglandin synthesis in inflammatory conditions is largely attributable to COX-2 **(Yu *et al.*, 2005) and Chen *et al.*, 2008).** Interestingly, the statistical correlation shown in (Table 4) revealed a strong positive correlation at 0.985 between COX2 concentration in synovial fluid and its concentration in blood. Moreover SAA showed a significant and positive correlation at 0.983 between its concentration in synovial fluid and its concentration in blood. These results highlight the potential usefulness of COX2 and SAA as inflammatory markers in horses with arthritis. Identically, **(Jacobsen *et al.*, 2006)** suggested that SAA is synthesized locally in the equine inflamed joint; and that, the marked local SAA synthesis suggests an important pathophysiological role in inflammatory arthritis. Here in this study it was concluded that COX2 and SAA havea potential usefulness as inflammatory

markers in horses, showing joint inflammation, when their concentrations were measured either in synovial fluid or in blood serum.

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