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Bull Vet Inst Pulawy 59, 129-134, 2015
DOI:10.1515/bvip-2015-0019

Changes in synovial fluid inflammatory mediators and cartilage biomarkers after experimental acute equine synovitis

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Received: October 10, 2014 *Accepted: February 26, 2015*

Abstract

The purpose of the study was to define transient changes in the concentration of inflammatory biomarkers and cartilage biomarkers in the synovial fluid of joints following experimentally induced acute equine synovitis. Acute synovitis was induced in eight skeletally mature mares by a sterile intra-articular injection of 1 mL of phosphate-buffered saline (PBS) containing 0.5 ng of lipopolysaccharide (LPS). The solution was injected into the right middle carpal joint. One mL of sterile PBS was injected into the left control joint. Synovial fluid was obtained at the baseline level and at 8, 24, and 168 h after injection. The levels of inflammatory biomarkers-prostaglandin E2 (PGE2), interleukin 1β (IL-1β), and tumour necrosis factor-α (TNF-α), and cartilage turnover biomarkers-collagenase-cleavage neoepitope of type II collagen (C2C) and C-terminal crosslinked telopeptide type II collagen (CTX-II) were detected with proper assays. Single injections of LPS raised the number of synovial white blood cells and concentrations of total protein, PGE2, IL-1β, TNF-α, C2C, and CTX-II. PGE2 and IL-1β rose sharply at 8 h, while TNF-α increased steadily through 8 h and 24 h, at that point; these three factors returned to the baseline level by 168 h. The time course of C2C and CTX-II concentrations peaked sharply at 24 h, and continued to be significantly elevated over the baseline level even at 168 h. Injections of LPS into the joints led to a temporal inflammatory response, which in turn increased local release of inflammatory biomarkers and significantly altered the concentrations of cartilage markers in the synovial fluid.

Keywords: horse, experimental synovitis, inflammatory mediators, biomarkers, synovial fluid.

Introduction

Synovitis plays a significant part in the pathophysiology of equine and human joint diseases (23, 21). In the past decades, research works have offered ample evidence indicating that synovitis is associated with symptoms such as pain, degree of joint dysfunction, and may even promote an increased level of cartilage degeneration (20). In synovitis, affected articular tissues produce inflammatory mediators, including cytokines and enzymes, which lead to the degradation of articular cartilage. These inflammatory mediators are secreted in excess of the concentrations required for normal metabolic homeostasis in the joint. During the metabolic process of articular cartilage turnover, cartilage macromolecules and/or fragments are released into synovial fluid (SF). However, cartilage destruction leads to an accumulation of these degradation products in the SF, which can be measured to analyse cartilage turnover. Inflammatory and cartilage mediators accumulated in the SF play important roles in the disruption of joint homeostasis, but also can be used as the biomarkers of joint disease utilising biochemical or immunochemical assays.

Collagen type II is the important component of articular cartilage. Collagenase-cleavage neoepitope of type II collagen (C2C) and C-terminal crosslinked telopeptide type II collagen (CTX-II) are the main degradation products in the process of cartilage destruction. Changes in the extracellular matrix (ECM) of articular cartilage during the progression of osteoarthritis (OA) can be attributed to multiple factors. Among them, inflammation plays an active role, affecting both the quantity and quality of ECMs (12). The factors that contribute to the catabolic processes in OA include interleukin 1β (IL-1β), tumour necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2), and various associated cytokines. These inflammatory factors have

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previously been shown to significantly increase the expression of matrix degrading proteins in chondrocytes, such as matrix metalloproteinases (MMPs) (7). In addition, these inflammatory mediators and enzymes have considerable effects on cartilage biomarker levels in the SF. So far, few studies have analysed the intra-articular inflammation effects on SF biomarker levels following the induction of transient localised sterile inflammation.

The pathophysiology of equine arthritis and joint disorders has been clinically relevant and extensively researched by veterinarians for many years. In addition, synovial fluid is much easier to obtain in horses than in humans and small animals. In the horse, intra-articular lipopolyssacharide (LPS) injections have been shown to cause marked transient synovitis (4). This model has frequently included the primary inflammatory reaction in order to evaluate the potential benefits of a particular therapeutic regimen (10, 26).

Thus equine synovial fluid biochemical biomarkers at four time points were investigated in this study. The biomarkers included inflammatory mediators (PGE_2, IL-1β, and TNF-α), which are implicated in joint disorders as bringing change to joint homeostasis, and cartilage matrix turnover biomarkers (C2C and CTX-II). The overall objective of this study was to examine the influence of acute synovitis on biomarker production in the SF over a 1 week period of time following LPS-induced acute synovitis. The results of this study will provide a better understanding of the molecular changes and tissue damage occurring at the level of cartilage in the course of acute synovitis.

Material and Methods

Eight skeletally mature, mixed Mongolian mares with clinically normal carpal joints (as evaluated by radiography) and no history of joint disease or lameness were used. The age of the horses was between five and eight years, and their body weight was between 330 kg and 502 kg. These horses were maintained in an isolated and ventilated stable with box stalls during the course of the study, and animal care was provided.

LPS from *Escherichia coli* O55:B5 (catalogue number L2880, lot 102M4017V; Sigma-Aldrich, USA) was diluted in phosphate buffered saline (PBS; pH 7.2), then injected into the right middle carpal joints in the volume of 1 mL. The final concentration of LPS was 0.5 ng/mL. At the baseline level, horses were sedated with detomidine hydrochloride (10 μg/kg intravenously, Sigma-Aldrich, USA) and methadone hydrochloride (0.1 mg/kg intravenously; Sigma-Aldrich, USA). Sterile PBS was injected into the left middle carpal joint of each horse as a control.

The attitude, rectal temperature, pulse, and respiratory rate of each horse were examined at the baseline and at 8, 24, and 168 h after injection. Lameness was scored at the same time using the American Association of Equine Practitioners' grading scale (19). The carpal circumference was measured using a tape at the level of the accessory carpal bone. All results were assessed and examined by the same observer.

At the baseline level and at 8, 24, and 168 h after injection, SF was aseptically aspirated. Part of each SF sample was examined cytologically and had its total protein concentration determined, while the remainder was centrifuged in plain tubes at 300 × g for 10 min, placed into aliquots, and subsequently stored at -80°C until further analysis.

The concentrations of total protein and white blood cell (WBC) count were measured with refractometry and a TC10 automated cell counter (Bio-Rad, USA) respectively. Differential neutrophil values were determined using cytospin and direct smear analysis.

The PGE_2 and TNF-α concentrations in SF samples were estimated with commercially available ELISA kits, these being the PGE_2 kit (Enzo Life Sciences, USA) and TNF-α kit (Thermo Fisher Scientific, USA). An equine IL-1β monoclonal antibody (RnDsystems, USA) was used to develop an equine IL-1β ELISA. All assays were performed according to the manufacturers' instructions. Concentrations of C2C were also determined using a commercial ELISA kit (IBEX, Canada). The assay was previously validated for use in the horse (3). CTX-II concentrations were estimated using a sandwich ELISA, which was on the basis of a monoclonal antibody against the EKGPDP linear six-amino-acid epitopes of CTX-II (Serum Pre-Clinical Cartilaps ELISA, IDS, USA), an ELISA based on the binding of two identical monoclonal antibodies to cross-linked fragments of type II collagen (2) and previously validated for use in equine SF samples (23).

Statistical analysis. All data was analysed *via* SPSS 18.0 statistical software (IBM, USA). The results are shown as the mean ± standard error. Statistical analyses were undertaken by one-way ANOVA and the Student's *t*-test for repeated measures. Significance level was set at $P < 0.05$.

Results

All horses had no lameness before injection. As expected, LPS injection resulted in a significant increase in the lameness scores and circumference of the carpal joint (Fig. 1). The lameness score reached significance as early as 4 h ($P < 0.05$) after injection of LPS and continued through 24 h, resolving in all horses by 168 h. Injections of PBS in the left middle carpal joints did not cause lameness. The appetite, pulse, respiration, and rectal temperature were within the normal limits.

Results from the analysis of SF are shown in Table 1. Compared to control joints, there was a sharp rise in PGE_2 and IL-1β concentrations at 8 h in LPS joints; TNF-α showed a more sustained rise at 8 h and 24 h (Fig. 2). The effect of time on concentrations of C2C and CTX-II was also different; they increased significantly by 24 h and remained increased at 168 h (Fig. 3). In control joints, there was a sharp increase in PGE_2 at 24 h. However, there were no significant changes in other biomarkers.

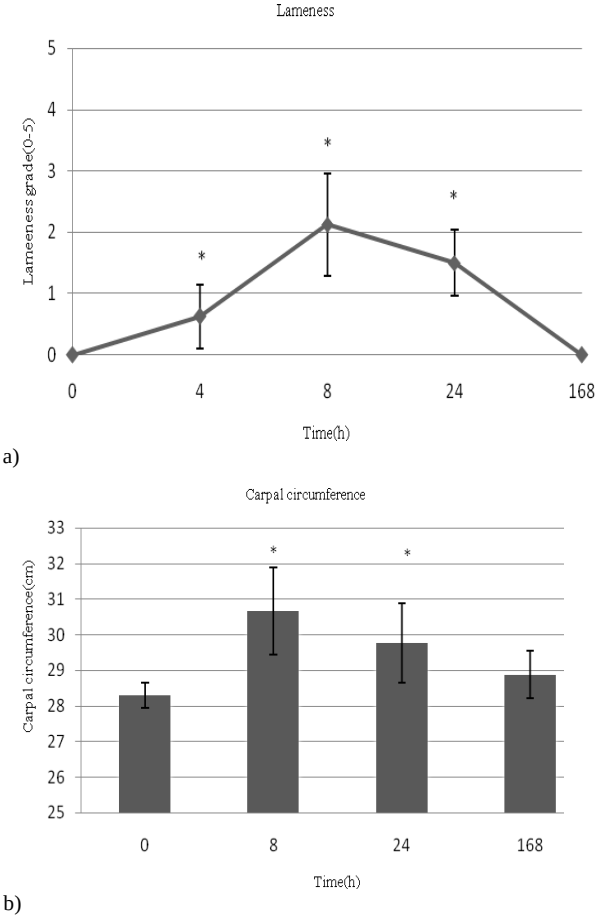


Fig. 1. Clinical results of LPS-induced acute synovitis. (a) lameness grade and (b) carpal circumference following induction of acute synovitis in the middle of carpal joints (n = 8). Synovitis was induced at t = 0 by intra-articular injection of 0.5 ng of *Escherichia coli* lipopolysaccharide. Data are shown as the mean ± standard error of the mean. * P < 0.05 compared with the baseline level (t = 0)

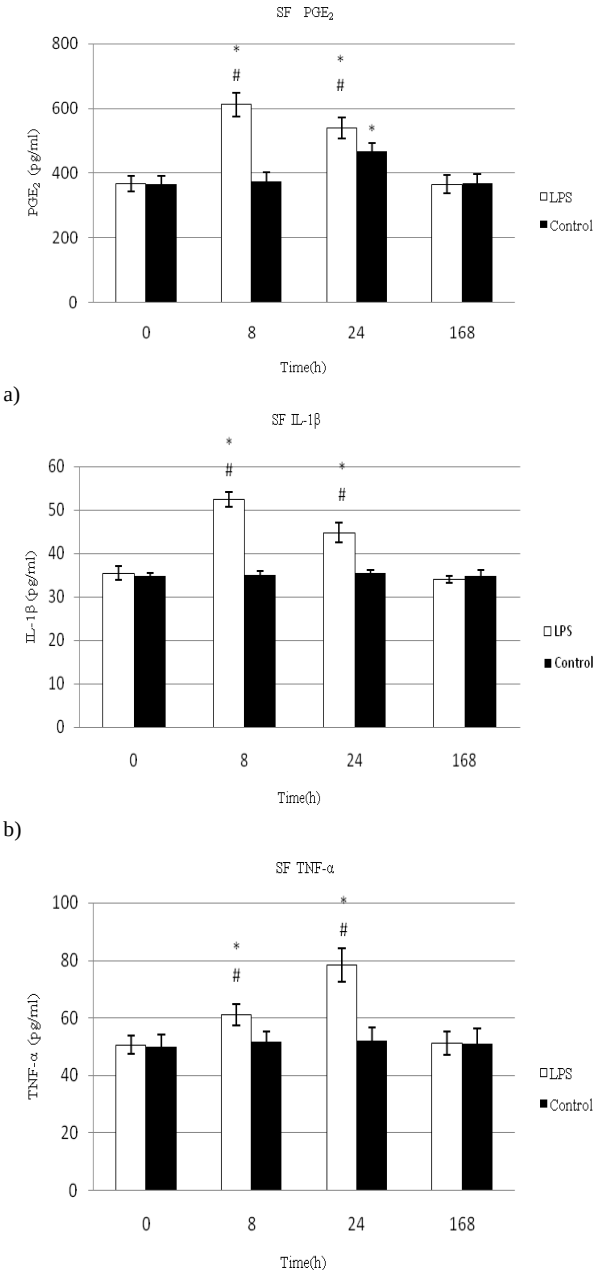
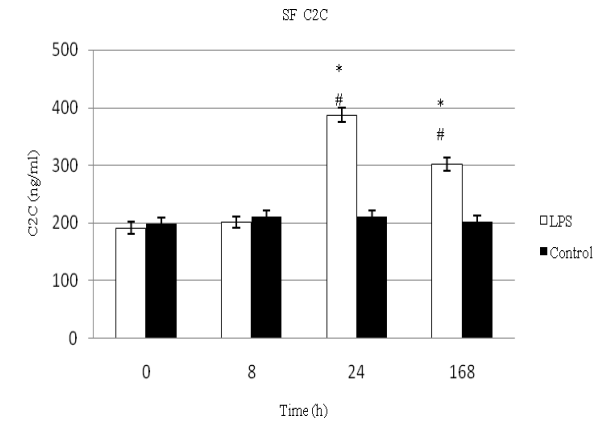


Fig. 2. Synovial fluid inflammatory biomarkers in LPS and control joints. Synovial fluid (SF) levels of (a) prostaglandin E_2 (PGE_2), (b) interleukin 1β (IL-1β), and (c) tumour necrosis factor-α (TNF-α) over time in inflamed intercarpal and control joints (n = 8). Synovitis was induced at t = 0 by intra-articular injection of 0.5 ng of *Escherichia coli* lipopolysaccharide. Data are shown as the mean ± standard error of the mean. * P < 0.05 compared with the baseline level (t = 0); # P < 0.05 compared with control group

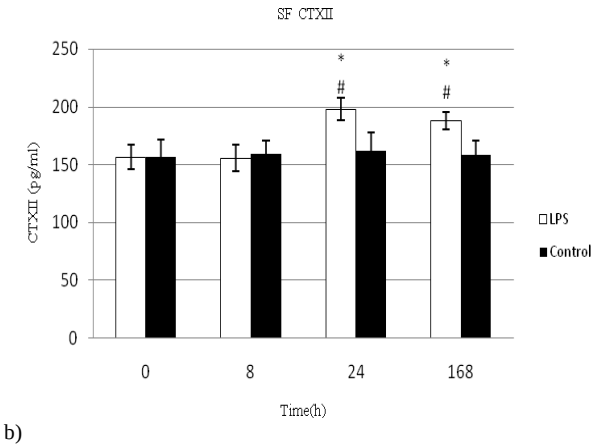
Table 1. The analysis of conventional synovial fluid after intra-articular injection of phosphate buffered saline or 0.5 ng lipopolysaccharide

Time(h) (after injection)	White blood cells (x10^9 cells/L)		Total protein (g/dl)		Neutrophils (%)	
	LPS	PBS	LPS	PBS	LPS	PBS
0	0.3 ± 0.09	0.3 ± 0.1	1.6 ± 0.2	1.6 ± 0.2	N/A	N/A
8	132.9*# ± 7.9	0.8 ± 0.1	4.5*# ± 0.6	1.7 ± 0.1	99.1*# ± 0.9	N/A
24	57.4* ± 10.6	42.9* ± 1.4	4.8*# ± 0.7	2.0* ± 0.3	81.4* ± 1.9	83.3* ± 1.9
168	0.5 ± 0.1	0.4 ± 0.7	1.8 ± 0.2	1.7 ± 0.2	N/A	N/A

Comparison of synovial fluid total protein, white blood cell counts, and differentiation over time after induction of acute synovitis. The data are shown as the mean ± standard error. (n = 8). * Significant difference from 0 h and 168 h (P < 0.001). # Significant difference from PBS at the same time (P < 0.001). N/A - no comparison was conducted given low white blood cell counts



a)



b)

Fig. 3. Synovial fluid concentrations of cartilage turnover markers in inflamed and control joints. Synovial fluid (SF) levels of (a) collagenase-cleavage neoepitope of type II collagen (C2C) and (b) C-terminal crosslinked telopeptide type II collagen (CTX-II) over time in inflamed intercarpal and control joints (n = 8). Synovitis was induced at t = 0 by intra-articular injection of 0.5 ng of *Escherichia coli* lipopolysaccharide. Data are shown as the mean ± standard error of the mean. * $P < 0.05$ compared with the baseline level (t = 0); # $P < 0.05$ compared with control group

Discussion

During symptomatic and structural progression of OA, synovial inflammation plays an important role. Moreover, synovitis has been shown to correlate with the severity of symptoms and the rate of cartilage degeneration (5). Synovial fluid biomarkers provide useful diagnostic information by allowing the detection of cartilage degradation during disease progression, thus reflecting disease-relevant biological activity and helping in real-time monitoring of therapeutic strategies for the disease (14). However, the magnitude and timing of the effects of synovial inflammation on SF marker concentrations have been subjected to attention relatively seldom.

In the present study, the synovitis model (0.5 ng of LPS) was used because it is a comprehensively-developed model of temporary synovitis in horses and a well-accepted model for inducing acute synovitis (4, 1). This reversible model of synovitis *in vivo* is useful for studying the pathophysiology of joint disease. Moreover, the horse model is a great translational model for estimating intra-articular inflammation with outcomes relevant to human OA.

Intra-articular inflammatory response was strong in LPS joints of our equine model at 8 h and 24 h, as shown by the significant rises in synovial WBC and neutrophil counts, and total protein content. The outcomes agree with previous studies using LPS models (4, 1). In control joints, a significant rise in SF parameters at 8 h, reflective of repeated arthrocentesis, was also observed. These parameters returned to the baseline level in all joints after one week, which is also consistent with previous studies (4), indicating that the LPS synovitis model is transient. This model may have limitations in that it may not reflect the chronic inflammatory processes of osteoarthritic joints.

Through analysis of inflammatory mediators in the SF, an induced inflammatory response was described. For instance, PGE_2 is considered to be a sensitive predictor of synovitis and is important for the maintenance of local homeostasis, mediation of inflammation, and sensitisation to pain (22). In LPS joints, the concentration of PGE_2 in SF elevated within the first 24 h and returned to the baseline level after one week, concurring with the results of previous studies (15). Models of inflammation in horses have also demonstrated a rise in IL-1β and TNF-α concentrations (6). In addition, LPS induces IL-1β and TNF-α release, factors strongly implicated in naturally occurring arthritis (17, 23). IL-1β induces and augments the pathological processes involved in inflammatory joint disease and stimulates chondrocytes and synovial cells to release enhanced amounts of PGE_2 and MMPs, such as collagenases and stromelysin (19). TNF-α exerts many of the same catabolic effects as IL-1β since it activates similar cell signalling pathways (9). In addition, IL-1β and TNF-α significantly up-regulate MMP-3 steady-state mRNA derived from human synovium and chondrocytes (8) and TNF-α has also been shown to induce MMP-13 expression in OA (9). In the synovial inflammatory process, these mediators interact with each other and probably contribute to cartilage turnover. The present study helps to elucidate the longitudinal effect of synovial inflammation on these inflammatory mediators *in vivo*.

Collagen II is a major component of the cartilage extracellular matrix, and C2C and CTX-II are considered to be markers of collagen II breakdown. The results showed a significant change in collagen II breakdown markers following LPS injection at 24 h, which persisted through the duration of the experiment. This suggests that inflammation induces the release of inflammatory mediators and enzymes, resulting in the cleavage of collagen II, which produces C2C and CTX-II. Moreover, the time of collagen II breakdown seems to be delayed during the course of cartilage breakdown, similar to previous results (4). The concentration of CTX-II in the SF increases following joint injury in dogs, rats, and humans (11, 13, 16).

Similarly, CTX-II has been shown to be of important value in evaluation of cartilage degradation (2). CTX-II may be very useful as a disease burden and/or prognostic marker. The current results indicate that a single inflammatory response can lead to changes in CTX-II in the SF of the horse for at least one week.

On the other hand, no significant changes in the level of these markers in the control joint were observed, with the exception of PGE_2, indicating that the response to LPS was much more profound than the response to repeated aspiration. PGE_2 is a very sensitive marker released primarily by the synovial membrane. Since the aspirations in this study were accomplished through the synovial membrane, aspiration may stimulate the membrane to produce PGE_2. In a previous study, repeated arthrocentesis led to the release of PGE_2 in the normal joint (25). Therefore, in our study repeated arthrocentesis likely resulted in an increase in PGE_2 concentration. In response to the repeated arthrocentesis, there may be slight increases in other marker levels, but they appear to be cleared within the lymph nodes rapidly.

All things considered, however, there were no other significant changes in control joints, indicating that the effect of arthrocentesis may be unimportant in the context of this LPS synovitis model. The overall response was acute, wearing off within a week, but the degree of inflammation is highly reflective of early OA. The observed trends are important for future clinical trials studying the treatment of this disease.

The results presented in this study demonstrate that acute synovitis has an effect on inflammatory and cartilage biomarkers in the SF. After the induction of inflammation, PGE_2, IL-1β, and TNF-α increased acutely during the first 24 h, returning to the baseline level by 168 h. Cartilage biomarkers also rose dramatically with increases of C2C and CTX-II occurring at a slightly later time and persisting longer. In conclusion, synovitis plays an important role in early OA, and the temporary model utilised in this paper will be useful in evaluating therapeutics through identifying biomarkers which change during the early stages of joint disease.

Conflict of Interests Statement: The authors declare no conflicts of interest.

Financial Disclosure Statement: This study was supported by the National Science and Technology Support Programme of China（2012BAD46B02-03).

Animal Rights Statement: The study was performed in accordance with the Ethical Committee for Animal Experiments (Northeast Agricultural University, Harbin, China).

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