Cytokine, catabolic enzyme and structural matrix gene expression in synovial fluid following intra-articular administration of triamcinolone acetonide in exercised horses

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Summary

Reason for performing study: The frequent use of intra-articular triamcinolone acetonide (TA) in performance horses warrants further study of the duration of as well as the beneficial and detrimental effects on gene expression associated with administration.

Objectives: To assess the effects of intra-articular administration of TA on the expression of selected anti- and proinflammatory and structural matrix genes following its administration into joints of exercised Thoroughbred horses and to correlate these effects with plasma and synovial fluid drug concentrations.

Study design: Block design experiment.

Methods: Eight exercised horses received a single intra-articular administration of 9 mg of TA. Synovial fluid samples were collected from the treated and contralateral joints prior to and up to 49 days following drug administration. Microarray and quantitative reverse transcription polymerase chain reaction analysis were used to assess changes in expression levels of various inflammatory and structural genes post drug administration.

Results: Drug concentrations in plasma and synovial fluid, were no longer quantifiable by 6 and 28 days following drug administration respectively. In total, the expression level of 5490 genes were significantly altered on microarray analysis, following intra-articular TA administration. Of the genes selected for further study by quantitative reverse transcription polymerase chain reaction analysis, significant changes in inflammatory genes (annexin type 1, cyclooxygenase-1 and tumour necrosis factor stimulated gene 6) and structural genes (collagen and aggrecan) were noted.

Conclusions: This study supports the use of synovial fluid as a biological matrix for studying the effects of corticosteroids on gene expression. For the majority of genes studied the effects on expression relative to baseline for both inflammatory and matrix genes were prolonged relative to plasma and synovial fluid TA concentrations. Downregulation of collagen gene expression warrants the careful use of TA in horses.

Keywords: horse; triamcinolone acetonide; gene expression; biomarker; inflammation; intra-articular

Introduction

Osteoarthritis is arguably one of the most common disease processes affecting performance horses [1]. Abnormal stress placed on otherwise normal or abnormal joints leads to inflammation and the release of various inflammatory cytokines and mediators [2]. If left unchecked, these inflammatory mediators may destroy joint structures, ultimately resulting in osteoarthritis [2]. To facilitate improved understanding of the pathogenesis and clinical management of osteoarthritis, molecular technology is increasingly being used to define and assess biomarkers of osteoarthritis in both man and animals [3–6]. For example, Kamm and colleagues [3] recently used microarray analysis to describe changes in gene expression in a model of experimentally induced osteoarthritis in horses.

Corticosteroids, such as triamcinolone acetonide (TA), are potent anti-inflammatory agents and are commonly used to treat performance related joint injuries, such as synovitis, capsulitis and osteoarthritis, in performance horses [7,8]. Following intra-articular (i.a.) administration, corticosteroids have been shown to be effective at inhibiting the production of inflammatory cytokines and in turn preventing permanent damage to cartilage and subsequent osteoarthritis. Corticosteroids have both genomic and nongenomic effects. With respect to the genomic effects, after binding to cytosolic receptors, the corticosteroid-receptor complex translocates into the nucleus where, in the case of inflammation, it downregulates proinflammatory genes and upregulates anti-inflammatory genes [9]. It would seem that because of their genomic mechanism of action, studying gene expression following corticosteroid administration would be an ideal means by which to assess the pharmacodynamics as well as the duration of effect of this class of drugs. Although corticosteroids are only detected for a short period of time in blood following i.a. administration, the detection time within the joint itself is much longer [10]. This prolonged residence time, along with the genomic mechanism of action, has led us to speculate that the effects of corticosteroids are likely to be prolonged relative to the time of drug detection in blood. However, there are currently no reports supporting this hypothesis in the horse.

Assessing gene expression may prove to be an objective approach for evaluating the anti-inflammatory effects of corticosteroids following i.a. administration and would complement additional studies of the clinical effects of this class of drugs in the horse. Furthermore, as previous studies describing changes in gene expression in horses with joint disease were conducted using synovium or articular cartilage, in the current study we sought to determine whether synovial fluid, which would be easier to collect yet presumably contain lower quantities of RNA, could serve as a suitable biological matrix for studies describing gene expression in joints following corticosteroid administration. Therefore, the purpose of the current study was to assess the effects of TA on the expression of selected inflammatory and structural genes in synovial fluid following its administration into joints of exercised Thoroughbred horses, and to correlate these effects with plasma and synovial fluid drug concentrations. It is important to note that this study describes the effect of TA on inflammatory mediators in exercised sound horses with presumably normal joints, and that the effects on these genes may be different in horses with inflammation or osteoarthritis. Nonetheless, this investigation represents a first step in the study of the effects of i.a. TA on gene expression in horses.
Gene expression and triamcinolone in horses

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Methods

Animals

Eight healthy racing-fit mature Thoroughbred horses including 4 geldings and 4 mares (3–6 years) were studied. Prior to and throughout the course of the study, horses were exercised 5 days a week using a protocol intended to simulate the strenuous exercise of race training. The exercise regimen for these horses consists of 3 days per week on an Equineciser® (5 min at walk; 30 min trot; 5 min walk) and 2 days per week on a high speed treadmill (Mustang 2200®) (Day 1: 5 min at 1.6 m/s; 5 min at 4 m/s; 5 min at 7 m/s; 5 min at 1.6 m/s all at 6% incline. Day 2: 2 min at 1.6 m/s; 4 min at 4.0 m/s; 2 min at 7.0 m/s; 2 min at 11.0 m/s and 5 min at 1.6 m/s all at 3% incline). All horses were subject to regular fitness testing, including weekly heart rate measurements and calculation of VO$_2$ (running velocity that elicits a heart rate 200 beats/min) and monthly measurements of end run plasma lactate concentrations, as a means by which to ensure that the fitness levels of the horses used in this study were comparable to those of the average racehorse (Supplementary item 1). Horses were not exercised on the day of or the day after synovial fluid collection. Two days after synovial fluid collection, horses were allowed to freely exercise in a round pen. Horses returned to their regular exercise regimen on Day 3 after drug administration.

Before beginning the study, horses were determined to be healthy based on physical examination, evaluation of complete blood count and a serum biochemistry panel that included aspartate aminotransferase, creatinine phosphokinase, alkaline phosphatase, total bilirubin, sorbitol dehydrogenase, blood urea nitrogen and creatinine. Blood analyses were performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis, using their standard protocols. Horses did not receive corticosteroids or any other i.a. medications for at least one year and any other medications performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis, were recorded prior to collection of synovial fluid. Two days after synovial fluid collection, horses were allowed to freely exercise in a round pen.

Collection of synovial fluid and plasma

Blood samples for determination of drug concentration were collected at Time 0 (prior to drug administration) and at 15, 30 and 45 min, and 1, 2, 3, 4, 5, 6, 8, 12, 18, 24, 36, 48, 72 and 96 h post administration. Subsequent samples were collected every other day until 49 days post administration. Prior to drawing each sample of blood for analysis of plasma drug concentrations, 10 ml of blood were aspirated and discarded from the catheter and T-Port extension set (combined internal volume ~2 ml). The catheter was flushed with 10 ml of a dilute heparinised saline solution (10 i.u/ml) following each sampling time. Catheters were removed following collection of the 24 h sample and the remaining samples were collected by direct venipuncture. Blood samples were collected into EDTA blood tubes and stored on ice until centrifugation at 3000 g for 10 min at -4°C. Plasma was then immediately transferred into storage cryovials and stored at -20°C until analysis (10–12 days following collection of the final sample).

Synovial fluid was aseptically collected from the right and left antebrachiocarpal joints. Prior to collection of synovial fluid, the area over the joint was clipped (Oster Golden Clippers) with a size 10 blade and scrubbed for 10 min alternating a 2% chlorhexidine solution and 70% isopropyl alcohol. The joint was then flexed and a total dose of 9 mg (1.5 ml) of triamcinolone acetonide (Vetalog) was administered aseptically into the dorsal aspect of the antebrachiocarpal joint. Nothing was injected into the right carpus. Prior to collection of synovial fluid, the area over the joint was wiped with alcohol. Synovial fluid was aseptically collected from the right and left antebrachiocarpal joints. Prior to collection of synovial fluid, the area over the joint was clipped (Oster Golden Clippers) with a size 10 blade and scrubbed for 10 min alternating a 2% chlorhexidine solution and 70% isopropyl alcohol. The joint was then flexed and a total dose of 9 mg (1.5 ml) of triamcinolone acetonide (Vetalog) was administered aseptically into the dorsal aspect of the antebrachiocarpal joint. Nothing was injected into the right carpus.

RNA extraction and quality assessment

For RNA isolation, 200 µl of synovial fluid was added to 1 ml of QiAozol lysis reagent®. The sample was vortexed, passed through a 20-gauge needle attached to a sterile syringe and subjected to chloroform extraction and centrifugation. Total RNA was purified using an miRNeasy Mini kit® and integrity assessed using an Experion Automated Electrophoresis System®. RNA samples with an RNA integrity number ≥8 and 260/280 and 260/230 ratios between 1.7 and 2.1 were utilised [11,12].

Microarray analysis

Equine specific microarrays (EquivGene-1.0-st®), containing expression profiling of 30,559 well-characterised genes using 504,603 probes, were utilised. To reduce biological noise as a result of genetic variability, each horse was analysed separately and served as its own control for comparison of baseline samples to each time point. Five biological replicates per time point were tested. Purified total RNA (5 µg) was used for cDNA synthesis in accordance with the Ambion WT Expression assay kit manufacturer’s protocol. In vitro transcription was used to incorporate biotin labels using the GeneChip WT Terminal Labeling system® and samples hybridised to the equine microarray. Arrays were washed and stained on a Fluidics Station 450® and scanned on a GeneChip Scanner 3000® in accordance with manufacturer’s protocols.

The microarrays were evaluated for differential gene expression using Transcriptome Analysis Console and for hybridisation quality control using Expression Console Software®. Briefly, Robust Multichip Analysis (RMA) was used for probe summarization and normalization of background-adjusted, normalized, and log-transformed perfect-match (PM) probe intensity values from the Cel raw data files. Comparison analysis was then performed in order to identify genes that were differentially expressed in the different treatment groups. For evaluation of the assay performance, the number of differentially expressed genes detected between different time points were compared with baseline samples. Based on the Transcriptome Analysis Console software user’s manual, genes with mean transformed ratios significantly less than −2 and larger than +2 were considered significantly regulated. A number of the significant genes were selected by filtering the genes using an ANOVA (P<0.05). Pearson’s correlation coefficient was used to calculate linear dependence between time point and baseline samples to evaluate the correlation coefficient, where 1 was a positive correlation and 0 was no correlation.

Quantification of mRNA using TaqMan Low Density Arrays

The top 18 candidate genes were selected from those identified as markers of inflammation in previous studies [3,13,14] and/or the genes that showed significant changes in gene expression following microarray analysis. Microarray data were validated by measuring the levels of specific mRNA in synovial fluid in the treated group with triamcinolone vs. control groups at various time points via Taqman Low Density Arrays (TLDA®), which are preloaded 384 well reverse transcription polymerase chain reaction analysis (rt-PCR) microfluidic cards. RNA was diluted to a concentration of 2 ng/µl for cDNA conversion using the QuantiTect Reverse Transcription Kit®. cDNA was then combined with Taqman Universal Mastermix® at a final concentration of 2x and then 100 µl of each sample was loaded onto the TLDA card.

Primers for candidate genes were designed and manufactured by Life Technologies®. Reference or ‘housekeeping’ genes were used to evaluate the effect of RNA integrity on the array and quantitative RT-PCR (qRT-PCR) performance. Based on stability across all samples studied [15] equine β2
microglobulin (β2M) was used as the endogenous control gene to normalise the qRT-PCR data and human 18s (hs18s) was used as an internal manufacturing control. Each sample was run in quadruplicate with 10 candidate genes per card. The TLDA cards were then run on a QuantStudio 12K Flex Software v1.2.2. Analysis was performed using ExpressionSuit Software 1.0.3 using Singleplex analysis.

Statistical analyses using commercially available software (StataC IC 13.1) were performed to assess significant differences in expression (fold change) between baseline and each time point in the corticosteroid and control groups. Data were analysed using a mixed effects analysis of variance, with the horse treated as a random effect and with group and time as fixed effects. Post hoc comparisons were performed with a Bonferroni multiple-comparison adjustment to preserve a nominal significance level of 0.05.

Results

Drug concentrations

Average plasma and synovial fluid TA concentrations with respect to time are shown in Figure 1. TA was below the limit of detection by 6 and 28 days post drug administration in plasma and synovial fluid (right antebrachiocarpal joint), respectively. TA was not detected in the left antebrachiocarpal joint at any time point sampled.

Synovial fluid biomarkers

Results of macroscopic analysis indicated no difference in synovial fluid colour or clarity relative to baseline. There were no significant changes, relative to baseline, in total protein or white blood cell count at any time point post drug administration. The volcano plot of differentially abundant transcripts generated from microarray analysis is depicted in Figure 2. From a total of 30,559 genes studied, 2734 were significantly upregulated and 2756 downregulated. Based on the results of the microarray analysis, the following pro- and anti-inflammatory and structural genes were selected for further analysis to quantify the change in expression relative to baseline (pretreatment): interleukin-1β (IL-1β), IL-1 receptor (IL1R1), tumour necrosis factor (TNF), phospholipase A2 (PLA2), cyclooxygenase-1 (COX-1), COX-2, annexin type 1 (ANAX-1), ANAX-2, collagen 1A1 (COL1A1), COL1A2, COL2, COL3A1, aggrecan (ACAN), cartilage oligomeric matrix protein (COMP), matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9 and TNF stimulated gene 6 (TSG-6).

No significant changes in IL-1β gene expression were noted. Significant changes in IL-1R1 expression were only noted in the TA treated group at 12 h (upregulated; P < 0.001) and 24 h (upregulated; P < 0.001) and 14 days (downregulated; P = 0.002) post drug administration (Figure 3a). TNF gene expression was significantly upregulated relative to baseline in the TA treated group at 12 h (P = 0.040) post administration (Figure 3b). Thereafter, significant downregulation of TNF gene expression was only observed at 21 days (P < 0.001) in the TA treated group. TNF gene expression was significantly downregulated relative to baseline in the control group at 24 h (P < 0.001) and 96 h (P = 0.013; Figure 3b). Changes in COX-2 expression were not significant, whereas COX-1 was significantly downregulated at 12 h (P < 0.001), 24 h (P < 0.001) and 96 h (P < 0.001) and again at Day 49 (P < 0.001) in the TA treated group and 24 h in the control group (P < 0.001; Figure 3c).

Annexin type 1 expression was significantly upregulated until 21 days (12 h: P = 0.025; 24 h: P = 0.001; 96 h: P = 0.003; 14 days: P = 0.003; 21 days: P = 0.017) and downregulated at 49 days (P = 0.038) in the TA treated group (Figure 3d). Conversely, ANAX-1 expression was downregulated in the control joints with significant differences (relative to baseline) at 12 h (P = 0.012) and 14 P = 0.007) and 21 days (P = 0.001; Figure 3d). ANAX-2 was only significantly different from baseline at 96 h and Day 14 in both the TA (upregulated; P = 0.010) and control (upregulated; P = 0.039 and P = 0.001) groups (Figure 3e). Changes in PLA2 gene expression were only significant relative to baseline levels in the TA treated groups (Figure 3f). Significant upregulation of the PL2A gene was observed at 12 h (P = 0.001), 24 h (P = 0.001) and 96 h (P = 0.001), and on Days 14 (P = 0.001) and 21 (P = 0.001) and downregulation was observed on Day 42 (P = 0.001).

Tumour necrosis factor stimulated gene 6 gene expression increased significantly relative to baseline levels immediately (12 h; P = 0.001) following TA administration and expression remained significantly elevated through 96 h (P = 0.001) and again at Day 49 (P = 0.036; Figure 4). Significant upregulation in TSG-6 expression, relative to baseline, was noted in the control group at 12 h (P < 0.001) and 24 h (P < 0.001) h.

Increases in MMP-1 gene expression were observed in the TA treated group until Day 14 post drug administration (P = 0.001; Figure 5a). Significant increases in MMP-1 gene expression, relative to baseline, were noted in the control group at 12 (P = 0.001), 24 (P = 0.001) and 96 h (P < 0.001; Figure 5a). Changes in MMP-3 expression were not significantly different from baseline in the TA treated or control groups at any time point post drug administration. Conversely, MMP-9 gene expression was notably increased at 12 h (P < 0.001), 24 h (P = 0.001), 72 h (P = 0.001) and 96 h (P = 0.001) post TA administration (Figure 5b). In the control group MMP-9 gene expression was significantly increased at 12 h (P < 0.001), 24 h (P = 0.001) and 96 h (P < 0.001; Figure 5c).

Significant changes in COL1A1 gene expression were noted only at 12 h (downregulation) in the TA (P = 0.040) and 96 h (upregulation) in the control group (P < 0.001; Figure 6a). COL1A2 expression was significantly downregulated at 12 h (P < 0.001), 24 h (P = 0.001) and 96 h (P < 0.001), and Day 42 (P = 0.031; Figure 6b). Significant downregulation of COL1A2 was observed at 12 h (P = 0.001), 24 h (P = 0.001) and 96 h (P < 0.001) h and upregulation on Day 14 (P = 0.032) in the control group (Figure 6b). COL2 gene expression was significantly downregulated at 12 h (P = 0.020), 24 h (P = 0.001), 72 h (P = 0.003) and 96 h (P = 0.008), and Day 21 (P = 0.005; Figure 6c). Significant changes in COL2 were noted in the control group. Significant downregulation of COL3A1 gene expression was noted until Day 21 (12 h and 24 h: P < 0.001; 72 h: P = 0.007; 96 h: P = 0.001; Day 14: P = 0.02; Day 21: P = 0.047 and Day 42: P = 0.018) in the TA treated group (Figure 6d). COL3A1 gene expression was downregulated at 24 h (P = 0.006) and upregulated at 96 h (P < 0.001) in the control group. ACAN was significantly downregulated (relative to baseline) in the treated group for at least 96 h post TA administration (12 h: P = 0.001; 24 h: P = 0.002; 72 h: P = 0.004; 96 h: P = 0.001; Figure 6e). Significant upregulation was noted in the control group at 24 h (P = 0.008) and 96 h (P = 0.003; Figure 6e). COMP levels were significantly downregulated, relative to pretreatment values at 24 h (P = 0.009) post TA administration (Figure 6f).

No significant changes in COMP expression were noted in the control group.

Discussion

Previous studies have described changes in inflammatory proteins or inflammatory gene expression in horses with either a diagnosis of naturally occurring osteoarthritis or in which inflammation had been induced experimentally [3,16-23]. This is the first in vivo report of changes in inflammatory gene expression, using microarray and qRT-PCR analysis,
following administration of a corticosteroid to horses. As this is the first study of its kind, rather than inducing inflammation to mimic an acute injury, we chose instead to start by assessing changes in gene expression following TA administration using horses enrolled in an intense exercise regimen, mimicking that of a racehorse in training. Although perhaps not as dramatic as would be expected in horses with an acute injury or osteoarthritis, changes in the biomarkers of inflammation have been demonstrated in horses enrolled in an intense exercise regimen in the absence of notable joint inflammation or osteoarthritis [14]. Therefore, as a first step in characterising the effects of corticosteroid administration on molecular biomarkers, in the current study we sought to quantify the expression of inflammatory and degradative genes in cells floating in the synovial fluid in exercised horses.

Interleukin-1β has been shown to play a large role in cartilage destruction following an inflammatory insult [24,25] and has been referred to as the master cytokine in human osteoarthritis. IL-1β induces transcripts involved in arachadonic acid metabolism, including COX-2, both in equine articular cartilage explants [26–28] and in vivo in rabbits [29]. In the current study, changes in IL-1β gene transcription were not significant following TA administration, which is in agreement with previous findings in synovium following administration of dexamethasone to rabbits with post traumatic osteoarthritis [30]. COX-2 gene expression has been reported to be significantly repressed in IL-1β stimulated equine articular cartilage explants following administration of TA and methylprednisolone [28]. In the current study, changes in COX-2 gene expression were not statistically significant following TA administration in horses. This could be attributed to the lack of significant changes in IL-1β gene expression or to the lack of inflammation in the joints of the horses studied here. It is well established that COX-2 expression is induced in the presence of inflammation, and therefore it is possible that greater suppression might have been observed had an inflammatory model been used in the current study, as was the case in the previously described study [28]. Conversely, COX-1, the constitutively expressed cyclooxygenase enzyme, was significantly downregulated following TA administration.

Annexin type 1 was significantly upregulated as a result of TA administration. ANAX-1, which is found in inflammatory cells, has been shown to increase in the presence of corticosteroids [31,32]. ANAX-1 subsequently inhibits PLA2, preventing the release of arachidonic acid [31,32]. Although ANAX-1 was upregulated and is known to inhibit PLA2, downregulation of PLA2 was not observed in the current study. As the proposed mechanism for inhibition of PLA2 is substrate depletion [33,34] and not alteration of gene expression, lack of downregulation is not unexpected. In this case, assessment of PLA2 protein concentrations would be a better indicator of the effects of ANAX-1 on PLA2. ANAX-1 also reportedly affects COX-2 gene expression, as noted by increases in expression of this enzyme in ANAX-1 knockout mice following exposure to an inflammatory stimulus [35]. In the current study, where a noninflammatory model was utilised, the increase in ANAX-1 expression in combination with lack of notable changes in COX-2 gene expression, further supports that changes in COX-2 gene expression may only be observed in the presence of inflammation, and supports further study of gene expression in a model of equine joint inflammation.

In addition to downregulating production of proinflammatory cytokines, corticosteroids have also been shown to upregulate expression of anti-inflammatory mediators [9]. TSG-6 protein, which is believed to be chondroprotective and to blunt the deleterious effects of MMPs [36,37], is
reportedly found at high levels in the synovial fluid of human patients with various forms of arthritis [38]. In the study reported here, changes in TSG-6 gene expression were significant in both the TA and control groups, suggesting that alterations in expression levels of this gene are a result of repeated arthrocentesis and not drug administration.

Articular cartilage destruction associated with osteoarthritis occurs as result of an imbalance between catabolic and anabolic processes [39]. Destruction of the cartilage occurs when catabolic processes predominate and is characterised by significant upregulation of MMPs [39]. Based on microarray analysis, in the current study only MMP-1, MMP-3 and MMP-9

Fig 3: Mean relative expression (compared to baseline) of (a) interleukin 1 receptor type 1 (IL1R), (b) tumour necrosis factor (TNF), (c) cyclooxygenase 1 (COX-1), (d) annexin 1 (ANAX-1), (e) annexin 2 (ANAX-2) and (f) phospholipase A2 (PLA2) in synovial fluid following intra-articular administration of 9 mg of triamcinolone acetonide (TA) in 8 exercised Thoroughbred horses. *Represents changes that are significantly different (P < 0.05) from baseline (pretreatment) values.
were affected by administration of TA and were selected for further study. As corticosteroid administration is generally reported to inhibit MMPs, the increase noted in the current study was unexpected. However, previous reports suggest that effects on MMP subtypes vary under different conditions [40]. Shimizu and colleagues [40] reported that synovial levels of certain MMP subtypes were only lowered in response to combination therapy with hyaluronic acid and corticosteroids, and not by treatment with corticosteroids alone. However, based on the finding of similar increases in nontreated control horses, the increase in MMP expression observed in the current study was probably due to inflammation induced by repeated arthrocentesis.

In osteoarthritic joints, destruction of the articular cartilage extracellular matrix, composed primarily of aggrecan and type II collagen, occurs. Subsequently, upregulation of collagen genes, including COL1A2, COL2, COL2 and COL3A1, is observed [41]. In horses, the effect of corticosteroid administration on the expression of collagen coding genes has been mixed. Richardson and Dodge [42] reported that high doses of corticosteroids significantly reduced expression of type II collagen in isolated articular chondrocytes however, in recombinant equine IL-1β stimulated articular cartilage explants, TA did not alter the IL-1β inhibition of genes coding for collagen or aggrecan [28]. The authors theorised that the lack of inhibition of collagen gene expression was probably due to the use of lower doses in the latter study [28]. Similar to that reported by Richardson and Dodge [42], in the current study significant downregulation of genes coding for COL1A2, COL2, COL3A1 and ACAN was observed following administration of TA. These findings are similar to those reported previously in in vitro studies of the effects of dexamethasone on collagen gene expression in rabbits [30]. Although changes in COL genes in the current study are in agreement with previous reports, it is important to note that whereas the study by Richardson and Dodge [42] demonstrated changes in chondrocytes, in the current study synovial fluid was used, and therefore the source of the increased COL, ACAN and COMP gene expression is not clear. Presumably, in the case of cellular infiltration of the synovial fluid the major cell type is inflammatory, which are not known to express COL, ACAN and COMP. However, Zhou and colleagues [5] demonstrated expression of COL, ACAN and COMP genes, albeit at dramatically lower levels relative to chondrocytes, in synovial fluid cells collected from bovine joints. It is also possible that in the current study the synovial fluid cells include chondrocytes that were dislodged during arthrocentesis. Further studies will be necessary to elucidate the source of the increased expression of these structural genes.

It is certain that repeated arthrocentesis, as occurred in the current study, has the potential to induce inflammation and potentially disrupt the extracellular matrix. This can make attributing changes in expression levels of inflammatory genes and genes coding for extracellular matrix proteins to either drug administration or repeated collection of synovial fluid slightly ambiguous. With respect to expression levels of extracellular matrix proteins, a lack of significant decreases in gene expression in the control group, along with a previous report that joint collagen metabolism and cartilage aggrecan turnover remained stable with repeated synovial fluid aspiration in horses [43], suggests that the changes in collagen gene expression noted here were drug induced. Further supporting this hypothesis is the increase in aggrecan turnover and collagen catabolism observed 1 week following i.a. TA (12 mg) administration in that same study [43].

There are a few notable limitations to the current study, perhaps the most important being the limited number of animals studied. If more animals had been included, it is possible that changes in gene expression might be found to be significant. Secondly, for this preliminary study, we chose not to use an inflammatory model that would mimic an acute inflammatory insult. Instead, horses undergoing an intense exercise...
regimen similar to the strenuous exercise of race training were used. Therefore, although changes were observed in the expression of inflammatory genes following TA administration, they may not be representative of an animal experiencing an acute inflammatory insult. A third limitation to the current study was the selection of synovial fluid as the matrix to assess changes in gene expression. As synovial fluid is generally acellular, in the absence of overt inflammation, it is not necessarily an ideal matrix for isolating RNA. Although we were able to

Fig 6: Mean relative expression (compared to baseline) of (a) collagen type 1A1 (COL1A1), (b) collagen type 1A2 (COL1A2), (c) collagen type 2 (COL2), (d) collagen type 3A1 (COL3A1), (e) aggrecan (ACAN) and (f) cartilage oligomeric matrix protein (COMP) in synovial fluid following intra-articular administration of 9 mg of triamcinolone acetonide (TA) to 8 exercised Thoroughbred horses. *Represents changes that are significantly different (P < 0.05) from baseline (pretreatment) values.
successfully isolate high quality RNA at most of the time points studied, the expression of various biomarkers might have been slightly different had articular cartilage or synovium been collected. However, as tissue collection from the joint is an invasive procedure, it was judged not appropriate for the current study. Finally, in the current study we chose to focus on gene expression and did not assess protein concentrations. Ideally, we would have been able to look at both, as increased gene expression does not always translate to increased protein concentrations. Unfortunately, the limited sample volume precluded assessment of both gene expression and protein concentrations, and as corticosteroids directly affect gene expression, presumably qRT-PCR would be a useful approach for assessing the direct effects of TA in equine athletes.

The current study furthers understanding of the anti-inflammatory nature of i.a. administration of TA. For the majority of genes studied, the effects on expression relative to baseline, for both inflammatory and matrix genes, were prolonged relative to plasma and synovial fluid TA concentrations; however, increases in expression of MMPs and downregulation of collagen genes warrant the careful use of TA in horses. This study also supports the use of synovial fluid as a biological matrix for studying the effects of corticosteroids on gene expression and supports further study in an inflammatory model in horses.

Authors’ declaration of interests

No competing interests have been declared.

Ethical animal research

This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

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Authorship

H. Knych contributed to conception and design, obtaining of funding, collection and assembly of data, analysis and interpretation of the data, and drafting, critical revision and final approval of the manuscript. M. Vidal contributed to design of study, collection and assembly of data, analysis and interpretation of the data, and drafting, critical revision and final approval of the manuscript. N. Chouicha contributed to collection and assembly of data, analysis and interpretation of the data, and review of manuscript. M. Mitchell contributed to collection and assembly of data, and review of manuscript. P. Kass contributed to data analysis and review of the manuscript.

Manufacturers’ addresses

Centaur Horse Walkers Inc, Mira Loma, California, USA.
Graber AG, Fahrwangen, Switzerland.
Oster Professional Products, McMinnville, Tennessee, USA.
Agri Laboratories Ltd, St Joseph, Missouri, USA.
Boehringer Ingelheim Vetmedica, St Louis, Missouri, USA.
Kendall/Tyco Healthcare, Mansfield, Massachusetts, USA.
Phenix Research Products, Chandler, North Carolina, USA.
Qiagen Inc., Valencia, California, USA.
BioRad Laboratories, Hercules, California, USA.
Affymetrix, Santa Clara, California, USA.
Life Technologies, Foster City, California, USA.
StataCorp LP, College Station, Texas, USA.

References

15. TaqMan Endogenous Control Assays to Select an Endogenous Control for Experimental Studies (Publication CO16806)) Life Technologies.


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Item 1: Average ± s.d. end run haemoglobin and lactate plasma concentrations and running speed required to elicit a heart rate of 200 beats/min in exercised Thoroughbred horses (n = 12) on 2 separate occasions.