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RESEARCH ARTICLE

A time-course evaluation of inflammatory and oxidative markers following high-intensity exercise in horses: a pilot study

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MacNicol JL, Lindinger MI, Pearson W. A time-course evaluation of inflammatory and oxidative markers following high-intensity exercise in horses: A pilot study. *J Appl Physiol* 124: 860–865, 2018. First published October 26, 2017; doi:10.1152/japplphysiol.00461.2017.—Exercise is a physiological stress resulting in reactive oxygen species and inflammatory mediators, the accumulation of which are thought to contribute to degenerative articular diseases. The horse is of particular interest in this regard as equine athletes are frequently exposed to repetitive bouts of high-intensity exercise. The purpose of this study was to provide a detailed description of the response of articular and systemic oxidative and inflammatory biomarkers following high-intensity, exhaustive exercise in horses. A group of horses (Ex) underwent repeated bouts of high-intensity exercise, at a target heart rate of 180 beats/min, until voluntary exhaustion. Baseline plasma and synovial fluid (SF) samples were taken 24 h before exercise and then at 0.5, 1, 2, 4, 8, and 24 h following exercise cessation. This time course was repeated in a group of nonexercised control horses (Co). Plasma and SF samples were analyzed for prostaglandin E_2 (PGE_2), nitric oxide (NO), total antioxidant status (TAS), and glycosaminoglycans (GAG). The Ex group had significantly higher plasma NO at 0.5, 1, and 2 h; and higher plasma PGE_2 at 0.5 and 1 h compared with Co. SF PGE_2 and GAG were also higher in Ex horses at 8 h compared with Co. It is concluded that high-intensity exercise in horses results in a rapid increase in systemic oxidative and inflammatory markers from 0.5 to 2 h after exercise, which is followed by local articular inflammation and cartilage turnover at 8 h postexercise.

NEW & NOTEWORTHY In horses, the influence of exercise systemically and within the articular space remains unclear and requires further detailed characterization. In this study, we identify that an acute bout of high-intensity exercise in horses induces systemic inflammation and oxidative stress within 30 min of exercise cessation, which lasts for ~2 h. Articular inflammation and cartilage turnover were also be observed within the equine carpal joint 8 h following exercise completion.

exercise; horse; inflammation; oxidative stress; physiology

INTRODUCTION

Exercise stress is associated with alterations in circulating inflammatory cytokines as well as increased markers of oxidative stress (30, 36). Elevations of creatine kinase (CK), lactate dehydrogenase, and malondialdehyde (MDA) were observed in human athletes following an 80-km running race, indicating not only significant tissue damage but also lipid peroxidation (19). Oxidative stress and inflammation are closely linked, and several reactive oxygen species (ROS), such as nitric oxide (NO), are also involved in inflammatory pathways. Both oxidative stress and inflammation can lead to damage at the level of the tissue, cell, protein, or DNA.

Although several physiological processes produce free radicals as by-products and this results in routine levels of oxidative stress, relatively little is known concerning when, where, and how much oxidative stress the body undergoes during physiologically taxing conditions, such as disease or exercise (44). Nevertheless, there is a degree of overlap between the cytokine response seen following exercise and the response to trauma (34). These similarities establish exercise as a physiologically traumatic event and underscore the need for further investigation into the body's adaptive response to it.

The response of equine athletes to acute, high-intensity exercise is still poorly understood. While it is known that mRNA expression of inflammatory cytokines including interferon-γ, tumor necrosis factor-α, interleukin-6, and interleukin-1 increase in plasma following exercise (25), the impact of these responses on functional changes in protein expression, and ultimately their contribution to articular changes in the highly mobile joints, remain unclear. This is an important question because articular changes leading to osteoarthritis (OA) can often result in the termination of a horse's athletic career. Articular inflammation in horses is associated with elevated local biomarkers of inflammation and oxidative stress (13, 20, 22). In particular, PGE_2 (7) and NO (17) are recognized as being key participants involved in the disease pathway. Several biomarkers of cartilage metabolism and turnover are also influenced by exercise (4, 11). Glycosaminoglycans (GAG) have been used as direct biomarkers of cartilage metabolism (31) and thus can be useful indicators of the response of cartilage to a particular stimulus such as exercise. It is clear that exercise can result in an upregulation of various biochemical compounds involved in joint disease; however, to the authors' knowledge, a comprehensive examination of the response of oxidative and inflammatory biomarkers to exercise in horses has yet to be undertaken.

The objective of this study was to characterize a time course of inflammatory and oxidative markers in both plasma and synovial fluid (SF) following an acute bout of high-intensity exercise in horses. It was hypothesized that a high-intensity bout of exercise would result in increased concentrations of plasma and SF biomarkers of inflammation and oxidative stress.

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MATERIALS AND METHODS

Experimental animals. All experimental procedures and protocols were approved by the Nutraceutical Alliance Animal Care Committee before the beginning of this study in accordance with the Ontario Animals for Research Act. Eight horses of mixed breed, gender, and age were used (Table 1). All horses were clinically normal and had no known history of chronic joint inflammation or lameness. Owners provided written informed consent before the inclusion of their horse in the study. The horses included in this study were used for pleasure riding and light lessons. The approximate workload of these horses was between 2 and 5 h per week consisting of walking (30%), trotting (55%), and cantering (10%) and skill work including low-level jumping and dressage (5%). Four horses were randomly assigned to an exercise group (Ex) and four to a control group (Co). *Exercise horse 1* only provided SF samples to the 1-h time point but provided blood samples at each sampling time. *Control horse 8* was removed from the study due to intractability with the procedures and was not replaced. Thus for SF in the Ex group $n = 4$ at baseline (BL), 0.5, and 1 h after which $n = 3$, and in the Co group $n = 3$ at all times for both blood and SF samples.

Exercise. Horses were galloped in pairs on a half-mile dirt track. They were each fitted with a heart-rate monitor (Polar Electro, Lachine, QC, Canada). They were galloped for one lap around the track, with rider encouragement, at near maximal effort (at a target heart rate of 180 beats/min) and then walked until their heart rates recovered to ~100 beats/min (~4 min of walking). This process was repeated until their time around the track increased by 10% over their fastest lap. Refer to Table 1 for the number of laps completed by each horse.

Sample collection. Baseline blood and SF samples were taken ~24 h before exercise (BL) and then 0.5, 1, 2, 4, 8, and 24 h after cessation of exercise. The sampling time course was also conducted in the unexercised Co group.

Blood samples. Approximately 30 min before venipuncture, Emla cream (2.5% lidocaine and 2.5% prilocaine; AstraZeneca, Mississauga, ON, Canada) was applied directly to the skin (from which the haircoat had been clipped) as a topical anesthetic. Blood samples were collected directly into sodium heparin vacutainers (Becton-Dickenson, Mississauga, ON, Canada) from the jugular vein immediately before arthrocentesis using a 21-G, 1-in. multiple sample needle. Blood samples were kept chilled until processing.

SF samples. The hair coat from an area ~4 × 5 cm was clipped over the left carpal joint. Emla cream was applied as a topical anesthetic on the skin ~30 min before the arthrocentesis. The carpal joint was sterilized using iodine scrub and 99% alcohol. One to three milliliters of SF were aspirated from the intercarpal joint using a 22-G, 1-in. needle into a sterile 3-ml syringe and transferred into a sodium heparin vacutainer. SF samples were kept chilled until processing.

Sample processing. Blood and SF samples were centrifuged at 6,000 *g* for 15 min. Effluent was transferred into Eppendorf tubes and stored at −20°C until analysis.

Table 1. *Description of horse age, gender, breed, and number of laps run during the exercise test*

Horse	Breed	Gender	Age	Group	Laps
1	Morgan	Gelding	17	Ex	3
2	Oldenberg	Mare	7	Ex	3
3	Thoroughbred	Gelding	8	Ex	4
4	Thoroughbred	Gelding	7	Ex	4
5	Halflinger X	Mare	5	Co	NA
6	Quarter horse	Gelding	7	Co	NA
7	Morgan	Gelding	16	Co	NA
8	Andalusian	Gelding	7	Co	NA

Ex, exercise group; Co, control group; NA, not applicable.

Analysis of biological samples. Plasma samples were analyzed directly, whereas SF samples were pretreated with hyaluronidase to improve assay precision (18). Hyaluronidase solution (4 mg/ml) was prepared in PBS. SF samples were thawed to room temperature and centrifuged for 10 min at 10,000 rpm. One-hundred microliters of supernatant were then mixed with 100 μl of hyaluronidase solution.

Samples were analyzed for NO (Griess Reaction; Molecular Probes, Eugene, OR), total antioxidant status (TAS; Cayman Chemical, Ann Arbor, MI), glycosaminoglycan (GAG; dimethyl methylene blue) (5, 14), and prostaglandin E_2 (PGE_2; DetectX Prostaglandin E_2 Enzyme Immunoassay; Arbor Assays, Ann Arbor, MI). These particular types of assays do not appear to be species specific and have previously been used in equine research (3, 8, 35).

Statistics. One-way repeated-measures ANOVA was performed for each treatment to determine the within group effect of time. Post hoc comparisons were made against baseline. Two-way repeated-measures ANOVAs were performed to determine between group treatment effects at each time point. When a significant *F*-ratio was obtained, the Holm-Sidak correction was applied. Outliers were defined as being 4 SD beyond the mean of their treatment group, and the effect of their removal is included in RESULTS. Data are presented as means ± SE. $P \leq 0.05$ was considered significant.

RESULTS

TAS. Co horses had significantly higher plasma TAS than the Ex horses at BL (2 ± 0.08 vs. 1.3 ± 0.029 mM; $P = 0.02$), likely due to the aberrantly low value of *horse 2* whose TAS concentration at BL was 0.5 mM. This value was more than 3 SD below the mean, and when it was removed from the analysis, no significant differences between the Co and Ex groups were found (Table 2).

SF TAS concentration ([TAS]) did not differ within or between groups.

NO. Plasma NO concentration ([NO]) was significantly higher in Ex horses at 1 h compared with BL (Table 2; $P = 0.04$). Plasma [NO] in the Ex group was significantly higher than in Co at 0.5 h ($P = 0.03$), 1 h ($P = 0.05$), and 2 h ($P = 0.02$) (Table 2; Fig. 1).

SF [NO] did not differ within or between groups.

PGE_2. Plasma PGE_2 concentration ([PGE_2]) in the Ex group was significantly higher than in the Co group at 0.5 h ($P < 0.001$), and 1 h ($P = 0.003$) (Table 2; Fig. 2).

SF [PGE_2] in the Ex group was significantly higher than Co at 8 h ($P = 0.03$) (Table 2; Fig. 3).

GAG. There was a significant increase in SF GAG concentration ([GAG]) in the Ex horses at 8 h compared with BL ($P = 0.02$) (Table 2; Fig. 4). No significant differences in SF [GAG] were observed between Ex and Co at any time. This was likely due, at least in part, to an aberrantly high SF [GAG] in horse 5 at 8 h (766 μg/ml), which was beyond 4 SD of the mean. When this point was removed, the Ex group SF [GAG] at 8 h was significantly higher than that of Co ($P = 0.003$) (Table 2; Fig. 4).

Plasma GAG levels did not differ within or between groups.

DISCUSSION

A growing body of evidence supports the hypothesis that high-intensity exercise contributes to a postexercise inflammatory state, mediated at least in part by a cytokine profile akin to that seen during sepsis or trauma (34, 36). Our data further support this paradigm and indicate a time course of transient systemic inflammation that was evident 30 min following

Table 2. *Plasma and SF markers of inflammation, oxidative stress, and cartilage breakdown in Co and Ex horses*

	Time, h						
	BL	0.5	1	2	4	8	24
P TAS, mM							
Co	2.2 ± 0.08	1.9 ± 0.05	1.8 ± 0.15	1.5 ± 0.04	1.8 ± 0.04	1.9 ± 0.06	2.1 ± 0.20
Ex	1.6 ± 0.12	1.7 ± 0.17	1.8 ± 0.17	1.7 ± 0.10	1.5 ± 0.14	1.8 ± 0.29	1.7 ± 0.12
SF TAS, mM							
Co	1.0 ± 0.17	1.7 ± 0.27	1.9 ± 0.61	2.4 ± 0.81	1.9 ± 0.40	2.3 ± 1.0	1.9 ± 0.70
Ex	1.7 ± 0.63	1.9 ± 0.26	0.7 ± 0.11	1.2 ± 0.32	1.3 ± 0.20	1.9 ± 0.24	2.6 ± 0.24
P NO, μM							
Co	0.8 ± 0.09	1.6 ± 0.40	1.5 ± 0.43	1.1 ± 0.36	1.2 ± 0.23	0.7 ± 0.09	0.9 ± 0.09
Ex	1.5 ± 0.27	2.7 ± 0.54†	3.0 ± 0.55*†	2.3 ± 0.20†	1.8 ± 0.13	2.0 ± 0.28	1.2 ± 0.28
SF NO, μM							
Co	1.6 ± 0.11	7.4 ± 3.38	2.1 ± 0.60	1.6 ± 0.23	5.2 ± 2.74	13.1 ± 11.73	5.0 ± 3.11
Ex	4.7 ± 1.71	4.8 ± 1.84	11.5 ± 4.56	2.4 ± 1.21	3.4 ± 0.70	3.6 ± 0.40	8.4 ± 5.06
P GAG, μg/ml							
Co	135 ± 12.1	138 ± 27.1	195 ± 24.5	146 ± 9.8	133 ± 43.8	93 ± 29.2	150 ± 89.5
Ex	97 ± 16.0	151 ± 20.6	106 ± 8.4	100 ± 27.1	199 ± 87.9	264 ± 69.6	301 ± 118.0
SF GAG, μg/ml							
Co	99 ± 4.2	141 ± 28.4	146 ± 12.2	102 ± 3.5	192 ± 55.2	99 ± 8.8	137 ± 42.1
Ex	79 ± 11.7	102 ± 23.4	105 ± 14.9	81 ± 30.3	76 ± 10.7	356 ± 133.2*†	197 ± 72.3
P PGE_2, pg/ml							
Co	46 ± 14.6	13 ± 13.3	28 ± 13.1	60 ± 4.4	56 ± 5.5	51 ± 13.7	52 ± 0.4
Ex	60 ± 4.6	66 ± 6.2†	61 ± 1.8†	63 ± 3.1	66 ± 1.7	60 ± 3.6	60 ± 1.3
SF PGE_2, pg/ml							
Co	79 ± 30.0	72 ± 24.5	111 ± 17.8	104 ± 17.4	123 ± 6.2	75 ± 9.3	88 ± 17.3
Ex	112 ± 16.1	187 ± 42.2	221 ± 86.6	129 ± 14.3	122 ± 8.1	263 ± 132.3†	130 ± 7.3

Values are means ± SE. For plasma (P): Ex, $n = 4$; Co, $n = 3$; for synovial fluid (SF): Ex, $n = 4$ until 1 h, $n = 3$ between 2 and 24 h; Co, $n = 3$. TAS, total antioxidant status; NO, nitric oxide; GAG, glycosaminoglycan. *$P \leq 0.05$, significantly different from baseline (BL). †$P \leq 0.05$, significantly different from Co.

exercise and that lasted for ~2 h. This was followed by a localized articular inflammation (as evidenced by elevated PGE_2) and increased accumulation of GAG in SF 8 h after cessation of exercise.

Biomarkers significantly influenced by high-intensity exercise in the current study included NO, PGE_2, and GAG. NO is a by-product of L-arginine metabolism (21) and can itself result in oxidative stress (42). In the current study, plasma NO was elevated in exercised horses between 0.5 and 2 h following exercise. This is consistent with previous reports of increased NO in horses at 5 and 30 min (1) and 2 h (24) postexercise. Skeletal muscle produces small amounts of NO during normal metabolism; however, during exercise this production is markedly amplified (37). The relative contributions of NO produced during and after exercise in the current study are not known. However, NO synthase, the enzyme responsible for catalyzing formation of active NO, is a key factor in sweat production in human athletes and is substantively upregulated during exercise (2). Thus it can be reasonably predicted that NO produced during exercise contributed to the elevated NO observed in the current study.

The role of elevated NO during and following high-intensity exercise requires further study. Exogenous provision of nitrate,

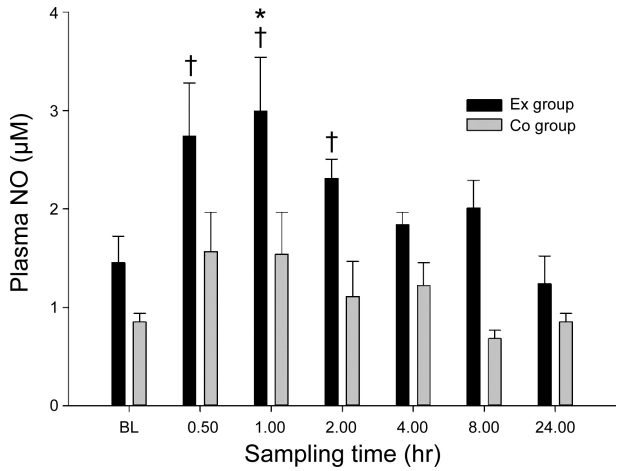

Fig. 1. Data are means ± SE of plasma nitric oxide (NO) concentration before and at selected time points following high-intensity exercise in exercised (Ex) horses ($n = 4$) and control (Co) horses ($n = 3$) that underwent the same sampling time course but were not exercised. *$P \leq 0.05$, significantly different from baseline (BL). †$P \leq 0.05$, significantly different from Co.

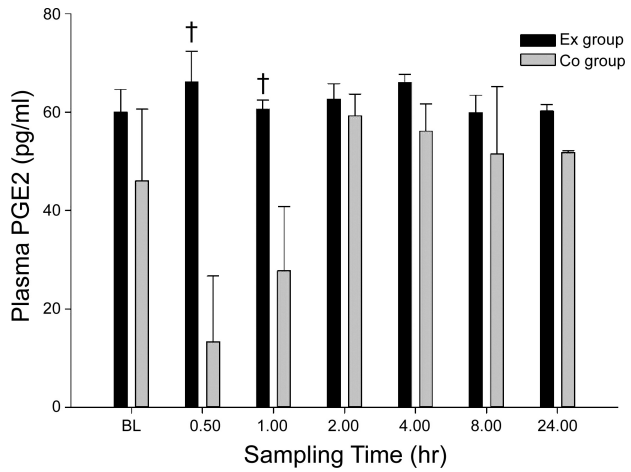

Fig. 2. Data are means ± SE of PGE_2 concentration before and at selected time points following high-intensity exercise in Ex horses ($n = 4$) and Co horses ($n = 3$) that underwent the same sampling time course but were not exercised. †$P \leq 0.05$, significantly different from Co.

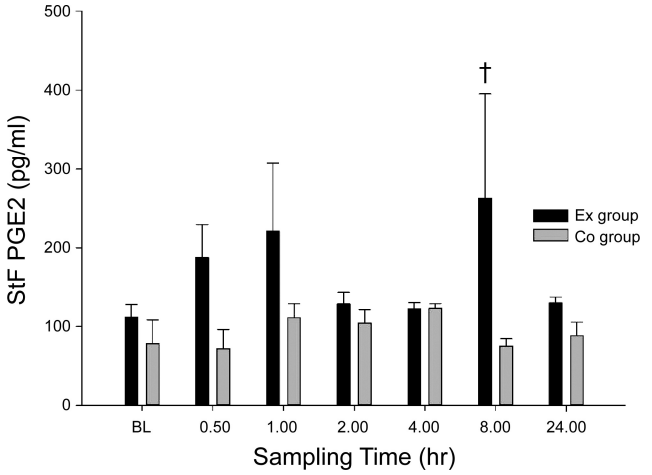

Fig. 3. Data are means ± SE of synovial fluid (SF) PGE_2 concentration before and at selected time points following high-intensity exercise in Ex horses (n = 4 until 1 h; n = 3 between 2 and 24 h) and Co horses (n = 3) that underwent the same sampling time course but were not exercised. †$P \leq 0.05$, significantly different from Co.

a stable precursor to NO, to human athletes results in marked improvement in maximal sprint and high-intensity intermittent running performance (41) and significantly improved maximal work rate in untrained humans undergoing 3 wk of sprint interval training (33). Furthermore, inhibition of NO synthase in humans blunted the beneficial effects of periodic whole body acceleration on recovery from unaccustomed eccentric exercise (28). In equines, a significant linear relationship between postexercise body temperature and NO metabolites can be observed (1) indicating a role of NO in thermal adaptation to exercise. These data suggest that NO production may play an important beneficial role in adaptation to and/or recovery from high-intensity exercise, which may be dampened by provision of exogenous antioxidant substances.

PGE_2 is a prototypical proinflammatory eicosanoid, which is a central chemical in the propagation and perception of inflammation and pain (15, 23). Alterations in both plasma and SF concentrations of PGE_2 were detected in our study. Plasma PGE_2 was elevated above the Co between 0.5 and 2 h following exercise. PGE_2 plays a complex role in adaptation to and recovery from high-intensity exercise. Studies in untrained humans corroborate our observed increase in postexercise plasma PGE_2 (29). Inhibition of this increase resulted in concurrent blunting of lipid mediators associated with the resolution of inflammation and recovery from exercise. This points to a direct mechanistic connection between elevated plasma PGE_2 postexercise and induction of physiological recovery from exercise.

PGE_2 in SF is an established biomarker of articular inflammation and joint disease (11, 22, 32). It is reported to be an excellent biomarker for the prediction of joint disease in horses (3) and is significantly elevated in the SF of equine joints that have undergone a traumatic injury (13). While the increase in plasma PGE_2 returned to preexercise levels by 2 h postexercise in the current study, SF PGE_2 did not peak until 8 h postexercise. The physiological significance of this increase is not completely understood and requires further study. We propose that the initial elevations in plasma levels of PGE_2 may have triggered inflammatory processes within the joint leading to PGE_2 production by synoviocytes and/or chondrocytes resulting in the lag time before the increase in SF PGE_2 became apparent. Future research investigating the impact of systemic PGE_2 production on the ensuing increase in SF PGE_2 will help to clarify whether the stimulus for joint inflammation following high-intensity exercise is chemical or mechanical in nature.

GAGs are structural breakdown products of the large aggregating proteoglycan molecules, which are present in most connective tissue. They play an essential role in maintaining hydrostatic tension in cartilage and are released during catabolism of proteoglycan. In SF, GAGs have been identified as potential biomarkers of proteoglycan breakdown (9). Proteoglycan fragments have been documented to increase in humans following various types of exercise (39) and in patients following traumatic injury to the knee, being elevated in some patients for up to 4 yr following an injury (27). However, reports of alterations in GAGs following exercise or injury are inconsistent throughout the literature. While no significant difference between SF GAG concentrations in equine OA-affected joints compared with unaffected contralateral joints are reported by some (12), others (11) report SF GAG to be significantly increased by both disease state and exercise in horses. There are several methodological differences between these studies which may account for the discrepancies concerning their results. The latter (11) used horses in which OA was induced by the surgical creation of an osteochondral fragment, perhaps controlling disease development and providing a more standardized environment than what would be possible when using clinical cases reported in the former (12). It is also possible that the use of the contralateral joint in OA-affected horses (12) cannot be regarded as representative of, or equivalent to, a healthy equine joint. In humans, there are no differences in SF GAG concentration before and 30–60 min after bouts of various forms of exercise (soccer, treadmill running, and road running) (38). However, based on our results the timing of these samples would not have been adequate to observe any potential SF GAG alterations. Our results indicate an ~8-h delay between the termination of exercise and a significant increase in SF GAG concentration. The physiological implications of the postexercise rise in SF GAG observed

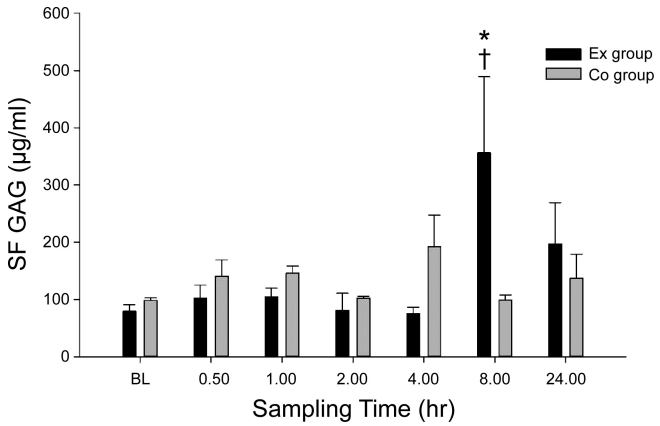

Fig. 4. Data are means ± SE of SF glycosaminoglycan (GAG) concentration before and at selected time points following high-intensity exercise in Ex horses (n = 4 until 1 h; n = 3 between 2 and 24 h) and Co horses (n = 3) that underwent the same sampling time course but were not exercised. *$P \leq 0.05$, significantly different from BL. †$P \leq 0.05$, significantly different from Co.

in our study are not known and should be explored in further research. Others have suggested that increased postexercise GAG in horses indicates accelerated breakdown of the cartilage matrix due to the chemical and/or mechanical stress imposed by exercise (11). However, it is not known to what extent proteoglycan synthesis is also occurring, a physiological process that is known to be increased with exercise (43). Future research should explore the net effect of exercise on proteoglycan synthesis and breakdown to better understand the overall effects of exercise on cartilage health.

In the current study, both PGE_2 and GAG took a full 8 h to become elevated following exercise. The protracted appearance of these biomarkers in SF compared with plasma may suggest that cell signaling requires time to be translated to cells within the joint in response to exercise. Alternatively, it is possible that the production or accumulation of articular inflammatory markers and breakdown products, instigated during exercise, is more gradual than what occurs systemically. Further characterization of other inflammatory markers and enzymatic indicators of cartilage turnover such as matrix metalloproteases and cartilage oligomeric matrix protein (6, 31) will be necessary to accurately characterize the local articular response to exercise.

Although exercise is a physiological stressor that induces systemic and localized oxidative stresses (10, 44), we found no changes in plasma or SF TAS concentrations. It is known that exercise of differing intensities results in varied measures of oxidative stress (26). Therefore, the lack of change in serum TAS could indicate that, although our exercise test was of a high-intensity, it was not sufficiently rigorous, or was too short in duration, to stress the antioxidant equilibrium within the body to such a degree that a deficit was apparent.

Limitations. This was a pilot study intended to help define the time course of measure in oxidative stress markers and inflammation markers in blood and SF; therefore, the lowest numbers of animals was used in each group while still meeting statistical and animal welfare criteria.

One may question if breed differences play into the present results. There are differences in skeletal muscle fiber type and myosin ATPase activity between different breeds of horses particularly thoroughbreds and other less athletic breeds (16, 40). These structural differences within the muscle of different equine breeds may result in functional differences in exercise capacity. Thus, to account for these breed differences, the exercise test employed in this study based fatigue on individual horses' exercise tolerance. Additionally, breed differences to the very high-intensity exercise, such as the type used in the current study would be minimal in comparison to the magnitude of the physiological responses observed in the postexercise phase.

Conclusion. An acute bout of high-intensity exercise in horses resulted in a time course of transient systemic inflammatory markers characterized by elevated levels of plasma NO and PGE_2 beginning approximately half an hour following exercise and lasting ~2 h. This systemic inflammatory state was followed by increased GAG and PGE_2 within the joint at 8 h. These results provide insight into the systemic and articular biochemical responses of horses to acute, repeated bouts of high-intensity exercise.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.I.L. and W.P. conceived and designed research; J.L.M., M.I.L., and W.P. performed experiments; J.L.M. and M.I.L. analyzed data; J.L.M. interpreted results of experiments; J.L.M. prepared figures; J.L.M. drafted manuscript; J.L.M., M.I.L., and W.P. edited and revised manuscript; J.L.M., M.I.L., and W.P. approved final version of manuscript.

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