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Original Research

## The Effect of Exercise and Nutritional Supplementation on Proinflammatory Cytokine Expression in Young Racehorses During Training

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## ABSTRACT

The inflammatory response to vigorous exercise ranges from the mild symptoms of delayed-onset muscle soreness to debilitating injuries affecting soft tissue, joint, and bone. Although there is a great deal of information available on the inflammatory response to exercise in human athletes, less information is available regarding the inflammatory response to exercise in young horses undergoing training for racing careers. Here, we assessed the cytokine response to exercise in a group of young Thoroughbred racehorses during their initial training. Because there is interest in non-pharmacologic approaches to control or ameliorate exercise-induced inflammation, we also examined the anti-inflammatory effect of a nutritional supplement fed to half of the horses undergoing training. Twenty-five Thoroughbred horses aged 2 years were followed through their initial race training. Peripheral blood samples were collected at various times during the exercise for the quantitation of lactic acid, oxidative stress, and inflammatory cytokine gene expression. There was an intensity-dependent effect of exercise on lactate, malondialdehyde, and proinflammatory cytokine gene expression. Although training itself was associated with an overall reduction in inflammatory markers, horses receiving the supplement exhibited further reductions in their indicators of inflammation. As such, this study provides novel evidence of nutritional supplementation reducing postexercise inflammation.

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### 1. Introduction

Lameness in horses is the most common cause of diminished athletic performance, as well as of economic and animal loss [1]. In the early 1980s, it was reported that 67.6% of the lost training days for racehorses was because of lameness [2]. Recent data indicated that more than 80%

of total days lost from training Thoroughbred racehorses aged 2 and 3 years were because of lameness issues [3]. In another study, race records showed that less than 80% of the 2-year-old racehorses in training completed their training and went on to race [1]. Although the causes of lameness are varied, increased attention has been focused on the role of inflammatory mediators in this process [4-6]. Inflammation is associated with the production of proinflammatory cytokines, which are small hormone-like proteins produced in response to external stimuli. Proinflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are induced by vigorous

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exercise [7-10]. The consequences associated with this increased expression of inflammatory mediators post-exercise can range from the mild symptoms of delayed-onset muscle soreness [11] to debilitating problems related to soft tissue, joint, and bone damage [12-14]. Although an acute inflammatory response is a part of the healing process, chronic inflammation can lead to tissue destruction, subsequent poor performance, and perhaps an end to the athletic career [15]. Although numerous studies have evaluated proinflammatory cytokine expression in response to different modes and intensities of exercise in humans [16-18], limited work has been done in horses [19-21]. In general, equine athletes exhibit evidence of increased inflammation subsequent to exercise as characterized by increased expression of mRNA for TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in peripheral blood mononuclear cells [21,22]. However, most of these studies have focused on treadmill-based exercise protocols typically involving a single bout of intensive exercise. The use of a treadmill allows for standardized testing, precise control of exercise parameters, and frequent blood collection and other sampling [23]; however, there are both physiological and locomotion differences when comparing exercise on the track with that on a treadmill [24]. Differences between treadmills can also affect some measurements of performance [25]. Because all young Thoroughbred horses are trained on tracks, we were interested in using this approach to assess proinflammatory cytokine gene expression during their exercise at a training facility.

Given the negative impact of exercise-induced inflammation, there is a widespread use of nonsteroidal anti-inflammatory drugs (NSAIDs) in racehorses [26-33]. A recent study reported that the plasma concentrations of NSAIDs were highest in injured horses [33]. Although anti-inflammatory medication may relieve muscle soreness in human athletes, they do not affect recovery of muscle function or markers of inflammation [34]. Besides concerns regarding the possibility that excessive use of NSAIDs may contribute to race-related injuries [33], long-term use of NSAIDs is associated with other adverse effects [35]. Thus, there is growing interest in nonpharmacologic approaches to ameliorate exercise-induced inflammation [36-39]. Various dietary supplements have been proposed to alleviate exercise-related injuries and illnesses [40-50]. Although many of these same compounds have been used in horses [37,48-58], their effect on exercise-induced proinflammatory responses has not been investigated in detail [55]. Here, we have examined the effect of a nutritional supplement on the proinflammatory response to exercise in 2-year-old Thoroughbred racehorses in training. A second population of 2-year-old Thoroughbred racehorses training at the same facility was not given the supplement, and these horses were included as control horses.

## 2. Materials and Methods

### 2.1. Horses

Twenty-five Thoroughbred horses aged 2 years were housed and trained at a farm in Darlington, MD. A dirt oval track of 1.0 km (total length) was used for training and

tests. A licensed trainer oversaw the training of the horses. All horses remained under routine management, with veterinary care provided by the farm's regular veterinarian. Breaking to saddle and basic preparatory fitness work began when the horses were aged 18-21 months which lasted for approximately 8 weeks. In general, the training of the 2-year-old horses consisted of 6 days/week slow cantering (7-9 m/s) for 4 weeks, fast cantering (9-11 m/s) for another month, and then continuation of fast cantering with galloping (14-15 m/s) three times a week. A daily exercise log was maintained, with specific mention of any days of missed training and the reason. Each horse was trained according to its individual abilities, as assessed by the trainer, to achieve each horse's optimal race potential. All training and testing of the horses occurred at the same training facility. All horses received the same daily rations. Health records were maintained for all horses, including specific information regarding injuries and treatments. The use of any medication was duly noted, and any horse receiving NSAIDs or other treatments within a week of the exercise test was excluded from testing.

### 2.2. Nutritional Supplement

The nutritional supplement (Table 1) tested is designed to reduce joint injuries by preventing inflammation and enhancing energy production. The supplement was fed to the horses ( $n = 13$ ) twice daily, with meals provided everyday throughout the study period. Control horses ( $n = 12$ ) received daily meals without the supplement. Horses were randomly assigned to treatment or control groups at the beginning of their training period and remained on treatment throughout the testing period.

### 2.3. Exercise Tests and Blood Sampling

The study consisted of four tests of increasing exercise intensity (Table 2) during the training period. These tests coincided with each of the phases of the training period. At the beginning of the training period, all horses were initially sampled at rest to assess baseline proinflammatory cytokine responses before training. Each test consisted of an initial warm-up period, followed by galloping at the specified speed and distance for that particular test, and then by a cool-down period (Table 2). The first test was performed before the beginning of the initial training period. All horses completed the tests on the scheduled day. The first peripheral blood samples (Pre) were collected within 1 hour of the horses beginning the exercise test. A second set of samples were collected within 4-5 minutes of the completion of the exercise test (Post). Owing to the nature of training on a track, it was not possible to collect

**Table 1**  
Supplement components<sup>a</sup>

1.6 g Curcumin (BCM-95)
1.6 g <i>Boswellia</i> (Bospure)
400 mg coenzyme Q10 (HydroQSORb)
4 g Glycine propionyl-L-carnitine HCL (GlycoCarn)
10 g D-Ribose

<sup>a</sup>Double diamond (peak performance), Equine Nutraceuticals, L.L.C., Franklin Lakes, NJ.

**Table 2**  
Exercise tests

	Date	Distance	Speed
Test 1	June 12	3.2 km	8.0 m/s
Test 2	July 31	400 m	13.3 m/s
Test 3	August 14	600 m	14.3 m/s
Test 4	August 21	1,000 m	14.2 m/s

all samples at the same time, but every effort was made to collect them within a few minutes of each other. A third set of samples were collected at 2 hours postexercise when the horses were in their stall (after 2 hours). Specific notations were made on the length and time of the workout session during which the sample was collected. This sampling schedule was repeated for each of the subsequent tests at the intervals indicated in Table 2. Each test consisted of an initial warm-up period, followed by the specified speed and distance. The final test was performed at the end of the training period.

#### 2.4. Blood Measures

Peripheral blood (~3 mL) was aseptically collected from the jugular vein into a sodium-heparinized evacuated tube using an 18-g needle and hub. Lactate was measured in plasma using an automated analyzer (Lactate Plus; Nova Biomedical, Waltham, MA). Results are expressed in millimeter concentrations. The Lactate Plus unit provides repeatable measures with precision, as has been reported for similar units when compared with traditional laboratory-based tools used for the assessment of blood lactate [59]. However, it has been suggested that such tools may not have precise linearity when used to assess lactate in blood samples obtained under extreme intensities of exercise. Because horses were pushed hard in the current design, yielding lactate values close to 20 mM, this potential limitation should be noted [60]. Malondialdehyde (MDA) was measured in plasma following the modified procedures of Jentzsch et al. [61], using reagents purchased from Northwest Life Science Specialties (Vancouver, WA). Specifically, 75  $\mu$ L of plasma was added to microcentrifuge reaction tubes, with the addition of 3  $\mu$ L of butylated hydroxytoluene in methanol to minimize ex vivo lipid peroxidation. Seventy-five microliters of 1-M phosphoric acid and 75  $\mu$ L of 2-thiobarbituric acid reagent was added to each reaction tube and mixed thoroughly. Samples and reagents were then incubated for 60 minutes at 60°C. Following incubation, tubes were removed, and the reaction mixture was transferred to a microplate. The absorbance was then read using a spectrophotometer at both 535 and 572 nm to correct for baseline absorption. MDA equivalents were calculated using the difference in absorption at the two wavelengths. Quantification was performed with a calibration curve using tetramethoxypropane in a stabilizing buffer. Samples were analyzed in duplicate on first thaw. The coefficient of variation was 5.6%. Results are expressed in micrometer concentrations.

Peripheral blood (~3 mL) was aseptically collected from the jugular vein into the PAXgene tube (Qiagen, Valencia,

CA) using an 18-g needle and hub. Once collected, the PAXgene tubes were inverted 20 times to ensure proper mixing; they were then kept at room temperature for 24 hours and then refrigerated until shipped. If held for more than 7 days, the tubes were stored frozen (-4°C) until shipped. Each tube was labeled with the following information: horse ID, date of collection (MM/DD/YY), and sample (pre, post, 2 hours).

#### 2.5. Gene Expression Analyses

Total RNA was isolated from the PAXgene tubes and analyzed for gene expression of proinflammatory cytokines and Lymphokine activated Killer (LAK) cell markers using real-time polymerase chain reaction and equine-specific primers and probes (Table 3) [62]. The relative quantitation method was used to analyze gene expression where the average of all the horses' initial pretraining samples was used as a calibrator against which all subsequent samples were compared [63]. The results were expressed as fold increases above that of the initial sample.

#### 2.6. Statistical Analysis

The effect of treatment and exercise on the lactate, MDA, and the expression of proinflammatory cytokines post-exercise was determined using a 3-way analysis of variance with exercise test, time (before, immediately after, and after 2 hours), and nutritional supplement included as the main variables. Results were considered significant at a  $P$  value  $\leq .05$  and power  $\geq .80$ . When necessary, data were transformed to obtain normality of distribution and equal variances.

### 3. Results

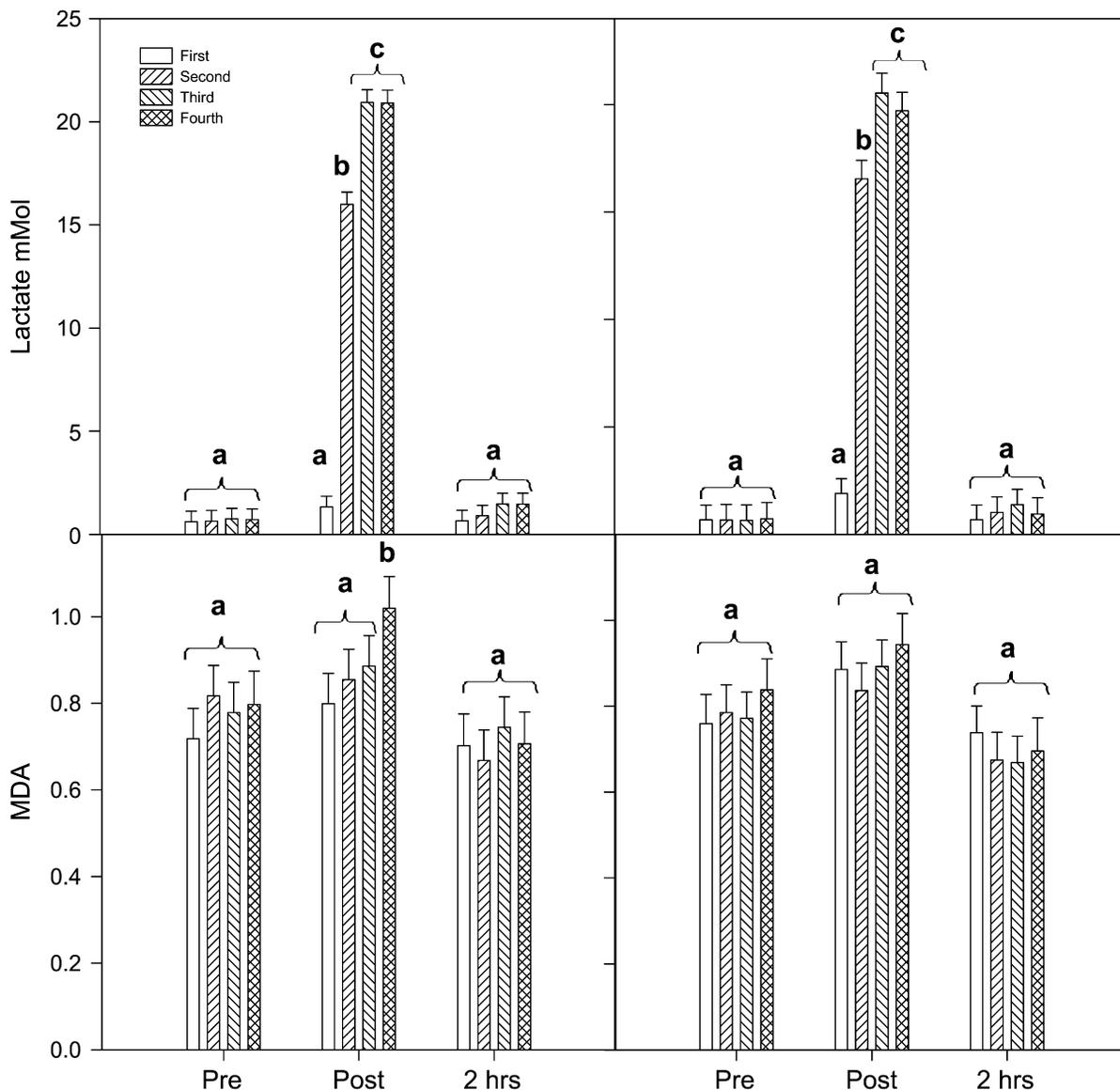
#### 3.1. Lactate and Lipid Peroxidation Postexercise

Figure 1 shows blood lactic acid (mM) and MDA ( $\mu$ M) concentrations throughout the exercise test for both the control (left panels) and supplemented (right panels) horses. As expected, lactic acid accumulation was observed immediately after exercise, which returned to pre-exercise levels after 2 hours. In general, there was an intensity-related increase in lactic acid production, with the third and fourth tests being different from the first and second test, and the second test being significantly different from the first. Although a similar pattern is observed for MDA, this intensity-related increase was only significant at the fourth test. The difference in the mean values between the control and supplement groups were not great enough to

**Table 3**  
RT-PCR analysis of gene expression

Gene Target	Function
IL-1	Proinflammatory cytokine
IL-6	Proinflammatory cytokine
TNF- $\alpha$	Proinflammatory cytokine
IFN $\gamma$	LAK cell marker
Granzyme B	LAK cell marker

IL-1, interleukin 1; IL-6, interleukin 6; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN $\gamma$ , interferon gamma; RT-PCR, real-time polymerase chain reaction.



**Fig. 1.** Exercise-induced expression of plasma lactate and malondialdehyde (MDA). Left panels: control. Right panels: supplement. Peripheral blood samples were collected directly into glass tubes at the times indicated, spun down, and frozen at  $-20^{\circ}\text{C}$  until further processed. Results are reported as millimeter for lactate and micrometer for MDA. Bars with the same letters are not significantly different.

exclude the possibility that the difference is just because of random sampling variability after allowing for the effects of differences in test and sampling time for lactate or MDA ( $P = .418$ ).

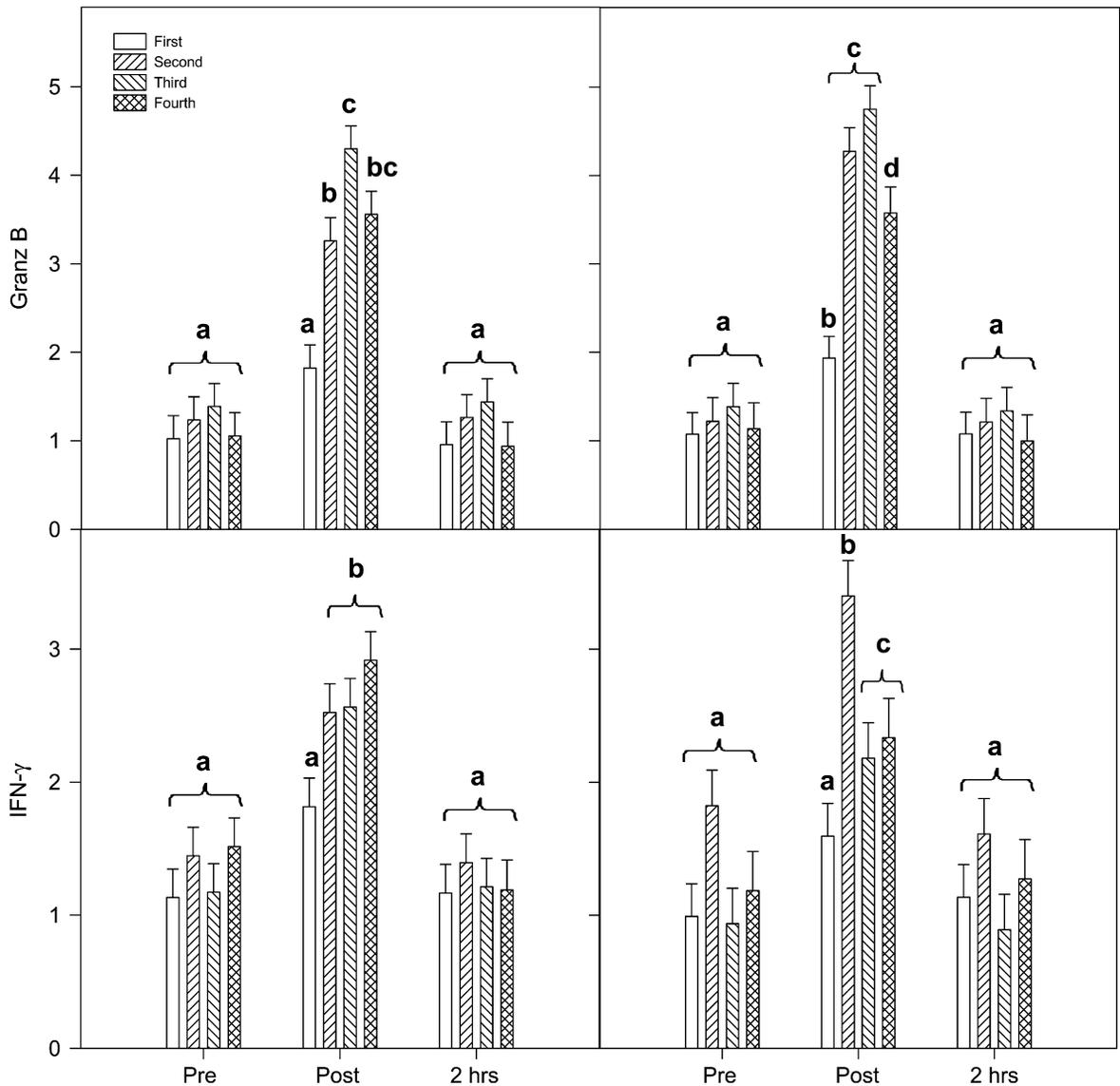
### 3.2. Immediate Postexercise Increase in LAK Cell Markers

We also observed an exercise-induced increase in granzyme B and interferon- $\gamma$  (IFN- $\gamma$ ) mRNA transcription (Fig. 2). Peak expression of both genes was seen immediately after the completion of the exercise test. This increase was intensity dependent with peak expression of IFN- $\gamma$  seen during the fourth test and granzyme B after the third test. Although the supplemented group (right panel) showed a lesser response at the latter two tests, this was

not significantly different from the control group (left panel).

### 3.3. Proinflammatory Cytokine Response Postexercise

There was an increase in proinflammatory cytokine IL-1 $\beta$  mRNA expression 2 hours after the exercise bout (Fig. 3), which was intensity dependent. There was an initial significant increase in TNF- $\alpha$  mRNA expression in the treated group following their first, low-intensity exercise bout. Afterward, there was no significant effect on TNF- $\alpha$  mRNA expression. There was also a significant decline in IL-1 $\beta$  gene expression at the Pre time point for those horses on the supplement, but not in the control group. We observed a similar decline in TNF mRNA expression in the



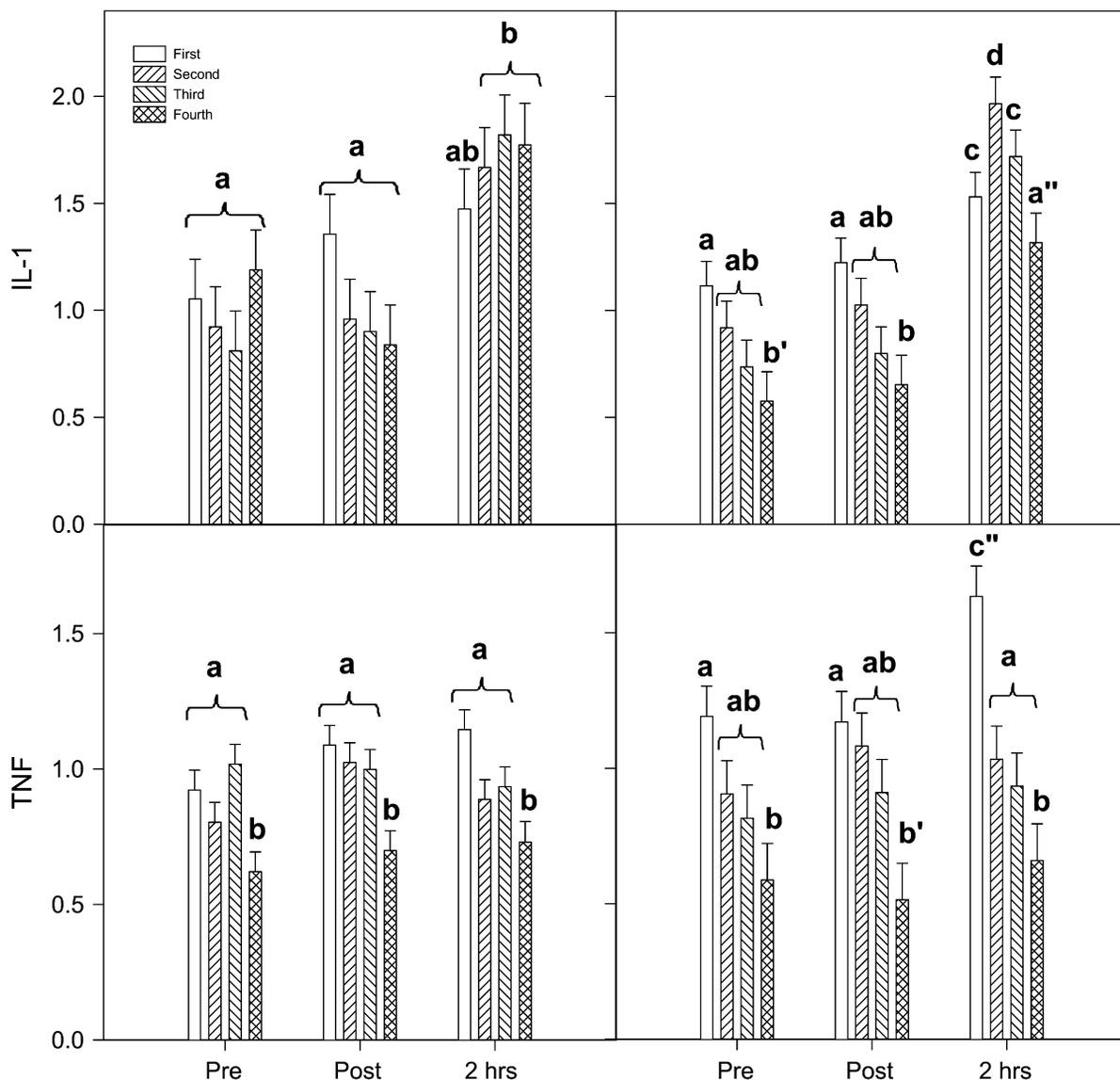
**Fig. 2.** Exercise-induced increase in LAK cell markers. Peripheral blood samples were collected directly into PAXgene tubes at the times indicated and later processed for real-time polymerase chain reaction (RT-PCR) analysis of granzyme B (Granz B) and interferon gamma (IFN $\gamma$ ) mRNA expression. Left panels: control. Right panels: supplement.

Pre time point of the supplemented group, but not the control group. There was a significant increase in IL-6 mRNA expression in both groups of horses over the six tests, although no specific effect of sample time (Fig. 4) was observed. However, TNF mRNA expression was inversely correlated with IL-6 mRNA expression ( $r = 0.253$ ,  $P < .001$ ).

#### 4. Discussion

Numerous studies have documented alterations in proinflammatory cytokine expression in response to exercise in humans [16-18,64]. Equine athletes likewise exhibit altered inflammatory responses subsequent to exercise [22,62,63,65-68]. The proposed mechanism for this exercise-induced inflammation is high-intensity training-induced muscle, skeletal, or joint trauma [14,69]. A role for

reactive oxygen species in exercise-induced damage to muscle has also been proposed [41,70,71]. Free radical generation can be estimated by the measurement of products of lipid peroxidation such as MDA whose level is increased in equine blood and tissues during exercise [72]. The accumulation of lactic acid is a functional measure of anaerobic metabolism associated with high-intensity exercise [73]. Together, these multiple insults activate circulating monocytes and other cells to produce proinflammatory cytokines [74]. These changes in proinflammatory gene expression occur in skeletal muscle and peripheral blood cells [62]. Peripherally produced cytokines can in turn augment or exacerbate localized inflammatory responses in joints and muscles, thus leading to an amplification of the inflammatory cascade [10]. Although most previous work has used treadmill-based exercise



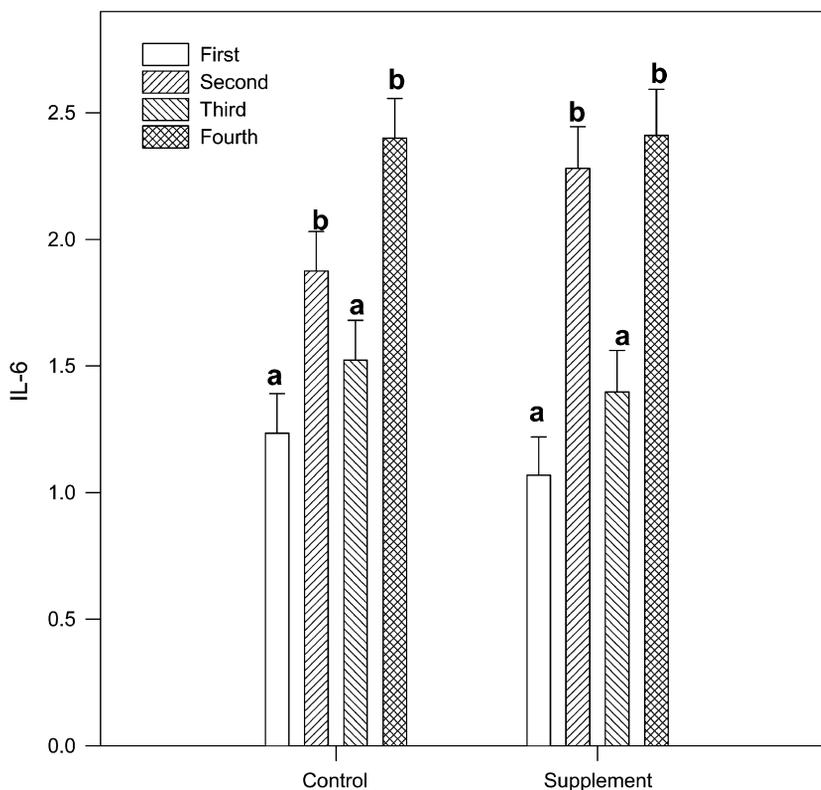
**Fig. 3.** Exercise-induced increase in proinflammatory markers. Peripheral blood samples were collected directly into PAXgene tubes at the times indicated and later processed for RT-PCR analysis of interleukin 1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA expression. Left panels: control. Right panels: supplement.

models, in this study, we have looked for similar effect in young Thoroughbreds exercising at a training facility.

For each of the tests performed, the initial effect of the exercise bout was the intensity-dependent accumulation of lactic acid. Because it was only possible to perform limited sampling on these horses, we could only detect lactate accumulation within 5 minutes of the completion of the speed portion of the exercise test. Thus, peak lactate production can only be estimated. Nevertheless, the levels of lactate in the blood sample were proportional to the effort expended in each of the tests, with maximal accumulation occurring in response to the higher speed tests. This relationship between peak lactate accumulation and exercise intensity is well established [73]. There was also an increase in MDA in the same postexercise sample, although this was only significant for the last test. A similar transient

increase in MDA was also observed in horses 5 minutes after high-speed treadmill exercise [75]. The role of reactive oxygen species in the mediation of exercise-induced oxidative damage to muscle has been well studied and is thought to contribute to the inflammatory response [72,76]. As each of the first four tests was of increasing intensity, it was not possible to use either of these measures as a means of assessing a conditioning effect. Instead, they provided evidence of the intensity of the exercise at the time of sample collection. There was no difference in lactate production between the third and fourth tests, likely reflecting the fact that they were performed at similar speeds.

Concurrent with the increase in lactate and MDA, we also observed a significant increase in two indirect markers for LAK activity in the horse, granzyme B and



**Fig. 4.** IL-6 mRNA expression increases over time. Peripheral blood samples were collected directly into PAXgene tubes at the times indicated and later processed for RT-PCR analysis of interleukin 6 (IL-6) mRNA expression. Tests with the same letter are not significantly different using a 2-way analysis of variance. There was no effect of treatment ( $P = .781$ ).

IFN- $\gamma$  [77], immediately after exercise. Increased LAK activity immediately following peak exercise has been reported for humans [64,78,79] and horses [67]. This increase in LAK function is likely the result of a repartitioning of the cells in response to exercise and the increased cytotoxic activity of the cells in response to increased cytokine or other mediator production [80-82]. The overall effect we observed was intensity-dependent, as there was no effect of the initial light exercise test on either marker, similar to what occurs to LAK function following low-intensity exercise in the horse [67]. There was no effect of exercise training on pre-exercise or baseline IFN- $\gamma$  and granzyme B expression throughout the training period. These results are consistent with data indicating that exercise training had no effect on natural killer (NK) cell activity in mice [83], although it is in contrast to other reports stating that exercise training could augment baseline NK cell function in humans and mice [84,85].

Two hours after completing the exercise tests, the horses exhibited increased expression of IL-1 $\beta$  mRNA in their peripheral blood. This parallels our previous results following treadmill- [62] and race-based exercise studies [86]. The expression of IL-1 $\beta$  was exercise intensity-dependent. This postexercise increase in IL-1 $\beta$  production is well documented [7-10]. The intensity-related association between cytokine production and markers of oxidative damage supports the notion that damage to muscle fibers stimulates the production of inflammatory cytokines [87].

Given the role of IL-1 $\beta$  in tissue repair [88], its increased expression postexercise is expected, although chronic or elevated expression of this cytokine can lead to degenerative changes and loss of function [69,88]. We did not see a significant increase in TNF- $\alpha$  mRNA expression at 2 hours after exercise, possibly because its expression had not yet peaked [62], although there is an overall trend for its expression to increase during the 2-hour sampling period. Limitations in the study design prevented us from collecting samples at later time points.

During the training period, there was a significant decrease in baseline expression of TNF- $\alpha$ , and this was also associated with an overall trend for decrease in the IL-1 $\beta$  baseline expression. This overall decline in proinflammatory cytokine expression was significant for horses receiving the supplement. It is known that adaptation to exercise leads to reduced inflammatory responses in trained human athletes [89,90]. Thus, exercise training is associated with an overall "anti-inflammatory state" [17,91]. A recent study reported that IL-6 and IL-6R were significantly upregulated in both humans and horses following training [92]. Here, too, we saw increased expression of IL-6 in both groups of horses. This upregulation of IL-6 expression in peripheral blood mononuclear cells in horses could reflect a mechanism that generated an anti-inflammatory environment, thereby reducing the expression of the proinflammatory cytokines. Our observation that IL-6 mRNA expression was inversely correlated

with proinflammatory cytokine mRNA expression is consistent with this proposed mechanism.

Because oxidative stress and inflammation have traditionally been associated with fatigue and impaired recovery from exercise [45], we evaluated the effect of the supplement on markers of inflammation (e.g., cytokines) and oxidative damage (MDA). The main difference between the supplemented horses and the control horses was the significant reduction in proinflammatory gene expression in the supplemented group. This was evidenced by both an overall decline in baseline IL-1 $\beta$  and TNF- $\alpha$  mRNA in the pre-exercise samples and a reduced postexercise increase in both IL-1 $\beta$  and TNF- $\alpha$  during subsequent exercise tests. The supplement contained *Boswellia* whose anti-inflammatory and proapoptotic activities have been well documented [93], with the primary mode of action appearing to be the inhibition of nuclear factor- $\kappa$ B [93,94]. Similarly, the anti-inflammatory activities of curcumin have been widely studied [95-97] and shown to affect intracellular signaling pathways involved in proinflammatory cytokine and other mediator production [98]. In vitro, curcuminoids neutralized free radical species generated by activated equine neutrophils portending a therapeutic possibility for equine pathologies associated with excessive inflammatory reactions [99]. Although there are no in vivo data for either curcumin or *Boswellia* in the horse, there is information regarding the other components of the nutritional supplement; coenzyme Q10 (CoQ10), glycine propionyl-L-carnitine (GPLC), and ribose. These nutrients have a fundamental role in cellular bioenergetics. GPLC transports fatty acids to the mitochondria for oxidation, CoQ10 is essential for adenosine-5'-triphosphate (ATP) production as a cofactor in the mitochondrial electron transport chain, and D-ribose is an intergral structural component of ATP. These three nutrients have been demonstrated to increase exercise capacity in humans [100-103] and equids [58,104], presumably by stimulating ATP production and recovery while supporting ATP energy substrates at the same time [105]. Daily ribose supplementation (0.07 g/kg BW, twice daily) for 14 days in the diet of exercising geldings resulted in lower blood ammonia-N and plasma lactic acid during recovery after a standardized exercise test. CoQ10 can also serve as a protective agent against lipid peroxidation [106] and has an anti-inflammatory activity [107]. There are no data in the literature on CoQ10 dosage in horses; our results indicated a marked increase from a baseline value of 0.187  $\mu$ g/mL to 0.407  $\mu$ g/mL at 30 days and 0.511  $\mu$ g/mL after 60 days at a daily dose of 800 mg [108]. Besides its role as a buffer in different tissues during ketosis and hypoxic muscular activity, carnitine protects membrane structures and reduces lactate production [109]. GPLC has been previously reported to lower resting concentrations of MDA, suggesting a potential antioxidant effect of this nutrient [110]. An anti-inflammatory function for carnitine has also been described in several model systems [111-113]. The concentration of carnitine in blood plasma of horses varies markedly between animals and with increasing exercise intensity [109]. Although acute exercise does not have a marked effect on the content of total carnitine in skeletal muscle, training elevates its total concentration in the middle gluteal muscle [114]. Oral supplementation with

10 g/d L-carnitine can elevate carnitine concentration in equine blood plasma [104]. The daily dose of carnitine used in this study was 8 g/d, which could achieve similar effects. Thus, the reduced proinflammatory cytokine response in the supplemented horses in this study likely reflected an adaptation to exercise, which was enhanced by the nutritional components present in the supplement. However, it is not possible to identify which specific component or components mediated this effect.

## 5. Conclusions

Although exercise-induced changes in cytokine gene expression have been widely studied using treadmill-based exercise tests, the cytokine response to race training had not been investigated in Thoroughbred racehorses. Here, we have shown that the exercise under race training conditions is associated with the temporal induction of cytokines characteristic of the initial elevation in LAK cell activity immediately after exercise and the subsequent expression of proinflammatory cytokines 2 hours later. These time- and intensity-dependent changes in cytokine gene expression parallel data from previous studies using treadmill-based exercise testing. We also observed signs of adaptation to exercise over the training period as indicated by an overall reduction in the expression of proinflammatory cytokines and increased expression of IL-6. Although dietary supplements have been used in horses in the past for various reasons, including performance enhancement, their effect on race training has not been investigated. The nutritional supplement used in the present study containing antioxidant nutrients and anti-inflammatory agents did not affect the immediate response to exercise; however, it was associated with an enhanced adaptation to exercise in terms of a significant reduction in proinflammatory cytokine expression before and after exercise. This underscores the potential for nutritional supplementation to reduce exercise-induced inflammatory pathologies in racehorses.

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