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Original Research

Effects of a Novel Dietary Supplement on Indices of Muscle Injury and Articular GAG Release in Horses

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ABSTRACT

This study determined the ability of an oral nutraceutical supplement to attenuate the oxidative stress and inflammation that occurs in muscles and joints with repeated bouts of high-intensity exercise in horses. The supplement, fed daily, was comprised of whole dried mushrooms, golden flaxseed, omega-3 fatty acids, plant-based enzymes, a melon-concentrate powder, and *Saccharomyces cerevisiae boulardii*. Ten horses participated in a partial cross-over design, with 7 horses completing the Control trial and 7 horses completing the supplement trial. Blood and synovial fluid samples (from the intercarpal joint) were taken before, and at 1 and 24 hours after a standardized, repeated high-intensity exercise test that was performed before supplementation and on the 22nd day of supplementation. At the end of the supplement trial exercise resulted in reduced concentrations of plasma markers of oxidative stress (decreased TBARS, with increased total antioxidant status and increased superoxide dismutase activity); there was no effect on plasma markers of muscle injury (creatine, creatine kinase, and aspartate aminotransferase) or inflammation (PGE_2, nitric oxide). Within synovial fluid, there was a tendency for increased superoxide dismutase activity, and decreased concentration of glycosaminoglycans. It is concluded that the supplement, when fed to horses as part of the normal diet for 23 days, was associated with reduced concentrations of markers of oxidative stress and inflammation in muscle and synovial fluid.

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1. Introduction

Historically, the main research focus on traditional (non-nutraceutical) nutritional supplements for horses has been on macronutrients (carbohydrates, fats, and protein or amino acids), trace minerals and electrolytes for their ability to improve health and performance. However, despite seemingly optimizing these aspects of nutrition, horses often do not appear to reach their genetic and/or physiological potential. Recent research efforts are increasingly directed towards examining the effects of nutraceuticals or functional foods on health, wellness, and physical and cognitive performance. These products are typically single animal or plant components, or blends of such components that are believed to exert wellness benefits that cannot be readily attributed to classical nutrition. Nutraceutical products often comprise a vast array of bioactive molecules that interact with numerous biochemical and signaling pathways in virtually every cell system within the body. The plant and animal parts from which these nutraceutical products are derived tend to have a long history of use in human and animal folklore medicine. At the same time, we have a poor understanding of these products because of their complex nature and myriad interactions. Therefore, research using such products is needed.

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There are numerous products in the veterinary nutraceutical supplements marketplace aimed at reducing the negative effects of oxidative stress and inflammation that occurs as a result of high-intensity exercise training. The rationale is that excessive oxidative stress can result in an exaggerated inflammatory response [1,2] and may be detrimental to normal healing of affected tissues [2]. At the same, it is recognized that inflammation is a normal part of the tissue repair, and that normal adaptation to normal stress (i.e., exercise training and competition) requires controlled inflammation that allows repair to occur without severe tissue destruction.

In horses, oxidative stress occurs during intense exercise [3–5] and endurance exercise [6,7] and is often associated with muscle membrane leakage. In animal studies, strenuous exercise that results in muscle microtrauma (ultrastructural damage) is characterized by leukocyte infiltration and an inflammatory response resulting from oxidative stress [1]. Part of the healing process to microtrauma involves the inflammation that occurs in response to the damage.

The present study tested the hypothesis that a nutraceutical supplement comprised of ingredients that confer antioxidative, anti-inflammatory, and immune-enhancing effects will attenuate the severity of oxidative stress and inflammation that occurs in skeletal muscle and joints as a consequence of high-intensity exercise training. The present study used a stress of repeated ~60 s gallop bouts with 4–5 minutes of walking rest between gallops. The supplement is comprised of mushrooms and yeast that are rich in immune-enhancing 1→3 and 1→6 β-glucans [8–13], a superoxide dismutase (SOD)–rich melon concentrate [14], golden flaxseed, and a microencapsulated fish oil rich in omega-3 oils (minimum 28% by weight including EPA and DHA) selected to confer anti-inflammatory effects [15,16]. The product also is comprised 20% by weight of a blend of nonanimal source enzymes containing a combination of proteases, peptidase, amylase, and lipase.

2. Materials and Methods

Ten privately owned healthy, adult horses of mixed breed and gender participated in the study (Table 1). Owners freely agreed for their horse(s) to participate and signed an informed consent. Horses were outdoor stabled, with access to large run-in shelters with free access to hay and water at all times. The study was performed during the fall of 2015 near Toronto, Canada. A horse health check and lameness examination were performed on animals selected for the study. Five of the horses had to be withdrawn from the study: two due to lameness, two at the owner's request, and one due to negative behavior associated with the arthrocentesis procedure. Therefore, five horses fully completed both the placebo and supplement trials (double crossover design), with two additional horses completing either the placebo trial or the supplement trial. Animal care and use procedures were approved by the Nutraceutical Alliance Animal Care Committee in compliance with the Animals for Research Act (Ontario) and the Canadian Council on Animal Care guidelines.

Horses (Table 1) were fed twice daily (~7 am and ~4 pm) an individually tailored ration designed to meet their nutritional needs based on their work, body condition, and nutritional analysis of forage. Horses had ad libitum access to hay and water. The basal diet for Daisy, Stella, Lance, Lilo, Sasha, Rosalie, and Snoopy was 250 g of a ration balancer (Equilibrium Equilizer, Agribrands Purina, Woodstock, ON, Canada) and 40-g trace mineralized salt (Canadian Stockman, Sifto, Mississauga, ON, Canada). The basal diet for Dixi, Lucus, Norman, and Reese was 1.5 kg of Happy Horse pelleted feed ration (W-S Feeds, Tavistock, ON, Canada), 1-kg beet pulp pellets (Sharp's Feed Mills, Guelph, ON, Canada), 700-g ground flaxseed (Sharp's Feed Mills, Guelph, ON, Canada) and 75 g of trace mineralized salt (Canadian Stockman, Sifto, Mississauga, ON, Canada). The ingredients in Equilibrium Equalizer are soy hulls, dehydrated alfalfa meal, soy oil, flaxseed, wheat shorts, lysine, vitamins, minerals, and flavoring

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Table 1
Characteristics of the horses that completed trials in the study.

Horse	Treatment	Initial, Final Mass, kg	Sex	Age, y	Breed	Exercise Bouts	Reason for Withdrawal
Sasha	C	583	M	6	Dx	4	Lameness
Lance	C	498, 491			Morgan	3	
	S	489, 466[a]				3	
Stella	S	511, 516	M	5	Px	3	
	C	480, 493				3	
Lilo	S	585, 587	M	7	OH	4	
Rosie	C	565, 566	M		TB	4	Behavior
Norman	C	571, 582	G	9	TB	4	
	S	586, 578				4	Owner request
Lucus	C	533, 535			TB	4	
	S	530, 528				4	
Dixi	S	472, 474	M	6	TB	4	
	C	467, 477				4	Owner request
Snoopy	S	597, 604	G	6	Paint	3	
Daisy	C	630, 629			TB	3	
Reese	S	613, 610	M	5	Dx	4	Lameness

Abbreviations: C, control; Dx, Dutch warmblood cross; G, gelding; M, mare; OH, Oldenburg Holsteiner cross; Px, Hafflinger pony cross; S, supplement; TB, thoroughbred.
[a] Not completing the morning meal, but completed the afternoon meal.

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(http://equipurina.ca/en/products/lines/equilibrium/equilizer/). The ingredients in the trace mineralized salt are sodium chloride (96.5% minimum), zinc 4,000 mg/kg, iron 1,600 mg/kg, manganese 1,200 mg/kg, copper 330 mg/kg, iodine 100 mg/kg, and cobalt 40 mg/kg. The ingredients of the Happy Horse pelleted feed ration are wheat shorts, alfalfa meal, soy hulls, soy oil, molasses, limestone F (pulverized), water (liquid pellet conditioner), fine salt, Pelltech (Borregaard LignoTech, Sarpsborg, Norway), horse premix (2% by weight) vitamins and minerals), Novin toxin binder, Santoquin—dry (Novus), biotin 2,000 mg/kg of premix, and vitamin E (500,000 IU/kg).

The supplement is a proprietary, dry powder/particulate blend of mushrooms (proprietary; Equilabs, Wootton, Northampton, UK), yeast (*Saccharomyces cerevisiae* strain SB20; Lallemand Animal Nutrition, Milwaukee, WI), a SOD-rich melon-concentrate (6.7% by weight; Lallemand Animal Nutrition, Milwaukee, WI), powdered golden flaxseed (Pizzey Ingredients, Russell, MB, Canada), a powdered microencapsulated fish oil rich in omega-3 fatty acids (minimum 28% by weight including EPA and DHA; Novotech, Ventura, CA), and a proprietary blend of nonanimal source enzymes (20% by weight of proteases, peptidase, amylase and lipase; Specialty Enzymes, Chino, CA). Horses on the supplement trials received a loading dose of 30 grams per day for 7 days, followed by 15 grams per day for the remainder of the supplementation period.

Before the beginning of the 23-day supplementation period, horses completed a standardized high-intensity exercise test. Horses were fitted with a heart rate monitor with electrode placement underneath the saddle and girth strap. Two or three horses were exercised together. Riders mounted the horses at approximately 10 AM, and the horses were warmed up for 20 minutes by walking, trotting, and cantering. The test was performed on firm dirt ½-mile course. The test comprised three or four bouts of ½-mile gallop with a 4-minute walking rest between each bout. The intensity, before and after supplementation test, was standardized with respect to distance (1/2-mile laps), heart rate (greater than 190 bpm), and speed (based on time required to complete ½-mile laps). The determination for 3 or 4 bouts was on the basis of time required to complete the ½-mile course: when the time required was $\geq$ 10% slower than the fastest bout (typically the second bout) then that was taken as the final bout. Heart rates were recorded at the end of each bout when the horse crossed the finish line. At the end of the final bout, horses were walked for 30 minutes to cool out, hosed off with water, and placed into a stall ($\sim$50 minutes after exercise) before sampling blood and synovial fluid from a sampling fore limb intercarpal joint at 60 minutes after exercise.

An overview of the study protocol is shown in Fig. 1. Every morning at $\sim$7 am, horses were fed their morning meal (with or without supplement as appropriate for the trial). On the morning (between 10 and 11 am) of the day preceding the exercise test, the hair coat overlying the blood and synovial fluid sampling sites was clipped, the sites aseptically prepared and jugular venous blood and synovial fluid samples were taken as described previously [17]. On the morning of the test, horses were fed at 7 AM, three hours before warm up for the exercise test. Ten minutes before obtaining the 60-minute postexercise samples, sites were aseptically prepared and jugular venous blood and synovial fluid sampled. The following day, at 24 hours after exercise, the process was repeated.

Blood was collected into one EDTA-containing (for complete blood count) and two heparin-containing (for plasma biochemistry) 7-mL blood collection tubes (Becton-Dickenson, Mississauga, ON). Synovial fluid was collected into a 7 mL, heparin-containing blood collection tube. One blood sample and the synovial fluid sample collected in heparin-containing tubes were centrifuged for 15 minutes at 3,000 g to remove cells. The supernatant was collected and split equally into two 1-mL cryovials. These samples were stored at -20°C until analyzed. The two remaining blood collection tubes were analyzed for CBC (Siemens Model Advia 2120) and chemistry (Johnson & Johnson, Ortho Clinical Diagnostics Model 5.1 Fusion) by Nassagaweya Veterinary Laboratory Services (Campbellville, ON, Canada).

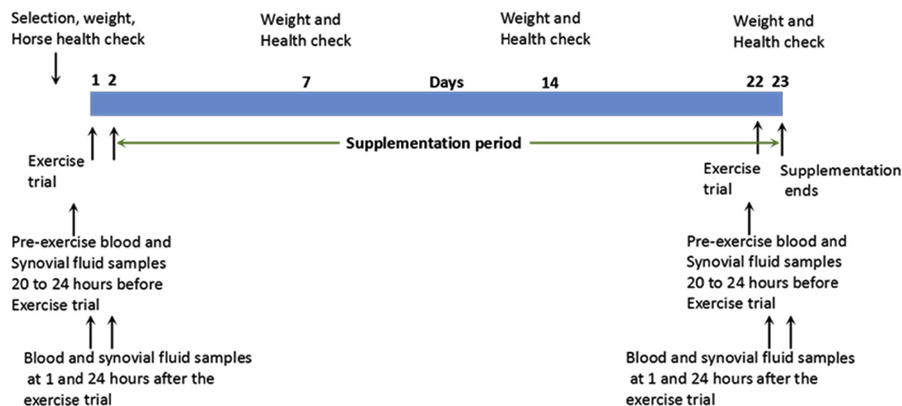

Fig. 1. Schematic overview of the protocol used for each horse.

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Table 2
Synovial fluid oxidative stress responses to high-intensity exercise in the Control and Supplement trials, comparing exercise responses and presupplementation with postsupplementation time points.

	Trial	Before Exercise	1 Hour After Exercise	24 Hours After Exercise
Presupplement				
TAS (mM)	Control	1.65 ± 0.048	1.65 ± 0.060	1.61 ± 0.055
SOD activity (IU/mL)	Control	223 ± 11	226 ± 15	212 ± 13
TAS (nM)	Supplement	1.83 ± 0.184	1.67 ± 0.285	1.74 ± 0.235
SOD activity (IU/mL)	Supplement	216 ± 11	222 ± 25	222 ± 11
Postsupplement				
TAS (mM)	Control[d]	1.610 ± 0.021	1.383 ± 0.143[d]	1.636 ± 0.033
SOD activity (IU/mL)	Control[b]	167 ± 20	172 ± 20[a]	186 ± 9.8[a]
TAS (mM)	Supplement[d]	1.63 ± 0.023	1.62 ± 0.090	1.72 ± 0.121
SOD activity (IU/mL)	Supplement[c]	182 ± 12	202 ± 6.7[e]	207 ± 24[e]

[a] Significantly different than time 0.
[b] Significantly different than presupplementation within trial.
[c] Significantly different than presupplementation trial.
[d] Tendency for difference from presupplementation within trial.
[e] Tendency for significant difference from pre-exercise mean.

A 200-μL aliquot of synovial fluid was treated with hyaluronidase to improve assay precision [18], resulting in 1:2 dilution. This hyaluronidase-treated synovial fluid was assayed for PGE_2 (DetectX Prostaglandin E_2 Enzyme Immunoassay Kit K051-H1, Arbor Assays), nitric oxide (Griess Reagent Kit for Nitrite Determination, G-7921, Molecular Probes), SOD activity (Superoxide Dismutase Assay Kit 7500-100 K, Trevigen), and total antioxidant status (TAS, Antioxidant Assay Kit 709001, Cayman Chemical). Untreated synovial fluid was used for measurement of glycosaminoglycan (GAG) concentrations using the method of Chandrasekhar et al. [19]. All assays were performed in duplicate. Plasma samples were analyzed in duplicate for thiobarbituric acid reactive substances (Parameter TBARS Assay KGE013, R&D Systems), PGE_2 (DetectX Prostaglandin E_2 Enzyme Immunoassay Kit K051-H1, Arbor Assays), nitric oxide (Griess Reagent Kit for Nitrite Determination, G-7921, Molecular Probes), SOD activity (Superoxide Dismutase Assay Kit 7500-100 K, Trevigen), and TAS (Antioxidant Assay Kit 709001, Cayman Chemical). All assays were performed using 96 well plates, and absorbances detected and recorded using a microplate plate reader (1420 Victor 2, Perkin Elmer; Fusion α, Perkin Elmer).

Data were analyzed with respect to presupplementation versus postsupplementation, exercise time points, and trial using a series of two-way analysis of variance (ANOVA). Within trial or exercise trial effects were examined using one-way repeated measures ANOVA. When a significant F-ratio was obtained the Holm–Sidak post hoc test was to identify different means. Statistical significance was accepted at $P \le .05$, and a statistical tendency was designated as $P > .05$ and $\le .10$. Tendencies ($.05 < P \le .10$ weak evidence against the null hypothesis in favor of the alternative) were also used to describe the data [20].

3. Results

3.1. Synovial Fluid Markers of Oxidative Stress

There was no significant effect of exercise on intercarpal joint synovial fluid markers of oxidative stress (Table 2). There was an effect of trial duration on SOD activity, which was lower at the end of the trial period before exercise with both control and supplement trials. In the Control trial, TAS tended ($P = .095$) to be lower 1 hour after exercise at the post-trial compared to pretrial. After 23 days of the supplement, synovial fluid SOD was higher than presupplementation, and there was a tendency ($P = .10$) for increased SOD activity postexercise compared to pre-exercise. TAS tended ($P = .059$) to be higher at 1 hour after exercise at the end of supplementation, compared to control, which showed a decrease ($P = .10$) at 1 hour postexercise compared to pre-exercise. In the supplement trial, compared to Control, SOD activity also tended ($P = .076$) to be greater at 1 and 24 hours postexercise postsupplementation test.

3.2. Synovial Fluid Markers of inflammation

There was no significant effect of exercise on synovial fluid markers of inflammation (Table 3). There was also no effect of time or trial on synovial fluid NO concentrations. In

Table 3
Synovial fluid markers of inflammation in response to high-intensity exercise in the control and supplement trials, comparing exercise responses and presupplementation with postsupplementation time points.

Trial	Before Exercise	1 Hour After Exercise	24 Hours After Exercise
PGE_2 (ng/L)			
Precontrol	202.9 ± 54.5	291.5 ± 72.7	245.8 ± 48.3
Postcontrol	368.5 ± 72.3	417.0 ± 72.7	311.7 ± 69.9[a]
Presupplement	184.4 ± 70.6	236.7 ± 66.2	258.5 ± 85.1
Postsupplement	356.7 ± 73.9	349.0 ± 51.8	236.5 ± 41.2
NO (μM)			
Precontrol	7.03 ± 1.33	15.1 ± 4.35	15.8 ± 4.59
Postcontrol	8.90 ± 0.71	43.8[b] ± 18.4	13.0 ± 2.52
Presupplement	11.7 ± 5.39	14.1 ± 5.24	20.5 ± 8.75
Postsupplement	8.61 ± 0.665	20.1 ± 6.42	29.0 ± 13.8
GAG (mg/L)			
Precontrol	290.3 ± 7.43	412.7 ± 61.5	418.7 ± 70.9
Postcontrol	342.5 ± 50.1	817.1[b] ± 329.0	309.3 ± 16.4
Presupplement	288.7 ± 8.08	774.8 ± 382.6	492.1 ± 136.0
Postsupplement	302.3 ± 7.14	411.2 ± 92.1	474.0 ± 143.4

[a] Significantly different than mean at 1 hour after exercise.
[b] Tendency for significant difference from pre-exercise mean.

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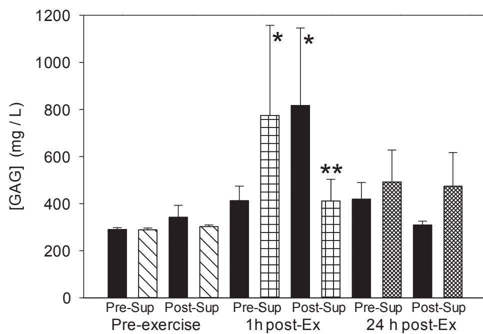

Fig. 2. Synovial fluid concentrations of glycosaminoglycans (GAG) before (Pre-Sup) and at the end of the supplementation period (Post-Sup). Controls: black bars; supplement: patterned bars. Values are mean ± SE. * indicates mean significantly different than before exercise. ** indicates postsupplementation mean tended (P = .078) to be different than presupplementation mean and to the control mean.

the Control trial, there was a tendency (P = .081) for increased PGE_2 in response to exercise (P = .073) and due to trial duration (P = .073). In the supplement trial, compared to the control trial, high-intensity exercise did not result in increased in PGE_2 at either postexercise time point.

Inflammation within the joint can lead to degradation of cartilage matrix resulting in release of GAGs. GAG was significantly increased at 1 hour postexercise in both Control trial exercise tests, and in the pretrial exercise test for the supplement (Fig. 2). In contrast, after 23 days of the supplement, synovial fluid GAG did not increase in response to exercise and tended (P = .078) to be lower after supplementation. In summary, there was a tendency for attenuation of PGE_2 concentrations after supplementation and no evidence of increased synovial fluid GAG, compared to control and to supplement pretrial high-intensity exercise tests.

3.3. Plasma Markers of Oxidative Stress

Within the Control trial there was no effect of exercise or trial duration on TAS, SOD and TBARS (Fig. 3). Two-way ANOVA comparing exercise and trial did not show any significant differences between control and supplement.

Within the supplement trial, there was no effect of exercise on oxidative stress markers. However, 23 days of supplementation resulted in significant increases TAS and SOD activity and a significant decrease in TBARS (Fig. 3) compared to presupplementation. SOD activity in both trials was similar in the presupplementation test. In contrast, at the end of the trial, SOD activity was greater in the supplement trial than in the Control trial. In summary, the supplement showed a significant decrease in TBARS associated with increased TAS and increased SOD activity.

3.4. Plasma Markers of Inflammation

There was a tendency (P = .073) for increased plasma PGE_2 in response to exercise (Table 4). There was a significant decrease in plasma NO 1-hour after exercise in the presupplementation Control trial. There was a tendency (P = .1) for reduced PGE_2 after 23 days of supplementation compared to the control trial. In summary, because exercise did not produce profound effect on markers of inflammation, despite evidence of oxidative stress, it was unlikely that there would be effects of supplementation.

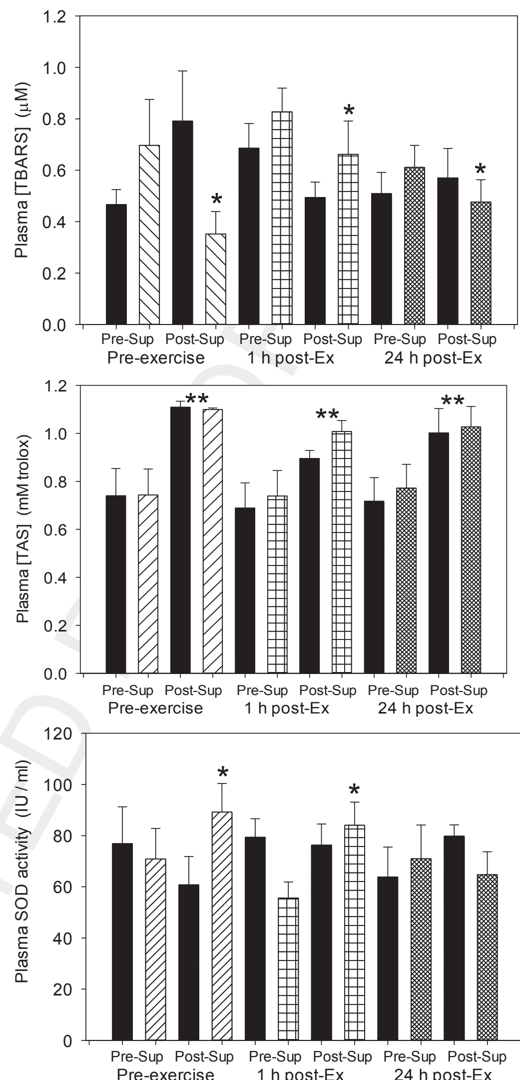

Fig. 3. Plasma markers of antioxidative status. Top panel: plasma concentration of plasma membrane lipid peroxidation products as represented by thiobarbituric acid (TBARS). Middle panel: plasma total antioxidant status as represented by trolox). Bottom panel: plasma superoxide dismutase (SOD) activity. * postsupplementation significantly different than presupplementation, within trial. ** postsupplementation test mean significantly different than presupplementation test means in both the control and supplement trials.

3.5. Plasma Markers of Skeletal Muscle Cellular Integrity

Exercise resulted in significant increases in plasma markers of muscle damage, both at 1 and 24 hours after exercise (Table 5). In both Control trials significant

Table 4
Plasma markers of inflammation in response to high-intensity exercise in the control and supplement trials, comparing exercise responses and presupplementation with postsupplementation time points.

Supplementation	Before Exercise	1 Hour After Exercise	24 Hours After Exercise
PGE_2 (ng/L)			
Precontrol	108.7 ± 27.5	331.9 ± 147.6	357.9 ± 156.2
Postcontrol	147.6 ± 21.6	440.7 ± 213.8	495.5 ± 246.7
Presupplement	223.0 ± 72.6	258.8 ± 77.7	232.1 ± 76.6
postsupplement	184.3 ± 53.9	337.7 ± 140.9	330.2 ± 159.2
NO (μM)			
Precontrol	52.6 ± 7.28	29.8 ± 5.29[a]	51.0 ± 7.89
postcontrol	58.3 ± 8.32	49.3 ± 5.38	58.2 ± 6.88
presupplement	47.6 ± 7.37	42.7 ± 5.98	45.6 ± 7.47
postsupplement	45.5 ± 7.96	51.9 ± 8.12	47.0 ± 7.70

[a] Significantly different than time 0 (pre-exercise).

Table 5
Plasma markers of skeletal muscle integrity in response to high-intensity exercise in the control and supplement trials, comparing exercise responses and presupplementation with postsupplementation time points.

Presupplement (n = 8) and Postsupplement (n = 7)	Before Exercise	1 Hour After Exercise	24 hours After Exercise
CK (IU/L)			
Precontrol	199.4 ± 17.1	585.1 ± 104.9[a]	452.4 ± 92.5[a]
Postcontrol	258.3 ± 44.2	698.6 ± 76.9[a]	681.9 ± 125.4[a]
Presupplement	228 ± 21.9	469 ± 90.8[a]	371 ± 89.5
Postsupplement	275 ± 28.5	641 ± 176[b]	1445 ± 721[b]
Creatine (μM)			
Precontrol	121.8 ± 14.1	147.1 ± 10.8[a]	118.5 ± 8.95
Postcontrol	113.3 ± 6.27	149.3 ± 5.71[a]	117.0 ± 7.02
Presupplement	100 ± 8.6	127 ± 10.7[a]	107 ± 9.9
Postsupplement	103 + 7.8	141 ± 9.1[a]	107 ± 9.1
AST (IU/L)			
Precontrol	382.6 ± 26.8	387.5 ± 21.7	459.4[a] ± 39.5
Postcontrol	342.7 ± 21.8	401.6 ± 42.2	524.9[a] ± 95.4
Presupplement	356 ± 30	398 ± 24	430[b] ± 58
Postsupplement	397 ± 60	455 ± 56	595 ± 182
Alk P (IU/L)			
Precontrol	171.9 ± 14.0	203.0 ± 16.1[a]	182.6 ± 13.2
Postcontrol	157.0 ± 17.3	187.7 ± 11.2[a]	161.0 ± 13.2
Presupplement	137 ± 12.8	159 ± 13.4[a]	146 ± 13.5
Postsupplement	148 ± 19.7	187 ± 25.5[a]	152 ± 23.3
GGT (IU/L)			
Precontrol	29.7 ± 3.06	27.9 ± 1.61	25.9 ± 2.10
Postcontrol	24.6 ± 4.26	34.7 ± 9.52	32.57 ± 8.95
Presupplement	26.9 ± 1.49	30.6 ± 1.39[a]	27.1 ± 2.06
Postsupplement	24.9 ± 2.55	40.0 ± 11.8	37.3 ± 11.0

[a] Significantly different than time 0 (preexercise).
[b] Tendency for mean to be different from time 0 (preexercise).

elevations for creatine kinase (CK) and aspartate aminotransferase (AST) persisted to 24 hours postexercise. After 23 days of supplementation, there were no significant elevations on average, although one horse had an elevated CK. There were no differences between the Control trials and supplementation trials.

3.6. Other Blood Parameters

Typical exercise effects were observed with respect to blood cells, white cell differentials, and red cell variables (data not reported). Exercise effects also occurred with plasma proteins, electrolytes, and other biochemical variables (data not reported). These were expected exercise responses. There were no effects of the supplement on these parameters.

4. Discussion

This is the first study to examine the effects of dietary supplementation designed to have combined antioxidant, anti-inflammatory, immune-enhancing product on systemic and synovial fluid oxidative stress and inflammation markers in horses that have performed exercise of sufficiently high intensity to cause oxidative stress and inflammation. This study showed that, compared to the Control trial, 23 days of supplementation with the product showed some evidence of decreased skeletal muscle injury and decreased intercarpal joint GAG release. The absence of effect of the supplement on red cells, white cells, and plasma biochemistry showed no evidence of adverse events to these blood systems and thus appeared safe over the 23 day period of use.

4.1. Effects in Articulating Joints

The exercise test was chosen for its ability to result in mild-to-moderate oxidative stress associated with mild-to-moderate muscle injury and inflammation [21,22]. It was also expected that this intensity of exercise would generate a mild inflammatory response within limb articulating joints, in agreement with previous research [23,24]. Excess production of reactive oxygen species (ROS, such as superoxide and hydrogen peroxide) within muscle or joints directly contributes to the inflammation of these tissues [21,25]. Oxidative stress and inflammation within joints is manifest by decreased TAS, increased markers of inflammation (PGE_2, NO), and cartilage matrix components (GAG) within synovial fluid [17]. In the present study, there was little evidence of oxidative stress within the intercarpal joint, with only a moderate decrease in TAS at 1 hour after exercise at the end of the Control trial (Table 2). The general absence of effect of exercise on markers of oxidative stress within the joint suggests that innate mechanisms for handling oxygen free radicals were adequate, even during the Control trial. It is of interest that in both the Control and supplement trials that SOD activity was reduced in the postsupplementation test compared to the postsupplementation test (Table 2). There is no obvious explanation for this result. Of greater interest, perhaps, is the upregulation of SOD activity that occurred at 1 hour after exercise after 23 days of the supplement. Determination of a cellular mechanism for this response requires further study.

The mild-to-moderate oxidative stress and inflammation within limb joints resulting from strenuous exercise can be attributed to the repeated, forceful impacts of the hoof with the terrain resulting in ground reaction forces travelling up the limb [26–28]. In addition, the repeated and forceful extensions and flexions around the joint causes stress to all joint structures including the synovial membrane and articular cartilage (meniscus). Inflammation within the joint can lead to degradation of

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cartilage matrix through multiple pathways including COX-2/PGE_2-mediated upregulation of aggrecanase/metalloproteinase activity [29,30]. Mild inflammation is required for normal healing; however, excessive inflammation may result in matrix breakdown rates greater than repair rates [31]. Degradation of the cartilage matrix results in the release of GAGs, and therefore, the concentration of GAGs within the joint postexercise is an indication of the rate and severity of matrix breakdown. In the present study, the inflammatory response within the intercarpal joint was negligible or mild at best, with respect to PGE_2 and NO (Table 3). Although there was evidence for a tendency to increased GAG release in the Control trial and in the presupplementation test of the supplement trial, the synovial fluid GAG concentration tended to be lower after supplementation than in the Control trial (Fig. 2). Further research is required to confirm or refute a beneficial effect.

4.2. Systemic/Muscle Effects

In the context of high-intensity exercise, increases in the plasma concentrations of enzymes/molecules present within skeletal muscle cells including CK, creatine, AST, alkaline phosphatase (Alk P), and gamma-glutamyl transferase are considered to be indicators of skeletal muscle damage [32,33]. In the present study, each exercise test resulted in mild-to-moderate muscle injury, as evidenced by increases in plasma creatine and all four enzymes at 1-hour after exercise (Table 5). The oxidative stress associated with intense exercise [3,4,34] can result in rapid increase in ROS that oxidize and damage cell membrane lipid components, with attendant loss of membrane integrity and appearance of muscle-specific enzymes in the blood [6,7,35]. During prolonged or strenuous exercise, MDA (the main component of TBARS) is strongly associated with increased circulating levels of muscle-specific proteins such as CK [5,36,37]. The concentrations of these markers in plasma rise after cessation of exercise, and the peak is proportional to the magnitude of muscle injury [38].

In the present study, a significant, systemic inflammatory response was not evident. In the control trial, significant elevations for CK and AST persisted to 24 hours after exercise; in contrast, after 23 days of the supplement, there were no significant elevations, and there were no differences between control trials and supplement trials. When plasma markers of oxidative stress (Fig. 3) are examined after 23 days of the supplement, there was a tendency for decreased TBARS postsupplementation at all time points, suggesting decreased lipid peroxidation of cell membranes. At the same time, TAS and SOD activity were both significantly increased; both of these would act to reduce the oxidative stress present at affected tissue sites [1].

Other researchers have also investigated the hypothesis that oral supplementation with SOD-rich products will reduce the severity of oxidative stress and damage associated with high-intensity exercise (reviewed by Carillon et al. [39]). As noted by Carillon et al. [39] it is highly unlikely that dietary SOD itself confers the observed effects, as SOD is degraded within the intestinal tract. Notin et al. [40] studied 2 groups of 12 Standardbreds in training, of which one group received an SOD-rich powdered melon concentrate similar to that present in the supplement used in this study. The trial group received the equivalent of 520 IU of SOD/day for 60 days, and the placebo group received the excipient with no active substances. On days 0, 30, and 60 of supplementation, the physiological responses to a standardized exercise test were assessed, including plasma creatine kinase, erythrocyte SOD activity, and blood resistance to hemolysis. In the "SOD" group, 60 days of supplementation resulted in a significant increase in the plasma resistance to hemolysis, and resting plasma CK remained unchanged but increased in the placebo group. The authors concluded that the melon-concentrate supplement may make erythrocyte and muscle cells membranes less permeable during the stresses associated with regular exercise training. Lamprecht and Williams [35] evaluated the effect of oral SOD-rich melon-concentrate supplement on responses to intense, exhaustive exercise in horses. Horses were fed the equivalent of 3,000 IU SOD/day of powder for 6 weeks. The authors reported no benefit on blood and synovial fluid markers of inflammation (PGE_2, chondroitin sulfate; gene transcripts for interferon-gamma, interleukin-10, and interleukin-1β in blood, and decreased plasma NO) and antioxidant status (SOD activity, total glutathione, glutathione peroxidase) in response to a single bout of repeated sprint exercise test at 6 weeks.

The benefits of dietary supplementation with the specific aim to upregulate the concentrations and activities of the enzymes capable of degrading ROS (SOD, catalase, glutathione peroxidase) in horses performing exercise remain unclear, with differences in dosage and outcomes. The present study supplemented melon concentrate at the equivalent of 2,600 IU SOD per horse per day but also provided beta-glucans and other ingredients that may have contributed to beneficial effects. It is also unlikely that it is not the exogenous SOD that is exerting the effects, but rather that other compounds within the SOD-rich melon concentrate is, either directly or indirectly, responsible for inducing antioxidative effects [39]. Orally supplemented SOD-rich products are typically encapsulated to protect against breakdown by gastric acids, they have low bioavailability (14%–22%; [41]) and once in the circulation are rapid elimination by the kidneys [39]. Encapsulated forms have been developed that are capable of eliciting in mice in vivo, and cells in vitro, antioxidative effects [42–44].

4.3. Limitations

The numbers of horses used in each group, and the attrition within each group due to exercise and other factors unrelated to the experiment, was a limiting factor. The results prevent firm conclusions but provide the basis for additional research. A possible limitation of the study, given that five of the horses participated in both trials, is that it is unknown whether 21 days of washout of the supplement was an inadequate duration of time for complete loss of effects of supplementation. However, because five of the seven horses that completed the supplement trial performed the control trial first, no

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washout required for these horses. It is therefore unlikely that washout duration for the two of seven horses that performed the supplement trial first was a factor that influenced the results of this study. The exercise test resulted in a hemoconcentration (increased plasma proteins and PCV) that persisted to the first hour after exercise consistent with numerous exercise studies (see [45,46] for reviews). The postexercise time points of 1 hour and 24 hours were chosen based on the literature [47–49] and on a pilot study that analyzed samples taken at 0.5, 1, 2, 4, 8, and 24 hours after exercise performed by us (M.I.L., J.M., and W.P., unpublished). These time points were proximal to when peak effects occurred, and taking only three samples in a 24-hour period minimized trauma and risk of inflammation due to the repeated arthrocentesis procedure [49].

4.4. Design of the Supplement

The supplement used in the present study was designed to exert main effects by influencing oxidative stress, inflammation, and immune function. In this section, we point to putative mechanisms for the benefits reported in the present study. Among the main ingredients of the supplement are an SOD-rich melon concentrate, yeast, mushrooms, and omega-3 fatty acids. The SOD-rich melon concentrate was selected based on reports of improved antioxidant status [42,50,51]. As discussed above, the results of supplementing SOD-rich melon concentrate to horses has produced mixed results, with indications of potential for increased oxidative status and reducing inflammation. The omega-3 fatty acids were chosen for their ability to reduce inflammation [52], although such effects have not been reported in horses there appear to be other health benefits including improved blood fatty acid profile [53], improved oxidative balance after exercise [34], and improved serum glucose:insulin ratios with lower cholesterol [54]. The yeast and mushrooms contribute beta-glucans which have immune-enhancing properties [11,55]. Beta-glucans are a class of polysaccharides derived from cell walls of yeast, fungi, algae, and oats. Horses consuming oats and oat products as a normal part of their diet will be receiving oat beta-glucans. Beta-glucans in general, when supplemented to the diet, enhance immune system functions via direct stimulation of macrophages, neutrophils, and NK cells [55]. Beta-glucan supplementation has been associated with improved defense against microbial challenges in vitro and in vivo [8,9,11], including elite athletes during periods of strenuous training [56]. In vitro, increased concentrations of proinflammatory monocytes and cytokines (IL-4, IL-5, and IFN-γ) compared to placebo [57] have led authors from the Davis group to conclude that beta-glucans have the potential to alter immune function after strenuous exercise.

5. Conclusions

In summary, 23 days of feeding horses a nutraceutical supplement designed to combat oxidative stress, inflammation, and enhance immune function provided some support that supplementation attenuated increases in muscle and joint markers of inflammation of oxidative stress after high-intensity exercise in horses. There is interplay between oxidative stress, inflammation, and immune function that has important implications for how and what we feed horses and when best to exercise train and compete horses. A nutritional supplement that reduces the oxidative stress associated with intense exercise should reduce the amount of skeletal muscle damage that occurs, with attendant reductions in inflammation and pain and with improved postexercise recovery.

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All authors contributed to the design and execution of the study, including sample and data analysis and the written manuscript. All authors have approved the final article.

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