ARTICLE

Dietary Cucumis melo Reduces Markers of Muscle and Articular Inflammation Following High-intensity Exercise in Horses

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ARTICLE INFO	ABSTRACT
	We evaluated the antioxidative and anti-inflammatory potential of daily oral supplementation with a proprietary powdered *Cucumis melo* pulp (CMP) on exercise-induced markers of articular and muscular oxidative stress and inflammation in 12 horses. Horses performed a high-intensity exercise test immediately prior to, and then following, 3 weeks of daily supplementation of 1 g powdered CMP (CMP; n=8). Controls (Co; n=8) underwent the same exercise and sampling regime but were not supplemented. Blood and synovial fluid (SF) samples were taken 24 h prior to exercise (BL), and at 1 and 24 h following exercise. Plasma and SF were analysed for prostaglandin E_2 (PGE$_2$), total antioxidant status (TAS), nitrite and superoxide dismutase (SOD) activity. SF was analysed for glycosaminoglycans (GAG), and plasma was analysed for thiobarbituric acid reactive substances (TBARS). Comparisons were made using repeated measures with the initial exercise test as a covariate. There was an increase in SF SOD activity in the CMP group. Compared to Co at 1 h, CMP reduced nitrite and GAG in SF, as well as maintained plasma TAS and lymphocyte levels. At 24 h, plasma PGE$_2$ and creatine kinase were lower in horses receiving CMP. Three weeks of supplementation with CMP reduced markers of articular and skeletal muscle oxidative stress and inflammation in response to high-intensity exercise in horses. Nutritive antioxidants may provide a useful adjunct to the daily nutrition plan of horses undergoing regular exercise training and competition.

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Abbreviations

A:G ratio	albumin:globulin ratio
ALB	albumin
AST	aspartate aminotransferase
BL	baseline
CK	creatine kinase
CMP	*Cucumis melo* pulp powder
Co	control
Cre	creatinine
GAG	glycosaminoglycans
HGB	hemoglobin
IU	International Units
LYM	lymphocytes
NRC	National Research Council
PGE$_2$	prostaglandin E$_2$
RBC	red blood cells
ROS	reactive oxygen species
SF	synovial fluid
SOD	superoxide dismutase
TAS	total antioxidant status
TBARS	thiobarbituric acid reactive substances.

1. Introduction

Horses that have performed moderate to high intensity exercise experience increased mitochondrial production of reactive oxygen species (ROS; oxygen free radicals) within muscle [1,2]. An accumulation of ROS appears to be a key contributor to the inflammatory response to intense exercise that is evident both in muscle [2] and in blood [1-7]. In horses, the magnitude and duration of oxidative stress markers in plasma and synovial fluid was proportional to the exercise intensity and duration [3,7,8]. One of the first studies to relate performance to inflammation was that of Davis et al. [9] using humans, in a trial where curcumin was provided as a nutritive antioxidant. Similar results were recently demonstrated when people consumed quercetin for 14 days [10]. Improved performance has also been shown to be a sequalae of reduced muscle damage [11], inferring that inflammation is associated with decreased performance. Yang et al. [12] showed within-game deterioration of performance has also been directly associated with increased muscle inflammation. Tanabe et al. [13]

demonstrated that dietary curcumin attenuates acute inflammation and muscle damage and could facilitate faster recovery. Taken together, these results demonstrated that the inflammation associated with the oxidative stress of exercise can impair performance and rate of recovery from normal exercise training bouts [14,15].

Within the joints, the stress of exercise training or conditioning is also often associated with increased ROS within synovial fluid and inflammation around and within the joints [16]. This is evidenced by elevated levels of synovial fluid markers of oxidative stress and inflammation such as prostaglandin E$_2$ (PGE$_2$) [17,18]. Excess production of ROS (such as superoxide and hydrogen peroxide) within joints directly contributes to the inflammation [17,19,20]. While a mild inflammation may be deemed a normal part of the beneficial adaptive responses to exercise, excessive inflammation contributes to muscle and joint pain, further tissue degeneration, and impaired exercise performance and wellness [21,22].

The incidence of degenerative joint disease, eventually leading to clinical osteoarthritis (OA), is high in young performance horses, often at a time when these athletes should be at the peak of their careers [2,8]. Increasingly, veterinarians, owners and trainers are using dietary or nutritive antioxidant supplements to mitigate inflammation associated with excess oxidative damage [23]. The dried and powdered pulp of a particular non-GMO strain[a] of *Cucumis melo* LC (cantaloupe or muskmelon) is rich in the antioxidant enzymes superoxide dismutase (SOD) and catalase [24-26] and has been shown to provide protection against administered pro-inflammatory compounds [25]. This *C. melo* pulp (CMP) also reduced markers of oxidative stress and improved antioxidant activity in humans [27], pigs [28], felines [29], mice [30] and horses [31]. In a recent study CMP was one of the ingredients in an oral supplement for horses that performed the same study as described herein [23]. They also reported reductions in the markers of muscle and synovial fluid oxidative stress and inflammation.

The objective of the current study was to investigate if CMP, one of the ingredients used in the previous study [23] was associated with antioxidant and anti-inflammatory effects when fed daily to horses for 3 weeks. It was hypothesized that CMP will result in reduced signs of muscle and/or articular markers oxidative stress and inflammation following high-intensity exercise.

2. Methods

2.1 Ethical Approval

All experimental procedures and protocols were approved

by the Nutraceutical Alliance Animal Care Committee (Campbellville, ON, Canada) prior to the beginning of this study in accordance with the Ontario Animals for Research Act and the Canadian Council on Animal Care guidelines. All horses were privately owned, and written informed consent was obtained from each owner prior to the start of the study.

2.2 Experimental Animals

Twelve horses of mixed breed, gender (6 mares, 6 geldings), and age (range 5 - 17, median 7.5) participated in this study (Table 1). Stratified assignment of horses to groups was performed so that groups were as uniform as possible according to body mass, body condition score, age, sex, breed and age. All horses were clinically normal and underwent weekly health checks which consisted of checking rectal temperature, heart rate, respiratory rate, hydration (by skin pinch), menace reflex, and gut sounds. Health checks were also performed on all horses within 24 h prior to each sampling day. All horses had no known veterinary history of chronic joint inflammation or lameness and were visibly sound on exercise days using the American Association of Equine Practitioners lameness exam scoring system. Prior to participation in the study, and during the study, all horses maintained a regular schedule of pleasure riding and light lessons. Horses were randomly assigned to a diet containing either 0 (Co; n=8) or 1 g (CMP; n=8) in their morning feed, based on the manufacturer's instructions. The supplement dosage was therefore in the range of 1.55 to 2.14 mg / kg body mass per day. The supplement provided approximately 2600 IU SOD per horse/day according to the manufacturer. All horses were fed a diet which met their nutritional requirements [32] and were housed at the same facility in a loose-housing system, with unlimited access to shelter, pasture, hay and water.

Table 1. Description of horse breed, age, gender, treatment, and round for control (Co, n=8) and supplemented (CMP, n=8) horses used in the current study

Horse	Breed	Gender	Age, yr	initial, final mass (kg)	Group	Round
1	Thoroughbred	Gelding	8	467, 477	Co	1
2	Quarter horse	Mare	5	565, 566	Co	1
3	Dutch warm-blood	Mare	5	585, 583	Co	1
4	Thoroughbred	Mare	13	571, 582	Co	2
5	Morgan	Gelding	16	504, 516	CMP	1
6	Oldenburg	Mare	7	591, 584	CMP	2
7	Quarter horse	Gelding	7	579, 585	CMP	3
8	Thoroughbred	Gelding	13	473, 466	CMP	3
9	Morgan	Gelding	17	506, 496	Co, CMP	2,1
10	Quarter horse / Haflinger	Mare	5	501, 508	Co, CMP	2,3
11	Thoroughbred	Gelding	7	541, 542	Co, CMP	2,1
12	Quarter horse	Mare	8	645, 647	Co, CMP	3,2

2.3 Test Material

The test material was a 1 kg sample of a fresh batch of CMP provided by Lallemand Animal Nutrition in a sealed vacuum pouch (registered as Melofeed® in Europe and as Enzalox™ in North America; Lallemand Animal Health, Montreal, Canada). The pouch was opened on the first day it was to be used as a supplement to the feed, and thereafter maintained in a sealed plastic container in a refrigerator (6 – 10°C). The dry, powdered product is stable for at least 2 years when maintained under these conditions. This sample provided adequate supplement for the entire study.

2.4 Study Design

This study was part of a larger 3-way crossover design [23] that was conducted over 3 rounds (Fig. 1); horses partic-
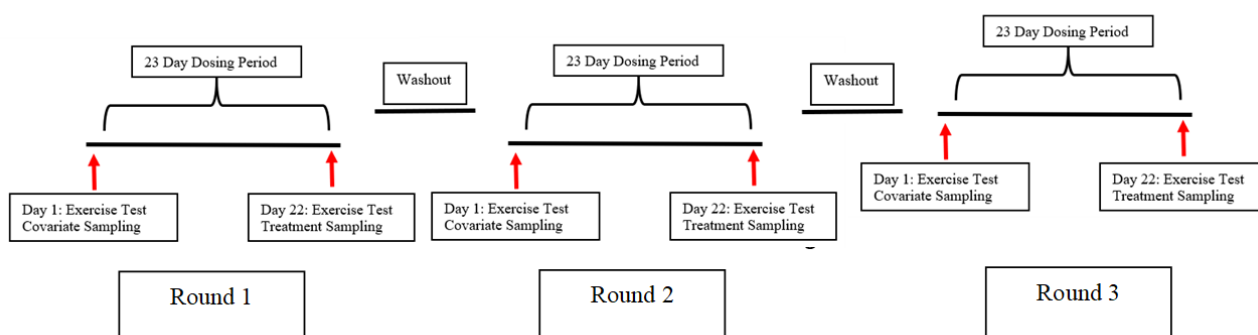

Figure 1. Schematic representation of the experimental schedule

ipated in a control round, a CMP round, and a round for another nutraceutical treatment with a 25-day duration for each round. Only the person feeding the horses knew if which horses received supplement, and horse owners were also blinded to the treatment, by consent. Each round consisted of a covariate sampling period, a dosing period, and a treatment sampling period. For each round, a high-intensity exercise test was performed on day 1 (covariate sampling period), followed by 23 days of supplementation (or placebo) provided in the morning meal (dosing period), and a second exercise test on day 22 (treatment sampling period). The purpose of the day 1 exercise test was to serve as a time-based control, as well as provide a baseline. This was incorporated into the statistical analysis as a covariate. Horses maintained their usual activity and training level for the entire duration of their participation in the study. Rounds were separated by a 3-wk washout period to minimize nutritional, biochemical and physiological carryover effects [33-35]. The order of the treatments was randomized across the cohort of horses (Table 1).

By design, it was intended that each horse was to complete each of three treatments trials: control, CMP and a blended product of which the results have already been reported [23]. However, as in most moderate-duration field-type studies, and particularly those with a high intensity exercise component with repeated arthrocentesis, there was some attrition of horses that started. It is important, physiologically and psychologically, that horses are calm and easily manageable for all procedures, else anxiety will be a major confounder of physiological variables. Thus, the first two rounds consisted of 6 horses per round (3 Co, 3 CMP), and the last round consisted of 4 horses (2 Co, 2 CMP), due to subject attrition unrelated to the study (see Table 1). Of the 12 horses, 4 participated in the control group (Co), 4 participated in the CMP group (CMP), and 4 participated in both (Co and CMP).

2.5 Exercise Test

Horses were exercised under saddle in groups of 2 or 3 matched according to speed and fitness at approximately 11 am. Three riders were used, of similar body mass (+/- 10 kg) and riding experience (competent, non-professional). Ambient conditions were dry, with relative humidity 50 and 70% and temperature 14 – 22° C. Heart rate was monitored during galloping by the riders using equine heart rate monitors (Polar Electro Canada, Lachine, QC, Canada). Horses were warmed up (at the walk, trot, and canter) for 20 min prior to the exercise test. The exercise test consisted of repeated single-lap gallops around a dry ½ mile dirt track at a target heart rate of 180 bpm, separated by approximately 4 min of walking until heart rates

decreased to approximately 100 bpm [36]. This process was repeated for 2-3 more laps (depending on the ability of the horse) until the horse's time around the track increased by 10% of their fastest lap. The first exercise test sequence performed by each horse was used to determine their maximal heart rate, speed, and ability. The mixed breed horses were able to complete 3 laps while the Thoroughbreds completed four. The number of laps was conserved for each horse for the subsequent exercise tests.

2.6 Sample Collection

Samples were obtained at baseline (BL), around 11 am the day prior to each of the exercise tests, then again at 1 and 24 h following the cessation of exercise. Timing of sampling was determined in a previous study that examined the time course of inflammation and oxidative stress markers in response to high intensity exercise in horses [36].

An area approximately 4 X 5 cm was clipped around the blood sampling site (left jugular groove), and synovial fluid sampling sites (the medial side of the left and right intercarpal joints) to reduce hair length to less than 2 mm. Topical anesthetic (Emla cream, 2.5% lidocaine, 2.5% prilocaine; AstraZeneca, Mississauga, ON, Canada) was applied to the clipped area approximately 30 min prior to sampling. Blood samples were collected directly into sodium heparin and EDTA-vacutainer tubes (Becton-Dickson, Mississauga, ON, Canada) from the jugular vein using a 21G 1.5" multiple sample needle (Becton-Dickson, Mississauga, ON, Canada). Blood samples were taken immediately prior to arthrocentesis and chilled on ice until processing, which occurred within a 2 h period.

Aseptic arthrocentesis was performed at each time point, alternating joints between samples to allow sufficient time for replenishment of SF. The left or right intercarpal joint was prepared using a stanhexidine / iodine scrub followed by 99% isopropyl alcohol. Approximately 1 mL of fluid was aspirated using a 22 G x 1" needle into a 3 cc sterile syringe. Aspirated fluid was immediately transferred into a sodium heparin vacutainer tube and chilled on ice until processing, which occurred within 2 h.

2.7 Sample Processing

Fresh blood samples were analyzed for biochemistry [albumin (ALB), albumin/globulin ratio (A:G), aspartate amino transferase (AST), creatinine (Cre), creatine kinase (CK)] (Johnson & Johnson, Ortho Clinical Diagnostics Model 5.1 Fusion) and complete blood count (Siemens Model Advia 2120, Nassagaweya Veterinary Laboratory Services, Campbellville, ON, Canada). Additional aliquots of heparinized blood and SF were centrifuged at 6000 x g

for 15 min. Supernatant was then transferred into Eppendorf tubes and stored at -20ºC until analysis.

Prior to analysis, SF samples were digested via hyaluronidase treatment to improve assay precision [37]. Hyaluronidase (Sigma-Aldrich, Oakville, ON) was suspended in PBS to a final concentration of 4 mg/ml solution. SF samples were prepared using a 1:1 dilution of hyaluronidase solution and sample. Mixed hyaluronidase:SF samples were agitated for 1 h, centrifuged at 1000 x g for 5 min, and the supernatant was removed and used in assays.

2.8 Sample Analyses

Samples were analysed for nitrite (Griess Reaction, Molecular Probes, Eugene OR), total antioxidant status (TAS; Cayman Chemical, Ann Arbor, MI), superoxide dismutase (SOD; Trevigen, Gaithersburg, MD), and prostaglandin E_2 (PGE$_2$; DetectX Prostaglandin E2 Enzyme Immunoassay, Arbor Assays, Ann Arbor, MI). Plasma was analysed for thiobarbituric acid reactive substances (TBARS; R&D Systems Inc., Minneapolis, MN), and untreated SF was analysed for glycosaminoglycans (GAG) [38] as detailed previously [20].

Table 2. Time, treatment, and treatment by time effects within plasma markers of oxidative stress and inflammation, as well as blood biochemistry and hematology parameters in response to high-intensity exercise in control (Co; n=8) and supplemented (CMP; n=8) horses

	BL	1 h	24 h	p_{time}	p_{trt}	$p_{trt*time}$
SF						
SOD IU/ml						
Co	166 ± 13.0	170 ± 13.0	187 ± 13.0	0.4	0.04	0.7
CMP	183 ± 13.0	204 ± 13.0	198 ± 13.2			
PGE$_2$ pg/ml						
Co	196 ± 31.6	227 ± 32.1	163 ± 31.6	0.04	0.3	0.2
CMP	171 ± 30.8	144 ± 30.5	128 ± 30.5[a]			
Plasma						
TAS mM						
Co	1.09 ± 0.023[a]	0.97 ± 0.023[b*]	1.06 ± 0.024[ab]	0.004	0.1	0.1
CMP	1.07 ± 0.022	1.04 ± 0.024	1.09 ± 0.023			
Blood						
A:G						
Co	0.92 ± 0.035	1.04 ± 0.053	0.91 ± 0.044	0.004	0.6	0.8
CMP	0.96 ± 0.033	1.04 ± 0.051	0.93 ± 0.043			
ALB, g/dL						
Co	31.4 ± 0.54	34.8 ± 1.65	31.9 ± 0.64	0.0062	0.6	0.3
CMP	32.2 ± 0.53[ab]	36.3 ± 1.62[a]	31.6 ± 0.65[b]			
AST, µmol/L						
Co	342 ± 25.8	399 ± 34.2	490 ± 74.2	0.013	0.7	0.4
CMP	401 ± 26.1	417 ± 33.2	468 ± 71.0			
CRE, U/L						
Co	117 ± 5.0[a]	137 ± 5.5[b]	119 ± 5.0[a]	<0.0001	0.9	0.7
CMP	114 ± 4.8[a]	139 ± 5.0[b]	121 ± 5.0[a]			
HGB, g/L						
Co	13.7 ± 0.55[a]	16.0 ± 0.59[b]	15.3 ± 0.55[b]	0.0005	0.7	0.4
CMP	14.0 ± 0.54[a]	15.9 ± 0.55[b]	14.5 ± 0.55[ab]			
RBC, 10^{12}/L						
Co	8.1 ± 0.29[a]	9.6 ± 0.33[b]	9.0 ± 0.29[ab]	0.0004	0.4	0.6
CMP	8.1 ± 0.30[a]	9.3 ± 0.29[b]	8.5 ± 0.30[ab]			
LYM, 10^9/L						
Co	2.9 ± 0.30[a]	4.8 ± 0.30[a]	3.0 ± 0.30[a]	≤ 0.006	0.002	0.01
CMP	3.0 ± 0.28	3.3 ± 0.30*	3.3 ± 0.30			

Marker concentrations are means ± SEM. BL = baseline sample taken approximately 24 h prior to the exercise test; SOD = superoxide dismutase; TAS = total antioxidant status; A:G = albumin to globulin ratio; ALB = albumin; AST = aspartate aminotransferase; CRE = creatinine; HGB = hemoglobin; RBC = red blood cells; LYM = lymphocytes.

Notes: [ab] Superscripts which differ denote significantly different values within treatment (p≤0.05).

* Denotes a significant difference between Co and CMP at the particular timepoint (p≤0.05).

2.9 Statistical Analysis

Data are presented as mean ± SEM unless otherwise stated. Data were analyzed with respect to pre- vs post-supplementation / placebo within each group, exercise time points, and trial using analysis of variance (ANOVA). Marker concentrations (Yijk) were subject to repeated measures ANOVA (PROC MIXED University Edition, SAS Institute Inc.) according to the following model:

$$Yijk = \mu + \beta \times initial + trti + timej + trt*timeij + \varepsilon ijk$$

where μ = overall mean, β = the covariate slope, initial = marker concentration during the covariate sampling period, trt = fixed effect of treatment (i = Co or CMP), time = repeated measure of time (j = 1 to 3) and εijk = the experimental error. Comparisons were also made between treatments at each sampling time using estimate statements. Least square means using a Tukey adjustment were employed to analyse the effects of time within group. Significance was set at $P \leq 0.05$. In the description of the results the term 'time effect' therefore indicates that the 3 weeks of supplement resulted in an effect that can be, and has been, attributed to the supplement. The term 'treatment effect' refers to differences between the control group and the treatment group at the second time point (end of trial).

3. Results

3.1 SF Markers of Oxidative Stress

No effects were observed for SF TAS (Table 2). There was an effect of trial duration on SOD activity, which was lower at the end of the trial period before exercise in the control trial but not the CMP trial (data not shown). There was an effect of treatment for SF SOD, with consistently higher SF SOD in the CMP group than the Co group (p = 0.04, Table 2).

3.2 SF Markers of inflammation

There were no effects of trial duration on SF markers of inflammation (pre-exercise comparisons). SF PGE$_2$ was significantly lower at 24 h post exercise in CMP resulting in an overall time effect (p = 0.04, Table 2). There were no significant differences between treatments at 1 h post-exercise (227 ± 32.1 pg/ml in Co vs 144 ± 30.5 pg/ml in CMP; p = 0.09). There was an effect of time for SF fluid nitrite (p = 0.04). Nitrite was lower in CMP (9 ± 7.7 μM) compared to Co (39 ± 7.7 μM) at 1 h (p = 0.01, Figure 2). Synovial fluid GAG was lower in CMP (344 ± 155 μg/ml) compared to Co (820 ± 130 μg/ml) at 1 h (p = 0.03, Figure 3).
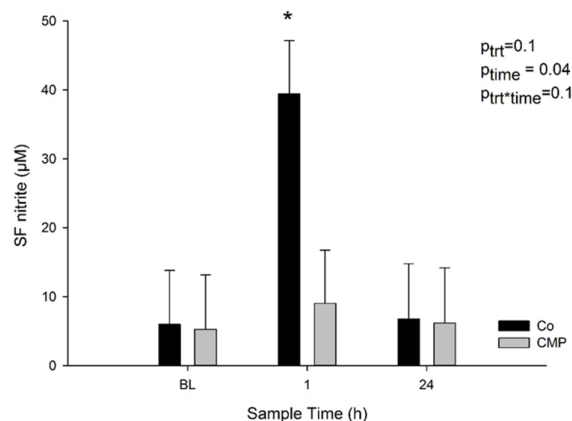

Figure 2. Mean ± SEM of synovial fluid (SF) nitrite (μM) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

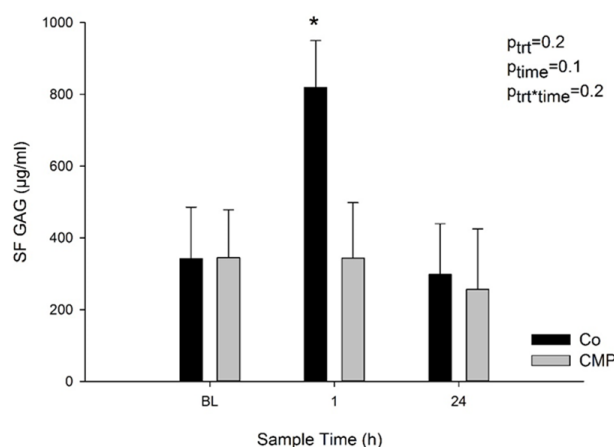*Note:* * Denotes significant difference between the Co and CMP (p≤0.05).

Figure 3. Mean ± SEM of synovial fluid (SF) glycosaminoglycans (GAG; μg/ml) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Note: * Denotes significant difference between the Co and CMP (p≤0.05).

3.3 Plasma Markers of Oxidative Stress

There were no effects of trial duration on plasma markers of oxidative stress (pre-exercise comparisons). There was an effect of time on plasma TAS (p = 0.004), and in the Co plasma TAS was lower compared to BL at 1 h (p = 0.01) and compared to CMP at 1 h (p=0.03, Table 2).

3.4 Plasma Markers of Inflammation

There were no effects of trial duration on plasma markers of inflammation (pre-exercise comparisons). Plasma PGE$_2$ was reduced in CMP (128 ± 30.5 pg/ml) compared to Co (163 ± 31.6 pg/ml) at 24 h (p = 0.04, Figure 4).
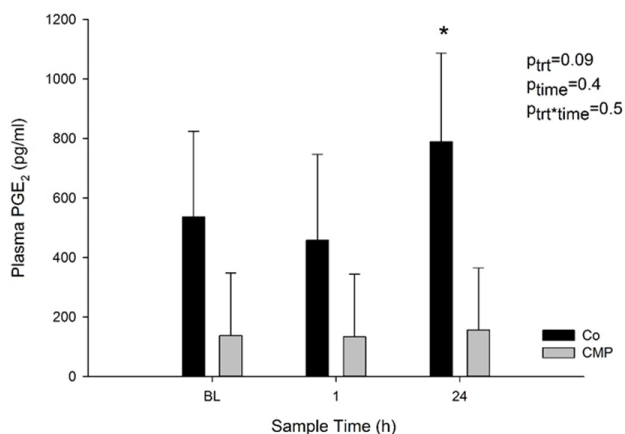
Figure 4. Mean ± SEM of plasma prostaglandin E2 (PGE$_2$; pg/ml) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Note: * Denotes significant difference between the Co and CMP (p≤0.05).

3.5 Markers of Blood Biochemistry

Time effects were observed for CRE, AST, ALB, A:G ratio, HGB, and RBC (Table 2). There was an effect of treatment (p = 0.02) and time (p = 0.02) for CK. In the Co CK was higher compared to BL at 24 h (665 ± 73.0 U/L vs 295 ± 76.8 U/L; p = 0.03) and CMP at 24 h (353 ± 77.6 U/L; p = 0.008, Figure 5).
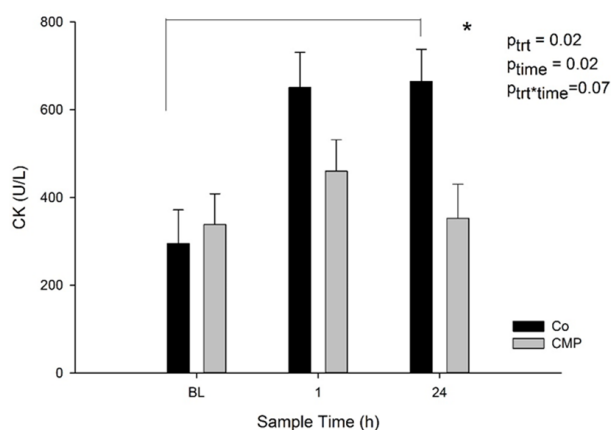

Figure 5. Mean ± SEM of creatine kinase (CK; U/L) in control (Co; n=8) and supplemented (CMP; n=8) horses 24 h prior to (BL), at 1 and 24 h following, a standardized high-intensity exercise test

Notes: Lines connect significantly different time points within the Co.
* Denotes significant difference between the Co and CMP (p≤0.05).

3.6 Markers of Immunity

Time (p = 0.004) and time by treatment (p = 0.01) effects were observed for blood lymphocytes (Table 2, LYM). In the Co, LYM was elevated at 1post-exercise, and this response was not observed in the CMP trial. In the CMP trial, there was no effect of exercise on the LYM response. There were no changes in any of the other hematological variables.

4. Discussion

This study provides evidence that dietary CMP behaved as a nutritive antioxidant and influenced the systemic and articular environment in horses undergoing and recovering from intense exercise. Given that muscle mass is about 40% of body mass, and the largest single tissue in the body [39] and that the horses exercised at high intensity using muscle, the systemic responses observed in the present study can only be attributed to skeletal muscle. Horses supplemented with CMP displayed attenuation of post-exercise increases in SF nitrite and GAG, as well as evidence of diminished plasma PGE$_2$ and CK. These results are consistent with reductions of oxidative stress and inflammation in joints and skeletal muscle after high intensity exercise. The results also confirm that antioxidant and anti-inflammatory responses observed in the equine joint oral supplement reported previously [23] may be attributed in part to CMP.

There was a general absence of effect of CMP supplementation on measures obtained at rest (pre-exercise samples on day 1 compared to pre-exercise on day 22). This suggests that in the absence of a measurable oxidative stress a physiological or biochemical adaptation to 3 weeks of supplementation was not readily apparent. The observed time effects in blood variables are consistent with physiological changes due to high-intensity exercise [7,36,40] indicating that the standardized exercise test was of suitable intensity and physiological stress. Additionally, in Co horses, plasma TAS was reduced 1 h following the exercise test indicating that the exercise was of sufficient intensity to tax and diminish antioxidant systems. Short intense bursts of physical effort in horses [3,5,40], endurance exercise in dogs [41] and horses [8,42], acute bouts of treadmill exercise in rats [43] and high-intensity leg cycling exercise in healthy adult humans [44] have also resulted in decreased plasma TAS.

In contrast, following 23 days of CMP the maintenance of plasma TAS at 1 h post-exercise suggests a reduction of oxidative damage, inflammation, and muscle membrane injury (evidenced by reduced plasma PGE$_2$ and CK 24 h following exercise). A nutritive antioxidant that mitigates excessive oxidative stress and inflammation [2,6,20,45] is appealing for endurance horses and horses that are required to compete for multiple days. However, care must be taken to also allow normal muscle repair and growth mechanisms to occur [46].

Lymphocyte concentrations and plasma TAS were maintained at baseline levels in CMP supplemented horses 1 h post-exercise, in contrast to the elevation seen at 1 h post-exercise in Co. Within the Co group, TAS was significantly decreased at the 1 h time point compared to BL. While there were no overt effects of CMP supplementation in horses at rest (pre-exercise comparison 22 days apart) CMP supplementation appears to have assisted in maintaining blood antioxidant potential and this became evident in response to the oxidative stress imposed by high intensity exercise. The mechanism is unclear as plasma SOD activity was unchanged in the CMP group, but perhaps indicating that activity was adequate for the demands. It is also possible that upregulated activities of glutathione peroxidase or catalase may have contributed to the increase in plasma TAS [25,47], and this needs to be investigated in future studies.

Ingestion of encapsulated forms of CMP reportedly mitigates the severity of diabetic neuropathy [26] and biomarkers associated with exercise stress [48]. The CMP is made from a melon with naturally very high SOD and catalase activities [26,49]. After ingestion in vegetal oil encapsulated form, as in the present study, the CMP exhibits anti-oxidant and anti-inflammatory properties *in vitro* and *in vivo* [24-26]. However the *in vivo* effects cannot be attributed to an absorption of SOD, catalase or other large protein molecules because these cannot be absorbed by the g.i. tract. Rather, dietary proteins and peptides undergo chemical modification and degradation to smaller peptides and amino acids when unprotected from the gastric environment (low pH, proteases, etc.), so it is not surprising that ingestion of unprotected CMP resulted in a loss of antioxidant parameters *in vivo* [19,24] consistent with SOD pharmacokinetics [50]. While the encapsulation (protection) of CMP protects proteins within the product from acid degradation in the stomach, the large protein molecules then present within the intestinal environment cannot be absorbed by transport systems of the intestinal epithelial cells. Based on this well known physiology, it can be concluded that the high SOD and catalase concentrations and activities of CMP cannot be directly responsible for the increased antioxidant potential in plasma and synovial fluid seen in the present study. This raises the question of how dietary CMP, when it passes through the stomach and enters the intestinal system largely intact, has the ability to induce systemic and local upregulation of antioxidant parameters. This is a topic of investigation by Lallemand (personal communication, Dr. Yannig Letreut, January 2017). What appears to be the case is that an indirect, and as yet unknown mechanism, induces the reported increases in endogenous antioxidant defense systems [24,29,49].

Excessive oxidative stress (levels of ROS that cannot be controlled by endogenous activities of oxygen free radical scavengers such as SOD, catalase and glutathione peroxidase) in skeletal muscle is associated with elevations in cytosolic calcium and membrane lipid peroxidation [46,51]. Membrane lipid peroxidation results in leakage of proteins into blood with the time course and magnitude proportional to the amount of muscle damage [52]. The exercise stress in the present and related studies [19,29] resulted in evidence of muscle damage as indicated by increases in plasma CK and AST in horses that did not receive CMP. Twenty-three days of supplementation with the blended supplement resulted in plasma CK and AST that were not significantly elevated following exercise and there was a tendency towards an upregulation in SF SOD 1 h following exercise [23]. The indications of reduced skeletal muscle damage in these studies are consistent with the reported antioxidant effects of CMP. An additive or synergistic effect of ingredients in the blended supplement may account for the more definitive antioxidant response seen previously [23].

Post-exercise muscle soreness, stiffness, reduced muscle force production and joint range of motion (DOMS – delayed onset muscle soreness) do not become apparent until approximately 24 to 48 h following exercise [52]. The mitigation of plasma PGE_2 24 h following exercise in the CMP group suggests CMP supplementation may have aided in attenuating post-exercise inflammation.

Proteoglycan fragments and increased GAG occur in SF following exercise [17] and also occur as a result of injury or applied irritants [20,53]. Increased SF GAG concentration is a marker of articular cartilage degradation. While the mechanism of exercise-induced increases in proteoglycan fragments and GAG are not fully understood, excessive mechanical loading [54] and / or inflammatory response [20] may be responsible. Thus, the blunted increase in post-exercise SF GAG noted in the CMP group compared to the Co 1 h post exercise in the current study indicates a reduction in cartilage breakdown post exercise. This was associated with evidence for an improved antioxidant potential of the synovial environment in CMP horses (increased SF SOD in the CMP; reduced SF nitrite 1 h post exercise). A more comprehensive examination of SF markers of antioxidant activity both at rest and following exercise are required to understand mechanisms by which nutritive antioxidant can mitigate marker of joint stress and inflammation.

The regulatory environment for dietary supplements differs by country or region. This CMP product has been registered and approved for use as an ingredient in animal feeds in the EU (under the name Melofeed), as a veterinary health product in Canada (under the name Enzalox)

and as an animal supplement in the United States (National Animal Supplements Council under the name Enzalox). It is important that dietary nutraceutical supplements are both safe and effective for the animals for which they are intended. The regulatory authorities have agreed that the product is safe under the intended conditions of use for food production animals and for horses, dogs and cats. The present study demonstrates efficacy of the CMP at dosages between 1.55 and 2.14 mg / kg body mass for horses.

Duration-response and dose-response studies with the CMP product have not been performed using horses, and the amount used was recommended on the basis of studies performed on production animals (cattle, swine) and approved for use by the European Food Safety Authority (EFSA). The duration selected is typical of that required for physiological and biochemical adaptations, although the time-course of response remains to be determined. It is also possible that a higher dosage may be associated with additional physiological benefit. It would also be useful to have mode of action studies that specifically examines mechanisms by which the supplement exerts the antioxidant effects at the level of muscle and in synovial joints.

5. Summary and Conclusions

Three weeks of supplementation with CMP reduced markers of articular and skeletal muscle oxidative stress and inflammation in response to high-intensity exercise in horses. There was an increase in SF SOD activity, and reduced SF nitrite and GAG 1 h following exercise in CMP horses. Plasma TAS and LYM levels in CMP horses were maintained 1 h following exercise, while PGE_2 and CK were reduced at 24 h. These data indicate that dietary CMP improved the oxidative and inflammatory responses following intense exercise. Future studies should seek to better characterize mechanisms of action of nutritive antioxidants and their roles in the physiological response to demanding exercise.

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