Brief Communication
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Assessment of Serum Myokines and Markers of Inflammation Associated with Exercise in Endurance Racing Sled Dogs

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Background: The endurance sled dog is the ultimate endurance athlete in which to examine the exercise-associated acute phase and myokine responses that might be related to changes in muscle metabolism and damage. An inciting cause for increased C-reactive protein has yet to be elucidated, which might involve interleukin-6 and other myokines.

Objectives: To examine concentrations of interleukin-6 (IL-6), interleukin-15 (IL-15), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α), and markers of the inflammatory response of exercise; monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein (CRP) before, during, and after an endurance racing event.

Animals: 26 sled dogs completing a 1650-km race.

Methods: In a prospective study, cephalic venipuncture was performed before racing, at the midpoint, and after racing. Body weight and serum CRP, MCP-1, IL-15, IL-8, IL-6, and TNF-α concentrations were evaluated using enzyme-linked immunoabsorbance-based assays or a luminex multiplex assay.

Results: There were no significant differences in concentrations of IL-6, IL-15, IL-8, or TNF-α at the 3 time points, whereas there were significant increases in MCP-1 (median and range—start: 86 pg/mL [30–1845]; midpoint: 179 pg/mL [53–730]; finish: 180 pg/mL [21–1294]; $P < .01$) and CRP (median and range—start: 18 μg/mL [11–58]; midpoint: 76 μg/mL [12–198]; finish: 60 μg/mL [12–170]; $P < .01$) at the midpoint and race finish. There was a significant linear relationship between MCP-1 and IL-6 ($R = 0.68$; $P < .01$).

Conclusions and Clinical Significance: The inflammatory response to exercise increases as measured by MCP-1 during and after endurance exercise in sled dogs. IL-6 appears to be associated with MCP-1; however, the reasons for increases in the acute phase response (CRP) cannot be attributed to IL-6 or other myokines. IL-6 and MCP-1 concentrations might be useful in future investigations of exertional rhabdomyolysis.

Key words: C-reactive protein; Interleukin-6; Myokine; Sled dog.

Contracting skeletal muscle cells play an active endocrine role in the regulation of metabolism and inflammation through production of cytokines in human endurance athletes competing in marathons, ultramarathons, and triathalons. These cytokines might be detected in serum and some have been associated with the exercise-induced acute-phase response (APR).[1] The acute-phase response is a rapid, nonspecific systemic response occurring secondary to many types of tissue injury and might be a physiologic protective mechanism during inflammatory events. The exact cause for the APR, has not been elucidated; although increased serum CRP concentrations occur in endurance racing huskies.[2,3] A consistent marker for the inflammation of exercise is often observed as increase in macrophage chemoattractant protein-1 (MCP-1).[4]

Three cytokines, now termed myokines, IL-6, IL-8, and IL-15, have been associated with release from contracting skeletal muscle. Of these three, IL-6 is the most extensively studied, whereas less is known about IL-15 and IL-8.[5] An exponential increase in circulating serum concentrations of IL-6 in response to prolonged exercise, followed by a decline in the postexercise period, is a consistent finding in human endurance athletes, and plays a role in acute inflammation in muscle as well as glucose metabolism.[1,6] IL-15 is an anabolic factor that is highly expressed in skeletal muscle and plays a role in muscle-adipose tissue "cross-talk," associated with fatty acid oxidation, and decreases with muscle atrophy.[5,7] Increase in IL-15 serum concentration during exercise might be because of an increased need for fatty acid oxidation and metabolism in contracting skeletal muscle. IL-15 production by type-II muscle fibres is influenced by strength training and resistance exercise with potential for detection of slight increase in serum after exercise.[8] IL-8, a chemokine, is a locally active angiogenic factor in skeletal muscle.[9] Increase in plasma concentrations during exercise response occurs inconsistently during strenuous running exercise in trained and untrained individuals.[5,10]

C-reactive protein is a marker of systemic inflammation and the APR in both humans and dogs, and IL-6, TNF-α, MCP-1, or all might be associated with this change.[4,5,11] The inflammatory response, although mild might be an indication of muscle inflammation and has the potential to be a marker of changes that might be associated with rhabdomyolytic events, as there is an inflammatory change observed in exertional rhabdomyolysis in sled dogs,[12] with invasion of damaged

Abbreviations:

APR	acute phase response
CRP	C-reactive protein
MCP-1	monocyte chemoattractant protein-1

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tissue by these inflammatory cells promoting with potential for increased TNF-α, IL-6, and MCP-1 production, which act as chemoattractants.[4]

The potential severe muscle fatigue/injury and pronounced CRP response in sled dogs competing in long-distance races suggest that they might serve as a model for the study of myokines, exercise-induced stress and muscle damage, as these dogs cover greater distances (80–100 miles/day) and are active for longer periods of time than human athletes. The aims of this study were 2-fold: to examine the myokine (IL-6, IL-8, and IL-15) and inflammatory/APR (TNF-α, IL-6, CRP, and MCP-1) responses in trained sled dogs participating in the 2011 Yukon Quest Sled dog race before, midrace, and at the end of the race, hoping to provide insight regarding metabolic and inflammatory changes in muscle associated with strenuous exercise that might one day lead to important biomarkers of exercise stress and muscle damage; and to examine associations between IL-6, MCP-1, and CRP and associations between body weight and IL-15, hoping to divulge the potential mechanisms for the robust CRP response in exercising sled dogs and to examine if IL-15 might be a marker of lean mass changes in exercising sled dogs that lose weight while racing.

Materials and Methods

Animals

All procedures complied with the standards set forth by the Cornell University Institutional Animal Care and Use Committee and the Yukon Quest Board of Directors Ethics and Animal Use Committee. Three teams (14 mixed-breed sled dogs/team) were enrolled in the study once owners provided informed consent regarding the study protocol. All dogs had a physical exam performed by a race veterinarian before the race start, at the midpoint of the race (Dawson City), and race finish (Fairbanks); and at each point the dogs were deemed healthy. Based on the Yukon Quest racing rules and regulations, dogs were not allowed to have any anti-inflammatory or immune-modulatory medications and random drug screening was performed to ensure this.

Each team of dogs had been in training for 5 months before the start of the race, running a minimum of 3,000 km during training over that time. All dogs were rested for at least 24 hours before collection of a prerace blood sample (collected from each dog 72 hours before the start of the race) with the longest distance being run by the teams before the initial blood draw being approximately 35 km. The race consisted of 1,600 km over varied terrain and under variable winter weather conditions. At the midpoint (approximately 700 km), each team was required to take a mandatory rest period of 36 hours.

Blood Sample Collection

A blood sample (5 mL) was collected from each dog via jugular or cephalic venapuncture and placed into a 7-mL red top plastic coagulation tube 72 hours before the start of the race (start), within 4 hours of arrival at the mandatory halfway rest stop for the 41 dogs that completed the first half of the race to the Dawson City check point (midpoint; 725 km), and within 6 hours after completion of the race (finish; 1,600 km) for the 28 dogs that completed the race. The tubes were centrifuged at 3,200 × *g* for 5 minutes within 30 minutes after blood draw and the serum was harvested and transferred to cryovials. All serum samples were immediately frozen (−20°C) and stored until after the race, at which time they were shipped on dry ice via overnight delivery to the primary investigator's laboratory where they were stored at −80°C for 4 months until analysis. Only 26 of the 28 dogs that completed the entire race had adequate quantity and quality (minimal visual hemolysis) of serum to perform analyses.

CRP ELISA

A canine CRP kit[a] that has been previously validated for use on canine serum was used to measure serum CRP concentrations. All samples from the same dog were analyzed in duplicate on the same plate and averaged. The kit was used in accordance with the manufacturer's suggestions with a minor modification being the use of only 2 μL of serum, rather than 10 μL, in an effort to remain in the linear portion of the standard curve set forth by the manufacturer's kit.[5] The intra-assay and interassay coefficients of variation (CV) for the ELISA kit were 6.5 and 7.2%, respectively, with a lower limit of detection (LLD) being 3.75 μg/mL and the upper limit of detection being 300 μg/mL, taking into consideration sample dilutions.

Cytokine Analysis

A canine cytokine kit (Millipore)[b] consisting of beads coated with antibodies against canine IL-6 (interassay CV = 15.8%; intra-assay CV = 3.9%; LLD = 12.1 pg/mL), IL-15 (interassay CV = 15.8%; intra-assay CV = 8.3%; LLD = 14.8 pg/mL), IL-8 (interassay CV = 15.2%; intra-assay CV = 15.6%%; LLD = 20.3 pg/mL), MCP-1 (interassay CV = 19.1%; intra-assay CV 13.8%; LLD = 8.6 pg/mL), and TNF-α (interassay CV = 20.0%; intra-assay CV = 13.2%; LLD = 0.32 pg/mL) was used.[13] The upper limits of detection for all cytokines/chemokines were between 10,000 and 50,000 pg/mL. Each serum sample from each sled dog was run in duplicate on the same plate, and a mean value was calculated based on standardized canine controls. All data was examined to assess whether the lower limit of detection was reached. In the case that a lower limit of detection was not met, in an effort to avoid statistical bias, a value was placed on that missing data point as one half of the lower limit of detection for that particular data point.[14,15]

Body Weights

Initial body weights were recorded using a veterinary platform digital standing scale[c] and weights were recorded for each dog. A 13.64 kg weight used to validate the weight obtained by weighing on 10 different occasions showed only a 1% coefficient of variation; therefore recorded body weights were not adjusted. At midpoint and finish, a commercial digital scale[d] was purchased and dogs were weighed while being held by the investigator. Validation of weight was performed with the investigator holding and not holding the 13.64 kg weight, which provided accuracy at a 0.7% intra-assay coefficient of variation, therefore no adjustments to final weights were made.

Statistical Analysis

All parameters except body weight failed normality testing using the Shapiro–Wilks test. Examination of data over time was assessed using a Friedman's paired repeated measures ANOVA and a posthoc Wilcoxon signed rank test for all significant parameters. Body weight data were normally distributed over time and analysed using repeated measures analysis of variance

with Tukey's posthoc analysis. Because of potential associations between myokines IL-15 and weight loss and IL-6 and CRP, MCP-1, or both, linear regression statistics were utilized to examine the data for associations. Significance for all variables was set at $\alpha = 0.05$.

Results

Of the 26 dogs that completed the race and had adequate serum sample for analysis, 8 were from team 1, 10 were from team 2, and 8 were from team 3. The finishing times of teams 1, 2, and 3, respectively, were 10 days, 14 hours, 45 minutes; 12 days, 7 hours, 15 minutes; and 13 days, 2 hours, 26 minutes. Physical examination of each of these dogs at the corresponding check points showed no illness or injury associated with racing. Data from the 28 dogs that completed the race showed a mean weight at the start of 23.2 ± 3.5 kg, which remained similar at the midpoint (24.0 ± 3.3 kg) and then decreased significantly between the midpoint and the finish (22.8 ± 3.5; $P = .012$). The median serum CRP concentration increased significantly from the start (18 ug/mL) to the midpoint (76 μg/mL), and remained elevated at the finish (60 μg/mL; $P < .001$). There was no significant difference in median serum CRP concentration between the midpoint and finish (Fig 1A).

The median serum MCP-1 concentration increased significantly from the start (85.9 pg/mL) to the midpoint (179 pg/mL) and remained elevated at finish (180 pg/mL: $P < .01$), with no significant differences between midpoint and race finish (Fig 1B). The median serum IL-6 (start 49.5 pg/mL, midpoint 40.8 pg/mL, and finish 42.0 pg/mL), IL-15 (start 56.9 pg/mL, midpoint 45 pg/mL, and finish 53.3 pg/mL), and IL-8 (start 3923 pg/mL, midpoint 3510 pg/mL, and finish 3764 pg/mL) concentrations did not differ significantly between the start, midpoint, and finish of the race (Fig 1C–E). Only 1 data point from IL-6 in a single dog was below the lower limit of detection. Measured TNF-α concentrations did not reach the detectable limit of the assay on at least 1 data point or more for 20 of the 28 dogs and a value of 0.16 pg/mL was given to these data points. There were no significant differences between median serum TNF-α concentrations across time points (Fig 1F).

Regression analysis was performed to examine the correlation between serum IL-6 concentrations and serum MCP and CRP concentrations at midpoint and finish. IL-6 and CRP showed no significant associations (midpoint, $R = 0.08$; finish, $R = 0.13$), whereas there were associations between IL-6 and MCP-1 at both the midpoint and finish ($R = 0.66$, $P < .01$; $R = .88$, $P < .01$: Fig 2A and B, respectively). Further examination of the association between MCP-1 and CRP at midpoint and finish showed no significant associations ($R = 0.33$, $P = .10$; $R = 0.22$, $P = .28$, respectively). Regression analysis between body weight and IL-15 showed no significant associations at midpoint ($R = 0.03$) and finish ($R = 0.02$).

Discussion

The sled dogs included in this study did not show significant systemic changes in serum myokine concentrations during or immediately after a 1,650-km race. These data are in accordance with a previous study, which showed no significant changes in serum IL-6 concentration in sled dogs after a 563-km race.[2] The lack of systemic increased in IL-6 might reflect metabolic differences between endurance trained sled dogs and human endurance athletes. Exercising sled dogs are able to utilize primarily fat and protein as their fuel source and therefore glycogen depletion, which has been associated with increased serum IL-6, might not be as prevalent as in human endurance athletes.[5,16]

No significant changes in serum concentrations of IL-15 and IL-8 were identified in the dogs enrolled in this study. These findings might be explained by the locally directed autocrine and paracrine activity of these myokines such that increased physical activity is not reflected by changes in systemic circulation.[5,9] IL-15 increases have been associated with muscle hypertrophy while decreases are associated with atrophy.[5] Although we expected a decrease because of the overall weight loss in the dogs, other factors might be involved in skeletal muscle IL-15 secretion being increased with increased muscle tissue fat oxidation. Therefore, in a species that preferentially utilizes fat more efficiently, IL-15 might not increase substantially in response to exercise. The data suggest that in sled dogs, these myokines have limited value as markers of metabolic changes influenced by endurance exercise. In addition, considering our timing for sample collection was within 4–6 hours of the cessation of exercise, it is entirely possible that there might have been modest increases or decreases in cytokines that were missed, and further investigation where blood is drawn at the cessation of an exercise bout might be far more revealing.

Our APR data are in agreement with a previous study demonstrating increased serum CRP concentrations in sled dogs after a 563-km and 1,850-km race.[2,3] Distance trained dogs also appear to have increased baseline CRP values when compared to normal canine values and baseline values established in our laboratory for healthy house pets.[2,3] The exact reasons for the higher baseline concentrations might be attributable to all teams having run within 48 hours of the prerace serum collection, which might have induced a mild acute phase response. Similar increases into the 10–25 μg/mL range have been observed in sprint sled dogs 24 hours after participating in a 16-mile sprint race.[17] One would also expect that other inciting causes, such as infection and inflammation related changes in IL-6 or TNF-α, might induce this CRP response; however, the lack of a detectable response of TNF-α and lack of detectable changes in IL-6 indicates that infectious or serious exercise-induced inflammation is not the source of elevated CRP.[18,19] Additional investigation is needed to determine the mechanisms of induction of the APR, which do not appear to be related to IL-6, as has been suggested in human endurance athletes.[1]

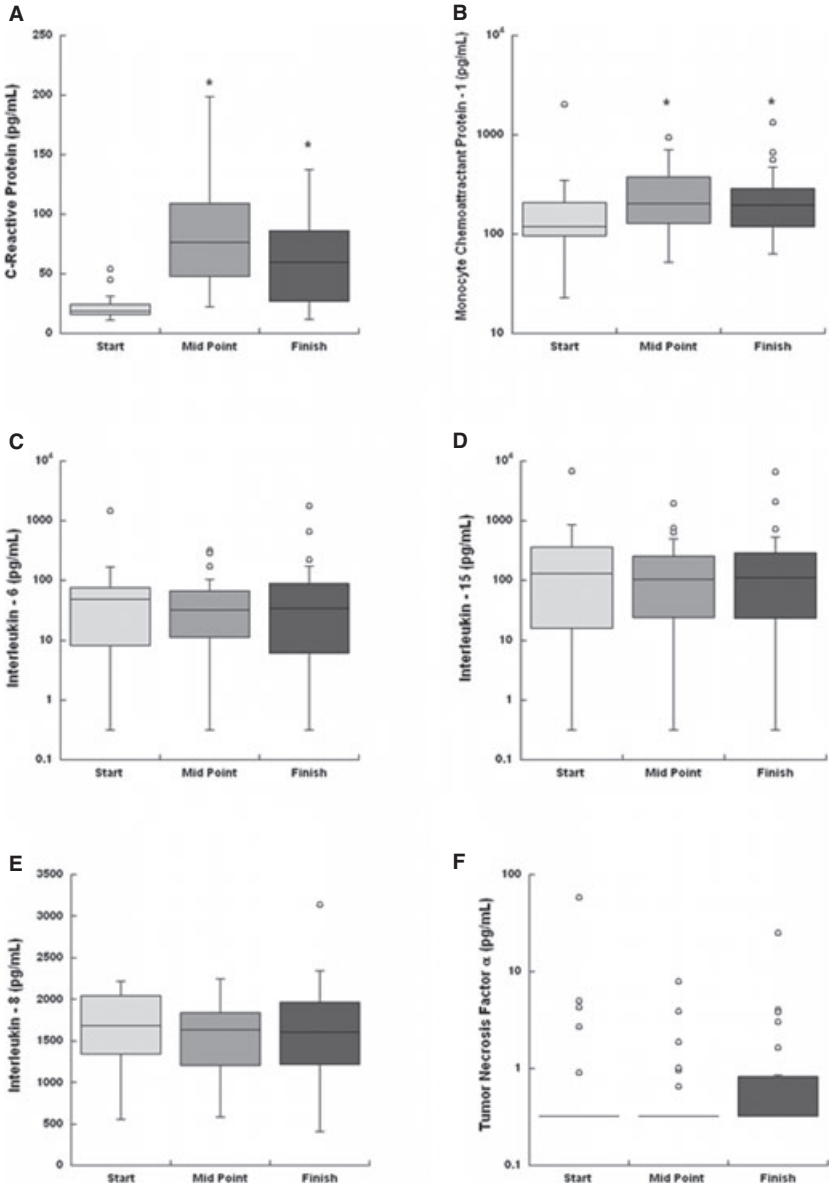


Fig 1. Box and whisker plots depicting the median serum CRP (A), MCP-1 (B), IL-6 (C), IL-15 (D), IL-8 (E), and TNF-α (F) from 26 sled dogs before the race start, at the midpoint, and at the finish of a long-distance endurance race. Box represents the 75th and 25th percentiles while whiskers extend to the 95th and 5th percentiles. Outliers are depicted as open circles beyond the 95th and 5th percentiles. * indicates a significant difference from race start (resting) values.

Another component of acute low grade muscle inflammation is the induction of MCP-1, which was increased at both the midpoint and at the race finish, indicating that there is a mild sustained inflammatory response. Because of the lack of a TNF-α response, we conclude that the inflammatory response is most likely originating from the skeletal muscle, which is commonly observed in human athletes undergoing endurance exercise.[20] Muscle damage is followed by a repair process involving the migration of macrophages into the muscle which can lead to a low mild increase in IL-6 attributable to release from macrophages.[21] Interestingly, there was a significant positive correlation between IL-6 and MCP-1 at both the half-way point and race finish. Considering these elevations, the use of MCP-1 or possibly IL-6 as markers of muscle inflammation in sled dogs might be a fruitful area of investigation in dogs suspected of acute/subacute rhabdomyolytic events.

In conclusion, the serum myokines often associated with endurance exercise in human athletes do not appear to be elevated systemically in canine endurance athletes, which may be attributable to inherent differences in metabolism and skeletal muscle biochemistry. In this and other studies, IL-6 was not associated with the APR as in some human studies, which might be because of either time of sample acquisition during exercise or a lack of a response in dogs. However, the

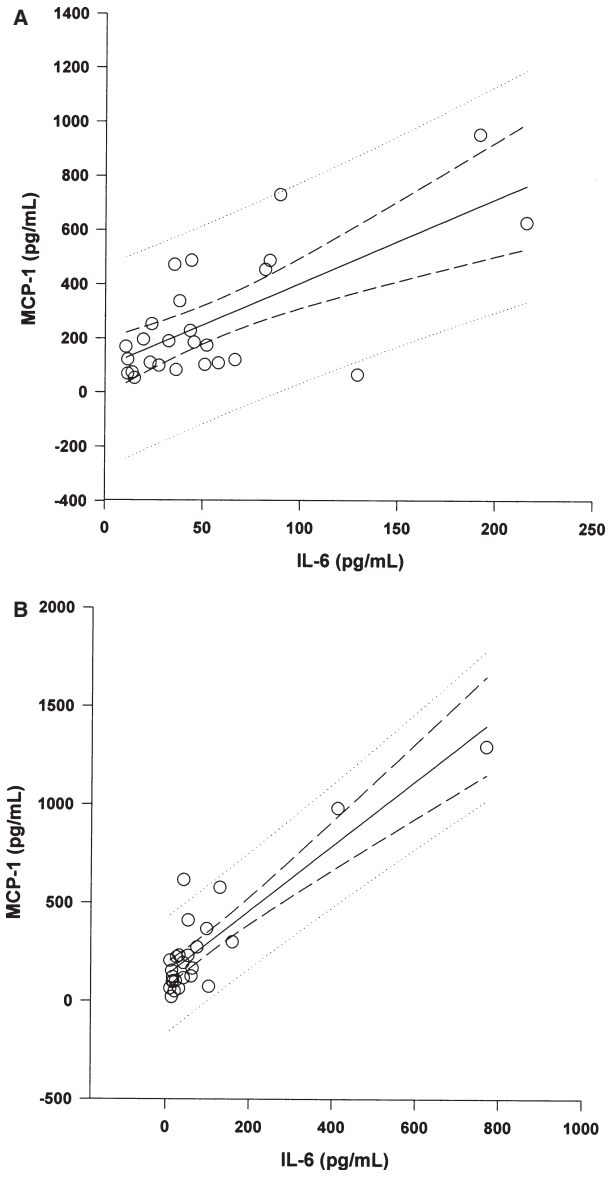


Fig 2. A. Regression analysis of MCP-1 and IL-6 from serum samples obtained at midpoint. A significant positive correlation was observed ($P < .01$, $r = 0.66$). B. Regression analysis of MCP-1 and IL-6 from serum samples obtained at race finish. A significant positive correlation was observed ($P < .01$; $r = 0.88$).

increase in serum MCP-1 and CRP suggest that mild inflammation and APR are present in endurance dogs. Therefore, in hindsight, to fully examine the inflammatory response of exercise, it would have been useful to examine interleukin-10 and interleukin-4, which are involved in dampening inflammation and promoting regeneration in skeletal muscle during exercise. These cytokines might be playing a significant role in the inflammatory response and further investigations in endurance dogs should include these cytokines in the analysis. A more complete analysis of this nature may hold the key to understanding the inflammatory response observed in our study by helping to differentiate normal physiologic response to exercise versus inflammation unrelated to exercise, possibly shedding light on the APR of exercise.

Footnotes

[a] Canine CRP ELISA, Tridelta PLC, Morris Plains, NJ
[b] Lincoplex canine cytokine multiplex, LINCO Research, St Charles, MO
[c] Bigtop Electronic Scale, Zeiss, Oberkochen, Germany
[d] 75444102BL CalMax Electronic Digital Scale, Oak Brook, IL

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

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