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Evaluation of plasma inflammatory cytokine concentrations in racing sled dogs

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Abstract — In human athletes significant changes in cytokine concentrations secondary to exercise have been observed. This prospective study evaluated the effect of a multi-day stage sled dog race on plasma concentrations of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-10 (IL-10). Samples from 20 dogs were harvested prior to and on days 2 and 8 of an 8-day race. Exercise resulted in significantly decreased TNF-α and IL-8 as well as increases of MCP-1, IL-6, and IL-10 concentrations (*P*-value between 0.01 and < 0.0001 for all parameters). The proportion of values for IL-2 that were below the detection limit increased from 40% on day 0 to 75% on day 2 and decreased on day 8 to 40% ($P = 0.04$). Racing sled dogs show cytokine-concentration changes that are different from those in humans.

Résumé — Évaluation des concentrations plasmatiques de cytokines inflammatoires chez des chiens de traîneau de course. Chez les athlètes humains, des changements importants des concentrations de cytokines secondaires à l'exercice ont été observés. Cette étude prospective a évalué l'effet d'une course de chiens de traîneau par étapes de plusieurs jours sur les concentrations plasmatiques des protéines-1 chimio-attractives des monocytes (MCP-1), du facteur-alpha nécrosant des tumeurs (TNF-α), d'interleukine-2 (IL-2), d'interleukine-6 (IL-6), d'interleukine-8 (IL-8) et d'interleukine-10 (IL-10). Des échantillons ont été prélevés sur 20 chiens avant la course et aux jours 2 et 8 d'une course de 8 jours. L'exercice a produit des valeurs significativement réduites de TNF-α et d'IL-8 ainsi qu'une hausse des concentrations de MCP-1, d'IL-6 et d'IL-10 (la valeur-*P* entre 0,01 et < 0,0001 pour tous les paramètres). La proportion des valeurs pour IL-2 qui étaient inférieures au seuil de détection a augmenté de 40 % le jour 0 à 75 % le jour 2 et a baissé le jour 8 à 40 % ($P = 0,04$). Les chiens de traîneau de course montrent des changements de la concentration des cytokines qui sont différents de ceux observés chez les humains.

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Introduction

In human athletic endurance medicine, measurement of cytokines is frequently performed to assess potentially negative effects of exercise (1,2). Similarly, as the ultramarathon model, sled dogs may also be negatively affected from exhaustive exercise (3,4). Various cytokines have been suggested to be associated with systemic inflammation and the acute phase response (APR) that could also be involved in muscle damage (5–7). However, to date, only few studies report cytokine concentration changes in racing sled dogs, related to endurance exercise (8,9). In addition, during previous studies the time at which blood was drawn (5 d after starting the race) and the prolonged exercise (over 160 km per day) (8,9) may not have been ideal for assessment of cytokine concentration changes. Furthermore, these endurance events are very different from typical human marathon or ultramarathon endurance racing due to the extreme distance covered. Different blood sampling times in shorter races may reveal additional information related to cytokine release during

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Table 1. Median and range of plasma concentrations (pg/mL) of MCP-1, TNF-α, IL-2, IL-6, IL-8, and IL-10

Parameter	Day 0	Day 2	Day 8
MCP-1	132.0 (55.4 to 240.5)	389.8 (159.6 to 1042.4)	255.7 (66.2 to 547.7)
TNF-α	0.91 (0.1 to 3.4)	0.21 (0.1 to 3.4)	0.9 (0.1 to 4.6)
IL-2	8.4 (3.8 to 44.3)	3.8 (3.8 to 37.6)	8.63 (3.8 to 47.8)
IL-6	10.75 (1.2 to 54.3)	14.7 (5.3 to 240)	20.32 (4.6 to 172)
IL-8	3738.55 (121 to 18 757)	324.9 (23.7 to 2332)	3102.7 (303.5 to 10 231)
IL-10	16.1 (1.9 to 107.6)	28.9 (3.0 to 151.8)	21.4 (3 to 312)

MCP-1 — monocyte chemoattractant protein-1; TNF-α — tumor necrosis factor alpha; IL — interleukin.

exercise, particularly since the prior studies in endurance racing sled dogs have not shown typical rises in interleukin-6 (IL-6) or interleukin-10 (IL-10) that are commonly associated with exercise (8,9). In human endurance athletes there is typically a rise in IL-6 and other inflammatory cytokines often termed the macrophage 1 (M1) response. This is thought of as a mild inflammation that is followed by a macrophage 2 (M2) response that relates to anti-inflammatory actions through the release of specific interleukins such as interleukin-10 (10,11).

The aim of this study was to determine the effect of daily endurance exercise during a mid-distance marathon stage racing event in racing sled dogs on plasma concentrations of pro-inflammatory cytokines [monocyte chemoattractant protein-1, (MCP-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and interleukin-8 (IL-8)], the anti-inflammatory cytokine IL-10 (IL-10), and the lymphocyte stimulating cytokine interleukin-2 (IL-2) using specific novel canine assays before racing, early in the race (day 2) and at the end of the race (day 8). We hypothesized that racing would result in significant changes in measured cytokine plasma concentrations on the different sampling days.

Materials and methods

Mixed breed racing sled dogs (n = 20) from 4 teams (T1 to T4), participating in the 2014 International Pedigree Stage Stop Sled Dog Race were enrolled. The study was approved by the Cornell University Institutional Animal Care and Use Committee (2014–0014). There were 5, 5, 4, and 6 dogs in T1, T2, T3, and T4, respectively. The kennel owners signed a consent form that described the study protocol. A full physical examination was performed on all dogs before the race, with all dogs proving to be healthy. Dogs were fed a mixture of commercial dog food supplemented with meat. The dogs selected for analysis participated in 5 of the 8 total days of the race with all dogs racing on days 1, 2, and 8 of the racing schedule. Dogs ran 52 to 75 km per racing day. All dogs were rested on day 3 as a scheduled travel day, and due to weather and trail conditions were also rested on day 7. The average racing time for each stage of the race was 3.5 to 5 h of continuous running with no significant resting periods. Dogs pulled a lightweight sled (80 to 95 kg) that included musher and supplies.

Blood sampling and analysis

Blood was sampled on day 0 between 12 and 1 pm prior to the race, day 2 between 2 and 3 pm and day 8 between 2 and 3 pm. Samples harvested on days 2 and 8 were taken within 30 min of arrival. At each time-point 6 mL of whole blood was collected via cephalic venipuncture using a 22-gauge needle into a 5 mL lithium heparin tube. Blood samples were protected from light and immediately centrifuged at 4000 × g for 10 min. Three aliquots of plasma were immediately stored on dry ice until transportation to the investigators' lab within 48 h, where they were immediately placed into a −80°C freezer.

Canine cytokine assays

All samples from an individual dog were run on the same plate to eliminate interplate variability. All kits were used according to the manufacturer's suggestions. The canine MCP-1 enzyme-linked immunosorbent assay (ELISA) was purchased and used within 1 mo of receiving the kits (Millipore, Temecula, California, USA). The interassay and intraassay coefficients of variation (CV) for the assay are 8.6% and 6.9%, respectively, with a lower limit of detection (LLOD) of 16.0 pg/mL. The canine IL-10 ultrasensitive assays were acquired and used within 1 mo of purchase (Mesoscale Discovery, Rockville, Maryland, USA); IL-10 [interassay coefficient of variation (CV) = 19.1%; intra-assay CV = 13.8%; LLOD = 5.9 pg/mL]. The canine electrochemoluminescent multiplexed cytokine kit [Proinflammatory Panel 3 (4-Plex)b] consisted of antibodies against canine TNF-α (interassay CV = 23.5%; intra-assay CV = 6.9%; LLOD = 0.17 pg/mL), IL-2 (interassay CV = 12.2%; intra-assay CV = 9.8%; LLOD = 7.6 pg/mL), IL-6 (interassay CV = 10.6%; intra-assay CV = 10.2%; LLOD = 2.4 pg/mL), IL-8 (interassay CV = 18.6%; intra-assay CV = 5.5%; LLOD = 1.3 pg/mL). Each 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 were examined to assess whether the LLOD was reached. In the case that a LLOD 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 (12,13).

Statistical analysis

Due to the skewed nature of many of the data sets, the median and ranges of plasma concentrations of the measured factors were calculated (Microsoft Excel 2013, Redmond, Washington, USA). Data for these parameters were analyzed over time using repeated measures analysis of variance (ANOVA) with the Proc MIXED procedure of SAS (User's Guide: Statistics Version 9.2; SAS Institute, Cary, North Carolina, USA). Fixed effects were

Table 2. Results of repeated measures ANOVA for differences in cytokine concentration over time presented as least squares geometric means and 95% CI. Fixed effects included time, gender and age. Kennel was included as a random effect in all models

	Least squares means* (95% CI)			*P*-value for fixed effects		
Parameter	Time 0	Time 2	Time 8	Time	Gender	Age
MCP-1	127.9[a] (93.64 to 174.8)	412.5[b] (288.2 to 590.3)	237.8[c] (161.3 to 350.7)	< 0.0001	0.3	0.91
TNF-α	0.811[a] (0.490 to 1.345)	0.243[b] (0.147 to 0.403)	0.935[a] (0.565 to 1.548)	< 0.0001	0.16	0.51
IL-6	10.27[a] (6.65 to 15.84)	20.80[b] (13.49 to 32.08)	20.13[b] (13.05 to 31.04)	0.01	0.07	0.03
IL-8	3011[a] (1609 to 5638)	310[b] (194 to 495)	2374[a] (1460 to 3860)	< 0.0001	0.24	0.98
IL-10	13.29[a] (4.41 to 40.02)	24.46[b] (7.79 to 70.66)	17.69[b] (5.88 to 53.27)	0.006	0.7	0.55

*Geometric mean. 95% CI — 95% confidence interval.
[a,b,c] Means with different superscript letters differ at level $P < 0.05$ (Tukey's *post hoc* test).

time, gender, and age. Kennel was included in each model as a random effect. Since measurements were unequally spaced in time, covariance structures tested included spatial (power law, Gaussian, spherical, exponential, linear) as well as unstructured. The covariance structure leading to the smallest Akaike Information criterion was chosen for each analysis. A Tukey's honest significant difference (HSD) test was performed to account for multiple comparisons when results for the effect of time reached significance ($P \leq 0.05$), least squares means were generated with the SLICE option in the LSMEANS statement of SAS. Because 52% of the values obtained for IL-2 were below the detection limit of the assay, data over time were dichotomized into 2 categories: those values above or below the detection limit. A Chi-square test was performed with the Proc FREQ procedure in SAS to analyze the proportion of samples below the detection limit.

Results

Of the 20 sampled racing dogs 10 were male and 10 were female, averaging 3.9 ± 1.9 y of age. Median (range) of plasma concentrations of the measured parameters are listed in Table 1 and results of repeated measures ANOVA in Table 2. All outcome variables were logarithmically transformed to satisfy the model assumptions for residuals. The covariance structure chosen for the analysis of repeated measures was unstructured for MCP-1 and IL-8, power law for TNF-α and IL-6 and exponential for IL-10. The *P*-value for the random effect of kennel was > 0.10 in all models. The proportion of values for IL-2 that were below the detection limit increased from 40% on day 0 to 75% on day 2 and decreased again on day 8 to 40% ($P = 0.04$).

Discussion

Racing resulted in significantly decreased circulating plasma concentrations of TNF-α, IL-2, and IL-8 on day 2 and significant increases in circulating MCP-1, IL-6, and IL-10 plasma concentrations on days 2 and 8. After the last day of the race, an overall increase to near pre-race circulating plasma concentrations for TNF-α, IL-8, and IL-2 were noted. The elevations of IL-6 and IL-10 and decreases in IL-2 and IL-8 concentrations in plasma are novel in racing sled dogs undergoing this type of racing.

The results of this study being different from prior studies in sled dogs (8,9) may have been influenced by various factors. First, the newer Mesoscale Discovery™ cytokine assays developed for canine species and used in our study appears to have lower sensitivity limits making data more reproducible across duplicates and potentially able to detect small changes that were not possible previously (8,9). Considering the differences in technologies, comparison of our results with past studies has to be performed with caution because past studies used fluorescent bead cytometry technology while the current study used fluorescent ELISA technologies.

Second, the stage stop format of racing with only 3 to 5 h of racing with regular rest periods is vastly different from typical ultra-marathon sled dog races that are up to 1500 km long with erratic exercise and resting periods, in which no changes in cytokines were observed after roughly 450 or 700 km of racing (8,9). In contrast, the dogs herein covered a total distance of approximately 300 km, with daily regular rest periods; this may affect plasma cytokine concentration changes. The early acquisition of samples on day 2, and the extra day of rest on day 7 before the final blood sample after day 8 of exercise may have influenced our results.

Third, it is possible that dogs in our study were trained differently and were exercised to a different level. Intensity of exercise can have a significant influence on the inflammatory response in humans (14). Indeed, trained athletes have shown lesser magnitude of changes in cytokine levels measured prior to and after exercise compared to non-athletes (15) and biochemistry values differed significantly among sled dog teams (16). However, the overall training schedule for these stage race dogs includes 30- to 60-mile runs, averaging 3 times a week. Therefore the exercise they undertook was similar to typical training — unlike endurance racing in which the actual race is often more intense than the pre-racing training regimen. In addition, there was no kennel effect herein: placement of teams were 4th, 9th, 11th, and 12th, suggesting that harder working dogs did not have different elevations of measured cytokines.

Fourth, and importantly, it is unclear if the measured plasma cytokine concentration changes are related to muscular origin, and therefore other sources must be considered. It is well recognized that increased gastrointestinal (GI) permeability and gastric erosions associated with inflammation are observed in competing sled dogs. Defects in gastric mucosa of sled dogs and other working dogs are common and can appear within 1 day of racing (17–19). This may have effects on immune function including increased leucocyte counts and potentially cytokine liberation (3,4). In addition, increased IL-6 concentrations in ironman racing have been associated with both muscular origin and increased GI permeability for endotoxins, resulting in systemic cytokine release, and onset of the acute-phase

response (20). Although no study has evaluated whether there is an association between increased IL-6 concentrations associated with erosive ulceration in exercising dogs, this cannot be ruled out; however, the overall cytokine profile showing decreases in major inflammatory cytokines (IL-8, TNF-α) with increases in anti-inflammatory cytokine IL-10 makes this pattern unique compared with pure inflammatory profiles observed in dogs with systemic inflammation due to sepsis (21).

None of the dogs in this study were diagnosed with clinical signs of rhabdomyolysis. Serum CK has been used in the past to detect exertional problems in sled dogs (23,24) with no consensus regarding its ability to be used effectively to definitively diagnose rhabdomyolysis, as CK concentrations above the reference range have also been reported without definitive evidence of rhabdomyolysis (24). While CK may be overly sensitive, it is an appropriate screening tool as a biomarker for rhabdomyolysis. A major limitation of our study was that the detected plasma cytokine concentration changes were not evaluated in relationship to plasma CK concentrations. However, the relatively small changes in cytokines suggest that any correlations in this population would not have been fruitful. The evaluation of cytokines in the current study aimed to potentially offer other markers (MCP-1, TNF-α, and IL-6) that may be more reliable. Yet, with only 2- to 3-fold rises or decreases in these markers we were unable to achieve this goal, suggesting that dogs do not have a similar response to humans during endurance exercise. These results suggest that sled dogs running similar distances to human marathon runners do not show prominent rises in inflammatory (IL-6) cytokines, further exemplifying that the (patho) physiology in racing sled dogs is likely different across species. Further studies examining endurance dogs early in exercise with and without exertional rhabdomyolysis may be more revealing.

The most convincing and repeatable evidence of activated macrophages was the heightened MCP-1 response during exercise as MCP-1 plasma concentrations more than doubled on day 2. Increased plasma MCP-1 concentrations are also described in human marathon runners (5). In contrast to the only previous study on MCP-1 in racing sled dogs (8), we found a decrease of plasma MCP-1 concentrations after racing on day 8 compared to day 2, while an elevated plasma concentration was maintained at the end of the previous study in which samples were taken on days 0, 5, and 10. This may be the result of different distances covered in these races, or the day of rest on day 7 before the day 8 blood collection in the dogs in this study.

IL-6 concentrations are consistently reported to increase in human endurance athletes, possibly secondary to hyperventilatory hypocapnia (25–27). Exercising muscle is likely the major source of IL-6 production in humans, supported by higher IL-6 concentrations in muscle compared to circulating blood (27). For the first time increased IL-6 plasma concentrations in canine endurance athletes are reported in our study, contradicting previous results (8,9). However, the IL-6 response is not typically a sustained response during exercise and these previous studies may have missed the acute, M1 macrophage-driven response phase. Although there was a modest increase in plasma IL-6 concentrations on days 2 and 8, we are unsure whether these are peak concentrations after exercise. The fact that both IL-6 and IL-10 plasma concentrations were elevated may be related to engagement of the M1 and M2 response, similar to human athletes, yet at a much lower level with species or fitness level playing a role in these responses.

Our study is the first to report IL-10 plasma concentrations in the canine endurance athlete. Besides increased IL-10 concentrations, a marked upsurge of additional immunomodulatory factors such as stress-related catecholamines has been described in humans, suggesting that each bout of exercise results in an anti-inflammatory environment (1). However, the latter is not necessarily true in sled dogs — at least, not to the extent it occurs in humans (28). The current study is corroborated by most previous reports from human endurance literature, in that an increase of IL-10 plasma concentrations is to be expected as a result of an anti-inflammatory process that takes place following exercise and is believed to be part of a protective and regenerative process. The IL-10 plasma concentrations returned to normal on day 8, possibly related to the natural M2-response that likely took place during the day of rest on day 7. This interpretation is supported by the fact that IL-6 plasma concentrations had increased again on day 8, likely a result of a new M1-response.

Decreases in plasma TNF-α concentration may be related to the anti-inflammatory cytokine IL-10, reported to inhibit TNF-α-release (29). Interestingly, we found that on day 2, circulating TNF-α plasma concentrations were decreased in face of IL-10 plasma concentration increases. Based on this combination and as previous studies did not find any changes or could not detect TNF-α (8,9), it is possible that decreased TNF-α plasma concentrations are normal in racing sled dogs. The detected rebound on day 8 could be based on a recovery mechanism similar to that observed in human ultramarathon runners (26).

IL-8 concentrations in human athletes are typically increased (5) and the only previous study evaluating IL-8 in sled dogs reported no significant changes (8). In contrast, we found a significant decrease in IL-8 plasma concentrations. As IL-8 is released in response to increased TNF-α (1) and we detected decreased TNF-α plasma concentrations, this could be an explanation for our findings. Another possible explanation could be induction of immune regulatory molecules, such as soluble IL-8 receptor, that is attenuated by day 8. In fact, soluble receptors for IL-6 and TNF-α may attenuate the availability of many of these cytokines for signaling. IL-2 plasma concentrations for many samples were below the detectable level in the current study. A significant decrease was found on day 2 compared to pre-race values when dichotomizing the samples as either above or below the detection limit. As in our study, a report on half-ironman triathletes described IL-2 as hardly detectable pre-race (2). Interestingly, day 2 showed suppression of inflammation and this crudely identified decrease in IL-2 plasma concentrations may be related to immune regulation and decreased lymphocyte counts, as observed in a study evaluating hematologic changes in racing sled dogs, since IL-2 is thought to stimulate propagation of lymphocyte populations (3). IL-2 plasma concentrations rebounded by day 8, similar to humans (30).

In conclusion, the current study showed that strenuous exercise in sled dogs induced significant changes in circulating

plasma cytokine concentrations. The sources of these cytokines were not determined. CVJ

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