Impacts of previous heatstroke history on physiological parameters eHSP72 and biomarkers of oxidative stress in military working dogs

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Abstract

Heatstroke (HS) is an acute, progressive life-threatening emergency. Animals, including military working dogs (IDFMWD), rapidly activate cytoprotective processes, e.g., heat shock proteins (HSPs) and antioxidative molecules, in response to heat stress. We hypothesized that serum HSPs (eHSP72) and oxidative stress markers would differ in IDFMWD with a history of HS compared with controls and thus could be used to detect susceptibility to recurrent HS. eHSPs concentration, oxidative stress markers, and systemic physiological parameters were studied in dogs with and without histories of HS, undergoing indoor or outdoor training. Treadmill physical performance tests (PPTs) were conducted indoors at 22 °C (groups C-I and HS-I) or outdoors under heat stress conditions of 36 °C; 60% humidity (groups C-O and HS-O). Pre-, immediately post-, and 45 min post-PPT heart rate (HR), respiratory rate, and rectal temperature (T_{re}) were recorded in all dogs. Likewise, blood samples were collected and eHSP72, venous blood gas analysis, and lactate and creatine kinase activity (CK) were assayed. Serum uric acid (sUA) and total serum redox potential (TRP) were measured only in the indoor group. Immediately post-PPT under both environmental conditions, T_{re} , HR, eHSP, sUA, and TRP (only measured in indoor PPT) significantly ($P < 0.05$) increased, whereas venous blood pH and bicarbonate decreased significantly ($P < 0.05$). Between groups comparisons demonstrated significant differences in basal HR and post-PPT T_{re} immediately after outdoor PPT. eHSP72 induction, CK, sUA, and serum TRP remained significantly higher in the HS group during post-PPT recovery. Taken together, animals with a history of HS have different results, and this signature of previous HS may predict altered heat sensitivity.

Keywords Heatstroke · Serum HSP72 · Low molecular weight antioxidants · Serum biochemical profile · Dogs

Introduction

Heat tolerance is a major concern in working dogs performing demanding physical activities in environmental conditions with extreme heat loads (Andress and Goodnight 2013; Stojših et al. 2014). Israel Defense Force military working dogs (IDFMWD) are heat-acclimatized and undergo a

controlled training regime involving gradually increasing physical activity, both indoors and out (Bruchim et al. 2014). Nevertheless, similarly to highly trained young athletes and soldiers, heat-related illness and its severe form, heatstroke (HS), sometimes occur (Andress and Goodnight 2013; Epstein et al. 1999; Kazman et al. 2013; Stojših et al. 2014; Wijerathne et al. 2016). During training, IDFMWD dogs are subjected to high metabolic loads and heat stress leading to an increase in body temperature (T_{re}). Despite the identical environmental conditions and physical effort, some dogs develop heat-induced illnesses, recurrent heat illness, or even fatal HS. Recurrent exertional HS episodes have been reported in young athletes, soldiers, manual laborers, and military working dogs, suggesting that heat intolerance has an individual predisposition, in contrast to pet dogs, where some of the factors affecting HS have been proposed to be associated with heat susceptibility (e.g., heavier breeds, non-athletic or brachycephalic breeds, retriever breeds, and obesity (Bruchim et al. 2006; Drobatz and Macintire 1996)). In highly trained, athletic dogs,

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it is unknown whether the environmental factors, namely the extreme heat load under which the dogs work are solely responsible for the occurrence of heat-related illness, or whether a lack of acclimatization, erroneous handling, or possibly innate physiological or genomic malfunctions, are also involved.

In human medicine, several heat tolerance tests (HTT) are used to assist informed return to duty. Decisions rely on clinical recovery (Moran et al. 2007) and HTT, designed to monitor physiological parameters, such as heart rate (HR), T_{re} , respiratory rate (RR), and sweating rate, before, during, and upon recovery from physical activity (Epstein et al. 2016; Kazman et al. 2013; Schermann et al. 2017; Stacey et al. 2017). Yet, the sensitivity (66%), specificity (77%), and overall accuracy (77%) of the HTTs are moderate at best (Schermann et al. 2017). Some data based on physiological measurements (HTT) have been used to predict future heat-related injury in humans (Schermann et al. 2017). There are no similar heat tolerance or intolerance predictors for working dogs. Considering that the death rate from heat-related illness in humans and dogs is as high as 50% (Argaud et al. 2007; Aroch et al. 2009; Bruchim et al. 2017; Carter 3rd et al. 2005; Segev et al. 2015), early identification of susceptible individuals is critical in order to prevent illness. Furthermore, regarding military working dogs, early detection will improve dog selection and the planning of training and work-loads.

Compendia of physiological and molecular data collected in hospitalized dogs and humans after HS of various degrees provide some clues to assessing disease severity and its prognostication (Bruchim et al. 2006; Epstein and Roberts 2011; Kazman et al. 2015; Lisman et al. 2014; Moran et al. 2006; Schermann et al. 2017). These include molecular signaling pathways that trigger the heat shock response (HSR)/cellular stress response, a highly conserved adaptive mechanism, protecting cells and tissues from excessive heat and other noxious stimuli, such as hypoxia, inflammation, and ischemic reperfusion injury (Horowitz 2002). Serum heat shock protein (eHSP) concentration has been evaluated as a biomarker of HS severity in monkeys (Dehbi et al. 2010), dogs (Bruchim et al. 2016), and humans subjected to heat stress (Walsh et al. 2001). In monkeys, eHSP72 concentration positively and significantly correlated with the severity of the heat insult, and in a study on 30 dogs with naturally occurring HS, eHSP72 recovery 24 h after the heat insult was significantly associated with survival (Bruchim et al. 2016). A study using dogs revealed that venous blood pH and several laboratory measures of hemostasis correlated with eHSP72 concentration during hospitalization (Bruchim et al. 2016). A 2-year follow-up of 15 military working dogs, from before the initiation of training (naïve dogs) and during training under conditions of high environmental heat stress, showed that the profound elevations in aerobic power, physical performance, and heat acclimation were accompanied by significant increases in eHSP72

and lymphocyte *hsp72* mRNA expression, before and after a PPT, suggesting that the training regime induces cellular adaptive processes (Bruchim et al. 2014).

Considering that heat stress increases the formation of reactive oxygen species (ROS) and induces cellular oxidative stress (Patton et al. 2018), other potential markers are linked to oxidative stress. Several biochemical and physiological hyperthermia-associated events promote free oxygen-derived radical formation (Flanagan et al. 1998). Exercise, an essential element in human and dog acclimation (Leon-Lopez et al. 2018), is characterized by increased oxygen consumption and lactic acidosis, and is accompanied by a proportionate increase in free radical production (Hinchcliff et al. 2000). HSPs reduce ROS and protect cells against oxidative stress (Wang et al. 2005; Wyttenbach et al. 2002).

Israeli Defiance Force military working dogs experiencing heat injury return to duty upon recovery from HS. In humans, previous HS may predict recurrent heat injury (Schermann et al. 2017). Thus, IDFMWD with a history of HS are a unique model enabling us to determine whether prior severe heat injury has a long-term impact on heat susceptibility. The hypothesis of this study was that eHSPs and antioxidative markers in dogs with a history of HS will be different from matched controls, and their responses to an environmental challenge will also differ. Our findings may advance the identification of a signature of heat sensitivity and tolerance/intolerance that will prevent heat injury.

Materials and methods

Dogs

The study included 22 healthy IDFMWD purebred Belgium Malinois military working dogs and was approved by the Commission of Animal Experiments Israel Ministry of Defense (12011). The dogs were deemed healthy based on history, physical examination, complete blood count, and serum chemistry. All dogs were well trained and acclimatized for at least 2 years. The dogs were divided into two groups: prior HS group and healthy match control. The diagnosis of HS is based on the consensus definition in the veterinary literature based on a history of strenuous exercise, exposure to a hot environment, or both, and acute onset of appropriate clinical signs (i.e., acute collapse and CNS dysfunction, increased body temperature, and tachypnea or tachycardia) in dogs that were otherwise healthy prior to the insult (Bruchim et al. 2016).

Each group was further divided into indoor or outdoor exercise groups, as follows:

1. Dogs with a history of exertional HS during military training or work (3–28 months before the trial)

- a. Indoor exercise (HS-I)
- b. Outdoor exercise (HS-O)
2. Control group—military dogs with no history of HS
 - a. Indoor exercise (C-I)
 - b. Outdoor exercise (C-O)

The military working dogs were held in ambient temperature kennels during the resting hours and were fed with high activity canine nutrition and free water access. The PPTs were initiated morning time at 8 a.m. Each dog, handled by his guide, was taken to the training acclimatized room (22 °C, 40% humidity) or to the outdoor training obstacle (36 °C, 60%), respectively, in which several physiological parameters were recorded including heart rate, rectal temperature (T_{re}), and respiratory rate (RR). Heart rate was measured by palpation of the femoral artery, whereas T_{re} was measured by fast responding electrical thermometer as previously described (Bruchim et al. 2014). Thereafter, each dog performed its regular treadmill training or outdoor training. Immediately at the end of the training session, the dogs physiological parameters were recorded (post-PPT). Dogs were running according to their physical performance level, which they used to do. No one of the dogs developed any clinical signs of heat-induced illness, collapse, or exhaustion during the PPTs.

There were no differences in age, sex, and body weight between groups, and all dogs underwent the same training program and military work.

Study design

Physical performance tests

In this experimental series, we studied whether eHSP72 level predicted susceptibility to heat-related illness.

The physical performance tests (PPT) were conducted twice, as follows:

1. Indoor—treadmill running in a climatic room (22 °C, 40% humidity, constant speed of 10 km/h with 10% inclination); 10 military working dogs, 5 with a history of HS, and 5 age- and body-weight-matched controls
2. Outdoor obstacles trail, under heat stress conditions (ambient temperature, 36 °C; relative humidity, 60%); 12 military working dogs, 4 with a history of HS, and 8 age- and body-weight-matched controls.

Blood collection

Cephalic venous blood samples were collected in serum, fluoride, separator tubes immediately before, immediately after, and 45 min after completion of each PPT. The sera were

separated within 30 min and immediately stored at −80 °C until eHSP 72 was analyzed in both inside and outside PPTs, while sUA, TRP, and creatine kinase measurements were performed only in the indoor PPT. Fluoride tubes were used for lactate measurement only in the indoor PPT. Blood was also collected in heparin-washed 1-ml syringes and stored on ice for blood gas analysis only in the indoor PPT.

eHSP72 measurement

Commercially available canine kits HSP72 High-Sensitivity (Stress Xpress®, Bioscience, Victoria, BC, Canada) were used to measure eHSP72 concentration, according to the manufacturer's instructions. Briefly, each sample was divided into three aliquots. Analyses were done in duplicates and the third aliquot was used for further analysis of TRP as described below. Samples were placed into the wells. The biotinylated antibody against HSP72 was added; the plate was sealed and incubated for 2 h at room temperature. The wells were then washed with a Tris-buffered solution, leaving only plate-bound HSP72, and were then stained with a horseradish peroxidase catalyzed reaction for 30 min. The stained solution was read by a spectrophotometer (i-MARK microplate reader, Bio-Rad, City, State, USA) at 450 nm. The amount of signal read by the spectrophotometer is directly proportional to the HSP concentration. The average optical density (OD) of each duplicate was calculated and used for statistical analysis. The normal range of HSP 72 was based on the control dogs and determined to be OD 0.09–0.15 (Bruchim et al. 2014).

Creatine kinase measurement

Blood samples for serum biochemistry were centrifuged within 30 min of collection, and sera were either analyzed immediately or refrigerated at 4 °C (pending analysis) that was performed within 12 h of collection or the samples were stored at −80 °C (pending analysis) using an auto analyzer (Cobas-Mira or Cobas Integra 400 Plus, Roche, Mannheim, Germany).

Cyclic voltammetry

The tested serum homogenate was placed in a well containing three electrodes: a working electrode (glassy carbon 3.3 mm in diameter), a reference electrode (Ag/AgCl), and an auxiliary electrode (platinum wire). A constant 100-mV/s electric potential (up to 1.3 V) was applied towards the positive pole, inducing an anodic current (Chevion et al. 2000; Ligumsky et al. 2005). Each wave on the current reflects the activity strength of several compounds sharing a similar ability to donate an electron. The determined potential value on the x-axis (E_p) (Fig. 1) indicates the presence of different types of low molecular weight antioxidants (LMWAs). The lower the

oxidation potential is, the higher is the ability to donate electron(s) (Kohen et al. 2000; Lenchner et al. 2017; Ligumsky et al. 2005). The concentration of the reducing agents is determined by the y -axis value (I_p) and is measured as the area under a certain wave curve (A_h) (Fig. 1). Low and high I_p/A_h values indicate low and high concentrations of the tested compound(s), respectively (Ligumsky et al. 2005).

Serum uric acid

A commercially available UA ELISA kit (Cayman Chemical, City, Michigan, USA) was used according to the manufacturer's instructions and as previously described (Tsukimori et al. 2008; Yadav et al. 2015). Briefly, the samples were placed into the wells. The biotinylated antibody against sUA was added and the sealed plate was incubated for 2 h at room temperature. The wells were then washed with a Tris-buffered solution, leaving only plate-bound UA and stained with a horseradish peroxide catalyzed reaction for 15 min. The stained solution was read by a spectrophotometer (Bio-Rad, i-MARK microplate reader, USA) at 540 nm. The average optical density (OD) of each duplicate is directly proportional to sUA concentration reported in mg/dl.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess the distribution of the continuous parameters. In order to compare the quantitative variables between two independent groups, the two-sample Student t test or the Mann-Whitney U test were used, for normally and non-normally distributed data, respectively. Assessment of the changes in continuous parameters (e.g., serum HSP 72, sUA) available at three time points (pre-, immediately post-, and 45 min post-PPT) was performed using the Friedman test, since some of the data were not normally distributed. For assessment of the differences in continuous parameters (e.g., HR and T_{re}), and for

data only available at two time points in each dog, before and after intervention, the paired t test or the Wilcoxon signed rank test was used, for normally and non-normally distributed data, respectively. All tests were 2-tailed, and $P \leq 0.05$ was considered significant. Statistical analyses were performed using a statistical software package (SPSS 21.0 for Windows, IBM, Chicago, IL, USA).

Results

Indoor physical performance

Signalment and physiological parameters

Ten purebred Belgian Malinois male dogs were included in the indoor PPT, five in each group (HS-I and C-I, respectively). The median time from the last HS event to the PPT was 5 months (range 3–28). There were no group differences ($P > 0.05$) in age, body weight, the pre-PPT physiological parameters (i.e., T_{re} , RR, and HR), and the treadmill running distance and duration between the study (HS-I) and control (C-I) groups. The mean treadmill distance and running time in all dogs were 5.8 ± 0.5 km and 36.6 ± 3.3 min, respectively, with no significant difference between the groups.

The immediate post-PPT T_{re} was significantly ($P < 0.001$) higher in both groups (HS-I, mean 41.00 ± 1.47 °C; C-I, mean 40.96 ± 0.79 °C) compared with the pre-PPT value (HS-I, mean 38.36 ± 0.28 °C; C-I, mean 38.48 ± 0.43 °C). The changes in T_{re} values over time within each group were significant ($P < 0.001$); however, no significant difference was found between groups (Fig. 2). The mean HR after the PPT was higher in both groups (HS-I, mean 118.20 ± 14.87 bpm; C-I, mean 114.40 ± 18.02 bpm) than before the PPT (HS-I, mean 81.20 ± 17.75 bpm; C-I, mean 84.80 ± 12.46 bpm). The change in HR values over time within each group was

Fig. 1 A typical voltammogram performed on the serum of a dog with a history of HS. Two typical anodic waves are noted as part of the anodic current. The first one is more prominent, and therefore, our study focused on it

Fig. 2 The change in the mean T_{re} over time within each group was significant ($*P < 0.001$); however, no significant difference was found between groups

significant ($P < 0.001$); however, no significant differences were found between groups. The median CK was significantly higher immediately after PTT ($P = 0.011$, median 148, range 145–666 vs. median 108, range 71–340 mg/dl, respectively) in the HS-I group compared with the C-I group.

Serum HSP72

The median eHSP72 levels in all indoor dogs increased significantly ($P = 0.008$) immediately post-PPT compared with the pre-PPT concentration (19.2 vs. 7.9 ng/ml; range 2.2–26.4 vs. 2.7–15.7, respectively), when groups were separated according to HS history; this change was only significant in the HS-I group ($P = 0.043$) (Fig. 3).

Venous blood gas, serum uric acid, lactate levels, and total redox potential

There was no difference in median serum lactate concentration pre- and immediately post-PPT indoor ($P = 0.074$, median 10.1 mg/dl, range 5.9–21.5 vs. median 14.2 ng/dl, range 9.5–36.5) with no significant difference between study groups in pH and HCO_3^- . sUA profile changed significantly over time in the three time points ($P = 0.003$, respectively) for all indoor dogs in the study. However, differences in sUA profile over time were only noted in the HS-I group but not in the C-I group ($P = 0.042$ vs. $P = 0.074$, respectively) (Fig. 4). Differences in TRP were only noted 45 min after PPT between groups, where it was significantly ($P = 0.012$) higher in the HS-I compared with the C-I (Fig. 5).

Fig. 3 Serum HSP 72 levels of HS-I and C-I groups at three time points: before PPT, after PPT, and 45 min after PPT. Serum HSP72 levels of both groups presented as ng/ml. Each box represents the median and the range. The change in median eHSP72 levels was only significant in the HS-I group ($*P = 0.043$)

Fig. 4 Significant difference in sUA concentration over time was only noted in the HS-I group ($P = 0.042$)

Outdoor physical performance

Signalment and physiological parameters

Twelve purebred Belgian Malinois dogs (males 7; females 5) underwent the outdoor PPT, 4 with a history of HS. The mean duration of the PPT in all dogs was 8.34 ± 2.02 min, with no differences between the groups. The mean post-PPT T_{re} (HS-O, mean 41.78 ± 0.35 °C; C-O, mean 40.86 ± 0.54 °C) was significantly ($P < 0.001$) higher in both groups compared to the pre-PPT T_{re} (HS-O group, mean 39.18 ± 0.37 °C; C-O, mean 38.58 ± 0.49 °C) with no significant difference between groups in the 3 measured time points. The mean HS-O group T_{re} was significantly higher than that of the C-O group, both

pre- and immediately post-PPT ($P = 0.046$ and $P = 0.013$, respectively) (Fig. 6).

The mean HR after PPT was higher in both groups (HS-O, mean 135.5 ± 26.3 bpm; HS-I, mean 126 ± 9.7 bpm) than before PPT (HS-O, mean 100 ± 3.2 bpm; C-O, mean 85.5 ± 10.24 bpm). The change in HR values over time within each group was significant ($P < 0.001$). The mean HR of the HS-O group was significantly higher than the mean HR of the C-O group before PPT ($P = 0.022$, 100 ± 3.2 bpm vs. 85 ± 10.2 bpm); however, no significant difference was found between groups after PPT (Fig. 7). Median CK did not change significantly after PPT, with no difference between groups ($P = 0.3$, CK before PPT HS-I, median 145 mg/dl, range 89–307 vs. C-I, 132 mg/dl, range 71–293 mg/dl post-PPT).

Fig. 5 There was no significant difference in TRP between groups, before and immediately after PPT; Ip level was only significantly higher in the HS-I group in recovery from PPT after 45 min, not in the C-I group ($P = 0.012$, median Ip $1.01E-06$ vs $7.32E-07$, respectively)

Fig. 6 The median rectal temperature (T_{re}) within each group before and after PPT outside. Each box represents the median and the range. The median T_{re} after PPT was significantly ($*P < 0.001$) higher than the T_{re} before PPT in both groups. The T_{re} before and after PPT were significantly higher in the HS-O group than in the C-O group ($**P = 0.013$ and 0.046 , respectively)

Serum HSP72 Levels

The median eHSP72 levels of the HS-O group, before PPT, immediately after PPT, and 45 min after PPT were 5.20 (range 5.12–5.35), 6.46 (range 6.2–7.13), and 5.78 (range 5.51–5.78), respectively. The median eHSP72 levels of the C-O, before PPT, immediately after PPT, and 45 min after PPT, were 5.27 (range 5.22–5.51), 6.2, (range 5.63–7.18), and 5.50 (range 5.45–6.27), respectively. The change in the median eHSP72 serum levels over time within each group was significant ($P < 0.001$); however, no significant difference was found between groups (Fig. 8).

Fig. 7 The median heart rate (HR) after PPT was significantly ($*P < 0.001$) higher after PPT in both groups. The change in the mean HR over time within each group was significant. The median HR before PPT was significantly ($**P = 0.01$) higher in the HS-O group

Discussion

The novel findings of this investigation are that dogs with a history of HS maintain stress markers, despite the physiological recovery. The biochemical signature was primarily of higher oxidative stress (HS-I) and significantly higher induction of eHSP72 post-PPT (HS-I). T_{re} was profoundly higher in the HS-O group before and after PPT increasing the vulnerability of the dogs when subjected to metabolic and environmental heat loads during training. These issues will be discussed below.

The IDFMWD dogs included in this study underwent rigorous training under intense environmental heat stress in the 2 years before the study. The HS group (HS-O and HS-I) suffered from HS events 3 to 28 months before the study, and all dogs showed a complete physiological recovery and underwent the same training as the control (C-O and C-I) dogs. Dogs with a history of HS running outdoors had significant increases in T_{re} immediately post-PPT compared with controls (C-O). Preceding the physical training program, environmental conditions, and fitness did not differ between the groups. The immediate post outdoor PPT mean T_{re} of the HS-O group was 41.8 °C compared with 40.6 °C in the C-O group, but the magnitude of T_{re} elevation post-PPT was similar in the both groups (Fig. 6). The consensus thermal threshold above which HS might develop in dogs is 41 °C (Hemmelgarn and Gannon 2013a, 2013b). In the present study, immediately post-PPT, in 7/22 dogs, the T_{re} was > 41 °C, and in one dog, it was above 42 °C, but none had clinical signs of heat-related illness or typical serum chemistry abnormalities (e.g., increased serum CK activity). In mongrel dogs following experimentally-induced HS, T_{re} exceeding 43 °C for 40 min are required to induce HS-related clinical signs (Shapiro et al. 1973). Likewise, the severity of the HS was positively correlated with the duration of maximal T_{re} (Shapiro et al. 1973). We noted that following strenuous physical activity military working dogs had marked hyperthermia ($T_{re} > 42$ °C), without clinical or laboratory abnormalities characteristic of HS, suggesting that acclimatization to high ambient temperatures and physical training can prevent overt HS, at least in part by increasing the temperature threshold for heat injury (Bruchim et al. 2014; Shapiro et al. 1973).

In the present study, in an agreement with our previous study (Bruchim et al. 2014), the post-PPT eHSP72 level was higher than the pre-PPT level following both indoor and outdoor exercise. Xiao et al. demonstrated that lymphocyte HSP 72 concentrations in humans with a history of HS before physical activity were elevated (Xiao et al. 2003) in the spring but not in the autumn. Ruell et al. showed that when comparing athletes with prior HS events to matched controls, a 1-h treadmill run induced a fourfold elevation in eHSP72 with no difference between study groups (Ruell et al. 2014). In the present study, a prominent, significant eHSP72 level increase

Fig. 8 Serum HSP 72 concentration in HS-O and C-O groups at three time points: before PPT, after PPT, and 45 min after PPT. Serum HSP72 concentration of both groups presented in ng/ml. Each box represents the median and the range. The change in median eHSP72 levels was significant between the pre- and post-PPT ($P < 0.0001$ in both)

post-PPT was only noted in the HS-I group but not in the C-I group (Fig. 3). HSP72 levels may be useful as a biomarker to predict individual susceptibility to stress, but because of the non-homogeneous responses, interspecies comparisons are hard to reconcile.

In the indoor PPT groups, we examined if dogs with prior HS sustained higher oxidative stress versus matched controls, by measuring sUA concentration and TRP evaluating LMWA. The sUA concentration in the HS-I group was significantly higher in the recovery phase post-PPT (Fig. 4). Concomitantly, this group (HS-I) demonstrated significantly higher TRP at the recovery time point compared with the C-I group.

Uric acid is a major antioxidant and has protective effects under certain conditions, associated with oxidative stress (e.g., hypertension and cardiovascular disease) (Sautin and Johnson 2008). The association between repetitive endurance exercise and lipid peroxidation, serum antioxidant capacity, and skeletal muscle damage were examined, and sUA concentration significantly increased post-exercise compared with controls (Hinchcliff et al. 2000).

The present study demonstrated a significant increase in sUA concentration during exercise in both C-I and HS-I. However, upon recovery, only HS-I maintained significantly higher sUA levels, indicating delayed recovery from a longer stress period (Fig. 4). In rats experimentally exposed to hyperthermic stress, sUA concentration increased significantly, likely due to tissue hypoxia (Kampa and Frascella 1977) perhaps from thermoregulatory activity.

Several possible mechanisms linking exercise and hyperthermia with oxidative stress and increased ROS production during exercise have been suggested, including lactic acidosis, hypoxia, shear stress, effects of hemoconcentration, and signaling pathways involving muscle osmoregulation (King et al.

2016). Serum LMWA was measured as a determinant of TRP in relation to the PPT, as described previously in humans (Kohen et al. 2000) and in 30 dogs that underwent gastric dilatation and volvulus (Lenchner et al. 2017) but not during exercise. In the present study, increased LMWA, expressed by TRP, was noted post-PPT in both the HS-I and C-I groups; however, the LMWA level at 45 min post-PPT was only significantly higher in the former, suggesting that while both groups had sustained oxidative stress during PPT, the C-I dogs were fully recovered 45 min post-PPT, whereas the HS-I group was not, possibly indicating an increased susceptibility and intolerance to oxidative stress due to the past HS or/and individual genetic differences. In the future, prospective studies in working dogs are warranted to assess if this test could be used to screen for individual susceptibility to heat-related illness.

The present study examined whether eHSP72, along with other oxidative stress parameters, can be used to predict susceptibility to heat-induced illnesses. Dogs with a history of HS showed significantly higher pre- and post-PPT T_{re} in the HS-O group, delayed recovery of oxidative stress markers, and a significantly greater increase in eHSP post-PT. However, stress-induced ΔT_{re} and HR did not differ in the HS and the C groups, suggesting that an epigenetic tag may be involved.

This study has several limitations. First, the small cohort size weakens the power of the statistical analyses and might have led to the insignificance and inconsistency of some results. Secondly, only single PPTs were conducted indoors and outdoors, and the effect of physical activity over an extended period was not assessed. Thirdly, the outdoor and indoor PPTs were recorded in 2 different years, though in the same season. The methods of the second PPT (an indoor series) were expanded; sUA and TRP were only examined in dogs that underwent the indoor PPT. Lastly, different dogs were used

for each PPT type. It would have been ideal to test the same dogs under the two different conditions; however, due to limitations set by the IDFMWD, this was impossible.

Our hypothesis that molecular and biochemical markers reflect previous HS injury is supported by the prominent induction of eHSP and CK and maintenance of oxidative stress parameters during the PPT-recovery period in the HS-I group. Although sUA and TRP were not measured in the outdoor PPT, higher pre- and immediately post-PPT T_{re} can also be considered as sensitive markers. Additional studies with larger numbers of dogs are required to accept or refute these findings.

Compliance with ethical standards

The study included 22 healthy IDFMWD purebred Belgium Malinois military working dogs and was approved by the Commission of Animal Experiments Israel Ministry of Defense (12011)

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