Final Report for the NSW Sporting Injuries Committee, Research and Injury Prevention Scheme

Study Title: Can plasma HSP70 be used to screen athletes at risk of injury from heatstroke?

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Fig 1. Runners in the City to Surf, Sydney. Every year some runners require treatment for heat illness.

Introduction

There are numerous instances of people collapsing during exertion dating back to antiquity, with an example documented in the Old Testament (II Kings 4:18-20) (Shibolet et al., 1976). Collapse of soldiers participating in military training is often the basis for reports of exertional heatstroke in the literature. A man aged 25 collapsed while on a march, and was admitted to hospital with a rectal temperature (T_{rec}) of 110°F. The patient's temperature remained elevated for four days, after which he appeared to improve; however, on the twelfth day he developed pneumonia and died (Malamud et al., 1946). This example demonstrates the serious nature of heatstroke, which has a mortality rate of around 10% even with vigorous treatment (Simon, 1993). More recently, athletes are better educated as to the dangers of heatstroke, but cases still occur. In 1988 in Australia, a 28 year old man collapsed with heatstroke after competing in an 8 km fun-run in hot conditions, and spent five months in intensive care, suffering severe rhabdomyolysis and the loss of one leg (Lee et al., 1990).

Exertional hyperthermia may range from a relatively mild disorder such as heat syncope, to the potentially fatal heatstroke. While earlier publications considered absence of sweating to be diagnostic for the disorder (heatstroke) in conjunction with central nervous system disturbance and body temperature above 40.6°C (Leithead and Lind, 1964), it is now considered that patients with lower temperatures can be at risk, and cessation of sweating is not necessarily diagnostic. Heat illnesses range from heat fatigue, heat rashes, disorders resulting from water or salt depletion, including heat exhaustion and heat cramps, heat syncope, and finally, the most serious disorder, heatstroke (Parsons, 2003).

A number of biochemical changes have been noted in athletes collapsing from exertional heat illness. Most investigations have been exploratory in nature, attempting to delineate the mechanisms leading to heatstroke. A variety of laboratory tests have identified abnormalities in the blood and plasma of patients with heat illness, including alterations in plasma phosphate (Knochel and Caskey, 1977), endotoxin, cytokines (Bouchama et al., 1991), and lymphocyte number (DuBose et

al., 2003). More recently the protective role of the heat shock proteins in the cell suggests that these proteins may play an important role in the aetiology of heat illness.

A field study by our research group has identified higher levels of plasma HSP72 in athletes with exertional heat illness compared with runners in the same race who finished the race with no symptoms (Ruell et al., 2006). Extracellular HSP72 found in the plasma is thought to be a danger signal for the organism, acting on immune cells and producing cytokines that promote an inflammatory reaction that may in extreme cases lead to heatstroke (Phillips, 2003). The novel aspect of the present project is that we have studied the HSP72 response during a standardized exercise test in a climate chamber. Our hypothesis was that HSP72 measurement can be used in a laboratory setting to distinguish those athletes at risk of developing exertional heat illness.

Methods

Subjects

Fifteen physically active, healthy men participated in the study which was approved by the University of Sydney Human Ethics Committee.

Heat Illness (HI) Group

In the heat illness group we have recruited and tested 7 subjects who have previously experienced an episode of heat illness. Criteria for inclusion in the HI group included treatment for heat stress during or after a running event by medical personnel as well as high temperature (>41.0°C), and/or rhabdomyolysis, and/or signs of neurological dysfunction such as loss of consciousness.

Further details on the HI group:

Subject 1: collapsed with a high temperature (41.9 ° C) during a running race 6 weeks prior to testing. He was medically treated onsite and discharged several hours later.

Subject 2: collapsed with a high temperature (41.7 ° C) following a marathon 3 months before testing. He was treated onsite and discharged 90 minutes later.

Subject 3: collapsed with a high temperature (42.9 ° C) in a running race 9 years before testing. He was treated on site and discharged 90 minutes later.

Subject 4: Two episodes of heat illness the year prior to testing. Both involved rhabdomyolysis. In the second episode he suffered severe symptoms (vomiting, muscle cramps, extreme nausea) and was hospitalised for 8 days.

Subject 5: Suffered 2 episodes of heat illness, 10 years and 2 years before testing. After the last episode, the runner was in a coma for 6 days, and spent 10 days in hospital.

Subject 6: Suffered collapse during a running race with a high temperature (41.9 C°).

Subject 7: Suffered 2 episodes of heat illness 2 years and 10 months prior to testing. The runner was treated both times and showed more serious symptoms of heat illness, with vomiting and mental disorientation.

Control Group

In the control group we have recruited and tested 8 subjects of similar age and aerobic fitness level as the subjects with heat illness. Characteristics of all the athletes tested are presented in Table 1. It should be noted that another 3 subjects were recruited and tested, but they were excluded from the final analysis either because of technical reasons (failure to appropriately record temperature during the exercise test), one subject fainted after cannula insertion, another subject did not meet the criteria for inclusion in the HI group.

Exercise tests

VO2 max test

Before the heat test, all subjects carried out a standard incremental exercise test to volitional exhaustion on a treadmill to determine the subjects maximal oxygen uptake (VO_2 max). Expired gases were collected using Douglas bags during the last minute of each work load and ventilation volume was measured with a Harvard dry gas meter and the volume was corrected to standardised temperature, pressure and dry gas. Oxygen and carbon dioxide analysers enabled the fractions of oxygen and carbon dioxide to be determined and a desk top computer programme was used to calculate the volume of oxygen used and the volume of expired carbon dioxide.

Heat test

The subject returned for the heat test approximately one week after the VO2max test. Before the heat test subjects were asked to refrain from vigorous physical activity and consuming caffeine and alcohol in the preceding 24 hours. A running velocity was chosen for the heat test equivalent to 72% of the subjects VO_2 max. The heat test was

carried out in a climate chamber set to 40% RH (relative humidity) and 30 ° C. On presentation to the laboratory subjects were weighed, asked to insert a disposable rectal probe 10 cm beyond the anal sphincter, then a cannula was inserted into an arm vein, and skin temperature sensors were placed on the chest, thigh, upper arm and calf. A blood sample was taken at rest then at 10 minute intervals during the exercise which finished either at 60 minutes, at exhaustion, or if the subject's temperature increased beyond 39.9°C. A further blood sample was taken 1 hour after exercise. The cannula was kept patent with sterile saline.

During the heat test, heart rate was measured every 10 minutes, and subjects were asked to rate their RPE (relative perceived exertion) and thermal comfort. Rectal temperature was taken every five minutes while skin temperature was acquired every minute. Sweat rate was estimated by weighing the subject and their clothes before and after exercise, with correction for sweat trapped in clothing, respiratory moisture loss and the water ingested during exercise.

During the heat test, heart rate was measured every 10 minutes, and subjects were asked their RPE and thermal comfort index. Rectal temperature was taken every five minutes while skin temperature was acquired every minute. Sweat rate was estimated by weighing the subject and their clothes before and after exercise, and the water ingested during exercise was corrected for.

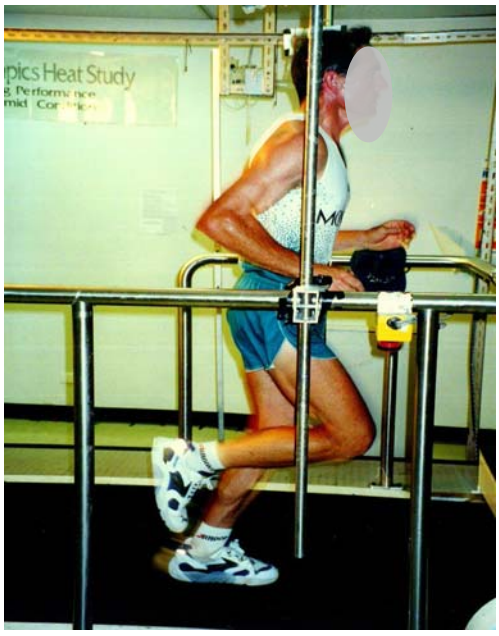

Figure 2. Athletes ran on the treadmill in the climate chamber during the VO_2max and prolonged exercise tests.

Biochemical analysis

Blood samples were collected into EDTA and serum tubes and immediately placed on ice. Samples for flow cytometry and for hematology measures were collected into an EDTA tube and kept at room temperature. The sample for lactate/glucose was collected into a heparinised tube and kept on ice.

The blood for plasma or serum HSP72, HSP72B' and HSP27 was centrifuged at 2,000g for 10 minutes, and the plasma or serum removed and stored at -85 °C for later analysis. Plasma HSP72 and HSP27 were analysed using R and D (MN, USA) kits catalogue numbers DYC1663 and DYC1580 respectively, while serum HSP72B' was analysed using an Assay designs (MI, USA) kit, catalogue number EKS-725. Haematology was performed using a Sysmex KX-21N instrument. Glucose/lactate was analysed using a Radiometer EML105. Monocyte and lymphocyte HSP72 were analysed by flow cytometry and mean fluorescence intensity (MFI) measured.

Statistics

Analysis was carried out using SPSS 16.0. Non-paired t-test was used to compare parameters such as weight, height, age, VO_2max and sweat rate. For other measures a 2 way ANOVA was conducted followed by simple contrasts to further analyse the time effect where it was significant. A significance level of $p<0.1$ was selected due to the relatively small numbers included in the study.

Results

Subjects

General characteristics of the subjects including age, weight, height and VO2 max are presented in Table 1. There was no difference between groups with respect to these characteristics. C is the control athletes while HI is the group with a prior episode of heat illness.

Table 1 *Characteristics of subjects*

Group	**n**	**Age (years)**	**VO2 max (ml/kg/min)**	**Height (m)**	**Weight (kg)**
C	8	32 ± 2	64 ± 2	1.80 ± 0.03	75 ± 3
HI	7	31 ± 3	62 ± 4	1.80 ± 0.02	80 ± 3

Hematology

Lymphocyte and monocyte cell number were analysed and the results are shown in Figure 3. Both showed a significant time effect ($p<0.05$). There was no difference between groups nor an interaction between groups and time for lymphocytes, while for monocytes there was no interaction but a significant difference between groups ($p<0.1$). Figure 3 shows that monocyte number was higher in the heat illness group. It should be noted that the monocyte number was at all times in the normal range (0.28-1.07 x 10^9/l for males).

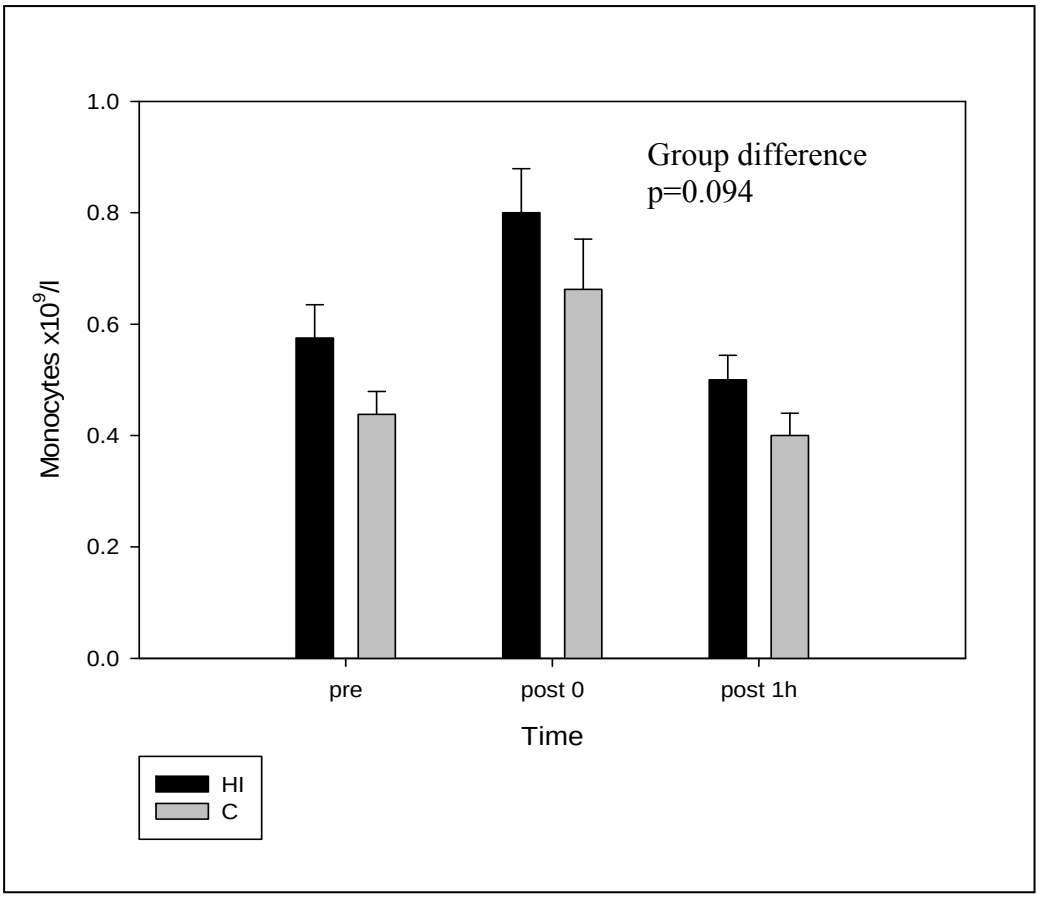

Figure 3. Monocyte cell number in both groups at 3 different times including pre, immediately post and 1hr post exercise. Monocyte cell count was different between groups. Numbers increased significantly at the end of exercise compared with rest ($p<0.001$). C=control, HI=heat illness group.

Lymphocyte and monocyte HSP72 were analysed by flow cytometry at three time points before and after exercise. Lymphocyte HSP72 showed a time effect ($p<0.05$), but no group effect nor interaction between group x time. Monocyte HSP72 is shown in Figure 4. Again there was a significant time effect, no interaction between time x group and monocyte HSP72 was lower in the HI group ($p<0.1$).

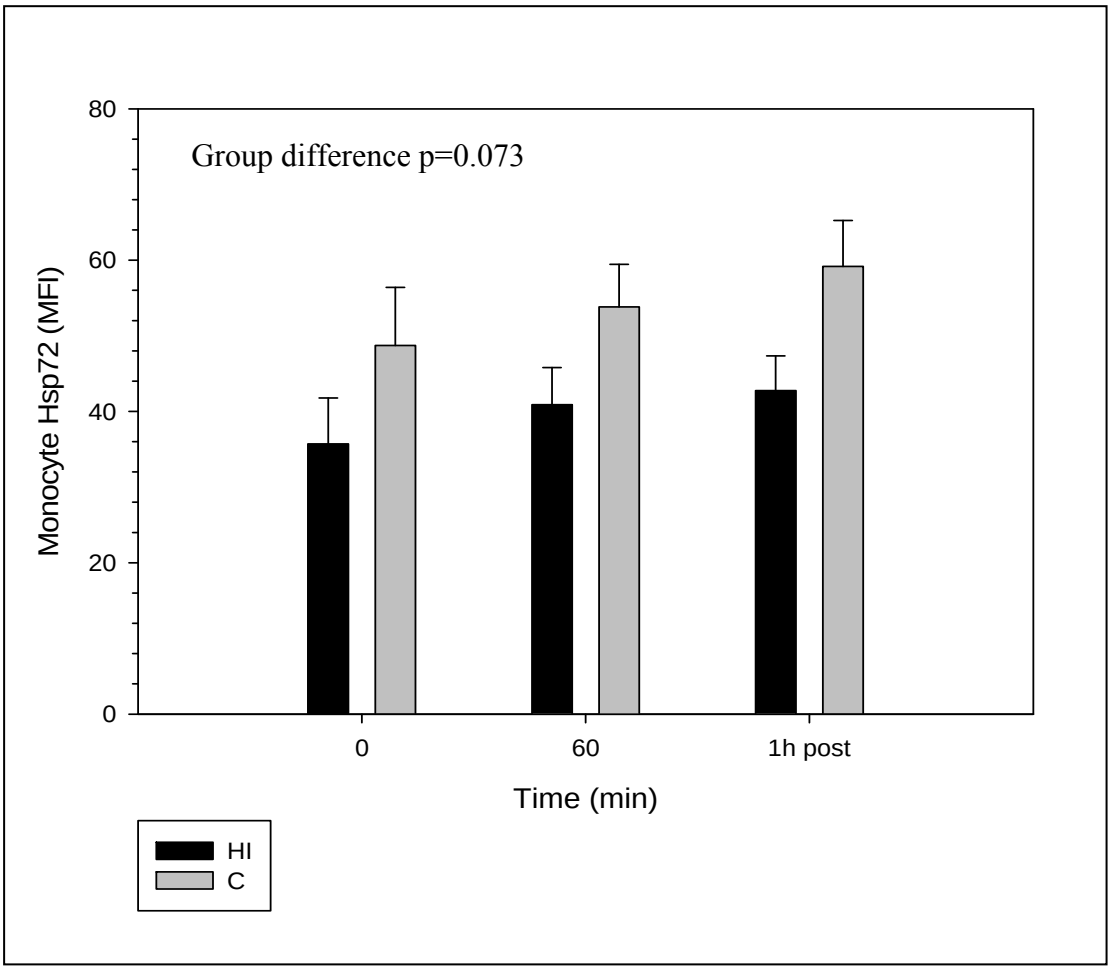

Figure 4. Monocyte HSP72 was significantly different between groups ($p<0.1$). The levels were lower in the HI group. C=control, HI= heat illness.

Plasma HSP72 was measured before, during and after exercise and the results are presented in Figure 5.

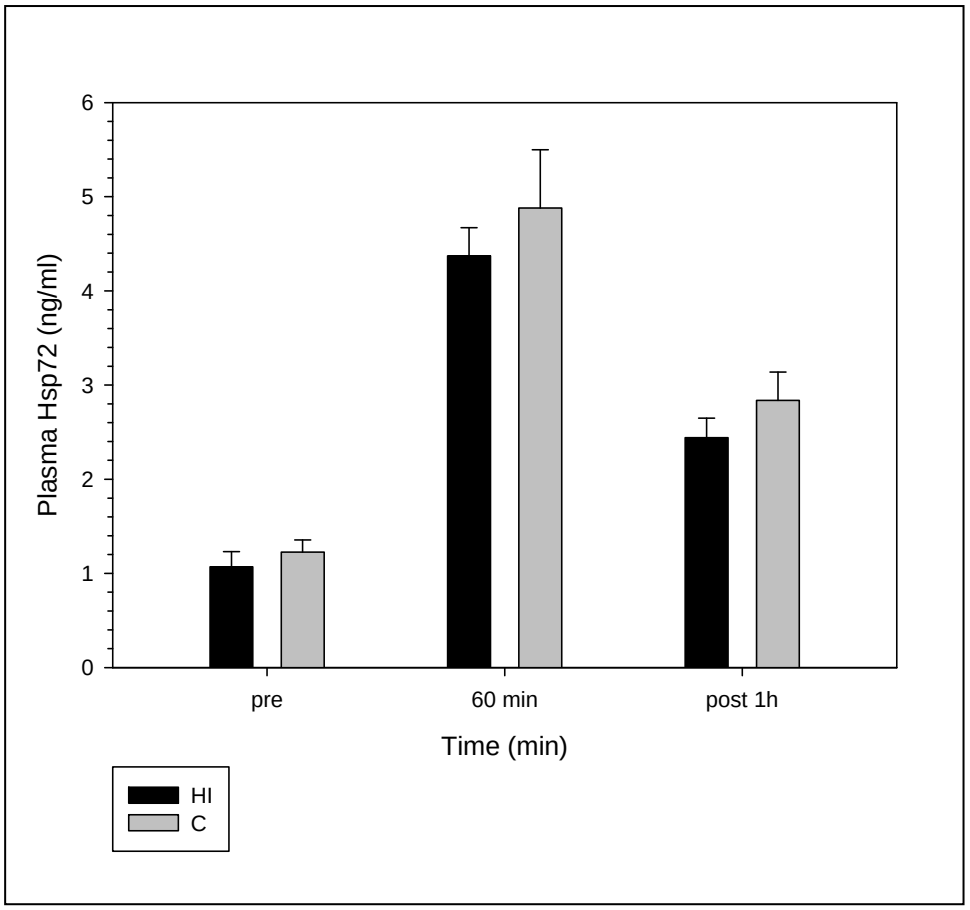

Figure 5. Plasma HSP72 was markedly increased with exercise, but the response was the same in both groups.C=control, HI = heat illness.

A variant, plasma HSP72B' has been discovered with different characteristics to HSP72. We analysed samples at three time points and the results are presented in Figure 6.

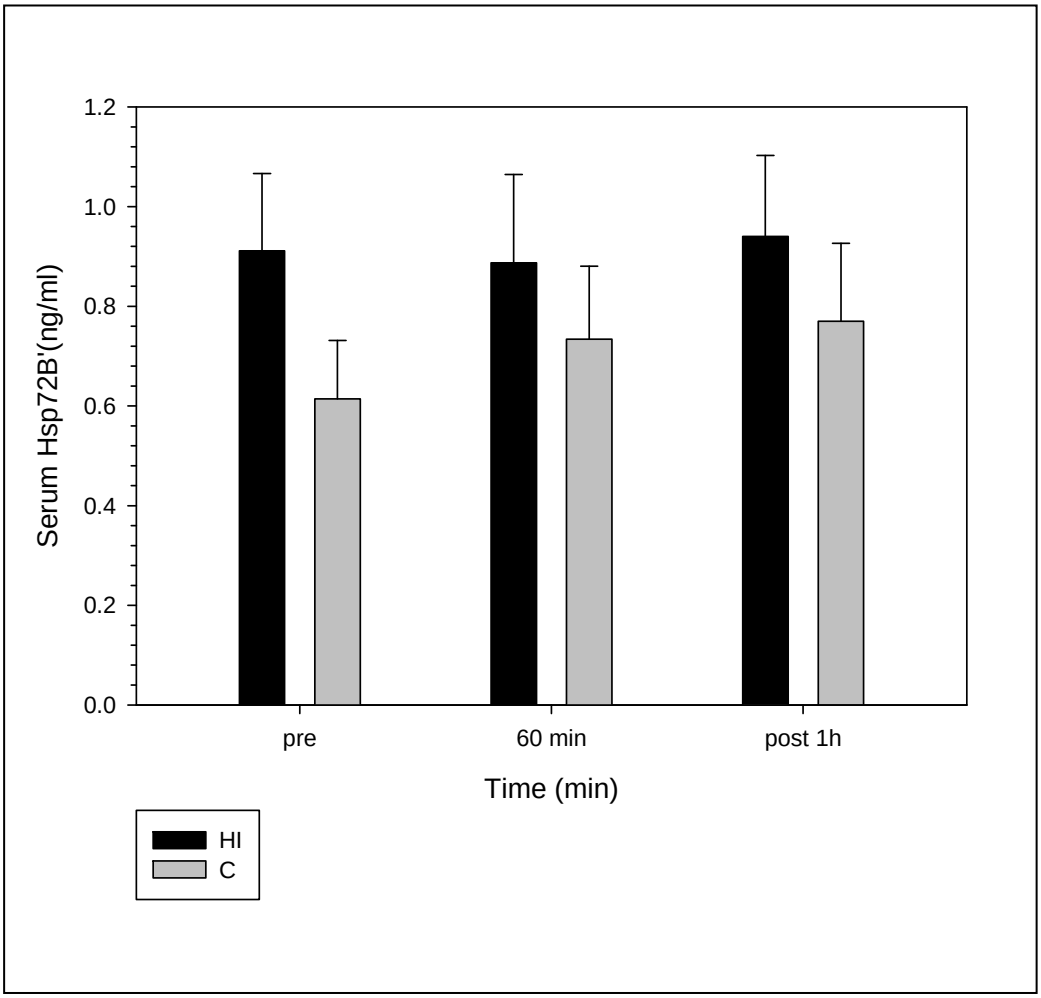

Figure 6. Serum HSP72B' in control runners (C) and those with a history of heat illness (HI). There was no effect of exercise and no difference between groups.

Plasma Hsp27 was analysed also at 3 time points, and a significant time effect was noted ($p<0.005$), with no interaction between group x time and no difference between groups (Figure 7).

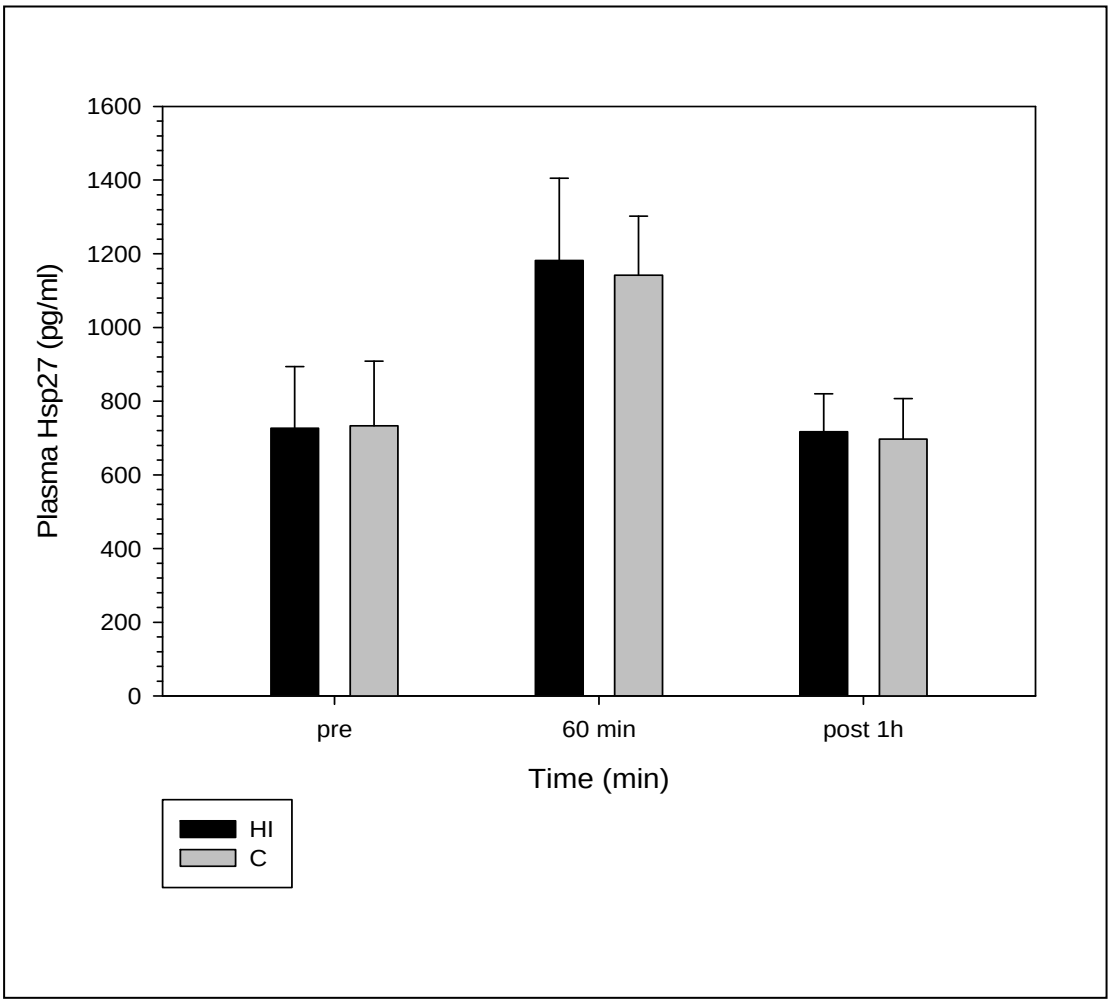

Figure 7. Plasma HSP27 did not differ between groups. C=control, HI=heat illness.

Other measures included glucose, haemoglobin, leucocyte, platelet number and sweat rate and these are presented in Table 2.

Variable	**C**	**HI**
Glucose (mM) pre	4.94 ± 0.4	5.09 ± 0.3
Glucose (mM) post	6.4 ± 0.3	6.3 ± 0.7
Hemoglobin (g/dl) pre	14.7 ± 0.3	14.3 ± 0.4
Hemoglobin (g/dl) post	15.7 ± 0.2	15.2 ± 0.5
Leucocyte ($x10^9$/l) pre	4.4 ± 0.2	4.7 ± 0.4
Leucocyte ($x10^9$/l) post	8.0 ± 0.3	8.7 ± 0.4
Platelets ($x10^9$/l) pre	190 ± 21	201± 16
Platelets ($x10^9$/l) post	275 ± 22	283 ± 22
Sweat rate (l/hr)	1.64 ± 0.1	1.53 ± 0.17

Table 2. Other biochemical/physiological measures. There was no difference between the groups. C= control and HI= heat illness group.

Discussion

There are many studies examining athletes collapsing from heat illness during the acute phase of their illness, and many physiological changes have been noted compared to athletes who did not collapse (Sutton and Thompson, 1998). Alterations to a number of blood constituents have been noted, including higher plasma HSP72 (Ruell et al., 2006), endotoxin and cytokine levels (Bouchama and Knochel, 2002), β endorphin (Kraemer et al., 2003), hyperkalemia and hypocalcemia (Moreau and Deeter, 2005). There are few studies examining heat illness patients following complete recovery.

Hence our study is somewhat unique in studying individuals with a prior history of heat illness/heatstroke. A novel finding of the present study was an increase in monocyte number in the HI group (Figure 3). Our results also indicate a lower level of monocyte HSP72 in the HI group (Figure 4). Our finding of increased monocytes in the HI group is useful in understanding the mechanism for the pathogenesis of heatstroke. A recent hypothesis is the dual pathway model of heatstroke which suggests that heatstroke is triggered by two distinct but connected pathways (Lim and Mackinnon, 2006). A robust immune system protects the organism from endotoxin leakage from the gut which occurs in response to exercise. Under this hypothesis, prolonged exercise training can compromise the immune system, leading to higher cytokine levels, lower anti-LPS levels and suppression of cell-mediated immunity. These individuals are more susceptible to heatstroke because their tolerance to endotoxemia is compromised. Our finding of higher monocyte levels in the HI group is in agreement with this model as monocytes produce proinflammatory cytokines, hence the HI group with a higher level of monocytes may be able to more quickly produce higher levels of cytokines such as tumour necrosis factor that are important in the pathogenesis of heatstroke.

Our study also found that monocyte HSP72 was lower in the HI group. Another study found a difference in the leucocyte HSP72 1 hour after exercise (Moran et al., 2006). This study examined heat intolerant individuals, many of whom were suspected to have suffered from heat illness, though details were not given. Lymphocyte HSP72 was the same pre and immediately post exercise, and lower one hour post exercise. Moran suggests that the lower HSP72 levels may be important for HI athletes who are

not as well protected against a subsequent acute heat stress. Our finding of both a higher monocyte number and a lower HSP72 content of the cells could be important firstly in that these athletes are able to produce a more rapid increase in pro-inflammatory cytokines, while at the same time these runners are not as protected from the deleterious effects of heat stress because of the lower amount of HSP72 in these cells.

Our hypothesis, that plasma HSP72 during a standard exercise test in the heat may be useful as a screening tool to identify runners most likely to suffer heat illness was not supported by the data. Assays for plasma HSP72, HSP27 and serum HSP72B' showed similar responses between the two groups. This study has been unique in measuring serum HSP72B' during exercising runners (Figure 6). It has been noted in microarray studies that the HSP70B′ gene is specifically affected by exertional heat injury, but not by exercise (Sonna et al., 2007). This study found no increase in HSP72B' with exercise. While there was no significant difference between groups, the numbers in this study are relatively small and it would be useful to analyse serum HSP72B' levels in a larger of runners with heat illness, as there appears to be a trend to higher levels of this protein in the HI group.

Another unique feature of the present study was inclusion of plasma HSP27 analysis. HSP27 is a small heat shock protein that has an important role in protecting muscle following maximal lengthening contractions (Paulsen et al., 2007). The role of HSP27 in the plasma during exercise has only recently been studied in patients with chronic fatigue syndrome during exercise, with lower values in the patients compared with normal control subjects (Jammes et al., 2009). In the present study plasma HSP27 was similar in both groups, being higher immediately following exercise compared with rest and with levels returning to baseline values 1 hour post exercise.

Limitations of the study

Our final number of runners with prior heat illness was 7. Initially we had hoped to recruit 12, but recruitment was slow and given the stringent requirements of out entry criteria we were not able to recruit more subjects.

Our study has chosen to recruit subjects who have collapsed from heat illness because we presume they are more likely to suffer from heat illness again. Some subjects do in

fact suffer from recurring episodes of serious heat illness, and this was the case for 3 of our subjects. It is possible that the occurrence of the heat illness episode induces changes to the individual that were not present before the episode.

Summary

This study underlines the morbidity associated with heatstroke as demonstrated by the medical history of the HI runners. Other studies have shown that runners succumbing to heatstroke are often ill, overweight, are less well-trained, unacclimatized to heat, may be dehydrated or have a history of heatstroke (Sutton, 1984). Increases in scientific knowledge, and development of new testing procedures are never a substitute for common sense. Proper preparation for running races (hydration and heat acclimatisation), choosing not to run if ill and holding races in cooler conditions are all measures that will significantly reduce the incidence of heat illness, and their importance should not be diminished because of their apparent simplicity.

Our study did not show that plasma HSP72 is useful in predicting athletes at risk of developing heat illness. The differences shown in monocyte number and monocyte HSP72 level, while not sufficiently large to be used for screening purposes are useful in understanding possible mechanisms in the development of heatstroke.

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