AUTOLOGOUS RED BLOOD CELL REINFUSION: EFFECTS ON STRESS AND FLUID REGULATION

ARMY RESEARCH INST OF ENVIRONMENTAL MEDICINE NATICK MA

R P FRANCESCONI ET AL. JUL 96

UNCLASSIFIED
**Report Title:** Autologous Red Blood Cell Reinfusion: Effects on Stress and Fluid Regulatory Hormones During Exercise-Heat Stress


**Performing Organization:** US Army Research Institute of Environmental Medicine, Natick, MA 01760-5007

**Report Date:** July 1986

**Number of Pages:** 23

**Summary:**

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AUTOLOGOUS RED BLOOD CELL REINFUSION:
EFFECTS ON STRESS AND FLUID REGULATORY
HORMONES DURING EXERCISE-HEAT STRESS

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Running Title: Erythrocythemia and hormonal responses

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Abstract

This study assessed the effects of induced erythrocythemia on stress and fluid regulatory hormones during exercise in the heat. Six unacclimated male subjects received approximately 600 ml of a sterile saline solution containing 50% v/v of autologous erythrocytes. Three heat stress tests (HST's) were attempted: one approximately 2 weeks prior to the reinfusion procedure, a second 48 h after the reinfusion procedure, and a third 1 wk later corresponding to 9 d subsequent to reinfusion. Each HST comprised three consecutive exercise and rest intervals of 45/15 min, respectively ($V_{O_2}$ = 2.0 L.min$^{-1}$, 1.56 m.s$^{-1}$, 6% incline, 35°C, 45% rh). Blood was withdrawn before the HST and 30 min into each exercise (EX) bout. In all 3 HST's plasma cortisol (PC) levels were significantly (p<.01) reduced during the first exercise bout when compared to pre-exercise levels, and then progressively increased during the second and third exercise intervals during HST 1. During HST 2 (48h post-infusion), however, PC levels were significantly (p<.05) reduced in two blood samples (EX 2,3) when compared to the same blood samples from HST-1 (pre-infusion). Plasma renin activity (PRA) and aldosterone (ALD) were significantly (p<.01) increased by the exercise/heat stress, but were unaffected by erythrocythemia either 48 h or 9 d subsequent to reinfusion. PRA and ALD were correlated (r=0.84, p<.001) under all conditions. We concluded from this study that acutely induced erythrocythemia reduced the stress response to consecutive exercise/heat intervals as manifested in PC responses during HST 2. However, alterations in the fluid regulatory hormones (ALD and PRA) were unaffected by erythrocythemia probably because total blood volume was not altered by reinfusion.

Key Words: cortisol, plasma renin activity, aldosterone, environmental stress, physical activity
Introduction

The use of autologous erythrocyte reinfusion to improve physical performance has been evaluated during several environmental and exercise paradigms. For example, Buick et al. (2) reinfused approximately 900 ml of autologous erythrocytes and demonstrated that both maximal aerobic power ($V_O^2_{max}$) and endurance capacity were significantly increased 24 hours after reinfusion in a normothermic and normoxic environment. Robertson and co-workers (19) confirmed these findings under normoxic conditions and extended the studies to demonstrate that exercise tolerance and $V_O^2_{max}$ under normobaric, hypoxic conditions were both significantly improved by autologous transfusion. The same group (18) reported that similar improvements were noted for female test subjects during cycle ergometry under normoxic conditions. Further, they (18) noted that the physiological advantages conferred by autologous reinfusion persisted for up to 2 weeks following the reinfusion.

Earlier, Bidani and Crandall (1) hypothesized that erythrocythemia resulting in an increase in hematocrit from normal (45%) to 60% effected an increased $V_O^2$ of approximately 30% and $VCO_2$ of about 25%. Gledhill (11) concluded in a recent review that increased $O_2$ delivery to working muscles should theoretically increase physical performance when the following criteria are met: incremented blood viscosity does not significantly reduce cardiac output, blood flow distribution is unaffected, and the oxidative capacity of the working muscles is not limiting. While potential increases in total blood and plasma volume may also be implicated in the increased physical capacity of induced erythrocythemia (14), Gledhill (11) argues that such effects may be
labile and are probably normalized after the first 24 h of reinfusion although prior investigations have not addressed these effects in reinfused subjects.

Despite the apparent interest in the use of autologous reinfusion to improve physical performance, we are unaware of any studies which have assessed the effects of reinfusion on the thermoregulatory and endocrinological responses to exercise in the heat especially during the first 48h and up to 9d following reinfusion. For several years we have studied the responses of plasma hormones to exercise-heat stress after the body fluid status of a test subject has been experimentally manipulated. Thus, we reported (8) that while hypohydration by 5% of body weight elicited significant elevations in plasma renin activity and aldosterone prior to and during exercise in the heat, these increments were attenuated following heat acclimation. These results were consonant with theory since heat acclimation is accompanied by significant increases in intravascular fluid volume (21,22). Analogously, we demonstrated (9) that heat acclimation also moderated the stress hormone response to exercise in the heat during hypohydration. Most recently, we have observed (10) that increased severity of hypohydration is accompanied by increased circulating levels of aldosterone, plasma renin activity, and cortisol. Our previous results generally indicated that experimental manipulations which increased blood/plasma volume (i.e. heat acclimation) tended to reduce the response of selected stress and fluid regulatory hormones to exercise in the heat while procedures which reduced blood/plasma volume (i.e. dehydration) increased the response of these hormones to the heat stress test.

In considering the beneficial effects of acute erythrocythemia induced by autologous erythrocyte infusion, it is apparent that the physiological strain
of exercise in the heat may be reduced by either improved $O_2$-$CO_2$ systemic transport or heat dissipation. If such benefits accrued subsequent to erythrocythemia, then responses of stress hormones to exercise in the heat may be attenuated particularly in unacclimated subjects. Alternatively, since the fluid regulatory hormone responses are dependent also on such variables as plasma osmolality, sodium levels, and oncotic pressure as well as blood/plasma volume, it is difficult to predict the intensity and the direction of the responses that may occur. Thus, the current study was designed to determine the effects of acutely induced erythrocythemia on the response of a representative stress hormone (plasma cortisol, PC) and fluid regulatory hormones (aldosterone, ALD and angiotensin I as determined by plasma renin activity, PRA) to a heat stress test. Unacclimated test subjects participated so that the subjective and physiological effects of heat acclimation did not mask the potential effects of the reinfusion.

Methods

Subjects Six adult, male test subjects (Ss) participated in this study; all were members of the same military unit, and thus were exposed to similar regimens of diet, activity, and environment through the duration of the study. Anthropometric measures for the experimental group were as follows (mean ± SD): age, 30 ± 7 yr; weight, 79 ± 9 kg, height, 182.3 ± 42 cm, and percent body fat, 15 ± 5. All test subjects received a written and oral description of the procedures and risks of the study, and signed a voluntary consent form signifying their agreement to participate. All volunteers reserved the right to withdraw from the study at any time without prejudice or retribution, but none elected to do so.
Phlebotomy and Reinfusion During the late fall and early winter two units of blood (900 ml) were collected from each volunteer; the collection of each unit was separated by at least 6 weeks. Phlebotomy, blood processing, storage, and ultimately, reinfusion were done by personnel on the staff of the Naval Blood Research Laboratory, Boston, MA. Blood was collected in citrate-phosphate-dextrose, and was stored at 4°C for 2-5 days. The erythrocytes were then separated by centrifugation and suspended in 40% W/V of glycerol, deep-frozen (-80°C) (23,24) and stored. At reinfusion the glycerolized erythrocytes were thoroughly washed (Haemonetics Blood Processor 115) and resuspended in a saline-glucose-phosphate solution; approximately 600 mls of solution with autologous erythrocytes (50% HCT) were reinfused over a 1 h time period.

Heat Stress Tests Three heat stress tests (HST's) were conducted at an environmental temperature of 35°C and relative humidity of 45% eliciting a WBGT of approximately 28°C. Each HST comprised a total of 180 min (3 repetitions of 45 min exercise, EX1, EX2, EX3, interspersed by 15 min rest) unless predetermined safety criteria or exhaustion were achieved. The exercise component of the HST involved walking (1.56 m.s⁻¹) on an inclined (6%) treadmill (~ 50% VO₂ max). During the rest intervals Ss were reweighed and rehydrated with sufficient cool water to maintain initial body weight. A program of scheduled rehydration was also employed during each exercise bout. The subjects wore only shorts, socks, and tennis shoes during each HST. Each HST was conducted on three separate occasions: the first or control HST was completed at least 6 weeks after the second phlebotomy, and approximately two weeks prior to the autologous reinfusion during the late spring season; the second was accomplished exactly 48 h after completion of the reinfusion
procedure; and the third HST one week following the second which corresponded to 9 d after the reinfusion procedure. Thus, the minimal time interval between HST's was one week so that partial heat acclimation effects were minimized.

**Blood Sampling** Indwelling Teflon catheters were inserted in a superficial arm vein prior to each HST; their patency was maintained by flushing with heparinized saline. Pre-exercise blood samples were obtained after the test subjects stood quietly in a moderate environment (antechamber, 20°C, 40%rh) for at least 20 min to control for postural effects on vascular fluid shifts (13). The remaining three blood samples were obtained 30 min into each exercise bout (EX1, EX2, EX3) of the respective HST while the subjects continued to walk. Blood was centrifuged (1000 g, 30 min), aliquotted, and frozen (-20°C) for subsequent analysis. Tests were conducted between 0700 and 1100 h to offset the effects of circadian variations on the dependent variables.

**Plasma Analyses** Samples were analyzed for plasma cortisol (PC) using commercially prepared test kits purchased from New England Nuclear Corp., Billerica, MA, according to standardized procedures outlined in their technical bulletin. Using these techniques intra-assay variability was just 2.5% and inter-assay variability was 7.3%. PC values are generally reported to range from 4-25 ug.100 ml⁻¹ depending importantly on the time of day at which the blood samples are drawn (15). Angiotensin I levels were assessed by quantitating plasma renin activity (PRA) using radioimmunoassay test kits also produced by New England Nuclear Corp. When converting enzyme and angiotensinasises are appropriately inhibited, it has been demonstrated that the accumulation of angiotensin I reflects plasma renin activity. Intra-assay
variability was determined to be 4% and inter-assay variability was 7.2% by these methods. Control levels of PRA for healthy normotensive men range from approximately 1.0-4.0 ng Angiotensin I formed per hour per ml plasma by this method. Aldosterone (ALD) levels were quantitated using radioimmunoassay test kits purchased from Diagnostics Products Corp, Los Angeles, CA, by methods outlined in their technical bulletin. Intra-assay variability was 9.3% by these methods and interassay variability was recorded at 11%. Expected values for normotensive adult men range from 5-31 ng.dl⁻¹ by these methods.

Statistics Repeated measures analyses of variance were performed followed by the application of Tukey's t test corrected for multiple group comparisons to determine the effects of exercise/heat stress on the variables of interest (16,17). During the pre-infusion HST one test subject did not complete the third exercise bout; therefore, a single calculated value was used for each variable (16, p.228). To determine the significance of effects of red cell reinfusion, Dunnett's t test (16, p.422) for paired, dependent data was performed and the results for the pre-infusion trial (HST 1) were compared with those of the 48 h post-infusion HST (HST 2) as well as the 9 d HST (HST 3). Correlation coefficients were calculated by linear regression analysis. For all statistical tests the null hypothesis was rejected at p<0.05.

Results

Fig. 1 illustrates the effects of erythrocyte reinfusion and exercise/heat stress on circulating levels of cortisol in these unacclimated test subjects. The results indicate that during all three HST's there occurred a reduction in PC levels between the pre-exercise and EX₁ sample which was significant for
the first (pre-infusion) HST (p<.05) and the second (48 h post-infusion) HST (p<.01). However, during the third (9 d post-infusion) HST PC was at an apparently low basal level in the pre-exercise sample (8.06 ug.dl⁻¹) and was further reduced to 6.52 ug.dl⁻¹ during EX1; however, the minimum difference necessary for significance was 4.36 ug.dl⁻¹. We have observed this decrease previously (9,10) and attributed the decrement to the normally occurring circadian reduction during this time of day. It is noteworthy that the cumulative effects of exercise in the heat apparently offset the anticipated continued circadian decline of PC until by the third exercise interval in the pre-infusion (HST 1) and 9 d post-infusion (HST 3) HST's PC levels were not significantly different from pre-exercise levels. However, in the 48 h post-infusion HST (HST 2) PC levels were persistently and significantly (p<.01) depressed during all 3 exercise bouts (from pre-exercise). The effects of induced erythrocythemia are best demonstrated if paired data are compared between the pre-infusion (HST 1) and 48 h post-infusion (HST 2) HST's. In these comparisons PC was significantly reduced at each of the exercise intervals in the 48 h post-infusion trial (HST 2) vs the respective sample of the pre-infusion (HST 1) trial (p<.005 EX1 and p<.05 EX2, EX3).

Values for aldosterone responses to reinfusion and exercise in the heat are noted in Fig. 2. The effects of exercise in the heat were apparent since by the second exercise bout during all three HST's ALD levels were significantly (p<.01) elevated over pre-exercise concentrations. During EX1, ALD levels (vs. pre-exercise) were significantly elevated during the pre-infusion (p<.05) and 48 h post-infusion (p<.01) HST's; however, despite an increment significance was not achieved during EX1 for the 9 d post-infusion trial (pre-exercise level = 24.28 ng.dl⁻¹, EX 1 level = 34.4 ng.dl⁻¹, minimum
difference for significance = 13.8 ng.dl⁻¹). Erythrocythemia, however, apparently had no effect on the ALD responses to exercise in the heat as no significant differences were noted in comparisons between pre- and 48 h or 9d post-infusion HST's.

Responses of plasma levels of PRA were remarkably consistent during all HST's with significant (p<.01) increments noted even during the first exercise period (vs pre-exercise). Further, these elevations persisted and for all three HST's the levels measured during EX 2 were significantly (p<.01) greater than those recorded during EX1 (HST 1, 7.91 vs 9.76; HST 2, 6.71 vs. 9.20; HST 3, 6.59 vs 9.02 ng.ml⁻¹.h⁻¹). While PRA was further elevated during the third exercise interval, the rate of increase had moderated for all three HST's. Figs. 2 and 3 illustrate apparently analogous responses of PRA and ALD to exercise in the heat and erythrocythemia; statistical analysis confirmed the correlation between these two covariables: n=72, r=.84, t=12.76, p<.001, Fig. 4.

Discussion

We have previously reported (9,10) that when euhydrated subjects exercise in the heat under conditions similar to those selected in the current experiments, physiological stress response as manifested in circulating cortisol levels is minimal. In fact, again in euhydrated subjects tested between 0700-1100 h, the decrement between the pre-exercise and the EX1, EX2 samples has been a consistent observation in our earlier studies (9,10). It is noteworthy that in the current investigation wherein the metabolic rate has been increased from approximately 30% (9,10) to about 50% Vo₂max, PC levels have increased in the pre-infusion and 9 d post-infusion trials by the third exercise interval. These observations imply that by the third exercise
interval, Ss were experiencing physical discomfort as a result of the combination of the physical work rate and environment. In fact, Follenius et al. (7) had earlier reported that heat stress induced an adrenocortical response only in those individuals who experienced physical discomfort during the exposure.

Alternatively, erythrocyte reinfusion apparently had represive effects on this cumulative stress response, and even during the first exercise interval plasma levels of cortisol during the second HST (48h post-reinfusion) were significantly reduced (approximately 24%) in comparison with the pre-infusion trial. Further, these reductions persisted throughout the 2nd and 3rd exercise bouts. This may be interpreted in terms of an attenuation of the physiological strain of this exercise/heat regimen following reinfusion. It had been previously demonstrated that the increased arterial oxygen content induced by erythrocythemia can translate to a reduced requirement for skeletal muscle blood flow (25,26), in turn permitting greater cutaneous perfusion for heat dissipation. In fact, we have reported (20) that erythrocythemia not only provided a thermoregulatory benefit during exercise in the heat but also elicited an 11% increase in maximal O₂ consumption. Thus, it is hypothesized that the reduced cortisol concentrations observed in the 48 h reinfusion trial are the result of an increased O₂ delivery capacity with perhaps a beneficial shift in cardiac output or redistribution of blood volume effecting improved thermoregulation.

Alternatively, levels of ALD and PRA were unaffected by the induced erythrocythemia during exercise-heat stress. We had originally hypothesized that an increased blood volume, anticipated particularly during the 48 h post-infusion HST (20), might alleviate the increments in ALD and PRA reported
during and immediately subsequent to exercise in the heat (3,5,12). However, our hemodynamic measurements demonstrated (20) that immediately prior to the second HST (48 h post-infusion) the acute erythrocythemia had elicited a marked (−7%) decrease in plasma volume when compared to the pre-infusion level, an observation which had not been reported previously. However, the compensatory decrement in plasma volume was offset by the increased erythrocyte volume so that total blood volume was unchanged. Thus, the normal response of the fluid regulatory hormones to exercise in the heat are not inconsistent. Indeed, despite the induced erythrocythemia, the results of the present experiment depicted in Figs. 2 and 3 are very similar to those which we have reported previously in euhydrated unacclimated men (8). In fact, the present results, with particularly sharp increases in PRA and ALD at each exercise interval in all 3 HST’s, confirm the intensity of these responses in unacclimated men. Our previous data (8,10), as well as that of other investigators (4-6), have indicated that the acquisition of heat acclimation moderates the intensity of the hormonal response to exercise in the heat. Clearly, induced erythrocythemia had no effects on the heat/exercise responses of these fluid regulatory hormones in our euhydrated, but unacclimated subjects.

We have concluded from this study that autologous reinfusion of 2 units of erythrocytes attenuated the stress response to exercise in the heat as manifested in PC levels. Further, induced erythrocythemia had no effects on the incremental response pattern of the fluid regulatory hormones ALD and PRA to the heat/exercise regimen. The latter observation is reflective of the fact that the anticipated increase in blood volume effected by the red blood cell infusion was compensated by a slight decrease in plasma volume. Thus,
the response pattern of the reinfused, euhydrated and non-acclimated subjects is consistent with our earlier observations of response profiles in non-reinfused, euhydrated, and unacclimated test volunteers.
References


7. Follenius, M., G. Brandenberger, B. Reinhardt, and M. Simeoni. Plasma aldosterone, renin activity, and cortisol responses to heat exposure in


Figure Legends

Fig. 1. Effects of acutely induced erythrocythemia and exercise in the heat on circulating cortisol levels. Means ± SEM are depicted for n=6 in all cases. Blood was removed after standing for 20 min in a moderate environment (20°C, 45% rh) for the pre-exercise sample, and 30 min into each exercise interval during the heat stress tests (1.56 m.s⁻¹, 6% incline, 35°C, 45% rh).

Fig. 2. Effects of erythrocythemia and exercise in the heat on circulating levels of aldosterone during exercise in a hot environment. All conditions and specifications are noted under Fig 1.

Fig. 3. Effects of erythrocythemia and exercise in the heat on circulating levels of plasma renin activity during exercise in the heat. All conditions and specifications are as noted under Fig. 1.

Fig. 4. Linear regression and scatter plot of values for PRA and ALD during all three heat stress tests and before and during all exercise bouts.
Acknowledgements and Disclaimers

The authors gratefully acknowledge the skilled technical assistance of Leslie Levine and Bruce Cadarette. Special gratitude is expressed to the test volunteers of the 10th Special Forces Group stationed at Ft. Devens, MA for their cooperation and enthusiasm. We thank Susan Henry and Diane Danielski for the word processing support.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense. Human subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Regulation 70-25 on Use of Volunteers in Research.
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