Eur J Appl Physiol (1995) 70:427–436

ORIGINAL ARTICLE

John A. Smith · Marysia Kolbuch-Braddon
Ian Gillam · Richard D. Telford
Maurice J. Weidemann

Changes in the susceptibility of red blood cells to oxidative and osmotic stress following submaximal exercise

Accepted: 7 November 1994

Abstract Red blood cell (RBC) susceptibility to oxidative and osmotic stress in vitro was investigated in cells from trained and untrained men before and after submaximal exercise. Whilst no significant change in peroxidative haemolysis occurred immediately after 1 h of cycling at 60% of maximal aerobic capacity ($\dot{V}O_{2max}$), a 20% increase was found 6 h later in both groups ($P<0.05$). The RBC osmotic fragility decreased by 15% immediately after exercise ($P<0.001$) and this was maintained for 6 h ($P<0.001$). There was an associated decrease in mean cell volume ($P<0.05$). Training decreased RBC susceptibility to peroxidative haemolysis ($P<0.025$) but it did not influence any other parameter. These exercise-induced changes were smaller in magnitude but qualitatively similar to those found in haemopathological states involving haem-iron incorporation into membrane lipids and the short-circuiting of antioxidant protection. To explore this similarity, a more strenuous and mechanically stressful exercise test was used. Running at 75% $\dot{V}O_{2max}$ for 45 min reduced the induction time of O_2 uptake (peroxidation), consistent with reduced antioxidation capacity, and increased the maximal rate of O_2 uptake in RBC challenged with cumene hydroperoxide ($P<0.001$). The proportion of high-density RBC increased by 10% immediately after running ($P<0.001$) but no change in membrane-incorporated haem-iron occurred. In contrast, treatment of RBC with oxidants (20–50 $\mu mol \cdot l^{-1}$) in vitro increased cell density and membrane incorporation of haem-iron substantially. These results showed that single episodes of submaximal exercise caused significant changes in RBC susceptibility to oxidative and osmotic stress. Such responses may account for the increase in RBC turnover found in athletes undertaking strenuous endurance training.

Key words Antioxidant · Erythrocytes · Free radicals · Exertion · Haematology

J. A. Smith · M. Kolbuch-Braddon · M. J. Weidemann
Division of Biochemistry and Molecular Biology,
School of Life Sciences, Faculty of Science,
Australian National University,
Canberra, ACT, 0200, Australia

I. Gillam
Department of Human Movement Science,
Royal Melbourne Institute of Technology,
PO Box 71 Bundoora, Victoria, 3083, Australia

R. D. Telford
Department of Physiology and Applied Nutrition,
Australian Institute of Sport,
PO Box 176, Belconnen, ACT, 2616, Australia

J. A. Smith (✉)
National Quality of Life Foundation,
c/o Department of Physiology and Applied Nutrition,
Australian Institute of Sport,
PO Box 176, Belconnen, ACT, 2616, Australia

Introduction

Efficient delivery and release of oxygen to exercising muscles by red blood cells (RBC) are important determinants of peak athletic performance. Many reports have suggested that intensive physical training accelerates the rate of RBC turnover and can, in some cases, lead to the so-called condition of sports anaemia which may result in a decreased capacity for physical activity (Smith 1995). Evidence that exercise may cause significant RBC damage and haemolysis has included reports of significant exercise-induced decreases in haemoglobin concentration and circulating RBC counts (Guglielmini et al. 1989), as well as reductions in the plasma concentrations of iron-binding proteins such as ferritin and haptoglobin (Selby and Eichner 1986). At the cellular level, a short period of training has been reported to elevate blood reticulocyte counts (Schmidt et al. 1989) and the activities of some RBC antioxidant enzymes (Ohno et al. 1988; Robertson et al. 1991). Mean RBC density, which increases as cells age, has been found to be lower in endurance athletes (Mairbäurl et al. 1983). While the mechanical trauma associated with footstrike has been suggested as a cause of intravascu-

lar haemolysis during running, low-impact activities such as cycling and swimming have also been shown to induce significant levels of RBC destruction (Schmidt et al. 1988; Selby and Eichner 1986).

Oxidative damage to various cell types occurs as a consequence of aerobic metabolism and exposure to oxygen through the generation of free radicals. The rate of this process may rise in proportion to the increased rate of whole body oxygen uptake (10 to 20-fold) that occurs during exercise (Gohil et al. 1988; Duthie et al. 1990). Because of their continual exposure to oxygen, and high content of haem-iron and polyunsaturated fatty acids, RBC are particularly susceptible to oxidative damage (Hebbel and Eaton 1989). Auto-oxidation of haemoglobin to met-haemoglobin, which occurs under resting conditions at the rate of $3\% \cdot day^{-1}$, is accompanied by free radical generation. These radicals may overwhelm antioxidant defences and attack polyunsaturated lipids and proteins in RBC. Association of haem-iron, which is normally confined to the cytosol, with membrane lipids may increase the vulnerability of RBC to oxidative stress substantially because of the potential of Fe^{2+} to short-circuit membrane vitamin-E (α-tocopherol) protection (Hebbel and Eaton 1989). Oxidative damage to the cell membrane may also perturb intracellular osmotic homeostasis and facilitate cellular dehydration (Snyder et al. 1981). In this way, oxidative and osmotic stress to RBC during exercise may increase their susceptibility to irreversible damage and destruction.

Single episodes of submaximal exercise have been reported to have substantial effects on the redox status of blood glutathione, although discrepancies in the magnitude and direction have been described (Gohil et al. 1988; Ji et al. 1993). In addition to oxidative damage, changes in RBC hydration may occur because submaximal exercise may decrease plasma volume through perspiration loss (Wilkerson et al. 1977). Intensive exercise has also been shown to trigger leakage of intra-erythrocyte K^+ (Hespel et al. 1986), and thus water, which increases cell density (Robertson et al. 1988). These changes may alter the deformability of RBC and so impair their ability to enter and move through the microcirculation (Clark 1988).

Because several studies have reported that single episodes of moderate exercise, and regular training, alter the antioxidant status of RBC (Gohil et al. 1988; Robertson et al. 1991), we have investigated whether a session of either moderate cycling or running, and/or endurance training alter the susceptibility of isolated RBC to oxidative and osmotic stress in vitro. Since these groups have reported that single episodes of submaximal exercise deplete certain RBC anti-oxidants and cause lipid peroxidation, we have used assays that measure their total anti-oxidant capacity and susceptibility to damage in vitro. As results found in the pilot study (group 1) were similar, albeit much smaller in magnitude, to those found in haemopathological conditions (Snyder et al. 1981), this similarity was explored in a second group using a more intense and mechanically stressful workload. The changes reported indicate, for the first time, that single episodes of submaximal exercise caused significant alterations in RBC properties consistent with increased susceptibility to oxidative and osmotic stress.

Methods

Human subjects and exercise protocols

Group 1

A group of 11 trained men [maximal oxygen uptake, $\dot{V}O_{2max}$ 71.4 (SEM 6.0) $ml \cdot min^{-1} \cdot kg^{-1}$, age 22 (SEM 4) years] and 9 untrained men [$\dot{V}O_{2max}$ 48.1 (SEM 9.0) $ml \cdot min^{-1} \cdot kg^{-1}$, age 22 (SEM 4) years] were recruited for the pilot study. The trained subjects were road cyclists competing at Australian national level who trained about $25\ h \cdot week^{-1}$. In contrast, the untrained subjects devoted less than $4\ h \cdot week^{-1}$ to physical activity. Each subject cycled continuously for 1 h at 60% of his predetermined $\dot{V}O_{2max}$ as described previously (Smith et al. 1990). The blood withdrawal and RBC isolation procedures have also been described in earlier studies (Smith et al. 1992). Blood samples were taken immediately before and after exercise, and 6 h postexercise.

Group 2

A group of 20 highly-trained men [$\dot{V}O_{2max}$ 71.5 (SEM 4.5) $ml \cdot min^{-1} \cdot kg^{-1}$, age 24 (SEM 5) years] and 6 untrained men [$\dot{V}O_{2max}$ 52.0 (SEM 6.0) $ml \cdot min^{-1} \cdot kg^{-1}$, age 24 (SEM 6) years] were recruited for the running study. Blood samples were taken immediately before and after exercise. The trained subjects were triathletes, cross-country skiers and runners competing at Australian national level and training for 10–20 h each week. The untrained subjects devoted less than 4 h each week to physical activity. Each individual ran on a treadmill (zero gradient) for 40 min at an exercise intensity equivalent to approximately 75% of his $\dot{V}O_{2max}$. At least a week before the test, the $\dot{V}O_{2max}$ of each subject was determined on a treadmill. A continuous incremental protocol was used in which the gradient of the treadmill was increased by 1% each minute until the subject was exhausted. For both groups, all exercise tests were commenced between 9 a.m. and 10 a.m.

Measurements

Unless otherwise stated, all reagents were purchased from Sigma (St Louis, Mo., USA).

Oxidative stress assays

The peroxidative haemolysis, oxygen uptake and luminol-amplified chemiluminescence assays were performed as described previously (Smith et al. 1992). RBC membranes (ghosts) were prepared by the method of Kuross et al. (1988). The protein content of the ghosts was measured with a protein assay kit obtained from Bio-Rad (San Lorenzo, Calif., USA). In membrane assays, ghosts (0.3 mg protein) were added to a total volume of 1.0 ml. Spectrophotometric methods were used to measure the concentrations of haem-iron (Kuross et al. 1988) and haemichrome (Campwala and Desforges 1982) in RBC ghosts.

Haematological parameters

Red and white blood cell counts, haematocrit, mean cell volume (MCV), haemoglobin concentration, mean cell haemoglobin concentration (MCHC), and mean cell haemoglobin (MCH) were determined using an automated haematology analyser (Coulter Electronics, Hialeah, Fla., USA). Met-haemoglobin concentration in RBC supernatants was measured spectrophotometrically (Winterbourn 1983).

Osmotic fragility

Heparinised whole blood (100 μl) was added to hypotonic phosphate-buffered saline (pH 7.4) in the concentration range of 0.2–0.5% (w/v) of saline (Simmons 1972). The tubes were mixed and incubated for 1 h at room temperature, mixed again and centrifuged for 5 min at 1000 *g* to pellet the ghosts. Supernatant optical density was measured by spectrophotometry at 540 nm.

Density fractionation and scanning

Red cells were fractionated on a self-forming Percoll (Pharmacia, Uppsala, Sweden)/diatrizoate density gradient using the original technique described by Vettore et al. (1980) with the modifications introduced by Snyder et al. (1983). Specifically, RBC were washed in phosphate buffered saline (PBS) three times before the additon of packed cells (200 μl) to the Percoll/diatrizoate mixture (10 ml) in a Corex tube. The suspension was mixed and centrifuged for 20 min at 35000 *g* at 4° C using an angle-rotor (SS-34) in a RC-2B centrifuge (Sorvall, USA).

Immediately after centrifugation, each tube was photographed, using an evenly illuminated light box as background, with 25 ASA film (Kodak Technical Pan). The film was exposed for 6 s at f16, developed in Kodak Technidol, and the continuous-tone negatives printed on multigrade paper (Ilford, Multigrade 3) using a zero-grade filter. The maximal density of the exposed image did not exceed an optical density of 4.0 which represented the upper linear detection limit of the laser densitometer. The density of each band was quantitated by scanning laser densitometry (LKB Ultrascan XL, Bromma, Switzerland). Individual density profiles were integrated using the Gelscan XL software package (LKB, Bromma, Switzerland).

Statistical analysis

A repeated measures ANOVA was used to analyse the multiple samples from group 1. Scheffé's post-hoc test was used to determine significant effects. Other exercise effects were analysed by Student's *t*-test for paired data. The significance level was set at 5%. Where no significant differences between the trained and untrained subjects were found the data were pooled for statistical analysis of the exercise effects.

Results

Oxidative responses

Group 1 (cycling)

Exposure of RBC to H_2O_2 (range 0–2 mmol·l^{-1}) induced peroxidative haemolysis in a concentration-dependent manner. Analysis of the mean pre-exercise values at a single concentration point in the linear region of the curve (1.25 mmol·l^{-1} H_2O_2) showed that the increase in peroxidative haemolysis that occurred after exercise only became significant at 6 h after the cycling test in both trained ($P<0.01$) and untrained ($P<0.05$) subjects (Fig. 1). The RBC from trained individuals were much more resistant to peroxidative haemolysis than cells from their untrained counterparts both before and after exercise ($P<0.025$; Fig. 1).

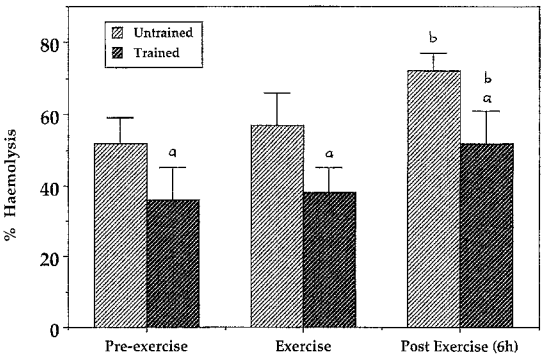

Fig. 1 The effects of a single episode of cycling, and training status, on red blood cell suspectibility to haemolysis induced by H_2O_2 treatment. **a** $P<0.025$ compared to untrained subjects; **b** $P<0.05$ compared to pre-exercise values

Group 2 (running)

As found with cycling, there was no significant change in the susceptibility of RBC to peroxidative haemolysis by H_2O_2 immediately after the running protocol ($n=15$; data not shown). To extend our investigation, the susceptibility of RBC to peroxidation in vitro (by organic hydroperoxides) was assessed by monitoring O_2 uptake and luminol-amplified chemiluminescence (LCL) in separate assays. The typical kinetic patterns of O_2 uptake and LCL following treatment of RBC with either lipophilic cumene hydroperoxide (Cum-OOH) or amphipathic *t*-butyl hydroperoxide (*t*-BuOOH) have been shown previously (Smith et al. 1992).

The O_2 uptake data for each individual are presented in four separate panels (Fig. 2a–d). These panels show the induction times (which are indicative of antioxidant capacity) and maximal rates of O_2 uptake (peroxidation) induced by exposure of RBC to either of the organic hydroperoxides at a concentration of 1.2 mmol·l^{-1}. Exercise caused significant decreases in the induction time in cells challenged with either Cum-OOH ($P<0.001$) or *t*-BuOOH ($P<0.05$; Table 1). With Cum-OOH only, the decreased induction time was accompanied by a significant increase in the maximal rate of O_2 uptake ($P<0.01$; Table 1). In some subjects ($n=9$), blood samples were also taken 24 h after exercise. There were no significant differences between the pre-exercise and 24-h postexercise induction times and the maximal rates of O_2 uptake in either Cum-OOH- or *t*-BuOOH-treated cells.

With LCL, however, no significant changes in the induction times or maximal rate of LCL (i.e. peak LCL)

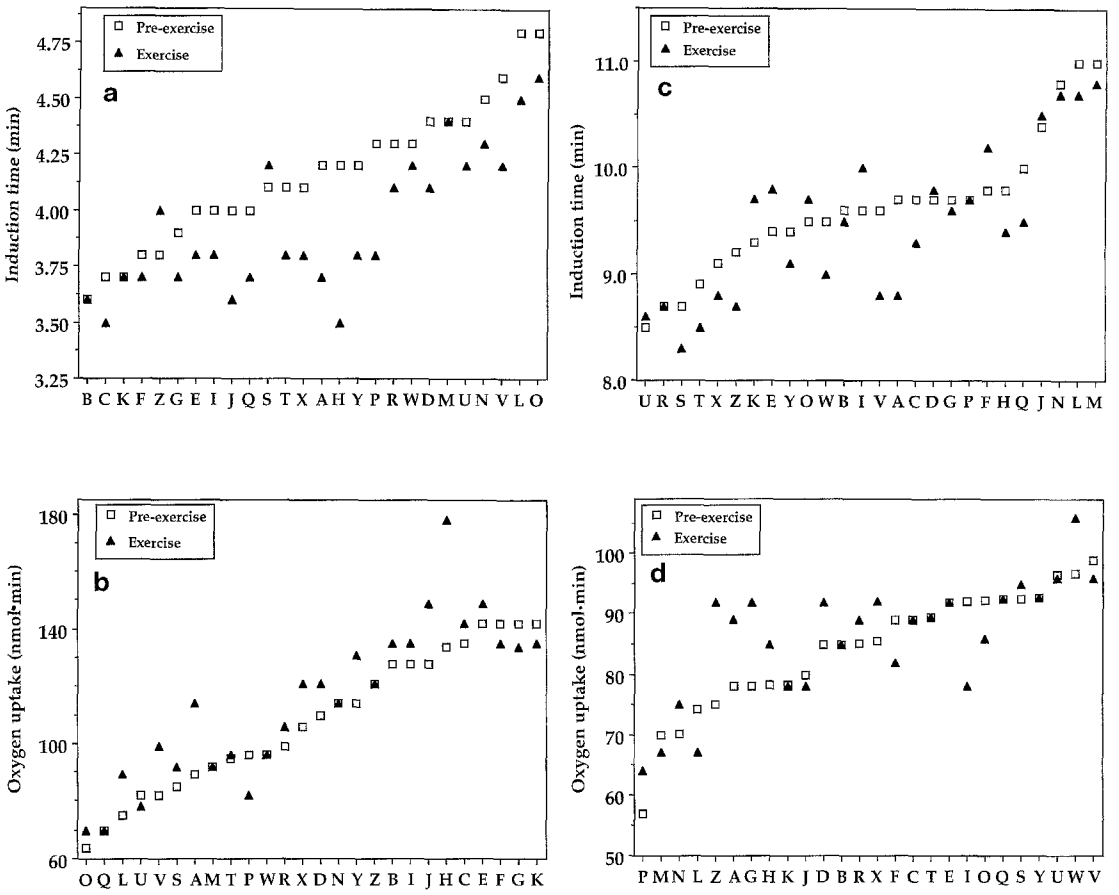

Fig. 2 The effects of treadmill running on red blood cell (RBC) peroxidation in each individual. *These panels* show each individual result, plotted in ascending order of the pre-exercise value, for (**a, c**) induction time and (**b, d**) maximal rate of O_2 uptake following exposure of isolated RBC to either (**a, b**) cumene hydroperoxide or (**c, d**) *t*-butyl hydroperoxide in vitro (1.2 mmol·l^{-1}). Each individual is identified by an *upper case letter*

were detected after exercise in response to either of the organic hydroperoxides at a concentration of 75 μmol·l^{-1} (Table 2). This was also the case when the integrated areas under the kinetic curves were analysed statistically (Table 2). There were no significant differences from pre-exercise values in any of the parameters measured in Cum-OOH treated cells isolated 24 h after exercise ($n=15$) although integrated LCL tended to decrease [rest: 206 (SEM 91.2)×10^6 cpm to 144 (SEM 73.9)×10^6 cpm, 24-h postexercise ($P<0.10$)]. However, in cells challenged with *t*-BuOOH, both peak and integrated LCL fell significantly at 24 h after exercise [peak LCL: rest 90.8 (SEM 27.7)×10^4 cpm to 68.2 (SEM 15.1)×10^4 cpm, 24-h postexercise ($P<0.01$) – integrated LCL: rest 186 (SEM 68.3)×10^6 cpm to 139 (SEM 40.5)×10^6 cpm, 24-h postexercise ($P<0.025$)]. The same trends were found when the cells were treated with higher concentrations (115 μmol·l^{-1} or 150 μmol·l^{-1}) of organic hydroperoxides. The induction times decreased, and peak and integrated LCL increased in proportion to concentration (data not shown)

Osmotic responses

Group 1 only

The osmotic fragility test measures the physical state of the membrane, which has been shown to be dependent

Table 1 Induction time and maximum rate of O_2 uptake immediately before and after 45 min of treadmill running. Red blood cells were treated with either cumene-hydroperoxide (*Cum-OOH*) or *t*-butyl-hydroperoxide (*t-BuOOH*) (1.20 mmol·l^{-1})

State	Cum-OOH Induction time (min)		Cum-OOH Max-rate (nmol·min^{-1})		*t*-BuOOH Induction time (min)		*t*-BuOOH Max-rate (nmol·l^{-1})	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
Pre-exercise	4.15	0.0632	108	4.41	9.58	0.129	85.5	1.25
Exercise	3.91	0.0686*	115	5.10*	9.43	0.143*	87.1	2.16

* $P<0.05$ compared to pre-exercise

Table 2 Chemiluminescence parameters determined immediately before and after 45 min of treadmill running. RBC were treated with either *Cum-OOH* or *t-BuOOH* (75 $\mu mol \cdot l^{-1}$); for definitions see Table 1

State	Induction time (mins) mean	SEM	Peak LCL ($\times 10^4$ cpm) mean	SEM	Integral LCL ($\times 10^6$ cpm) mean	SEM
	Cum-OOH					
Pre-exercise	21.9	1.02	119	14.4	185	16.7
Exercise	22.0	0.87	122	12.2	177	15.5
	t-BuOOH					
Pre-exercise	18.4	1.09	103	11.0	172	12.7
Exercise	17.3	0.98	94.2	8.29	163	10.8

Table 3 Haematological parameters measured immediately before and after submaximal exercise*. *MCV* Mean cell volume, *MCHC* mean cell haemoglobin concentration, *MCH* mean cell haemoglobin

State	MCV (fl) mean	SEM	MCHC ($g \cdot dl^{-1}$) mean	SEM	MCH (pg) mean	SEM
	Cycling					
Pre-exercise	89.8	0.886	33.96	0.381	29.50	0.402
Exercise	89.0	0.896*	34.50	0.376	30.23	0.358
Postexercise (6 h)	89.1	0.830*	33.70	0.565	29.84	0.380
	Running					
Pre-exercise	90.3	0.372	34.30	0.137	30.90	0.157
Exercise	90.3	0.353	34.30	0.137	30.91	0.313

* $P<0.05$ compared to pre-exercise

on the surface area:volume ratio of the cell (Beutler et al. 1982). When the mean responses of the trained and untrained subjects were analysed statistically at one concentration (0.40% saline) in the linear region of the curve, no differences attributable to training were found either before or after exercise. The mean 15% decrease found immediately after exercise ($P<0.001$) was maintained for at least 6 h ($P<0.001$; Fig. 3).

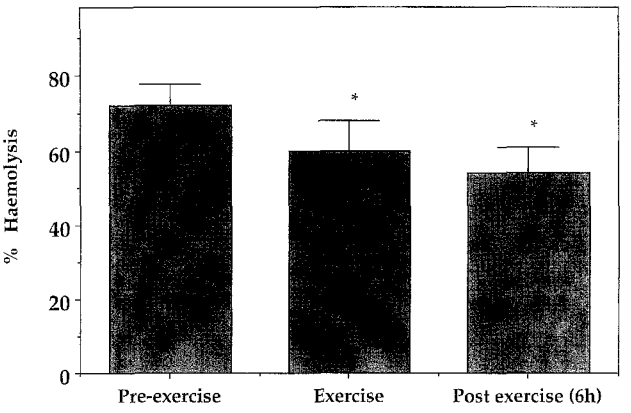

Fig. 3 The effects of a single episode of cycling on red blood cell susceptibility to haemolysis induced by osmotic stress. * $P<0.001$ compared to pre-exercise

Haematological parameters

Groups 1 and 2

The exercise-induced decreases in osmotic fragility found after cycling were accompanied by small but significant decreases in MCV ($P<0.05$). There were no significant changes in MCH or MCHC (Table 3). In contrast, there were no significant changes in any of these haematological parameters immediately after exercise in individuals undertaking the running protocol

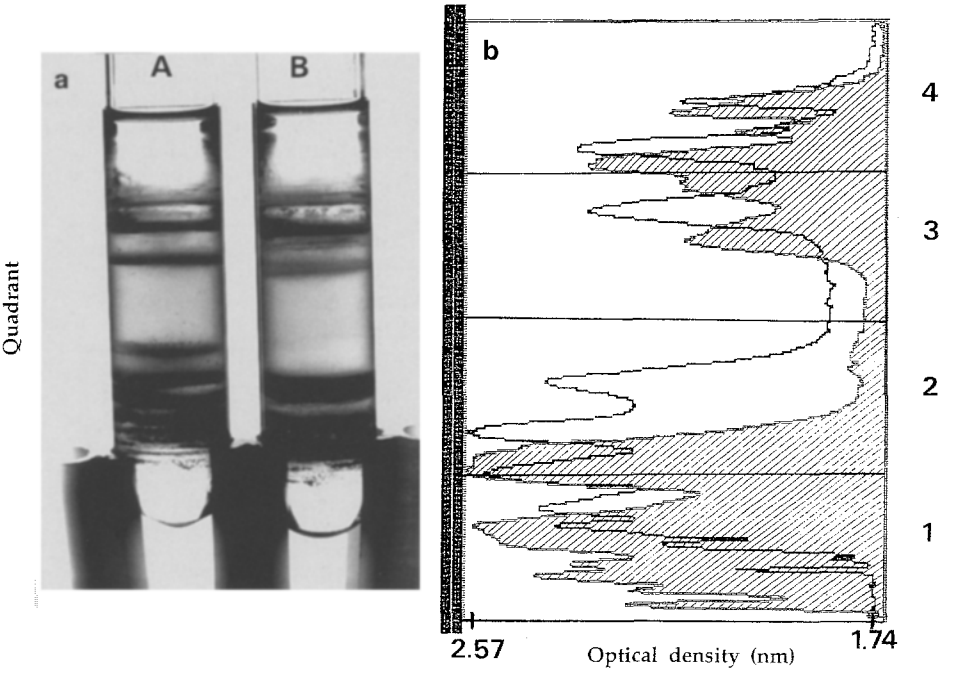

Fig. 4a Photograph of **a** typical individual profile of red blood cell density distribution immediately before and after treadmill running. The laser densitometry scan of the photograph (**b**) represents an overlay of the profiles analysed immediately before (*A unhatched*) and after exercise (*B hatched*). Each profile was analysed by division of the *panel into four equal quadrants* [*4 (top) – 1 (bottom)*] and calculation of the relative percentages of cells within each area to determine shifts in density caused by exercise

(Table 3). There were no significant differences between the trained and untrained groups.

Effect on RBC density

Group 2 only

Figure 4 shows a typical individual profile of density-fractionated RBC before and after exercise, together with a laser scan of the gradient. The percentage of RBC found in the densest quadrant of the gradient increased by 10% immediately after exercise ($P<0.001$) and this shift was accompanied by a similar decrease in the percentage of low-density RBC in quadrants 3 and 4 (Fig. 5a). In the trained group before exercise (i.e. at rest), there was a significantly lower percentage of low-density cells in quadrant 4 in RBC isolated before exercise ($P<0.001$) accompanied by uniform increases in the percentages of cells in the other three quadrants, which were, however, not significant statistically (Fig. 5b). There were no significant differences in the exercise responses between the trained and untrained subjects.

We also examined whether the exercise-induced increase in RBC density could be reproduced by conditions that are known to cause oxidative damage in vitro. Normal RBC isolated from nonexercised subjects were treated with phenazine methosulphate (PMS; 50 $\mu mol \cdot l^{-1}$) for 45 min to simulate the auto-oxidation of haemoglobin (Hebbel et al. 1989). There was a much greater increase in the percentage of high-density cells in PMS-treated cells compared to those induced by exercise (Fig. 6). This increase was even greater in RBC that were challenged with a low concentration (20 $\mu mol \cdot l^{-1}$) of Cum-OOH instead of PMS.

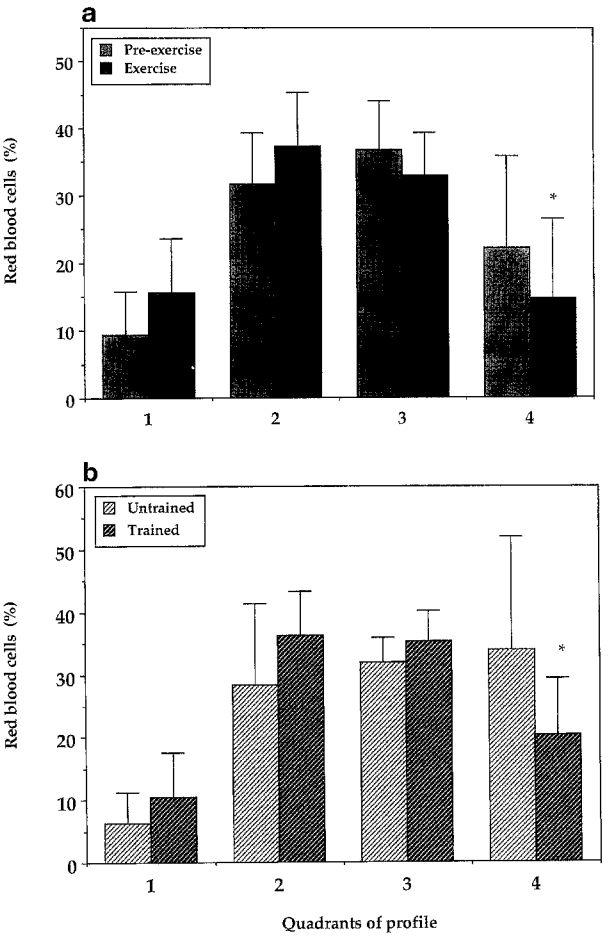

Fig. 5 The short- and long-term effects of exercise on mean (SEM) red blood cell (RBC) density. (**a**) The density distribution of RBC immediately before and after running (* $P<0.001$). (**b**) The relative distribution of RBC density in cells isolated from nonexercised untrained and trained men (* $P<0.001$)

Role of membrane-bound haem-iron

Group 2 only

We tested whether the exercise-induced changes may be due to free radical generation catalysed by an increase in haem-iron bound to the membrane. This hypothesis was proposed because RBC isolated from individuals with β-thalassaemia or sickle cell anaemia have also shown increased vulnerability to peroxidative haemolysis and decreased susceptibility to osmotic fragility when compared to RBC isolated from normal individuals (Snyder et al. 1981). Sickled cells have been shown to contain excessive concentrations of haem-iron that is truly membrane-incorporated (Kuross et al. 1988). No significant changes in membrane-bound haem-iron were found in ghosts prepared from RBC isolated either immediately after treadmill running (Table 4) or 3 h later (data not shown), irrespective of whether this was measured by spectrophotometric or chemiluminescent methods. Furthermore, no significant changes in

Table 4 Individual measurements of membrane-associated haem-iron before and after treadmill running. Haemichrome, haem-iron and luminol-amplified chemiluminescence (*LCL*) were measured in washed ghosts using the procedures described in the Methods. The *LCL* was initiated by adding lipophilic cumene hydroperoxide (75 $\mu mol \cdot l^{-1}$) to the reaction mixture (1.0 ml) which contained the ghosts (1.0 mg protein) and luminol (225 $\mu mol \cdot l^{-1}$) in Hanks' balanced salt solution. *nm* Not measured

Subject	Haemichrome ($\mu mol \cdot l^{-1} \cdot mg^{-1}$ protein)		Haem-iron ($nmol \cdot mg^{-1}$ protein)		Peak-LCL ($cpm \times 10^4$)	
	Rest	Exercise	Rest	Exercise	Rest	Exercise
1	0.408	0.507	1.00	1.02	5.00	4.40
2	0.426	0.474	1.96	2.55	2.90	7.10
3	0.419	0.429	1.48	1.52	3.78	3.98
4	0.341	0.352	2.64	3.12	21.5	16.5
5	0.297	0.308	6.89	6.15	8.96	12.8
6	0.308	0.264	7.67	6.99	24.2	22.6
7	nm	nm	nm	nm	19.1	19.6
8	nm	nm	nm	nm	12.0	9.9

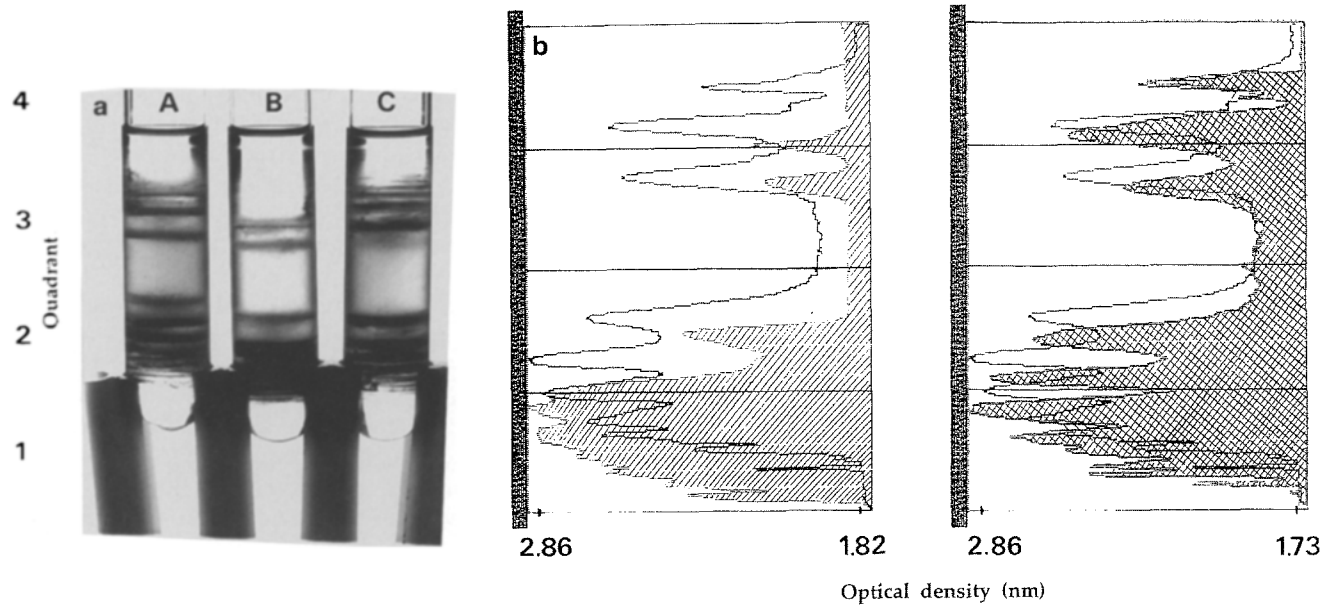

Fig. 6a, b Changes in red blood cell density caused by oxidative stress in vitro. The photograph (**a**) shows untreated cells (*A*) and cells challenged with either 20 $\mu mol \cdot l^{-1}$ cumene hydroperoxide (*B*) or 50 $\mu mol \cdot l^{-1}$ phenazine methosulphate (*C*). In the laser densitometry scan (**b**), the *left panel* shows an overlay of the untreated cells (*unhatched*) and cells challenged with cumene hydroperoxide (*hatched*). The *right panel* compares cells treated with phenazine methosulphate (*cross-hatched*) to untreated cells (*unhatched*). Untreated cells were incubated in phosphate-buffered saline under the same conditions as the cells treated with oxidants (see Methods). The profiles were analysed using the procedures described in the legend to Fig. 4

met-haemoglobin concentration were detected after exercise [pre-exercise 0.78 (SEM 0.78)% to exercise 0.24 (SEM 0.26)%; $n=8$]. In contrast to the negligible effect of exercise, cells stressed oxidatively with PMS (50 $\mu mol \cdot l^{-1}$; $n=4$) showed a substantial increase in membrane-associated haem-iron [control 1.27 (SEM 0.53) $nmol \cdot mg^{-1}$ to PMS 1.66 (SEM 0.64) $nmol \cdot mg^{-1}$] accompanied by peak and integrated LCL signals (integrals not shown) that were twofold stronger in washed ghosts from PMS-treated [86.6 (SEM 33.0) $\times 10^4$ cpm] than those from untreated [47.6 (SEM 9.60) $\times 10^4$ cpm] cells.

Discussion

Our results showed that single episodes of submaximal exercise increased RBC susceptibility to oxidative stress in vitro. Anti-oxidant depletion would seem to be the most likely explanation because membrane-bound haem-iron did not change. Paradoxically, the vulnerability of RBC to osmotic stress decreased after exercise, and this was associated with decreased MCV and an increase in high-density cells. The cumulative effects of exercise-induced oxidative damage may conribute, along with mechanical and osmotic stress, to an increased rate of RBC turnover in endurance athletes. The presence of a younger RBC subpopulation (Weight et al. 1991), which may increase endurance capacity, could explain why RBC from trained men are more resistant to peroxidative, but not to osmotic, stress compared to cells from untrained men. The oxidative and osmotic results will be discussed separately.

Oxidative stress

Depletion and/or uncoupling of antioxidant protection is the most plausible explanation for the increased susceptibility of RBC to peroxidation in vitro after exercise. Moderate cycling for 1 h has been shown to deplete the concentration of reduced glutathione (GSH) in whole blood by 60% and increase oxidised glutathione (GSSG) by 100% in recreational athletes (Gohil et al. 1988). Replenishment of GSH during recovery (Gohil et al. 1988) is consistent with increased GSSG reductase activity detected in RBC immediately after a similar workload (Ohno et al. 1986). Depletion of ascorbate and α-tocopherol may also occur, because dietary supplementation with these anti-oxidants has been found to abolish the exercise-induced increase in RBC density and the decrease in induction time of O_2 uptake of RBC challenged with Cum-OOH in vitro (I. Gillam and J. A. Smith, unpublished data).

The elevated rate of O_2 consumption postexercise, which may not return to basal levels until 12 h after a similar workload (Bahr and Sejersted 1991), could contribute, along with secondary oxidative reactions, to the delayed increase in RBC susceptibility to peroxidative haemolysis. Increases in serum lipid peroxidation markers have not been found to be detectable in serum until 6 h after downhill running (Maughan et al. 1989) and while RBC vulnerability to peroxidative haemolysis has been found to increase significantly 24 h after a

half-marathon, no increase was detected immediately after the race despite a significant fall in blood GSH concentration (Duthie et al. 1990). Decreases in the activities of free radical detoxification enzymes such as GSH peroxidase may be responsible for the increased susceptibility of RBC to peroxidation. Replacement of oxidatively-damaged polyunsaturated fatty acids by plasma exchange (Chiu et al. 1989) with species such as arachidonate during the 6-h period following exercise may also increase the vulnerability of RBC to lipid peroxidation (Clemens and Waller 1987).

Antioxidant capcity may have to fall below a critical threshold before RBC become susceptible to oxidative damage. We attempted to simulate the effects of exercise-induced oxidative stress in vitro by treating RBC from nonexercised subjects with a low concentration of the superoxide-generating reagent PMS (50 $\mu mol \cdot l^{-1}$) (Hebbel et al. 1989). While the oxidative stress of exercise was insufficient to cause haem-iron incorporation into RBC membranes, this phenomenon occurred, and was associated with increased LCL, when membranes from RBC treated with PMS were challenged with Cum-OOH. Thus, single episodes of exercise produce only minor oxidative damage to RBC compared to that found in pathological situations such as β-thalassaemia which have been found to be associated with substantial incorporation of haem-iron into the membrane (Snyder et al. 1981; Hebbel and Eaton 1989). Because senescent cells, which constitute only a minute fraction of the total RBC population, may be the most susceptible to exercise-induced oxidative stress, assays that can analyse anti-oxidant status and other footprints of oxidative damage at the single cell level are required to identify the affected RBC subpopulation.

Our results add to the mounting evidence that adaptive RBC responses to repeated exercise-induced oxidative stress may occur in athletes. Moderate endurance training has been found to increase the RBC activities of GSSG reductase, GSH peroxidase and catalase (Ohno et al. 1988; Mena et al. 1991; Evelo et al. 1992). The activities of GSH peroxidase and catalase have been shown to increase progressively with training distance in endurance runners and the concentrations of α-tocopherol and GSH in RBC become higher in trained runners (Robertson et al. 1991). One study with rats has suggested that RBC produced under conditions of accelerated erythropoiesis and/or metabolic rate have reduced survival times (Landaw 1988). This mechanism may explain the results of Weight et al. (1991) who have shown that, by injecting ^{51}Cr-labelled autologous RBC back into donors, these cells survived for 114 days in sedentary people compared to only 74 days in athletes running 50–129 km every week. Thus athletes contain, on average, a younger RBC population.

Osmotic stress

Red blood cells have been described as normally being biconcave discocytes with a large surface area to volume ratio that gives them strong flexibility (Clark 1988). With each trip through the microcirculation, these cells shrink and swell, which may lead to damage associated with wear-and-tear and this may be exacerbated by exercise. There are two mechanisms that may lead to decreased RBC osmotic fragility coupled with decreased MCV and increased density after submaximal exercise: (1) cellular dehydration; and (2) protein crosslinking, which may reinforce the ability of the cytoskeleton to withstand hypotonic shock. As there is no evidence to support the latter suggestion cellular dehydration is the most likely explanation. While K^+ leakage has normally been associated with decreased osmotic fragility (Snyder et al. 1981), the exercise intensities used here were too low to reduce intra-erythrocyte K^+ concentration which occurs only when the exercise intensity exceeeds 80% $\dot{V}O_{2max}$ (Hespel et al. 1986). Failure to detect a larger decrease in MCV is indicative of this because a stable intracellular K^+ content has been suggested to be critical to the maintenance of cell volume (Clark 1988). Because the increase in the proportion of high-density cells was only 10%, calculation of MCV and MCHC in the entire population may mask subtle changes in subpopulations. Thus, analysis of volume and haemoglobin concentration in individual cells by flow cytometry, or a similar technique, or in subpopulations isolated from density gradients, may resolve this apparent conflict in our data. Increased RBC density may restrict the transport of O_2 in the microcirculation to working muscles during exercise because older (high-density) RBC have been shown to be less deformable under shear stress than younger (low-density) cells (Morse and Warth 1990). Therefore, regular exercise may accelerate the RBC ageing process. Our recent work has shown that the percentage of blood reticulocytes detected by flow cytometry is two to three times higher in some groups of athletes compared with untrained subjects (R. D. Telford et al. unpublished data). Schmidt et al. (1988) have reported that even 3 weeks of endurance training increased the blood reticulocyte count substantially. These reports are consistent with accelerated removal of the oldest cells during athletic training.

Our results suggest that the percentage of RBC with the lowest density decreases chronically in triathletes and runners compared to the profiles obtained with untrained subjects. Cycling, running and cross-country skiing, in contrast, have been reported to cause a loss of high-density cells (Mairbäurl et al. 1983). The reason for the discrepancy between these studies is not clear, but it may involve the type and intensity of training the subjects were undertaking when tested (i.e. whether ageing of the cells exceeded the rate of destruction and replacement, or vice versa). In agreement with our results, participation in a half-marathon was found to

cause a significant increase in RBC density (Robertson et al. 1988), suggesting that a subpopulation of RBC aged or lost fluid during the race. The changes in MCV and RBC density found after exercise in our study were unlikely to have been caused by changes in plasma osmolality. It has been reported that decreases in MCV detected after a marathon did not correlate with increased plasma osmolality (Staubli and Roessler 1986). RBC do not respond to changes in plasma osmolality that are as small as 12% (Van Beaumont and Rochelle 1974). It has been shown that similar workloads do not cause increases in plasma osmolality that exceed this threshold (Gore et al. 1992).

It has been suggested that transient shrinkage of RBC during exercise may be a compensatory response to increased haematocrit that enables RBC to maintain a state of optimal deformability (Staubli and Roessler 1986). Atrial natriuretic peptide, which has been shown to be secreted into the circulation during exercise (Follenius and Brandenberger 1988), has been reported to increase membrane flexibility (Zamir et al. 1992) and this hormone may optimise the deformability of the high-density cells and, thus, O_2 delivery to working muscles. In this way, hormones released into the circulation during exercise may be able to modulate the RBC response to oxidative and osmotic stress (Kanaley and Ji 1991).

Sickled RBC have been shown to be more susceptible than normal cells to oxidative damage (Hebbel and Eaton 1989). Sickle cell trait (the heterozygous form, HbAS) has been shown to increase the risk of sudden death during intense physical exertion, perhaps through irreversibly-sickled cells causing vascular obstruction (Das et al. 1993). A recent report has suggested that HbAS cells are more susceptible than normal cells to oxidation during exercise and that the percentage of dense RBC membranes increases substantially in HbAS, but not normal (HbAA), cells after exercise (Das et al. 1993). The authors did not investigate whether these so-called dense membranes contained aggregates of incorporated haem-iron.

In summary, our results are consistent with the hypothesis that the increased oxidative and osmotic stress encountered by RBC during repeated episodes of aerobic exercise may shorten their life-span substantially. This would, in turn, increase erythropoietic demand and may account for the lower mean age of the RBC population found in distance runners (Weight et al. 1991). Testing of this hypothesis would require isolation of RBC of various ages and their exposure to the oxidative and osmotic treatments used in this study.

Conclusion

Our results have shows that, while a single episode of submaximal exercise increases the susceptibility of RBC to oxidative stress, regular endurance training may confer a protective effect. Furthermore, acute exercise decreases MCV and increases the percentage of high-density RBC. These responses are both characteristic of cells that may have aged, perhaps, through irreversible oxidative damage to major membrane components. While the causal mechanisms are not consistent with incorporation of haem-iron into the membrane, the most plausible explanation involves depletion of specific anti-oxidants and/or uncoupling of cooperative interactions between the different anti-oxidants and free radical detoxification enzymes. When anti-oxidant protection falls below a critical threshold, oxidative damage of susceptible proteins and lipids may then occur.

Acknowledgements We are grateful to the subjects who paticipated in this study. Financial support was provided by the Faculties Research Found of the Australian National University and the National Quality of Life Foundation. Both J. A. Smith and I. Gillam were recipients of Australian Postgraduate Research Awards and I. Gillam was also a recipient of an Australian Institute of Sport Research Fellowship.

References

Bahr R, Sejersted OM (1991) Effect of intensity of exercise on excess postexercise O_2 consumption. Metabolism 40:836–841

Beutler E, Kuhl W, West C (1982) The osmotic fragility of erythrocytes after prolonged liquid storage and after reinfusion. Blood 59:1141–1147

Campwala HQ, Desforges JF (1982) Membrane-bound hemichrome in density-separated cohorts of normal (AA) and sickled (SS) cells. J Lab Clin Med 99:25–28

Chiu D, Kuypers F, Lubin B (1989) Lipid peroxidation in human red cells. Semin Hematol 26:257–276

Clark MR (1988) Senescence of red blood cells: problems and progress. Physiol Rev 68:503–553

Clemens MR, Waller HD (1987) Lipid peroxidation in erythrocytes. Chem Phys Lipids 45:251–268

Das SK, Hinds JE, Hardy RE, Collins JC, Mukherjee S (1993) Effects of physical stress on peroxide scavengers and sickle cell trait erythrocytes. Free Radic Biol Med 14:139–147

Duthie GG, Robertson JD, Maughan RJ, Morrice PC (1990) Blood antioxidant status and erythrocyte lipid peroxidation following distance running. Arch Biochem Biophys 282:78–83

Evelo CTA, Palmen NGM, Artur Y, Janssen GME (1992) Changes in blood glutathione concentrations, and in erythrocyte glutathione reductase and glutathione-S-transferase activity after running training and after participation in contests. Eur J Appl Physiol 64:354–358

Follinius M, Brandenberger G (1988) Increase in atrial natriuretic peptide in response to physical exercise. Eur J Appl Physiol 57:159–162

Gohil K, Viguie C, Stanley WC, Brooks GA, Packer L (1988) Blood glutathione oxidation during human exercise. J Appl Physiol 64:115–119

Gore CJ, Scroop GC, Marker JD, Catcheside PG (1992) Plasma volume, osmolarity, total protein, and electrolytes during treadmill running and cycle ergometer exercise. Eur J Appl Physiol 65:302–310

Guglielmini G, Casoni J, Patracchini M, Manfredini F, Grazzi G, Ferrari M, Conconi F (1989) Reduction of Hb levels during the racing season in nonsideropenic professional cyclists. Int J Sports Med 10:352–356

Hebbel RP, Eaton JW (1989) Pathobiology of heme interaction with the erythrocyte membrane. Semin Hematol 26:136–149

Hebbel RP, Ney PA, Foker W (1989) Autoxidation, dehydration, and adhesivity may be related abnormalities of sickle erythrocytes. Am J Physiol 256:C579–C583

Hespel P, Lijnen P, Fiocchi R, Denys B, Lissens W, M'Buyamba-Kabangu JR, Amery A (1986) Cationic concentrations and transmembrane fluxes in erythrocytes of humans during exercise. J Appl Physiol 61:37–43

Ji LL, Katz A, Fu R, Griffiths M, Spencer M (1993) Blood glutathione status during exercise: effect of carbohydrate supplementation. J Appl Physiol 74:788–792

Kanaley JA, Ji LL (1991) Antioxidant enzyme activity during prolonged exercise in amenorrheic and eumenorrheic athletes. Metabolism 40:88–92

Kuross SA, Rank BH, Hebbel RP (1988) Excess heme in sickle erythrocyte inside-out membranes: possible role of thiol oxidation. Blood 71:876–882

Landaw SA (1988) Factors that accelerate or retard red blood cell senescence. Blood Cells 14:47–67

Mairbäurl H, Humpeler E, Schwaberger G, Pessenhofer H (1983) Training-dependent changes of red blood cell density and erythrocytic oxygen transport. J Appl Physiol 55:1403–1407

Maughan RJ, Donelly AE, Gleeson M, Whiting PH, Walker KA, Clough PJ (1989) Delayed-onset muscle damage and lipid peroxidation in man after a downhill run. Muscle Nerve 12:332–336

Mena P, Maynar M, Gutierrez JM, Maynar J, Timon J, Campillo JE (1991) Erythrocyte free radical scavenger enzymes in bicycle professional racers: adaptation to training. Int J Sports Med 12:563–566

Morse PD, Warth JA (1990) Direct measurement of the internal viscosity of sickle erythrocytes as a function of cell density. Biochim Biophys Acta 1053:49–55

Ohno H, Sato Y, Yamashita K, Doi R, Arai K, Kondo T, Taniguchi N (1986) The effect of brief physical exercise on free radical scavenging enzyme systems in human red blood cells. Can J Physiol Pharmacol 64:1263–1265

Ohno H, Yahata T, Sato Y, Yamamura K, Taniguchi N (1988) Physical training and fasting erythrocyte activities of free radical scavenging enzyme activities in sedentary men. Eur J Appl Physiol 57:173–176

Robertson JD, Maughan RJ, Davidson RJL (1988) Changes in red cell density and related indices in response to distance running. Eur J Appl Physiol 57:264–269

Robertson JD, Maughan RJ, Duthie GG, Morrice PC (1991) Increased blood antioxidant systems of runners in response to training load. Clin Sci 80:611–618

Schmidt W, Maassen N, Trost F, Boning D (1988) Training-induced effects on blood volume, erythrocyte turnover, and haemoglobin oxygen-binding properties. Eur J Appl Physiol 57:490–498

Schmidt W, Maassen N, Tegtbur U, Braumann KM (1989) Changes in plasma volume and red cell formation after a marathon competition. Eur J Appl Physiol 58:453–458

Selby GB, Eichner ER (1986) Endurance swimming, intravascular haemolysis, anemia, and iron depletion: new perspective on athletes anemia. Am J Med 81:791–794

Simmons A (1972) Technical hematology. Lippincott, Philadelphia

Smith JA (1995) Exercise, training and red blood cell turnover. Sports Med 19:9–31

Smith JA, Telford RD, Mason IB, Weidemann MJ (1990) Exercise, training and neutrophil microbicidal activity. Int J Sports Med 11:179–187

Smith JA, Baker MS, Weidemann MJ (1992) Free radical generation in hydroperoxide-treated erythrocytes monitored continuously by luminol-amplified chemiluminescence. Biochem Int 28:1009–1020

Snyder LM, Sauberman N, Condara H, Dolan J, Jacobs J, Szymanski I, Fortier NL (1981) Red cell membrane response to hydrogen peroxide-sensitivity in hereditary xerocytosis and in other abnormal red cells. Br J Haematol 48:435–444

Snyder LM, Leb L, Piotrowski J, Sauberman N, Liu SC, Fortier NL (1983) Irreversible spectrin-haemoglobin crosslinking in vivo: a marker for red cell senescence. Br Haematol 53:379–384

Staubli M, Roessler B (1986) The mean red cell volume in long distance runners. Eur J Appl Physiol 55:49–53

Van Beaumont W, Rochelle RH (1974) Erythrocyte volume stability with plasma osmolarity changes in exercising man. Proc Soc Exp Biol Med 145:240–243

Vettore L, De Matteis MC, Zampini P (1980) A new density gradient system for the separation of human red blood cells. Am J Hematol 8:291–297

Weight LM, Byrne MJ, Jacobs P (1991) Haemolytic effects of exercise. Clin Sci 81:147–152

Wilkerson JE, Gutin B, Horvath SM (1977) Exercise-induced changes in blood, red cell, and plasma volumes in man. Med Sci Sports 9:155–158

Winterbourn CC (1983) Reactions of superoxide with hemoglobin. In: Greenwald RA (ed) CRC handbook of methods for oxygen radical research. CRC Press, Boca Raton, Fla., pp 137–141

Zamir N, Tuvia S, Riven-Kreitman R, Levin S, Korenstein R (1992) Atrial natriuretic peptide: direct effects on human red blood cell dynamics. Biochem Biophys Res Comm 188:1003–1009