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Gwozdziński, Krzysztof; Pieniazek, Anna; Tabaczar, Sabina; Jegier, Anna; Brzeszczynska, Joanna

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Investigation of oxidative stress parameters in different life span erythrocyte fractions in young untrained men after an acute exercise

Running title: Oxidative stress in different life span erythrocytes after an exercise

Krzysztof Gwozdziński¹, Anna Pieniazek², Sabina Tabaczar¹, Anna Jegier³, Joanna Brzeszczynska¹

¹ Department of Molecular Biophysics, University of Lodz, Poland

² Department of Medical Biophysics, University of Lodz, Poland

³ Department of Sport Medicine, Medical University of Lodz, Poland

Corresponding author:

Krzysztof Gwozdziński

Department of Molecular Biophysics

University of Lodz,

Pomorska 141/143,

90-236, Lodz, Poland

Tel.: +48 42 635 44 52; Fax.: +48 42 635 44 73;

e-mail: kgwozdz@biol.uni.lodz.pl

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New Findings**What is the central question of this study?**

What is the influence of a single bout of exercise on the properties of erythrocyte fractions at different age?

What is the main finding and its importance?

A single bout of exercise in untrained men induced oxidative stress in erythrocytes and had an influence on antioxidant defense in these cells. Old erythrocytes were more sensitive to oxidative damage than young and middle-age cells. Higher level of glutathione in old erythrocyte fractions did not protect them against oxidative stress. It seems that exercise may promote the removal of old erythrocytes from the circulation.

Abstract

The objective of study was to establish the role of exercise-induced oxidative stress in the erythrocyte fractions: young (YF), middle-aged (MAF) and old (OF) of young untrained men after acute exercise. Blood samples were collected before exercise, immediately and one hour after exercise. Maximum wattage was 292 ± 27 W and exercise time duration was 8.73 ± 0.9 min.

Different optical properties and oxidative stress parameters were found in each erythrocyte fraction. Total thiols in YF and MAF after exercise and after one hour rest were similar to values before exercise, however, in OF (32.7 ± 9.8 nmol/mgHb) the concentration was lower in comparison to YF (55.5 ± 3.2 nmol/mgHb), MAF (56.8 ± 7.7 nmol/mgHb) and increased one hour later ($p<0.0002$).

The glutathione concentration was higher in OF (8.4 ± 0.4 nmol/mgHb) than in YF (4.5 ± 0.6 nmol/mgHb) and MAF (4.8 ± 0.5 nmol/mgHb) ($p<0.0002$) and did not change neither after exercise nor one hour later.

In OF, the peroxides level was higher after exercise (1.2 ± 0.2 nmol/mgHb) and one hour later (1.1 ± 0.2 nmol/mgHb), when compared with samples before exercise (0.9 ± 0.1 nmol/mgHb) ($p<0.05$). Similar results were observed in YF and MAF.

The TBARS (thiobarbituric acid reactive substances) level was approx. 2.5 fold higher in OF (0.19 ± 0.04 nmol/mgHb) when compared with YF (0.07 ± 0.01 nmol/mgHb) and MAF (0.08 ± 0.02 nmol/mgHb) ($p<0.0002$) and was increased after exercise remaining unchanged after one hour later. In YF and MAF, no difference in TBARS level was detected after exercise one hour later. No difference in membrane fluidity was observed in all fractions.

Erythrocytes OF appeared to be more sensitive for cellular oxidative damages.

Introduction

Regular exercise prevents the development of diseases associated with oxidative stress (Kruk, 2007; Durstine et al., 2013). Athletes possess better free radical defense probably due to regular oxidative stress which is a result of regular physical activity and exercises. (Cazolla et al. 2003; Tong et al. 2012). There are a lot of studies that have investigated exercise-induced oxidative stress. A single bout of exercise often leads to an acute oxidative stress, which may result in an increased level of oxidized molecules and macromolecules (Powers & Jackson 2008; Powers et al. 2011). Factors such as: time, intensity, duration and subject's individual (genetic and physical) differences may impact the degree of oxidation. The concentration levels of TBARS (thiobarbituric acid reactive substances) and carbonyl groups after exercise have been shown to be correlated with an increase in reactive oxygen species (ROS) production in blood (Mrakic-Sposta et al. 2012). Skeletal muscle and blood (erythrocytes and leukocytes) are the major sources of ROS generation during exercise (Powers & Jackson 2008; Jackson, 2008; Nikolaidis & Jamurtas 2009). However, ROS are also produced by tissues such as heart and lungs (Nikolaidis & Jamurtas, 2009).

Erythrocytes, more than other cells are exposed to mechanical damage, biochemical disturbance and damage associated with oxidative stress (Petibois & Deleris, 2005). The most common alteration of erythrocytes, as a result of oxidative stress, is membrane lipid and protein peroxidation, which may destabilize the cell's membrane skeleton, cause changes in transmembrane transport and indirectly affect the cell life span. An efficient antioxidant system is therefore required to maintain the redox balance, preventing any changes affecting structural and functional properties of red blood cells. After exhaustive aerobic and anaerobic exercise, oxygen radicals are produced in larger quantities and their neutralization by the endogenous antioxidant system may be insufficient (Alessio et al. 2000). Previous studies found an increased level of thiobarbituric acid reactive substances and a significant decrease of membrane protein thiol groups one hour after strenuous exercise (Brzezczynska et al. 2008; Gwozdzinski et al. 2013). One such detrimental change is an increase in erythrocyte's membrane rigidity as a result of lipid oxidation after a single bout of maximal and submaximal exercises (Berzosa et al. 2011). Similarly, in our previous report we showed that one hour after exercise, lipid membrane fluidity in the outer region of lipid bilayers is significantly decreased (Brzezczynska et al. 2008). Our work has also shown that acute exercise generates oxidative stress in erythrocytes and plasma (Brzezczynska et al. 2008, Gwozdzinski et al. 2013). Moreover, it should be noted that erythrocyte ageing is accompanied by a decrease in cell volume increase of haemoglobin content and cell density (Schmidt et al. 1987).

Changes in the aging cells may have a significant impact on their response to increased oxidative stress during exercise and thus on their proper functioning.

The aim of this study was to investigate the effects of acute physical exercise on the oxidative stress process in the erythrocytes of young sedentary healthy man subjects. Taking into account that the redox balance in aging cells is disturbed in favour of the oxidation processes (Pandey, 2013), we examined whether the age of the erythrocytes has an effect on the changes resulting from oxidative stress, induced by exhaustive exercise. Oxidative stress markers such as protein thiol groups and lipid peroxidation products were measured and compared. Moreover, fluidity of red blood cell membrane and optical properties of RBCs from three populations were investigated.

Material and methods

Chemicals

TMA-DPH (1,6-diphenyl-1,3,5-hexatriene), DAUDA (11-(5-dimethylaminonaphthalesulphonyl)-undecanoic acid, 4,4'-dithiodipyridine (Aldrithiol), DTNB (5,5'-dithio-bis(2-nitro-benzoic acid)), xylanol orange and Percoll were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals were obtained from POCh (Gliwice, Poland).

Subjects and protocol

Eight healthy, untrained (i.e. not performing any regular physical activity) males (mean age, 21 ± 3 years; mean height, 180 ± 5 cm; mean mass, 80 ± 9.5 kg; mean body mass index, 24 ± 3.5 kg/m²) volunteered to participate in this study. The volunteers were clinically healthy, according to a medical doctor's examination, and did not meet any of the following criteria: resting blood pressure higher than 140/90 mm Hg, resting heart rate higher than 90 beats/min, smoking, antioxidant supplementation and medicaments usage. All subjects gave an informed consent to blood withdrawal. The experiments have been performed in accordance with the rules of the Declaration of Helsinki and conformed to the ethical principles set by the Belmont Report, Ethical Principles and Guidelines for the Protection of Human Subjects of Research. Approval for investigation was

obtained from the Bioethical Committee of the Medical University of Lodz. All participants signed an Informed Consent Form prior to enrolment into the study.

Experimental Procedures

The tests and blood samples collection were performed in the morning, after an overnight fasting. Subjects refrained from drinking alcohol and performing strenuous exercise for 24 hours prior to testing.

An exhaustive bout of exercise was performed on a friction-brake cycle ergometer (Monark, Sweden). The initial load was set to 60 Watts and the subjects were told to pedal at 60 rpm (revolutions per minute) for 1 min, the workload was increased by 30 Watts every minute. The test was terminated when the subjects expressed their exhaustion. The heart rate at the peak of exercise was higher than 90% of the expected maximal heart rate in relation to the subjects' age. Maximal heart rate was estimated using the formula "220- age". Individual response to this exercise was HR_{max} (maximal heart rate) 195 ± 12 beats/min, and maximum wattage (W_{max}) was 292 ± 27 W, maximum wattage per kilogram 3.43 ± 0.57 (W/kg). During exercise, the heart rate was measured using ECG (system Case 16 Marquette DRG comp). Total work performed during exercise amounted to 92782 ± 16465 J. The average exercise time duration was 8.73 ± 0.9 min.

After exercise, subjects remained seated for 1 hour, and were allowed to drink water only. Venous blood samples were collected from an antecubital vein to heparinised tubes three times: before exercise, immediately after exercise (in less than 20 seconds from the end of exercise) and one hour after the exercise. The concentration of the erythrocytes and mean corpuscular volume (MCV) were determined in the collected blood samples using auto-analyser. For other experiments, the blood samples were centrifuged and the plasma was separated. The erythrocytes (RBC) were washed three times with phosphate buffered saline (PBS, pH 7.4) and the cells were suspended in PBS to a hematocrit of 40%.

Density separation of erythrocytes

The erythrocytes were fractionated into three fractions: young, middle-aged and old by the Percoll discontinuous density gradient. Dulbecco Modified Eagle medium was used to prepare different densities of Percoll (Prall et al. 1998). This resulted in a gradient containing 35%, 40%, 45%, 50%, 55%, 65%, 80% and 100% of Percoll. In the centrifuge tubes the density of Percoll gradually decreased from 80% (bottom) to 35% (top). Erythrocyte samples were carefully layered on the top of the prepared gradient. Centrifugation was carried out in a swing-out rotor at 1200 x g for 30 min at 20°C. Erythrocyte density increased as a function of age and thus in the Percoll density gradient, the fractions containing young erythrocytes were stratified at the top of gradient, the middle-aged were below the young fraction and the eldest were on the bottom layer (Fig. 1). Each fraction was carefully collected to separate tubes. Erythrocytes were then washed with PBS at 4°C in order to remove Percoll and processed for the different assays.

Cytometric assay

The erythrocytes from each fraction were analyzed with flow cytometry (LSR II. Becton, Dickinson, excitation laser 488 nm, SSC measured using 488/10 bandpass filter). Two parameters were analyzed by simultaneous separate detection of forward scatter (FSC-A) low angle and side scatter (SSC-A) right angle of light. The light scattered near the forward direction (low angle) is expected to be proportional to the size (volume) of the particle and is independent of the cell refractive index and shape, whereas scattering at right angle depends on the cell shape and internal properties of the scattering particles (Marczak & Jozwiak, 2008). FSC/SSC is a dual parameter contour plot histogram proportional to the total cell diversity. Data were displayed in the form of diagrams of cell numbers vs. light scatter. Each measurement was done for 30,000 cells. For each set of histograms, the percentage of shape changes in erythrocytes was calculated.

Light microscope analysis

The microscopy analysis of the erythrocyte fractions was performed under phase contrast at 100× magnification using Nikon Eclipse Ti-U inverted research microscope. For analysis the samples of erythrocytes were suspended in PBS buffer.

Thiols concentration

The thiol group concentration in the erythrocytes was determined by the spectrophotometric method with 4,4'-dithiodipyridine (Aldrithiol) (Grassetti & Murray Jr., 1967). Erythrocytes (hematocrit 1%) were mixed with phosphate buffer (10 mM with 1% SDS, pH=8.0). After mixing, an initial absorbance (A_0) was determined at 324 nm and then the 4,4'-dithiodipyridine (3 mM) was added. The samples were then incubated, for the first 15 min at 37°C and the next 15 min at room temperature. Subsequently, the absorption (A_1) was measured. The ΔA ($A_1 - A_0$) is a measure of thiol groups. The thiol group content was calculated in nmol/mg Hb using 2-thiopyridon extinction coefficient $\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \times \text{cm}^{-1}$.

Glutathione concentration

The reduced glutathione (GSH) concentration in the erythrocyte fractions was determined by Ellman's method (Ellman, 1959). The erythrocytes were deproteinized by the addition of 25% TCA and centrifuged (600×g for 10 min). Phosphate buffer (0.5 M, pH 8.0) and DTNB (5,5'-dithio-bis(2-nitro-benzoic acid)) (5 mM) were added to the cleared supernatant (1:0.1:1). The formation of 5-thio-2-nitrobenzoic acid, which is proportional to GSH concentration was monitored at 412 nm. The concentration of glutathione was calculated using 5-thio-2-nitrobenzoic acid extinction coefficient $13.6 \text{ mmol}^{-1} \times \text{l} \times \text{cm}^{-1}$ and expressed in nmol/mg Hb.

Hydroperoxides

The amount of hydroperoxides in the erythrocyte fraction was measured using spectrophotometric method with xylenol orange (Gay & Gebicki, 2000).

A working solution (mixture at 1:100 ratio of 25 mM ammonium iron (II) sulfate dissolved in 2.5 M sulphuric acid and 125 μ M xylenol orange with 100 mM sorbitol) was added to the samples. After 30 min of incubation in the dark, the violet-coloured complex was quantified spectrophotometrically at 560 nm against blank. The amount of hydroperoxides was calculated using calibration curve for H_2O_2 (concentration range 0–20 mM) and expressed in nmol/mg Hb.

Lipid peroxidation

Lipid peroxidation in the erythrocyte fractions was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Rice-Evans et al. 1991). The erythrocytes were mixed with 20% (w/v) trichloroacetic acid, 2% BHT in ethanol and 0.37% (w/v) 2-thiobarbituric acid in 0.25 M HCl (1:1:0.0075:1 ratio). The samples were heated to 100°C for 10 min and centrifuged (600 \times g 10 min 20°C). The absorbance of the supernatant was measured at 532 nm against blank. The TBARS content was calculated using extinction coefficient for MDA, $\epsilon=1.56\times 10^5 M^{-1}\times cm^{-1}$ and expressed in nmol TBARS/mg Hb.

Hemoglobin concentration

The concentration of hemoglobin was measured by the Drabkin method (Drabkin, 1946). The erythrocytes were added to Drabkin reagent at 1:200 ratio. After a 15 min incubation at the room temperature the absorption of hemoglobin was measured at 540 nm against blank. The hemoglobin concentration was calculated from the extinction coefficient $\epsilon = 0.044 M^{-1}\times l\times cm^{-1}$ and mol mass of hemoglobin 64 000 g/mol.

Measurement of membrane fluidity

Membrane fluidity was measured by fluorescence spectroscopy and evaluated on the basis of fluorescence anisotropy of TMA-DPH (1,6-diphenyl-1,3,5-hexatriene) and DAUDA (11-(5-dimethylaminonaphthalesulphonyl)-undecanoic acid) probes. The erythrocyte suspension (diluted with PBS to hematocrit of 0.04%) was incubated with the fluorescence probes at final concentration 10^{-6} M, at the room temperature for 10 min. The fluorescence was measured with Perkin-Elmer LS-50B

spectrofluorimeter. The excitation and emission wavelengths were: $\lambda_{\text{ex}} = 365$ nm and $\lambda_{\text{em}} = 425$ nm for TMA-DPH, and $\lambda_{\text{ex}} = 365$ nm and $\lambda_{\text{em}} = 471$ nm for DAUDA. The fluorescence anisotropy was obtained from the following equation (Van der Meer, 1988)

$$r = \frac{I_{vv} - I_{vh} \cdot G}{I_{vv} + 2I_{vh} \cdot G}$$

where I_{vv} and I_{vh} represent the components of the light intensity estimated respectively parallel and perpendicular to the direction of the vertically polarized excitation light and G is the correction factor ($G = I_{vh}/I_{hh}$).

Statistical analysis

All measurements were expressed as mean \pm standard deviation (SD). Data normality was tested using the Shapiro-Wilk test and variance homogeneity was verified with Levene test. The significance of the differences between couples of means was estimated using one-way ANOVA and post hoc Tukey test. All statistical calculations were made with the use of STATISTICA.PL v.10. (StatSoft) and StatsDirect (StatsDirect Limited).

Results

The three obtained erythrocyte fractions showed different optical properties (Fig. 2). Fraction II consisting of middle-aged cells seemed to be the most homogeneous, while fraction III seemed to be the most heterogenic and consisted of the oldest and most dense cells. In addition, light microscopy measurements showed changes in more than 40% of old fraction erythrocytes (Fig. 3) with a high level of echinocytes (approx. 30%).

Afterwards, the oxidative stress parameters in hemolysates obtained from the erythrocyte fractions before, after and 1-hour after post exercise recovery were determined. The old erythrocyte fraction III was characterized by the lowest concentration of thiols before exercise (Fig. 4A). However, in the subsequent exercise and after 1h of rest, the thiols concentration was increased ($p < 0.01$). No significant difference in thiols in hemolysates of fraction I and II following exercise and 1h post exercise was observed as compared to the levels detected before the exhaustion (Fig. 4A).

Glutathione concentration in the old erythrocytes was significantly higher than in young and middle-aged erythrocytes (marked per mg hemoglobin). However, the level of GSH in all three fractions did not change significantly during exercise and after 1 hour rest post exercise as compared to the level measured before exercise (Fig. 4B).

Oxidative stress in cells was also measured by the concentration of hydroperoxides and the end products of lipid peroxidation which react with thiobarbituric acid (TBARS). In the old erythrocytes, the concentration of hydroperoxides was significantly higher than in young and middle age fractions ($p < 0.001$). This fraction, immediately after exercise, had a significant ($p < 0.002$) increase of hydroperoxide and after 1 hour of rest post exercise ($p < 0.05$) as compared to the hydroperoxide level before exercise. In addition, the increasing tendency in the level of hydroperoxides was observed in young, middle-aged and old erythrocytes after exercise and after 1 hour of rest post exercise (Fig. 5A). Similarly, lipid peroxidation was 2.5 times higher (concentration of TBARS) in the old erythrocytes than in the young and middle-aged fractions. This was the only fraction with a significant increase of lipid peroxidation after exercise ($p < 0.05$) and after 1 hour of rest post exercise. There was no difference in the level of products reacting with thiobarbituric acid in young and middle-aged erythrocytes after exercise and after a rest (Fig. 5B).

The oxidation of membrane lipids led us to investigate the erythrocyte membrane fluidity after exercise by measuring the fluorescence anisotropy. Two fluorescence probes TMA DPH and DAUDA, located respectively near the polar and hydrophobic regions of lipid layer were applied. No difference in lipid membrane fluidity close to the polar region in young, middle-aged and old fractions of erythrocytes was recorded at every time point of the experiment after exercise (Fig. 6A). The hydrophobic region of the phospholipids also did not yield any changes in the membrane fluidity in erythrocytes fractions (Fig. 6B). However, an increasing trend was observed in young and middle-aged erythrocytes immediately after exercise.

Discussion

The relationship between exercise and oxidative stress has commonly been viewed as a detrimental phenomenon. Oxidative stress certainly has the ability to result in physiological damage, however, an optimal level of prooxidant may act as the stimulus for the upregulation of antioxidant defenses. Although the role of oxidative stress in exercise-induced adaptations, and in human physiology remains to be elucidated, it becomes clear that ROS production may be crucial for optimal adaptive potential and physiological function of the body. In this respect, the ROS produced during exercise should rather be viewed as a positive mechanism because it stimulates the growth of antioxidant potential and activation of regeneration processes. For example, induced adaptive mechanism was reported as the beneficial effects of regular exercise in reducing oxidative stress and inflammation in aged rats (Goto et al. 2007).

Nevertheless, exercise is well accepted to increase oxidative stress due to free radical production, as $VO_2\text{max}$ (maximal oxygen consumption) becomes elevated 10- to 15-fold above the resting condition. Malondialdehyde, lipid hydroperoxides and protein carbonyls were documented to increase after both exhaustive aerobic and isometric exercises (Alessio et al. 2000; Sepehri et al. 2014), indicating wide-spread oxidative damage due to exercise. Although the data from many different studies seem to support the notion of exercise-induced oxidative damage (Bloomer et al. 2008), not all results are consistent (Duthie et al. 1990). The studies concerning oxidative stress in relation to exercise are complex and multifactorial, and therefore various modes for exercise intensity, duration, and length of time may have different effects and may produce varied results through the adaptive responses (Goto et al. 2007).

It has been shown that long, regular training lead to the improvement of physiological parameters such as $VO_2\text{max}$, maximal workload, heart rate (rest and exercise), hemoglobin's affinity for oxygen and indicates adaptive mechanisms (Schmidt et al. 1988).

On the other hand, our study showed short acute exercise may induce oxidative stress in the erythrocytes of sedentary young men indicated by significant increase of hydroperoxides and TBARS levels in the old fraction of erythrocytes when compared with the young and middle-aged counterparts (Fig. 6A) and stay in line with previous reports (Aslan et al. 1998; Brzezczynska et al. 2008). An hour after exercise, we have also found a significant increase of peroxide in young and middle fractions of erythrocytes when compared with the results of the blood samples collected before exercise. However, in the old fraction an increase of hydroperoxides level was observed just after exercise (Fig. 6B).

While examining the parameters of oxidative stress, a significant decrease in total thiols in the old fraction of erythrocytes was noted as compared to young and middle-age before exercise. Surprisingly, this level of thiols significantly increased after exercise and one hour later (Fig. 4A). We also observed that the level of total thiols did not change after exercise and after 1 hour of recovery post exercise in young and middle-aged fractions. Unchanged level of thiols in plasma was observed both in sedentary individuals and athletes after acute exercise (Zinellu et al. 2007, Vezzoli A et al. 2016). Similar results were obtained in plasma proteins post exercise (Inayama et al. 2002). However, an increase in the thiol levels in plasma after exercise and 1 hour post exercise recovery were observed (Gwozdziński et al. 2013; Nakagami et al. 2009), whereas moderate exercise induced loss of serum free cysteine and proteins thiol oxidation (Sen, 1995; Inayama et al. 2002).

To further support our results, it is also important to keep in mind that erythrocytes are exposed intermittently to high concentration of oxygen. Higher content of poly-unsaturated fatty acids in the lipid bilayer and the presence of hemoglobin increases erythrocyte sensitivity to the redox environment (Clemens & Waller, 1987). Previous study found reversible changes in haemoglobin structure after an acute single bout of exercise in young untrained men (Gwozdziński et al. 2013). It is also possible that changes in haemoglobin structure may have influence on the affinity of oxygen to this hemoprotein. It has been reported that exercise changed haemoglobin-oxygen characteristic in the blood of volunteers (Schmidt et al. 1988).

Oxidation of hemoglobin and methemoglobin generates strong oxidizing agents (Jeney et al. 2013), whereas blood flow in vessels may in addition activate leukocytes, neutrophils and other phagocytic cells, which can further release ROS (Lehoux, 2006; Hsieh et al. 2014). At the same time superoxide and nitric oxide may be released by vascular endothelium (Laurindo, et al. 1994). The elevated generation of free radicals this way induces peroxidation in cellular membranes affecting cellular function. Under normal physiological conditions activation of antioxidant network and elimination of unwanted ROS is therefore of great importance for cell survival. In the present study, there was evidence of increased GSH content correlated with erythrocyte age (Fig. 4B). Our result was consistent with the result of Ghashghaeinia et al. (2012), who reported that erythrocyte antioxidant status (GSH) increases with the erythrocytes age (Ghashghaeinia et al. 2012). Several studies have shown that glutathione concentration in erythrocytes may be altered after exercise (Sahlin et al. 1991; Laires et al. 1993). It has been suggested that the decrease of GSH in the plasma after exercise reflects its consumption by skeletal muscle (Kretzschmar & Muller, 1993). Duthie et al.

(1990) reported that (GSH) was decreased immediately after an exercise in erythrocytes, which become susceptible to lipid peroxidation. However, (Robertson et al. 1991) reported that the protective antioxidant capacity of blood is enhanced in endurance runners and the improvement in the blood antioxidant potential may be correlated with the physical activity. In this respect lifestyle strategies may play a very important role in increasing individual health and longevity. Therefore, an insignificant change of the GSH level in erythrocytes and plasma may further support the importance of the regular exercise on blood antioxidants capacity (Vezzoli et al. 2016). It is possible that the discrepant results in a concentration of glutathione in erythrocytes obtained by researchers may be associated with different oxygen saturation in the blood samples (Hutler et al 2000). Our analyses also indicated that there are age-associated alterations in the erythrocyte defense capacity as a protective and/or adaptive mechanism from elevated oxidative stress in aging cells.

Human erythrocytes have a definitive 120 days long lifespan (Bratosin et al. 1995; Bosman et al. 2008). In physiological circumstances, senescent erythrocytes (nearing the 120 day old stage) induce immune process activation (Bosman et al. 2005) associated with changes in their structure and function of the band 3 protein (Bosman et al. 2008). This change may affect not only CO₂ transport through the body but also oxidation processes of cellular components in aging cells. It has been reported that senescent erythrocytes possess a higher density and a smaller size than their young counterparts (Bratosin et al. 1995). Furthermore, the aging is also correlated with significant changes of erythrocytes shape (Pinkofsky, 1997) but also with oxygen affinity to hemoglobin (Schmidt et al. 1987). The dissociation curve in old erythrocytes was shifted to the left in comparison to the young ones.

Using flow cytometry, we showed different optical properties of young, middle-aged and old fractions of erythrocytes (Fig. 2). The use of light microscopy enabled the identification of various forms of erythrocytes in the third fraction. This fraction was the most heterogenic and contains a high level of echinocytes. Our results are in accordance with the literature, which supports the notion of echinocytes formation as a result of the oxidative stress induced RBC aggregation decrease (Reinhart & Singh, 1990).

Additionally, an increase in the plasma membrane stiffness correlated with erythrocytes age was observed. These analyses demonstrated as well that fluidity decreases after exercise and increases slightly one hour after exercise. These changes may result from mechanical and/or oxidative stresses in the membrane after exercise but also may be associated with senescence

processes including optical properties alterations. Another evidence which supports the ROS role in erythrocytes changes comes also from our earlier study (Brzezczynska et al. 2008). This study proves that acute exercise induces oxidative stress in all RBCs fractions and results in a decrease of membrane fluidity, however this also leads us to propose that changes in Hct, or RBC rigidity or viscosity are physiological adaptive modifications which occur during many kinds of exercises (Tsuda et al. 2003). For example, aging cell with higher rigidity is more likely to undergo damage and then death or macrophage uptake.

Another pathophysiologic concept explains the muscle injury-related cytokines role, which is focused on recovery/survival but not adaptation. This concept is considered to be “protective”, and occurs in response to excessive physical/physiological stress. Large quantities of proinflammatory IL-1beta and/or IL-6, and/or TNF-alpha induce systemic inflammation impacting on immune function. IL-6 is responsible for HSP72 activation in the muscle (Henstridge et al. 2014). IL-6 is also one of the causal factors of oxidative stress in RBCs (Peak, 2002), which together with IL-8 activates neutrophil activity.

This study mainly assessed oxidative stress markers in different fractions of RBC in young sedentary subjects after a single bout of exercise but was not compared for example with regular exercise model, which may have a significantly different impact on pro- and antioxidants activation, erythrocytes adaptive or regenerative properties and senescence. Additionally, a relatively small sample size indicates that our analysis can be treated as a pilot study for future investigations with larger numbers of participants.

Conclusion

On the basis of the collected data, we showed that short acute exercise of untrained subjects may induce oxidative stress in the erythrocytes. Although the old aged erythrocytes had the highest levels of erythrocyte glutathione, they also showed the highest levels of TBARS and hydroperoxide. This data implies that an elevated level of GSH did not protect the cells against oxidative stress. Furthermore, these results show that the old fraction is more sensitive to oxidative damage than the young and middle-aged ones.

Conflict of interest

The authors declare that there are no conflicts of interest.

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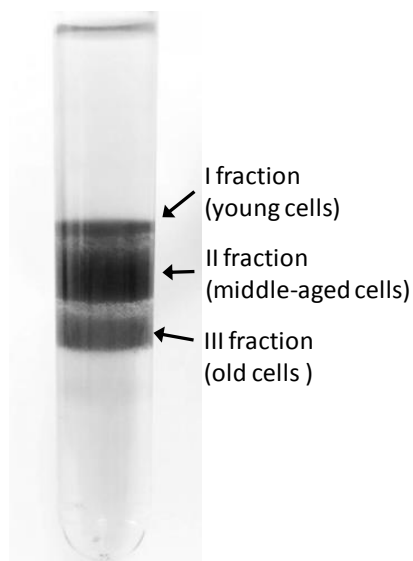


Fig. 1. Picture of the tube with erythrocytes separate into three fractions.

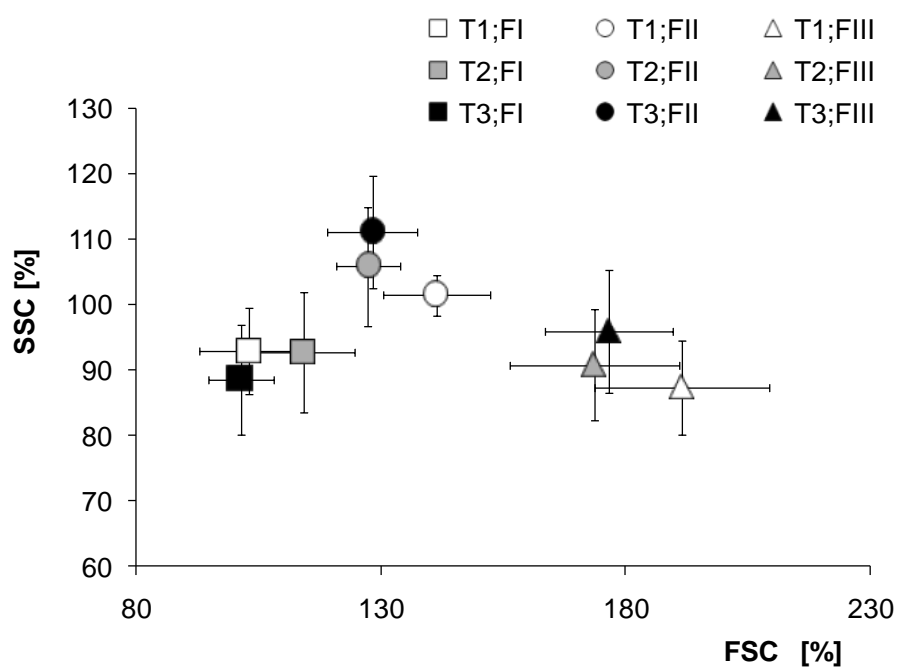


Fig. 2. The effect of exercise on optical properties of erythrocytes in different fractions. T1- pre-exercise; T2- post-exercise; T3- 1h recovery; F (I-III) – fractions of erythrocytes

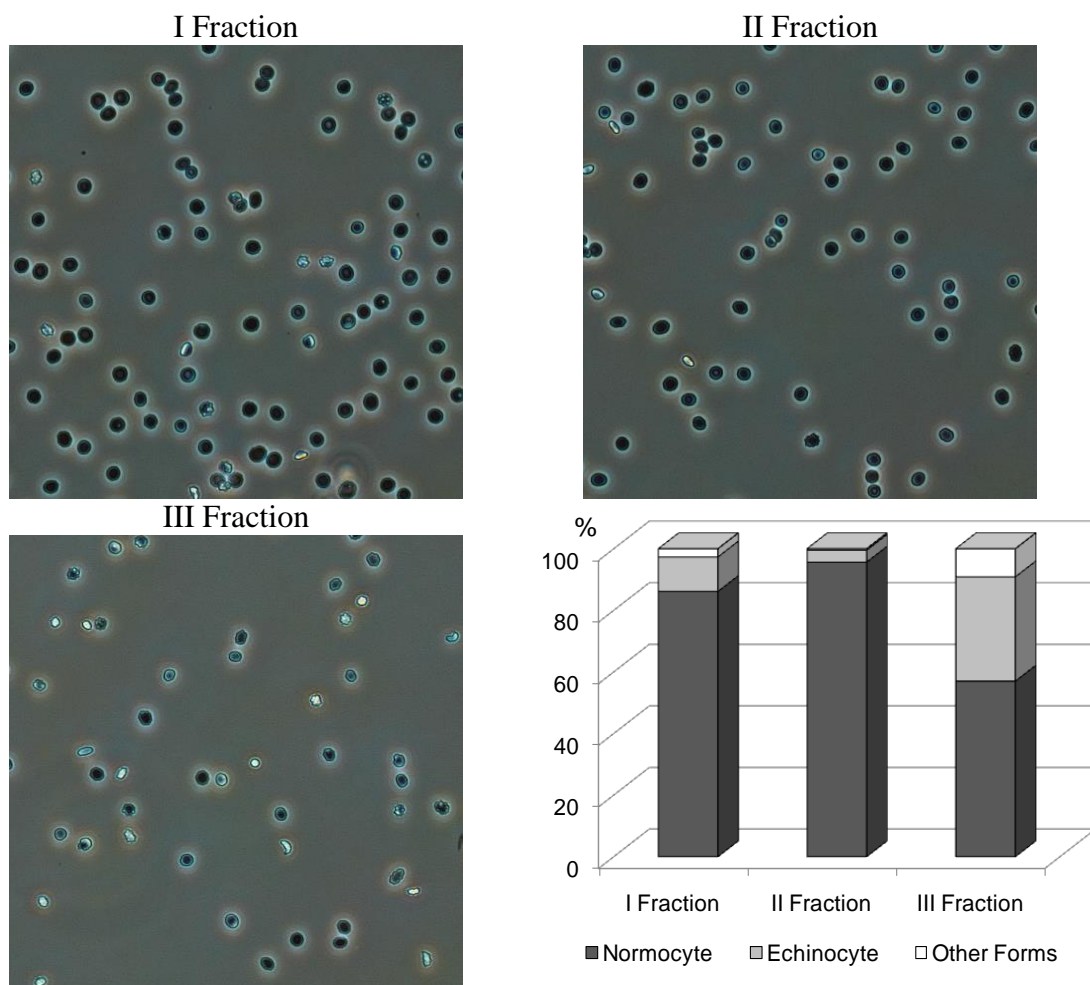


Fig. 3. Microscopy images of young, middle-aged and old fractions of erythrocytes and percentage of normocytes, echinocytes and other forms of erythrocytes.

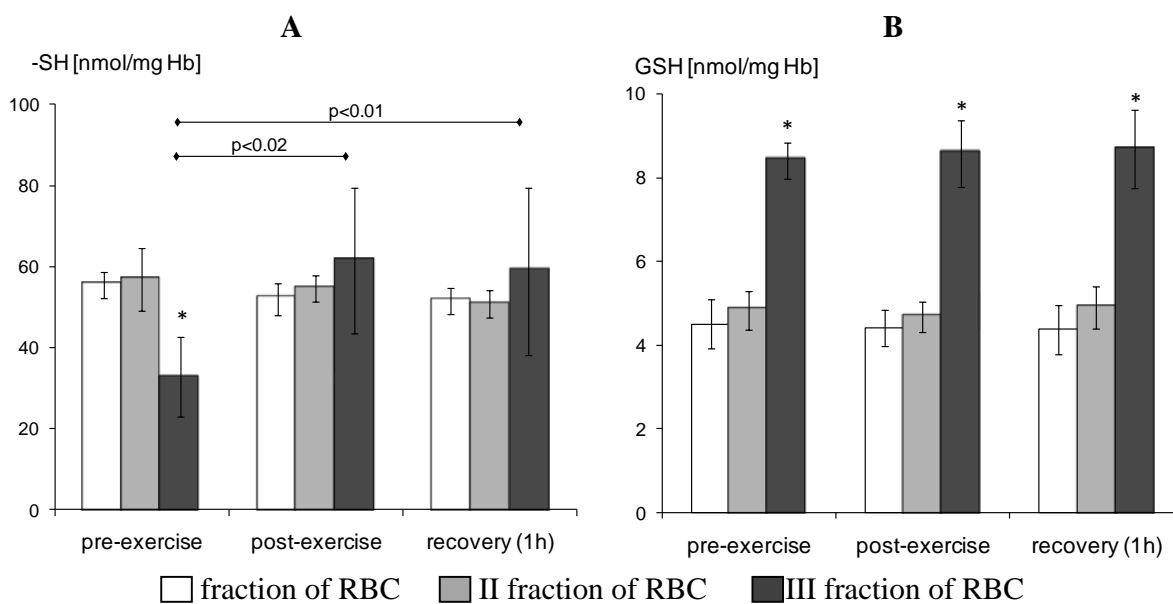


Fig. 4. (A) The -SH groups level and (B) the GSH concentration in young, middle-age and old fractions of erythrocytes before and after exercise, and after 1 hour recovery post exercise (n=8).

Significant differences of -SH level: * I and II fraction vs. III fraction ($p < 0.0002$) in pre-exercise and significant differences of GSH concentration: * I and II fraction vs. III fraction ($p < 0.0002$) in pre-exercise, post-exercise and recovery (1h).

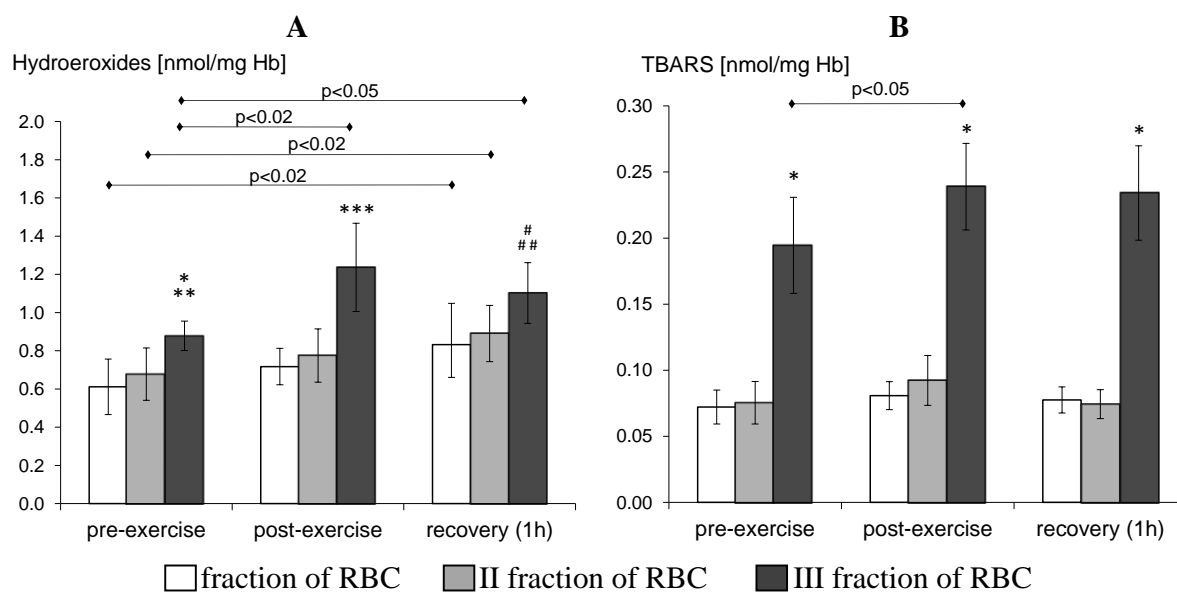


Fig. 5. (A) The hydroperoxides and (B) TBARS level in different fractions of erythrocytes before and after exercise as well as after 1 hour of rest post exercise (n=8).

Significant differences of hydroperoxides level: * I fraction vs. III fraction ($p < 0.02$) and ** II fraction vs. III fraction ($p < 0.001$) in pre-exercise, *** I and II fraction vs. III fraction ($p < 0.0002$) in post-exercise, # I fraction vs. III fraction ($p < 0.01$) and ## II fraction vs. III fraction ($p < 0.05$) in recovery (1h)

Significant differences of TBARS level: * I and II fraction vs. III fraction ($p < 0.0002$) in pre-exercise, post-exercise and recovery (1h) (n=8).

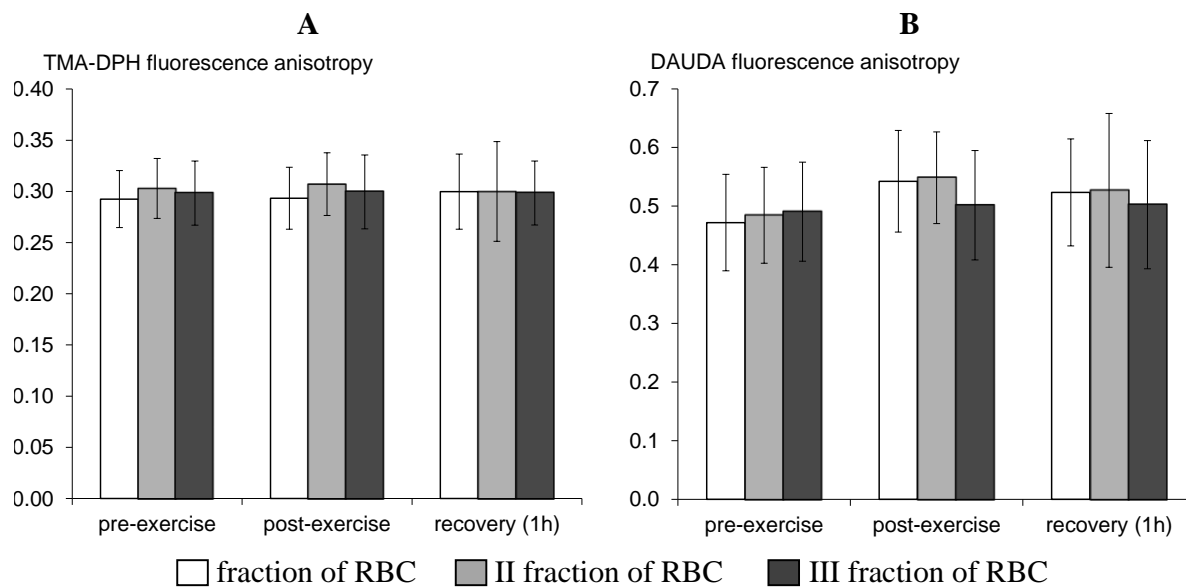


Fig. 6. (A) The TMA-DPH (B) DAUDA anisotropy in different fractions of erythrocytes before and after exercise as well as after 1 hour of recovery post exercise.