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Original article

Does endurance training improve red blood cell aging and hemorheology in moderate-trained healthy individuals?

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Abstract

Objective: To examine the impact of a 6-week endurance training on red blood cell (RBC) aging and deformability of healthy participants to detect possible improved hemorheological and performance-related adaptations.

Methods: A total of 31 participants (17 females and 14 males) performed a 6-week moderate training protocol (three 1-h running sessions per week at 70% of maximal heart rate). Blood was sampled before and after the training. RBCs from each participant were fractioned according to density and age into 4 RBC subfractions. Subfractions were examined for changes of RBC properties, including aging distribution, RBC deformability, RBC microparticles, and phosphatidylserine concentrations. RBC and plasma nitrite levels were measured as indicators of nitric oxide metabolism.

Results: Aerobic performance, peak oxygen consumption, ventilatory thresholds, velocity at the aerobic–anaerobic threshold, and lactate at exhaustion improved after training. The relative amount of both young RBCs and old RBCs increased, and the amount of the main RBC fraction decreased. Phosphatidylserine externalization and RBC-derived microparticles decreased. Overall deformability expressed as shear stress required to achieve half-maximum deformation to theoretical maximal elongation index at infinite shear stress improved in unfractioned RBCs ($p < 0.001$). Nitrite decreased in total ($p = 0.001$), young ($p < 0.001$), main ($p < 0.001$), and old ($p = 0.020$) aged RBCs and in plasma ($p = 0.002$), but not in very old RBCs.

Conclusion: These results indicate that non-endurance-trained healthy participants benefit from a regular moderate running training program because performance-related parameters improve and a younger RBC population with improved RBC properties is induced, which might support oxygen supply in the microcirculation.

Keywords: Cellular adaptation; Hemorheology; Nitric oxide; Performance; RBC physiology and aging

1. Introduction

The major function of red blood cells (RBCs) relates to facilitating the exchange of oxygen and carbon dioxide with the surrounding tissue in the microcirculation.¹ Indeed, RBCs need to deform to flow through small capillaries, which sometimes have a smaller diameter than the RBCs themselves.¹ The average life span of RBCs is about 120 days but may vary with health condition, hormonal status, or physical activity

(PA) level. Approximately 200 billion RBCs are newly formed each day, meaning that RBCs of different ages are present in the circulation.²

It has been shown previously that RBC deformability decreases with RBC aging.^{3,4} Because RBCs lack a nucleus, their capacity of antioxidative defense and damage removal is limited. To maintain their functionality, RBCs can remove oxidized and damaged proteins by membrane shedding and vesiculation.⁵ Because the concomitant loss of enclosed water is proportionally greater than the loss of proteins, RBC volume decreases and RBC density increases with aging.^{5,6} In addition to the removal of nonfunctioning cell components,

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microparticles (MPs) derived from RBCs can still exhibit functional proteins and are assumed to play a role in cell-to-cell communication and in the induction of adaptations through internal and external stimulations, such as exercise.⁷

PA is one factor that can modify the age distribution of the RBC population, and it has been shown that endurance athletes possess a greater number of more flexible and low-density young RBCs.⁸ Increased RBC deformability is suspected to play a crucial role for athletic performance.⁹ RBC deformability depends on the surface-to-volume ratio, internal viscosity, and membrane elasticity, which can be affected by other factors such as by dehydration, cytoskeletal protein distribution, hemoglobin concentration, or RBC aging.^{10,11} It has been demonstrated that nitric oxide (NO) synthesized by a RBC-NO synthase may S-nitrosylate α - and β -spectrins of the RBC cytoskeleton, which ultimately leads to an improvement of membrane flexibility and thus whole RBC deformability.¹² Therefore, improvement of RBC and plasma NO bioavailability has been proposed to play a major role in physical performance because it enhances vasodilatation and RBC deformability, which leads to a more efficient oxygen supply in the microcirculation.

The aim of this study was to investigate the effects of a 6-week endurance training program on the RBCs aging distribution and their deformability. Previous studies suggest that RBC deformability is positively influenced by endurance exercise, primarily by increased formation of new RBCs with higher deformability,^{4,13} and that aerobic exercise leads to adaptations in the RBC aging proportion in patients with cardiovascular diseases.¹⁴ Because these studies showed improvements in aerobic performance, formation of a younger RBC population, and improved deformability, we aimed to examine these effects in a healthy population. We hypothesized that even a comparable short, 6-week training intervention would lead to fast adaptations in the RBC population, which in turn would lead to an increased numbers of young RBCs and an overall rejuvenation of the entire RBC population.

2. Methods

2.1. Ethics

The protocols used in this study were approved by the Ethics Committee of the German Sport University Cologne. These protocols align with the Declaration of Helsinki, and all participants gave written informed consent to participate in this study.

2.2. Participants

A total of 31 participants (17 females, 14 males) were included in this study. All participants were healthy, moderately fit, and nonsmokers. Further inclusion criteria were: age 18–32 years; body mass index 18–26 kg/m², central European, no severe cardiovascular diseases and <4 h of endurance training per week. Baseline anthropometric data were as follows: age = 23.9 ± 3.3 years, height = 174.2 ± 8.8 cm, and weight = 68.0 ± 11.5 kg (mean ± SD).

2.3. Performance diagnostics

To determine the endurance capacity of the participants, an exercise test was performed on a treadmill using a previously used incremental stepwise protocol.¹⁵ Lactate concentrations were measured using EBIOplus (EKF Diagnostic Sales GmbH, Magdeburg, Germany). Running speed at the 4 mmol/L lactate threshold was calculated via a calculation matrix.¹⁵ Peak oxygen consumption (VO_{2peak}) was measured using a ZAN 300 spirometer (ZAN Austria E.U., Winkling, Austria) during the test. Heart rate (HR_{max}) was measured using a heart rate monitor (Polar Electro GmbH, Büttelborn, Germany). The individual maximal heart rate (HR) was determined as the highest value measured during the exercise test. The VO_{2peak} was determined as the average of the highest values measured over the last 30 s. The following classical criteria were used to check that all participants reached their VO_{2peak}: (1) plateau of VO₂ achieved, (2) HR_{max} achieved (220 – age (±10%)), (3) inability to maintain the effort, and (4) VCO₂/O₂ ratio of >1:1. Performance diagnostics were conducted before and after the training. First ventilatory threshold (VT1) and second ventilatory threshold (VT2) were determined based on the protocol of Beaver et al.¹⁶ and Schneider et al.¹⁷ and were expressed as a percentage of VO_{2peak}. Time to exhaustion (TTE) was determined as the time from the start of the exercise test until the stoppage of the exercise test (when participants were completely exhausted).

2.4. Training protocol

Participants were not endurance trained but practiced combat sports, horse riding, court sports, and recreational activities such as yoga and surfing. In addition to their normal activities and training, participants were asked to run for 1 h 3 times a week at 70% of HR_{max} for 6 weeks. Total training volume of the participants thus ranged 3–10 h per week, depending on their other recreational activities (3–5 days per week). A 1-day rest period between the endurance training sessions was predefined for regeneration and adaptation purposes. To ensure that participants followed training intensity protocols, HR monitors (Polar Electro GmbH) were provided. All participants completed the training protocol over the training period with high compliance, which covered all exercise activities, including the type of sport and its duration, as well as subjective exertion (Borg scale) to control and monitor all training and recreational exercise activities. Additionally, the training protocol enabled participants to control their individual progress at the predefined heart rate and running time. Exercise sessions were performed individually or in training groups having similar endurance capacity. In addition, a professional trainer was provided, who offered associated training such as training advice or individual training.

2.5. Blood sampling and experimental protocol

2.5.1. Blood sample collection

Venous blood samples were taken before and after training. Blood was anticoagulated using either sodium heparin or Na₃-citrate (BD Vacutainer; Becton Dickinson, Franklin Lakes,

NJ, USA). Pretraining samples were taken within 1 week before training. Post-training samples were taken within 1 week after training. Blood removal was performed so that every participant had the same time interval between the pre-training and post-training blood removal. RBCs were separated according to age using a discontinuous Percoll density centrifugation (Percoll; Amersham Biosciences, Uppsala, Sweden) according to the protocol of Bizjak et al.¹⁸ Briefly, heparinized blood samples were centrifuged at 3500 *g* for 1 min and the buffy coat was removed.

2.5.2. RBC fractionation

Plasma supernatant was collected into 1.5 mL microcentrifuge tubes (Eppendorf GmbH, Wesseling-Berzdorf, Germany) for plasma nitrite analysis and stored at -80°C until measurement. RBCs were isolated, washed with a 9-fold volume of isotonic buffer originating from an established protocol by Bosch et al.⁵ (GASP-buffer containing albumin and glucose: 9 mmol/L Na_2HPO_4 , 1.3 mmol/L NaH_2PO_4 , 140 mmol/L NaCl, 5.5 mmol/L glucose, and 0.8 g/L BSA pH 7.4), and centrifuged (3500 *g*, 1 min, at 4°C). The supernatant was removed, reference samples of unfractionated RBCs were taken (total RBCs), and the RBC pellet was resuspended 1:1 in SAH-buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing BSA: 26.3 g/L BSA, 132 mmol/L NaCl, and 4.6 mmol/L KCl, and 10 mmol/L HEPES; pH = 7.1). A total of 600 μL of washed and resuspended RBCs were cautiously applied on 5 Percoll layers diluted in SAH buffer. Percoll layers were built up as previously described by Bosch et al.,⁵ and the density of Percoll solutions (Amersham Biosciences) was adapted to yield the optimal fractionation success. Briefly, 2 mL of the respective Percoll solutions with an increasing density of 1.064 g/mL, 1.066 g/mL, 1.068 g/mL, 1.072 g/mL, and 1.076 g/mL were layered manually, beginning with the densest solution. Layers were built cautiously and dropwise to minimize mingling of the different dense solutions. Centrifugation was performed at 3700 *g* for 25 min at 4°C , with the lowest possible slowing-down process of the centrifuge used to minimize RBCs clumping or mingling. After centrifugation, 4 different RBC fractions assembled in the respective Percoll phases and were obtained by careful collection of each fraction. Obtained fractions were classed into young (≤ 1.064 g/mL), main (1.065–1.071 g/mL), old (1.072–1.076 g/mL), and very old (> 1.076 g/mL) RBCs. The volume percentage of each fraction was determined, and isolated RBCs were subsequently used for the following analyses.

2.5.3. Aging-related parameters

Hemograms were performed to determine the mean cellular volume (MCV) in whole blood after blood removal as well as in the subpopulations after fractionation using the hematology analyzer Sysmex Digitana KX-21N (Sysmex, Horgen, Switzerland).

Concentrations of externalized phosphatidylserine (PS) were determined with an Annexin V enzyme-linked immunosorbent assay (IBL International, Hamburg, Germany) for the

main fraction and the very old fraction according to the manufacturer's kit manual.

2.5.4. RBC deformability

RBC deformability was measured using the Laser-Assisted Optical Rotational Cell Analyzer (LORCA, RR Mechatronics, Hoorn, the Netherlands) as described in detail elsewhere.^{18,19} Briefly, RBCs were mixed with a viscous polyvinylpyrrolidone solution (RR Mechatronics, Hoorn, the Netherlands; viscosity of 30 cP; 1:250 ratio), and deformability was measured at 37°C at 9 increasing shear stresses (0.3–50.0 Pa). An elongation index (EI) was calculated by the LORCA software (RR Mechatronics) from the vertical and horizontal radius of the ellipsoidal diffraction pattern. The theoretical maximal EI at infinite shear stress (EI_{max}) and the shear stress that is required to achieve half-maximum deformation ($\text{SS}^{1/2}$) were calculated using a nonlinear regression according to Baskurt et al.²⁰ Any increase in EI_{max} and/or decrease in $\text{SS}^{1/2}$ indicates increased RBC deformability.²⁰ The $\text{SS}^{1/2}$ to EI_{max} ratio, which is a more robust approach in reflecting alterations in deformability,¹⁹ was calculated to complete deformability outcomes. A low ratio represents high deformability.¹⁹

2.5.5. Nitrite analysis

Measurement and analysis of nitrite were performed with a chemiluminescence detector (CLD 88e; EcoPhysics, Duernten, Switzerland) and the Chart FIA software (EcoPhysics) described in detail elsewhere.^{14,18} For RBC nitrite measurement, total RBCs and all subfractions were immediately mixed with preservation solution (800 mmol/L $\text{K}_3[\text{Fe}(\text{CN})_6]$, 100 mmol/L N-ethylmaleimide, 10 V-% Nonidet P-40, 90 V-% aqua dest.) in a 5:1 ratio, snap frozen, and stored at -80°C until measurement. Measurement of nitrite content was performed according to Hendgen-Cotta et al.²¹ Briefly, for measurement of RBC nitrite content, samples were mixed with methanol in a 1:2 ratio for protein precipitation and centrifuged at 21,000 *g* for 10 min at 4°C . Clear supernatant was separated from the pellet and collected in 1.5-mL microcentrifuge tubes (Eppendorf GmbH). The concentration of RBC nitrite was determined by injecting triplicates of 100 μL of each supernatant into an acidified tri-iodide solution that reduced nitrite to NO gas. The resulting NO was purged by a NaOH trap and transported into an ozone-based chemiluminescence NO detector via a constant helium gas-stream. Measured NO was analyzed with the Chart FIA software (EcoPhysics), and a calibration curve derived from an aqueous solution with a known nitrite solution was used to calculate the RBC nitrite concentration. Plasma nitrite samples were directly injected without further processing and analyzed as described. RBC nitrite concentration of the sample was corrected for nitrite levels of methanol and preservation solution.

2.5.6. RBC MP analysis

For RBC MP isolation, anticoagulated Na_3 -citrate blood was prepared according to the method of Jansen et al.²² and MP concentration was measured via flow cytometry as described in

detail elsewhere.²³ Briefly, after purification using sequential centrifugations, MPs were incubated with fluorescein isothiocyanate–annexin V (Beckman Coulter, Brea, CA, USA) and phycoerythrin-conjugated anti-CD235a (glycophorin A, clone 11E4B-7-6, IgG1; Beckman Coulter) monoclonal antibodies to identify MPs derived from RBCs. As an isotypic control, immunoglobulin G1 (IgG1) was used (679.1Mc7; Beckman Coulter). The Megamix kit (size-calibrated fluorescent microspheres of 0.5 μm , 0.9 μm , and 3.0 μm (Biocytex, Marseille, France)) was used to obtain a reproducible gating for MPs, and Flow-Count™ fluorospheres (Beckman Coulter) were used to determine absolute concentration of MPs. Sample data were acquired on an FC500 MCL flow cytometer (Beckman Coulter) using the CXP acquisition software (Beckman Coulter). Both forward scatter and side scatter were set in a logarithmic gain and acquired at a low flow rate for 5 min for all samples.

2.6. Statistics

All data were tested for normality of distribution using the Shapiro-Wilk test. If data fulfilled the requirements for a normal distribution, a paired *t* test was conducted to reveal differences between pre- and post-testing. If requirements for a normal distribution were not met, Wilcoxon tests were conducted to reveal differences between pre- and post-testing. Statistical analyses were performed using the GraphPad Prism 5 software package (La Jolla, CA, USA). Differences were considered as significant with $p < 0.05$, unless otherwise marked. All data are expressed as mean \pm SD.

3. Results

3.1. Performance data

Endurance training significantly improved $\text{VO}_{2\text{peak}}$ ($p < 0.001$; Fig. 1A) and VT_1 ($p = 0.0046$; Fig. 1B). Changes in VT_2 (Fig. 1C) were not statistically significant. Peak lactate values at exhaustion significantly decreased after training compared with before ($p = 0.025$; Fig. 1D). TTE (Fig. 1E) was not significantly affected by the training. Running velocity at the 4 mmol/L lactate threshold significantly increased after training compared to before training ($p = 0.004$; Fig. 1F).

3.2. RBC fractions

The relative distribution of the 4 different RBC subfractions that were determined according to their densities revealed that the relative amount of young RBCs (< 1.064 g/mL) significantly increased after training from pretraining to post-training ($p = 0.002$; Fig. 2A). The relative amount of the main fraction (1.065–1.071 g/mL) significantly decreased ($p < 0.001$), and the relative amount of the old fraction (1.072–1.076 g/mL) significantly increased after training ($p = 0.021$). The relative amount of the very old fraction (> 1.076 g/mL) did not show any difference between the pre- and post-training groups ($p = 0.799$).

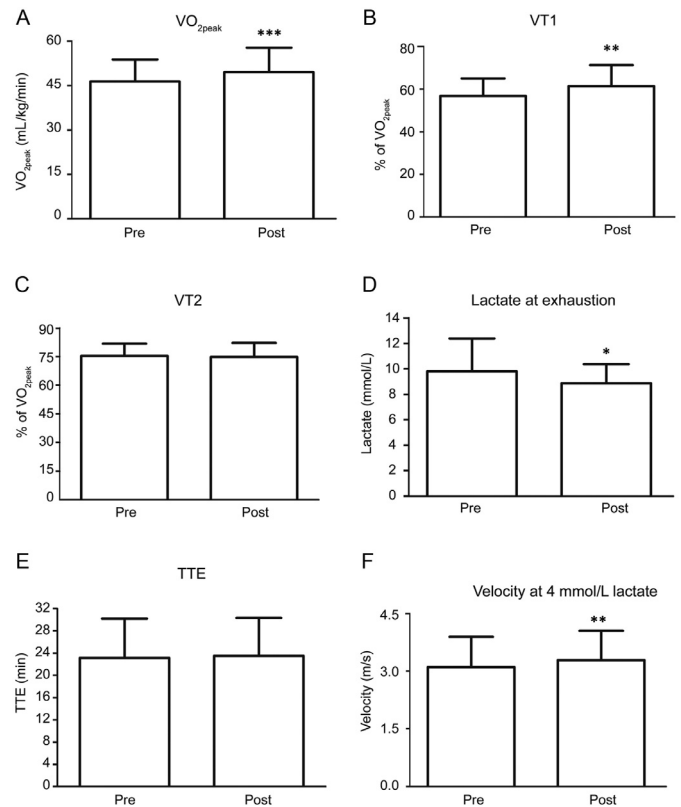


Fig. 1. Performance parameters before (Pre) and after (Post) a 6-week endurance training. (A) $\text{VO}_{2\text{peak}}$ increased significantly; (B) Percentage of VT_1 in relation to $\text{VO}_{2\text{peak}}$ increased significantly; (C) Percentage of VT_2 in relation to $\text{VO}_{2\text{peak}}$ remained unchanged; (D) Lactate concentrations at exhaustion decreased significantly; (E) Paired *t* tests revealed no change in running duration until exhaustion; (F) Participants could maintain higher velocities at the 4 mmol/L lactate threshold. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to pre-training. TTE = time to exhaustion; $\text{VO}_{2\text{peak}}$ = peak oxygen consumption; VT_1 = first ventilatory threshold; VT_2 = second ventilatory threshold.

3.3. RBC MPs, PS externalization, and MCV

MPs deriving from RBCs significantly decreased from before to after training ($p = 0.044$; Fig. 2B). Annexin V concentrations as a 1:1 determinant for PS externalization decreased in the main RBC population ($p = 0.028$) and in the very old RBC fraction ($p = 0.022$) from pretraining to post-training (Fig. 2C). The MCV was highest in the young fraction and lowest in the very old fraction, whereas MCV in the old fraction increased after training ($p = 0.035$; Fig. 2D). The MCV of the total RBCs did not change after training.

3.4. RBC deformability

The EI_{max} of the total RBCs decreased after training compared with pretraining values ($p = 0.039$; Fig. 3A). The $\text{SS}^{1/2}$ of the total RBCs significantly decreased after training ($p < 0.001$), as did the $\text{SS}^{1/2}$ to EI_{max} ratio ($p < 0.001$), indicating greater RBC deformability. The deformability of subfractions did not show any significant difference between the pretraining and post-training groups (data not shown).

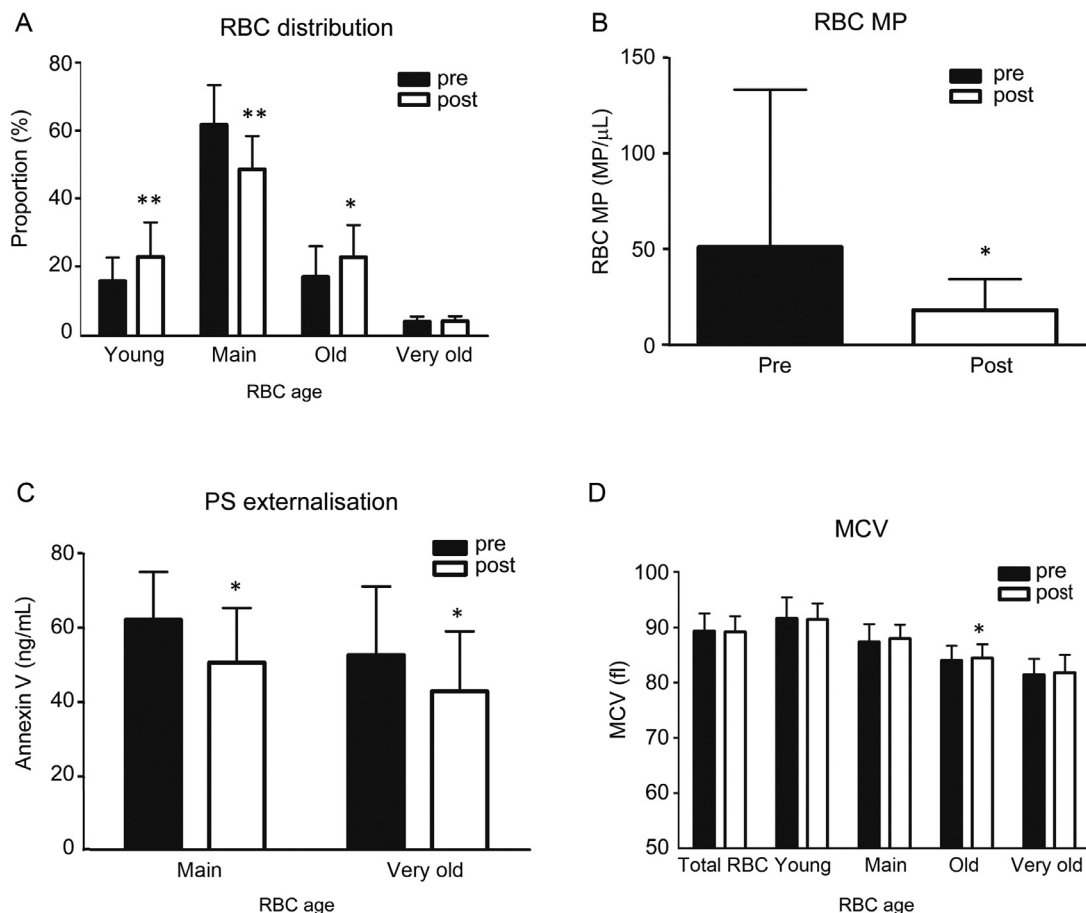


Fig. 2. RBC aging parameters before (Pre) and after (Post) a 6-week endurance training. (A) For RBC subfraction distribution, statistical analyses revealed that training resulted in a significant increase in the young and old fractions with a concomitant decrease of the main fraction. The very old fraction was not affected by the training; (B) Microparticles derived from RBCs decreased after training compared with Pre; (C) PS externalization represented by Annexin V concentration decreased in the main fraction as well as in the very old fraction; (D) MCV of the old fraction significantly increased. **p* < 0.05, ***p* < 0.01, compared to pretraining. MCV = mean cellular volume; MP = microparticle; PS = phosphatidylserine; RBC = red blood cell.

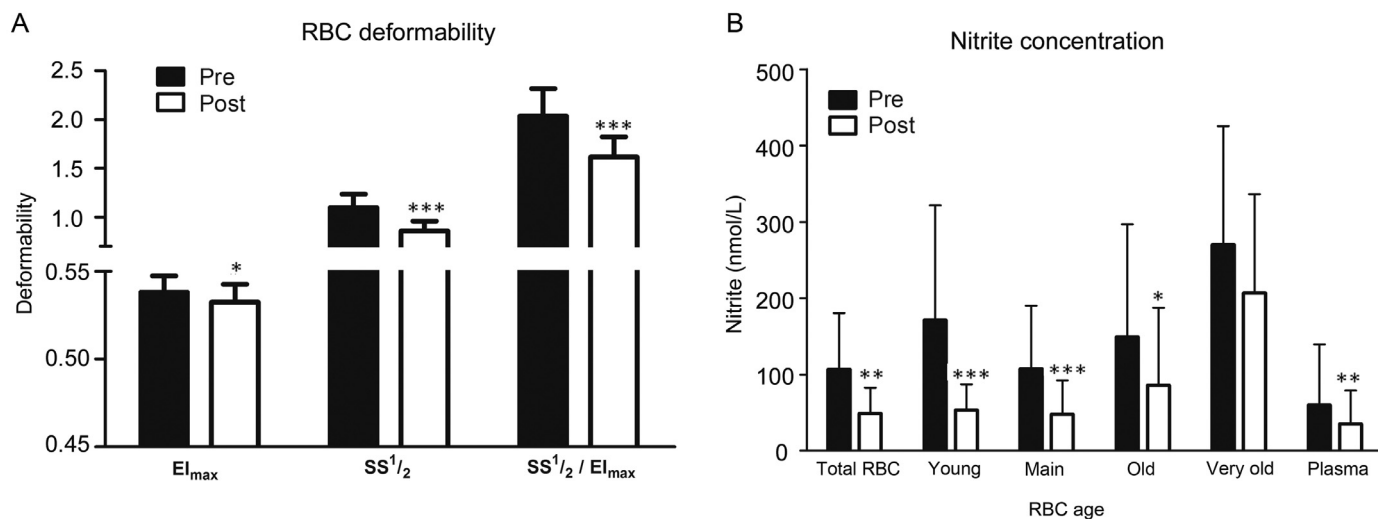


Fig. 3. RBC deformability and nitrite concentrations of subfractions before (Pre) and after (Post) a 6-week endurance training. (A) For the EI_{max} , statistical analyses revealed a decrease in total RBCs. The shear stress required to achieve $SS^{1/2}$ was markedly decreased in total RBCs. The calculated $SS^{1/2}$ to EI_{max} ratio decreased significantly between pre- and post-training; (B) Nitrite decreased in post-training in total RBCs and in all RBC samples except for the very old fraction and for the plasma nitrite. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with pretraining. EI_{max} = theoretical maximal elongation index at infinite shear stress; RBC = red blood cell; $SS^{1/2}$ = half-maximum deformation.

3.5. NO metabolism

In total RBCs, the nitrite concentration significantly decreased from pre- to post-training data ($p = 0.001$; Fig. 3B). Nitrite concentrations decreased in young RBCs ($p < 0.001$), main RBC fraction ($p < 0.001$), and old fraction ($p = 0.020$) in the post-training group. Nitrite levels remained unaltered in the very old RBC fraction ($p = 0.065$). Fig. 3B shows that plasma nitrite decreased from pretraining to post-training data ($p = 0.002$).

4. Discussion

This study sought to evaluate the effects of a 6-week running training program on RBC aging and distribution, and on RBC deformability in young, moderately fit, and moderately healthy untrained participants. RBC deformability is usually thought to be of major importance for athletic performance in providing sufficient oxygen to the working muscle because RBCs have to pass through capillaries with diameters smaller than their own.^{1,9} In contrast with resting and nonexercising healthy individuals, whose blood profiles and aging parameters do not change significantly over time,²⁴ trained individuals usually exhibit increased RBC deformability,^{8,15} increased RBC turnover (with a decreased RBC life span), and a concomitantly greater proportion of young RBCs.^{25,26}

However, the interdependence of possible training-induced adaptations on the RBC population and hemorheological properties has not been examined thus far. The present study shows for the first time that a 6-week endurance training program involving submaximal running leads to a significant increase in the number of young RBCs, improved aerobic performance, and improved overall RBC deformability in individuals who are not endurance trained. The 6-week training period resulted in increased aerobic endurance performance with respect to VO_{2peak} , VT1, and velocity measured at the 4 mmol/L lactate threshold. Furthermore, lactate concentration measured at exhaustion decreased. These results were very much expected, because the positive effects of regular aerobic endurance training are well-documented.²⁴

Regular endurance exercise leads to an upregulation of catecholamines, cortisol, growth hormones, and insulin-like factors that stimulate bone marrow activity and increase erythropoiesis.¹ Erythropoiesis leads to a greater number of reticulocytes, which in turn increases the number of young RBCs.²⁴ This adaptation in response to the endurance training could be observed with an increased proportion of the young as well as the old RBC subpopulation. Interestingly, the relative proportion of the main RBC fraction significantly decreased concomitantly after the training in the present study, which was also observed in a previous training study with participants who had type 2 diabetes.¹⁴ A possible explanation might be related to the fact that regular endurance training accelerates RBC aging; thus, the formation of both young and old RBCs is favored by an overall increased RBC turnover. Mairbäurl et al.⁴ showed that trained individuals have more young, low-density cells with high 2,3-diphosphoglycerate concentrations and fewer old, high-density RBCs with

impaired functionality. A greater proportion of young RBCs might contribute to improved oxygen transport and release in the microcirculation. These observations were recently confirmed by Tomschi et al.,⁹ whose study tested highly trained, moderately trained, and low-trained individuals in relation to their RBC age distribution. Highly trained individuals exhibited more young RBCs and overall improved hemorheological properties. Thus, the relatively large number of RBCs in the main fraction that was measured before training in the present study might have matured faster in response to the regular endurance exercise. This finding might explain the significantly increased number of old RBCs. Weight et al.²⁵ observed an increased RBC turnover and reduced RBC life span (65–75 days) in highly active runners, indicating an overall rejuvenated RBC population in endurance-trained athletes.²⁷ Therefore, although a nonexercising control was not integrated into the design of the current study, regular endurance training seems to accelerate the selective elimination of rigid and old RBCs via the reticuloendothelial macrophage system.^{14,27}

Adaptations of RBCs could be observed already after 6 weeks of training in the present study. RBC-derived MPs, whose formation can, among other things, be induced by high shear stress,²⁸ decreased after training, as did phosphatidylserine externalization, which acts as a removal signal for the reticuloendothelial system and RBC degradation.²⁹ Both reduced RBC-derived MPs and decreased phosphatidylserine externalization indicate reduced RBC senescence and later removal with chronic endurance exercise. These findings suggest that RBC adaptations during exercise possibly lead to less frequent membrane shedding, which could reduce circulating MP.^{7,30} Regular endurance training could, therefore, improve the MP profile, as was assumed in a previous cross-sectional study.³¹

Increased formation of young RBCs and the faster removal of high-density old RBCs likely strengthens the entire RBC system, because old RBCs show increased membrane exocytosis to discard oxidative waste products.^{32,33} This process in turn leads to a loss of cytosolic components, decreased MCV, decreased RBC deformability, and overall weakened RBC function.³³ The successful separation with intact RBCs was indicated by MCV levels of total RBCs being in the normal range. The MCV decreased from the young to very old RBCs, thus confirming previous findings.¹⁸ The MCV increased in the old fraction after training. It was speculated that this increase in RBC volume could be favorable^{14,18} because of the concomitant loss of volume during the aging process. A cell with an increased MCV might be capable of removing an increased amount of damaged and oxidized protein through an increased capacity of vesiculation. However, this hypothesis remains to be tested.

Total RBCs showed higher deformability values after training. Smith et al.⁸ found increased RBC deformability in world-class endurance athletes and ascribed this finding to the increased RBC turnover with a higher proportion of younger RBCs. The EI_{max} describes the theoretical maximum deformability at infinite shear stress.³⁴ The EI_{max} is generally higher in endurance-trained athletes,⁸ but can temporarily decrease

with unusually high training loads.³⁵ In this study, the EI_{max} values of total RBCs decreased after the training, indicating impaired maximum deformability, which is in accordance with previous studies.¹¹ Increased lactic acid production and/or enhanced oxidative stress during training sessions could have contributed to the decrease in the EI_{max} .^{11,36} It is known that longer endurance training is needed to improve erythrocyte metabolism.³⁶ Previous observations showed that a 12-week moderately intense treadmill walking program increased overall deformability.³⁷ However, although our training program decreased the EI_{max} , we also observed a decrease in the $SS^{1/2}$, which indicated an overall improved deformability. This apparently inconsistent finding could be explained by the faster RBC aging process that was induced by training. Both results might point to more young RBCs in the circulation, which are more flexible (reduced $SS^{1/2}$), but might show overall reduced EI_{max} because of an altered surface-to-volume ratio. The increase of the young fraction lowered overall EI_{max} , as was observed in previous studies, which showed that young RBCs exhibit a lower EI_{max} compared with the main fraction owing to high cellular volume and ongoing membrane remodelling.^{14,18} Therefore, an $SS^{1/2}$ to EI_{max} ratio was calculated, which is a more robust approach in reflecting alterations of deformability and covers 2 of the important parameters of RBC rheological properties.¹⁹ The markedly reduced ratio, indicating improved deformability, supports the assumption of training-induced positive effects on the hemorheological system.

A crucial parameter for increasing endothelial and hemorheological functionality is the main vasodilator NO.^{12,38} Nitrite represents the primary oxidation product of NO³⁹ and, thus, functions as a valid marker of NO synthesis. In the present study, NO concentrations significantly decreased in plasma, total RBCs, and the young, main, and old subfractions. Multivarious reaction pathways of NO exist in the RBCs, in which NO can be oxidized to form nitrite and finally nitrate³⁹ or can result in the formation of RxNOs, which includes S-nitrosothiols or N-nitrosamines.²¹ Because bioactive NO in the microcirculation could be produced by several cells,⁴⁰ the reason for the observed decrease in plasma nitrite can only be speculated. A decrease might result either by endothelial or RBC adaptations, but a specific interdependence with observed decreased RBC nitrite remains speculative. It was previously shown that especially very old and barely functioning RBCs exhibit high NO concentrations.¹⁸ It was suggested that the increased NO concentrations found in RBCs could counteract impaired RBC deformability, possibly by increasing NO-mediated modification of the cytoskeleton, which was shown to improve RBC deformability and mechanical properties.¹² The findings of reduced nitrite and improved deformability might, therefore, be explained by 2 hypotheses: (1) the hemorheological adaptations caused by the endurance training are independent of NO adaptations in the RBC metabolism, or (2) less NO is needed to improve deformability owing to an improvement of antioxidative defenses with training and overall better RBC health, which might be caused by a rejuvenated and younger RBC population. To address these questions,

further studies are needed to solve the possible interactions and elucidate the complex *in vivo* situation.

Different techniques exist to separate RBCs according to age, which exhibit different advantages and disadvantages. The use of Percoll density centrifugation as a separation medium is one established method to separate RBCs, but continuous or discontinuous gradient, as well as low-speed,¹⁸ high-speed,²⁹ or counterflow centrifugation,⁵ are applied. Therefore, an established and widely accepted protocol is missing. This factor limits the transferability of the present study to other RBC aging studies. In addition, the only reliable method of measuring the exact RBC aging is *in vivo* labelling and calculation of the biological half-life of RBCs, whereas the separation in this study only yielded “young to very old” RBCs, which could disguise further RBC aging properties after training. This study used buffer systems established by Bosch et al.,⁵ which led to RBCs with different buoyant densities, normally expected to be around 1.07–1.11 g/mL.⁴ Therefore, although blood profile and microscopic approaches were used to determine cell integrity, an impact of these buffers on *in vitro* RBCs shrinking or swelling cannot fully be excluded. In addition, although previous studies did not show any aging effects in the untrained populations,^{4,18,24} a nonexercising control group might be integrated in prospective studies to determine the differences in RBC aging alterations between training and resting individuals.

5. Conclusion

It was previously shown that highly endurance-trained athletes have a younger RBC population with improved deformability and oxygen transport/release properties compared to the average population.^{3,9} In endurance-trained athletes, younger RBC populations with a lower mean age are induced by enhanced shear stress in the cardiovascular system and by increased oxidative stress, which limit the average RBC aging and increase RBC turnover.²⁴ Therefore, faster RBC aging seems like a promising way for the blood system to adapt faster to changed exercise activity by increasing old RBC removal and continuously increasing the number of younger RBCs with improved properties. However, the time needed for erythropoietic adaptations of RBCs, the impact of the exercise intensity or form of endurance sport, and the influence of a younger RBC population on athletic performance are still under debate. The present study found that a 6-week, moderately intensive endurance training program leads to improved aerobic capacity in young, moderately fit participants who had not previously had endurance training. The benefits of the program included a generally younger RBC population and increased overall RBC deformability. Therefore, the present study supports the idea that blood rheology and the distribution of the RBC population can be rapidly influenced by endurance training, but their interdependence with increased aerobic capacities remains to be investigated. It should be noted that, although the present study supports beneficial RBC adaptations through moderate endurance running, research involving prolonged training periods, different training intensities (e.g.,

high-intensity marathon running), and alternative exercises with weight-bearing activities (e.g., floor exercises in gymnastics) that may impact increased foot hemolysis are needed to give more insights into the long-term adaptation processes.

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Authors' contribution

DAB and FT designed the study, performed the experiments, data collection, and data analysis, and drafted the manuscript; GB performed the experiments and helped to coordinate the training; EN and MR performed MP experiments and analysis; PC performed MP analysis and helped to draft the manuscript; WB helped to draft and to edit the manuscript; MG designed the study and helped to draft the manuscript. All authors read and approved the final version of the manuscript, and agreed with the order of presentation of the authors.

Competing interest

The authors declare that they have no competing interests.

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