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Original article

# Spinning, oxidative damage and hemolysis in athletes

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ABSTRACT

*Background:* The aim of this study is to put into evidence and verify the correlation between hemolysis and oxidative damage in subjects who practice spinning.
*Methods:* A total of 12 volunteers, aged 35 ± 5 years were enrolled in the present study (60 min) before and (60 min) after practicing spinning. The measurement of lipid peroxidation products and the hemolysis assays conducted on blood samples of athletic subjects, and also the red blood cell morphological study.
*Results:* The obtained data evidence that there are significant differences at least 60 min after training: in malonyldialdehyde value (0.12 ± 0.05 nmol/ml) and in malonyldialdehyde value with 2,2′-azobis (2-amidinopropane) dihydrochloride (0.22 ± 0.05 nmol/ml), in hemolysis data up to both 80 min (oxidative lysis) and 120 min (2,2′ azobis (2-amidinopropane) dihydrochloride lysis) confirmed by microscopical analysis.
*Conclusion:* The described data on red blood cell hemolysis, after exercise-induced oxidative damage (malonyldialdehyde), and the degradation kinetic under action of 2,2′-azobis (2-amidinopropane) dihydrochloride can be described according to a multistage process with multiple contemporaneous equilibria. The final red blood cell echinocytic form could describe the end product of process. Consequently authors hypothesize a relationship between spinning exercise, plasma membrane oxidation and hemolysis susceptibility.



## 1. Introduction

Physical exercise was used as a model of the physiological parameter modulating the free radical production to examine the effects of exercise-induced oxidative modifications on the physico-biochemical properties of erythrocyte membrane.[1]

Intra-vascular hemolysis is one of the most emphasized mechanisms for degradation of erythrocytes during and after physical activity. Exercise-induced oxidative stress has been proposed among the different factors explaining exercise-induced hemolysis.[2] Since it has been suggested that lipid peroxidation (Malondialdehyde (MDA)) following free radical overproduction may be one of the causes of physical exercise-induced oxidative damage and hemolysis in athletes.[3]

The rate of lipid peroxidation, the antiradical defense in red blood cells, the levels of cholesterol and phospholipids, and resistance of erythrocytes to hemolysis were studied in cyclic and non-cyclic sportsmen. It was found that intensity of lipoperoxidation, anti-radical protection in erythrocytes and stability of erythrocytes to hemolytic depended on adaptation to the amount and character of regular muscular activities. The findings allow the above parameters to be recommended in the comprehensive evaluation of the functional status of sportsmen.[4]

Human RBCs (Red Blood Cell) were incubated in air at 37 °C as a 10% suspension in buffered saline solution, they were stable and little hemolysis was observed in 5 h (3.1 ± 0.9% hemolysis). When a water-soluble radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (final concentration 50 mM), was added to the RBC suspension, it induced hemolysis in a time-dependent manner. In this experimental condition, the onset of oxidative hemolysis was within 120–150 min. AAPH (50 mM) also induced lipid peroxidation in the RBC suspension reflected by the generation of MDA. The MDA content of resting RBCs was 1.4 ± 0.4 nmol/2 × $10^9$ RBCs. AAPH caused lipid peroxidation of RBCs in a time-dependent manner. The MDA content was increased to 4.8 ± 0.3 nmol/2 × $10^9$ RBCs 180 min after incubation with AAPH.[5]

The hemolysis curves for erythrocyte suspensions incubated with AAPH at different concentrations (12.5, 25, 50 mV) were shown. In absence of AAPH, human erythrocytes incubated in air at 37 °C as a 10% suspension in PBS (phosphate-buffer saline)

*Abbreviations:* MDA, malonyldialdehyde; RBC, red blood cell; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; PBS, phosphate-buffer saline; TBARS, thiobarbituric acid reactive substances.

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remained stable and little hemolysis was observed for 6 h (6.8 ± 0.5%), and the similar kinetic curve of hemolysis in AAPH-untreated erythrocytes occurred. When AAPH was added to the erythrocyte suspension, the hemolysis occurred quickly after an inhibition period and AAPH-induced hemolysis in a typical time- and concentration-dependent manner was evidenced. The curve also revealed sigmoidal kinetics, showing a lag phase which was progressively shorter with an increase in AAPH concentration. Intensive process of lipid peroxidation took place in the AAPH-mediated. The intensity of lipid peroxidation was assessed by measuring the formation of MDA. As shown the MDA levels in the erythrocytes in the control group were 2.5 ± 0.3, 3.1 ± 0.2, and 4.5 ± 0.6 nmol/mg Hb, respectively, at 2, 4 and 6 h. When the erythrocytes were incubated with FSH (water-soluble feruloyl oligosaccharides) alone, MDA formation was maintained at a background level similar to that in the AAPH-untreated samples (data not shown). The MDA content was significantly increased by 400%, 1397%, 1191%, respectively, at 2, 4 and 6 h after incubation with 25 mM AAPH when compared to respective control. AAPH caused lipid peroxidation of erythrocytes in a time-dependent manner.[6]

Authors investigated whether exercise related hemolysis is associated with alterations of RBC membrane. At high intensity exercise (cycling) "Triathlon" total protein concentration (TP) and hematocrit (HCT) increased ($p < 0.05$) whilst MDA [2.80 (2.65/3.20) vs 3.13 (2.78/3.31) nmol ml (-1)] increased ($p < 0.05$). Corresponding changes were found at low (non-running) intensity exercise.[7]

Supporting measurements of lipid peroxidation showed an increase in thiobarbituric acid reactive substances immediately after exercise ($p < 0.05$) and at 1 h of recovery ($p < 0.001$).[1]

Spinning is an aerobic/anaerobic group of stationary bicycle. The words "Spinning", "Spin" and "Spinner" are registered trademarks.[8] It was imported from the United States to Europe in 1995. Born in indoor preparation for road cycling (indoor cycling), it undergoes an evolution technique thanks to a personal trainer, Johnny Goldberg from Los Angeles, California, USA.[8] Spinning is practiced at various pedaling speed, usually with the aid of an appropriate background music that accompanies from moment to moment the pedaling speed. The lesson takes place in groups, with the guidance of an instructor, who riding as well, dictates the rhythm of pedaling. The Spinning program is implemented by instructors certified "Johnny G Spinning Instructor" – JGSI.[8]

At present in literature there is only one article on spinning which induces oxidative damage (as MDA). Immediately after spinning a significant increase in TBARS (thiobarbituric acid reactive substances) (from 16.5 ± 2 to 25 ± 2 nmol MDA/mL serum) was observed ($p < 0.05$) compared to both those at rest and control group (not regularly exercised).[9]

The aim of this study is to test the effect of spinning load on oxidative damage (AAPH-induced MDA), oxidative hemolysis, RBC morphology study and relationship between athletes before and after exercise and in control samples and in AAPH treated samples (evaluating MDA levels).

## 2. Materials and methods

### 2.1. Athletes

Twelve healthy and physically active volunteers: 7 males, 40 ± 5 years old, body weight 70 ± 5 kg and height 1.70 ± 0.07 mt, body mass index (BMI) 25 and 5 females, 40 ± 5 years old, weight 60 ± 5 kg body and height 1.60 ± 0.07 mt, body mass index (BMI) 21, trained subjects, not engaged in competitive sports, were enrolled and studied. The study was approved by the University of Calabria Ethics Committee and all participants provided written informed consent upon entry into the study. The changes in skin mechanical parameters 60 min after training, expressed as either increase or decrease of skin elevation during instrumental measurements, are described in subjects practicing spinning (60 min) before and (60 min) after physical exercise. The volunteers performed each spinning session listening to a compilation of music that lasted 50.5 min and was composed of 11 tracks. Each track corresponded to a specific phase of the session, the phases being labeled as warm-up, sitting, seated climbing, jumping and winning, based on the official spinning program manual.[8] In addition to the *music* protocol the participants were assessed to maintain a pedal stroke cadence that had previously been established for each track, training is performed in 1 h. The diet followed by all subjects is based on a Mediterranean-type power,[10] these data have been acquired through a specific questionnaire to learn about the eating habits of the same subjects. They were admitted to the study after personal and familiar anamnesis and medical check-up. The exclusion criteria were: acute or chronical infections, allergy, hypersensitivity or allergy to not steroidal anti-inflammatory drugs (FANS) or medicines. They were not using for 15 days any topical agent on the test areas and were free of pathological findings on their arms.

### 2.2. Assay for lipid peroxidation products

Measurement of malonyldialdehyde (MDA) is widely used as an indicator of lipid peroxidation.[11] MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts, including cross-linked products. The TBARS method is commonly used to measure MDA in biological samples.[12] The ALdetect (MDA – specific) Lipid Peroxidation Assay Kit [*AK-171*] (Enzo Life Sciences) method is designed to assay free MDA, the assay conditions are useful to minimize interference from other lipid peroxidation products, such as 4-hydroxyalkenals. The AK-171 Assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole (NMPI) with MDA at 45 °C. One molecule of MDA reacts with 2 molecules of NMPI to yield a stable carbocyanine dye. The AK-171 assay is specific for MDA because 4-hydroxyalkenals do not produce significant color at 586 nm under the conditions of the assay.[13] Blood samples were obtained from healthy human volunteers by venipuncture into heparinized tubes and centrifuged at 2000 g for 10 min a 4 °C. The plasma was removed and packed erythrocytes were washed three times with phosphate-buffer saline (PBS; 10 mM sodium phosphate, 135 mM NaCl, ph 7.4). The buffy coat of white cells was removed. The washed erythrocytes were suspended in PBS in a final hematocrit of 5%,[5] that is the same experimental protocol used for determination of lipid peroxidation in red blood cells with addition of the water-soluble exogenous starter 2,2′-azobis (2-amidinopropane) dihydrochloride (*AAPH*) (50 mM) (alternative Sigma–Aldrich®) a pro-oxidant useful to generate free radicals.

### 2.3. Assay system for hemolysis

For each measurement, $1 \times 10^6$ red blood cells were collected and washed three times with phosphate-buffer saline (PBS). In experimental protocol initially RBC were pre-incubated at 37 °C for 5 min in a water bath and successively two different series of microtest-tubes were performed: the first one treated with 20 volumes of distilled sterile $H_2O$ (I) and second one treated with the same volume of an azo compound solution AAPH (50 mM) diluted in PBS at pH 7.4 (II). Hemolysis assay was incubated up to different times (20 or 40 or 80 or 120 or 180 min). At the concentration of 50 mM, AAPH induces a hemolysis that occurs roughly after 2 h of exposure, and only for higher concentrations, hemolysis is observed earlier.[14] The extent of hemolysis was spectrophotometrically determined.[5] At specific intervals the reaction was taken out, diluted to 20 volumes with isotonic saline buffer and centrifuged (1000 g for

10 min). The percentage of hemolysis at different incubation intervals was determined by measuring the absorbance of the supernatant of the erythrocytes at 540 nm (II) and compared with that of complete hemolysis (I) according to literature[5] "Enzo Life Sciences".

### 2.4. RBC morphology study

The state of RBCs membrane were determined through a typical histological method defined May-Grunwald from Merck (Germany). This method is divided in several step, in fact initially 5 μl of RBC were put down on the slide and the cover-slide must stick to the blood drop with an angle of 45°, successively carried out a blood crawl. The slide for 5 min to dry; then the slide is fixed by addition of May-Grunwald die three times. Three 15 min staining cycles are made, each slide is washed with tap water at room temperature and dried. Finally the cover-slide is soldered with Canadian balsam. Red blood cells morphology was captured by images advanced 3.2 on Motic AE21 microscope and M580 camera.

### 2.5. Statistical analysis

Data are expressed as mean ± SD. The one-way analysis of variance ANOVA and Tukey's test for coupled data at each time were performed. At each time twelve observations were performed. Database managements and statistical analysis were performed using GraphPad Prism 6 (for Windows). Values were considered statistically significant for $p < 0.05$ level.

## 3. Results

In blood samples of enrolled subjects, we noticed a statistically significant increase in MDA value and in MDA-AAPH value at least 60 min after training. The MDA content of RBCs is 0.12 ± 0.05 $2 \times 10^9$ RBCs and the MDA-AAPH content of RBCs is 0.22 ± 0.05 nmol/ml $2 \times 10^9$ RBCs. We reported both results as shown in Fig. 1. Lipid peroxidation and consequently a major formation of TBARS are supported by these soluble radical initiators AAPH to confirm our analysis. Successfully on these samples, we examined if time incubation influences the MDA analysis. Several incubation times (0, 60, 90 min) were considered both in the presence and absence of AAPH. Results show that lipid peroxidation measurement significantly increases to 90 min in presence of AAPH respect to lipid peroxidation measurement of MDA only (Fig. 2). So AAPH caused and incremented lipid peroxidation of RBC

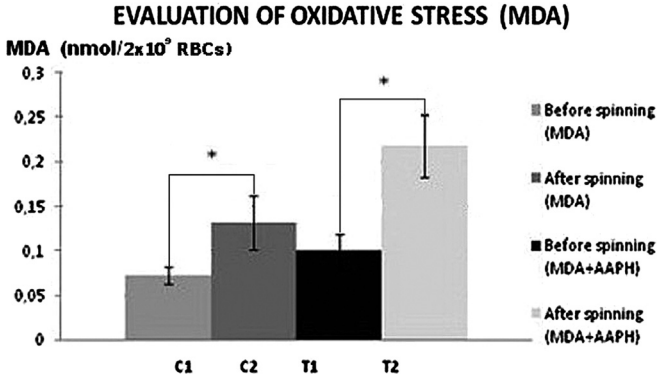



**Fig. 1.** Lipid peroxidation on the kinetic of human RBCs and effects of AAPH (50 mM) before and after exercise ($t = 120$ min). Each point is the mean ± S.E.M. of twelve independent determinations. Comparison with all references according to one-way ANOVA and Tukey post hoc test * ($p < 0.05$).

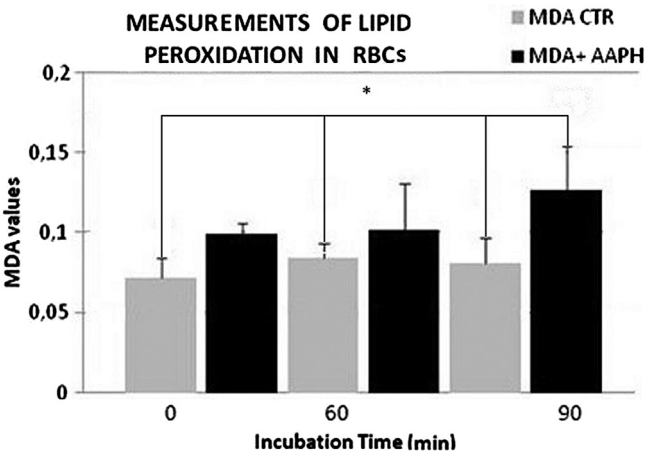



**Fig. 2.** Effect of AAPH (50 mM) on the kinetic of human RBCs lipid peroxidation at several times (0,60,90 min) of incubation. Each point is the mean ± S.E.M. of twelve independent determinations. Comparison with all references according to one-way ANOVA and Tukey post hoc test * ($p < 0.05$).

in spinning subject in a time-dependent manner according to literature data.[5] On the base of the described data about the effect of AAPH on MDA levels in RBC membranes we ascertained that the AAPH concentration 50 mM is useful to induce an accelerated hemolysis of RBC from spinning athlete blood. The effect of AAPH 50 mM on hemolysis of control RBC is preliminarily verified as described in Fig. 3.

The value of % hemolysis correlated to incubation's time of blood sample as referred in Fig. 4. Oxidative lysis in PBS isotonic buffer after oxidative damaging induced by standardized exercise of RBCs membrane occurs. The increase is statistically significant only at 80 min after spinning, as ascertained by one-way ANOVA and Tukey post hoc test for coupled data at each incubation time (Fig. 4A). Instead the results obtained by oxidative and AAPH treatment (50 mM final concentration) in samples from the same spinning athletes is significant at 120 min by comparison of RBC samples from before and after spinning athletes. The statistical analysis was performed by previously described method (Fig. 4B).

The oxidative stress is confirmed by RBC images compared in Fig. 5. They show marked modifications of erythrocyte membranes with an increase in the volume of red blood cells observed in hemolytic condition between before and after exercise especially at 120 min and 180.

## 4. Discussion

At present only A.M. Cardoso et al 2012 studied the effect of standardized spinning activity on MDA production in human blood of spinning athletes. Other kind of functional and structural damages of human RBC in athletes performing non-standardized physical exercises were not simultaneously studied from the viewpoints of spontaneous RBC lysis, AAPH–induced hemolysis and by morphological methods.

In the present research spinning activity monitoring was conducted three times a week and the recovery period of subjects was very short. So, in the sport activity of healthy subjects done before and after physical training, we observed significant differences in ROS productions and evaluated as nanomolar equivalents of MDA. Furthermore, the oxidative stress by radical water-soluble initiator (AAPH) confirms the structural modifications of RBCs as function of increased time. The MDA values obtained with AAPH (50 mM) treatment are in good accordance to oxidative stress of RBCs,[11] so

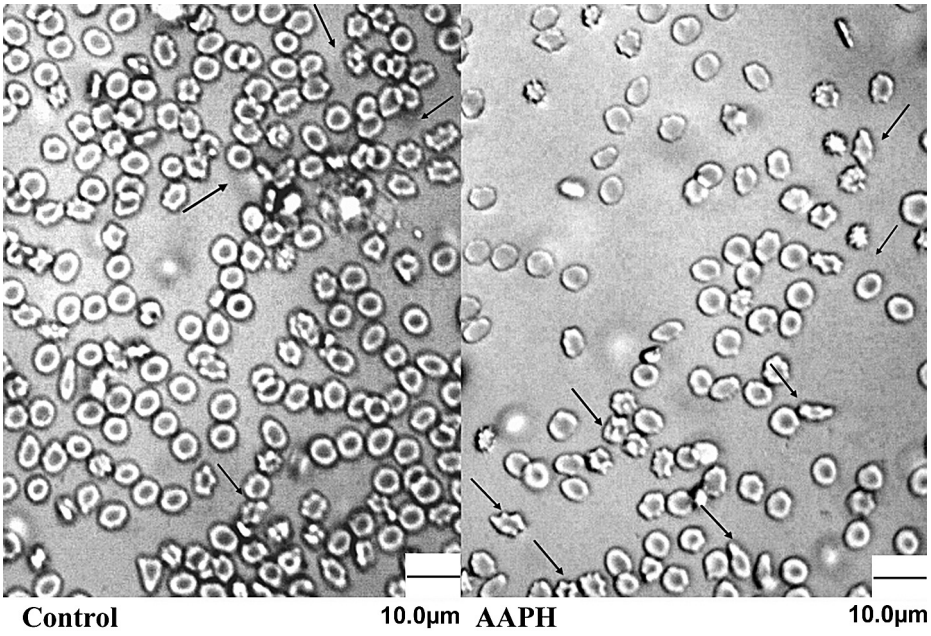



**Fig. 3.** Preliminary study of AAPH (50 mM) effect of control RBC on hemolysis at 80 min of incubation. The → symbol indicates the main groups of crenated RBCs. The — symbol shows the scale bar of micrographs.

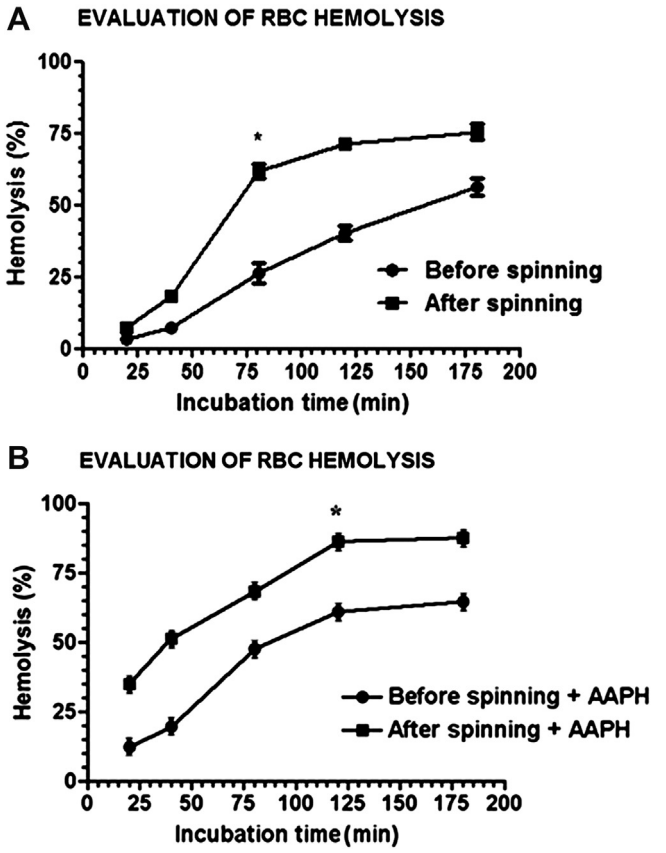
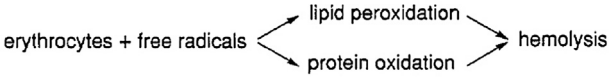


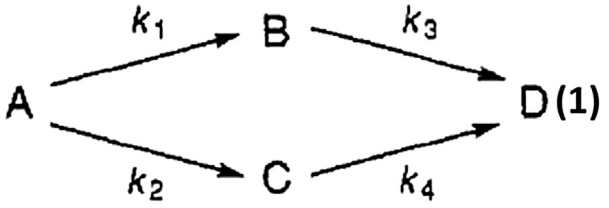

**Fig. 4.** Effect of AAPH − induced hemolysis in sportive human RBC at various times. Each point is the mean ± S.E.M. of twelve independent determinations. Comparison both at the same timepoint between after spinning vs before spinning (A) and after spinning + AAPH vs before spinning + AAPH (B). According to one-way ANOVA and Tukey post hoc test * ($p < 0.05$).

two types of analysis (one done with only AAPH before and after training and one done at several incubation times) evidence that sportsmen after training show a large increase of lipid peroxidation products in both categories. In assay system of hemolysis, at the different times of incubation, a large hemolysis percentage is present at 120 min also in the presence of AAPH that increased after physical exercise, as also confirmed by microscopical study (see Figs. 3 and 5).

Consideration of the participation both of lipid peroxidation and band 3 oxidation (protein) in erythrocytes membranes, a competitive model for the hemolysis reaction by free radicals can be illustrated as follows:




From the above-mentioned results, the binding of oxidized lipids to oxidized band 3 may occur. In the present study, however, the interaction between peroxidized lipids and oxidized band 3 is not taken into consideration for simplicity.




In this reaction, A, B, C, and D correspond to intact, swollen by lipid peroxidation, swollen by band 3 oxidation, and ruptured (hemolyzed) erythrocytes, respectively. $k_1$ and $k_2$ are rate constants for membrane swelling, and $k_3$ and $k_4$ are for membrane rupturing or forming of hemolytic holes. When [A], [B], [C], and [D] are substituted for the concentration of hemoglobin corresponding to each state, the integrated rate equation for [D] is as follows, from the boundary conditions $[A] = [A]_0$ and $[B] = [C] = [D] = 0$ at $t = 0$:

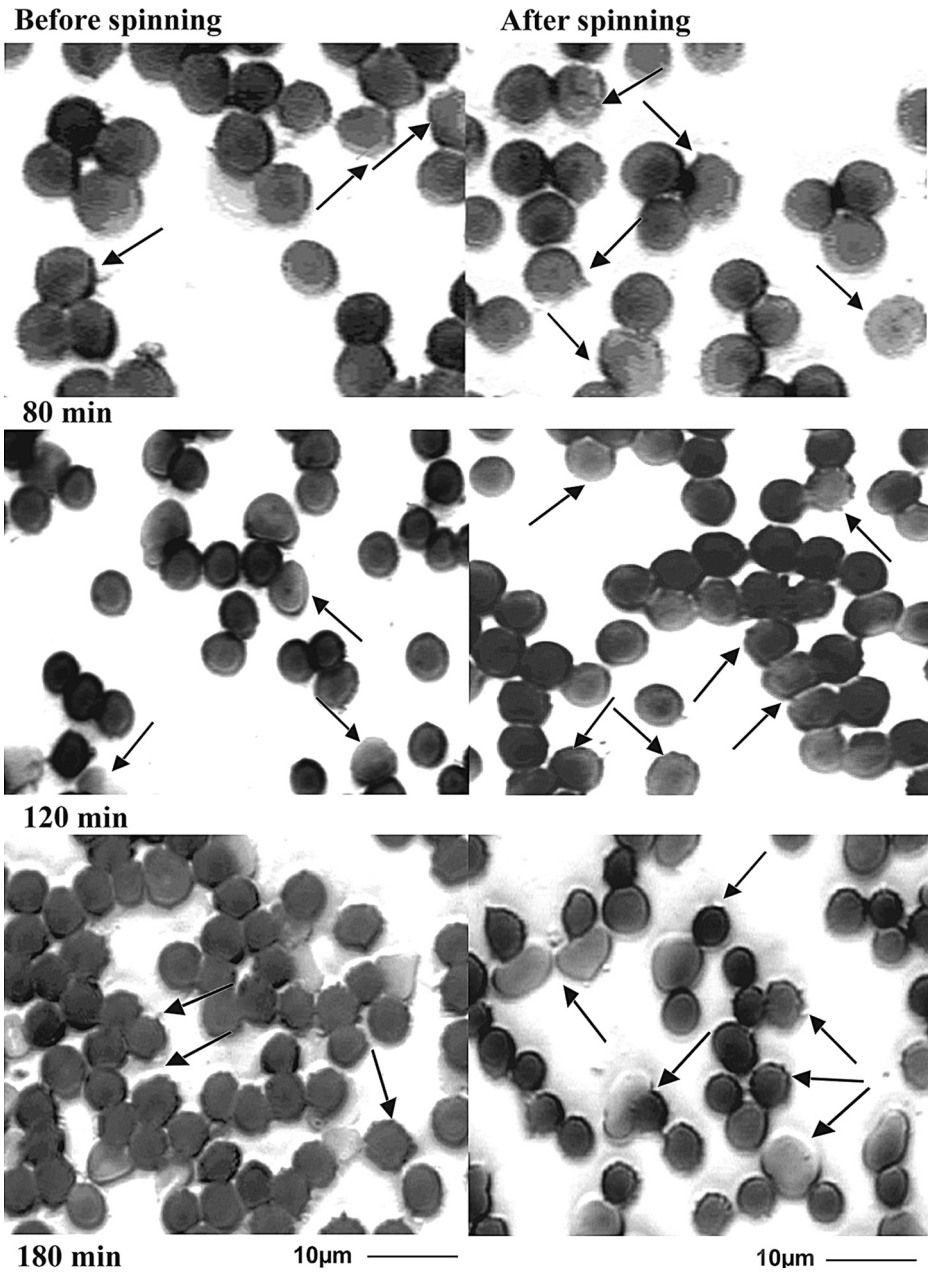



**Fig. 5.** Typical microscopy images at different haemolysis time (80, 120 and 180 min) of RBCs before and after practicing sport. The → symbol indicates the main groups of crenated RBCs. The — symbol shows the scale bar of micrographs.

$$[D] = [A]_0\left\{1 - \frac{k_1}{k_1+k_2-k_3}e^{-k_3t} - \frac{k_2}{k_1+k_2-k_4}e^{-k_4t} + \frac{k_1k_3+k_2k_4-k_3k_4}{(k_1+k_2-k_3)(k_1+k_2-k_4)}e^{-(k_1+k_2)t}\right\} \quad (2)$$

where $[A]_0$ is the initial concentration of hemoglobin. Thus, from Eq. (2), the relative values of the rate constant for the hemolysis induced by radical can be determined so as to give the smallest mean square error between observed and calculated values.[15]

*In vitro* oxidative damage of normal human erythrocytes is induced by AAPH. In the whole process of AAPH-initiated oxidation, hemolysis occurred quickly after the lag time. The rate of hemolysis is dose-dependent correlated with AAPH concentration.

Morphological alteration in the erythrocytes from a smooth discoid to an echinocytic form was observed by Wang et al[6] Erythrocyte shape observation by scanning electron microscopy.

As observed by Sato et al,[15] we can say that crenated erythrocytes are accompanied by redistribution of band 3 proteins. This indicates that band 3 proteins were topologically redistributed and clustered to form holes in the membrane. The EMI (eosin-5-maleimide)-labeled erythrocytes were observed by "fluorescence microscopy" (Olympus BH-2 photomicroscope) and with a confocal scanning laser microscope (Lasertec Co. Ltd., 1LM21H).

## 5. Conclusions

The effect of spinning load is studied as oxidative damage (spontaneous MDA production and AAPH-induced MDA), or

oxidative hemolysis. Indeed morphological analysis of RBC from spinning athletes before and after activity as in the separated observations made by different research groups[6,15] was never examined before. According to results authors hypothesize a relationship between spinning exercise, RBC plasma membrane oxidation and hemolysis susceptibility.

In conclusion, the exercise (at least after 60 min) applied to tested athletes, causes in them intra-vascular hemolysis (120 min hemolysis data). This process is associated with in vivo alterations in RBC membranes similar to those found in inherited anemic diseases.

We hypothesize these structural and possibly also functional alterations of the RBCs are result of an increased susceptibility of the cells to physical and/or chemical stress rather than compression of capillaries during the foot strike as the primary contributor to exercise related hemolysis.[7]

## Conflicts of interest

All authors have none to declare.

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