DELAYED URIC ACID ACCUMULATION IN PLASMA PROVIDES ADDITIONAL ANTI-OXIDANT PROTECTION AGAINST IRON-TRIGGERED OXIDATIVE STRESS AFTER A WINGATE TEST

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ABSTRACT: Reactive oxygen species are produced during anaerobic exercise mostly by Fe ions released into plasma and endothelial/muscle xanthine oxidase activation that generates uric acid (UA) as the endpoint metabolite. Paradoxically, UA is considered a major antioxidant by virtue of being able to chelate pro-oxidative iron ions. This work aimed to evaluate the relationship between UA and plasma markers of oxidative stress following the exhaustive Wingate test. Plasma samples of 17 male undergraduate students were collected before, 5 and 60 min after maximal anaerobic effort for the measurement of total iron, haem iron, UA, ferric-reducing antioxidant activity in plasma (FRAP), and malondialdehyde (MDA, biomarker of lipoperoxidation). Iron and FRAP showed similar kinetics in plasma, demonstrating an adequate pro-/antioxidant balance immediately after exercise and during the recovery period (5–60 min). Slight variations of haem iron concentrations did not support a relevant contribution of rhabdomyolysis or haemolysis for iron overload following exercise. UA concentration did not vary immediately after exercise but rather increased 29% during the recovery period. Unaltered MDA levels were concomitantly measured. We propose that delayed UA accumulation in plasma is an auxiliary antioxidant response to post-exercise (iron-mediated) oxidative stress, and the high correlation between total UA and FRAP in plasma (R-Square = 0.636; p = 0.00582) supports this hypothesis.

KEY WORDS: anaerobic metabolism, exercise, haemoglobin, lipid peroxidation, xanthine oxidase

INTRODUCTION

Skeletal muscle overproduces reactive oxygen species (ROS) during contractile activity, compared to the lower – but still significant – levels found at rest [1]. However, depending on the prevalent energy-demanding activity (aerobic/endurance or anaerobic/resistance), intensity, and duration, intracellular ROS sources other than mitochondria might be significantly involved in the process [2]. The enzyme xanthine oxidase, found in muscle and vascular endothelium, is activated in response to the intermittent (pseudo) ischaemia-reperfusion that occurs during anaerobic/resistance exercise coupled with severe energy depletion in exhausted fast-twitch type-II fibres (AMP accumulation) [3]. This enzyme has long been known to catalyze the conversion of hypoxanthine (from inosine) into uric acid (UA) by the purine catabolic pathway, with the concomitant generation of superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [4]. In turn, $O_2^{\cdot-}$ and H_2O_2 yield the highly reactive hydroxyl ($HO^{\cdot}$) and lipid-alkoxyl ($RO^{\cdot}$) radicals via several iron-catalyzed reactions. In the worst-case scenario, iron overload in plasma and muscles has been associated with strenuous/intense exercise [5].

Although the link between iron availability and oxidative stress is presently well established, sources of iron overload in plasma during/after exercise are still unclear. Several hypotheses have been raised, including rhabdomyolysis, haemolysis, and/or iron release from ferritin/transferrin stocks [6]. Biochemical changes in iron homeostasis have been associated with injuries and illness following overtraining periods, chronic inflammation, impaired athletic performance, early fatigue, myalgia and myopathies [7].

Under intense muscle workload, higher rates of ROS production demand appropriate antioxidant responses to avoid exaggerated oxidative stress and its harmful consequences [8]. Antioxidant defences must cover all stages of the injurious action of ROS in bio-

Abbreviations
AUC, area under curve; FRAP, ferric-reducing activity in plasma; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; UA, uric acid.

logical systems: prevention, interception/scavenging, and repair [9]. Among several antioxidants in plasma, UA has been suggested as a powerful biological iron-chelating agent to provide protection against oxidative injury [10]. There is significant current interest in redox biomarkers in plasma and their relationship to exercise and other physiological alterations and/or pathologies, such as atherosclerosis, obesity, and cardiopathies [11]. Moreover, fast, simple, and low-cost analytical tests have been prioritized in sports science for practical and logistic reasons. This work aimed to evaluate the relationship between UA and plasma markers of oxidative stress after an exhaustive anaerobic 30 s cycling sprint test (Wingate test).

MATERIALS AND METHODS ▪▬▬▬▬

Subjects. Seventeen male undergraduate students (age, 23.2 ± 5.3 years; height, 176.2 ± 2.1 cm; weight, 80.8 ± 8.9 kg) were invited to participate in the study. All subjects were experienced in cycling activity and were physically active for 6 months prior to the study (at least three times a week). The exercise protocol and all other experimental procedures were approved by the Institutional Ethical Committee and are in agreement with the Declaration of Helsinki (1964). All 17 subjects had not undergone any systemic or topical therapeutic treatment for at least 2 months (not even anti-inflammatory medication), and had no history of smoking, alcohol use, obesity, or systemic disease. Volunteers were instructed to avoid vigorous physical activity at least 6 h before the Wingate test, as well as drinking caffeinated substances (e.g., coffee, cocoa, mate, caffeine-based drinks, guarana, and cola) or alcohol within 24 h prior to the tests.

Chemicals

All chemicals were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA), except those used for usual solutions, including specific buffers, which were purchased from Labsynth (Diadema, SP, Brazil). Chromatography-quality solvents were obtained from Merck (Düsseldorf, Germany), and all stock solutions and buffers were prepared with Milli-Q purified water (Millipore, Billerica, MA, USA).

Experimental protocol

The protocol for the Wingate test was that described by Dotan and Bar-Or [4], and was carried out with a Cybex cycle ergometer, Model Metabolic Systems (Division of Lumex, Ronkonkoma, NY, USA). After each set of maximal effort, the workload was adjusted to accommodate the active recovery mode (no resistance, 80 rpm, for 3 min).

Sample collection and analyses

Blood samples (5 mL) were withdrawn from the forearm cubital vein of the volunteers immediately before, 5 min after, and 60 min after the Wingate test, using EDTA-containing Vacutainer® kits. Heparinized tubes were avoided for blood sampling since physiological haem

iron was also measured in plasma. Blood samples were immediately centrifuged for 5 min at $4 \times g$ (RT), and plasma fractions were isolated and stored at -80°C until analysis.

Biochemical analysis

Ferric-reducing activity of plasma (FRAP)

The ferric-reducing activity of plasma (FRAP) test was performed by replacing the Fe^{2+}-chelating agent 2,4,6-tripyridyl-S-triazine (TPTZ) with its analogue 2,3-bis(2-pyridyl)-pyrazine (DPP) [12]. Control analytical assays with standard $Fe^{2+/3+}$ ions confirmed the stoichiometric equivalence between the two chelating agents (data not shown). Briefly, the reactant mixture for the FRAP assay contained 10 mM DPP (stock solution prepared in 40 mM HCl) and 20 mM $FeCl_3$ in 0.30 M acetate buffer (pH 3.6). To 200 μL of FRAP reactant mixture, 10–20 μL of sample and 40–30 μL of distilled water were added (total volume, 250 μL). Absorbance at 593 nm was recorded for 4 min in a 96-well microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA USA) to determine the rate of Fe^{2+}-DPP complex formation as compared to a standard curve. Total FRAP values were determined from the area under curves between 0 and 60 min ($AUC_{0-60min}$).

Total iron determination

The total iron concentration in plasma was assayed using a specific biochemical kit from Doles-Bioquímica Clínica (Brazil). The method is based on the detection at 560 nm of the Fe^{2+}:ferrozine complex formed from ferritin and released iron ions from plasma transferrin by the reducing agents 0.36 M hydroxylamine chloride, 0.10 M glycine, 14 mM thiosemicarbazide, and 0.50 mM octylphenoxy-polyethoxyethanol, at pH 2.2. Total iron released in plasma was determined from the area under curves between 0 and 60 min ($AUC_{0-60min}$).

Haem iron determination

Total haem iron content in plasma (from haemoglobin, myoglobin and other haem proteins) was assayed by a method based on haem iron oxidation by the ferricyanide anion contained in a solution of 0.10 M KH_2PO_4, 60 mM $K_3[Fe(CN)_6]$, 77 mM KCN, and 82 mM Triton X-100. Haem iron cyanide is stoichiometrically detected at 540 nm, using haemoglobin as a standard curve [13]. Total haem iron released in plasma was calculated by determining the area under curves between 0 and 60 min ($AUC_{0-60min}$).

Uric acid determination

Plasma uric acid (UA) content was assayed with a biochemical kit from BioClin-Quibasa. In the assay mixture, H_2O_2 produced from UA in the presence of uricase (to form allantoin) is coupled with *p*-hydroxybenzoate and 4-aminoantipyrine oxidation catalyzed by peroxidase to form a pinkish chromophore detected at $\lambda = 505$ nm. Total UA released in plasma was calculated by determining the area under the curves between 0 and 60 min ($AUC_{0-60min}$).

Lipid peroxidation determination

Lipid peroxidation was measured by MDA derivatization with TBA followed by malondialdehyde (MDA) derivatization with thiobarbituric acid (TBA) followed by high-performance liquid chromatography (HPLC) analysis [14]. The total MDA content was first released from cellular compartments in a mixture of 50 μL of plasma with 250 μL of 30% methanol for 15 min (4°C) in an ultrasound bath. Subsequently, 100 μL of 0.50% BHT was added to the samples to avoid oxidation reactions in the following steps. MDA was then converted into a pinkish chromophore by derivatization with 600 μL of 0.4% TBA (in 0.20 M HCl) for 30 min at 95°C, under constant mixing. The sample was then filtered (MilliPore nylon membranes, 0.45 μm pore size, 13 mm diameter) and injected (20 μL) in a Shimadzu SCL10A HPLC system equipped with LC10AD pumps and fluorescence (RF-10AXL) detector. The MDA-TBA adduct was isocratically eluted by a 65:35 mixture of 25 mM phosphate buffer (pH 6.5) and methanol 30% through a 0.39 $\times$ 30 cm μBondapack C18 column and detected by fluorescence (excitation/emission $\lambda = 515/555$ nm; retention time $\sim$ 6 min). The total MDA peak areas of the samples were compared with a standard curve obtained with 1,1,2,2-tetraethoxypropane (also in 30% methanol). Total MDA released in plasma was calculated by determining the area under curves between 0 and 60 min (AUC$_{0\text{-}60min}$).

Statistical analysis

All data are presented as the mean values of at least triplicate experiments. Biochemical assays were analyzed by multivariate analysis of variance (MANOVA), helpful when more than one dependent variable is evaluated at a time (iron, haem iron, uric acid, FRAP, and MDA). Greenhouse-Geisser corrections were applied when necessary, as well as the post-hoc Bonferroni test. MANOVA allows us to test hypotheses regarding the effect of one or more independent variables on two or more dependent variables (significance $p < 0.05$). SPSS and OriginPro 8 software (v8.0725/B725; OriginLab Corporation, Northampton, MA, USA) were used for statistical analyses and graph preparation, respectively.

RESULTS

Data from the Wingate test (mean $\pm$ SD) are indicative of the anaerobic effort performed by the young athletes during the Wingate test: peak anaerobic power, 363.5 ± 30.8 W; anaerobic power, 173.1 ± 14.3 W; total workload, 5193 ± 430 J; and specific total workload, 59.5 ± 6.8 J·kg^{-1}.

One-way, repeated measure MANOVA revealed the effect of time on biomarkers of oxidative stress [Wilks' lambda $= 0.32$, $F_{(10,56)} = 4.39$; $p < 0.001$, $\eta^2 = 0.44$]. Univariate analyses revealed the effect of time on plasma iron [$F_{(2,32)} = 7.27$; $p < 0.005$, $\eta^2 = 0.31$; Figure 1A-B], uric acid [$F_{(2,32)} = 6.03$; $p < 0.01$, $\eta^2 = 0.27$; Figure 2A-B], and haem iron [$F_{(2,32)} = 4.30$; $p < 0.05$, $\eta^2 = 0.21$; Figure 3A-B]. On the other hand, no significant effect of time on MDA [Greenhouse-Geisser corrected, $F_{(1.18,25.78)} = 1.14$;

$p > 0.05$, $\eta^2 = 0.07$; Figure 4A-B] was observed. FRAP only showed a slight tendency for time-dependent responses and consistently showed similar kinetics as plasma Fe during the 0–60 min time span [Greenhouse-Geisser corrected, $F_{(1.13,18.14)} = 3.38$; $p = 0.078$, $\eta^2 = 0.18$; Figure 5A-B]. Post-hoc tests with Bonferroni corrections revealed that iron concentration immediately after the exercise protocol (t5) was higher than before the exercise (t0) and after 60 min (t60), whereas no difference in iron content between t0 and t60 was found. Regarding UA, post-hoc tests indicated higher concentration at t60 after exercise when compared to the plasma concentration before (t0) and 5 min after the Wingate protocol (t5). Uric acid content in plasma was unaltered between t0 and t5 after exercise. Regarding haem iron concentration, despite univariate analysis having indicated an effect of time, post-hoc tests with Bonferroni corrections revealed no difference with time. Box plots are presented in Figures
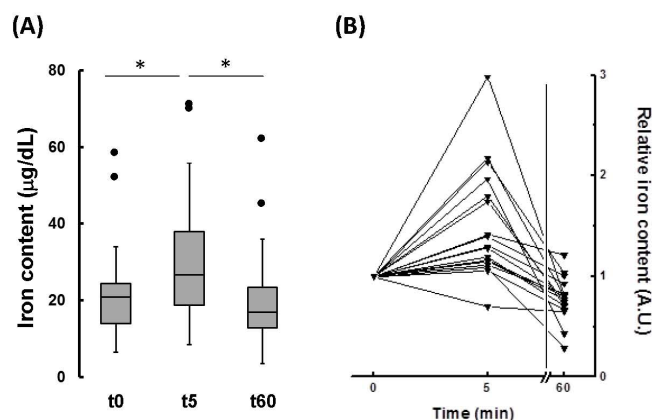

(A) **(B)**

FIG. 1. IRON CONTENT IN PLASMA (μg·dL^{-1}) OF MALE UNDERGRADUATE STUDENTS BEFORE (T0), 5 (T5) AND 60 MIN (T60) AFTER A WINGATE TEST, EXPRESSED AS: (A) BOX PLOT OF REAL VALUES; AND (B) INDIVIDUAL RELATIVE VALUES (T0 = 1 UNIT, IN ORDER TO OBSERVE INDIVIDUAL VARIATIONS).
Note: *$p < 0.05$; n = 17, (•) Outlier data of the IQR = 1.5*(Q3-Q1), where: IQR = interquartile range; Q3 = 75% quartile; and Q1 = 25% quartile.
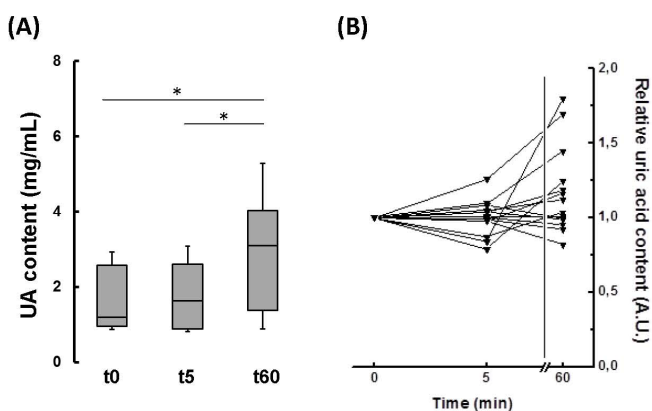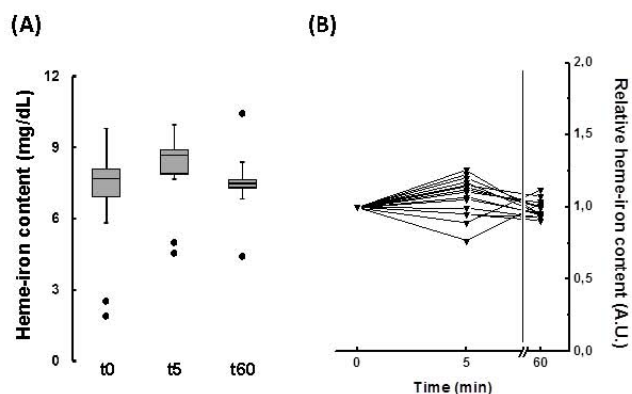

(A) **(B)**

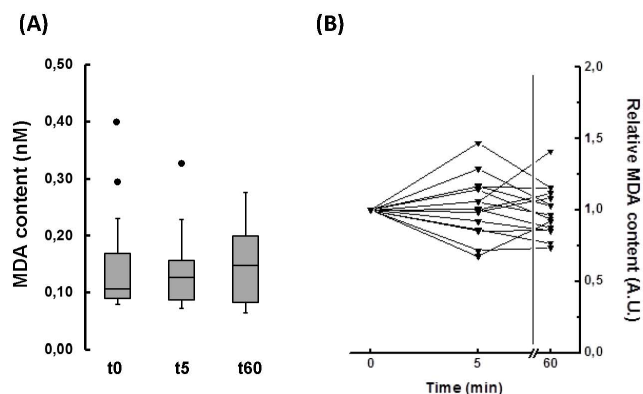**FIG. 2.** URIC ACID CONTENT IN PLASMA (mg·mL^{-1}) OF MALE UNDERGRADUATE STUDENTS BEFORE (t0), 5 (t5) AND 60 MIN (t60) AFTER A WINGATE TEST, EXPRESSED AS: (A) BOX PLOT OF REAL VALUES; AND (B) INDIVIDUAL RELATIVE VALUES (t0 = 1 UNIT, IN ORDER TO OBSERVE INDIVIDUAL VARIATIONS)
Note: *$p < 0.05$; n = 17.

FIG. 3. HAEM IRON CONTENT IN PLASMA (mg · dL^{-1}) OF MALE UNDERGRADUATE STUDENTS BEFORE (t0), 5 (t5) AND 60 MIN (t60) AFTER A WINGATE TEST, EXPRESSED AS: (A) BOX PLOT OF REAL VALUES; AND (B) INDIVIDUAL RELATIVE VALUES (T0 = 1 UNIT, IN ORDER TO OBSERVE INDIVIDUAL VARIATIONS)
Note: *$p < 0.05$; n = 17, (•) Outlier data of the IQR = 1.5*(Q3-Q1), where: IQR = interquartile range; Q3 = 75% quartile; and Q1 = 25% quartile
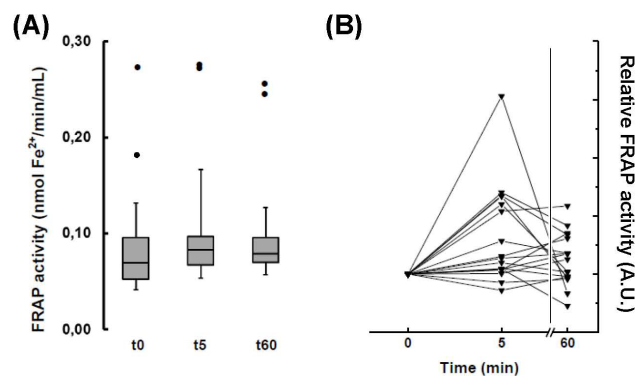

FIG. 4. MALONDIALDEHYDE CONTENT IN PLASMA (nM) OF MALE UNDERGRADUATE STUDENTS BEFORE (t0), 5 (t5) AND 60 MIN (t60) AFTER A WINGATE TEST, EXPRESSED AS: (A) BOX PLOT OF REAL VALUES; AND (B) INDIVIDUAL RELATIVE VALUES (T0 = 1 UNIT, IN ORDER TO OBSERVE INDIVIDUAL VARIATIONS)
Note: *$p < 0.05$; n = 17, (•) Outlierr data of the IQR = 1.5*(Q3-Q1), where: IQR = interquartile range; Q3 = 75% quartile; and Q1 = 25% quartile

FIG. 5. FERRIC-REDUCING ACTIVITY OF PLASMA (FRAP, in nmol Fe^{2+}·min^{-1}·mL^{-1}) OF MALE UNDERGRADUATE STUDENTS BEFORE (t0), 5 (t5) AND 60 MIN (t60) AFTER A WINGATE TEST, EXPRESSED AS: (A) BOX PLOT OF REAL VALUES; AND (B) INDIVIDUAL RELATIVE VALUES (t0 = 1 UNIT, IN ORDER TO OBSERVE INDIVIDUAL VARIATIONS).
Note: *$p < 0.05$; n = 17, (•) Outlier data of the IQR = 1.5*(Q3-Q1), where: IQR = interquartile range; Q3 = 75% quartile; and Q1 = 25% quartile

1B-5B for better evaluation of data distribution with time. All baseline biochemical markers were normalized to 1.0 at t0 (as relative values) in Figures 1B-5B to better evidence individual post-exercise changes.

In order to analyze the integral antioxidant responsive capacity of subjects for exercise, areas under the curves in Figures 1B–5B (AUC$_{0-60min}$) were calculated within the 0–60 min interval and expressed as Fe$_{TOTAL}$, FRAP$_{TOTAL}$, MDA$_{TOTAL}$, and UA$_{TOTAL}$. Furthermore, the prevalence of pro-oxidative events over antioxidant responses during the experimental period (0–60 min) was estimated by calculating the ratio (Fe$_{TOTAL}$)/(FRAP$_{TOTAL}$) over (MDA$_{TOTAL}$) (Figure 6A). Based on our integrative approach, the extension of pro-oxidative events in plasma – expressed by the (Fe$_{TOTAL}$)/(FRAP$_{TOTAL}$) ratio – showed a satisfactory correlation with MDA$_{TOTAL}$ (Adj. R-Square = 0.594; inset, Figure 6A). Using an identical integrative approach, a relatively high degree of correlation between FRAP$_{TOTAL}$ activity and UA$_{TOTAL}$ in plasma was measured during the 60-min time span (Adj. R-Square = 0.636; inset, Figure 6B).

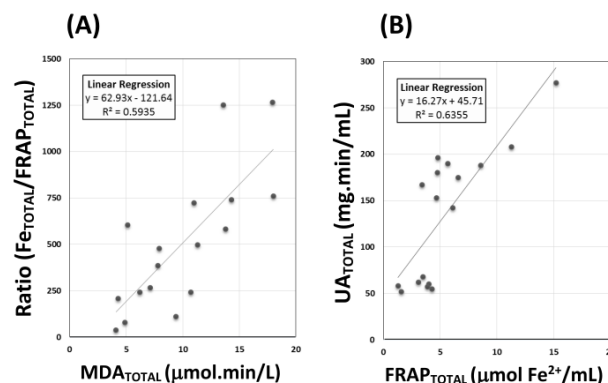**FIG. 6.** LINEAR CORRELATIONS BETWEEN: (A) (Fe$_{TOTAL}$)/(FRAP$_{TOTAL}$) RATIO AND (MDA$_{TOTAL}$), AND (B) BETWEEN (UA$_{TOTAL}$) AND (FRAP$_{TOTAL}$), BASED ON CALCULATIONS OF AUC FROM 0–60 MIN
Note: Inset box: linear regression and statistical parameters (n = 17)

DISCUSSION

Iron is a crucial microelement for several physiological/biochemical processes in the human body and regarding exercise, the disruption of iron homeostasis can lead to reduction in oxygen transport capacity, diminished energy supply for muscle contractions, and oxidative stress [15]. As shown in Figure 1A-B, the Wingate test induced massive releases of iron in plasma of subjects, but those levels were normalized during the recovery period (5–60 min). The observed lower iron content after 60 min may result from the induction of efficient antioxidant systems against the oxidative stress imposed by the anaerobic exercise. No evidence of rhabdomyolysis or haemolysis was reported here, since haem iron contents did not vary significantly on pre-/post analysis (post-hoc tests with Bonferroni corrections; Figure 3A-B).

The ferric-reducing activity in plasma (FRAP) evaluates the presence of metal ligands in plasma that form redox inactive complexes [Fe(L)]$^{n+}$, which, thereby, inhibit the formation of aggressive ROS

from deleterious Fenton-type and other radical reactions [12]. As shown in Figure 5A-B, FRAP values showed tendencies to increase immediately after the Wingate test (p = 0.078), which were still observed in the recovery period (t60). Although the FRAP assay quantifies a bulk of substances with Fe-chelating capacities in plasma, other antioxidants could also be depleted in the plasma under harmful oxidative stress. Therefore, compensatory effects of different antioxidants (some partially induced, others depleted) could account for the insignificant variation of FRAP in subjects within the time span (p = 0.078). Nevertheless, the kinetics of FRAP variation in plasma statistically match those found for plasma iron concentration within the same period (Figures 1A-B and 5A-B). One-way, repeated measure MANOVA revealed that, altogether, pro-oxidant agents (iron and haem iron) and antioxidants (FRAP and UA) are orchestrated in plasma after the Wingate test, which accordingly limited the levels of lipid peroxidation in plasma (MDA, Figure 6A).

Interestingly, the concentration of UA – one of the major antioxidants in human plasma – did not vary immediately after exercise, but showed a late onset accumulation (29% compared to baseline; p = 0.0135) during the following recovery period (Figure 2A-B). Thus, we can assume that the antioxidant accumulation of UA in plasma was important to sustain FRAP activity during the recovery period (from 5 to 60 min after exercise). This hypothesis was reinforced by the relatively high correlation between UA_{TOTAL} released in plasma and $FRAP_{TOTAL}$ (Adj. R-Square = 0.636; inset, Figure 6B). The reasons for the delayed increase of UA concentration in plasma (renal activity, kinetics of UA release, etc.) deserve further investigation. Otocka-Kmiecik et al. observed higher FRAP in human plasma following maximal aerobic exercise on a treadmill (i.e., more aerobic-type exercise) [16]. An increase in UA was also detected in their study as a long-lasting process which, accordingly, showed high correlation with FRAP. However, in agreement with our results, Otocka-Kmiecik et al. also observed unaltered levels of TBA-reactive substances (TBARS). We believe that monitoring oxidative stress markers depends greatly on the time of sampling, in a similar way as the classic biomarkers of muscle damage, creatine kinase and lactate dehydrogenase activities in plasma/blood [17]. Moreover, Kabasakalis et al. (2013) stated that oxidative damage was short-lived and different biomarkers of oxidative stress could prevail depending on time: (i) DNA oxidation products 1 h after high-intensity;

or (ii) MDA, immediately after moderate and intense exercises [18]. It is worth mentioning that aldehyde derivatives from lipid peroxidation – most of the TBARS compounds, including MDA – promptly react with proteins and nucleic acids in plasma to form adducts that, thus, interfere in the precise determination of free MDA concentration in plasma [19]. Finally, it is possible that the Wingate protocol applied was not intense enough to induce maximal (or detectable) redox imbalances in all subjects, especially considering simple, fast, and low-cost assays as proposed here. We aimed to monitor exercise-associated oxidative stress by simple methods in order to discuss (or start discussing) sampling and redox analysis *in situ*, which would provide crucial information for adequate post-exercise supplementation, energy supply, etc. However, an evaluation regarding method sensibility, technique costs, and data accuracy undoubtedly deserves further investigation.

CONCLUSIONS

The late response of UA following the exhaustive Wingate test is here suggested as a secondary antioxidant frontline that limits iron availability, sustains higher antioxidant activity (FRAP), and limits lipid peroxidation in plasma during the recovery period.

Conflict of interests: The results of the present study do not constitute endorsement of any products by the authors or by other organizations. The authors herewith have no competing interests.

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