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Reduced serum concentrations of reactive oxygen and nitrogen species following strenuous exercise in the heat are not associated with an upregulation in serum antioxidative capacity

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Reduced serum concentrations of reactive oxygen and nitrogen species following strenuous exercise in the heat are not associated with an upregulation in serum antioxidative capacity

Abstract

Introduction: Reactive oxygen and nitrogen species (RONS) are produced as a response to exercise and play a part in guiding the adaptive response to physical training. Exercise in the heat has been shown to further increase accumulation of RONS in systemic circulation; however, a high antioxidative response or heat acclimatization mechanisms could attenuate this response. In a previous study, we found reduced RONS concentrations in trained athletes following strenuous cycling in the heat. Therefore, this secondary analysis assessed whether these reduced RONS serum concentrations are induced by an increased serum antioxidant capacity. **Methods:** Twelve male cyclists (VO_{2peak} : $60 \pm 4 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) completed a 60-minute constant workload trial (55% peak power output, ambient temperature $30.4 \pm 0.6^\circ\text{C}$) with and without ice vest in a randomized order. The core body temperature (T_{core}) was measured by an ingestible capsule. Blood samples were collected before and after each trial to determine superoxide dismutase (SOD) and catalase (CAT) activity, total antioxidant capacity (TAC) and RONS. Due to the absence of between-group differences, data of both conditions were pooled. **Results:** T_{core} statistically increased ($p < 0.001$) over the experimental trials ($+6.0 \pm 1.6\%$, effect size (ES) = 5.6). Concentrations of RONS ($-17.2 \pm 15.5\%$, $p < 0.001$, ES = 1.0) and TAC ($-8.9 \pm 22.9\%$, $p = 0.04$, ES = 0.7) statistically decreased, while the activity of CAT ($+15.5 \pm 84.0\%$, $p = 0.90$, ES = 0.04) and SOD ($+9.2 \pm 58.7\%$, $p = 0.98$, ES = 0.01) remained unchanged. **Conclusions:** Reduced serum RONS concentrations after strenuous cycling in the heat were not associated with upregulation of serum antioxidant capacity in trained athletes. The prooxidant-antioxidant balance may rather be regulated at a myocellular level and should be further assessed in future studies.

Keywords

antioxidants, catalase, reactive oxygen species, superoxide dismutase, sports medicine, total antioxidant capacity

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Article

Reduced serum concentrations of reactive oxygen and nitrogen species following strenuous exercise in the heat are not associated with an upregulation in serum antioxidative capacity

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Abstract: Introduction: Reactive oxygen and nitrogen species (RONS) are produced as a response to exercise and play a part in guiding the adaptive response to physical training. Exercise in the heat has been shown to further increase accumulation of RONS in systemic circulation; however, a high antioxidative response or heat acclimatization mechanisms could attenuate this response. In a previous study, we found reduced RONS concentrations in trained athletes following strenuous cycling in the heat. Therefore, this secondary analysis assessed whether these reduced RONS serum concentrations are induced by an increased serum antioxidant capacity. Methods: Twelve male cyclists ($\dot{V}O_{2\text{peak}}$: $60 \pm 4 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) completed a 60-minute constant workload trial (55% peak power output, ambient temperature $30.4 \pm 0.6^\circ\text{C}$) with and without ice vest in a randomized order. The core body temperature (T_{core}) was measured by an ingestible capsule. Blood samples were collected before and after each trial to determine superoxide dismutase (SOD) and catalase (CAT) activity, total antioxidant capacity (TAC) and RONS. Due to the absence of between-group differences, data of both conditions were pooled. Results: T_{core} statistically increased ($p < 0.001$) over the experimental trials ($+6.0 \pm 1.6\%$, effect size (ES) = 5.6). Concentrations of RONS ($-17.2 \pm 15.5\%$, $p < 0.001$, ES = 1.0) and TAC ($-8.9 \pm 22.9\%$, $p = 0.04$, ES = 0.7) statistically decreased, while the activity of CAT ($+15.5 \pm 84.0\%$, $p = 0.90$, ES = 0.04) and SOD ($+9.2 \pm 58.7\%$, $p = 0.98$, ES = 0.01) remained unchanged. Conclusions: Reduced serum RONS concentrations after strenuous cycling in the heat were not associated with upregulation of serum antioxidant capacity in trained athletes. The prooxidant-antioxidant balance may rather be regulated at a myocellular level and should be further assessed in future studies.

Keywords: antioxidants, catalase, reactive oxygen species, superoxide dismutase, sports medicine, total antioxidant capacity.

1. Introduction

Oxidative stress is defined as an imbalance between reactive oxygen and nitrogen species (RONS) formation and the antioxidant defense system [1, 2]. RONS including superoxide ($O_2^{\cdot-}$) and nitric oxide (NO), hydrogen peroxide (H_2O_2), hydroxyl radical (OH \cdot), or peroxynitrite (ONOO \cdot) have been associated with the pathogenesis of many diseases, e.g. diabetes, hypertension or arteriosclerosis [3–5]. However, RONS are also linked to adaptive responses to exercise, such as enhanced calcium release and muscle function [6], skeletal muscle remodeling [7] and to the expression of an oxidative phenotype in muscles [8, 9]. Therefore, oxidative stress plays an ambiguous role in human physiology and needs to be considered carefully with regard to the biological context in which it occurs [10].

In the context of exercise, systemic RONS accumulation appears to be dependent on the type, mode, duration and intensity of exercise as well as athletes' training and nutrition status [10, 11]. In addition, adverse environmental conditions, such as heat exposure, have been reported to exacerbate plasma/serum oxidative stress responses along with both elevated core body temperature (T_{core}) and/or dehydration [12–14]. While skeletal muscle, specifically NADPH oxidase and potentially xanthine oxidase, is considered a major contributor to RONS accumulation during exercise [8, 15, 16], hyperthermia may also trigger other potential mechanisms [17, 18]. For example, acute increases in blood viscosity due to marked dehydration (>2% of body mass) may augment vascular shear stress and lead to RONS release from NADPH or xanthine oxidases in the vessel wall [17, 19]. Furthermore, sweat loss and peripheral vasodilation may also reduce splanchnic blood flow, potentially increasing systemic RONS accumulation [20, 21].

Oxidative stress may be blunted by enhanced antioxidant capacity, either due to high antioxidant nutritional intake [22] or due to involvement in regular exercise training [23]. Thus, exercise-induced increases in the plasma antioxidant capacity and the plasma activity of endogenous enzymes such as superoxide dismutase (SOD) or catalase (CAT) have been reported to enhance the body's ability to detoxify RONS over-accumulation [14,23]. Similarly, it has recently been suggested that the development of heat acclimatization is likewise accompanied by and potentially even requires distinct oxidative signaling processes inducing a more antioxidative environment [24–27].

In a previous study [28], we investigated whether per-cooling through an ice vest attenuated inflammatory and oxidative stress responses induced by strenuous exercise (60-minutes cycling at $\sim 68\%$ $\dot{V}O_{2peak}$) and heat exposure ($30.4 \pm 0.6^\circ C$). Interestingly, we were somewhat surprised to see reductions in serum RONS concentrations both after exercise with and without per-cooling intervention, a phenomenon that has scarcely been described so far. Similarly to this finding, Kaldur et al. [24] observed reduced oxidative stress indices after 162 ± 48 minutes of low-to-moderate intensity treadmill running in the heat ($42^\circ C$) in heat-acclimatized participants but not in non-acclimatized ones. In contrast, many other investigations have previously reported increased instead of reduced levels of oxidative stress following exercise and heat exposure [12–14, 29]. Since in our previous investigation, all participants were endurance-trained athletes ($\dot{V}O_{2peak}$: 60 ± 4 ml \cdot kg $^{-1}$ \cdot min $^{-1}$), and the intervention took place in the summer months of the northern hemisphere, we speculated that this surprising finding might be related to participants' training and acclimatization status. Thus, the participants might have had sufficient systemic endogenous antioxidant capacities to buffer RONS accumulated during exercise in the heat, potentially explaining the lower serum RONS concentrations measured post-exercise [23, 24]. Therefore, the aim of this secondary analysis was to evaluate a set of biomarkers representing the serum antioxidant response in order to test our hypothesis that systemic RONS concentrations might be reduced after exercise and heat exposure due to a systemic upregulation of antioxidant capacities in trained and partly acclimatized athletes.

2. Materials and Methods

2.1. Participants

Twelve male cyclists or triathletes (age = 26 ± 5 yrs, height = 181 ± 7 cm, body mass = 75.0 ± 4.8 kg, $\dot{V}O_{2\text{peak}} = 60.3 \pm 4.0$ mL · kg⁻¹ · min⁻¹, PPO = 419 ± 40 W, previous training volume 8 ± 3 h · wk⁻¹) participated in the study. All athletes were at least partly heat acclimatized, since testing was performed in the summer months of the northern hemisphere, with daytime temperatures reaching values $\geq 25^\circ\text{C}$ [30]. Their performance level was classified as trained based on the measured $\dot{V}O_{2\text{peak}}$ [31]. Prior to all testing, the medical history of all participants was assessed using a standardized questionnaire, and athletes provided their written informed consent. The study was conducted in accordance with the declaration of Helsinki and approved by the local university's ethical committee (176/2018).

2.2. Experimental Designs

Three separate testing sessions were conducted in this randomized-crossover trial: a preliminary ramp test in thermoneutral conditions to assess peak power output (PPO) and peak oxygen uptake ($\dot{V}O_{2\text{peak}}$), as well as two 60-minute constant workload trials in a hot environment. These two trials were performed in a randomized and counterbalanced order while wearing a vest with and without ice packs. Tcore was assessed by an ingestible telemetric capsule (e-Celsius™; BodyCap, Caen, France). In addition, in order to assess serum RONS concentration, the activity of SOD and CAT as well as total antioxidative capacity (TAC), venous blood samples were collected before and immediately after the trials.

2.3. Procedures

By keeping a 24-hour food record, participants were required to keep the dietary intake constant before each trial, while abstaining from alcohol, caffeine, dietary supplements and strenuous exercise 24 hours preceding each test. In addition, testing was carried out at the same time of the day (± 1 h), and laboratory visits were separated by at least two days. A detailed description of performance testing was presented elsewhere [28]. Briefly, PPO and $\dot{V}O_{2\text{peak}}$ were assessed in a ramp test performed on a cycle ergometer in thermoneutral ambient conditions ($21.0^\circ\text{C} \pm 0.3^\circ\text{C}$ and $49\% \pm 9\%$ humidity). For the constant workload trials, after five minutes of familiarization at 100 W, participants completed 60 minutes at 55% of individualized PPO (230 ± 22 W) in an ambient temperature of $30.4^\circ\text{C} \pm 0.6^\circ\text{C}$ ($38\% \pm 7\%$ humidity). During both constant workload trials, participants wore the same lightweight softshell vest (Regatta Professional, Manchester, UK; 285 g) once with and once without inserting ten cooling pads (Active.Pad 3x6; EMCOOLS Sports GmbH, Traiskirchen, Austria; 1,730 g). For analysis of blood markers, venous blood samples were drawn from the antecubital vein into sterile K2-EDTA tubes for blood cell counts and into serum separation tubes (BD, Plymouth, UK) for the assessment of (anti-)oxidative markers. After storage for 10 minutes at room temperature for blood clotting, the serum separation tubes were centrifuged at 1000 g for 10 minutes at 4°C (Heraeus® Multifuge® 3 L-R, Kendro Laboratory Products, Newton, USA). Immediately after centrifugation, serum was separated into 1 mL aliquots and stored at -80°C for further analysis.

2.4. Measures from Blood Analysis

Hemoglobin and hematocrit were analyzed by fluorescent flow cytometry (Sysmex KX-21N; Sysmex Corporation, Kobe, Japan). Serum SOD and CAT activity as well as TAC were assessed by a laboratory specialized in clinical routine diagnostics (Labor Dr. Wisplinghoff, Cologne, Germany) using Superoxide dismutase Colorimetric Activity Kit (ABIN2866576; Antibodies Online, Aachen, Germany), Catalase Assay Kit (KA0884; Abnova, Taipei City, Taiwan) and ImAnOx® (TAS/TAC) Kit (Immundiagnostik AG, Bensheim, Germany), respectively. Serum concentrations of RONS were assessed using Ox-

iSelect™ In Vitro ROS/RNS Assay Kits (STA-347; Cell Biolabs, Inc., San Diego, USA) as described previously [32]. Briefly, free radicals (i.e. NO, H₂O₂, ONOO⁻ and peroxy radical) in the samples react with a specific probe that is converted into the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). Therefore, fluorescence intensity is proportional to RONS levels in the samples. The samples were analyzed in duplicates using a microplate reader (Multiscan™ FC; Thermo Scientific™, Waltham, USA), and the mean was used for statistical analysis. Concentrations of all markers were corrected for plasma volume change due to fluid loss, as calculated from changes in hemoglobin and hematocrit [33].

2.5. Statistical Analysis

Residual histograms, residual plots and Q-Q-plots were visually checked for homoscedasticity and normality prior to statistical analysis. Data were analyzed by two-way repeated-measures analyses of variance (ANOVA) using the *afex* package in R [34]. Main effects for time were explored using Tukey's HSD test from the *emmeans* package. Consequently, *p*-values were reported along with Cohen's effect sizes (ES) and 95% confidence intervals determined by means of the *effsize* package, using Hedge's correction for small sample sizes. Furthermore, the *stats* package was used to assess associations between the changes in the dependent variables across both conditions by means of the Pearson product-moment correlation coefficient *r* (95% confidence intervals). For all tests, statistical significance was accepted at *p* < 0.05. All data are presented as mean ± standard deviation (SD).

3. Results

The blood lactate accumulation was reported elsewhere and did not differ between the conditions [28]. Similarly, as no statistical interaction effects between conditions in all parameters (including concentrations of RONS and TAC and activity of CAT and SOD) except for Tcore (*p* = 0.004) were observed, the two conditions were pooled for the current secondary analysis.

3.1. Core Temperature, Body Mass and Blood Parameters

Tcore statistically increased in both conditions, by +5.1 ± 1.1% (*p* < 0.001; ES = 5.74 [2.56–8.92]) with per-cooling and by +7.0 ± 1.4% (*p* < 0.001; ES = 6.29 [2.88–9.71]) without per-cooling, respectively. Overall, body mass statistically decreased by -2.6 ± 0.8 % (*p* < 0.001, ES = 0.36 [0.31–0.41]). Both hematocrit and hemoglobin statistically increased by +4.5 ± 4.2% (*p* < 0.001, ES = 0.52 [0.32–0.72]) and +5.1 ± 3.7% (*p* < 0.001, ES = 0.64 [0.43–0.84]) respectively. Plasma volume decreased by -7.7 ± 5.4%. The detailed results are shown in Table 1 or can be found elsewhere [28].

Table 1. Pooled values (mean ± standard deviation) of core body temperature (Tcore), body mass and blood parameters immediately before (Pre) and after (Post) 60 minutes of cycling in the heat.

Variable	Pre	Post
Tcore (°C)	36.8 ± 0.3	39.0 ± 0.4
Body mass (kg)	75.0 ± 5.0	73.1 ± 4.8
Hematocrit (%)	42.6 ± 3.6	44.5 ± 3.1
Hemoglobin (g · dL ⁻¹)	14.3 ± 1.1	15.1 ± 1.1

3.2. RONS and Antioxidant Markers

Changes in RONS and antioxidative capacity are displayed in Figure 1. Concentrations of RONS statistically decreased by -17.2 ± 15.5% (*p* < 0.001, ES = 0.96, [0.53–1.39]). Concentrations of TAC also statistically decreased by -8.9 ± 22.9% (*p* = 0.04, ES = 0.73 [0.05–1.41]).

The activity of CAT ($+15.5 \pm 84.0\%$, $p = 0.90$, $ES = 0.04 [-0.49-0.56]$) and SOD ($+9.2 \pm 58.7\%$, $p = 0.98$, $ES = 0.01 [-0.50-0.51]$) remained statistically unchanged.

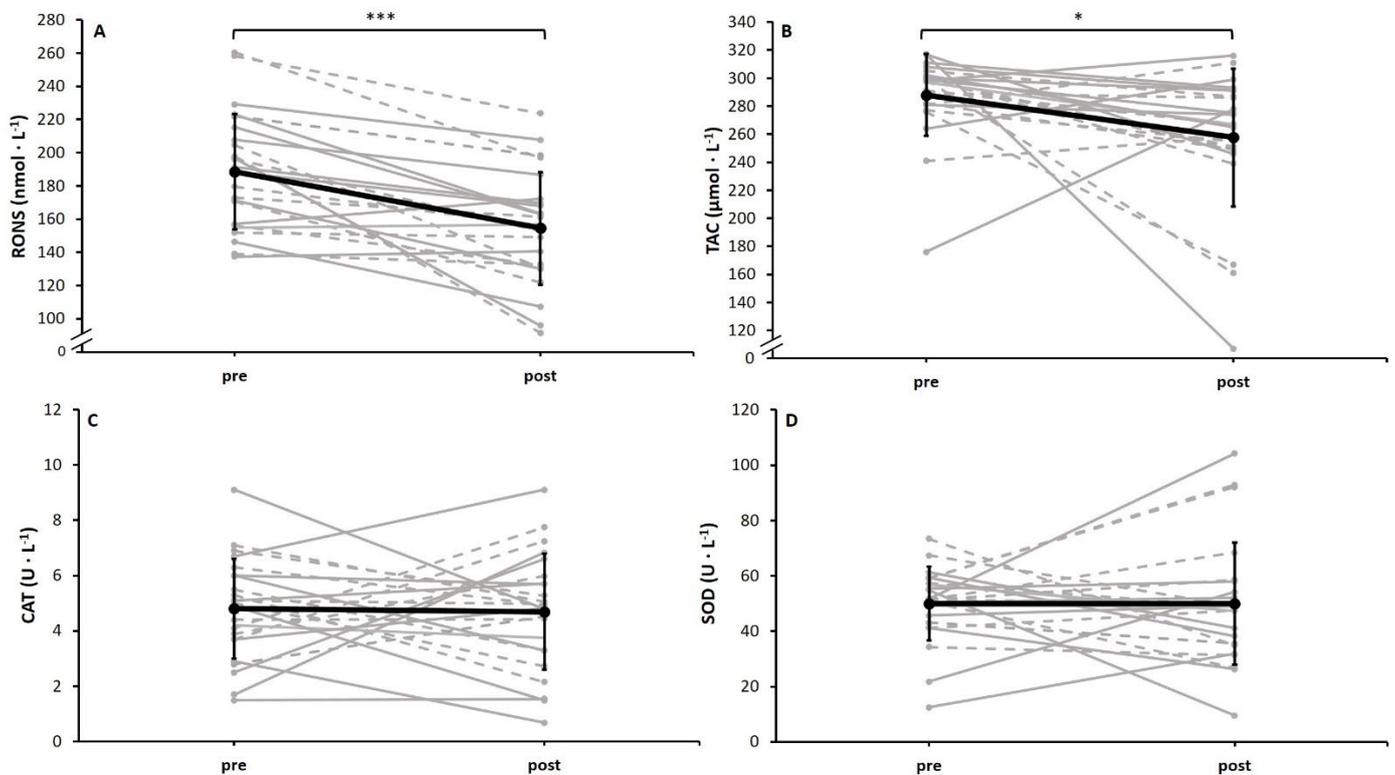


Fig. 1. Serum concentrations of (A) reactive oxygen and nitrogen species (RONS), (B) total antioxidative capacity (TAC) and serum activity of (C) catalase (CAT) and (D) superoxide dismutase (SOD) before and after 60-min cycling at 55% peak power output (PPO) in an ambient temperature of 30°C. Dotted grey line = without cooling, full grey line = with ice vest cooling, full black line = pooled mean \pm standard deviation (SD) of both conditions. Statistical difference between pre and post concentrations (repeated-measures ANOVA, main effect for time): * $p < 0.05$, *** $p < 0.001$.

No systematic statistical associations between the changes in RONS, antioxidant status, Tcore and body mass were found.

Table 2. Pooled correlations (Pearson's r along with 95% confidence interval) between the changes in RONS and antioxidant status as well as Tcore and body mass loss.

	Tcore		Body mass loss	
	r (95% CI)	p	r (95% CI)	p
RONS ($\text{nmol} \cdot \text{L}^{-1}$)	0.16 (-0.26, 0.53)	0.45	0.01 (-0.39, 0.41)	0.95
TAC ($\mu\text{mol} \cdot \text{L}^{-1}$)	-0.41 (-0.70, -0.00)	0.05	-0.12 (-0.50, 0.30)	0.58
CAT ($\text{U} \cdot \text{L}^{-1}$)	0.03 (-0.38, 0.43)	0.90	-0.14 (-0.52, 0.28)	0.50
SOD ($\text{U} \cdot \text{L}^{-1}$)	0.33 (-0.09, 0.65)	0.12	0.07 (-0.35, 0.46)	0.76

Abbreviations: CAT, catalase; RONS, reactive oxygen and nitrogen species; SOD, superoxide dismutase; TAC, total antioxidative capacity; Tcore, body core temperature.

4. Discussion

The aim of this study was to investigate whether serum RONS concentrations after cycling in the heat are reduced due to a systemic upregulation of antioxidant capacities in trained and partly acclimatized athletes. This study was based on previous novel findings, where we found reduced serum RONS concentrations after 60-minutes of cycling exercise in the heat. In contrast to our hypothesis, however, the activity of CAT and SOD remained statistically unchanged after exercise cessation, while TAC was even statistically reduced. Furthermore, changes in RONS concentrations were not associated with changes in TAC or CAT and SOD activity.

Decreased systemic levels of RONS after exercise and heat exposure are somewhat surprising since heat and dehydration are known to increase oxidative stress with and without exercise [12, 17, 18, 35, 36]. Indeed, we observed a T_{core} increase of $+2.2 \pm 0.6^{\circ}\text{C}$ ($+6.0 \pm 1.6\%$) and a body mass decrease of $-2.0 \pm 0.6 \text{ kg}$ ($-2.6 \pm 0.8\%$). This is similar to other studies which observed changes in (anti-) oxidative parameters when investigating physical exercise and heat exposure [12, 13, 29]. On the other hand, plasma antioxidant capacities have been shown to be upregulated in trained [23] and especially also in heat acclimatized athletes [24]. In a study by Kaldur et al. [24], the oxidative stress index, determined by the ratio of total peroxide concentration and total antioxidant capacity, decreased after an acute bout of exercise in the heat after ten days of heat acclimatization. Similarly, the athletes in our study were well-trained and at least partly acclimatized; therefore, this may possibly be an explanation for the reduction of RONS concentrations after cycling in heat exposure.

To determine if the systemic antioxidant capacity was increased, serum activity of CAT and SOD as well as concentrations of TAC were determined. Especially CAT activity has previously been shown to increase following endurance exercise in hot environments, at least in trained athletes ($\dot{V}O_{2\text{peak}} = 61.4 \pm 2.8 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) [23]. Thus, an increase in plasma CAT activity immediately after 45-minutes of treadmill running in hot ($32.4 \pm 0.5^{\circ}\text{C}$) as opposed to thermoneutral conditions ($11.6 \pm 0.8^{\circ}\text{C}$) has been observed, probably to counteract increased plasma RONS concentrations [23]. Similarly, plasma antioxidant capacity, albeit analyzed with different methods, has also been shown to increase post-exercise [14]. However, despite similar exercise conditions and participant characteristics, in the current investigation, CAT and SOD activity did not statistically increase following the intervention, and concentrations of TAC were even statistically reduced. While it can be speculated that reductions of non-enzymatic antioxidant capacity may result from immediate RONS scavenging or shifting to other tissues such as skeletal muscle [37], the investigated antioxidant enzymes in serum do not seem to contribute to reduced RONS levels. Therefore, it can be concluded that decreased RONS concentrations in this study are not caused by an increase in serum antioxidant capacity for buffering peroxide and superoxide radicals and that other mechanisms must be considered, possibly in immune cells [29] and likely in skeletal muscles [38].

Notably, RONS production after acute exercise is thought to originate to a considerable extent from skeletal muscles [8, 15, 16]. Therefore, to prevent the risk of oxidative damage, myocytes also have an extensive network of antioxidant defense mechanisms to reduce RONS levels [39]. This network includes the intracellular expression of enzymatic antioxidants, like SOD, CAT, glutathione peroxidase and non-enzymatic antioxidants [40]. Thus, in contrast to our approach to find a systemic upregulation of antioxidant capacity as previously reported [14, 23], it is plausible that in well-trained and partly acclimatized athletes, RONS buffering already takes place at an intra- and myocellular level, as most antioxidants exert their properties intracellularly [41]. Likewise, these systems have been previously shown to be upregulated with regular training [8, 42]. Therefore, further investigations should clarify if reduced RONS accumulation in trained athletes may already be due to regulation of antioxidant capacities in skeletal muscles.

It has also been shown that while measuring antioxidant activity and capacity of blood plasma reflects tissue antioxidants [43], plasma antioxidant capacity does not necessarily reflect the true skeletal redox state and redox-sensitive protein signaling of working muscle [44, 45]. Indeed, the antioxidant capacity of plasma rather gives a comprehensive gross redox perspective of all organs and tissues and was, therefore, considered important in the context of the current study. However, as no markers of oxidative damage were measured in this study, it is not possible to assess whether the reduced levels of RONS also resulted in lower levels of oxidative damage.

When interpreting our data, it is important to keep in mind that this was a secondary analysis, which was limited by the design of the initial study, including handling of blood samples. We acknowledge that to gain a better insight into total antioxidant capacity, it would be beneficial to include non-enzymatic antioxidants and to use more advanced methods to detect changes in antioxidant status and oxidative stress, especially at the cellular level. However, these analyses require specific handling of blood directly after sampling. Furthermore, the kinetics of RONS production and regulation of antioxidative capacity should be considered. As blood sampling was only performed directly before and after the exercise intervention, it is not possible to describe the kinetics of these markers during or post-exercise. In a study by Michailidis et al. [46], it was shown that depending on the measured redox marker, optimal blood sampling time for peak magnitude of change varied between directly and 4-hours post-exercise. However, while possibly not of the greatest magnitude, changes in parameters were already visible directly post-exercise. Thus, a measurement immediately after exercise cessation does offer the possibility to detect changes in systemic RONS accumulation and antioxidative capacity in the current study. Lastly, our original study did not involve a control group. Previous studies have shown that compared to athletes, untrained subjects are unable to complete trials at the same exercise conditions. Therefore, external loading relating to intensity and duration would have to be adapted, leading to reduced comparability [36]. However, to gain more insights into the influence of heat acclimatization status and to examine more long-term adaptations, a comparison of trained athletes before and after a period of heat acclimatization would be promising.

5. Conclusions

The setting of intense cycling and heat exposure in the current study led to reduced serum RONS concentrations, which were not associated with upregulation of serum total antioxidant capacity or SOD and CAT activity. Due to partial heat acclimatization and a high training status, RONS overaccumulation may potentially be regulated at a (myo-) cellular level by other defense mechanisms in these athletes. However, this hypothesis needs to be supported by further investigations. On that basis, our initial results may serve as an opener for future debates and research examining pro-oxidant-antioxidant, balance not only on a systemic but also on an intracellular and myocellular level. Furthermore, the impact of training status as well as heat acclimatization on serum RONS concentrations and antioxidant capacity should be further examined.

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