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Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability

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Abstract

Aim: The aim of this study was to reveal the role of reactive oxygen and nitrogen species (RONS) in exercise adaptations under physiological *in vivo* conditions and without the interference from other exogenous redox agents (e.g. a pro-oxidant or antioxidant).

Methods: We invented a novel methodological set-up that exploited the large redox interindividual variability in exercise responses. More specifically, we used exercise-induced oxidative stress as the 'classifier' measure (i.e. low, moderate and high) and investigated the physiological and redox adaptations after a 6-week endurance training protocol.

Results: We demonstrated that the group with the low exercise-induced oxidative stress exhibited the lowest improvements in a battery of classic adaptations to endurance training (VO_2 max, time trial and Wingate test) as well as in a set of redox biomarkers (oxidative stress biomarkers and antioxidants), compared to the high and moderate oxidative stress groups.

Conclusion: The findings of this study substantiate, for the first time in a human *in vivo* physiological context, and in the absence of any exogenous redox manipulation, the vital role of RONS produced during exercise in adaptations. The stratification approach, based on a redox phenotype, implemented in this study could be a useful experimental strategy to reveal the role of RONS and antioxidants in other biological manifestations as well.

Keywords antioxidants, exercise adaptations, free radicals, oxidative stress, trainability, variability.

During the last two decades, great advances have been made in unravelling the molecular and biochemical 'signals' that regulate various biological processes. An analogous progress has also taken place in the exercise field. Vigorous research has enriched the repertoire of the potential molecular 'exercise signals' that stimulate and mediate adaptations.[1–4] Reactive oxygen and nitrogen species (RONS) represent one of the most prominent 'new entries' in the long list of the 'exercise signals'. In fact, it is nowadays acknowledged that exercise-induced RONS are important signalling molecules regulating several adaptations.[5–9]

Our current knowledge on the role of RONS in exercise adaptations stems predominantly from *in vitro*, *ex vivo* and *in situ* experiments that mechanistically implicated RONS in biochemical cascades

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associated with specific adaptations.[10] The relevant *in vivo* studies have typically followed a different methodology: researchers administered a generic (e.g. N-acetylcysteine, vitamins C and E) or, more rarely, a targeted antioxidant (e.g. allopurinol, apocynin and mitoQ) and evaluated the subsequent outcomes, which were attributed to the attenuated exercise-induced oxidative stress.[11–17] Despite the fact that many seminal discoveries in exercise redox biology have been accomplished using these methodological strategies, there are also limitations surrounding these approaches. *In vitro*, *ex vivo* and *in situ* preparations have most frequently utilized supraphysiological doses of RONS,[18] while the extrapolation of *in vitro* findings into the complex *in vivo* biology suffers from inevitable interpretational pitfalls.[19] In experiments using *in vivo* models, the generic antioxidants administered are pleiotropic compounds exerting multiple redox-independent biological effects,[20,21] while several 'targeted' antioxidants have also off-target effects.[22] In the light of the above, it would be useful to invent a methodological set-up that could delineate the role of RONS in exercise adaptations under physiological *in vivo* conditions and without interference from exogenous redox manipulations, such as the administration of an antioxidant.

Towards this aim, we sought to exploit the large interindividual variability in redox responses after exercise, previously reported by our group.[23] More specifically, we hypothesized that the use of exercise-induced oxidative stress as classifier could provide vital information about the potential role of RONS in exercise adaptations. Yet, an important threat when stratifying and enrolling participants based on their initial value for a given biological trait (in our case exercise-induced oxidative stress) is regression to the mean.[24,25] This artefact takes place when a group with an extreme average value during a first measurement tends to obtain a less extreme value (i.e. tendency towards the mean) on a subsequent measurement, in the absence of any treatment. We encountered this artefact by performing a duplicate pre-treatment measurement (described in detail in Margaritelis *et al.*[26]), an approach that has been reported to efficiently minimize regression to the mean.[27,28] By implementing this novel methodological design, we investigated whether the degree of exercise-induced oxidative stress after acute exercise regulates the physiological (both aerobic and anaerobic) and redox adaptations in the long-term.

Results

The physiological characteristics of all initially enrolled participants ($N = 100$) are presented in Table 1. The first acute cycling exercise session, as expected, increased urine F_2-isoprostanes compared to baseline ($P < 0.001$). In particular, the average increase was equal to 40% (ranging from −25 to +120%) and the coefficient of variation was equal to 83%, signifying a large variation in redox responses after exercise among the 100 individuals. The physiological characteristics of the three experimental groups formed based on their exercise-induced oxidative stress levels ($N = 36$) are presented in Table 1. Daily energy, macronutrient and antioxidant intake did not differ among the three groups ($P > 0.05$ for all parameters, Table 1). Acute exercise-induced changes in urine F_2-isoprostane levels were different among the three groups even after minimizing regression to the mean (i.e. after the second acute exercise session; $P < 0.001$). This fact validates the choice of exercise-induced oxidative stress as a classifier for the three groups. Finally, an inverse correlation ($r = -0.71$, $P < 0.001$) was found between resting F_2-isoprostane levels and the exercise-induced F_2-isoprostane changes in the 36 enrolled participants.

Performance

A significant group × condition interaction was found for VO_2max ($P = 0.001$), time trial ($P < 0.001$) and Wingate test ($P < 0.001$; Table 2, Fig. 1). With regard to VO_2max, the low oxidative stress group exhibited the lowest improvement (i.e. 12%) compared to the high and moderate oxidative stress groups (i.e. 19% and 17% improvement respectively). With regard to the time trial and similar to the VO_2max, the low oxidative stress group exhibited a blunted improvement (i.e. 9%) compared to the high and moderate oxidative stress groups (i.e. 22 and 24% improvement respectively). In the Wingate test, the low oxidative stress group exhibited again the lowest improvement (i.e. 7%) compared to the high and moderate oxidative stress groups (i.e. 15 and 12% increase respectively).

Lipid peroxidation and protein oxidation

A significant group × condition × time interaction was found for F_2-isoprostanes ($P = 0.022$) and protein carbonyls ($P < 0.001$; Table 3, Figs 2 and 3). The resting F_2-isoprostane levels decreased by 6% in the high oxidative stress group, by 16% in the moderate group and by 8% in the low oxidative stress group. Following training, the acute exercise-induced increases in F_2-isoprostanes were lower in the high (from 62 to 49%) and moderate (from 37 to 21%) oxidative stress groups. On the contrary, the low oxidative stress group exhibited an increase from 4 to 10%. Similarly, following training the resting protein

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Table 1 Physiological characteristics and dietary energy analysis of the participants (mean ± SD)

	Before stratification	After stratification			
	N = 100	High (*N* = 12)	Moderate (*N* = 12)	Low (*N* = 12)	*P*
Age (years)	23.49 ± 2.60	23.25 ± 2.45	22.91 ± 2.60	22.75 ± 2.92	0.897
Weight (kg)	73.34 ± 7.78	72.25 ± 7.99	74.33 ± 7.81	70.08 ± 7.45	0.224
Height (cm)	177.00 ± 5.37	180.6 ± 5.4	175.5 ± 3.5	176.5 ± 4.4	0.022*
Body Fat (%)	13.31 ± 3.44	14.44 ± 4.05	14.79 ± 3.75	12.86 ± 3.34	0.414
Resting HR (bpm)	66.73 ± 5.12	67.66 ± 4.67	67.91 ± 3.96	66.41 ± 4.71	0.681
Energy (kcal/day)		2640 ± 308	2591 ± 319	2692 ± 287	0.578
CHO (% energy)		53.35 ± 4.54	52.13 ± 3.82	53.68 ± 4.15	0.731
Protein (% energy)		14.63 ± 2.44	14.00 ± 1.83	15.78 ± 2.07	0.449
Fat (% energy)		32.02 ± 5.54	33.87 ± 4.42	30.54 ± 5.32	0.490
Vitamin C (mg)		118.5 ± 12.0	121.0 ± 16.1	119.5 ± 15.1	0.782
Vitamin E (mg)		8.11 ± 1.23	7.91 ± 1.41	8.24 ± 1.69	0.691
Selenium (μg)		72.53 ± 2.81	70.21 ± 3.01	74.40 ± 3.21	0.349

CHO, carbohydrate.
*Significant difference between high and moderate oxidative stress groups.

Table 2 Performance changes after the 6-weeks cycling training (mean ± SD)

	Pre-training	Post-training	G	C	G × C
VO_2max (ml kg^{-1} min^{-1})					
High	45.04 ± 5.64	53.47 ± 5.49*#	0.270	<0.001	0.001
Moderate	46.12 ± 4.73	53.90 ± 3.95*#			
Low	43.99 ± 6.44	49.17 ± 5.65*			
Wingate test (W)					
High	732.6 ± 149.4	841.5 ± 156.2*	0.293	<0.001	<0.001
Moderate	702.2 ± 125.6	785.8 ± 140.4*			
Low	681.0 ± 125.3	727.3 ± 129.3*			
Time trial (W)					
High	260.7 ± 31.1	317.1 ± 31.0*#	0.173	<0.001	<0.001
Moderate	247.9 ± 33.9	304.7 ± 29.3*			
Low	251.4 ± 28.7	275.0 ± 27.3*			

G, group (high, moderate, low exercise-induced oxidative stress); C, condition (pre-, post-training).
*Significant difference compared to pre-training in the same group.
#Significant difference compared to the low oxidative stress group at the same time point.

carbonyl levels decreased by 14% in the high oxidative stress group, by 19% in the moderate group and by 5% in the low oxidative stress group. Moreover, post-training, the acute exercise-induced increases in protein carbonyls were reduced in the high (from 60 to 23%) and moderate (from 49 to 12%) oxidative stress groups. As was the case with F_2-isoprostanes, the low oxidative stress group exhibited an increase in protein carbonyls from 13 to 17%.

Glutathione and antioxidant enzymes

A significant group × condition × time interaction was found for glutathione (*P* = 0.041), superoxide dismutase (*P* = 0.002) and glutathione peroxidase (*P* = 0.043), whereas no interaction was found for catalase (*P* = 0.444; Table 3, Figs 4 and 5). The resting glutathione levels increased in the high oxidative stress group by 18%, in the moderate group by 8%, while no difference was found in the low oxidative stress group. Remarkably, post-training the low oxidative stress group exhibited significantly lower resting glutathione levels compared to the other two groups. The acute exercise-induced decreases in glutathione levels reduced in the high oxidative stress group from 30 to 5% and in the moderate group from 23 to 15%, while the corresponding reduction in the low oxidative stress group was from 8 to 4%.

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Table 3 Redox biomarker changes during the 6-wk cycling training

	Pre-training		Post-training								
	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise	*G*	*C*	*T*	*G* × *C*	*G* × *T*	*C* × *T*	*G* × *C* × *T*
F_2-isoprostanes											
High	484 ± 155†	776 ± 235§¶	448 ± 131*†	661 ± 168$^{*\#\S}$	<0.001	0.002	<0.001	0.193	<0.001	0.044	0.022
Moderate	713 ± 123$^{\#}$	967 ± 154§	591 ± 87$^{*\#}$	717 ± 121$^{*\#\S}$							
Low	880 ± 84	911 ± 189	803 ± 71*	881 ± 134§							
Pr. carbonyls											
High	0.52 ± 0.14	0.80 ± 0.16§	0.43 ± 0.15$^{\#}$	0.51 ± 0.17$^{*\#\S}$	<0.001	<0.001	<0.001	0.027	0.003	<0.001	<0.001
Moderate	0.51 ± 0.12$^{\#}$	0.75 ± 0.17§	0.41 ± 0.10$^{*\#}$	0.46 ± 0.09$^{*\#\S}$							
Low	0.67 ± 0.12	0.74 ± 0.12§	0.63 ± 0.11*	0.73 ± 0.11§							
GSH											
High	2.97 ± 0.66	1.99 ± 0.33§¶	3.40 ± 0.50$^{*\#}$	3.21 ± 0.41$^{*\#\S}$	<0.001	<0.001	<0.001	0.001	0.003	0.001	0.041
Moderate	3.35 ± 0.64$^{\#}$	2.56 ± 0.46§	3.49 ± 0.60$^{\#}$	2.95 ± 0.51$^{*\#\S}$							
Low	2.54 ± 0.53	2.27 ± 0.41§	2.49 ± 0.47	2.38 ± 0.42							
SOD											
High	1249 ± 365	5005 ± 1161§	1501 ± 353$^{*\#}$	2485 ± 886*§	0.115	<0.001	<0.001	0.170	0.920	<0.001	0.002
Moderate	1121 ± 342	4440 ± 1193§	1300 ± 305*	2583 ± 790*§							
Low	1017 ± 294	3741 ± 1193§	1045 ± 259	2861 ± 1003*§							
Catalase											
High	143.1 ± 38.4	250.4 ± 67.4§	166.8 ± 39.2*	188.5 ± 27.3*§	0.438	0.001	<0.001	0.463	0.017	<0.001	0.444
Moderate	153.1 ± 34.9	290.7 ± 96.4§	175.3 ± 27.2$^{*\#}$	195.8 ± 29.2*§							
Low	138.9 ± 33.0	300.2 ± 82.5§	145.0 ± 29.1	191.9 ± 34.2*§							
GPx											
High	32.64 ± 5.25	45.79 ± 5.51$^{\#\S}$	37.42 ± 5.23$^{*\#}$	39.25 ± 3.92*§	0.001	0.998	<0.001	0.402	0.388	<0.001	0.043
Moderate	34.53 ± 5.65$^{\#}$	43.18 ± 6.91§	36.36 ± 5.02*	39.03 ± 2.86*§							
Low	28.18 ± 3.34	37.56 ± 6.35§	32.47 ± 5.22*	37.38 ± 5.04							

GSH, glutathione; SOD, superoxide dismutase; GPx, glutathione peroxidase; G, group (high, moderate, low exercise-induced oxidative stress); C, condition (pre-, post-training); T, time (pre-, post-exercise).

*Significant difference compared to the pre-training condition in the same group at the same time point.

#Significant difference compared to the low oxidative stress group in the same time point at the same condition.

§Significant difference compared to pre-exercise in the same group at the same condition.

¶Significant difference compared to the moderate oxidative stress group in the same time point at the same condition.

†Significant difference compared to the other two groups in the same time point at the same condition. Units: F_2-isoprostanes (pg mg^{-1} $creatinine^{-1}$); protein carbonyls (nmol mg^{-1} $protein^{-1}$); GSH (μmol g^{-1} Hb^{-1}); SOD (U g^{-1} Hb^{-1}); catalase (U g^{-1} Hb^{-1}); GPx (U g^{-1} Hb^{-1}).

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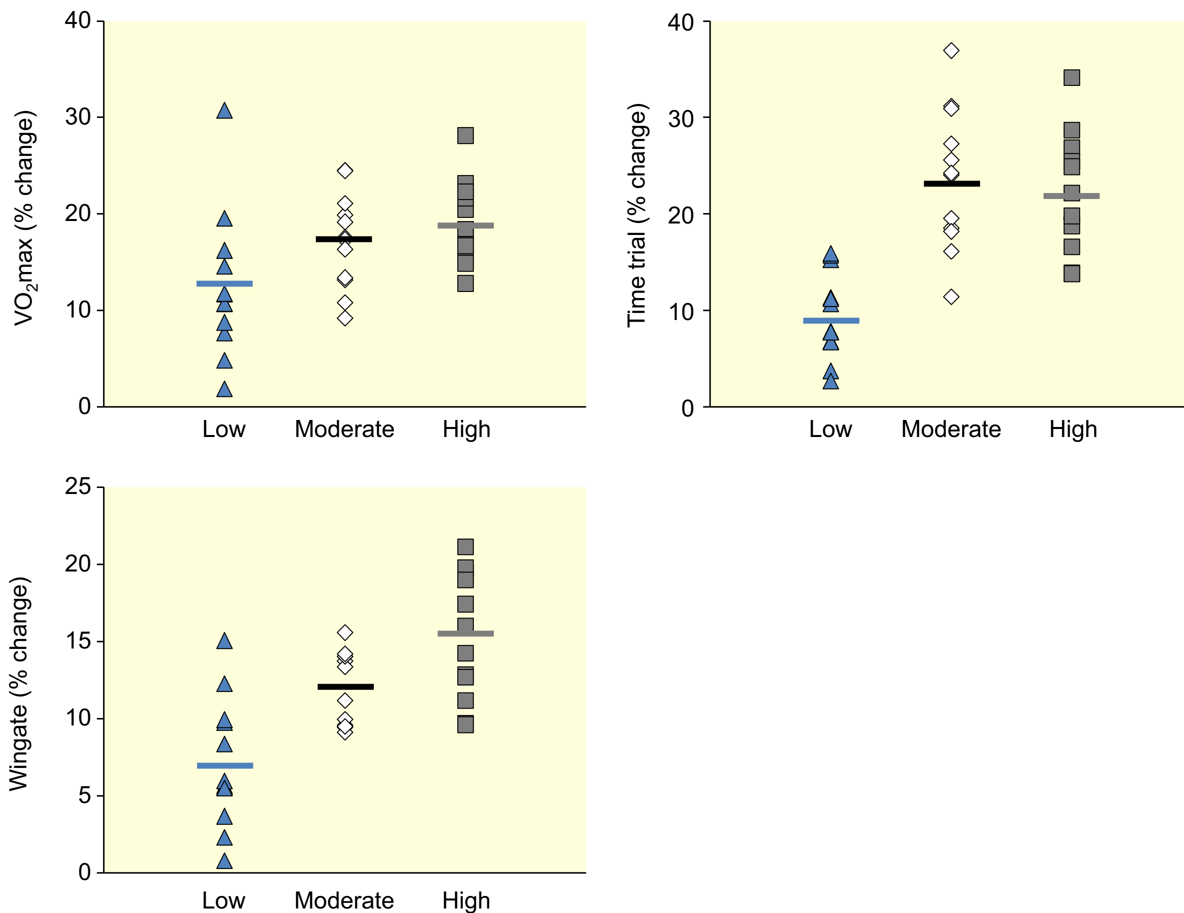

Figure 1 Performance adaptations in the three experimental groups.

With regard to the superoxide dismutase, the high and moderate oxidative stress groups exhibited a significant increase in resting activity levels after the chronic exercise protocol by 22 and 20% respectively. On the contrary, the low oxidative stress group exhibited a minor non-significant increase of 4%. Interestingly, post-training the high oxidative stress group exhibited significantly greater resting superoxide dismutase activity compared to the low oxidative stress group. The acute exercise-induced increases in superoxide dismutase activity showed a great reduction in the high oxidative stress group from 317 to 64% and in the moderate oxidative stress group from 305 to 101%, while a much lower reduction was found in the low oxidative stress group from 272 to 180%.

With regard to the glutathione peroxidase, all groups exhibited a significant increase in resting activity levels after the chronic exercise protocol. In particular, the high oxidative stress group exhibited an 16% increase, the moderate oxidative stress group exhibited an 6% increase and the low oxidative stress group exhibited an 16% increase. It is noteworthy that post-training the high oxidative stress group showed significantly greater resting glutathione peroxidase activity compared to the low oxidative stress group. The acute exercise-induced increases in glutathione peroxidase activity showed the greatest reduction in the high oxidative stress group from 43 to 6%, while the reduction in the moderate group was from 26 to 8% and in the low group from 34 to 17%.

Regarding catalase, despite the fact that no group × condition × time interaction was found, it is worth mentioning that the resting activity levels after the chronic exercise protocol increased significantly only in the high and moderate oxidative stress groups by 20 and 19%, respectively, while a slight 7% increase was found in the low oxidative stress group.

Discussion

By exploiting the large redox interindividual variability, we report that classical adaptations to endurance training are, at least in part, regulated by the degree of exercise-induced oxidative stress. Over the years, reductionist and muscle-centric approaches have focused more on the molecular underpinning signals

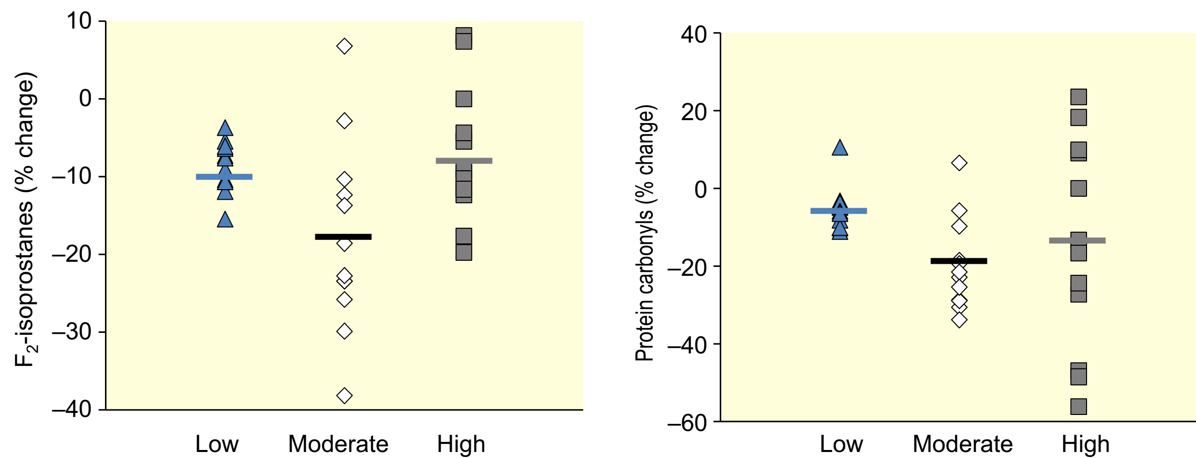

Figure 2 Resting oxidative stress biomarker adaptations in the three experimental groups.

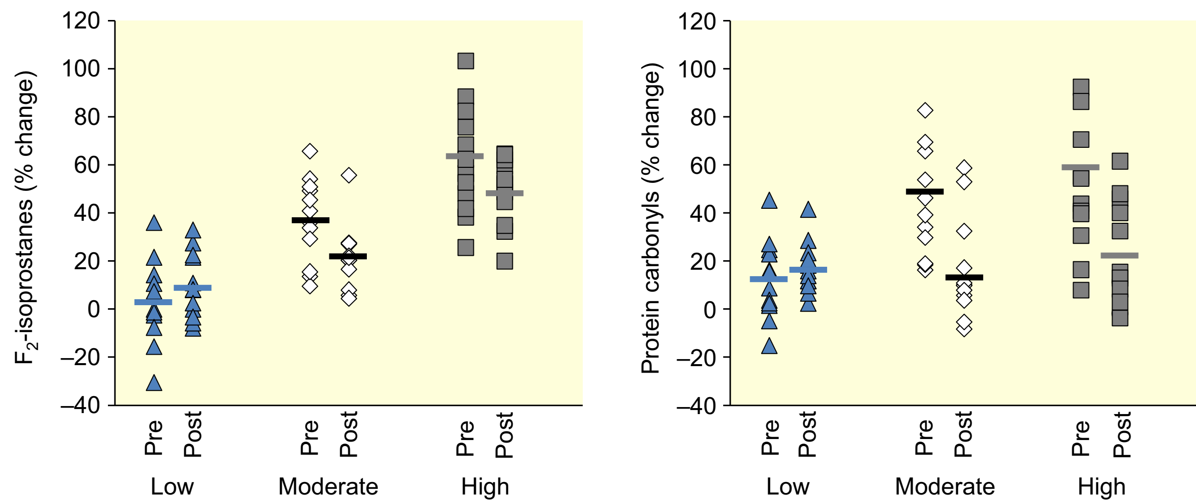

Figure 3 Acute exercise-induced oxidative stress biomarker changes pre- and post-training in the three experimental groups.

that regulate exercise adaptations and, in this context, RONS have been increasingly recognized as essential signalling molecules driving adaptations.[29–39] Our findings support the role of RONS produced during exercise as important 'exercise signals', yet, using a novel *in vivo* methodological approach.

The originality of the present work compared to all previous studies is that we investigated the *in vivo* role of RONS in the absence of any exogenous redox manipulation (i.e. administration of a pro-oxidant, antioxidant or RONS inhibitor). In particular, we exploited redox individuality, which is a readily observed phenomenon, but at the same time, widely underappreciated in redox literature.[23,40] More specifically, we used exercise-induced oxidative stress levels as a classifier to form three experimental groups, which were subsequently subjected to a 6-week endurance training protocol. The central hypothesis was that if RONS are indeed necessary for adaptations, then individuals with different levels of exercise-induced oxidative stress will also exhibit different adaptations. In line with our hypothesis, the low oxidative stress group (i.e. the group with the lowest RONS production after acute exercise) generally experienced the lowest adaptations in both physiological and redox characteristics. In fact, in some cases the adaptations developed were not even the half of the adaptations seen in the groups that experienced the moderate and the highest increases in RONS after acute exercise (e.g. 9% improvement in time trial in the low oxidative stress group vs. 22 and 24% improvement in the moderate and high oxidative stress groups respectively). Moreover, the absence of differences between the high and moderate oxidative stress groups highlights the fact that beyond a particular 'threshold' the magnitude of the RONS production

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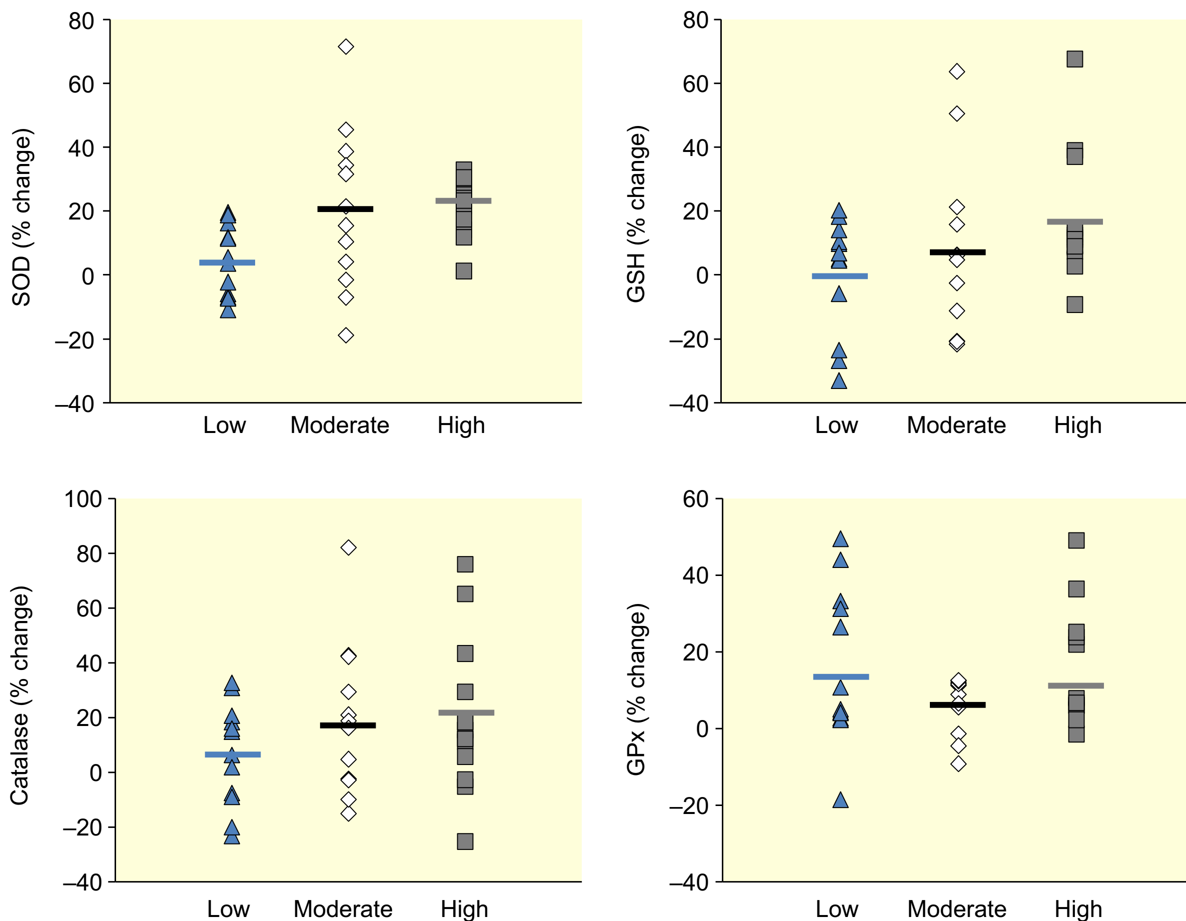

Figure 4 Resting antioxidant profile adaptations in the three experimental groups.

is not a key aspect. In other words, the 'exercise signal' effects of RONS are genuine, yet can be gained in response to either a moderate or a high production rate. Collectively, performance measurements show that exercise-induced oxidative stress regulates both aerobic (VO_2max and time trial) and anaerobic (Wingate test) adaptations to endurance training.

Exercise-induced oxidative stress regulates adaptations in performance

Nowadays, it is well established that endurance exercise improves cardiorespiratory fitness, an important predictor of cardiovascular mortality.[41] In the context of the present study, we measured VO_2max, which is considered the gold standard measure of cardiorespiratory fitness. Both baseline values and trainability of VO_2max after chronic exercise are generally characterized by wide interindividual variability. Despite that heritability can explain a large part of this variability (i.e. estimated at an almost 50%),[42] other intrinsic and acquired physiological and biochemical factors, such as the expansion of red blood cell volume and the improvement in stroke volume, also contribute to this cardiorespiratory fitness phenotype.[43] Herein, we investigated the role of RONS production in VO_2max adaptations and found that trainability of VO_2max after a chronic endurance protocol is determined, at least in part, by redox processes. More specifically, all groups exhibited significant gains in VO_2max, which were comparable to the gains reported previously in the literature in a similar population (i.e. approx. 16% VO_2max increase in young adult males).[44] However, the low oxidative stress group experienced the lowest improvements (i.e. 12% increase) compared to the moderate and the high oxidative stress groups (i.e. 17 and 19% respectively). A similar pattern was also observed in performance adaptations, assessed in the time trial and the Wingate test, with the low oxidative stress group exhibiting half (in Wingate) or less than half (in time trial) improvements compared to the other two groups. On this basis, we could rationally assert that the hotly debated issue of high and low responders in exercise adaptations[45,46] may have a redox background.

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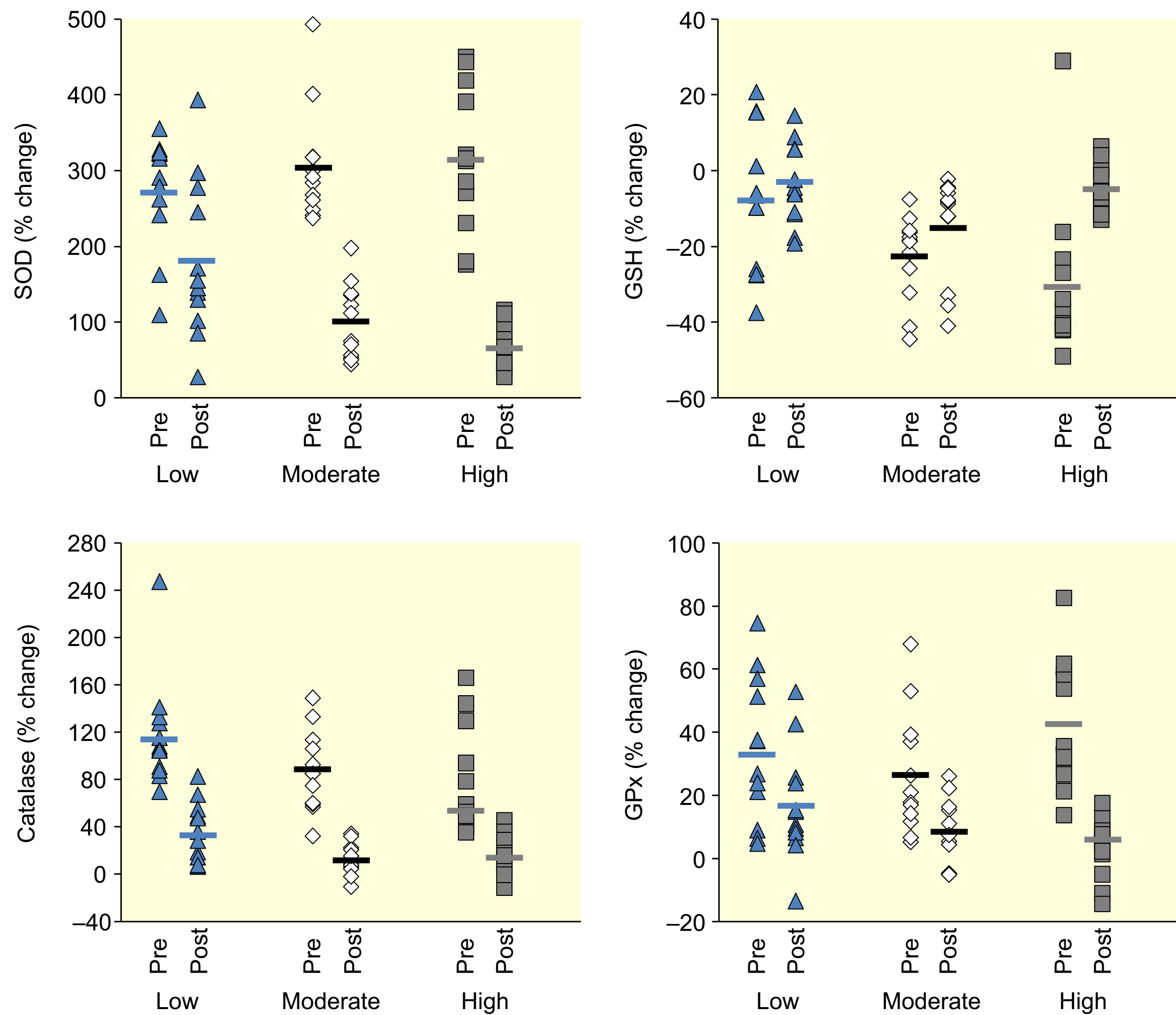

Figure 5 Acute exercise-induced antioxidant profile changes pre- and post-training in the three experimental groups.

The role of RONS in exercise performance adaptations has predominantly been investigated via molecular and/or biochemical markers and more rarely through *in vivo* integrated measurements. Despite that these markers provide crucial information about the mechanistic and compartmentalized redox signalling pathways that regulate specific adaptations,[10] it should be stressed that the molecular mechanisms often do not satisfactorily explain how the phenomena at the higher organizational level (i.e. organism) are possible and often cannot accurately predict what the higher-level phenomena will actually be at any given instance.[43,47] This is the reason why we have focused on physiology and measured a battery of classic adaptation biomarkers to exercise training (VO_2max, time trial and Wingate test). Apparently, analogous limitations exist in our case too. By performing a correlation analysis between the F_2-isoprostane changes and the adaptations in performance measures across the 36 trainees, we found moderate correlation coefficients for VO_2max ($r = 0.32$, $P = 0.05$), Wingate ($r = 0.46$, $P < 0.05$) and time trial ($r = 0.41$, $P < 0.05$). These moderate correlations can be explained by the 'nonlinear' communication between the distinct layers of biological organization (i.e. from molecular and cellular to organism and whole-body level).[10,48] More specifically in our case, the redundancy and in-built reserve capacity of redox processes (e.g. the suppression or the complete inhibition of an upstream redox signal does not always translate to a blunted downstream redox response) along with the emergent properties of a complex biological system could explain the results of our correlation analysis. Hitherto, the most common *in vivo* methodology to investigate the role of RONS in exercise adaptations is to utilize exogenous antioxidants aimed to neutralize the exercise-induced RONS production. Nevertheless, using this strategy, the subsequent outcomes

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cannot be safely attributed to the reduced RONS production due to the pleiotropic nature of antioxidants.[49] Thus, we applied a stratification approach to bypass the effects of the redox interventions on the biological system under study.

Although we acknowledge that the degree of exercise-induced oxidative stress was dependent on the resting levels of oxidative stress ($r = -0.71$, between resting F_2-isoprostane levels and the exercise-induced F_2-isoprostane changes in the 36 participants), we believe that the ultimate driving force for the different response to training was the distinct levels of oxidative stress produced by the three groups during each exercise session. This is also in line with the current consensus, which supports that the main driver for adaptations is the molecular events that occur during and shortly after a single session of exercise that will, over the period of weeks, 'accumulate' to cause a change in physiological capacity (e.g. VO_2max).[2] In addition, it is worth mentioning that before training (i.e. baseline) no difference was found among the three oxidative stress groups in all three performance tests (i.e. VO_2max, Wingate, time trial; Table 2). To our opinion, this indicates that the different resting redox state of the groups did not exert any significant role on exercise performance before the start of the training programme. This also ensured that the participants of the three groups made a similar 'exercise effort' that triggered the adaptive responses.

Exercise-induced oxidative stress regulates adaptations in redox metabolism

Exercise training induces a wide variety of redox adaptations, best exemplified by the increased resting levels of low molecular antioxidants and increased activity of antioxidant enzymes[50] as well as by the reduced magnitude of exercise-induced changes in oxidative stress biomarkers.[51,52] Our findings are in line with this consensus. However, these beneficial adaptations were predominantly seen in the high and moderate oxidative stress groups. For instance, resting glutathione levels and superoxide dismutase activity increased following the 6-weeks endurance training in the high oxidative stress group by 18 and 22%, and in the moderate oxidative stress group by an 8 and 20% respectively (Fig. 4). On the contrary, the low oxidative stress group did not exhibit any significant change in resting glutathione levels, while a minor 4% increase was found in superoxide dismutase activity. This was also the case for the adaptations in the acute exercise-induced changes in redox profile. More specifically, the low oxidative stress group exhibited a blunted adaptation (pre- to post-training) in the acute oxidative stress and antioxidant changes compared to the other groups (Figs 3 and 5). The above evidence suggests an improved redox profile in the high and moderate oxidative stress groups, which was hampered or completely absent (i.e. no change in resting glutathione levels) in the low oxidative stress group.

Urine F_2-isoprostanes as classifier and the use of systemic redox biomarkers

F_2-isoprostanes were used as the classification measure, because they are generally regarded the reference biomarker for oxidative stress assessment, and their urine concentration provides an integrated view of the redox state of an organism.[52] More specifically, arachidonic acid, the precursor molecule of F_2-isoprostanes, is mostly found in esterified forms in membranes and lipoproteins of almost all tissues, instead of a free form. Hence, F_2-isoprostanes are initially produced esterified and are then hydrolysed to free forms and released into the circulation through the action of specific enzymes, such as phospholipase A2 and platelet activating factor acetylhydrolase.[53] Afterwards, F_2-isoprostanes are further metabolized in the circulation and/or excreted in the urine. Consequently, the different rates of RONS production at rest, along with the different rates of clearance (i.e. renal function), may explain the basal redox variability among participants. The high levels of F_2-isoprostanes detected in urine after acute exercise may have been produced in virtually all tissues (including the kidneys) of the human body.[52]

We preferred a systemic oxidative stress biomarker, as the physiological adaptations evaluated herein are not determined by the function of one tissue only. Instead, they depend on an integrated set of blood, neuromuscular, metabolic and cardiovascular functions.[44] In addition, we preferred to measure F_2-isoprostanes in urine instead of plasma because of the short half-life of F_2-isoprostanes in plasma (<20 min).[54] The marked increases in F_2-isoprostane levels detected after the acute exercise sessions indicate that there was enough time for F_2-isoprostanes to accumulate in urine during the 45-min exercise period and justify our choice to collect urine as the classifier specimen instead of plasma.

For the determination of the training-induced redox adaptations, a battery of redox biomarkers (i.e. lipid peroxidation and protein oxidation biomarkers, enzymatic and non-enzymatic antioxidants) was assessed in different biological matrices (i.e. plasma, erythrocytes and urine). This option provides the opportunity to acquire a more comprehensive redox perspective.[55] Although oxidative stress biomarkers have been criticized as indirect markers of RONS production that do not provide mechanistic insights, in the context of the

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present study, F_2-isoprostanes have been used as diagnostics of oxidative stress and in order to pinpoint a likely redox component in a physiological process (i.e. exercise adaptations).[56]

Conclusion

Exercise triggers various biological events that act as signals for adaptations, such as mechanical load, neuronal activation and hormonal and metabolic fluctuations.[57] Most of the evidence presented in this study indicates that the RONS produced during exercise seem to be an additional essential 'signal' for adaptations. More specifically, our first *in vivo* data denote that exercise-induced oxidative stress regulates, at least in part, adaptations in performance and redox metabolism. These findings were obtained using a novel methodological approach that does not disturb human metabolism (e.g. by administration of pleiotropic antioxidants). We suggest that the stratification of individuals, based on oxidative stress levels in response to a stimulus, could be a valuable experimental strategy to reveal the role of RONS and antioxidants in biology.

Materials and methods

Participants

One hundred ($N = 100$) recreationally active young male volunteers (18–29 years old) participated in the study. Subjects were excluded from the study, if they reported musculoskeletal injury that would limit their ability to perform the exercise sessions. Participants were asked to recall whether they had participated in regular resistance or aerobic training or in unaccustomed exercise (e.g. soccer, competitive running, high-impact aerobics) during the period before the study entry. Individuals who reported such activities were precluded from the study. Smoking and consumption of antioxidant supplements the days prior to the study were additional exclusion criteria. Participants were also instructed to abstain from any strenuous exercise during the study and were advised to refrain from anti-inflammatory or analgesic medications. A written consent was obtained from all participants, after they were informed for the procedures and risks of the study. The procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. The overall project was reviewed and approved by the institutional review board (#83022/2015).

Study design

The study design is illustrated in Fig. 6.

Phase 1. The participants ($N = 100$) arrived at the laboratory at two different occasions between 08:00 and 10:00 after an overnight fast, in order to avoid the effect of diet on redox status (e.g. sulphur-containing nutrients increase glutathione levels). During the first visit, physiological measurements were performed (body fat was assessed with the use of skinfold callipers) and, then, participants performed a VO_2max test. During the second visit, at least 3 days later, they performed an acute cycling session at 70% Wmax. Spot urine samples were collected before and immediately after the acute exercise session in order to determine the level of exercise-induced oxidative stress by measuring F_2-isoprostanes.[52] Next, participants were sorted by increasing levels of exercise-induced oxidative stress (i.e. participant No. 1 was the participant with the lowest oxidative stress and participant No. 100 was the participant with the highest oxidative stress).

Three groups were formed ($n = 12$ individuals per group): (i) the low oxidative stress group, consisting of participants with the lowest levels of exercise-induced oxidative stress plus those experiencing reductive stress (i.e. participants No. 1–12 in the sorting scale), (ii) the moderate oxidative stress group, consisting of participants with moderate levels of exercise-induced oxidative stress (i.e. participants No. 44–55 in the sorting scale), and (iii) the high oxidative stress group, consisting of participants with the highest levels of exercise-induced oxidative stress (i.e. participants No. 89–100 in the sorting scale). In order to bypass the regression to the mean artefact, the three aforementioned groups were subjected to a second acute cycling session 1 week after the first session.[26] To ensure no interference from diet, participants were asked to record their diet 3 days before the first cycling session and to follow the same diet 3 days before the second session.

Similarly to the first acute session, spot urine samples were collected before and immediately following the second acute session to determine the level of exercise-induced oxidative stress. The value of this second measurement was subsequently used as the 'true' baseline value for the three experimental groups. Blood samples were also collected at the same time points for the evaluation of the rest redox parameters. The second session was followed by a time trial and a Wingate test to evaluate performance.

Phase 2. The three experimental groups (i.e. low, moderate and high oxidative stress) carried out a 6-week cycling training programme. At the end of the training period, another VO_2max test, an acute exercise session, a time trial and a Wingate test

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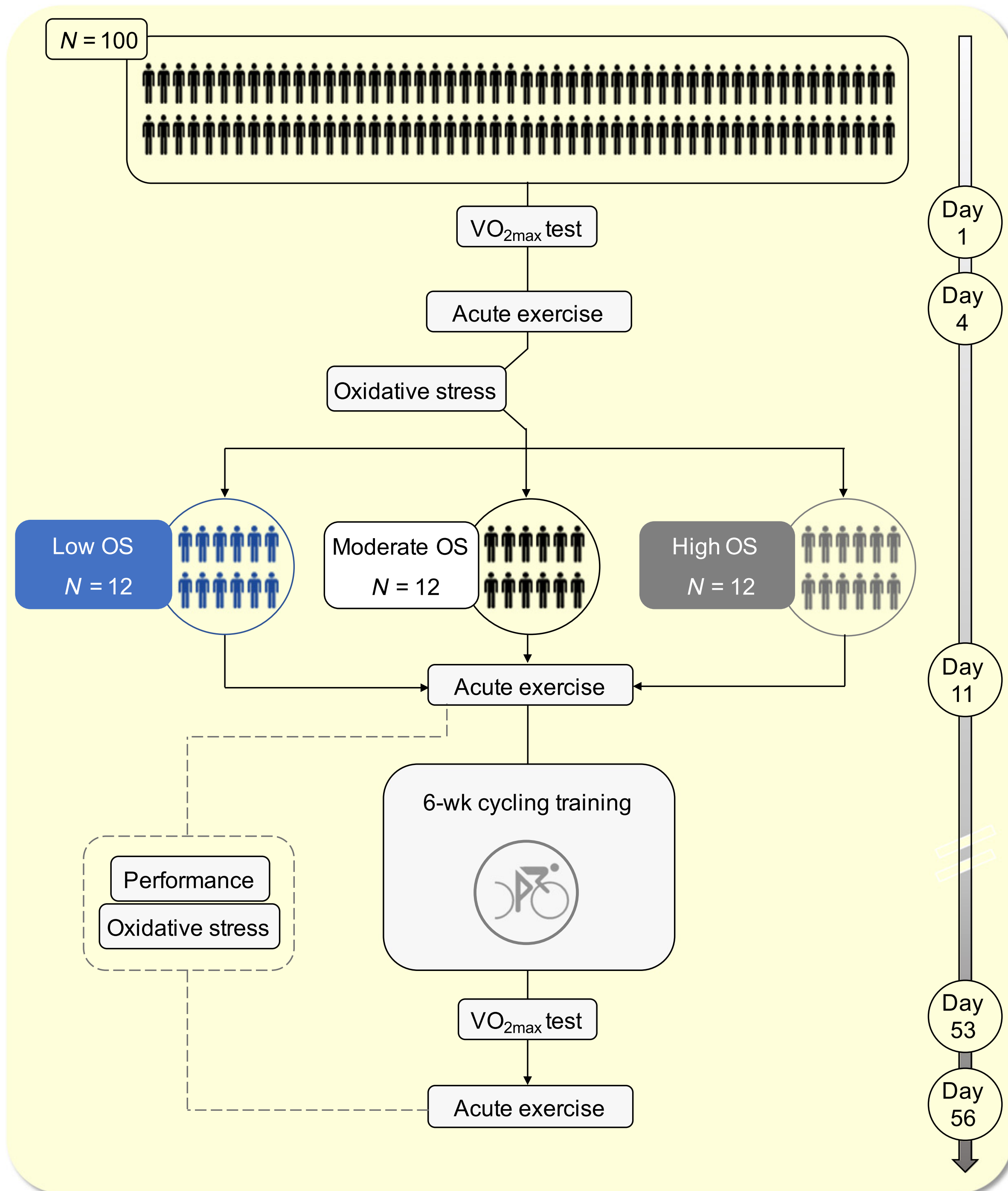

Figure 6 Study design.

were performed similarly to phase 1. The VO_2max test measured after training was used to calculate the new relative intensity for each participant, due to the adaptations that attained during the 6-weeks training period. Before and immediately after the acute exercise session, urine and blood samples were collected. To avoid any effect of diet on performance, participants were asked to follow the same diet 3 days before the acute exercise session, as was recorded at phase 1.

Exercise protocols

All exercise protocols were performed on cycle ergometers (Monark, Vansbro, Sweden). The VO_2max test was performed as described in Kuipers *et al.*[58]

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More specifically, after a 5-min warm-up period at 100 W, workload increased by 50 W every 2.5 min until heart rate reached 160 bpm. Then, workload increased by 25 W every 2.5 min until exhaustion. The test was terminated when three of the following four criteria were met: (i) volitional fatigue, (ii) a lower than 2 mL kg^{-1} min^{-1} increase in VO_2 despite an increase in workload, (iii) a respiratory exchange ratio greater than or equal to 1.10 and (iv) heart rate within 10 bpm of the predicted maximal heart rate (220–age). Respiratory gas variables were measured via a metabolic cart (Quark b2, Cosmed, Italy), which was calibrated before each test using standard gases of known concentration. Wmax was calculated according to the formula: $W_{max} = W_{out} + (t/150) \times 25$, where W_{out} was the workload of the last completed stage and t was the time in seconds of the final uncompleted stage.[59] W_{max} was used as a reference value to ensure that all subjects cycled at a similar relative intensity (70% W_{max}) during the two acute exercise sessions and during the 6-weeks training programme.

The acute exercise session was merged with the time trial and constituted a slightly modified version of the protocol B in Jeukendrup *et al.*[59] More specifically, participants cycled for 45 min with 60 rpm at an intensity corresponding to their 70% W_{max} (i.e. the acute exercise protocol). Subsequently, participants provided urine and blood samples and then returned to the cycle ergometer and were urged to perform as much work as possible for 15 min (i.e. time trial). The average amount of power produced during this period (W_{15}) was used as criterion measure of aerobic performance. The Wingate test was briefly as follows: subjects were pedalling with no brake on flywheel as fast as they could and within 3-s, a load equal to 7.5% body weight was dropped instantly on the flywheel and the subjects had to maintain maximal pedalling for 30 s. The power output produced during the Wingate test was used as a criterion measure of anaerobic performance. The 6-weeks cycling training protocol consisted of three exercise sessions per week, lasting 45 min per session at an intensity corresponding to the 70% W_{max} of each participant.

Collection and handling of samples

Urine spot samples were collected in a container and were purified using solid-phase extraction cartridges. For standardizing urine dilution, creatinine levels were measured using a commercially available kit. Blood samples were drawn from a forearm vein, collected in EDTA tubes and centrifuged immediately at 1370 *g* for 10 min at 4°C, and the plasma was collected. The packed erythrocytes were lysed with 1:1 (v/v) distilled water, inverted vigorously and centrifuged at 4000 *g* for 15 min at 4°C. All body fluid samples were stored at −80°C and thawed only once before analysis.

Redox measurements

Urine F_2-isoprostanes, plasma protein carbonyls and erythrocyte glutathione, superoxide dismutase, catalase and glutathione peroxidase were determined as previously described.[60]

Statistical analysis

The normality of distribution of all dependent variables was examined by the Kolmogorov–Smirnov test. Student's *t*-test for paired samples was performed to investigate the effect of acute exercise on the F_2-isoprostane levels in all initially enrolled individuals ($N = 100$). After stratification, the three groups formed ($n = 12$/per group) were compared for differences in physiological characteristics by one-way analysis of variance (ANOVA). In addition, two separate two-way ANOVA tests [group (high, moderate and low oxidative stress) × time (pre- and post-exercise)] were used to compare the effect of the two acute exercise sessions on the F_2-isoprostane levels between the three groups and a two-way ANOVA [session (first and second) × time (pre- and post-exercise)] for each group to examine whether regression to the mean affected our results. Furthermore, an analysis of variance with repeated measures was used to analyse the physiological [group (high, moderate and low oxidative stress) × condition (pre- and post-chronic exercise)] and redox adaptations [group (high, moderate and low oxidative stress) × condition (pre- and post-chronic exercise) × time (pre- and post-exercise)] that took place during the 6-weeks training programme. When a significant interaction was obtained, pairwise comparisons were performed through the Sidak test. When sphericity was violated, the Greenhouse–Geisser correction was applied. Finally, a correlation analysis between the F_2-isoprostane changes and the adaptations in performance measures across the 36 trainees was performed. Data are presented as mean ± standard deviation (SD) and the level of significance was set at $a = 0.05$. The SPSS version 21.0 was used for all analyses (SPSS, Chicago, IL, USA).

Data in the figures show per cent changes. We did not perform statistical analyses on these data, as per cent changes are just ratios, and, therefore, may lead to misleading results.[61] Thus, we performed statistical analyses using the raw data, while the figures presenting the per cent changes were used complementarily in order to better illustrate the findings of the study. We acknowledge that this is not a common practice

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for presenting research data (i.e. Tables with raw data and statistics accompanied by figures with per cent changes). Yet, we believe that this 'joint' presentation more comprehensively confers the main message of our study.

Conflict of interest

The authors declare no conflict of interest.

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