Impact of off-road competitive motocross race on plasma oxidative stress and damage markers

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ABSTRACT

Objective: To analyze the impact of an off-road motocross heat on plasma levels of oxidative stress and damage, blood leukocyte counts and urine catecholamine concentration.

Methods: Plasma contents of total (TGSH), reduced (GSH), oxidized (GSSG) glutathione, %GSSG, malondialdehyde (MDA), protein carbonyl and –SH groups, total antioxidant status (TAS) and uric acid (UA) as well as blood neutrophil and lymphocyte counts were evaluated in ten male top-level riders before, immediately and one hour after a simulated competitive motocross race. 24-hours urine adrenaline, noradrenaline and dopamine concentrations were also measured.

Results: Motocross heat resulted in an increase in plasma oxidative stress and damage (p<0.05). This was observed by significant increase in %GSSG, TAS, MDA and carbonyls as well as by decrease in –SH after the race. There was a significant increase in both plasma UA and urine catecholamine concentration after the race (p<0.05). Blood neutrophil counts increased at zero and one hour after exercise (p<0.05). Lymphocyte counts increased from baseline to zero hours, although decreased from baseline and zero to one hour post exercise (p<0.05).

Conclusion: The data reinforce the notable metabolic and hormonal demands imposed by motocross, resulting in a condition of enhanced plasma oxidative stress and damage.
INTRODUCTION
Motorcycling is a motor sport modality that includes several disciplines such as speed, enduro, trial and motocross. Several particular features in off-road motocross notably increase the physical and physiological demands for the riders. These include the irregular terrain, dirt and sharp turns that lead to the so-called “arm pump” – a muscular stress suited on upper limbs as several enduring isometric and/or eccentric muscular contractions are necessary to absorb shocks caused by roughness and motorbike handling during the constant surface driving and landings. Given that motor sports are representative of psycho-emotional stress activities, further catecholamine release is expected during this type of exercise. Notwithstanding the notably elevated number of isometric contractions, the mean oxygen consumption previously reported in motocross are suggestive of the elevated metabolic aerobic contribution for performance. In this regard, it is tempting to hypothesize that these characteristics, isolated or in combination, may favor the enhanced reactive oxygen and nitrogen species (RONS) generation. This so-called oxidative stress, results from an imbalance between RONS production such as superoxide (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$) and peroxinitrite (ONOO$^-$) and the ability of body antioxidant systems to counteract RONS. The consequent muscle oxidative damage, which has been widely reported following different types of physical exercise, predominantly during eccentric exercise, contributes to temporarily loss of the exercising capacity of muscle for force production compromising exercising capacity.

To date and to the best of our knowledge, there is no study analyzing the impact of motocross on oxidative stress and damage biomarkers. Thus, the purpose of the present study was to examine the effect of a motocross heat on plasma content of oxidative stress (total, reduced and oxidized glutathione, total antioxidant status – TAS) and damage (malondialdehyde - MDA, sulphhydryl – SH and carbonyls). Levels of urine catecholamine, plasma UA and blood leukocyte counts were also determined.

METHODS
Subjects
Ten top-elite non-smoker male off-road motocross riders with international experience participated in this research after being informed about the aims, experimental protocol and giving writing consent to participate. This protocol was approved by the Ethical Committee of Faculty of Sport, University of Porto, Portugal, which followed the Declaration of Helsinki for research with humans. All subjects were previously familiarized with the settings carried out in this study.

For 4 weeks prior to data collection, subjects refrained from additional vitamin dietary supplementation. The riders were instructed not to introduce appreciable deviations from their normal eating habits during this period. The participants were tested within one week and the experimental protocol included a laboratory and a field test session separated by three days (Fig 1).

Laboratory and field evaluations
After prediction of the %body fat through skin-fold equation, the riders performed an incremental treadmill (Quasar-Med, Nussdorf, Germany) test until voluntary exhaustion to determine maximal oxygen uptake (VO$_2$max), maximal heart rate (HRmax) and anaerobic ventilatory
threshold (Lan). Expired respiratory gas fractions were measured using an open circuit breath-by-breath automated gas-analysis system (Cortex, Metalyzer, 3 B, Leipzig, Germany).

HR was measured using a HR monitor (Vantage NV, Polar Electro, Kempele, Finland). Capillary blood was collected from the ear lobe and immediately analyzed using an electroenzymatic device (YSI® 1500S, Yellow Springs, OH).

Participants underwent a simulated competitive heat in a 1.5 Km motocross circuit. Changes induced by race effort on biochemical markers (capillary blood lactate, urine catecholamine contents, plasma oxidative stress and damage) were analyzed before, immediately and one hour after the end of the race. HR and blood lactate concentration were outlined throughout the heat.

Catecholamine measurements

Urine catecholamine levels were determined before and after (24-hour continuous urine sample) the heat by means of HPLC (Waters, model 600) with electrochemical detection, using a commercial Kit, from Chromsystems (ref. 6000). Dihydroxybenzylamine (Sigma Chemical) was used as the internal standard. All steps related to the procedures of sample preparation, extraction and elution were performed according to the manufacturer instructions.

Blood lactate

During the race, each rider stopped for only 15 sec at 10th and 20th minute to collect a 30μL blood sample that was immediately analyzed for lactate concentration as described. Blood lactate concentration was also measured immediately before and after the end of the race.

Venous blood sampling and preparations

Blood samples (5mL) were withdrawn three times during the experimental protocol from the antecubital vein. The first blood drawn was taken at rest and the other two at zero (immediately after the end of the race) and after one hour. During blood drawn, no tourniquet constriction was used in order to minimize potentially enhanced oxidative stress induced by an ischaemia-reperfusion maneuver. All samples were taken using plastic syringes, placed in EDTA-containing tubes and immediately centrifuged during 10 minutes at 1500 rpm. An aliquot of whole blood was separated for leukocyte counts. From the remaining blood, plasma was separated into several aliquots and rapidly frozen at –80°C for later biochemical analysis of glutathione, -SH, MDA, carbonyls and UA contents.

White blood cell counts

Leukocyte count were assessed by an automatic cell counter (Horiba ABX Micros 60; ABX Diagnostics, Montpellier, France) calibrated with an ABX Minocal (ABX Diagnostics); the intra-assay coefficient of variation (CV) determined on five replicates of each leukocyte measurement was <1%. Whole blood smears on glass slides (VBS 655/A Microscope - Biosigma) were used for white blood cell differential analysis. Smears were stained using Wright coloring (Merck) and air-dried. Cell differentials were performed using an Olympus microscope equipped with 1000X oil immersion lens. Specifically, the leukocyte counts including neutrophils, eosinophils, basophils, monocytes and lymphocytes were recorded.

Oxidative stress and damage markers

UA was determined by an enzymatic method at 550nm using a commercial kit (Horiba ABX A11A01670, Montpellier, France) according to the specifications of the manufacturer.
Total antioxidant status (TAS) was measured spectrophotometrically at 600nm using a commercial kit (Randox NX2332 Crumlin, UK).

Plasma TGSH and GSSG measurements were spectrophotometrically determined at 412nm as previously described by Tietze.\textsuperscript{3} TGSH and GSSG concentrations were established based on calibration curves made with commercial standards. Reduced (GSH) and percentage of oxidized glutathione (%GSSG) were calculated as follows: GSH=TGSH-GSSG; %GSSG = (GSSG/TGSH)*100.

The plasma content of oxidative modified sulfhydryl protein groups was quantified by spectrophotometric measurement at 414 nm according to the method proposed by Hu.\textsuperscript{4}

Lipid peroxidation was spectrophotometrically measured at 535nm by determining the levels of lipid peroxides as the amount of thiobarbituric acid reactive substances (TBARS) formed according to Rohn \textit{et al}\textsuperscript{5} with some modifications.

Protein carbonyl derivatives were assayed according to Robinson \textit{et al}\textsuperscript{6} with some modifications. A certain plasma volume (V) containing 20µg of protein was derivatized with dinitrophenylhydrazine (DNPH). After diluting the derivatized proteins in TBS, a 100ml volume was slot-blotted into a Hybond-PVDF membrane. Immunodetection of carbonyls was then performed using rabbit polyclonal anti-DNP (1:2000; V0401 DakoCytomation). Bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier’s instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software 4.3.1 (BioRad). Optical density results were expressed as percentage variation of control values. Protein content was spectrophotometrically assayed using bovine serum albumin as standard according to Lowry \textit{et al}.\textsuperscript{7}

\section*{Statistics}
Descriptive measures were calculated for all variables. ANOVA for repeated measures was used to compare biochemical variables between baseline, 0 and 1 hour. The Pearson correlation coefficient was used to analyze the inter-correlations between UA and TAS. Statistical Package for the Social Sciences (SPSS Inc, version 12.0) was used for all analysis. The significance level was set at 5%.

\section*{RESULTS}
The anthropometric and physiological characteristics of participants are shown in Table 1. At the day of the heat, temperature and relative humidity levels were within the range of 18-22°C and 60-65%, respectively.

\begin{table}[h]
\centering
\begin{tabular}{ll}
\hline
Variables & Mean±SD\
\hline
Age (yr) & 28.3 ± 7.9 \\
Mass (kg) & 71.1 ± 7.0 \\
Height (cm) & 169.8 ± 4.0 \\
% Body fat & 14.9 ± 3.3 \\
HRmax (beats.min\textsuperscript{-1}) & 198.3 ± 4.4 \\
VO\textsubscript{2}max (L.min\textsuperscript{-1}) & 3.8 ± 0.4 \\
VO\textsubscript{2}max (mL.kg\textsuperscript{-1}.min\textsuperscript{-1}) & 53.5 ± 3.7 \\
Lan vent (mL.kg\textsuperscript{-1}.min\textsuperscript{-1}) & 42.0 ± 5.6 \\
\hline
\end{tabular}
\caption{Characteristics of the subjects}
\end{table}

HR, heart rate; VO\textsubscript{2}, oxygen consumption; Lan vent, ventilatory threshold.
As can be depicted from Table 2, elevated HR and estimated oxygen consumption were observed during the race.

**Table 2 Main characteristics of the race.**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>30min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRmax lab (beats.min⁻¹)</td>
<td>198.3 ± 4.4</td>
</tr>
<tr>
<td>HRmax field (beats.min⁻¹)</td>
<td>197.7 ± 8.0</td>
</tr>
<tr>
<td>HR average (beats.min⁻¹)</td>
<td>180.7 ± 9.5</td>
</tr>
<tr>
<td>%VO₂max</td>
<td>90.8 ± 6.8</td>
</tr>
<tr>
<td>Lactate concentration (mM)</td>
<td>5.4 ± 1.2</td>
</tr>
</tbody>
</table>

Values are mean and SD; HRmax lab, maximum heart rate obtained in the laboratory test; HRmax field, maximum heart rate obtained during the race; HRmax average, average heart rate; %VO₂max, estimated intensity of the race from the corresponding heart rate determined in the lab treadmill running test and expressed in %VO₂max.

As can be depicted from figure 2, plasma carbonyl levels increased both at zero and at one-hour post exercise. Plasma lipoperoxidation expressed as MDA significantly rose immediately after motocross heat and returned to initial values after one hour (p<0.05). Sulphydryl protein groups (-SH) significantly decreased both immediately and one hour after the end of the race (p<0.05). Motocross race induced a significant decrease in TGSH and GSH levels, whereas GSSG and %GSSG increased. These changes were observed both immediately and one hour after the race (p<0.05). There was a significant rise in plasma UA as well as in TAS from baseline to zero and one hour after motocross race (Table 3). Significant correlation was found between TAS and UA levels (r=0.72; p<0.001).

**Table 3 Plasma contents of total (TGSH), reduced (GSH), oxidised (GSSG), %GSSG, malondialdehyde (MDA) and sulfhydryl protein groups (-SH), total antioxidant status (TAS) and uric acid (UA) at baseline, immediately and one hour after the race.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>0 hour</th>
<th>1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGSH (nmol/mg prot)</td>
<td>0.234 ± 0.05</td>
<td>0.187 ± 0.02 *</td>
<td>0.183 ± 0.04 *</td>
</tr>
<tr>
<td>GSH (nmol/mg prot)</td>
<td>0.216 ± 0.03</td>
<td>0.142 ± 0.06 *</td>
<td>0.146 ± 0.03 *</td>
</tr>
<tr>
<td>GSSG (nmol/mg prot)</td>
<td>0.018 ± 0.005</td>
<td>0.045 ± 0.006 *</td>
<td>0.037 ± 0.003 *</td>
</tr>
<tr>
<td>%GSSG</td>
<td>7.82 ± 0.54</td>
<td>24.95 ± 1.02 *</td>
<td>19.99 ± 1.05 *</td>
</tr>
<tr>
<td>MDA (nmol/g prot)</td>
<td>324.79 ± 13.73</td>
<td>394.63 ± 11.91 *</td>
<td>303.43 ± 16.76</td>
</tr>
<tr>
<td>-SH (mmol/g prot)</td>
<td>6.21 ± 0.22</td>
<td>5.35 ± 0.18 *</td>
<td>4.04 ± 0.29 *</td>
</tr>
<tr>
<td>TAS (mM)</td>
<td>1.51 ± 0.05</td>
<td>1.83 ± 0.06 *</td>
<td>1.86 ± 0.05 *</td>
</tr>
<tr>
<td>UA (mg/dl)</td>
<td>6.36 ± 0.35</td>
<td>7.34 ± 0.46 *</td>
<td>7.26 ± 0.53 *</td>
</tr>
</tbody>
</table>

Values are mean and SEM; * vs. Baseline (p<0.05)

Blood leukocyte and neutrophil counts were markedly higher at zero and one hour post exercise when compared to pre-exercise levels (p<0.05). Although lymphocyte count significantly rose at zero hours after exercise, they decreased below rest values after one-hour post exercise (Table 4).

**Table 4 Changes in blood leukocyte, neutrophil and lymphocyte counts before, immediately and one hour after simulated competitive motocross race.**
A significant increase in the 24-h urine catecholamine (adrenaline, noradrenaline and dopamine) concentration was found after the race when compared to rest values (p<0.05) (Figure 3).

DISCUSSION

Specific motocross effort implies several particular acute physiological changes such as increased cardiac output and blood flow, augmented catecholamine release, high contractile isometric and eccentric demands and importantly relies on aerobic metabolism. Given that these are predisposing conditions for pro-oxidant redox changes in human body, we tested the hypothesis that a simulated motocross heat induces alterations in plasma markers of oxidative stress and damage. Our data demonstrated for the first time that a single competitive heat resulted in an increased expression of oxidative stress and damage markers in plasma, urine catecholamines and significant changes in neutrophil and lymphocyte counts.

The participants in this study are high-level professional motocross pilots involved in daily specific training sessions. Several studies in the literature reported that chronic exercise might attenuate the increased oxidative stress and damage caused by severe acute stimuli. This fact has been attributed to the increase endogenous up-regulation of both enzymatic and non-enzymatic antioxidants and/or a more tightly coupled electron transport system allowing fewer electrons to escape from mitochondrial electron transport chain and thus form O$_2^-$ radicals (this for mostly aerobic exercises). Therefore, and considering the training status of the pilots, the lack of changes in the markers of oxidative stress and damage was initially unlikely to exclude.

As an estimated 1-5% of the total VO$_2$ results in the formation of O$_2^-$ and given the high level of VO$_2$ accompanying motocross heat, it is not surprising that the biomarkers of oxidative stress and damage had increased. In addition, other concurrent factors can influence cellular and blood antioxidant status. For example, stress hormones undergoing autoxidation and circulating neutrophils-induced oxidative burst can contribute to the observed blood oxidative stress and damage. The influence of isometric exercise-mediating muscular ischemia-reperfusion like events on the formation of RONS has also been reported. Considering the specific physiological demands imposed by motocross, none of these potential RONS sources should be ruled out in the current study. However, it is important to note that under the technical constrains of the present study we cannot conclusively demonstrate a casual link between any of those potential sources and the increased plasma oxidative stress and damage found.

Considering that %GSSG is a sensitive marker of enhanced oxidative stress closely related to brisk cellular redox changes, the significant increase from baseline to 0h and 1h post exercise seems to demonstrate an additional free radical production and an overwhelming of antioxidant capacity induced by the race. The increase in %GSSG observed immediately after the end of the heat suggests that during this stress period, and despite tissues GSH oxidation and compensatory GSH importation from plasma to protect cells against enhanced RONS production, hepatic exportation of GSH to circulation was insufficient to maintain its concentrations. Consequently, the levels of plasma TGSH decreased, regardless the rise observed in plasma GSSG. The increased %GSSG was likely related to both marked plasma GSH oxidation and also to GSSG exportation from the tissues, due to its toxic effect and destabilizing action on cell redox status...
when present at high concentrations. Indeed, off-road motocross-induced tissue oxidative stress seems to lead to incapacity of the liver for keeping up sufficient GSH exportation to maintain their plasma levels and consequently to plasma redox status.

Surprisingly, we found that plasma TAS significantly increased after the race, which may indicate compensation in response to intense exercise. Previous studies have shown that half-marathon in trained male runners and treadmill running until exhaustion also induced increase in total antioxidant capacity. Considering that TAS assay only measures the antioxidant capacity of the aqueous blood compartment, which relies mostly on protein (10–28%), UA (7–58%) and ascorbic acid (3–27%), the increase in TAS observed immediately after exercise seems to reflect and/or be influenced, at least partially, by the significant increase observed in UA, as suggested by the elevated correlation found between TAS and UA (r=0.72; p<0.001). In fact, although being an end product of the purine nucleotide system, UA scavenge OH− radicals as well, and there is evidence that it may be an important biological scavenger against free radicals in human plasma and in skeletal muscle during and after acute hard exercise. This well-known free radical quenching action of UA might have contributed in this particular case to an attenuation of the rise in plasma oxidative damage.

During high intensity exercise and muscle ischemic conditions, the purine nucleotide system is extremely active and the elimination of adenosine monophosphate (AMP) causes a build-up of hypoxanthine in skeletal muscle and in plasma. Despite some may be converted back to AMP during rest and at lower exercise intensities, hypoxanthine is also converted to UA generating O2−. Given the lactate concentrations measured in the present study after exercise (Table 3), possible lactate-induced inhibition of renal UA clearance may also further contribute, at least partially, for the rise in UA after the race. The observation that plasma UA levels increased in response to motocross race is consistent with the findings from other studies using exercise. In this regard, it is likely that the observed increased oxidative stress and damage during the intense exercise that off-road motocross represents might have the contribution, at least partially, of xanthine oxidase free radical generating system.

In the present study, the pro-oxidative condition induced by motocross and suggested by the increase in %GSSG was accompanied by the significant accumulation of lipid peroxidation and protein oxidation by-products in plasma (table 3 and figure 2, respectively). Accordingly, the race also induced a significant reduction in plasma sulfhydryl residues (-SH), indicating increased disulfide linkages (-S-S-) from both proteins and GSH.

As previously described by others, the present data showed that motocross induced a leukocytosis dependent on neutrophilia, which can be ascribed to the mobilization of blood cells from marginal pools by hemodynamic redistribution and augmentation that resulted from exercise-related metabolic conditions, such as enhanced catecholamine secretion imposed by motocross. Regardless some controversy on the involvement of neutrophils in exercise-induced oxidative stress, previous studies using chemiluminescence techniques had shown that intense exercise was able to increase the capacity of neutrophils for RONS generation. Nevertheless, the causal link between the increased oxidative damage and neutrophilia observed in the present study should be cautiously established, as we did not measure the levels of neutrophil activation.

In accordance with others, data from the present study reported higher lymphocyte counts immediately after exercise and a marked lymphocytopenia during the subsequent hours after the end of exercise. Although Steensberg et al observed that, even in a study in which high levels of apoptosis-inducing factors were generated, such as cortisol and isoprostanes, lymphocyte apoptosis did not contribute to post-exercise lymphocytopenia, others suggested that apoptosis may partially account for the transient loss of lymphocytes after intense exercise with
consequent immunosuppression. Moreover, as hormonal changes such as catecholamine overproduction during exercise have been described to be responsible for inducing apoptosis, the increased dopamine release observed in the present study might likely be a contributor as well.

In conclusion, the data from the present study reinforce the notable metabolic and hormonal demands imposed by motocross to the riders, which result in a condition of plasma oxidative stress and damage. The specific nature and the contribution of the different RONS sources for this oxidative burst need to be clarified. Further studies in this field are needed in order to analyze whether more accurate and suited redox balances, obtained after antioxidant supplementation-based strategies, could be useful for the pilots and for performance, particularly during fully intense training periods.

ACKNOWLEDGMENTS
We thankfully acknowledge to all the riders who participated in this study. The authors are also grateful to Dr Henrique Reguengo from the Department of Clinical Analysis of the Faculty of Pharmacy, University of Porto for his kind support in urine catecholamine assays. We also thank Dr Rocha e Costa for his kind help in blood cell counts.

FIGURE LEGENDS
Figure 1. Scheme summarizing the general lines of the experimental protocol.

Figure 2. Effect of motocross heat on the content of plasma carbonyl derivatives obtained from 8 independent subjects. Immediately below the histogram, the protein carbonyl formation panel shows a representative pattern of anti-denitrophenyl (DNP)-specific interaction with DNP for each moment as described in METHODS. Values (mean and SEM) are expressed as percentage of baseline * p<0.05 Baseline vs. other moments.

Figure 3. Mean 24-h urinary epinephrine, norepinephrine and dopamine excretion rates in riders before and after the race. Values are mean and SEM. Measurements were made at rest (throughout 24-h before the race) and during the immediate 24-h period following the simulated competitive race *p<0.05, Before vs. after.

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24 Cioca DP, Watanabe N, Isobe M. Apoptosis of peripheral blood lymphocytes is induced by catecholamines. *Jpn Heart J* 2000;41:385-98.
What is already known on this topic?
Despite exercise-induced enhanced pro-oxidant redox changes is a widely studied phenomenon, the impact of this specific exercise effort – off-road motocross race – on plasma markers of oxidative stress and oxidative damage is not known.

What this study adds?
The results of this study demonstrated that an off-road motocross heat elicited increased levels of plasma oxidative stress and damage. Although the direct effects of enhanced oxidative stress on exercise performance are inconclusive, the results of the present study can be useful as a starting point for further works on the possible benefit of antioxidant supplementation on motocross performance.
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