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Urinary Levels of 8-Hydroxydeoxyguanosine as a Marker of Oxidative Damage in Road Cycling

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We have determined the urinary 8-hydroxydeoxyguanosine (8-OHdG) levels of eight professional cyclists during a 4-day and a 3-week stage races. Monitoring of heart rates was used to establish zones corresponding to different intensities of exercise. The urinary 8-OHdG excretion, expressed by body weight, increased significantly in the first day or the first week of each race, respectively, and did not show further increases thereafter. Maximum 8-OHdG levels were reached in parallel to longer times spent at high intensities of exercise. Urinary excretion of creatinine increased with exercise, and changes in 8-OHdG levels were not detected when corrected by creatinine excretion. Serum glutathione concentrations did not change significantly at any point during exercise. We conclude that road cycling courses with an oxidative damage to DNA, which is sustained as long as the exercise is repeated. Both adaptation of antioxidant defenses and a decreased capacity to maintain a high intensity of effort may contribute to explain the absence of progressive increases in 8-OHdG excretion. The results of this study also confirm that the correction procedure using the amount of creatinine excreted should not be used when studying effects of exercise on urinary 8-OHdG.

Keywords: DNA damage; Exercise; Oxidative stress; Urine

INTRODUCTION

The energy demand during physical exercise causes an increased oxygen uptake and supply to active tissues, which may increase the rate of reactive oxygen species (ROS) production and lead to lipid peroxidation and oxidative modifications of proteins and DNA.[1] The organism is naturally equipped with antioxidant protection systems to cope with the harmful effects of ROS and elevations in antioxidant defenses can occur during exercise, although it is not clear whether these responses prevent exercise-induced oxidative stress. Some studies report increased lipid peroxidation after exercise[2,3] but others provide no evidence.[4,5] These conflicts may arise from methodological differences, especially in the use of the TBARS assay to detect malondialdehyde.[2,6] Variations in exercise duration and intensity also seem to contribute to inconsistent results[7] and training may influence susceptibility to oxidative injury by enhancing some components of the antioxidant system.[3,8]

Oxidative damage to DNA is generally measured by levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) and excretion of 8-OHdG reflects the integrated rate of oxidative DNA damage and the repair of DNA in the whole body. Data obtained by different authors suggest that oxidative stress induced by a single bout of exercise does not increase consistently the oxidative DNA damage. Thus, exercise has been shown to induce DNA damage to polymorphonuclear leucocytes one day after half-marathon[9] and the ratio of 8-OHdG to creatinine excretion increases after a marathon race.[10] The urinary excretion of 8-OHdG did not change significantly during the 3-day period after acute exercise to exhaustion on a bicycle ergometer[11] and Hartmann *et al*.[12] reported no changes in leucocyte 8-OHdG levels after short-distance triathlon running in trained subjects.

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Contradictory reports on the long-term effects of exercise also exist. Levels of 8-OHdG do not differ between controls and long-distance runners[13] and Viguie *et al.*[4] observed no change in 8-OHdG excretion for three days after 90 min of moderate exercise at 65% peak O_2 uptake. In contrast, Poulsen *et al.*[14] reported an increase in the urinary excretion of 8-OHdG after a 30-day program of 8–11 h of vigorous exercise and increases in long-distance runners during participation in an 8-day training camp[15] or a 4-day race period[16] have also been observed. Discrepancies may arise from poor optimization of analytical procedures[17] or the use of creatinine as a reference marker.[18]

Professional road cycling is an extreme endurance sport with competitions ranging from 1-day to 22-day races in which cyclists cover up to 3500 km. Long duration and high intensity, with a high participation of aerobic metabolism,[19] make this sport an optimal model to study the oxidative damage induced by exercise. Our study aimed to investigate changes in the urinary excretion of 8-OHdG in professional cyclists participating in multi-day stage races and its relationship to the duration of exercise and intensity of effort quantified by telemetric heart rate monitors.[19,20]

MATERIALS AND METHODS

Subjects and Procedures

The subjects of this study were eight professional road cyclists (mean age 25.5 ± 1.7 yr). The study protocol conformed to the guidelines of the Helsinki Conference for research on human subjects and written informed consent was obtained prior to the study. Each subject was a highly trained cyclist who had been previously competing for at least three seasons. The total training performed during a year by these cyclists ranges from 30,000 to 40,000 km.

Laboratory Tests

Four weeks before participating in the first multi-stage race, subjects reported to the laboratory to have their body fat measured according to Jackson and Pollock.[21] Next, each subject was required to perform an incremental exercise test on a bicycle ergometer (Ergometrics 900; Ergo-line, Barcelona, Spain) following a ramp protocol until exhaustion. Starting at 100 W, the workload was increased by 25 W/min and pedaling cadence was kept constant at 90–105 rpm. Heart rate was continuously recorded with a telemeter (Sport Tester Xtrainer, Polar, Kempele, Finland). Gas exchange data were obtained using an automated breath-by-breath system (CPX Medical Graphics; St Paul, Minn). Each subject's anaerobic threshold was determined from plots of the ventilatory equivalents for $O_2(V_E \cdot VO_2^{-1})$ and $CO_2(V_E \cdot VCO_2^{-1})$ vs. time. The threshold was selected as the minute just before the occurrence of a sustained increase in $V_E \cdot VO_2^{-1}$ without a sustained increase in $V_E \cdot VCO_2^{-1}$.[20,22] Data were used to establish three heart-rate zones that corresponded to the following intensities of exercise: Anaerobic (AN), which was over the individual anaerobic threshold (this IAT was around 90% of VO_{2max}), Intense Aerobic (IA), which was between 60 and 90% of VO_{2max}, and Moderate Aerobic (MA), which was under 60% of VO_{2max}.

Field Tests

Field testing was conducted in the 1999 Vuelta Ciclista a España (3-week) and in a local 4-day race. The 3-week race covered a total of 3427 km, 3329 km in 19 in-line stages and 98 km in three individual time trial stages. The 4-day race covered 528.5 km, 519 km in in-line stages and 9.5 km in one individual time-trial stage. Body weight was measured daily. During each day's racing, subjects wore a Polar Sports Tester heart rate monitor for the determination of field heart rate. Data were downloaded and subsequently analyzed by a specific software.

Urine was collected for a 16 h-period from 17–18 h p.m to 9–10 h a.m. In the 3-week race, samples were taken on days 7, 14 and 21 of the competition. In the 4-day race, samples were taken every day. The urine samples were collected separately in plastic bottles. The volume of the urine was measured and aliquots were stored at −40°C. Venous blood samples were collected from the cubital vein with minimal stasis, and immediately treated for glutathione analysis as described later, or centrifuged for 10 min at 3000*g*. Aliquots of plasma were frozen at −80°C.

Biochemical Analysis

The urinary concentration of 8-OHdG was measured by high performance liquid chromatography using an electrochemical detector (HPLC–ED) with a column switching method according to Loft and Poulsen.[23] For analysis, thawed urine samples (2 ml) were acidified with 40 µl HCl and frozen at −20°C for precipitation of uric acid and other solutes. After centrifugation at 3000 rpm for 5 min, 34 µl of 2 M NaOH was added to each tube containing 1.7 ml of the supernatant. To 95 µl aliquots of the treated urine, 5 µl of a solution containing 8-OHdG (Sigma, St Louis, MO) 0, 800, or 8000 nM and 100 µl of 1 M Tris buffer pH 7.9, were added. A 25 µl sample was applied to a LiChrospher 100 RP-18 column (4.0 mm ID × 4 mm as a guard column + 4.0 mm ID × 150 mm, Merck, Darmstadt, Germany) and eluted with acetonitrile (2.5% v/v)

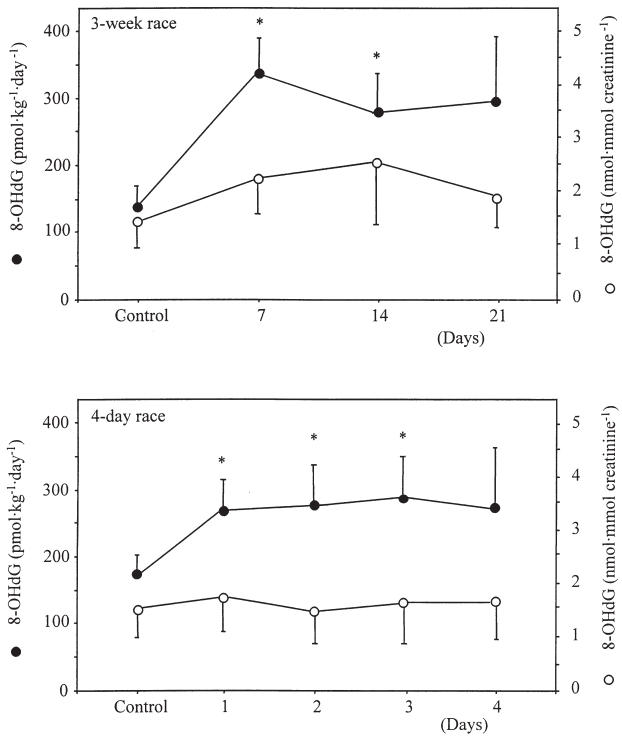

FIGURE 1 Urinary excretion of 8-OHdG during the races. Data are means ± SEM ($n = 8$) and were obtained every seven days (3-week race) or daily (4-day race) from the day prior to the beginning of the race (control). *$p < 0.05$ significantly different from control.

and methanol (1.5% v/v) in 10 mM borate buffer pH 7.9 containing EDTA (5 mg/ml) at 1 ml/min. The retention time of 8-OHdG was determined with an UV detector at 254 nm. Sixty seconds before the retention time of 8-OHdG a Valco automatic 6-port valve switched the effluent to a 2 cm Hamilton cation-exchange column. Sixty seconds later a switch of the valve brought the effluent on a Nucleosil C_{18} 3 μm column. The effluent of this column was monitored by an ESA Coluchem II electrochemical detector (Bedford, MA) equipped with a 5011 cell set at 100 mV (electrode 1) and 230 mV (electrode 2). The chromatographic system was controlled by means of Merck–Hitachi D-6000 chromatographic software.

Blood samples for the determination of total glutathione were collected in 5-ml vacutainers containing 7.5 mg Na_2EDTA. To 1 ml blood and equal volume of 20% (v/v) perchloric acid was added immediately. After centrifugation at 3000*g* for 10 min the acidic protein-free supernatants were stored at −40°C. Aliquots of deproteinized extracts were neutralized with a solution containing 0.4 M *N*-morpholioinopropanesulfionic acid and 2 mM EDTA adjusted at pH 6.75 with KOH 1 M and glutathione was determined using a cyclic oxidation–reduction method.[24]

Hemoglobin and hematocrit were analyzed using a Technicon Hemalog (Technicon España, Madrid, Spain) and percentage changes in plasma volume were estimated using the procedure of Dill and Costill.[25] Plasma activity of creatine kinase (CK) was determined with the use of a commercial kit (Sigma Chemical, St. Louis, MO). The creatinine concentration in urine was analyzed by an alkaline picrate method using a Hitachi 704 Autoanalyzer (Hitachi Ltd, Tokyo, Japan).

Statistical Analysis

Results are expressed as means ± standard error of means (S.E.M.) for all data. All parameters studied

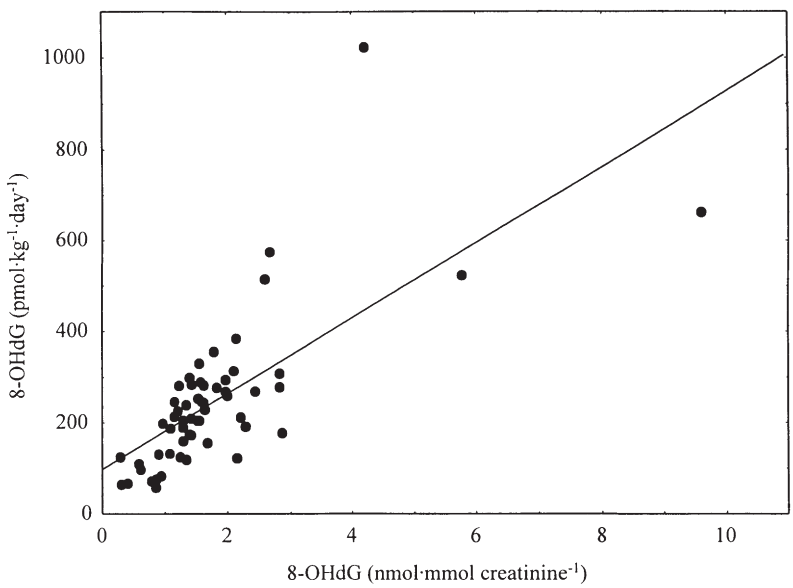

FIGURE 2 Relationship between daily urinary excretion of 8-OHdG expressed per kg body weight and the ratio of 8-OHdG to creatinine in spot urine samples. Data correspond to the different sampling period in both the 3-week and the 4-day races. $y = 82.97x + 97.02$, $r = 0.71$.

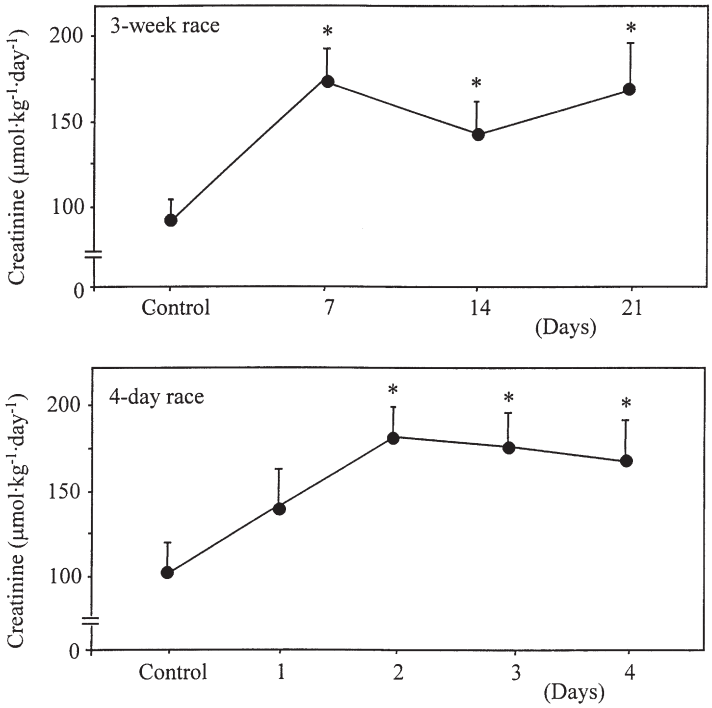

FIGURE 3 Urinary creatinine excretion during the races. Data are means ± SEM ($n = 8$) and were obtained every seven days (3-week race) or daily (4-day race) from the day prior to the beginning of the race (control). *$p < 0.05$ significantly different from control.

were tested for significance by one way analysis of variance (ANOVA) followed by the Newman–Keuls test. *P*-values of less than 0.05 were considered to be significant statistically. Relations between selected variables were evidenced by Pearson product moment correlations. A SPSS + vers. 4.0 statistical software (Chicago, IL) was used.

RESULTS

Effects of Exercise on Urinary 8-OHdG Excretion

Body weight decreased non-significantly by 2.0% during the 3-week race and by 1.8% during the 4-day race. The daily urinary excretion of 8-OHdG expressed as a function of body weight, increased significantly during the first week (+121%) of the 3-week race and the first day (+47%) of the 4-day competition and did not show further increase thereafter (Fig. 1). No significant changes in 8-OHdG were detected when levels in spot urine samples were corrected by creatinine excretion (Fig. 1). Although the urinary excretion of 8-OHdG expressed per kg body weight and the ratio of 8-OHdG to creatinine in spot urine samples were related significantly (Fig. 2), correlation was rather poor when levels increased. As shown in Fig. 3, creatinine excretion was elevated by 83% in the first week of the 3-week competition and by 33% in the first day of the 4-day race. Values remained significantly increased at any of the following sampling periods (Fig. 3).

TABLE I Serum GSH and CK

Period	GSH (mmol/l)	CK (IU/l)
	3-Week race	
Baseline	9.7 ± 0.5	93 ± 18
1st Week	10.2 ± 0.6	214 ± 50*
2nd Week	9.9 ± 0.8	487 ± 37*
3rd Week	9.6 ± 0.6	260 ± 51*
	4-Day race	
Baseline	7.3 ± 1.2	88 ± 24
4th Day	8.7 ± 0.8	179 ± 46*

Values are means ± SEM ($n = 8$). Data were obtained every 7 days (3-week race) or at the end of the race (4-day race). Baseline values correspond to the day prior to the beginning of the race (control). *$p < 0.05$ Significantly different from control.

Changes in Serum GSH and CK

Post-exercise values of blood variables were corrected for changes in plasma volume. Serum GSH concentration did not change significantly at any point during the 3-week race (Table I). Serum CK activity increased progressively for the first two weeks (+130 and +423%, respectively, vs. control) and decreased thereafter (+179% vs. control) (Table I). At the end of the 4-day competition GSH concentration did not differ significantly from pre-race values (Table I). Serum CK activity had increased by 103% (Table I).

TABLE II Time and percentage of participation at different intensities of exercise during the races

	AN		IA		MA		
Period	Min/day	%	Min/day	%	Min/day	%	
			3-Week race				Kms/week
Baseline	9 ± 1	5.3 ± 0.6	39 ± 4	23.2 ± 2.3	120 ± 8	71.3 ± 1.9	495
1st Week	42 ± 14*	19.1 ± 3.1*	95 ± 6*	43.6 ± 2.5*	81 ± 7	37.3 ± 2.1*	1012
2nd Week	21 ± 6*	7.2 ± 2.1	141 ± 6*	48.1 ± 1.7*	131 ± 8	44.7 ± 2.8*	1259
3rd Week	12 ± 4	4.9 ± 1.4	101 ± 3*	39.3 ± 1.1*	144 ± 6	56.0 ± 2.1*	1156
			4-Day race				Kms/day
Baseline	3 ± 1	1.3 ± 0.5	18 ± 5	10.7 ± 13.3	147 ± 10	87.5 ± 4.7	78
1st Day†	9 ± 2*	72.6 ± 14.2*	3 ± 2*	24.2 ± 6.0*	0.4 ± 0.1*	3.2 ± 0.6*	9.5
2nd Day	35 ± 11*	14.4 ± 4.4*	120 ± 17*	49.4 ± 6.9*	88 ± 13*	36.2 ± 5.6*	170
3rd Day	20 ± 7*	7.2 ± 2.5*	155 ± 16*	56.5 ± 5.5*	99 ± 10*	36.1 ± 3.7*	182
4th Day	28 ± 10*	10.6 ± 3.8*	130 ± 11*	49.8 ± 4.0*	103 ± 36*	39.4 ± 1.6*	167

Data are means ± SEM ($n = 8$). AN, anaerobic; IA, intense aerobic; MA, moderate aerobic Baseline values correspond to the training week prior to the beginning of the races. *$p < 0.05$ Significantly different from control. † Individual time trial.

Time and Percentage of Participation at Different Intensities of Exercise

Table II shows time and percentage of participation at different intensities of exercise and distance run in both races. The percentage of participation at IA was elevated during all the stages of the 3-week competition. The percentage at AN increased markedly during the first week and decreased thereafter, with an opposite evolution for the percentage at MA. During the first day of the 4-day race (an individual time trial), the percentage at AN was markedly elevated, with a decrease in MA. Percentage at AN decreased for the following days, while that at IA and MA was elevated (Table II).

Analysis of individual data corresponding to the 3-week competition is shown in Table III. Subject 5, with the lower 8-OHdG excretion in the last week, was the only one able to spend more than 10% of time at AN, while maximal DNA damage corresponded to the cyclist with a percentage of time at AN under 2% (subject 4). Both increase and reduction of 8-OHdG excretion were observed from the first week of the race in subjects who spent 2–7% of time at AN.

TABLE III Individual data for urinary 8-OHdG excretion and percentage of participation in high intensities of exercise for the 3-week race

	8-OHdG ($pmol\,kg^{-1}day^{-1}$)		AN (%)	
Subject	A	B	A	B
1	522	132	26.7	6.4
2	574	124	24.3	6.7
3	293	329	12.2	2.0
4	281	1022	16.5	1.6
5	198	65	29.2	13.1
6	312	119	8.7	3.0
7	205	283	19.4	3.1
8	109	245	16.8	2.9

AN, anaerobic exercise; A: 1st week; B: 3rd week.

DISCUSSION

Although a number of studies have analyzed physiological variables of professional athletes, most have relied upon laboratory measurements, and little information is available concerning the organic responses during competitions. Quantitative data on the intensity of the exercise are required for an adequate interpretation of the physiological demands and organic responses during events such as multi-day stage races.[20] The intensity of an exercise may be monitored by measurement of heart rate, which shows a linear relationship with oxygen uptake and this, in turn, is known to correlate with oxidative DNA damage.[26] In road cycling, the availability of reliable telemetric heart rate monitors has made possible the use of heart rate as an accurate indicator of exercise intensity.[19,20] Results obtained in our study concerning the average total amount of time spent in the different heart rate zones and the relative contribution of each zone demonstrate that during a multi-stage cyclist race, aerobic metabolism is mainly involved,[20] with a relevant contribution of high intensity exercise (AN phase).

The exact value of normal DNA oxidation levels is subject to considerable debate and an international group of experts, the European Standards Committee on Oxidative DNA Damage (ESCODD), has been established to provide recommendations on optimizing procedures and standardization of this assay.[17] In any case, HPLC with electrochemical detection is the most universally applied methodology for the measurement of 8-OHdG and is so far the method generally employed when studying effects of exercise.

Data obtained by different authors do not show consistently an increased oxidative DNA damage by prolonged exercise. Discrepancies could partly arise from the method used to express 8-OHdG urinary excretion. Because the collection of urine for prolonged periods may present some practical problems, spot urine samples have been sometimes collected for creatinine and a correction procedure using the amount of creatinine excreted has been used as a reference marker. However, confirming previous data by other authors,[23] we have found that the excretion of 8-OHdG per kg body weight correlates rather poorly with the ratio of 8-OHdG to creatinine in a spot urine sample, and the latter does not change significantly during multi-stage races. Standardization by creatinine excretion has been suggested to be probably valid for longitudinal studies where subjects are their own controls,[18] although this makes sense only if the amount of its excretion is stable. As shown in this study, participation in road cycling courses with an increased urinary creatinine excretion. Our results coincide with a report on long distance runners by Okamura *et al.*,[15] who suggested that an enhanced muscle and other tissue degradation and/or an accelerated energy metabolism might cause an increase in urinary creatinine excretion. Both factors could be involved in the present study, because urinary creatinine rose earlier than serum enzymes indicating tissue damage. In any case, it can be concluded that the correction procedure using the amount of creatinine excreted should not be used when studying effects of exercise on urinary 8-OHdG.

The excretion of 8-OHdG based on the body weight of subjects increased significantly on the first week of the 3-week race. This was probably an early event because values were already elevated on the first day of the 4-day race, and no further increase was observed on later stages. Oxidative damage could be related to the increased intensity of exercise when compared to the pre-race periods, as shown by the increased amount of time spent in the anaerobic heart rate zone (over the IAT) and its relative contribution during these stages. In the case of the 4-day race, the first day was a 9.5 km individual time trial, in which subjects try to sustain a near-to-maximum workload with an important contribution of high intensity exercise.

The pattern of change in urinary 8-OHdG during the races was similar to the previously found change in long-distance runners during participation in a training camp[15] or during a 4-day competition.[14] In both cases, there was an increase in oxidative damage as long as the exercise was repeated, although it was not accumulated. A previous study has also reported that in multi-stage cyclist race, lipid peroxidation is increased significantly at the end of the first stage and, although return partially to basal values, still remains significantly elevated at the end of the final stage.[8]

These results have been taken as indicative of an adaptative process during repeated exercise. In fact, antioxidant enzymes are significantly increased after cycling 2800 km in 20 days[8] and a 10-week endurance training elevates significantly plasma and erythrocyte glutathione peroxidase.[27] Viguie *et al.*[4] have shown that single bouts of submaximal exercise (90 min at 65% peak O_2 uptake) result in sufficient oxidant stress to significantly reduced GSH in plasma, but changes on consecutive days of exercise indicate no evidence of persistent or cumulative exercise effects[4] and GSH increases by 20 weeks of training have been reported.[28] The fact that in our study plasma GSH levels did not decrease during participation in the races could also point to an adaptation of antioxidant status.

Changes in markers of damage during competitions and field studies should however be interpreted with caution. In our study, serum CK was also significantly increased for the first two weeks of the 3-week race, but then decreased markedly. This could be taken as an indication of adaptation process,[16] but the possibility exists of a decrease in the intensity of exercise because of the high demands imposed to the organism by participation in an extreme endurance sport, which would result in less oxidative damage. This is supported by the fact that time spent at AN was markedly decreased during the second and third week of the 3-week race and the 2nd to 4th day of the 4-day race, suggesting a lack of capacity to maintain the levels of effort reached at the beginning of the competition. In fact, analysis of individual data during the 3-week race supports both explanations. The only cyclist able to spent more than 10% of time at AN during the third week was the one with the lower 8-OHdG excretion, indicating that subjects with more efficient adaptation mechanisms are more able to sustain considerable higher loads for longer period. On the other hand, when compared to the first week of the race, both increases and decreases were observed for 8-OHdG excretion in subjects who spent a limited time at AN.

An additional fact to be considered is that sources of 8-OHdG in urine could include not only repair of DNA damage, but also degradation of nuclei and mitochondria from cell turnover as well as the cellular desoxynucleotide pool reported.[18] With the ongoing massive muscle damage indicated by the increase in CK, degradation of nuclei and mitochondria could be a significant contributor, but the questions of the quantitative and qualitative importance of these factors compared to DNA remains to be elucidated.[29]

In summary, we conclude that road cycling courses with an oxidative damage to DNA that is sustained as long as the exercise is repeated. Both adaptation of antioxidant defenses and a decreased capacity to maintain a high intensity of effort may contribute to explain the absence of progressive increases in 8-OHdG excretion. The results of this study also confirm that the correction procedure using the amount of creatinine excreted should not be used when studying effects of exercise on urinary 8-OHdG.

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