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Effects of Repeated Bouts of Long-duration Endurance Exercise on Muscle and Urinary Levels of 8-hydroxy-2'-deoxyguanosine in Moderately Trained Cyclists

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Abstract

The purpose of this study was to determine the effects of repeated bouts of long-duration endurance exercise on both muscle and urinary levels of oxidative DNA damage in moderately trained individuals. Seven moderately trained male cyclists participated in this study. All participants repeated two sessions consisting of a 5 hour cycling period (equivalent to approximately 52% $\dot{V}O_{2peak}$) followed by a 15 hour rest, then a 40 km time trial. During the sessions, participants were instructed to take water *ad libitum* and to consume a standard sports drink consisting of 0.12 g·kg⁻¹ body weight·hr⁻¹ of carbohydrates. For each session, 24 hour urine output was collected on the day before the 5 hour exercise, and also between the 5 hour exercise and 40 km time trial, in addition to between days 1–5 post-exercise. Subsequently, muscle and urinary levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) were determined using high performance liquid chromatography with electrochemical detection. No significant alterations were observed between two sessions at the muscle or urinary levels of 8-OHdG. These results suggest that repeated bouts of exercise with a 7 day wash-out period did not lead to an accumulation of DNA damage products after a second 5 hour stationary cycling bout.

Keywords

exercise intensity; ultra-endurance exercise; oxidative stress; DNA oxidation; apoptosis

Introduction

Numerous studies have demonstrated damage associated with a generation of free radicals, reactive oxygen and nitrogen species in lipids, proteins, and DNA in tissues and blood from animals and humans following different kinds of exercise (Mahoney et al., 2005;

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Quadrilatero et al., 2010). Mitochondria abundant in the Type I fibers (slow twitch, oxidative) in the skeletal muscle appears to be a major site for the generation of the reactive oxygen and nitrogen species (Finkler et al., 2013). It is widely accepted that many types of exhaustive/high intensity endurance exercise (Mastaloudis et al., 2004; Radak et al., 2000) and eccentric exercise (Fogarty et al., 2013; Satchek et al., 2003), including unaccustomed exercise, all induce DNA damage that is rapidly cleared from the body with appropriate rest/recovery. On the other hand, it is also demonstrated that exercise that does not overly stress the body, such as regular training (Radak et al., 2002), accustomed exercise and low to moderate intensity exercise (Bloomer et al., 2005) do not typically elicit DNA damage. Furthermore, there is accumulating evidence that dietary antioxidants (Davison et al., 2005; Fogarty et al., 2013; Harms-Ringdahl et al., 2012; Muñoz et al., 2010; Satchek et al., 2003) and creatine supplementation (Rahimi, 2011) as an ergogenic aid, and caloric restriction (Hofer et al., 2008) appear to attenuate exercise-induced oxidative DNA damage.

It has been known that increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) serve as a biomarker of oxidatively damaged DNA by reactive oxygen and nitrogen species in the mitochondria (Kasai and Nishimura, 1984; Loft et al., 1992). The magnitude of the oxidative DNA damage appears to rely on the balance of free radical generation and the capability of antioxidant systems (Wagner et al., 2011). Repeated endurance exercise and training are considered to attenuate the oxidative stress by enhancing antioxidant defense mechanisms including antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase (Rowlands et al., 2012; Wagner et al., 2011). Moreover, moderate endurance exercise has been considered to be beneficial to increase the gene expression of antioxidant enzymes (Gomez-Cabrera et al., 2008). In line with those phenomena, DNA repairing enzymes such as human 8-oxoguanine DNA glycosylase1 and oxidized purine-nucleoside triphosphatase can be augmented after endurance exercise in order to protect against exercise-induced DNA damage (Radák et al., 2000, 2007, 2013).

With regard to the local muscle tissue 8-OHdG level, only a few studies have described augmented 8-OHdG levels in the vastus lateralis muscle following a single bout of eccentric exercise with regard to muscle 8-OHdG level in humans (Fogarty et al., 2013; Radak et al., 1999b;). In contrast, some studies on rats have reported attenuated 8-OHdG levels in the gastrocnemius muscle after 8 weeks of treadmill running (Radak et al., 2002) and 9 weeks of swimming (Radak et al., 1999a). A canine study has shown no changes of 8-OHdG levels in the gastrocnemius muscle after 7 hours of treadmill running compared with the control group (Okamura et al., 1997). In terms of urinary 8-OHdG level, the excretion of urinary 8-OHdG is considered to reflect the dynamics of oxidative DNA damage and repair from all cells *in vivo* (whole body level) (Loft et al., 1992). Concerning urinary 8-OHdG levels following endurance exercise, previous studies have described increased urinary 8-OHdG excretion after 60 minutes of cycling exercise at 70% VO_2 peak (Orhan et al., 2004), and following the completion of a running marathon (Tsai et al., 2001) and a 2 day ultramarathon race (Miyata et al., 2008). Contrary to those data, other reports have shown no alterations of the urinary 8-OHdG excretion after a single bout of intense exercise (treadmill running, cycling, and a 20 km run)(Sumida et al., 1997a), a 4 day and 3 week stage road cycling race (Almar et al., 2002), and a 894 km relay trail run (Rowlands et al., 2011).

Taken collectively, although the changes of muscle or urinary 8-OHdG are documented, to date, there have been no reports examining the alterations in comparison of muscle with urinary 8-OHdG levels (comparative evaluation at both local tissue and whole-body levels) following repeated bouts of long-lasting endurance exercise in humans.

The purpose of this study was to determine the effects of two repeated bouts of long-duration endurance exercise (5 hour cycling exercise plus a 40 km time trial) on oxidative DNA damage at both muscle and urinary levels in moderately trained cyclists, including the assessment of the repeatability of exercise performance. It was hypothesized that there would be no cumulative effects at the muscle or urinary levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) between two repeated sessions consisting of a 5 hour cycling period followed by a 15 hour rest, then a 40 km time trial, since moderately trained cyclists have training adaptation and are accustomed to prolonged cycling exercise.

Methods

Participants

Seven moderately trained male cyclists participated in this study. The descriptive characteristics of the participants are shown in Table 1. None of the participants reported a history of regular exogenous antioxidant supplementation or other drugs with antioxidant properties. All participants provided written informed consent prior to the current study. The protocol of the present study was reviewed and approved by The University of Montana Institutional Review Board.

Preliminary Exercise Testing

With a graded exercise protocol (starting at 95 watts and increasing 35 watts every three minutes), lactate threshold and peak oxygen uptake ($\text{VO}_{2\text{peak}}$) were determined using an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA). During the exercise testing, expired gases were collected and analyzed at 15 second intervals using a metabolic cart (Parvomedics, Inc., Salt Lake City, UT), which was calibrated with a 3L calibration syringe and medical gases of known concentrations (15.2% O_2 , 5.17% CO_2 , N_2 -balance).

VO_2 peak and lactate threshold were selected to evaluate participants' fitness levels. The criteria for VO_2 peak included achieving at least two of the following three criteria; a plateau in VO_2 , respiratory exchange ratio (RER) ≥ 1.1 and volitional exhaustion. In terms of determination of the lactate threshold, finger-tip capillary blood (20 μl) was obtained in the last 30 seconds of each 3 minute stage during the incremental test. Subsequently, blood lactate was determined using spectrophotometry (Milton Roy Spectronic 401, Rochester, NY).

Body Composition Assessment

Using hydrodensitometry, body density was assessed and corrected for estimated residual lung volume. Net underwater weights for each participant were recorded with a digital scale

(Exertech, Dresbach, MN). Based on body density, the percent of body fat was eventually computed with the Siri equation (Siri, 1993).

Experimental Procedures

Figure 1 represents the schematic diagram of the experimental procedure in the present study. All participants repeated two sessions consisting of a 5 hour cycling period followed by a 15 hour rest, then a 40 km time trial. The two experimental sessions were carried out on the different occasions separated by 7 days (Only one subject had 8 days) to ensure complete recovery. The participants reported to the laboratory at 07:30 am on the day of the trial. Each participant was instructed to consume a standard breakfast consisting of 2854 kilojoules of energy (16.5 g fat, 117.1 g carbohydrate, and 17.1 g protein). After breakfast, the participants rested in the lab until 10:00 am to allow for digestion, at which time a muscle biopsy from the vastus lateralis was taken (Pre)(Slivka et al., 2008). The participants then performed 5 hour cycling exercise at an intensity of 50% of their peak power output (approximately equal to 52% VO_2 peak). During the exercise, the participants were instructed to take water *ad libitum* and to consume a standard sports drink consisting of 0.12 $\text{g}\cdot\text{kg}^{-1}$ body weight $\cdot\text{hr}^{-1}$ of carbohydrate at the beginning of each hour in an effort to better maintain a euglycemic state. In order to monitor exercise intensity relative to individual VO_2 peak, expired gases were analyzed during the final ten minutes of each hour.

Immediately following the 5 hour cycling exercise, a second biopsy was obtained from a separate incision made approximately 2 cm proximal to the initial biopsy site on the same leg (Post). Immediately after and again two hours following the Post biopsy, each participant received feedings of dextrose (1.8 $\text{g}\cdot\text{kg}^{-1}$ body weight).

Four hours following the Post biopsy, a third biopsy was carried out (4 hour Post) at a separate site approximately 2 cm from the previous incision. All participants then consumed a meal composing of 3707.0 kilojoules of energy (22.3 g fat, 134.5 g carbohydrate, and 36.8 g protein). The participants were allowed to ingest provided snacks (3401 kilojoules of energy), which were also consumed during the subsequent trial. Therefore, each trial within an individual participant was isocaloric. The participants stayed in the lab overnight in order to closely control activity and diet. Each participant was instructed to replicate exactly the same diet for the same period of time until days 1–5 post-exercise during the second session.

On the following morning (15 hours after the completion of the 5 hour exercise bout), a fourth muscle biopsy was obtained (15 hour Post) at a different incision made 2 cm proximal from the previous biopsy. The participants then completed a simulated flat 40 km time trial on the same Velotron cycle ergometer with RacerMate® custom course software (RacerMate Inc., Seattle, WA).

Muscle Biopsies

Muscle biopsies were obtained from the vastus lateralis muscle of the same leg in a randomized order for each trial (Slivka et al., 2008). Each successive biopsy specimen on the same leg was taken from a separate incision, 2 cm proximal to the previous biopsy.

Muscle samples were immediately immersed into liquid nitrogen after removing any excess blood, connective tissue or fat and stored at -80°C until subsequent analyses.

Urinary Sample Collection

Twenty-four hour urine output was collected on the day before the 5 hour submaximal exercise and days up to 5 post-exercise to determine urinary 8-OHdG excretion, a marker of whole body DNA oxidation. Fifteen-hour urine output was collected between 5 hour-endurance exercise and the 40 km time cycling trial. By adding 2 M HCl, urinary samples were adjusted to the pH of 4–5, and 15 ml aliquots were kept frozen at -20°C (Germadnick et al., 1997) until subsequent analysis.

Determination of Muscle 8-OHdG Levels

The determination of 8-OHdG levels in the muscle tissue was based on the protocol previously described by Bolin et al. (2004). Analysis of the levels of 8-OHdG, expressed as the ratio of 8-OHdG/2-deoxyguanosine (2-dG) was performed as follows. 8-OHdG and 2-dG were resolved by high-performance liquid chromatography (HPLC) with a reverse phase YMCbasic column (4.6 mm $\times$ 150 mm; particle size 3 μm) (YMC Inc., Wilmington, NC) and quantified using a CoulArray electrochemical detection system (ESA Inc., Chelmsford, MA). An isocratic mobile phase consisting of 100 mM sodium acetate, pH 5.2, 4% methanol (HPLC Grade) diluted in water passed through C18 Sep-Pak cartridges (Waters Corp., Milford, MA) was utilized to elute the nucleosides from the column. The mobile phase was filtered using 0.2 μm nylon filters and degassed by sonication before use with the HPLC. Potentials of the four coulometric analytical cells of the CoulArray system placed in series were as follows: 50, 125, 175, 200, 250, 380, 500, 700, 785, 850, 890, and 900 mV. Calibration curves were generated from standards of 2-dG ranging from 100 ng to 2 μg (0.350–7.01 nmol) and 8-OHdG (Cayman Chemical Company, Ann Arbor, MI) ranging from 5 to 100 pg (17.6–353 fmol). The calibration curve for 8-OHdG was created based on the peak area of the 8-OHdG standard, eluting at 9.0 min, in the 250 mV channel. The calibration curve for 2-dG was created based on the sum of peak areas of the 2-dG standard, eluting at 7.8 min, in the 850, 890, and 900 mV channels. The electrochemical cells were allowed to equilibrate and wake up standards (three times the highest concentration of calibration standards) were injected before calibration standards as suggested by ESA in order to obtain highest sensitivity. The amount of 8-OHdG and 2-dG was calculated by comparing the peak area and sum of peak areas, respectively, from the 10 μl injection of reconstituted enzymatic hydrolysate of the DNA sample with the calibration curves for 8-OHdG and 2-dG. Levels of 8-OHdG in the samples were expressed relative to the content of 2-dG [e.g. the molar ratio of 8-OHdG to 2-dG (fmol of 8-OHdG/nmol of 2-dG)]. Data were recorded, analyzed, and stored using CoulArray software for Windows (ESA Inc., Chelmsford, MA).

Determination of Urinary 8-OHdG Levels

By modifying a protocol of Germadnick et al. (1997), urinary 8-OHdG levels were determined using HPLC with electrochemical detection. Each sample had at least one freeze-thaw step in order to lyse bacterial cells and was centrifuged at 1500 g for 5 min before precipitates were removed. By solid-phase extraction, the supernatant underwent a

cleanup procedure. In the solid-phase extraction, the Bond Elut columns C18/OH (C18/OH, 500 mg, 3 ml; Varian Inc., Harbor City, CA, USA) were pretreated with 10 ml of methanol, 5 ml of water and 10 ml of 50 mM KH_2PO_4 , pH 7.5 (buffer A). A 2-ml volume of urine premixed with 1 μl of 8-OHdG standard ($1 \text{ mg} \cdot \text{ml}^{-1}$) was applied to the first column. The column was washed with 3 ml of buffer A and 3 ml of 5% methanol in buffer A. 8-OHdG was eluted with 3 ml of 15% methanol in buffer A. The eluate then was diluted with 5 ml of water, mixed and applied to another conditioned C18/OH column. The column was dried under vacuum and the absorbed material was eluted with 1.5 ml of 20% methanol in buffer A. Using a SpeedVac® concentrator SVC200H (Savant, Holbrook, NY, USA), methanol in the samples were evaporated for 1.5 h and subsequently filled up with buffer A in order to give a final volume of 1.5 ml. Subsequently, 20 μl of the prepared elute was injected into the HPLC system (ESA Inc., Chelmsford, MA).

Determination of Proteinuria and Specific Gravity

Urinary total protein concentration was determined using a colorimetric assay (BioAssay Systems, Hayward, CA). Urinary specific gravity was determined with a refractometer (ATAGO, Bellevue, WA) in order to evaluate each individual's hydration status over the experimental period.

Statistical Procedures

A two-way (time $\times$ session) Analysis of Variance (ANOVA) with repeated measures was performed on all dependent variables. When significance was observed, the location of the difference was determined using the Tukey's *post hoc* test with a statistical package (Statistica V5.1 for Windows, Statsoft, Tulsa, OK, USA). For ANOVA with repeated measurements, effect sizes were calculated with omega-squared (ω^2) (Kirk, 2007). Interpretation of the effect size ω^2 is as follows: $0.01 \leq \omega^2 < 0.06$ = small effect; $0.06 \leq \omega^2 < 0.14$ = moderate effect; $\omega^2 \geq 0.14$ = large effect (Kirk, 2007). The acceptable level of statistical significance was set at $p < 0.05$, including the 95% confidence interval (95% CI). All results are described as mean $\pm$ SD.

Results

Muscle 8-OHdG Levels

No significant main effects for time ($p = 0.291$), session ($p = 0.698$) or interaction ($p = 0.148$) were identified for muscle 8-OHdG levels [(fmol/2dG(nmol))] over the experimental periods (Figure 2).

Urinary 8-OHdG Levels

Over two experimental sessions, there were no significant main effects for time ($p = 0.760$), session ($p = 0.317$) or interaction ($p = 0.772$) in urinary 8-OHdG excretion ($\text{ng} \cdot \text{mL}^{-1}$, Figure 3-A). Similarly, no main effects for time ($p = 0.156$), session ($p = 0.358$), or interaction ($p = 0.707$) were found when expressed relative to creatinine ($\text{ng} \cdot \text{mg creatinine}^{-1}$, Figure 3-B). In contrast, a significantly higher 8-OHdG secretion was detected at Pre when expressed relative to 24 h urinary volume ($\mu\text{g} \cdot \text{day}^{-1}$) (main effect for time, 95%CI: 0.69 to 8.34, $p < 0.05$; $\omega^2 = 0.11$), with no main effect for session ($p = 0.113$) or interaction ($p = 0.659$) (Figure

3-C). There was a trend toward a higher urinary creatinine excretion ($\text{mg}\cdot\text{dL}^{-1}$) at Day 1 (main effect for time: $p=0.074$) compared with Pre between two sessions, although a main effect for session ($p=0.933$) or interaction ($p=0.685$) were not achieved (Figure 3-D). Urinary volume ($\text{mL}\cdot\text{day}^{-1}$) was significantly higher at Pre compared with other time points (main effect for time, 95%CI: 133.1 to 2121, $p<0.05$; $\omega^2=0.09$), with no main effect for sessions ($p=0.113$) or interaction ($p=0.659$)(Figure 3-E).

Hydration Status

The values of urine specific gravity ($\text{g}\cdot\text{mL}^{-1}$) were similar over the experimental periods, describing no main effects for time ($p=0.217$), session ($p=0.869$) or interaction ($p=0.802$) (Figure 3-F), which shows the well-hydrated condition (euhydration condition: 1.020; Armstrong, 2012).

Urinary Total Protein

Urinary total protein excretion ($\text{mg}\cdot\text{dL}^{-1}$) tended to be higher on Day 1 (main effect for time, $p=0.070$) than Pre between two sessions (main effect for session, $p=0.089$; interaction: $p=0.726$, Figure 4-A), while all values were within a normal upper limit ($<10\text{ mg}\cdot\text{dL}^{-1}$ or $<150\text{ mg}\cdot\text{day}^{-1}$; Helzer-Julin et al. 1988). In addition, total protein excretion expressed relative to creatinine ($\text{mg}\cdot\text{g creatinine}^{-1}$) was also similar between two sessions, demonstrating no main effects for time ($p=0.445$), session ($p=0.104$) or interaction ($p=0.624$)(Figure 4-B).

Discussion

The main findings in the present study were that 5 hour stationary cycling were not enough to induce DNA damage and repeated bouts of exercise with a 7 day wash-out period between bouts did not lead to an accumulation of DNA damage products after a second 5 hour stationary cycling bout. To our knowledge, this study was the first to evaluate oxidative DNA damage in both muscle and urinary (whole body) 8-OHdG levels after repeated bouts of long-duration endurance exercise in humans, including the assessment of the repeatability of exercise performance.

Exercise-induced DNA Oxidation

It has been demonstrated that exhaustive/high intensity endurance exercise (Mastaloudis et al., 2004; Radak et al., 2000) and eccentric exercise (Fogarty et al., 2013; Sacheck et al., 2003) induce oxidative DNA damage. On the other hand, it has been shown that regular training (Radak et al., 2002), accustomed exercise, and low to moderate intensity exercise (Bloomer et al., 2005) elicit no DNA damage. Although DNA repair enzymes and antioxidant enzymes were not measured in the present study, there is accumulating evidence concerning the activities of DNA repair enzymes and antioxidant enzymes after aerobic exercise. For example, Bloomer et al. (2005) have reported that moderate duration and/or intensity aerobic exercise may not be sufficient to elicit an increase in 8-OHdG, possibly due to the rapid repair of DNA following oxidation (Radak et al., 2002, 2003), as an increase in the activity of certain DNA repair enzymes has been observed following acute aerobic exercise (Radak et al., 2003). Other studies have demonstrated the more prominent

antioxidant capacity of Type I fibers (slow twitch, oxidative) compared with that of Type II fibers (fast twitch, glycolytic)(Radak et al., 2001). Some human studies have reported that endurance exercise and training, accompanied with a more prominent involvement of Type I fibers, promotes the prevention of nuclear DNA damage by up-regulating the repairing enzyme activity such as human 8-oxoguanine DNA glycosylase I and oxidized purine-nucleoside triphosphatase (Radak et al., 2007). This is supported by the fact that in endurance-trained individuals, the Type I fibers are more noticeable because of abundant mitochondrial density compared with the Type II fibers (Coyle et al., 1995). Thus, subjects of the present study who are regularly involved in endurance training may have an enhanced adaptive response, which results in a protective effect against oxidative stress and attenuates the extent of apoptosis (e.g., DNA fragmentation and caspases activity) (Radak et al., 2013). Moreover, it is suggested that the degree of endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase in the skeletal muscle are increased in endurance-trained athletes (Rowlands et al., 2012; Wagner et al., 2011).

Muscle 8-OHdG Levels and Apoptosis

In terms of muscle 8-OHdG levels in humans, only a few studies have reported increased DNA damage in the vastus lateralis muscle after a single bout of eccentric exercise (Fogarty et al., 2013; Radak et al., 1999b). However, a canine study has described no alterations of the 8-OHdG levels after 7 hours of treadmill running (Okamura et al., 1997). Studies on rats have shown the reduction of the 8-OHdG levels in the gastrocnemius muscle after 8 weeks of treadmill running (Radak et al., 2002) and 9 weeks of swimming (Radak et al., 1999a) compared with the control group. In the current study, no changes were observed at any of the time points where DNA damage was evaluated, which could in part be explained by the reason that moderately trained cyclists may have had training adaptation and been accustomed to prolonged exercise (Bloomer et al., 2005).

Whether or not DNA damage directly results from oxidative damage, or whether it is derived from a part of the process of apoptosis remains elusive. While there are several studies examining the effects of acute exercise on skeletal muscle apoptosis in humans (Kerksick et al., 2008; Mahoney et al., 2005; Quadrilatero et al. 2010), only two studies have dealt with endurance exercise (Mahoney et al., 2005; Quadrilatero et al. 2010). For instance, Mahoney et al. (2005) reported ultra-structural muscle damage and inflammation at 48 hours as well as apoptotic myonuclei 3 h postendurance exercise (high-intensity cycling) in untrained healthy males. Furthermore, Quadrilatero et al. (2010) demonstrated no alteration of apoptotic signaling and DNA fragmentation in human skeletal muscle following prolonged moderate-intensity aerobic exercise in 8 recreationally active individuals (7 men and 1 woman). Although apoptotic signaling and DNA fragmentation were not examined in the present study, oxidative DNA damage accounted for by muscle 8-OHdG levels were also unchanged following repeated sessions of long-duration moderate endurance exercise over all time points. Taken collectively, it is needed to be clarified whether long-duration moderate aerobic exercise alters apoptotic signaling in conjunction with DNA damage in moderately trained individuals, as the underlying mechanism remains inconclusive

regarding how and why apoptosis is induced in adult skeletal muscle after exercise (Peters et al., 2006; Phaneuf & Leeuwenburgh, 2001).

Urinary 8-OHdG levels

The excretion of urinary 8-OHdG is considered to reflect the dynamics of oxidative DNA damage and repair from all cells *in vivo* (Loft et al., 1992). In comparison with the actual site of oxidant generation (e.g., the active skeletal muscle), the measurements of both hematological and urinary biomarkers could be diluted (Bloomer et al., 2005). Furthermore, oxidative stress as reflected by hematological and urinary biomarkers could be independent of oxidants produced within contracting muscle or attributed to a disparity in the baseline antioxidant status between samples (e.g., blood, urine, and muscle tissue) (Fogarty et al., 2013). In the present study, no alterations of urinary 8-OHdG excretion were observed over the post-exercise period. The exercises performed in the current study may have been insufficient to induce significant oxidative DNA damage at the urinary level. Some studies have shown no accumulation of oxidative DNA damage accounting for urinary 8-OHdG excretion after a single bout of intense exercise (treadmill running, cycling, and a 20 km run) (Sumida et al., 1997a), a 4 day and 3 week stage road cycling races (Almar et al., 2002), and an 894 relay train run (Rowlands et al., 2011). On the other hand, several studies have demonstrated increased urinary 8-OHdG excretion after 60 min of cycling exercise at 70% VO_2 peak (Orhan et al., 2004), a running marathon (Tsai et al., 2001) and a two-day ultramarathon race (Miyata et al., 2008). Possible reasons for these discrepancies may in part be due to the differences of exercise mode, intensity, duration and fitness level of the individuals. Another explanation may be related to the use of a creatinine-adjustment, taking into consideration variability in urine sampling collected as spot or 24 hour urine. Previous studies have used a creatinine-adjustment for 24 hour urinary 8-OHdG excretion at the steady-state or after aerobic exercise (Sumida et al., 1997a; Tsai et al., 2001). As shown in the results, creatinine excretion was higher in Day 1 compared to Pre. Therefore, our expression of urinary 8-OHdG was in agreement with the previous findings by Okamura et al. (1997) and Almar et al. (2002), which suggest that a creatinine-adjustment should not be used when determining the effect of exhaustive endurance exercise, as creatinine excretion is not stable under these conditions. In line with those explanations for the potential reason of the results in the present study, it is possible that the considerable inter-subject variability, assay variability, and small sample size may lead to a Type II error. Thus, care must be taken when interpreting exercise-induced DNA oxidation, given the variability of results, the low number of observations and the lack of additional post-exercise biopsy sampling times to detect cumulative effects of DNA damage. Further work is required to confirm these findings.

Technical considerations

Based on a previous observation in our lab, moderately trained individuals could not maintain more than 55–65% VO_2 peak of cycling exercise for long duration (5 hours). Thus, 5 hours of cycling exercise at 50% peak power output was equivalent to 52% VO_2 peak was chosen in the present study, which was considered as an upper limit for long-duration steady-state exercise. As another technical consideration, the 5th muscle biopsy after time trial was not collected to determine potentially accumulated DNA damage. The

reason was that time trials of the present study were used to assess participants' exercise performance. According to Marino et al. (2002), the advantage of time trial protocols is to allow for the assessment of exercise performance rather than exercise capacity. In addition, the findings obtained from self-paced trials represent the ability of individuals to reliably reproduce exercise performance rather than their exercise capacity.

In light of rest and recovery periods between the 1st and 2nd sessions, sufficient time should be considered to avoid any carry-over effects. In the present study, a 7 day recovery period was carried out between the two experimental sessions. In this regard, the timed courses of exercise-induced DNA damage have been inconsistent as described by most previous studies, which demonstrated no changes or increased hematological and urinary levels of 8-OHdG at several days and returned to the baseline within 7 days after endurance exercise (e.g., 30 minutes of continuous cycling at 70% VO_2 peak, Bloomer et al, 2005; 50 km ultramarathon, Mastaloudis et al., 2004; 60 minutes at 70% VO_2 peak, Orhan et al., 2004). In contrast, few studies demonstrated elevated DNA damage that persisted longer than 7 days following prolonged exercise (e.g., 42 km marathon, average run time 3 hours, Tsai et al., 2001). Thus, a 7-day recovery period after a first 5 hour stationary cycling bout in the present study could have been reasonable. Further investigation is needed to answer the question of how much degree of exercise intensity and duration influences time courses of DNA damage.

With regard to effects of carbohydrate supplementation on oxidative DNA damage after exercise training, de Sousa et al. (2011) have recently reported that ingestion of a carbohydrate beverage resulted in less DNA damage and attenuated the acute post-exercise inflammation response, providing better recovery during training. While carbohydrate supplementation in the present study were used to maintain an euglycemic state during exercise, whether it could be effective in attenuating the marked increase of exercise-induced DNA oxidation and consequently reducing disturbances at muscle and whole body levels cannot be concluded. Therefore, further studies are warranted to confirm those phenomena.

In conclusion, the results of the present study indicated that 5 hours of stationary cycling appears not to be enough to induce DNA damage and repeated bouts of exercise with a 7 day wash-out period between bouts would not lead to an accumulation of DNA damage products after a second 5 hour stationary cycling bout in moderately trained individuals. Furthermore, the responses from repeated bouts of exercise could demonstrate the repeatability of exercise performance.

Perspectives

Even though there may be adaptive responses based on the training status of the present participants, further research is required to clarify the optimal exercise mode, intensity and duration, promising as a means of maximizing the benefits of moderate endurance exercise. These data may help in the interpretation of repeated endurance exercise tests by defining the variations in oxidative stress accounted for by damaged DNA under standardized conditions. In this respect, additional research is needed to make sure whether repeated bouts of long-duration moderate endurance exercise upregulates DNA repair and antioxidant

enzyme activities, which consequently lead to attenuated oxidative DNA damage or apoptosis.

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1st session: long-duration endurance exercise (5 hours cycling exercise plus 40 km time trial), including muscle biopsy and urine collection
2nd session: long-duration endurance exercise (5 hours cycling exercise plus 40 km time trial), including muscle biopsy and urine collection

Figure 1.
The schematic diagram of the experimental procedures. *Time point supplemented with sports drink at beginning of each hour ($0.12 \text{ g} \cdot \text{kg}^{-1} \text{ body weight} \cdot \text{hr}^{-1}$) and expired gases collected at the end of each hour. BREAKFAST: 2584 kilojoules of energy (16.5 g fat, 117.1g carbohydrate, and 17.1 g protein), GLU: feedings of dextrose ($1.8 \text{ g} \cdot \text{kg}^{-1} \text{ body weight}$), MEAL: solid food meal (3707 kilojoules of energy; 22.3 g fat, 134.5 carbohydrate, and 36.8 g protein), PRE: before exercise, ITM: Intermediate, and TT: simulated 40 km cycling time trial.

Figure 2. Muscle 8-hydroxy-2'-deoxyguanosine (8-OHdG) expressed relative to deoxyguanosine (2dG) [8-OHdG (fmol)/2dG(nmol)] between the 1st and 2nd sessions. PRE: before 5 hour exercise, 0 h Post-Ex: immediately after 5 hour exercise, 4 h Post-Ex: 4 hour post-exercise, 15 h Post-Ex: 15 hour post-exercise. All data express mean $\pm$ SD.

Figure 3.

Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), creatinine, urinary volume, and specific gravity in comparison between the 1st and 2nd sessions. ITM stands for intermediate (between 5 hour exercise and 40 km time trials) at which 15 hours of urine was collected. (A) Non-normalized urinary 8-OHdG excretion (ng·mL⁻¹); (B) normalized urinary 8-OHdG excretion expressed relative to creatinine (ng·mg creatinine⁻¹); (C) normalized urinary 8-OHdG excretion expressed relative to 24 hour urinary volume (g·day⁻¹); (D) urinary specific gravity (g·mL⁻¹); (E) creatinine excretion (μg·dL⁻¹); (F) 24 hour urinary volume

(mL·day⁻¹). *Significantly different from Pre (main effect for time, $p < 0.05$). All data show mean $\pm$ SD.

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Figure 4. Urinary total protein excretion expressed as (A) $\text{mg} \cdot \text{dL}^{-1}$; (B) $\text{mg} \cdot \text{g creatinine}^{-1}$ between two sessions. All data demonstrate mean $\pm$ SD.

Table 1

Descriptive characteristics of the subjects

Characteristic	Men (N=7)
Age (yr)	29.9±9.9
Height (cm)	181.0±6.5
Body weight (kg)	76.1±7.2
BMI (kg·m ⁻²)	23.3±2.3
Body fat (%)	16.1±3.4
VO ₂ peak (l·min ⁻¹)	4.54±0.48
VO ₂ peak (ml·kg ⁻¹ ·min ⁻¹)	60.0±5.1
VO ₂ at Tlact (l·min ⁻¹)	2.60±0.46
% VO ₂ peak at Tlact	57.1±7.2

BMI=Body mass index; Tlact=Lactate threshold; % VO₂peak at Tlact=(VO₂ at lactate threshold/VO₂peak) × 100