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Effect of Repeated Exercise on Urinary 8-Hydroxy-deoxyguanosine Excretion in Humans

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The purpose of this study was to investigate the effect of repeated exercise on oxidative damage to DNA in 10 well trained long distance runners who participated in an 8-day training camp. The average running distance during the camp was 30 ± 3 km/day. The amount of urinary 8-hydroxy-deoxyguanosine (8-OHdG) excretion was used to estimate the oxidative DNA damage. Urine samples were collected for both a 3-day control period as well as throughout the camp. Blood samples were drawn after overnight fasting both before and after the camp. Urinary 8-OHdG excretion was significantly increased during the camp compared to the control period (265.7 ± 75.5 vs. 335.6 ± 107.4 pmol/kg/day, $P < 0.05$). The content of 8-OHdG in the lymphocyte DNA on the day after finishing the camp did not differ from that before the camp. Plasma TBARS, LDH, CK, CK-MB, and myoglobin significantly rose after the camp ($P < 0.05$). The plasma β-carotene levels tended to rise after the camp, while the plasma α-tocopherol levels increased significantly after the camp ($P < 0.05$). These results indicate that repeated exercise augments oxidative stress and that DNA is also injured by exercise-induced reactive oxygen species. However, the oxidative damage to DNA is not accumulated by consecutive exercise, although it is sustained as long as the exercise is repeated.

Keywords: Oxidative stress, lymphocyte 8-OHdG content, TBARS, β-carotene, α-tocopherol

INTRODUCTION

Because exercise increases oxygen consumption, it is now accepted that the generation of reactive oxygen species (ROS) is increased by exercise.[1,2] Other proposed mechanisms for the generation of exercise-induced ROS include a xanthine oxidase catalyzed reaction[3,4] and the contribution of leukocytes.[5]

The exercise-induced increase in ROS is thought to cause oxidative damage to several types of tissue.[1,2,6] It was reported that DNA could be a target for ROS.[7–10] The urinary excretion of 8-hydroxy-deoxyguanosine (8-OHdG) has been used to estimate the magnitude of oxidative DNA damage. It has been reported that oxidative damage to DNA increased in propor-

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tion to the species-specific basal metabolic rates.[11,12] Loft *et al.* showed that oxidative DNA damage which was estimated by the urinary excretion of 8-OHdG correlated with oxygen consumption in humans.[13]

These results suggest that ROS generated by exercise may oxidize DNA. Little is known, however, as to whether or not exercise induces oxidative DNA damage. Alessio *et al.*, showed that the urinary excretion of 8-OHdG/creatinine ratio increased by 130% 10 hours after a marathon race.[14] This result indicates that vigorous exercise such as a marathon race may injure DNA. It remains to be elucidated, however, as to whether or not consecutive exercise induces oxidative DNA damage.

Inoue *et al.* reported that the nuclear 8-OHdG content in lymphocytes was significantly reduced and the urinary excretion of 8-OHdG tended to increase after a single bout of exercise, thus suggesting that the repair mechanism for the DNA damaged by exercise-induced ROS may be augmented by exercise.[15]

We therefore monitored the changes in urinary 8-OHdG excretion and lymphocyte 8-OHdG content during a training camp in which the athletes all ran 30 km a day on average.

MATERIALS AND METHODS

Subjects

Ten well trained male long distance runners (20.1 ± 1.4 yr., weight 56.8 ± 3.2 kg, height 168.9 ± 5.1 cm, body mass index 19.9 ± 1.3 kg/m^2) participated in the study. The study protocol conformed to the guidelines of the Helsinki Conference for research on human subjects. Written informed consent signed by each subject was also obtained prior to the study.

Experimental Design

The subjects participated in an 8-day training camp. They ran 30 ± 6 km/day on average during the camp. As shown in Figure 1, the running distance varied day by day. Other exercise besides running and jogging was also included in the training menu. A three day sedentary period was provided as a control period. There was about 1 week between the control and the camp periods.

Venous blood samples were taken after overnight fasting on the first day of the control period (cont) and both on the first day (Day 1: D1) and on the morning after the last day of the camp (Day 9: D9).

Urine samples were collected for 24 hours during both the 3-day control period and throughout the camp. The urine samples were stored in a refrigerator until all collection samples of the day had been completed. Afterwards the volume of the urine was measured and the aliquots of the urine were stored at –80°C until the analyses were performed.

Analyses

The urinary concentrations of 8-OHdG were measured by high performance liquid chromatography using an electrochemical detector (HPLC-ECD) with a column switching method according to Loft *et al.*[16] with some modifications. The frozen urine samples were thawed and then centrifuged at 3,000 rpm for 10 min. To each tube con-

Day	1	2	3	4	5	6	7	8	9
6:00	Blood	14 km run		13 km run	14 km run	7 km run	7 km run	13 km run	Blood
8:00									
10:00		WT 5 km jog	WT 5 km jog	WT 5 km jog	WT 5 km jog	WT 5 km jog	WT 5 km jog		
noon									
14:00									
16:00	16 km run	22 km run	BG 5 km run	16 km run	25 km run	BG 5 km run	30-40 km run		
18:00									

FIGURE 1 Training menu during the camp. Blood: blood sampling, WT; weight training, BG; ball game. The training on the afternoon of day 8 was canceled due to bad weather.

taining 1 ml of the supernatant and to each tube containing 1 ml of 8-OHdG standard solution, 1 ml of 1 M Tris-HCl buffer pH7.9 was added. After mixing thoroughly, these samples were filtered (Ekicrodisc Acro LC, 13, 0.45 μm, German Science, Tokyo, Japan). A 50 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 × 250 mm, Merck, Darmstadt, Germany) and eluted with acetonitrile (2.5%, v/v) and methanol (1.5%, v/v) in 10 mM borate buffer pH 7.9 containing EDTA (5 μ/ml) at 1 ml/min. The retention time of 8-OHdG was determined with an UV detector at 254 nm. The collection of 60 seconds effluent was started 30 seconds before the retention time of 8-OHdG, which was retained temporarily in an ion exchange resin 2619F (Hitachi, Tokyo) packed in a column (4.0 mm × 10 mm), and a LiChrospher 100 RP-18 column (4.0 mm ID × 75 mm). The retained effluent was eluted then introduced to a LiChrospher 100 RP-18 column (4.0 mm × 250 mm) with acetonitrile (2.5%, v/v) and methanol (1.5%, v/v) in 100 mM phosphate buffer containing EDTA (5 μ/ml) at 1.0 ml/min. The effluent was assayed by an ECD (ECD-100, Eicom, Tokyo) set at 750 mV. The 8-OHdG was prepared following the procedure described by Kasai *et al.*[8]

The creatinine concentrations in the urine were analyzed by an alkaline picrate method using a kit (Creatinine-HR, Wako Pure Chemical, Osaka, Japan).

The 8-OHdG content in the lymphocytes was measured basically according to the method reported by Yamamoto *et al.*.[17] The samples of lymphocytes were prepared using LeucoPREP (Becton Dickinson, Lincoln Park, NJ). Lymphocyte DNA extracted with DNA Extractor WB (Wako Pure Chemical) was dissolved in 100 μl of 1 mM EDTA and then heated at 95°C for 5 min. After standing in ice for 5 min, 1 μl of 2 M sodium acetate pH 4.5 and 50 μg of nuclease P1 (Sigma, St. Louis, MO) were added. The 16 μl of 1 M Tris-HCl buffer pH 7.5 and 1.14 units of alkaline phosphatase (type III, Sigma) were added to each sample and then heated at 37°C for 1 h. The samples were then centrifuged at 13,000 rpm for 3 min at 4°C.

The supernatant of 100 μl was applied to a LiChrospher 100 RP-18 column (4.0 mm ID × 150 mm) and eluted with acetonitrile (1.5%, v/v) and methanol (1.5% v/v) in 100 mM phosphate buffer pH 2.2 containing EDTA (5 μg/l) at 1 ml/min. The amount of deoxyguanosine (dG) was determined from the absorbance at 290 nm measured with an UV monitor (L-4250, UV-VIS detector, Hitachi, Japan) and the amount of 8-OHdG was measured with an ECD (Coulochem II, ESA, Bedford, MA) equipped with an analytical cell (model 5011, detector 1: 150 mV, detector 2: 300 mV) and a guard cell (model 5020, 350 mV). The content of 8-OHdG in lymphocyte DNA was expressed as the ratio of the peak area of 8-OHdG to the peak area × 10^6 of dG. The dG was purchased from Wako Pure Chemical.

The concentrations of plasma all-trans-β-carotene were measured with an HPLC according to Ben-Amotz *et al.*[18] The plasma α-tocopherol concentrations were determined with an HPLC according to Igarashi.[19]

The concentrations of serum thiobarbiturate reactive substances (TBARS) were measured using the TBA method (Lipid Peroxide Test Wako, Wako Pure Chemical). The serum samples were also analyzed for creatine kinase (CK) with the UV-NAC method (Merck Auto CK, Kanto Kagaku, Tokyo), its isozyme MB type (CK-MB) with immunoinhibition method (Merck Auto CK-MB, Kanto Kagaku), lactate dehydrogenase (LDH) using a kit based on Wroblewski-LaDue method (LDH-HR, Wako Pure Chemical), and myoglobin (Mb) with RIA-PEG method (Myoglobin Kit, Dai-ichi, Tokyo). The serum low-density-lipoprotein concentrations were determined using the heparin-Ca^{++} precipitation method.

Statistics

A one way analysis of variance was applied and where significance was observed Fisher's pro-

tected least significant difference test was employed. The D1 and D9 values were compared by a paired t test. $P < 0.05$ was considered to be significant.

RESULTS

Serum Enzymes and Mb (Fig. 2)

The hematocrit values did not differ and thus no correction of the plasma or serum levels was performed (cont 44.1 ± 3.1, D1 44.2 ± 2.1, and D9 42.0 ± 2.8, respectively). The serum LDH levels significantly differed from each other and the D9 level was the highest (control 304 ± 31, D1 378 ± 66, D9 557 ± 53 IU/l). The concentrations of serum Mb were significantly elevated from the control and D1 (37 ± 7 and 49 ± 15 ng/ml, respectively) to D9 levels (67 ± 14 ng/ml). The CK levels were significantly higher D9 (1349 ± 749 IU/l) than either D1 or the control value (152 ± 38 and 365 ± 222 IU/l, respectively), while the D1 value did not differ from the control value. The CK-MB level of D9 (30 ± 14 IU/l) was significantly higher than those of both the control (9 ± 2 IU/l) and D1 (14 ± 5 IU/l).

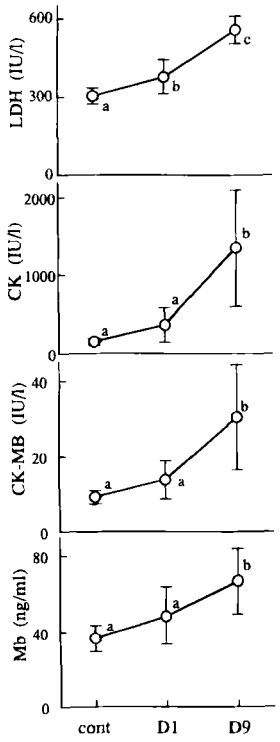

FIGURE 2 Serum enzymes and myoglobin. Values are the means ± SD, n = 10. The values not sharing the same characteristics were significantly different. $P < 0.05$ by Fisher's PLSD. cont; control, D1; Day 1, D9; Day 9.

Plasma TBARS

The plasma TBARS concentrations significantly declined from the control (2.5 ± 0.3 nmol/ml) to D1 (2.0 ± 0.3 nmol/ml), but then significantly rose D9 (2.7 ± 0.3 nmol/ml). No difference was found between the values of the control and D9.

Lymphocyte 8-OHdG (Fig. 3)

The lymphocyte 8-OHdG/dG ratios did not change after the camp.

Urinary 8-OHdG Excretion (Fig. 4)

Figure 4a depicts the daily 8-OHdG excretion in the urine. The amount of 8-OHdG excreted on day 8 was less than that of the other days of the camp. Fig. 4b represents the average amount of 8-OHdG excreted in the urine during the control and the camp period and this amount was significantly larger during the camp than for the con-

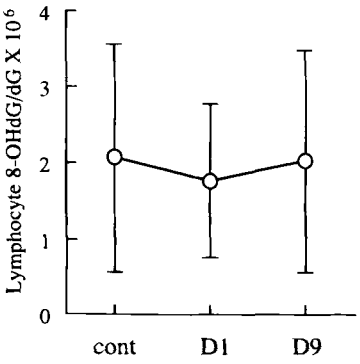

FIGURE 3 Lymphocyte 8-OHdG/dG. See legend to Figure 2 for details.

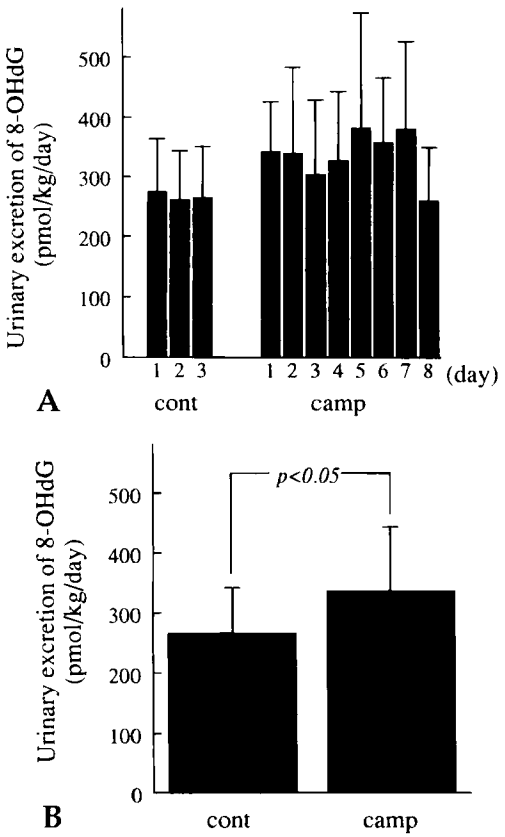

FIGURE 4 The urinary excretion of 8-OHdG. Values are the means ± SD, n = 10.

trol period (265.7 ± 75.5 vs. 335.6 ± 107.4 pmol/kg/day).

Urinary Creatinine Excretion

The amount of creatinine excreted into the urine during the camp was significantly larger than that during control period (29.5 ± 2.1 vs. 24.4 ± 4.1 mg/kg/day).

Plasma all-trans-β-carotene and α-tocopherol (Fig. 5)

The plasma all-trans-β-carotene concentrations tended to rise after the camp, however, no significant changes were observed during the study period. The plasma α-tocopherol levels on both

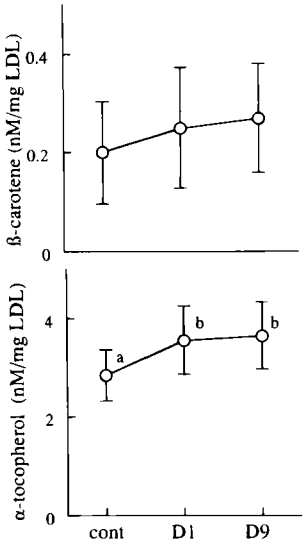

FIGURE 5 Plasma all-trans-β-carotene and α-tocopherol. See legend to Fig. 2 for details.

D1 (3.56 ± 0.68 nM/mgLDL) and D9 (3.65 ± 0.67 nM/mgLDL) were significantly higher than those of the control (2.85 ± 0.52 nM/mgLDL).

DISCUSSION

The results of the present study showed that repeated heavy exercise augmented the urinary excretion of 8-OHdG, thus suggesting that oxidative DNA damage was induced by consecutive heavy exercise. Regarding the oxidative damage to nucleic acids caused by exercise-induced ROS, Viguie *et al.*[20] and Witt *et al.*[21] reported that neither 3 consecutive days of 60 min of exercise at 60% VO_2max nor 3 consecutive days of 90 min exercise at 65% VO_2max affected the amount of 8-hydroxyguanosine excretion into the urine. They thus concluded that the exercise employed in their studies did not induce any oxidative RNA damage. With regard to the exercise-induced DNA damage, Alessio et al. showed that the ratio of 8-OHdG/creatinine in the urine significantly increased 10 h after a marathon race.[14] They thus suggested that a single bout of heavy exercise such as a marathon race might thus induce oxida-

tive damage. We observed that the urinary excretion of 8-OHdG was less on the last day of the camp in the present study (Fig. 4a). We interpret this decrease to be due to the cancellation of the training because of bad weather. This finding suggests that the oxidative DNA damage correlates to the intensity and the duration of exercise. In addition, although no exercise-induced damage to DNA was accumulated, such damage was considered to have been sustained during the camp.

The amount of 8-OHdG excreted into the urine in our subjects both the control (265.7 ± 75.5 pmol/kg/day) and the camp period (335.6 ± 107.4 pmol/kg/day) were comparable to the previously reported level of 200–300 pmol/kg per 24 h.[22]

The urinary parameters are usually standardized based on the amount of creatinine excreted in the urine when the collection of 24-h urine is not possible. Because we collected all urine during the study period, we expressed the excretion of 8-OHdG based on the body weight of the subjects. In the present study, however, the amount of urinary excretion of creatinine was increased during the camp. The amounts of urinary excretion of 8-OHdG standardized by creatinine excretion were not significantly different between the control period (11.0 ± 0.9 nmol/g creatinine) and the camp period (11.3 ± 1.0 nmol/g creatinine) in this study. An increase in the creatinine excretion into the urine is thought to be associated with an increase in the muscle mass, an enhanced muscle or other tissue breakdown or an accelerated energy metabolism. In the present study, the body weight of the subjects did not change during the camp, thus an association of an increase in muscle mass was not likely. It thus seems to be reasonable to assume that an enhanced muscle and other tissue degradation and/or an accelerated energy metabolism might thus cause the increase in urinary creatinine excretion. The former possibility is strongly supported by the increases in serum CK, CK-MB, LDH and myoglobin levels after the camp. The latter hypothesis is also quite possible because the subjects exercised so hard during the camp. The correction procedure using the amount of creatinine excreted would make sense if the amount of its excretion was stable. We therefore chose not to correct the excretion of 8-OHdG by creatinine excretion and thus concluded that an increased degree of oxidative damage to DNA accounted for the increase in urinary 8-OHdG excretion.

The amount of 8-OHdG excreted in the urine indicates the amount of repaired DNA previously injured by ROS. Inoue *et al.* reported that a single bout of swimming significantly decreased the content of 8-OHdG in the lymphocyte DNA, while the urinary excretion of 8-OHdG tended to increase but the difference was not significant.[15] They speculated that exercise might thus stimulate the repair of DNA which had been damaged by ROS generated by exercise.

In the present study, we might expect that the content of 8-OHdG in the lymphocyte DNA was reduced after the camp because the urinary excretion of 8-OHdG significantly increased during the camp period. The ratio of 8-OHdG/dG in the lymphocyte DNA, however, showed no change between the D1 and D9 levels. We assume that this apparent discrepancy may be due to the fact that the blood samples were drawn the day after the last day of the camp. The elimination of 8-OHdG from DNA might thus have been augmented after each training session at the camp as Inoue *et al.* suggested so that we could find a decrease in the content of 8-OHdG in the lymphocytes if we had collected the lymphocytes immediately after the training. We thus suspect that the generation of 8-OHdG continued the night before the blood was sampled. This assumption seems to be supported by the reports showing that the exercise-related tissue damage was more severe 4 h after exercise than immediately after exercise.[23] Another possibility could be that the increased urinary excretion of 8-OHdG could be due to cells other than lymphocytes or from deoxyGTP in the DNA precursor pool of nucleotides rather than from DNA itself.[24]

The plasma level of LDH in the serum significantly increased and the levels of CK, CK-MB, and Mb also tended to increased on D1 in comparison to those on the control day. Because there was about 1 week between the control day and D1 and the subjects exercised freely during the period, it might thus be possible to assume that the exercise training during that period might contribute to the increase in the serum enzyme levels. Of course, none of them exercised as hard as during the training camp, however, we thus consider that the observed increase in these serum enzyme values before entering the camp were most likely related to the subjects' pre-camp exercise.

It has been reported that antioxidant vitamins protect some types of tissue from exercise-related oxidative stress.[25–29] In the present study, both plasma β-carotene and α-tocopherol status showed no sign of deficiency. The present study showed that the plasma concentrations of α-tocopherol expressed per LDL significantly increased while the β-carotene showed no changes during the camp. The plasma levels of vitamin E were reported to decreased in the muscles with endurance exercise training.[30–32] Pincemail *et al.* reported that the mobilization of tocopherol into the blood occurred following intense cycling to exhaustion in humans.[33] In this study, the provision of these above described vitamins by the meal might be sufficient to maintain their plasma levels. The exact reason as to why the plasma α-tocopherol increased, however, remains to be clarified.

Loft *et al.* reported that the intake of vitamin C, vitamin E and β-carotene was not associated with the urinary excretion of 8-OHdG in humans.[16] Studies on large doses of the antioxidant nutrients on exercise-induced oxidative DNA damage should thus be done in future.

In conclusion, the present study demonstrated that the urinary 8-OHdG excretion significantly increased during a training camp which included 30 km of running per day. These data suggest that repeated exercise induces oxidative damage to DNA. Such damage, however, is not accumulated, although it continues as long as the exercise is repeated. Further studies are required to evaluate the pathological significance of these findings and to also determine whether or not the antioxidants could reduce the oxidative DNA damage induced by exercise.

Acknowledgments

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