

This article was downloaded by: [88.148.122.85]

On: 07 August 2015, At: 12:35

Publisher: Taylor & Francis

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: 5 Howick Place, London, SW1P 1WG



Free Radical Research

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/ifra20>

Effect of elite physical exercise by triathletes on seven catabolites of DNA oxidation

D. Villaño^a, C. Vilaplana^a, S. Medina^a, R. Cejuela-Anta^b, J. M. Martínez-Sanz^b, P. Gil^c, H.-G. Genieser^d, F. Ferreres^a & A. Gil-Izquierdo^a

^a Department of Food Science and Technology, CEBAS-CSIC, Murcia, Spain

^b Department of Physical Education and Sport, Faculty of Education, University of Alicante, Alicante, Spain

^c General University Hospital of Alicante, Spain

^d BIOLOG Life Science Institute, Bremen, Germany

Published online: 29 Jul 2015.



[Click for updates](#)

To cite this article: D. Villaño, C. Vilaplana, S. Medina, R. Cejuela-Anta, J. M. Martínez-Sanz, P. Gil, H.-G. Genieser, F. Ferreres & A. Gil-Izquierdo (2015) Effect of elite physical exercise by triathletes on seven catabolites of DNA oxidation, *Free Radical Research*, 49:8, 973-983

To link to this article: <http://dx.doi.org/10.3109/10715762.2015.1025388>

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at <http://www.tandfonline.com/page/terms-and-conditions>

ORIGINAL ARTICLE

Effect of elite physical exercise by triathletes on seven catabolites of DNA oxidation

D. Villaño¹, C. Vilaplana¹, S. Medina¹, R. Cejuela-Anta², J. M. Martínez-Sanz², P. Gil³, H.-G. Genieser⁴,
F. Ferreres¹ & A. Gil-Izquierdo¹

¹Department of Food Science and Technology, CEBAS-CSIC, Murcia, Spain, ²Department of Physical Education and Sport, Faculty of Education, University of Alicante, Alicante, Spain, ³General University Hospital of Alicante, Spain, and ⁴BIOLOG Life Science Institute, Bremen, Germany

Abstract

The oxidized nucleoside 8-hydroxy-2'-deoxyguanosine has been widely studied as a marker of DNA oxidation; however, data on the occurrence of other metabolites in plasma that are related to DNA damage are scarce. We have applied an improved, sensitive, robust, and reliable method, involving solid phase extraction and ultrahigh-performance liquid chromatography (UHPLC)–tandem mass spectrometry (MS/MS), to the precise quantitation of seven metabolites in the plasma of 15 elite triathletes after a 2-week training program. All compounds were eluted in the first 1.6 min, with limits of detection and quantification ranging between 0.001 and 0.3 ng.mL⁻¹ and 0.009 and 0.6 ng.mL⁻¹, respectively. Four compounds were detected in plasma: guanosine-3'-5'-cyclic monophosphate, 8-hydroxyguanine, 8-hydroxy-2'-deoxyguanosine, and 8-nitroguanosine. After two weeks of training, 8-hydroxyguanine exhibited the highest increase (from 0.031 ± 0.008 nM to 0.036 ± 0.012 nM) ($p < 0.05$), which could be related to the enhanced activity of DNA-repairing enzymes that excise this oxidized base. Increased levels of guanosine-3'-5'-cyclic monophosphate and 8-hydroxy-2'-deoxyguanosine were also observed. In contrast, levels of 8-nitroguanosine ($p < 0.05$) were significantly reduced, which might be a protective measure as this compound strongly stimulates the generation of superoxide radicals, and its excess is related to pathologies such as microbial (viral) infections and other inflammatory and degenerative disorders. The results obtained indicate an induced adaptive response to the increased oxidative stress related to elite training, and point to the benefits associated with regular exercise.

Keywords: oxidative stress, DNA oxidation biomarkers, sport, 8-hydroxy-deoxyguanosine, 8-hydroxyguanine

Introduction

Oxidative damage to DNA by free radicals includes a large variety of mechanisms and final products [1]. Mechanisms of DNA and RNA repair involve the excision of damaged bases or nucleotides and liberation of deoxy- and oxy-nucleotides and nucleosides, which circulate in plasma and are further excreted in urine [2]. Among the DNA bases, guanine has the lowest reduction potential, and is therefore the best electron donor and is preferentially oxidized [3]. The catabolites most studied are 8-hydroxy-2'-deoxyguanosine (8-OH-dGuo) and 8-hydroxyguanosine (8-OH-Guo), and they are generally used as markers of oxidative modifications to DNA and RNA respectively [4]. Other breakdown products formed under inflammatory conditions are 8-nitroguanine (8-NO₂-Gua) and its corresponding nucleoside 8-nitroguanosine (8-NO₂-Guo) [1].

In contrast, nucleotides such as guanosine-3'-5'-cyclic monophosphate (cGMP) play an important role in the cellular response to stress: nitric oxide (NO) acts as a transduction signal, activating cGMP, which is involved in the regulation of some protein-dependent kinases with important effects in the vascular and neuronal systems [5]. Its nitrated derivative, 8-nitroguanosine 3',5'-cyclic monophosphate (8-NO₂-cGMP), is produced in cells by RNOS

and acts as a signaling molecule, reacting with sulfhydryl groups of redox sensor proteins in an adaptive response to the increased oxidative stress in cells. Different analytical methods have been used to determine these compounds, mostly based on chromatographic and immunoassay techniques. Some authors have applied ELISA immunoassay techniques to identify metabolites, but have encountered problems derived from the lack of selectivity of the antibody in the urine matrix [6]. The method employed most widely to measure urinary 8-OH-dGuo is HPLC-MS/MS, due to its high sensitivity, specificity, and time-saving characteristics [7].

In the present study, we have developed a new method for the evaluation of seven DNA catabolites, to assess the effect of physical training on the redox status of the organism. Exercise increases oxygen consumption in muscle fibers during contraction, which, together with the induced injury of muscle tissues, leads to excessive production of RNOS. In fact, an increase in oxidative stress and DNA damage during acute strenuous exercise has been described, for example, during 42-km marathon runs [8]. On the other hand, mild, intense, and continuous intense exercise can induce an adaptive response, increasing the efficiency of endogenous antioxidant systems [9]. Besides, it has been demonstrated that long-term physical exercise

has beneficial effects, such as reducing the incidence of cardiovascular diseases, cancer, and diabetes [10].

The objective of our study is to determine the levels of these markers of DNA damage in triathletes before and after intense elite training for 14 days. We have used a new, sensitive, robust, and reliable method to evaluate—for the first time—seven biomarkers of DNA damage simultaneously, using solid phase extraction (SPE) as the extraction procedure and UHPLC–MS/MS as the acquisition and detection system.

Material and methods

Chemicals and reagents

The 8-NO₂-Guo, 8-OH-Gua, cGMP, 8-NO₂-Gua, and the external standards 8-azidoadenosine (8-N3-Ado) and 8-phenylthioadenosine (8-PT-Ado), were purchased from the Biolog Life Science Institute (Bremen, Germany). The 8-OH-dGuo and 8-OH-Guo were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA), while 8-NO₂-cGMP was obtained from the Biolog Life Science Institute (Bremen, Germany). The LC-MS solvents were purchased from J.T. Baker (Phillipsburg, New Jersey, USA), and the ultra-high quality (UHQ) water was produced using a Millipore water purification system. Reagents such as acetic acid, sodium hydroxide, and ammonium acetate were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). Two types of SPE cartridges were used in this study: the ISOLUTE cartridge (ENV+, 50 mg, 1 ml) from Biotage (Uppsala, Sweden), and the OASIS WAX cartridge (30 mg, 30 µm, 1 ml) from Waters (Milford, Massachusetts, USA).

In vivo study and sample collection

The individuals participating in the sample collection were 15 Caucasian triathletes (5 amateur training women and 10 elite training men), aged 19–21 years, from the University of Alicante (Spain). They were non-smokers, had stable food habits, and did not receive any medication during the study. The study was approved by the Bioethics Committee of the University Hospital of Murcia, in accordance with the Declaration of Helsinki, and all participants gave written informed consent.

The physical characteristics, dietary intake, and training loads during the intervention are detailed in Table I. The volunteers consumed a constant diet during the two weeks before the intervention and until its conclusion, to avoid any interference of the diet with the oxidative stress events. The diet was accurately designed and overviewed by nutritionists, using specific software for the calculation of the dietary parameters and caloric intake (software available on the website <http://www.invesalia.es/evaluacion/>, with the additional assistance of the Spanish and USDA databases <http://www.bedca.net/and> <http://www.nal.usda.gov/fnic/food-comp/search/>). The subjects were instructed to eat only the food provided to them by the nutritionists. Daily and weekly averages of the 24-h calorie intake were calculated.

Anthropometric measurements were carried out according to the International Society of Advancement of Kinanthropometry (ISAK) and all were performed by the same internationally certified anthropometrist (level 2 ISAK), as described in Medina et al. [11].

The quantification of the training program was performed to evaluate its effects on physiological adaptation and subsequent performance. In our work, the training load quantification was performed using the “Objective-load scale” (ECOs) developed by Cejuela Anta and Esteve-Lanao [12]. The training load that a triathlete supports is an indication of his/her performance level. The method used allowed the quantification of the training loads in the sport of triathlon (swim, bike, run, and transitions), which are determined by the difficulty in maintaining technique, delayed muscle soreness, typical workout density, and energy cost of each separate sport. The values of the daily and weekly training were determined and summarized to assess the training load (ECOs) of each

Table I. Dietary parameters, physical characteristics, and training loads of the triathletes.

Dietary parameters during the intervention period		
Energy intake (kcal d ⁻¹)	2446.3 ± 528.5	
Carbohydrate (g d ⁻¹)	268.7 ± 81.2	
Dietary fiber (g d ⁻¹)	21.4 ± 8.3	
Sugars (g d ⁻¹)	100.9 ± 28.8	
Proteins (g d ⁻¹)	108.5 ± 35.6	
Total lipids (g d ⁻¹)	110.4 ± 4.7	
SFA ^a (g d ⁻¹)	31.6 ± 2.8	
MUFA ^b (g d ⁻¹)	56.7 ± 0.1	
PUFA ^c (g d ⁻¹)	16.4 ± 0.7	
Vitamin C (mg d ⁻¹)	156.9 ± 30.9	
Vitamin A (µg d ⁻¹)	2198.7 ± 1090.8	
Vitamin E (mg d ⁻¹)	17.5 ± 5.0	
Vitamin D (mg d ⁻¹)	869.9 ± 167.2	
Iron (mg d ⁻¹)	17.9 ± 4.2	
Selenium (mg d ⁻¹)	126.4 ± 33.1	
Physical characteristics	Baseline	2-week training
Weight (kg)	62.3 ± 10.5	62.3 ± 10.5
Height (m)	1.7 ± 0.1	1.7 ± 0.1
BMI ^d (kg m ⁻²)	21.7 ± 0.7	21.7 ± 0.6
Total fat (kg)	9.0 ± 0.4	8.9 ± 0.1
Lean weight (kg)	26.1 ± 7.5	25.6 ± 7.0
Subscapular skinfold (mm)	11.2 ± 2.2	11.5 ± 2.8
Tricipital skinfold (mm)	12.6 ± 5.2	14.1 ± 6.2
Bicipital skinfold (mm)	7.9 ± 3.5	7.3 ± 3.6
Ileocrestal skinfold (mm)	15.9 ± 5.4	15.1 ± 2.8
Supraspinal skinfold (mm)	11.7 ± 3.7	11.7 ± 3.9
Abdominal skinfold (mm)	19.8 ± 4.7	19.6 ± 5.7
Thigh skinfold (mm)	21.1 ± 8.7	20.2 ± 8.8
Calf skinfold (mm)	11.9 ± 4.1	11.7 ± 3.1
Training loads	Baseline	2-week training
ECOs ^e	38 ± 4	1201 ± 577

^aSaturated fatty acids.

^bMonounsaturated fatty acids.

^cPolyunsaturated fatty acids.

^dBody Mass Index.

^eECOs, Objective-load scale (this parameter has been defined in the text).

volunteer, depending on their physical characteristics and the intensity of the training program (Table I).

Human blood was collected in heparin sampling tubes and centrifuged to separate the plasma from the cells. These samples were collected in fasting conditions before (ECOs 38) and after (ECOs 1201) two weeks of intense training.

The plasma was first deproteinized. A preliminary study of different methods of deproteinization (ZnSO₄, acetonitrile addition) as well as non-deproteinization of samples (according to Lam et al.) [7] was performed, by spiking plasma with a pool of catabolites (100 nM c.f. each) and evaluating the recovery after each type of plasma pretreatment. Protein precipitation with two volumes of cold acetonitrile gave less interference and good recoveries for 8-OH-Guo, but ZnSO₄ (26.68 mg per 1 mL plasma) provided better recoveries for most of the target compounds of this study. Therefore, ZnSO₄ was selected for deproteinization of the samples (Table II). After addition of ZnSO₄, the samples were centrifuged at 10000 rpm for 5 min and the supernatant collected for analysis.

Solid phase extraction of DNA catabolites from human plasma

Prior to the analysis of the plasma samples of the triathletes, the SPE conditions were optimized for the quantification of DNA catabolites. We prepared the standard of 8-OH-Gua and 8-NO₂-Gua by dissolving it in NaOH 0.1 N, in the minimum quantity needed to avoid basic hydrolysis of the compound. Once dissolved, the following dilutions were performed in Milli-Q water, and we checked the stability of the solution during the day of analysis. The other standards were prepared in Milli-Q water.

We determined the efficiency of extraction of the SPE columns ISOLUTE and OASIS WAX, based on polarity and the principles of ion-exchange chromatography, respectively, since the DNA catabolites show complex retention mechanisms on solid phases due to their chemical nature.

The SPE extraction of DNA catabolites with ISOLUTE cartridges was performed as described by Lam et al. [7] for 8-OH-dGuo, with some modifications for the detection of additional compounds. This method was used for the extraction of 8-PT-Ado, 8-N3-Ado 8-OH-dGuo, 8-OH-Guo, and 8-OH-Gua (on the basis of the recoveries

obtained, shown in the Results section). Deproteinized plasma (1 mL) was diluted with deionized water at a ratio of 1/1. ISOLUTE (Env+, 1 mL, 50 mg) cartridges (Biotage, Japan) were preconditioned with 1 mL of methanol and equilibrated with 1 mL of deionized water. Samples were then introduced into the cartridges and drawn through at a flow rate of 1 mL/min. The cartridges were washed twice with 300 µL of H₂O and the compounds were eluted twice with 300 µL of 20% (v/v) acetonitrile in methanol.

Extraction with OASIS WAX cartridges (1 cc, 30 mg) (Waters) was performed based on the method of Martens-Lobenhoffer et al. [13], developed for cGMP determination. In our assay, it was adapted for the extraction of cGMP, 8-NO₂-cGMP, 8-NO₂-Guo, and 8-NO₂-Gua. The SPE cartridge was first conditioned with 1 mL of methanol and equilibrated with 1 mL of deionized water. Plasma samples (1 mL) were diluted with 2% (v/v) acetic acid at a ratio of 1/1, and the mixture was drawn through the SPE column. The columns were washed first with 1 mL of 2% (v/v) acetic acid and then with 1 mL of methanol. The cartridges were left to dry for 2 min and the analytes were eluted with 1 mL of 5% ammonia in methanol.

In both types of extraction, the eluents obtained were dried under speed-vacuum, reconstituted in 50 µL of mobile phase B (2.5 mM acetic acid/ammonium acetate pH 3.9), and then sonicated for 3 min before being transferred to UHPLC vials. A dilution factor of 20 was applied, and therefore, we were able to detect lower concentrations of standards in the samples than those detailed in LOD and LOQ.

The extraction capacities of each type of SPE column were assessed by the addition of 10 µL of a pool of catabolites at different concentrations to 990 µL of plasma before SPE (the final concentration of each catabolite was between 5 and 0.05 nM). In parallel, 990 µL of plasma underwent SPE and was spiked with the same amount of the pool afterwards. The percentage recovery was calculated as $(\text{Area}_{\text{PRE}}/\text{Area}_{\text{POST}}) \times 100$, where Area_{PRE} and Area_{POST} represent the samples with compounds added before and after SPE, respectively.

UHPLC-MS/MS analysis

Chromatographic analyses were carried out with a UHPLC coupled to a 6460 QqQ-MS/MS (triple quadrupole mass spectrometer) (Agilent Technologies,

Table II. Effect of the plasma deproteinization agents on the recovery of DNA catabolites, expressed as percentage (%) of the total amount of catabolite added.

Compound	ZnSO ₄	Acetonitrile	No Deproteinization
8-Hydroxy-2'-deoxyguanosine	87.8	85.2	160.1
8-Hydroxyguanine	105.1	n.d.	72.1
8-Hydroxyguanosine	76.3	102.0	156.5
8-Nitroguanine	83.0	n.d.	n.d.
8-Nitroguanosine	49	n.d.	n.d.
8-NO ₂ -cGMP	123	105.1	85.1
cGMP	66.0	82.0	298.0
8-Azidoadenosine	87.0	96.2	102.3
8-Phenylthioadenosine	84.1	63.7	149.1

Waldbronn, Germany) equipped with an electrospray ionization (ESI) source. The separation of analytes was performed on a Kinetex HILIC column (100 × 2.10 mm), packed with 1.7 μm particles, from Phenomenex (Torrance, USA). The column temperatures were 27°C (left) and 27°C (right). The mobile phases were adapted from a previous study [14]. Different tests were conducted with the mobile phase B of the method; by varying the percentages of the organic phase, we were able to shorten the elution time with respect to the method mentioned above, with better peak resolution. Finally, the mobile phases employed were (A) acetonitrile, and (B) 2.5 mM acetic acid/ammonium acetate buffer of pH 3.9. The gradient conditions started with 70% A and 30% B for 2 min, then the fraction of B was raised to 95% in 6 min, and the post-run time was 2 min. The flow rate and the injection volume were 0.2 mL min⁻¹ and 20 μL, respectively. The MS analysis was performed in the multiple reaction monitoring (MRM) mode, using negative or positive ESI, depending on the analyte. The source-optimized parameters were as follows: gas temperature: 340°C, gas flow: 10 L min⁻¹, nebulizer: 50 psi, sheath gas temperature: 300°C, sheath gas flow: 10 L.min⁻¹, capillary voltage: 2500 V, and nozzle voltage: 2000 V. The dwell time was 25 ms for all MRM transitions. The MS parameters (ion optics, capillary exit voltage) and the fragmentor and collision energies were optimized for each compound. The mass transitions, parent ions to product ions, collision energies, fragmentor potentials, and ESI mode for the analytes are described in Table III. Data acquisition and processing were performed using MassHunter software version B.04.00 (Agilent Technologies).

Optimization of the method for the determination of DNA catabolites

The method was optimized according to ICH guidelines [15] for the DNA catabolites that had not previously been described, as well as for the two external standards, 8-PT-Ado and 8-N3-Ado, used for quantification. The limits of detection and quantification were established by the signals measured from samples spiked with known, low concentrations of analyte, which yielded signal-to-noise ratios greater than 3 and 10, respectively. The linearity of the assay for each compound was determined by 10-point standard curves and linear regression analysis (from 200 ng.mL⁻¹ to 0.001 ng.mL⁻¹). The intraday (*n* = 9) and interday (*n* = 9) precision and accuracy were calculated over three days for three different concentrations of each metabolite within the linear interval of the procedure. The recovery was determined at different concentrations by comparing the measured concentration values obtained for the standards spiked in plasma samples prior to SPE, with those obtained in plasma samples spiked after SPE, as described above.

Once the parameters had been optimized, we employed an external calibration curve as well as two internal standards

Table III. Precursor ion, product ions, and preferential MRM transitions for quantification and conditions optimized for fragmentation of the seven DNA catabolites and two internal standards (8-azidoadenosine and 8 phenylthioadenosine) in the analysis by UHPLC/QqQ-MS/MS.

Analyte	Precursor Ion (m/z), [MH] ⁺	Product Ion(s) (m/z)	Quantification transition (m/z)	Dwell time (ms)	Fragmentor (V)	Collision Energy (V)	Polarity	SPE cartridge
8-Hydroxy-2'-deoxyguanosine	284.3	167.8	284.3 → 167.8	25	110	0	Positive	ISOLUTE
8-Hydroxyguanine	168.1	140.3	168.1 → 140.3	25	80	15	Positive	ISOLUTE
8-Hydroxyguanosine	298.0	207.7; 165.2	298.0 → 207.7	25	80	0	Negative	ISOLUTE
8-Nitroguanine	195.1	178; 166.6; 150.9; 136.9; 61.0	195.1 → 178	25	80	5	Negative	OASIS WAX
8-Nitroguanosine	327.1	194.8; 178.1; 152.9	327.1 → 194.8	25	110	10	Negative	OASIS WAX
8-NO ₂ -cGMP	389.0	194.7	389.0 → 194.7	25	110	0	Negative	OASIS WAX
cGMP	346.0	151.9	346.0 → 151.9	25	110	0	Positive	OASIS WAX
8-Azidoadenosine	309.1	176.8; 149.0; 151.1	309.1 → 176.8	25	80	0	Positive	ISOLUTE
8-Phenylthioadenosine	376.1	244.0	376.1 → 244.0	25	100	0	Positive	ISOLUTE

(8-PT-Ado and 8-N3-Ado) for quantification of the catabolites in plasma samples of the triathletes.

Statistical analysis

The data are expressed as mean \pm SD. Statistical comparison of the concentrations of DNA catabolites in the samples of triathletes before and after the intensification of the training load was performed using the paired Student *t*-test. Statistical analyses were performed using the SPSS 17.0 software package (LEAD Technologies Inc., Chicago, US), and the level of statistical significance was set at $P < 0.05$.

Results

Optimization of the procedure of analyte extraction

Seven catabolites and two internal standards were optimized with our method. Their identities were confirmed according to their molecular masses, the characteristic MS/MS fragmentation patterns, and the retention times, compared to the corresponding standards. Quantitative MRM transitions were obtained in the negative or positive ESI mode, depending on the response of each analyte. Additional, complementary MRM transitions (qualifier transitions) were also selected, for further confirmation of the compounds (Table III). The retention times of the catabolites were between 0 and 2 min (Figures 1 and 2).

Fundamental parameters for the optimization of the method were determined, such as the recovery, sensitivity, linearity, and precision (Table IV). The linearity was derived from 10-point calibration curves and the responses were linear over the range of 200 to 0.001 ng.mL⁻¹, with correlation coefficients (R^2) higher than 0.99. The lowest LOD and LOQ values obtained were 0.0010 ng.mL⁻¹ (for 8-OH-Gua) and 0.0092 ng.mL⁻¹ (for cGMP), respectively. The intraday coefficient of variation (CV %) for each compound ranged from 0.2 to 12% (except for 8-NO₂-Gua, with 29%) and the interday CV % ranged from 1.7 to 17% (except for 8-OH-Gua, with 26%) (Table V). The recovery for each catabolite was tested at concentrations between 0.05 nM and 5 nM, within the range of the values expected in plasma. The ISOLUTE cartridges, due to their hydrophobic nature, are specific for the extraction of polar analytes from aqueous samples. In contrast, the weak cation exchange OASIS WAX columns exert interactions based on ion charge; with an acidic pH, it is possible to retain compounds that are then eluted at basic pH by neutralizing the ion exchange resin [13]. By applying both procedures, we were able to extract from the plasma all the compounds of interest. The ISOLUTE cartridges provided better recoveries (42 to 87%) of 8-OH-dGuo, 8-OH-Gua, 8-OH-Guo, 8-N3-Ado, and 8-PT-Ado (Table VI). The OASIS WAX cartridges yielded better values, ranging from 70 to 155%, for cGMP, 8-NO₂-cGMP, 8-NO₂-Gua, and 8-NO₂Gua.

Levels of DNA catabolites in triathletes subjected to intense elite training

Our aim was to determine the effect of intense elite physical exercise on the generation of DNA catabolites in the plasma of triathletes. Plasma samples were subjected to two types of SPE, with the OASIS WAX and ISOLUTE cartridges described above, to ensure the extraction and subsequent identification of all compounds of interest.

Human plasma samples from volunteers at the baseline (ECO 38) and after two weeks of training (ECO 1201) were analyzed. External calibration curves were generated for each analyte, in the range from 100 nM to 0.2 nM. Four analytes were detected in the plasma of the triathletes: cGMP, 8-OH-dGuo, 8-OH-Gua, and 8-NO₂-Gua (Figure 3). The highest baseline levels were 0.027 ± 0.01 nM for cGMP and 0.031 ± 0.008 nM for 8-OH-Gua. After two weeks of intensified training, 8-OH-Gua exhibited the highest increase, reaching a mean value of 0.036 nM, which was statistically significant ($p < 0.05$). We also observed increased levels of cGMP (from 0.027 nM to 0.036 nM) and 8-OH-dGuo (from 0.016 nM to 0.018 nM), albeit non-significant. In contrast, a significant reduction (45%, $p < 0.05$) in the concentration of 8-NO₂-Gua occurred, from 0.016 nM to 0.009 nM.

Discussion

The present work is focused on evaluating the influence of exercise on the levels of DNA catabolites in plasma.

To date, the degree of DNA damage has been assessed by the measurement of 8-OH-dGuo in biological fluids by LC-MS or ELISA methods [6,14]. Scarce data are available on other products of the oxidation of nucleic acids. In our study, we have adapted an analytical method validated for 8-OH-dGuo for the simultaneous determination of several DNA catabolites in triathletes, to assess—for the first time—the influence of intense elite training on DNA oxidative damage. Our method consists of sample pretreatment with SPE, followed by chromatographic separation with a HILIC column, by UHPLC-MS/MS. In recent years, hydrophilic interaction chromatography (HILIC) has been reported as a good alternative to reverse-phase liquid chromatography (RPLC) for the separation of highly polar compounds such as nucleotides and their derivatives [6,14]. In our experience, SPE is essential to facilitate the subsequent detection and quantification of metabolites.

All compounds eluted in the first six minutes highlight the improved technical throughput—compared to HPLC methods developed previously for the determination of DNA nucleotides and analogs—of our ultrafast UHPLC-MS/MS method [16].

Regarding the SPE procedures, most recovery levels were above 70%, similar to those found in the literature [7,13,17]. Our UHPLC method is much more sensitive compared to other previously described methods (e.g., LOQ for cGMP 0.025 nM vs 1 nM, and LOD for 8-OH-dGuo 0.028 ng.mL⁻¹ vs 0.2 ng.mL⁻¹) [13,14,18].

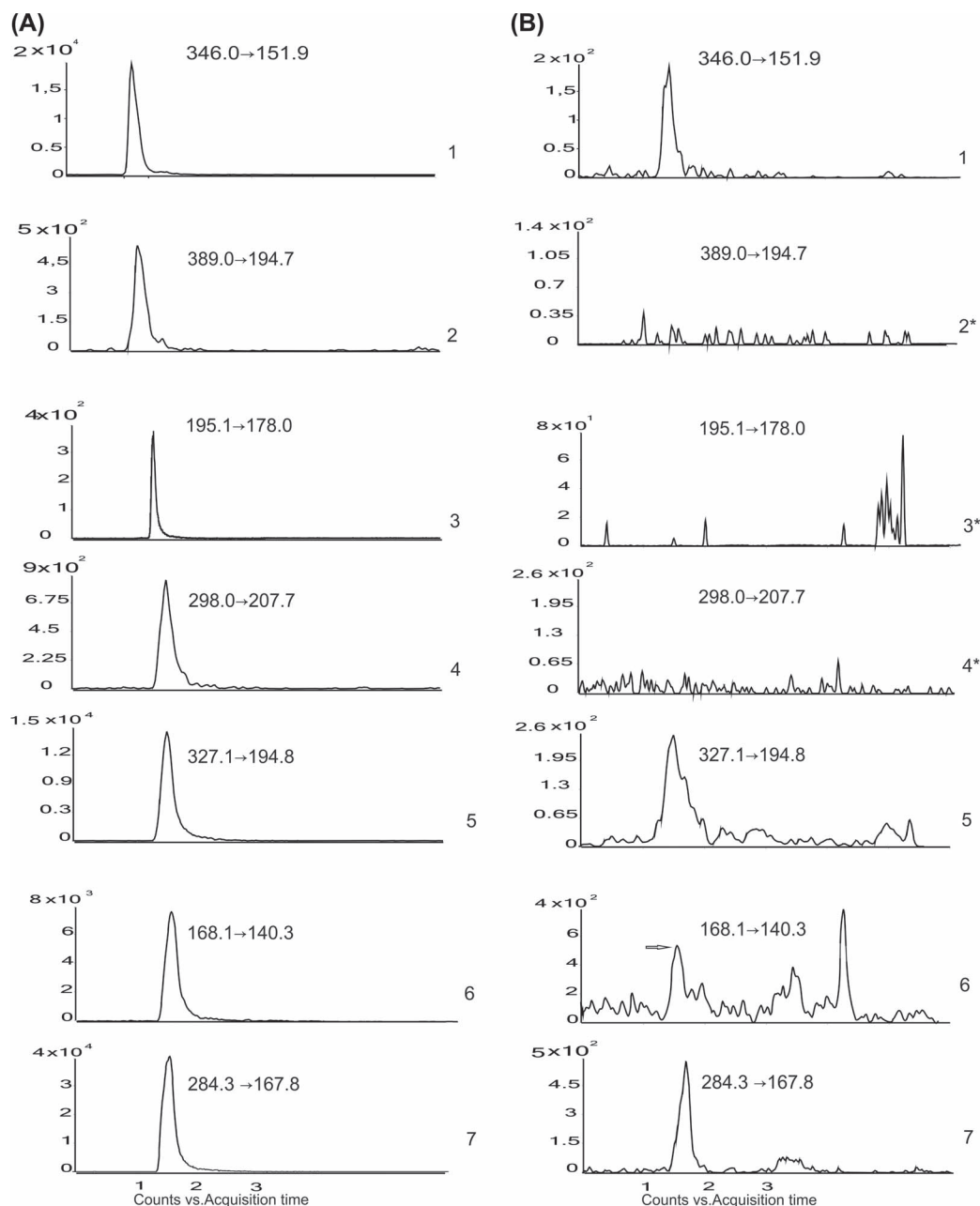


Figure 1. UHPLC-QqQ-MS/MS chromatograms of the seven compounds at the MRM transitions for quantification. A) Standards in H_2O B) compounds detected in plasma samples of triathletes: (1) cGMP (2) 8- NO_2 -cGMP (3) 8-Nitroguanine (4) 8-Hydroxyguanosine (5) 8-Nitroguanosine (6) 8-Hydroxyguanine (7) 8-Hydroxy-2'-deoxyguanosine. *not detected.

Of the seven metabolites optimized, only four were present in the plasma samples of the triathletes; 8-OH-Guo, 8- NO_2 -cGMP, and 8- NO_2 -Gua were not detected. However, this does not mean that they did not exist in these samples; they could have been present at trace levels below the LODs of our method (Table IV).

8-Nitroguanine is a product of nitrate DNA lesion, and concentrations of this compound have been reported in cells of different organs and tissues of animal models [19] and in humans with cancer or degenerative diseases [20]. However, and to the best of our knowledge, no data are available for human plasma samples. The same occurs with the 8- NO_2 -cGMP, which is a second messenger generated during stress conditions and implicated in redox

signaling in different processes, as in the cardiovascular system [21]. No data of its presence in human plasma has been reported.

8-Hydroxyguanosine has been reported as the most examined of the RNA oxidation products, and the procedures such as HPLC-MS that are currently used to measure this compound have been applied to human urine and cerebral spinal fluid (CSF) samples [22], but no data are available for human plasma samples.

In our study, cGMP levels tended to increase in response to intense elite exercise (Figure 3), although not significantly so, probably due to interindividual variability. Similarly, increased levels of cGMP were found after three months of exercise training in metabolic syndrome patients

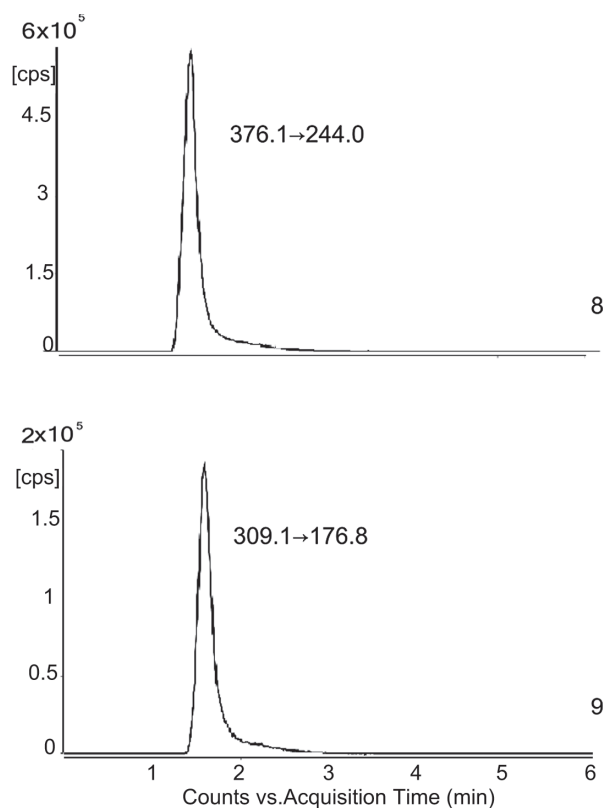


Figure 2. UHPLC-QqQ-MS/MS chromatograms of the internal standard compounds in H₂O at the MRM transitions for quantification. (8) 8-Phenylthioadenosine (9) 8-Azidoadenosine.

[23] and in subjects with elevated cardiovascular risk after 12 weeks of training [24], as well as in physically trained athletes after acute exercise [25].

Blood flow increases markedly during exercise to meet oxygen demands. This response is regulated by vasodilators such as nitric oxide (NO) that exerts its action through the signaling molecule cGMP. The circulating levels of cGMP may reflect NO synthase activity and are a marker of NO action. An increase in NO availability is related to an improvement of vascular function and a reduced risk of acute coronary events in metabolic syndrome patients [26]. Activation of cGMP inhibits the contraction of smooth muscle, thus increasing blood flow and decreasing blood pressure. Also, plasma levels of cGMP have been

correlated with those of atrial natriuretic peptide (ANP), since the latter is able to increase the release of the intracellular second messenger cGMP. The level of the hormone ANP increases with exercise, as it regulates water and sodium balance and helps to reduce blood pressure, as a mechanism of protection against the excessive efforts of the cardiac muscle [27]. Concentrations of this natriuretic hormone were raised after exercise in subjects with congestive heart failure, and this was related to better survival [28]. Correlations were found between the increase in the ANP and cGMP concentrations in plasma after a treadmill test, in both athletes and sedentary people [25]. Measurement of plasma cGMP after exercising on an ergometric bicycle was related to improved early diagnosis of patients with asymptomatic left ventricular dysfunction or heart failure with minimal symptoms [29]. In our study, the tendency observed toward an increment in cGMP levels in triathletes may reflect the positive adaptive responses of the organism to the increased vascular and cardiac demands with exercise. The magnitude of this increase could be dependent on the intensity of the training and the physical fitness of the elite triathletes.

After the 2-week training program, 8-OH-Gua had increased significantly in the triathletes, by up to 98% from baseline levels (Figure 3). This compound has been described as an excision product derived from the activity of DNA-repairing enzymes. There are two ways to repair damaged DNA: base excision repair (BER) and nucleotide excision repair (NER). Oxidized bases are mainly repaired via BER—by the action of glycosylase enzymes that cleave the glycoside bond, liberating a range of products. The enzyme 8-hydroxyguanine DNA glycosylase (OGG1) is responsible for the excision of 8-OH-Gua. The other main substrate of OGG1 is 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Both compounds are excised from DNA, with similar excision kinetics. The activity of this enzyme is enhanced upon aerobic exercise (such as a marathon race or a single bout of exercise in a treadmill test), thus increasing 8-OH-Gua levels [30,31]. Hard training implies an increase in oxidative stress, and DNA repair pathways are launched, generating greater amounts of 8-OH-Gua, as seen in our study. This process is of great relevance due to the mutagenic potential of 8-OH-Gua,

Table IV. Analytical features of the method: linearity, limit of detection (LOD), and limit of quantification (LOQ) for each analyte.

Analyte	Calibration curve	Coefficient of regression (R ²)	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)
8-Hydroxy-2'-deoxyguanosine	y = 9422.3x - 8998.6	0.9967	0.0071	0.0079
8-Hydroxyguanine	y = 98.748x + 106.02	0.9992	0.001	0.0106
8-Hydroxyguanosine	y = 189.53x - 101.59	0.9973	0.0299	0.035
8-Nitroguanine	y = 7.4613x + 21.453	0.9963	0.0959	0.3491
8-Nitroguanosine	y = 3655.7x - 2642.4	0.9971	0.0033	0.0164
8-NO ₂ -cGMP	y = 204.29x - 313.24	0.9988	0.3215	0.6430
cGMP	y = 10808x - 9331.6	0.9978	0.0046	0.0092
8-Azidoadenosine	y = 227734x - 55522	0.9966	0.0184	0.0184
8-Phenylthioadenosine	y = 164991x - 83681	0.9987	0.0225	0.045

Table V. Intraday and interday coefficients of variation (RSD).

Compound name	Intraday		Interday	
	Measured (ng.ml ⁻¹)	RSD (%)	Measured (ng.ml ⁻¹)	RSD (%)
8-Hydroxy-2'-deoxyguanosine	87.78 ± 8.30	9.25	89.42 ± 12.35	13.97
	20.91 ± 0.75	3.57	19.79 ± 3.39	17.12
	3.30 ± 0.15	4.50	3.35 ± 0.33	9.96
8-Hydroxyguanine	44.14 ± 4.98	11.27	23.46 ± 2.13	9.07
	2.67 ± 0.20	9.79	2.54 ± 0.37	14.42
	0.43 ± 0.07	17.18	0.26 ± 0.07	26.67
8-Hydroxyguanosine	160.41 ± 12.42	7.74	182.22 ± 10.48	5.75
	74.99 ± 5.83	7.77	81.10 ± 6.09	7.51
	17.69 ± 0.91	5.16	17.33 ± 0.83	4.77
8-Nitroguanine	132.82 ± 1.04	0.78	358.32 ± 49.03	13.68
	66.15 ± 2.08	3.15	115.96 ± 15.61	13.46
	1.95 ± 0.58	29.96	61.14 ± 3.26	5.33
8-Nitroguanosine	95.00 ± 0.18	0.19	87.33 ± 4.13	4.73
	21.95 ± 0.92	4.19	20.69 ± 1.60	7.71
	3.30 ± 0.18	5.53	2.99 ± 0.15	5.06
8-NO ₂ -cGMP	104.52 ± 5.91	5.66	106.05 ± 5.73	5.40
	27.57 ± 3.22	11.68	26.61 ± 0.46	1.74
	4.00 ± 0.10	2.47	3.61 ± 0.31	8.63
cGMP	25.19 ± 0.85	3.37	25.06 ± 2.91	11.61
	4.23 ± 0.19	4.45	3.76 ± 0.43	11.52
	2.80 ± 0.16	5.71	2.30 ± 0.32	13.88
8-Azidoadenosine	83.47 ± 6.61	7.92	74.70 ± 10.26	13.74
	3.98 ± 0.41	10.29	3.60 ± 0.11	3.13
	2.31 ± 0.27	11.86	1.95 ± 0.27	13.60
8-Phenylthioadenosine	27.19 ± 0.85	3.37	8.46 ± 1.47	17.34
	4.23 ± 0.19	4.45	3.97 ± 0.65	16.25
	2.80 ± 0.16	5.71	2.06 ± 0.29	14.23

which could lead to GC transversion to TA unless repairs are made before DNA replication [32]. In our study, the upregulation of DNA repair mechanisms, and the consequent release of this by-product into plasma for further excretion, indicate positive induced adaptation to regular exercise.

The oxidation product most studied in order to describe changes in DNA stability related to physical activity is 8-OH-dGuo. Its levels showed a mild increase but were not changed significantly by the triathletes' 2-week elite training regime (Figure 3).

Despite 8-OH-dGuo being the DNA damage biomarker most widely studied, differences between chromatographic analytical methods and ELISA-based methods do not help to achieve consistent and comparable results, and for this reason, the European Standards Committee on Urinary Lesions Analysis (ESCUA) was created to assess differences between analytical methods and laboratory results [33]. In addition to the methodological sources of variability, they pointed out some human-subject factors that may also bear influence in the different data reported by literature on urinary 8-OH-Guo, and the effect of sport. Some authors did not observe significant changes in urinary 8-OH-dGuo until four days after a short-distance marathon [34], whilst others still found increases 14 days after the race [35] or after 30 days of intensive military training of 8–11 h/day [36]. In the case of competitive ultra endurance exercise—prolonged, vigorous exercise

for days, such as the ultramarathon (2 days: 40 km, 90 km) or super marathon (4-day race: 93 km, 120 km, 56 km, 59 km)—urinary 8-OH-dGuo increased in the first few days but declined to baseline afterwards [37,38]. Most studies of non-competitive endurance exercise (<4 h) and periods of intensified training showed no changes in 8-OH-dGuo levels, or increase levels that returned to baseline afterwards [39,40]. On the other hand, a study was performed with cancer patients, who showed reduced urinary excretion of 8-OH-dGuo when moderate exercise was conducted, compared with a non-significant increase with intense exercise [41]. It has been hypothesized that levels of the modified nucleoside 8-OH-dGuo and the modified

Table VI. Recoveries (%) of the analytes at different spiked concentrations in plasma (5, 2, 1, 0.05 nM; *n* = 12), in triplicate, with both types of SPE cartridges.

	ISOLUTE	OASIS WAX
8-Hydroxy-2'-deoxyguanosine	84–87	17–18
8-Hydroxyguanine	87	77–114
8-Hydroxyguanosine	76.25	
8-Nitroguanine		155
8-Nitroguanosine	49	70–80
8-NO ₂ -cGMP	123	110
cGMP	48–75	78–89
8-Azidoadenosine	69–87	11–28
8-Phenylthioadenosine	42–85	13–25

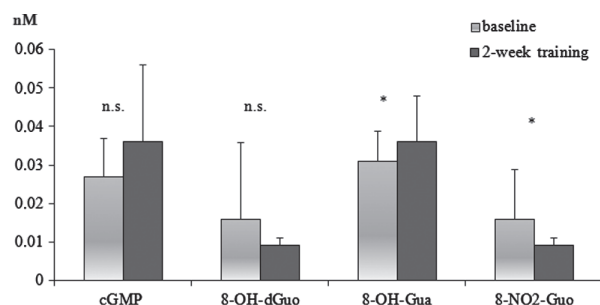


Figure 3. DNA catabolites (cGMP, 8-OHdG, 8-OHGua, and 8-NO₂-Guo) determined in triathletes following the 2-week training program. Bars with an asterisk show statistically significant differences, at * $P < 0.05$.

base 8-OH-Gua are reflective of different repair pathways (BER vs NER) [42]. In the latter study, 42 cancer patients showed a mean increase of 50% over the control in the 24 h-urinary excretion of 8-OH-Gua, but the level of 8-OH-dGuo remained almost equal in the two groups, similar to our results with the trained athletes.

We observed a significant reduction in the 8-NO₂-Guo levels in triathletes after the training period, compared to the non-training situation. Up to now, there are no studies available that relate this compound with physical activity, and this is the first time that this marker has been determined *in vivo* in human plasma. 8-NO₂-Guo is a product of the oxidative damage caused to nucleic acids by peroxynitrite, and it can be considered a potential indicator of nitrative stress during infections and inflammation [43]. It has been linked to redox-based signal transduction, and it strongly stimulates the generation of free radicals such as superoxide. Due to this property, it has a physiological role in the protective response to infections, but also in pathological events, as a mutagenic compound. Increased levels of 8-NO₂-Guo have been reported in tumoral tissues and cells [44]. Thus, we hypothesize that the decrease in the levels of 8-NO₂-Guo, observed in our triathletes after intense moderate training, might be protective, supporting the current evidence on the positive effects of sustained physical activity.

Conclusions

Our results show an adaptive response of the organism to exercise, mirrored by a significant increase in the excision of the oxidized base 8-OH-Gua by the DNA repair mechanisms. In contrast, the levels of mutagenic compounds such as 8-NO₂-Guo were significantly reduced. We have also observed a tendency of signaling molecules such as cGMP to increase in concentration, for the protection of vascular and muscle tissues against the demands of exercise. Hence, the available data indicate that regular exercise induces a free-radical adaptive mechanism, with increased activity of DNA repair systems and enhanced resistance to oxidative stress. Differences observed in the literature may be due to the study design, training status

of the subjects, duration and intensity of the exercise, or DNA repair activity. These molecular mechanisms might explain the beneficial cardiovascular effects of exercise training against lifestyle-dependent diseases. For this, a new technique has proved to be useful for the assessment of the pattern of oxidatively damaged DNA in triathletes after a two-week elite training program. We have optimized a protocol for the evaluation of metabolites implicated in DNA oxidation, using a fast and reliable UHPLC-MS/MS method. Our method is very sensitive and shows good recoveries within a six-minute run, with ultrafast detection of the compounds in the first 1.6 min.

Acknowledgements

The authors are grateful to the triathlete volunteers that participated in the study and to Dr. David Walker, for the review of the English grammar and style of the current report.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

The study received financial support from the national funding agencies through the projects AGL2011-23690 (CICYT), CSD007-0063 (CONSOLIDER-INGENIO 2010 "Fun-c-Food"), and CSIC 201170E041 (Spanish Ministry of Economy and Competitiveness). Sonia Medina is grateful to the CICYT for a research contract (AGL2011-23690).

References

- [1] Cooke MS. Special issue on DNA oxidation: mechanisms, measurement and consequences. *Free Radic Res* 2012;46:365–366.
- [2] Mateos R, Bravo L. Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). *J Sep Sci* 2007;30:175–191.
- [3] Dizdaroglu M, Jaruga P. Mechanisms of free radical-induced damage to DNA. *Free Radic Res* 2012;46:382–419.
- [4] Nunomura A, Moreira PI, Castellani RJ, Lee HG, Zhu X, Smith MA, Perry G. Oxidative damage to RNA in aging and neurodegenerative disorders. *Neurotox Res* 2012;22:231–248.
- [5] Ihara H, Sawa T, Nakabeppu Y, Akaike T. Nucleotides function as endogenous chemical sensors for oxidative stress signaling. *J Clin Biochem Nutr* 2011;48:33–39.
- [6] Hosozumi C, Toriba A, Chuesaard T, Kameda T, Tang N, Hayakawa K. Analysis of 8-hydroxy-2'-deoxyguanosine in human urine using hydrophilic interaction chromatography with tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2012;893–894:173–176.
- [7] Lam PM, Mistry V, Marczylo TH, Konje JC, Evans MD, Cooke MS. Rapid measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine in human biological matrices using

- ultra-high-performance liquid chromatography-tandem mass spectrometry. *Free Radic Biol Med* 2012;52:2057–2063.
- [8] Wagner KH, Reichhold S, Neubauer O. Impact of endurance and ultraendurance exercise on DNA damage. *Ann N Y Acad Sci*. 2011;1229:115–123.
- [9] Radak Z, Chung HY, Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic Biol Med* 2008;44:153–159.
- [10] Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev* 2008;88:1243–1276.
- [11] Medina S, Dominguez-Perles R, Cejuela-Anta R, Villano D, Martinez-Sanz JM, Gil P, et al. Assessment of oxidative stress markers and prostaglandins after chronic training of triathletes. *Prostaglandins Other Lipid Mediat* 2012;99:79–86.
- [12] Cejuela Anta R, Esteve-Lanao J. Training load quantification in triathletes. *JHSE* 2011;6:218–232.
- [13] Martens-Lobenhoffer J, Dautz C, Bode-Boger SM. Improved method for the determination of cyclic guanosine monophosphate (cGMP) in human plasma by LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010;878:487–491.
- [14] Rodriguez-Gonzalo E, Garcia-Gomez D, Carabias-Martinez R. Development and validation of a hydrophilic interaction chromatography-tandem mass spectrometry method with on-line polar extraction for the analysis of urinary nucleosides. Potential application in clinical diagnosis *J Chromatogr A* 2011;1218:9055–9063.
- [15] ICH. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. 2005.
- [16] Cohen S, Jordheim LP, Megherbi M, Dumontet C, Guitton J. Liquid chromatographic methods for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy: a review. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010;878:1912–1928.
- [17] Cohen S, Megherbi M, Jordheim LP, Lefebvre I, Perigaud C, Dumontet C, Guitton J. Simultaneous analysis of eight nucleoside triphosphates in cell lines by liquid chromatography coupled with tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;877:3831–3840.
- [18] Peoples MC, Karnes HT. Recent developments in analytical methodology for 8-hydroxy-2'-deoxyguanosine and related compounds. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005;827:5–15.
- [19] Pinlaor S, Yongvanit P, Prakobwong S, Kaewsamut B, Khoontawad J, Pinlaor P, Hiraku Y. Curcumin reduces oxidative and nitrative DNA damage through balancing of oxidant-antioxidant status in hamsters infected with *Opisthorchis viverrini*. *Mol Nutr Food Res* 2009;53:1316–1328.
- [20] Sawa T, Ohshima H. Nitrative DNA damage in inflammation and its possible role in carcinogenesis. *Nitric Oxide* 2006;14:91–100.
- [21] Nishida M, Toyama T, Akaike T. Role of 8-nitro-cGMP and its redox regulation in cardiovascular electrophilic signaling. *J Mol Cell Cardiol* 2014;73:10–17.
- [22] Jacob KD, Noren Hooten N, Trzeciak AR, Evans MK. Markers of oxidant stress that are clinically relevant in aging and age-related disease. *Mech Ageing Dev* 2013;134:139–157.
- [23] Gomes VA, Casella-Filho A, Chagas AC, Tanus-Santos JE. Enhanced concentrations of relevant markers of nitric oxide formation after exercise training in patients with metabolic syndrome. *Nitric Oxide* 2008;19:345–350.
- [24] Alfieri AB, Rodriguez-Plaza LG, Cubeddu LX. Effects of short- and long-term exercise on urinary cGMP excretion in healthy subjects and in patients with coronary artery disease. *J Cardiovasc Pharmacol* 2000;35:891–896.
- [25] Poveda JJ, Riestra A, Salas E, Cagigas ML, Lopez-Somoza C, Amado JA, Berrazueta JR. Contribution of nitric oxide to exercise-induced changes in healthy volunteers: effects of acute exercise and long-term physical training. *Eur J Clin Invest* 1997;27:967–971.
- [26] Hellsten Y, Nyberg M, Jensen LG, Mortensen SP. Vasodilator interactions in skeletal muscle blood flow regulation. *J Physiol* 2012;590:6297–6305.
- [27] de Almeida JC, Alves CL, de Abreu LC, Sato MA, Fonseca FL, de Mello Monteiro CB, et al. Involvement of the atrial natriuretic peptide in cardiovascular pathophysiology and its relationship with exercise. *Int Arch Med* 2012;5:4.
- [28] Larsen AI, Hall C, Aukrust P, Aarsland T, Faris P, Dickstein K. Prognostic usefulness of an increase of N-terminal proatrial natriuretic peptide during exercise in patients with chronic heart failure. *Am J Cardiol* 2003;92:91–94.
- [29] Jakob G, Mair J, Pichler M, Puschendorf B. Ergometric exercise testing and sensitivity of cyclic guanosine 3',5'-monophosphate (cGMP) in diagnosing asymptomatic left ventricular dysfunction. *Br Heart J* 1995;73:145–150.
- [30] Radak Z, Apor P, Pucsock J, Berkes I, Ogonovszky H, Pavlik G, et al. Marathon running alters the DNA base excision repair in human skeletal muscle. *Life Sci* 2003;72:1627–1633.
- [31] Radak Z, Bori Z, Koltai E, Fatouros IG, Jamurtas AZ, Douroudos, II, et al. Age-dependent changes in 8-oxoguanine-DNA glycosylase activity are modulated by adaptive responses to physical exercise in human skeletal muscle. *Free Radic Biol Med* 2011;51:417–423.
- [32] Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. *J Biol Chem* 1992;267:166–172.
- [33] Barregard L, Moller P, Henriksen T, Mistry V, Koppen G, Rossner P Jr, et al. Human and methodological sources of variability in the measurement of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine. *Antioxid Redox Signal* 2013;18:2377–2391.
- [34] Hartmann A, Pfuhrer S, Dennog C, Germadnik D, Pilger A, Speit G. Exercise-induced DNA effects in human leukocytes are not accompanied by increased formation of 8-hydroxy-2'-deoxyguanosine or induction of micronuclei. *Free Radic Biol Med* 1998;24:245–251.
- [35] Tsai K, Hsu TG, Hsu KM, Cheng H, Liu TY, Hsu CF, Kong CW. Oxidative DNA damage in human peripheral leukocytes induced by massive aerobic exercise. *Free Radic Biol Med* 2001;31:1465–1472.
- [36] Poulsen HE, Loft S, Vistisen K. Extreme exercise and oxidative DNA modification. *J Sports Sci* 1996;14:343–346.
- [37] Miyata M, Kasai H, Kawai K, Yamada N, Tokudome M, Ichikawa H, et al. Changes of urinary 8-hydroxydeoxyguanosine levels during a two-day ultramarathon race period in Japanese non-professional runners. *Int J Sports Med* 2008;29:27–33.
- [38] Radak Z, Pucsock J, Boros S, Jospai L, Taylor AW. Changes in urine 8-hydroxydeoxyguanosine levels of super-marathon runners during a four-day race period. *Life Sci* 2000;66:1763–1767.
- [39] Inoue T, Mu Z, Sumikawa K, Adachi K, Okochi T. Effect of physical exercise on the content of 8-hydroxydeoxyguanosine in nuclear DNA prepared from human lymphocytes. *Jpn J Cancer Res* 1993;84:720–725.
- [40] Pilger A, Germadnik D, Formanek D, Zwick H, Winkler N, Rudiger HW. Habitual long-distance running does not enhance urinary excretion of 8-hydroxydeoxyguanosine. *Eur J Appl Physiol Occup Physiol* 1997;75:467–469.
- [41] Allgayer H, Owen RW, Nair J, Spiegelhalder B, Streit J, Reichel C, Bartsch H. Short-term moderate exercise programs reduce oxidative DNA damage as determined by high-performance liquid chromatography-electrospray ionization-mass spectrometry in patients with colorectal carcinoma following primary treatment. *Scand J Gastroenterol* 2008;43:971–978.

- [42] Rozalski R, Gackowski D, Roszkowski K, Foksinski M, Olinski R. The level of 8-hydroxyguanine, a possible repair product of oxidative DNA damage, is higher in urine of cancer patients than in control subjects. *Cancer Epidemiol Biomarkers Prev* 2002;11:1072–1075.
- [43] Akuta T, Zaki MH, Yoshitake J, Okamoto T, Akaike T. Nitrate stress through formation of 8-nitroguanosine: insights into microbial pathogenesis. *Nitric Oxide* 2006;14:101–108.
- [44] Kaneko K, Akuta T, Sawa T, Kim HW, Fujii S, Okamoto T, et al. Mutagenicity of 8-nitroguanosine, a product of nitrate nucleoside modification by reactive nitrogen oxides, in mammalian cells. *Cancer Lett* 2008;262:239–247.