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DNA catabolites in triathletes: effects of supplementation with an aronia–citrus juice (polyphenols-rich juice)

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In this study we analyzed whether our aronia–citrus juice (ACJ, the composition is based on a mixture of 95% citrus juice with 5% of *Aronia melanocarpa* juice), rich in polyphenols, and physical exercise had an effect on seven catabolites of DNA identified in plasma and on a urine isoprostane (8-iso-$PGF_{2\alpha}$). Sixteen elite triathletes on a controlled diet for triathlon training (45 days) were used in this clinical trial. Our results show a decrease in the 8-hydroxy-2′-deoxyguanosine concentration due to chronic physical exercise. The ACJ intake and physical exercise maintained the guanosine-3′,5′-cyclic monophosphate plasmatic concentrations and decreased the concentration of 8-hydroxyguanine as well as urinary values of 8-iso-$PGF_{2\alpha}$. Finally, we observed a significant increase in the 8-nitroguanosine levels in triathletes after ACJ intake, compared to the placebo stage. It is concluded that the combination of the intake of ACJ, rich in polyphenolic compounds, with adequate training was able to influence the plasmatic and urinary values of oxidative stress biomarkers. This suggests a positive effect on the oxidative damage and potential associations with DNA repair mechanisms.

1. Introduction

Interventions aimed at the discovery of potential effects of dietary polyphenols intake have shown significant reductions in the oxidative DNA damage as well as in the lipid peroxidation damage, although such findings generate controversy.[1] Fruit phenolic compounds may directly scavenge superoxide and other reactive oxygen species (ROS) such as hydroxyl and peroxyl radicals,[2] although also it has been reported that the polyphenols, rather than being direct antioxidants, act as xenobiotics and stimulate the hermetic cellular response that leads to higher endogenous antioxidant production (indirect action).[3] Oxidative DNA damage in a tissue or population of cells may in part be due to oxidative stress (OS) or may derive from a deficit in the repair system dealing with oxidative modifications.[4] The endogenous products of DNA damage (in the cell) can be released by diffusion or transport into the extracellular space for subsequent distribution in the blood circulation to the liver and excretory organs.[5] Under OS, the DNA bases are prone to oxidation, a process which includes a large variety of mechanisms and final products.[6] For example, interaction of $HO^{\bullet}$ (hydroxyl radical) with the nucleobases of the DNA strand, such as guanine (G), leads to the formation of 8-hydroxyguanine (8-OH-Gua) or its 2′-deoxynucleoside form (8-hydroxy-2′-deoxyguanosine, 8-OH-dGuo).[7] The most-studied catabolites are 8-OH-dGuo and 8-hydroxyguanosine (8-OH-Guo) and they are generally used as markers of oxidative modifications to DNA and RNA, respectively.[8]

DNA can also be damaged by reactive nitrogen species (RNS), undergoing mainly nitration and deamination of purines.[6] However, it should be mentioned that nucleotide modifications, both oxidative and nitrosative, may not be simply chemical damage and also may be physiologically-relevant phenomena which allow the cells to activate the versatile cell signaling necessary for adaptive responses to the various chemical stresses. For example, cGMP (guanosine-3′,5′-cyclic monophosphate) plays an important role in the cellular response, through its regulation of some protein-dependent kinases with important effects in the vascular and neuronal systems.[9–11] A critical role of *in vivo* nitric oxide (NO) is the activation of soluble guanylate cyclase; stimulation of guanylate cyclase leads to the synthesis of this biologically-

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important second messenger, cGMP. The circulating levels of cGMP may reflect NO synthase (iNO) activity and are a marker of NO action.[9] An increase in NO is important regarding the damage repair/remodeling of the skeletal muscle, which might be important in delayed muscle soreness.[12] In addition, the nitrated derivative of cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-NO_2-cGMP, produced in cells by RNS),[9] has been implicated in redox signaling in different processes, as in the cardiovascular system during stress conditions.[13]

Nitration of G residues at the C8 position is proposed to occur under conditions of increased nitrative stress, such as inflammation.[14] The first nitration product to be identified was 8-nitroguanine (8-NO_2-Gua); its *in vivo* formation may be an important source of apurinic sites arising from peroxynitrite (ONOO–) production.[15] Another catabolite deriving from nitration is 8-nitroguanosine (8-NO_2-Guo), a product of the oxidative damage caused to nucleic acids by ONOO–, which can be considered a potential indicator of nitrative stress during infections and inflammation.[16] Moreover, 8-NO_2-Guo may not be simply a damaged nucleoside. It may be a potent redox cofactor that intensifies oxyradical generation by various NADPH/reductase-like enzymes and thus participates in diverse physiological events.[17]

In a previous human intervention trial, evidence for the protective effects of an anthocyanin/polyphenols-rich fruit juice (700 mL per day of juice provided 197.9 mg per L of total anthocyanins) was provided, since it reduced oxidative DNA damage and gave a significant increase in reduced glutathione, when compared to the controls.[18] Specifically, in athletes, urinary 8-OH-dGuo excretion decreased following four days of vegetable juice intake.[19] In the case of professional athletes, dietary supplementation with red orange extract (containing anthocyanins, flavanones, hydroxycinnamic acids, and ascorbic acid) was able to protect against oxidative DNA damage.[20] Our group has previously evaluated the effects of acute physical training on the levels of markers of DNA damage in the plasma of triathletes; there was an adaptive response of the organism, mainly in the DNA repair pathway.[11] In another work, the intake of aronia–citrus juice (ACJ, 95% citrus juice with 5% aronia juice (*Aronia melanocarpa*)) for six months (300 mL per day) produced a decrease in the level of 8-OH-dGuo in metabolic syndrome patients.[21] In addition, ACJ (200 mL per day) and physical exercise showed a synergistic effect due to the increased bioavailability of flavonoids in triathletes,[22] and ACJ consumption (250 mL per day) was found to be associated with the excretion of metabolites that could have effects on human health.[23] Based on the foregoing, we wished to analyze whether chronic physical exercise and ACJ intake show an effect on oxidation metabolites of DNA, identified in plasma by UHPLC-QqQ-MS/MS. We also studied the isoprostane (IsoP) 8-iso-$PGF_{2\alpha}$ (8-iso-prostaglandin $F_{2\alpha}$), a representative marker of lipid peroxidation,[24,25] with the aim of determining the physiological modifications, in relation to DNA catabolites, after the juice intake by triathletes.

2. Materials and methods

2.1 Physical characteristics of participants

The recruitment started on 28th and 29th October 2010 and was complete on 24th and 25th March 2011. Sixteen Caucasian triathletes (6 training women and 10 training men), aged 19–21 years, from the University of Alicante (Spain) agreed to participate in the project. All subjects fulfilled the following eligibility criteria: non-smokers, had stable food habits, and did not receive any medication (particularly the absence of acute administration of anti-inflammatory drugs) during the experimental procedure. The study was approved by the Bioethics Committee of the University Hospital of Murcia, in accordance with the Declaration of Helsinki, and all participants signed written informed consent. The physical parameters of the triathletes were controlled during the entire assay. The anthropometric measurements were performed according to the International Society of Advancement of Kinanthropometry (ISAK: http://www.isakonline.com) by the same internationally-certified anthropometrist (level 2 ISAK) – to minimize the technical error of measurement. The body composition was determined by GREC Kineanthropometry consensus,[26] using a model consisting of: total fat by Withers' formula;[27] lean weight by the procedure described in ref. 28; and residual mass by the difference in the weight (Table 1).

Table 1 Physical and metabolic characteristics and training loads of the elite triathletes

Physical characteristics of triathletes	Male triathletes (n = 10)		Female triathletes (n = 6)	
	Baseline	Weeks training[a]	Baseline	Weeks training[a]
Year (yr)	19.0 ± 1.7	19.0 ± 1.5	21.0 ± 3.0	21.8 ± 3.0
Weight (kg)	69.7 ± 6.2	69.7 ± 6.1	54.8 ± 12.2	54.8 ± 6.07
Height (m)	1.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
BMI[b] (kg m^{-2})	22.2 ± 1.0	22.1 ± 2.07	21.2 ± 4.1	21.2 ± 2.35
Total fat (kg)	9.2 ± 2.8	8.8 ± 2.6	8.7 ± 4.1	8.9 ± 2.05
Lean weight (kg)	31.4 ± 2.1	30.5 ± 2.8	20.8 ± 3.6	20.6 ± 2.4
Subescapular skinfold (mm)	9.6 ± 3.0	9.5 ± 1.9	12.7 ± 6.7	13.4 ± 3,85
Tricipital skinfold (mm)	8.9 ± 3.0	9.7 ± 2.1	16.3 ± 2.3	17.7 ± 4.6
Bicipital skinfold (mm)	5.4 ± 2.4	4.7 ± 1.0	10.3 ± 2.8	9.8 ± 1.4
Ileocrestal skinfold (mm)	12.0 ± 2.6	11.6 ± 3.5	19.7 ± 4.5	17.2 ± 4.8
Supraespinal skinfold (mm)	9.0 ± 2.6	7.9 ± 2.1	14.3 ± 6.5	10.9 ± 3.1
Abdominal skinfold (mm)	16.4 ± 8.0	12.9 ± 5.4	23.1 ± 5.9	21.6 ± 5.0
Thigh skinfold (mm)	14.9 ± 4.4	11.2 ± 2.8	27.2 ± 5.2	25.5 ± 6.6
Calf skinfold (mm)	9.0 ± 3.0	8.0 ± 2.3	14.8 ± 3.8	14.1 ± 2.4

Data are expressed as the mean ± standard deviations. [a] The data of weeks training column are results from: control-training, placebo, ACJ, and control post-training. [b] Body mass index.

2.2 Dietary intake of participants

The dietary habits of the triathletes were controlled during the entire assay. The diet was kept constant during the study (Table 2), to avoid any interference. The calculation of the dietary parameters and caloric intake was accurately designed and overviewed during the experimental intervention by nutritionists, and specific planning diets software. The dietary assessment and planning for our volunteers were estimated based on their energy needs, calculated by the basal energy equation for individuals over 18 years of age, according to the Institute of Medicine.[29] Energy expenditure by physical activity was calculated according the standard resting metabolic rate.[30] In addition, dietary planning for the nutrient and water requirements before, during, and after training was based on different recommendations for triathletes[31] and sportsmen/women.[32] The nutritionist delivered the diet plan to each of the triathletes with all instructions. The data were calculated using software available on the website http://www.easydiet.es, with the additional assistance of the Spanish and USDA databases http://www.bedca.net/ and http://www.nal.usda.gov/fnic/foodcomp/search/. Triathletes were responsible for preparing their meals according to the given diet plan. Dietary information was obtained *via* 24 h recall.[33] The athletes were requested to complete a questionnaire 24 hours prior to each provision of urine and plasma, in which they described in detail all foods and drinks consumed during this 24-hour period. If the dietary guidelines were not met, the athletes were oriented by nutritionists to adjust their nutrient intake.

2.3 Aronia citrus juice and placebo beverage

The juice composition was based on a mixture of citrus juice (95%) with 5% *Aronia melanocarpa* juice, based on a drink model developed before.[34] The composition was developed on an industrial pilot scale with organoleptically-acceptable criteria, to mimic the flavonoids composition of the original beverage. Supplementation with this natural fruit juice has been used in other studies, as described in the Introduction,[21–23,35] the daily dose being 200 mL to 250 mL in healthy subjects. One serving of the juice corresponds to 240 mL according to the FDA (U.S. Food and Drug Administration), but in this study it was adjusted to 200 mL, to adapt to the caloric requirements of the triathletes. It is important to mention that one serving (200 mL) of ACJ did not make an important caloric or nutritional contribution since it only represented 2.6% of the diet, its content of phytochemical compounds being much more relevant. The nutrient composition and caloric supply of the ACJ as well as the contents of flavanones, flavones, and anthocyanins are summarized in Table 3. Of the phenolic compounds, 68% were flavanones, flavones, or anthocyanins, while hydroxycinnamates represented approximately 28%.

The placebo beverage composition was based on a mixture of water, authorized red dye, flavoring agent and sweetener, its sensory characteristics being adjusted so that they were similar to those of the ACJ.[21]

2.4 Training load

The training load quantification was performed using the Objective Load Scale (ECOs).[36] The method used in the present work allowed the quantification of the training loads in triathlon (swim, bike, run, and transitions). Our study was designed according to the training season (which lasts approximately five months) before the start of the competition season; thus, the protocol was adapted to 145 days. The values of daily and weekly trainings have been summarized to assess the ECOs of each volunteer (Fig. 1), depending on their physical

Table 2 Dietary parameters and caloric intake of the triathletes during the study

	Male triathletes	Female triathletes
Energy intake (kcal)	2820.0 ± 241.2	2072.6 ± 223.4
Carbohydrate (g d^{-1})	326.1 ± 63.5	211.3 ± 43.9
Dietary fiber (g d^{-1})	27.3 ± 7.4	15.5 ± 4.4
Sugars (g d^{-1})	121.3 ± 33.9	80.5 ± 18.3
Proteins (g d^{-1})	133.7 ± 12.9	83.5 ± 9.0
Total lipids (g d^{-1})	113.7 ± 13.3	107.1 ± 14.4
SFA[a] (g d^{-1})	33.5 ± 6.5	29.6 ± 4.4
MUFA[b] (g d^{-1})	56.5 ± 5.5	56.6 ± 7.5
PUFA[c] (g d^{-1})	16.9 ± 2.7	15.9 ± 6.7
Vitamin C (mg d^{-1})	178.9 ± 71.9	135.0 ± 60.4
Vitamin A (μg d^{-1})	2970.0 ± 913.9	1427.4 ± 573.1
Vitamin E (mg d^{-1})	21.0 ± 5.6	13.9 ± 3.4
Vitamin D (mg d^{-1})	988.0 ± 47.5	751.6 ± 163.0
Iron (mg d^{-1})	20.9 ± 2.4	14.9 ± 2.6
Selenium (mg d^{-1})	149.8 ± 21.5	103.0 ± 17.4

Data are expressed as the mean ± standard deviations. [a] Saturated fatty acids. [b] Monounsaturated fatty acids. [c] Polyunsaturated fatty acids.

Table 3 Nutritional and phenolic composition of the aronia–citrus juice

ACJ	200 mL
Energy intake (kcal)	76
Proteins (g)	0.9
Carbohydrate (g)	18
Fat (g)	0.06
Flavanones (mg)	
Eriocitrin	22.9 ± 0.16
Hesperidin	27.08 ± 0.28
Flavones (mg)	
Vicenin-2	1.18 ± 0.04
Diosmetin-6,8-di-*O*-glucoside	15.5 ± 0.38
Diosmin	<0.5
Anthocyanins (mg)	
Cyanidin 3-*O*-galactoside	30.16 ± 0.20
Cyanidin 3-*O*-glucoside	2.62 ± 0.04
Cyanidin 3-*O*-arabinoside	18.36 ± 0.40
Cyanidin 3-*O*-xyloside	2.22 ± 0.03
Total anthocyanins	53.4 ± 0.70
Hydroxycinnamic acids (mg)	
Neochlorogenic acid	39.44 ± 0.34
Chlorogenic acid	29.38 ± 0.26
∑Quercetin derivatives[a] (mg)	8.62 ± 0.26

The values are means ± standard deviation (n = 3, expressed as mg per 200 mL of juice).[34a] Quercetin derivatives were quantified as the sum of quercetin 3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside.

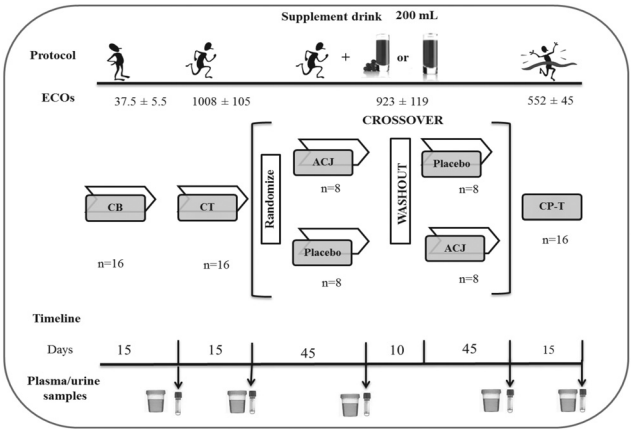

Fig. 1 Study design. This was a randomized, double-blind, and placebo-controlled crossover study. Sixteen athletes, randomly divided into two groups, were assigned to supplementation with either 200 mL of ACJ or 200 mL of placebo. After 45 days of supplementation and a 10-day washout period, the beverages were swapped during the same period (45 days). Three controls were used: baseline control (C-B), control-training (C-T), and control post-training (CP-T), with a duration of 15 days. The samples (urine/blood) were collected at rest and under fasting conditions, on the last day of each stage. The training load was quantified by the Objective Load Scale (ECOs).

characteristics and the intensity of the training program (the ECOs data presented in this work are the average of the individual ECOs of the triathletes). To better understand the scale used to quantify the training load, ref. 36, 37 should be consulted. The training loads developed by triathletes in the present work were similar to those found in other studies.[38,39]

2.5 Study design

We hypothesized that ACJ supplementation would have a positive effect on our volunteers, as previously assessed for oxidative biomarkers during the training period.[21–23] The primary outcome measure was the change in the values of the metabolites of DNA identified in plasma samples by UHPLC-QqQ-MS/MS, from the baseline (pre-training) until the end of the five-month training period (increase of ECOs and beverage intake). The secondary outcome measures of interest were a urinary lipid oxidation biomarker (8-iso-$PGF_{2\alpha}$), physical and metabolic characteristics, dietary parameters and caloric intake, and training loads of the elite triathletes. This study had a randomized, double-blind and placebo-controlled crossover design (Fig. 1).

2.5.1 Randomization and intervention. The allocation order of beverages was produced using a computer-generated simple randomization with consecutive codes linked to the preparation of the placebo or ACJ. The volunteers remained blinded throughout the study. An impartial outsider who was not involved in the study helped to select the randomization code and indicated the assignment order. The researchers responsible for the outcome measurements remained separate from the randomization process and remained unaware of the allocation order throughout the study and during data analysis. Before the supplementation with ACJ, both plasma/urine samples were collected as controls: the first was the control baseline (C-B) with low training loads (minimal ECOs) and the second control (control-training: C-T) started with an increase in ECOs, both periods lasting 15 days. During the following stage, the subjects were randomly divided into two groups: each received a supplement of 200 mL of ACJ or placebo. The drink intake was 15 minutes after the subjects had finished their training, to improve the bioavailability of the flavanones in the ACJ.[22] The two groups consumed ACJ or placebo for 45 days. Ten days were utilized as the washout period without drink intake, but the training and the same diet were maintained. Subsequently, the supplementation was repeated, swapping the two groups according to the corresponding drink intake while maintaining their ECOs. After the crossover period, the control post-treatment (CP-T) without supplementation was started for the last 15 days of the study (active recovery phase), with the objective of analyzing the post-training adaptation while maintaining the training diet without ACJ. The dietary intake of the volunteers was controlled and did not change during the whole training and nutritional trial (Table 2).

2.6 Sample collection and preparation

Human blood was collected in heparin sampling tubes and centrifuged to separate the plasma from the cells. The blood samples were collected at rest and under fasting conditions, at the end of each stage (Fig. 1). One milliliter of plasma was deproteinized; subsequently, solid phase extraction with ISOLUTE cartridges was performed as described previously.[11] Twenty-four-hour urine samples were collected at the end of each stage. They were collected in sterile and clear polystyrene pots with screw caps and were protected from light. In the present experiment, urinary IsoP was assayed using the method described previously.[40] All samples collected were immediately frozen (−80 °C) to preserve the sample integrity until the time of the analysis.

2.7 Chemicals and reagents

8-Nitroguanosine (8-NO_2-Guo), 8-hydroxyguanine (8-OH-Gua), guanosine-3′,5′-cyclic monophosphate (cGMP), 8-nitroguanine (8-NO_2-Gua), and 8-nitroguanosine-3′,5′-cyclic monophosphate (8-NO_2-cGMP) were purchased from the Biolog Life Science Institute (Bremen, Germany). 8-Hydroxy-2′-deoxyguanosine (8-OH-dGuo) and 8-hydroxyguanosine (8-OH-Guo) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA) (Fig. 2). The IsoP, 8-iso-$PGF_{2\alpha}$ was purchased from Cayman Chemicals (Ann Arbor, MI, USA). 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. The β-glucuronidase, type H2 from *Helix pomatia*, and bis–tris (bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl)-methane) were from Sigma-Aldrich (St Louis, MO, USA). Reagents such as acetic acid, sodium hydroxide, and ammonium acetate were purchased from Panreac (Castelar del Vallés, Barcelona, Spain). The SPE cartridges used were

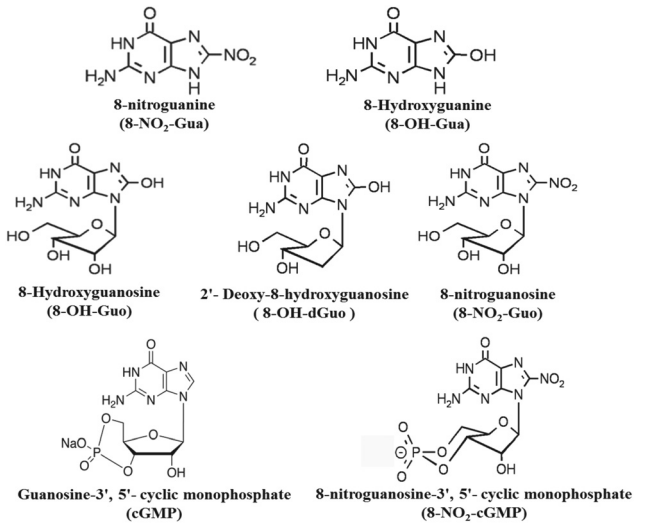

Fig. 2 Chemical structures of the seven DNA oxidation catabolites analyzed in this study.

the ISOLUTE cartridge (ENV+, 50 mg, 1 mL) from Biotage (Uppsala, Sweden), and the Strata X-AW, 100 mg per 3 mL SPE cartridge, from Phenomenex (Torrance, CA, USA).

2.8 UHPLC-QqQ-MS/MS analysis

The samples were analyzed according to the methods described previously.[11,40] Chromatographic analyses were carried out with a UHPLC coupled to a 6460 QqQ-MS/MS (triple quadrupole mass spectrometer) (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization (ESI) source. The separation of DNA analytes was performed on a Kinetex HILIC column (100 × 2.10 mm), packed with 1.7 μm particles, from Phenomenex (Torrance, USA).[11] The urine samples were analyzed on an ACQUITY UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm; Waters), using the set-up described previously.[40] Data acquisition and processing were performed using Mass Hunter software version B.04.00 (Agilent Technologies, Walbronn, Germany). The identification was confirmed according to their pseudomolecular ion, the characteristics of the MS/MS fragmentation product ions, and the retention time relative to the corresponding authentic markers. The mass spectral information on the seven DNA catabolites and 8-iso-$PGF_{2\alpha}$ has been summarized previously.[11,40]

2.9 Statistical analysis

The concentrations of DNA catabolites in the different stages were calculated as nM in plasma. The 24 h urine was used for the absolute calculation of the amount of IsoP excreted (μg per 24 h). The data are shown as mean ± SD, as well as the quartiles (upper values 75%, median 50%, and lower values 25%), of the concentrations of DNA metabolites in plasma throughout the study. Because the baseline data of the two phases did not differ, data from both groups were pooled into one placebo or ACJ treatment. For DNA concentrations, a Friedman's non-parametric repeated measure analysis of variance (ANOVA) was used to compare the concentrations in the different stages, since the normality and/or equal variance tests failed. When a significant difference was found in the ANOVA, a pair-wise comparison was performed using the Wilcoxon signed rank test with Bonferroni correction. *A posteriori*, the sample size was calculated using the value r, calculated by $r = Z/\sqrt{N}$, in which Z is the Z-score that SPSS produce, and N is the size of the study on which Z is based.[41] An r value of 0.1, 0.3, or 0.5 was considered to show a small, moderate, or large effect, respectively. In the specific case of 8-iso-$PGF_{2\alpha}$, the assumption of homogeneity of variance was tested and was satisfactory; thus, the results were examined by one-way ANOVA followed by Tukey's honestly significant difference test. For the statistical analyses, an adjusted P value of <0.05 was considered to be significant. The statistical analyses were carried out using the SPSS 21.0 software package (LEAD Technologies Inc. Chicago, USA).

3. Results and discussion

3.1 Anthropometric variables and training performance

The kineanthropometric measurements, performed following the International Working Group of Kineanthropometric procedure, did not yield differences between the experimental groups (Table 1). The training loads of the triathletes ranged from 37.5 ± 5.5 to 1008 ± 105 ECOs.

3.2 Qualitative analysis

Previous results[11,21,23,35] led us to investigate the effect of ACJ intake on seven DNA metabolites in plasma samples of triathletes, which could be related to the development of different disorders and mutagenic processes. Three of the catabolites analyzed 8-NO_2-Gua, 8-OH-Guo, and 8-NO_2-cGMP, were below the limit of detection/quantification in most of the samples and therefore were described as not detected (n/d). But, this does not mean that they did not exist in these samples; they could have been present in trace amounts below the LODs of the method used.[11] Three of the catabolites, 8-OH-dGuo, 8-NO_2-Gua, and 8-OH-Gua, were detected but in some stages were n/d; only cGMP was detected in all stages (Table 4). The catabolite 8-OH-dGuo showed a non-significant increase between the first two controls (from 0.016 nM in C-B to 0.018 nM in C-T) and in the next stage was n/d. Thereby, we have observed a major effect of chronic physical exercise on this catabolite, linked to a decrease in its level in plasma. The predominant detectable oxidation product of DNA bases *in vivo* is 8-OH-dGuo.[42] Also, it has been hypothesized that the levels of the modified nucleoside 8-OH-dGuo are indicative of different repair pathways, namely base excision repair and nucleotide excision repair.[43] The absence of cumulative effects may, in part, have been due to the adaptive responses induced by long-term, regular training – which enhances endogenous antioxidant defense and DNA repair systems to prevent exercise-induced DNA damage.[44–46] On the other hand, 8-iso-$PGF_{2\alpha}$ was detected in all stages.

Table 4 Plasmatic concentrations of the DNA metabolites and excretory values of 8-iso-$PGF_{2\alpha}$ in the different stages of the study

	8-NO_2-Guo[a]		8-OH-Gua[a]		8-OH-dGuo[a]		cGMP[a]		8-iso-$PGF_{2\alpha}$[b]	
Stages	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C-B	0.016	0.013	0.031	0.008	0.016	0.013	0.027	0.010	3.2	0.7
C-T	0.009	0.002	0.036	0.012	0.018	0.016	0.036	0.020	2.7	0.5
Placebo[c]	n/d	—	0.021	0.014	n/d	—	0.016	0.014	2.5	0.5
ACJ[c]	0.046	0.012	n/d	—	n/d	—	0.041	0.032	2.1	0.6
CP-T	n/d	—	0.015	0.003	n/d	—	0.028	0.025	1.6	0.4

[a] The data are shown as mean ± standard deviations (SD) in nM per 24 h. [b] The data are shown as mean ± standard deviations (SD) in μg per 24 h. [c] Average of the two plasma samples in the crossover period (placebo/ACJ). Abbreviation: C-B: control baseline, C-T: control training, ACJ: aronia–citrus juice, CP-T: control post-treatment; n/d: not detected.

3.3 Quantitative analysis

The only catabolite detected in all stages was cGMP, which ranged from ~0.016 ± 0.014 to ~0.041 ± 0.032 nM. There was statistically-significant variation in the cGMP concentration, according to the Friedman test: χ^2 (4) = 11.867, P = 0.018. *Post hoc* analysis with the Wilcoxon signed-rank test was conducted with the application of a Bonferroni correction, resulting in a significance level set at $P < 0.005$. When the Bonferroni correction was applied to our results the significance levels were not adjusted to $P = 0.005$; thus, only the Wilcoxon signed-rank test was carried out to compare the ACJ and placebo stages. This test revealed that the ACJ intake stage differed significantly from the placebo stage; $Z = -2.100$, $P = 0.036$, $r = 0.525$, statistical power (SP) = 0.502 (Fig. 3A), suggesting an effect of ACJ intake on plasma levels of cGMP. In the literature, polyphenol-rich foods (*e.g.* berries and citrus fruits) have been shown to improve endothelium-dependent vasodilation, assessed by flow-mediated dilation, *via* increased plasma NO bioavailability in healthy individuals.[1] It is reported that, similar to a green tea polyphenol (epigallocatechin gallate), the citrus polyphenol hesperetin stimulates PI3K (phosphatidylinositol 3-kinase), which results in activation of the downstream serine kinases Akt (protein kinase B) and AMPK (adenosine monophosphate-activated protein kinase) that phosphorylate and activate eNOS, producing NO in the vascular endothelium.[47,48] Cyclic GMP acts as a second messenger, producing smooth muscle relaxation and vasodilation,[49] since it can bind to cyclic nucleotide-gated ion channels and to target proteins like protein kinases (*e.g.* protein kinases A and G). Protein kinase G (cGMP-dependent protein kinase or PKG) plays a role in cell division and smooth muscle relaxation (vasodilation).[50] In addition, blood flow increases markedly during exercise, to meet oxygen demands. This response is regulated by vasodilators such as NO – that exerts its action through the signaling molecule cGMP.[11] In relation to this, we now provide evidence of the effect of the intake of ACJ (rich in polyphenols) during a training period with regard to maintenance of the plasmatic cGMP levels, suggesting a potential positive effect on the vascular system during training.

Also, we observed a significant increase in the 8-NO_2-Guo levels (χ^2 (2) = 9.556, P = 0.008) in the triathletes after ACJ intake, compared to the placebo stage and C-T (n/d). *Post hoc* analysis with the Wilcoxon signed-rank test showed that the values were higher in the ACJ stage (Fig. 3B), although only the C-T stage ($Z = -2.803$, $P = 0.005$, $r = 0.700$, SP = 0.80) differed significantly with the Bonferroni correction ($P < 0.016$). With

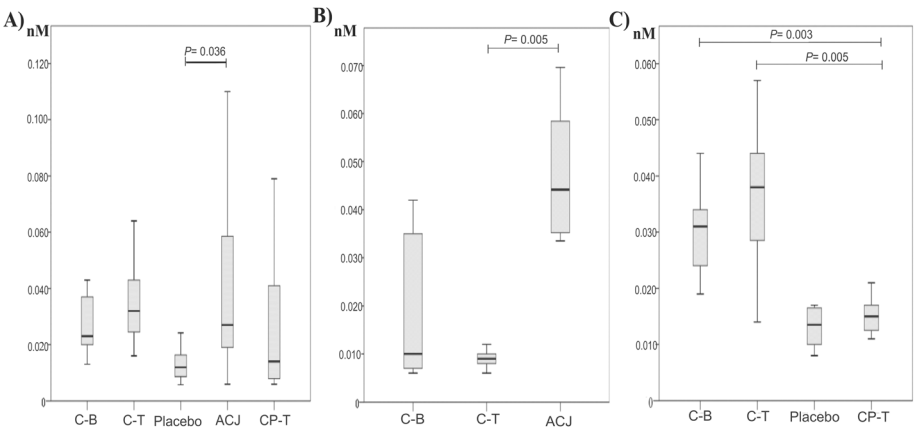

Fig. 3 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of the concentrations of DNA metabolites in plasma throughout the study (nM). Friedman's ANOVA and *post hoc* analysis with Wilcoxon signed-rank tests (with a Bonferroni correction) were conducted. (A) cGMP ($P < 0.05$, only the ACJ and placebo stages were compared), (B) 8-NO_2-Gou ($P < 0.016$), and (C) 8-OH-Gua ($P < 0.005$). Abbreviations: C-B: control baseline, C-T: control training, ACJ: aronia–citrus juice, CP-T: control post-treatment.

acute physical activity the plasmatic levels of this catabolite showed a significant reduction (from 0.016 nM in C-B to 0.009 nM in C-T), thus suggesting a positive effect of sustained physical activity.[11] In our study, with chronic exercise, 8-NO_2-Guo was undetectable in two of the stages (Table 4). Despite the inter-individual variability observed regarding the values of this catabolite, ACJ intake produced a significant increase of 8-NO_2-Guo in the plasma of these triathletes. To the best of our knowledge, there are no studies available relating this compound to juice intake and physical activity *in vivo*. 8-NO_2-Guo is a product of the oxidative damage caused to nucleic acids by ONOO– and it can be considered a potential indicator of nitrative stress during infections and inflammation processes.[16] Nevertheless, 8-NO_2-Guo may not be simply a damaged nucleoside. It may be a potent redox cofactor that intensifies oxyradical generation by various NADPH/reductase-like enzymes, thus participating in diverse physiological events.[17] Polyphenols activate signaling pathways related to cellular stress that result in increased expression of genes encoding cytoprotective proteins.[3] Flavonoids may be prooxidant or antioxidant depending on the concentration and structure of the polyphenol as well as the cellular redox environment.[42,51] Citrus polyphenol hesperidin is a phenolic compound containing hydroxyl groups that may generate ROS through autoxidation.[47] The increase in 8-NO_2-Guo due to ACJ intake reflects the participation of the constituents of this beverage (*e.g.* polyphenols and/or the nutritional biomarkers associated with its intake) in increased redox activity. Also, it is noteworthy that ACJ, in addition to their phytochemicals, contains a variety of vitamins, minerals, and fiber that appear to have biological activities and health benefits.[2] Therefore, we are developing further research to clarify the positive influence that the intake of functional fruit juices and polyphenols could have on athletes.

In contrast to the above-mentioned results concerning the increased concentrations of the DNA catabolites with ACJ intake, 8-OH-Gua was n/d in this stage (Table 4). We observed significant differences among C-B, C-T, placebo, and CP-T with the Friedman test: χ^2 (4) = 10.441, P = 0.034. The Bonferroni correction of the results from the Wilcoxon test gave $P < 0.005$, showing that the CP-T value was statistically lower than those of C-B ($Z = -2.934$, P = 0.003, r = 0.734, SP = 0.783) and C-T ($Z = -2.824$, P = 0.005, r = 0.706, SP = 0.752) (Fig. 3C). This catabolite has been described generally as a marker of oxidative modifications to DNA and RNA.[8] Indirectly, the polyphenols from ACJ may stimulate endogenous antioxidant defense systems; for example, NF-E_2 related factor 2 (Nrf_2) is a transcription factor that controls the production of antioxidant enzymes such as catalase and glutathione peroxidase.[52] Phenolic compounds may contribute to beneficial health effects since they can also "repair" damage to DNA.[53] A study using *in vitro* 8-OH-Gua as a marker of OS showed that flavonoids can act as antioxidants at physiological levels of 1 μM or lower – but not all flavonoids have the same activity, depending on their structure.[54] In addition, fluid replacement following dehydration (caused by an exercise endurance session) appeared to have positive effects on the maintenance of physiological homeostasis and alleviation of DNA damage.[55] This suggests that ACJ intake helped to decrease DNA damage due to its effect on the hydration status, since its intake occurred after the training session. Moreover, the chronic physical exercise caused the concentration of plasmatic 8-OH-Gua in the CP-T stage to decline significantly, compared with C-B and C-T, thus showing an association of this catabolite with chronic physical exercise. The intake of ACJ and physical exercise decreased the plasmatic levels of 8-OH-Gua, which suggests a positive effect against DNA oxidation. Thus, once again, we observed an adaptive response induced by long-term regular training, supporting current evidence on the positive effects of sustained physical activity.[44–46]

Finally, IsoPs are considered to be "gold standard" biomarkers of endogenous lipid peroxidation and oxidative stress.[24] The DNA and lipid biomarkers are the biomarkers of oxidative stress reported most frequently in the literature.[25] Since 8-iso-$PGF_{2\alpha}$ is one of the most-abundant IsoP isomers formed *in vivo*,[24] we analyzed it together with the seven DNA catabolites with the aim of determining the possible antioxidant role of compounds from ACJ.[21,23,35] Prior to conducting the ANOVA, the assumption of homogeneity of variances was tested and was satisfactory, based on Levene's F test: F (4, 40) = 0.531, P = 0.714. The ANOVA yielded a statistically-significant effect: F = 8.878, P = 0.000, η^2 = 0.470, SP = 0.998. As with 8-OH-Gua, the 8-iso-$PGF_{2\alpha}$ levels were also lower in the ACJ intake stage (2.1 ± 0.6 μg per 24 h, P = 0.006), as well as in the CP-T (1.6 ± 0.4 μg per 24 h, P = 0.000), compared with the CB (3.2 ± 0.7 μg per 24 h). Thus, a possible antioxidant role of the compounds from ACJ has been shown, since the values of the OS biomarkers (RNA/DNA/lipidic) in the biological samples of the elite triathletes showed statistically-significant changes during the study.

4. Conclusions

This study provides new insights into the link between the intake of a functional juice rich in polyphenols (ACJ, one 200 mL serving in the diet) and chronic physical exercise (two external stimuli), and their influence on plasmatic concentrations of DNA oxidation catabolites and on urinary 8-iso-$PGF_{2\alpha}$ in elite athletes. The ingestion of the bioactive compounds found in ACJ – flavanones, flavones, and anthocyanins, among others – seems to be sufficient to influence the plasmatic concentrations of DNA catabolites and biomarkers of lipid peroxidation in athletes during training, suggesting a positive effect on the protection of DNA and lipids against oxidation as well as a potential association with DNA repair mechanisms. However, further studies with greater numbers of volunteers are necessary to clarify how ACJ compounds influence physiological functions.

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