

Under Increased Hydrogen Peroxide Conditions, the Antioxidant Effects of Pequi Oil (*Caryocar brasiliense* Camb.) to Decrease DNA Damage in Runners are Influenced by Sex, Age and Oxidative Stress-related Genetic Polymorphisms

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ABSTRACT

Context: Exhausting exercise, increasing reactive oxygen species, can overwhelm the endogenous antioxidant system's capacity, resulting in oxidative damage to DNA. Deficient antioxidant defenses, influenced by certain genetic polymorphisms, may contribute. **Aims:** We aimed to investigate whether carotenoid-rich oil from pequi (*Caryocar brasiliense*) could decrease DNA damage in athletes submitted to increased hydrogen peroxide (H_2O_2) conditions and in those less genetically favored by antioxidant defenses. **Methods and Material:** Runners' blood (N = 125) was analyzed after races under the same environment, type, intensity and length of weekly training conditions, before and after 14 days of pequi-oil supplementation. DNA damage was assessed by comet assay before and after H_2O_2 exposure, with gene polymorphisms of MnSOD Val9Ala, CAT -21A/T, GPx-1 Pro198Leu, del{GSTM1}, del{GSTT1}, ACE and Haptoglobin. **Results:** Without additional oxidative stress imposed by H_2O_2 , pequi oil was particularly efficient reducing DNA damage for women, age group of 20-40 years, distance of 8-10 Km and genotypes MnSOD Val/Ala, CAT TT, GPx-1 Pro/Leu, GSTM1 null, GSTT1 non-null, ACE DD and II and Hp1F-2. For treatment with H_2O_2 at 0.25 mM, pequi oil resulted in decreased DNA damage only for running 16-21 Km; for treatment with 1 mM, decrease was for 20-40 years and genotypes GPx-1 Pro/Pro and ACE ID. **Conclusions:** Pequi oil's effect on exercise-induced DNA damage was therefore influenced by sex, age and genetic polymorphisms, indicating that: long-distance races can be harmful, mainly for older athletes, due to oxidative stress above organism adaptability; genotypes showed different responses; under increased H_2O_2 conditions, GPx-1 Pro/Pro and ACE ID genotypes were more responsive to antioxidant supplementation.

Key-words: reactive oxygen species; hydrogen peroxide; exercise-induced oxidative stress; exercise-induced DNA damage; comet assay; gene polymorphisms related to oxidative stress

Key Messages: the present study can help broaden knowledge of how antioxidant supplementation affects exercise-induced DNA damage and how individual athletic genetic makeup can affect the way athletes respond to antioxidant supplementation against exercise-induced DNA damage.

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INTRODUCTION

Hydrogen peroxide (H_2O_2) is an important representative reactive oxygen species (ROS) that arises during the aerobic respiration process and from other cellular sources.^[1] If not controlled, the reaction of H_2O_2 with transition metals can provoke oxidative stress in the

cells and may result in DNA damage, mutagenesis and cell death.^[1,2] Under normal circumstances, H_2O_2 and other ROS are neutralized by an elaborate antioxidant defense system of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and numerous non-enzymatic antioxidants.^[2] However, oxidative stress can be caused by excessive production

of ROS, deficient antioxidant defenses, or a combination of both.^[3]

As exercise increases oxygen consumption, it can cause an imbalance between ROS and antioxidants, leading to oxidative stress.^[4] Although physical training induces beneficial adaptations, exercise, especially under unaccustomed intensity or duration, may increase ROS production, exceeding endogenous antioxidant system capacity and often resulting in oxidative stress, even in trained individuals.^[4,5] Because the byproducts of oxidative phosphorylation reactions can diffuse from mitochondria reach nuclear DNA and induce damage,^[6] this type of exercise can result in DNA strand breaks and oxidatively damaged bases in DNA.^[7] Many athletes and even those in regular exercise programs consume antioxidant supplements to avoid enhanced production of ROS.^[8] However, it has been shown that antioxidant supplements can inhibit beneficial adaptive responses associated with improved athletic performance. Recommending antioxidant supplements should thus only be for cases when exhaustive exercise causes oxidative stress and cell damage.^[9-11]

Additionally, many potentially significant genetic variants related to oxidative stress have already been identified.^[12,13] Among them, several single nucleotide polymorphisms (SNPs) in the antioxidant enzyme genes and null polymorphisms in glutathione S-transferases (GSTs) genes have been reported to produce altered or absent levels or activities of those enzymes, leading to lowered protection against oxidative stress.^[14,15] In such circumstances, ROS may interact with cellular biomolecules, such as DNA, with potentially serious consequences for the cell.^[16] Similarly, activation of the renin angiotensin system has been associated with increased vascular superoxide anion production,^[17] so the insertion/deletion polymorphism of the angiotensin I-converting enzyme (ACE) gene can influence vascular oxidative stress. Moreover, the ability of the serum glycoprotein haptoglobin (Hp) to block hemoglobin-induced oxidative stress and damage is reportedly phenotype dependent.^[18]

Since the prudent recommendation for physically active individuals is a diet rich in antioxidants from natural foods,^[10] natural antioxidant supplementation can prevent exercise-induced damage in athletes who exercise strenuously and exceed their endogenous antioxidant defenses^[9] or even for those who were born less genetically favored by the antioxidant defense system. In previous studies, our group demonstrated that carotenoid-rich oil from pequi (*Caryocar brasiliense*

Camb.), a fruit found in the Brazilian Cerrado, had anti-inflammatory properties, besides reducing arterial pressure, exercise-induced damages, lipid peroxidation and anisocytosis.^[19-22] Although the protective effects of pequi oil are unquestionable, some of these responses were influenced by genetic polymorphisms related to oxidative stress and inflammatory markers.

The extent of DNA damage in trained individuals is small compared to that of untrained individuals, suggesting that adaptation to aerobic endurance training can reduce effects of oxidative stress, including DNA damage.^[7] Thus, we aimed to investigate whether pequi oil could diminish exercise-induced DNA damage of trained runners' leucocytes submitted to increased hydrogen peroxide conditions and/or in athletes less genetically favored by the antioxidant defense system, using comet assay and the following polymorphisms: Val9Ala in mitochondrial targeting sequence of MnSOD gene (NCBI, refSNP ID: rs1799725), -21A/T in promoter region of CAT gene (NCBI, refSNP ID: rs7943316), Pro198Leu of GPx-1 gene (NCBI, refSNP ID: rs1050450), del{GSTM1}, del{GSTT1}, ACE I/D and Hp.

SUBJECTS AND METHODS

Study design and participants

The trial was conducted after preclinical and toxicological tests in mice.^[23] Volunteers of both genders (76 men, 49 women) and different age groups (15 to 67) were recruited in high schools, colleges, universities, clubs and companies in Brasília (Federal District/Brazil). The inclusion/exclusion criterion used for the runners was that they had at least a 4,000 m run performance, keeping the race of the same type, intensity and length of weekly training, to guarantee no additional physical stress beyond the habitual, to avoid differences in training amount or intensity and consequent increased oxidative stress. They participated in two races of the same route and time, before (control group) and after (treatment group) ingestion of 400 mg of pequi oil in capsules supplied daily for 14 consecutive days. This daily ingestion took into account data from pequi literature and the maximum daily dose of provitamin A carotenoids (25 mg) recommended by the National Agency for Sanitary Surveillance (ANVISA). Each athlete participated as control and treatment, being compared in the statistical tests with him(her)self. There was no significant change in daily routine, training or lifestyle of runners between the first and second race, except for ingestion of pequi-oil capsules.

The races were outdoors on flat tracks, under the same environmental conditions, and athletes chose the distance they would cover (4-21 Km), according to type, intensity and length of their weekly training; both races for each athlete were the same distance and inside the same time. After the first race, they were instructed to take capsules for 14 days during or immediately after lunch until the second race.

This study was conducted according to the guidelines in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee for Health Sciences Faculty Research, University of Brasília, and the National Commission for Ethics in Research (CONEP), number 0.001668/2005-18. All subjects gave written informed consent.

Preparation of capsules

Pequi fruit was obtained in natura from markets in Brasília and surroundings. The internal mesocarp was peeled to obtain pulp, extracted by cold maceration using chloroform as solvent, submitted to evaporation under reduced pressure for solvent removal and dried at high vacuum. Pequi oil, whose relative composition is shown in Table 1,^[24-27] was then incorporated in Aerosil q.s.p., so that users ingested a daily dose of 400 mg. Capsule production was patented as PI0601631-6 (National Institute of Industrial Property – INPI).

Procedures and measurements

Blood samples were drawn with EDTA immediately after races in two rounds: (1) race without pequi-oil supplementation and (2) race after ingestion of 400 mg of pequi oil in capsules

supplied daily for 14 consecutive days. About 5 mL of peripheral blood was collected by venipuncture, using Vacutainer tubes with EDTA as anticoagulant, to verify DNA damage by comet assay (single-cell gel electrophoresis, SCGE) and genes' polymorphisms. Blood was collected in situ, being immediately processed for comet assay.

Comet assay

The comet assay (alkali method) was performed according to Singh et al. (1988)^[28] with few modifications, as previously reported.^[29,30]

Antioxidant enzymes, GST M1, GST T1, Ace and Haptoglobin genotyping

DNA was isolated from buffy-coat layer using the Blood genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, England). DNA samples underwent amplification in MJ PTC-100 (MJ. Research Inc.).

Mn-SOD, CAT and GPX1 genotypes were determined by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assays performed as described respectively by Mitrunen et al. (2001),^[31] Ukkola et al. (2001)^[32] and Zhao et al. (2005).^[33] GSTM1 and GSTT1 fragments were amplified simultaneously as proposed by Chen et al (1996),^[34] using β -globin as positive control. The absence of an amplification product combined with the presence of a positive control band (268 bp DNA fragment of β -globin) indicated the null (variant) type for both polymorphisms. DNA fragments containing I/D polymorphism in intron 16 of the ACE gene were amplified by PCR as previously described by Rigat et al. (1992),^[35] using DMSO (dimethyl sulfoxide)

Table 1. Relative composition of pequi (*Caryocar brasiliense* Camb.) pulp-oil capsules.

Fatty Acids ^[*,23]		Carotenoids ^[24-27]
Saturated (%)	Unsaturated (%)	Types (mg/100 g)
Palmitic (41.78)	Mono-unsaturated	Provitamin A (6.26-11.5)
Stearic (1.28)	Oleic (54.28)	Lycopene (1.12-2.08)
Araquidic (0.12)	Palmitoleic (0.67)	
	Bi-unsaturated	
	Linoleic (1.36)	
	Tri-unsaturated	
	Linolenic (0.51)	
Total (43.18)	Total (56.82)	Total (6.75-28.66)

*Present study

as recommended by Odawara et al. (1997),^[36] to avoid mistyping DD genotype. Hp genotypes were determined by allele-specific PCR as described by Yano et al. (1998).^[37] The PCR and PCR-based RFLP products were resolved in non-denaturing polyacrylamide gels stained with silver nitrate.

Statistical analyses

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 17.0. Data were expressed as mean \pm SEM (standard error of mean) and values of $p < 0.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Differences between sexes, GSTM1 and GSTT1 genotypes were evaluated by the Independent Samples T-test or the Mann-Whitney U-test, while differences among age groups, distance covered and other genotypes was assessed by ANOVA or the Kruskal-Wallis test. For significant ANOVA results, the Tukey HSD post-hoc test was chosen for multiple comparisons; for significant Kruskal-Wallis results, Mann-Whitney U test was used. The stratification of subjects by age was: adolescents (15-19 year-olds), young adults (20-40 year-olds) and middle-aged/elderly adults (41-67 year-olds), following age criteria for reference values of biochemical parameters; for clinical purposes some reference values are different for ages up to 19 years old.^[38] The Paired Samples T-test or the Wilcoxon test verified differences before-after pequi-oil supplementation. Possible correlations between the parameters sex/age groups, sex/covered distance and age groups/covered distance were analyzed through Chi-square correlation test.

To verify differences between control and treatment slides, the Friedman and Wilcoxon tests were used. Differences were considered significant at $p < 0.05$. Interactions between two genetic markers in the comet assay results were also analyzed through Multivariate Analyses of Variance.

Because the population genetics data have been previously reported,^[22] they were not presented here.

RESULTS

Significant differences between genders appeared for the control slides before pequi-oil supplementation ($p = 0.005$) and for those treated with 0.25 mM H_2O_2 after (0.025). For age groups, these differences were

seen on control slides after pequi ($p = 0.003$) and on slides treated with 0.25 mM H_2O_2 before ($p = 0.018$) and after ($p = 0.033$). Control showed differences for 15-19 and 20-40 years ($p = 0.002$) and 20-40 and ≥ 41 years ($p = 0.032$). Treated slides before pequi showed differences for 15-19 and ≥ 41 years ($p = 0.025$) and 20-40 and ≥ 41 years ($p = 0.007$); after pequi, for 15-19 and 20-40 years ($p = 0.034$), and 20-40 and ≥ 41 years ($p = 0.048$). Concerning distance covered, these differences appeared on control slides after pequi ($p = 0.005$) and on treated slides before ($p = 0.006$) pequi. Control slides showed at 4-5 and 16-21 Km ($p = 0.011$), for 6-7 and 8-10 Km ($p = 0.011$), for 6-7 and 16-21 Km ($p = 0.030$), and 8-10 and 16-21 Km ($p = 0.007$); for treated slides, for 4-5 and 6-7 Km ($p = 0.031$), 4-5 and 16-21 Km ($p = 0.000$), 6-7 and 8-10 Km ($p = 0.019$), and 8-10 and 16-21 Km ($p = 0.013$). Although no significant difference was detected by the Kruskal-Wallis test for the slides treated with 1 mM H_2O_2 , the Mann-Whitney U test detected significant differences between 6-7 and 8-10 Km after pequi-oil supplementation. A significant positive correlation existed between age and distance covered ($p = 0.000$) (data not showed).

Disregarding the influence of the studied genetic polymorphisms, DNA damage tended to fall after pequi-oil supplementation, except in group 15-19 years and in most slides treated with H_2O_2 at 0.25 mM. Significant decreases were observed for control slides of the total group ($p = 0.003$), women ($p = 0.002$) (Fig. 1), 20-40

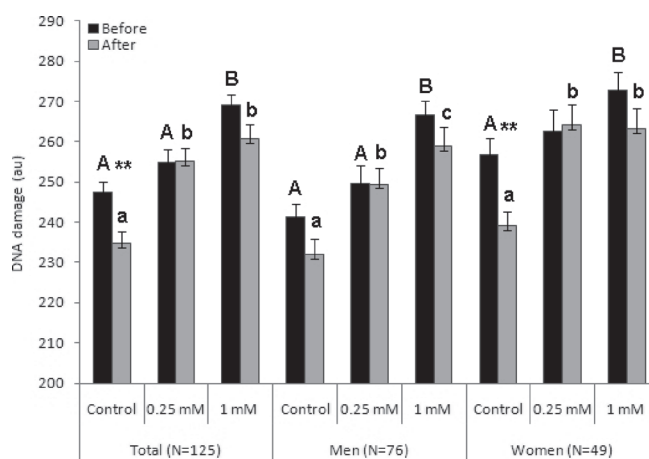


Figure 1. Influence of pequi-oil supplementation on the total and gender groups.

au = arbitrary units. The data correspond to the means and to the standard error of mean (SEM). The capital letters indicate significant differences between control and treatments before pequi, while the lower-case letters indicate these differences after pequi. Different letters indicate significant differences. Asterisks indicate highly significant (** $p < 0.01$) differences in the comparison of before-after values.

years ($p = 0.002$) (Fig. 2) and distance 8-10 Km ($p = 0.017$) (Figure 3); for the 1 mM H_2O_2 treatment in age group 20-40 years (Fig. 2); and for those treated with 0.25 mM H_2O_2 at distance 16-21 Km (Fig. 3). H_2O_2 concentrations and DNA-damage levels correlated positively in both,

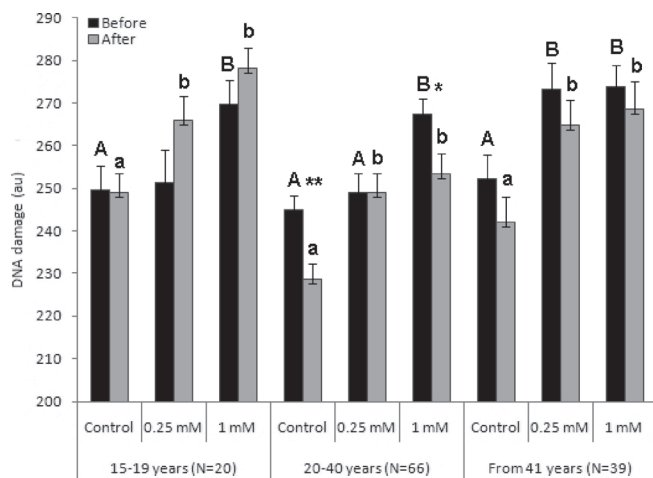


Figure 2. Influence of pequi-oil supplementation on the age groups. *au = arbitrary units. The data correspond to the means and to the standard error of mean (SEM). The capital letters indicate significant differences between control and treatments before pequi, while the lower-case letters indicate these differences after pequi. Different letters indicate significant differences. Asterisks indicate significant (* $p < 0.05$) and highly significant (** $p < 0.01$) differences in the comparison of before-after values.*

before and after pequi-oil supplementation, particularly for 1 mM H_2O_2 concentration, which significantly increased DNA damage when compared to control (Fig. 1). This trend remained in analyses by age groups (Fig. 2) and distance covered (Fig. 3), except for 16-21 Km, where before pequi the greatest DNA damage was observed for 0.25 mM H_2O_2 concentration (Fig. 3).

Regarding genetic polymorphisms (Table 2), significant differences among the genotypes before pequi-oil supplementation were showed only for GPx-1 ($p = 0.033$) in slides treated with 0.25 mM H_2O_2 . Kruskal-Wallis test did not detect significant differences on control slides among Ace genotypes, but Mann-Whitney U test showed significant differences between DD and ID genotypes ($p = 0.046$). After pequi-oil treatment, significant differences among genotypes were showed by Ace polymorphism ($p = 0.002$) for slides treated with 1 mM H_2O_2 , these differences appeared for DD and ID ($p = 0.024$) genotypes and ID and II genotypes ($p = 0.002$). For the same H_2O_2 concentration, differences among the GPx-1 genotypes showed by ANOVA were at p-value of 0.054, but Tukey HSD test detected significant differences between Pro/Pro and Pro/Leu genotypes ($p = 0.044$). Pequi-oil supplementation resulted in significant DNA damage decrease for control slides of MnSOD Val/Ala ($p = 0.0004$), CAT T T T ($p = 0.026$),

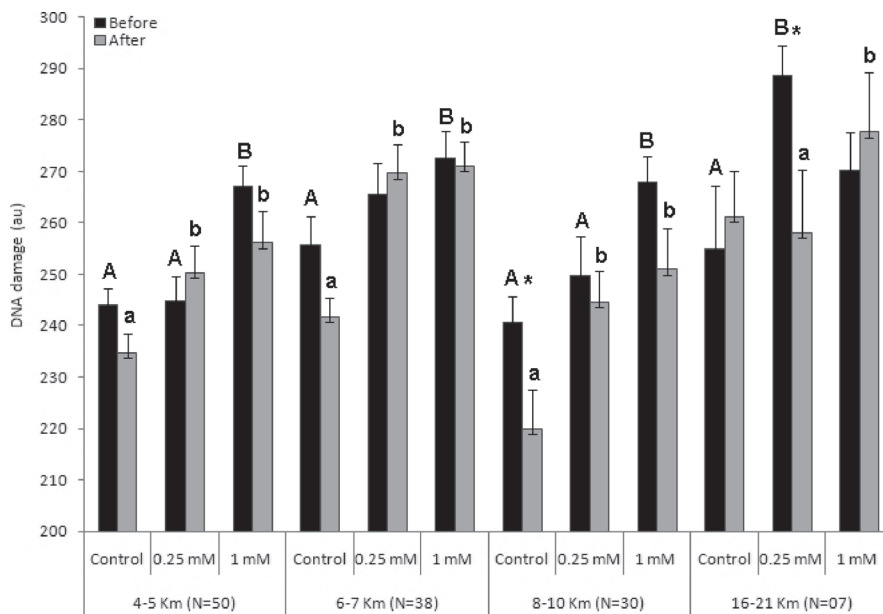


Figure 3. Influence of pequi-oil supplementation on the distance covered (Km). *au = arbitrary units. The data correspond to the means and to the standard error of mean (SEM). The capital letters indicate significant differences between control and treatments before pequi, while the lower-case letters indicate these differences after pequi. Different letters indicate significant differences. Asterisks indicate significant (* $p < 0.05$) differences in the comparison of before-after values.*

Table 2. Influences of MnSOD, CAT, GPx-1, GSTM1, GSTT1, ACE and of Haptoglobin (Hp) genes' polymorphisms on exercise-induced DNA damage before and after pequi oil supplementation

Genetic Markers	N (%)	Comet assay (DNA damage - au)					
		Control		H ₂ O ₂ (0.25 mM)		H ₂ O ₂ (1 mM)	
		Before	After	Before	After	Before	After
MnSOD							
Val/Val	21 (12.2)	245.81 ± 6.09	240.19 ± 4.69	261.76 ± 9.59	260.48 ± 8.77	275.81 ± 5.75	263.24 ± 5.64
Val/Ala	100 (58.1)	246.39 ± 2.88*	232.60 ± 3.29	251.74 ± 3.64	254.02 ± 3.54	267.66 ± 3.00	260.69 ± 4.20
Ala/Ala	04 (2.3)	279.25 ± 13.88	261.00 ± 12.80	292.50 ± 17.25	257.75 ± 17.97	271.50 ± 10.48	245.25 ± 17.84
P-Values		0.664	0.296	0.105	0.546	0.511	0.327
CAT							
AA	23 (18.4)	249.70 ± 5.79	240.22 ± 5.23	252.26 ± 7.65	259.96 ± 7.17	273.13 ± 6.59	264.04 ± 6.48
AT	53 (42.4)	245.91 ± 4.33	233.30 ± 4.89	252.00 ± 5.52	254.75 ± 5.07	268.85 ± 3.90	260.11 ± 6.37
TT	49 (39.2)	247.80 ± 3.89*	233.84 ± 4.19	258.84 ± 5.28	253.51 ± 5.19	267.61 ± 4.18	259.57 ± 5.05
P-Values		0.866	0.774	0.630	0.775	0.755	0.946
GPx-1							
Pro/Pro	61 (48.8)	244.21 ± 3.79	231.61 ± 4.53	246.08 ± 4.61	253.07 ± 4.84	267.03 ± 3.48*	252.10 ± 5.08
Pro/Leu	50 (40.0)	250.34 ± 3.68*	237.94 ± 3.78	261.00 ± 5.52	258.84 ± 4.97	270.06 ± 4.63	270.04 ± 5.28†
Leu/Leu	14 (11.2)	250.29 ± 9.81	237.36 ± 7.61	270.00 ± 10.03	251.71 ± 8.56	275.14 ± 6.51	264.14 ± 10.85
P-Values		0.503	0.895	0.033	0.655	0.622	0.054
GSTM1							
Null	81 (64.8)	248.01 ± 3.33*	235.19 ± 3.52	255.15 ± 4.59	253.06 ± 4.14	270.32 ± 3.24	257.54 ± 4.81
Non-null	44 (35.2)	246.11 ± 4.14	234.05 ± 4.70	253.95 ± 4.82	259.20 ± 5.08	267.00 ± 4.44	266.30 ± 4.67
P-Values		0.940	0.914	0.858	0.364	0.545	0.199

GSTT1							
Null	17 (13.6)	254.29 ± 8.36	236.00 ± 5.18	257.35 ± 8.51	257.59 ± 5.84	268.35 ± 7.41	266.29 ± 8.50
Non-null	108 (86.4)	246.25 ± 2.70*	234.59 ± 3.15	254.31 ± 3.73	254.85 ± 3.62	269.28 ± 2.80	259.73 ± 3.87
P-Values		0.290	0.617	0.762	0.772	0.904	0.653
ACE							
DD	45 (36.0)	254.09 ± 4.50*	241.51 ± 3.39	252.53 ± 5.38	255.71 ± 5.45	271.13 ± 4.23	265.80 ± 6.00
ID	61 (48.8)	241.52 ± 3.52†	232.46 ± 4.15	253.95 ± 5.06	254.25 ± 4.56	267.97 ± 3.83*	250.25 ± 4.35†
II	19 (15.2)	250.05 ± 6.60*	226.32 ± 9.73	262.42 ± 9.09	257.21 ± 8.67	268.26 ± 6.89	281.68 ± 10.31‡
P-Values		0.128	0.439	0.627	0.947	0.852	0.002
Hp							
1F-1F	08 (4.7)	239.75 ± 10.82	225.12 ± 17.16	241.00 ± 11.81	238.13 ± 13.99	266.25 ± 9.93	234.88 ± 19.45
1F-1S	08 (4.7)	247.50 ± 9.86	228.75 ± 9.24	270.38 ± 10.78	245.12 ± 10.21	264.75 ± 9.99	255.00 ± 12.31
1S-1S	20 (11.6)	250.15 ± 6.71	244.50 ± 5.43	254.60 ± 9.56	258.10 ± 9.36	274.45 ± 7.31	262.85 ± 9.44
1F-2	20 (11.6)	253.50 ± 7.85*	232.50 ± 4.51	247.70 ± 8.89	259.80 ± 7.84	270.40 ± 7.13	251.25 ± 7.82
1S-2	35 (20.3)	245.20 ± 4.39	234.91 ± 6.49	256.14 ± 5.16	259.89 ± 5.91	268.89 ± 4.59	272.69 ± 6.15
2-2	34 (19.8)	246.03 ± 4.93	233.97 ± 5.04	257.03 ± 7.63	252.44 ± 6.04	267.29 ± 5.06	259.79 ± 6.60
P-Values		0.890	0.620	0.672	0.426	0.957	0.152

au = arbitrary units. Data are expressed as mean ± SEM (standard error of mean). P-values of control "before", H₂O₂ 0.25 mM "before" and "after" and H₂O₂ 1 mM "before", MnSOD and CAT; control "before", H₂O₂ 0.25 and 1 mM "before" and "after" GPx-1; H₂O₂ 0.25 "before", H₂O₂ 1 mM "before", H₂O₂ 0.25 "before", H₂O₂ 1 mM "before" and "after". Hp were generated by ANOVA. The Kruskal-Wallis test was used to generate the p-values of control and H₂O₂ 1 mM "after" both MnSOD and CAT; control "after" GPx-1; control "before" and "after" Ace; and control "before" and "after" and H₂O₂ 0.25 "after" Hp. The Mann-Whitney U test was used to generate the p-values of control before and after, and H₂O₂ 1 mM after GSTM1; and control and H₂O₂ 1 mM both "after", while the Independent Samples T-test generated the p-values of H₂O₂ 0.25 "before" and "after", H₂O₂ 1 mM "before" GSTM1; and control "before", H₂O₂ 0.25 "before", and H₂O₂ 1 mM "before" GSTT1. Asterisks indicate significant differences in the comparison of before-after values detected by the Paired Sample T-Test or Wilcoxon matched pairs test. The symbols indicate significant differences between two specific genotypes: † compared to the first genotype of each genetic marker; ‡ compared to the second genotype of each genetic marker by the Tukey HSD post-hoc test or the Mann-Whitney U test.

GPx-1 Pro/Leu ($p = 0.028$), GSTM1 null ($p = 0.011$), GSTT1 non-null ($p = 0.012$), Ace DD ($p = 0.039$) and II ($p = 0.036$), and Hp 1F-2 ($p = 0.020$) genotypes. For slides treated with H_2O_2 , significantly lower DNA damage after pequi-oil treatment only showed for genotypes GPx-1 Pro/Pro ($p = 0.020$) and Ace ID ($p = 0.010$) at concentration of 1 mM (Table 2).

The two H_2O_2 treated groups were also significantly more damaged than controls in most analyses of the genetic polymorphisms (Friedman, $p < 0.05$), except for MnSOD Ala/Ala, CAT AA, Hp 1F-1F, 1F-1S, 1S-1S and 1F-2 before pequi, and for MnSOD Val/Val and Ala/Ala, GPx-1 Leu/Leu, Hp 1F-1F, 1F-1S and 1F-2 after pequi. However, the Wilcoxon test showed significant differences between control and treatment with 0.25 mM H_2O_2 before pequi for genotypes MnSOD Ala/Ala ($p = 0.046$), CAT TT (0.031), GPx-1 Leu/Leu ($p = 0.008$), Ace ID (0.015); between control and treatment with 1 mM H_2O_2 significant differences emerged for MnSOD Val/Val ($p = 0.001$) and Val/Ala ($p = 0.000$), CAT AA ($p = 0.003$) and AT ($p = 0.000$), GPx-1 Pro/Pro ($p = 0.000$) and Pro/Leu ($p = 0.000$), GSTM1 null ($p = 0.000$) and non-null ($p = 0.001$), GSTT1 null ($p = 0.026$) and non-null ($p = 0.000$), Ace DD ($p = 0.005$), ID ($p = 0.000$) and II ($p = 0.003$), Hp 1S-1S ($p = 0.007$), 1F-2 ($p = 0.015$), 1S-2 ($p = 0.001$) and 2-2 ($p = 0.006$); and between the two H_2O_2 treatments for MnSOD Val/Ala ($p = 0.000$), CAT AT ($p = 0.002$), GPx-1 Pro/Pro ($p = 0.000$), GSTM1 null ($p = 0.006$) and non-null ($p = 0.029$), GSTT1 non-null ($p = 0.001$), Ace DD ($p = 0.003$) and Hp 1F-2 ($p = 0.018$). After pequi-oil supplementation, significant differences between control and 0.25 mM H_2O_2 treatment appeared for most genotypes of each polymorphism, except for MnSOD Val/Val and Ala/Ala, GPx-1 Leu/Leu, Hp 1F-1F and 1S-1S; the same happened between control slides and 1 mM H_2O_2 treatment, except for Hp 1F-1F and 1S-1S. The two H_2O_2 treatments significantly differed after pequi only for genotypes MnSOD Val/Ala ($p = 0.042$) and Ace II ($p = 0.039$) (data not showed).

Although no interaction between the genetic markers was showed by Multivariate Analysis of Variance, in the tests between subjects and effects, there was a significant interaction effect between CAT and GSTT1 ($p = 0.012$) polymorphisms in the control before pequi, where greatest damage was presented by CAT AT/T1 null genotype carriers and least damage by CAT AA/T1 null genotypes (Fig. 4). After pequi, this effect disappeared.

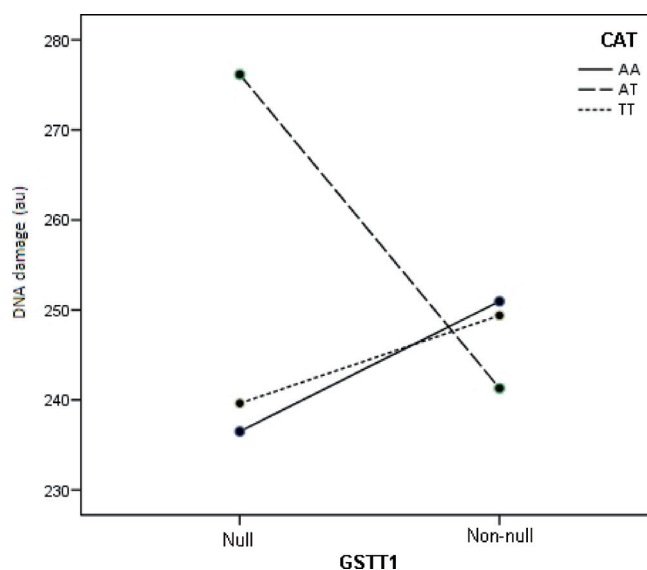


Figure 4. Significant interaction effect between CAT and GSTT1 polymorphisms in the control slide before pequi-oil supplementation. au = arbitrary units.

DISCUSSION

Experimental studies include randomized placebo-controlled, case control, quasi-experimental and before-after studies; ours was designed to be a before-after study. Before-after studies have been validated in the scientific literature,^[39] and although they can present some limitations compared to randomized placebo-controlled studies, we followed all the steps to guarantee quality control and the validation of our study: (1) only trained sportsmen were included; (2) although the athletes had a variable degree of training intensity, the distance that they would cover was chosen according to the type, intensity and length of weekly training, guaranteeing no additional physical stress beyond what they were accustomed to; (3) the volunteers ran the same route and distances in both races in the same sample time interval and under the same environmental conditions; (3) the only change in the athletes' routine between the two races was supplementation with pequi oil; (4) results obtained in previous reports assessing biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) corroborate this affirmation, since they did not exceed the reference values.^[20] We cannot exclude differences in relative intensity experienced by the athletes completing a distance in both races with the same running time, because we did not measure oxygen uptake or heart rate parameters. However, the significant differences in DNA damage before-after the two races are more likely to be

related to the pequi-oil supplementation and/or its interaction with genetic factors than to differences in relative intensity, as will be further discussed below.

Endurance exercise increases whole body oxygen consumption 10-20 fold, which at the level of the skeletal muscle increases 100-200 fold. This increase in oxygen utilization may result in the production of ROS at rates that exceed the body's detoxification capacity.^[7] Thus, there has been growing interest in exercise-induced DNA damage due to its potential involvement in various diseases, since oxidatively damaged DNA has been implicated in carcinogenesis, ageing process, lifestyle-related diseases and age-related degenerative diseases.^[40-42] Exercise-induced production of reactive oxygen and nitrogen species has been shown to cause oxidative stress to skeletal muscle, heart and other organs. As an adaptive response to exercise, antioxidant defense systems are upregulated, restoring intracellular prooxidant-antioxidant homeostasis.^[11,43] As a result, humans involved in regular exercise have shown reduced oxidative damage to DNA during physical exertion at accustomed or even excessive intensity.^[7,43] Using the comet assay technique, various forms of exercise - high intensity, short duration; moderate intensity, moderate duration; and moderate intensity endurance - have all been demonstrated to produce the same relative amounts of DNA damage.^[7] The hydrogen peroxide used in the present study was to increase oxidative stress. The choices of 0.25 and 1 mM were based on our previous results: peripheral blood leukocytes of untrained healthy individuals treated with 0.25 mM H₂O₂ showed DNA damage similar to that suffered by trained athletes after running a race.^[29,30,44]

Physical exercise involves the entire body in terms of energy metabolism, hormonal mobilization and action, signal transduction process, immunological responses and adaptations^[43] and other reports with lower or equal intervention times have also shown physiological effects, with significant changes in the biochemical markers used.^[45-48] Pequi oil contains several carotenoids,^[24-27] recognized as an effective antioxidant under low PO₂ conditions^[2] such those undergone by various tissues and organs during races. In addition, it has been reported that meals rich in monounsaturated fatty acids, as provided by the Mediterranean diet and by pequi-oil supplementation, increases plasma carotenoids,^[46] which can improve antioxidant defense in situations of increased oxidative stress. In this context, without additional oxidative stress imposed by H₂O₂, results indicated that the protective antioxidant effect of pequi oil against exercise-induced

DNA damage was influenced by sex, age, distance covered and all the studied genetic polymorphisms. So, previous reports associating regular exercise with reduced oxidative damage to DNA during physical exertion, independently of its intensity, may perhaps be mistaken in applying the findings to all age groups. This study can also broaden knowledge of how antioxidant supplementation affects exercise-induced DNA damage and how individual athletic genetic makeup can affect the way athletes respond to antioxidant supplementation against exercise-induced DNA damage.

A low dosage of toxins may increase the body's tolerance for greater toxicity^[43] and the reaction of H₂O₂ with transition metals imposes an oxidative stress condition on cells, which can result in DNA damage, permanent growth arrest and apoptosis.^[1,2] Before pequi-oil supplementation, results suggested that the tolerance for an increased oxidative stress and consequent DNA damage happened only with H₂O₂ at up to 0.25 mM; the treatment with H₂O₂ at 1 mM increased oxidative stress above the capacity of the organism to adapt. However, this tolerance did not occur for age group above 41 years, distances above 10 Km and the genotypes MnSOD Ala/Ala, CAT TT and ACE ID. Because aging is characterized by a general decline in physiological function,^[49] results indicate that long-distance races can be harmful, mainly for older athletes carrying one or more of these genotypes, due to increased oxidative stress above adaptability. In contrast, after pequi-oil supplementation this pattern of tolerance changed; there was no tolerance for either H₂O₂ concentration, except at the distance of 16-21 Km (which also presented significantly decreased DNA damage after pequi-oil supplementation) and genotypes MnSOD Val/Val, GPx-1 Leu/Leu and Hp 1F-1F and 1S-1S. However, in the case of the group of 20-40 years, the effectiveness of the pequi oil in significantly reducing exercise-induced DNA damage followed a type of inverted monotonic dose response curve (significant results only for control and H₂O₂ at 1 mM). So could the individuals in this age group practice regular physical activity above accustomed intensity or duration without overwhelming the capacity of the endogenous antioxidant system, as long as they ingest antioxidants? This question needs further investigation. In addition, runner response to pequi-oil in decreasing exercise-induced DNA damage was influenced by all the studied genetic polymorphisms. Although the genotypes of GPx-1 and Ace polymorphisms responded differently with and without increased oxidative stress forced by 1 mM H₂O₂ treatment, under increased

H₂O₂ conditions GPx-1 Pro/Pro and ACE ID genotypes have been more responsive to antioxidant supplementation.

MnSOD (EC 1.15.1.1) is a mitochondrial enzyme catalyzing the dismutation reaction of superoxide radicals (O₂^{•-}) to H₂O₂, which is coded by a nuclear gene located on chromosome 6q25.3. The enzyme is synthesized with a mitochondrial targeting sequence (MTS), which is cleaved in the mitochondrial matrix to produce the active enzyme. The valine to alanine substitution in the MnSOD MTS induces a conformational change from an α-helix to a β-sheet, which has been reported to change mitochondrial processing efficiency, affect the transport of MnSOD to mitochondria, and to decrease MnSOD efficiency against oxidative stress, being associated with diseases related to oxidative stress and abnormal free radical defense mechanisms.^[50] Given that our research was carried out with athletes and that MnSOD is under selective pressure, results suggest that MnSOD heterozygosis can favor defense against oxidative stress. This suggestion is based on our previous reported results, since the Val/Ala genotype presented the lowest damage to tissues and the lowest lipid peroxidation levels.^[20] Moreover, this group had a better response to pequi oil against exercise-induced DNA damage in this study. Also, it has been suggested that an overexpression of MnSOD could increase production of H₂O₂, which, if not subsequently neutralized and converted to H₂O and O₂, could contribute to further generations of ROS.^[51] In this way, MnSOD heterozygosis could favor mainly those in athletes less genetically favored by the CAT and GPx-1 antioxidant defenses.

CAT (EC 1.11.1.6) is an enzyme whose main role involves controlling H₂O₂ concentrations in human cells, converting H₂O₂ into H₂O and O₂.^[52] Although no effects on catalase expression, catalase activity or association with disease/pathological changes have been reported,^[53] results indicated that there are differences among CAT genotypes and more investigations are needed, mainly because pequi-oil significantly decreased DNA damage only in athletes carrying the TT genotype, which also presented no tolerance to increased oxidative stress imposed by H₂O₂ at 0.25mM. Moreover, there was a significant interaction between subject's effects linking CAT and GSTT1, where, in presence of GSTT1 null genotype, extent of DNA damage depended on the presence or absence of the CAT variant allele. Additionally, results indicated that, under situations of increased oxidative stress such those imposed by H₂O₂ at 1mM, the GPx-1 polymorphism seemed to influence DNA damage more than CAT polymorphism. Because GPx-

1 enzyme (EC 1.11.1.9) also detoxifies H₂O₂ using glutathione in its reduced form (GSH) as co-substrate, and the variant Leu allele has been implicated in GPx-1 activity, which becomes less responsive to stimulation, being associated with some kinds of cancer,^[33] we would have expected a lower or non-responsive reaction of Leu/Leu genotype for the pequi-oil supplementation. However, under increased oxidative stress, such as treatment with H₂O₂ at 1mM, the Pro/Leu genotype was more unresponsive to the supplementation than Leu/Leu genotype. Moreover, after pequi, the Leu/Leu genotype showed tolerance to the increased oxidative stress promoted by the 0.25mM H₂O₂ treatment, which was not observed for Pro/Leu genotype. Thus, results indicated that, at least for DNA damage, GPx-1 heterozygosis could be more harmful to subjects submitted to increased oxidative stress, even consuming natural antioxidants.

The glutathione S-transferases GSTM1 and GSTT1 genes code for the cytosolic enzymes GST-μ (mu) and GST-θ (theta) (EC 2.5.1.18), respectively, which catalyze reactions involving the conjugation between reduced glutathione (GSH) and a variety of electrophilic compounds, including xenobiotics and products of oxidative stress.^[54] Because the null genotypes result in deficiency of the GST activities,^[14] environmental exposure to cytotoxic and genotoxic agents could increase cancer risk for those individuals carrying GST deletions. Although no significant differences between the genotypes of these polymorphisms were observed in this study, non-null genotypes showed less DNA damage than null genotypes in both races. Thus, under xenobiotic exposure differences could be significant. On the other hand, in healthy individuals, GSTM1 homozygous nulls have been reported ranging from 22-67% worldwide in different ethnicities,^[55,56] and some gradients and intra-ethnic differences have already been reported.^[14,54] This considerable range of variation can indicate distinctive processes of natural selection and adaptation to variable environmental conditions. Therefore, our results suggested a possible selective advantage for GSTM1 null genotype, since there was a higher frequency of this genotype in the athletes' group. Furthermore, this null genotype was significantly more responsive to pequi-oil supplementation than the non-null genotype, and similar results occurred with the GSTT1 non-null genotype.

Haptoglobin is an integral part of the immune acute phase response, which binds free hemoglobin (Hb), preventing oxidative damage and modulating immune function. The complex Hp-Hb also functions as a

scavenger of nitric oxide (NO), a free radical vital in basal blood flow regulation and vascular homeostasis, regulating NO bioavailability and vascular homeostasis.^[18] Several functional differences reported between Hp phenotypes could have important biological and clinical consequences. These differences are explained by a phenotype-dependent modulation of oxidative stress and prostaglandin synthesis.^[18,57] Hp polymorphism is associated with the prevalence and clinical evolution of many inflammatory diseases.^[58] It has also been investigated with leukemia, and the Hp1-1 phenotype associated with CML, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).^[58,59] Recently, Hp polymorphism has also been suggested as a determining factor in runners' performance.^[44] In our previous studies, deviation from HWE was mainly due to higher than expected frequency of Hp1F-1F and 1S-1S contrasting with lower frequency of Hp1F-1S.^[22] Hp1F-1S and 1S-2 have been associated with higher lipid peroxidation,^[44] while Hp1S-1S presented significantly different results from Hp1F-1S.^[22] Moreover, in this study only Hp1F-1F and 1S-1S presented tolerance to increased oxidative stress after pequi-oil supplementation, while only Hp1F-2 reduced DNA damage significantly after pequi oil. Hp type frequency differs greatly among ethnic groups, which suggest that particular populations are susceptible to particular diseases.^[18] However, results indicated that differences in biological responses among the Hp*1 alleles cannot be treated as a single block in association studies.

In conclusion, runner response to the pequi oil in decreasing exercise-induced DNA damage was influenced by sex, age and all the studied genetic polymorphisms, indicating that: (1) long-distance races can be harmful, mainly for older athletes, due to increased oxidative stress above organism adaptability, even with antioxidant supplement use; (2) there were differences in genotypes' responses; and (3) under increased H₂O₂ conditions, GPx-1 Pro/Pro and ACE ID genotypes were more responsive to antioxidant supplementation.

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CONFLICTS OF INTEREST

Nothing to declare

AUTHOR CONTRIBUTIONS

All authors contributed to this work. ALMV was principal investigator and takes primary responsibility for the paper. ALMV, MNKG and CKG were responsible for planning the study; CKG and MNKG coordinated it and arranged funding; ALMV, PCZ, AKA, GSL performed laboratory work; and ALMV was responsible for statistical analysis, data interpretation and writing the manuscript.

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