

# Dietary açai modulates ROS production by neutrophils and gene expression of liver antioxidant enzymes in rats

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Açai (*Euterpe oleracea* Mart.) has recently emerged as a promising source of natural antioxidants. Because increased oxidative stress and impaired antioxidant defense mechanisms are important factors in the development of diabetic complications and many health claims have been reported for açai, the present study was undertaken to evaluate the possible protective effects of açai on the production of reactive oxygen species by neutrophils and on the liver antioxidant defense system in control and streptozotocin-induced diabetic rats. Diet supplementation with 2% açai was found to increase mRNA levels for gamma-glutamylcysteine synthetase and glutathione peroxidase in liver tissue and to decrease reactive oxygen species production by neutrophils. Compared to control animals, diabetic rats exhibited lower levels of mRNA coding for Zn-superoxide dismutase, glutathione peroxidase and gamma-glutamylcysteine synthetase and higher levels of reactive oxygen species production by neutrophils, thiobarbituric acid-reactive substances and carbonyl proteins in hepatic tissues. Although açai supplementation was not effective in restore gene expression of antioxidant enzymes in diabetic rats, it showed a protective effect, decreasing thiobarbituric acid-reactive substances levels and increasing reduced glutathione content in the liver. These findings suggest that açai can modulate reactive oxygen species production by neutrophils and that it has a significant favorable effect on the liver antioxidant defense system under physiological conditions of oxidative stress and partially revert deleterious effects of diabetes in the liver.

**Key Words:** açai, antioxidant enzymes expression, neutrophils, diabetes, rats

Açai berry is the fruit of the *Euterpe oleracea* Mart. palm tree, a species that is native to the Amazon region. In recent years, açai has been the subject of much attention due to its high antioxidant capacity and its role as a “functional food”.<sup>(1,2)</sup> Açai is currently one of the main export products of the Amazon region; it is widely distributed and commercialized as frozen pulp, juice or wine.<sup>(3)</sup> Biochemical studies have revealed that açai is rich in phytochemicals, especially polyphenols such as anthocyanins, proanthocyanidins and other flavonoids.<sup>(4)</sup> A variety of assays show that açai pulp has a high *in vitro* antioxidant capacity, especially against superoxide and peroxy radicals; its antioxidant capacity is the highest of any fruit reported to date in the literature.<sup>(5)</sup>

Recent studies have shown that supplementation of the diet with açai pulp improved lipid and oxidative stress biomarker profiles in sera of hypercholesterolemic rats<sup>(6)</sup> and that it increased the longevity of flies fed a high-fat diet through the activation of transcription of genes coding for proteins that function in oxida-

tive stress response pathways.<sup>(7)</sup> A clinical trial has shown that consumption of açai pulp and juice resulted in a significant increase in the plasma antioxidant capacity of healthy human volunteers.<sup>(8)</sup> Açai pulp also showed a protective effect against hydrogen peroxide-mediated damage to lipids and proteins in the cerebral cortex, hippocampus and cerebellum of rats<sup>(9)</sup> and against DNA damage induced by doxorubicin (DXR) in liver and kidney of mice.<sup>(3)</sup> Furthermore, açai extract showed antiproliferative and proapoptotic activity against C-6 rat brain carcinoma cells,<sup>(10)</sup> as well as anti-inflammatory properties demonstrated by its ability to inhibit the activity of cyclooxygenases (COX)-1 and COX-2 in cell culture.<sup>(5)</sup> Many of these effects are attributed to the polyphenol fraction of açai.

Diabetes mellitus is a chronic metabolic disorder that affects more than 170 million people worldwide.<sup>(11)</sup> Diabetes is characterized by hyperglycemia and insufficient insulin production or action and is associated with damage to and dysfunction of several body tissues, altering quality of life and longevity.<sup>(12)</sup> Oxidative stress plays a central role in the pathogenesis and development of the complications of diabetes. Disease progression is usually accompanied by increased production of reactive oxygen species and/or reduction in the efficiency of antioxidant defense systems.<sup>(13)</sup> The mechanisms involved in the increased oxidative stress associated with diabetic complications are partially known; they include activation of transcription factors, formation of advanced glycation end products and activation of protein kinase C.<sup>(14)</sup>

Many *in vivo* and *in vitro* studies have been conducted in conjunction with the search for new treatments for the control of diabetes. Currently used therapies include insulin and several synthetic anti-diabetic drugs used alone or in combination, and many of the available anti-diabetic drugs possess a number of adverse side effects.<sup>(15)</sup> The fact that management of diabetes without causing side effects is still a challenge has generated a growing interest in the use of natural antioxidants as a strategy to reduce the occurrence of diabetic complications. Dietary compounds such as polyphenols can play an important role in the improvement of antioxidant status because they are able to neutralize reactive oxygen species (ROS), acting as metal ion-chelating agents and enzyme modulators.<sup>(16)</sup> These compounds can therefore protect against complications of diabetes caused by increase in oxidative stress. However, the effect of açai on diabetes-induced oxidative stress is still unknown.

The aim of this study is to investigate *in vivo* the effects of açai supplementation on ROS production in neutrophils and on

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oxidant/antioxidant balance and regulation of gene expression of antioxidant enzymes in livers of control and diabetic rats, representing normal physiological conditions and conditions of potentially high oxidative stress.

## Materials and Methods

**Reagents and açai pulp.** DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO). Pasteurized açai pulp (*Euterpe oleracea* Mart.) was obtained from Icefruit Comércio de Alimentos Ltda. (Tatuí, SP, Brazil). For the phytochemical and antioxidant capacity determination analyses, the pulp was centrifuged (2,000 g at 4°C for 15 min) to remove solid residues and lipid, and the supernatant was collected and filtered.

**Phytochemical composition of açai pulp.** The total polyphenol content of açai pulp was determined by the Folin-Ciocalteu method as described by George *et al.*<sup>(17)</sup> Briefly, 2.5 mL of Folin reagent diluted in distilled water (1:10) was added to 500 µL of the diluted sample or of a standard solution of gallic acid. The blank consisted of distilled water. After 2 min at room temperature, 2 mL of 7.5% sodium carbonate solution was added and mixed vigorously. After incubation at 50°C for 15 min, the mixture was placed in an ice bath. Absorbance at 760 nm relative to the blank was determined. All analyses were performed in triplicate. Total polyphenol content was expressed in milligrams of gallic acid equivalent (GAE) per 100 g of fresh pulp.

Total anthocyanin content was determined by the differential pH method.<sup>(18)</sup> Diluted samples were added to 0.025 M chloride buffer (pH 1.0) and 4.0 M sodium acetate buffer (pH 4.5). Absorbances were determined simultaneously as absorption maxima for the visible light spectrum and at 700 nm after incubation in the dark for 30 min at room temperature. Total anthocyanin content was expressed in milligrams of cyanidin-3-glucoside equivalent per 100 g of fresh pulp. A molar absorptivity of 26,900 M<sup>-1</sup>cm<sup>-1</sup> and a molecular mass of 449.2 g/mol were used for cyanidin-3-glucoside.

**DPPH-radical-scavenging activity.** The DPPH Radical Scavenging Activity of açai pulp was determined using a modified method of Brand-Williams *et al.*<sup>(19)</sup> In short, 100 µL of different concentrations of açai pulp (0, 1, 2.5, 5 and 10%) and the standard antioxidant Trolox (0, 50, 75, 100, 125, 150, 175 and 200 mg/L) were added to 3.9 mL of 60 µM DPPH dissolved in 80% methanol. The mixture was homogenized and kept in the dark for 30 min at room temperature. The absorbance of the solution at 515 nm was determined. Methanol (80%) was used as a blank. Antioxidant activity was determined by the reduction in absorbance of the DPPH radical at 515 nm; the percentage inhibition was determined according to the formula below.

$$\% \text{ Scavenging activity} = (1 - A_{\text{Sample 515}}/A_{\text{Control 515}}) \times 100$$

**Animals and experimental design.** Female Fisher rats, weighing on average 180 g, were obtained from the Experimental Nutrition Laboratory of the Federal University of Ouro Preto (UFOP). The animals were habituated in polypropylene boxes, maintained in an environment controlled for temperature, light and humidity and given food and water *ad libitum*. The Ethics Committee on Animal Use of the Federal University of Ouro Preto approved all animal procedures. The animals were divided into four groups, control (C), açai (A), diabetic (D), diabetic + açai (DA), according to the treatment received. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (35 mg/kg body weight) in 0.1 M of citrate buffer, pH 4.5. Rats in groups C and A were injected with vehicle only. The animals were considered diabetic if blood glucose was higher than 15 mM 72 h after STZ injection. Animals in groups C and D were fed a

standard diet (AIN-93);<sup>(20)</sup> those in groups A and DA were given the standard diet with 2% (w/w) açai pulp added. After 30 days of treatment, the animals were fasted overnight, anesthetized with isoflurane and euthanized by exsanguination. The liver was removed, immersed in liquid nitrogen and immediately stored at -80°C for subsequent analysis.

**Glucose, insulin and fructosamine plasma levels.** Glucose, fructosamine and insulin levels were determined by the Kits Labtest (Lagoa Santa, MG, Brazil) and the Ultra-Sensitive Rat Insulin Elisa Kit (Crystal Chem, Downers Grove, IL), respectively.

### ROS production by neutrophils

**Isolation of neutrophils.** Blood was obtained by exsanguination of the brachial plexus and collected in heparinized tubes. Neutrophils were isolated using two different density gradients, Monopaque (d = 1.08) and Leucopaque (d = 1.12), in accordance with the procedures described by Bicalho *et al.*<sup>(21)</sup> with minor adjustments. The cell viability of each sample was greater than 95% as determined by the exclusion test with trypan blue.

**Chemiluminescence assay.** To measure ROS production, chemiluminescence assays were carried out as described by Chaves *et al.*<sup>(22)</sup> For each assay, 1 × 10<sup>6</sup> neutrophils were incubated in Hank's solution, pH 7.4, with 500 µL of luminol (10<sup>-4</sup> M). Photon emission was determined each minute for 30 min using a luminometer (Lumat, LB 9507, Berthold, Germany). The values were expressed as relative units of light/min (RLU/min).

**Antioxidant defenses and oxidative stress biomarkers in liver homogenate.** Catalase (CAT) activity was determined according to Aebi,<sup>(23)</sup> whose method is based on the enzymatic decomposition of H<sub>2</sub>O<sub>2</sub> observed spectrophotometrically at 240 nm for 5 min. Ten µL of homogenate supernatant was added to a cuvette containing 100 mM phosphate buffer (pH 7.2) and the reaction was initiated by the addition of 10 mM H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxide decomposition was calculated using the molar absorption coefficient 39.4 M<sup>-1</sup>cm<sup>-1</sup>. The results were expressed as activity per milligram of protein. One unit of CAT is equivalent to the hydrolysis of 1 µmol of H<sub>2</sub>O<sub>2</sub> per min.

The total glutathione content of liver homogenates was determined using a kit (CS0260) from Sigma (St. Louis, MO). This assay uses a kinetic method based on the reduction of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) to TNB (5-thio-2-nitrobenzoic acid), which can be determined spectrophotometrically at 412 nm.

The level of thiobarbituric acid reactive substances (TBARS) was estimated by the method of Buege and Aust.<sup>(24)</sup> Liver homogenate supernatants were mixed with TCA (28% w/v in 0.25 N HCl), TBA (1% in 0.25 M acetic acid) and BHT (125 mM in ethanol), heated for 15 min at 95°C and then placed in an ice bath. Precipitated material was removed by centrifugation, and the absorbance of the sample at 535 nm was determined. The TBARS level was calculated using the molar absorption coefficient of MDA (154,000 M<sup>-1</sup>cm<sup>-1</sup>).

Carbonyl protein levels were determined according to the method described by Levine.<sup>(25)</sup> Each sample was precipitated with 10% (w/v) TCA. After centrifugation, the precipitate was treated with 10 mmol of DNPH in 2 N HCl, incubated in the dark for 30 min and then treated with 10% TCA. After centrifuging, the precipitate was washed twice with ethanol/ethyl acetate (1:1) and dissolved in 6% SDS. Absorbance was determined at 370 nm. The results were expressed in nmol of DNPH incorporated/mg of protein. The content of DNPH incorporated was calculated using the molar absorption coefficient of DNPH (22,000 M<sup>-1</sup>cm<sup>-1</sup>). Total protein content was determined according to the method described by Lowry *et al.*,<sup>(26)</sup> using bovine serum albumin (BSA) as a standard.

**Real time quantitative RT-PCR assay.** Total RNA was isolated from liver tissue of rats using the RNeasy Total RNA Isolation System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. cDNA was synthesized from

2 µg of total RNA with random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), following the manufacturer's recommendations. Real-time PCR was performed with the *Power SYBR*<sup>®</sup> Green PCR Master Mix reagent (Applied Biosystems, Foster City, CA) in a final reaction volume of 12 µL; the reaction included 1 µL of cDNA and 0.5 µL of each primer (forward and reverse, 10 µM). The primers for PCR were designed according to the published nucleotide sequences<sup>(27)</sup> for CAT, glutathione peroxidase (GPx), gamma-glutamylcysteine synthetase (γ-GCS), Zn-superoxide dismutase (SOD), Mn-SOD and GAPDH. The reactions were carried out under the following conditions: 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (primer annealing and product extension). The specificity of the products obtained was confirmed by analysis of dissociation curves of the amplified product. The data obtained were analyzed using the comparative C<sub>T</sub> method. Target gene expression was determined relative to the expression of the endogenous GAPDH gene. All analyses were performed in triplicate.

**Statistical analysis.** Data were expressed as mean ± standard deviation (SD). Data were subjected to the Kolmogorov–Smirnov test for normality. The Student's *t* test was used for data with normal distributions. Differences were considered significant for *p*<0.05. All analyses were conducted using the software GraphPad Prism version 5.00 for Windows (San Diego, CA).

## Results

**Phytochemical composition and DPPH radical-scavenging activity.** Table 1 shows the total polyphenol and anthocyanin content of açai pulp. The ability of four different concentrations of açai pulp to neutralize the DPPH radical was determined. The ability of a given sample to reduce the absorbance of DPPH is indicative of its capacity to neutralize free radicals. All concentrations tested showed a high radical neutralization capacity, similar to that of the standard antioxidant Trolox in the range of 50 to 200 mg/L.

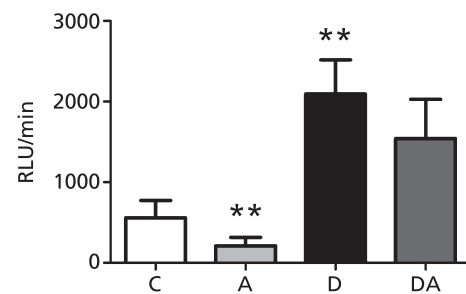
**Glucose profile, body weight and liver weight.** The results presented in Table 2 show that diabetic rats had reduced body weight, increased glucose and fructosamine levels and reduced insulin levels compared to control animals (Table 2). Glucose levels in diabetic rats were 4.4 times higher than in control animals, and insulin levels were 4.4 times lower. Dietary supplementation with 2% açai pulp did not affect the levels of glucose, insulin, fructosamine, or body weight in diabetic and control rats. There was no significant difference in liver weight in animals in the different groups.

**ROS production by neutrophils.** To investigate the antioxidant properties of açai pulp *in vivo*, ROS production was measured in neutrophils isolated from peripheral blood of experimental animals. Relative to neutrophils from control rats, neutrophils from diabetic rats showed a 3.7-fold increase in ROS production; however, ROS production in neutrophils of diabetic animals that received a diet supplemented with açai did not show

**Table 1.** Content of total phenolics and total anthocyanins and DPPH radical-scavenging activity of açai pulp

Compounds	Concentration
Total phenolic (mg GAE/100 g)	118.3 ± 0.96
Total anthocyanins (mg/100 g)	28.36 ± 0.69
DPPH radical scavenging activity	Inhibition %
Açai (v/v)	
1%	20.91 ± 0.72
2.5%	35.09 ± 0.54
5%	54.71 ± 0.32
10%	78.16 ± 0.94
Trolox (mg/L)	
50	19.57 ± 0.44
75	29.43 ± 0.11
100	38.04 ± 0.22
125	45.42 ± 0.55
150	55.36 ± 0.94
175	69.10 ± 1.09
200	78.34 ± 1.21

DPPH, 2,2-diphenyl-1-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid. Values are expressed as mean ± SD. All mean values are from triplicate determinations. Total phenolics are expressed in milligrams of gallic acid equivalents (GAE) per 100 g of açai pulp.



**Fig. 1.** Effects of açai supplementation on ROS production by neutrophils of control and diabetic rats. The data are expressed as mean ± SD (*n* = 8). C, control group; A, açai group; D, diabetic group; DA, diabetic + açai group. \*\*Significant at *p*<0.01 with respect to control group.

a significant difference from the control. Addition of açai pulp to the diet of the non-diabetic animals caused an approximately 2.6-fold reduction in ROS production by neutrophils relative to the control group (Fig. 1).

**Antioxidant defenses and biomarkers of oxidative stress.** To evaluate the effects of dietary supplementation with açai pulp on hepatic antioxidant defenses, total glutathione level and CAT activity were determined. Açai pulp supplementation increased

**Table 2.** Effects of açai pulp on glucose profile, body and liver weight in control and diabetic rats

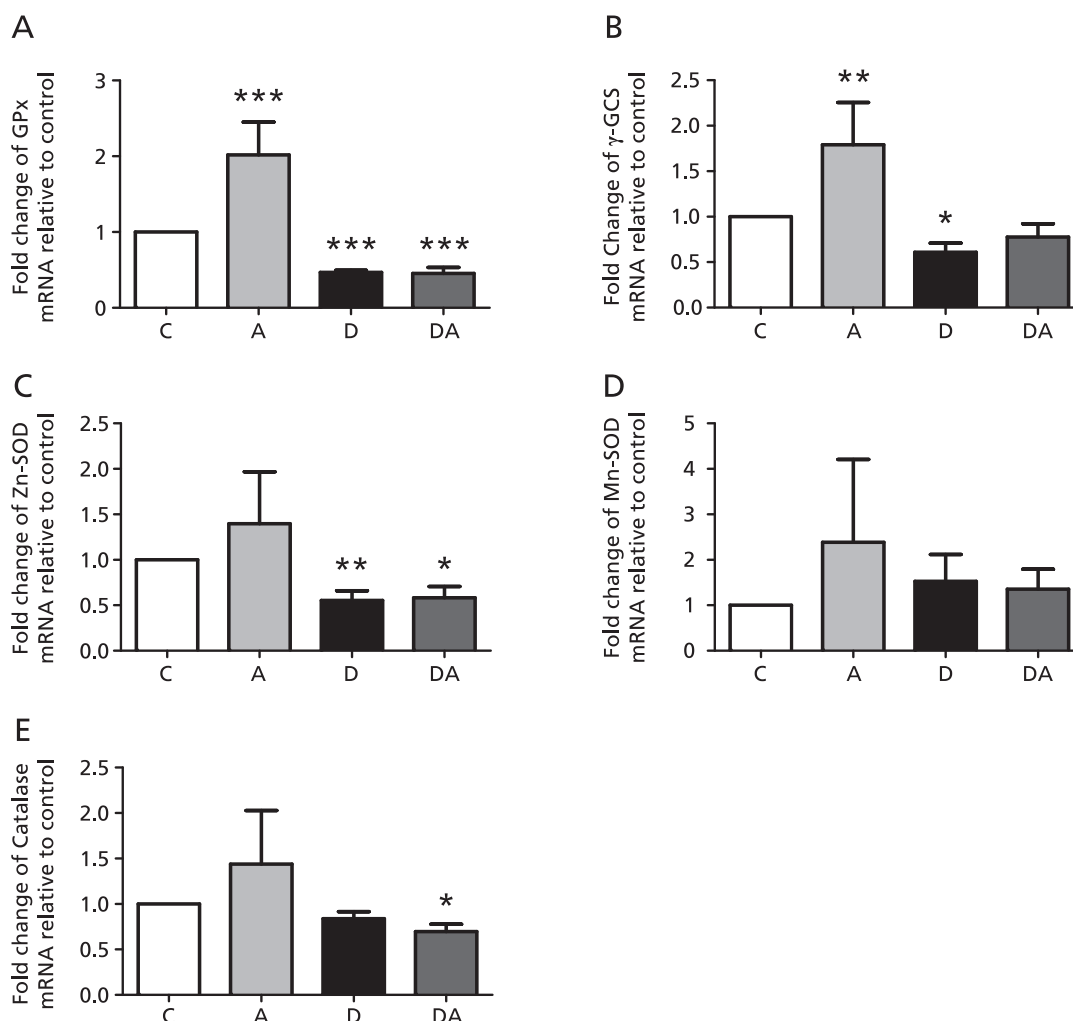
Variables	Experimental Groups			
	Control	Açai	Diabetic	Diabetic + Açai
Body weight (g)	199.24 ± 19.87	203.13 ± 25.33	130.00 ± 10.77***	126.25 ± 11.3***
Glucose (mmol/L)	5.36 ± 0.89	5.68 ± 0.94	23.52 ± 3.06***	24.67 ± 2.57***
Plasma insulin (pmol/L)	65.20 ± 45.80	88.21 ± 42.99	14.71 ± 4.31*	12.54 ± 7.49**
Fructosamine (µmol/L)	7.77 ± 1.38	8.35 ± 1.00	16.01 ± 2.81***	16.27 ± 2.39***
Liver weight (g)	4.83 ± 0.63	4.86 ± 0.75	4.94 ± 0.41	4.85 ± 0.41

Values are expressed as mean ± SD (*n* = 8). \*Significant at *p*<0.05 with respect to control group. \*\*Significant at *p*<0.01 with respect to control group. \*\*\* Significant at *p*<0.001 with respect to control group.

**Table 3.** Effects of açai pulp on antioxidant defenses and on biomarkers of oxidative stress in control and diabetic rats

Variables	Experimental Groups			
	Control	Açai	Diabetic	Diabetic + Açai
Catalase (U/mg protein)	102.62 ± 18.69	92.38 ± 10.06	91.66 ± 16.76	81.27 ± 20.02
Glutathione (nmol/mL)	31.82 ± 10.17	51.43 ± 8.08**	41.96 ± 16.27	54.21 ± 10.27**
Carbonyl protein (nmol/mg protein)	4.72 ± 1.98	2.75 ± 0.46*	9.79 ± 2.14**	8.31 ± 1.70*
TBARS (nmol/mg protein)	0.34 ± 0.03	0.38 ± 0.06	0.56 ± 0.18*	0.40 ± 0.07#

TBARS, thiobarbituric acid-reactive substances. Values are expressed as mean ± SD ( $n = 8$ ). \*Significant at  $p < 0.05$  with respect to control group. \*\*Significant at  $p < 0.01$  with respect to control group. #Significant at  $p < 0.05$  with respect to diabetic group.



**Fig. 2.** Effects of açai supplementation on mRNA expression of liver antioxidant enzymes in control and diabetic rats. (A) glutathione peroxidase, (B) gamma-glutamylcysteine synthetase, (C) Zn-superoxide dismutase, (D) Mn-superoxide dismutase, (E) Catalase. The data are expressed as mean ± SD ( $n = 6$ ). C, control group; A, açai group; D, diabetic group; DA, diabetic + açai group. \*Significant at  $p < 0.05$  with respect to control group, \*\*Significant at  $p < 0.01$  with respect to control group, \*\*\*Significant at  $p < 0.001$  with respect to control group.

the total hepatic glutathione content approximately 1.6- and 1.7-fold in control and diabetic rats, respectively. CAT activity did not show significant differences between groups.

TBARS levels and carbonyl protein content are widely used as biomarkers of lipid peroxidation and oxidative modification in proteins, respectively.<sup>(28)</sup> Compared to control animals, diabetic rats showed an increase of 1.6-fold in TBARS levels and 2.1-fold in carbonyl protein levels. Adding 2% açai pulp to the diet significantly reduced TBARS levels in diabetic rats to levels similar to those observed in the controls. Supplementation with açai also

reduced the levels of carbonyl protein 1.7-fold relative to the controls (Table 3).

**Real-time reverse-transcriptase-polymerase chain reaction.** To investigate the molecular mechanism of the antioxidant effects of açai pulp on the liver, expression of the genes for the antioxidant enzymes  $\gamma$ -GCS, GPx, CAT, Zn-SOD and Mn-SOD was evaluated by qRT-PCR. The results in Fig. 2 show that adding açai pulp to the diet significantly increased the expression of  $\gamma$ -GCS and GPx, approximately 1.8- and 2-fold, respectively, relative to the control. Diabetic rats showed decreases in  $\gamma$ -GCS,

GPx and Zn-SOD expression compared to control animals. The mRNA levels for  $\gamma$ -GCS in diabetic rats that received açai supplementation did not differ from those in controls, but CAT expression was significantly lower in these animals than in controls. The mRNA levels for Mn-SOD were not significantly different between groups (Fig. 2).

## Discussion

ROS are continually formed as by-products of aerobic metabolism; these compounds are known to carry out physiological functions as well as to have deleterious effects.<sup>(29)</sup> Under physiological conditions, ROS are effectively neutralized by enzymes of the antioxidant defense system and glutathione (GSH)-related enzymes, including SOD, CAT, GPx and  $\gamma$ -GCS.

Overproduction of ROS results in oxidative stress, which can cause significant damage to cellular proteins, lipids and DNA. Oxidative stress has been associated with the development and progression of complications of diabetes.<sup>(13)</sup> Therefore, this study aimed to investigate the effects of açai supplementation on ROS production in neutrophils and on oxidant/antioxidant balance and gene expression of hepatic antioxidant defense system enzymes in control and diabetic rats.

The liver is the principal organ involved in oxidative and detoxification processes. In the initial stages of many diseases, oxidative stress biomarkers are elevated in the liver.<sup>(30)</sup> Experimental data show that the liver is subject to damage mediated by ROS in diabetes.<sup>(31,32)</sup> The results presented here show that an increase in hepatic oxidative stress in diabetic rats led to a reduction in the expression of mRNAs coding for the antioxidant enzymes Zn-SOD, GPx and  $\gamma$ -GCS. Because changes in the hepatic oxidant/antioxidant balance can affect the translocation of transcription factors sensitive to the redox state to the nucleus, the decrease in the mRNA levels of antioxidant enzymes that occurs in diabetes may be due to the oxidation of transcription factors that are responsible for initiating the transcription of antioxidant enzymes.<sup>(33)</sup>

SOD converts the superoxide anion into hydrogen peroxide, which is neutralized in the presence of water and molecular oxygen by the activity of CAT and GPx. The isoforms of SOD are located in different cell compartments. CuZn-SOD is found in the cytosol and in the nucleus, while Mn-SOD is the mitochondrial isoform. Our data corroborate recent studies that show decreased Zn-SOD mRNA and protein expression in the livers of diabetic rats,<sup>(33)</sup> mRNA expression for Mn-SOD did not change under the same conditions.<sup>(34)</sup> These results suggest that CuZn-SOD is more sensitive than Mn-SOD to oxidative stress caused by diabetes.

Several studies have shown decreased GPx activity in hepatic tissue of diabetic rats.<sup>(31,32,35)</sup> Nevertheless, the reported effects of oxidative stress caused by diabetes on the gene expression of GPx vary considerably. Matsunami *et al.*<sup>(36)</sup> found an increase in GPx mRNA expression in livers of diabetic rats. Another study reported no differences in the hepatic levels of mRNA for GPx in diabetic rats compared to controls.<sup>(34)</sup> These variations may be explained by differences in the experimental conditions, such as time since onset of diabetes and age of the experimental animals.

Açai supplementation was not effective in reverse these changes on gene expression in liver tissue of diabetic rats. Similarly, Sadi *et al.*<sup>(33)</sup> and Sadi and Guray<sup>(34)</sup> evaluated the effects of supplementation with the antioxidants vitamin C and alpha lipoic acid on gene expression of antioxidant enzymes in the STZ-induced diabetic rat liver tissues and also found no differences in both mRNA and protein expressions of Zn-SOD and GPx between diabetic control group and the diabetic supplemented groups.

The cellular redox environment is influenced by the production and removal of ROS.<sup>(37)</sup> The increase in ROS seen in diabetes can affect cellular signaling pathways and gene expression. Thus, even if exogenous antioxidants may provide benefit in attenuating

oxidative stress, it becomes difficult to provide antioxidant in concentrations sufficient to completely restore physiologic redox status, since these cannot be regenerated enzymatically as glutathione. In this way, since patterns of gene expression have been altered by oxidative stress in diabetes, may not be possible to reverse this process and restore normal patterns of gene expression.<sup>(38)</sup> This may be the cause for which açai supplementation did not have a substantial effect on liver antioxidant enzymes gene expression in diabetic rats, and however, led to changes in the mRNA levels of antioxidant enzymes in control rats.

Polyphenols are the main phytochemical components found in açai pulp, most notably flavonoids and anthocyanins. The predominant anthocyanins in açai are cyanidin-3-glucoside and cyanidin-3-rutenoside.<sup>(3,4,39,40)</sup> Others anthocyanins are found in minor amounts as cyanidin-3-sambubioside, peonidin-3-glucoside and peonidin-3-rutenoside. Major non-anthocyanin polyphenolic components found in açai include flavonoids such as homorientin, orientin, isovitexin, quercetin and procyanidins, phenolic acids and lignans.<sup>(2,4,40,41)</sup>

Many dietary polyphenols have antioxidant activity, and this activity is generally attributed to their ability to directly neutralize pro-oxidant reactive species. Experimental data indicate that polyphenols can offer indirect protection against oxidative stress through the activation of gene transcription for enzymes that make up the endogenous antioxidant defense system.<sup>(42)</sup> However, the effect of açai consumption on the expression of mRNAs coding for antioxidant enzymes has not been investigated. This study demonstrated the induction of gene expression of the hepatic antioxidant enzymes  $\gamma$ -GCS and GPx by dietary supplementation with açai pulp. Our results suggest that açai plays a role in the up-regulation of the endogenous antioxidant defense system and has a potential protective effect against hepatic oxidative stress *in vivo*. In this regard, many reports show that modulation of antioxidant enzymes by flavonoids such as procyanidins and quercetin may be also important in their antioxidant effects in liver cells.<sup>(43,44)</sup>

The induction of expression of the antioxidant defense system enzymes by polyphenols obtained from the diet mainly results from activation mediated by the transcription factor Nrf2 through interaction with the antioxidant response element (ARE), which is found in the promoter region of many genes that are induced by changes in the redox state.<sup>(42)</sup>

*In vivo* experiments demonstrated the induction of mRNA expression of enzymes CuZnSOD, GPx and CAT, accompanied by an increase in Nrf2 protein levels in heart tissue of control rats following oral administration of phenolic acids for 14 days.<sup>(45)</sup>

Moreover, recent studies have reinforced the role of activation of the antioxidant response element by quercetin and other flavonoids in stimulating  $\gamma$ -GCS gene transcription in COS-1 and HepG2 cells.<sup>(46)</sup> This enzyme catalyzes the rate-limiting step in the synthesis of glutathione, the most prevalent endogenous cellular antioxidant.

Glutathione is an intracellular reducing agent that plays a central role in antioxidant defense by detoxifying ROS directly or by a mechanism catalyzed by GPx. Several studies have demonstrated induction of liver GSH by phytochemical agents.<sup>(47,48)</sup> Our results show that açai supplementation increases total hepatic glutathione levels in control and diabetic rats. The high glutathione content found in the liver after açai supplementation may reflect an increase in the antioxidant status of this tissue. Roig *et al.*<sup>(49)</sup> have demonstrated that the main detoxification pathway for lipid peroxidation products involves glutathione conjugation. Consequently, the increase in hepatic glutathione levels may have significantly contributed to the reduction of TBARS levels in diabetic rats receiving diets supplemented with açai. Açai supplementation also decreased hepatic carbonyl protein levels in control rats; this reduction may be associated with increased hepatic antioxidant status in these animals, evidenced by the induction of antioxidant enzyme expression in this organ.

Although diabetes increases cellular ROS levels by a variety of mechanisms, an important source of increased ROS in diabetes is the NADPH oxidase of neutrophils.<sup>(50)</sup> Activation of neutrophils leads to the production of ROS through oxidative metabolism; this involves activation of the NADPH oxidase enzyme complex, which catalyzes the reduction of oxygen to superoxide ion. It has been shown that the hyperglycemia associated with diabetes mellitus results in the activation of neutrophils<sup>(51)</sup> and that this activation contributes to an increase in oxidative stress that is partly responsible for diabetes complications. Our results corroborate this hypothesis in that neutrophils from diabetic rats showed a significant increase in the production of ROS, while neutrophils from diabetic rats supplemented with açai did not differ from controls in ROS production. Açai supplementation also reduced ROS production by neutrophils of control rats. Recent *in vitro* studies have demonstrated an inhibitory effect of açai on ROS production by neutrophils isolated from healthy humans.<sup>(2,4,52)</sup> Here, we show for the first time that açai supplementation significantly reduces ROS production in neutrophils *in vivo*. These data indicate a possible modulating effect of açai on ROS production in neutrophils.

In this study, the intraperitoneal administration of STZ effectively induced diabetes mellitus in rats. The STZ-induced diabetes experimental model displays most of the complications of diabetes that are mediated by oxidative stress.<sup>(13)</sup> As expected, diabetic rats showed significant increases in plasma glucose, reduction in insulin levels and increased levels of fructosamine, a biomarker used to determine the degree of protein glycosylation in diabetes. Diabetes induced by STZ also led to a sharp reduction in body weight. The weight loss typically associated with diabetes is due to increased muscle catabolism.<sup>(48)</sup> Although açai supplementation did not promote significant changes in the glucose profiles of diabetic rats, the results obtained in relation to oxidant/antioxidant balance indicate that açai consumption may play a protective role against diabetes complications associated with oxidative stress.

In conclusion, this study demonstrates that dietary supplementation with açai pulp not only acts as an antioxidant but also can modulate ROS production by neutrophils and improve the liver oxidant/antioxidant balance by the induction of mRNA expression of antioxidant enzymes under physiologic condition of oxidative stress. However, antioxidant properties of açai pulp *in vivo* appear to involve different mechanisms for the observed effects in different physiologic and pathologic condition of oxidative stress. Such differences may be due to changes in cellular signaling pathways and gene expression patterns related to diabetes.

Due to the complexity of the antioxidant defense system and involvement of multiple pathways in the increase ROS formation

in diabetes, may be particularly difficult a dietary antioxidant reverse the adverse effects of oxidative stress. Although it may not be possible to completely reverse diabetic complications, açai attenuated oxidative stress in the liver through the reduction of TBARS and increased glutathione content.

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## Abbreviations

A	açai group
ARE	antioxidant response element
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
C	control group
C <sub>T</sub>	threshold cycle
COX	cyclooxygenase
D	diabetic group
DA	diabetic + açai group
DNPH	2,4-dinitrophenylhydrazine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5-dithiobis(2-nitrobenzoic acid)
DXR	doxorubicin
GAE	gallic acid equivalent
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSH	reduced glutathione
GPx	glutathione peroxidase
MDA	malondialdehyde
Nrf2	NF-E2-related factor-2
qPCR	quantitative reverse transcription polymerase chain reaction
ROS	reactive oxygen species
STZ	streptozotocin
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
γ-GCS	gamma-glutamylcysteine synthetase

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