



## Trabajo Original

### Neuroprotective effect of Sapucaia nuts (*Lecythis pisonis*) on rats fed with high-fat diet *Efecto neuroprotector de la castaña de sapucaia (Lecythis pisonis) en ratones sometidos a la dieta de cafetería*

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#### Abstract

**Background:** *Lecythis pisonis* Cambess is commonly known as "castanha de sapucaia" in Brazil. Chemical composition studies revealed that this nut is an excellent source of anti-oxidant minerals and of essential lipids.

**Objective:** The aim of the present study is to assess the anti-oxidant and anti-inflammatory effect of *Lecythis pisonis* Cambess on the brain tissue of Wistar rats.

**Material and methods:** The animals were divided in four experimental groups (n = 6), total of forty-eight rats. Treatments included the standard diet (AIN-93G) and high-fat food, supplemented with *Sapucaia* nut from 14 to 28 days. The gene expression markers TNF- $\alpha$ , NFkB, ZnSOD and HSP-72 were defined through reverse transcriptase polymerase chain reaction (rtPCR). The anti-oxidant effect was assessed through the thiobarbituric acid-reactive substances (TBARS) and the measurement of the activity performed by superoxide dismutase enzymes.

**Results:** Accordingly, the gene expression of the inflammatory markers NFkB (p65) and TNF- $\alpha$  was lower in rats fed on diets supplemented with "sapucaia", and they presented significant difference in the Tukey test (p < 0.05). The heat-shock HSP-72 protein and the ZnSOD enzyme raised the gene expression and showed significant statistical difference (p < 0.05) in both groups fed on Sapucaia nut-based diet.

**Conclusion:** Thus, the nutritional properties of the Sapucaia nuts perform important neuroprotective activities because they modulated the anti-oxidant activity and the brain tissue inflammatory process in the assessed animals.

#### Key words:

Brain tissue. Oxidative stress. Inflammation. Gene expression.

#### Resumen

**Introducción:** la *Lecythis pisonis* Cambess es conocida popularmente en Brasil como "castaña de sapucaia". Estudios de su composición química revelaron que esta castaña es una excelente fuente de minerales antioxidantes y de lípidos esenciales.

**Objetivo:** evaluar los efectos antioxidantes y anti inflamatorios en el tejido cerebral de ratones Wistar.

**Material y métodos:** los animales fueron distribuidos aleatoriamente en cuatro grupos experimentales (n = 6) totalizando 48 ratones. Los tratamientos fueron conducidos por un periodo de 14 a 28 días con dietas estándar AIN-93G y de cafetería con castaña de sapucaia. La expresión génica de los marcadores TNF- $\alpha$ , NFkB, ZnSOD y HSP-72 fue determinada por la reacción en cadena de la polimerasa cuantitativa tras transcripción inversa (qPCR). La actividad antioxidante también fue verificada por la determinación de las especies reactivas del ácido tiobarbitúrico (TBARS) y por mensuración de la actividad de la enzima superóxido dismutasa.

**Resultados:** la expresión génica de los marcadores inflamatorios NFkB (p65) y TNF- $\alpha$  fue menor para los grupos de ratones que consumieron las dietas enriquecidas con sapucaia con diferencia significativa por el test de Tukey (p < 0,05). La proteína de choque térmico HSP-72 y la enzima ZnSOD presentaron aumento de la expresión génica con diferencia estadística significativa (p < 0,05) para ambos grupos que consumieron sapucaia en sus dietas.

**Conclusión:** las propiedades nutricionales de la castaña de sapucaia ejercieron importante actividad neuroprotectora por modular la actividad antioxidante y el proceso inflamatorio en los tejidos cerebrales de los animales evaluados.

#### Palabras clave:

Tejido cerebral. Estrés oxidativo. Inflamación. Expresión génica.

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## INTRODUCTION

Diets based on high saturated fat levels may lead to redox disequilibrium in the body since the lipid-oxidation metabolism is a strong generator of reactive oxygen species (ROS) (1). The brain is particularly susceptible to the deleterious ROS effects due to its high metabolic rate and to its low cell regeneration capacity in comparison to that of other organs (2,3).

The ROS perform intracellular signaling within the immune system, since the phagocytic cells are activated under oxidative stress conditions. The redox disequilibrium acts directly in the Transcription Factor NF- $\kappa$ B (NF $\kappa$ B) by increasing the inflammatory process mediators (4).

The accumulation of damages caused by redox disequilibrium may lead to degenerative diseases as well as to mitochondrial dysfunction and apoptosis (5). However, the human body has antioxidant systems that seek to preserve cell integrity and neutralize these harmful effects. These systems work in synchrony in order to protect the cells against oxidative molecules (6).

As a further defense system, the body increases the activity of the HSP 70 protein family and produces an anti-inflammatory effect under metabolic stress conditions, since these proteins act in the NF $\kappa$ B regulation point and, consequently, inhibit the expression of inflammatory markers such as TNF- $\alpha$  and IL-1 $\beta$  (7).

The literature has shown significant results related to the ingestion of nuts and its beneficial effects on human health due to its nutritional components (6,8). Overall, oilseeds are rich in unsaturated fatty acids, in sulfur-containing amino acids, and in minerals such as selenium, magnesium, manganese, zinc, iron and copper. They also contain satisfactory concentrations of vitamin C and E, which together may change specific processes related to the regulation of cellular differentiation, to DNA protection, and to the regulation of inflammatory responses and of those associated with oxidative stress (6,9,10-12).

The Brazilian biome has several species that need to be subjected to further scientific studies. Brazil or Pará nuts and cashew nuts are the Brazilian chestnuts most consumed by the population and they are both rich in minerals and in essential lipids. Although the sapucaia chestnut is not well known, it is rich in nutrients and it is mainly composed of essential lipids and of important minerals such as zinc, iron, copper, manganese, magnesium and selenium.

In light of the foregoing, it is worth exploring foods able to minimize or prevent diseases related to the interaction between the oxidative stress and the molecular mechanisms involved in neurodegeneration. Thus, the aim of the present study is to assess the antioxidant and anti-inflammatory action capacity of Sapucaia nut in the brain tissue of rats.

## MATERIALS AND METHODS

### PLANT MATERIAL

Sapucaia nuts were collected from five naturally occurring trees at the campus of the Federal University of Viçosa, which is located

(20°76' S and 42°86') in the region of Zona da Mata Mineira, in Southeastern Minas Gerais State. Five fruits were collected from each tree and ten chestnuts were extracted from each fruit. The study of the chestnut chemical composition was conducted in our laboratory (13).

### ANIMALS AND DIETS

The current study was approved by the Ethics Committee on Animal Use at the Federal University of Viçosa (CEUA-UFV) (protocol 77/2014). The experiment was conducted in accordance with the standards established by Law 11794 and the Normative Resolutions issued by CONCEA/MCTI.

The biological assay comprised 48 male albino Wistar rats of the species *Rattus norvegicus*. The newly weaned animals came from the Central Animal Laboratory of the Biological Sciences Center (CCB - Centro de Ciências Biológicas) at the Federal University of Viçosa. The animals were randomized in four groups of six animals. They were kept in a controlled environment, with temperature ranging from 22 to 25 °C, and light-dark cycles of 12 hours per day. They were given *ad libitum* food and water for 14 and 28 days according to the group the animal was inserted in.

A consumption pattern of three sapucaia nuts per day has been established based upon studies according to which a nut portion per day can have beneficial effects on the body. Calculations were carried out to simulate this intake pattern in rats. They were performed based on the nutritional information of sapucaia nuts obtained in a study conducted by our research group (13). One male individual with body weight 70 kg receiving 2,000 kcal per day was taken as reference. According to the information about the chemical composition of sapucaia, three nuts equals 93.08 kcal, corresponding to 4.65% of the 2,000 daily kcal. Therefore, the daily consumption of 15 g of sapucaia nuts represents 4.65% of the total energy value of a diet.

Four treatments were conducted for 28 days, namely: Standard diet (AIN-93G) (14) with caloric density of 3.95 kcal/g; standard diet added with sapucaia nut (AIN-93G + SAP) at the ratio of 24 g of nuts per 1,000 grams of diet (4.65% of total caloric value of the diet), keeping the same caloric density of the AIN-93G (3.95 kcal/g); high-fat diet (HFD) consisting of ham pâté, bacon, bologna sausage, cornstarch cookie, chocolate powder, potato sticks, whole milk powder, and commercial feed at the ratio of 2: 1: 1: 1: 1: 1: 1, with caloric density of 6.92 kcal/g; high-fat diet added with sapucaia nuts (HFD + SAP), at the ration of 52.2 g of nuts per 1,000 grams of diet (4.65% of the total caloric value of the diet), keeping the same caloric density of the High-fat diet (6.92 kcal/g). The rats had *ad libitum* access to the diets and to water. The diets were weekly prepared and kept refrigerated at 4 °C to prevent oxidation. At the end of each experiment period (14 and 28 days) and after fasting for 12 hours, the animals were euthanized under anesthesia with ketamine (25 mg/kg IM) and xylazine (2 mg/kg IM). The brain tissues were collected, transported in a container with liquid nitrogen and stored in ultrafreezer (-80 °C).

## GENE EXPRESSION

With respect to the total mRNA extraction, Trizol reagent (Invitrogen, CA, USA) was used in 100 mg of brain tissue previously macerated in liquid nitrogen in order to obtain its homogenization, according to the manufacturer's recommendations. Concentration and purity were assessed by Multiskan Go spectrophotometer (Thermo Scientific, DE, USA) and the integrity of the mRNAs was assessed by agarose gel electrophoresis. The recovered mRNA was treated with RNase-free Dnase (Promega). The M-MLV Reverse Transcriptase kit (Invitrogen, CA, USA) was used in the cDNA synthesis, according to the manufacturer's protocol. The cDNA was used to determine the expression of the mRNA of the TNF- $\alpha$ , NF $\kappa$ B (p65), ZnSOD, HSP-72 markers, and the GAPDH gene was used as reference.

The quantitative real-time polymerase chain reaction (qPCR) technique was used with the Sybr Green 2X Master Mix reagent (Applied Biosystems, CA, USA) for relative quantification. The final volume of each reaction was 10  $\mu$ L, wherein: 2  $\mu$ L of cDNA; 0.8  $\mu$ L of oligonucleotides mixture at 2.5  $\mu$ M (sense and antisense), 5.0  $\mu$ L of Sybr Green 2X Master Mix reagent, and 2.2  $\mu$ L of ultra-pure water for each gene. The herein used qPCR protocol consisted of 15 min at 95  $^{\circ}$ C, then 40 cycles at 95  $^{\circ}$ C (15 s), 60 $^{\circ}$ C (30 seconds) and 72  $^{\circ}$ C (30 s), which were followed by melting curve analysis. The samples were analyzed in four biological and two technical replicates quantified in independent runs.

Negative controls (NTC) were held in two technical replicates replacing the cDNA samples by the same volume of water in the reaction. The "AB Step One Real Time PCR System" (Applied Biosystems) equipment was used to run the experiment. The relative quantification of the gene expression was performed according to the  $2^{-\Delta\Delta CT}$  method suggested by Livak (15).

The pairs of oligonucleotides used to amplify the genes of interest were: NF $\kappa$ B (p65) (Fw 5'-CTTCTGGGCCATATGTGGAGA-3') and (Rw 5'-TCGCACTTGTAAACGGAACG-3'), TNF- $\alpha$  (Fw 5'-GCCGATTGCCATTTACATACC-3') and (Rw 5'-GGACTCCGTGATGTCTAAGTAG-3'), HSP 72 (Fw 5'-AGGCCAACAGATCACCATC-3') and (Rw 5'-TAGGACTCGAGCGCATTCTT-3'), ZnSOD (Fw 5'-GAGCAGAA-GGCAAGCGGTGAA-3') and (Rw 5'-CCACATTGCCAGGTCTC-3'), GAPDH (Fw 5'-GGTTGCTCCTGTCCTC-3') and 5' CTGTTGCTGTAGCCATATTC -3'). The oligonucleotides were designed based on the gene sequences of the Wistar *Rattus norvegicus* found in the GenBank, using the Primer 3 plus software. The experiment followed the MIQE guidelines established for studies that use the qPCR technique in real time (16).

## DETERMINING THE LIPID PEROXIDATION AND THE ENZYME ACTIVITY OF THE BRAIN SUPEROXIDE DISMUTASE

The lipid peroxidation analysis was determined through the formation of malondialdehyde (MDA), which is a by-product of the oxidation of fatty acids, by testing thiobarbituric acid reactive substances (TBARS). A 200 mg sample was collected from each brain, and it was then macerated and homogenized under cooling

in Tris-HCl 0.01 M buffer, pH 7.4, at the ratio of 5 mL buffer per 500 mg tissue, and centrifuged at 10,000 g, for 15 minutes, at 4  $^{\circ}$ C. The supernatants were used to determine the total protein content, according to the method by Bradford (17), as well as the enzymatic antioxidant activity of the superoxide dismutase (SOD) and the lipid peroxidation.

The SOD antioxidant activity was quantified as relative units, wherein one SOD unit was defined as the amount of enzymes that inhibit by 50% the pyrogallol oxidation rate. We used 20  $\mu$ L of brain tissue homogenate, 8  $\mu$ L of 24  $\mu$ mol/L pyrogallol and 4  $\mu$ L of 30  $\mu$ mol/L catalase in the reaction medium, and added 200  $\mu$ L Tris-HCl-EDTA 0.01 M buffer (pH 7.4). The absorbance reading was taken at 420 nm and the results were expressed as U of SOD/mg of protein (18).

In order to measure the lipid peroxidation, a reaction containing 400  $\mu$ L of brain tissue homogenate, 1 ml of 20% trichloroacetic acid, and 400  $\mu$ L of 1.6% thiobarbituric acid incubated at 95  $^{\circ}$ C for 60 minutes was performed. Then, it was added with 1.6 mL n-butanol and centrifuged at 3000 rpm/10 min. The reading was performed at 510, 532, and 560 nm. The calculation of the final values used the following equation suggested by Pyles (19) in order to minimize the interference of heme pigments and that of hemoglobin in the MDA dosage:

$$MDA_{532} = 1.22[A_{532}] - (0.56) (A_{510}) + (0.44) (A_{560})$$

The molar absorptivity coefficient  $E_0 = 1.56 \times 10^5 \text{ mol L}^{-1} \text{ cm}^{-1}$  was used to measure the malondialdehyde concentration, and the results were expressed as nmol of MDA per protein milligrams (20).

## STATYSTICAL ANALYSES

The Kolmogorov-Smirnov test was performed and the nonparametric Kruskal-Wallis test was used to compare multiple variances. The Wilcoxon test was used to compare two independent groups whenever the distributions showed no normality. ANOVA variance analysis was performed whenever the variables showed normal distribution and it was followed by Tukey test (Sigma Plot 11.0), which was applied to all treatments. The sample size calculation was performed with the Sample Size Determination in Health Studies11 software (80% effect and  $p < 0.05$ ) and the 48 animals (100 g average weight) were randomized into four groups ( $n = 6$ ). The significance level was 95% with  $p < 0.05$ . The results were expressed as mean  $\pm$  standard deviation.

## RESULTS

### ASSESSING THE LIPID PEROXIDATION AND THE ANTIOXIDANT ACTIVITY OF THE SUPEROXIDE DISMUTASE

The lipid peroxidation (MDA) and the antioxidant enzyme activity expressed in SOD units were measured in order to assess

the protective effect of the chestnut inclusion in the herein used diets. All the groups who received sapucaia nut showed lower concentrations of nmol MDA/mg of PTN regardless of the diet and the evaluated treatment time (Table I).

The diet administration time (14 and 28 days) influenced the lowest concentration of nmol MDA/mg of brain PTN in the animals ingesting sapucaia nuts, regardless of the diet type. The animals subjected to high-fat diet alone showed higher MDA concentrations.

The antioxidant enzyme activity expressed in SOD units was higher and statistically different in the animals that received high-fat diet added with sapucaia nut and the dietary management time did not affect the enzyme activity, which remained stable between 14 and 28 days of experiment.

### THE EXPRESSION OF THE GENES INVOLVED IN THE ANTI-INFLAMMATORY AND ANTIOXIDANT RESPONSE OF THE BRAIN TISSUE

In order to evaluate the impact of inserting sapucaia nuts in the diets, we used the RTq-PCR technique to assess the relative gene expression of the mRNAs in comparison to that of the control groups of markers involved in the inflammatory process and in the antioxidant activity of the brain (Fig. 1). The NFkB (p65) gene expression was lower in the groups of rats that consumed the sapucaia-enriched diets. The time influenced the lower expression of this marker in the group fed with HFD + SAP.

The TNF- $\alpha$  gene expression was lower among rats fed with sapucaia-enriched diets. Both the AIN-93G+SAP and the HFD+SAP showed no statistically significant difference in the TNF- $\alpha$  gene expression in the different treatment times.

According to the assessment of the HSP-72 gene expression, the group fed with HFD + SAP showed higher and different values according to the diet administration times. The 28<sup>th</sup> day showed the highest expression of this protein.

ZnSOD showed increased gene expression in both SAP groups and the SD + SAP group showed greater values according to the diet administration time. However, the time did not affect the increased expression of this gene in the HFD + SAP group.

### DISCUSSION

Studies support the hypothesis that sugar-and-lipid-rich diets as well as the oxidative DNA damage may contribute to the development of neurodegenerative diseases (11,12). The current study found increased oxidative stress in the brain tissue of rats. It was evidenced by the increased malondialdehyde (MDA) concentrations and by the decreased superoxide dismutase (SOD) concentration in the brain tissue of rats fed with high-fat diets.

The final lipid peroxidation product has high toxicity and generates inhibitory action on the protection enzymes, thus affecting the antioxidant defense mechanism (1,21). Therefore, pro-oxidants play a significant role in the pathogenesis of inflammatory processes. There are many mediators that initiate and amplify the inflammatory response such as the pro-inflammatory cytokines, the tumor necrosis factor (TNF- $\alpha$ ), and the macrophages (21,22).

There are no published studies evaluating antioxidant and anti-inflammatory activities using sapucaia nuts. However, the results of studies that used walnuts and other chestnuts showed some similarities to our study.

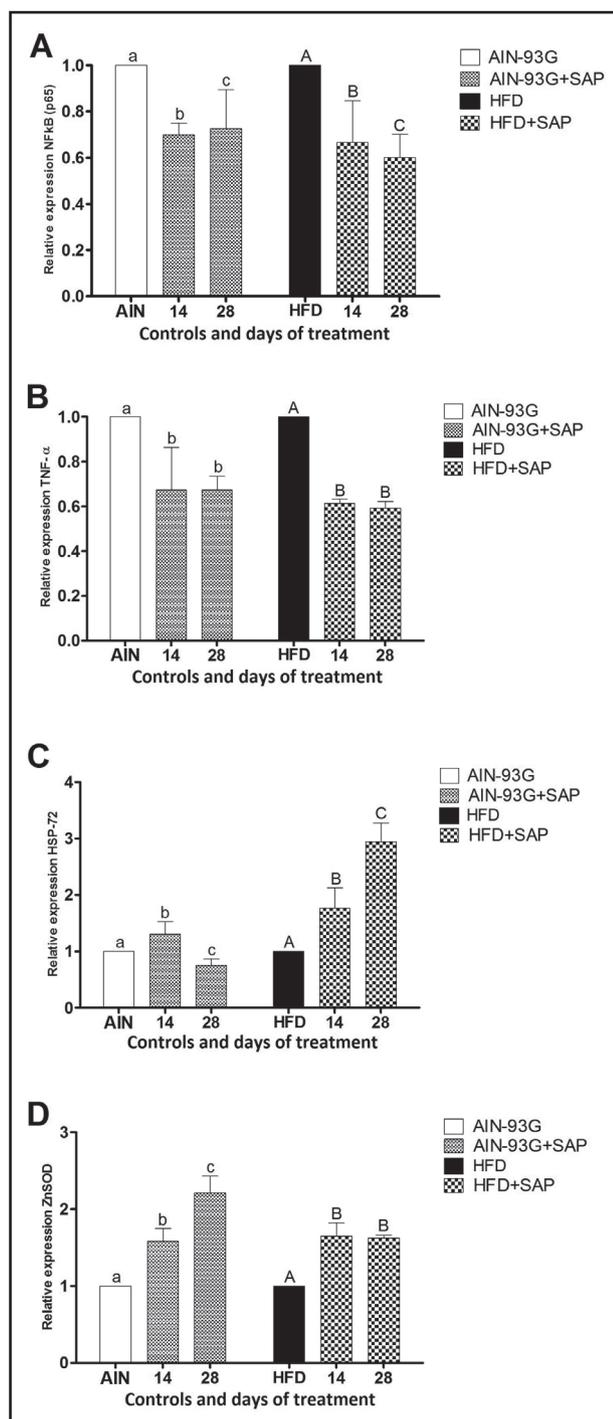
The results showed that the *in vivo* antioxidant activity of sapucaia nuts promoted protective effect against the attack of free radicals. The assessment of the effects of HFD + SAP intake for a period of 14 and 28 days found interference in the MDA concentration reduction and in the increase in the superoxide dismutase enzyme, and it minimized the lipid peroxidation effects on the brain of the animals (Table I). The diet administration time showed that the brain tissue showed increased superoxide dismutase activity and, therefore, lower ROS concentration after only two weeks of treatment with sapucaia.

These effects may be attributed to the metabolic interaction of unsaturated fatty acids, magnesium, manganese, selenium, copper, iron and zinc -already identified by studies about the chemical composition of sapucaia- and the possible phenolic composites and tocopherol that may be found in this nut (13,23-25). Studies performed with walnuts also found antioxidant activities, which have been positively correlated with the bioactive compounds in the walnuts, namely: minerals, tocopherol, fibers and phytochemicals; which could work as protective agents in inflammatory and oxidative stress processes (26-28).

**Table I.** Mean brain concentrations of malondialdehyde (MDA) (nmol MDA / mg of PTN) and of superoxide dismutase (SOD) (U SOD / mg of PTN) at 14 and 28 days of treatment for each diet (AIN-93G, AIN-93G + SAP, HFD, HFD + SAP) administered to the animals

Groups	MDA		SOD	
	14 days	28 days	14 days	28 days
AIN-93G	0.84 ± 0.02 <sup>aA</sup>	0.89 ± 0.04 <sup>aA</sup>	6.07 ± 0.30 <sup>aA</sup>	8.56 ± 0.33 <sup>bA</sup>
AIN-93G+SAP	0.67 ± 0.02 <sup>aB</sup>	0.60 ± 0.01 <sup>bB</sup>	9.56 ± 0.57 <sup>aB</sup>	11.91 ± 0.37 <sup>bB</sup>
HFD	1.01 ± 0.03 <sup>aC</sup>	1.21 ± 0.02 <sup>bC</sup>	4.14 ± 0.51 <sup>aC</sup>	7.01 ± 0.34 <sup>bC</sup>
HFD+SAP	0.67 ± 0.02 <sup>aB</sup>	0.70 ± 0.01 <sup>bD</sup>	12.17 ± 0.29 <sup>aD</sup>	11.13 ± 0.51 <sup>aB</sup>

Means (n = 6) followed by different lowercase letters in the same line and by capital letters in the same column show statistically significant difference according to the Tukey test (p < 0.05). AIN-93G (standard diet), AIN-93G + SAP (standard diet + sapucaia), HFD (high-fat diet), HFD + SAP (high-fat diet + sapucaia), PTN (proteins).



**Figure 1.**

Averages of the mRNA relative gene expression (calibrator sample,  $y = 1$ ) of NfκB (p65) (A), TNF- $\alpha$  (B), HSP-72 (C) and ZnSOD (D) by qPCR for the animals fed with AIN-93G + SAP for 14 and 28 days. Means followed by different letters (lowercase for AIN-93G and capital to HFD) are significantly according to Tukey's test ( $p < 0.05$ ).

The inflammatory process and the oxidative stress may be controlled by endogenous defense mechanisms. The superoxide

dismutase is described as the first defense line of the body to combat reactive oxygen species (ROS), since it performs the dismutation of superoxide anions produced by mitochondria under stressful situations (29).

The results of the ZnSOD gene expression showed its significant increase, which was observed in all the groups fed with nut-enriched diets. It allows inferring that the nut was effective in contributing to the antioxidant response, since the gene expression of this enzyme was higher in HFD + SAP than in HFD. It was also higher among animals subjected to the AIN-93G + SAP in comparison to the control group. The brain tissue is quite susceptible to the deleterious effects of ROS due to its high metabolic rate in comparison to other tissues. Thus, the presence of nuts in the diet has shown to be very effective in increasing the gene expression and the SOD activity (Fig. 1).

The oxidative stress caused by the high-fat diet tends to increase the gene expression of inflammatory process mediators such as TNF- $\alpha$  and NfκB (p65). It also affects other molecular components involved in it, such as the heat shock protein (30,31). The results showed that the rats treated with sapucaia-enriched diets presented significantly higher levels of HSP-72 expression than those fed with control diets alone (HFD and AIN-93G). It indicates that sapucaia may have played an important role in the modulation of this gene response.

The HSP-72 heat shock protein is part of the HSP-70 family. These proteins are able to inhibit intracellular signaling pathways such as the NfκB (p65) and to generate low production of pro-inflammatory cytokines in response to this inhibition (32). The results of the current study showed lower expression of the NfκB (p65) transcription factor and this fact may attributed to the higher HSP-72 expression.

As these factors are interconnected, the NfκB (p65) inhibition caused by the HSP-70 family in the group of rats treated with HFD + SAP also caused low TNF- $\alpha$  gene expression. The TNF- $\alpha$  gene expression was lower among the animals treated with HFD + SAP and with AIN-93G + SAP in comparison to those treated with HFD and with AIN-93G, respectively.

In short, the increased HSP-72 expression observed in the groups fed with nut-enriched diets interfered with the NfκB (p65) cascade activation, which, in turn, significantly contributed to reduce the TNF- $\alpha$  expression, since the tumor necrosis factor alpha is a pro-inflammatory agent whose expression is driven by the NfκB (p65) (31,32). Thus, we may deduce that there was modulation in the target genes and that it affected the expression of proteins in the assessed tissue.

## CONCLUSION

The antioxidant and anti-inflammatory effects found in the current study may be correlated with the nutritional composition described in the sapucaia nut. The antioxidant properties of sapucaia played an important role in the modulation of the inflammatory process and it allowed suggesting the neuroprotective effect of this nut.

The significant reduction in the gene expression of the NF $\kappa$ B (p65) and TNF- $\alpha$  inflammatory agents, combined with the significant ZnSOD and HSP-72 increase, showed how a food may be effective in the health prevention and protection processes.

Another important finding concerns the effects found according to the week of treatment, since 14 days of sapucaia nut-enriched diet showed very impressive results in both the antioxidant activity and the inflammation reduction.

Despite the relevance of the herein presented findings, it is worth highlighting the need for further studies about this nut in order to better understand the mechanisms involved in these responses.

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