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Polyphenol rich fruit attenuates genomic instability, modulates inflammation and cell cycle progression of offspring from fatty acid intake maternal

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ABSTRACT

The aim of this study was to investigate the effect of juçara (*Euterpe edulis Mart.*) supplementation on the maternal trans fatty acids intake in the livers of 21-day-old offspring. In order for this to happen, histopathological analysis, cytogenetic status, inflammation (COX-2 and TNF-alpha) and cell cycle progression were investigated in this setting. On the first day of pregnancy, female rats were distributed into four groups, as follows: control diet (C), control diet with 0.5 % juçara supplementation (CJ), diet enriched with hydrogenated vegetable fat, rich in TFAs (T), or T diet supplemented with 0.5 % juçara (TJ) during pregnancy and lactation. Juçara pulp induced liver regeneration in newborns exposed to maternal trans fatty acids. A significant decrease in the number of micronucleated hepatocytes was observed in animals exposed to trans fatty acids and treated with juçara. COX-2 and TNF immunoexpression was reduced in animals treated with juçara pulp. Furthermore, a decrease of Ki-67 immunoexpression was detected after treating trans fatty acids intake with juçara. Taken together, our results demonstrate that juçara pulp is able to prevent tissue degeneration and mutagenicity because it decreases inflammation and cell cycle control induced by maternal trans fatty acids in liver cells of rat offspring.

1. Introduction

Nowadays, obesity represents a public health problem around the world, especially in western countries. Unfortunately, the pathobiological condition not only affects these fat-consuming individuals, but also causes impacts to future generations [1]. It has been established that a maternal high-fat diet, rich in saturated and trans fatty acids, promotes metabolic disorders such as obesity, diabetes and cancer [2–4]. Perinatal exposure to high-fat diet alters the intrauterine environment leading to permanent phenotypic alterations that can persist throughout the life of individuals [5]. Such a condition has been demonstrated in humans and experimental models [6–8]. It is triggered by metabolic programming induced by high-fat diet that in turn promotes changes in gene expression and physiologic abnormalities in organs and tissues closely related to fatty acid metabolism, such as liver. Further scientific investigation is important in order to clarify if, and to what extent, liver of offspring is susceptible to high fat diet. Such information will provide new knowledge regarding the newborns during gestation and lactation.

Bioactive compounds have been identified as promising modulators of inflammation, genome stability and oxidative stress [9,10]. Juçara palm fruit (Euterpe edulis Mart.) is a species native of the Atlantic Forest located in Brazil. This fruit is rich in cis-unsaturated fatty acids, and dietary fiber; such components are an important source of polyphenols [11]. These bioactive compounds have potential to exert some antioxidant activity, as well as to modulate cell cycle regulatory proteins, and inflammatory host response [12]. Studies investigating the effect of fruits rich in phenolic compounds during gestation and lactation in the offspring have been little explored in literature so far, especially maternal trans fatty acids condition.

Thus, the aim of this study was to investigate the effect of juçara supplementation on the maternal trans fatty acids in the liver of 21-day-old offspring using experimental models in rats. For this

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Table 1
Score criteria for histopathological changes in liver adopted in this study (17).

Score	Structural changes
0	Tissue structure preserved
1	Mild degeneration and congested vessels.
2	Increased of eosinophilic, presence of congested vessels and inflammatory cells, and vacuolization.
3	Severe degeneration, necrosis and loss of structure.

purpose, histopathological analysis, cytogenetic status, inflammatory status (COX-2 and TNF-alpha) and cell cycle progression were investigated in this setting.

2. Materials and methods

2.1. Animals and experimental design

All experimental procedures adopted in this study were approved by the Experimental Research Committee of the Federal University of Sao Paulo (Protocol number 859814). All animals were kept under controlled conditions of light (12:12 h light-dark cycle and temperature (°C), with ad libitum water and food.

A total of twelve week-old female Wistar rats of first-order parity were left overnight to mate. Copulation was verified the following morning by the presence of sperm in vaginal smears. On the first day of gestation, female rats were isolated in individual cages and randomly distributed into four groups: control diet (C diet, C group); control diet supplemented with juçara 0.5 % freeze-dried powder (CJ diet, CJ group); diet enriched with hydrogenated vegetable fat (T diet, T group); or T diet supplemented with 0.5 % juçara freeze-dried powder (TJ diet, TJ group). The experimental design was established in a previous study conducted by our research group [11].

The diets were prepared according to the recommendations of the American Institute of Nutrition (AIN-93 G) [13] and all had similar caloric and lipid content. The source of lipids for the C and CJ diets was soybean oil; the principal source for the T and TJ diets was partially hydrogenated vegetable fat rich in TFAs. The CJ and TJ diets were prepared by adding 5 g/kg of juçara freeze-dried powder to each diet.

Juçara pulp (Euterpe edulis Mart.) was obtained from the agroecological Project Juçara/IPEMA - Institute of Permaculture and Ecovillages of the Atlantic (Ubatuba, SP, Brazil) - and then, freeze-dried to powder using a lyophilizer. Diets were stored at −20 °C. The chemical characterization and determination of total polyphenols of juçara was conducted in a previous study, by our research group [14]. Diets were maintained during pregnancy and lactation. After birth, litter sizes were adjusted to eight pups that remained with their mother. The pups were weighed and measured (nasoanal length) at birth and on postnatal days (7, 14, and 21). After 21 days the offspring were decapitated.

2.2. Histopathological analysis

Histopathological changes in liver - including tissue degeneration, congested vessels, steatosis and inflammation - were analyzed by the semi-quantitative method (Table 1) according to Aguiar et al. [15] with some modifications.

2.3. Cytogenetic assay

After completing the experimental period, the micronucleus test was performed in liver tissue. Paraffin sections (3 μm) were stained by Feulgen and counterstained with Fast Green (Sigma Aldrich™, USA) [16]. A total of one thousand hepatocytes were analyzed per animal [17]. Slides were scored blindly using a light microscope with a 100x immersion objective.

Table 2
Total number of rats in all groups according to degree of liver histological changes.

Groups	Number of animals	0	1	2	3
Control	5	5	0	0	0
Juçara	5	5	0	0	0
Trans fatty acids + Juçara 0.5 %*	5	0	4	1	0
Trans Fatty acids*	5	1	2	2	0

Semi quantitative scoring evaluation in liver histology (According to Aguiar Jr et al. (17).
* Significantly different when compared with its respective control group ($p < 0.05$).

2.4. Immunohistochemistry for TNF-alpha, COX-2 and ki-67

Sections of 3 μm were cut from the same paraffin blocks as used for histopathological analysis, mounted on silanized slides, and kept in an oven at 56–60 °C overnight. Next, the slides were deparaffinized in xylene for 15 min twice at room temperature, dehydrated in absolute ethanol, and washed under running water. Antigen retrieval was performed in citrate buffer, pH 6.0, for 40 min in a steamer. The slides were left to stand at room temperature for at least 20 min and washed under running water. Endogenous peroxidase was blocked by incubation in 3 % hydrogen peroxide (4 washes of 5 min each), followed by washing under running water and incubation in 3 % skim milk (Molico, Nestlé, São Paulo, Brazil) in PBS, pH 7.4, for 30 min. All slides were incubated with the goat polyclonal anti-TNF-alpha antibody (Santa Cruz Biotechnology Inc, CA, USA) or anti-COX-2 antibody (Santa Cruz Biotechnology Inc, CA, USA), anti-ki-67 monoclonal antibody (Novocastra, USA) both diluted 1:200 in BSA at 4 °C overnight in a moist chamber. Next, the slides were incubated with the biotinylated secondary antibody of the LSAB kit (Dako, Glostrup, Denmark) in a moist chamber for 30 min at room temperature. After washing with PBS, the slides were incubated with streptavidin conjugated to peroxidase of the same kit for 30 min at room temperature, followed by washing in PBS. The reaction was developed with 3.3′-diaminobenzidine (DAB Liquid) (DakoGlostrup, Denmark) as chromogen at room temperature. The sections were washed in distilled water, counterstained with Carazzi'shematoxylin, and mounted in Entellan resin (Merck, Jacarepaguá, RJ, Brazil). Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells as described elsewhere [18]. These values were used as labeling indices.

2.5. Statistical analysis

All the data are expressed as mean ± standard deviation (SD). Kruskal-wallis non-parametric test was performed followed by Dunn's test. Statistical analysis was performed using Graph Pad Prism™ 6.0 program. $p<0.05$ was considered to be significant.

3. Results

3.1. Histopathological analysis

Histopathological analysis was conducted on the rat liver of offspring exposed to trans fatty acids and treated with juçara. Table 2 shows the degree of histopathological changes found in this setting. Under microscopic evaluation, liver tissues from the control group presented ordinary architecture based on hepatocytes, sinusoidal vessels, and portal field preserved (Fig. 1a). In the group exposed to maternal trans fatty acids, a total of two animals presented degenerative vacuoles in hepatic tissues, as well as presence

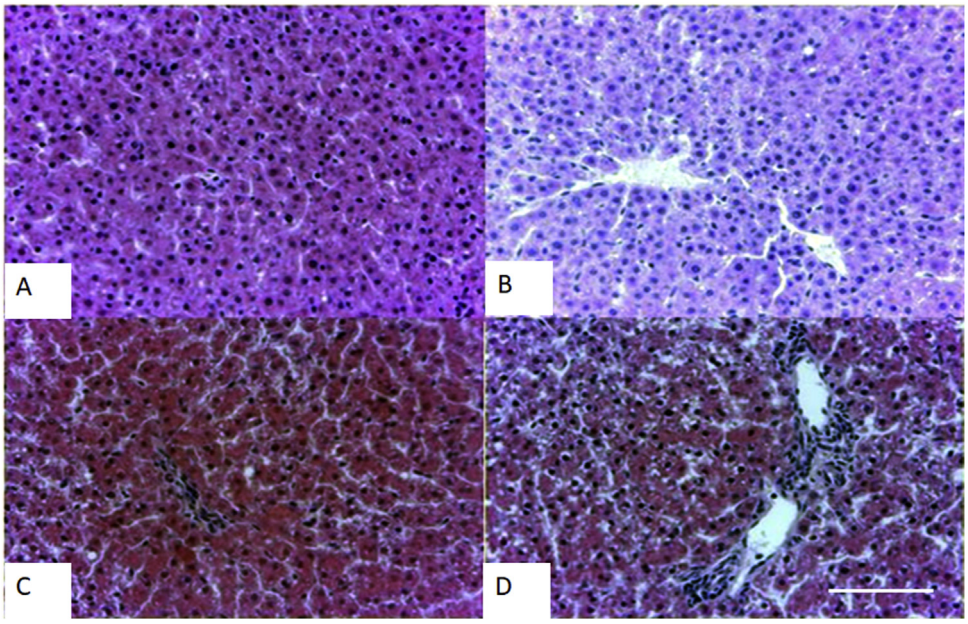

Fig. 1. Photomicrographs from A. control group; B. Juçara group; C. Trans fatty acids and juçara; D. Trans fatty acids. H.E. stain, Bar =36 μm.

Table 3
Mean + S.D. of Micronucleus frequency and cytotoxic parameters (pyknosis an karyolysis) in liver cells of offspring exposed to maternal trans fatty acids and treated with juçara pulp.

Groups	Micronucleus
Control	0.2 ± 0.4
Juçara	0.4 ± 0.4
Trans fatty acids + Juçara 0.5 %*	0.8 ± 0.7
Trans fatty acids*	1.6 + 1.0*

* Significantly different when compared with its respective control group ($p < 0.05$).

Table 4
Numerical data from immunohistochemistry for TNF-alpha of rat offspring exposed to maternal trans fatty acids and treated with juçara pulp.

Groups*	Score 0	Score 1	Score 2	Score 3
Control	4	1	0	0
Juçara	2	2	1	0
Trans fatty acids + Juçara 0.5 %**	1	2	2	0
Trans fatty acids**	0	1	2	2

* Total number of animals.
** $p < 0.05$ when compared to respective control.

Table 5
Numerical data from immunohistochemistry for COX-2 of rat offspring exposed to maternal high fat diet and treated with juçara pulp.

Groups*	Score 0	Score 1	Score 2	Score 3
Control	5	0	0	0
Juçara	1	3	1	0
Trans fatty acids + Juçara 0.5 %**	1	1	1	2
Trans fatty acids**	1	2	2	0

* Total number of animals.
** $p < 0.05$ when compared to respective control.

of inflammatory cells in the lobular central bands (Fig. 1b). Such abnormalities were categorized as number 2 for these animals. In the group exposed to juçara and maternal trans fatty acids, apparent normal architecture of liver tissues presenting mild changes was found when compared to the group exposed to trans fatty acids (Fig. 1c). One animal presented degenerative vacuoles. A total of four animals were categorized as number 1 and one animal as number 2 in this group. In the group treated with juçara, no remarkable histopathological differences were noticed when compared to negative control group. All animals were categorized as number zero.

3.2. Cytogenetic assay

Micronucleus test showed that trans fatty acids were able to induce micronuclei in rat liver cells of offspring as a result of chromosomal breakage or loss. Nevertheless, a significant decrease in the number of micronucleated hepatocytes was observed in animals exposed to trans fatty acids and treated with juçara when compared to animals only exposed to trans fatty acids. Such findings are demonstrated in Table 3.

3.3. Immunohistochemistry

TNF-alpha immunomarker was detected in the cytoplasm of rat liver cells (Fig. 2). In the negative control group, weak immunoreactivity for TNF-alpha was found. On the other hand, the majority of liver cells were positive for TNF-alpha immunomarker in the group exposed to trans fatty acids. Juçara pulp was able to reduce the TNF-alpha levels when compared to maternal trans fatty acids group, being significant ($p < 0.05$) between groups. The numerical results can be better visualized in Table 4.

When COX-2 immunoreactivity was investigated, the same findings were observed when compared to TNF-alpha immunoexpression. COX-2 was detected in a granular pattern in the cytoplasm of liver cells (Fig. 3). Animals from control group demonstrated the lack of positivity of the COX-2 immunomarker. Therefore, the immunoreactivity was considered negative for this group. However, a high expression of COX-2 was found in the liver cells of offspring exposed to maternal trans fatty acids when compared to negative control group. Animals treated with juçara pulp decreased immunoexpression of COX-2 compared to maternal trans fatty acid group. Such findings are demonstrated in Table 5.

Ki-67 immunoexpression was detected in the nucleus of rat liver cells (Fig. 4). Control group did not show Ki-67 positive cells. Nevertheless, rats' offspring treated with maternal fatty acid diet showed

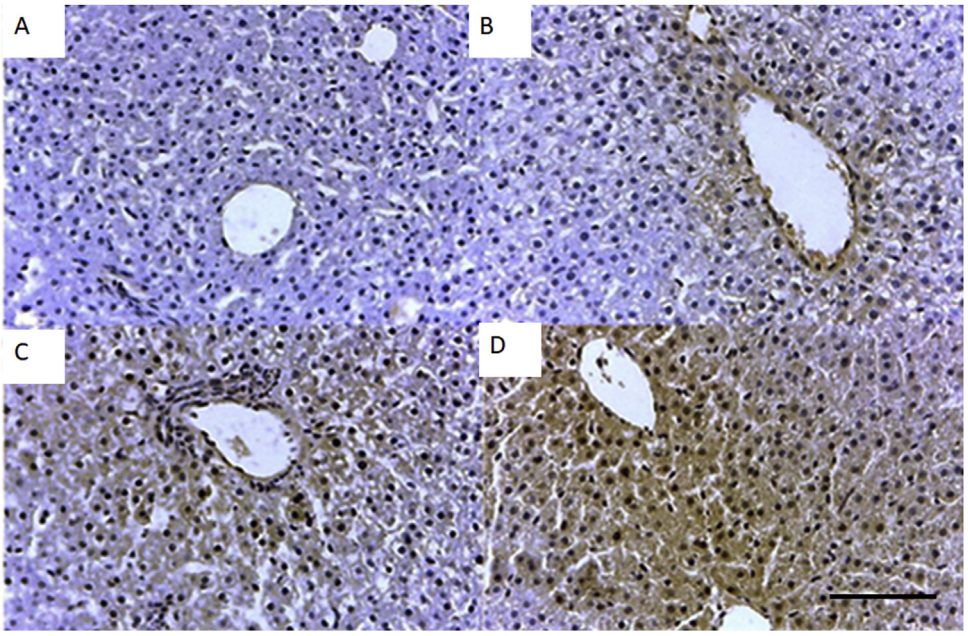

Fig. 2. Immunohistochemistry for **TNF-alpha**. Photomicrographs from A. control group; B. Juçara group; C. Trans fatty acids and juçara; D. Trans fatty acids. Immunohistochemistry stain, Bar =42 μm.

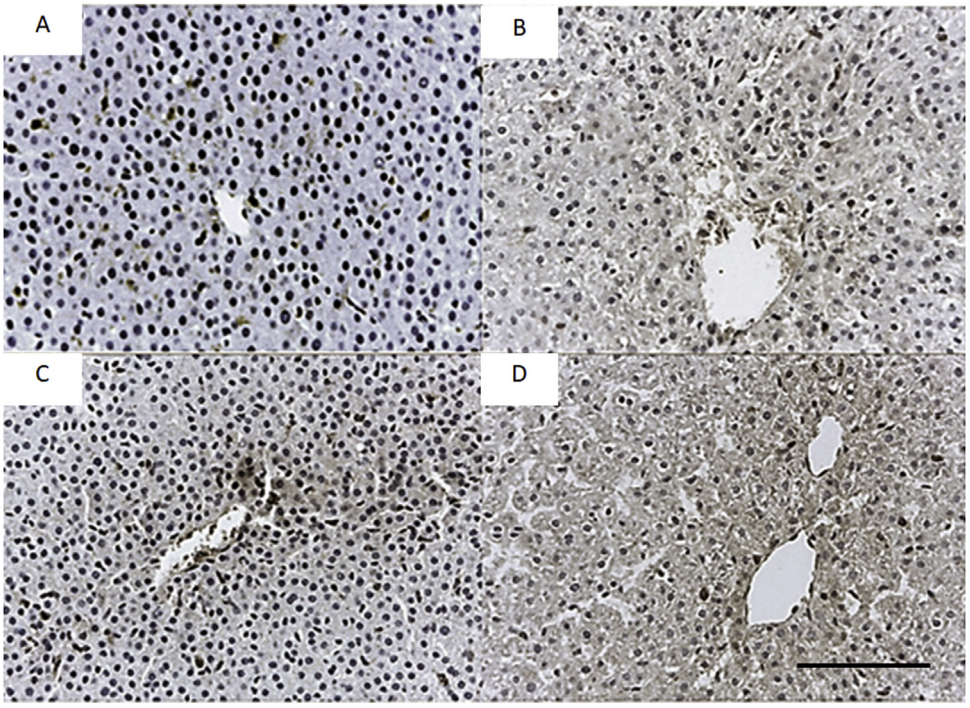

Fig. 3. Immunohistochemistry for **COX-2**. Photomicrographs from A. control group; B. Juçara group; C. Trans fatty acids and juçara; D Trans fatty acids. Immunohistochemistry stain, Bar =36 μm.

an increase of Ki-67 immunoexpression when compared to negative control group. Juçara decreased Ki-67 levels since statistically significant differences ($p < 0.05$) were detected when compared to trans fatty diet group. The treatment with juçara did not show remarkable changes. Such findings are presented in Table 6.

4. Discussion

The aim of this work was to evaluate if juçara pulp is able to protect against the noxious activities induced by trans fatty

Table 6
Numerical data from immunohistochemistry for ki-67 of rat offspring exposed to maternal high fat diet and treated with juçara pulp.

Groups*	Score 0	Score 1	Score 2	Score 3
Control	4	1	0	0
Juçara	4	1	0	0
Trans fatty acids + juçara 0.5 %**	3	2	0	0
Trans fatty acids**	0	4	1	0

* Total number of animals.
** $p < 0.05$ when compared to respective control.

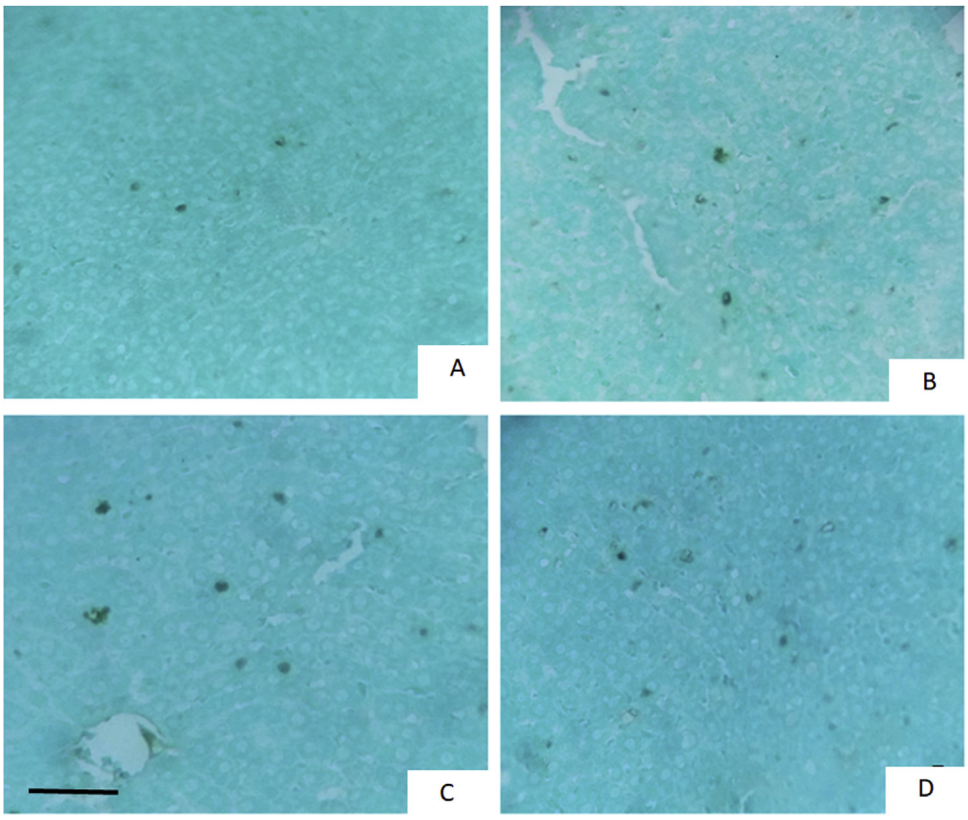

Fig. 4. Immunohistochemistry for **ki-67**. Photomicrographs from A. control group; B. Juçara group; C. Trans fatty acids and juçara; D Trans fatty acids. Immunohistochemistry stain, ×40 magnification. Bar =42 μm.

acids maternal intake on offspring after lactation. For this purpose, histopathological analysis, cytogenetic monitoring, inflammatory cytokines and cell cycle progression were evaluated in this study. To the best of our knowledge, there are no studies that have addressed the issue so far.

Histopathological analysis from liver revealed that newborns exposed to maternal trans fatty acids presented tissue degeneration as a result of cellular vacuolization, congested vessels and tissue degeneration. However, animals treated with juçara pulp improved the histopathological changes induced by maternal trans fatty acids as depicted by reduction of congested vessels and cellular vacuolization. Therefore, we assume that juçara pulp protects the liver of rat offspring against trans fatty acids induced by damage in the liver tissue. Nevertheless, some authors have revealed that juçara is not able to restore liver function in mice after consuming high fat diet [19]. In fact, is it known that maternal fat diet induces steatogenic and fibrogenic effect in liver of rodents [20]. However, other authors did not find any histopathological changes induced by high fat diet in the liver of rat offspring [21]. Such data are dependent on experimental design as well the rodent strain chosen. It has been established that intrauterine exposure to high-fat diet, rich in saturated and trans fatty acids, is able to alter genetic programs in multiple organs such as liver [1]. Taken as a whole, our data reveal that juçara pulp improved histopathological changes induced by liver tissue in rat offspring exposed to trans fatty diet.

To further elucidate the role of juçara pulp on liver cells, we performed additional experiments focusing genetic status, inflammation and cell cycle progression for a better understanding of which biological mechanisms are closely involved in the process, especially to explain how liver regeneration occurs after treating liver with juçara pulp at cellular and/or molecular level.

We decided to evaluate genomic damage since it plays a crucial role in mutagenesis and carcinogenesis. It has been well documented that genomic damage is responsible for environmental exposure to mutagens, carcinogens, and other inherited genetic factors closely related to xenobiotic metabolism and DNA repair deficiency [22]. Micronucleus detection characterizes genomic instability in eukaryotic cells [22]. The detection of an elevated frequency of micronuclei in living tissues indicates increased risk of developing chronic degenerative diseases [23]. Our results demonstrated that maternal trans fatty acids increased the incidence of micronuclei in liver cells of offspring. Juçara pulp was able to reduce the number of micronucleated cells. Ki-67 immunoexpression was high in the liver of rats' offspring treated with trans fatty acids, and juçara decreased Ki-67 levels. Some authors have postulated that significant G0/G1 are found in the livers of offspring having a maternal high fat diet [24]. The increase of DNA content has been associated with increased proliferative activity and/or the presence of inflammatory cells [25]. We suggest that juçara pulp exerted antimutagenic effect on liver cells exposed to trans fatty acids. Probably, the bioactive compounds found in the juçara pulp promoted genome stability and cell cycle control against noxious scenario induced by trans fatty diet in the offspring liver rat. It could have happened due to the strong antioxidant activity exerted by juçara pulp. However, further studies are necessary to elucidate the issue, especially focusing on the cell cycle regulatory proteins and apoptosis process.

Accumulating evidence suggests that inflammation induces high expression of a variety of cytokines, including TNF-alpha, and cyclooxygenase (COX)-2 [26]. COX-2 is induced by a variety of stimuli such as growth factors, proinflammatory mediators and hormones [27]. It has been established that maternal high-fat diet promotes low-grade chronic inflammatory response. This condi-

tion is associated with some pathophysiological consequences [1]. Several studies using animal models have reported that perinatal exposure to a high-fat diet induces inflammatory host response in liver cells. It was shown in a lactating high-fat mice model that inflammatory markers IL-6 and TNF-α were significantly induced [20]. Maternal high-fat diet increased TNF-α in serum as well as constituting one of the risk factors for fatty liver development [28]. Some authors have revealed that maternal trans fatty acids can promote adverse effects on offspring, such as an increase of the tumor necrosis factor-α (TNF-α) mRNA expression, the plasminogen activator inhibitor-1 (PAI-1) mRNA expression, and TNF receptor-associated factor-6 (TRAF-6) protein in the adipose tissue of 21-day-old offspring [29]. Our results demonstrated that rat offspring exposed to maternal trans fatty diet increased the levels of TNF-α in their liver cells. However, exposure to juçara pulp was able to reduce the TNF-α immunoreactivity in liver cells. By comparison, a previous study conducted by our research group demonstrated that juçara pulp reduced the levels of TNF-α expression in adipose tissue in the rat offspring exposed to maternal trans fatty diet [11]. Similarly, whereas our results demonstrated that, in liver cells of rat newborns, maternal trans fatty acids induced overexpression of COX-2, juçara pulp was able to decrease COX-2 immunoexpression. Considering the results mentioned previously, our results demonstrate that juçara pulp is able to decrease TNF-α and COX-2 immunoexpression in liver cells. The action exerted by juçara attenuates the inflammatory response induced by maternal trans fatty acids in liver tissue of the rat offspring.

In summary, our results demonstrate that juçara pulp is able to prevent tissue degeneration, mutagenicity, inflammation and cell cycle control induced by maternal trans fatty acids in liver cells of rat offspring, immediately after lactation.

Declaration of Competing Interest

None declared.

Acknowledgements

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