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Original Article

The effect of consumption of purple grape juice in the gestational period on oxidative stress parameters in *Wistar* rat foetuses

Isabel Cristina Teixeira Proença[1], Tamires Marques de Abreu[1], Jessica Pereira Marinho[1], Malena Rostirola Miri[1], Gustavo Fernandes Vasques[1], Luiz Fernando Lopes[1], Manuela dos Santos[1], Vanessa Duarte Ortiz[2], Patrick Türck[2], Alex Sander da Rosa Araujo[2], Claudia Funchal[1], Daniela Pochmann[1], Caroline Dani[1]

[1]Biochemistry Laboratory, Research Center, Methodist University Center-IPA, Porto Alegre, RS, Brazil; [2]Department of Physiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

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Abstract: This study aimed to evaluate the effects of purple grape juice consumption in pregnancy on oxidative stress parameters in *Wistar* rat fetuses. Twenty-four pregnant rats were divided into five groups: control group, indomethacin group (received a single dose of indomethacin in DG20), group grape juice DG14 (received an amount for 14 days/first and second gestational trim), group grape juice DG20 (received a dose throughout the gestational period), group grape juice two doses (received two doses, at morning and afternoon). On the 20th day of pregnancy (DG20), rats were anesthetized, and a cesarean section was performed to obtain the fetuses. A sample of liver, heart, and total brain of fetuses was collected for oxidative stress analyses. Values $P<0.05$ were considered significant. In fetuses' heart, we observed that the grape juice two dose group decreased sulfhydryl and increased SOD. In the liver, the grape juice decreased TBARS and SOD. There was a decrease in carbonyl and sulfhydryl in the indomethacin and grape juice one dose groups in the brain. We conclude that indomethacin altered oxidative stress parameters only in the fetal brain, and grape juice was presented as an important modulator of antioxidant capacity when consumed in a dose.

Keywords: Grape juice, offspring, pregnancy, indomethacin, oxidative stress

Introduction

Pregnancy is considered a moment of extreme importance in women's life. In this period, physiological and nutritional changes occur, which can alter maternal metabolism [1]. Also, currently, there is an increase in exposure to free radicals and oxidative stress [2, 3]. As a result, fetal circulation is different from the circulation of the newborn. At this stage, the placenta is responsible for the nutrition of the conceptus [4]. However, some organs such as the liver, heart, and brain of the fetus have already been functional since the beginning of development [5]. Therefore, both pre-gestational and gestational nutritional statuses are fundamental, as they are causally related to fetal growth and the development of specific pathologies of this period [6, 7].

In this sense, it is worth emphasizing the importance of maintaining good eating habits [1], especially foods capable of acting in the body's chemical reactions generating health benefits at different stages of life. These should receive special attention [8]. A balanced diet in macro and micronutrients is related to comorbidity prevention, avoiding the need for the use of contraindicated drugs during pregnancy [9], such as nonsteroidal anti-inflammatory drugs (NSAIDs) because these induce an imbalance in the synthesis of prostaglandins. Therefore, the number of studies related to NSAIDs and the consumption of polyphenols with the closure of the fetal arteriosus duct (DA) [10-12]. Furthermore, the vasoconstriction process activated by the use of NSAIDs during pregnancy is related to an increase in the production of reactive oxygen species (ROS) altering cell homeo-

stasis [13], since the increased oxygen demand and energy substrate generation favor the process of oxidative stress during pregnancy [14]. However, studies are rare in the literature that demonstrates this interaction in foetal tissues.

However, several studies have analyzed the influence of maternal intake of foods with antioxidant and anti-inflammatory potential in offspring [15-18]. In this sense, the grape and its derivatives become a reference for its high concentration in phenolic compounds [19, 20]. A recently published experimental study from our group observed a decrease in damage to proteins (carbonyls) in daughters' hippocampus after maternal consumption of grape juice during pregnancy, demonstrating the benefits of these foods in transgenerational models [21].

In this sense, further studies about the polyphenols intake during pregnancy and its action on the offspring's health are needed. Therefore, this study aimed to evaluate the effects of chronic consumption of purple grape juice at different periods and doses during pregnancy on oxidative stress parameters in the liver, heart, and total brain of Wistar rat fetuses.

Materials and methods

Animals

For this study, 22 female rats and 11 male rats of the species (*Rattus norvegicus*), *Wistar*, were heterogenic, approximately three months of age, from the bioterium of the Methodist University Center-Porto Alegre Institute. All animals were fed commercial feed and water at will and housed in plastic cages in the experimental rooms of the Bioterium itself, which have a temperature of 22±1°C, humidity between 60-80%, and light/dark cycle of 12 hours. It is noteworthy that males were used only for mating with females. All experimenters' procedures were performed with the approval of the Ethics Committee on The Use of Animals (CEUA) of the Methodist University Center IPA, under protocol number 003/2017 and 001/2018.

Diet and grape juice

The palleted commercial food consisted of 20.5% protein (predominantly soybean), 54% carbohydrates, 4% fat, 4.5% fiber, 7% ash, and 10% moisture. The integral purple grape juice of the Vitis labrusca Bordeaux species was kindly donated by Nova Aliança. The juices were from the same harvest (2017) and the same batch and were previously analyzed. The total phenolic compounds (2.08±0.05), total flavonoids (2.58±0.002), catechin (15.41±0.59), epicatechin (3.08±0.06), epigallocatechin (0.33±0.005), epigallocatechin gallate (4.13±0.05) and gallic acid (4.92±0.11) were quantified.

Experimental design

The rats were placed for mating, in proportion to two females for one male per box in the late afternoon, and the vaginal smear was performed on the morning of the following day. Gestational day 0 (DG0) was determined by the presence of sperm in the vaginal smear, confirming pregnancy [22]. After the confirmation of pregnancy, pregnant rats were randomly divided into five experimental groups, as follows:

Group 1 (control), composed of five rats who received a daily morning administration of water at the dose of 7 µL/g of animal weight orally (gavage) from gestational day 0 (DG0) until the 20th day (DG20); Group 2 (indomethacin), composed of four rats who received a daily morning administration of water at the dose of 7 µL/g of animal weight orally (gavage) from DG0 and a single administration of indomethacin (Aspen Pharma, Durban, South Africa) at the dose of 10 mg/kg of animal weight orally (gavage) on the 20th day of pregnancy (DG20); Group 3 (DG14), composed of four rats who received a daily morning administration of integral purple grape juice at the dose of 7 µL/g of animal weight orally (gavage) from gestational day 0 (DG0) until the 14th day (DG14) characterizing first and second trimester; Group 4 (DG20), composed of four rats who received a daily morning administration of whole purple grape juice at the dose of 7 µL/g of animal weight orally (gavage) from gestational day 0 (DG0) until the 20th day (DG20); Group 5 (2 doses), composed of five rats who received two daily administrations of grape juice at the dose of 7 µL/g of animal weight each, one in the morning and one in the afternoon orally (gavage) from gestational day 0 (DG0) until the 20th day (DG20).

During treatment (pregnancy), the rats and their food ration were weighed daily on a digital scale (Crystal 200, Gibertini, Italy). Four hours after of administration of the last dose of gavage, the rats were anesthetized with an association of ketamine (75 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally, and a cesarean section was performed to obtain the foetuses, which were euthanized before the first breath. After this procedure, the mothers were euthanized by cardiac puncture (exsanguination). Confirmation of euthanasia was performed by the evaluation of respiratory and cardiac arrest. At the end of this procedure, fetuses' liver, heart, and total brain were extracted.

Oxidative stress parameters

Oxidative stress parameters were evaluated in the liver, heart, and total brain of foetuses. The tissues were homogenized in a 1,5% KCl solution and frozen (-20°C) until oxidative stress analysis.

Lipid peroxidation levels

The levels of thiobarbituric acid reactive substances (TBARS) were used to evaluate lipid peroxidation. The TBARS assay was performed according to the method described by Ohkawa et al. [23] Briefly, 50 µL of 8.1% sodium dodecyl sulfate (SDS), 375 µL of 20% acetic acid (pH 3.5), and 375 µL of 0.8% thiobarbituric acid (TBA-Merck, Darmstadt, Germany) were added to 200 µL of the sample and then incubated in a boiling water bath for 60 min. After cooling, the mixture was centrifuged (1000 g, 10 min). Next, the supernatant was removed, and absorbance was read at 535 nm on a spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments, Alma Parck, Lutterworth, UK). Commercially available malondialdehyde was used as a standard. Results were expressed as nmol/mg protein.

Levels of oxidatively damaged proteins: The oxidation of serum proteins (carbonyls) was evaluated based on a reaction with 2,4-dinitrophenylhydrazine (DNPH-Sigma, St. Louis, USA) [24]. Briefly, a sample containing 1 mg of protein was reacted with 10 mM 2,4-dinitrophenylhydrazine for 30 min and subjected to protein precipitation with 10% TCA (Trichloroacetic Acid), followed by centrifugation (11,000 g, 3 min, 4°C). Next, the pellet was washed with ethanol:ethyl acetate (1:1) and centrifuged (11,000 g, 3 min, 4°C) three times. Then, pellets were suspended in 6 M guanidine hydrochloride (20 mM KH_2PO_4, pH 2.4) and read using a spectrophotometer (380 nm). Finally, blank samples were reacted with 2 M HCl instead of 10 mM 2,4-dinitrophenylhydrazine, and were run in parallel. Results were expressed as nmol carbonyl/mg protein.

Measurement of total sulphydryl groups

Quantifying the concentration of total sulfhydryl groups provides an idea of the level of oxidative attack on plasma proteins and is based on the reaction with 5,5'-Dithio-Bis (2-Nitrobenzoic Acid) [25]. Briefly, 50 µL of tissue or serum was mixed with 980 µL of PBS and 35 µL of 30 mM Tris/3 mM EDTA (pH 8) in a microplate well. After reading baseline absorbances, samples were reacted with 10 µL of 5,5'-dithiobis-(2-nitrobenzoic Acid) (10 mM in ethanol) for one hour. Absorbance was determined spectrophotometrically at 405 nm. The obtained values were compared with those obtained with a cysteine standard curve, and results were expressed as nmol-SH/mg protein.

Measurement of antioxidant enzyme activity: Using the method described by Aebi et al., catalase activity (CAT) was determined using the absorbance of hydrogen peroxide at 240 nm (25°C) [26]. The results were expressed as CAT/mg protein units. Superoxide dismutase (SOD) activity was determined by spectrophotometry based on a decrease in the autocatalytic adrenochrome formation rate at 480 nm [27]. Enzymatic activity was expressed as SOD/mg of protein units

Protein dosage

Proteins were quantified using the Lowry et al. [28] method, using bovine serum albumin at the standard concentration of 1 mg/mL.

Statistical analysis

The values were determined as parametric or non-parametric, using the Kolmogorov-Sminorv. Non-parametric data were analyzed by Kruskal Wallis, followed by post-test Dunn's, being expressed as the median and interquartile interval. Parametric data were analyzed by ANOVA from a pathway, followed by post-test

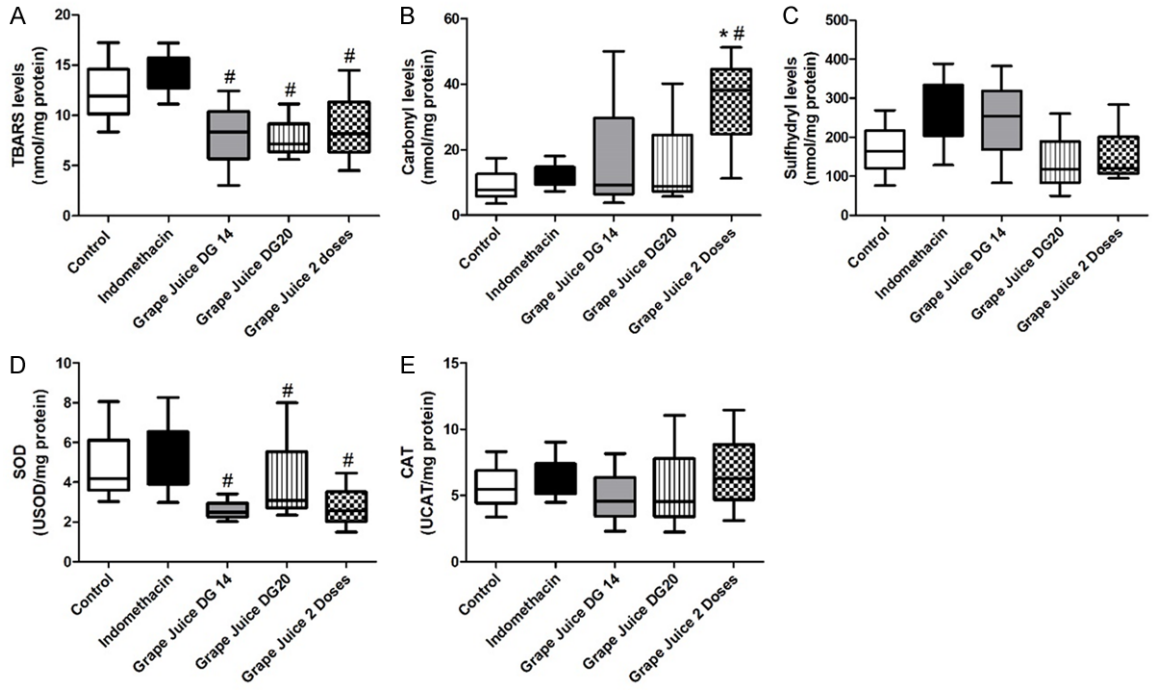

Figure 1. Analysis of cellular damage parameters and antioxidant capacity. Lipid peroxidation levels (TBARS) (A). Levels of protein oxidation (carbonyls) (B). Levels of sulfhyidryls grouping (C). Levels of activity of the enzyme superoxide dismutase (SOD) (D). Levels of catalase enzyme activity (CAT) (E) in the liver of *Wistar* rat fetuses that were treated with purple grape juice during the gestational period. Data expressed in median and interquartile interval. *Difference between these groups in relation to the control group. #Difference between the groups in relation to the indomethacin group. Statistical difference according to ANOVA of a pathway, with post Dunn's test, P<0.05.

Tukey, being expressed as the average and standard error. Values (P≤0.05) indicate a significant difference. All analyses were performed using sigma software Plot version 11.0.

Results

About the oxidative stress parameters, we observed that grape juice consumption during the pregnancy reduced the lipid peroxidation in the liver of fetuses (P≤0.05) (**Figure 1A**). In oxidative protein damage, we observed that grape juice two doses increased compared to the control and indomethacin groups (P≤0.05) (**Figure 1B**). Concerning antioxidant enzymes, we observed that the intake of grape juice in the first trimesters of pregnancy or two doses decreased enzyme activity superoxide dismutase compared to the indomethacin group (P≤0.05) (**Figure 1D**). However, there were no significant differences in sulfhydryl levels and catalase activity (**Figure 1C** and **1E**).

Regarding the analysis of these parameters in the heart of foetuses we observed that grape juice and indomethacin could not alter lipid damage markers and proteins (**Figure 2A** and **2B**). However, the intake of 2 doses of grape juice decreases sulfhydryl amount. It increases superoxide dismutase activity compared to the one dose grape juice group (P≤0.05) (**Figure 2C** and **2D**), no significant difference was observed in the catalase enzyme (**Figure 2E**).

Regarding the brain tissue of fetuses, we observed that the grape juice two doses decreased TBARS levels (P≤0.05) (**Figure 3A**). Indomethacin and grape juice one dose decreased compared to the control groups, DG14 grape juice, and group 2 doses for carbonyl levels (P≤0.05) (**Figure 3B**). About the sulfhydryl group, indomethacin group, and grape juice, one dose showed a decrease compared to the control groups, DG14 grape juice, and group 2 doses (P≤0.05) (**Figure 3C**). However, both juice and indomethacin did not alter SOD and CAT activity (**Figure 3D** and **3E**).

Discussion

Studies by our research group have demonstrated the importance and benefits of consuming grape juice during pregnancy [18, 21].

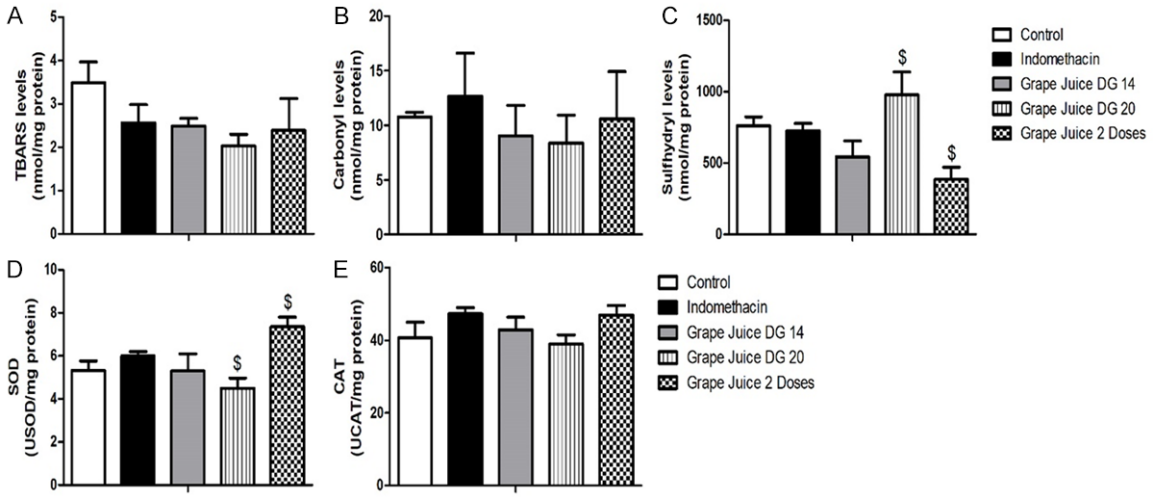

Figure 2. Analysis of cellular damage parameters and antioxidant capacity. Lipid peroxidation levels (TBARS) (A). Levels of protein oxidation (carbonyls) (B). Levels of sulfhyidryls grouping (C). Levels of activity of the enzyme superoxide dismutase (SOD) (D). Levels of catalase enzyme activity (CAT) (E) in the heart of *Wistar* rat fetuses that were treated with purple grape juice during the gestational period. Data expressed on average and standard error. $Difference between these groups grape juice DG20 and grape juice 2 doses. Statistical difference according to ANOVA of a pathway, with Tukey's post-test, P<0.05.

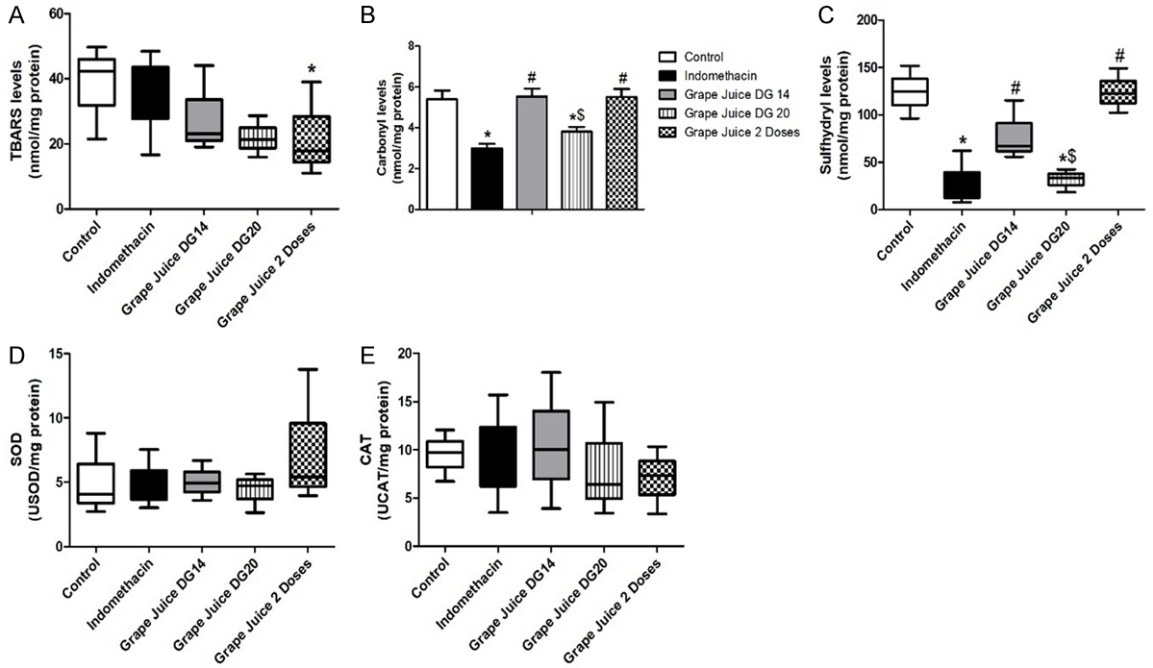

Figure 3. Analysis of cellular damage parameters and antioxidant capacity. Lipid peroxidation levels (TBARS) (A). Levels of protein oxidation (carbonyls) (B). Levels of sulfhyidryls grouping (C). Levels of activity of the enzyme superoxide dismutase (SOD) (D). Levels of catalase enzyme activity (CAT) (E) in the total brain of *Wistar* rat fetuses that were treated with purple grape juice during the gestational period. Data expressed in median and interquartile interval and carbonyls expressed on average and standard error. *Difference between these groups in relation to the control group. #Difference between the groups in relation to the group indomethacin. $Difference between the grape juice groups. Statistical difference according to ANOVA of a pathway, with post test of Dunn's or Tukey, P<0.05.

Given this, our study evaluated the consumption of grape juice at different stages and doses during the gestational period, evaluating fetuses' liver, heart, and brain tissue. This study in-

vestigated the relationship between maternal food consumption and the impacts caused on the offspring compared with indomethacin (an anti-inflammatory drug) during the gestational period. As a result, we can see that grape juice decreased lipid peroxidation in the liver, increased carbonyl and decreased superoxide dismutase, increased sulfhydryl levels in the heart. In addition to reducing lipid peroxidation, protein damage and altering sulfhydryl levels in brain tissue, and demonstrating that maternal nutrition influences the redox homeostasis of the offspring [18, 21, 29].

By evaluating, in detail, the biomarkers of oxidative stress in the liver of fetuses, we observed a decrease in lipid peroxidation in the groups whose mothers ingested grape juice. This result corroborates Vega et al. [30], who observed protection from lipid peroxidation in fetuses of mothers who received resveratrol and a low-protein diet during pregnancy. This protection demonstrated that grape compounds could protect against the damage caused by an inadequate diet. Also, in the liver, we observed that grape juice 2 doses, ingested in excess, increased protein oxidation compared to the control group. In a recent experimental study, ethanol exposed during pregnancy increased protein oxidation levels compared to control, demonstrating that the fetal liver is an organ affected by maternal ingestion [31]. Thus, possibly justifying that the excess doses of foods even rich in polyphenols should be controlled during pregnancy, as they can alter the redox homeostasis in fetal liver tissue. However, the literature already described that the consumption of grape juice during the gestational period decreased the levels of carbonyl in the mothers' liver. However, it could not protect against the damage caused by the ingestion of a diet rich in lipids [29].

Regarding the antioxidant defenses in the liver of fetuses, we observed a decrease in the superoxide dismutase enzyme activity in the groups that consumed grape juice compared to the indomethacin group. Corroborating our study, Vega et al. [30] also observed a decrease in SOD in the liver of fetuses of mothers who ingested a low-protein diet with resveratrol administration compared to the control group. One explanation for this occurrence is that antioxidants play the role of the enzyme, acting as "SOD-like", thus generating a decrease in enzyme activity [32].

Our study observed changes in the antioxidant defense system in the heart of fetuses generated by the consumption of grape juice in two doses, with a decrease in the content of sulfhydryls and an increase in SOD compared to the grape juice group DG20. Furthermore, Tarryadkins et al. [33], evaluated the influence of a low-protein diet during pregnancy followed by a recovery from low birth weight through breastfeeding. After 3 and 22 days of birth, the authors observed an increase in catalase expression and CuZnSOD decrease in the hearts of puppies with weight recovery. The results were more significant at 22 days after birth, positively reflecting maternal nutrition's influence on the antioxidant capacity. But it is important to mention that the adequate amount of grape juice ingested during pregnancy did not influence cell damage markers such as TBARS and Carbonyl. Also, it didn't alter the activity of antioxidant enzymes in fetuses' heart, as there were no differences in the control group.

Indomethacin is an NSAID widely used in AD constriction induction models [34-36]. Still, the counterpoint, although contraindicated, is a drug widely used during pregnancy to combat fever, pain, and inflammation due to a lack of clarification of its adverse effects [37]. However, the results of this drug on the fetus are rare in the literature since the main organ involved in the metabolization of this drug is the maternal liver, and there is a passage, even in a short period, to the fetus through the placenta [38, 39]. In our study, we observed that indomethacin did not damage lipids and proteins, nor did it alter the antioxidant activity in the liver and heart of fetuses. Perhaps the explanation is the dose and time of action since the drug was administered four hours before the cesarean. However, it showed a relative change in the fetal brain.

Brain tissue is rich in polyunsaturated fatty acids, making it highly susceptible to free radicals [40]. Our study observed that grape juice in 2 doses decreased lipid peroxidation in the fetal brain compared to the control group. Previous studies by our group have already highlighted the beneficial effects of grape juice on brain tissue, corroborating our findings [20, 41, 42]. However, regarding protein oxidation, we observed that both indomethacin and DG20 grape juice decreased levels in brain tissue compared to the control group. The effects of

indomethacin on protein oxidation in the brain have not been described in the literature. However, indomethacin increased carbonyl levels in gastric tissue in an experimental study [43]. As for the decrease in the oxidation of proteins generated by grape juice, Gabardo et al. [44], evaluated the effects of grape juice on brain tissue and observed a decrease in carbonyl levels in the hippocampus after in vitro incubation of grape juice and also protection of grape juice against increased damage from protein oxidation generated by temozolomide (TMZ) in the cortex, hippocampus and cerebellum.

Regarding the analysis of the antioxidant capacity in the fetal brain, we only observed changes in the levels of the sulfhydryl group, where indomethacin decreased the levels of non-enzymatic activity. Hilal et al. [45] evaluated the effect of indomethacin at different concentrations in brain tissue of male rats, and observed a significant decrease in glutathione reductase (GSH), as well as in the activity of SOD and CAT, demonstrating the genotoxic effects of this drug's anti-inflammatory drugs. However, unlike this study, we did not observe changes in the activity of superoxide dismutase and catalase enzymes. In our research, grape juice also decreased non-enzymatic activity levels. This result differs from another study from our group that demonstrated that grape juice consumption during pregnancy did not change sulfhydryl levels in rats' cortex, hippocampus, and cerebellum [42]. However, grape juice associated with gavage stress generated an increase in sulfhydryl in the cerebral cortex, demonstrating that grape juice and other important factors from this period contribute to the alteration of oxidative stress biomarkers [42].

Assessing the results obtained in our study, we can conclude that indomethacin administered at the end of pregnancy changed oxidative stress parameters only in fetal brain tissue. Moreover, it was realized that the consumption of grape juice during pregnancy could reduce or modulate oxidative damage and antioxidant defenses in fetuses. Thus, we can define that the daily and moderate consumption of grape juice promotes better results when compared to the data obtained in the groups that consumed only 14 days of gestation or ingested the drink in two doses. Despite this, further studies should be encouraged, including knowing the effects of using anti-inflammatory drugs for a more extended period and their action on the offspring. Despite this, we can say that the consumption of grape juice by pregnant women helps balance the antioxidant defense in their offspring.

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Disclosure of conflict of interest

None.

Address correspondence to: Caroline Dani, Post-Graduation Biosciences and Rehabilitation Program, Biochemistry Laboratory, Research Center, Methodist University Center-IPA, Cel. Joaquim Pedro Salgado, 80, 90420-060, Porto Alegre, RS, Brazil. Tel: +55-5133161298; E-mail: carolinedani@yahoo.com.br

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