



Research report

Metabolic and feeding behavior alterations provoked by prenatal exposure to aspartame [☆]

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ABSTRACT

The use of artificial sweeteners has increased together with the epidemic growth of obesity. In addition to their widespread use in sodas, artificial sweeteners are added to nearly 6000 other products sold in the US, including baby foods, frozen dinners and even yogurts. It has been suggested that the use of nonnutritive sweeteners can lead to body weight gain and an altered metabolic profile. However, very few studies have evaluated the effects of maternal consumption of artificial non-caloric sweeteners on body weight, feeding behavior or the metabolism of offspring in adult life. In this study, we found that animals exposed to aspartame during the prenatal period presented a higher consumption of sweet foods during adulthood and a greater susceptibility to alterations in metabolic parameters, such as increased glucose, LDL and triglycerides. These effects were observed in both males and females, although they were more pronounced in males. Despite the preliminary nature of this study, and the need for further confirmation of these effects, our data suggest that the consumption of sweeteners during gestation may have deleterious long-term effects and should be used with caution.

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Introduction

The global prevalence of obesity continues to remain high and is directly associated with a dramatic increase in healthcare expenses as medical costs to treat obese patients are 42% higher than for normal-weight patients. The annual costs of obesity for the medical system in the United States alone is estimated to be as high as \$147 billion (Finkelstein, Trogdon, Cohen, & Dietz, 2009; Ogden et al., 2006). Therefore, the need to better understand factors that are associated with weight gain and obesity is an important public health as well as an economic issue.

The increased prevalence of obesity has been attributed to the increased dietary intake of high-fat foods, sugar and other caloric sweeteners, and reduced physical activity (Andersen, 2000). To counterbalance this problem, individuals have replaced sugar for artificial non-caloric sweeteners in an attempt to lose or maintain weight. Recently, however, a number of studies have questioned the long-term effectiveness of replacing sugar with artificial sweeteners. Increased Non-nutritive sweetener (NNS) consumption is associated with an increase in body weight (Fowler et al., 2008; Mattes & Popkin, 2009; Stelman & Garfinkel, 1988) and the increased prevalence of obesity (Fowler et al., 2008; Popkin & Nielsen, 2003; Yang,

2010). Furthermore, the use of saccharin or aspartame is associated with an increased sensation of hunger (Appleton & Blundell, 2007; Blundell & Green, 1996; Lavin, French, & Read, 1997; Rogers, Carlyle, Hill, & Blundell, 1988). However, these were performed in adult animals and, to our knowledge, very few studies have evaluated the effects of the maternal consumption of artificial non-caloric sweeteners on body weight, feeding behavior or metabolism of pups.

It has been reported that maternal diet composition and quality have a major influence on the metabolic development of offspring. Maternal obesity and over-nutrition may alter the hypothalamic leptin sensitivity (Férézou-Viala et al., 2007) and increase the expression of hypothalamic appetite-regulating peptides in the offspring. In addition, dams that consumed ad libitum high-fat (HF) or high-carbohydrate (HC) diets during gestation and lactation gave birth to pups with altered production and action of hypothalamic neuropeptides, such as neuropeptide-Y, and other molecules involved in the regulation of appetite and metabolism (Beck, Kozak, Moar, & Mercer, 2006; Cerf et al., 2010; Chang, Gaysinskaya, Karatayev, & Leibowitz, 2008; Chen & Morris, 2009; Kozak, Burlet, Burlet, & Beck, 2000; Kozak et al., 1998; Kozak, Richy, & Beck, 2005; Morris & Chen, 2009; Page, Malik, Ripple, & Anday, 2009). Thus, it is clear that maternal nutrition and environment during early development may markedly affect behavior and metabolism later in life (Jackson, Burdge, & Lillcrop, 2010) as well as the differentiation of the neuroendocrine system that regulates energy homeostasis which begins during the pre-natal period (Grove, Grayson, Glavas, Xiao, & Smith, 2005). Yet, few studies have investigated maternal

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diet and metabolism of offspring using artificial sweeteners during pregnancy. Thus, the aim of our study was to determine the effect of chronic consumption of artificial sweeteners during pregnancy on the metabolism and feeding behavior of pups as they reach adulthood.

Materials and methods

For the present study, we used 16 adult female Wistar rats (90 days of age at the beginning of the treatment), weighing 150–200 g and their offspring. The animals were divided into 4 groups; control (receiving water), sucrose (45 g/L), saccharin (1.35 g/L) and aspartame (2 g/L). The experimentally-naïve animals were housed in groups of three to five, according to their groups, in home cages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (lights on between 7:00 and 19:00 h), at a room temperature of 22 ± 2 °C. The rats had free access to food (standard rat chow) and water (or the different solutions). The compounds were administered in the drinking water as the only source of water for 30 days. Subsequently, the females were placed with a male, in a ratio of 3 to 1, in order to mate. Animals were then monitored and approximately 5 days prior to delivery, the dams were isolated. The animals continued receiving the treatment until giving birth. After birth all animals received free access to water and standard lab chow and all litters were standardized at 8 animals per mother. At 21 days post-partum the pups were separated from the dams and divided by sex. The animals were left undisturbed until the start of behavioral procedures with free access to standard chow and tap water. At 112 days of age the animals were killed by decapitation in order to perform the biochemical evaluations. No more than two animals per sex per litter were used per experiment. All studies were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS) and of the Federation of Brazilian Societies for Experimental Biology.

Behavioral procedures

Habituation to the new foods

From 60 days of age, rats were habituated to a novel environment containing new foods. During this period, they were placed in a lighted rectangular box (40 cm × 15 cm × 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot Loops® (Kellogg's® – pellets of wheat, cornstarch and sucrose) were placed at one end of the box. The animals were habituated to this environment during 5 days, 3 min each day, under food restriction (80% of habitual ingestion of standard lab chow) in order to avoid the neophobic effect of presenting a food other than the usual rat chow. After the habituation session, the animals were fed ad libitum and exposed to a 3-min test session, 24 h later. Time spent until the initiation of eating and the number of ingested Froot Loops® were evaluated in each trial and in the test session. A protocol was established so that when the animals ate part of the Froot Loops® (e.g., 1/3 or 1/4), this fraction was considered.

Open-field test

At 70 days of age the animals were submitted to the open-field test which consisted of an open wooden arena (60 × 40 cm) with 12 equally-divided areas measuring 15 × 13.3 cm and fifty-centimeter high wall. The front wall was made of glass, which allowed the observation of the animal by the researcher. The open-field test was originally described to study emotionality (reviewed by Prut & Belzung, 2003) and has been extensively used to evaluate motor

activity, having the ability to detect and quantify both increased and decreased motor activity due to pharmacological and non-pharmacological factors (Frussa-Filho et al., 2010). The number of line crossings and the time in the central area were recorded for a 5 min period as a measurement of locomotor activity.

Elevated plus maze

At 73 days of age the animals were evaluated for anxiety by being submitted to the elevated plus maze that consisted of two opposing open arms (48.5 × 10 cm), two opposing enclosed arms with no roof (48.5 × 9.5 × 49 cm), and an open area (13 × 10 cm) in the center. The maze was elevated 50 cm above the floor. The behavioral test was conducted in the same observational room using red light illumination. The animal was placed in the center of the plus maze, facing one of the open arms, and remained in the apparatus for 5 min. Rats tend to avoid unprotected areas of a novel environment. Therefore, they will typically explore the arms with walls, avoiding the open arms. When the animal stays longer in the closed arms, and rarely ventures out to the open arms, this behavior is interpreted as anxious-like (Bertoglio & Carobrez, 2010); thus the number of entries and the time spent in the open or enclosed arms were recorded to assess anxiety.

Chocolate and lab chow consumption

To evaluate the feeding behavior of treated animals when they had free access to a high caloric sweet food, 1 male and 1 female animal per litter were chosen at random and from the ages of 80 to 110 days the animals were exposed to a free choice between standard chow and chocolate. The consumption of both foods was measured on a daily basis.

Blood collection and evaluation of abdominal fat

At 112 days of life the animals were killed by decapitation between 10:00 and 16:00 h, and the trunk blood was collected using EDTA, centrifuged at 4 °C at 1000 g, and plasma separated and stored at –80 °C until analysis. The epididymal and retroperitoneal fats were dissected and weighed on a precision scale.

Plasma lipids and glucose (blood biochemistry)

Total cholesterol (TC), high-density lipoprotein cholesterol (HDL), and triglyceride analyses were performed on EDTA plasma collected. TC and triglycerides were measured by enzymatic method kits (Wiener Lab, Argentina). HDL concentrations were measured by a system for selective precipitation of Low and Very Low Density Lipoproteins (LDL and VLDL) and HDL cholesterol measurement in the supernatant, using an end-point reaction, as described in the HDL Kit (Labtest, Brazil). Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald formula. Plasma glucose was measured by the glucose oxidase method (Wiener Laboratories, Rosario, Argentina). All analyses were performed on a Spectra Max M5 autoanalyzer.

Statistical analyses

Results are expressed as mean and standard deviation. All data were analyzed by one-way ANOVA; a post hoc Duncan test was employed when appropriated to compare the differences between the groups. Since the present study presented multiple comparisons, we applied the Bonferroni correction to diminish the false discovery rate and $P < 0.001$ was considered statistically significant. All data were analyzed using SPSS software, made by IBM, New York.

Results

Fluid consumption was evaluated during treatments and each animal was found to consume approximately 30 mL fluid/day (data not shown). Figure 1 demonstrates that males from the groups whose mothers were treated with sucrose and saccharin did not present any increase in the number of entries in the closed arms [$F(3,26) = 3.46$, $p = 0.03$] (Fig. 1A) of the apparatus. On the other hand, females from the group whose mothers were treated with aspartame showed a reduced number of entries in the closed [$F(3,36) = 11.47$, $p < 0.001$] and open arms [$F(3,36) = 6.60$, $p < 0.001$] (Fig. 1B). As also shown in Fig. 1, both males and females spent more time in the closed arms [males, $F(3,26) = 134$, $p < 0.001$; females, $F(3,36) = 135$, $p < 0.001$] and less time in the open arms [males, $F(3,26) = 29.94$, $p < 0.001$; females, $F(3,36) = 34.33$, $p < 0.001$] (Fig. 1C and D, respectively). In order to assess whether these effects were due to altered motor activity, the open-field test was performed, which did not show any differences between the groups (data not shown).

Using repeated measures ANOVA, male pups whose mothers were treated with aspartame Froot Loops® ate more compared to the saccharin-treated group as shown for both within subjects $F(12,104) = 3.98$ ($p = 0.001$) and between subjects effect $F(3,26) = 6.89$ ($p < 0.001$), Fig. 2A. In addition, female animals whose mothers were treated with aspartame ate more Froot Loops® during the five-day trial compared to all other groups [within subjects effects $F(12,168) = 4.54$, $p < 0.001$; between subjects effects, $F(3,42) = 7.27$, $p < 0.001$, 2B]. In the test session, offspring from mothers treated with aspartame showed increased Froot Loops® consumption,

for both males and females [males, $F(3,26) = 39.8$, $p < 0.001$; females, $F(3,42) = 63.66$, $p < 0.001$].

There was no statistically significant increase in the consumption of calories from chocolate in the aspartame group [$F(3,15) = 4.32$, $p = 0.022$], when this type of food was offered chronically (Fig. 3). As food consumption was measured in home cages with 3 to 4 animals per cage, data for males and females were analyzed together.

As shown in Fig. 4A, an increase in weight gain was observed in males whose mothers were treated with aspartame and saccharin [$F(3,26) = 21.6$, $p < 0.001$]; the same result was not observed for females whose mothers were treated with aspartame [$F(3,36) = 3.05$, $p = 0.041$]. However, Fig. 4B shows that the increase in gonadal fat depots in males of the sucrose and aspartame groups is not statistically significant [$F(3,26) = 3.29$, $p = 0.036$] and the same effect was also observed in the retroperitoneal fat depots in the females of the saccharin group [$F(3,36) = 3.36$, $p = 0.029$].

Plasma lipids values are presented by group in Fig. 5. An increase in cholesterol and LDL in the sucrose and aspartame treated groups was determined for both in males [$F(3,26) = 19.02$, $p < 0.001$]; [$F(3,26) = 15.80$, $p < 0.001$] and females [$F(3,36) = 8.38$, $p = 0.001$]; [$F(3,36) = 12.57$, $p < 0.001$] as shown in Fig. 5A and B. It was also observed that females from the saccharin-treated group presented increased LDL [$F(3,36) = 12.57$, $p < 0.001$]. Males of the sucrose and aspartame groups presented an increase in triglycerides [$F(3,26) = 20.82$, $p < 0.001$] compared to the control group (Fig. 5D). Finally, plasma glucose levels were increased in the group whose mothers were treated with aspartame, as shown in Fig. 6 [males, $F(3,26) = 11.18$, $p < 0.001$, females, $F(3,36) = 20.57$, $p < 0.001$].

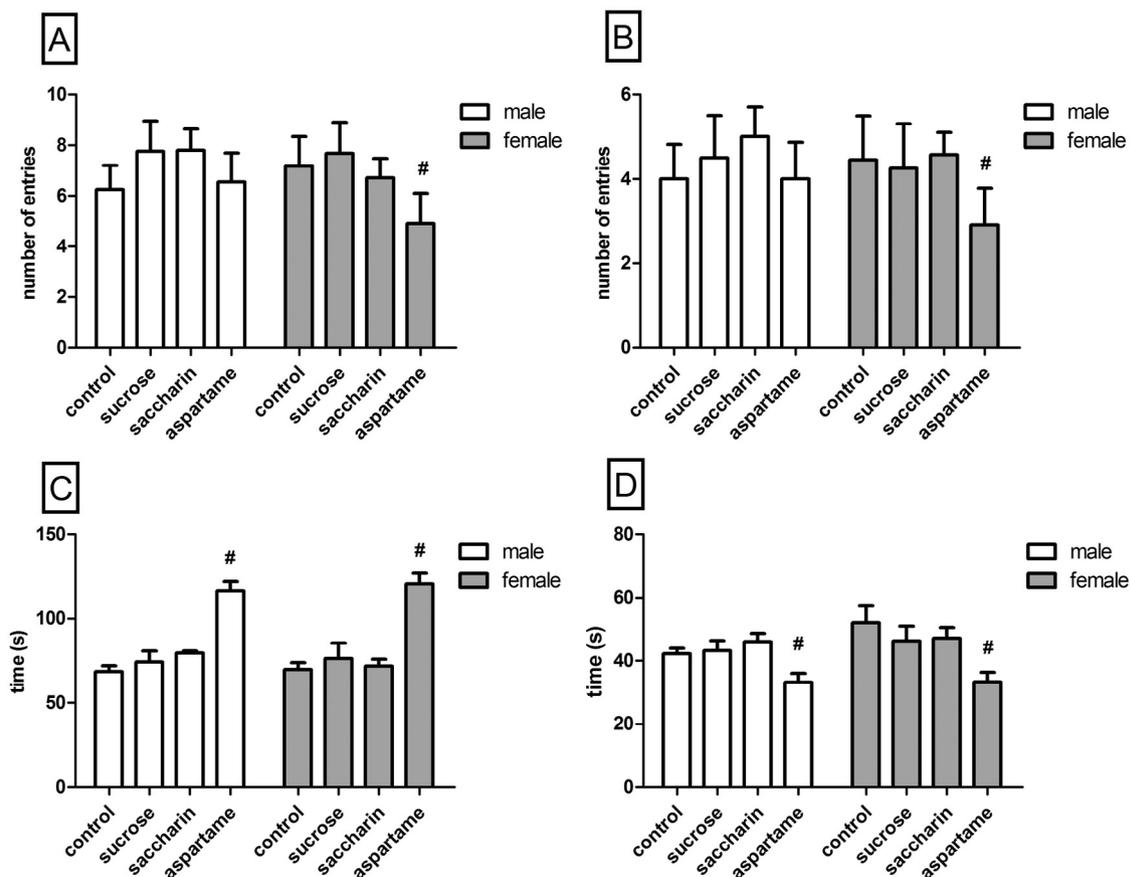


Fig. 1. Effect of chronic consumption of sweeteners by the dam on adult offspring performance in the plus maze test: (A) number of entries on closed arms; (B) number of entries in open arms; (C) time spent in the closed arms; (D) time spent in the open arms. # different from all other groups.

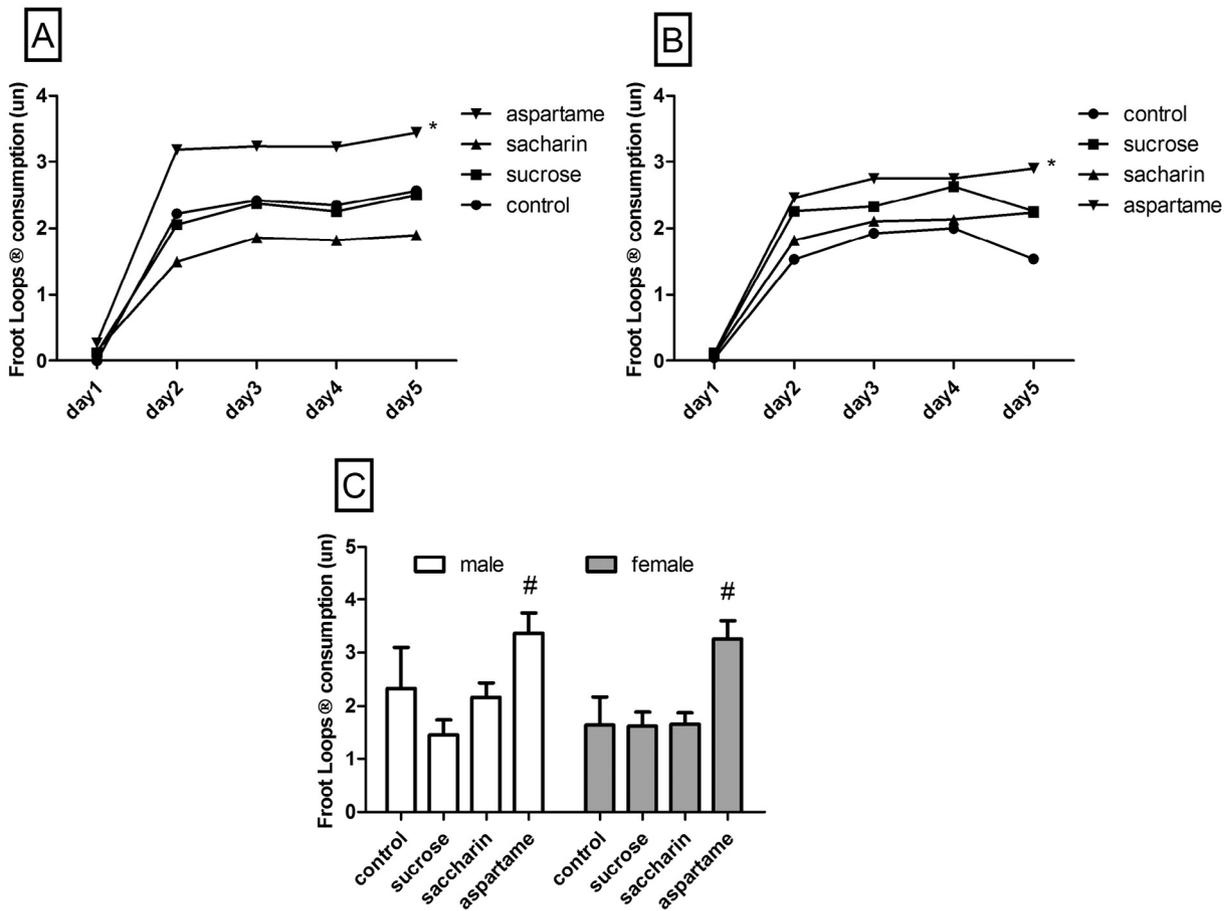


Fig. 2. Effect of chronic consumption of sweeteners by mother on feeding behavior of adult offspring: (A) Froot Loops® consumption (units) in males during trials; (B) Froot Loops® consumption (units) in females during trials; (C) Froot Loops® consumption (units) in the test. * Different from control group; # different from all other groups.

Discussion and conclusion

As the global prevalence of obesity continues to increase, it is important to determine factors that promote excess weight gain. Recent studies have associated the increase in food consumption and consequently in weight gain with the increase in the use of NNS (Fowler et al., 2008; Mattes & Popkin, 2009; Yang, 2010). The present study provides additional support to this idea by demonstrating that

offspring of dams that consumed aspartame during pregnancy, when evaluated as adults, showed increased consumption of palatable foods and gained more weight, as well as exhibited more anxiety-like behavior, when compared to controls. Additionally, offspring of dams that consumed aspartame also had higher plasma glucose, total and LDL-cholesterol when compared to control subjects. In the case of the sucrose group, an increase in total cholesterol and LDL cholesterol was observed, as well as an increase in serum triglycerides in males.

Recent studies have suggested that consumption of artificial sweeteners may lead to an increased risk of excessive weight gain, metabolic syndrome, type 2 diabetes, and cardiovascular disease (Duffey, Steffen, Van Horn, Jacobs, & Popkin, 2012; Lutsey, Steffen, & Stevens, 2008; Yang, 2010). Several large-scale prospective cohort studies have found a positive correlation between artificial sweetener use and weight gain in adults (Colditz et al., 1990; Fowler et al., 2008; Stellman & Garfinkel, 1988) and in children (Berkey, Rockett, Field, Gillman, & Colditz, 2004; Blum, Jacobsen, & Donnelly, 2005; Striegel-Moore et al., 2006). Consistent with the findings of our study, artificial sweeteners appear to have more effects in males than in females (Berkey et al., 2004). However, these similarities must be viewed with a great deal of caution, given the fact that these studies were performed in consumers of sweeteners, and that effects were not evaluated in the consumers’ offspring.

With regards to feeding behaviors and dietary intake, recent studies suggest that artificial sweeteners do not activate the food reward pathway in the same manner as natural components as there is a lack of a caloric contribution to eliminating the post-ingestive

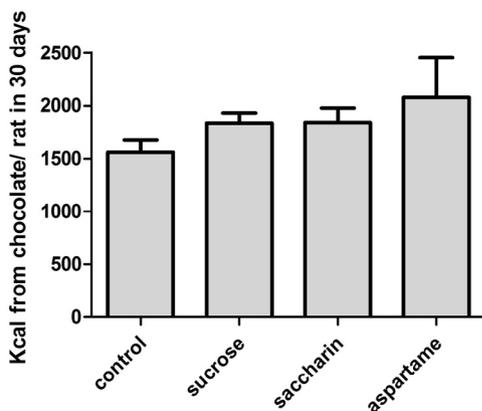


Fig. 3. Effect of chronic consumption of sweeteners by mother on homecage chocolate consumption by adult offspring.

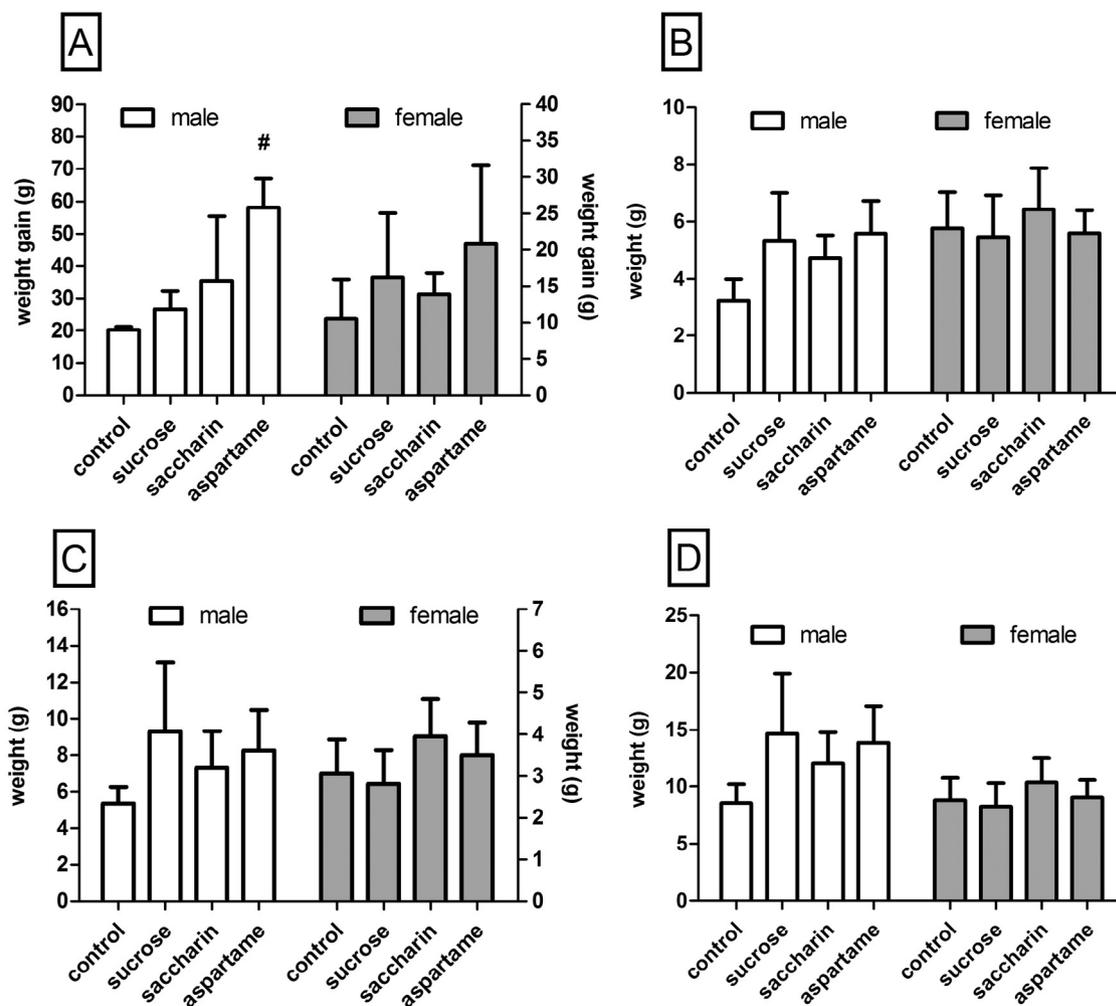


Fig. 4. Effect of chronic consumption of sweeteners by mother on adult offspring weight gain and fat after 30 days of free access to chocolate: (A) weight gain of males is represented on the left Y scale and of females is on the right Y scale; (B) Gonadal fat weight; (C) retroperitoneal fat weight; (D) total abdominal fat weight. # different from all other groups.

component. The absence of complete satisfaction, due to the failure to activate this post-ingestive factor, enhances food-seeking behavior, which may contribute to obesity. Despite the fact that aspartame is usually completely hydrolyzed (Rycerz & Jaworska-Adamu, 2013), when pregnant animals are subjected to chronic, high levels of this sweetener, some may reach the fetus. In animals that were subjected to aspartame throughout their prenatal development, their ability to activate the post-ingestive factor of the food reward pathway may be affected, or underdeveloped, losing, at least in part, this hypothalamic control of food ingestion, especially concerning sweet foods. We found that animals whose mothers consumed aspartame during pregnancy consumed more energy than those born to mothers fed a standard diet, both during short-term and in a long-term exposure to palatable food. These findings suggest that animals chronically subjected to aspartame during the prenatal period could have developed a less efficient mechanism of satiety or a distinct mechanism of reward related to palatable food, which could lead to compensatory overeating and positive energy balance later on in life, even if the exposure to the sweetener occurred in the past, during the intra-uterine life. However, due to the novelty of the subject, further studies must be performed in order to confirm or deny the present findings.

We found that animals with perinatal exposure to aspartame developed an anxiety-like behavior as adults. Similar to our findings, other studies have demonstrated that perinatal decreases in serotonin, which may be generated by chronic exposure to high levels of Phe can lead to increased anxiety-like behavior in adults (Gross et al., 2002). In fact, studies have suggested that, in rodents, chronic treatment with aspartame (Christian et al., 2004; Dow-Edwards, Scribani, & Riley, 1989) and its exposure prenatally may cause behavioral differences and learning impairment, suggesting the possibility of an effect on pathways related to learning and development in the brain (Humphries, Pretorius, & Naude, 2008). Specifically, a single dose of 200 mg/kg of aspartame can increase plasma tyrosine and Phe levels by 142% and 62%, respectively in rats (Yokogoshi, Roberts, Caballero, & Wurtman, 1984). Furthermore, tyrosine will increase as a breakdown by-product of Phe in the liver (Fernstrom, 1988; Stegink, Filer, & Baker, 1988) and the aspartame-induced elevated levels of phenylalanine could potentially accumulate in the brain, leading to changes in regional brain concentrations of neurotransmitters (Abdel-Salam, Salem, & Hussein, 2012). In addition, it has been demonstrated that a tryptophan-deficient diet during the neonatal period can also lead to an increase in anxiety-like behavior in adult life (Zoratto, Fiore, Ali, Laviola, & Macri, 2013). Our results are consistent with other studies that have

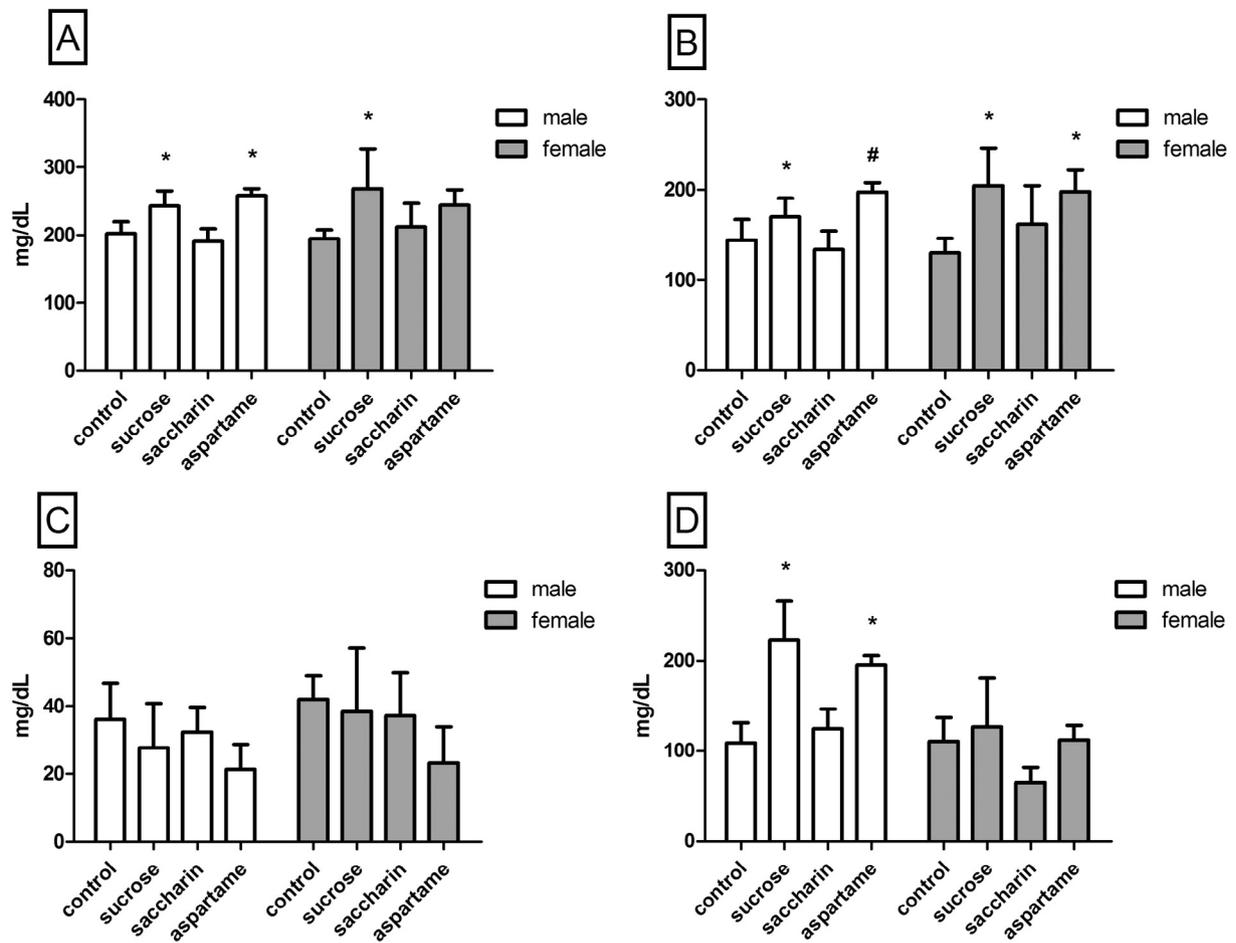


Fig. 5. Effect of chronic consumption of sweeteners by mother on adult offspring lipid profile: (A) total cholesterol; (B) LDL; (C) HDL; (D) triglycerides. * Different from control group; # different from all other groups.

found an increase in the consumption of sweet foods in association with anxiety-like behavior (Ely et al., 1997; Silveira et al., 2000). Thus, aspartame and its components could potentially disrupt a wide range of processes in the body. This disruption, if occurring during fetal development, could lead to permanent alterations in brain circuits and in the metabolism of these neurotransmitters.

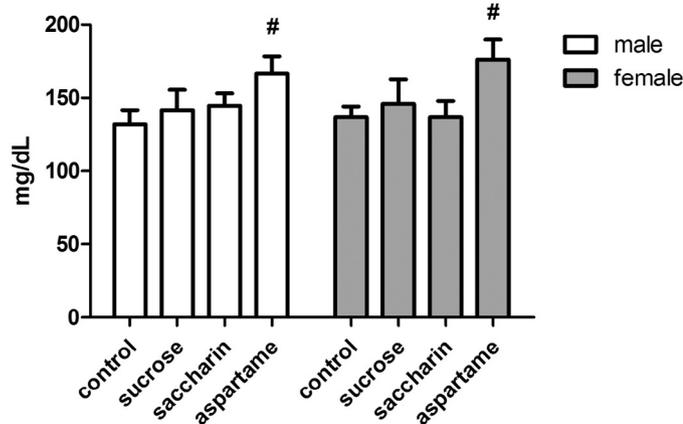


Fig. 6. Effect of chronic consumption of sweeteners by mother on adult offspring serum glucose. # Different from all other groups.

Regarding post-natal metabolism, we found an increase in the plasma glucose levels in the animals whose mothers were treated with aspartame, both males and females. It is generally agreed that Phe is actively transported across the placenta (Pueschel, Boylan, Jackson, & Piasecki, 1982), resulting in an increase in placental Phe dependent on maternal consumption. In rodents, high levels of accumulated Phe may be converted into other metabolites (Gazit, Ben-Shlomo, Ben-Shachar, Karnieli, & Katz, 1998; Scriver, Kaufman, Eisensmith, & Woo, 1995) that could interfere with normal metabolism (Gazit, Ben-Abraham, Rudin, & Katz, 2003; Gazit et al., 1998). In rodents, phenylalanine hydroxylase activity is absent until the end of gestation (Tourian, Treiman, & Carr, 1972; Yeoh et al., 1988). It is improbable that the concentration of phenylalanine in the brains of offspring is as high as that found in hyperphenylalaninemia, but a small chronic alteration in neuronal glucose homeostasis during prenatal development could lead to permanent changes in this parameter. Taken together, these disturbances could explain the mechanism underlying the perturbation in glucose homeostasis observed in our study.

In conclusion, we present one of the first studies showing the effects of gestational aspartame ingestion on offspring health as animals were found to develop a higher susceptibility to altered metabolic parameters, as well as augmented risk factors for cardiovascular disease. These effects were observed both in males and females, although they were more pronounced in males. This study is one of the first to indicate a possible deleterious effect of artificial sweeteners consumption during gestation. Still, other studies are needed to confirm and elucidate the mechanisms involved in the development of these effects.

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