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Effects of *Ilex paraguariensis* (yerba mate) treatment on leptin resistance and inflammatory parameters in obese rats primed by early weaning

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ABSTRACT

Aims: We evaluated the effects of yerba mate treatment over 30 days on body weight, food intake, hypothalamic leptin action and inflammatory profile in adult rats that were weaned early.
Main methods: To induce early weaning, the teats of lactating rats were blocked with a bandage to interrupt milk access for the last 3 days of lactation (EW group). Control offspring had free access to milk throughout lactation. On postnatal day (PN) 150, EW offspring were subdivided into: EW and M groups were treated with water and mate aqueous solution (1 g/kg BW/day, gavage), respectively, for 30 days. Control offspring received water by gavage. On PN180, offspring were killed.
Key findings: EW group presented hyperphagia; higher adiposity; higher NPY and TNF-α expression in the ARC nucleus; higher TNF-α and IL-1β levels in the adipose tissue; and lower IL-10 levels in the adipose tissue. These characteristics were normal in M group. As expected, the leptin injection in control offspring caused lower food intake. However, EW group exhibited no change in food intake after the leptin injection, indicating leptin resistance. In contrast, M group had a normal response to the leptin injection.
Significance: Thirty days of mate treatment prevented the development of hyperphagia, overweight, visceral obesity and central leptin resistance. This beneficial effect on the satiety of M offspring most likely occurred after the improvement of inflammatory markers in the hypothalamus and adipocytes, which suggests that *Ilex paraguariensis* plays an important role in the management of obesity by acting on the inflammatory profile.
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Introduction

Nutritional, hormonal or environmental alterations in early life can contribute to the onset of some diseases in adulthood, including obesity, type 2 diabetes, dyslipidemia and cardiovascular diseases. This phenomenon is known as metabolic programming (Barker, 2003; Moura et al., 2008); more recently, it has been termed developmental plasticity (Gluckman and Hanson, 2007). Our group showed that pharmacological and physical early weaning models cause overweight, higher visceral adiposity, hyperphagia, central leptin resistance, hypertriglyceridemia, insulin resistance and other metabolic diseases in offspring at adulthood (Bonomo et al., 2007; Moura et al., 2009; Lima et al., 2011, 2014).

An imbalance between energy intake and energy expenditure, such as a hypercaloric diet or sedentary lifestyle, leads to increased body fat storage, resulting in obesity (Riccardi et al., 2004). The regulation of energy balance occurs in specific regions of the central nervous system, including the hypothalamus, which is capable of controlling body weight (BW) through the regulation of energy intake and expenditure (Schwartz et al., 2000; Morton et al., 2006). Obesity is associated with a higher proinflammatory status in the hypothalamus with an increase in the IκB kinase-β/nuclear factor-κB (IKKβ/NFκB) pathway (De Souza et al., 2005). Zhang et al. (2008) showed a link between hypothalamic leptin resistance and the IKKβ/NFκB pathway when they observed that this pathway increases the expression suppressor of cytokine signaling 3 (SOCS3), which has an inhibitory action on the signaling leptin pathway, in the hypothalamus. SOCS3 is stimulated by tumor necrosis factor-alpha (TNF-α), an activator of IKKβ/NF-κB. In addition, the signal transducer and activator of transcription 3 (STAT3) of classical leptin signaling, which increases SOCS3 transcription, is controlled by NF-κB.

Beverages such as *chimarrão* (green dried leaves prepared with hot water), *tererê* (green dried leaves prepared with cold water) and mate tea (roasted leaves prepared with hot water or used to produce soft drinks) that contain yerba mate, a native plant from subtropical regions, are widely consumed in South America (Bastos et al., 2007; Heck and de Mejia, 2007). Studies have shown the potential beneficial effects of *Ilex paraguariensis* on obesity; these effects are most likely due to the plant's biologic compounds, such as flavonoids (quercetin and rutin), phenolic acids (chlorogenic and caffeic acids), caffeine and saponins. Even when combined with a high-fat diet, *Ilex paraguariensis* seems to contribute to

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the loss of BW and to improve insulin resistance (Arçari et al., 2009). Recently, we showed that yerba mate treatment in obese rats primed by early weaning was able to normalize hyperphagia, BW, total body fat, and neuropeptide Y (NPY) and SOCS-3 content in the hypothalamus (Lima et al., 2014).

Obesity is a chronic and slightly inflammatory state, and this inflammation seems to be directly related to the development of leptin resistance, particularly in the hypothalamus. Arçari et al. (2011) showed that a high-fat diet caused an up-regulation of the TNF-α and interleukin 6 genes in the liver of the obese mouse, and these genes were down-regulated via the NF-κB pathway after 8 weeks of yerba mate extract treatment, indicating that yerba mate may have an anti-inflammatory properties. Considering that yerba mate can influence the inflammatory profile but has not been evaluated in the hypothalamus or adipose tissue, we examined whether the leptin resistance observed in adult obese rats that were programmed by early weaning normalized the inflammatory cytokine profile in the plasma, hypothalamus and adipose tissue after 30 days of yerba mate treatment by observing the effects on food intake, fat mass and central leptin action.

Material and methods

Animals

Procedures involving the animals and their care conformed to institutional guidelines (CEUA/017/2009 and CEUA/057/2011) and were in compliance with national and international laws and guidelines for the use of animals in biomedical research. The experiments were performed to minimize the number of rats and their suffering, following the ethical doctrine of the three "R"s — reduction, refinement and replacement. Wistar rats were kept under well-controlled conditions of temperature (25 ± 1 °C) with artificial light–darkness cycles (lights on at 0700 h and lights off at 1900 h). Virgin female rats (3 months old) were caged with male rats at a ratio of 2:1 for 5 days. After mating, each female rat was placed in an individual cage with free access to food and water until delivery. Because pregnant rats produced 10 to 12 pups, we only used dams that produced a litter size of 10 pups to avoid the influence of litter size on the programming effect. At birth, to maximize lactation performance, the litters were adjusted to 6 male pups per dam.

Experimental design: programming by early weaning

On the 1st postnatal (PN) day, 20 lactating rats were randomly separated into two groups: EW (early weaning, $n = 10$) — mothers were lightly anesthetized with thiopental (0.06 mg/ml/100 g) and wrapped with a teat bandage (physical barrier) to interrupt lactation for the last 3 days of the period; and C (control, $n = 10$) — mothers with pups that had standard weaning, i.e., a 21-day lactation period. The EW and C groups had free access to a standard rodent diet, and pups had free access to drinking water. To facilitate food intake by the pups, the food pellets were placed directly inside the cage.

After weaning, the EW and control offspring had free access to water and a standard diet. Their BW was recorded until PN 180, and the 24-hour food intake was evaluated with animals in individual cages after 12 h of fasting.

Oral treatment with the aqueous solution of yerba mate

The roasted yerba mate solution was prepared fresh each day by dissolving instant mate tea powder (Leão Jr, Curitiba-PR, Brazil - lot A326/06) in distilled water (330 mg/ml) using a homogenizer. Previously, a sample of this lot was analyzed by our group (Kaezer et al., 2012) and described by Lima et al. (2014). The total phenolic (4.33 ± 0.01 g l^{-1}) content was estimated using the Folin–Ciocalteu method. The chlorogenic acid (610 ± 15 mg l^{-1}), caffeine (508 ± 79 mg l^{-1}), theobromine (99 ± 11 mg l^{-1}), quercetin and rutin (both undetected) contents in the sample were quantified using high-performance liquid chromatography. The analysis of soluble powder yerba mate indicated 41.20 ± 80 mg l^{-1} chlorogenic acid, 21 ± 44 ml l^{-1} caffeine and 8.57 ± 10 mg l^{-1} theobromine; the concentrations of quercetin and rutin were not determined.

On PN 150, four EW offspring from each litter were randomly subdivided into the following two groups: EW + Mate (M, $n = 20$) — rats received 1 g/kg BW of yerba mate aqueous solution (Lima et al., 2014); and EW + water (EW, $n = 20$) — rats received pure water. The C offspring ($n = 20$) were also randomly chosen from each litter and received pure water. The animals received mate tea or water once per day for 30 days by intragastric gavage to guarantee total ingestion. On PN 180, all animals were killed by quick decapitation with no prior anesthesia (because anesthesia affects hormone and lipid metabolism). Blood, brain tissue and retroperitoneal and subcutaneous white adipose tissues (RWAT and SWAT, respectively) were collected. Plasma and tissue samples were frozen at −80 °C until analysis.

Leptin resistance test — feeding study

A leptin resistance test was performed using a recombinant mouse leptin (PeproTech, Rocky Hill, NJ, USA) dissolved in saline (0.9% wt./vol.) and injected as a bolus at a dose of 0.5 mg/kg BW intraperitoneally as previously reported (Passos et al., 2004; Bonomo et al., 2007). On PN 180, the rats in the C, EW and M groups were divided into the following groups: leptin (CLEP, EWLEP and MLEP, respectively) or saline (CSAL, EWSAL and MSAL, respectively). We used 10 pups for each group. The animals were placed into individual cages with free access to water and were fasted for 12 h before the test. After injection, each rat was returned individually to its cage with access to 100 g of standard chow and placed in a dark room. Food intake was measured by weighing the food at 1, 3 and 5 h after the leptin or saline injection.

Body composition evaluation

Total fat mass (g), body mass (g), body fat (%) and trunk fat (%) were measured by dual-energy X-ray absorptiometry (DXA). The rats were anesthetized with an intraperitoneal injection of a 2:1 solution of ketamine hydrochloride (Cetamin®, 50 mg/ml) and xylazine hydrochloride (Xilazin®, 20 mg/ml) at a dose of 0.1 ml/100 g BW and then subjected to DXA (Lukaski et al., 2001) using a Lunar DXA 200368 GE instrument (Lunar, Wisconsin, USA) with specialized software (Encore 2008, Version 12.20 GE Healthcare). The DXA technician was blinded to the experimental protocol.

Microdissection of the hypothalamic arcuate (ARC) nucleus

The punch technique was used to obtain the ARC nucleus using the bregma as a reference (Palkovits, 1973; Paxinos and Watson, 2007; Helena et al., 2009). Briefly, two subsequent sections of 1000 μM were made: from −0.6 mm to −1.6 mm for PVN microdissection (not used) and from −1.6 mm to −2.6 mm for microdissection of the ARC nucleus. The dissection of the ARC nucleus was performed in the second section using a 2-mm 'square puncher' needle that was centered on the third ventricle, approximately 1 mm dorsal to the base of the brain (Franco et al., 2012). After removal of the ARC nucleus, the tissue samples were kept at −80 °C until the performance of western blot assays.

Western blot analysis

To obtain cell extracts, the ARC nucleus was homogenized in 50 μl of ice-cold RIPA buffer pH 7.4 (50 mM TRIS, 150 mM NaCl, 0.1% SDS, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphatase, 5 mM-EDTA and 1% Triton X-100) with complete/EDTA-free protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). After homogenization, the ARC was stored at −20 °C. NPY,

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