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Involvement of serotonergic pathways in gastric dysmotility induced by fat burning nutritional supplements in mice

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ABSTRACT

Fat burners are a category of nutritional supplements that are claimed to increase the metabolism and promote greater energy expenditure, leading to weight loss. However, little is known about the side effects on gastrointestinal motility. In this study, we evaluated the effect of ingestion with a fat burner named Thermbuterol® (THERM) on the gastric motility and food behavior of mice. THERM compounds were identified using nuclear magnetic resonance (NMR). Mice received variable doses of THERM (10, 50, 100 or 300 mg/kg, p.o.) or NaCl 0.15 M (control). Gastric emptying (GE) was assessed using the phenol red technique. Another set of mice was pretreated with intraperitoneal administration of hexamethonium (HEXA, 10 mg/kg), prazosin (PRAZ, 0.25 mg/kg), propranolol (PROP, 2 mg/kg), parachlorophenylalanine (PCPA, 300 mg/kg) or ondansetron (ONDA, 50 μg/kg) 30 min before THERM treatment for evaluation of GE. We assessed the gastrointestinal responsiveness *in vitro* as well as THERM's effects on food behavior. Caffeine was the major compound of THERM, identified by NMR. THERM 100 and 300 mg/kg decreased GE compared to the respective controls. Pretreatment with PRAZ or PROP did not prevent gastric dysmotility induced by THERM 100 mg/kg. However, the pretreatment with HEXA, ONDA or PCPA prevented GE delay induced by THERM. *In vitro*, THERM relaxed contractions in strips of longitudinal gastric fundus and duodenum. THERM also increased food intake, which was prevented by PCPA and ONDA treatments. THERM decreased GE of a liquid and increased food intake in mice, a phenomenon mediated by the autonomic nicotinic receptors and serotoninergic receptor.

1. Introduction

The percentage of overweight people in society is increasing, closely related to a more sedentary lifestyle, prompting greater efforts to reduce weight to improve health. These efforts involve several combinations of diet and exercise programs to combat obesity (Vaughan et al., 2014; Bo et al., 2020; Guo et al., 2020). Fat burners, or thermogenics, are increasingly used as nutritional supplements to combat the continuing epidemic of obesity (Okla et al., 2017). Fat burners are a category of nutritional supplements that are claimed to increase the metabolism and promote greater energy expenditure, leading to weight loss (Jitomir et al., 2008; Ratamess et al., 2016; Campbell et al., 2020).

Fat burners are formulated with various ingredients of natural or synthetic origin. These products act in the body as appetite modulators or metabolism accelerators, acting to reduce food intake (de Oliveira et al., 2017). Ephedrine preparations are among the most popular, but there are reports of adverse effects caused by ephedrine (Kim et al., 2008b). Therefore, the United States Food and Drug Administration (FDA) issued a regulation prohibiting the sale of all nutritional supplements containing ephedrine due to tolerability concerns (Haller et al., 2002; Zhang et al., 2018). Since then, new products such as "ephedrine-free" dietary supplements have been introduced in the world market. Although these supplements are not supposed to contain ephedrine alkaloids, they often incorporate various sources of caffeine and other botanical extracts

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whose compounds exhibit pharmacological activities including fat oxidation and increased total daily energy expenditure, promoting body fat reduction (e.g., *p*-synephrine, forskolin, and yohimbine) (Gurley et al., 2015; Gutiérrez-Hellín and Del Coso, 2016; Jo et al., 2016).

However, despite great research efforts of the pharmaceutical industry, the possible side effects of fat burners remain inconclusive (Vogel et al., 2015). Greater incidence of acute kidney injury and proteinuria, acute liver dysfunction and cardiovascular disorders (high blood pressure, high heart rate and acute myocardial infarction) has been associated with the use of fat burners (da Silva et al., 2014; Ferreira et al., 2020). Despite the relative scarcity of studies in animal models and limited evidence of their tolerability and efficacy for use in humans, interest in dietary supplements continues to increase.

Obesity is associated with chronic and low-grade inflammation as well as neuronal, endocrine and adipocyte factors that interact in the regulation of food intake and energy storage (Halpern et al., 2004; Guo et al., 2020; Zhou et al., 2020). Food intake is influenced by gastrointestinal mechanisms involving gastric accommodation and emptying, digestion and absorption of nutrients, mechanisms that are regulated by gut-brain axis and mediated by serotonin (5-hydroxytryptamine [5-HT]) (Hayes et al., 2004; González-Arancibia et al., 2016; D'Agostino et al., 2018; Choi et al., 2020).

Serotonin is a monoamine that has various functions in both neuronal and non-neuronal systems. In the central nervous system, 5-HT regulates mood, behavior, appetite, gastrointestinal motility and energy expenditure (Mazda et al., 2004; Manousopoulou et al., 2016; Oh et al., 2016; D'Agostino et al., 2018; Choi et al., 2020). 5-HT_3 receptors are located in many brain areas, especially areas involved in the vomiting reflex, such as the area postrema and the solitary tract nucleus (Mazda et al., 2004). Consistent with their role in emesis, 5-HT_3 receptors are also involved in information transfer in the gastrointestinal tract, while in the enteric nervous system they regulate gut motility and peristalsis (McLean et al., 2007; Coates et al., 2017). Studies have provided strong evidence of physiological roles of 5-HT_3 receptors in several important processes, including hematopoiesis, bone metabolism and metabolic homeostasis (Voronova et al., 2011; Amireault et al., 2011; Burke and Heisler, 2015; Oh et al., 2016). Thus, this serotoninergic pathway may be the target of nutritional supplements (Manousopoulou et al., 2016; Oh et al., 2016; Choi et al., 2020).

To investigate possible adverse gastrointestinal effects of using fat burners, the present study was designed to evaluate the effects of oral administration of a thermogenic nutritional supplement, called Thermbuterol® (THERM), on important gastrointestinal parameters in mice, specifically on gastric emptying. The contractile behavior of strips isolated from the stomach fundus and duodenum were also evaluated to confirm whether such effects coincide with the *in vivo* effects of this fat burner in the upper segments of the gastrointestinal tract. Additionally, we evaluated the effect of THERM on solid food intake, since changes in gastrointestinal motility can influence animals' appetite.

Thus, our hypothesis was that the use of THERM can influence gastrointestinal motility and eating behavior and that the possible effects may be related to serotonin signals through the gut-brain axis.

2. Materials and methods

2.1. Chemical composition of THERM by nuclear magnetic resonance (NMR)

Two hundred 50 mg of the powdered thermogenic nutritional supplement (Thermbuterol®, Sei Pharmaceuticals, Miami, USA) were dissolved in 500 μL of $CDCl_3$ and placed into NMR tube with an external diameter of 5 mm. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation spectroscopy (HMBC) were performed with a Bruker Ascend™ 400 spectrometer at 300.0 K. Chemical shifts were reported on a ppm scale and referenced using tetramethylsilane resonance (TMS) (Okaru et al., 2020).

2.2. Animals

The study was conducted using female Swiss mice (n = 150, 30–40 g body weight) obtained from the animal vivarium of Federal University of Vale do São Francisco (Petrolina, Pernambuco, Brazil). The animals were maintained in a temperature-controlled room (23–25 °C) with a 12/12 h light/dark cycle and free access to food and water until the experiment began. All the experimental procedures were performed in accordance with the rules of the Brazilian National Council for Control of Experimentation with Animals and were reviewed by and had prior approval from our local animal ethics committee (protocol no. 023240408).

2.3. Ovariectomy procedure

Bilateral ovariectomy was performed one week before starting administration of the investigated compounds. The animals were anesthetized intramuscularly with ketamine (100 mg/kg) plus xylazine (5 mg/kg) both from Syntec® (Santana de Parnaíba, São Paulo, Brazil), and after 10 min bilateral ovariectomy was performed (Souza et al., 2019).

2.4. Evaluation of gastric emptying of liquid test meal

After one week, the gastric emptying (GE) was measured in mice, which were subjected to 18 h fasting period, with free access to water only, until 2 h before the experiment. The mice were randomly treated orally with THERM at 10, 50, 100 or 300 mg/kg (experimental group) dissolved in a NaCl 0.15 M solution (0.1 ml per animal). Mice in a separate group were each treated with 0.1 ml of the vehicle solution (NaCl 0.15 M, control group). All mice were fed a liquid test meal (0.3 ml, 0.5 mg/ml of phenol red in 5% glucose solution) 30 min later. Afterward, at 10 min intervals, the mice were sacrificed by cervical dislocation, and their stomach contents were emptied and measured. Briefly, after laparotomy, the gut was quickly ligated to divide it into two consecutive segments: stomach and small intestine. The volume of each segment was calculated by soaking it in a graduated cylinder that contained 20 ml of NaOH (0.1 N). After the homogenization of each segment, the proteins were precipitated with 200 μl of 20% trichloroacetic acid. After centrifugation for 20 min (2800 rpm), 600 μl of the supernatant was added to 800 μl of 0.5 N NaOH. Samples were read spectrophotometrically at 560 nm to construct dilution curves by plotting the dye concentrations against optical densities. The amount of dye emptied by the stomach was expressed as a percentage (Silva et al., 2015).

2.5. Assessment of the effect of adrenergic and serotonergic pathways on liquid GE

To assess neurotransmission involved in the present GE delay induced by THERM, other mice received an intraperitoneal (i.p.) injection (1 ml/kg) of one of the following agents: hexamethonium (10 mg/kg), prazosin (PRAZ, 0.2 mg/kg), propranolol (PROP, 2 mg/kg), or ondansetron (ONDA, 50 μg/kg). Another group received *p*-chlorophenylalanine (PCPA, 100 mg/kg) once daily for the duration of 3 days (total of 300 mg/kg of PCPA). All these antagonists were purchase on Sigma Chemical Co, St Louis, MO, USA. These doses were based on the previous findings of Fioramonti et al. (1993), Capasso et al. (2004), and Souza et al. (2013). After 30 min (hexamethonium, PRAZ, PROP, or ONDA) or three days (PCPA subset) of pharmacological pretreatment, the mice were randomly subjected to the control or THERM (100 mg/kg, the lowest dose for prompt GE delay) treatment, fed the test meal, and sacrificed 10 min later for GE assessment as described above.

2.6. Evaluation of smooth muscle contractility in vitro

The *in vitro* experiments were performed on longitudinal strips isolated from the gastric fundus and duodenum of the mice, sacrificed by cervical dislocation. After laparotomy, the stomach and an approximate

8 cm duodenal segment were removed, excised, and immersed in perfusion medium (Tyrode's solution) at room temperature. The stomach was opened along the lesser curvature, and its contents were rinsed with the solution. The gastric fundus was cut into strips with an approximate length of 10 mm and width of 3 mm, respecting the direction of the longitudinal smooth muscle, with a maximum of two strips from each mouse's stomach. To obtain duodenal strips, the duodenum was cut into approximately 10 mm cylindrical segments. The gastric and duodenal strips were set up under 1 g tension in a 5 ml tissue bath filled with Tyrode's solution. Isometric contractions were recorded using an isometric transducer coupled to an acquisition system (PowerLab, AD Instruments, Bella Vista, Australia). The Tyrode solution with the tissues was continuously maintained at 37 °C and bubbled with a carbogen mixture (5% CO_2 in O_2) (Silva et al., 2015).

The gut strips were prompted to contract in response to a contractile stimulus consisting of a high potassium ion (K^+) concentration (60 mM). In the steady state of a given sustained contraction, concentration-effect curves were obtained by exposing preparations to increasing concentrations of THERM (0.1–1000 μg/ml), which was added cumulatively to the organ bath (5 min for each concentration). Control preparations received only the vehicle (NaCl 0.15 M) for an identical experimental interval.

2.7. Assessment of the influence of serotonergic pathways on food intake behavioral changes induced by THERM

The animals were kept in individual cages for fasting for 18 h, during which each mouse had free access to filtered water. Then the animals were randomly treated (i.p.) with vehicle (NaCl 0.15 M, 0.1 ml), ondansetron (ONDA, 50 μg/kg) or *p*-chlorophenylalanine (PCPA, 100 mg/kg), administered once daily during three days (total of 300 mg/kg of PCPA). After 30 min, all animals were treated orally with NaCl 0.15 M (0.1 ml) or THERM 100 mg/kg. Thirty minutes later vehicle or THERM treatments, was offered 30 g of standard pellet feed (Presence Rats and Mice® - Agribands Purina do Brasil Ltda, containing 2.93 kcal/g of metabolizable energy, 20.5% crude protein and 7% crude fat on a dry matter basis) and food intake was recorded over a period of 12 h (between 9:00 a.m. and 9:00 p.m.). Food intake was calculated by subtracting the amount of food remaining in the hopper after 4 h and 12 h, adjusted for the amount of spillage (Sutton et al., 2008; de Oliveira et al., 2019).

2.8. Statistical analysis

Data are presented as mean ± standard error of mean (SEM) of each group (n = 5–7 mice). Inhibitory concentration (IC_{50}) values of THERM were calculated by interpolation from semi-logarithmic plots, reported as geometric means (95% confidence interval). For multiple-group comparison, one-way analysis of variance (ANOVA) followed by the Tukey test was used. *In vitro* data were compared using two-way ANOVA followed by the Holm-Sidak test, as appropriately indicated. Values of $P < 0.05$ were considered statistically significant. All the statistical analyses were performed using the Prism 6.0 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Chemical composition profile of THERM by nuclear magnetic resonance (NMR)

In the present study, we observed three singlets in the low field (3.41–4.00 ppm) and one singlet in the aromatic region (7.51 ppm) in the THERM ^{1}H NMR spectrum (Table 1).

For low field signals, the integration area was compatible with the presence of methyl protons for all resonances indicated in that region, while the integration area for the singlet at 7.51 ppm was compatible with aromatic hydrogens. The ^{13}C NMR spectrum also showed low field spectral lines (27.95–33.59 ppm), compatible with the presence of methyl groups, signals of aromatic carbons (107.64–148.75 ppm) and two signals of carbonyl groups (151.76 and 155.76 ppm) (Table 1). The data confirmed the presence of caffeine (Fig. 1) as the main chemical compound in THERM.

Table 1
NMR spectroscopic data of THERM (δ ppm, $CDCl_3$).

^{1}H	^{13}C	^{1}H–^{13}C HMBC	^{13}C (HMDB)[a]
3.41. *s*. 3H	27.93	C-2 and C-6	27.88
3.58. *s*. 3H	29.75	C-2 and C-5	29.70
3.99. *s*. 3H	33.58	C-4 and C-8	33.57
-	107.64	-	107.51
7.51. *s*. 1H	141.39	C-4. C-5 and C-14	141.57
-	148.75		148.67
-	151.76		151.66
-	155.46		155.32

[a] Human Metabolome Database (2018).

3.2. Effects of THERM on the percentage of gastric dye emptied by the stomach in awake mice

The amount of the administered dye content emptied by the stomach was significantly lower ($P < 0.05$, Fig. 2) in mice that were previously treated with THERM 100 mg/kg (30.5 ± 3.0%) or THERM 300 mg/kg (30.7 ± 2.7%) than in mice that were treated only with vehicle (50.2 ± 2.6%, control group), THERM 10 mg/kg (50.1 ± 4.0%) or THERM 50 mg/kg (52.1 ± 3.4%).

3.3. Mechanism involved in GE delay induced by THERM

In relation to the respective control group, hexamethonium prevented ($P > 0.05$) the reduction of GE induced by THERM (53.7 ± 3.0 vs. 44.0 ± 2.8%, Fig. 3). On the other hand, in relation to their control groups, respectively, neither prazosin (55.1 ± 5.9 vs 36.6 ± 3.5%) nor propranolol (54.0 ± 2.8 vs. 25.2 ± 1.5%) prevented ($P < 0.05$) that THERM-induced GE delay (Fig. 3). There are no differences ($P > 0.05$) in fluid GE in animals treated with THERM, prazosin + THERM or propranolol + THERM (30.5 ± 3.0%, 36.6 ± 3.5% and 25.2 ± 1.5%, respectively).

Moreover, PCPA prevented the THERM-induced delay in fluid GE (46.2 ± 5.8 vs. 50.9 ± 4.4%, Fig. 4). In addition, Fig. 4 shows that ondansetron also prevented the reduction of THERM-induced GE (43.9 ± 2.9 vs. 57.4 ± 3.8%). It is worth mentioning that the GE was increased (Fig. 4, $P < 0.05$) in animals pretreated PCPA + THERM and ondansetron + THERM (50.9 ± 4.4% and 57.4 ± 3.8%, respectively) in relation animals treated only with THERM alone (30.5 ± 3.0%).

3.4. Effects of THERM on gastrointestinal contractility in vitro

In the gastric fundus (Fig. 5A) and duodenum (Fig. 5B), THERM in a concentration range of 0.1–1000 μg/ml inhibited basal contractions. The median inhibitory concentration (IC_{50}) of THERM in the fundus was 177.7 (130.4–242.0) μg/ml, while in the duodenum it was 34.4 (3.0–394.1) μg/ml.

Fig. 5C shows that when compared to the contraction induced by KCl 60 mM/l in fundic strips, the maximal relaxation values were −84.6 ± 11.1% in response to THERM 1000 μg/ml, and in duodenal strips this relaxing effect was −22.0 ± 4.7%, (Fig. 5D).

3.5. The effect of THERM on food intake of mice in vivo

THERM 100 mg/kg increased ($P < 0.05$) food intake compared to the control group at 4 h (511.6 ± 113.5 vs. 1096.0 ± 110.4 mg/g body weight, Fig. 6A) and 12 h (5135.0 ± 299.00 vs. 6715.0 ± 435.3 mg/g body weight, Fig. 6B). Moreover, PCPA prevented ($P > 0.05$) the THERM-induced increase in food intake at 4 h (1625 ± 219 vs. 2013 ±

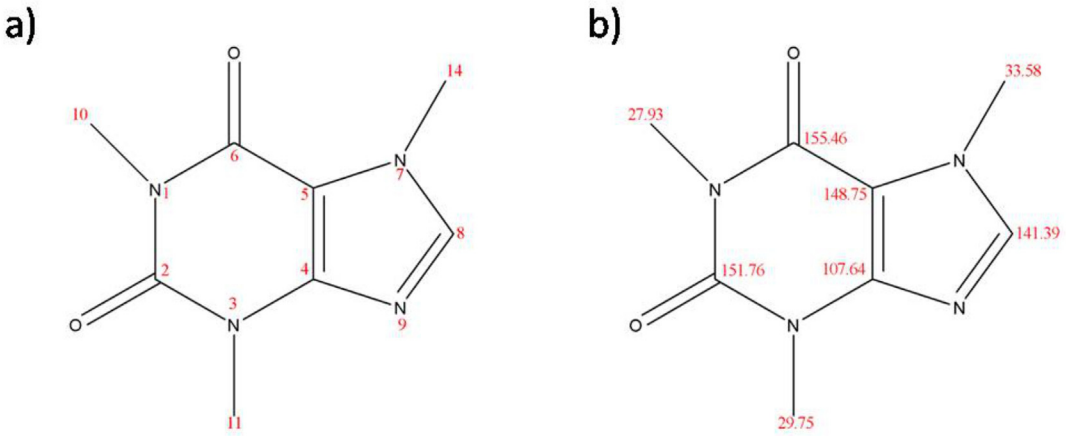

Fig. 1. Majority chemical compound of THERM (caffeine). a) Molecule enumeration; b) ^{13}C NMR shifts at $CDCl_3$ in ppm.

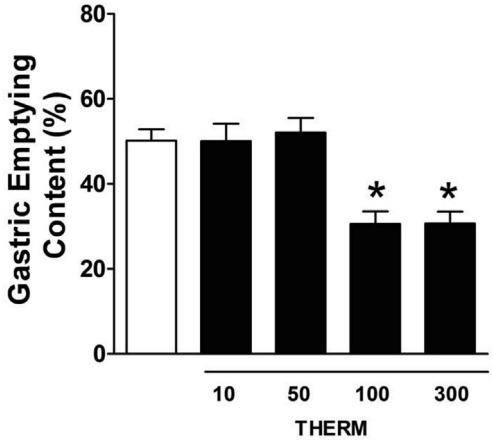

Fig. 2. Effects of NaCl 0.15 M (▫) or THERM (■ - 10, 50, 100 or 300 mg/kg, experimental group) on the percentage of gastric dye emptied by the stomach in awake mice. Each subset consisted of 6 mice. $^{*}P < 0.05$, compared with respective control group (ANOVA followed by Tukey test).

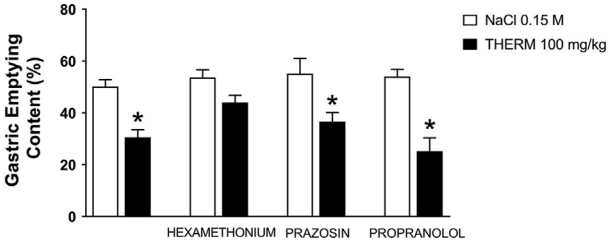

Fig. 3. Effects of autonomic nicotinic and adrenergic pathways on the percentage of gastric dye emptied by the stomach in awake mice treated with NaCl 0.15 M (▫) or THERM (■). Comparison of effects of pretreatment with hexamethonium (10 mg/kg), prazosin (0.2 mg/kg) or propranolol (2 mg/kg). Each subset consisted of 6 mice. $^{*}P < 0.05$, compared with respective control group (ANOVA followed by Tukey test).

358.9 mg/g body weight, Fig. 6A) and 12 h (7988 ± 991 vs. 7474 ± 541 mg/g body weight, Fig. 6B). In addition, as shown Fig. 6A, ondansetron also prevented ($P > 0.05$) the increase of THERM-induced food intake (1506 ± 258.2 vs. 1520 ± 179.5 mg/g body weight) at 4h and 12 h (7410 ± 687.7 vs. 6707 ± 411.3 mg/g body weight, Fig. 6B). However, compared with control, PCPA and ondansetron pretreatment increased ($p < 0.05$) food intake at 4 h (Fig. 6A), this PCPA effects remains during 12 h (Fig. 6B).

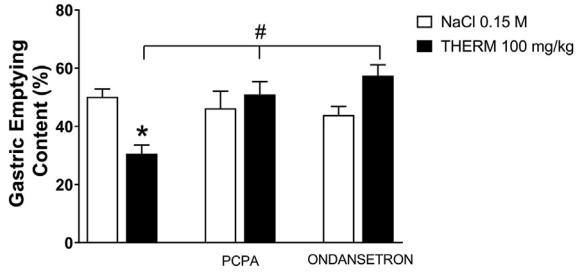

Fig. 4. Effects of serotonergic pathways on the percentage of gastric dye emptied by the stomach in awake mice treated with NaCl 0.15 M (▫) or THERM (■). Comparison of effects of pretreatment with ρ-chlorophenylalanine (PCPA, 300 mg/kg) or ondansetron (50 μg/kg). Each subset consisted of 6 mice. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$, vs. group THERM-100 mg/kg only (ANOVA followed by Tukey test).

4. Discussion

Thousands of people self-medicate with dietary supplements containing unknown quantities of pharmacologically active compounds. These poorly regulated substances can cause real harm to people (Brooks et al., 2016). The monitoring of these products depends directly on the available analytical methods to detect the presence of adulterant substances in various types of samples (Viana et al., 2016). In addition, the diversity of the composition and presentation of the nutritional supplements available in the market impose the use of diverse and sometimes complex preparation/extraction steps of the analytes for the analysis of the real composition of samples, e.g., by high-performance liquid chromatography coupled to diode array detection (HPLC-DAD), gas chromatography-mass spectrometry (GC-MS) or nuclear magnetic resonance (NMR) analysis (Viana et al., 2016; Neves and Caldas, 2017; Zhao et al., 2018).

NMR, a research tool traditionally used for structural elucidation, is now being used frequently for metabolomics and chemical fingerprinting. Its stability and inherent ease of quantification have been exploited extensively to identify and quantify bioactive components in foods and sports nutritional supplements (Ramakrishnan and Luthria, 2017; Zhao et al., 2018; Okaru et al., 2020).

A similar profile as the THERM ^{1}H NMR spectrum was obtained by del Campo et al. (2010). In present study, they attributed the low field signals to the *N*-methyl groups and the signal at 7.83 ppm to the aromatic hydrogen from caffeine molecules. The 1D and 2D spectral analysis (Table 1) as well as the similarity of the spectral data of THERM ^{1}H NMR with literature data confirmed the presence of caffeine (Fig. 1) as the main chemical compound in THERM. Powdered pure caffeine and

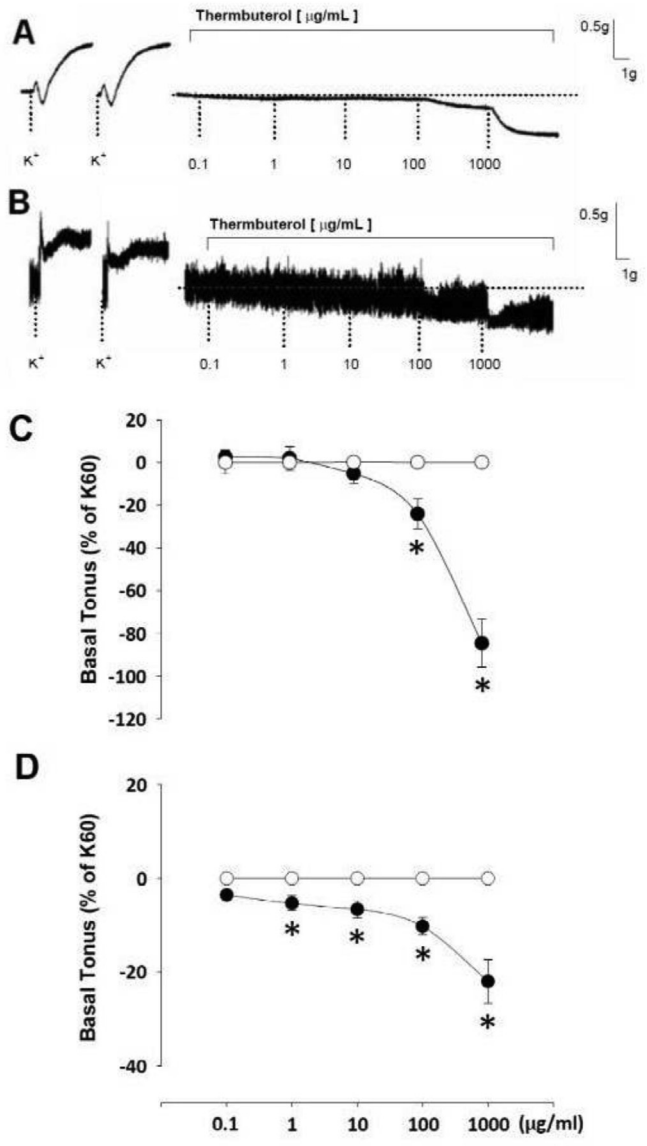

Fig. 5. Effects of THERM (●) or NaCl 0.15 M (○) on gastrointestinal contractility *in vitro*. Effect of the cumulative addition of THERM [0.1–1000 µg/mL] on spontaneous tonus and contractions induced by KCl (60 mM/L) in strips of (A) stomach fundus and (B) duodenum. (C) Concentration-effect curves of the responses induced by 100 µg/l THERM (•, black circle) or Vehicle (◦, white circle) on stomach fundus contractility or duodenum contractility (D). Results are expressed as percent change of the initial KCl contraction and are shown as mean and SEM (n = 6). $^{*}P < 0.05$, compared with respective control group (NaCl 0.15 M) after two-way ANOVA followed by the Holm-Sidak test for multiple comparisons.

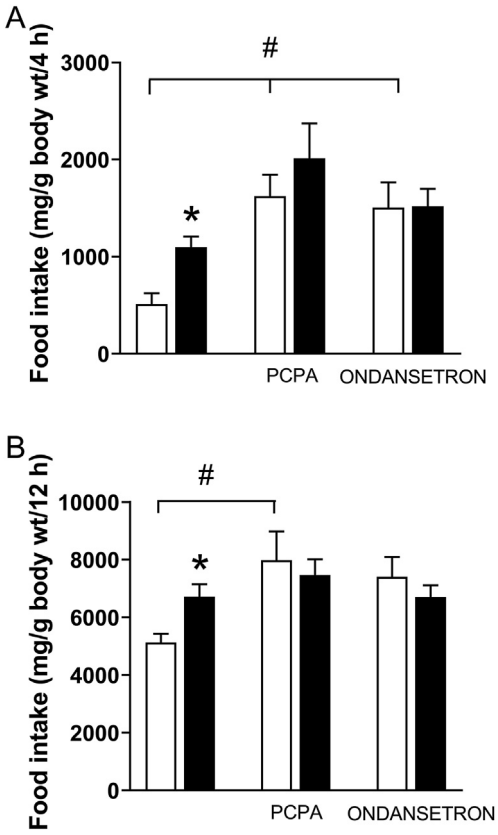

Fig. 6. Effects of serotonergic pathways on the cumulative solid food intake during 4 h (Figs. 6A) and 12 h (Fig. 6B) in awake mice treated with NaCl 0.15 M (▫) or THERM (■). Comparison of effects of pretreatment with ρ-chlorophenylalanine (PCPA, 300 mg/kg) or ondansetron (50 µg/kg). Each subset consisted of 6 mice. $^{*}P < 0.05$, compared with respective control group (ANOVA followed by Tukey test). $^{\#}P < 0.05$, vs. control group (vehicle) only (ANOVA followed by Tukey test).

adrenergic amines are also likely to be added intentionally as adulterants in products for physical fitness and weight loss. In Brazil, caffeine has been found in other nutritional supplements marketed for weight loss and physical fitness (Viana et al., 2016). Caffeine is, perhaps, the most researched thermogenic or ergogenic substance, and has been reported to significantly increase total daily energy expenditure, facilitate body fat reduction, improve alertness, and enhance physical performance (Jo et al., 2016; Ratamess et al., 2016; Clark et al., 2019; Kliszczewicz et al., 2019).

In fact, when taken regularly, caffeine has several performance-enhancing benefits. But in humans, excessive amounts of caffeine (>2000 mg) can give rise to significant toxic effects, including tachycardia, severe hypertension, arrhythmia, seizures, nausea, vomiting, and even death. However, individuals sensitive to caffeine can exhibit adverse effects at lower doses (Miyata et al., 2020; Duncanson et al., 2018).

Considering that some studies have reported the influence of estradiol and progesterone on gastric motility and intestinal transit (Chen et al., 1995; Heitkemper et al., 2002), an ovariectomy was performed on the females in this study (Souza et al., 2019). Then, to evaluate the effect of THERM on the gastrointestinal motility of mice, we analyzed the functionality of the gastrointestinal tract by *in vivo* and *in vitro* assessment, as described by Silva et al. (2015). We show the amount of the administered dye content emptied by the stomach, measured 10 min after the administration of a dye-marked liquid test meal, was lower (Fig. 2) in mice that were previously treated with THERM 100 mg/kg or THERM 300 mg/kg, suggesting that THERM 100 and 300 mg/kg induced GE delay.

Caffeine is generally known as a stimulant of gastric secretion (Liszt et al., 2017), and the increase in gastric secretion may be related to decreased THERM-induced GE (Hunt and Knox, 1972; Cooke, 1974). However, in this study, in relation to the animals treated with THERM (100 mg/kg), pretreatment with omeprazole (20 mg/kg), a proton pump (H^+,K^+-ATPase) inhibitor, did not affect the reduction of THERM-induced emptying (30.5 ± 3.0% vs. 35.5 ± 6.3%, $P > 0.05$). Moreover, as described by Kamiya et al. (2011), we found that pretreatment with omeprazole did not interfere in GE (50.2 ± 2.6% vs. 56.6 ± 3.6%, $P > 0.05$). These results indicate that the GE delay induced by THERM is not related to the gastric secretion rise, suggesting that the oral administration of THERM at 100 mg/kg induces some gastrointestinal dysmotility in mice.

Based on the findings described above, we investigated the possible role of some classic neurotransmitters involved in the inhibition of gastric motility induced by THERM, we found the autonomic nicotinic blocker hexamethonium prevented the reduction of GE induced by THERM (Fig. 3), indicating participation of the autonomic nicotinic ganglion in this delay of GE induced by THERM. The synaptic endings of the autonomic nervous system in the gastrointestinal tract release parasympathomimetic agents responsible for increasing of intestinal motility, such as acetylcholine. On the other hand, stimulation of the sympathetic terminals releases noradrenaline, which decreases intestinal contractility (Hansen, 2003).

To assess the possible participation of the adrenergic pathway in this study, we examined the effect of adrenergic receptor antagonists on THERM-induced GE delay. We found that blockade of α_1 and $\beta_{1,2}$ receptors did not prevent that THERM-induced GE delay (Fig. 3). These results indicate that the decrease in liquid GE induced by THERM is not mediated by α_1, β_1 or β_2-adrenergic receptors, suggesting that 5-HT is involved in THERM-mediated GE delay.

Serotonin modulates gastric motility via a variety of 5-HT receptor subtypes. Regional and functional differences among 5-HT receptor subtypes can trigger contraction or relaxation of gastrointestinal motility (Komada and Yano 2007; Mawe and Hoffman, 2013; McLean et al., 2007). Other studies have found that pharmacological 5-HT_3 receptor antagonism and 5-HT_4 receptor agonism stimulate GE in rats (Ito et al., 1996; Yamano et al., 1997; Tonini, 2005; McLean et al., 2007). In the present study, PCPA, a competitive inhibitor of the enzyme tryptophan hydroxylase (responsible for the conversion of *L*-tryptophan into 5-HT), prevented the THERM-induced delay in fluid GE (Fig. 4).

Many actions attributable to the 5-HT_3-receptor have been described in both the peripheral and central nervous systems, and clinical trials have shown the potential use of these 5-HT_3 receptor antagonists to treat a number of disorders of the gastrointestinal tract and central nervous system (McLean et al., 2007; Coates et al., 2017). The activation of the 5-HT_3-receptor is involved in gastrointestinal transit delay (Lin and Chen, 2003). The 5-HT_3 receptor antagonist, ondansetron, enhanced GE in conscious rats and prevented GE delay induced by cisplatin (Miyata et al., 1995). Ondansetron prevented gastric relaxation induced by intraduodenal infusion of glucose, an effect related to the role of 5-HT in mediating intestinal feedback inhibition of GE (Raybould et al., 2003). The present finding was the ondansetron also prevented the reduction of THERM-induced GE (Fig. 4). Interestingly, the ondansetron binding 5-HT_3 receptors in the rat vagus nerve and cerebral cortex (Ito et al., 1995) and ondansetron 30 μM both reduced gastric relaxation induced by vagal stimulation in the isolated stomach of guinea pigs (Desai et al., 1994), indicating possible autonomic correlation between the vagus nerve and 5-HT_3 receptors, so that hexamethonium (autonomic nicotinic blocker) and ondansetron (5-HT_3 antagonist) possibly impaired the present THERM's effects on GE in mice. Thus, we hypothesize that such phenomenon may involve autonomic nicotinic receptors and 5-HT_3 serotonergic pathways.

The tone of the proximal stomach influences the flux of liquids through the gastroduodenal junction (Jucá et al., 2011). It is plausible that THERM modulates the smooth muscle contractility of the gastrointestinal tract. In relation of the *in vitro* studies, we observed that THERM inhibited basal contractions of the gastric fundus and duodenum (Fig. 5). Such decreases in the contractility of the gastric fundus and duodenum induced by THERM can contribute to the reduction of GE of liquids (Kelly, 1980).

In addition to controlling GE, hypothalamic serotonergic neurons are involved in controlling food intake (Costall et al., 1986; Serrano et al., 2011). For this reason, the 5-HT signaling pathway in the central nervous system is the target of drugs designed to combat obesity. These drugs influence GE and increase thermogenesis and satiety, helping to increase the weight loss mediated by dietary supplements (Saraç et al., 2006; Xu and Chen, 2008; McGlashon et al., 2015).

In the present study, THERM increased food intake at 4 h and 12 h (Fig. 6). This effect of THERM on solid food intake was prevented by treatments with PCPA or ondansetron in both periods. Moreover, we found that the PCPA and ondansetron pretreatment increased food intake (Fig. 6A). These findings suggest that 5-HT_3 receptors are involved in mediating the anorexigenic activity of 5-HT by controlling feeding behavior (Leon et al., 2019; Holt et al., 2017; Li et al., 2015; Hammer et al., 1990). In fact, sibutramine, an inhibitor of serotonin-noradrenaline reuptake, reduces food intake and increases GE (Halford et al., 1995; Xu and Chen, 2008). Considering that when stimulated the 5-HT_3 receptors participate in positive feedback, increasing the release of 5-HT (Schwörer and Ramadori, 1998), and that *Citrus aurantium* and/or caffeine increase the biosynthesis of 5-HT (Jiang et al., 2014; Jaffe et al., 2004), we do not rule out the possibility that THERM's effect is mediated by 5-HT-like activity in 5-HT_3 type receptors, explaining the blocking of THERM effects after using PCPA or ondansetron in mice. Future studies should be conducted to investigate the long-term effects of THERM on food consumption and body weight.

Methylxanthines and adrenergic stimulants, such as synephrine and caffeine, are commonly added to nutritional supplements due to their stimulation of metabolism, thermogenesis and energy expenditure, effects that contribute to the desired weight loss (Ratamess et al., 2016; Clark et al., 2019; Kliszczewicz et al., 2019). However, caffeine, due to its activity in the intracellular mobilization of calcium, inhibition of phosphodiesterases and antagonism at adenosine receptors, triggers a series of effects in the central nervous system, such as the release of neurotransmitters like norepinephrine, dopamine and serotonin in regions of the central nervous system responsible for controlling gastrointestinal motility and food intake (Nehlig et al., 1992; Okada et al., 1999; Goitia et al., 2016). Since caffeine was found to be the main constituent of THERM, a pilot study was carried out, and we found that in relation to the control group, caffeine at 30 mg/kg decreased liquid GE (50.2 ± 2.6 vs. 22.9 ± 2.8%, $P < 0.05$, ANOVA followed by the Tukey test), including an intensity of reduction in GE similar to the values found with THERM 100 mg/kg ($P > 0.05$). In adult mice, caffeine decreased the gastric fundus smooth muscle basal tone to ~85% of the response obtained with sodium nitroprusside (Kim et al., 2005). Caffeine also decreased the basal tone and amplitude of phasic smooth muscle contractions in the gastric antrum of adult mice (Kim et al., 2008a). These effects may be related with GE delay. In fact, in rats caffeine induces GE delay of liquid and reduces esophageal, gastric and intestinal muscle tone (Welsh et al., 2015). The resulting gastrointestinal dysmotility may be due to the fact that caffeine diminishes slow waves of the gastric corpus circular muscle and of interstitial cells of Cajal from jejunum. Slow waves are important physiologically because they impose a periodic depolarization/repolarization cycle on membrane potentials of smooth muscle cell alterations (Hashitani et al., 2005; Jin et al., 2009). These results support that caffeine present in THERM can inhibit gastrointestinal motility in mice. Caffeine can also stimulate the local release of 5-HT, an effect described in mesenteric mast cells (Jaffe et al., 2004). Thus, the involvement of a serotonergic pathways in the present phenomenon should be considered.

Despite the limitations of the present study, mainly because THERM is a substance with several ingredients, the gastrointestinal effects described here cannot be attributed only to caffeine, since the activity of other components, such as *Citrus aurantium* L. and/or synephrine, can play a role a role in spasmolytic activities (Fang et al., 2009; Ahangarpour et al., 2011; Wu et al., 2016). Despite this, there are significant indications that this thermogenic agent has strong effects on gastrointestinal function, which can culminate in possible signs of dyspepsia in individuals who use this product.

5. Conclusions

THERM starting at a dose of 100 mg/kg reduced liquid gastric emptying, an effect mediated by autonomic nicotinic receptors and 5-HT_3 serotonergic pathways. THERM also decreased gastric and duodenal contractility *in vitro*. Moreover, THERM increased the food intake mediated by the 5-HT_3 serotonergic pathways. Caffeine was the major component of THERM and it decreased gastric emptying delay.

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CRediT authorship contribution statement

Luciano N. de Sousa: conceived the experimental design, conducted the experimental assays. **Débora S. Paraguassú Sant'ana:** conducted the experimental assays. **Rildo G. Siqueira dos Santos:** conducted the experimental assays. **Anita Eugênia A. dos Santos Ribeiro:** conducted the experimental assays. **Camila F. da Costa:** conducted the experimental assays. **Ana Paula de Oliveira:** Formal analysis, assisted in the NMR analysis, Writing - original draft, drafted the manuscript and revised it for important intellectual content. **Jackson Roberto G. da Silva Almeida:** conceived the experimental design, Formal analysis, assisted in the NMR analysis, Writing - original draft, drafted the manuscript and revised it for important intellectual content. **Davi M. Jucá:** conceived the experimental design, Writing - original draft, drafted the manuscript and revised it for important intellectual content. **Moisés Tolentino:** conceived the experimental design, Writing - original draft, drafted the manuscript and revised it for important intellectual content. **Armênio A. dos Santos:** Writing - original draft, drafted the manuscript and revised it for important intellectual content. All the authors have read and approved the final version of the manuscript. **Raimundo C. Palheta Junior:** conceived the experimental design, conducted the experimental assays, Writing - original draft, drafted the manuscript and revised it for important intellectual content.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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