Research Article

Satietogenic Protein from Tamarind Seeds Decreases Food Intake, Leptin Plasma and *CCK-1r* Gene Expression in Obese Wistar Rats

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Keywords

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Abstract

Objective: This study evaluated the effect of a protein, the isolated Trypsin Inhibitor (TTI) from *Tamarindus indica* L. seed, as a CCK secretagogue and its action upon food intake and leptin in obese *Wistar* rats. **Methods:** Three groups of obese rats were fed 10 days one of the following diets: Standard diet (Labina®) + water; High Glycemic Index and Load (HGLI) diet + water or HGLI diet + TTI. Lean animals were fed the standard diet for the 10 days. Food intake, zoometric measurements, plasma CCK, plasma leptin, relative mRNA expression of intestinal CCK-related genes, and expression of the *ob* gene in subcutaneous adipose tissue were assessed. **Results:** TTI decreased food intake but did not increase plasma CCK in obese animals. On the other hand, TTI treatment decreased *CCK-1R* gene expression in obese animals compared with the obese group with no treatment ($p = 0.027$). Obese animals treated with TTI presented lower plasma leptin than the non-treated obese animals. **Conclusion:** We suggest that TTI by decreasing plasma leptin may improve CCK action, regardless of its increase in plasma from obese rats, since food intake was lowest.

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Introduction

Obesity is considered a worldwide epidemic, characterized by excessive accumulation of body fat due to hyperplasia and/or hypertrophy of fat cells [1]. For the complex genetic-environmental etiology, obesity has a range of specific pathological consequences. It can cause disorders in the hormonal metabolism, with the appearance of most different diseases such as arterial hypertension, atherosclerosis, carcinomas, type 2 diabetes, obesity hypoventilation syndrome, pulmonary embolism, heart failure, infertility, and propensity for falls [2, 3].

A close relationship between diet and the hormonal metabolism is involved in the control of hunger and satiety [4]. The presence of food in the gastrointestinal tract contributes not only to appetite modulation but also to the regulation of energy intake and metabolism, mediated by various hormones. In this sense, the gastrointestinal tract presents different peptide-secreting cells. Among the important peptides secreted by these cells is pre-pro-cholecystikinin (pre-pro-CCK), which by the action of the serine endopeptidase proprotein convertase subtilisin/kexin (PCSK) transforms it into its different active forms [5, 6].

The active forms of CCK differ in the number of amino acids in their structure and are found in the plasma, gastrointestinal tract, and brain [6–8]. They act at these sites by means of specific receptors, such as type 1 CCK receptor (CCK-1R or CCKAR) in the gastrointestinal tract, and type 2 CCK receptor (CCK-2R or CCKBR) in the brain [9]. The active isoforms of CCK, when binding to the CCK-1R present in the intestine and vagal afferent nucleus (VAN), send signals of activation via the vagus nerve to the nucleus of the solitary tract, thus activating the anorectic neuropeptides α -MSH and CART that induce the sensation of satiety [9].

Another hormone involved in modulating appetite in the long term is leptin, which is secreted by adipocytes, is responsible for the control of food intake via acting on neuronal cells of the hypothalamus. This action of leptin, as well as that of CCK, promotes the reduction of food intake and the increase in energy expenditure, thus also regulating neuroendocrine function and metabolism of carbohydrates and fats [10].

The actions of CCK and leptin have been studied in satiety, with research demonstrating that the effects of these hormones may vary when considering eutrophic and obese experimental models or humans [11, 12].

McLaughlin et al. [13–15], in their satiety studies, have shown that the increase in food intake of obese *Zucker* rats might probably be due to CCK resistance. Concerning leptin, although obese individuals present high concentrations of this hormone since it is directly proportional to the number of adipocytes, no reduction in appetite was observed, which also suggests a resistance condition [16].

Studies have found that CCK also has synergistic and functional activity in the secretion of other hormones, causing increase or inhibition of important peptides related to satiety, such as leptin or ghrelin, thus raising satiety [17, 18]. Additionally, in obesity, it was observed that the increase in plasma leptin concentrations caused a reduction in the sensitivity of CCK receptors in the VAN [19].

In addition, Barrachina et al. [20] and Matson et al. [21] suggest a functional synergistic interaction between leptin and CCK, and strategies to reduce the effects of leptin in resistant obese individuals seem to be a way to improve the action of CCK in reducing weight gain.

Experimental models have been used to study obesity, especially those with genetically susceptible animals [22, 23]. Although genetic mutations cause obesity in animals, obesity in humans is not only genetically determined [24]. In this sense, diet-induced obesity models (DIO) may have a great value, given its similar etiology to human obesity [25, 26].

As hormones discussed above present a clear relationship with the genesis of obesity, studies with molecules that act contributing to their better functioning are essential. Bioactive proteins, such as trypsin enzyme inhibitors, have been studied, showing beneficial health

effects related to the modulation of protein-mediated responses [27]. In nutrition, studying the anti-obesity and/or satiety potential of substances or molecules present in food is of great importance, given the relevance that obesity presents for human health.

Recent research by our group demonstrated that an isolated trypsin inhibitor from *Tamarindus indica* L. seed (TTI) presents anti-obesity and anti-inflammatory activities [28, 29]. Ribeiro et al. [28] analyzed the influence of TTI in an experimental model of satiety and found that the inhibitor was able to reduce food intake and weight gain of eutrophic animals, possibly by raising plasma CCK. Carvalho et al. [29] tested TTI activity in obese animals with metabolic syndrome and found that there was a decrease in food consumption and tumor necrosis factor alpha (TNF- α), without reduction of weight gain; however, plasma CCK was not analyzed.

The classic study by Spannagel et al. [30] described pancreatic proteases that inhibit this CCK secretion in the intestine due to proteolytic inactivation of a CCK-releasing peptide. The trypsin-sensitive luminal CCK-releasing factor (LCRF) functions as an intraluminal regulator of intestinal. Thus, when pancreatic proteases are inhibited, LCRF remains active and stimulates CCK secretion.

Apart from these, few types of research attempted to analyze the effect of substances upon CCK in the context of obesity treatment, evaluating their features in other important hormones involved in the control of hunger and satiety. Studies with trypsin inhibitors that act as CCK secretagogues have confirmed their action on peripheral and central appetite control only in eutrophic conditions [28, 31–34].

In this study we tested the effect of TTI, a recognized CCK secretagogue in lean animals [28, 31–34], on food intake, nutritional status assessment, expression of intestinal CCK-related genes, and CCK plasma concentrations as well as on the expression of *ob* gene and leptin plasma concentrations in obese rats.

Material and Methods

Tamarind Seeds

Tamarind was obtained and botanically identified by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) seed bank in Natal, RN, Brazil, and its seeds were obtained by removing them from the interior of the fruit. The seeds were kept at ambient temperature (23–25 °C) and humidity of 50 $\pm$ 5%.

Animals

Male adult *Wistar* rats, weighing around 400 g, obese according to the Lee Index (≥ 300) [35] comprise the experimental group. Obesity was previously induced using the high glycemic index and load diet (HGLI diet) in rats with 4 weeks of age for 17 weeks. Male adult lean *Wistar* rats were used as controls in the experiment. All animals were kept under standard lighting conditions (12 h light / 12 h dark); temperature (23–25 °C) and humidity (50 $\pm$ 5%); receiving water and food ad libitum.

Experiments were performed in accordance with the Laboratory Animals Care and Use Guide of the National Institutes of Health and after approval by the Ethics Committee on the Use of Animals of Potiguar University (CEUA – UnP) under the protocol number 012/2015.

Diets

Diets used in this study were Labina produced by Purina®, and the HGLI diet – glycemic index 77.6; glycemic load 38.8 – was produced by our group using condensed milk, sugar and Labina® (1:0.2:1), according to the methodology described by Carvalho (unpublished results). The HGLI diet was the same used to induce and maintain obesity in the experimental animals.

TTI Isolation

The methodology was established in the Laboratory of Chemistry and Function of Bioactive Proteins (LQFPB) at Federal University of Rio Grande do Norte State, Brazil, and adapted by Carvalho et al. [29].

Tamarind seeds were peeled from the cotyledon and fine-grained to a flour (40 mesh). Then, the protein fractions were extracted using different saturation ranges (F1: 0–30%; F2: 30–60%; F3: 60–90%) of ammonium sulfate. The protein fraction saturated at 30–60% of ammonium sulfate (F2), presented the highest anti-tryptic activity and was subjected to an enrichment process by affinity chromatography on a trypsin-sepharose column (GE Health Care, Waukesha, WI, USA), according to the methodology described by Carvalho et al. [29].

Protein fractions obtained by affinity chromatography were dialyzed against water, lyophilized, and subjected to trypsin inhibition assays, using 1.25 mmol/l BAPNA as substrate, as described by Kakade et al. [36]. Protein was determined according to Bradford [37] using bovine serum protein (BSA) as standard.

The electrophoresis was performed to confirm the isolation of TTI as described by Laemmli [38]. The gel was subjected to a constant voltage with 50 V in the first hour; and after that, voltage was increased to 150 V until the end of the run. The gel was then submerged in a solution of distilled water (30 mL), acetic acid 10% (5 mL), and methanol (20 mL). After 30 min, it was withdrawn from the fixative solution and stayed overnight in a dye solution composed of methanol (10 mL) and colloidal Coomassie Blue (40 mL).

Experimental Design

Obese *Wistar* rats were individually and randomly distributed into three groups ($n = 5$). Additionally, five lean *Wistar* rats were selected to compose a fourth group. All groups had five days of adaptation to establish the conditions of the experiment and pattern of food consumption of each animal. On the first and last day of the experiment, nutritional status assessment (Lee index) and food intake were evaluated. Animals were then submitted for 10 days to one of the following diets and treatments:

1. Lean group/standard diet: lean animals receiving standard diet + 1 mL of water by gavage.
2. Obese/HGLI diet: obese animals receiving HGLI diet + 1 mL of water by gavage, i.e. obese animals with no treatment;
3. Obese/standard diet: obese animals receiving standard diet + 1 mL of water by gavage, i.e. obese animals with standard treatment;
4. Obese/HGLI diet plus TTI: obese animals receiving HGLI diet + 1 mL of TTI (25 mg/kg) by gavage i.e. obese animals receiving TTI as treatment.

All diets were orally given to animals, while water and TTI were administered by oral gavage at the same time over a period of 10 days. Animals fasted 6 h before oral administration of diets. On the 11th day of the experiment, animals fasted for 8–12 h before the diet was given and after 1 h from gavage, the rats were anesthetized with 250 mg of tiletamine (hydrochloride) and 250 mg of zolazepam (hydrochloride) and then euthanized for collection of blood, gut, and subcutaneous fat.

Food Intake and Nutritional Status Assessment

Food intake was evaluated 1 h after diet administration. The diets were weighed before and after treatment. Animals fasted 6 h before oral administration of diets. The initial food intake (day 1) for each animal was considered the mean of the 5 days before treatment and the final food intake the mean of the 10 days of the treatment (day 10).

The zoometric measurements for nutritional status assessment were done on the first day of the treatment before the oral administration of the diets and on the last day of treatment 1 h after treatment administration. The Lee index was calculated according to Bernardis and Patterson [35] by the relation between the cubic root of the body weight in grams divided by the naso-anal length in centimeters, with a cut-off value ≥ 300 for the diagnosis of obesity. All measures were conducted by trained researchers, aiming to meet the criteria of scientific authenticity, as recommended by Novelli et al. [39].

Plasma CCK and Leptin Concentrations

Plasma CCK and leptin were determined on the 11th day of the experiment. Blood was collected by the hepatic portal vein, and the plasma was separated by centrifugation at 3,000 rpm for 15 min at 4 °C. Plasma was used for the determination CCK and leptin according to the manufacturers' recommended protocol. CCK was determined by immunoassay with Phoenix[®] Kit (EK-069—04; Phoenix Pharmaceuticals, Inc., Burlingame, CA 94010, USA), while leptin was assayed using the Leptin ELISA Rat Kit (Millipore[®]-EZRL-83K; Merck KGaA, Darmstadt, Germany).

Relative mRNA Expression of Intestinal CCK-Related Genes and Subcutaneous Adipose Tissue ob Gene

The evaluation of relative mRNA expression of intestinal CCK-related genes and the *ob* gene in subcutaneous adipose tissue was done on the 11th day of the experiment.

Fig. 1. Food intake and Lee index of *Wistar* rats in the four groups during the 10-day experiment. **A)** The initial food intake (day 1) for each animal was considered the mean of the 5 days before treatment and the final food intake the mean of the 10 days of the treatment (day 10). Kruskal-Wallis with Dunn's post-hoc test, $p > 0.05$. **B)** The zoometric measurements were done on the first day of the treatment and on the last day. The dashed line indicates the cut-off value for obesity, according to the Lee index (≥ 0.300). Two-way ANOVA with Dunn's post-hoc test, $p > 0.05$. Groups: standard diet + 1 mL of water by gavage (lean/standard diet); high glycemic index and load diet + 1 mL of water by gavage (obese/HGLI diet); standard diet + 1 mL water by gavage (obese/standard diet); high glycemic index and load diet + 1 mL of TTI at 25 mg/kg by gavage (obese/HGLI diet + TTI). HGLI diet: Mixture composed of Labina[®], condensed milk and sugar (1:1:0.21); Standard diet: Labina[®] chow. TTI, *Tamarindus indica* L. seed-isolated trypsin inhibitor.

The first portion of the small intestine and the subcutaneous fat was collected, and total RNA was extracted using TRIzol[®] reagent (Thermo Fisher Scientific, Wilmington, DE, USA), from 100 mg of fresh tissue sprayed in crucibles with purified liquid nitrogen, using the Mini PureLink[®] RNA module (Thermo Fisher Scientific), according to the manufacturer's protocol.

Total RNA quantification was done with 1 μ L of total RNA sample using a NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher Scientific).

cDNA synthesis was performed from the total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol in a MyCycler[™] thermocycler (Bio-Rad, Philadelphia, PA, USA).

RT-qPCR was performed for the following genes: pro hormone convertase-1 (*PCSK1*, Rn00567266_m1), *pre-pro-CCK* (*CCK*, Rn005663215_m1), *CCK-1R* (*CCKAR*, Rn00562164_m1), *CCK-2R* (*CCKBR*, Rn00565867_m1), leptin *ob* (*Lep*, Rn00565158_m1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Rn01775763_g1), all from Thermo Fisher Scientific, using the TaqMan assay. RT-qPCR assays were done using a real-time 7500 fast PCR system (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method normalized for *GAPDH* gene, according to Livak and Schmittgen [40], and results are presented as fold changes compared to the mean values of the lean control group.

Statistical Analysis

The sample size was calculated according to the difference between the treatments considered significant (25%) and the variation coefficient (10%) with a power of 90%. Data were analyzed for normality using the Shapiro-Wilk test. The Lee index variable was parametric, and two-way ANOVA test with Dunn's post-hoc

Table 1. Plasma CCK of the obese and lean Wistar rats in the four studied groups after the 10-day experiment

Groups	Plasma CCK, ng/mL mean (dp)
Lean/standard diet	2.46 (0.15)
Obese/HGLI diet	2.39 (0.19)
Obese/standard diet	2.43 (0.29)
Obese/HGLI diet + TTI	2.71 (0.28)

Groups: Standard diet + 1 mL of water by gavage (lean/ standard diet); high glycemic index and load diet + 1 mL of water by gavage (obese/HGLI diet); Standard diet + 1 mL water by gavage (obese/ standard diet); high glycemic index and load diet + 1 mL of TTI at 25 mg/kg by gavage (obese/HGLI diet + TTI). HGLI diet: mixture composed of Labina®, condensed milk and sugar (1:1:0.21); Standard diet: Labina® chow. TTI, *Tamarindus indica* L. seed-isolated trypsin inhibitor. Kruskal-Wallis test with Dunn's post-hoc test, $p > 0.05$.

test was used to verify differences between studied groups. The food intake, plasma CCK, and leptin variable were nonparametric, and Kruskal-Wallis with Dunn's post-hoc test was used. Differences between the relative mRNA expression were tested using the Mann-Whitney test. Graph Pad Prism, version 5.0 (Graph Pad Software, San Diego, CA, USA), was used for statistical analyses. For all analyses, p values of less than 0.05 were considered significant.

Results

TTI Isolation with Affinity Chromatography on Trypsin-Sepharose

TTI showed 39700.58 IU/mg of protein and 100% inhibition for trypsin. TTI isolation was confirmed by 12.5% SDS-PAGE, with a protein band of approximately 20 kDa.

TTI Effect on the Food Intake and Lee Index

According to Figure 1A, obese animals treated with TTI decreased food intake in a similar way as lean animals. Interestingly, obese animals treated with TTI when compared to those with no treatment, reduced food intake ($p = 0.0127$) but, in all the other groups the food intake is statistically equal. As shown in Figure 1B, lean animals maintained the zoometric measurements during the 10 days of the experiment. All studied animals in the three other groups were obese from the beginning to the end of the experiment. Although not significant, obese animals treated with TTI showed a slight reduction of the Lee index, even if they were fed with the HGLI diet.

TTI Effect on Plasma CCK and Relative mRNA Expression of Intestinal CCK-Related Genes

TTI did not increase plasma CCK in obese animals; Table 1 shows the plasma values of CCK for the four groups analyzed in the study. Relative mRNA expression of the pre-pro-CCK, CCK-2R and PCSK1 genes involved in CCK secretion did not change in response to TTI treatment in obese animals (Fig. 2A, C, D). The relative mRNA expression of CCK-1R significantly decreased in response to TTI in obese animals when compared to obese animals with no treatment ($p = 0.027$) (Fig. 2B). In obese animals, the standard diet also decreased the relative mRNA CCK-1R expression to values even lower than those found in the lean animals ($p = 0.027$) (Fig. 2B). Moreover, the standard diet reduced mRNA pre-pro-CCK in the obese animals when compared to those with no treatment and those that received TTI (Fig. 2A).

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Fig. 2. Relative mRNA expression of genes involved with CCK in the intestine of obese and lean *Wistar* rats in the four studied groups after the 10-day experiment. Groups: standard diet + 1 mL of water by gavage (lean/standard diet); high glycemic index and load diet + 1 mL of water by gavage (obese/HGLI diet); standard diet + 1 mL water by gavage (obese/standard diet); high glycemic index and load diet + 1 mL of TTI at 25 mg/kg by gavage (Obese/HGLI diet + TTI). HGLI diet: mixture composed of Labina®, condensed milk and sugar (1:1:0.21); Standard diet: Labina® chow. TTI, *Tamarindus indica* L. seed Isolated Trypsin Inhibitor. *CCK*, Pre-pro-Cholecystokinin; *PCSK1*, pro protein convertase subtilisin/kexin1; *CCK-1R*, type 1 CCK receptor; *CCK-2R*, type 2 CCK receptor and *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase. Mann-Whitney test was used to compare the studied groups. Letters indicate $p < 0.05$, **a**: comparing with the lean/standard diet group; **b**: comparing with the obese/HGLI diet group; **c**: comparing with the Obese/Standard diet group; **d**: comparing with the obese/HGLI diet + ITT group.

TTI Effect on Plasma Leptin and Relative mRNA Expression of the ob Gene in the Subcutaneous Adipose Tissue

Lean animals in the standard diet presented the lowest mean plasma leptin concentrations, as expected ($p = 0.027$). We observed that 60% of the obese animals receiving TTI ($n = 3$) had very low leptin concentrations under the minimum detection concentration (<0.1 ng/mL). Thus, only two obese animals presented with detectable plasma leptin of 0.2 ng/mL

Table 2. Plasma leptin of the obese and lean *Wistar* rats in the four studied groups after the 10-day experiment

Groups	Plasma leptin, ng/mL mean (dp)
Lean/Standard diet	0.15 (0.06) [#]
Obese/HGLI diet	0.54 (0.11)
Obese/Standard diet	0.48 (0.19) [#]
Obese/HGLI diet + TTI	0.20 ^{##}

[#] $n = 1$ animal under the detection concentration (0.1 ng/mL).
^{##} $n = 3$ animals in this group under the detection concentration (0.1 ng/mL); standard deviation was not calculated. Dunn's post-hoc test indicated significant a difference between the lean/standard diet and obese/HGLI diet. Groups: standard diet + 1 mL of water by gavage (lean/standard diet); high glycemic index and load diet + 1 mL of water by gavage (obese/HGLI diet); Standard diet + 1 mL water by gavage (obese/standard diet); high glycemic index and load diet + 1 mL of TTI at 25 mg/kg by gavage (obese/HGLI diet + TTI). HGLI diet: mixture composed of Labina®, condensed milk and sugar (1:1:0.21); Standard diet: Labina® chow. TTI: *Tamarindus indica* L. seed- isolated trypsin inhibitor. Kruskal-Wallis test with Dunn's post-hoc test, $p = 0.027$.

Fig. 3. Relative mRNA expression of the leptin *ob* gene in subcutaneous fat in obese and lean *Wistar* rats in the four groups studied after 10 days of experiment. Groups: standard diet + 1 mL of water by gavage (lean/standard diet); high glycemic index and load diet + 1 mL of water by gavage (obese/HGLI diet); standard diet + 1 mL water by gavage (obese/standard diet); high glycemic index and load diet + 1 mL of TTI at 25 mg/kg by gavage (obese/HGLI diet + TTI). HGLI diet: Mixture composed of Labina®, condensed milk and sugar (1:1:0.21); Standard diet: Labina® chow. TTI, *Tamarindus indica* L. seed-isolated trypsin inhibitor. Mann-Whitney test was used to compare the studied groups. Letters indicate $p < 0.05$, **a**: comparing with the lean/standard diet group; **b**: comparing with the obese/HGLI diet group; **c**: comparing with the obese/standard diet group; **d**: comparing with the Obese/HGLI diet + ITT group.

of this hormone, which is also very low, but detectable by the test utilized. Thus, comparison of the mean plasma leptin concentrations between studied groups is unfeasible. In contrast, 100% of the obese animals receiving the HGLI diet had plasma leptin above the detection concentrations (Table 2).

There was no significant effect of the tested diets/treatments on the relative mRNA expression of the leptin *ob* gene from subcutaneous fat (Fig. 3). As expected, lean animals presented a significantly lower relative mRNA expression of the leptin *ob* gene when compared to the obese animals with no treatment ($p = 0.021$).

Discussion

Previous studies conducted by our group demonstrated that TTI has a satietogenic and anti-inflammatory effect [28, 29]. We isolated TTI from tamarind seeds and observed the same inhibitory and molecular mass characteristics as reported previously by Ribeiro et al. [28] and Carvalho et al. [29]. We administered TTI to a group of obese animals fed with a HGLI diet and compared this group with obese animals under conventional treatment (standard diet), with obese animals without treatment (maintaining the HGLI diet), and with lean animals receiving standard diet. In the present study, the Lee index, resembling human BMI, was used [35] as a standard for the diagnosis of nutritional status assessment [2].

On the first and last days of the experiment food intake and zoometric measurements of all animals were done. In 10 days of experiment, TTI decreased food intake of obese animals when compared to those with no treatment, confirming the findings of the study of Carvalho et al. [29] who already showed that TTI had reduced food intake even when obese animals were offered an unbalanced diet causing obesity and its associated comorbidities.

In addition, after 10 days we observed that lean and obese animals maintained their previous nutritional zoometric status. However, animals receiving TTI showed a discrete reduction in weight gain, probably due reductions of the food intake, and, consequently, a slight decrease in the Lee index, while animals in the other groups tended to continue increasing weight. Sprague-Dawley rats with diet-induced obesity treated with exogenous CCK-8 showed weight reduction only after 23 days of experimentation [41]. Accordingly, we hypothesize that, with a more extended treatment period, reduction of the Lee index would be more consistent.

In the present study, we also evaluated plasma CCK and the relative mRNA expression of essential genes to its production in order to test if TTI could increase plasma CCK in obese animals, as previously observed in eutrophic animals [28]. However, TTI did not increase plasma CCK in obese animals.

Studies with obese rats have shown that these animals present CCK resistance, whereas eutrophic animals present regular production and response to CCK. Interestingly, although obese animals presented reduced sensitivity to the satietogenic effect of CCK, they maintained the sensitivity to other CCK functions such as gastric emptying and intestinal transit [42, 43]. However, data on CCK secretagogues and mRNA expression of CCK-related genes in obese rats are not yet available.

In the present study, the relative mRNA expression of CCK-related genes was evaluated. We found no effect of TTI on the relative mRNA expression of *PCSK1* and *CCK-2R* genes. Moreover, when analyzing the relative mRNA expression of *CCK-1R*, we observed that obese animals treated with standard diet significantly showed a reduced relative mRNA expression of this gene when compared to the other obese animals. TTI was also able to decrease *CCK-1R* relative mRNA gene expression in obese animals when compared to those with no treatment. To our knowledge, there are no other studies in obese animals investigating genes involved in CCK secretion.

Barrachina et al. [20] suggested the existence of a functional synergistic interaction between leptin and CCK leading to early suppression of food intake involving CCK-1R receptors and activation of capsaicin-sensitive afferent fibers sending signals to paraventricular nucleus of the hypothalamus neurons as part of the central neural pathway underlying the mechanism of action of leptin-CCK interaction.

These results suggest that the CCK-1R receptor in the intestine may also be involved in leptin-CCK-induced food suppression as previously established for the CCK-1R receptor in vagal afferent fibers [20, 42, 44, 45]. Future studies on TTI should clarify whether or TTI treatment activates vagal afferent neurons.

Among studies with trypsin inhibitors, only the study of Komarnytsky et al. [32] observed duodenal CCK postprandial mRNA expression. In this study, eutrophic *Wistar* rats were treated with protease inhibitors from potato and presented, in accordance with the observed plasma increase in CCK, a 2.4-fold increase in pre-pro-CCK (Genbank X01032) when compared to the control group. This finding was in contrast our data as we did not find any difference in *pre-pro-CCK* relative mRNA expression in response to TTI in obese animals.

By analyzing the data presented, we hypothesize that obesity influences the TTI effect upon CCK resulting in divergent results in eutrophic and obese rats with respect to plasma concentrations and the relative mRNA expression of the genes involved in CCK production. Studies with nutritionally unbalanced diets, offered for extended periods to animals with or without obesity, have demonstrated a reduction in the sensitivity to satiety hormones, including GLP-1 and CCK in addition to a postprandial decrease in their circulating concentrations [43, 46].

It is known that trypsin inhibitors cause a reduction in food intake and consequently induce weight loss in eutrophic animals, even when given for short periods. These effects are commonly attributed to a secretagogue effect upon CCK [28, 31–34].

Unfortunately, in obese animals, these effects do not seem to be the same, given the resistance and/or decrease in the secretion or action of CCK caused by the excessive increase of adipose tissue [11, 42]. Nevertheless, in the present study, TTI decreased food intake within 10 days of the experiment.

The influence of obesity on the secretion of hormones that act on the modulation of the CCK response, e.g. leptin, is still controversially discussed [20, 21]. There seems to be a close synergetic relationship between the effect of CCK and the concentration of leptin in obese animals [18–21, 47–49].

We therefore also investigated in the present study if TTI has an effect upon on plasma leptin concentrations and on the mRNA expression of the *ob* gene. We expected that obese animals presented with high leptin plasma concentrations, since obesity commonly leads to hyperleptinemia [12]. Accordingly, we observed that lean animals (lean/standard diet) consuming a nutritionally adequate diet had low concentrations of plasma leptin) very close to the minimum value detectable by the method used. In untreated obese animals (obese/HGLI diet) leptin concentrations were at about four times higher than in lean animals. These findings corroborate studies in obese humans and rodents showing that leptin concentrations in obese subjects can be five times higher than in lean subjects [50, 51]. In 60% of animals treated with TTI (obese/HGLI diet + TTI), leptin concentrations were under the detection limit, and in the remaining 40% they were considerably lower than in obese (HGLI diet group) which is an interesting result as these animals were still obese and consumed an obese/HGLI diet (the diet that was used to induce and maintain obesity in the experimental animals). Moreover, only 20% of obese animals submitted to the standard diet reduced plasma leptin to undetectable concentrations. Surprisingly, a substance known to stimulate CCK production (TTI) is thus more effective in reducing leptin plasma levels than the conventional treatment of obesity by standard diet. Carvalho et al. [29] showed that TTI reduced TNF- α levels, which, like leptin, is also produced by fat tissue, regardless of whether weight loss occurred or not.

One of the limitations of the present study is that we only determined the nutritional status assessment, and not adiposity. By determining adiposity, the characterization of the relationships between lipogenesis and reduction of leptin would be even clearer. Further studies should focus on that.

TTI was able to reduce plasma leptin but did not reduce the relative mRNA expression of the *ob* gene when compared with the obese group with no treatment. Nevertheless, obese animals treated with standard diet and with HGLI diet plus TTI showed no significant differ-

ences in the expression of this gene compared to lean animals. The action of TTI might explain this plasma reduction on genes that are involved in other pathways regulating leptin expression, post-transcriptional changes or even act on the leptin receptor.

Also studies with hormones showed divergences between plasma and expression values, especially when involving a single candidate gene, given the complexity in the regulation of expression. A study evaluating the sensitivity of obese animals to peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) found plasma reductions of these hormones, which was not corroborated by gene expression [52]. Concerning leptin, little is known about the transcription pathways that regulate its specific expression in adipocytes [53].

Matson et al. [21] suggested the search for strategies to decrease the effects of leptin in resistant obese individuals, which is the way to improve the efficiency of CCK in weight reduction. This effect is not entirely dependent on the eating behavior but on a synergistic effect of the peripheral action of CCK and the of central action of leptin.

In a study to analyze leptin resistance in the afferent vagal nerve (AVN) in DIO *Zucker* rats [19], the authors found that leptin resistance in the AVN was also associated with a reduced sensibility to CCK, thus altering the expression of receptors related to satiety in the AVN. The authors suggested that this was a result of increased signaling to orexigenic pathways and decreased signaling from intestinal anorexic hormones, finally leading to hyperphagia [19].

Additionally, Buyse et al. [54] indicated that vagal leptin receptors in the stomach may be activated by locally produced leptin and may serve to increase postprandial satiety induced by the CCK-1R. Leptin is considered to promote CCK-1R uptake via increased gene expression of the *CCK-1R*. Thus, amelioration of hyperleptinemia by TTI could also cause a down-regulation of mRNA expression of *CCK-1R*.

Another hypothesis that can be raised by the present results is that TTI can act as a hormone, binding to specific receptors and stimulating the production of anorexigenic neuropeptides. A bioactive peptide (trypsin inhibitor) with hypoglycemic action was observed in *Momordica charantia* (bitter melon) [55]. This peptide presents 68 amino acid residues and the insulin receptor binding protein of *M. charantia* (mcIRBP). It acts by activating insulin receptor signaling pathways and stimulating both the uptake and release of glucose in rat cells [56, 57]. Confirming these data, mcIRBP-19 was observed to be the peptide insulin receptor binding motif of mcIRBP with a similar ability for glucose uptake [58].

The present study investigating with the relative mRNA expression of intestinal CCK-related genes in obese rats is in agreement with several studies and considering the synergism of CCK and leptin in the control of appetite. We believe that the reduction of food intake in obese animals induced by TTI, as observed in the present study and in the study by Carvalho et al. [29], is the result of a TTI-dependent decrease of plasma leptin to values even lower than those detected in lean animals. Both studies demonstrated that TTI might have effects that are comparable to those induced by a standard, nutritionally adequate diet. The results are particularly interesting as TTI was administered to obese animals still consuming the HGLI diet, which is unbalanced.

To the best of our knowledge, this is the first report investigating the effects of a trypsin inhibitor on plasma CCK and mRNA expression of key genes related to CCK expression in obese animals. Our data suggests that TTI, by decreasing plasma leptin, improves CCK action, regardless of its increased values in plasma of obese rats, since food intake and *CCK-1R* expression were lower in the TTI-treated group when compared with no-treatment group. Therefore, the effects of TTI should be further investigated in obese models.

Translating a standard experimental diet to a balanced clinical diet for obese humans is difficult, as obese individuals may be reluctant in changing food habits. Thus, TTI, after successfully passing further animal studies, might be investigated as a therapy adjuvant, which may help to improve hormone production related to satiety.

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Disclosure Statement

The authors declare no conflict of interest.

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