Original Communication

Effects of Maternal Flaxseed Supplementation on Female Offspring of Diabetic Rats in Serum Concentration of Glucose, Insulin, and Thyroid Hormones

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Abstract: *Objective*: This study aimed to evaluate the effect of maternal consumption of flaxseed flour and oil on serum concentrations of glucose, insulin, and thyroid hormones of the adult female offspring of diabetic rats. *Methods*: Wistar rats were induced to diabetes by a high-fat diet (60%) and streptozotocin (35 mg/kg). Rats were mated and once pregnancy was confirmed, were divided into the following groups: Control Group (CG): casein-based diet; High-fat Group (HG): high-fat diet (49%); High-fat Flaxseed Group (HFG): high-fat diet supplemented with 25% flaxseed flour; High-fat Flaxseed Oil group (HOG): high-fat diet, where soya oil was replaced with flaxseed oil. After weaning, female pups (n = 6) from each group were separated, received a commercial rat diet and were sacrificed after 180 days. Serum insulin concentrations were determined by ELISA, the levels of triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) were determined by chemiluminescence. *Results*: There was a significant reduction in body weight at weaning in HG (−31%), HFG (−33%) and HOG (44%) compared to CG ($p = 0.002$), which became similar by the end of 180 days. Blood glucose levels were reduced in HFG (−10%, $p = 0.044$) when compared to CG, and there was no significant difference between groups in relation to insulin, T3, T4, and TSH after 180 days. *Conclusions*: Maternal severe hyperglycemia during pregnancy and lactation resulted in a microsomal offspring. Maternal consumption of flaxseed reduces blood glucose levels in adult offspring without significant effects on insulin levels and thyroid hormones.

Keywords: Flaxseed, diabetes, high fat diet, programming, thyroid hormones

Introduction

Diabetes is a metabolic disorder with increasing prevalence rates in many countries in recent years. According to recent data, it is estimated that about 387 million individuals worldwide have diabetes, and an increase to 592 million is expected for 2035 [1]. The occurrence of diabetes in women of reproductive age is a worrying trend, since studies indicate that fetal exposure to a hyperglycemic intrauterine environment may be associated with an increased risk of metabolic disorders at birth, which may have an influence over lifetime [2, 3]. Studies have shown that metabolic programming caused by diabetes during pregnancy increases the risk of offspring developing metabolic alterations in adult life [4, 5].

Among the most frequent endocrine diseases are diabetes and thyroid disorders [6]. Thyroid hormones (TH) significantly influence glucose metabolism and the development of insulin resistance [7], and changes in thyroid function have been observed in diabetic patients [8, 9]. These changes are reflected through modifications in the levels of TH thyroxine (T4), triiodothyronine (T3) and thyroid-stimulating hormone (TSH) [10]. Animal studies also observe histological changes in the thyroid and in TH levels in diabetes models [11, 12]. Diabetic rats induced by streptozotocin (STZ) have central hypothyroidism features in uncontrolled diabetes mellitus type 1, with reduction of T3, T4, and TSH. However, this is reversible upon metabolic control by insulin treatment [13].

It has also been reported that endocrine function can be influenced by nutritional and hormonal factors. Dutra et al. [14] observed a reduction in secretion of TSH and a thyroid hyperfunction in the offspring of mothers submitted to protein malnutrition during lactation. Just like Passos et al. [15], who found that thyroid function can be programmed in the offspring of mothers who received treatment with leptin in pregnancy and during the lactation period.

Recent studies found a relationship between omega-3 supplementation in the prevention of adverse effects of fetal programming, where essential fatty acids act beneficially, assisting in the control of parameters such as blood glucose, lipid metabolism [16, 17], blood pressure, and body fat [18]. Flaxseed is notable for its chemical composition, being a vegetable-origin source rich in essential omega-3 fatty acids with approximately 50–55% of total lipids as alpha linolenic acid and 15–18% as alpha linoleic acid. Flaxseed has a nutritional composition of approximately 23–28% protein, 28–35% fiber (10% soluble and 30% insoluble), 35–45% fat, and 3–6% minerals [19, 20]. Additionally they contain high levels of lignan SDG (secoisolariciresinol diglucoside), a phenolic compound with antioxidant and anti-inflammatory properties [21].

Studies show that consumption of flaxseed and their derivatives provides beneficial health effects by influencing a reduction in risk factors associated with several disorders and for being a source of essential fatty acids that contribute to the normal development of the nervous system [22, 23], acting, among other factors, in the prevention of cardiovascular disease and diabetes [24, 25].

Therefore, in view of the scarcity of studies addressing the relationship between omega-3 fatty acid sources, such as flaxseed, and TH, this study aims to determine the influence of the maternal consumption of a diet supplemented with flaxseed flour and oil by diabetic rats during pregnancy and lactation in serum concentrations of glucose, insulin, and TH of their adult female offspring.

Methods and materials

Ethical aspects

The present work is characterized as an experimental research study and was approved by the Ethics Committee on Animal Research of the Rectory of Research and Graduate Studies at the Fluminense Federal University under No 035/2010. All experimental procedures performed follow the standards set by the Conventional Guide to Animal Experimentation (NIH Publication No. 85-23, revised 1996) and the national recommendations made by Arouca Law (11.794/2008).

Experimental design

Female Wistar rats (n = 30), 90 days of age, at the Experimental Nutrition Laboratory, School of Nutrition, Fluminense Federal University, were separated into 2 groups: Control Group (CG) (n = 6), fed a casein-based diet, and High-fat Group (HG) (n = 24), fed a high-fat diet (60% of total calories from lipids and 14% from protein) for the purpose of inducing insulin resistance.

After 3 weeks of a high-fat diet, HG animals received an intraperitoneal injection of streptozotocin (STZ) at a low dose (35 mg/kg) [26] dissolved in a vehicle solution (sodium citrate 0.01 M, pH = 4.5). The CG received an intraperitoneal injection containing only the vehicle solution. The groups continued to receive their respective experimental diets for another week, ending at a total of 4 weeks of exposure to the dietary standards adopted.

Diabetes was confirmed by plasma glucose concentration higher than 300 mg/dL [26]. Female rats were separated for mating for a period of 15 days, with a proportion of 2 female : 1 male. Once pregnancy was confirmed, the animals were randomly divided into the following groups: CG, n = 6, diet based on casein; HG, n = 6, high-fat diet based on casein; High-fat Flaxseed Group (HFG, n = 6): high-fat diet based on casein supplemented with 25% flaxseed flour in order to get the appropriate percentage of recommended dietary fiber; High-fat Flaxseed Oil Group (HOG, n = 6): high-fat diet based on casein, with the total amount of soybean oil replaced by flaxseed oil (Table I).

The animals were fed diets according to experimental group assignment throughout the period of gestation and lactation. Litter size was adjusted at random to 6 female pups per litter to assure adequate and standardized nutrition until weaning [27]. We used the sample of 6 animals per group according to an established pattern for quantitative studies [28]. After weaning at 21 days, female pups (n = 6) from each group were separated and received a specific commercial rat chow diet (22% protein, Nuvilab®, Nuvital LTDA, Paraná, Brazil) until the animals reached 180 days of life. Sex-specific effects have been described in animal models of fetal programming, with females appearing to be more sensitive to some programming effects, the reason why we use females in our study [29].

During the experiment, the animals were kept in polypropylene cages at 22 °C and controlled periods of light/dark (12/12 h), receiving water and food *ad libitum*. At 180 days, the animals fasted for 6 h and were anesthetized with an intraperitoneal injection of Thiopentax® (sodium Thiopental 1G, Cristália Chemicals Pharmaceutical LTDA, Brazil) to 5% (0.15 mL/100 g BW) before being sacrificed. Blood samples were collected by cardiac puncture and placed in tubes without anticoagulant. Blood samples were centrifuged (Sigma centrifuge) at 3500 rpm for

Table I. Composition every 1000 g of rations that were offered during pregnancy and lactation period (19% protein-AIN-93G).

Ingredient/Diet	Control	High-fat	High-fat + flaxseed oil	High-fat + flaxseed flour
Casein (g)*	190	230	230	200
Flaxseed[Ж]	0	0	0	250
Corn starch (g)*	539,486	299,486	299,486	229,486
Sucrose (g) [‡]	100	100	100	100
Soybean oil (mL)**	70	70	0	0
Flaxseed oil (ml)[Δ]	0	0	70	0
Lard (g)[¥]	0	200	200	170
Fiber (g) [††]	50	50	50	0
Vitamin mix (AIN93-G) (g)[§]	10	10	10	10
Mineral mix (AIN93-G) (g)[§]	35	35	35	35
L-Cystine (g)[Φ]	3	3	3	3
Choline bitartrate (g)[Φ]	2,5	2,5	2,5	2,5
Tert-Butilhydroquinone (g)	0,014	0,014	0,014	0,014
Total (g)	1000	1000	1000	1000
Energy (kcal/kg)	**3950**	**4950**	**4950**	**4954,7**
Carbohydrate (% of Kcal)	**64**	**32**	**32**	**31,6**
Protein (%d of Kcal)	**19**	**19**	**19**	**19,3**
Fat (% of Kcal)	**17**	**49**	**49**	**49**

AIN-93G = American Institute of Nutrition-93G; *Comércio e Indústria Farmos Ltda. (Rio de Janeiro RJ, Brasil); [†]Maizena da Unilever Bestfoods Brasil Ltda. (Mogi Guaçu, SP, Brasil); [‡]União (Rio de Janeiro, RJ, Brasil); **Liza da Cargill Agricultura Ltda. (Mairinque, SP, Brasil); [††]Microcel da Blanver Ltda. (Cotia, SP, Brasil); [¥]Sadia Comercial Ltda; [§]PragSoluções Comércio e Serviços Ltda-ME (Jáu, SP, São Paulo); [Φ]M. Cassab Comércio e Indústria Ltda. (São Paulo, SP, Brasil); [Ж]Mãe terra (São Paulo, SP, Brasil); [Δ]Giroil Agroindustria LTDA (Santo ¢ngelo, RS, Brasil).

15 min to obtain the serum, which was stored at −80 °C for later analysis. Thyroid glands were dissected and weighed on an analytical digital scale for comparison of absolute and relative weight between the study groups. Relative gland weight was calculated by adjusting to every 100 g of body weight.

Experimental diets

The experimental diets were prepared at the Experimental Nutrition Laboratory (LabNE), School of Nutrition, Fluminense Federal University. A vitamin and mineral mix was added to all diets, consistent with the recommendations of the Committee on Laboratory Animal Diets, 1979, modified according to the recommendations of AIN-93 [30]. Diets were prepared for two stages of the experiment: the initial diet for the induction of diabetes in rats to be mated and the subsequent diet offered during the pregnancy and lactation phases.

The initial high-fat experimental diets for the induction of diabetes were obtained as described by Correia-Santos *et al.* [31], the high-fat diet differs from the control by the addition of lard. The macronutrient values of the diets of animals during the pregnancy and lactation stages were modified to contain 19% protein (AIN-93G) (Table I) to meet nutritional requirements. The HFG diet was composed of 25% flaxseed, which acts as a source of fibers and lipids. The HOG diet had the total amount of soybean oil replaced by flaxseed oil. CG and HG diets were similar to those offered at the time of induction, only with changes in protein percentage.

Food intake and body weight of offspring

To assess variations in weight gain during the experiment, the animals were weighed weekly, along with the supply of chow, using an analytical digital scale (precision 0.01 g, Filizola®, Técnica Industrial Oswaldo Filizola, Sao Paulo, Brazil). To calculate the intake of each animal, individual consumption was obtained by subtracting the remains of what was offered, and the result was divided by the number of animals in each cage.

Determination of serum levels of T3, T4, TSH, and insulin

Serum insulin concentrations were determined by ELISA using a commercial kit (RADPK-81 K, Millipore). The levels of T3, T4, and TSH were determined by chemiluminescence and measured by a commercial kit using Immulite® 2000 analyzer (Siemens, Erlangen, Germany).

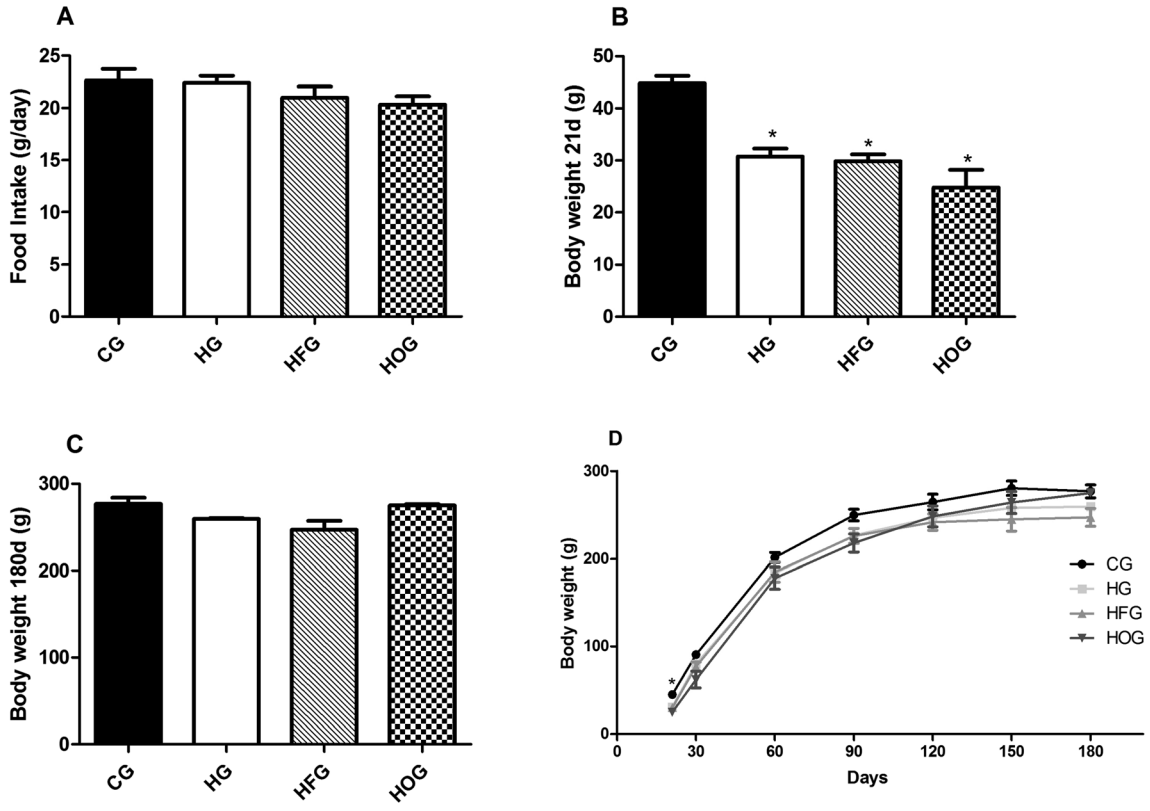

Figure 1. (A) Mean daily food intake of female offspring throughout the experiment. (B) Body weight of female offspring at weaning. (C) Body weight of female offspring at 180 days. (D) Changes in the body weight gain of female offspring from weaning at the end of 180 days. CG: Control Group (n = 6), HG: High-fat Group (n = 6), HFG: High-fat Flaxseed Group (n = 6), HOG: High-fat Flaxseed Oil Group (n = 6); *p < 0,05.

Statistical analysis

The data were presented as means ± standard error. Normal distribution of values was analyzed using the Kolmogorov–Smirnov test. For groups that were within the normal distribution, one-way ANOVA was applied. Kruskal–Wallis test was used for nonparametric distributions. The established significance level was $p < 0.05$, calculated using GraphPadPrism versão 5.03 for Windows (GraphPad Software, San Diego, California, USA).

Results

Daily food intake of the groups was similar throughout the whole experiment, with no significant difference compared to CG (p = 0.298) (Figure 1A). Body mass at weaning of the offspring of diabetic mothers from HG, HFG, and HOG was significantly lower when compared to CG (−31, −33, and −44%, respectively; p = 0.002; Figure 1B). Although CG values were superior to other weight groups throughout the study, these demonstrated an evolution of similar body weight gain, and after 180 days, no significant differences in animal body weight were identified (p = 0.066; Figure 1C, 1D).

It was observed that the study groups showed no significant difference in absolute thyroid weight at 180 days (p = 0.705; Figure 2A). Similarly, no significant differences were found in relation to relative weight (p = 0.751; Figure 2B).

Blood glucose levels of animals whose mothers received a high-fat diet supplemented with flaxseed (HFG) were significantly lower (−10%) in comparison with CG (p = 0.044), while the other groups showed similar levels to CG (Figure 3A). In relation to serum insulin, groups showed no significant difference in comparison with CG (p = 0.660; Figure 3B).

Serum levels of T3 and T4 of the groups were similar at 180 days (Figure 4A, 4B), with no significant difference compared to CG (p = 0.06 and p = 0.11, respectively), similarly to TSH levels (p = 0.473; Figure 4C).

Discussion

Studies have shown that metabolic programming caused by diabetes during pregnancy increases the risk of offspring

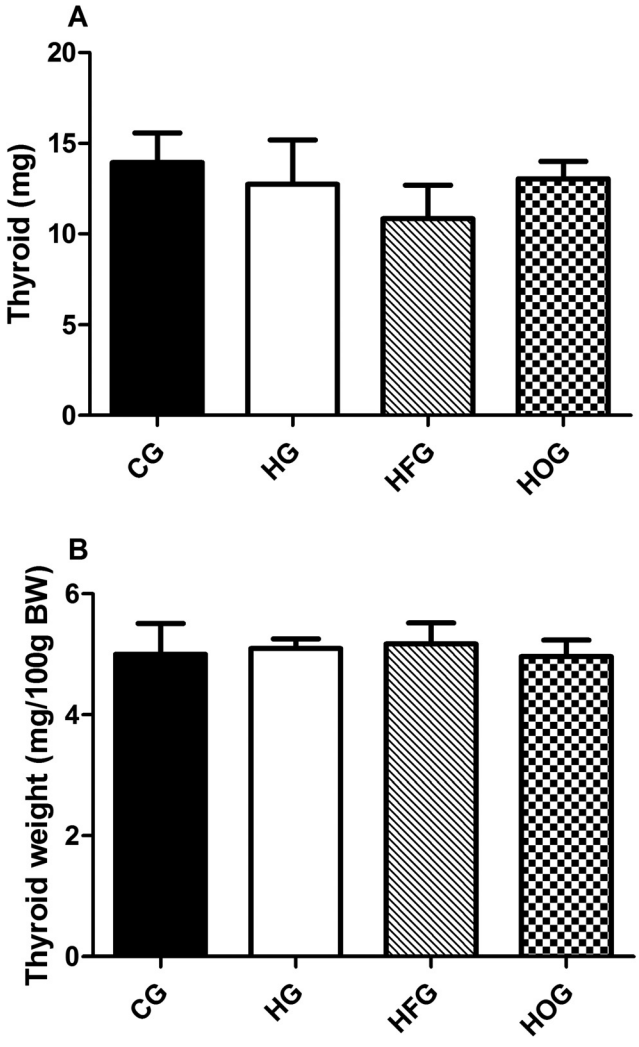

Figure 2. Absolute (A) and relative weight (B) thyroid female offspring at 180 days. Values expressed as mean ± SEM. CG: Control Group (n = 6), HG: High-fat Group (n = 6), HFG: High-fat Flaxseed Group (n = 6), HOG: High-fat Flaxseed Oil Group (n = 6).

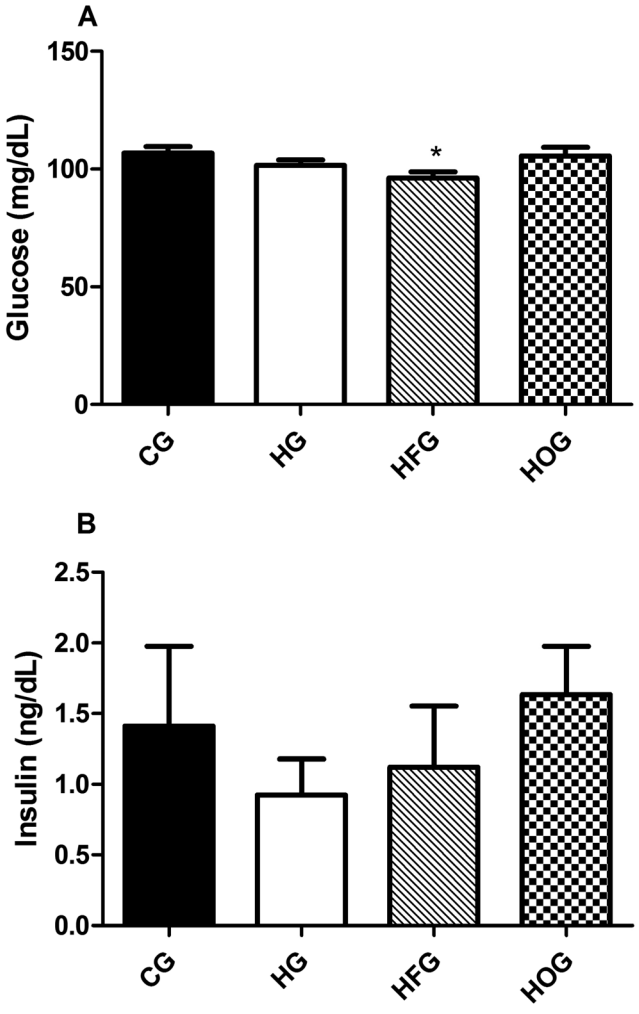

Figure 3. Values of serum glucose (A) and insulin (B) female offspring at 180 days. Values expressed as mean ± SEM. CG: Control Group (n = 6), HG: High-fat Group (n = 6), HFG: High-fat Flaxseed Group (n = 6), HOG: High-fat Flaxseed Oil Group (n = 6); *p < 0,05.

developing metabolic alterations in adult life [4, 5]. Similarly, it has been observed that obesity and a maternal diet characterized by high energy density are also risk factors for metabolic disorders during intrauterine development [31, 32, 33]. Therefore, this study sought to assess adult animals exposed perinatally to a hyperglycemic environment, and verify that the consumption of flaxseed flour or flaxseed oil during this same period can exert some hormonal or metabolic influence.

Initially it was found that food intake of animals monitored from weaning until day 180 was similar in all experimental groups. This can be explained by the fact that after weaning, all animals of different experimental groups began to consume commercial chow, thus all were exposed to the same dietary patterns, with no difference in external stimuli that could cause alterations in consumption.

During the study period, body weight of animals was measured periodically. It was initially found that the body mass of all animals from diabetic mothers showed significantly lower values compared to animals from healthy mothers. These results are in agreement with the literature [34, 35], where weight is lower in the offspring of diabetic rats, especially at birth and after weaning. Maternal diabetes leads to a greater availability of fetal glucose and insulin, and therefore contributes to the development of macrosomic fetuses [36]. However, the presence of severe hyperglycemia limited fetal growth, resulting in microsomic fetuses [37]. Maternal hyperglycemia alters glucose uptake of the fetus, the process creates overstimulation of pancreatic beta cell activity that affects the secretion of insulin, leading to changes in fetal growth and development [38]. Throughout and at the end of the experiment the animals showed no significant difference in body mass in

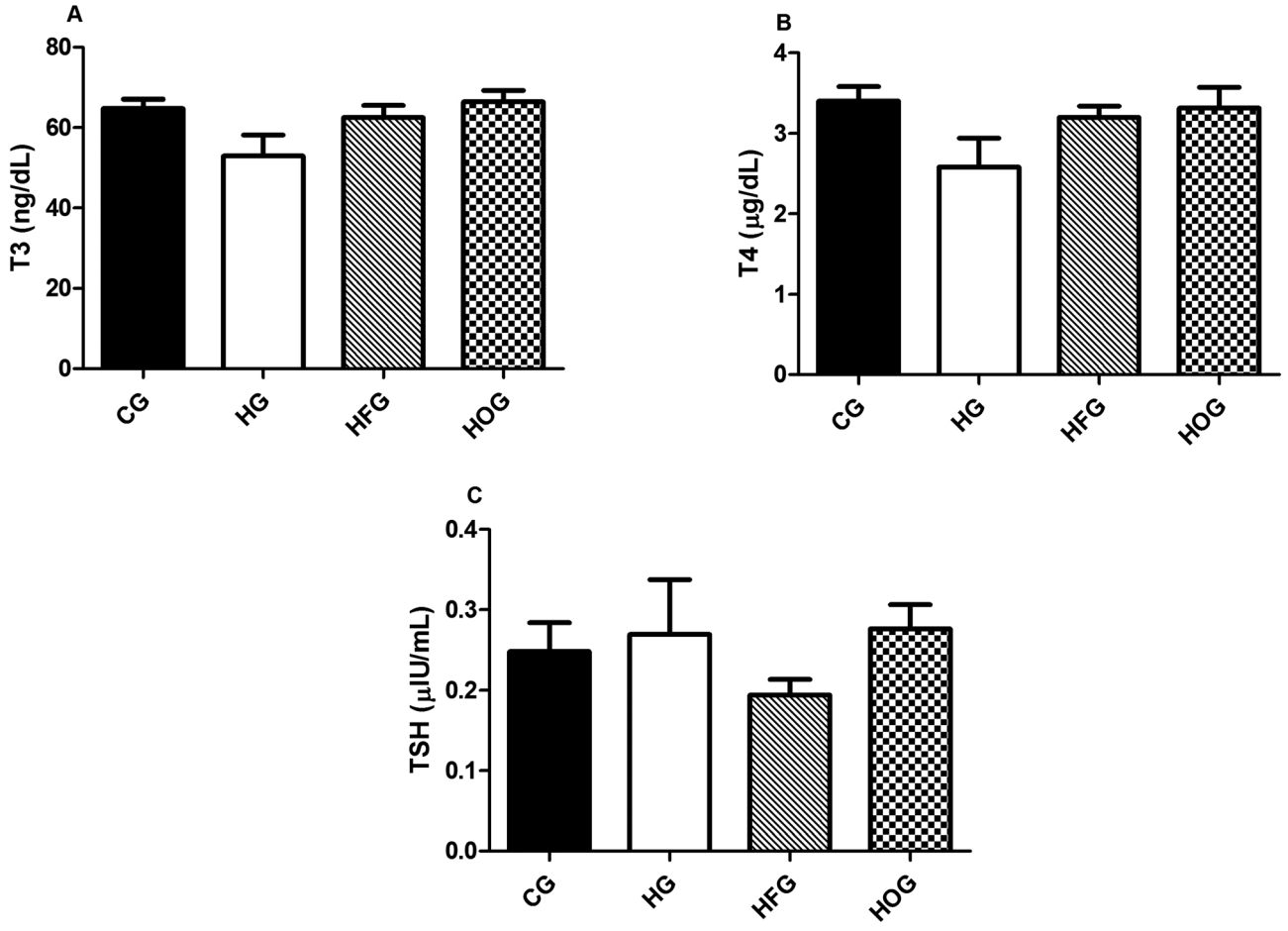

Figure 4. (A) Serum levels of 3,5,3-triiodothyronine (T3) of female offspring at 180 days. (B) Serum levels of thyroxine (T4) of female offspring at 180 days. (C) Serum levels of thyroid-stimulating hormone (TSH) of female offspring at 180 days. Values expressed as mean ± SEM. CG: Control Group (n = 6), HG: High-fat Group (n = 6), HFG: High-fat Flaxseed Group (n = 6), HOG: High-fat Flaxseed Oil Group (n = 6).

view of the standardization of the diets at weaning, enabling similar weight gain.

In this study, food intake and body mass gain of animals were not influenced by the addition of flaxseed flour or oil to the high-fat diet offered to mothers during pregnancy and lactation. These results are different compared to other studies, possibly due to the additional factor of maternal diabetes and its interference with fetal development, as the exposure to severe maternal hyperglycemia is related with lower weight at birth, also affecting postnatal growth [39], in addition to offering diets with high fat, modifying the quality and level of exposure to dietary lipids during initial periods of life. In a study by Fernandes *et al.* [22], the offspring from the diet group supplemented with flaxseed had a lower body mass at weaning and after 50 days. The influence of flaxseed in weight gain was also observed in a study by Figueiredo *et al.* [24], where the offspring from mothers who consumed flaxseed during lactation had a higher body mass at weaning and in adulthood at 180 days of life.

Different experimental models have been used in diabetes research, and STZ has been one of the most used chemicals for this purpose. In this work, the use of a high-fat diet associated with the specific actions of STZ was used to determine the development of hyperglycemia in rats that were used as matrices [31]. At 180 days of age, the offspring from these rats had similar glucose levels, except in animals whose mothers received a high-fat diet with added flaxseed, these animals showed reduced blood glucose values compared to the CG. Regarding insulin values, no difference was observed among the groups, only a percentage reduction in HG (−34%) and HFG (−20%) when compared to CG. The numerical differences observed in HG may be related to exposure of the fetus to severe maternal hyperglycemia as it serves as a stimulus for increased secretion of insulin and overstimulation of pancreatic beta cells, which results in the altered function of these cells and reduced insulin production [40].

The literature reports that the offspring of rats offered a flaxseed-supplemented diet during lactation showed no

change in blood glucose levels at weaning, but at 180 days blood glucose levels were reduced when compared to the control. Similarly, insulin levels were also influenced, and were found to be reduced during weaning and elevated during adulthood [24]. It was concluded that the consumption of flaxseed during lactation predisposes to increased sensitivity to insulin, a fact that would be related to lower blood glucose levels identified, similar to the results observed in this study. Morre *et al.* [41] reported that SDG, a substance present in flaxseed, influences the reduction of blood glucose in diabetic rats through its antioxidant activity. Regarding the addition of flaxseed oil to the maternal diet, no significant effect on glycemic adult offspring was identified. Guarda et al. [42] did not observe differences in glucose levels of offspring at 21 days by offering the rats a fat diet with added flaxseed oil during lactation.

The animals used in this study were exposed to maternal hyperglycemia during gestation and lactation, when diabetic mothers had significantly higher values than the controls (CG: 90 ± 5.4 mg/dL; HG: 438.5 ± 3.5 mg/dL; HOG: 405.7 ± 14.8 mg/dL; HFG: 422 ± 24.3 mg/dL; $p < 0.001$). However, characteristics of long-term exposure could not be observed in the paired animals. The data obtained here are the most divergent findings in the literature regarding the programming of diabetes. In general, studies related to this subject identify insulin resistance and occasional glucose level variations in adult animals [34]. However, comparative results are difficult to obtain due to the variety of methodologies used in these studies. The severity of diabetes varies according to the dosage of the drug, with notable differences in the route of administration and timing of intervention for the induction process, as such factors may affect the results obtained over the short and long term [43]. The clinical picture of insulin resistance in most studies that attempt to reproduce and resemble this disease is not fully representative since the origin of metabolic changes is different, given that in humans this disease is caused by multiple factors.

Studies have reported the occurrence of thyroid dysfunction in diabetic patients [44]. Lambadiari *et al.* [45] found a correlation between levels of TH and insulin sensitivity, fasting plasma glucose levels and postprandial glucose levels in euthyroid subjects in the early stages of diabetes mellitus, identifying that an increase in these hormones even within the normal range has a positive association with insulin resistance indices in early stages of diabetes. In this study, TH levels were not significantly different between groups, but there was a reduction of 18% of T3 and T4 in 24% of the HG group when compared to the group of animals derived from healthy mothers. Viewing protocols used to induce diabetes, it was expected that when adult offspring present metabolic changes the fetal programming, and consequently TH levels, could be affected, since the literature reports that offspring of diabetic rats have upper blood glucose levels, reduced insulin, T3, and T4 glandular and plasmatic [46]. In a study by Ahmed *et al.* [11] that identified an increase in T4, reduction in T3 and depletion of TSH in diabetic rats with streptozotocin-induced diabetes, the authors suggest that the conversion to T3 is reduced in diabetes in peripheral tissues.

These short-term changes in TH after administration of STZ can be related to thyroid peroxidase that has little activity in the initial stages of this diabetogenic drug induction [47]. Evaluating the long-term effect, STZ did not cause significant changes in TSH levels, which is also identified as normalization of T4 levels, highlighting greater fluctuations of insulin deficiency in the initial period of animals, or their most evident effects are short-term [48]. This fact may explain the results observed for TH in this study, evaluated over a long period, and the fact that after 180 days the animals showed no changes in the values for blood glucose and insulin, which can be established as a favorable condition for the maintenance of normal levels of TH presented by these animals that were exposed to maternal hyperglycemia. In rodents, STZ administration involves the reduction of T3 concentration and total and free T4 [49]. Metabolic changes resulting from gestational diabetes did not have a clear effect on thyroid function of the animals used in the present study. Few studies correlate the consequences of diabetes induced by STZ to complications in TH long-term offspring, a fact that makes it difficult to compare the results of this study.

Regarding TH results of this research, we find no significant influence concerning maternal consumption of flaxseed flour and oil, but is worth mentioning that the addition of these compounds to the maternal diet also was not reflected in negative changes, while the results of these parameters were similar to those in long-term healthy rats offspring. In contrast to the results found in this study, Figueiredo *et al.* [50] found that consumption of flaxseed by healthy rats during lactation programmed the function of the thyroid, altering TH metabolism of the adult offspring. When a diet containing 25% flaxseed was offered during lactation only, newborn animals from the flaxseed group showed reduced levels of T3 and high TSH levels. At 180 days the levels of T3 and TSH normalized, but levels of T4 were reduced, suggesting the inappropriate action of TSH on the thyroid gland. The mechanism by which the consumption of flaxseed may influence TH and TSH remains unclear.

Observing the influence of dietary quality on endocrine mechanisms through changes in the balance of n-6 and n-3 offered, Clandinin et al. [51] found that male offspring that began to consume diet supplemented with DHA, after 3 weeks of consumption had significantly elevated TSH levels. Similarly, Takeuchi et al. [52] determined the effect

of the fatty acid composition of the diet on serum levels of TH by comparing lard, safflower oil, and flaxseed. They reported reduced T3 levels in the group using lard, source of saturated fatty acids in relation to other groups characterized by mono- and polyunsaturated lipids. These studies demonstrate that the consumption of saturated fat contributes to reducing the levels of TH, as opposed to a fat source of n-6/n-3 and suggested that such modifications may also be related to the conversion mechanism, not just to the secretion of these hormones. Similar characteristics may not be significant in the results presented in this study by the proposed order to verify the nutritional intervention n-3 sources in the long-term fetal programming, with the offspring consuming commercial chow during the post weaning period and throughout life adult.

Conclusions

In summary, the present study aimed to evaluate the influence of flaxseed flour and oil on adult female offspring of diabetic rats, which were obtained through an experimental model induced by STZ and a high-fat diet. Influences of the hyperglycemic state of pregnant rats were observed in the weaning weight of the offspring, resulting in a microsomic offspring, but were not significant to the other parameters observed in the long term. However, despite exposure to an unfavorable environment of development, long-term offspring remained normoglycemic when kept on a standard diet. All other parameters, including insulin, TH, and TSH, were unchanged by maternal dietary treatment. Since there is no evidence that explains a possible mechanism of action of flaxseed in thyroid function, there is a need for more research related to this topic, as well as its relationship with omega-3 fatty acids. This research aims to contribute to new findings to this area and to serve as a proposal for future research.

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Conflicts of interest
The authors declare that they have no conflicts of interest.

Ethical approval
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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