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Peripubertal soy isoflavone consumption leads to subclinical hypothyroidism in male Wistar rats

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Original Article

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Abstract

Exposure to endocrine-disrupting chemicals during critical windows of development may lead to functional abnormalities in adulthood. Isoflavones are a flavonoid group of phytoestrogens that are recognized by their estrogenic activity and are highly abundant in soybean. Since the thyroid gland presents estrogen receptors and infants, toddlers and teenagers may consume isoflavones from soy-based infant formula and beverages as alternatives to animal milk, we propose to investigate the potential effects of relevant concentrations of soy isoflavones in the regulation of the hypothalamic–pituitary (HP) thyroid axis using peripubertal male rats as an experimental model. Thirty-two 23-day-old male rats were exposed to 0.5, 5, or 50 mg of soy isoflavones/kg from weaning to 60 days of age, when they were euthanized, and the tissues were collected to evaluate the mRNA expression of genes involved in the regulation of the HP thyroid axis and dosages of thyroid hormones (THs). Serum TSH concentrations were increased, while alterations were not observed in serum concentrations of triiodothyronine and thyroxine. Regarding mRNA gene expression, *Mct-8* was increased in the hypothalamus, *Mct-8*, *Thra1*, and *Thrb2* were decreased in the pituitary, and *Nis* and *Pds* were reduced in the thyroid. In the heart, *Mct8* and *Thrb2* were increased, and *Thra1* was decreased. In the liver, *Mct8*, *Thra1*, and *Thrb2* were decreased. These results suggest that the consumption of relevant doses of soy isoflavones during the peripubertal period in males may induce subclinical hypothyroidism, with alterations in the regulation of the HP thyroid axis, modulation of TH synthesis, and peripheral alterations in TH target organs.

Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous compounds capable of altering the functioning of the endocrine system and consequently affecting important physiological processes even at low doses[1–6]. There are several compounds with potential EDCs, including natural substances, such as phytoestrogens[1,7]. Isoflavones are a flavonoid group of phytoestrogens that are recognized by their estrogenic activity[8,9] and are highly abundant in soybeans[10]. For this reason, there are known benefits of soy isoflavone consumption during menopause to ameliorate the effects caused by the decline in endogenous estradiol[11] and many other potential beneficial effects, such as decreased risk of cancer, type 2 diabetes, myocardial injury, reduction in the levels of cholesterol, reduction in blood pressure, etc.[12].

However, exposure to EDCs during critical windows of development, which includes events from gametogenesis and fertilization to the end of puberty, can lead to functional abnormalities in adulthood, mainly related to the endocrine system[13]. In this sense, there is a potential of human exposure during these critical phases, since isoflavones were present in soy-based infant formula and in breast milk after the consumption of soy by the mother[10,14,15]. In addition, soy is also used for the preparation of beverages commonly consumed by all families, including toddlers and teenagers, as an alternative to animal milk[16].

The estrogenic effects of EDCs are generally discussed based on classically responsive structures, such as the hypothalamic–pituitary–gonadal axis itself and on tissues such as breast and bones[17]. However, the thyroid gland has estrogen receptors[18] that, when stimulated, are correlated with the increased production of thyrotropin (TSH) and triiodothyronine[19]. In this sense, the assessment of thyroid function in individuals exposed to soy isoflavones remains relevant, although exposure to soy isoflavones is known to reduce iodine uptake by thyrocytes in vitro[20], suggesting the iodine fortification of soy-based infant formula[21].

Thyroid hormones (THs, triiodothyronine and tetraiodothyronine) play an important role during fetal development and central nervous system maturation but are also equally central for controlling events related to growth, metabolism, and the cardiovascular system[22]. The serum levels of THs are finely regulated by the hypothalamic–pituitary–thyroid (HPT) axis through stimulatory and inhibitory events[22]. The hypothalamus secretes thyrotropin-releasing hormone (TRH), which stimulates the pituitary gland to produce and secrete TSH, which in turn acts on the thyroid gland, promoting the synthesis and release of THs[23,24]. Finally, THs enter cells through membrane transporters to perform their action[25].

The incidence of hypothyroidism, hyperthyroidism,[26] and cancer[27] is increasing worldwide, and exposure to EDCs may be related to this[28–30]. Thus, considering the potential risk of human exposure during critical windows of development, we proposed to investigate the potential effects of relevant concentrations of soy isoflavones in the regulation of the HPT axis using peripubertal male rats as an experimental model.

Materials and methods

Ethical standards

All procedures were performed according to the recommendations of the Brazilian College of Animal Experimentation and approved by the Comite de Etica em Uso de Animais of the Universidade Estadual do Centro-Oeste (CEUA/UNICENTRO # 005/2017 and 019/2017).

Experimental design

The experimental design was based on a prepubertal protocol from the Endocrine Disrupting Screening and Testing Advisory Committee (EDSTAC) and includes the evaluation of EDC effects on thyroid function[31]. Pubertal development is evaluated by monitoring the separation of the preputial membrane and the externalization from the glands of the penis by gentle handle manipulation. This event is a mark of male rat puberty and is correlated with increased levels of testosterone reached during the peripubertal period[32]. Soy isoflavone treatment was administered from PND23 to PND60, which comprises the developmental phases of weanling (PND23), peripuberty (PND40), and young adult (PND60)[33]. Considering the different phases of rat life in comparison to humans, the experimental period corresponded to a human being exposed from 6 months to 18 years of age[33].

Diet during all experimental periods

Fifteen days before mating, couples were maintained with rat chow commercially prepared without soybeans [mineral matter (max) 6.2%, crude protein (min) 14%, ether extract 4%, crude fiber 4.2% and carbohydrates 71.7%; from the components: corn, wheat bran, albumin, corn oil, canola oil, dicalcium phosphate, sodium chloride, calcium carbonate, mineral premix, vitamins and amino acids; PragSoluções Biociências, Jaú, SP, Brazil] until the end of the experiment.

Mating

Twenty 90-day-old female Wistar rats were mated in a monogamous couple, and the beginning of gestation (gestational day, GD1) was confirmed by vaginal smear containing spermatozoa. At postnatal day 4 (PND4), the litters were culled to eight pups per female and kept at this proportion until weaning (PND21).

Soy isoflavone exposure

At PND23, the male offspring (32 animals; 1 or 2 pups/little allocated in different groups of treatments; 8 animals/group) were divided into four groups receiving 0, 0.5, 5, or 50 mg of soy isoflavones/kg of body weight diluted in corn oil (*Glycine max L.*, soy extract standardized to 40% isoflavones, Florien, Brazil). The control group received only corn oil. The treatment was administered from PND23 to PND60 by gavage once a day. The animals were weighed, and the dose was calculated with a volume of 0.25 mL for 100 g of body weight.

Euthanasia

At PND60, the euthanasia procedure was performed under general anesthesia through the administration of 60 mg/kg ketamine, 10 mg/kg xylazine, and 3 mg/kg acepromazine intraperitoneally.

Housing

The animals were kept in polypropylene boxes (43×43×20 cm) with a layer of 5 cm of shavings, with rat chow commercially prepared without soybeans (PragSoluções Biociências, Jaú, SP, Brazil) and water ad libitum under a 12:12 h light/dark and controlled-temperature room (23 ± 1°C) at the animal facility of the Laboratory of Reproductive Toxicity, Universidade Estadual do Centro-Oeste.

Weekly body weight gain

Weekly body weight gain was calculated by subtracting the body weight at the end of the week from the body weight at the beginning of the week.

Blood and tissue harvest

The blood was collected by cardiac puncture under deep anesthesia, placed in a tube containing silica blasted into the tube wall as a clot activator and centrifuged at 3,500 rpm (Excelsa II 206 BL, São Paulo, SP, Brazil) for 15 min, and the serum was separated and maintained at −80°C until hormonal analysis. After euthanasia, the hypothalamus, pituitary, thyroid, heart, and liver were rapidly excised, immediately frozen in liquid nitrogen at −196°C, and kept frozen at −80°C until analysis by real-time quantitative polymerase chain reaction (RT–qPCR).

Reverse transcription followed by RT–qPCR

The tissues (hypothalamus, pituitary, thyroid, liver, and heart) were pulverized in nitrogen liquid, and total RNA was extracted by the guanidine-phenol–chloroform method[34] using Trizol® reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. The total RNA concentration was estimated by the optical density of the solution, measuring absorbance at 260 nm, and purity was determined by the ratio A260 nm/280 nm. After quantification, the samples were subjected to electrophoresis in a 1.2% agarose gel in TBE buffer for analysis of RNA integrity and then stored at −80°C. A total of 2.5 µg of RNA was reverse transcribed using oligo(dT) with the resources of GoScript® Reverse Transcription System kit (Promega, Madison, USA) according to the manufacturer's instructions. For each reaction, 10 µL of cDNA was obtained with an estimated final

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Table 1. Primers used for RT–qPCR analyses and GenBank accession numbers

Gene	Primers sequences (5'-3')	GenBank
Trh (thyrotropin releasing hormone)	F:AACTCTACCCAGCCAGTTTGC R: GCATCCTGGAGTCTGCGAAGT	NM_013046.3
Slc16a2 (solute carrier family 16 member 2)	F: TGCCCTTGGTTACTTCGTCC R: CAGGAATGAGAGGACCTGCAA	NM_147216.1
Thrα1 (thyroid hormone receptor alpha isoform 1)	F: ACCTCCGCATGATCGGGGC R: CCTGATCCTCAAAGACCTC	NM_001017960.1
Thrα2 (thyroid hormone receptor alpha isoform 2)	F: TGAAGGCTGCAAGGGTTTCT R: GCACTGGTTACGGGTGACTT	NM_031134.2
Thrb2 (thyroid hormone receptor beta isoform 2)	F: TGAAGGCTGCAAGGGTTTCT R: GCACTGGTTACGGGTGACTT	NM_001270854.1
Trhr (thyrotropin releasing hormone receptor)	F: TGCTAACTACAGTGTGGCCC R: AACTGGGTCCATTCTTCTCGG	NM_013047.3
Tshb (thyroid stimulating hormone beta)	F: GGCAAACTGTTTCTTCCCAA R: GTTGGTTTTGACAGCCTCGT	NM_013116.2
Tshr (thyroid stimulating hormone receptor)	F: CGCATTCCAGGGACTATGCAA R: GTGGAAGACACGTCTAGCAAA	NM_012888.1
Slc5a5 (solute carrier family 5 member 5)	F: TCTTGCCGATCTTCTACCGC R: ATGTCCAACCCGGTCACTTG	NM_052983.2
Slc26a4 (solute carrier family 26 member 4)	F: CAAGTGGGTTCTTGCCTCCT R: TTGGTGGCGTAGACTTTCCC	NM_019214.1
Tpo (thyroid peroxidase)	F: CACGGCTTACCAGGCTACAA R: GCCTCCCAACCAGACATCAA	NM_019353.2
Myh6 (myosin heavy chain 6)	F: ACAAGGTTAAAAACCTGACAGAGG R: TACTGTTCTGCTGACTGATGTCAA	NM_017239.2
Dio1 (deiodinase type 1)	F: GCCATTCCCCTGCTGTAACT R: CCGTCAGTCCAAAGCCATCT	NM_021653.4
Dio2 (deiodinase type 2)	F: ACGCCTACAAACAGGTTAAATTGG R: CCGTCTTCTCTGAGGCACAA	NM_031720.3
Dio3 (deiodinase type 3)	F: GCCCGTTGGTGCTCAATTTT R: CTGTGGGATGACGTAGGGTG	NM_017210.4
Esr1 (Estradiol receptor 1)	F: CCATATCCGGCACATGAGTA R: TGAAGACGATGAGCATCCAG	NM_012689.1
Esr2 (Estradiol receptor 2)	F: CTCACGTCAGGCACATCAGT R: TGTGAGCATTCAGCATCTCC	NM_012754.1
Gper1 (G protein-coupled estrogen receptor 1)	F: CCCTTGACAGGCCACATAGT R: CTCCGTGCTGTCTGGTATGA	NM_133573.1
Rpl19 (ribosomal protein L19)	F: CAATGAAACCAACGAAATCG R: TCAGGCCATCTTTGATCAGCT	NM_031103.1

F, forward; R, reverse.

concentration of 250 ng/μL. Gene expression was evaluated by the RT–qPCR method from reverse transcription using a Platinum SYBR Green PCR Master Mix Kit (Life Technologies, CA, USA) according to the manufacturer's instructions. Amplification was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Singapore), and the PCR conditions were as follows: 50°C (2 min), 95°C (2 min), and 40 cycles of 95°C (15 s) and 60°C (30 s). At the end of the reaction, the dissociation curve was performed to confirm the specificity of the reaction. The mean cycle threshold (Ct) values were automatically determined by StepOneTM Software v2.3 (Applied Biosystems), and quantification was performed by the $2^{-\Delta\Delta Ct}$ method, as described by[35]. The quantification was calculated by the $2^{-\Delta\Delta Ct}$ method using ribosomal protein L19 (*Rpl19*) as an endogenous control. All primers used were previously standardized in previous publications of our group[36,37] and are described in Table 1.

Serum hormone concentrations and lipid profile

The serum hormonal dosages of triiodothyronine (T3) and thyroxine (T4) were determined by chemiluminescence using the Siemens ADVIA Centaur test kit (Siemens, Dublin, Ireland). The TSH concentration was determined using a Luminex xMap (Milliplex MAP rat pituitary panel, Billerica, MA, USA). All doses were performed according to the manufacturer's instructions. The serum dosages of triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein

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Table 2. Body weight gain

Isoflavones (mg/kg)	Weeks				
	1	2	3	4	5 until the end
0	1.82 (± 0.05)	2.87 (± 0.09)	2.83 (± 0.07)	3.02 (± 0.09)	3.85 (± 0.09)
0.5	1.79 (± 0.02)	2.54 (± 0.03)	2.35 (± 0.03)	3.01 (± 0.02)	3.45 (± 0.03)
5	1.55 (± 0.02)	2.38 (± 0.04)	2.33 (± 0.04)	3.03 (± 0.03)	3.15 (± 0.05)
50	1.77 (± 0.04)	2.78 (± 0.04)	2.61 (± 0.02)	3.16 (± 0.03)	3.67 (± 0.05)

Data are expressed as the mean ± standard error of the mean; MANOVA (weeks *vs* groups $p > 0.05$).

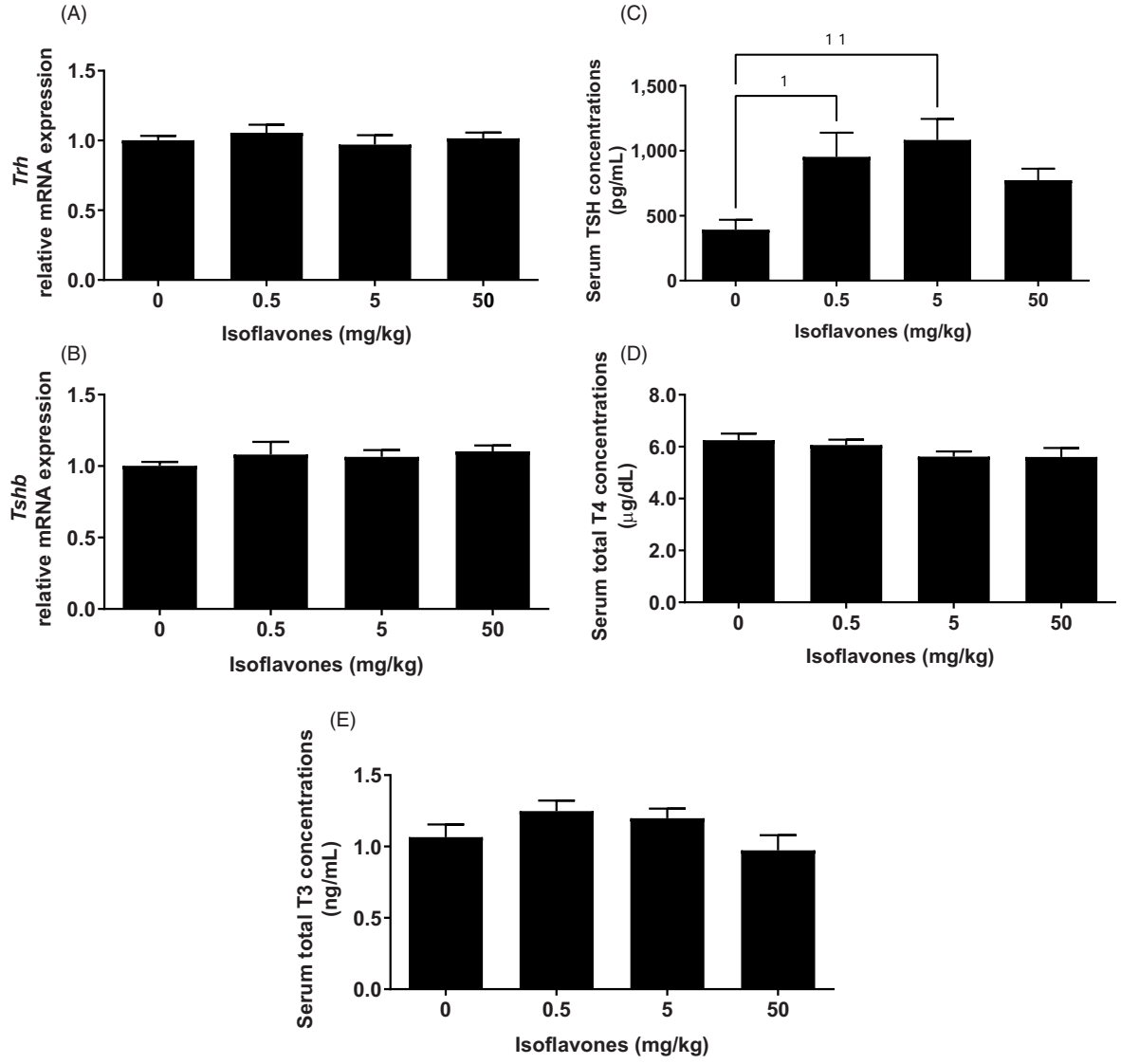

Fig. 1. Stimuli of the hypothalamus-pituitary-thyroid axis. Relative expression of transcripts of (A) *Trh* in the hypothalamus, (B) *Tshb* in the pituitary gland and serum concentrations of (C) TSH, (D) T4, and (E) T3 in male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. *Rpl19* was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunn's post-hoc test); *$p < 0.05$ and **$p < 0.01$ *vs*. the control group. *Trh*, thyrotropin releasing hormone; *Tshb*, thyroid stimulating hormone beta; TSH, thyrotropin; T3, triiodothyronine; T4, thyroxine; *Rpl19*, ribosomal protein L19; BW, body weight; SEM, standard error of the mean.

(LDL) cholesterol, and very low-density lipoprotein (VLDL) cholesterol were measured by an enzymatic colorimetric method with commercial kits from Labtest (Labtest Diagnostica SA, Brazil).

Statistical analysis

The variables were first submitted to the Kolmogorov–Smirnov normality test and homoscedasticity by the Bartlett test. The parameters were evaluated by ANOVA followed by Dunnett's

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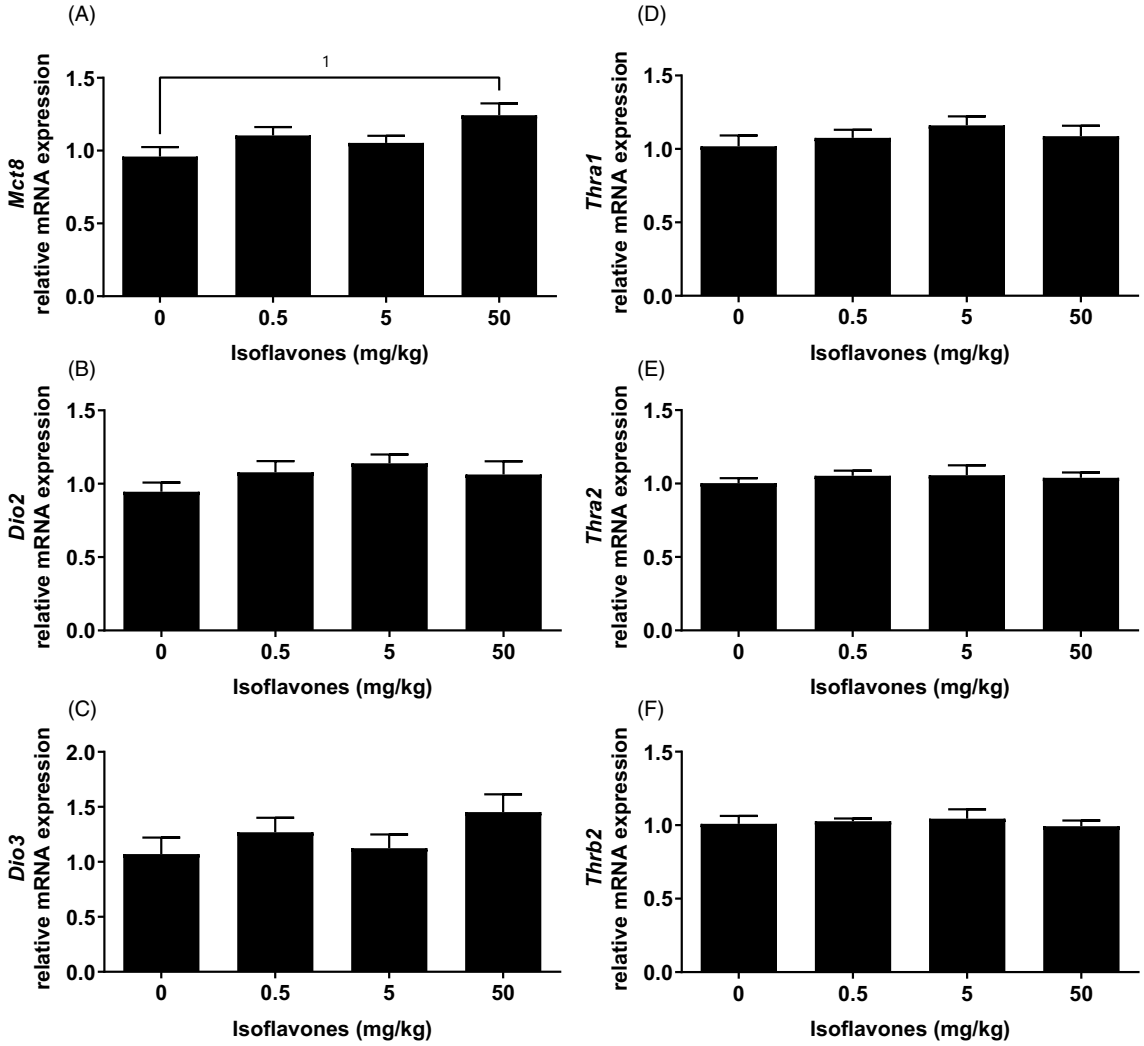

Fig. 2. Negative feedback in the hypothalamus. Relative expression of transcripts of (A) *Mct-8* (B) *Dio2,* (C) *Dio3, (D) Thra1,* (E) *Thra2,* and (F) *Thrb2* in the hypothalamus of male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. *Rpl19* was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's post-hoc test); *$p < 0.05$ *vs*. the control group. *Mct-8*, monocarboxylate transporter 8; *Dio2*, iodothyronine deiodinase 2; *Dio3*, iodothyronine deiodinase 3; *Thra1*, thyroid hormone receptor alpha 1; *Thra2*, thyroid hormone receptor alpha 2; *Thrb2*, thyroid hormone receptor beta 2; *Rpl19*, ribosomal protein L19; BW, body weight; SEM, standard error of the mean.

posttest. Body weight gain was evaluated by multivariate analysis of variance for repeated measures. The Pearson correlation coefficient (r) was used to measure the linear correlation between the two variables. The linear correlation ranged from −1 to 1 and was classified as strong ($|r > 0.7|$), moderate ($|0.5 < r < 0.7|$), or weak ($|0.3 < r < 0.5|$). When $|0 < r < 0.3|$, there is no linear correlation between the variables. Only strong linear correlation was considered in this study. Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA) was used for the analyses. A significant difference was considered when the *P* value was less than 0.05.

Results

Weekly body weight gain

The weekly body weight gain increased with age but was not altered by treatment (Table 2).

HPT axis stimulatory events

The stimulatory coordinated events of the HPT axis were evaluated through the analysis of the genes involved in the production of TRH, TSH, and THs (*Trh* in the hypothalamus, *Tshb* in the pituitary, and *Tshr* in the thyroid), as well as the serum dosages of TSH, T4, and T3. Regarding gene expression, peripubertal exposure to isoflavones did not affect the mRNA levels of *Trh* or *Tshb* at any dosage (Fig. 1A-B). The serum concentrations of TSH were increased in the groups treated with 0.5 ($p < 0.05$) or 5 ($p < 0.01$) mg isoflavones/kg of BW (Fig. 1C). Both serum concentrations of T4 and T3 were not affected by treatments (Fig. 1D-E).

Negative feedback of the HPT axis in the hypothalamus and pituitary

Negative feedback events of the HPY axis occur by the action of THs modulating the production and secretion of TRH and TSH.

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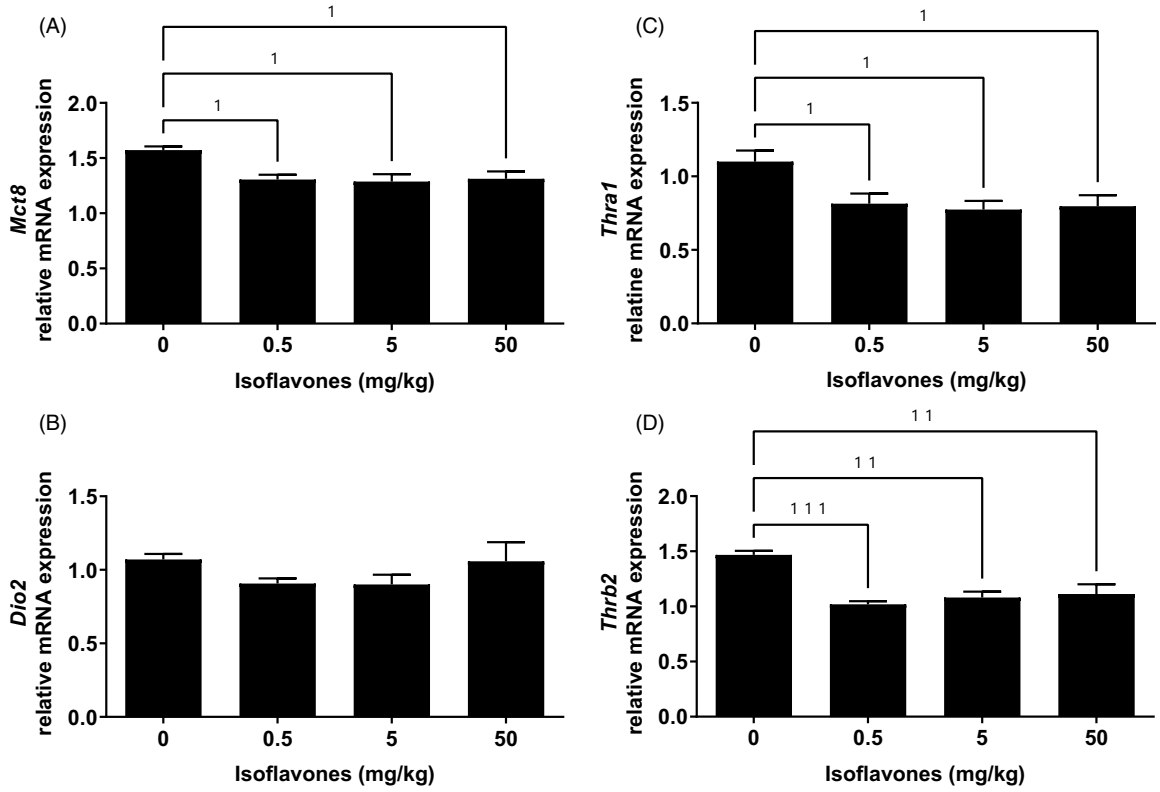

Fig. 3. Negative feedback in the pituitary. Relative expression of transcripts of (A) *Mct-8* (B) *Dio2,* (C) *Thra1,* and (D) *Thrb2* in the pituitary of male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. *Rpl19* was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's posttest); *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs*. the control group. *Mct-8*, monocarboxylate transporter 8; *Dio2*, iodothyronine deiodinase 2; *Thra1*, thyroid hormone receptor alpha 1; *Thrb2*, thyroid hormone receptor beta 2; *Rpl19*, ribosomal protein L19; BW, body weight; SEM, standard error of the mean.

In this manner, we evaluated a series of signaling events mediated by the transcript expression of transporters (*Mct-8*), enzymes (*Dio2, Dio3*), and specific receptors (*Thra1, Thra2*, and *Thrb2*) in the hypothalamus and pituitary. In the hypothalamus, peripubertal exposure to isoflavones increased the expression of *Mct-8* in the group treated with 50 mg of isoflavones/kg of BW (Fig. 2 A; $p < 0.05$). All other parameters were not affected by the treatments (Fig. 2 B-F). In the pituitary, peripubertal exposure to isoflavones decreased the mRNA levels of *Mct-8* ($p < 0.05$), *Thra1* ($p < 0.05$), and *Thrb2* ($p < 0.01$) in all treated groups (Fig. 3A, C and D), while *Dio2* was not affected by treatment (Fig. 3B).

Regulation of thyroid gland hormone production

TH synthesis and secretion are regulated by the expression of particular genes in the thyroid gland: TSH binds to the Tsh receptor (*Tshr*), which stimulates iodine uptake by NIS (sodium/iodide symporter, encoded by the *Slc5a5* gene). In the apical membrane, pendrin (PDS, encoded by the *Slc26a4* gene) and TPO (thyroperoxidase) participate in TH synthesis, which is secreted in the basal membrane by the Mct8 transporter[24]. In this sense, the relative transcript expression of these genes was evaluated to verify the effects of peripubertal isoflavone exposure on the regulation of TH production. The transcript expression of *Nis* was reduced in the groups that received 0.5 or 5 mg of isoflavones/kg of BW (Fig. 4A; $p < 0.05$). The transcript expression of *Pds* was reduced in the groups that received 0.5 mg of isoflavones/kg of BW (Fig. 4B; $p < 0.05$). The transcript expression of *Tshr* (Fig. 4C), *Tpo* (Fig. 4D), and *Mct8* (Fig. 4E) was not altered by treatment. There was a strong positive correlation between *Tshr* and *Nis* mRNA relative expression (Pearson correlation coefficient $r = 0.88$; $p < 0.0001$; Fig. 4F).

Effect of THs on peripheral organs

The heart and liver are peripheral T3-target tissues and were chosen to be evaluated after peripubertal exposure to different concentrations of soy isoflavones. In the heart, the transcript expression of *Mct8* and *Thrb2* was increased in the group treated with 50 mg of isoflavones/kg of BW (Fig. 5A and E, $p < 0.05$). The transcript expression of *Thra1* was decreased in the group treated with 5 mg of isoflavones/kg of BW (Fig. 5C, $p < 0.05$). The relative transcript expression of *Dio2*, *Thra2*, and *Myh6* was not affected by treatment (Fig. 5B, D and F). There was a strong positive correlation between *Dio2* mRNA relative expression and *Mct-8* (Pearson correlation coefficient $r = 0.85$; $p < 0.0001$; Fig. 6A), *Thra1* (Pearson correlation coefficient $r = 0.75$; $p < 0.0001$; Fig. 6B), and *Myh6* (Pearson correlation coefficient $r = 0.78$; $p < 0.0001$; Fig. 6C).

In the liver, the transcript expression of *Mct8* (Fig. 7A; $p < 0.05$), *Thra1* (Fig. 7C; $p < 0.05$), and *Thrb2* (Fig. 7D; $p < 0.01$) was decreased after exposure to 0.5 mg of isoflavones/kg of BW. The expression of *Dio1* and *Dio3* was not altered (Fig. 7B-E).

The lipid profile was evaluated in the serum by measuring the levels of triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, and VLDL cholesterol (Fig. 8A-E). Total cholesterol was decreased after exposure to 50 mg of isoflavones/kg of BW

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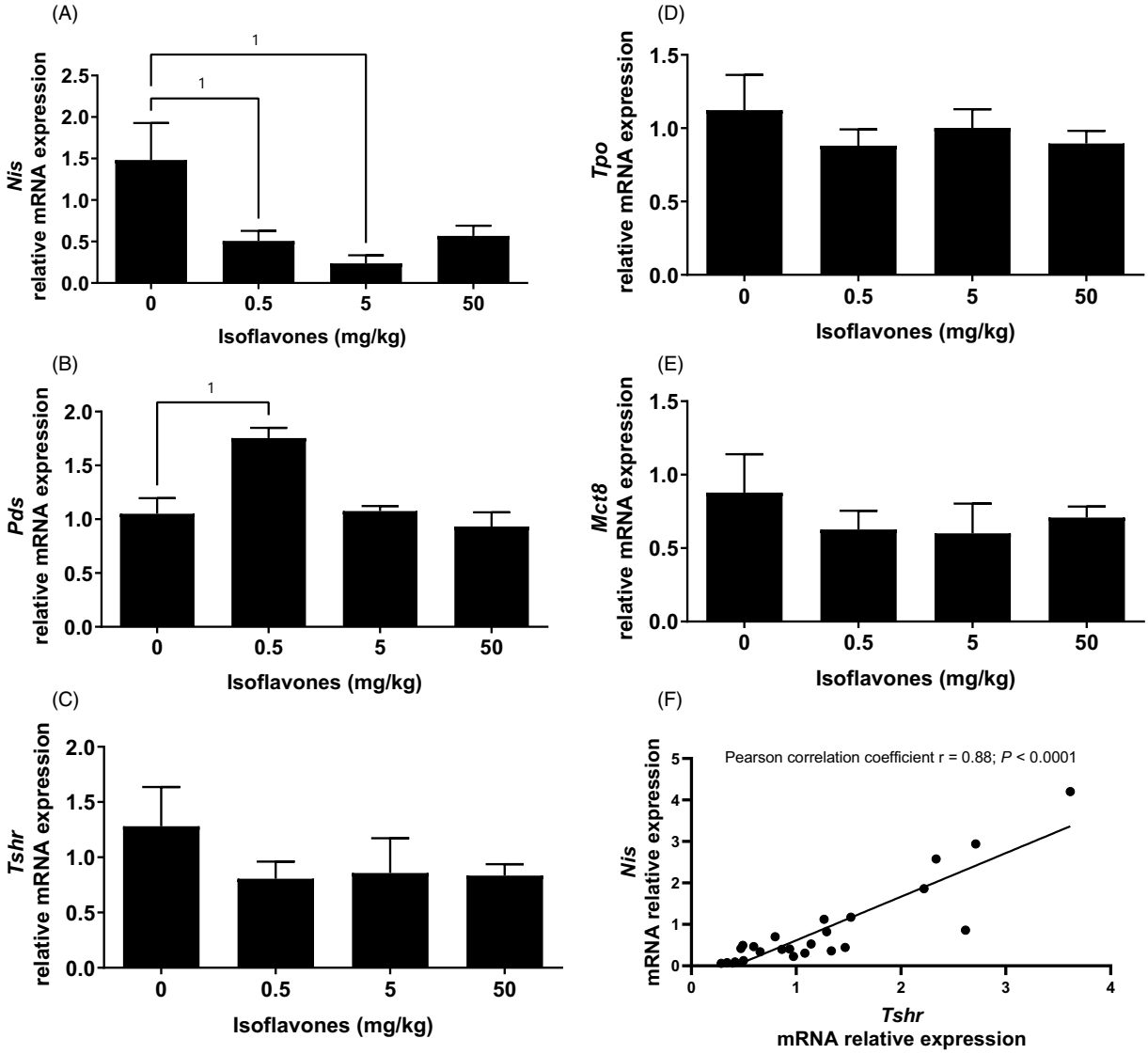

Fig. 4. Regulation of thyroid gland hormone production. Relative expression of transcripts of (A) *Nis*, (B) *Pds*, (C) *Tshr*, (D) *Tpo*, and (E) *Mct-8* in the thyroid of male rats treated with 0.5, 5, or 50 mg isoflavones/kg of BW during the prepubertal period. (F) Correlation between *Nis* and *Tshr* mRNA relative expression (Pearson correlation coefficient r = 0.88; p < 0.0001). *Rpl19* was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's post-hoc test); *p < 0.05 *vs*. the control group. *Nis*, sodium/iodide symporter; *Pds*, pendrin; *Tpo*, thyroid peroxidase; *Mct-8*, monocarboxylate transporter 8; *Rpl19*, ribosomal protein L19; *Tshr*, thyroid stimulating hormone receptor; BW, body weight; SEM, standard error of the mean.

(Fig. 8B), HDL cholesterol was increased after exposure to 0.5 mg of isoflavones/kg of BW (Fig. 8C), and LDL cholesterol was decreased after exposure to 0.5 and 50 mg of isoflavones/kg of BW (Fig. 8D). Triglycerides and VLDL cholesterol were not altered (Fig. 8A and 8E).

Expression of estrogen receptors in the thyroid gland

The transcript expression of *Esr1*, *Esr2*, and *Gper1* was evaluated in the thyroid glands of animals after peripubertal exposure to different concentrations of soy isoflavones and was not affected by treatment (Fig. 9A-C).

Discussion

Isoflavones are a flavonoid group of phytoestrogens[8,9] that are highly abundant in soybeans[10] and stimulate estrogen receptors[8,9]. The thyroid gland presents the estrogen receptors ESR1, ESR2, and GPER1[18], and the exogenous administration of estradiol benzoate increases serum concentrations of TSH and triiodothyronine[19]. In addition, estrogen receptors are modulated in thyroid disorders, such as several types of thyroid cancer[38] and autoimmune thyroiditis[39]. Thus, considering (1) the increased consumption of soy products by the general population, including infants and teenagers, as an alternative to animal milk[16] and (2) the higher susceptibility of the HPT axis during windows of development[31] and the increase in thyroid disorders worldwide[26,27], we investigated the hypothesis that soy isoflavone consumption during the peripubertal period may cause disturbances in the HPT axis and in TH-target organs in male Wistar rats as an experimental model.

The doses used in this study were 0, 0.5, 5, or 50 mg of soy isoflavones/kg of BW per day, which is estimated to be 0, 0.07, 0.7, or 7 mg/kg per day, respectively, for the human equivalent dose[40]. The infant exposure to total isoflavones from soy-based formula varies

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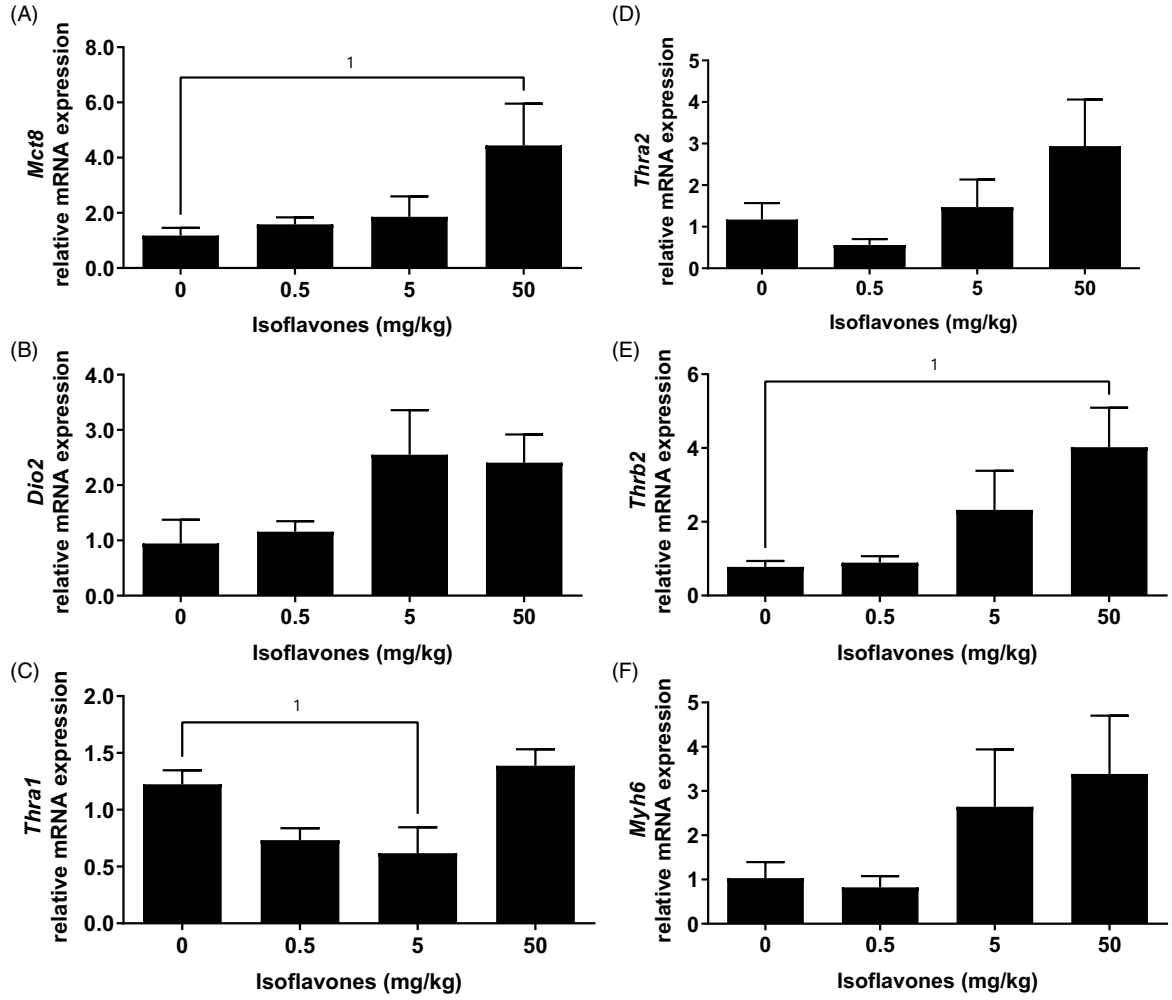

Fig. 5. Peripheral T3-target systems – Heart. Relative expression of transcripts of (A) *Mct-8,* (B) *Dio2,* (C) *Thra1,* (D) *Thra2,* (E) *Thrb2*, *and* (F) *Myh6* in the hearts of male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. *Rpl19* was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's post-hoc test); *$p < 0.05$ *vs*. the control group. *Mct-8*, monocarboxylate transporter 8; *Dio2*, iodothyronine deiodinase 2; *Thra1*, thyroid hormone receptor alpha 1; *Thra2*, thyroid hormone receptor alpha 2; *Thrb2*, thyroid hormone receptor beta 2; *Myh6*, myosin heavy chain 6; *Rpl19*, ribosomal protein L19; BW, body weight; SEM, standard error of the mean.

from 4.5 to 8.0 mg/kg of BW per day[41]. In a matter of concern, an infant exclusively fed soy-based formulas is exposed to a constant level of phytoestrogens that may correspond from 6- to 11-fold higher than the dose when hormonal deregulation of the menstrual cycle is detected in premenopausal women[41].

In this study, weekly body weight gain was not affected by isoflavone consumption, which was also observed in other studies[42,43]. The serum TSH concentrations were increased in the groups treated with 0.5 and 5 mg of soy isoflavones/kg of BW, while no alterations were observed in serum concentrations of triiodothyronine and thyroxine. This scenario is compatible with subclinical hypothyroidism[44]. In adults, subclinical hypothyroidism frequently progresses to hypothyroidism and is associated with dyslipidemia, insulin resistance, and cardiovascular disturbances[45]. However, the necessity of hormonal replacement therapy and the long-term effects of subclinical hypothyroidism in childhood are still debated[46]. In general, children with subclinical hypothyroidism present normal linear growth, but metabolic abnormalities, alterations in blood pressure, and alterations in cardiac morphology have also been reported[46]. In this sense, subclinical hypothyroidism in childhood is not a condition to be underestimated.

It is supposed that during subclinical hypothyroidism, this increased TSH is necessary to maintain adequate levels of THs because the thyroid gland responds little to its stimulation[47]. The first step of TH synthesis is the activation of TSHR in the thyroid gland[48]. TSH binds to TSHR, increasing intracellular cAMP and culminating with the induction of sodium iodide symporter (NIS) transcription[49]. In this study, we observed a downregulation of *Nis* transcript expression in the groups treated with 0.5 and 5 mg soy isoflavones/kg. Reduction in NIS protein expression was observed after soy isoflavones exposure[20]. Additionally, we evaluated the transcript expression of the estrogen receptors *Esr1*, *Esr2*, and *Gper1* in the thyroid gland, but they were not affected by isoflavone consumption.

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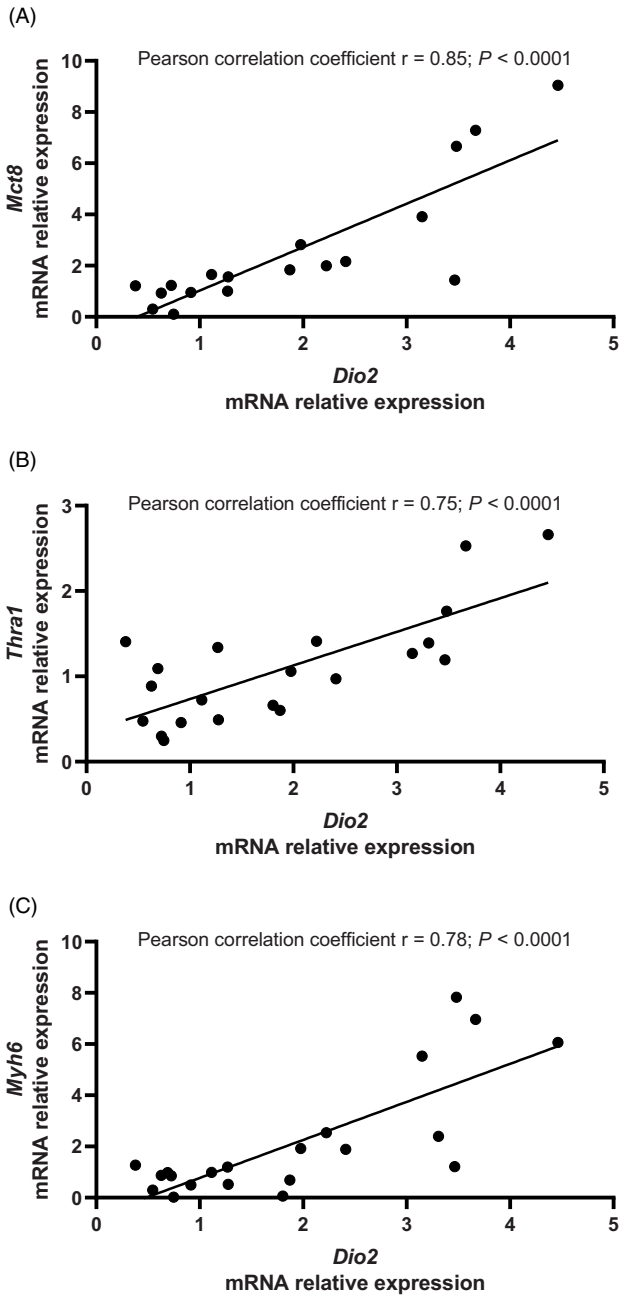

Fig. 6. Peripheral T3-target systems – Correlation in the Heart. Correlation between *Dio2* mRNA relative expression and (A) *Mct-8,* (B) *Thra1*, and (C) *Myh6* mRNA relative expression. *Mct-8*, monocarboxylate transporter 8; *Dio2*, iodothyronine deiodinase 2; *Thra1*, thyroid hormone receptor alpha 1; *Myh6*, myosin heavy chain 6.

In relation to the other genes evaluated in the thyroid gland (*Pds, Tpo, and Mct-8*), only mRNA of *Pds* was altered, with an increase in the group treated with 0.5 mg of soy isoflavones/day. Pendrin is an anion exchanger present in the apical membrane of thyrocytes, among other sites, that participates in apical iodide efflux[50]. The regulation of the PDS gene may be influenced by several factors, including TSH, iodide, immunoglobulin, and insulin[51], and apical iodide efflux in the thyroid is also performed by cystic fibrosis transmembrane conductance regulator and anoctamin 1[52]. The transcript expression of *Pds* observed in this study was not correlated with TSH or *Nis* expression, and considering that the level of participation of each iodide transporter in the apical membrane of thyrocytes is unclear[52], the role of soy isoflavones in *Pds* expression is also unclear.

Although the serum concentrations of TSH increased, the relative mRNA expression of *Trh* and *Tshb* was not altered by soy isoflavone exposure. It is possible that the increased TSH is due to posttranscriptional mechanisms of synthesis and secretion, such as an increased translational rate, increased mRNA half-life, and/or rearrangements of the cytoskeleton[53].

The transport of THs across the blood–brain barrier to achieve the hypothalamus and pituitary occurs through MCT-8 transport[54]. The reduced relative mRNA expression of *Mct8* in the pituitary of soy isoflavone-treated groups may influence the expression of *Thra1* and *Thrb2*, both transcripts regulated by THs[55]. However, the decreased expression of thyroid receptors plus decreased intracellular THs under physiological conditions are expected to downregulate *Tshb* transcript expression[55]. Additionally, any alterations in the expression of deiodinases, which could interfere with TH metabolism, were identified. There are no studies reporting the regulation of thyroid receptors in the pituitary during subclinical hypothyroidism, but two mechanisms may be hypothesized. First, other factors, such as steroid receptor coactivator-1 (SRC1)[56], MED1,[57] and/or GATA2[58], also regulate the expression of the *Tshb* gene. Second, possible activation of the immune system during subclinical hypothyroidism maintains elevated levels of TSH independent of the regulation of the HP thyroid axis[59]. To corroborate this hypothesis, epithelial follicular rat thyroid cells (FRTL) treated with genistein, one of the major components of soy isoflavones, present an increase in the protein expression of cleaved thyroglobulin fragment P40[20], which is considered immunoreactive[60]. This P40 fragment may be associated with the development of autoimmune thyroiditis[61]. A novel class of *Tshb*, *Tshbv*, produced by peripheral blood leukocytes and bone marrow hematopoietic cells and stored in intracellular secretory vesicles in macrophages was identified[62]. In autoimmune thyroiditis, especially Hashimoto's thyroiditis, high levels of Tshbv in peripheral blood leukocytes were identified[63]. This Tshbv is able to induce the synthesis of THs independently of the regulation of the HP thyroid axis[64].

The effects of soy isoflavone consumption during the peripubertal period were also evaluated as important targets for THs, heart, and liver. THs participate in cardiac remodeling, maintenance of electrophysiological functions and contractility, with important cardioprotective effects in the heart[65]. The adverse effects of thyroid dysfunction on the cardiovascular system are well documented, and it is known that minor changes in TH levels or subclinical thyroid dysfunction increase the risks of vascular morbidity and mortality[66]. THs act via genomic and nongenomic mechanisms in the heart, mediated or not by TH receptor isoforms α and β[66], and the actions carried out by *Thra1* are predominant in cardiac muscle[67]. In the group exposed to 50 mg of soy isoflavones/kg group, increased expression of *Mct8* and *Thrb2* were observed. The key importance of TH transporters in the heart is evidenced by high levels of cardiovascular abnormalities in patients with MCT8 deficiency[68]. In ThrβKO mice, a decreased heart rate was observed, possibly by indirect action of this receptor in the expression of genes related to ion channels, such as

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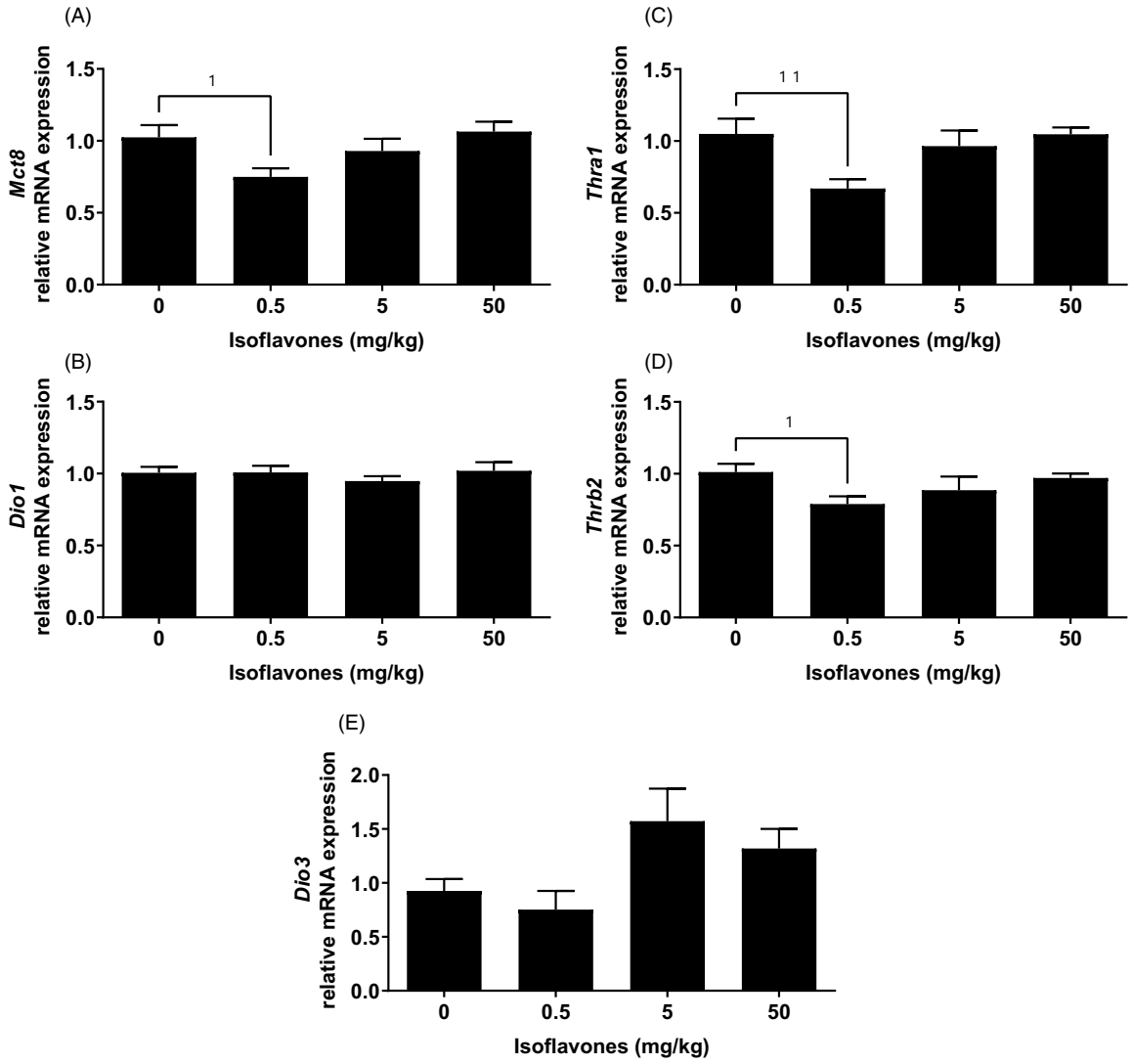

Fig. 7. Peripheral T3-target systems – Liver. Relative expression of transcripts of (A) *Mct-8,* (B) *Dio1,* (C) *Thra1,* (D) *Thrb2*, and (E) *Dio3* in the liver of male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. *Rpl19* was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's post-hoc test); *$p < 0.05$ and **$p < 0.01$ *vs*. the control group. *Mct-8*, monocarboxylate transporter 8; *Dio1*, iodothyronine deiodinase 1; *Dio3*, iodothyronine deiodinase 3; *Thra1*, thyroid hormone receptor alpha 1; *Thrb2*, thyroid hormone receptor beta 2; *Rpl19*, ribosomal protein L19; BW, body weight; SEM, standard error of the mean.

SERCA2 and phospholamban, altering calcium cycling in cardiomyocytes[69]. Additionally, *Thrβ* may be a negative regulator of *Thrα* effects in the heart[70]. In the group treated with 5 mg of soy isoflavones/kg, it was a reduction in the mRNA expression of *Thra1*. Another gene evaluated in the heart was *Myh6*, which encodes myosin heavy chain-α, an important target gene of TH modulation of contractility and cardiac structure[71]. The exposure to soy isoflavones did not affect its mRNA expression.

Finally, the effects of peripubertal exposure to soy isoflavones were evaluated in the liver. THs are important in the control of energetic metabolism, with actions in lipogenesis, beta-oxidation, cholesterol, and carbohydrate metabolism[72], stimulating the expression of enzymes involved in lipogenesis, reducing the synthesis of VLDL and LDL, while increasing the expression of LDL and HDL receptors[73]. Hypothyroidism is associated with impaired lipid metabolism and high levels of LDL and triglycerides[72,74]. Subclinical hypothyroidism may also be associated with an increased risk of developing nonalcoholic fatty liver disease and fibrosis[74]. The group exposed to 0.5 mg of soy isoflavones/kg presented reduced mRNA expression of *Mct8*, *Thra1,* and *Thrb2*, reduced LDL, and increased HDL cholesterol serum levels, an opposite effect expected in hypothyroidism. The group exposed to 50 mg of soy isoflavones/kg presented reduced levels of total cholesterol serum levels. Decreased cholesterol was also observed in thirteen-month-old Wistar rats treated with isoflavones and it was associated with increased cholesterol degradation into bile acids in the liver by triiodothyronine action[75].

It is interesting to note that there was no relationship between the dose of isoflavone and the effect for any of the parameters

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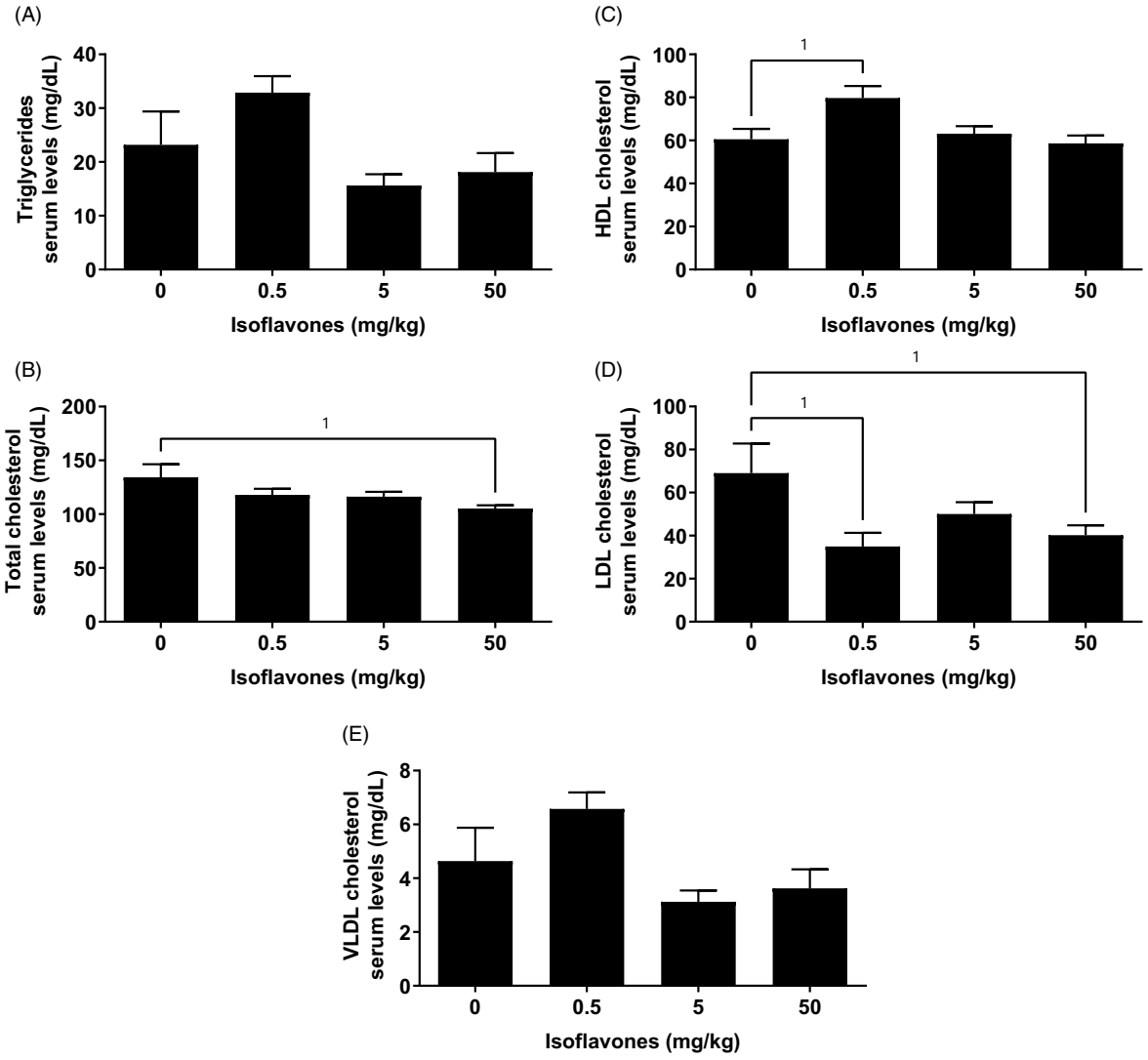

Fig. 8. Liver and nonfasting lipid profile. Lipid profile of (A) triglycerides, (B) total cholesterol, (C) HDL cholesterol, (D) LDL cholesterol, and (E) VLDL cholesterol in the serum of male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's post-hoc test); *$p < 0.05$ *vs.* the control group. VLDL, very low-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; BW, body weight; SEM, standard error of the mean.

evaluated. In addition, the group that received the lowest dose had the highest frequency of alterations. For hormones, it is more common to observe a U-dose response (maximum responses at low and high doses) or inverted U (maximum effects observed at intermediate doses) than a linear response. This is because a small increase in hormone levels can generate a higher increase in the receptor occupancy rate than a hormonal variation at higher levels[76].

In this study, we found that although exposure to isoflavones causes changes in serum TSH levels, there is an adaptation in the thyroid gland (reduced expression of *Nis* transcripts) and heart (correlation between the transcripts of *Dio2* with *Mct8*, *Thra1*, and *Myh6*). This adaptation may be related with the mechanism known as allostasis and its correlation with exposure to endocrine disruptors was previously reviewed by our group[77]. In general, in allostasis, both mechanisms involved in the synthesis and release of THs and the expression and activity of deiodinases and receptors are modulated to obtain the best response to the triggering stressor event[78].

The period used for soy isoflavone exposure comprised a phase of development of high plasticity. It is possible that both permanent and transient responses from this axis were triggered.

Conclusion

In conclusion, these results suggest that the consumption of relevant doses of soy isoflavones during the peripubertal period in males may induce subclinical hypothyroidism, with alterations in the regulation of the HPT axis, modulation of TH synthesis, and peripheral alterations in TH target organs. In this sense, the uses of soy isoflavones during critical windows of development, even with iodine supplementation, should be discussed.

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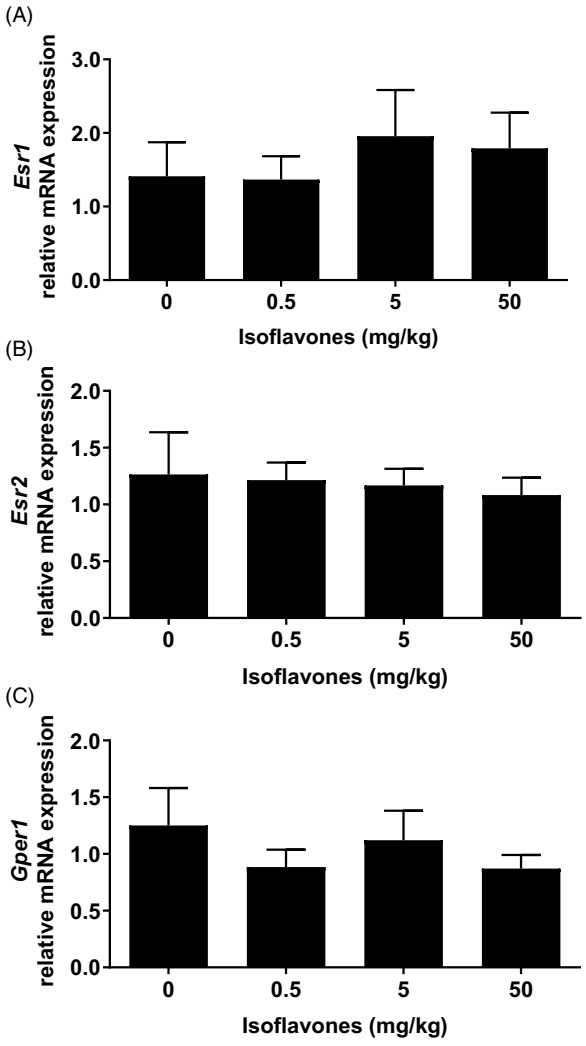

Fig. 9. Expression of estrogen receptors in the thyroid gland. Relative expression of transcripts of (A) *Esr1,* (B) *Esr2*, and (C) *Gper1* in the thyroid of male rats treated with 0.5, 5, or 50 mg of isoflavones/kg of BW during the prepubertal period. Rpl19 was used as an internal control. Data are expressed as the mean ± S.E.M. (one-way ANOVA followed by Dunnett's post-hoc test). *Esr1*, estradiol receptor 1; *Esr2*, estradiol receptor 2; *Gper1*, G protein-coupled estrogen receptor 1; *Rpl19*, ribosomal protein L19; BW, body weight; SEM, standard error of the mean.

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Compliance with ethical standards.

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

Ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and approved by the Universidade Estadual do Centro-Oeste, Ethical Committee for Animal Research (protocols # 005/2017 and 019/2017).

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