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# Immune stimulation improves endocrine and neural fetal outcomes in a model of maternofetal thyrotoxicosis

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ABSTRACT

The potentiation of the immune system in pregnant rats was performed with Complete Freund's Adjuvant [CFA; 20 μl, subcutaneous at gestation day (GD) 18] in experimentally-induced hyperthyroidism by Levo-thyroxine (L-T4; 10 μg/100 g of b.w., intraperitoneal from GD 2 to 17). The potential effects on the fetal neuroendocrine function were evaluated by observing some histopathological investigations in pregnant rats and measuring some biochemical parameters in dams and their fetuses at GD 20. In hyperthyroid group, an increase in maternofetal serum thyroxine (T4), triiodothyronine (T3) and a decrease in thyrotropin (TSH) levels were noticed, while the concentrations of fetal serum growth hormone (GH) and insulin-like growth factor-1 (IGF1) levels were increased at tested GD with respect to control and CFA groups. Moreover, the activity of uterine and placental myeloperoxidase (MPO) was increased ($P < 0.001$) in CFA and CFA-treated hyperthyroid groups in respect to control or hyperthyroid groups, respectively. The gestational thyrotoxicosis led to some histopathological lesions in uterine and placental tissues characterized by severe degeneration in trophoblast spongioblast cell layer with congestion, mild congested blood vessels in the endometrium and deficient in spiral artery remodeling. Although, the elevation in fetal serum transforming growth factor-beta (TGFβ) and cerebellar monoamines [norepineprine (NE), epinephrine (E), dopamine (DA) and 5-hydroxytryptamine (5-HT)] was observed, the reduction in fetal serum tumor necrosis factor-alpha (TNFα) and adipokines (Leptin and adiponectin) was detected. Treatment of dams with CFA showed an obviously reversing and protecting effect against hyperthyroid perturbations. Thus, the maternal CFA can be used in treatment of the fetal neuroendocrine dysfunctions.



## 1. Introduction

Thyroid hormones (THs) are vital both for the physiological sequence of gestation, the optimal differentiation of the embryonic organs especially the placenta [1,2], and fetal/neonatal brain development [3,4]. Also, these hormones are important regulators of growth factors [5,6], adipocytokines [7], and developing brain monoamines [8,9]. Conversely, the GH, IGF, TNFα and TGFβ could mediate the actions of developing THs [10,11]. On the other hand, maternal thyroid dysfunction may cause pregnancy complications and diseases in the fetus/child [12]. The early hyperthyroidism in rats modifies thyroid states and causes some malformations such as decrease in body, brain and cerebellar weight [13]. It also alters the levels of GH & IGF1 [14], TGFβ [15], TNFα [16], adiponectin & leptin [17] and biogenic amines in developing brain [18]. It causes irreversible dysfunction of the brain if not corrected shortly after the birth [19–21].

Maternal immune responses have been shown to influence the embryo's tolerance to teratogens [22]. Also, maternal immunostimulation using CFA could previously protect against thyroid disorders [23,24] and teratogenesis caused by chemicals or diabetes [25,26]. It causes non-specific immune stimulation including activation and migration of the immune cells (like macrophages and lymphocytes) to the uterine and placental tissue [27,28]. Also, the fetal trophoblast cells in human and rodent pregnancies invade into the uterine wall and aid to placental development and function by transforming the maternal spiral arteries [29]. These were found essential in development of embryo and inhibition of teratogenesis [28].

Because of CFA is a strong adjuvant capable of stimulating cellular immune responses [24], the present study aimed to determine whether the administration of CFA during the gestational period in L-T4-induced maternal hyperthyroidism may enhance the development of fetal neuroendocrine system. The study extended not only to follow the changes in the activities of maternofetal thyroid axis, and fetal GH/IGF1, adipocytokines and cerebellar monoamines but also to view the changes in the activity of MPO and histogenesis of the placental and uterine tissues at GD 20. In this regard, we used rats because their placenta is very similar to the human placenta [30]. The developing rat

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brain is highly sensitive to TH disturbance [31,32]. Notably, rat is an attractive animal model for the human condition of congenital hypothyroidism [33] because the rat brain at birth is at the same stage as the human brain at 5–6 months of gestation [34].

## 2. Materials and methods

### 2.1. Experimental animals

Mature white albino rats (*Rattus norvegicus*, Wistar strain) were purchased from the National Institute of Ophthalmology, Giza, Egypt. This study was carried out on 40 mature virgin females weighting about 170–190 g and 20 mature males for mating only. They were kept under observation in the department animal house for 2 weeks to exclude any intercurrent infection and to acclimatize the new conditions. The animals were housed in stainless steel separate bottom good aerated cages at normal atmospheric temperature (23 ± 2 °C). They were fed a standard rodent pellet diet manufactured by an Egyptian company producing oil and soap as well as some vegetables as a source of vitamins. Tap water was provided and the rats were allowed to drink ad libitum. The rats were exposed to constant daily 12 h light:12 h darkness each (lights on at 06:00 h) and 50 ± 5% relative humidity. Generally, all the animal procedures were in accordance with the general guidelines of animal care and the recommendations of the Canadian Council on Animal Care [35]. All efforts were made to minimize the number of animals used and their suffering.

### 2.2. Mating and fertilization

To determine the estrus cycle, the vaginal smear of each virgin female was examined daily. Three types of cells, leukocytes, epithelial and cornified cells, were observed in photomicrographs of unstained vaginal smear. As reported by Marcondes et al. [36], the proportion of the three types of cells was used for the determination of the estrous cycle phases. A proestrus smear consists of a predominance of nucleated epithelial cells; an estrous smear primarily consists of anucleated cornified cells; a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells; a diestrus smear primarily consists of a predominance of leukocytes. Proesterous females were left for one night to copulate with the normal males (2 females with one male). Early next morning (before 7 am), copulation was checked by examining the outer surface of the vagina for the presence of a vaginal plug formed by coagulation of semen (white clotting, sperm clot). When such a grayish-white clot blocking the mouth of vagina was detected, this day was considered as the first day of gestation. Then, the pregnant females were transferred into separate cages from males to start the experiment.

### 2.3. Experimental schedule and samples collection

On 1st day of pregnancy (GD 1), the adult female rats were allocated into 4 groups 10 rats each. The first group was designed as control receiving only normal saline while the second group was designed as hyperthyroid group which received L-T4 (GlaxoWellcome, Germany) (10 μg/100 g of body weight, intraperitoneal) [37] daily in saline from GD 2 to 17. The third group was designed as CFA (Sigma-Aldrich)-treated group which received 20 μl subcutaneously [25] at multiple sites on the inside of the hind legs at GD 18. The final group was designed as CFA-treated hyperthyroid group which received L-T4 from GD 2 to 17 and treated with CFA at GD 18. We injected the CFA at the end of gestation to avoid the stress and abortion because of the dams were very nervous (gestational hypertension) due to L-T4 administration. Two days later, animals were euthanized in the early morning before the delivery, the maternal and fetal blood samples were collected and centrifuged at 15,000 ×*g*. Uterus and placenta were weighed then parts were kept in 0.5% hexadecyltrimethylammoniumbromide (HTAB)/50 mM phosphate buffer (pH 6.0) while other parts were fixed in 10% neutral buffered formalin for study of the general histological structure (hematoxylin and eosin stain). Their slides were examined under a light microscope for the presence of any histological changes. On the other hand, fetal cerebellum was homogenized in 75% aqueous HPLC grade methanol by using a Teflon homogenizer (Glas-Col, Terre Haute, USA). All sera samples and tissues were stored at −70 °C. All reagents were of the purest grades commercially available.

### 2.4. Maternofetal hormonal examination

Maternofetal serum T4 [38], T3 [39], TSH [40], fetal GH [41] and IGFI [42] levels were estimated quantitatively by RIA in biochemistry department, faculty of medicine, Cairo University, Egypt. The kits were obtained from Calbiotech INC (CBI), USA.

### 2.5. Fetal TGFβ and adipocytokines examination

Serum TGFβ, leptin, adiponectin and TNFα levels were detected by ELISA, measured with a microplate reader (Spectra Max 190—Molecular Devices, Sunnyvale, CA, USA) in biochemistry department, faculty of medicine, Cairo University, Egypt. Commercial kits were utilized for the measurement of TGFβ, leptin and adiponectin (ELISA kit-Millipore, St. Charles, MO, USA). TNFα kit was purchased from Invitrogen Corporation 542 Flynn Road, Camarillo, CA 93012 (USA).

### 2.6. Maternal examinations

#### 2.6.1. Uterine and placental MPO

The activity of MPO was determined according to the method of Krawisz et al. [43]. Within each group, uterine and placental tissues were weighed and minced in 1 ml of 0.5% HTAB in 50 mM phosphate buffer (pH 6.0, 200 mg/1 ml). The minced tissue was homogenized three times (power set at 4) with a Polytron homogenizer (13.500 rpm for 1 min) on ice. The homogenate was sonicated on ice for 10 s, freezed (−20 °C)-thawed (immersion in warm water 37 °C) three times, and centrifuged at 20 000 ×*g* for 15 min at 4 °C to remove insoluble material. The supernatant was transferred to a 96-well plate (7 μl per well, triplicate each samples). The enzyme reaction was carried out by adding 200 μl of phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml *O*-dianisidine hydrochloride (Sigma-Aldrich) and 0.0005% $H_2O_2$. The kinetics of absorbance changes at 460 nm was measured at 0, 30 and 60 min in a microtiter reader. It was expressed in units/mg of wet tissue, 1 unit being the quantity of enzyme able to convert 1 μmol of $H_2O_2$ to water in 1 min at room temperature. Its activity/min was calculated from a standard curve using purified peroxidase enzyme (Sigma-Aldrich).

#### 2.6.2. Histopathological examination

The fixed uterine and placental tissues were processed according to Bancroft and Gamble [44]. These samples were washed in running water, dehydrated in ascending graduated ethyl alcohol, cleared in xylene, and embedded in paraffin wax. Tissue sections (5 μm) were stained by Heamatoxylin and Eosin stain at the Pathology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt. The slides were evaluated for the degree of injury and improvement in the uterine and placental tissues, and spiral artery.

### 2.7. Fetal cerebellar monoamines examination

The monoamines concentrations were estimated according to the fluorometric method described by Ciarlone [45]. These measurements were performed in National Research Center, Egypt. The fluorescence was read at excitation 380 nm for NE, 360 nm for E, 320 nm for DA and 355 nm for 5-HT, as well as the emission by Hitachi (F3010

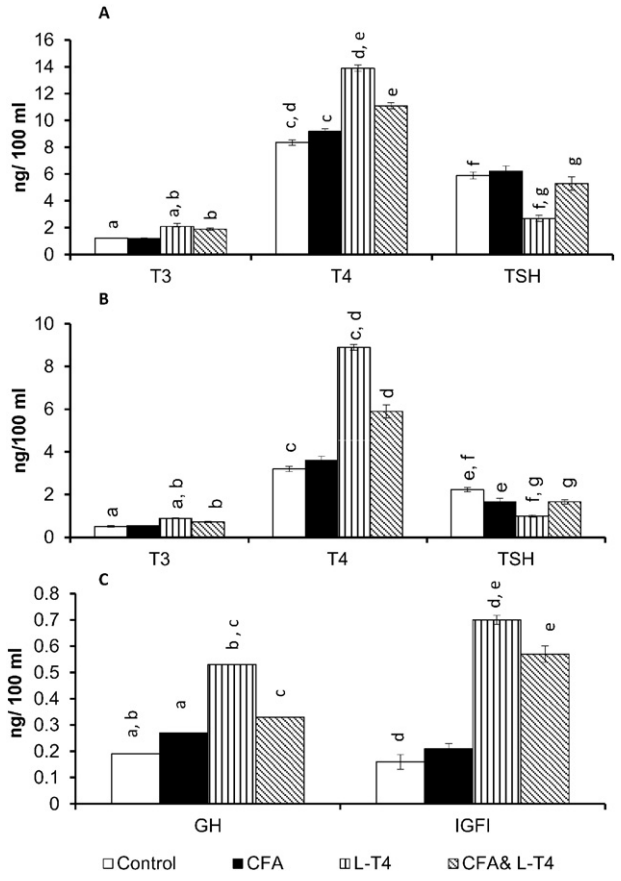


**Fig. 1.** Effect of CFA on L-T4-induced changes in the levels of maternofetal thyroid axis and fetal GH/IGF-1 axis at GD 20: (A) Maternal T3, T4 and TSH, (B) Fetal T3, T4 and TSH, (C) Fetal GH and IGF. Degree of statistical significance between rat groups in *A* was $p < 0.001$ at a, b, d, e, f and g but $p < 0.05$ at c; $p < 0.001$ at a, b, c, d, f and g but $p < 0.001$ at e in *B*; $p < 0.001$ at b, c, d and e but $p < 0.05$ at a in *C*.

model) spectrophotofluorometer was 480 nm for NE, E & DA, and 470 nm for 5-HT.

### 2.8. Statistical analysis

Data were analyzed using one-way ANOVA (SPSS, version 20; Chicago, IL). When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups were performed by using Tukey's test.

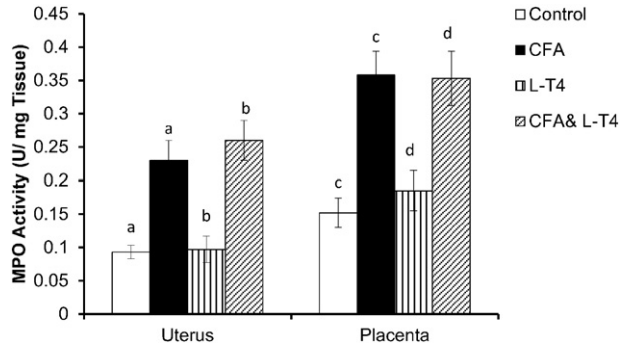


**Fig. 3.** (A) Effect of CFA or CFA & L-T4 on uterine and placental MPO. CFA versus control or CFA & L-T4 versus CFA could increase the activity of MPO in both of uterus and placenta. Degree of statistical significance was $p < 0.001$ at a, b, c, and d.

## 3. Results

### 3.1. Effect of CFA on L-T4-induced changes in the levels of maternofetal serum thyroid markers and fetal serum GH/IGF1 at GD 20

In dams, the administration of L-T4 resulted in a marked increase ($P < 0.001$) of T3 and T4 levels and a profound decrease ($P < 0.001$) of TSH level with respect to control. CFA could reverse the effect of L-T4 on the THs and TSH (Fig. 1A). On the other hand, CFA-treated hyperthyroid group showed a decrease ($P < 0.001$) in the T3 and T4 levels compared to non-treated hyperthyroid group. In contrast, the level of TSH was increased ($P < 0.001$) in the former compared to the second group.

In fetuses, the elevation ($P < 0.001$) in T3, T4, GH and IGF1 levels and diminution ($P < 0.001$) in TSH level were observed in hyperthyroid group in comparison to the control group (Fig. 1B & C). Conversely, there was reduction ($P < 0.001$) in T3, T4, GH and IGF1 levels which was accompanied by increase ($P < 0.001$) in TSH values of CFA-treated hyperthyroid group as compared to their levels in the non-treated hyperthyroid group.

### 3.2. Effect of CFA on L-T4-induced changes in the levels of fetal serum cytokines and adipokines markers at GD 20

The concentration of TGFβ was found to be increased ($P < 0.001$) in CFA and hyperthyroid groups, although the concentration of TNFα was found to be decreased in CFA ($P < 0.01$) and hyperthyroid ($P < 0.001$) groups if compared to their levels in the age-matched normal control (Fig. 2A). Conversely, CFA-treated hyperthyroid group showed an increase ($P < 0.001$) in the concentration of TNFα even though the concentration of TGFβ was not changed when compared to respective non-treated hyperthyroid values.

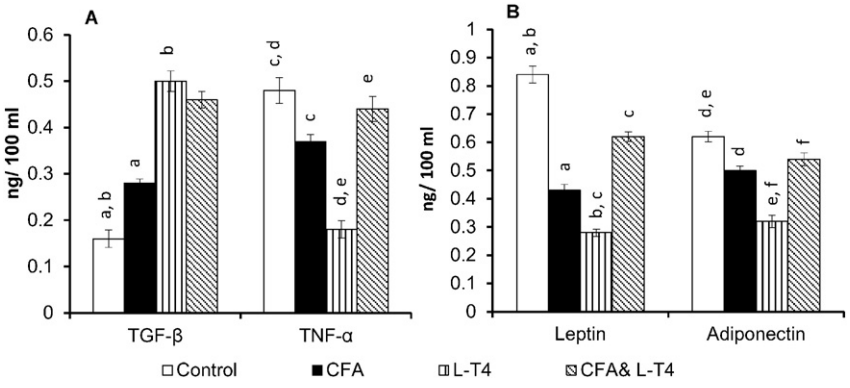


**Fig. 2.** Effect of CFA on L-T4-induced changes in the levels of fetal cytokines (A) and adipokines (B) at GD 20: Degree of statistical significance was $p < 0.001$ at a, b and c, d and e in *A*; $p < 0.001$ at a, b, c, e and f but $p < 0.01$ at d.

For adipokines, there was attenuation ($P < 0.001$) in leptin and adiponectin values of hyperthyroid group in comparison with their corresponding control one (Fig. 2B). In contrast, their elevations ($P < 0.001$) were noticed in CFA-treated hyperthyroid group in respect to non-treated hyperthyroid one.

### 3.3. Effect of CFA and L-T4 on the activity of MPO in both uterine and placental tissues at GD 20

In CFA and CFA-treated hyperthyroid groups, the activity of MPO in these tissues was increased ($P < 0.001$) in comparison to the control and non-treated hyperthyroid groups, respectively (Fig. 3). MPO activity in non-treated hyperthyroid group did not show any changes ($P < 0.05$) with respect to its own control.

### 3.4. Effect of CFA on L-T4-induced histopathological changes in uterus and placenta at GD 20

For placenta (Fig. 4, A-D), the control group showed normal architecture of trophoblast giant cell, spongioblast, and labyrinth layers. Hyperthyroid group indicated severely degenerated cells with congestion in trophoblast spongioblast layer. In CFA group, some irregularly enlarged trophoblast giant cells was observed with mild degenerated trophoblast spongioblast, while CFA-treated hyperthyroid group showed normal

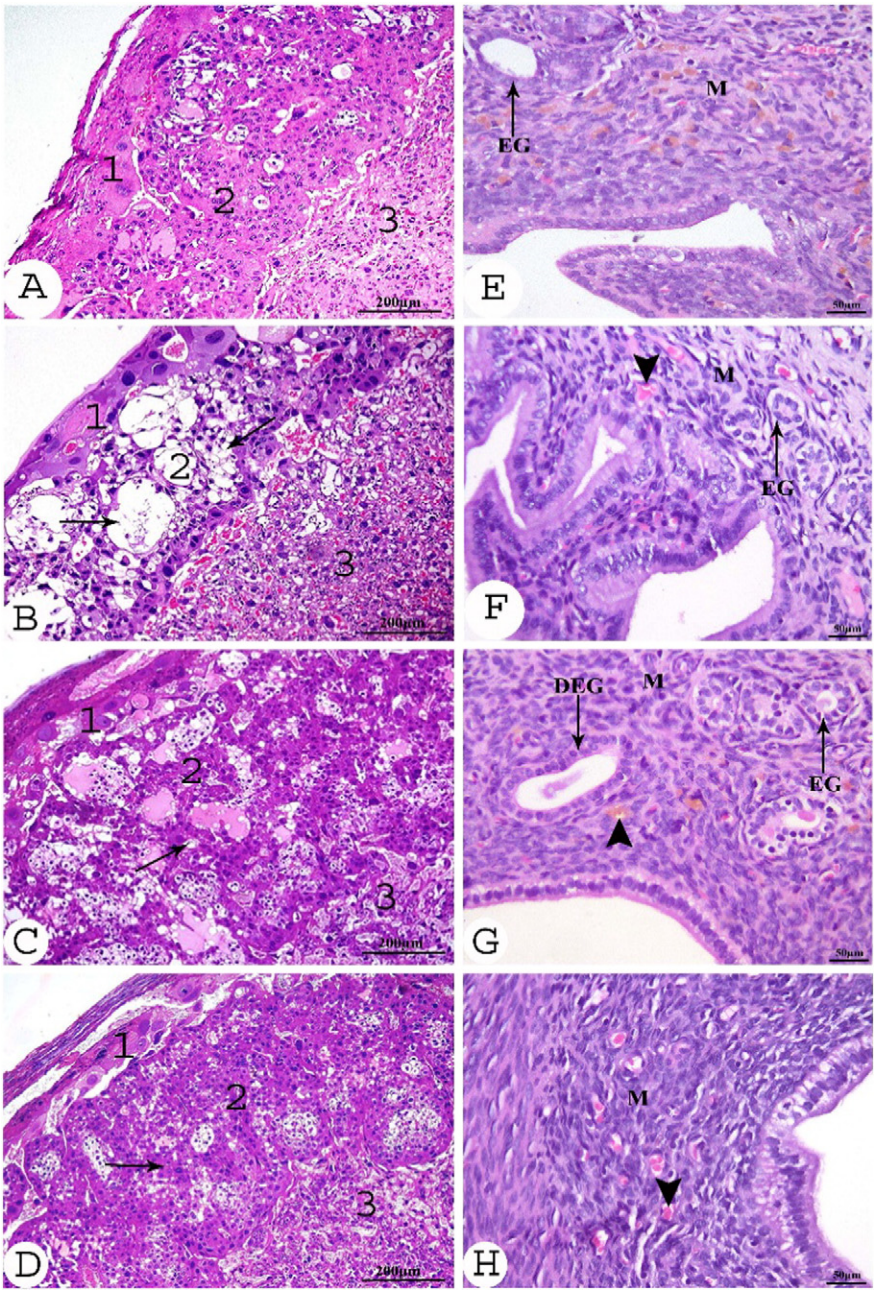


**Fig. 4.** *Placenta* of control group (A) showing regular cells (typical cells and nuclear size) in all layers (1, trophoblast giant cell layer; 2, spongioblast; and 3, Labrynith layers). Placenta of hyperthyroid group (B) showing trophoblast spongioblast layer having severely degenerated cells with congestion (arrow). Placenta of CFA group (C) has mildly degenerated trophoblast spongioblast (arrow) with some irregular enlarged trophoblast giant cell. Placenta of hyperthyroid treated group (D) showing normal architecture with enlarged cells of spongiotrophoblast and mild degeneration (arrow). Uterus of control group (E) shows normal endometrium architecture while that of hyperthyroid group (F) shows mild congested blood vessels in the endometrium. Uterus of CFA group (G) shows dilated endometrial gland and congested blood vessels while hyperthyroid treated group (H) shows normal architecture with some congested blood vessels in the endometrium. Where, DEG is dilated endometrial gland; EG is endometrial gland; M is dilated endometrium and arrowhead is congested blood vessels. H&E stain.

architecture with enlarged and mildly degenerated spongiotrophoblast cells. For the uterus (Fig. 4, E-H), the control group showed normal endometrium architecture, while that of hyperthyroid group showed mildly congested blood vessels in the endometrium. The uterus of CFA group indicated dilated endometrial gland and congested blood vessels, though CFA-treated hyperthyroid group showed normal architecture with some congested blood vessels in the endometrium.

On the other hand, the early cytotrophoblast invasion of spiral artery (spiral artery remodeling) was observed in control group (Fig. 5A). Hyperthyroid group showed a deficient spiral artery remodeling (the blood vessels retained its muscular and elastic tissue together with endothelial lining; Fig. 5B). The remodeling of spiral artery (cytotrophoblast invasion with losing of the smooth muscle cell and elastic tissue) was mild in CFA group (Fig. 5C) and moderate in hyperthyroid treated group (Fig. 5D).

### *3.5. Effect of CFA on L-T4-induced changes in the levels of fetal cerebellar monoamines at GD 20*

In hyperthyroid group, the elevation in NE, E and 5-HT was $P < 0.001$ while this elevation in DA was $P < 0.01$ if compared with the control one (Fig. 6). Their levels in hyperthyroid group were reversed after treatment with CFA where the reduction was observed in NE ($P < 0.05$), E and 5-HT ($P < 0.001$), and DA ($P > 0.05$).

## 4. Discussion

The administration of L-T4 to adult female rats during pregnancy induced a profound gestational and fetal thyrotoxicosis at GD 20 where the elevation in maternofetal serum THs and the reduction in TSH levels were recorded. This may be attributed to the passive transfer of maternal T4 to their fetuses by placental retinol binding protein transthyretin (TTR, TH distributor proteins) [46] or transfer of maternal thyroid antibodies [thyroglobulin (TgAb) and thyroperoxidase (TpAb)] into the fetal compartment to stimulate the fetal thyroid by binding thyrotropin receptor (TSHR) [47,48]. It is obvious that the fetal thyroid functions may directly affected by maternal pituitary–thyroid axis (PTA) where TSH stimulates T4 secretion from the thyroid gland via TSHR which stimulates adenylate cyclase catalyzed cAMP production [49] and regulates thyroidal T4 monodeiodination [32,50]. Since TSH is a regulator of T4 level, decreased T4 after CFA treatment was associated with increased TSH level. We also observed that the dams of hyperthyroid group were more active and nervous (gestational hypertension). Nearly, the same thought was supposed by Krassas et al. [51]. In accordance with the stimulatory effect of L-T4 on THs, fetal serum GH, IGF1and TGFβ levels were increased. The situation was different for fetal serum TNFα, leptin and adiponectin where their levels were decreased at the examined day in comparison to the control group. This disorder might reflect their important interaction with thyroid axis. In consistence, the reduction in TNFα could be referred to the high levels of TGFβ [52] or GH and IGF1 [32] which were known as inhibitors for proinflammatory cytokines. Likewise, administration of T3 decreased the concentration of these adipokines, as well as a reduction in their gene expression [17]. It can infer that the administration of maternal L-T4 might impair the physiological maturation of these markers via the fetal hypothalamic–pituitary–thyroid axis (HPTA) dysfunction.

One more interesting observation in our study is that the treatment with CFA indicated an antagonistic effect on T4-induced hyperthyroidism where the elevation in maternofetal serum THs levels and the reduction in maternofetal serum TSH level and in fetal serum adipokines (leptin and adiponectin) levels were reversed. Also, this treatment could ameliorate the fetal serum GH, IGF1 and TGFβ levels, and normalize the fetal serum TNFα level at the tested GD. These data reinforced by several possible mechanisms where the hyperthyroidism could be delayed by CFA [23] inducing T helper 1 (Th1) cytokines [such as interferon-γ (IFNγ)] [53,54] and modulating Th1/Th2 responses [55] to suppress iodide uptake and TH synthesis [56]. Also, the adaptive action of maternal CFA treatment seems to be sufficient to maintain and enhance the defense mechanisms in the fetuses where no any abortion case was recorded. There is general agreement that the maternal immune stimulation can protect the embryos from the teratogenic insults by increasing the TGFβ2 mRNA expression at the fetomaternal interface [57] and decreasing the mRNA expression of TNFα in embryos [58]. Another possibility is that the ameliorations and normalizations in the present markers might be essential in early development of the embryo, fuel availability during pregnancy, and inhibition of the pregnancy loss [5,59–61]. It can be suggested that the maternal immune stimulation by CFA may be important not only for the fetus but also for the health of the mother.

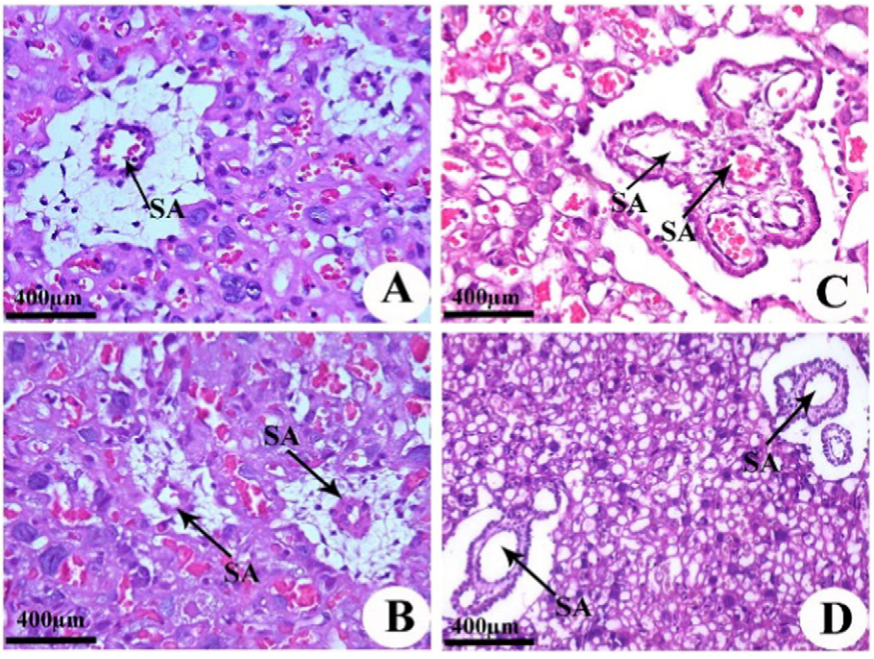


**Fig. 5.** Spiral artery of placental trophoblast in control group (A), hyperthyroid group (B), CFA group (C) and hyperthyroid treated group (D). Where, SA is spiral artery. H&E stain.

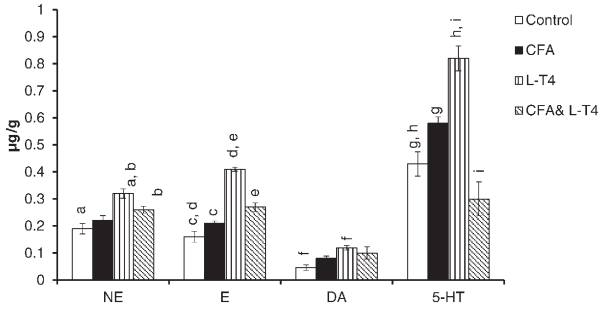


**Fig. 6.** Effect of CFA on L-T4-induced changes in the levels of fetal cerebellar monoamines (NE, E, DA and 5-HT). At GD 20. CFA & L-T4 could decrease the levels of NE, E and 5-HT in comparison to the CFA group. The decrease in DA was not statistically significant. Degree of statistical significance was $p < 0.001$ at a, d, e, h and i, $p < 0.01$ at f and $p < 0.05$ at b, c & g.

The conspicuous perturbations in hyperthyroid group led to some histopathological deterioration in uterine and placental tissues characterized by severe degeneration in spongiotrophoblast cell layer with congestion, and mild congested blood vessels in the endometrium at GD 20. The impairment in spiral artery remodeling was also observed in this group. These alterations reflected the abortion in some dams (two cases only). It has been postulated that abnormal TH levels (hyperthyroid state) caused a malplacentation [62], produced a significant changes in the uterine vasculature [63] and in the proliferation of trophoblast cells [64], impaired the remodeling of spiral artery [65] and facilitated the antibody dependent cell mediated cytotoxicity, the lymphocytic infiltration and the thyrocytotoxicity [66]. This may be explained by the occurrence of death and/or removal of invasive trophoblast cells during and after delivery [67]. Indeed, the appearance of immune cells in placenta after maternal immunostimulation with CFA indicated a probable trans-placental migration of these cells or cytokines to the embryo [55,68]. This stimulation could have beneficial effects on the embryo development [26]. The enlarged spongiotrophoblast in CFA-treated hyperthyroid group might be responsible for restoring normal development of the embryo. Also, the remodeling of the placental spiral arteries and trophoblast invasion are considered to be initiated by natural killer (NK) cells and macrophages to produce cytokines, chemokines and pro- and anti-angiogenic factors [29]. In parallel, the elevation of MPO level (a marker of leukocyte accumulation; [69]) in CFA-treated hyperthyroid group may reflect the disappearance of the histopathological changes in placental and uterine tissues of hyperthyroid group. These data probably support the conclusion, reached by Odobasic et al. [70] that MPO limits the pathological tissue inflammation and plays an important role in intracellular pathogen killing. These explanations strengthen the possibility that the stimulation of uterine and placental immune cells supported the maternal intrauterine vascular system during pregnancy and caused a protective effect against hyperthyroidism.

Further analysis in our study revealed a positive relationship between fetal cerebellar monoamines, THs, growth factors and adipocytokines in the control group. This balance was essential for normal brain development [8,32]. Alternatively, the maternofetal thyrotoxicosis could upregulate the secretion of monoamines compared to the control values. Concomitantly, a pronounced elevation in their levels [32] and in the expression of their receptors were depicted previously [71,72]. This may be mediated the neurodevelopmental toxicity through the maternofetal hormonal imbalance, and this observation is consistent with the results of Ahmed et al. [32] in hyperthyroid rat. On the other hand, treatment with CFA could decrease the concentrations of monoamines but the reduction in DA was the only non-significant at the examined day in comparison to the hyperthyroid group. In similar, Ilani et al. [73] proved that DA acts as a neuronal mediator for T cell activation. The importance of these improvements is attributed to a reduction in THs after CFA treatment. It is relevant to purpose that the treatment with CFA can ameliorate the hyperthyroid status.

## 5. Conclusion & Future direction

Third conclusions can be drawn from these data. The first is that the maternofetal thyrotoxicosis by L-T4 appears to alter the developing HPTA, GH/IGF and cytokines/adipokines axes. The second is that the maternal treatment with CFA seems to reduce or prevent the fetal

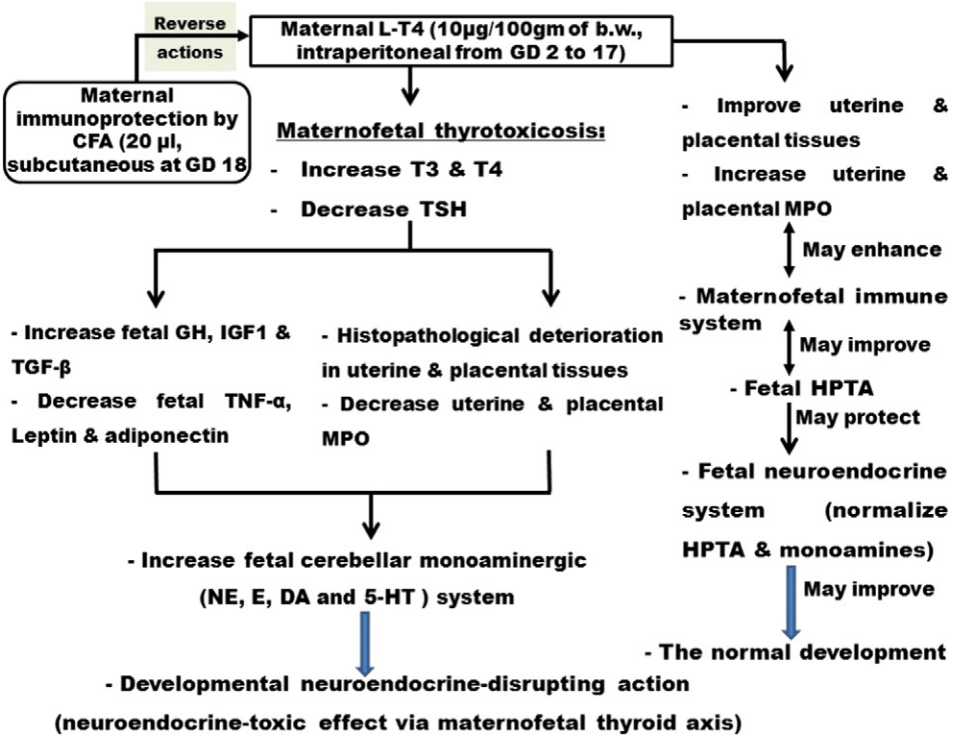


**Fig. 7.** Schematic diagram of the protective effect of CFA on LT4-induced hyperthyroidism and the developmental neuroendocrine homeostasis.

neuroendocrine dysfunction via regulating HPTA and monoamines. The final conclusion is that the maternal protection by CFA can be applied in treatment of maternofetal thyrotoxicosis and fetal neuroendocrine disorders (Fig. 7). More interestingly, this protection is dependent on dose, duration, developmental period, and the species involved, as well as the exact mechanism of this improvement is still unclear. Thus, more information is needed to determine the multiple mechanisms of CFA against the neurotoxic effect of maternofetal thyrotoxicosis. This may support development of new strategies to improve clinical immune responses.

## Conflict of interest

The authors declare that no competing financial interests exist.

## Acknowledgments

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## References

[1] A.J. Forhead, A.L. Fowden, Thyroid hormones in fetal growth and prepartum maturation, J. Endocrinol. 221 (2014) R87–R103.
[2] G.C. Micke, T.M. Sullivan, D.J. Kennaway, J. Hernandez-Medrano, V.E. Perry, Maternal endocrine adaptation throughout pregnancy to nutrient manipulation: consequences for sexually dimorphic programming of thyroid hormones and development of their progeny, Theriogenology 83 (2015) 604–615.
[3] G. León, M. Murcia, M. Rebagliato, M. Álvarez-Pedrerol, A.M. Castilla, M. Basterrechea, et al., Maternal thyroid dysfunction during gestation, preterm delivery, and birthweight. The Infancia y Medio Ambiente Cohort, Spain, Paediatr. Perinatol. Epidemiol. 29 (2) (2015) 113–122.
[4] K. Sánchez-Huerta, J. Pacheco-Rosado, M.E. Gilbert, Adult onset hypothyroidism: alterations in hippocampal field potentials in the dentate gyrus are largely associated with anesthesia-induced hypothermia, J. Neuroendocrinol. 27 (2015) 8–19.
[5] R.G. Ahmed, Early weaning PCB 95 exposure alters the neonatal endocrine system: thyroid adipokine dysfunction, J. Endocrinol. 219 (3) (2013) 205–215.
[6] P. De Vito, E. Candelotti, R.G. Ahmed, P. Luly, P.J. Davis, S. Incerpi, et al., Role of thyroid hormones in insulin resistance and diabetes. Immun., Endoc. & Metab, Agents Med. Chem 15 (2015) 86–93.
[7] X.L. Gao, H.X. Yang, Y. Zhao, Variations of tumor necrosis factor-α, leptin and adiponectin in mid-trimester of gestational diabetes mellitus, Chin. Med. J. 121 (2008) 701–705.
[8] Z. Aszalós, Some neurologic and psychiatric complications in endocrine disorders: the thyroid gland, Orv. Hetil. 148 (7) (2007) 303–310.
[9] R.G. Ahmed, Perinatal 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure alters developmental neuroendocrine system, Food Chem. Toxicol. 49 (2011) 1276–1284.
[10] R.G. Ahmed, S. Incerpi, Gestational doxorubicin alters fetal thyroid–brain axis, Int. J. Devl. Neurosci. 31 (2013) 96–104.
[11] L. Saranac, H. Stamenkovic, T. Stankovic, I. Markovic, S. Zivanovic, Z. Djuric, Growth in Children With Thyroid Dysfunction. In Current Topics in Hypothyroidism With Focus on Development pp 119–134. Ed E Potluková-.Rijeka, Intech Open Access Publisher, Croatia, 2013.
[12] P.N. Taylor, O.E. Okosieme, L. Premawardhana, J.H. Lazarus, Should all women be screened for thyroid dysfunction in pregnancy? Women's Health (Lond. Engl.) 11 (3) (2015) 295–307.
[13] J.M. Lauder, Effects of Thyroid State on Development of rat Cerebellar Cortex, in: G.D. Grave (Ed.), Thyroid Hormone and Brain Development, Raven Press, New York 1977, pp. 235–254.
[14] R. Rosato, D. Lindenbergh-Kortleve, J. van Neck, S. Drop, G. Jahn, Effect of chronic thyroxine treatment on IGF-I, IGF-II and IGF-binding protein expression in mammary gland and liver during pregnancy and early lactation in rats, Eur. J. Endocrinol. 146 (2002) 729–739.
[15] B. Kristensen, L. Hegedüs, H.O. Madsen, T.J. Smith, C.H. Nielsen, Altered balance between self-reactive T helper (Th)17 cells and Th10 cells and between full-length forkhead box protein 3 (FoxP3) and FoxP3 splice variants in Hashimoto's thyroiditis, Clin. Exp. Immunol. 180 (1) (2015) 58–69.
[16] M. Niyazoglu, O. Baykara, A. Koc, P. Aydoğdu, I. Onaran, F.D. Dellal, et al., Association of PARP-1, NF-κB, NF-κBIA and IL-6, IL-1β and TNF-α with graves disease and graves ophthalmopathy, Gene 547 (2) (2014) 226–232.
[17] R.A.M. de Luvizotto, A.F. do Nascimento, M.T. de Síbio, O. RMC, S.J. Conde, A.P. Lima-Leopoldo, et al., Experimental hyperthyroidism decreases gene expression and serum levels of adipokines in obesity, Sci. World J. (2012) 1–7.
[18] O.M. Ahmed, R.G. Ahmed, A.W. El-Gareib, A.M. El-Bakry, S.M. Abd El-Tawab, Effects of experimentally induced maternal hypothyroidism and hyperthyroidism on the development of rat offspring: II-the developmental pattern of neurons in relation to oxidative stress and antioxidant defense system, Int. J. Dev. Neurosci. 30 (6) (2012) 517–537.
[19] C.C. Wong, M.S. Leung, Effects of neonatal hypothyroidism on the expressions of growth cone proteins and axon guidance molecules related genes in the hippocampus, Mol. Cell. Endocrinol. 184 (1–2) (2001) 143–150.
[20] R.G. Ahmed, Hypothyroidism and brain developmental players, Thyroid Res. J. 8 (2) (2015) 1–12.
[21] R.G. Ahmed, Editorials and commentary: maternofetal thyroid action and brain development, J. Adv. Biol. 7 (1) (2015) 1207–1213.
[22] D. Yitzhakie, A. Torchinsky, S. Savion, V. Toder, Maternal immunopotentiation affects the teratogenic response to hyperthermia, J. Reprod. Immunol. 45 (1) (1999) 49–66.
[23] M. Kita, L. Ahmad, R.C. Marians, H. Vlase, P. Unger, P.N. Graves, et al., Regulation and transfer of a murine model of thyrotropin receptor antibody mediated graves' disease, Endocrinology 140 (1999) 1392–1398.
[24] R.A. O'Connor, X. Li, S. Blumerman, S.M. Anderton, R.J. Noelle, D.K. Daltonx, Adjuvant immunotherapy of experimental autoimmune encephalomyelitis: immature myeloid cells expressing CXCL10 and CXCL16 attract CXCR3+ CXCR6+ and myelin-specific T cells to the draining lymph nodes rather than the central nervous system, J. Immunol. 188 (2012) 2093–2101.
[25] K. Punareewattana, S.D. Holladay, Immunostimulation by complete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, or interferon-gamma reduces severity of diabetic embryopathy in ICR mice, Birth Defects Res. A Clin. Mol. Teratol. 70 (1) (2004) 20–27.
[26] M. Renee Prater, K.L. Zimmerman, C.L. Laudermilch, S.D. Holladay, Prebreeding maternal immunostimulation with Freund's complete adjuvant reduces placental damage and distal limb defects caused by methylnitrosourea, Am. J. Reprod. Immunol. 55 (2) (2006) 145–155.
[27] J.S. Hunt, M.G. Petroff, T.G. Burnett, Uterine leukocytes: key players in pregnancy, Semin. Cell Dev. Biol. 11 (2) (2000) 127–137.
[28] S. Savion, S. Gidon-Dabush, A. Fein, A. Torchinsky, V. Toder, Diabetes teratogenicity is accompanied by alterations in macrophages and T cell subpopulations in the uterus and lymphoid organs, Int. Immunopharmacol. 4 (10–11) (2004) 1319–1327.
[29] F. Spaans, B. Melgert, C. Chiang, T. Borghuis, P.A. Klok, P. de Vos, et al., Extracellular ATP decreases trophoblast invasion, spiral artery remodeling and immune cells in the mesometrial triangle in pregnant rats, Placenta 35 (8) (2014) 587–595.
[30] E. Cardonick, A. Iacobucci, Use of chemotherapy during human pregnancy, Lancet Oncol. 5 (2004) 283–291.
[31] O.M. Ahmed, A.W. El-Gareib, A.M. El-Bakry, S.M. Abd El-Tawab, R.G. Ahmed, Thyroid hormones states and brain development interactions, Int. J. Dev. Neurosci. 26 (2) (2008) 147–209.
[32] O.M. Ahmed, S.M. Abd El-Tawab, R.G. Ahmed, Effects of experimentally induced maternal hypothyroidism and hyperthyroidism on the development of rat offspring: I. The development of the thyroid hormones-neurotransmitters and adenosinergic system interactions, Int. J. Dev. Neurosci. 28 (6) (2010) 437–454.
[33] C. MacNabb, E. O'Hare, J. Cleary, A.P. Georgopoulos, Varied duration of congenital hypothyroidism potentiates preservation in a response alteration discrimination task, Neurosci. Res. 36 (2) (2000) 121–127.
[34] S.P. Porterfield, C.E. Hendrich, The role of thyroid hormones in prenatal and neonatal neurological development-current perspectives, Endocr. Rev. 14 (1993) 94–106.
[35] E.D. Olfert, B.M. Cross, A.A. McWilliam, (Eds), In Guide to the Care and Use of Experimental Animals, 1, CCAC, Ottawa, Ontario, Canada, 1993.
[36] F.K. Marcondes, F.J. Bianchi, A.P. Tanno, Determination of the estrous cycle phases of rats: some helpful considerations, Braz. J. Biol. 62 (4A) (2002) 609–614.
[37] A.N. Bruno, M.S. Carneiro-Ramos, A. Buffon, D. Pochmann, F.K. Ricachenevsky, M.L. Barreto-Chaves, et al., Thyroid hormones alter the adenine nucleotide hydrolysis in adult rat blood serum, Biofactors 37 (1) (2011) 40–45.
[38] C. Thakur, T.C. Saikia, R.N. Yadav, Total serum levels of triiodothyronine (T3), thyroxine (T4) and thyrotropin (TSH) in school going children of Dibrugarh district: an endemic goiter region of Assam, Indian J. Physiol. Pharmacol. 41 (2) (1997) 167–170.
[39] M. Maes, K. Mommen, D. Hendrickx, D. Peeters, P. D'Hondt, R. Ranjan, et al., Components of biological variation, including seasonality, in blood concentrations of TSH, TT3, FT4, PRL, cortisol and testosterone in healthy volunteers, Clin. Endocrinol. (Oxford) 46 (5) (1997) 587–598.
[40] S.J. Mandel, G.A. Brent, P.R. Larsen, Levothyroxine therapy in patients with thyroid disease, Ann. Intern. Med. 119 (6) (1993) 492–502.
[41] A.T. Reutens, Evaluation and application of a highly sensitive assay for serum growth hormone (GH) in the study of adult GH deficiency, J. Clin. Endocrinol. Metab. 80 (2) (1995) 480–485.
[42] M.J. Dauncey, A. Morovat, B.T. Rudd, R.A. Shakespear, Dissociation between plasma concentrations of thyroxine and insulin-like growth factor-I, Exp. Physiol. 75 (5) (1990) 729–731.
[43] J.E. Krawisz, P. Sharon, W.F. Stenson, Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models, Gastroenterol 87 (6) (1984) 1344–1350.
[44] J.D. Bancroft, M. Gamble, Theory and Practice of Histological Techniques, 6th ed. PA. Churchill Livingstone/Elsevier, Philadelphia, 2008.
[45] A.E. Ciarlone, Further modification of a fluorometric method for analyzing brain amines, Microchem. J. 23 (1978) 9–12.
[46] K.A. Landers, H. Li, V.N. Subramaniam, R.H. Mortimer, K. Richard, Transthyretin-thyroid hormone internalization by trophoblasts, Placenta 34 (8) (2013) 716–718.
[47] M.F. Correia, A.T. Maria, S. Prado, C. Limbert, Neonatal thyrotoxicosis caused by maternal autoimmune hyperthyroidism, BMJ Case Rep. (2015) http://dx.doi.org/10.1136/bcr-2014-209283.
[48] O.E. Okosieme, J.H. Lazarus, Important considerations in the management of graves' disease in pregnant women, Expert. Rev. Clin. Immunol. 11 (8) (2015) 947–957.

[49] T. Ando, R. Latif, T.F. Davies, Thyrotropin receptor antibodies: new insights into their actions and clinical relevance, Best Pract. Res. Clin. Endocrinol. Metab. 19 (1) (2005) 33–52.
[50] J.P. Walsh, A.P. Bremner, P. Feddema, P.J. Leedman, S.J. Brown, P. O'Leary, Thyrotropin and thyroid antibodies as predictor of hypothyroidism: a 13-year, longitudinal study of a community-based cohort using current immunoassay techniques, J. Clin. Endocrinol. Metab. 95 (2010) 1095–1104.
[51] G. Krassas, S.N. Karras, N. Pontikides, Thyroid diseases during pregnancy: a number of important issues, Hormones (Athens) 14 (1) (2015) 59–69.
[52] M. Kitamura, T. Sütö, T. Yokoo, F. Shimizu, L.G. Fine, Transforming growth factor-beta 1 is the predominant paracrine inhibitor of macrophage cytokine synthesis produced by glomerular mesangial cells, J. Immunol. 156 (8) (1996) 2964–2971.
[53] C. Mauri, R.O. Williams, M. Walmsley, M. Feldmann, Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis, Eur. J. Immunol. 26 (1996) 1511–1518.
[54] S.M. McLachlan, Y. Nagayama, B. Rapoport, Insight into Graves' hyperthyroidism from animal models, Endocr. Rev. 26 (2005) 800–832.
[55] M. Varedi, H. Shiri, A. Moattari, G.H. Omrani, Z. Amirghofran, Hyperthyroid state or in vitro thyroxine treatment modulates TH1/TH2 responses during exposure to HSV-1 antigens, J. Immunotoxicol. 11 (2) (2014) 160–165.
[56] K. Yamazaki, K. Suzuki, E. Yamada, T. Yamada, F. Takeshita, M. Matsumoto, et al., Suppression of iodide uptake and thyroid hormone synthesis with stimulation of the type I interferon system by double-stranded ribonucleic acid in cultured human thyroid follicles, Endocrinology 148 (7) (2007) 3226–3235.
[57] M. Gorivodsky, A. Torchinsky, I. Zemliak, S. Savion, A. Fein, V. Toder, TGF beta 2 mRNA expression and pregnancy failure in mice, Am. J. Reprod. Immunol. 42 (2) (1999) 124–133.
[58] I. Ivnitsky, A. Torchinsky, M. Gorivodsky, I. Zemliak, H. Orenstein, S. Savion, et al., TNF-alpha expression in embryos exposed to a teratogen, Am. J. Reprod. Immunol. 40 (6) (1998) 431–440.
[59] S. Savion, E. Zeldich, H. Orenstein, J. Shepshelovich, A. Torchinsky, H. Carp, et al., Cytokine expression in the uterus of mice with pregnancy loss: effect of maternal immunopotentiation with GM-CSF, Reproduction 123 (3) (2002) 399–409.
[60] R.G. Ahmed, Developmental adipokines and maternal obesity interactions, J. Adv. Biol. 7 (1) (2015) 1189–1206.
[61] E. Candelotti, P. De Vito, R.G. Ahmed, P. Luly, P.J. Davis, J.Z. Pedersen, et al., Thyroid hormones crosstalk with growth factors: old facts and new hypotheses, Immunol. Endocrinol. Metab. Agents Med. Chem. 15 (2015) 71–85.
[62] J.B. Laoag-Fernandez, H. Matsuo, H. Murakoshi, A.L. Hamada, B.K. Tsang, T. Maruo, 3,5,3′-triiodothyroninedown-regulates Fas and Fas ligand expression andsuppresses caspase-3 and poly (adenosine 5′-diphosphate-ribose) polymerase cleavage and apoptosis in early placental extravillous trophoblasts in vitro, J. Clin. Endocrinol. Metab. 89 (2004) 4069–4077.
[63] C.A. Souza, N.M. Ocarino, J.F. Silva, J.N. Boeloni, E.F. Nascimento, I.J. Silva, et al., Administration of thyroxine affects the morphometric parameters and VEGF expression in the uterus and placenta and the uterine vascularization but does not affect reproductive parameters in gilts during early gestation, Reprod. Domest. Anim. 46 (2011) e7–e16.
[64] E.S. Freitas, E.D. Leite, C.A. Souza, N.M. Ocarino, E. Ferreira, G.D. Cassali, et al., Histomorphometry and expressions of Cdc47 and caspase-3 in hyperthyroid rats uteri and placentas during gestation and postpartum associated with fetal development, Reprod. Fertil. Dev. 19 (2007) 498–509.
[65] P.B. Navas, A.B. Motta, M.B. Hapon, G.A. Jahn, Hyperthyroidism advances luteolysis in the pregnant rat through changes in prostaglandin balance, Fertil. Steril. 96 (2011) 1008–1014.
[66] T.P. Foley Jr., The relationship between autoimmune thyroid disease and iodine intake: a review, Endokrynol. Pol. 43 (1) (1992) 53–69.
[67] M.J. Soares, D. Chakraborty, M.A. Karim Rumi, T. Konno, S.J. Renaud, Rat placentation: an experimental model for investigating the hemochorial maternal–fetal interface, Placenta 33 (2012) 233–243.
[68] L.V. Sharova, A.A. Sharov, P. Sura, R.M. Gogal, B.J. Smith, S.D. Holladay, Maternal immune stimulation reduces both placental morphologic damage and down-regulated placental growth-factor and cell cycle gene expression caused by urethane: are these events related to reduced teratogenesis? Int. Immunopharmacol. 3 (7) (2003) 945–955.
[69] K. Miyakoshi, H. Ishimoto, O. Nishimura, S. Tanigaki, M. Tanaka, T. Miyazaki, et al., Role of leukocytes in uterine hypoperfusion and fetal growth retardation induced by ischemia–reperfusion, Am. J. Physiol. Heart Circ. Physiol. 280 (3) (2001) H1215–H1221.
[70] D. Odobasic, A.R. Kitching, Y. Yang, K.M. O'Sullivan, R.C.M. Muljadi, K.L. Edgtton, et al., Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function, Blood 121 (2013) 20.
[71] G.A. Mason, S.C. Bondy, C.B. Nemeroff, C.H. Walker, A.J. Prange Jr., The effects of thyroid state on beta-adrenergic and serotonergic receptors in rat brain, Psychoneuroendocrinol 12 (4) (1987) 261–270.
[72] M. Bauer, P.C. Whybrow, Thyroid hormone, neural tissue and mood modulation, World J. Biol. Psychiatry 2 (2) (2001) 59–69.
[73] T. Ilani, R.D. Strous, S. Fuchs, Dopaminergic regulation of immune cells via D3 dopamine receptor: a pathway mediated by activated T cells, FASEB J. 18 (13) (2004) 1600–1602.