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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 hyperthyroidism [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

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