Maternal thyroid dysfunction affects placental profile of inflammatory mediators and the intrauterine trophoblast migration kinetics

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Abstract

The objective of the present study was to evaluate the gene and immunohistochemical expression of inflammatory mediators involved in the immune activity and the intrauterine trophoblast migration of the placentas in hypothyroid and L-thyroxine (L-T4)-treated rats. A total of 144 adult female rats were divided equally into hypothyroid, L-T4-treated, and euthyroid (control) groups. Hypothyroidism was induced by daily administration of propylthiouracil. Rats were killed at 0, 10, 14, 15, 16, 17, 18, and 19 days of gestation. We evaluated the depth of interstitial and endovascular intrauterine trophoblast invasion and the immunohistochemical expression of interferon-γ (INFγ), migration inhibitory factor (MIF), and inducible nitric oxide synthase (NOS2 (iNOS)). The gene expression of Toll-like receptor 2 (Tlr2) and Tlr4, INFγ, Mif, tumor necrosis factor (Tnf (Tnfa)), Il10, Nos2, matrix metalloproteinase 2 (Mmp2) and Mmp9, and placental leptin was also measured in placental disks by real-time RT-PCR. The data were analyzed using a Student-Newman-Keuls (SNK) test. Hypothyroidism reduced the endovascular and interstitial trophoblast migration, and the expression of TLR4, INFγ, MIF, interleukin 10 (IL10), NOS2, MMP2 and MMP9, and placental leptin, while increased the expression of TLR2 (P<0.05). T4-treated rats not only increased the expression of IL10 and NO2 but also reduced the expression of TNF and MIF at 10 days of gestation (P<0.05). However, at 19 days of gestation, expression of INFγ and MIF was increased in T4-treated group (P<0.05). Excess of T4 also increased the gene expression of Mmp2 at 10 days of gestation (P<0.05), but reduced the endovascular trophoblast migration at 18 days of gestation (P<0.05). Hypothyroidism and excess of T4 differentially affect the immune profile and the intrauterine trophoblast migration of the placenta, and these effects are dependent on the gestational period.

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Introduction

The maternal immune response and the migration of trophoblast cells in the maternal–fetal interface are critical determinants of the success or failure of pregnancy in both humans and rodents (Koga et al. 2009, Hammer 2011). Changes in the pro- and anti-inflammatory cytokines in the maternal–fetal interface and placenta failure due to extensive trophoblastic invasion or superficial trophoblastic invasion can cause miscarriage, premature birth, intrauterine growth restriction, and pre-eclampsia in women as well as in experimental animal models (Coulam 2000, Toder et al. 2003, Zhang et al. 2007, Koga et al. 2009, Hammer 2011, Soares et al. 2012). Therefore, basic research in this area has attempted to evaluate the molecular mechanisms that control the placental immune system and the trophoblast invasion in physiological and pathological conditions (Rosario et al. 2008, Soares et al. 2012). However, there are few studies that have evaluated the effects of endocrine disorders on the placental immunology and on the migration of trophoblast cells, particularly in hypo- and hyperthyroidism.

Thyroid hypofunction affects fetal–placental development, impairing the decidualization, vasculization, and development of the placenta, increasing apoptosis, and reducing the proliferation of trophoblast cells (Shafrir et al. 1994, Morrish et al. 1997, Galton et al. 2001, Silva et al. 2012). However, several of the pathological processes observed in hypothyroidism may result from changes in the placent al pro- and anti-inflammatory cytokine profile (Koga et al. 2009). In contrast, hyperthyroidism increases reproductive efficiency in experimental animal models (Serakides et al. 2001, Freitas et al. 2007) and causes significant changes in the uterine vasculature (Souza et al. 2011) and in the proliferation of trophoblast cells (Freitas et al. 2007). It is important to mention that placental changes similar to those observed in hypo- and hyperthyroidism may also be influenced by the migration kinetics of the trophoblast cells (Knöfler 2010, Hammer 2011, Chakraborty et al. 2011, Soares et al. 2012).
The maternal immune system is suppressed during pregnancy. A significant reduction in cellular immunity (Weinberg 1987, Raghupathy 1997) and predominance of anti-inflammatory cytokines, such as interleukin 4 (IL4), IL10, and inducible nitric oxide synthase (iNOS), are essential conditions for the success of pregnancy (Coulam 2000, Toder et al. 2003). This is crucial to prevent rejection of the fetus (Murphy et al. 2004). However, while immune system suppression is essential during pregnancy, it increases susceptibility to various infectious agents (Kim et al. 2005).

Bacterial antigen recognition by trophoblast cells is mediated by multiple membrane receptors, especially Toll-like receptors (TLRs; Flo et al. 2002). Stimulation of TLR2 and TLR4 results in apoptosis and production of inflammatory cytokines by trophoblast cells respectively (Koga et al. 2009). However, little is known about the influence of thyroid dysfunction on the expression of these receptors. It has been observed that TLRs are associated with pregnancy complications, such as pathogenesis of pre-eclampsia, preterm delivery, and even the failure of fetal development (Koga & Mor 2010, Xie et al. 2010). Some pro-inflammatory cytokines, such as interferon γ (INFγ) and the macrophage migration inhibitory factor (MIF), are also important components of the placental immune response. INFγ stimulates the phagocytic activity of macrophages and trophoblast giant cells against microorganisms (Ashkar et al. 2000, Kim et al. 2005). MIF stimulates the expression of a wide variety of pro-inflammatory cytokines, such as tumor necrosis factor (TNF (TNFα)), INFγ, IL2, IL6, and IL8 (Faria et al. 2010, Cardaropoli et al. 2012), stimulates angiogenesis and cell proliferation, and suppresses apoptosis (Amin et al. 2003, Viganò et al. 2007, Faria et al. 2010, Cardaropoli et al. 2012). One hypothesis is that the expression of TLRs and inflammatory cytokines in trophoblast cells are impaired in hypothyroidism. This would compromise the fetal-placental development not only by influencing the permanence of the fetus in the uterine environment but also by facilitating infection by pathogens.

Inflammatory cytokines may also influence the invasion of trophoblast cells toward the decidua through its effects on the extracellular matrix remodeling and on the vascular compartment of the mesometrial decidua (Cartwright et al. 1999, Ain et al. 2003). Cartwright et al. (1999) demonstrated that the motility and invasion of the trophoblast cells is highly dependent on the NOS produced by trophoblasts in vitro. Silva et al. (2012) observed changes in the glycogen cell population as well as in trophoblast giant cells in the placenta of rats with hypothyroidism, suggesting that there is a failure in the migration kinetics of these cells toward the decidua.

Invasive trophoblasts fulfill numerous functions including anchoring the placenta to the maternal tissue, hormone secretion, modulation of decidual angiogenesis and lymphangiogenesis, and remodeling of maternal uterine spiral arteries (Knöfler 2010, Chakraborty et al. 2011). Research has shown that several growth factors, angiogenic, cytokines, and proteases control the trophoblastic cell migration kinetics (Soares et al. 2012). Among these factors are the matrix metalloproteinase 2 (MMP2) and MMP9, and placental leptin (Knöfler 2010, Soares et al. 2012). However, the influence of these factors on the dynamics of trophoblast cells in the maternal-fetal interface remains poorly understood and requires further studies (Ain et al. 2003). The failure of the migration kinetics of trophoblast cells in humans or model organisms with hypothyroidism also remains unproven, as does the participation of MMPs and placental leptin in this process in vivo.

Based on these previous findings and hypotheses, the objective of the work presented was to study the placental gene expression of TLRs, the immunohistochemical and gene expression of pro- and anti-inflammatory cytokines, and the migration kinetics of trophoblast cells in animals with induced thyroid dysfunction.

Materials and methods
All experimental procedures were approved by the Institutional Ethics Committee in Animal Experimentation at the Universidade Federal de Minas Gerais (protocol no. 239/2009).

Induction of thyroid dysfunction and mating
A total of 144 adult female Wistar rats were used in this study. Rats were housed in cages with six rats per cage in a 12 h light:12 h darkness cycle. They were fed with commercial rat chow containing 22% crude protein, 1.4% calcium, and 0.6% phosphorus. Food and water were provided ad libitum.

After a 7-day adaptation period, the rats were randomly divided into three groups (control, hypothyroid, and thyroxine (T4)-treated) with 48 rats per group. Hypothyroidism was induced by administration of propylthiouracil (PTU) diluted in 5 ml distilled water, in accordance with the method of Silva et al. (2004), using an orogastric probe (1 mg/animal per day). The T4-treated rats received L-T4 diluted in 5 ml distilled water, as described by Serakides et al. (2001), using an orogastric probe (50 μg/animal per day). The rats from the control group received 5 ml distilled water/day as a placebo.

Five days after treatment initiation, female rats from all groups were subjected to vaginal cytology to monitor the estrous cycle. Six rats from each group were also killed with an overdose of anesthetic for blood collection, measurement of free T4, and confirmation of the induction of thyroid dysfunction. The rats in proestrus were kept in plastic cages with adult male rats for 12 h during the night. After this period, vaginal smears were obtained on the next morning to detect spermatozoa. Copulation was confirmed by the presence of spermatozoa in vaginal cytology and that day was considered to be day 0 of gestation. After copulation, the female rats were kept individually in plastic cages. Rats in the hypothyroid, T4-treated, and control groups continued to receive PTU, T4, and water, respectively, by an orogastric probe throughout the experimental period.
**Hormone analysis**

At 0, 10, 14, 15, 16, 17, 18, and 19 days of gestation, six rats from each group were killed by an overdose of anesthetic (2.5% Tionembutal; Abbott). At 0 and 19 days of gestation, blood was collected from the rats and serum was separated by centrifugation and stored at -20 °C for the measurement of free $T_3$ and $T_4$, which was performed using the chemiluminescence ELISA technique with commercial kits and in accordance with the manufacturer’s instructions (IMMULITE, Siemens Medical Solutions Diagnostics, Malvern, PA, USA). The intra- and inter-assay coefficients of variation were 4 and 7% respectively.

**Necropsy and material collection**

At necropsy, the uterus was collected together with the placenta and fetuses. Six placental disks along with the decidua and metrial gland per rat were fixed in 4% paraformaldehyde for 20 h and were processed using a routine paraffin inclusion technique. To perform immunohistochemistry and morphological measurements, histological sections (4 µm) of placental disks along with the decidua and metrial gland were obtained and placed on silanized slides.

Three placental disks without decidua and metrial gland per rat were also dissected, snap frozen in liquid nitrogen, and stored at -80 °C for use in the evaluation of the gene expression of Tlr2 and Tlr4, Inter, Mif, Tnf, Il10, Nos2, Mmp2, and Mmp9, and placental leptin using real-time RT-PCR. Placental disks were formed only by syncytiotrophoblast, spongiosotrophoblast, and placental labyrinth.

**Immunohistochemistry and morphological measurements**

The biotin–streptavidin–peroxidase (Streptavidin Peroxidase, Lab Vision Corp., Fremont, CA, USA) technique was used for immunohistochemistry. Automated staining was performed using a retrieval pressure cooker (Majik Vision, Inc., Sunnyvale, CA, USA). The antigen retrieval procedure was performed using a retrieval pressure cooker (Marienfeld, Tuttlingen, Germany) in a 0.01 M sodium citrate buffer, pH 6, at 121 °C for 15 min. After cooling to room temperature, the sections were incubated in 3% hydrogen peroxide to block endogenous peroxidase, blocking serum (Ultra Vision Dualendomark, Lab Vision Corp.), and streptavidin–peroxidase. Incubation with the secondary antibody (goat biotin, Lab Vision Corp.) was performed for 45 min. The chromogen diaminobenzidine (DAB substrate system, Lab Vision Corp.) was performed for 45 min. The sections were counterstained with Harris hematoxylin. A negative control was included by replacing the primary antibodies with IgG. Harris hematoxylin. A negative control was included by replacing the primary antibodies with IgG.

Rat invasive trophoblast cells were detected using the anticytokeratin AE1/AE3 antibody. The depth of intrauterine endovascular and interstitial trophoblast cell invasion was used to determine an invasion index according to Rosario et al. (2008). The endovascular trophoblast invasion was evaluated by measuring the distance of endovascular cytokeratin-positive cell location relative to the trophoblast giant cell layer of the chorioallantoic placenta/total distance from the trophoblast giant cell layer to the outer mesometrial surface of the uterus. The interstitial trophoblast invasion was measured by the distance of interstitial cytokeratin-positive cell location relative to the trophoblast giant cell layer of the chorioallantoic placenta/total distance from the trophoblast giant cell layer to the outer mesometrial surface of the uterus. The depth of intrauterine trophoblast cell invasion was evaluated from a histological plane at the center of each placentation site perpendicular to the flat fetal surface of the placenta. Sample sizes for the analyses were at least five placental sites from at least five different animals per treatment group. All analyses were performed on placental disks at 14, 15, 16, 17, and 18 days of gestation.

The immunostaining intensity and stained area of INFγ, MIF, and NOS2 in the spongiosotrophoblast layer and placental labyrinth were evaluated at 14 and 19 days of gestation. To determine the immunostaining intensity and stained area, images from six and eight random fields per each layer at 14 and 19 days of gestation, respectively, were photographed with an Olympus BX-40 microscope and the Spot Color Insight digital camera (SPOTTM, Sterling Heights, MI, USA), and the immunostaining intensity and stained area were determined using WCIF ImageJ Software (Media Cybernetics Manufacturing, Rockville, MD, USA). Color deconvolution and thresholding of the images were performed. To ensure the objectivity of the procedure, the mean was obtained from the evaluation of three placentical disks per animal. Data from each placentical disk were archived, analyzed, and expressed as the integrated density and stained area in pixels.

**Real-time RT-PCR**

Total mRNA from placental disks was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. A total of 1 µg RNA was used for cDNA synthesis using the SuperScript III Platinum Two-Step qPCR Kit with SYBR Green (Invitrogen). The qRT-PCRs were conducted in a Smart Cycler II thermocycler (Cepheid, Inc., Sunnyvale, CA, USA). To quantify the cDNA generated by RT, real-time PCR with SYBR Green I was performed using SYBR Green PCR Master Mix in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Life Technologies). For negative controls, we used a complete DNA amplification mix in which the target cDNA template was replaced with water. Amplifications were performed using the default cycling conditions: enzyme activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 60 s. To assess the linearity and efficiency of PCR amplification, standard curves for all transcripts were generated using serial dilutions of cDNA. A melting curve was obtained for the amplification products to ascertain their melting temperatures. The samples were assayed in triplicate and then a gel was run with the reaction product to confirm the gene amplification. The PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, where the values from the samples were averaged and calibrated in relation to β-actin CT values. The primers were as follows (Table 1).
The evaluation of the gene expression of the inflammatory mediators was performed at 10, 14, and 19 days of gestation. The evaluation of the gene expression of Mmp2 and Mmp9 and placental leptin was performed at 10, 14, and 18 days of gestation.

**Statistical analysis**

Significant differences in the mean values between the experimental groups were determined by one-way ANOVA. The Student–Newman–Keuls test was used to compare data between groups, and the differences were considered to be significant if $P<0.05$.

**Results**

**Induction of thyroid dysfunction**

Hypothyroidism and excess of T4 during the entire period of the pregnancy was confirmed by assaying the free T3 and T4 levels in serum on days 0 and 19 of gestation and by the evaluation of symptoms. The rats treated with PTU displayed free T3 and T4 levels lower than those of the control group ($P<0.05$; Fig. 1) and clinical characteristics of lethargy. The T4-treated rats exhibited higher free T4 levels compared with control rats ($P<0.05$; Fig. 1) and showed clinical characteristics of aggressiveness.

**Immunohistochemical expression of INFy, MIF, and NOS2**

Regardless of the experimental group, the placenta showed expression of INFy, MIF, and NOS2 in three layers (syncytiotrophoblast, spongiotrophoblast, and placental labyrinth), at 14 and 19 days of gestation. The immunohistochemical expression of INFy and MIF was nuclear and/or cytoplasmic, unlike the NOS2 expression that was only cytoplasmic, with a more intense expression at 14 days of gestation compared with 19 days of gestation (Figs 2, 3 and 4).

Hypothyroidism reduced the area and intensity of INFy immunohistochemical expression at 14 and 19 days of gestation in the spongiotrophoblast layer.

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**Table 1** List of genes with primer sequences.

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<th>Gene</th>
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<th>Accession number</th>
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<td></td>
<td>Reverse, 5'-TTCTGAGAGGCGACGCCTGCTG-3'</td>
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<td>Tlr4</td>
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<td>NM_019178.1</td>
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<td></td>
<td>Reverse, 5'-AACCGAGGCGACGCCTGCTG-3'</td>
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Figure 1 (A and B) Free T3 and T4 levels (mean ± S.D.) in the plasma of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 0 and 19 days of gestation ($^{*}P<0.05$).
Figure 2 INFγ expression in the placenta of pregnant rats from the control, hypothyroid, and thyroxine (T₄)-treated groups at 14 and 19 days of gestation. (A) Immunohistochemical images of INFγ expression (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar = 12 μm). (B and C) Reduction in the area and intensity of INFγ expression in the spongiotrophoblast layer of the hypothyroid group compared with the control group at 14 and 19 days of gestation. Increase in the area and intensity of INFγ expression in the placental labyrinth of T₄-treated group compared with the control group at 19 days of gestation (*P<0.05).
Figure 3 MIF expression in the placenta of pregnant rats from the control, hypothyroid, and thyroxine (T4)-treated groups at 14 and 19 days of gestation. (A) Immunohistochemical images of MIF expression (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar = 12 μm). (B and C) Increase in the area and intensity of MIF expression in the spongiotrophoblast layer of the hypothyroid and T4-treated group compared with the control group at 14 days of gestation. Reduction in the hypothyroid group and increase in the T4-treated group with regard to the area and intensity of MIF expression in the spongiotrophoblast layer compared with the control group at 19 days of gestation (*P<0.05).
Figure 4 NOS2 expression in the placenta of pregnant rats from the control, hypothyroid, and thyroxine (T4)-treated groups at 14 and 19 days of gestation. (A) Immunohistochemical images of NOS2 expression (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar = 12 µm). (B and C) Reduction in the area and intensity of NOS2 expression in the spongiotrophoblast layer and placental labyrinth of the hypothyroid group compared with the control group at 14 days of gestation. Increase in the area and intensity of NOS2 expression in the placental labyrinth of the T4-treated group compared with the control group at 14 days of gestation (*P < 0.05).
when compared with the control rats \((P<0.05; \text{Fig. 2})\). This was different from the \(T_4\)-treated rats, which exhibited an increase in the area and intensity of INF\(\gamma\) immunohistochemical expression in the placental labyrinth at 19 days of gestation when compared with the control group \((P<0.05; \text{Fig. 2})\).

In relation to the MIF expression, the hypothyroid group showed an increase in the area and intensity of immunohistochemical expression in the spongiotrophoblast layer compared with the control group at 14 days of gestation, but this was not observed at 19 days of gestation. Instead of that, at 19 days of gestation, a reduction in the area and intensity of MIF immunohistochemical expression in the spongiotrophoblast layer was observed in the hypothyroid rats compared with the control rats \((P<0.05; \text{Fig. 3})\). \(T_4\)-treated rats showed an increase in the area and intensity of immunohistochemical expression of MIF in the spongiotrophoblast layer at 14 and 19 days of gestation compared with the control rats \((P<0.05; \text{Fig. 3})\). In placental labyrinth, at 14 and 19 days of gestation, there was no difference on the MIF immunohistochemical expression between the groups \((P>0.05; \text{Fig. 3})\).

At 14 days of gestation, the hypothyroid animals showed a reduction in the area and intensity of NOS2 immunohistochemical expression in the spongiotrophoblast layer and placental labyrinth compared with the control group \((P<0.05; \text{Fig. 4})\). The \(T_4\)-treated rats, differing from the hypothyroid group, showed an increase in the area and intensity of immunohistochemical expression of NOS2 in the placental labyrinth at 14 days of gestation compared with the control rats \((P<0.05; \text{Fig. 4})\).

**Gene expression of TLR2 and TLR4**

At 14 days of gestation, the placental disks of hypothyroid rats showed an increased expression of mRNA for Tlr2 compared with the control rats \((P<0.05)\). The \(T_4\)-treated group showed no differences in gene expression of Tlr2 compared with the control group in any of the gestational periods \((P>0.05; \text{Fig. 5A})\).

The hypothyroid group had decreased Tlr4 mRNA expression at 10, 14, and 19 days of gestation compared with the control rats \((P<0.05; \text{Fig. 5B})\). The \(T_4\)-treated group showed no differences in Tlr4 gene expression compared with the control group in any of the gestational periods \((P>0.05; \text{Fig. 5B})\).

**Gene expression of the pro-inflammatory cytokines INF\(\gamma\), MIF, and TNF**

Hypothyroid rats showed reduced expression of mRNA for INF\(\gamma\) at 10 and 14 days of gestation compared with the control rats \((P<0.05)\). This was different from the \(T_4\)-treated rats, which exhibited increased INF\(\gamma\) gene expression compared with the control group at 19 days of gestation \((P<0.05; \text{Fig. 6A})\).

In contrast to the expression of INF\(\gamma\), the hypothyroid rats showed no differences in gene expression of Mif in relation to the control rats at any of the gestational time points \((P>0.05; \text{Fig. 6B})\). The \(T_4\)-treated group showed a decrease in the mRNA expression of Mif compared with the control group at as early as 10 days of gestation, but this was not observed at 19 days of gestation. At 19 days of gestation, an increase in Mif gene expression was observed in the \(T_4\)-treated rats compared with the control rats \((P<0.05; \text{Fig. 6B})\).

Regarding Tnf gene expression, the hypothyroid rats showed no differences compared with the control rats at any of the gestational time points \((P>0.05)\), as opposed to the \(T_4\)-treated rats, which showed a decrease in mRNA expression of Tnf compared with the control group at 10 days of gestation \((P<0.05)\) (Fig. 6C). At 14 and 19 days of gestation, there were no differences in gene expression.
expression of Tnf between the T₄-treated and control groups (P>O.05; Fig. 6C).

Gene expression of the anti-inflammatory cytokines Il10 and Nos2

Hypothyroid rats showed a decrease in Il10 gene expression at 14 days of gestation compared with the control rats (P<O.05; Fig. 7A). T₄-treated rats, in contrast, showed an increase in gene expression of Il10 compared with the control rats at 10 and 19 days of gestation (P<O.05; Fig. 7A).

Hypothyroid rats also showed a reduction in Nos2 mRNA expression compared with the control rats at 14 days of gestation (P>O.05; Fig. 7B). The T₄-treated group showed no differences in Nos2 gene expression when compared with the control group at any of the gestational ages tested (P>O.05; Fig. 7B).

Figure 6 Relative expression of gene transcripts for Infy (A), Mif (B), and Tnf (C) (mean±s.d.) in the placentas of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 10, 14, and 19 days of gestation (*P<O.05, **P<O.01, and ***P<O.001).

Figure 7 Relative expression of gene transcripts for Il10 (A) and Nos2 (B) (mean±s.d.) in the placentas of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 10, 14, and 19 days of gestation (*P<O.05, **P<O.01, and ***P<O.001).
Gene expression of MMP2 and MMP9
Hypothyroid rats showed a decrease in the Mmp2 mRNA expression at 14 days of gestation compared with the control rats (P<0.05; Fig. 8A). The T4-treated rats, however, showed an increase in the mRNA expression of Mmp2 compared with the control rats at 10 days of gestation (P<0.05; Fig. 8A).
Hypothyroid rats also showed a decrease in Mmp9 mRNA expression compared with control rats at 18 days of gestation (P<0.05; Fig. 8B). The T4-treated group showed no differences in gene expression of Mmp9 compared with the control group in any of the gestational periods (P>0.05; Fig. 8B).

Gene expression of placental leptin
Similar to the expression of Mmp2, hypothyroid rats showed a decrease in the mRNA expression of placental leptin compared with the control rats at 14 days of gestation (P<0.05; Fig. 8C). The T4-treated group showed no differences in the gene expression of placental leptin compared with the control group in any of the gestational periods (P>0.05; Fig. 8C).

Kinetics of intrauterine trophoblast migration
At 16 and 17 days of gestation, the placental disks of the hypothyroid rats showed a reduction in the endovascular trophoblast invasion index compared with that of control rats (P<0.05; Figs 6 and 7). The same was also observed at 15 and 17 days of gestation in relation to the interstitial trophoblastic invasion index (P<0.05; Fig. 9 and 10).
A reduction in endovascular trophoblast invasion was observed in the T4-treated rats compared with the control group at 18 days of gestation (P<0.05; Fig. 9A), with no significant differences at other gestational ages.
Regarding interstitial trophoblastic invasion, the T4-treated group showed no significant differences compared with the control group in any of the gestational periods (P > 0.05; Fig. 9B).

**Discussion**

Hypothyroidism and excess of T4 affected the placental immune profile and the migration kinetics of trophoblast cells toward the decidua differently, and these effects were dependent on the gestational period. The changes observed in this study in the placent al immunological profile in animals with hypothyroidism and excess of T4 mirror, at least in part, the fetal–placental changes seen in women and in animals with thyroid dysfunction (Shafrir et al. 1994, Morrish et al. 1997, Galton et al. 2001, Freitas et al. 2007, Souza et al. 2011, Silva et al. 2012). Our study also confirmed the hypothesis of Silva et al. (2012) that the migration of trophoblast cells is affected by thyroid dysfunction, particularly in hypothyroidism.

The high expression of the Tlr2 gene observed in the placenta of the hypothyroid animals is in agreement with the increase in apoptosis observed by Silva et al. (2012) in rat placentas at 14 days of gestation. TLR2 expression induces apoptosis of trophoblast cells (Koga et al. 2009). In contrast, the lower Tlr4 mRNA levels in the placentas of the hypothyroid animals may have resulted in the decreased gene and/or protein expression of INFy, IL10, MIF, and NOS2 also observed in these animals. The expression and activation of TLR4 in trophoblast cells induces the expression and release of several pro- and anti-inflammatory cytokines (Koga et al. 2009). These results demonstrate that changes in the expression of TLRs in women and pregnant animals may result not only from infection-related processes (Koga et al. 2009) but also from changes in endocrine profiles.

Interestingly, 14 days of gestation, which is the time point at which there is greater apoptosis of trophoblast cells in hypothyroid animals (Silva et al. 2012) accompanied by increased gene expression of Tlr2, was the time point at which there was a reduction in the gene and/or protein expression of the anti-inflammatory cytokines IL10 and NOS2 (Koga et al. 2009). Changes in the profile of these cytokines affect the fetal–placental environment (Toder et al. 2003). During fetal development, the establishment of an anti-inflammatory placental immune system is essential for the success of pregnancy (Coulam 2000, Toder et al. 2003). The reduced expression of NOS2 also promotes oxidative stress at the maternal–fetal interface, which is a cause of miscarriage, stillbirth, and premature birth (Rosario et al. 2008). The high serum levels of progesterone in the hypothyroid rats, as observed by Hatsuta et al. (2004), may also have favored the low NOS2 gene and protein expression levels observed. Mouse macrophages under the influence of a high dose of progesterone show a reduced expression of NOS2 (Miller et al. 1996).

The low level of INFy gene and protein expression observed in hypothyroid animals may also affect fetal–placental development. INFy directly influences the function of uterine natural killer cells, which are
involved in immune function and placental vascular dynamics (Hu & Cross 2010). Hypothyroid animals have alterations in placental vascularization (Silva et al. 2012). Furthermore, INFy stimulates the phagocytic activity of macrophages and trophoblast giant cells against microorganisms (Ashkar et al. 2000, Kim et al. 2005). Consequently, it is suggested that hypothyroidism may facilitate the pathogenic infection of a fetus and, thus, the development of pregnancy complications.

Hypothyroidism also reduced the invasion of both intrauterine endovascular trophoblast cells and interstitial trophoblast cells. The invasive trophoblast cells, which originate from the junction zone, participate in uterine vascular remodeling and in the secretion of the hormones (Ain et al. 2003, Caluwaerts et al. 2005, Konno et al. 2007). Cartwright et al. (1999) demonstrated that the motility and invasion of the trophoblast cells is highly dependent on the NOS produced by trophoblasts in vitro. Hypothyroid rats exhibit reduced mRNA and protein expression of NOS2 at 14 days of gestation. The precise coordination of the process of uterine vascular remodeling is critical to the success of pregnancy, because it ensures the proper delivery of nutrients to the fetus and prevents the exposure of the fetus to the deleterious effects of reactive oxygen species (Burton 2009). Thus, our results verify and explain, at least in part, the reproductive disorders found in hypothyroid rats (Silva et al. 2012). In previous research, our research group observed that pregnant hypothyroid rats subjected to the same protocol of induced thyroid dysfunction had reduced placental weight and smaller fetuses without showing any change in the total number of fetuses or in the fetal mortality rate (Silva et al. 2012). Thus, reduction in placental weight and smaller fetuses in hypothyroid rats can be a consequence of changes in the gene and protein expression of NOS2 and INFy, as these inflammatory mediators affect placental vascularization (Cartwright et al. 1999, Hu & Cross 2010).

In contrast, our research group observed that pregnant T4-treated rats had a higher conception rate without changing the fetal weight and viability (Freitas et al. 2007). It is suggested that the maintenance of the fetal weight and viability is a result of increased production of proangiogenic factors and placental lactogen by the placenta of these animals (J F Silva, N M Ocarino, R Serakides, unpublished data).

The hypothyroid animals showed reduced gene expression of Mmp2 and Mmp9 and placental leptin, unlike the T4-treated animals that had an increase in the mRNA expression of Mmp2. MMP2 and MMP9, which are mainly produced by trophoblast cells in the maternal–fetal interface, allow for cellular migration by degrading extracellular matrix proteins (Lala & Chakraborty 2003, Varanou et al. 2006). These results are in agreement with the findings of Oki et al. (2004), which demonstrated that the deficiency of thyroid hormones in vitro reduces the mRNA expression of MMP2 and MMP3, fetal fibronectin and integrins by human extravillous trophoblasts. The expression of MMP2 and MMP9 has also been found to be stimulated by placental leptin (Gambino et al. 2012), corroborating with the results found in the present study.

Placental leptin is a key hormone in the placenta (Gambino et al. 2012). Research has shown that it induces the proliferation of trophoblast cells and inhibit apoptosis. Furthermore, it regulates the fetal growth and development by affecting the process of chondrogenesis and osteogenesis (Masuzaki et al. 1997, Señarís et al. 1997, Henson et al. 1998, Gambino et al. 2010, 2012, Miko et al. 2011). Hypothyroid rats show a reduction in the proliferation of trophoblast cells, an increase in apoptosis, and a reduction in fetal development (Silva et al. 2012). Thus, the reduction in the gene expression of placental leptin as well as of Mmp2 and Mmp9 in hypothyroid rats explains the failure in the migration kinetics of the trophoblast cells that was observed in these animals.

The higher plasma levels of progesterone in hypothyroid rats (Hatsuta et al. 2004) also reduce the migration of trophoblast cells. Progesterone reduces the secretion of gelatinase and MMP2 and MMP9 by trophoblasts (Shimonovitz et al. 1998, Dai et al. 2003, Spencer et al. 2004, Miko et al. 2011). The progesterone-induced blocking factor (PIBF), which has been identified in humans and mice, was originally described as a molecule induced by progesterone to mediate its effects during pregnancy (Szekeres-Bartho et al. 1985). It was found that PIBF reduces placental expression of leptin and its receptor, showing another route by which progesterone also affects the trophoblastic invasion (Miko et al. 2011).

Excess of T4 promoted an anti-inflammatory environment in the middle of gestation. It reduced the expression of the genes encoding TNF and MIF, which are pro-inflammatory cytokines, in addition to increasing the expression of IL10 and NOS2. Although we have not observed changes in the expression of TLR2 and TLR4 in the T4-treated animals, further research to evaluate the expression of other TLRs is necessary. Other TLRs such as TLR3, TLR6, and TLR9 are also expressed by trophoblast cells and can stimulate the release of inflammatory cytokines (Koga et al. 2009). The lower expression of TNF observed in T4-treated animals may be due to reduced Mif gene expression. MIF, directly or indirectly through its receptor CD74, promotes the expression of a variety of pro-inflammatory cytokines, including TNF (Faria et al. 2010, Cardaropoli et al. 2012).

We suggest that the increased gene and protein expression of MIF and INFy in T4-treated animals at 19 days of gestation may be related to the preterm delivery observed in these animals (Navas et al. 2011). Delivery is characterized by an influx of inflammatory cells into the myometrium (Koga et al. 2009). This pro-inflammatory environment promotes the contraction of the uterus, the expulsion of the fetus, and the rejection of the placenta (Koga et al. 2009). Delivery also occurs in a hypoxic environment, being that low oxygen tension
in vitro enhances the expression of MIF mRNA and protein in explants of chorionic villi (Ietta et al. 2007).

The lower endovascular trophoblast invasion observed in the T₄-treated rats at 18 days of gestation may also be related to the premature delivery observed in these animals (Navas et al. 2011). This may be explained by the occurrence of death and/or removal of invasive trophoblast cells during and after delivery (Soares et al. 2012). This is important not only for the proper delivery of the fetus but also for the health of the mother and the success of subsequent pregnancies (Soares et al. 2012). Increased intrauterine invasion of trophoblast cells during late gestation accompanied by a failure of their removal from the uterine decidua, in contrast to what was observed in T₄-treated rats, is a pertinent cause of retained placenta, dystocia, and post partum hemorrhage in women and domestic animal species and can be fatal (Tantbirojn et al. 2008, Rosario et al. 2009).

We conclude that hypothyroidism affects the maternal immune function by compromising the development of an anti-inflammatory environment in the maternal–fetal interface and affects intrauterine trophoblast migration. Hypothyroidism affects the expression of the Tlr2 and Tlr4 genes and reduces the gene and/or protein expression of IL10, Nos2, Infγ, Mif, Mmp2 and Mmp9, and of placentin leptin in experimental animals. Excess of T₄, in contrast, promotes an anti-inflammatory environment in the middle of gestation by reducing the gene expression of TNF and MIF and increasing the gene and/or protein expression of IL10 and Nos2. Excess of T₄ also increases the gene expression of MMP2 in the middle of pregnancy and the gene and protein expression of INFγ and MIF in the late pregnancy.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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