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

Juneo Freitas Silva, Natália Melo Ocarino and Rogéria Serakides

*Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, Minas Gerais, Brazil*

*Correspondence should be addressed to R Serakides; Email: serakidesufmg@gmail.com*

## 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-$T_4$)-treated rats. A total of 144 adult female rats were divided equally into hypothyroid, L-$T_4$-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 $\gamma$ (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* (*Tnfα*)), *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 an 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 ($P<0.05$). $T_4$-treated rats not only increased the expression of IL10 and NOS2 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 $T_4$-treated group ($P<0.05$). Excess of $T_4$ 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 $T_4$ differentially affect the immune profile and the intrauterine trophoblast migration of the placenta, and these effects are dependent on the gestational period.**

*Reproduction* (2014) **147** 803–816

## 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 placentation 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, vascularization, 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 placental 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).

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0374
Online version via www.reproduction-online.org

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 (NOS2 (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 placentas 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 ($T_4$)-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 $T_4$-treated rats received L-$T_4$ 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 $T_4$, 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, $T_4$-treated, and control groups continued to receive PTU, $T_4$, 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*, *Infy*, *Mif*, *Tnf*, *Il10*, *Nos2*, *Mmp2* and *Mmp9*, and placental leptin using real-time RT-PCR. Placental disks were formed only by syncytiotrophoblast, spongiotrophoblast, and placental labyrinth.

## Immunohistochemistry and morphological measurements

The biotin–streptavidin–peroxidase (Streptavidin Peroxidase, Lab Vision Corp., Fremont, CA, USA) technique was used for immunohistochemistry. Antigenic recovery using a retrieval solution was performed for 20 min. Histological sections were incubated overnight in a humidified chamber with the primary antibody. The primary antibodies and their dilutions included anti-cytokeratin AE1/AE3 (Clone AE1–AE3, Dako, St Louis, MO, USA; 1:100), anti-INFγ (AB9657, Abcam, Cambridge, UK; 1:200), anti-MIF (AB7207, Abcam; 1:500), and anti-iNOS (AB15323, Abcam; 1:100). The sections were incubated stepwise for 30 min with each of the following solutions: blocking endogenous peroxidase, blocking serum (Ultra Vision Block, 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 used for visualization. Sections were counterstained with Harris hematoxylin. A negative control was included by replacing the primary antibodies with IgG.

Rat invasive trophoblast cells were detected using the anti-cytokeratin 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 placentation 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 spongiotrophoblast 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 placental disks per animal. Data from each placental 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).

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

| Gene | Primer sequences | Accession number |
|---|---|---|
| *Tlr2* | Forward, 5′-CTGGAGAGGCCAGCCCTGGT-3′<br>Reverse, 5′-CTCTGGCCATGCAGGCGAGG-3′ | NM_198769.2 |
| *Tlr4* | Forward, 5′-GGGGCAACCGCTGGGAGAGA-3′<br>Reverse, 5′-AACCAGCGGAGGCCGTGAGA-3′ | NM_019178.1 |
| *Infy* | Forward, 5′-AGTGCTACACGCCGCGTCTT-3′<br>Reverse, 5′-AGTGTGCCTTGGCAGTAACAGCC-3′ | NM_138880.2 |
| *Mif* | Forward, 5′-AACACCGTCCTCCGGCCGTC-3′<br>Reverse, 5′-GGCGCGGGGAACATTGGTGT-3′ | NM_031051.1 |
| *Tnf* | Forward, 5′-AGCCCGTAGCCCACGTCGTA-3′<br>Reverse, 5′-CGGTGTGGGTGAGGAGCACG-3′ | NM_012675.3 |
| *Il10* | Forward, 5′-GGCCATTCCATCCGGGGTGA-3′<br>Reverse, 5′-AAGGCAGCCCTCAGCTCTCG-3′ | NM_012854.2 |
| *Nos2* | Forward, 5′-TGGTCCTGCAGGCTCACGGT-3′<br>Reverse, 5′-ACTCGAGGCCACCCACCTCC-3′ | NM_012611.3 |
| *Mmp2* | Forward, 5′-TGGGCCCTCCCCTGATGCTG-3′<br>Reverse, 5′-AGCAGCCCAGCCAGTCCGAT-3′ | NM_031054.2 |
| *Mmp9* | Forward, 5′-TGCACCACCTTACCGGCCCT-3′<br>Reverse, 5′-CAGCGCCCGACGCACAGTAA-3′ | NM_031055.1 |
| Placental leptin | Forward, 5′-CTGGAGACCCCTGTGCCGGT-3′<br>Reverse, 5′-GCCTGGCGGATACCGACTGC-3′ | NM_013076.3 |
| β-actin | Forward, 5′-TCCACCCGCGAGTACAACCTTCTT-3′<br>Reverse, 5′-CGACGAGCGCAGCGATATCGT-3′ | NM_031144.2 |

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 $T_4$ during the entire period of the pregnancy was confirmed by assaying the free $T_3$ and $T_4$ levels in serum on days 0 and 19 of gestation and by the evaluation of symptoms. The rats treated with PTU displayed free $T_3$ and $T_4$ levels lower than those of the control group ($P<0.05$; Fig. 1) and clinical characteristics of lethargy. The $T_4$-treated rats exhibited higher free $T_4$ 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

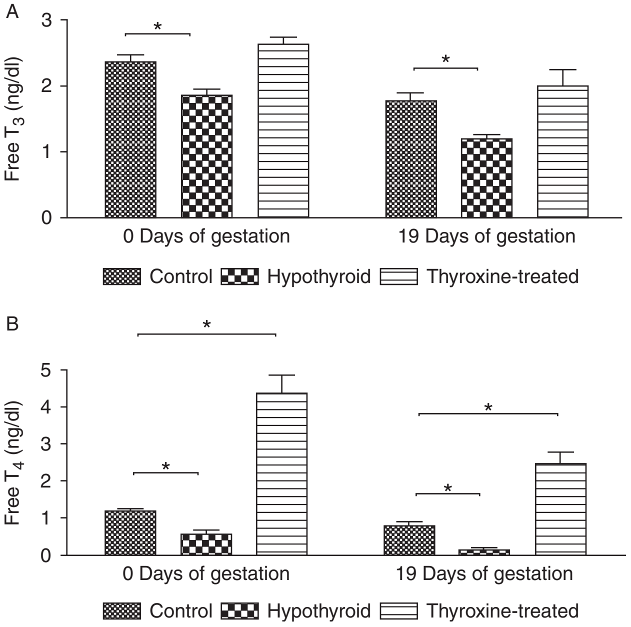

**Figure 1** (A and B) Free $T_3$ and $T_4$ levels (mean ± S.D.) in the plasma of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 0 and 19 days of gestation (*$P<0.05$).

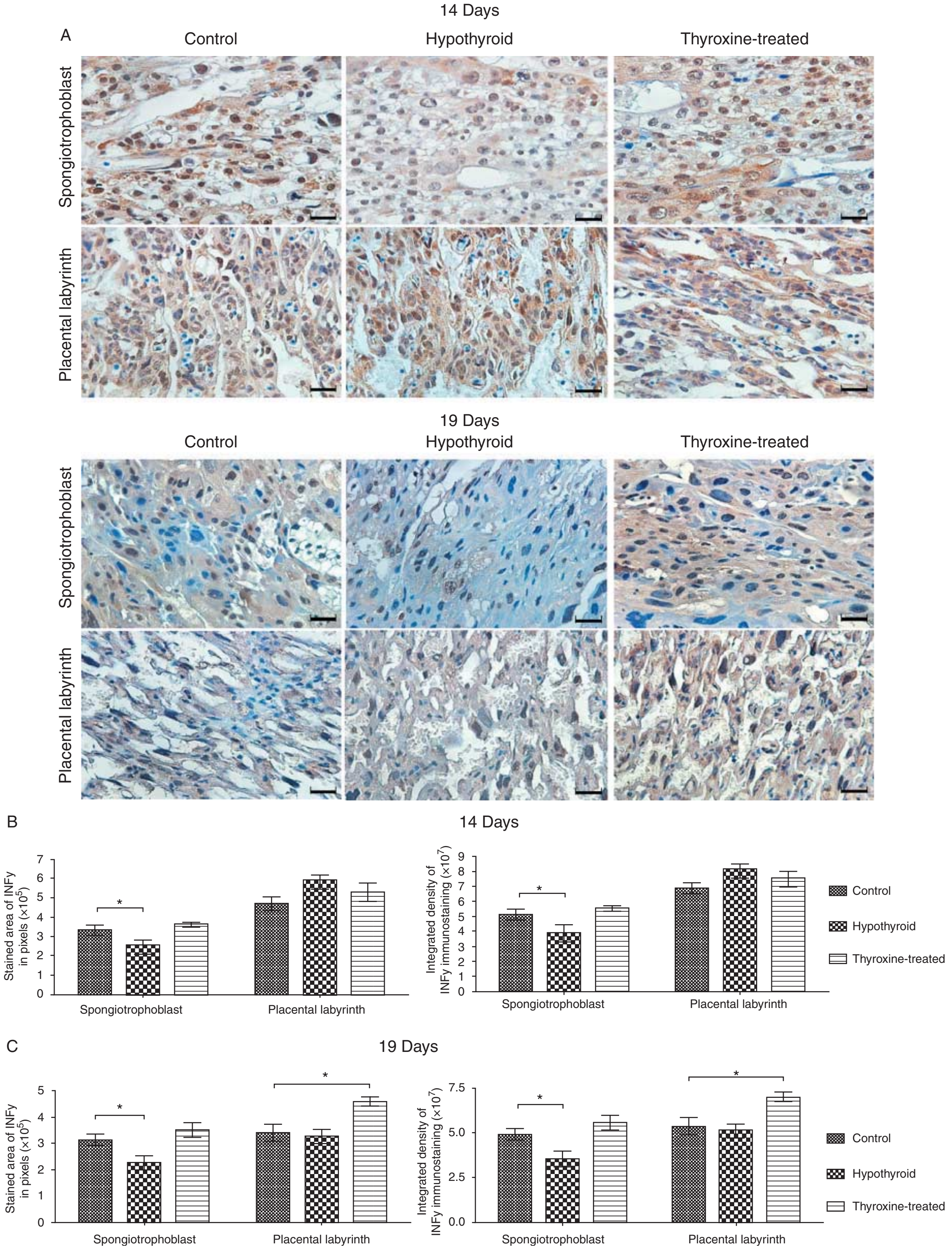

**Figure 2** INFy expression in the placenta of pregnant rats from the control, hypothyroid, and thyroxine ($T_4$)-treated groups at 14 and 19 days of gestation. (A) Immunohistochemical images of INFy expression (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar=12 µm). (B and C) Reduction in the area and intensity of INFy 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 INFy expression in the placental labyrinth of $T_4$-treated group compared with the control group at 19 days of gestation (*$P<0.05$).

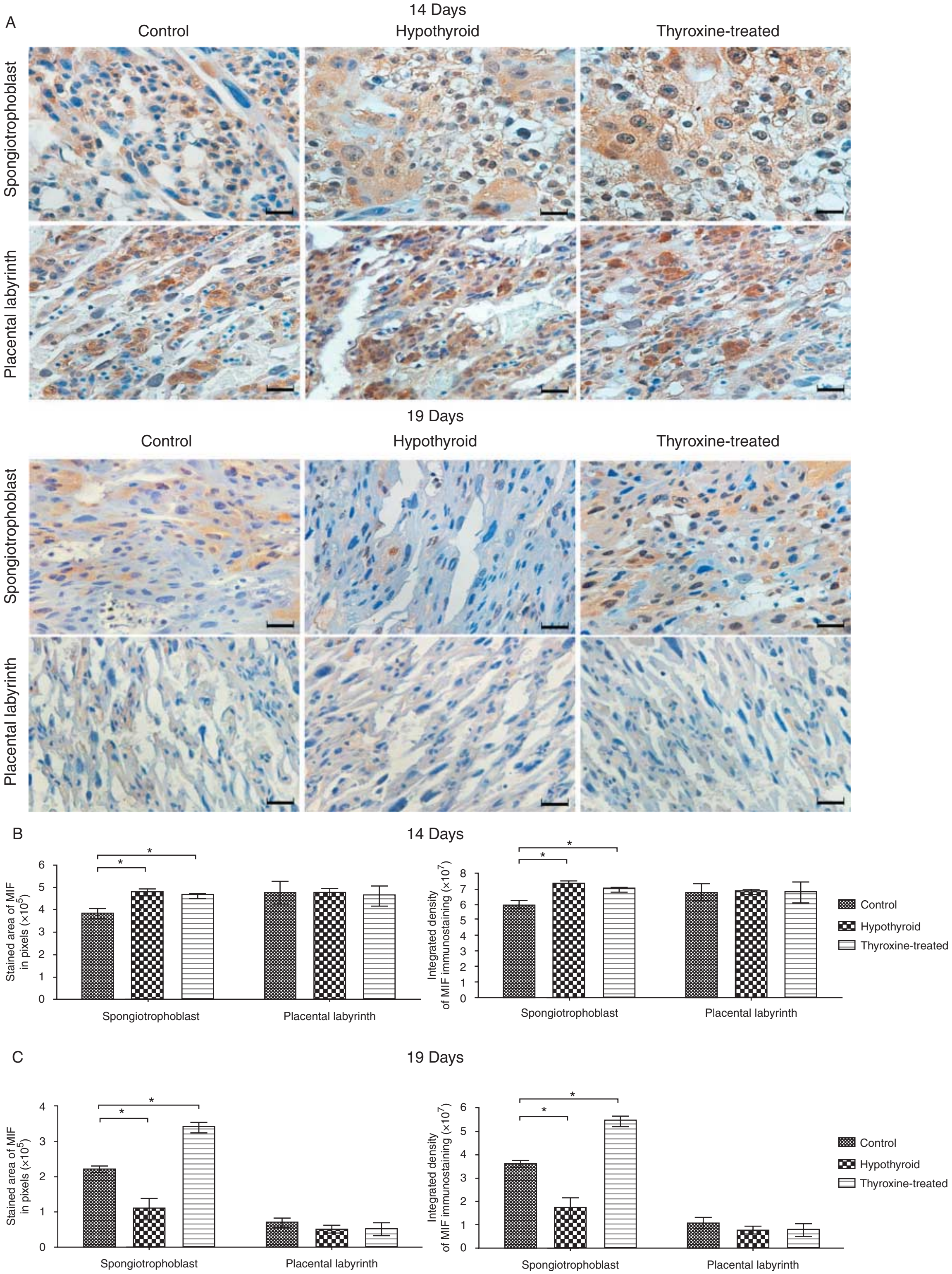

**Figure 3** MIF expression in the placenta of pregnant rats from the control, hypothyroid, and thyroxine ($T_4$)-treated groups at 14 and 19 days of gestation. (A) Immunohistochemical images of MIF expression (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar=12 μm). (B and C) Increase in the area and intensity of MIF expression in the spongiotrophoblast layer of the hypothyroid and $T_4$-treated group compared with the control group at 14 days of gestation. Reduction in the hypothyroid group and increase in the $T_4$-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 (*$P<0.05$).

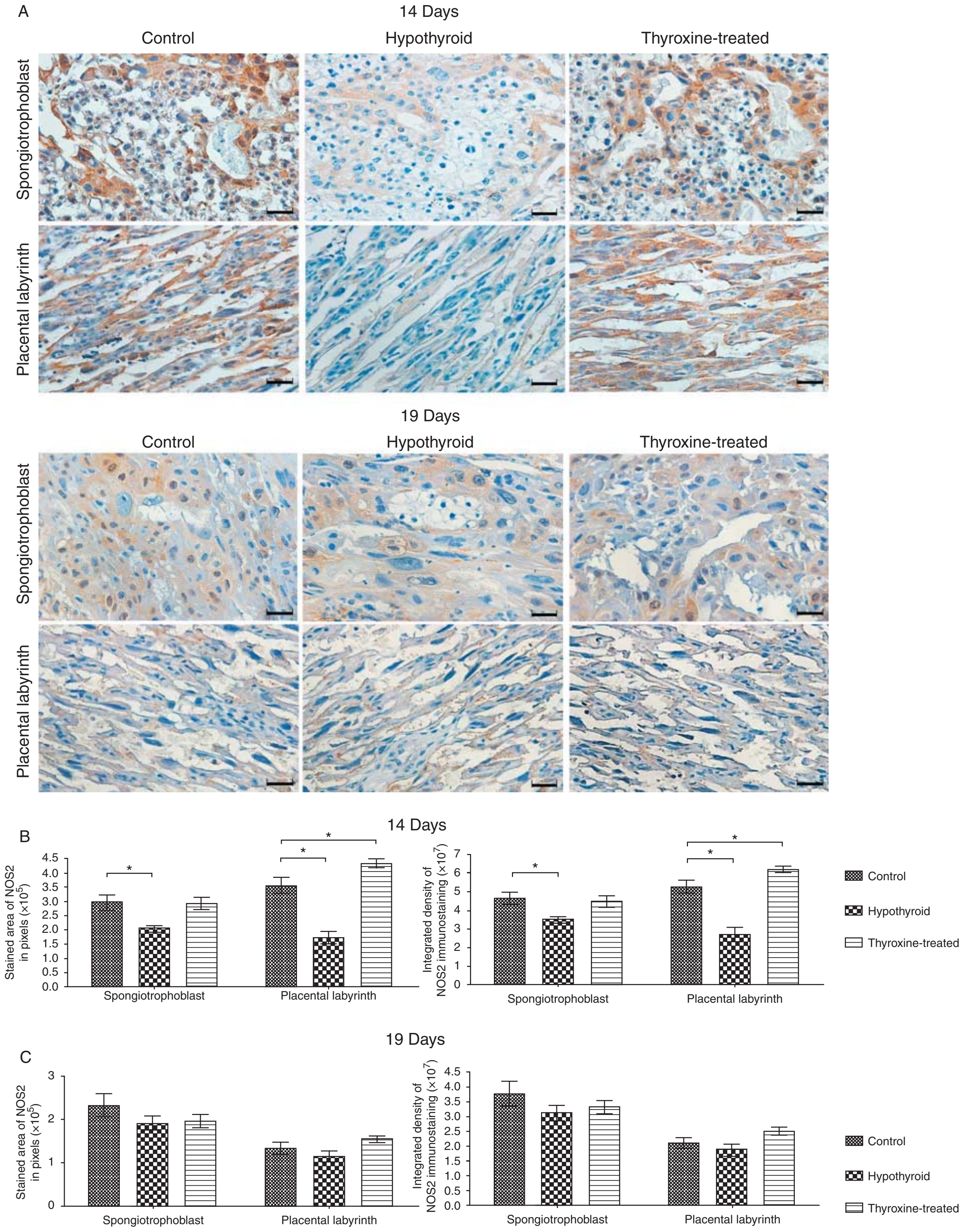

**Figure 4** NOS2 expression in the placenta of pregnant rats from the control, hypothyroid, and thyroxine ($T_4$)-treated groups at 14 and 19 days of gestation. (A) Immunohistochemical images of NOS2 expression (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar=12 μm). (B and C) Reduction 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 $T_4$-treated group compared with the control group at 14 days of gestation (*$P<0.05$).

when compared with the control rats ($P<0.05$; Fig. 2). This was different from the $T_4$-treated rats, which exhibited an increase in the area and intensity of INFγ immunohistochemical expression in the placental labyrinth at 19 days of gestation when compared with the control group ($P<0.05$; 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 ($P<0.05$; 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 ($P<0.05$; Fig. 3). In placental labyrinth, at 14 and 19 days of gestation, there was no difference on the MIF immunohistochemical expression between the groups ($P>0.05$; 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 ($P<0.05$; 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 ($P<0.05$; 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 ($P>0.05$; Fig. 5A).

The hypothyroid group had decreased *Tlr4* mRNA expression at 10, 14, and 19 days of gestation compared with the control rats ($P<0.05$; 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 ($P>0.05$; Fig. 5B).

### Gene expression of the pro-inflammatory cytokines Infγ, Mif, and Tnf

Hypothyroid rats showed reduced expression of mRNA for *Infγ* 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 *Infγ* gene expression compared with the control group at 19 days of gestation ($P<0.05$; Fig. 6A).

In contrast to the expression of INFγ, the hypothyroid rats showed no differences in gene expression of *Mif* in relation to the control rats at any of the gestational time points ($P>0.05$; 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 ($P<0.05$; 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

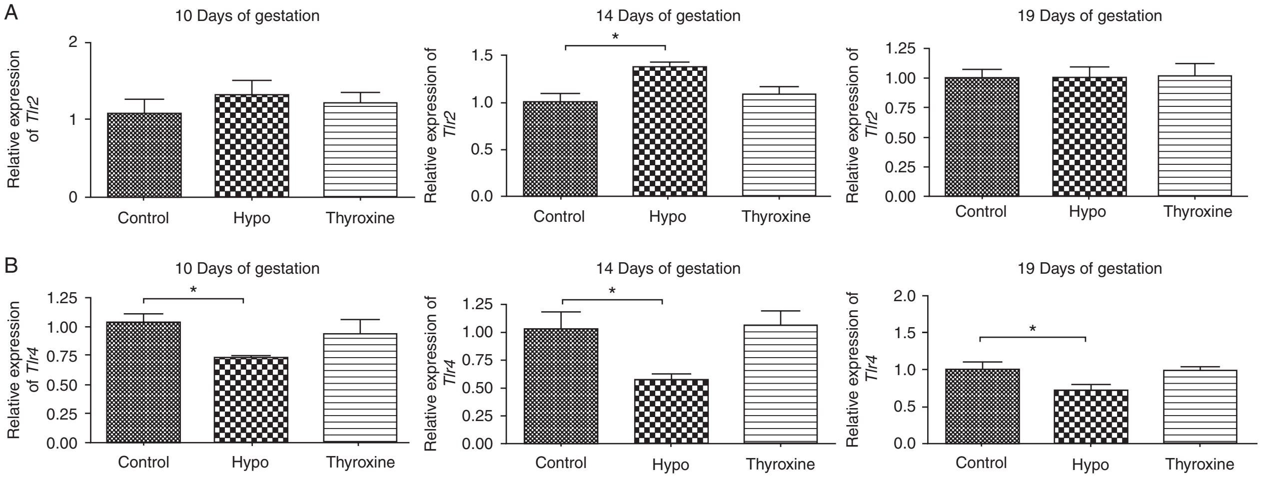

**Figure 5** Relative expression of gene transcripts for *Tlr2* (A) and *Tlr4* (B) (mean ± S.D.) in the placentas of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 10, 14, and 19 days of gestation (*$P<0.05$).

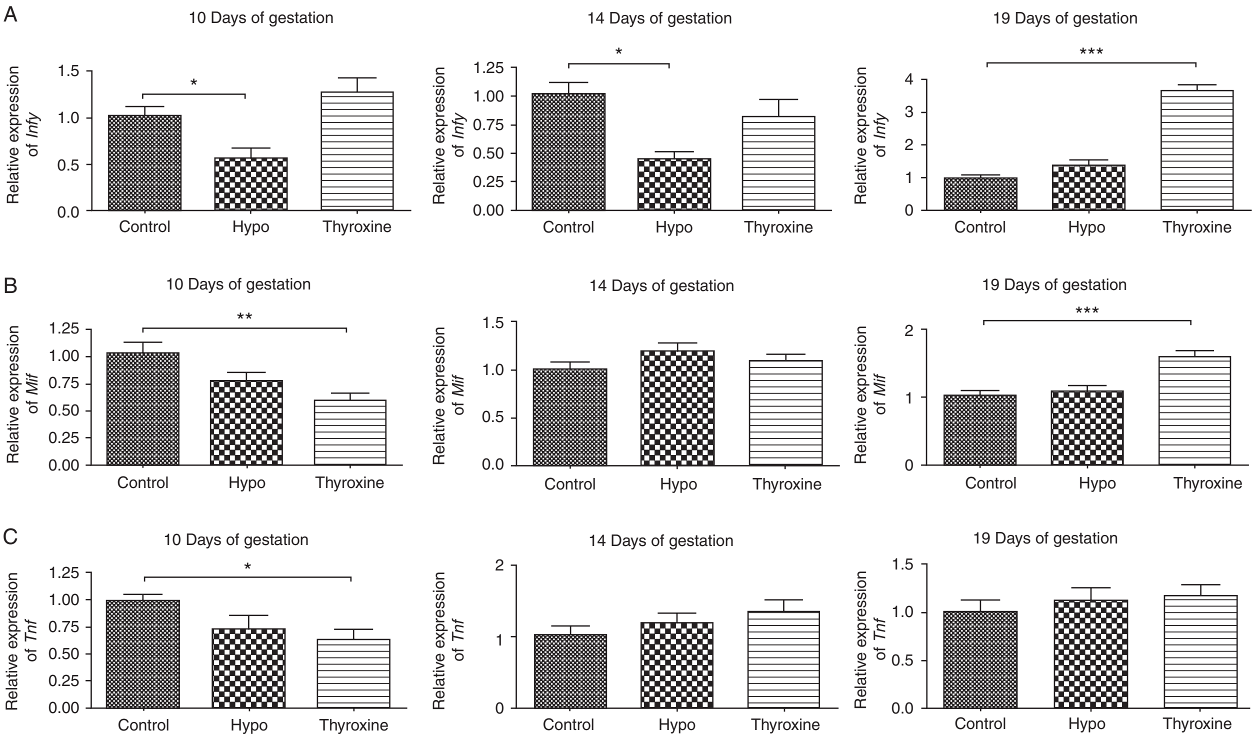

**Figure 6** Relative expression of gene transcripts for *Infy* (A), *Mif* (B), and *Tnf* (C) (mean ± s.d.) in the placentas of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 10, 14, and 19 days of gestation (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

expression of *Tnf* between the $T_4$-treated and control groups ($P>0.05$; 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 ($P<0.05$; Fig. 7A). $T_4$-treated rats, in contrast, showed an increase in gene expression of *Il10* compared with the control rats at 10 and 19 days of gestation ($P<0.05$; Fig. 7A).

Hypothyroid rats also showed a reduction in *Nos2* mRNA expression compared with the control rats at 14 days of gestation ($P>0.05$; Fig. 7B). The $T_4$-treated group showed no differences in *Nos2* gene expression when compared with the control group at any of the gestational ages tested ($P>0.05$; Fig. 7B).

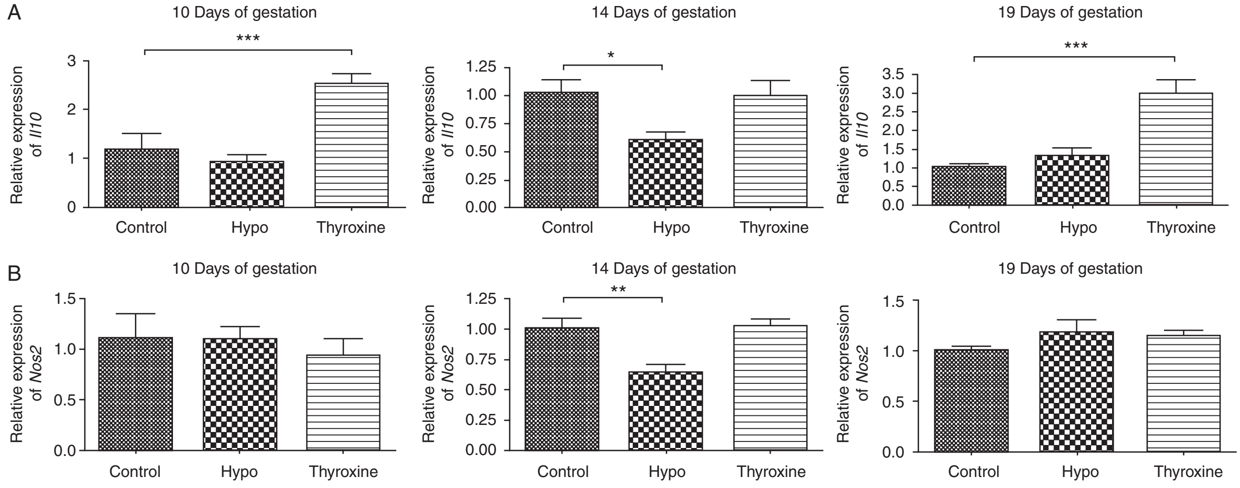

**Figure 7** Relative expression of gene transcripts for *Il10* (A) and *Nos2* (B) (mean ± s.d.) in the placentas of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 10, 14, and 19 days of gestation (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

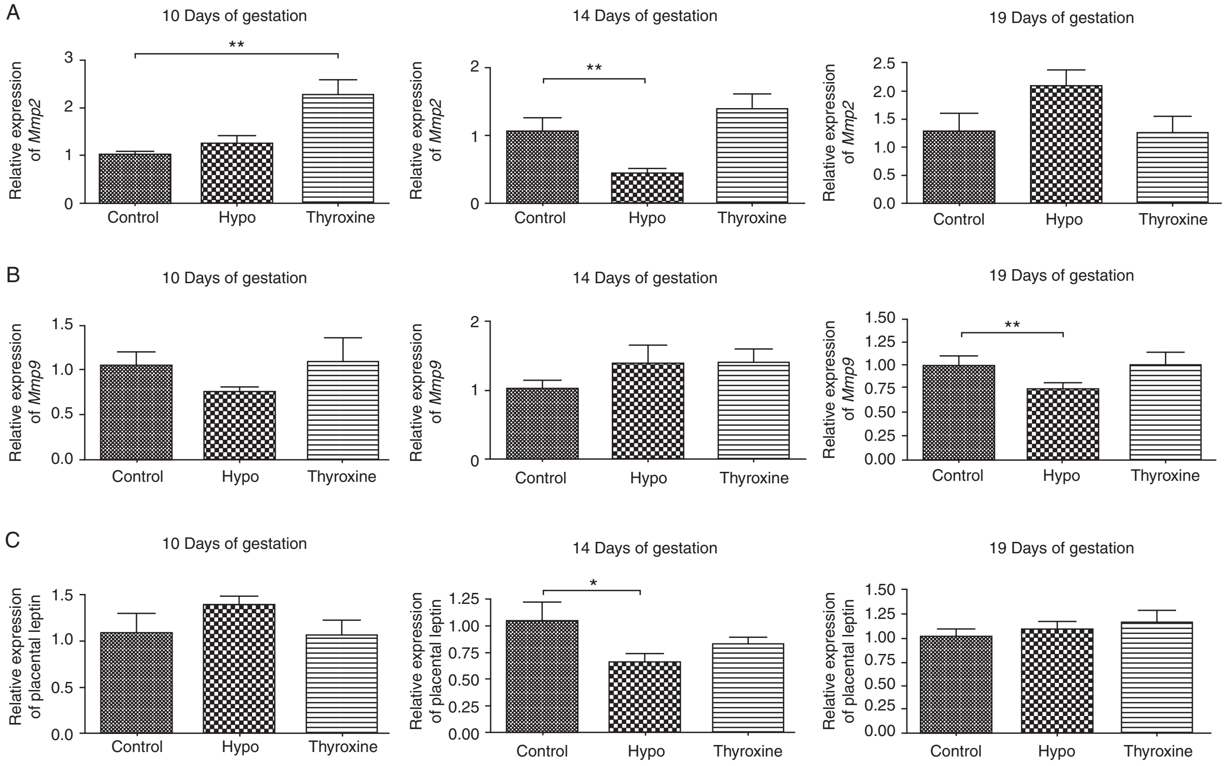

**Figure 8** (A, B and C) Relative expression of gene transcripts for *Mmp2* (A), *Mmp9* (B), and placental leptin (C) (mean ± S.D.) in placenta of pregnant rats from the control, hypothyroid, and thyroxine-treated groups at 10, 14, and 18 days of gestation (*$P<0.05$).

## 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 $T_4$-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 $T_4$-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 $T_4$-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 $T_4$-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

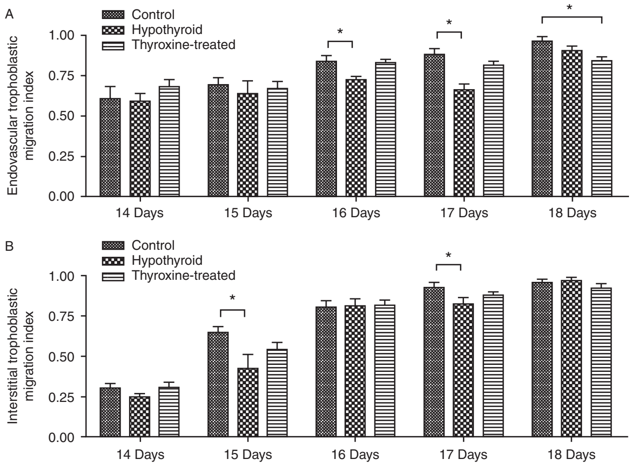

**Figure 9** (A and B) Migration index (mean ± S.D.) of endovascular (A) and interstitial (B) trophoblast cells in the decidua of pregnant rats of the control, hypothyroid, and thyroxine-treated groups at 14, 15, 16, 17, and 18 days of gestation (*$P<0.05$).

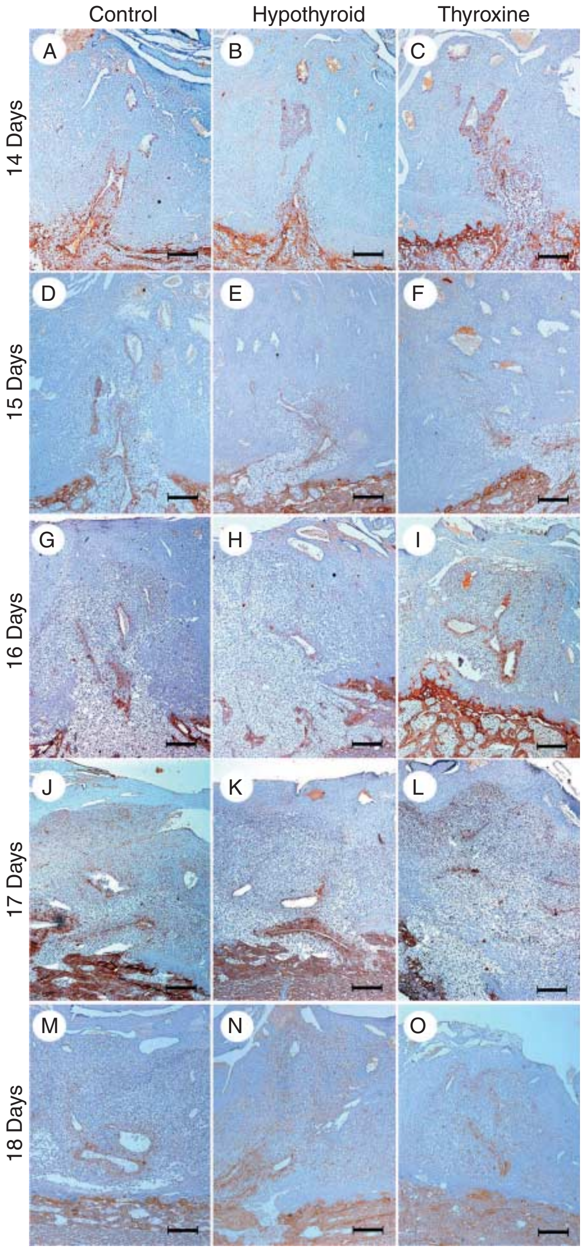

**Figure 10** (A, B, C, D, E, F, G, H, I, J, K, L, M, N and O) Immunohistochemical images of the migration kinetics of interstitial and endovascular trophoblast cells in the decidua of pregnant rats of the control, hypothyroid, and thyroxine ($T_4$)-treated groups at 14 (A, B and C), 15 (D, E and F), 16 (G, H and I), 17 (J, K and L), and 18 (M, N and O) days of gestation. (G, H and I) Lower endovascular trophoblast invasion in hypothyroid group (H) compared with control (G) and $T_4$-treated (I) groups at 16 days of gestation. (J, K and L) Lower interstitial trophoblastic invasion in the hypothyroid group (K) compared with control (J) and $T_4$-treated (L) groups at 17 days of gestation (streptavidin–biotin–peroxidase, Harris hematoxylin, scale bar = 360 μm).

($P > 0.05$). Regarding interstitial trophoblastic invasion, the $T_4$-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 $T_4$ 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 placental immunological profile in animals with hypothyroidism and excess of $T_4$ 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 INFγ, 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 INFγ gene and protein expression observed in hypothyroid animals may also affect fetal–placental development. INFγ 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 $T_4$-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 pro-angiogenic 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 $T_4$-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 inhibits 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 $T_4$ 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 $T_4$-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 $T_4$-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 $T_4$-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 $T_4$-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 $T_4$-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 placental leptin in experimental animals. Excess of $T_4$, 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 $T_4$ 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.

## Funding

This work was supported by grants from Fundação de Amparo à Pesquisa de Minas Gerais (Fapemig), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Pró-Reitoria de Pesquisa (PRPq) of the Universidade Federal de Minas Gerais.

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Received 12 August 2013

First decision 4 September 2013

Revised manuscript received 10 February 2014

Accepted 17 February 2014