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# Plasma concentrations and placental immunostaining of interleukin-10 and tumor necrosis factor- $\alpha$ as predictors of alterations in the embryo-fetal organism and the placental development of diabetic rats

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## Abstract

Interleukin-10 (IL-10) appears to be the key cytokine for the maintenance of pregnancy and inhibits the secretion of inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). However, there are no studies evaluating the profile of these cytokines in diabetic rat models. Thus, our aim was to analyze IL-10 and TNF- $\alpha$  immunostaining in placental tissue and their respective concentrations in maternal plasma during pregnancy in diabetic rats in order to determine whether these cytokines can be used as predictors of alterations in the embryo-fetal organism and in placental development. These parameters were evaluated in non-diabetic (control; N = 15) and Wistar rats with streptozotocin (STZ)-induced diabetes (N = 15). At term, the dams (100 days of life) were killed under anesthesia and plasma and placental samples were collected for IL-10 and TNF- $\alpha$  determinations by ELISA and immunohistochemistry, respectively. The reproductive performance was analyzed. Plasma IL-10 concentrations were reduced in STZ rats compared to controls ( $7.6 \pm 4.5$  vs  $20.9 \pm 8.1$  pg/mL). The placental scores of immunostaining intensity did not differ between groups ( $P > 0.05$ ). Prevalence analysis showed that the IL-10 expression followed TNF- $\alpha$  expression, showing a balance between them. STZ rats also presented impaired reproductive performance and reduced plasma IL-10 levels related to damage during early embryonic development. However, the increased placental IL-10 as a compensatory mechanism for the deficit of maternal regulation permitted embryo development. Therefore, the data suggest that IL-10 can be used as a predictor of changes in the embryo-fetal organism and in placental development in pregnant diabetic rats.

Key words: IL-10; Mild diabetes; Placenta; Pregnancy; Streptozotocin; TNF- $\alpha$

## Introduction

Virtually all known cytokines have been found to be expressed in the human placenta, although their temporal pattern of expression is still not completely understood (1). Therefore, a balance in the levels of various cytokines leading to a

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compatible local immune balance has been shown to be of considerable importance for a successful pregnancy (2).

Interleukin-10 (IL-10) appears to be the key cytokine for the maintenance of pregnancy due to its protective effect on the fetal-placental unit, since it inhibits the secretion of inflammatory cytokines such as IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon-gamma (IFN- $\gamma$ ) (3). Together with IL-4 and IL-13, IL-10 appears to modulate trophoblast invasion and to favor placental development (3). It is also thought to influence the activation and cytokine-secreting profile of uterine natural killer (uNK) cells, which are important for successful pregnancy (4). During normal pregnancy, uNK cells play an important role in maintaining early pregnancy by interacting with trophoblast cells and thereby controlling implantation and placentation (5).

Biologically active TNF- $\alpha$  is synthesized and released by gestational tissues, and women with gestational diabetes release large amounts of this cytokine in response to an increase in glucose (6).

During pregnancy, the presence of IL-10 in certain cell types is necessary for integrated development between the maternal-fetal and placental organisms, while TNF- $\alpha$  interferes with embryonic and fetal development in diabetic women. Despite these essential markers, no studies have evaluated the profile of cytokines IL-10 and TNF- $\alpha$  in diabetic rat models. Hence, the objective of the present study was to analyze the immunostaining for IL-10 and TNF- $\alpha$  in placental tissue and their respective concentrations in maternal plasma of pregnant rats with mild diabetes in order to determine whether these biological markers can be used to detect changes in the embryo-fetal organism and in placental development.

## Material and Methods

### Animals and experimental groups

Newborn female Wistar rats obtained from São Paulo State University (UNESP), São Paulo State, Brazil, were maintained in an experimental room under controlled conditions of temperature ( $22 \pm 2^\circ\text{C}$ ) and humidity ( $50 \pm 10\%$ ), and on 12-h light/dark cycle with *ad libitum* access to commercial diet (Purina<sup>®</sup> rat chow, Brazil) and tap water. At birth, the newborns (approximately 6.0 g) were randomly divided into two experimental groups: non-diabetic (control) animals and animals with streptozotocin (STZ)-induced diabetes. Procedures and animal handling were performed in accordance with the guidelines of the Brazilian College of Animal Experimentation and the experiment reported here was authorized by the Ethics Committee for Animal Research at Universidade Estadual de São Paulo (Brazil).

### Diabetes induction

Diabetes was induced with STZ (Sigma Chemical Company, USA) in newborn females on the day of birth. STZ was dissolved in 0.1 M sodium citrate buffer, pH 4.5, and subcutaneously administered at the dose of 100 mg/kg. Non-diabetic rats received citrate buffer subcutaneously.

### Pregnancy period

The animals were mated after reaching the age of adults. Gestational day 0 (GD0) was defined as the day when sperm was seen in the vaginal smear. On GD0, only diabetic rats with blood glucose between 120 and 300 mg/dL (mild diabetes), and control rats with blood glucose lower than 120 mg/dL were included in the experiment groups (7). Blood glucose levels were monitored during pregnancy in the morning after the animals received food *ad libitum* overnight using a standard glucometer (Johnson & Johnson<sup>®</sup>, Brazil). In addition, glucose and insulin tolerance tests were performed to confirm the diabetic status during pregnancy. The oral glucose tolerance test (OGTT) was performed on day 17 of pregnancy and the insulin tolerance test (ITT) on day 15 of pregnancy. The animals were sacrificed at two different times (day 11 or day 21 of pregnancy) to determine plasma levels of IL-10 and TNF- $\alpha$  (R&D Systems, USA) by ELISA according to the kit protocol. The measurements were made in duplicate on the same day. The intra-assay coefficient of variation was 2.1%. In addition, on day 21 of pregnancy the uterine contents were withdrawn to count implantation and the number of live fetuses, and the placentas were obtained, fixed in 4% formaldehyde, and embedded in paraffin for immunohistochemical analysis of IL-10 and TNF- $\alpha$ .

### Immunohistochemistry

One placenta from each rat ( $N = 15$  per group) (8) was used for the immunohistochemical analysis of IL-10 and TNF- $\alpha$ . Tissue was cut into 5- $\mu\text{m}$  sections, deparaffinized and rehydrated in a graded alcohol series using standard procedures. Antigens were then recovered from tissue using EDTA solution, pH 8.0, in a hot water bath at  $96^\circ\text{C}$ . Endogenous peroxidase activity was blocked by a 10-min incubation with universal block (Dako, USA; Dual Endogenous Enzyme Block code: S2003). Nonspecific binding sites were blocked by incubation with 3% skim milk. Slides were incubated with specific antibodies (IL-10, polyclonal goat anti-rat antibody, 1:100 dilution, code: AF-519, and TNF- $\alpha$ , polyclonal goat anti-rat antibody, 1:100 dilution, code: AF-510-NA; R&D Systems), or with the appropriate dilution of bovine serum

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albumin (BSA) overnight for the negative control. A Vectastain Elite kit (Vector Laboratories, USA) was used to visualize antibody binding. The intensity of immunolocalization in each placental zone was analyzed by two independent readers and averaged. Signal intensity was scored as follows: 1, not detectable; 2, weak; 3, moderate; 4, high (8).

### Statistical analysis

Data are reported as means  $\pm$  SD, and groups were compared by the *t*-test. The intensity of immunostaining was analyzed by the Friedman test followed by the chi-square test for the analysis of prevalence. The level of significance was set at  $P < 0.05$  in all analyses.

## Results

### Confirmation of diabetic status

On days 0 and 14 of pregnancy, there was a significant increase in the mean glycemia values of diabetic rats compared to control. Glucose intolerance and insulin resistance were confirmed by altered OGTT and ITT results. The diabetic rats presented alteration of the glycemic curve altered at four times in the OGTT and at two times on the ITT (data not shown).

### Maternal plasma IL-10 and TNF- $\alpha$ concentrations and reproductive outcomes

The control group showed a significantly increased plasma IL-10 concentration at the end compared to the middle of pregnancy. In the STZ group, plasma IL-10 concentrations remained unchanged as pregnancy developed. When compared to the control group, STZ rats showed a statistically significant decrease in plasma IL-10 concentration on days 11 and 21 of pregnancy. No change in TNF- $\alpha$  concentration was observed between groups at either time (Table 1).

The mean numbers of live fetuses and implantations were significantly decreased, and the mean pre- and post-implantation loss rates were increased in the diabetic group compared to the control group (Table 1).

### Immunohistochemical detection of placental IL-10 and TNF- $\alpha$

Figure 1 shows the immunohistochemical detection of IL-10 and TNF- $\alpha$  in placental tissues. Placentas from diabetic dams did not show gross morphological disarrangements (Figure 1C and D). In both control and diabetic placentas, IL-10 and TNF- $\alpha$  were localized in decidua and trophoblast lineages analyzed (trophoblast giant cells and spongiotrophoblast; Figure 1).

The distribution of IL-10 and TNF- $\alpha$  in control placentas was similar to the diabetic placentas. The arrows show the immunostaining cells and the arrowheads the negative cells. There was no significant difference in mean immunostaining intensity scores for IL-10 (Figure 1G) and TNF- $\alpha$  (Figure 1H) between groups in any of the regions studied ( $P > 0.05$ ). The score analysis of the protein in both groups revealed that TNF- $\alpha$  expression increased when IL-10 increased in decidua and spongiotrophoblast areas, showing a balance between these cytokines. The giant cell IL-10 expression showed a tendency to decrease the immunostaining compared to TNF- $\alpha$ , but there was no statistically significant difference.

## Discussion

In this study, the mild diabetes model was confirmed by the presence of increased serum glucose levels observed at the beginning and in the middle of pregnancy, and by the presence of glucose intolerance and insulin resistance. Despite the presence of insulin resistance in diabetic rats, our results showed no differences between groups at the times analyzed regarding plasma TNF- $\alpha$  levels. Studies of diabetic pregnant women have shown an increase in serum levels of TNF- $\alpha$  compared to healthy pregnant women (9-12). Thus, our results do not agree with the literature data for women but do show that TNF- $\alpha$  is not a good predictor of insulin resistance or maternal-fetal changes in this model of diabetic rat pregnancy.

The decrease of plasma IL-10 concentrations on days 11 and 21 of pregnancy in diabetic rats is related to impaired embryo-fetal development. The damage to early embryonic development was confirmed by the failure of implantation indicated by pre-implantation with a reduced number of implantations and by the post-implantation losses indicated by an increased reabsorption rate and a reduced number of live fetuses, in this case indicating placental damage. The decrease in IL-10 concentration in term pregnancies is consistent with the results obtained by Kuzmicki et al. (13) in women with gestational diabetes. However, a study by Atégbo et al. (9) revealed an increase in IL-10 levels in diabetic pregnant women. The literature reports that there is a greater IL-10 production in uncomplicated pregnancies as compared to those with pathologies such as spontaneous abortion and pre-eclampsia (14-16), thus supporting the role played by IL-10 in the maintenance of normal pregnancy.

Additionally, we observed that the control group showed a significant increase in plasma IL-10 concentration at the end relative to the middle of pregnancy, while in the STZ group the concentration of this cytokine remained unchanged during the entire pregnancy. There are divergent results regarding IL-10 concentrations during normal human pregnancy. Kruse et al. (17) and Power et al. (18) have reported increasing plasma IL-10 levels with increasing pregnancy duration, in agreement with the results obtained in our study. Vassiliadis et al. (19), however, have reported that IL-10 concentrations did not change with pregnancy duration. Holmes et al. (20) proposed that the possible explanation for differing results may be the small number of studies and the use of inappropriate study methodologies. However, there are no reports in the literature concerning the concentrations of these cytokines in experimental and clinical studies of diabetic pregnancy.

In the present study, analysis of immunostaining intensity showed no alterations in STZ rats and prevalence analysis showed that the IL-10 expression followed TNF- $\alpha$  expression. In the control group, when there was increased IL-10 immunostaining increased TNF- $\alpha$  was also observed, showing a balance between these cytokines in the placental tissue. Similarly, this balance was also seen in the diabetic group. Despite changes in the maternal organism, these placental cytokines provided an appropriate environment for embryo-fetal development, thus showing that both IL-10 and TNF- $\alpha$  acted locally in the placenta while plasma concentrations of these cytokines did not change in the maternal organism.

The data presented here permit us to conclude that plasma IL-10 levels in diabetic rats are related to damage during early embryonic development; however, the increase in placental IL-10 as a compensatory mechanism in the setting of a deficit in maternal regulation allowed appropriate embryo-fetal development. Thus, IL-10 may be used as a predictor of alterations in the embryo-fetal organism and in the placental development in pregnant diabetic rats.

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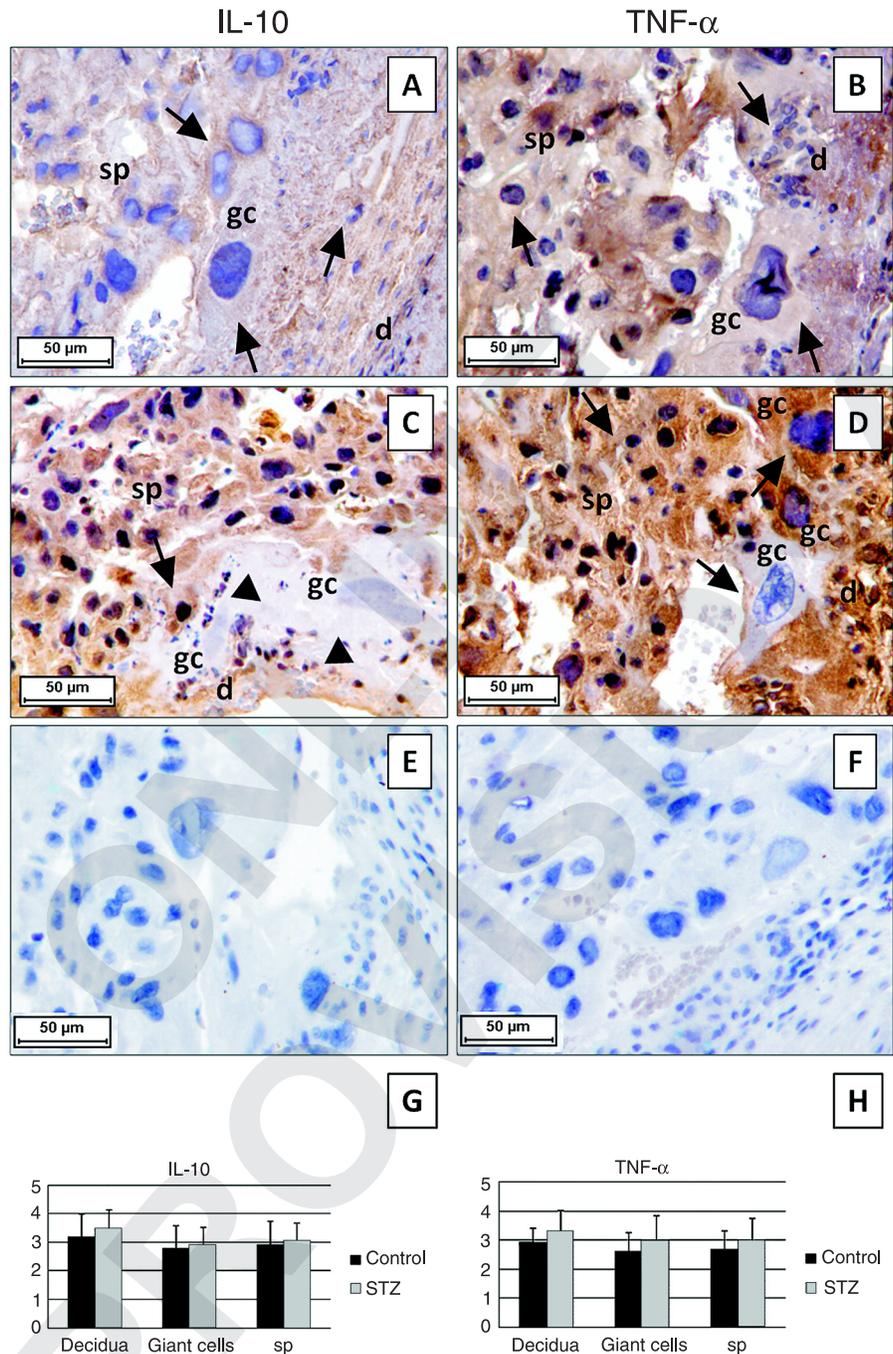
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**Table 1.** Plasma concentrations of interleukin-10 (IL-10) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in rats with mild streptozotocin (STZ)-induced diabetes and non-diabetic rats (control) on days 11 and 21 of pregnancy, and reproductive performance by day 21 of pregnancy.

	Control (N = 15)	STZ (N = 15)
Cytokine concentrations		
IL-10 (pg/mL)		
Day 11 of pregnancy	14.5 $\pm$ 8.9	4.6 $\pm$ 6.6*
Day 21 of pregnancy	20.9 $\pm$ 8.1#	7.6 $\pm$ 4.5*
TNF- $\alpha$ (pg/mL)		
Day 11 of pregnancy	2.3 $\pm$ 0.9	2.3 $\pm$ 0.9
Day 21 of pregnancy	3.1 $\pm$ 1.7	2.7 $\pm$ 0.9
Reproductive performance		
Number of implantations	13.4 $\pm$ 1.4	10.4 $\pm$ 2.8*
Number of live fetuses	12.8 $\pm$ 1.6	8.7 $\pm$ 3.4*
Pre-implantation loss (%)	5.7	16.1*
Post-implantation loss (%)	4.0	21.3*

Data are reported as means  $\pm$  SD. \*P < 0.05 compared to control on the same day (t-test). #P < 0.05 compared to day 11 of pregnancy in the same experimental group (t-test).



**Figure 1.** Immunohistochemical detection of interleukin-10 (IL-10; A,C) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; B,D) staining in late-gestation placentas (day 21) from control (A,B) and diabetic (STZ; C,D) rats. IL-10 and TNF- $\alpha$  staining was detected in all trophoblast lineages studied. Arrows show the immunostaining cells and arrowheads the negative cells. Negative controls (E,F). One placenta from each dam (N = 15) was assessed for staining intensity in the decidua (d), trophoblast giant cells (gc) and spongiotrophoblast (sp). Immunostaining intensity was assessed by semiquantitation of IL-10 (G) and TNF- $\alpha$  (H) on an arbitrary, four-point scale (1 = not detectable, 2 = weak, 3 = moderate, and 4 = high). Data are reported as means  $\pm$  SD (Friedman test). No significant differences were observed between groups regarding specific trophoblast lineages.