Increased expression of STAT3 and SOCS3 in placenta from hyperglycemic rats

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Abstract

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is activated by interleukin (IL)-6 and IL-10 that generate nearly opposing responses. The suppressor of cytokine signaling 3 (SOCS3) is the negative regulator of STAT3 and plays an important role in the negative regulation of the inflammatory process. Evidence has shown the importance of STAT3 and SOCS3 during implantation and normal pregnancy. However, little is known about the relationship of both factors under hyperglycemic condition. The aim of this study was to evaluate the placenta regions exhibiting immunopositivity for STAT3 and SOCS3 in hyperglycemic rats, as well as correlate these proteins with IL-10 and IL-6 levels. It was observed increased expression of STAT3 at the labyrinth (approximately 47% of increase compared to control) and junctional zone (approximately 32% of increase compared to control) from hyperglycemic placentas. Similar results were observed to SOCS3 (approximately 71% -labyrinth- and 53% -junctional zone- of increase compared to control). The levels of IL-10 were augmented at hyperglycemic placentas (approximately 1.5 fold of increase) and they were positively correlated with the increase of STAT3 at the labyrinth and SOCS at junctional zone. Therefore, under hyperglycemic conditions, the relation between STAT3 and SOCS3 was changed, leading to unbalance of the cytokine profile.

Introduction

Cytokines are constantly secreted by the placenta, controlling several physiological and immunological functions during pregnancy, including implantation, placentation and parturition.[1] After binding to their specific receptors, cytokines' actions occur through activation of specific pathways. The Janus kinase (JAK) and the transcription family of proteins (STATs), known as the JAK-STAT pathway has an important role during pregnancy and may be activated by several cytokines.[2] Among STATs family, STAT3 is considered a pleiotropic transcriptional factor in several cell types. STAT3 can be activated by both interleukin 6 (IL-6) and interleukin 10 (IL-10), generating opposing cellular responses. While IL-6 signaling results in transient activation of STAT3, IL-10 signaling results in sustained STAT3 activation, suppressing the expression of pro-inflammatory genes.[3] STAT3 has been described as a vital regulator for placentation and therefore to reproduction. STAT3 also seems to have a positive influence on the invasion, proliferation and/or differentiation in the first stages of pregnancy.[4,5]

STAT3 activates many intracellular transcription effectors, for example, the expression of a protein from the family of cytokine signaling suppressors (SOCS). Among this family, SOCS3 is particularly sensitive to JAK/STAT modulation, and SOCS3 represents the most enduring form of JAK/STAT signaling inhibition.[6] Compared with IL-6 signal transduction, IL-10 pathway seems to be much less sensitive to the inhibitory activity of SOCS3.[2] SOCS3 expression is observed in the placenta, independent of the labor status.[7] However, decreased SOCS3 seems to be an important mechanism by which inflammatory cytokines enter into a positive loop that occurs during delivery.

Maternal hyperglycemic disturbances during pregnancy is a recognized risk for maternal and perinatal adverse outcomes.[8] Indeed, hyperglycemia may interfere with the placental profile of cytokine expression,[9] as well as with the signaling pathways modulated by those cytokines. Therefore, hyperglycemia may differently regulate SOCS3 and JAK/STAT activity, in a tissue and manner specific condition, as demonstrated in pulmonary epithelial cells,[10] macrophage,[11] vascular smooth muscle cells,[12] among others.

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Besides the importance of SOCS and the transcription factor STAT3 has already been described in the placenta, little is known about the expression and distribution of STAT3 and SOCS3 under hyperglycemic condition. Evidence shows that SOCS3 mRNA was not changed in placentas from normoglycemic and women with gestational diabetes,[13] but the protein expression was not assessed on this tissue, making these interpretations inconclusive.

Therefore, in the present study, the expression and distribution of STAT3 and SOCS3 in placental tissues from hyperglycemic rats were histologically evaluated, as well the correlation of these proteins with IL-10 and IL-6 levels were evaluated.

Materials and Methods

Animals

All procedures were performed in

accordance with the Guiding Principles in the Care and Use of Animals, adopted by the Brazilian College of Animal Experimentation. The study design was approved by the Committee of Ethics in Animal (CEUA) from the Federal University of Mato Grosso (UFMT), under the number 23108.120946/2015-83.

Twelve 10-12 weeks-old female Wistar rats, were obtained from colonies maintained at the Central Animal Facilities from UFMT and they were kept in a temperature-controlled environment (21±1°C), maintained at a 12-h light/dark cycle, and given free access to tap water and standard rat food. The rats were randomly distributed into 2 experimental groups: hyperglycemic (n=6) and control (n=6). A single dose of streptozotocin (STZ, Sigma Chemical Co St. Louis, Missouri), dissolved in citrate buffer (10 µM, pH 6.5) was delivered to develop hyperglycemia (40 mg/kg body weight, i.p.). The respective control group (n=6) received vehicle in a similar way and volume. Severe hyperglycemia (>300 mg/dL) or normoglycemia (<120 mg/dL) was confirmed 3 days prior to the mating period, after 6 h fasting, using a digital glucometer (Accu-Check Active®- Roche, Basel, Switzerland).

Following hyperglycemic or sham treatment, each female was housed with a male for copulation. Vaginal smears were taken daily, and the day on which spermatozoa were found in the vaginal smear was designated gestational day 0. At 21st gestational day, rats were anesthetized with 3% sodium pentobarbital (50 mg/Kg body weight, i.p.). Subsequent to laparotomy for removal of placentas, rats were killed by pneumothorax. Fetuses were removed and killed by placement in a CO_2 chamber.

Immunohistochemistry

Immunohistochemistry was performed according to a previously established protocol.[14] Placentas were fixed in Methacarn solution (60% methanol, 30% chloroform, 10% acetic acid), for 3 h, at 4°C, under constant agitation. Later, placentas were dehydrated, clarified in xylene and embedded in paraffin (Paraplast; Sigma-Aldrich, St. Louis, MO, USA). Sections of 5 µm thickness were cut from near the midline region of placentas and adhered to glass slides, previously treated with 0.1% poly-L-lysine (Sigma-Aldrich). Sections were deparaffinized and rehydrated. Antigen retrieval was performed just for SOCS3 by incubating the sections in 10 mM acetate sodium buffer (pH 6.0), at 95ºC, for 15 min. The sections were then treated with 3% (v/v) hydrogen peroxide (H_2O_2) in PBS, for 30 min, to block endogenous peroxidase activity. Each of the succeeding steps was followed by a thorough rinse with PBS. All steps were performed in a humid chamber, and care was taken to avoid the dehydration of the sections. Nonspecific staining was blocked by immersion in Cas-Block solution (ThermoFisher Scientific, Waltham, MA, USA) and goat serum (Gibco, Waltham, MA, USA) for 30 min (both). Sections were incubated with the primary antibody anti-STAT3 (catalog #sc-7179, rabbit polyclonal, reactive to mouse, rat and human; Santa Cruz Biotechnologies Inc., Dallas, TX, USA) and anti-SOCS3 (catalog #sc-9023, rabbit polyclonal, reactive to mouse, rat and human, Santa Cruz Biotechnologies Inc.), diluted 1:100 and 1:250, respectively, in PBS containing 0.3% (v/v) Tween 20, overnight at 4°C. After extensive rinsing in PBS, all sections were incubated for 1 h at room temperature with biotin-conjugated goat anti-rabbit IgG (Rockland Immunochemicals, Inc. Pottstown, PA, USA) diluted 1:500 (for both) in PBS. The peroxidase reaction was visualized using NovaRED kit (Vector Laboratories Inc., Burlingame, CA, USA). After immunostaining, sections were lightly stained with Mayer's hematoxylin (Merck Millipore, Burlington, MA, USA). For each immunohistochemical reaction, controls were performed by incubating the sections with PBS or by omitting the primary antibody. Sections were examined in a Zeiss Axioskop 2 microscope, and images were captured using a digital camera (Canon, Tokyo, Japan) and KS 100 3.0 software (Zeiss, Oberkochen, Germany). Marked-cells and unmarked-cells were scored for each captured field, using the Image-Pro-Plus software (Media Cybernetics, Silver Spring, MD, USA), and were expressed as a percentage of the total number of cells counted in that specific field. The results expressed the percentage relative to marked cells. Samples were considered positive when more than 5% of cells stained positive and strongly positive when more than 5-fold of increase was observed, compared to control.

Measurement

Placenta were removed, cleaned from connective tissue and membranes, and frozen in liquid nitrogen. After pulverization, the placentas were homogenized in lysis buffer (10× Ripa buffer, 100 mM $Na_4P_2O_7$, 20 mM NaF, 100 mM Na_3VO_4, 100 mM PMSF, and protease inhibitor cocktail), quickly sonicated and centrifuged (1500 rpm), for 5 min. After centrifugation, protein concentration was determined by the Bradford assay. Proteins were used to quantify IL-10 and IL-6 cytokines (1200 µg and 450 µg, respectively), using commercial kits (cat. IL-10 #555134; cat. IL-6 #550319; BD Biosciences Pharmingen, San Diego, CA, USA), by ELISA, according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM and "n" represents the number of animals used in the experiments. Statistical analysis was performed using Graph Pad Prism 4.0 (GraphPad Software Inc.). Statistical analyses were performed by Student's *t*-test, compared to the control group. Correlation between cytokines; glycemia and STAT3; SOCS3 were done by Pearson correlation coefficient (r) and linear regression analysis (R). Values of $P<0.05$ were considered statistically significant.

Results

STAT3 and SOCS3

Immunohistochemistry showed nuclear stain for STAT3 in all evaluated placenta regions (Figure 1 A,B). Placentas from hyperglycemic rats displayed increased STAT3 immunopositivity in the labyrinth region (%), compared to control rats (71.4±5.6 *vs* 24.7±5.0, respectively; P=0.0005; Figure 1 C,D and Figure 2A). In this placental region, cytotrophoblast and syncytiotrophoblast cells were the main targets for STAT3, especially under hyperglycemic condition.

In the junctional zone, placentas from hyperglycemic rats displayed increased STAT3 immunopositivity (%), compared to control rats (84.6±3.0 *vs* 52.7±6.9, respectively; P=0.0028; Figure 1 E,F and Figure 2B). Spongiotrophoblast and giant cells were targets for STAT3 in this region and hyperglycemia considerably increased STAT3 stain in this region.

The following step was to investigate SOCS3 distribution through the placenta and immunohistochemistry analysis revealed cytoplasmatic targets protein for SOCS3 in all placenta regions (Figure 3 A,B). Placentas from hyperglycemic rats displayed significant augmented SOCS3 immunopositivity in the labyrinth region (%), compared to control rats (73.3±6.8 *vs* 1.9±1.0, respectively; P=0.0001; Figure 3 C,D and Figure 4A). Accordingly, in the junctional zone, SOCS3 distribution (%) was further enhanced in placentas from hyperglycemic rats, compared to control rats (79.2±1.2 *vs* 26.5±12.3, respectively; P=0.0052; Figure 3 E,F and Figure 4B). The most abundant cytoplasmic SOCS3 stain was observed in cytotrophoblast and syncytiotrophoblast, in the labyrinth; and in the spongiotrophoblast cells, in the junctional zone (Figure 3 D,E). Interestingly, SOCS3

stain was abundant in the cytoplasm region, making it difficult to define borders between cells.

Cytokine expression and correlation

IL-6 (Figure 5A) and IL-10 (Figure 5B) placental levels were measured in the entire placenta, and these cytokines are augmented in the hyperglycemic rats, compared to those in the controls. Correlations were observed between IL-10 and labyrinth zone with STAT3 (r=0.640; P=0.046; R^2= 40.9%) and SOCS3 (r=0.811, P=0.015; R^2= 65.8%; Table 1). No correlation was observed with IL-6. A strong correlation was observed between glycemia and STAT3 (junctional zone r=0.813; P=0.004; R^2= 63.1%, and labyrinth r=0.859; P=0.001; R^2=73.9%). With regard to SOCS3, a strong (junctional zone, r=0.844, P=0.008; R^2=71.3%) and very strong (labyrinth, r=0.958; P=0.000; R^2= 91.7%) correlation was observed (Table 1).

Discussion

Pregnancy represents a unique immunological state where any imbalanced immune responses against fetus antigens can result in disturbances related to pregnancy.[9,15] A pro-inflammatory state is expected in the final stage of pregnancy, by a progressive enhancement of Th1 immunity,[16] favoring the production of cytokines playing a vital role in the initiation of parturition.[7] The perfect immunological balance during pregnancy is mediated through a complex mechanism, including the release of specific cytokines, according to the gestational stage, controlled by hormones, proteins and transcription factors. Among them, STAT3 is a transcription factor expressed both in the maternal and fetal compartments during placenta development, as demonstrated in murine tissue.[17] STAT3 is also required during the pre-implantation period and decidualization.[4,17-20] Hyperglycemia promotes a specific pattern of STAT3 activation, as demonstrated in cardiomyocytes,[21] as well as in glomerular and tubulointerstitium.[22]

In view of the above, it remains to be demonstrated how STAT3 participates in the placental modulation in late stages of pregnancy and how maternal hyperglycemia would affect this expression pattern. Currently, evidence shows that STAT3 is observed in immature trophoblasts, favoring trophoblast invasion and that STAT3 modulation should be lost in term placentas, providing an adequate environment for the last period of gestation.[23] Here, we showed that during hyperglycemia, IL-10 and IL-6 levels were augmented in placental tissue, simultaneously with augmented STAT3 and

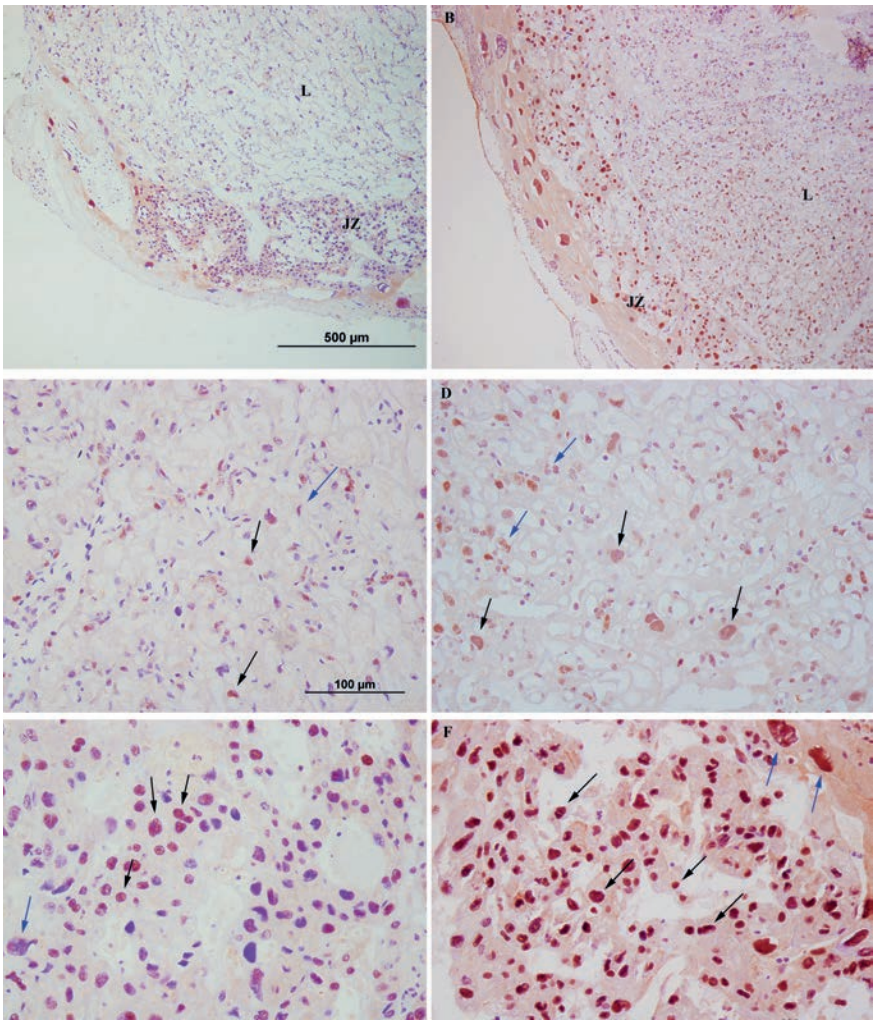

Figure 1. Immunohistochemistry for STAT3. Overview of the placenta regions stained for STAT3 from control (A) and hyperglycemic (B) groups; A-B pictures were taken with 10x magnification. C-D) Immunoreaction in labyrinth region from control (C) and hyperglycemic (D) groups. The arrows indicate the main target cells for STAT3 in labyrinth region: cytotrophoblast (black arrows) and syncytiotrophoblast (blue arrows). E-F) Immunoreaction in the junctional zone from control (E) and hyperglycemic (F) groups. The arrows indicate the main target cells for STAT3 in junctional zone: spongiotrophoblast (black arrows) and giant cells (blue arrows); C-F pictures were taken with 40x magnification and used to count the stained cells. Placentas from hyperglycemic rats (n=5) or control (n=6) were evaluated. Sections were treated with anti-STAT3 (1:100) and biotin-conjugated goat anti-rabbit IgG (1:500). Negative control sections were incubated in the absence of the primary antibody. JZ, junctional zone; L, labyrinth.

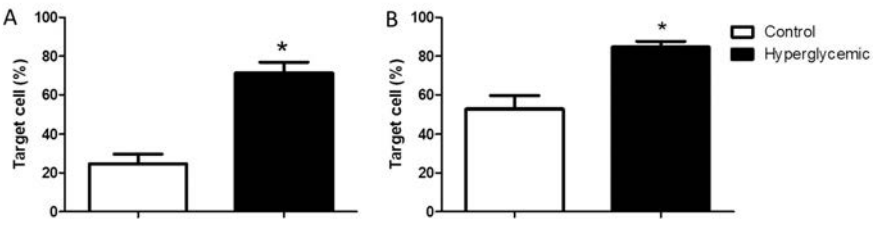

Figure 2. Hyperglycemia increases STAT3 immunopositivity in the placental labyrinth region (A) and junctional zone (B). Representative graphs showing mean ± SEM for target cells in each group. The statistical comparison was performed with Student's t-test. * P<0.05 *vs* control group. Placentas from hyperglycemic rats (n=5) or control rats (n=6) were evaluated. Sections were treated with anti-STAT3 (1:100) and biotin-conjugated goat anti-rabbit IgG (1:500).

SOCS3 immunopositivity in the labyrinth and junctional zone.

IL-10 is recognized as a regulatory cytokine, and among its actions, IL-10 limits the release of proinflammatory cytokines.[14] Augmented IL-10 placental levels, because of hyperglycemia has been already described in placental extravillous layer,[24] along with the release of other cytokines, building an inflammatory environment. The same pattern was also observed in placentas from type 2 diabetes mellitus patients.[25] Th2 and Treg lymphocytes are important sources for IL-10 production.[26,27] A recent study evaluated the peripheral T-cell profile in the third trimester from gestational diabetes patients. Higher expression of Th2, Treg and also Th17 lymphocytes was observed within these patients and this pattern persisted even after the delivery.[16,28] However, during active labor, the concentrations of cytokines belonging to the IL-10 family, which includes IL-10, IL-20, IL-22, IL-28A, should be significantly decreased in comparison with third-trimester levels,[29] as demonstrated in placentas from healthy women. Therefore, augmented IL-10 levels are an event more expected during the middle stage of pregnancy when the maternal organism, under the influence of several hormones, makes a shift favoring Th2 lymphocytes.[28] One possibility is that under hyperglycemic conditions, the maternal organism fails to reduce IL-10 and other Th2-derived cytokines, affecting the delivery process as well as fetal maturation.

IL-10 elicits a sustained activation of STAT3,[3] a process involving tyrosine phosphorylation and consequent activation of JAK tyrosine kinases, migration to the nucleus where this transcription factor initiates the gene regulation favoring an anti-inflammatory cascade. Indeed, STAT3 is essential for all known IL-10 functions.[30] Activation of STATs, particularly STAT3, classically regulates functions such as cell proliferation, cell cycle progression, apoptosis, angiogenesis and immune evasion, and has been implicated in events related to pregnancy such as placentation, embryonic development, organogenesis, innate immunity, adaptive immunity and cell growth regulation.[29] Interestingly, a predominant nuclear stain for STAT3 was observed in the present study, indicating the activated state of this transcription factor. STAT3 immunopositivity was previously described in the cytoplasm from cytotrophoblast and syncytiotrophoblast cells, whereas nuclear STAT3 immunopositivity was observed only in term villous trophoblast, both in the mid- and term-pregnancy.[31] Conversely from what would be expected in a normoglycemic pregnancy, increased STAT3 acti-

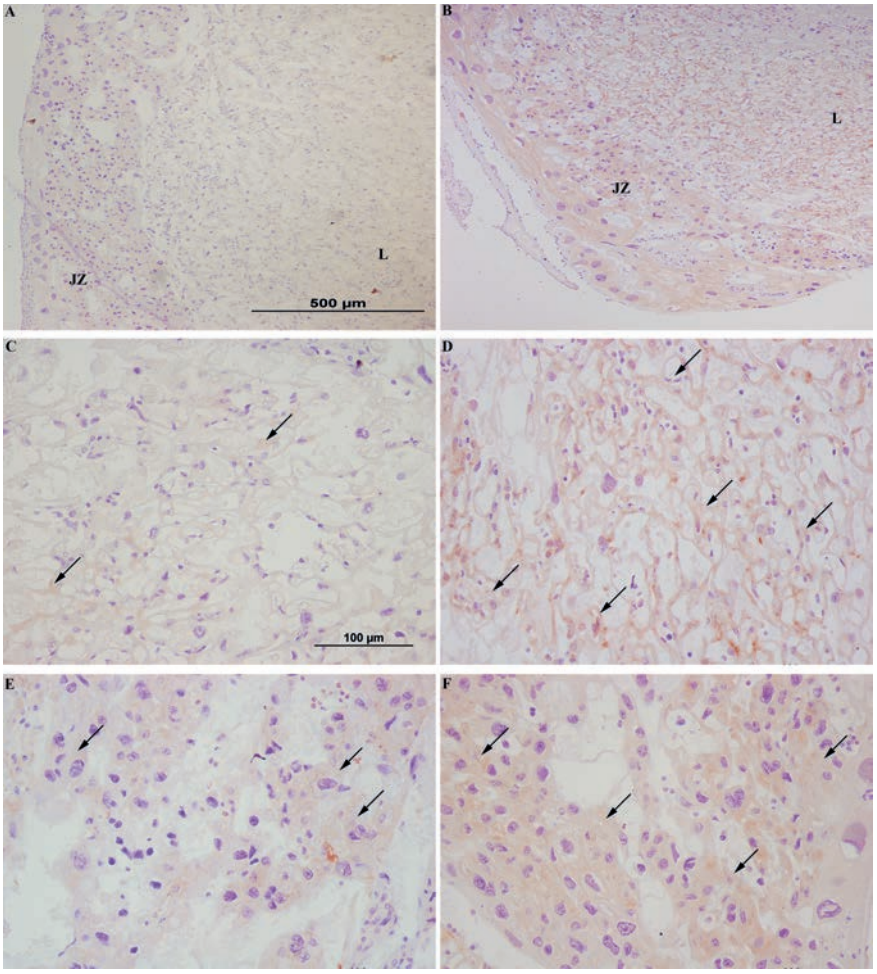

Figure 3. Immunohistochemistry for SOCS3. Overview of the placenta regions stained to SOCS3 from control (A) and hyperglycemic (B) groups; A-B pictures were taken with 10x magnification. C-D) Immunoreaction in labyrinth region from control (C) and hyperglycemic (D) groups. E-F) Immunoreaction in the junctional zone from control (E) and hyperglycemic (F) groups. The arrows indicate that the primary marking occurs in all cytoplasm of the cells. C-F pictures were taken with 40x magnification and used to count the cells stained. Placentas from hyperglycemic rats (n=5) or control (n=6) were evaluated. After antigen retrieval, sections were treated with anti-SOCS3 (1:250) and biotin-conjugated goat anti-rabbit IgG (1:500). Control sections were incubated with PBS or with the secondary antibody (omitting the primary antibody - *not shown*). JZ, junctional zone; L, labyrinth.

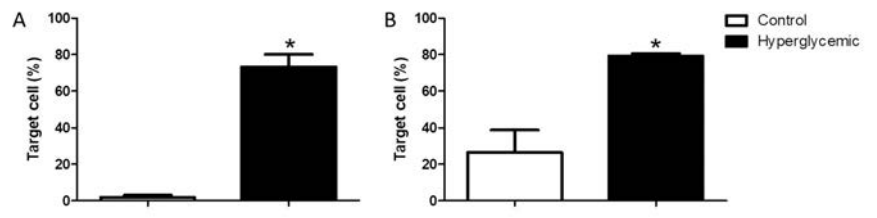

Figure 4. Hyperglycemia increases SOCS3 immunopositivity in the placental labyrinth region (A) and junctional zone (B). Representative graphs showing mean ± SEM in each group. The statistical comparison was performed with Student's t-test. *P<0.05 *vs* control group. Placentas from hyperglycemic rats (n=5) or control rats (n=6) were evaluated. After antigen retrieval, sections were treated with anti-SOCS3 (1:250) and biotin-conjugated goat anti-rabbit IgG (1:500). Control sections were incubated with PBS or with the secondary antibody (omitting the primary antibody).

Table 1. Cytokines' correlation and linear regression.

		Glycemia	IL-10	IL-6	STAT3 jz	STAT3 labyrinth	SOCS3 jz	SOCS3 labyrinth
Glycemia	r	1	0.593	0.124	0.813*	0.859*	0.844*	0.958*
	P		0.071	0.732	0.004	0.001	0.008	0.000
	R^2		-	-	63.1%	73.9%	71.3%	91.7%
IL-10	r	0.593	1	0.074	0.563	0.640*	0.811*	0.650
	P	0.071		0.830	0.090	0.046	0.015	0.058
	R^2	-		-	-	40.9%	65.8%	-
IL-6	r	0.124	0.074	1	0.175	0.112	0.265	0.079
	P	0.732	0.830		0.628	0.759	0.525	0.840
	R^2	-	-		-	-	-	-

jz, junctional zone; R, Pearson correlation coefficient; R^2, linear regression; *P<0.05.

vation was found both in the labyrinth region and junctional zone in term-placentas from hyperglycemic pregnant rats during our evaluations.

IL-10 also induces the expression of the SOCS3.[32] This protein acts as an intra-cellular inhibitory regulator of cytokines signaling through the STATs pathway, consequently decreasing the inflammatory cytokine production. The suppressor effects of SOCS3 is larger on the IL-6R than IL-10R, and therefore, the IL-10R appears refractory to the effects of all SOCS family members.[33] Hyperglycemia increases SOCS3 immunopositivity in the placental labyrinth region and junctional zone and IL-10 is strongly correlated with SOCS3 at the junctional zone. As shown before, the pro-inflammatory state expected in the third trimester of pregnancy is extremely important and the production of cytokines plays a vital role in the initiation of parturition.[7] Therefore, increased production of negative regulator of cytokine production, SOCS3, could lead to an imbalance in the regulated production of pro-inflammatory cytokines, resulting in fetal-placental dysfunction. During murine placenta development, SOCS3 was detected in the mesometrial decidua, endothelium of maternal blood vessels and in the giant trophoblast cells, from day 9 to 14 of pregnancy.[29] Here, SOCS3 was expressed in all placental regions, but hyperglycemia contributed to overexpression of SOCS3 in the entire placenta. It is recognized that SOCS3 is essential for embryonic development since genetic deletion of SOCS3 in murine models resulted in embryonic lethality due to placental insufficiency.[34] In this regard, the labyrinth and spongiotrophoblast layers in the SOCS3-null placenta were poorly formed whilst trophoblast giant cells were increased in number and in size, resulting in a model that is embryonically lethal. In the opposite direction, SOCS3 overexpression favors a shift towards systemic Th2 responses.[35] Similarly, during hyperglycemia, augmented placental SOCS3 sustained IL-10 expression, as demonstrated here. Human placenta, collected during delivery from healthy women, revealed suppressed SOCS expression, providing a mechanism by which inflammatory cytokines enter into a positive feedback loop of inflammatory changes leading to membrane rupture, cervical ripening, myometrial contraction, and delivery.[36] However, SOCS3 regulatory role may suffer important alterations during pathologic conditions. For example, placentas from preeclamptic women presented decreased SOCS3 expression in the villous tissues, favoring a pro-inflammatory environment and reducing endogenous anti-inflammatory activity.[37] Conversely, the augmented expression of SOCS3 usually culminates in the reduction of IL-6 concentrations. However, over-expression of SOCS3 in trophoblast cells promotes the increase of IL-10 production.[38]

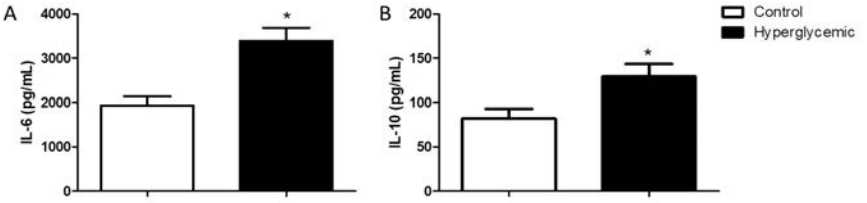

Figure 5. Hyperglycemia increases placental levels of IL-6 (A) and IL-10 (B). Representative graphs showing means ± SEM in each group. The statistical comparison was performed with Student's t-test. *P<0.05 *vs* control group. Placentas from hyperglycemic rats (n=5) or control rats (n=6) were evaluated.

Therefore, it is expected that IL-6 and IL-10 concentrations are inversely and positively related to SOCS3 expression, respectively. However, our results showed that upon hyperglycemic conditions, IL-6 placental concentration was augmented, despite SOCS3 up-regulation. Augmented IL-6 may be a direct effect of the increased hyperglycemic levels, through activation of other transcription factors involved in IL-6 production, by passing SOCS3 regulation. Indeed, greater activation of NF-κB, along with augmented IL-6 and tumor necrosis factor-alpha (TNF-α) expression was observed in placentas from hyperglycemic rats,[39] suggesting that other transcription factors may have an overlapping SOCS3 modulatory role.

Some important limitations should be acknowledged. This study was designed to study the endpoint of pregnancy, in a rat model. In the future, it will be interesting to address the temporal modification of STAT3/SOCS3 and IL-10 during pregnancy, and how the glycemic levels may play a role in their regulation. It is also possible that other transcription factors may be somehow involved in the differential cytokine expression. Finally, experimental models are useful to study maternal-related disturbances, but future studies conducted in human samples may further strengthen the findings from the present study.

Taken together, the results reinforce the importance of STAT3/SOCS3 regulatory function in placentas at the end stage of pregnancy in uncomplicated pregnancies. Additionally, under hyperglycemic conditions, IL-10 augmented levels were observed simultaneously with augmented IL-6 levels. It seems that under hyperglycemia, up-regulation of STAT3/SOCS3 was not enough to guarantee a shift favoring the production of pro-inflammatory cytokine, classically induced by Th1 lymphocytes, as desirable in the late stage of

pregnancy. This fact may be evidence that upon hyperglycemia, other transcription factors may be simultaneously activated, favoring the augmented production of pro-inflammatory factors

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