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The Role of IL-6, IL-10 and IFN- γ mRNA in Women with Recurrent Abortion

Wasan A. Bakir*, Zynab S. Abdul-gany*, and Aseel faeeg*

*Iraqi Center for Cancer and Medical Genetic Research, Al-Mustansiryia University, Baghdad, Iraq

Summary:

Human pregnancy appears to be an immunological paradox, in that the fetus represents a semi-allograft developing in the potentially hostile environment of the maternal immune system. One important mechanism involves the down-regulation of the cellular immune response, which has been shown to be dependent upon the suppression of T-helper (Th)1 and T-cytotoxic (Tc)1 cells,which produce interleukin -2, interferon -γ, and tumor necrosis factor -β, and the up- regulation of Th2 and Tc2 cells, which produce IL-4, IL-6, IL-10 and IL-13The aim of this study to investigate the possible immunological basis of recurrent abortion by studying the expression of IL-6, IL-10, IFN- γ in trophoblasts by using in situ hybridization (ISH) technique. Methods: using IL-6, IL-10 and IFN as biomarkers of Th1 and Th2 cytokines. Paraffin embedded blocks from trophoblasts tissue of each patient for determination available cytokines. Results: IL-6 increased in recurrent spontaneous abortion (RSA) and IL-10 was higher in control group than in recurrent spontaneous abortion while RSA showed a high percentage of IFN-γ in comparison with control. The expression of IFN-γ decreases whereas IL-6 and IL-10 increase with increasing gestational age. Conclusions: IFN-γ, IL-6 and IL-10 may be biomarkers of Th1/Th2 immune status during pregnancy. Defective IL-10 expression in women with RSA and non-RSA might be the possible defect in Th2 cytokines production in these patients.

Introduction:

Recurrent spontaneous abortion (RSA) is one of the important complications in pregnancy, a multiple factors have been observed in the pathology of it such as genetic, endocrine, infectious autoimmune defects and anatomical abnormalities, but still about 40-60% of cases with unknown etiology which was mainly attributed to the immune factors (1).

The implantation process of the human placenta involves an invasion of maternal endomatrium by the trophoblasts surrounding the blastocyst to which there is a cellular reaction in the endometrium (2). The cytokines may play an important role in the development of pregnancy (3).

The endometrial lymphocytes play some roles in the maintenance of pregnancy via immune mediators such as cytokines (4).

The T helper 1 (Th1) type cytokine secretion such as IFN-γ, IL-2 and TNF and Th1/Th2 ratio exert deleterious effects on pregnancy, inhibiting fetal growth and development. On the other hand, Th2 cytokines secretions such as IL-10, IL-4, IL-6 and IL-13 have been associated with successful pregnancy (5).

Interleukin -6 promoting hematopoietic processes and stimulating the B cell differentiation and facilitating the T- cell growth and differentiation in type Th2 (6, 7, 8). Interleukin – 10 was proposed to be a factor that might protect the semi-allogeneic fetus from maternal allo-recognition and rejection by driving the maternal (both local and systemic) immune reaction toward a Th2-type immune response (9), IL-10 is believed to play a major role in directing Th0 cell differentiation toward a Th2 phenotype (10), IL-10 inhibits pro-inflammatory cytokines production including IL-1β, TNF-α and IFN-γ (11, 12), therefore prevents the development of Thl-type immune reactions deleterious for the maintenance of pregnancy (13).

Furthermore, IFN-γ produces many biological

effects inducting immune cells (14). It increases macrophage activity to destroy bacteria and tumor cells, stop the viral replication and used in the chemotherapy of cancer because of its killing of tumor cells (15, 16, 17).

The aim of this study to investigate the possible immunological basis of recurrent abortion by studying the expression of IFN-γ, IL-6 and IL-10 in trophoblasts using ISH technique. And investigate the Th1 and Th2 cytokines throughout the pregnancy, using IFN-γ, IL-6 and IL-10 as biomarkers of Th1 and Th2.

Materials and Methods:

One hundred and nineteen women were recruited at the Obstetrics and Gynecology department of Al-Kadhimyia Teaching Hospital in Baghdad. Included recurrent spontaneous abortion (RSA); non-RSA (first and second abortion) and successful pregnancy (full term) as a control groups.

Trophoblastic tissue was collected from the evacuation of retained pieces during the procedure of curettage and placed in 10% formalin. Two to three paraffin embedded blocks were prepared for each patient. Staining with haematoxyline and eosin was carried out to decide which block can be used in the study (only sections that contained trophoplastic tissues were included in this study). These cases were subjected for in situ hybridization technique and with the different markers.

- Biotin – Labeled DNA probe for human IL-6 (8 µg/10070 µl ddH2O) Probe size: 349 bp (Maxim Biotech, Inc., U.S.A).
- Biotin-labeled DNA probe for human IFN-γ, (8 µg/100 µl ddH2O). Probe size: 249bp (Maxim Biotech, Inc., USA).
-Biotin-labeled DNA probe for human IL-10, (8 µg/100 µl ddH2O). Probe size: 223bp (Maxim Biotech, Inc., USA).

Scoring:

Hybridization /Detection System will give an intense blue –black color at the specific sites of the hybridization probe in both positive test tissues. Counting the number of positive trophoblastic cells, which gave a blue-black nuclear staining under the light microscope, counting of positive cells was done with the assistance of histopathologist. The extent of the ISH signal in the villi was determined in 10 fields (XI00 magnification). The percentage of positively stained villi was calculated for each case by taking the mean of the percentages of the positively stained villi in the 10 fields.

Statistical analysis:

Statistical analysis was performed using ANOVA test to determine whether the means were equal among three groups. p value of < 0.05 was considered statistically significant.

Results:

The mean percentage of IL-6 increased significantly in recurrent spontaneous (RSA) and the expression of IL-10 by trophoblasts tissue was significantly higher in pregnancy (control group) than in recurrent spontaneous abortion RSA while (RSA) showed a high percentage with a highly significant difference ($p>0.001$) of IFN-γ in comparison with control the mean (Figure 1, Table 1).

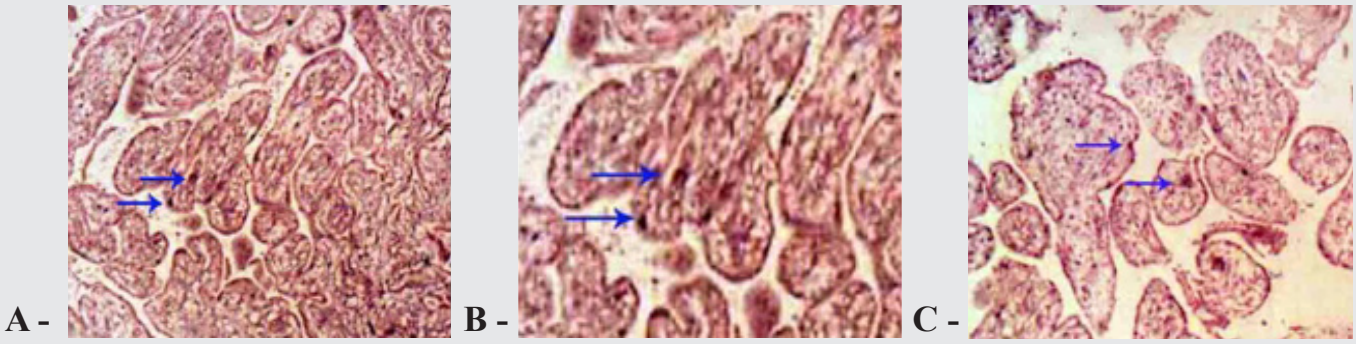

Figure 1: Detection of IL-6, IL-10 and IFN-γ mRNA in studied groups by in situ hybridization (ISH). Staining of IL-8; IL-10 and IFN-γ mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. (A) Tissue from patient with RSA shows positive IL-6 hybridization signals. Demonstrates the heterogenous nuclear staining pattern (arrows) (X100). (B) Patient with RSA shows positive IL-10 hybridization signals (C) Tissue from successful pregnancy shows positive IFN-γ hybridization signals.

Table (1): Comparison between the mean percent of the expression of IL-6, IL-10 and IFN-γ mRNA (ISH assay) in the trophoblasts of studied groups.

Variable	Group	n=73	Mean± SE	F test p value	Sig. between groups
IL-6	RSA	39	79.7±3.1	<0.01	RSA- Non. RSA**
	Non- RSA	20	52.1 ±2.03		RSA- Con
	Con	14	55.3 ±5.5		Non. RSA- Con
IL-10	RSA	39	25.9 ±1.9	<0.01	RSA- Non. RSA**
	Non- RSA	20	45.9 ±3.2		RSA- Con**
	Con	14	75.3 ±2.5		Non. RSA- Con**
IFN-γ	RSA	39	87.3 ±2.5	<0.01	RSA- Non. RSA**
	Non- RSA	20	54.1±1.3		RSA- Con**
	Con	14	±1.831.7		Non. RSA- Con**

*=*significant difference (p<0.05);*
**= *highly significant difference (p<0.01)*

A highly significant difference (p<0.001) between groups in the ratio of IFN-γ to IL-10 and IFN- γ to IL-6 for the ISH when compared between group RSA and control group and between RSA and non-RSA ,and a highly significant difference (p<0.001) when compared between non- RSA and control group, as shown in table (2).

Table (2): Comparison between IFN-γ/IL-10, IL-6/IL-10 ratio and three groups by used different techniques (ANOVA test analysis).

Variable	Group	n=73	Mean ± SE	F test P value	Sig. between groups
IFNγ/IL-10	RSA	39	3.7 ±1.8	<0.01	RSA- Non. RSA**
	Non-RSA	20	1.5±0.3		** RSA- Con
	Con	14	0.7± 0.04		Non. RSA- Con**
IFNγ/IL-6	RSA	39	3.2±0.3	<0.01	RSA- Non. RSA**
	Non-RSA	20	0.8±0.05		RSA- Con**
	Con	14	0.3±0.02		Non. RSA- Con**

*=significant difference (p<0.05);
**= highly significant difference (p<0.01)

The mean percentage of IL-6 in trophblastes tissues has been shown to increase significantly throughout pregnancy, in contrast, the mean percentage of IFNγ decreased significantly during pregnancy.

But the IL-10 increased significantly at 30-40 weeks (Table 3).

Table (3): Comparison between the expression of mRNA of (IL-6; IL-10 and IFN-γ) in trophoblasts of women in first, second trimester abortion and control groups.

Variable	weeks	n=73	Mean ± SE	F test *p* value	
IL-6	10-20	31	18.8±1.1	>0.05	0.001
	20-30	25	34.1±9.1		
	30-40	17	68.3±4.1		
IL-10	10-20	31	33.4±2.7	<0.01	0.001**
	20-30	25	39.4±1.7		
	30-40	17	85.2±1.3		
IFN-γ	10-20	31	75.3±2.2	<0.01	0.001**
	20-30	25	60.8±7.8		
	30-40	17	30.8±3.1		

** =highly significance difference (p<0.001)

Discussion:

The concentration of IL-6 in tissue increase progressively up to the term of pregnancy, however IL-6 appears to increase more from the middle of pregnancy. High level of IL-6 has been shown in the placenta, amniotic cells and deciduas of pregnancies complicated by preterm rupture of the membranes, intrauterine infection and prematurity (18). Also IL-6 may have an anti-infammatory action, thus it can be induce the secretion of hCG by trophoblasts, leading to an increase in the production of progesterone which activate the release of Th2 cytokines such as IL-4, and to suppress Th1 cytokines (19).

The level of IL-10 significantly higher with successful pregnancy in this study, could be explained by previous study that showed that IL-10 production was significantly lower in patients with recurrent miscarriage as compared with normal pregnancy or spontaneous abortion cases (20). Interleukin - 10 plays a positive role in the prevention of spontaneous pregnancy failure in a mouse model; the injection of IL-10 into abortion-prone mice resulted in the prevention of fetal wastage (21). Interleukin - 10 has emerged as an important Th2-type cytokine in the maintenance of normal pregnancy. Since it is directly involved in down-regulating Th1-type activity by inhibiting IFN-γ production, IL-10 has been proposed to play an important immunoregulatory role in pregnancy by maintaining a bias away from the detrimental Th1-type of reactivity (22).

The ratio of IFN-γ/IL-10 in situ expression in women with RSA which was significantly higher in trophoblasts tissue than that of successful pregnancy (control).

The pro-inflammatory cytokine, IFN-γ was targeted as a reflective for type 1 immune response, because of its Th1 polarizing effect due to its potential role in generating Th1 cells, mediating their effects functions and regulating Thl/Th2 balance (23). On the other hand, IL-6 and 1L-10 were targeted as a reflective for Type 2 immune response because it is an important anti-inflammatory cytokine contributing to the outcome of pregnancy due to its important modulatory effects against the pro-inflammatory cytokines (22, 24).

The increased level of IFN-γ at the beginning pregnancy may be related to its active role in the process of implantation. IFN-γ act on the proliferation of cytotrophoblasts (25) and can be secreted by syncytiotrophoblasts and have an autocrine effect on these cells. Also induce the production of cytokines, growth factors and hormones such as human chorionic gonadotropin (hCG), which stimulate the corpus luteum and the production of steroid hormones (e.g. estrogen and progesterone. This production will be ensured by the embryo after the first trimester of pregnancy (26).

The IFN-γ appear to be down- regulated from the second to third trimester, probably in order to control apoptosis of endothelial cells of the spiral arteries (25). And immune reactions against the fetus (27, 28, 29). High concentration of IFN-γ -appear to be involved in miscarriage by inducing immune reaction against fetus in pregnant women (28).

IFN-γ/IL-10 ratio with RSA was significant higher than that non-RSA. This results might be indicating that IFN-γ the cause of RSA and the expression of IFN-γ in this group of women was significantly lower than that of the RSA group, indicating that other factors might play a role in the development of miscarriage; activated macrophages were shown to be present in the decidua of some spontaneous abortion cases (30) indicating that in human decidua, macrophages might be activated and produce TNF-α (31) that could be the cause of abortion in these women. Also we suggest that, this was the case in the first abortion in women with RSA, furthermore, this macrophage-derived TNF-α stimulates NK cells to produce IFN-γ which further activates the macrophages, as occurs in the early defense response to infectious agents (32) this produced IFN-γ will further increase Thl polarization in the subsequent miscarriages, as the Thl polarization appears to increase with the chronicity of the immune response due to the presence of T memory cells (33).

The ratio of IFN-γ/IL-6 showed that there is a switch of Th1/Th2 around the middle of pregnancy. The role of Th1 cytokines during the placental implantation phase and up to the first half of pregnancy, and a role of Th2 cytokines from the middle to the end of pregnancy. It is possible that Th1might decrease

with the progression of pregnancy to avoid immune reaction to the fetus, and in fact Th2 take over to prevent Th1 cytokine release (34).

In conclusion IFN-γ expression is increased in women with RSA and non-RSA compared with successful pregnancy, indicating that Thl cytokines might well be implicated in adversely affecting pregnancy. The ratio of IFN-γ: IL-10 was found to be highly in aborted women indicating that there is a shift in Thl: Th2 ratio which might play a role in pregnancy failure.

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دراسة معدلات كل من الزنك، الحديد، السيلينوم، والنحاس في مصول سرطان المثانة

د.وسن عبد الاله باقر*، زينب سعد عبد الغني *، اسيل فايق*

*المركز العراقي لبحوث السرطان والوراثة الطبية، الجامعة المستنصرية

الخلاصة:

ثلاث وسبعون امرأة، تم تقسيمهن إلى ثلاثة مجاميع: مجموعة إجهاض تلقائي متكرّر (RSA) وعددهن 39 امرأة ومجموعة - إجهاض تلقائي غير متكرّر (non-RSA) وعددهن 20 امرأة و المجموعة الثالثة هي مجموعة سيطرة (حمل ناجح) وعددهن 14 امرأة حيث تم جمع نماذج من النسيجِ المغذي للجنين (التروفوبلاست) من كل المرضى وكذلك مجموعه السيطرة.

أن مستويات كلا من IL-6, IFN-γ, IL-10 تم التحقق منها باختبار التهجين الموضعي والكشف عن المجسات المهجنة (ISH) .

أظهرت النتائج وجود زيادة معنوية في IL-6 في مجموعة الإجهاض المتكرر التلقائي وIL-10 يزداد زيادة معنوية في مجموعة السيطرة مقارنة بمجموعتي RSAو non- RSA وهناك زيادة معنوية في IFN γ- في مجموعة الإجهاض المتكرر التلقائي وغير المتكرر مقارنة بمجموعة السيطرة وهذا يؤكد دورهم في حدوث الإجهاض مقارنة ب IL-10 الذي يعتبر الأساس لحمل ناجح. إن ال IFN -γ يقل بزيادة أشهر الحمل بينما ظهور IL-6وIL-10 يزداد عند زيادة أشهر الحمل.

إن ال IFN -γ و IL-6وIL-10 يعتبرون كمؤشر على Th1/Th2 خلال الحمل. النقص في IL-10 في حالة الإجهاض المتكرر التلقائي والإجهاض الغير متكرر يعني نقص في المناعة وإنتاج السايتوكاين .