

# Detection of IL-8, IL-10 and IFN- $\gamma$ mRNA in trophoblast tissues of recurrent spontaneous abortion using *in situ* hybridization

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## **Abstract**

**Background:** Th1-type cytokines secretion such as IFN- $\gamma$ , and Th2 cytokines such as IL-10, have been shown to exert deleterious effects on pregnancy, inhibiting fetal growth and development.

**Aim:** Measurement of the locally concentrations of selected Th1 and Th2 cytokines in women with a history of recurrent spontaneous abortion (RSA) at the time of abortion using *in situ* hybridization technique.

**Methods:** A total of one hundred and nineteen women, ranged from the mean age (23.9 – 28.5)years, were enrolled in the current study and were further classified into three categories: Group A- Recurrent spontaneous abortion (RSA): n= 62 women, with a mean age of (28.5 + 0.68);Group B- non- recurrent spontaneous abortion (non-RSA): n= 34 women, with a mean age of (26.4  $\pm$  0.85)and group C- Control (successful pregnancy): n= 23 women, with a mean age of (23.9  $\pm$  0.88). From each patient and control, placental tissues were collected. Trophoblasts tissues (an image for the local microenvironment) were screened to determine their *in situ* levels of IL-10 and IFN- $\gamma$  based on cDNA probes (for *in-situ* hybridization, ISH).

**Results:** There was a significant increase in the level of IL-10 within trophoblast tissues biopsies exclusively from women with successful

pregnancies (group C) ( $p < 0.001$ ). On the other hand, IFN- $\gamma$  was found predominantly expressed in trophoblast tissue biopsies of patients with RSA whether IHC or ISH were conducted ( $p < 0.05$ ). Accordingly, only trophoblast tissues biopsies from patients with RSA revealed a significant increase in the ratio of IFN- $\gamma$ /IL-10 levels expressed as determined by *in situ* hybridization in comparison to the same ration calculated from trophoblasts tissues of women with successful pregnancies (group C) ( $p < 0.001$ ). as marker for Th2 immune response, during successful pregnancies. Furthermore, the current study failed to demonstrate a significant difference in the tissue levels of IL-8 between RSA and control group ( $p > 0.05$ ) and no significant different between non-RSA and control ( $p > 0.05$ ), (always  $p < 0.05$ ).

**Conclusion:** These outcomes may further support the possible exisance of an immune response that orchestrates abortive phenomena and the possible protective role of IL-10.

**Keywords:** Recurrent spontaneous abortion (RSA), *in-situ* hybridization, ISH, cytokine,IL-8,IL-10,IFN  $\gamma$ .

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## **Introduction**

Recurrent spontaneous abortion is one of the important complications in pregnancy, its incidence is 0.5–1%, and the etiology of RSA is varied, and includes maternal or paternal chromosomal aberrations, uterine anatomic abnormalities, endocrine disorders, infections, and reproductive autoimmune defects.

However, the etiology is undetermined in 40–60% of women with recurrent abortion<sup>(1, 2)</sup>.

Successful 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<sup>(3, 4)</sup>. 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)1cells, which produce interleukin (IL)-2, interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\beta$ , and the up- regulation of Th2 and Tc2 cells,

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which produce IL-4, IL-6, IL-10 and IL-13<sup>(5-8)</sup>.

Previous investigations of Th1/Th2 immune responses during pregnancy were able to show that a distinct shift towards Th2-type reactions occurs, especially at the feto-maternal interface<sup>(9-12)</sup>.

On the other hand, Th1-type cytokine secretion such as IFN- $\gamma$  and Th1/Th2 ratio has been shown to exert deleterious effects on pregnancy, inhibiting fetal growth and development<sup>(13)</sup>.

In addition, IL-8 may be indirectly stimulated via endotoxin-induced inflammatory cytokine, such as IFN- $\gamma$ , TNF- $\alpha$  and IL-1- $\alpha$ , these cytokines are known to up regulate IL-8 expression in hemopoietic cells<sup>(14)</sup>. IL-8 displays both inflamm-atory and growth -regulating properties<sup>(15)</sup>, but is notable for its selective chemotaxis, degranulation, and activation of neutrophils<sup>(16)</sup>. During pregnancy IL-8 induced activation of neutrophils, collagenase and elastase activity in intrauterine environment has been imp-licated in mechanisms of rupture of fetal membranes<sup>(17)</sup> and cervical ripening<sup>(18, 19)</sup>.

#### **Subjects, materials and methods**

One hundred and nineteen women attending the Obstetrics and Gynecology department of Al-Kadhimiya Teaching Hospital in Baghdad between December 2004 and August 2005 were the subject of this study. Included recurrent spontaneous abortion (RSA); non-RSA (first and second abortion) and successful pregnancy (full term) as a control groups.

The gestational age was calculated for each patient from data of the last menstrual period.

These one hundred and nineteen women were grouped into three groups:

**Group A:** the study group included 62 pregnant ladies all of whom gave a history of previous 3-6 consecutive

abortions. History was taken from the patients taking into consideration their hospital records in addition to their previous medical reports (all of them had no family history of genetic disease).

**Group B:** included 34 pregnant ladies with incomplete abortion for the first time or second time.

**Group C:** included 23 pregnant ladies had at least two previous normal pregnancies taken as comparison group. All this was done under the supervision of a senior gynecologist.

Trophoblastic tissue was collected from the evacuation of retained pieces during the procedure of curettage and placed in 10% formaldehyde. 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 trophoblastic tissue were included in this study). These cases were subjected for in situ hybridization protocols with the different markers included in this study.

In situ hybridization (ISH) using DNA Probe Hybridization/Detection System In situ kit (Maxim Biotech, Inc., USA). For the detection of IL-8, IFN- $\gamma$  and IL-10, the biotinylated DNA probe hybridize to the target sequence (IL-8 or IFN- $\gamma$  or IL-10 mRNA sequence), then a streptavidin-AP (streptavidin-alkaline phosphatase) conjugate is applied followed by addition of the substrate bromo-chloro-indolyl-phosphate/nitro-blue tetrazolium (BCIP/NBT), which yields an intense blue-black signal appears at the specific site of the hybridized probe. This directly streptavidin-AP conjugate linked to the biotinylated probe provides a rapid and highly sensitive detection method. The procedure included the following steps: Paraffin embedded sections were cut into 5  $\mu$ m thickness, placed on

Fisherbrand positively charged slides and left overnight to dry at room temperature. In each ISH run negative control slides were included which were obtained by omitting the probe and using hybridization solution only, this was undertaken under identical test conditions (on the same slide). Another negative control slides were obtained by using RNase pretreatment, which abolished the hybridization signals, this was performed by placing 1-2 drops of RNase A onto the tissue sections and incubating the slides at 37°C for 2hr according to <sup>(20,21)</sup>. Poor tissue quality or target RNA degradation may give false negative results or poor signal. This could be verified by using a probe to an abundant RNA target like the probe of a housekeeping gene which is a sequence or gene product that is constitutively expressed in most tissue types such as actin or tubulin. In each ISH run the biotinylated housekeeping gene probe in a dilution of 1:3 by the hybridization solution, was used. reactive lymphocytes within the tissue were considered as internal positive control for IFN- $\gamma$  detection . Placental tissue obtained from the women who had had elective pregnancy termination, was considered as a positive control tissue for IL-10 detection using ISH. Placental tissue obtained from the women with normal vaginal delivery, was considered as a positive control tissue for IL-8 detection using ISH . The expression of IL-8, IFN- $\gamma$  and IL-10 mRNA was measured by the same scoring system, by counting the number of positive trophoblastic cells, which gave a blue-black (BCIP/NBT) nuclear staining under the light microscope, counting of positive cells was done with the assistance of histopathologist. The

number of villi cells that were positively and negatively stained was expressed as a ratio. The extent of the ISH signal in the villi was determined in 10 fields (X100 magnification). In each field the total number of villi were counted and the extent of nuclear staining of the cytotrophoblast and syncytiotrophoblast in a given villous was graded as 3, (75-100%); 2, (25-75%); or 1, (<25%). The total staining score was divided by the number of whole villi per field in 10 fields. These scores (between 1 and 3) were added for each field, and a score between 10 and 30 was gained for each sample. The scorer was blinded to the clinical diagnosis of the tissues at the time of assessment, and tissues were independently assessed by two observers, 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.

### **Results**

In (Table1), Chi-square test of significant was conducted to examine the association between IL-8 ;IL-10 and IFN- $\gamma$  mRNA expression in trophoblasts tissue in the three groups of investigated women ,it was found that highly significant association ( $p<0.001$ ) between them in the three scoring levels. The results showed that percentages of IL-8 and IFN- $\gamma$  were elevated in 94.3% (33/35) and 82.9%(29/35) ,respectively in women with RSA (group A). It was found that 90% (9/10) of women in control group showed highest level of IL-10 and 62.9%(22/35) of women with RSA have moderated level (score 2).

**Table 1: Comparison of the prevalence of IL-8, IL-10 and IFN-γ mRNA (ISH assay) in trophoblasts depend on the scoring level.**

	score <sup>a</sup>	Groups			Total (n=61)	Chi-Square P value
		A(n=35) No (%)	B(n=16) No (%)	C(n=10) No (%)		
IL-8	2	2 (5.7)	16 (100)	5 (50)	23	0.000**
	3	33 (94.3)	0	5 (50)	38	
IL-10	1	13 (37.1)	0	0	13	0.000**
	2	22 (62.9)	16 (100)	1 (10)	39	
	3	0	0	9 (90)	9	
IFN-γ	1	0	0	5 (50)	5	0.000**
	2	6 (17.1)	16 (100)	5 (50)	27	
	3	29 (82.9)	0	0	29	

Score<sup>a</sup>: 1<25%; 2(25-74) %; 3(75-100) %

ANOVA test analysis in table (2) showed that the expression of IL-10 by trophoblasts tissue ,was significantly higher in successful pregnancy (group C) than in recurrent spontaneous abortion (group A) (p<0.001) the mean ± SE (88.5±2.5), mean versus (25.5±0.9); whereas (group A) showed a high value with a highly significant difference (p>0.001)of IFN-γ in

comparison with control (group C), the mean ± SE (84.3±1.7; 25.7±1.9 ,respectively). In women with RSA group it was found that IL-8 was highly significant different from that in non-RSA groups (85.2±1.3 versus 63.0±2.1) and there was no significant difference (p>0.05) between RSA group's patients and controls group (67.3±7.5).

**Table 2: Comparison between the mean percent of the expression of IL-8, IL-10 and IFN-γ (ISH assay) in the trophoblasts of studied groups.**

Variable	Group	n=61	Mean± SE	F test p value	Sig. between groups	
					groups	P value
IL-8	A	35	85.2 ± 1.3	<0.01	A –B	0.000**
	B	16	63.0 ±2.1		A –C	0.122
	C	10	67.3 ±7.5		B – C	0.933
IL-10	A	35	25.5 ±0.9	<0.01	A –B	0.000**
	B	16	51.9 ±2.1		A –C	0.000**
	C	10	88.5 ±2.5		B – C	0.000**
IFN-γ	A	35	84.3 ±1.7	<0.01	A –B	0.000**
	B	16	52.1±2.03		A –C	0.000**
	C	10	±1.970.7		B – C	0.000**

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

**Discussion**

In the current study, we evaluated the expression of IL-10, IFN-γ and IL-8 in human placental tissues (trophoblastic tissue), from all three

groups: RSA, non-RSA and Control (successful pregnancy).

Much of the work on spontaneous abortions in humans has focused on the

analyses of maternal responses and local changes that occur following abortion. Evidence from studies on murine and human pregnancy points to a strong association between maternal Th2-type (IL-4, IL-6, IL-10) immunity and successful pregnancy on the one hand and between Th1-type (IL-2 and IFN- $\gamma$ ) immune reactivity and pregnancy loss on the other<sup>(22)</sup>. Moreover, during pregnancy, IL-8 is produced by a variety of cells, mainly monocytes/ macrophages<sup>(23)</sup>. IL-8 induced activation of neutrophils and elastase activity in the intrauterine environment has been implicated in the mechanisms of rupture of fetal membrane and cervical ripening<sup>(24)</sup>.

The pro-inflammatory cytokine, IFN- $\gamma$  was targeted as a reflective for type 1 immune response in this study, because of its Th1 polarizing effect due to its potential role in generating Th1 cells, mediating their effects functions and regulating Th1/Th2 balance<sup>(25)</sup>. On the other hand, IL-10 was targeted in this study 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<sup>(26-28)</sup>.

The current study demonstrated that 29/35 (82.9%) of the cases in RSA (group A) showed high level of IFN- $\gamma$  *in situ* expression, with a highly significant difference ( $p < 0.001$ ) from those with non-RSA (group B) in whom the expression of IFN- $\gamma$  was in moderated level, 16/16 (100%) of cases and from those with successful pregnancy (group C) the expression of IFN- $\gamma$  was 5/10 (50%) in moderated level and 5/10 (50%) in lowest level, no cases found to express high level. A part from the causes of this significant increase in the expression of IFN- $\gamma$  in women with recurrent abortion, revision was made for the previous

studies that examined the association between Th1 type cytokines and recurrent abortion. First studies in Hill's laboratory<sup>(29)</sup> had shown that peripheral blood mononuclear cells (PBMC) of women with a history of RSA when stimulated with a trophoblast antigen extract produced significantly higher concentrations of the Th1 cytokines, IFN- $\gamma$  and TNF- $\alpha$ , as compared with normal pregnancy<sup>(30,31)</sup>. This results in agreement with the similar local study that showed, highly significantly increased ( $p < 0.001$ ) expression of IFN $\gamma$  in women with RSA compared with first abortion or elective pregnancy termination<sup>(32)</sup> and when compared with successful pregnancy<sup>(33,34)</sup> because of that IFN- $\gamma$ , have been hypothesized to play a role in pathogenesis of recurrent abortion.

Highly significantly increase ( $p < 0.001$ ) *in situ* expression of IL-10 was found in women with successful pregnancy (group C). It was found that 9/10 (90%) of women in groups C in highest level of IL-10 compared with RSA (group A) which was no cases found in high level but 22/35 (62.9%) was in moderate level and was found 16/16 (100%) of women with non - RSA was in moderate level. This result is consistence with similar local study by that showed, highly significantly increased ( $p < 0.001$ ) expression of IL-10 in women with first abortion or elective pregnancy termination compared with RSA<sup>(32)</sup>; This significantly lower IL-10 expression could be attributed to defect in Th2 and Tc2 cells at the feto-maternal interface or to the accumulation failure of Th2 cells at the implantation site in women with RSA<sup>(35)</sup>. Several lines of evidence suggest that IL-10 may play a major role in influencing the activity of the placental trophoblast, which has been proposed as a key cell type in regulating the fetal immunoprotection<sup>(36-38)</sup>.

Since it is directly involved in down-regulating Th1-type activity by inhibiting IFN- $\gamma$  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<sup>(39, 28)</sup>.

There are many confounding studies held the notion on the balance of Th1 and Th2 cells at the circulation and implantation site, expressing them as a ratio of Th1/Th2 cytokines, so that, another dimension was added to the results of this study when it examined the ratio of IFN- $\gamma$ /IL-10 in situ expression in women with RSA which was significantly higher ( $p < 0.001$ ) in trophoblasts tissue, and about (19.1 times) than that of successful pregnancy (group C). This significantly high IFN- $\gamma$ /IL-10 ratio lends further support to the findings in this study as it was in consistency with the previous studies (41-34).

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