EARLY PREDCTION OF UNEXPLAINED RECURRENT PREGNANCY LOSS: COMPARATIVE STUDY BETWEEN TNF-∞ VERSUS IFN- γ

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ABSTRACT:

Objective: The objective of this study was to compare the predictive value of TNF-∞ versus IFN-γ to predict the cases of unexplained recurrent pregnancy loss in early pregnancy (6-8 weeks of gestations).

Study design: A total of 120 patients included in this study had a systematic investigations for causes of recurrent pregnancy loss, included, chromosomal karyotyping, hysterosalpigogram (HSG), endometrial biopsy, thyroid function tests, coagulation profiles, hormonal assay and pelvic ultrasound. A total of 10 ml venous blood was withdrawn from all participated patients, serum was separated and kept in the freezer at -80°C until assayed. TNF- ∞ and IFN- γ were measured in duplicate, using Enzyme Linked Immunosorbent Assay (ELISA).

Results: 98 out of 218 pregnant patients (45%) were excluded from this study due to presence of known causes of recurrent pregnancy loss. There was no statistically significant differences regarding age, body weight (BMI) and number of previous abortions between both groups. The serum TNF- ∞ levels of ≤ 200 pg/ml were found in 89 pregnant patients (74.2%), 75 of them (84.3%) had pregnancy loss during first and second trimesters of pregnancy with (sensitivity of 86.4%, specificity of 94.6%, PPV of 81%, NPV of 96%, and diagnostic accuracy of 92%). On the other hand, the serum TNF- ∞ levels of ≥ 600 pg/ml were found in 31 pregnant patients (25.8 %), 4 of them (12.9%) had pregnancy loss during first and second trimesters of pregnancy with less sensitivity, specificity, PPV, NPV, and less diagnostic accuracy to predict the pregnancy outcomes in the first and second trimester of pregnancy. Meanwhile, the serum IFN- γ levels of ≤ 0.1 ng/ml were found in 58 pregnant patients (48.3 %), 6 of them (10.3%) had pregnancy loss during first and second trimesters of pregnancy with sensitivity of 31.8%, specificity of 38.3%, PPV of 21.8%, NPV of 27.6%, and diagnostic accuracy of 9%. While, the serum IFN – γ levels of ≥ 1.8 ng/ml were found in 62 pregnant patients (51.7%), 3 of them (4.8%) had pregnancy loss during first and second trimesters of pregnancy with less sensitivity, specificity, PPV, NPV, and less diagnostic accuracy to predict the pregnancy outcomes in the first and second trimester of pregnancy.

Conclusion: In conclusion, plasma level of IFN-γ marker seems to be the less predictor of the early prediction of pregnancy loss. In contrast, TNF- ∞ at (cut- off ≤ 200 pg/ml) was found to be the most sensitive marker to predict early pregnancy loss in patients with a history of unexplained recurrent pregnancy loss in the first 6-8 weeks of gestation with high diagnostic accuracy of 92%.

KEYWORDS:

Unexplained recurrent pregnancy loss — Early prediction
TNF-∞ — IFN- γ

INTRODUCTION:

Recurrent spontaneous pregnancy loss defined as a loss of three or more consecutive pregnancies, affects 1-3% of pregnant women (Clifford et al., 1997). Transvaginal ultrasound scan, ß-hCG estimation (Al-Azemi et al., 2003), evaluation of CA-125 level has been reported to have a predictive role for pregnancy outcomes in patients with recurrent miscarriage (Predanie, 2000). Miscarriage was initially believed to happen exclusively due to abnormal villous development (Bouie et al., 1976). However, recent studies reported that, early pregnancy failure is a disorder of the placenta, and that the villous changes described in previous studies are not the cause, but rather the conse-quences of miscarriage (Jauniaux & Burton, 2005). Defective placen-tation is mainly characterized by a thinner and fragmented trophoblast shell, reduced cytotrophoblat invasion of the endometrium and incomplete plugging of the lumen at the tips of the spiral arteries and these changes occurred in two third of early pregnancy loss (Hustin, 1990 & Jauniaux et al., 1994). These changes lead to absence of physio-logical changes in most spiral arteries, resulting in premature onset of the maternal circulation through-out the entire placenta and early fetal demise (Jauniaux et al., 2006). It has been reported that, there are primary and secondary causes of oxidative stress leading to early pregnancy failure (Hempstock et al., 2003). The primary causes are mainly chromosomal abnormalities in almost 50% of cases (Greenwold et al., 2003). Secondary causes involve immunelogical factors including cytokines such as tumor necrosis factor-alpha (TNF-∞) and interferon gamma (INF- γ). However, so far their role in trophoblast- decidual interaction in normal and abnormal first trimester remain unclear (Jauniaux et al., 2006). Tumor necrosis factor- ∞ (TNF-∞) and interferon-gamma (IFN-γ) are pro-inflammatory cytokines exerting deleterious effects on pregnancy. Activation of neutrophils and overproduction of cytokines by the placenta in response to local ischemia are most likely to play an important role in the development of endothelial change, associated with pre-eclampsia and fetal demise (Vural et al., 2010).

Previous studies have shown that the circulating levels of TNF- ∞ and IFN- γ are higher in patients with a subsequent miscarriage compared with those with normal term pregnancy, suggesting that these cytokines may be potentially important factors in cases of recurrent pregnancy loss (Makhseed et al., 2000 & Baxter et al., 2001). However little is known about the role of TNF-∞ and INF- γ in patients with unexplained recurrent pregnancy loss.

Therefore, the objective of this study was to compare the predictive value of TNF-∞ versus IFN-γ to predict the cases of unexplained recurrent pregnancy loss in early pregnancy (6-8 weeks of gestations).

SUBJECTS AND METHODS:

A total of 218 patients in early pregnancy (6-8 weeks of gestation) as diagnosed by ultrasound with positive fetal heart activities, regular gestational and yolk sac were recruited from the patients attending the high risk clinic of the department of Obstetrics and Gynecology at EL-Minia Maternity and Children University Hospital from April, 2009 to

December, 2011. All patients had a history of at least three previous consecutive pregnancy loss. All patients gave a verbal consent before they are participated in this study. Patients with a history of well known causes of recurrent pregnancy loss (n = 98) were excluded from this study.

All patients included in this study had a systematic investigations for causes of recurrent pregnancy loss, included, chromosomal karyotyping, hysterosalpigogram (HSG), endometrial biopsy, thyroid function tests, coagulation profiles, hormonal assay and pelvic ultrasound. A total of 10ml venous blood was withdrawn from all participated patients. Serum was separated and kept in the freezer at -80°C until assayed. TNF- ∞ and IFN-γ were measured in duplicate, using Enzyme Linked Immunosorbent Assay (ELISA), using commercial kits according to the manufacture's instructions (Genzyme, Cambridge, MA, USA). Absorbance was measured at wave lengths of 450 nm and 550 nm using a Titertek Multiskan Plus plate reader. A standard curve was constructed for each cytokine. The limit of detection of these assays was 12.7 pg/ml for TNF- ∞ and 10.3 pg/ml for IFN- γ.

STATISTICAL ANALYSIS:

Statistics were calculated using Arcus Soft Ware (Cambridge, UK). The most of the data were found to be non-normally distributed and hence the non-parametric Mann-Whitney U-test was applied to compare differences between studied groups. Student t-test was used to determine the significance. Value of $p < 0.05$ was considered significance. Sensitivity, specificity, positive and negative predictive values with 95% confidence intervals (CIs), and diagnostic accuracy were also calculated.

RESULTS:

Our results have shown that, 98 out of 218 pregnant patients (45%) were excluded from this study due to presence of known causes of recurrent pregnancy loss. 28 patients (28.6%) due to chromosomal abnormalities, 23 patients (23.4%) due to antiphospholipid syndrome, 21 patients (21.4%) due to uterine anomalies, 14 patients (14.3%) due to coagulation defects, 9 patients (9.2%) due to thyroid disorders, and 3 patients (3.1%) due to infections. 79 pregnant patients (66%) had pregnancy loss during first and second trimester of pregnancy, and 41 pregnant patients (34%) have continued their pregnancies, 5 patients (12.2%) had preterm labour, and the other 36 pregnant patients (87.8%) were delivered healthy normal babies at full term.

Data of maternal, obstetrics and other patient's characteristics in patients with recurrent pregnancy loss and control groups are shown in table 1. There was no statistically significant differences regarding age, body weight (BMI) and number of previous abortions between both groups. The serum TNF- ∞ levels of ≤ 200 pg/ml were found in 89 pregnant patients (74.2%), 75 of them (84.3%) had pregnancy loss during first and second trimesters of pregnancy with (sensitivity of 86.4%, specificity of 94.6%, PPV of 81%, NPV of 96%, and diagnostic accuracy of 92%) as shown in table 2. On the other hand, the serum TNF- ∞ levels of ≥ 600 pg/ml were found in 31 pregnant patients (25.8%), 4 of them (12.9%) had pregnancy loss during first and second trimesters of pregnancy with less sensitivity,

specificity, PPV, NPV, and less diagnostic accuracy to predict the pregnancy outcomes in the first and second trimester of pregnancy (sensitivity of 21.4%, specificity of 24.9%, PPV of 5.2%, NPV of 17.4%, and diagnostic accuracy of 13%) as shown in table2.

Meanwhile, the serum IFN- γ levels of ≤ 0.1 ng/ml were found in 58 pregnant patients (48.3%), 6 of them (10.3%) had pregnancy loss during first and second trimesters of pregnancy with sensitivity of 31.8%, specificity of 38.3%, PPV of 21.8%, NPV of 27.6%, and diagnostic accuracy of 9% as shown in table 2. While, the serum IFN – γ levels of ≥ 1.8 ng/ml were found in 62 pregnant patients (51.7%), 3 of them (4.8%) had pregnancy loss during first and second trimesters of pregnancy with less sensitivity, specificity, PPV, NPV, and less diagnostic accuracy to predict the pregnancy outcomes in the first and second trimester of pregnancy (sensitivity of 22.3%, specificity of 23.1%, PPV of 11.8%, NPV of 16.4%, and diagnostic accuracy of 5%) as shown in table 2.

Table (1): Patient's characteristics in recurrent pregnancy loss and control groups.

Parameters	Group (1) Recurrent pregnancy loss (n = 79)	Group (2) Control (n = 41)	P – Value
Age (year) (Mean ± SD)	32.6 ± 4.7	34.2 ± 5.3	0.56*
Weight (BMI) (Mean ± SD)	33.8 ± 5.1	32.4 ± 4.9	0.63*
Number of previous abortion (Mean ± SD)	5.2 ± 1.9	4.8 ± 3.2	0.49*

* = means not significant.

Table (2): Serum cut-off points for TNF-∞ and IFN- γ and their sensitivity, specificity, positive, negative predictive values and diagnostic accuracy for predicting the pregnancy loss at (6-8 weeks of gestation) in patients with a history of recurrent unexplained pregnancy loss.

Parameters	# of patients (%)	# of pregnancy loss (%)	Sensitiv. (%) (95% CIs)	Spec. (%) (95%CIs)	PPV(%) (95%CIs)	NPP(%) (95%CIs)	DA (%)
TNF- ∞ ≤ 200 pg/ml	89/120 (74.2%)	75/89 (84.3 %)	86.4 % (59.2-91.6)	94.6 % (82.6-96.3)	81 % (67-86.7)	96 % (87.2-98.6)	92 %
≥ 600 pg/ml	31/120 (25.8%)	4/31 (12.9 %)	21.4 % (11.6-32.8)	24.9% (15.7-33.8)	5.2 % (3.1-13.4)	17.4 % (11.9-27.6)	13 %
IFN- γ ≤ 0.1 ng/ml	58/120 (48.3%)	6/58 (10.3 %)	31.8 % (18.6-41.5)	38.3 % (17.2-36.5)	21.8 % (13.2-26.5)	27.6 % (18.1-35.8)	9 %
≥ 1.8 ng/ml	62/120 (51.7%)	3/62 (4.8%)	22.3 % (11.9-34.2)	23.1 % (15.1-31.7)	11.8 % (9.8-21.6)	16.4 % (14.8-31.2)	5 %

Sensitiv = Sensitivity Spec = Specificity PPV = Positive predictive value
NPP = Negative predictive value DA = Diagnostic accuracy

DISCUSSION:

Recurrent pregnancy loss (RPL) is a pleiotrophic condition with several un-identified causes until now. However, different cytokines production are account as an important contributing factor (Calleja-Agias et al., 2011). This study has shown that, TNF-∞ was significantly decreased in patients who will went into pregnancy loss when it measured early in pregnancy (6-8 weeks of gestation) than in patients with the same obstetric history but they completed their pregnancy successfully. In addition this study demonstrates also that, when the serum level of TNF-∞ of ≤ 200 pg/ml (cut-off point) will carry the highest prediction value of pregnancy loss during first and second trimester of pregnancy, particularly in cases of un-explained recurrent pregnancy loss with high sensitivity, specificity, and high diagnostic accuracy up to 92%. It has been reported that, higher plasma levels of TNF-∞ in pregnant women compared with non-pregnant, and in cases of miscarriage with normal karyotype, its levels were higher than control (Calleja- Agius et al., 2012).

The previous findings are in contrast with our results, which revealed a very low levels of TNF-∞ in plasma of patients that had pregnancy loss during first and second trimester of pregnancy than control group. However our results are in consistent with other study which found that plasma levels of TNF-∞ was higher in patients with recurrent pregnancy loss than control (Bates et al., 2002). But the difference between our study and the previous studies in that, in our study we have used TNF-∞ level as an early predictor marker for detecting the subsequent pregnancy loss, while the other studies measured TNF-∞ levels in patients already with missed or recent pregnancy loss in comparison with control.

Moreover, it has been reported that, the pattern of cytokine production varies throughout pregnancy (Tranchot-Diallo et al., 1997). Therefore, we have to take such variations into consideration when comparing our results with other studies where the samples were taken at different trimesters of pregnancy. TNF-∞ is an important immunoregulatory cytokine, which may be produced in T-helper 1, or T-helper 2- type responses and is well known to have different effects depending on gestational age.

Moreover, it has been mentioned that TNF-∞ stimulates the programmed cells death of human primary villous trophoblast (Yui et al., 1994). Our results also have shown that, the plasma levels of TNF-∞ of ≥ 600 pg/ml in patients with a history of recurrent miscarriage has no predictive role for prediction of pregnancy loss later in pregnancy with less sensitivity, specificity, with diagnostic accuracy not exceeded 13%. It has been shown that, TNF-∞ production is suppressed at the mRNA level during early pregnancy and a significant increase does not occur until the eight month of pregnancy (Tranchot-Diallo et al., 1997). TNF-∞ production in late pregnancy is implicated in the induction of labor (Vince et al., 1992). Moreover placental production of soluble receptors has been shown to modulate TNF- ∞ function in pregnancy (Austgulen et al., 1992).

It has been reported that, TNF- ∞ production was decreased in normal first trimester samples compared with non-pregnant control, although this did not reach statistical significance. However, in women with recurrent pregnancy loss, this decrease was accentuated. Also no differences were observed between the level of TNF-∞ in those recurrent pregnancy loss patients who successfully completed their pregnancy compared with those who went on to miscarry (Bates et al., 2002). Our results regarding IFN-γ are in consistent with the previous study, where the level of IFN-γ has shown no any predictive value to predict the occurrence of pregnancy loss in patients with a history of unexplained recurrent pregnancy loss. However, other study has mentioned that the plasma level of IFN- γ was higher in patients with pregnancy loss than control normal pregnancy (Marzi et al., 1996).

In conclusion, plasma level of IFN- γ marker seem to be the less predictor of the early prediction of pregnancy loss. In contrast, TNF- ∞ was found to be the most sensitive marker to predict early the pregnancy loss in patients with a history of unexplained recurrent pregnancy loss in the first 6-8 weeks of gestation with high diagnostic accuracy of 92%. However, we need a further big controlled randomized studies to evaluate the role of theses markers in the early prediction of pregnancy loss in patients with a history of unexplained recurrent pregnancy loss.

REFERENCES:

1. Al-Azemi M, Ledger WL, Diejomaoh M, Mousa M, Makhseed M, Omu A. (2003): Measurement of inhibin-A and inhibin pro-ac in early human pregnancy and their role in the prediction of pregnancy outcome in patients with recurrent pregnancy loss. Fertil. Steril. 80:1473-1479.

2. Austgulen R, Liabakk NB, Brockhaus M, Espevik T. (1992): Soluble TNF receptors in amniotic fluid and in urine from pregnant women. J Reprod. Immunol. 22:105-116.

3. Bates MD, Quenby S, Takakuwa K, Johnson PM, Vince GS. (2002): Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss. Hum. Reprod. 9: 2439-2444.

4. Baxter N, Sumiya M, Cheng S, Erlich H, Regen L, Simons AJA. (2001): Recurrent miscarriage and variant alleles lymphotoxin alpha genes. Clin. Exp. Immunol. 3:529-534.

5. Bouie J, Philippe E, Giroud A, Boue A. (1976): Phenotypic expression of lethal chromosomal anomalies in human abortuses. Teratology. 1: 3-19.

6. Calleja-Agius J, Jauniaux E, Pizzey AR, Muttukrishna S. (2012): Investigation of systemic inflammatory response in first trimester pregnancy loss failure. Hum. Reprod. 27 (2): 349-357.

7. Calleja-Agius J, Muttukrishna S, Pizzey AR, Jauniaux E. (2011): Pro- and anti-inflamm-atory cytokines in threatened miscarriage. Am. J. Obstet. Gynecol. 83.e-83.e16.

8. Clifford K, Rai R, Regan I. (1997): Future pregnancy outcome in unexplained recurrent first trimester miscarriage. Hun. Reprod. 12: 387-389.

9. Greenwold N, Jauniaux E, Gulbis B, Hempstock J, Gervy C, Burton GJ. (2003): Relationship among maternal serum endocrinology, placental karyotype, and intervillous circulation in early

pregnancy failure. Fertil. Steril. 6: 1373-1379.

10. Hempstock J, Jauniaux E, Greenwold N, Burton GJ. (2003): The contribution of placental oxidative stress to early pregnancy failure. Hum. Pathol. 12: 1265-1275.

11. Hustin J. (1990): Histological study of the materno-embryonic interface in spontaneous abortion. Placenta. 11: 477-486.

12. Jauniaux E, and Burton GJ. (2005): Pathophysiology of histological changes in early pregnancy loss. Placenta. 2-3: 114-123.

13. Jauniaux E, Poston L, Burton GJ. (2006): Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. Hum. Reprod. Update. 6: 747-755.

14. Jauniaux E, Zaidi J, Jurkovic D, Campbell S, Hustin J. (1994): Comparison of color Doppler features and pathological findings in complicated early pregnancy. Hum. Reprod. 12: 2432-2437.

15. Makhseed M, Raghupathy R, Azizieh F, Farhat R, Hussam N, Bandar A. (2000): Circulating cytokine and CD30 in normal human pregnancy and recurrent spontaneous abortions. Hum. Reprod.9: 2011-2017.

16. Marzi M, Vigono A, Trabattoni D, et al. (1996): Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. Clin. Exp. Immunol.106: 127-133.

17. Predanic M. (2000): Differentiating tubal abortion from viable ectopic pregnancy with serum CA-125 and ß-hCG determinations. Fertil. Steril. 73: 522-525.

18. Tranchot-Diallo J, Gras G, Parnet-Mathieu F, Benveniste O, Morce D, Roques P, Milliez J, Chaouat G, Dormont D. (1997): Modulations of cytokine expression in pregnant women. AM.J. Reprod. Immunol. 37: 215-216.

19. Vince GS, Shorter S, Starkey P, Humphreys J, Clover L, Wilkins T, Sargent I, Redman C. (1990): Localization of tumor necrosis factor production in cells at the maternal/fetal interface in human pregnancy. Clin. Exp. Immunol. 88: 174-180.

20. Vural P, Degirmencioglu S, Saral NY, Demirkan A, Akgul C, Yildirim G, Issever H, Erogglu H. (2010): Tumor necrosis factor-∞, interleukin-6, and interleukin-10 polymorphisms in pre-eclampsia. J.Obstet. Gynecol. 36 (1): 64-71.

21. Yui J, Garcia-Lloret M, Wegmann TG, Guillbert LJ. (1994): Cytotoxicity of tumor necrosis factor-∞ (TNF-∞) and gamma-interferon (IFN-ɣ) against primary human placental trophoblast. Placenta. 15: 819-826.

التنبؤ المبكر لحالات الإجهاض المتكرر والغير مفسر : دراسة مقارنة بين معامل النخر الورمي – الفا مقارنة بالانترفيرون – جاما

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أقسام *الباثولوجيا الإكلينيكية وأمراض النساء والتوليد –**
كلية طب المنيا

- هدفت هذه الدراسة إلي مقارنة القيمة التنبؤية المبكرة لمعامل النخر الورمي – ألفا مقارنة بالانترفيرون – جاما للتنبؤ بحدوث الإجهاض في حالات الإجهاض المتكرر الغير مفسر وذلك خلال الأسابيع الأولي من الحمل (6 – 8 أسبوع من فترة الحمل).
- اشتملت هذه الدراسة علي عدد (120) مريضة تم لهن جميع الإجراءات والفحوصات التي قد تؤدي إلي حدوث الإجهاض المتكرر . تم سحب عينة الدم (10 مل) من جميع المرضي وتم فصل العينات وتجميد البلازما تحت درجة – 80 درجة مئوية .
- تم قياس معامل النخر الورمي – ألفا وكذلك الانترفيرون – جاما باستخدام طريقة الأليزا.
- أظهرت النتائج استبعاد عدد 98 مريضة (45%) من الدراسة نتيجة لوجود سبب أو أكثر من الأسباب المعروفة التي قد تؤدي إلي الإجهاض المتكرر.
- أظهرت النتائج عدم وجود فروق ذات دلالة إحصائية بالنسبة للسن والوزن وعدد مرات الإجهاض السابقة بين المجموعتين . عند مستوي أقل من ≥ 200 بيكوجرام / مل وجد أن معامل النخر الورمي ألفا موجود في عدد (89) مريضة (74.2%) . (75) مريضة منهن (84.3 %) حدث لهن إجهاض أثناء الفترة الأولي أو الثانية من الحمل بنسبة حساسية بلغت (86.4%) وخصوصية (94.6%) وقيمة تنبؤية موجبة بنسبة (81.0 %) وقيمة تنبؤية سالبة بنسبة (96.0%) ودقة تشخيصية بنسبة (92.0%) . وعلي الجانب الآخر وعند مستوي ≤ 600 بيكوجرام / مل كان موجوداً في (31) مريضة (25.8%) (4) منهن (12.9%) حدث لهن إجهاض خلال الفترة الأولي أو الثانية من الحمل بقيمة حساسية وخصوصية وتنبؤية موجبة وسالبة وكذلك دقة تشخيصية أقل . وفي نفس الوقت وجد أن الانترفيرون – جاما عند مستوي ≥ 0.01 نانو جرام / مل في عدد (58) مريضة (48.3%) (6) منهن (10.3%) حدث لهن إجهاض في خلال الفترة الأولي أو الثانية من الحمل بنسبة حساسية بلغت (31.8%) وخصوصية بنسبة (38.8%) وقيمة تنبؤية موجبة بنسبة (21.8%) وقيمة تنبؤية سالبة بنسبة (27.6%) ودقة تشخيصية (9%) بينما عند مستوي ≤ 1.8 نانو جرام / مل وجدت في عدد (62) مريضة حامل (51.7%) ثلاثة منهن فقط (4.8%) حدث لهن إجهاض خلال الفترة الأولي والثانية من الحمل بنسبة حساسية وخصوصية وقيمة تنبؤية موجبة وسالبة ودقة تشخيصية أقل .

وخلصت هذه الدراسة إلي أن معامل الانترفيرون – جاما ليس له دقة تشخيصية للتنبؤ بحدوث الإجهاض في مرضي الإجهاض المتكرر والغير مفسر وعلي العكس فإن معامل النخر الورمي – ألفا عند قيمة نقطة حدة تقاطع (Cut - of Point ≥ 200 بيكو جرام / مل) وجد أن له قيمة تنبؤية عالية للتبؤ المبكر لحدوث الإجهاض في حالات الإجهاض المتكرر والغير معروف السبب مع دقة تشخيصية عالية تصل إلي نسبة (92.0%) .