# The *In Situ* Hybridization Expression of Fas and Fas Ligand (FasL) in Trophoblastic tissue of Aborted Women Compared with Normal Pregnancy.

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**Summar**

**Background:** Estimation of the *in situ* expression of Fas and Fas ligand (FasL) in aborted women compared with normal human pregnancy .
**Patients and Methods:** A technique utilizing *in situ* hybridization(**ISH)** was performed to detect and determine the *in situ* expression of Fas and Fas ligand (FasL) mRNA using paraffin embedded sections of curettage samples obtained from 42 women, who were divided into two groups: 30 women with first trimester abortion and 12 women with induced abortion as control.
**Results:** The levels of the *in situ* expression of both Fas and Fas ligand (FasL) mRNA were found to be highly significant increased in group 1 as compared with group 2 ($p<0.01$), with a significant positive correlation between Fas; and abortion in group I , FasL & abortion in group I , and between these two parameters(Fas & FasL) ($p<0.01$) in group1 .
**Conclusions:** The increasing expression of Fas and FasL trophoblasts might influence pathogenesis of first trimester abortion.
**Key words:** Fas and Fas-Ligand in trophoblasts, In Situ Hybridization.

**Introduction**:

The fetus is a unique individual, whose genetic makeup is equally derived from both the mother and father. The fetus can be considered a semiallograft to the mother's immune system and consequently should generate a maternal immune rejection response (1) .Since Fas and FasL were originally implicated in the removal of activated peripheral T cells following an immune response (2), it was suggested that one of the possible mechanisms by which maternal T cells may be tolerized to paternal alloantigens is by the Fas/FasL system. Indeed, trophoblast cells isolated from mouse placentas were shown to induce apoptosis in a mouse T cell line that expresses Fas, but not in a Fas deficient T cell line. In contrast, trophoblast cells purified from the placentas of homozygous *gld* (generalized lymphoproliferative disease) mice, which do not express functional FasL, did not induce apoptosis in the Fas-expressing T cell line (3). Similarly, (Kauma *et al* 1999) demonstrated that a FasL-expressing first trimester trophoblast cell line was able to induce Fas-mediated apoptosis in activated lymphocytes *in vitro* (4).

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However, several *in vivo* studies have reported that cells expressing membranal FasL were rejected when transplanted into allogenic animals (5, 6), questioning the role of the Fas/FasL system in immune privilege. This may be due to the overexpression of FasL in transplanted cells engineered to express membranal FasL. Indeed, the overexpression of membranal FasL has been shown to be associated with inflammation, neutrophil activation and rejection (7).In present study, attempted to delineate the relation between the *in situ* expression of Fas and Fas ligand (FasL) and first trimester abortion and compar between the expression of these two parameter in aborted women and normal women pregnancy .

**Patients and Methods:**
**Patients:** The study included (42) women from three hospitals in Baghdad (Al-Kadhmiya, Al Ulwiya and Al- Yarmook hospitals). Patients' ages ranged between (18-36) years with a mean of ($27.9 \pm 1.08$) year. They were separated into two groups:
Group 1: Thirty pregnant woman who where presented with spontaneous incomplete first trimester abortion. All gave a history of 1-6 previous consecutive miscarriages. None of them had any significant medical disease, family history of genetic disease, or anatomical uterine abnormality.
Group 2: (12) pregnant women who had at least three normal previous pregnancies, undergoing elective termination of an apparently normal

pregnancy in the first trimester for a maternal indication under the approved consent of two senior gynecologists and a physician.

Sera from all women in the two groups were negative for specific IgM and IgG for Rubella Virus, human Cytomegalovirus, and *Toxoplasma gondii* and negative for specific IgM for Herpes Simplex virus; *Chlamydia trachomatis;* Syphilis; antiphospholipid; anticardiolipin, and antinuclear antibody.

**Samples:** From each woman, two to three samples were taken from different sites of the placental tissue during evacuation curettage operation; thus, 2-3 paraffin embedded blocks were prepared for each patient. Sections from each block were stained with heamatoxylin and eosin for histopathological examination (only the sections contained trophoblastic tissues were included in the study).

***In situ* hybridization:** For *in situ* hybridization technique (ISH), DNA Probe Hybridization/Detection System in situ kit (Maxim Biotech, Inc., USA) was used.Kit contents included: biotinylated housekeeping gene probe, hybridization solution (ready to use), protein block (20X), detergent wash buffer (20X), RNase A (15 *µg/* ml), streptavidin-AP conjugate, substrate (BCIP/NBT), and lyophilized proteinase K (4 mg); which is dissolved in a 2 ml DNase and RNase free dilution buffer to form 10X proteinase K, then diluted by deionized water to IX proteinase K.The probes were biotin-labeled DNA probes for human FAS (318 bp), and human FasL (250 bp), (Maxim Biotech, Inc., USA).Tissue sections were deparaffinized in Xylene for 5 minutes and rehytlrated through a series of ethanol dilutions. After digestion with IX proteinase K at 37°C for 15 minutes, the sections were quickly dehydrated in ethanol. Hybridization was carried out by applying 10 µl hybridization mixture (0.8 µl of heat denatured biotin-labeled DNA probe diluted in 9.2 µl hybridization solution) per slide. After overnight incubation, the slides were soaked for 10 minutes in IX detergent wash at 37 °C, followed by RNase A treatment at 37 °C for 30 minutes, and then the slides were washed for 5 minutes in IX protein blocking buffer. The biotin-labeled hybrids were detected with streptavidin-alkaline-phosphatase conjugate, and an enzyme-substrate chromogen (bromo-chloro-indolyl-phosphate/ in nitro-blue-tetrazolium salt) BCIP/NBT, yielding an intense blue-black signal that appears at the specific site of the hybridized probe. The slides were counterstained with nuclear last red stain.

Evaluation of ISH signal: The expression of both Fas and FasL mRNA was measured by the same scoring system, by counting the number of positive decidual and Trophoblastic cells, which gave a blue-black (BCIP/NBT) nuclear staining under the light microscope. The extent of the ISH signal in the villi was determined in 10 fields (100Xmagnification). In each field the total number of villi were counted and the extent of nuclear staining of the cytotrophoblast and syncytiotrophoblast in a given villus 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. For the purpose of comparison, 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 (8).

Inferential statistics: (9)These were used to accept or reject the statistical hypotheses, they include Chi-square ($\chi^2$),Student test (t-test)and Pearson Correlation (r).

Note: The comparison of significant (P-value) in any test were:

S= Significant difference (P<0.05).

HS= Highly Significant difference (P<0.01).

NS= Non Significant difference (P>0.05).

**Results:**

The expression of Fas and FasL was detected by ISH technique. Tables (1) and (2) Chi-square test was conducted to examine the association between Fas and FasL mRNA expression in trophoblasts tissue in the two groups of investigated women ,it was found that highly significant association ($p$<0.01) between them in the three scoring levels. The results showed that percentages of Fas and FasL were elevated in 40% (12/30) and 56.7%(17/30) ,respectively in aborted women. It was found that 50% (6/12) of women in control group showed low level and 50% (6/12) in moderated level of Fas and FasL. The t-test analysis table (3)and (4) show the highly significant difference ($p$<0.01) in the mean percentages of Fas and FasL *in situ* expression respectively in the villus trophoblasts of aborted women compared with control.In addition, the study demonstrated highly significant positive correlation ($P$<0.01) between Fas; FasL and abortion and between Fas and FasL demonstrated in Table 5 and Figure (1, 2, and 3). The expression of Fas and FasL was heterogeneous blue-black nuclear staining in trophoblastic cell, as shown in Figure (4 and 5).

**Table (1): Relationship between ISH% Fas grades in studied groups.**

| Studied groups | | ISH% Fas grades | | | Total | Comparison of Significant | |
|---|---|---|---|---|---|---|---|
| | | <25 | 25-74 | 75-100 | | P-value | Sig. |
| Aborted women | N | 0 | 18 | 12 | 30 | 0.00 | Highly Sig. (P<0.01) |
| | % | 0 | 60 | 40 | 100 | | |
| Control | N | 6 | 6 | 0 | 12 | | |
| | % | 50 | 50 | 0 | 100 | | |
| Total | N | 6 | 24 | 12 | 42 | | |
| | % | 14.3 | 57.1 | 28.6 | 100 | | |

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**Table (2): Relationship between ISH% FasL grades in studied groups.**

| Studied groups | | ISH% FasL grades | | | Total | Comparison of Significant | |
|---|---|---|---|---|---|---|---|
| | | <25 | 25-74 | 75-100 | | P-value | Sig. |
| Control | N | 6 | 6 | 0 | 12 | 0.00 | Highly Sig. (P<0.01) |
| | % | 50 | 50 | 0 | 100 | | |
| Aborted women | N | 0 | 13 | 17 | 30 | | |
| | % | 0 | 43.3 | 56.7 | 100 | | |
| Total | N | 6 | 19 | 17 | 42 | | |
| | % | 14.3 | 45.2 | 40.5 | 100 | | |

**Table (3): Mean distribution of ISH% Fas among studied group**

| Studied group | N | Mean± Std. Error | SD | Mini. | Maxi. | Comparisonof significant | |
|---|---|---|---|---|---|---|---|
| | | | | | | P-value | Sig. |
| Control | 12 | 23.33±2.36 | 8.18 | 10 | 35 | 0.00 | Highly Sig. (P<0.01) |
| Aborted women | 30 | 70.17±2.52 | 13.78 | 40 | 95 | | |
| Total | 42 | | | | | | |

**Table (4): Mean distribution of ISH% FasL among studied group**.

| Studied group | N | Mean± Std. Error | SD | Mini. | Maxi. | Comparison of significant | |
|---|---|---|---|---|---|---|---|
| | | | | | | P-value | Sig. |
| Control | 12 | 23±2.39 | 8.28 | 10 | 36 | 0.00 | Highly Sig. (P<0.01) |
| Aborted women | 30 | 77.47±2.26 | 12.40 | 46 | 96 | | |
| Total | 42 | | | | | | |

**Table (5): Pearson correlation (r) between parameters among aborted women.**

| Pearson Correlation | | ISH % Fas | ISH % FasL |
|---|---|---|---|
| Abortion No. | r | 0.669 | 0.737 |
| | P-value | 0.00 | 0.00 |
| | Sig. | *P*<0.01** | *P*<0.01** |
| ISH % Fas | r | | 0.732 |
| | P-value | | 0.00 |
| | Sig. | | *P*<0.01** |

**= highly significant difference

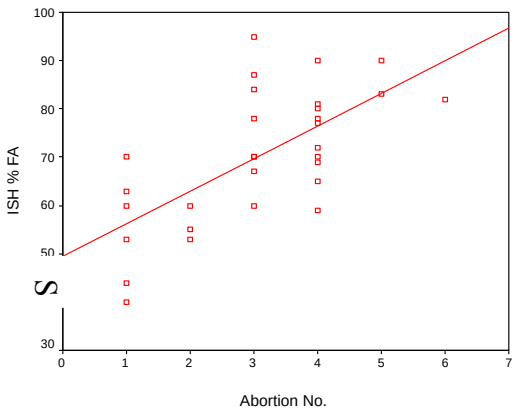


**Figure (1): Pearson correlation (r) between abortion number & ISH % Fas among aborted women.**

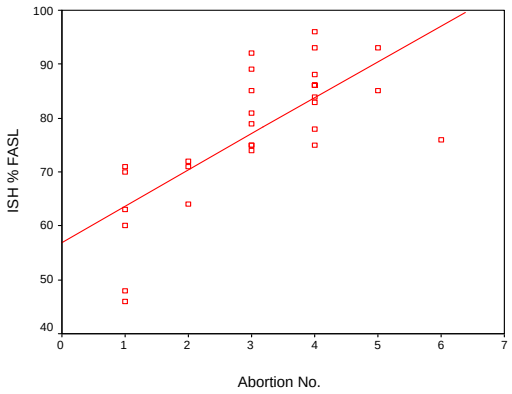


**Figure (2): Pearson correlation (r) between abortion number & ISH % FasL among aborted women.**

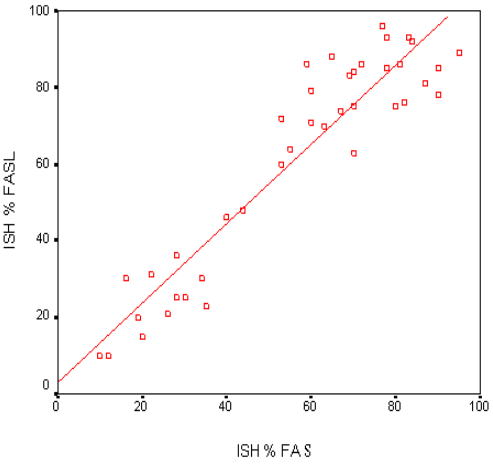


**Figure (3): Pearson correlation (r) between ISH % Fas & ISH % FasL among aborted women.**

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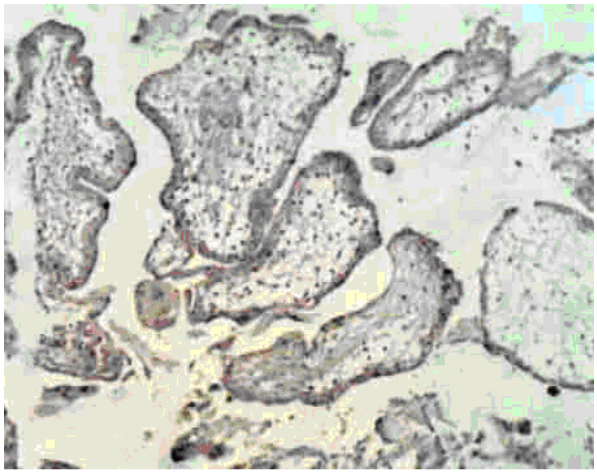

**Figure (4): Detection of Fas and FasL in studied groups by in situ hybridization (ISH). Staining of Fas and FasL mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from aborted women shows positive Fas hybridization signals.**

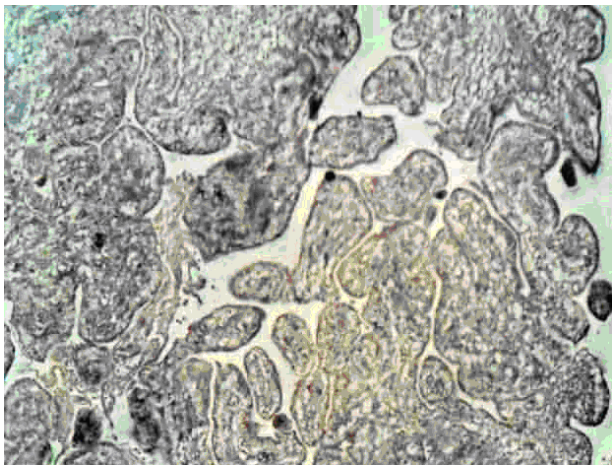

**Figure (5): Detection of Fas and FasL in studied groups by in situ hybridization (ISH). Tissue from aborted women shows positive FasL hybridization signals.**

**Discussion:**

To the best four a knowledge the present study is the first locally conducted in situ hybridization study to determine Fas and FasL as parameters that reflect the apoptotic process in aborted women.This study using the in situ hybridization method clearly demonstrate that the percentage of Fas and FasL in normal human placental tissue was 50% in moderated level and 50% in lowest level. This result is supported by a previous finding that was demonstrated in successful pregnancies, binding of trophoblast-associated FasL to Fas-expressing activated maternal T lymphocytes that invade the trophoblast during implantation induces apoptosis in Fas-bearing maternal T cells, allowing fetal trophoblast to invade into the myometrium while escaping immune recognition. The Fas-expressing invading trophoblasts also might undergo apoptosis from FasL-expressing maternal T cells, limiting the extent of myometrial invasion (10). Disturbed Fas-mediated apoptosis is involved in the pathogenesis of preeclampsia (**11)**. Although Fas-FasL system may play a role in maintaining human pregnancy, aberrant activation of this system in trophoblast, the major cell type at the maternal-fetal interface of placenta, may invoke pathologic changes in placenta (12, 13, 14, 15). Fas/FasL probably plays an active role in maternal immunotolerance to the fetus, the absolute necessity of trophoblast FasL expression for maternal immunotolerance of the fetus has not been demonstrated (16). In this study the in situ hybridization of Fas and FasL clearly showed that the localization of Fas and FasL was elevated in first trimester aborted women. This result indicated the highly placenta expression of Fas and FasL,which are thought to be associated with trophoblast apoptosis that causes abortion. Previous reports have suggested that placental apoptosis and altered expression of Fas and Fas ligand in trophoblast might influence pathogenesis (11, 17, 18). Another study showed the overexpression of membranal FasL is be associated with inflammation, neutrophil activation and rejection (7). In addition, in the current study the mean percentage of Fas and FasL showed a highly significant difference in women with abortion compared with control. A part from the causes of this significant increase in the expression of Fas and FasL in aborted women, revision was made for the previous studies that showed the human reproduction is remarkably inefficient, with more than half of spontaneous conceptions failing to complete the first trimester. However, little is known on the molecular events that take place at the implantation site during abortion (19, 20). Furthermore, this study demonstrated a highly significant positive correlation ($p<0.01$) between FAS; FASL and abortion and between these two markers. These results may explain the role of Fas and FasL which is an apoptotic pathway that secreted by the placenta and might induce implantation failure.

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