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The Role of HLA-G, Natural Killer Cells and CD8$^+$ T Cells in Tubal Tissue: The Comparison of Non-Pregnant, Intrauterine Pregnancy and Ectopic Pregnancy

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Abstract

Purpose: The goal of the study was to investigate the expression of HLA-G, CD16, CD56, CD8, TNF-α , IFN-γ in ectopic pregnancy (EP) by immunohistochemistry.

Methods: The study was conducted on non-pregnant women (NP) (n=10), women with intrauterine pregnancy (IUP) (n=7) and EP (n=10). Immunostaining evaluated by HSCORE analysis of HLA-G, CD16, CD56, CD8, TNF-α, IFN-γ in tubal tissues were compared.

Results: HLA-G showed stronger staining in IUP than other groups (p=0.001). The expression of CD16 was lower in IUP than EP (p=0.005). The expression of CD56 and CD8 was low in NP, while CD8 was highest in EP (p=0.001 and p=0.001). The highest expression for TNF-α was shown in EP, while IFN-γ expression was highest in IUP (p=0.016 and p=0.001, respectively).

Conclusion: The expression of HLA-G from trophoblast in EP is insufficient. The phenotypic characteristics of natural killer cells are different in EP than IUP and the activation of cytotoxic T cell is increased. TNF-α promotes the formation of EP.

Keywords: Ectopic pregnancy, HLA-G, natural killer cells, CD8-Positive T-Lymphocytes

Introduction

Ectopic pregnancy (EP) is defined as the implantation of the fertilized ovum outside the uterine cavity. Approximately 98% of pregnancies implant in the fallopian tube. Despite the advances in contemporary medicine, EP still remains as an important cause of maternal morbidity and mortality worldwide. Studies about the etiology of EP have shown that, corruption in tubal transport and changes leading to implantation in tubal environment cause embryo to implant in fallopian tube [1].

HLA-G belongs to nonclassical HLA class I family. It has originally been discovered on the extravillous cytotrophoblast at the maternal-fetal interface. HLA-G supports the immun mechanisms that protects the fetus from maternal alloimmune reactions. In intrauterin pregnancies, trophoblasts expressing paternal alloantigens should invade into the maternal uterine tissue in order to form placenta. HLA-G has an inhibitor role against CD8+ T and natural killer (NK) cells' desidual cytotoxic effect, limiting the trophoblast invasion [2]. Uterine

NK cells (uNK) are phenotypically and functionally different from circulating NK cells. Peripheral blood NK cells have a low expression of CD56 NK marker and are CD16 surface cell antigen positive (CD56dim, CD16+). On the other hand, uNK cells phenotypically has a high expression of CD56 and are CD16 negative (CD56bright, CD16-) [3]. While, the CD56dim population are mostly cytotoxic, CD56bright population is an intense cytokine producer [4]. uNK, especially in the early period of pregnancy, has roles in control of trophoblast invasion, uterine vascular remodelling and protection against uterine infections [5]. IFN-γ, TNF-α are important cytokines expressed by NK cells and decidual CD8+ T cells on maternal-fetal surface in decidualization process and vascular remodelling and they limit the trophoblast invasion [6,7].

There are limited studies about formation of EP in fallopian tube or immunological explanation of changes in fallopian tube in intrauterine pregnancies (IUP). Emmer et al. [8], have shown that HLA-G expression continues in trophoblastic tissue in EP. Also, von Rango et al. [9], have shown in the implantation area of fallopian tube, CD56bright and CD16- NK cells are barely existed in EP. Lascarin et al. [10], have shown that in EP, uNK cells are incompenent to limit the trophoblast invasion because of the low expression of cytotoxic mediators.

We also wanted to show the relationship of these cells and mediators and their varying expression levels in the formation of EP in one study. The aim of this study is to investigate the effects of immune mechanisms in EP, by comparing the immunohistochemical HLA-G, CD16, CD56, CD8, TNFα, IFNγ levels in fallopian tube of IUP, EP and non-pregnant (NP) women.

Materials and methods

This prospective study was conducted on Merkez Efendi State Hospital, Department of Obstetrics and Gynecology. It was approved by Ethics Committee of Celal Bayar University. All the participants provided informed consent. Tubal tissue samples were obtained from 10 NP (group 1) women during tubal ligation, 7 women with IUP (group 2) during curettage and tubal ligation, and during 10 salpingectomy due to EP (group 3). Chronic inflammatory diseases, history of ectopic pregnancy, tubal surgery, acute or chronic infection, smoking, immune system disease, immunsupressive drug were exclusion criterias. IUP consisted of women who had unwanted pregnancies (pregnant women who wished curettage) and had at least one live-born child. In IUP, CRL (crown-rump length) was shorter than 10 weeks (abortion is not allowed if CRL is more than 10 weeks) and positive fetal heart rate was observed with transvaginal ultrasound. Obtained tubal tissue samples were fixed with suitable fixatives for histochemical and immunohistochemical procedures.

Histopathological evaluation

Tubal isthmus tissue samples were evaluated in all groups. Tubal isthmus which is outside the site of implantation was considered in tuba with EP. For immunohistochemical analyses, 4µm thick sections were used for the primary antibodies; IFγ (SC 8308, Santa Cruz), CD-8 (SC 7188, Santa Cruz), TNF-α (SC 52746, Santa Cruz), CD-56 (SC 7326, Santa Cruz), CD-16 (SC20052, Santa Cruz) and HLA-G (SC21799, Santa Cruz) all diluted at 1/100. In brief, the deparaffinization procedure was accomplished in xylene for 1 hour. Rehydration was done in sequential descending alcohol series for 2 minutes each. After leaving in distilled water for 5 minutes, the tissues were delineated on the object slide, washed in phosphate buffered

saline (PBS) for 10 minutes, and than left in trypsin for 15 minutes. The primary antibody was then applied in an incubator at 57°C and washed with PBS. Afterwards the biotinylated secondary antibody was applied and washed with PBS before incubating with the enzyme conjugate and 3,3-diaminobenzidine tetrahydrochloride(DAB). Then sections were counterstained with Mayer's hematoxylin (Zymed Laboratories) and mounted with entellan.

Immunostaining was evaluated semiquantitatively by HSCORE analysis. All slides were examined and photographed with Olympus C-5050 digital camera integrated Olympus BX51 light microscope. Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak but detectable staining), 2 (moderate staining), and 3 (intense staining). An HSCORE value was derived for each specimen by calculating the sum of the percentage of cells for the nuclear and cytoplasmic immunoreaction of the tuba sections that was stained at each intensity category multiplied by its respective score, by means of the formula H-score=$\sum$Pi (i+1), where i=intensity of staining with a value of 1, 2, or 3 (weak moderate or strong respectively) and Pi is the percentage of stained cells for each intensity, varying from 0 to 100%. For each slide, ten different fields were evaluated microscopically at 200x magnification. HSCORE evaluations were performed independently by at least two investigators blinded to the source of the samples as well as to each other's results. The average score of both was utilized.

Statistical Analysis

The statistical package SPSS for Windows 15.0 (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL) was used to analyze the data. Mean and standard deviations was used to describe data. Statistical comparisons between three groups were performed using the Kruskal-Wallis and p values < 0.05 were accepted as significant. The Mann-Whitney U test with bonferroni correction was used to determine which pairs of the groups are significant different and p values <0.016 were considered to be statistically significant.

Results

Age didn't show any significant difference between NP (34.29±2.92), IUP (34.14±3.43) and EP groups (30.71±2.87) (p=0.072). The median gestational week was 7 weeks (range 6-8) in IUP. The median and standard deviation of immunochemical HSCORE values are presented (Table 1, Figure 1). HLA-G immunoreactivity was higher in IUP than NP and EP. CD16 was significantly lower in IUP than EP, there was no significant difference between other pair of groups. CD56 immunoreactivity was significantly lower in NP than other groups. In each pair of groups for CD8, there was significant difference and EP showed the strongest staining, while NP showed the weakest. EP showed the highest expression for TNF-α, but significant difference was established in comparison with NP, which shows the lowest expression. IUP showed significant higher expression compared to other two groups. p values of pair of the groups are presented (Table 2). Immunohistochemical analyses of the groups in tubal tissue are presented (Figure 2).

Discussion

We aimed to investigate the patho-physiology of tubal pregnancy through immune mechanisms. For this purpose, we analyzed tubal tissue samples of NP, IUP ve EP immuno-histochemically. HLA-G, CD56, CD8, IFN-γ and not statistically significant but TNF-α was higher in IUP than NP.

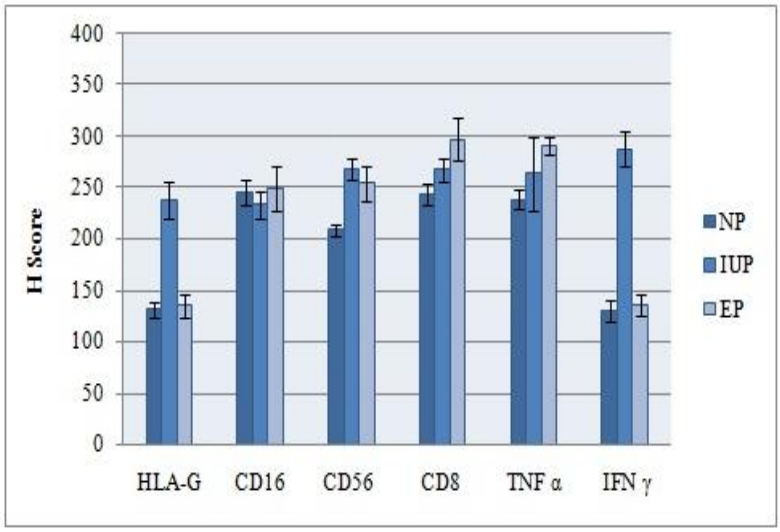

Figure 1: The HSCORE values of immunohistochemical analyses forthe groups in tubal tissue are presented. NP; non-pregnant, IUP; intrauterine pregnancy and EP; ectopic pregnancy represent. Bars represent standart deviations.

Table 1: Histopathological evaluation parameters in the groups.

	Non-pregnant (n=10)	**Intrauterine pregnancy (n=7)**	**Ectopic pregnancy (n=10)**	**P value**
HLA-G	130.71 ± 8.36	237.00 ± 17.73	134.57 ± 11.95	0.001
CD16	245.85 ±12.23	233.28 ± 13.03	248.42 ±21.93	0.006
CD56	208.71±6.15	267.85 ±11.30	253.85 ± 17.76	0.001
CD8	243.28 ± 9.67	267.14 ±11.75	297.14 ± 21.01	0.001
TNF-α	238.14 ±9.58	263.14 ±35.84	290.71 ±8.61	0.016
IFN-γ	129.42 ±10.92	287.14 ±17.26	135.14 ±10.23	0.001

Data are presented as mean±SD (standard deviation) .

Table 2: Comparison between the pairs of the groups. NP; non-pregnant, IUP; intrauterine pregnancy and EP; ectopic pregnancy represent.

	NP-IUP P value	**IUP-EP P value**	**NP-EP P value**
HLA-G	0.002*	0.002*	0.564
CD16	0.124	0.005*	0.021
CD56	0.002*	0.073	0.002*
CD8	0.015*	0.009*	0.002*
TNF-α	0.201	0.338	0.002*
IFN-γ	0.002*	0.002*	0.276

* $p<0.016$ When pairs of the groups compare with bonferroni correction

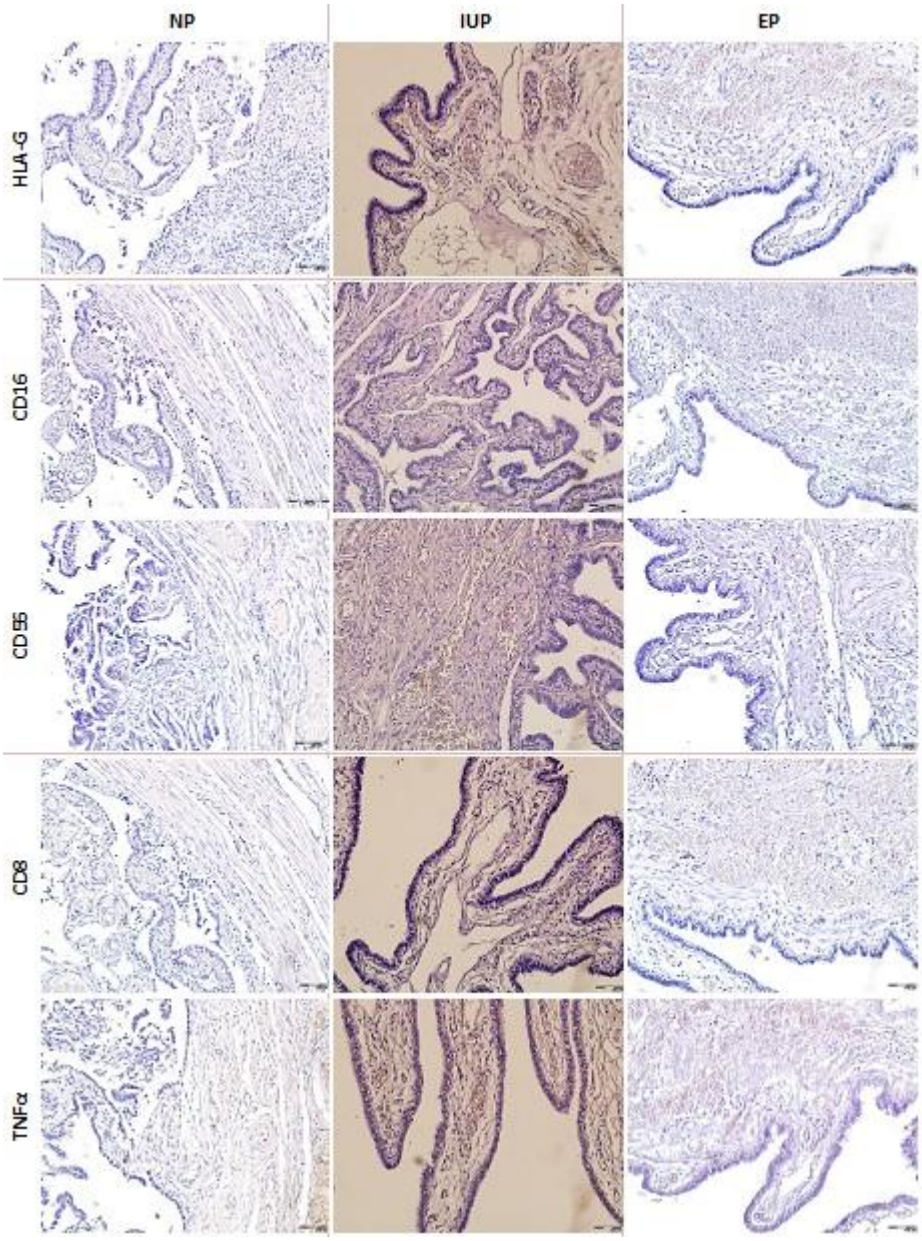

Figure 2: Immunohistochemical analyses of the groups in tubal tissue are presented. NP; non-pregnant, IUP; intrauterine pregnancy and EP; ectopic pregnancy represent. The magnification is 200x.

In EP, compared to IUP, CD8+, CD16+ and not istatistically significant but TNF-α were higher, while IFN-γ, HLA-G and not istatistically significant but CD56+NK cells were lower. This showed a different cytotoxic T and NK cell profile from healthy pregnant women's maternal tissue.

Trophoblasts have different mechanisms in invasion to implantation area and regulation of immune response. Extravillous trophoblasts (EVT) do not express major histocompatibility complex (MHC) class I and II, they express non-classic MHC class I especially HLA-G. Furthermore, it's a molecule that almost only expressed in immune privileged tissues in adults like cornea, thymus, erythroblasts, macrophage-dendritic cells [11]. HLA-G has a

membrane-bound (HLA-G1-HLA-G4) and soluble forms (sHLA-G5 –sHLA-G7) [12]. HLA-G shows its tolerance to immune system cells through immunglobulin-like transkript (ILT) receptor 2 (ILT-2), ILT-4 and KIR2DL2 (CD158d) receptors on NK cells [13]. In our study, in IUP group, though it is away from implantation area which has EVT cells which are the main source of HLA-G, we have established significantly more HLA-G positive cells compared to other groups. This lead to the conclusion that, HLA-G is transferred by trogocytosis to contacted cells. Trogocytosis is the rapid transfer of plasma membrane and membrane proteins between cells via cell contact. This non-spesific transfer makes especially MHC class I and II molecules transfer from cell to cell [14]. Caumartin et al. [15], showed that HLA-G is transferred especially to NK cells with this mechanism, and this makes the cell acquire supressive characteristic. Besides, sHLA-G5 and sHLA-G7 are soluble forms of HLA-G and they can have an inhibitor effect by reaching far from the origin. Although they are far from the fetus, this mechanism can explain the high HLA-G levels in fallopian tubes in IUP. CD56 level was high and CD16 level was low, which are uNK determinants. IFN-γ, expressed from uNK cells to regulate fetus implantation, angiogenesis and endometrial vascular remodelling, is high in IUP compared to other groups [16]. TNF-α is an important inflammatory cytokine expressed by activated immune cells (especially macrophage, NK and cytotoxic T lymphocytes) to inhibit the migration of EVT in order to abort the embryo in the early stages of pregnancy which has anomaly [17]. These cytokine levels were also lower in IUP compared to EP as expected.

In NP group, HLA-G levels were low due to absence of fetal growth. CD56 levels were low and CD16 levels were high in NK cells, which was consistent with previous studies about NP tubal tissue [3]. The dominant T cell phenotype in NP tubal tissue is CD8 T lymphocyte [18]. But CD8 expression was significantly lower in this group compared to other groups. IFN-γ and TNF-α, inflammatory cytokines were low in NP, as expected.

In EP group, although there was an implanted embryo in fallopian tube, HLA-G levels were significantly lower compared to IUP, and close to NP. This lead us to the conclusion that HLA-G is an important molecule for pathogenesis of tubal pregnancy. Fetal trophoblasts are the most important HLA-G source [12]. There is an implanted fetus in tubal pregnancy outside the uterus, so high HLA-G levels are expected. It's stated that high TNF-α levels we have found doesn't affect HLA-G production of EVT cells [17]. If cytokines doesn't affect HLA-G expression, there could be a problem in HLA-G production in EVT. The corruption in the immun mechanisms against semi-allogeneic fetus can cause the fetus to implant to tuba instead of uterus. As we have detected in our study, the same level of HLA-G in EP and NP, supports the existence of a problem in HLA-G expression by EVT. In EP, CD16 levels were significantly high and CD56 levels were low, although not istatistically significant in NK cells than IUP. In some studies, CD56Bright NK cells were absent in tubal tissue of NP and EP [3,9] On the contrary, there are some studies that support the existence of CD56Bright, CD16-cells in tubal tissue [10]. In our study, we detected CD56+cells in NP and EP tubal tissue, but the levels of these cells were lower in IUP. IFN-γ, which is essential for healthy pregnancy and expressed by uNK cells in the early stages of pregnancy, were at almost the same level with NP group and significantly lower than IUP [16]. TNF-α levels were higher in EP group compared to others. TNF-α is an apoptotic anti-tumor

inflammatory cytokine that has a negative effect on embryo development. Bauer et al. [17], stated that, TNF-α inhibits trophoblasts invasion and migration via plasminogen activator inhibitor-1 but it doesn't affect EVT cells' HLA-G expression, moreover, it doesn't induct EVT cells' apoptosis either. The other impact that TNF-α has on tuba is to make the ciliary cell disappear [19]. As we have found out in our study, high TNF-α levels has an important role on embryo's implantation to tubal tissue by inhibiting migration and continuous develepmont of embryo outside the uterus without being influenced.

Most of the CD8+ cells in the maternal tissue are antigen-presented effector memory cells [20], on the other hand, NK cells are CD56Bright, CD16-, which are specified for healthy pregnancy [21]. It can be assumed that effector CD8 memory cells are activated by high levels of systemic cytokines triggered by a viral infection during or after coitus. A viral infection also could have caused NK cells, dominant cell type in maternal tissue, to supress the fetal protecting CD56Bright phenotype and to bring out the anti-viral CD16+ phenotype. Activated CD8+ and NK cells secretes IFN-γ and TNF-α [6,7]. Monocytes, the tissue form of macrophages, are the responsible immune cells of TNF-α production and their levels are higher in tubal pregnancy than healty pregnancy [22]. This could have caused the adhesions molecules, which are not present in tuba and facilitates the inflammatory cell migration, to increase and the fetus to implant to fallopian tube. The low levels of IFN-γ in EP' tubal tissue compared to IUP, which activates the MHC molecule expression from the cells, can be related to low HLA-G levels in EP. High levels of TNF-α detected in EP' tubal tissue is an significant indicator of the host's response to inhibit the fetal migration and invasion. It had ben showed by Bauer et al. [17], that TNF- α, a strong apoptotic cytokine, did not induced the apoptosis in EVT. High levels of TNF-α that we have detected in our study can explain the early implantation of fetus, because of the inhibition of migration abilities of EVT and the development of fetus, because of being unaffected by the apoptotic signals which causes tubal rupture.

Conclusion

Expression of HLA-G, an important molecule for fetal protecting maternal tolerance, is not sufficient in EP. The phenotype characteristics of NK is different in EP than IUP and the activation of cytotoxic T cell is increased. Increase of TNF-α expression, is one of the changes in the tubal enviroment which causes embryo to implant in tuba. We believe that the data we have obtained by larger populations and further moleculer techniques, should be explicated.

Acknowledgments

None

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author's Contributions:

F Eskicioglu: Project development, Data management, Data analysis, Manuscript writing/editing

T Gokmen: Data collection, Data analysis

S Taskend: Data collection

References

1-Shaw JL, Dey SK, Critchley HO, Horne AW (2010) Current knowledge of the aetiology of human tubal ectopic pregnancy. Hum Reprod Update 16: 432-434

2-Pröll J, Bensussan A, Goffin F, Foidart JM, Berrebi A, Le Bouteiller P (2000) Tubal versus uterine placentation: similar HLA-

G expressing extravillous cytotrophoblast invasion but different maternal leukocyte recruitment. Tissue Antigens 56: 479-491.

3-Vassiliadou N, Bulmer JN (1998) Characterization of tubal and decidual leukocyte populations in ectopic pregnancy: evidence that granulated lymphocytes are absent from the tubal implantation site. Fertil Steril 69: 760–767.

4-Szekeres-Bartho J (2008) Regulation of NK cell cytotoxicity during pregnancy. Reprod Biomed Online 16: 211–217.

5-van den Heuvel MJ, Xie X, Tayade C, et al (2005) A review of trafficking and activation of uterine natural killer cells. Am J Reprod Immunol 54: 322–331.

6-Ashkar AA, Croy BA (2001) Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy. Semin Immunol 13: 235–241.

7-Tilburgs T,Strominger JL (2013) CD8+ effector T cells at the fetal-maternal interface, balancing fetal tolerance and antiviral immunity. Am J Reprod Immunol 69: 395–407.ET AL

8-Emmer PM, Steegers EA, Kerstens HM, et al (2002) Altered phenotype of HLA-G expressing trophoblast and decidual natural killer cells in pathological pregnancies. Hum Reprod 17: 1072-1080.

9-von Rango, R.U., Classen-Linke, I., Kertschanska, S., Kemp, B., Beier, H.M (2001) Effects of trophoblast invasion on the distribution of leukocytes in uterine and tubal implantation sites. Fertil. Steril 76, 116–124.

10-Laskarin G, Redzovic A, Vukelic P, et al (2010) Phenotype of NK cells and cytotoxic/ apoptotic mediators expression in ectopic pregnancy. Am J Reprod Immunol 64: 347-358.

11-Carosella ED, Favier B, Rouas-Freiss N, Moreau P, Lemaoult J (2008) Beyond the increasing complexity of the immunomodulatory HLA-G molecule. Blood 111: 4862-4870.

12-Park GM, Lee S, Park B, et al (2004) Soluble HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis. Biochem Biophys Res Commun 313: 606-611.

13- López-Botet M, Navarro F, Llano M (1999) How do NK cells sense the expression of HLA-G class Ib molecules? Semin Cancer Biol 9: 19-26.

14- LeMaoult J, Caumartin J, Daouya M, et al (2007) Immune regulation by pretenders: cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells. Blood 109: 2040-2048.

15- Caumartin J, Favier B, Daouya M, et al (2007) Trogocytosis-based generation of suppressive NK cells. EMBO J 26: 1423-1433.

16- Murphy SP, Tayade C, Ashkar AA, Hatta K, Zhang J, Croy BA (2009) Interferon gamma in successful pregnancies. Biol Reprod 80: 848-59.

17- Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD, Knöfler M (2004) Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. J Clin Endocrinol Metab 89: 812-822.

18-Ulziibat S, Ejima K, Shibata Y, et al (2006) Identification of estrogen receptor beta-positive intraepithelial lymphocytes and their possible roles in normal and tubal pregnancy oviducts. Hum Reprod 21: 2281-2289.

19-McGee ZA, Jensen RL, Clemens CM, Taylor-Robinson D, Johnson AP, Gregg CR (1999) Gonococcal infection of human fallopian tube mucosa in organ culture: relationship of mucosal tissue TNF-alpha concentration to sloughing of ciliated cells. Sex Transm Dis 26: 160-165.

20-Tilburgs T, Schonkeren D, Eikmans M, et al (2010) Human decidual tissue contains differentiated CD8+ effector-memory T cells with unique properties. J Immunol 185: 4470-4477.

21-Hanna J, Goldman-Wohl D, Hamani Y, et al (2006) Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nat Med 12: 1065-74.

22-Eskicioğlu F, Özdemir AT, Turan GA, Gür EB, Kasap E, Genç M (2014) The efficacy of complete blood count parameters in the diagnosis of tubal ectopic pregnancy. Ginekol Pol 85: 823-827.