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The role of cytokines in first trimester pregnancy losses with fetal chromosomal anomaly

Rola cytokin w utratach ciąż z nieprawidłowościami chromosomalnymi w pierwszym trymestrze

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Abstract

Objectives: *The contribution of local inflammation to the pathophysiology of abnormal choromosomally miscarriages remains unclear. The objective of this study was to investigate the inflammatory response at the maternofetal interface of women presenting with first trimester miscarriage with abnormal choromosomally.*

Material and methods: *Level of TNF-α , IL-6 ve IL-17 were asseyed using ımmunohistochemistry technique at decidual and placental bed biopsy samples from 23women with electıve termınatıon of pregnancy, 21euploid and 18 aneuploid missed miscarriages.Immunostainig for TNF-α , IL-6 ve IL-17 has been evaluated semi-quantitatively by 'quickscore' method*

Results: *We found that the intensity of TNF-α staining was high in the miscarriage group, and this has been found in previous studies. Unlike some previous studies, the intensity of IL-6 staining was higher in the miscarriage groups only in decidual glandular epithelium. The intensity of IL-6 staining was found to be higher in the miscarriage group with chromosome anomaly than in the miscarriage group without chromosome anomaly. There was no significant difference in IL-17 levels between any of the groups.*

Conclusions: *Cytokines are considered to play an important role in the maintenance of pregnancy,but the exact mechanism between them and the mutual regulation relationship were not been fully understood,which need our further study.*

Key words: **cytokines** / **miscarriage** / **ımmunohistochemical** /

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Streszczenie

Cel pracy: *Zależność między lokalnym stanem zapalnym a patofizjologią poronień ciąż nieprawidłowych chromosomalnie pozostaje niejasna. Celem tego badania była ocena odpowiedzi zapalnej u kobiet z poronieniem w pierwszym trymestrze ciąży z nieprawidłowymi chromosomami.*

Materiał i metoda: *Poziom TNF-α, IL-6 i IL-17 oznaczano metodą immunohistochemii z biopsji kosmówki i doczesnej od 23 kobiet z elektywną terminacją ciąży, 21 euploidalnych i 18 aneuploidalnych poronień zagrażających. Barwienie na TNF-α, IL-6 i IL-17 oceniono metodą półilościową tzw. QuickScore.*

Wyniki: *Siła barwienia TNF-α była wysoka w grupie z poronieniami, co już wcześniej zostało opisane w innych badaniach. Inaczej niż w poprzednich badaniach, siła barwienia Il-6 była wyższa w grupie z poronieniami ale tylko w nabłonku gruczołowym doczesnej. Siła barwienia IL-6 była wyższa w grupie z poronieniami z nieprawidłowościami chromosomowymi niż w grupie poronień prawidłowych ciąż. Nie znaleziono żadnych istotnych różnic w poziomie IL-17 pomiędzy grupami.*

Wnioski: *Uważa się, że cytokiny odgrywają ważną rolę w utrzymaniu ciąży ale dokładny mechanizm wzajemnych oddziaływań nie jest w pełni poznany i wymaga dalszych badań.*

Słowa kluczowe: **cytokiny** / **poronienia** / **immunohistochemia** /

Introduction

Approximately 15% of all pregnancies result in pregnancy loss [1]. The causes for approximately 50% cases of repeated pregnancy loss remain unknown [2]. It has been previously determined that approximately 60% of pregnancy loss cases that occur during the first trimester are associated with chromosomal anomalies [3]. Moreover, most chromosomal anomalies are associated with abnormal trophoblast invasion, which occurs in the uterine decidua [4].The secondary causes of pregnancy loss may involve maternal leukocytes and other immune factors, including cytokines (i.e., tumor necrosis factor alpha [TNF-α]) [5]; however, their roles in trophoblast-decidual interaction during normal and abnormal first trimester pregnancies remain unclear.

Extravillous trophoblast (EVT) cells invade the uterine decidua and myometrium (interstitial EVT) throughout the first half of pregnancy, accumulate around uterine spiral arteries and facilitate uterine spiral artery remodeling [6]. Failure of EVT invasion and spiral artery remodeling has been implicated in several pregnancy complications, including early and late miscarriages [7]. Despite their importance, the mechanisms that control EVT invasion and spiral artery remodeling are not currently understood.

The inflammatory process in the feto-maternal interface is critical for successful implantation and a full-term pregnancy [8]. This inflammatory reaction is regulated by cytokines. Immunity is regulated by CD (4+) T-helper (Th) lymphocytes. CD (4+) Th cells are classified as Th-1 and Th-2 cells,depending on the cytokines they produce. Recently, CD (4+) Th cells that were characterized by interleukin (IL)-17 production, named Th-17, were discovered [9]. TNF-α is a cytokine produced by Th-1 cells. According to previous studies, TNF-α affects pregnancy loss [10], placental invasion [11], and apoptosis [12]. However, the absolute roles of TNF-α and other cytokines in trophoblast invasion remain controversial [13].

IL-6 is a cytokine produced by Th-2 cells. The role of IL-6 in regulating EVT invasion is unclear. In one study, IL-6 has been observed to stimulate invasion [14], while no effects of IL-6 on EVT invasion were observed in another study [15].

IL-17, a proinflammatory cytokine, induces the secretion of many inflammation mediators; ; in particular,neutrophil activation is one of the functions of IL-17 [16]. Investigators of previous studies have utilized placental tissue in cases of pregnancy loss without knowledge of the karyotype or have focused on women presenting with recurrent miscarriages. Information about the impact of the karyotype in the conceptus on placental and systemic inflammatory responses in early pregnancy failures is currently limited. Therefore,in the present study using immunohistochemical methods, we aimed to compare, the secretion of TNF-α, IL-6, and IL-17 at the feto-maternal interface in placental and decidual tissue from cases of elective abortions and early pregnancy loss with or without a chromosome anomaly.

Material and methods

All samples for this study were approved by the University of Şifa Ethics Committee and informed consent was obtained from all patients. A total of 62 pregnant women who underwent dilation and curettage procedures at the Şifa University Department of Obstetrics and Gynecology were included in this study. Chorionic and decidua samples from women

who underwent elective abortions (n = 23) and missed abortions (n = 39) were obtained during curettage. The products of conception obtained from all patients who underwent elective removal of conception products were karyotyped at a commercial laboratory (Şifa, Izmir, Turkey) using standard culturing, suspension harvest, and G-band analysis methodology as previously described [17]. Twenty-one of the 39 miscarriages included in the study group had a normal karyotype, while 18 had an abnormal karyotype (i.e., 8 trisomies, 6 triploidies, and 4 monosomy X).

The inclusion criteria of the study group included a crown-rump length (CRL) corresponding to a gestational age of 6–11 weeks with transvaginal ultrasonography results and a negative fetal heart rate (FHR). The control group consisted of patients with unwanted pregnancies or pregnant women who wished to have curettage. In the control group, a CRL of <10 -gestational weeks.(curettage is not allowed if the CRL is >10 weeks) and a positive FHR were observed. All women were nonsmokers,

aged 19–33 years (mean age =27.6 years), and not using any medication.Furthermore, all women had a normal body mass index (BMI) of 20-24 kg/m^2 and a history of regular menstrual cycles. The exclusion criteria for both groups included the presence of chronic inflammatory disease, acute and chronic infections, diabetes mellitus, collagen tissue disease, an extrauterine gestational sac, vaginal bleeding, a recent history of anticoagulant and antiaggregant treatment, a history of recurrent miscarriage, or an unknown last menstrual period (LMP).

Fetal death has been diagnosed by transvaginal ultrasonography and confirmed by repeated ultrasonography prior to the dilation and curettage procedures. The chorionic villi and maternal decidua were separated and cleaned. The placental and decidual tissues were fixed in a 10% buffered formalin solution and embedded in paraffin. The blocks were then cut into 4-µm thick serial sections. The first tissue sections were stained with hematoxylin-eosin using histochemical techniques and the second tissue sections were stained by TNF-α, IL-6, IL-17 antibodies using immunohistochemical techniques.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used for immunohistochemical staining. The tissue samples were stored at 60ºC overnight and dewaxed by xylene for 30 min. After dehydration with ethanol, the sections were washed with distilled water and treated with 2% Trypsin (ab970, Abcam, Cambridge, UK) at 37ºC for 15 min and incubated in a 3% H_2O_2 solution for 15 min to inhibit endogenous peroxidase activity. The sections were then incubated for 32 min at room temperature with primary antibodies for TNF-α NB600-587, 1:150; Novus Biologicals, Littleton, USA),for 20 min at room temperature with primary antibodies for IL-6 (sc-130326, 1:100; Santa Cruz Biotechnology, California,USA), and 35 min for IL-17 (sc-7927, 1:100; Santa Cruz Biotechnology, California,USA).A rabbit linker for TNF-α and IL-17 and a mouse linker for IL-6 were allowed to stand 15 min. In subsequent steps, the sections were processed for 20 min with 100 µL of Envision FLEX/HRP2.The sections were stained brown with 0.01% hydrogen peroxide for 10 min in a solution of 3,3'-diaminobenzidine (Sigma Chemical Co). In the final step, the sections were stained for 1 min with hematoxylin, dehydrated, and made transparent with xylol. The sections were then coated with balsam to allow evaluations with the light microscope.

All immunohistochemical staining processes, including deparaffinization and antigen retrieval, were performed using a Dako LV-1 automated immunostaining system (Dako, Glostrup,Denmark).(Cytoplasmic stains were considered positive for all antibodies). Each immunostained section was analyzed semiquantitatively using a modified 'quick score' method [18] to account for both the intensity of staining (i.e., 0=negative, 1=weak, 2=moderate, and 3=strong) and the percentage of positive cells for each staining intensity (i.e., 1=0–25%, 2 = ≤25–50%, 3=50–75%, and 4= ≥75–100%).

The glandular epithelium, decidual stromal cells, decidual spiral artery, and placental villous stroma cells were all scored separately. For each slide, 10 different fields were evaluated microscopically at 200X magnification. All entire sections were assessed by a single operator (HP) who was blinded to the origin of the sample. The intensity and percentage scores were then multiplied and scores from all tissue sections were added to give a possible total score range of 0–12. For example, for a given cell type, negative staining of 20% (0 × 1 = 0), weak staining in 40% (1 × 2 = 2), and moderate staining in 40% (2 × 2 = 4) would give a total score of 0 + 2 + 4 = 6.

Statistical Analysis

Data are presented as means ± standard deviations. Differences between the decidua and villous expression of cytokines in missed abortions and elective termınatıon of pregnancy (ETP) were analyzed by a Fisher's two-tailed exact test corrected chi-square. Statistical comparisons between the groups were performed using the Mann–Whitney U test. P values <0.05 were accepted as significant.

Results

Based on the karyotyping of the products of conception, 21 missed abortions had a normal karyotype, 18 missed abortions had a abnormal karyotype (including 8 trisomies, 6 triploidies, and 4 monosomy X), and 23 elective abortions had a normal karyotype. There were no differences in maternal age, BMI, parity, and ethnic distributions between groups with or without a chromosomal abnormality. The fetal sex ratio was similar among all groups. The mean gestational age was 9.5–11.2 weeks in all groups. The intensity and prevalence of TNF-α, IL-6, and IL-17 expressions were evaluated at 3 randomly selected points at 4 different locations (i.e., decidual stroma, placental villous stroma, decidual spiral arterioles, and decidual glandular epithelium) in all cases. Cytoplasmic stains were considered positive for all antibodies.

TNF-α

TNF-α protein staining in the villous samples was higher in the missed abortion group compared to the ETP group (p=0,0001; Table I; Figure 1A). TNF-α staining in the villous tissue was significantly lower in the ETP group (0.74 ± 0.91 Table I; Figure 1B) and higher in the miscarriage group without a chromosomal anomaly (3,86±3,76; Figure 1A, 1B) compared to the miscarriage group with a chromosomal anomaly (1,12±1,53; Table I).

IL-6

No differences were observed in IL-6 expressions in the villous samples. IL-6 staining was stronger for glandular epithelial cells in the decidua in women with missed abortions, particularly in those with a chromosomal abnormality, compared women in the the elective abortion group (p=0.003; Table II; Figure 2).

IL-17

No differences in IL-17 expression were observed in the villous, decidual stroma, decidual spiral artery, and decidual glandular epithelial cells in all groups (Table III).

Discussion

Although the fetus is partly allogeneic to the mother, it is not frequently rejected by the maternal immune system. According to current studies, controlled immune cell access is an important component of the feto-maternal interface and is likely to play a role in the immune regulation and protection of the fetus

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Table I. Modified quick score method values of TNF-α protein in decidual stroma, placental vıllous stroma, decidual spiral artery, decidual glandular epithelium.

TNF-α		Missed Abortion (NC)	Missed Abortion (ANC)	ETP	P-value
Decidual Stroma	(Mean ± S.D.)	7,14 ± 4,21	3,65 ± 3,16	1,43 ± 1,37	0,0001
Placental Vıllous Stroma	(Mean ± S.D.)	3,86 ± 3,76	1,12 ± 1,53	0,74 ± 0,91	0,002
Decidual Spiral Artery	(Mean ± S.D.)	1,95 ± 1,83	0,59 ± 0,71	0,57 ± 1,20	0,002
Decidual Glandular Epithelium	(Mean ± S.D.)	4,48 ± 4,47	3,29 ± 2,93	1,17 ± 1,61	0,006

Missed Abortion (NC): Missed abortion with normal chromosomally
Missed Abortion (ANC): Missed abortion with abnormal chromosomally
ETP: Elective Termination of Pregnancy

Table II. Modified quick score method values of the IL-6 in decidual stroma, placental vıllous stroma, decidual spiral artery, decidual glandular epithelium.

IL-6		Missed Abortion (NC)	Missed Abortion (ANC)	ETP	P-value
Decidual Stroma	(Mean ± S.D.)	6,00 ± 4,55	5,65 ± 4,67	6,74 ± 4,95	0,878
Placental Villous Stroma	(Mean ± S.D.)	1,52 ± 2,73	0,76 ± 1,52	1,17 ± 2,18	0,950
Decidual Spiral Artery	(Mean ± S.D.)	1,33 ± 1,82	1,00 ± 1,62	1,83 ± 2,06	0,356
Decidual Glandular Epithelium	(Mean ± S.D.)	0,43 ± 0,97	0,82 ± 1,08	0,04 ± 0,21	0,016

Missed Abortion (NC): Missed abortion with normal chromosomally
Missed Abortion (ANC): Missed abortion with abnormal chromosomally
ETP: Elective Termination of Pregnancy

Table III. Modified quick score method values of the IL-17 in decidual stroma, placental vıllous stroma, decidual spiral artery, decidual glandular epithelium.

IL-17		Missed Abortion (NC)	Missed Abortion (ANC)	ETP	P-value
Decidual Stroma	(Mean ± S.D.)	1,00 ± 1,05	1,06 ± 0,96	0,35 ± 0,57	0,020
Placental Vıllous Stroma	(Mean ± S.D.)	0,24 ± 0,54	0,18 ± 0,39	0,17 ± 0,57	0,668
Decidual Spiral Artery	(Mean ± S.D.)	0,14 ± 0,65	0,29 ± 0,58	0,13 ± 0,45	0,210
Decidual Glandular Epithelium	(Mean ± S.D.)	1,24 ± 1,22	2,18 ± 2,29	1,00 ± 1,81	0,125

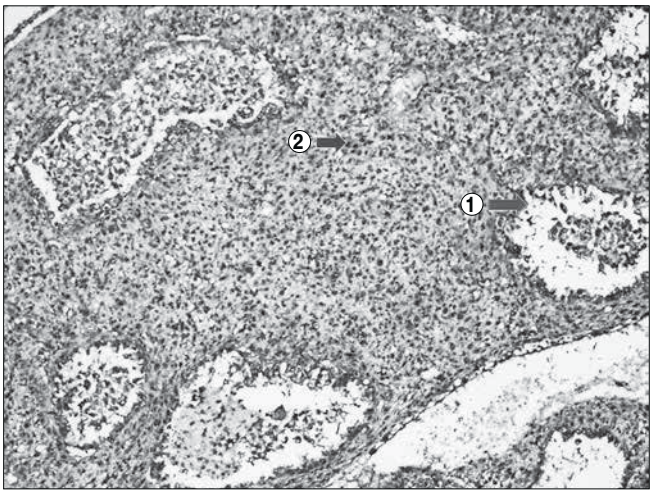
Missed Abortion (NC): Missed abortion with normal chromosomally
Missed Abortion (ANC): Missed abortion with abnormal chromosomally
ETP: Elective Termination of Pregnancy

from a maternal immune attack [19]. In the present study, we assumed that the best way to understand the pathological events in abnormal pregnancy loss with or without a chromosomal anomaly would be to observe the interactions inside the tissues where all these processes occur. In animal studies, Th-1 immunity has been reported to contribute to implantation failure and fetal resorption [20] by affecting local vasculogenesis and hindering the generation of proper spiral arteries [21], whereas the Th-2 cytokines secreted at the feto-maternal interface have proved to be beneficial for maintaining pregnancy by suppressing cellular cytotoxicity [22]. However, as observed in the present study, exaggerated Th-17 immunity primarily has a negative impact during the early stages of pregnancy.

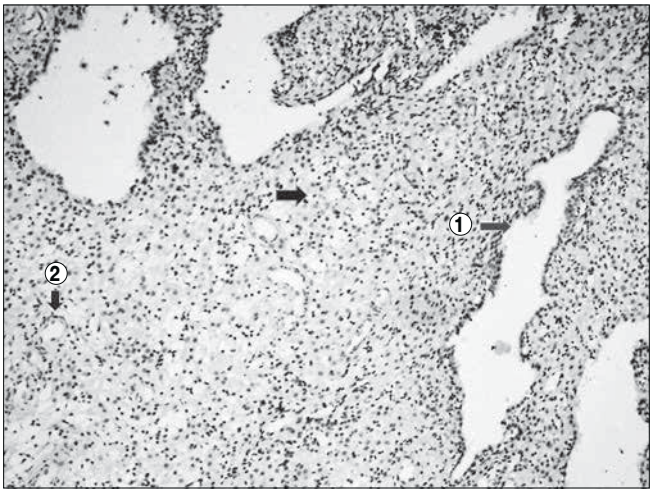
Agius et al. demonstrated an increased maternal systemic inflammatory response with an imbalance in the Th-1/Th-2 ratio in the maternal circulation of patients with pregnancy loss with a normal karyotype [23]. However, they found an increased secretion of the inflammatory cytokine TNF-α and its soluble receptors, TNF-R1 and TNF-R, as well as the anti-inflammatory cytokine IL-10 in villous samples from the same group in cases with an abnormal karyotype compared to those presenting with a normal karyotype. According to these data, the karyotype of the conceptus has a direct impact on the secretion of cytokines by the villous tissue. In the present study, we found that the intensity of TNF-α staining in villous and decidual structures was much higher in the pregnancy loss group than in the Elective Termination of Pregnancy (ETP) group, although the intensity of TNF-α staining in the pregnancy loss group without a chromosome anomaly was higher than in the group with a chromosome anomaly. (The increase in chromosome aberrations alters placental morphology and function, including vascularity, size, and shape).

There are few reports regarding the role of IL-6 in miscarriages and the related results vary significantly. The role of IL-6 in regulating trophoblast invasion is currently not clear. In one study, IL-6 was observed to enhance trophoblast invasion [17]. In fact, a significant if not major role of Th-2 cells is to downregulate Th-1 immunity by suppressing TNF-α and other factors related to Th-1 type cytokine production [24]. Euploid and aneuploid samples from early miscarriages (gestational age ≤12 + 6 weeks) [25] and late miscarriages (gestational age ≥13 weeks) [26] were assessed semiquantitatively. There were no differences in immunostaining between euploid and aneuploid samples between the early and late miscarriage groups. Conversely, Yamada et al. [27] revealed that the fetal karyotype is affected by the cytokine levels present in the maternal circulation in cases of recurrent miscarriages. In the present study, we found that the intensity of IL-6 staining was higher in only the pregnancy loss group with a chromosomal anomaly when compared to the miscarriage group without a chromosomal anomaly and the elective abortion group.

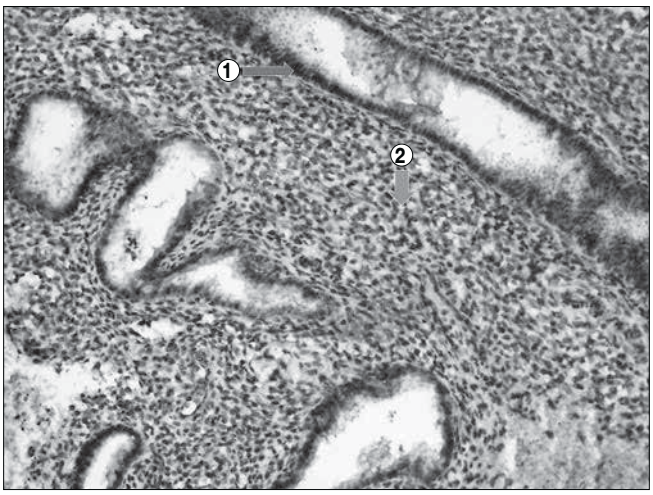
Th-17 cells are derived from CD (4+) T cells when stimulated with the transforming growth factor (TGFb) in the presence of IL-6 [28]. In the absence of IL-6, these cells are more likely to become T regulatory cells (Tregs). This is critical in determining the differentiation of CD (4+) T cells into either Tregs or proinflammatory. Th-17 cells have coexisted with neutrophils in patients who had inevitable abortions. Until very recently, Th-17 cells have only received limited attention in the fields of pregnancy and infertility. However, Wang et al. [29] have recently demonstrated increased levels of Th-17 cells in the peripheral



Rycina 1A. Third degree (**1**) staining in the decidual glandular epithelium and third degree (**2**) staining in the decidual stromal cells with tumor necrosis factor-α (TNF-α) in a patient who had a miscarriage without a chromosome anomaly (DAP×100).



Rycina 1B. Negative staining (**1**) in the decidual glandular epithelium, spiral arterioles (**2**), and decidual stromal cells with TNF-α in a patient who underwent elective pregnancy termination (DAP, ×100).



Rycina 1C. Second degree (**1**) staining in the decidual glandular epithelium and first degree (**2**) staining in the decidual stromal cells with interleukin (IL)-6 in a patient who had a miscarriage with a chromosome anomaly (DAP×100).

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blood and decidua of women with recurrent miscarriages as compared to those with normal pregnancy, which reveal that IL-17 helps maintain pregnancy during the early pregnancy period. In this present study, exaggerated Th-17 immunity has the most negative impact during the early stages of pregnancy. However, we did not find any differences in the intensity of IL-17 staining in either the decidua or the villous tissue in the miscarriage and elective abortion groups.

Thus, the intensity of TNF-α staining was high in the miscarriage group, which is similar to that noted in previous studies. However, the intensity of TNF-α staining was higher in the miscarriage group without a chromosome anomaly than in the group with a chromosome anomaly. Unlike that noted in some previous studies, the intensity of IL-6 staining was higher only in the decidual glandular epithelium in the miscarriage groups. Moreover, the intensity of IL-6 staining was found to be higher in the miscarriage group with a chromosome anomaly than in the miscarriage group without a chromosome anomaly. There was no significant difference in IL-17 levels between any of the groups.

In summary, cytokines are considered to play an important role in the maintenance of pregnancy and may mediate the effect of the karyotype of the conceptus via placental and systemic inflammatory responses, thus resulting in early pregnancy failure. However, the exact mechanism between the cytokines and the mutual regulation relationships are not fully understood and should be investigated in future studies.

Authors' contribution:

1. Esin Kasap – concept, artcicle draft, study design analysis and interpretation of data, acquisition of data, writing article, corresponding author.
2. Serap Karaarslan – concept, article draft, study design, acquisition of data, revised article critically.
3. Mine Genc – analysis and interpretation of data, writing article, revised article critically.
4. Esra B. Gur – writing article, revised article critically.
5. Nur Sahin – revised article critically.
6. Serkan Guclu – revised article critically.

Authors' statement

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