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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

Immunohistochemical study of the immunological markers IL-1β and IL-6 in placental tissues in recurrent pregnancy loss

OBJECTIVE **To examine the relationship between the expression of the cytokines interleukin-6 (IL-6) and IL-1β in human endometrial tissues in the case of recurrent pregnancy loss (RPL) of unexplained etiology.** METHOD **The RPL study group consisted of 30 women who miscarried at least three times during the first trimester of pregnancy, and the control group consisted of 30 women who underwent elective termination of pregnancy during the first trimester. The abortion material was studied using immunohistochemical methods on specimens taken from the decidua parietalis, decidua basalis and trophoblast. Monoclonal antibodies against IL-1β and IL-6 were used. Statistical Package for Social Sciences (SPSS), version 25 was used for the statistical analysis.** RESULTS **IL-1β and IL-6 expression was examined in the decidua basalis, decidua parietalis and trophoblast sections in both the RPL and the control groups. No difference was observed between the RPL and the control groups in IL-1β expression. Regarding IL-6, a difference in color intensity was detected between the two groups in the decidua basalis (p=0.048) and the trophoblast (p=0.044), but not in the decidua parietalis.** CONCLUSIONS **The study results did not support the involvement of IL-1β expression in RPL, but IL-6 expression appeared to have a role in its pathogenesis.**

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ΑΡΧΕΙΑ ΕΛΛΗΝΙΚΗΣ ΙΑΤΡΙΚΗΣ 2022, 39(6):812–818

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Ανοσοϊστοχημική μελέτη ανοσολογικών δεικτών σε ιστούς πλακούντα επαναλαμβανόμενης απώλειας κύησης: IL-1β, IL-6

Περίληψη στο τέλος του άρθρου

Key words

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Recurrent pregnancy loss (RPL) is defined as the occurrence of two or more failed clinical pregnancies before the pregnancy reaches 20 weeks of gestation. Approximately 5% of healthy women during their reproductive period undergo spontaneous abortion.[1] In half of the cases of RPL the cause is unrecognized, and the highly heterogeneous condition makes clarification of etiology difficult.[2] The etiology of RPL has not yet been fully identified, but known risk factors include parental chromosomal abnormalities, specific uterine anatomical abnormalities, hypothyroidism,

diabetes mellitus (DM), and antiphospholipid antibody syndrome. Other risk factors include additional endocrine disorders, thrombophilia, immunological anomalies, infections, and environmental factors.[3,4] In the cases where no cause has been identified, no specific treatment can be recommended,[5] and studies have been conducted in an attempt to clarify the immunological mechanism of RPL.[6–10]

T cells produce cytokines, which are crucial intercessors of signals between cells of the immune system and other cells. Immunological tolerance may be affected whenever modification takes place in the production of cytokines by T cells.[11] The maintenance of pregnancy is based on the homeostatic balance between Th1 and Th2 cytokines; higher expression of Th2 cytokines is observed in normal pregnancies, and pregnancies with a negative outcome are usually associated with higher expression of Th1 cytokines.[12]

Interleukin-6 (IL-6) is a cytokine which has a pleiotropic effect on T cell differentiation and a crucial role in the inflammatory response.[13] IL-6 is a 21–28 kDa glycoprotein, which is produced by various cells, but its primary sources are macrophages, T-lymphocytes and monocytes.[14] The gene encoding human IL-6 has been mapped on the short arm of chromosome 7 (7p21), containing 212 amino acids.[15] The expression of this gene is observed in human endometrial tissues,[16] and IL-6 is a multifunctional cytokine that is produced in the luminal epithelium in a periodical way. During the periods of implantation and menstruation, the levels of production are the highest, and conversely the levels of IL-6 are comparatively low in the proliferative phase, and remain steady all through the secretary phase.[17] A considerable number of polymorphisms in the IL-6 gene have been identified, a few of which are reported to modify its expression. The anti-inflammatory functions of IL-6 are well known, but its possible predictive value in pregnancy outcome remains to be clarified.[14] Raised levels of IL-6 are evident in the altered cytokine profiles of RPL, unexplained infertility, preterm delivery and preeclampsia.[13] Numerous factors control the expression of IL-6, one of which is IL-1.[17]

IL-1 is a pleiotropic, pro-inflammatory cytokine related to cell development, and two forms have been identified, IL-1α and IL-Iβ, which in most cases are difficult to separate biologically.[18] The IL-1 family of cytokines contains 11 proteins (IL-1F1 to IL-1F11) encoded by 11 definite genes in humans and rodents.[19] The IL-1 gene cluster is a 430 kb part of chromosome 2 (2q12-21). IL-1 is produced by monocytes, macrophages, and epithelial cells at the period of fetal-maternal attachment and through early pregnancy, and appears to regulate trophoblast invasion and tissue repair.[20–22] The IL-1 system comprises two agonists (IL1α and IL-1β), two cell-surface receptors (IL1R1 and IL1R2), an accessory protein (IL1RAcP), and a naturally occurring antagonist.[23] As IL-1 can serve as a co-stimulator for Th2 cell generation in both mice and humans, it is possible that the IL-1 system affects the modulation of Th1/Th2 cytokine production and the production of interferon-gamma (IFN-γ), interceded by natural killer (NK) cells and T cells.[24] IL-1 protein production can be modified by single-nucleotide polymorphisms (SNPs) placed in the promoter region of the IL-1 gene group that directly affects transcription, which, in sequence, might be associated with RPL. IL-1β promoter region variants IL1-511C and IL1B-31T with meaningful growth have been identified in women with a history of RPL. Specifically, IL-1β is related to effective embryo implantation, ectopic pregnancy, and preterm birth. IL-1β is expressed in the endometrium, and its expression is raised during the secretory phase of the menstrual cycle, and it is also present in the embryo.[25]

Various factors may be responsible for RPL, but the specific biochemical pathways that lead to spontaneous abortion are still unknown. The purpose of this study was to investigate a possible connection between early pregnancy loss and the cytokines IL-1β and IL-6.

MATERIAL AND METHOD

The study was conducted with a group of 30 women aged 29–41 years, who had miscarried at least three times in the first trimester of pregnancy (the RPL group), and a control group of 30 women aged 27–39 years old who underwent elective termination of pregnancy during the first trimester. The miscarriages in the RPL group were of unidentified etiology.

After informing each patient and obtaining their signed consent in the study, the abortion material was collected and processed.

Pathology examination and immunohistochemistry

The procedures of histopathological examination of the abortion tissue and the process of immunohistochemistry were performed as described in previous studies.[9,10] The immunohistochemical staining was conducted twice for each of the two antibodies reviewed, antibodies IL-1β (Santa Cruz) and IL-6 (Santa Cruz), which were in a dilution of 1:100.

Microscopic evaluation

Microscopic assessment was carried out on the cells of the middle trophoblast, the decidua basalis and the decidua parietalis of the material from repeated miscarriage and optional abortion. Overall, 60 specimens were examined. The intensity of staining was measured as negative (–), weak (+), moderate (++) and strong (+++), by three separate reviewers.

Statistical analysis

Statistical analysis was conducted using the Mann-Whitney test, after checking for distribution, which was not normal. The level of significance was set at 0.05.

RESULTS

No differences were identified on the initial macroscopic examination for placental lesions or malformations between the specimens from the RPL and the control groups.

On staining for IL-1β, minor differences were observed between the RPL and the control group, as shown in figures 1 and 2. Specifically, the colored cells of the decidua parietalis were less dispersed, and fewer colored cells were observed in the decidua basalis in the RPL group. The trophoblast was negative for staining in both groups. The minor differences detected in the immunohistochemical staining of the decidua basalis, decidua parietalis and trophoblast between the two groups were not statistically significant (tab. 1).

Differences were observed between the two groups in the staining for IL-6, as shown in figures 3 and 4. No statistically significant difference was detected in the immunohistochemical staining of the decidua parietalis between the two groups. Intense coloring was observed in the decidua basalis of both groups, and the colored area was more extensive in the RPL group (p=0.048). The syncytial staining was negative in the trophoblast of both groups, but the mesenchyme (mainly the capillaries) was more strongly stained in the RPL group (p=0.044) (tab. 1).

DISCUSSION

Recurrent miscarriage can be caused by numerous factors and constitutes a major challenge in the area of reproductive medicine. One study has demonstrated that the

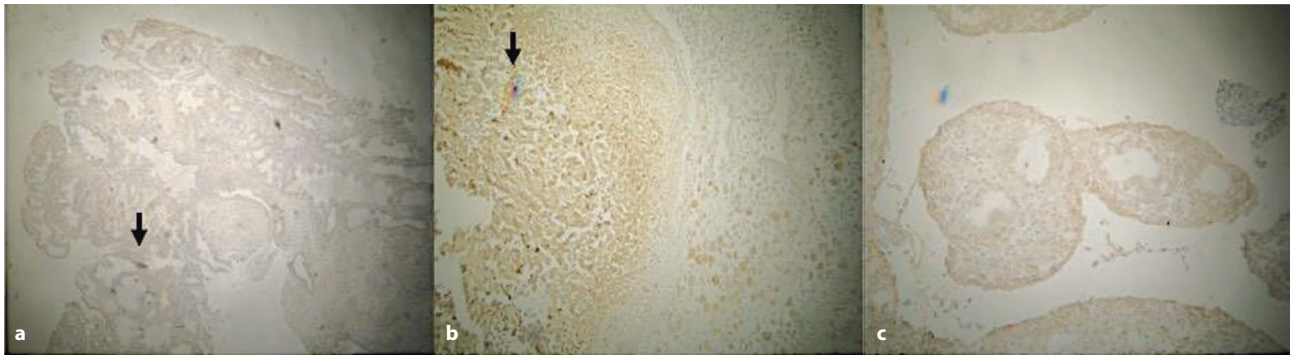

Figure 1. Immunohistochemical examination for IL-1β, control group: (a) Decidua parietalis, showing minor cell dispersion; (b) decidua basalis, showing positive-colored areas with some degree of disorganized external connective tissue and numerous strongly positive cells; (c) trophoblast, showing negative result.

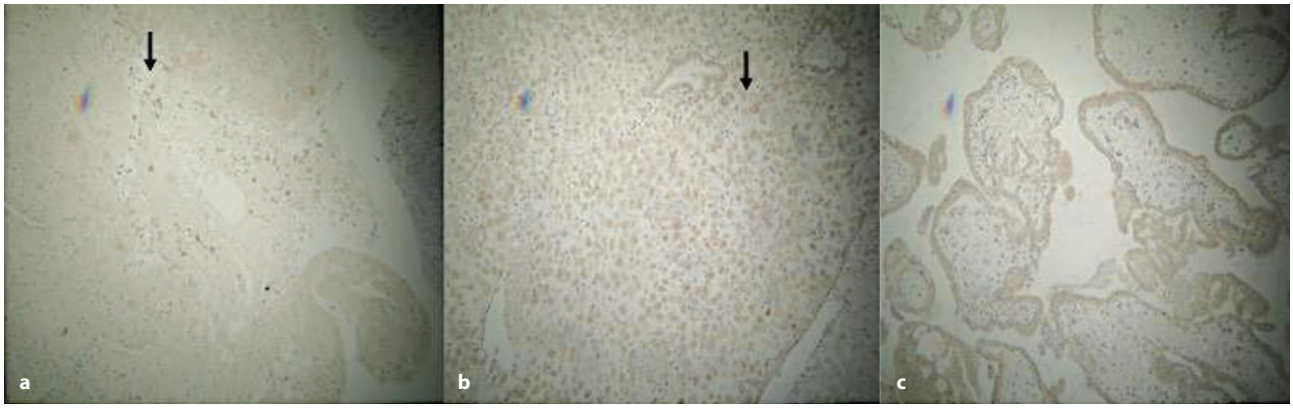

Figure 2. Immunohistochemical examination for IL-1β, RPL group: (a) Decidua parietalis showing minor dispersion of colored cells; (b) decidua basalis showing colored cells (not indicative of cellular decay) arranged in small groups; (c) trophoblast (negative).

Table 1. Immunohistochemical examination for IL-6 and IL-1β: Intensity of staining in decidua and trophoblast tissues from recurrent pregnancy loss group (RPL, n=30) and control group (n=30).

	RPL group decidua basalis	Control group decidua basalis	p-value	RPL group decidua parietalis	Control group decidua parietalis	p-value	RPL group trophoblast	Control group trophoblast	p-value
IL-1β									
(–)	20 (66.7%)	16 (53.3%)		22 (73.3%)	18 (60.0%)		12 (40.0%)	18 (60.0%)	
(+)	8 (26.7%)	8 (26.7%)	0.351	8 (26.7%)	12 (40.0%)	0.273	14 (46.6%)	10 (33.3%)	0.381
(++)	2 (6.7%)	6 (20.0%)		0 (0%)	0 (0%)		4 (13.3%)	2 (6.7%)	
(+++)	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
IL-6									
(–)	4 (13.3%)	3 (10.0%)		14 (46.7%)	14 (46.7%)		19 (63.3%)	19 (63.3%)	
(+)	5 (16.7%)	12 (36.4%)	*0.048*	8 (26.7%)	10 (33.3%)	0.776	6 (20.0%)	11 (36.7%)	*0.044*
(++)	7 (23.3%)	10 (33.3%)		8 (26.7%)	6 (20.0%)		5 (16.7%)	0 (0%)	
(+++)	14 (46.7%)	5 (16.7%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	

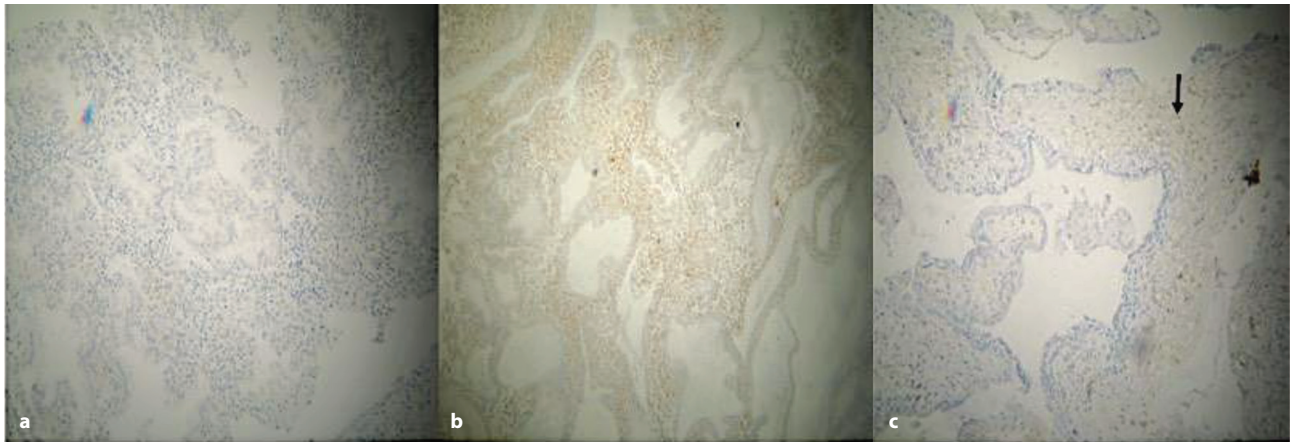

Figure 3. Immunohistochemical examination for IL-6, control group: (a) Decidua parietalis (negative); (b) decidua basalis showing intense cell coloring; (c) trophoblast showing negative syncytium, dispersed colored mesenchymal cells.

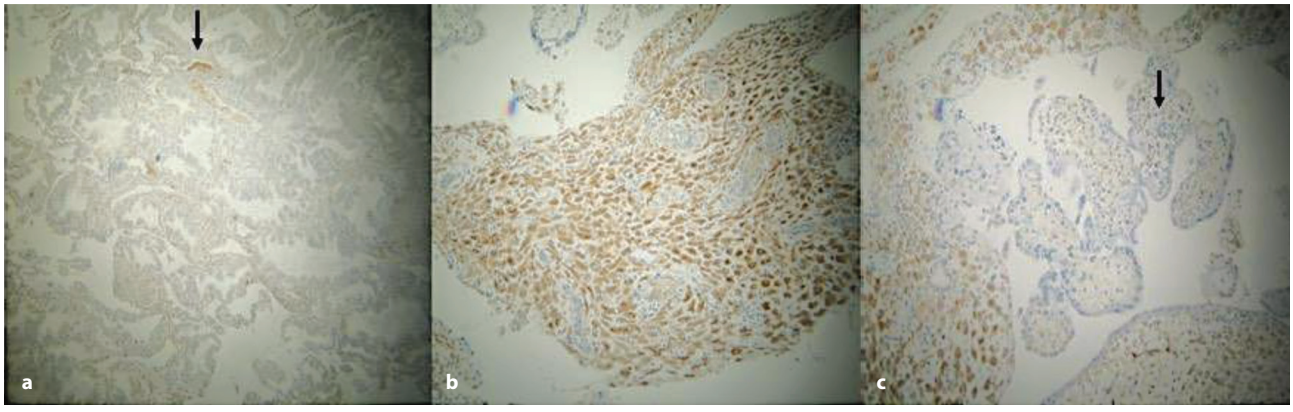

Figure 4. Immunohistochemical examination for IL-6, RPL group: (a) Decidua parietalis showing mesenchyma slightly colored; (b) decidua basalis showing various degrees of intense cell coloring; (c) trophoblast showing negative syncytium, positive coloring of mesenchyma.

increased expression of the immunological factors UCHL1, CD68 and CD14 are associated with RPL.[7] The present study examined the possible involvement of IL-1β and IL-6 in the immunological mechanism of spontaneous abortion of the first trimester. Reports have been published on the immunological profile of women who experienced RPL, but only a few on the involvement of the cytokines IL-1β and IL-6 in spontaneous abortion, although some studies have examined the effects of the many polymorphisms of these cytokines in abortion.

During pregnancy, an inflammatory response is essential for both embryo implantation and timely onset of labor. Pregnancy is a situation of immune activity, with a firmly preserved equilibrium between pro- and anti-inflammatory pathways. Loss of this balance may have an adverse impact on the placenta and subsequently on the fetus.[26] The immune cells located at the touch point between the placenta and the uterus are subject to a level of adjustment by the maternal immune cells.[27] These cells, apart from promoting placental functioning and growth, also minimize the potential of the placenta to attack the fetus.[22]

Several studies have demonstrated the connection of IL-1β with effective embryo implantation, but also with ectopic pregnancy, and early birth.[28] The IL-1β expression in the endometrium is temporally and spatially connected with embryonic implantation.[29] In a serum study, low levels of IL-1β were associated with positive pregnancy outcome (including live birth), while in other studies a negative correlation was demonstrated between IL-1β and embryonic growth.[25] Conversely, other studies showed that reduced IL-1β mRNA in the decidua was associated with unexplained RPL. A relationship between polymorphism in the promoter region of the IL-1β gene and RPL was shown to be associated with Th1 immunity to trophoblast.[24] IL-1β is a co-stimulator for Th2 cell family, and a Th1 immune response to trophoblast antigens may be due to low levels of IL-1β at the fetal-maternal interface.[30] In our study, no difference was observed in IL-1β cell expression in the decidua basalis, decidua parietalis and trophoblast of women with RPL compared with the control group. These results differ from previous studies concerning the IL-1β cell expression in women with RPL.

Increased urine expression of pro-inflammatory cytokines is correlated with RPL,[31] with IL-6 having a major role in embryonic implantation.[17] In an experimental study the expression of IL-6 was found elevated in mice that suffered a miscarriage.[32] It was also shown that the secretion of IL-6 was higher in women in miscarriage and control groups than in a non-pregnant group, but no difference was detected between women with RPL and healthy women.[33] Conversely another study showed that the plasma levels of IL-6 were lower in a miscarriage group than in a control group.[34] The investigation of the possible association of IL-6-634C/G polymorphism with RPL risk revealed that the polymorphic G allele increases the risk of miscarriage in comparison with the C allele.[35] Regarding the decidua basalis and trophoblast, higher intensity of staining for IL-6 was observed in the RPL group in this study, compared to the control group. No difference was detected in the expression of IL-6 in the decidua basalis between the two groups.

Cytokines should be studied further in relation to RPL as, in other conditions, cytokines have been the basis of successful application in immunotherapies over the last few years.[36] It should therefore be expected that in the coming years they could be used in the management of RPL.

In conclusion, according to our study, although the sample was small the expression levels of IL-6 showed differences between the RPL and the control group, suggesting a possible involvement of IL-6 in the pathogenetic mechanism of RPL. In contrast, IL-1β appeared to have no association with RPL. Further research is necessary to confirm these findings in women with RPL, and to explore the possible therapeutic application.

ΠΕΡΙΛΗΨΗ

Ανοσοϊστοχημική μελέτη ανοσολογικών δεικτών σε ιστούς πλακούντα επαναλαμβανόμενης απώλειας κύησης: IL-1β, IL-6

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ΣΚΟΠΟΣ Εξέταση της σχέσης μεταξύ της έκφρασης των κυτταροκινών ιντερλευκίνης-6 (IL-6) και ιντερλευκίνης-1β (IL-1β) σε ανθρώπινο ενδομήτριο ιστό επαναλαμβανόμενης απώλειας κυήσεων χωρίς αιτιολογία. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Την πειραματική ομάδα αποτέλεσαν 30 γυναίκες που απέβαλαν κατά το πρώτο τρίμηνο της κύησης και η ομάδα ελέγχου αποτελείτο από 30 γυναίκες που είχαν διακόψει εκλεκτικά την εγκυμοσύνη τους κατά το πρώτο τρίμηνο της κύησης. Το υλικό των αποβολών υποβλήθηκε σε επεξεργασία και τα δείγματα μελετήθηκαν, χρησιμοποιώντας ανοσοϊστοχημικές μεθόδους. Τα δείγματα ελήφθησαν από τις περιοχές του τοιχωματικού φθαρτού, του βασικού φθαρτού και της τροφοβλάστης. Χρησιμοποιήθηκαν μονοκλωνικά αντισώματα κατά της IL-1β και της IL-6. Για τη στατιστική ανάλυση εφαρμόστηκε το λογισμικό πρόγραμμα Statistical Package for Social Sciences (SPSS), έκδοση 25.0. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Η παρούσα έρευνα εξέτασε την έκφραση της IL-1β και της IL-6 σε ιστοτεμάχια των ανωτέρω περιοχών τόσο στην ομάδα ελέγχου όσο και στην πειραματική ομάδα. Λαμβάνοντας υπ' όψιν την IL-1β, δεν παρατηρήθηκαν διαφορές μεταξύ της πειραματικής ομάδας και της ομάδας ελέγχου. Αντίθετα, ανιχνεύτηκε διαφορά στην ένταση της χρώσης μεταξύ των δύο ομάδων όσον αφορά στην IL-6 στον βασικό φθαρτό (p=0,048) και στην τροφοβλάστη (p=0,044). Δεν ανιχνεύτηκε στατιστικά σημαντική διαφορά στον τοιχωματικό φθαρτό. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Αν και η εν λόγω μελέτη δεν υποστήριξε μια πιθανή εμπλοκή της έκφρασης της IL-1β στην επαναλαμβανόμενη απώλεια κύησης, η έκφραση της IL-6 φάνηκε να εμπλέκεται στους παθογενετικούς μηχανισμούς της.

Λέξεις ευρετηρίου: Επαναλαμβανόμενη απώλεια κύησης, Ιντερλευκίνη-1β, Ιντερλευκίνη-6

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