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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

Evaluation of VEGF, BCL-2, BCL-6 by immunohistochemistry in the endometrial tissue of patients with recurrent pregnancy loss

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ΑΡΧΕΙΑ ΕΛΛΗΝΙΚΗΣ ΙΑΤΡΙΚΗΣ 2021, 38(2):224–230

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OBJECTIVE **To investigate the role of vascular endothelial growth factor (VEGF), B-cell lymphoma 6 protein (BCL-2) and BCL-6 in recurrent pregnancy loss (RPL).** METHOD **Histopathological examination was made of the uterine contents of two groups of women, a RPL group consisting of 20 women aged 35 to 42 years, who miscarried during the first trimester of gestation and a control group of 20 women, aged 27 to 39 years, who had undergone elective termination of pregnancy during the first trimester. The histopathological specimens, derived from the decidua basalis, decidua parietalis and trophoblast, were studied by immunohistochemical methods, using monoclonal antibodies against VEGF, BCL-2 and BCL-6. Statistical analysis was conducted by the Mann-Whitney test.** RESULTS **A statistically significant difference was detected in the expression of BCL-2, BCL-6 and VEGF between the RPL and the elective abortion group, in the decidual cells. In addition, significant difference was observed between the two groups in the expression of both BCL-2 and BCL-6 in the trophoblastic cells, but there was no difference in the VEGF expression.** CONCLUSIONS **This is the first study to document an increased expression of VEGF, BCL-2 and BCL-6 simultaneously, by immunohistochemistry, in the endometrial tissue of patients with RPL. The comprehension of the behavior of these biomarkers could lead to better understanding of the specific pathways involved in RPL. This knowledge could be beneficial for the development of therapeutic methods in the field of recurrent miscarriages.**

Αξιολόγηση των δεικτών VEGF, BCL-2, BCL-6 με ανοσοϊστοχημεία στον ενδομήτριο ιστό ασθενών με επαναλαμβανόμενη απώλεια κύησης

Περίληψη στο τέλος του άρθρου

Key words

BCL-2
BCL-6
Recurrent pregnancy loss
VEGF

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Recurrent pregnancy loss (RPL) is defined as 3 consecutive miscarriages prior to the 20th week of gestation, and it affects up to 5% of couples. Multiple etiological factors have been investigated, such as antiphospholipid syndrome and parental chromosomal abnormalities, but the majority of RPL cases, almost 75%, are idiopathic.[1]

Vascular endothelial growth factor (VEGF) is a heparin binding endothelial cell-specific mitogen that acts as a regulator for angiogenesis. With a single intraarterial bolus administration of VEGF in a rabbit ischemic limb, revascularization was achieved, indicating that it could also act as a healing factor.[2] Genetic polymorphisms of VEGF have been included in the possible etiology of RPL; for example, the VEGF-1154 G/A polymorphism was significantly prevalent among aborted fetuses and their mothers.[3] VEFG is also expressed in placental macrophages (Hofbauer cells), which are located near the trophoblastic cells and fetal capillaries. These cells play an important role in the placental formation and angiogenesis, thus their significance for maintaining a normal gestation.[4]

Proteins of the B-cell lymphoma-2 (BCL-2) family are crucial for controlling apoptosis.[5] They mediate mitochondrial

apoptosis by regulating the permeability of the outer membrane. The functions of BCL-2 proteins are pro-apoptotic and anti-apoptotic.[6] The apoptotic gene polymorphisms are linked to RPL. The association of BAX-248G/A with RPL susceptibility showed that the maternal genes are involved in programmed cell death.[7]

B-cell lymphoma 6 protein (BCL-6) is a molecule that controls B-lymphocyte development, regulating their proliferation and inhibiting differentiation to plasma and memory cells. BCL-6 is essential for the transformation of B cells into B-cell lymphoma.[8] A recent study compared women with suspected endometriosis and abnormal endometrial BCL-6 expression, with women pre-treated by laparoscopy or medical suppression with gonadotropic releasing hormone (GnRH) agonist. The women with abnormal BCL-6 expression had worse reproductive outcomes following embryo transfer, including as high rate of miscarriages.[9]

Previous studies thus showed that these biomarkers might participate in the pathophysiology of recurrent pregnancy loss. In this immunohistochemical study, our purpose was to investigate whether VEGF, BCL-2 and BCL-6 expression in the endometrial tissues was altered in women with RPL, compared with those with normal early pregnancy.

MATERIAL AND METHOD

Approval by the Bioethics Committee of the School of Medicine of the Aristotle University of Thessaloniki was acquired for this study. The RPL group consisted of 20 women aged 35 to 42 years, who miscarried during the 1st trimester of gestation, and the control group consisted of 20 women, aged 27 to 39 years, who underwent elective termination of pregnancy during the first trimester of gestation. All 20 women in the RPL group had a history of at least 3 prior first trimester miscarriages of unexplained etiology (all laboratory examinations were normal, including parental karyotype, intrauterine structural study, luteal phase endometrial biopsy, hormone levels, cervical culture, lupus anticoagulant testing and antibodies to cardiolipin and phosphatidylserine). All the samples of uterine contents were collected and subjected to immunohistochemical analysis after obtaining informed consent from the patients.

Pathology-examination: Tissues

Tissues were collected immediately after miscarriage or elective abortion and washed with distilled water for removal of mucus and blood. The tissues were then studied under a stereoscope, so that the decidua, villus chorion and parts of the embryo could be distinguished and examined for formation abnormalities or placental lesions. Specimens with formation abnormalities or placental lesions were excluded from the study. Samples were collected from the decidua and the villus chorion and stabilized in an aqueous solution of neutral formalin 10% v/v for 12–24 hours. Following this, the samples were placed in an automatic machine for fixation, dehydration, xylene clarification and paraffin embedding. The paraffin-embedded blocks were cut into 3 mm sections and transferred to positively charged and properly prepared glass plates, which were kept in an oven, at 37–40 °C for 30–45 min, then stained with hematoxylin-eosin solution (Harris). The stained sections were examined with a microscope and the most suitable were selected for immunohistochemical study.

Pathology-examination: Immunohistochemistry

In all specimens, the decidua basalis was identified using the antibody cytokeratin (CK7), which is positive in trophoblastic cells. For discrimination between decidual and trophoblastic cells at the fetomaternal interface, duplicate sections were stained with a monoclonal antibody against prolactin for visualization of decidual cells (fig. 1).

The unstained specimens were further processed using an automatic machine (Bond Max) that carried out the following procedures. First, deparaffinization in xylene, then immersion in absolute alcohol in decreasing concentrations, 100%, 96% and 70% v/v consecutively, and then rinsing with distilled water. Antigen retrieval was performed by incubation at various temperatures, depending on the antibody under examination.

The specimens were first rinsed with PBS buffer, then incubated in H_2O_2 for 5 min to quench endogenous peroxidase activity, and finally rinsed again with PBS buffer. The specimens were then covered with a solution of the primary tonic monoclonal antibody, one of the three used in our study: VEGF (low pH solution 1:50, Dako), BCL-2 (high pH, solution 1:200, Dako), and BCL-6 (high pH,

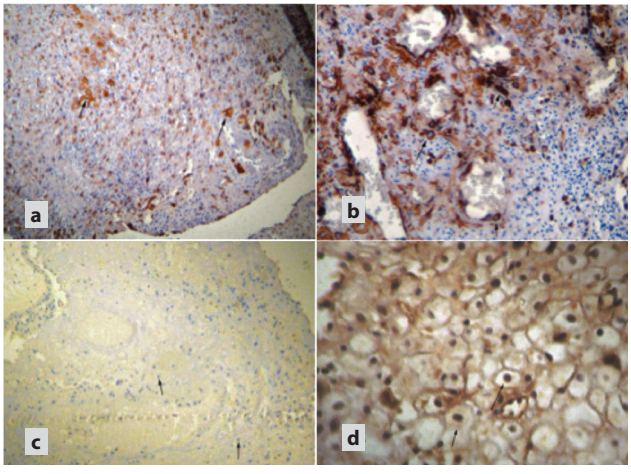

Figure 1. (a) Implantation site – CK7. Detection of trophoblastic cells (×40). **(b)** CK7. Detection of trophoblastic cells (×71). **(c)** Implantation site – prolactin. Detection of decidual cells (×16). **(d)** Implantation site – prolactin. Detection of decidual cells (×160).

solution 1:50, Leica). Finally, the specimens were washed using WAS solution.

For the detection of immunohistochemical staining, the specimens were firstly immersed in post-primary solution. After being washed, the specimens were immersed in polymere solution and then in chromogen diaminobenzidine (DAB) solution, and stained with Hematoxylin by the automatic processor. The specimens were rinsed in tap water and dehydrated with escalating densities of ethanol solution (70%, 96%, and 100% v/v consecutively) and xylene, then covered with tape, placed in glass plates and immersed in Canada balsam. This immunohistochemical staining procedure was repeated for each of the antibodies examined in this study.

Microscopic evaluation

Microscopic evaluation was made by two independent researchers on the cells of the intermediate trophoblast, the decidua basalis and the decidua parietalis, using an optical Zeiss™ microscope, and photographs were taken using a Contax™ camera, attached to the microscope. The intensity of staining was evaluated as negative (–), weak (+), moderate (++) and strong (+++).

Statistical analysis

The results were checked for their significance using the Mann-Whitney test.

RESULTS

No VEGF expression was detected in the decidual cells of the elective abortion (control) group, and the rest stained weakly. Negative expression for BCL-2 and BCL-6 was observed in the decidual cells of 15 specimens and low BCL-2 and BCL-6 expression in 5 specimens of the control group. In the decidual cells of the RPL group, negative VEGF expression was detected in 4 cases, low expression in 4 cases, moderate expression in 7 cases and strong expression in 3 cases. In the same group, negative BCL-2 expression was observed in 2 specimens, low in 4 and moderate in 10 specimens. Similarly, BCL-6 expression was negative in 5 cases, weak in 9 and moderate in 3 cases; 25% of RPL specimens displayed weak BCL-6 staining with scattered stained cells, in the extracellular matrix of their decidual cells. Regarding VEGF, the decidua basalis was stained moderately in sites of new capillary generation. Many VEGF stained vessels were also detected at the same sites.

In the trophoblastic samples of the control group, 6 specimens were negative and 14 stained weakly for VEGF. In the RPL group, 8 specimens presented negative staining, 6 weak, 1 moderate and 5 strong staining for VEGF. In the same group, 4 cases showed no expression of BCL-2, 6 cases displayed weak and 10 staining of moderate intensity. No expression of BCL-2 was detected in trophoblastic cells in 15 cases of the control group, and the other 5 cases presented a weak expression. No expression of BCL-6 in the trophoblast was detected in 15 cases, and weak expression in 5 cases of the control group. In the RPL group, BCL-6 expression was negative in 4 cases, weak in 8 and moderate in 8 cases (figures 2, 3 and 4).

BCL-2, BCL-6 and VEGF immunostaining was independent of gestational week or age of the patients. The Mann-Whitney test demonstrated a statistically significant difference between the two groups in the expression of BCL-2, BCL-6 and VEGF in the decidual cells. Statistically significant difference was also detected in both BCL-2 and BCL-6 expression in the trophoblastic cells between the two groups, but not in VEGF expression (tables 1, 2).

DISCUSSION

It is an undeniable fact that the dysregulation of VEGF, BCL-2 and BCL-6 plays a significant role in the pathogenesis of RPL. VEGF and connexin 43 (Cx43) appear to be significant for the angiogenesis and the development of the placenta, and thus alterations in their expression may contribute to RPL. The expression of Cx43 and VEGF in the villi and decidua of women with RPL were found

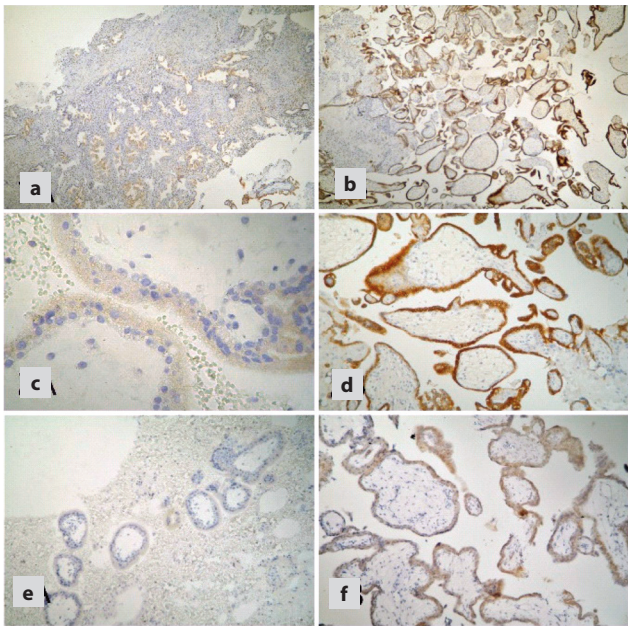

Figure 2. Immunohistochemical evaluation of B-cell lymphoma-2 protein (BCL-2) expression. Decidua parietalis: **(a)** Programmed abortion (control) (×16); **(b)** Miscarriage (×16). Trophoblast: **(c)** Programmed abortion (control) (×160); **(d)** Miscarriage (×16). Decidua basalis: **(e)** Programmed abortion (control) (×40); **(f)** Miscarriage (×40).

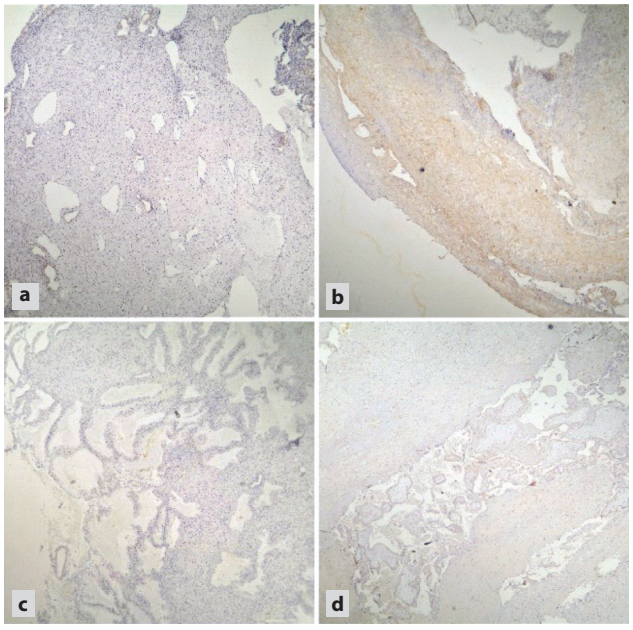

Figure 3. Immunohistochemical evaluation of B-cell lymphoma-6 protein (BCL-6) expression. Decidua parietalis: **(a)** Programmed abortion (control) (×16); **(b)** Miscarriage (×16). Decidua basalis and the creation of trophoblasts: **(c)** Programmed abortion (control) (×16); **(d)** Miscarriage (×16).

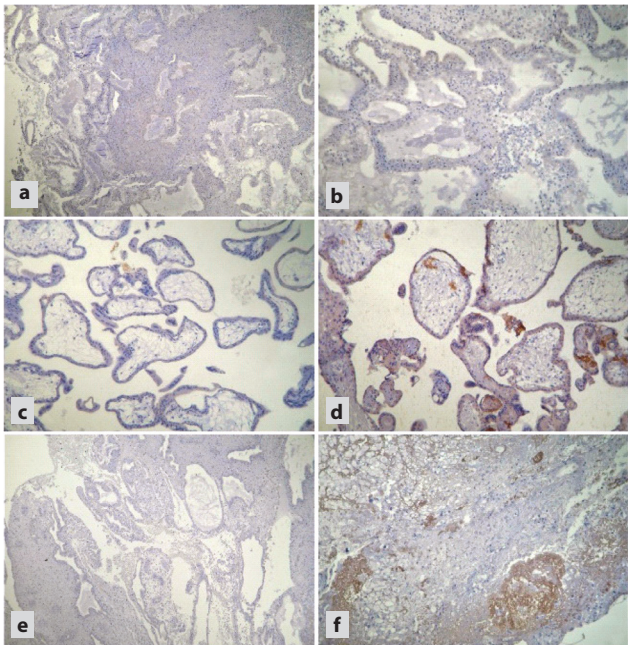

Figure 4. Immunohistochemical evaluation of vascular endothelial growth factor (VEGF) expression. Decidua parietalis: **(a)** Programmed abortion (control) (×16); **(b)** Miscarriage (×16). Trophoblast: **(c)** Programmed abortion (control) (×40); **(d)** Miscarriage (×40). Decidua basalis: **(e)** Programmed abortion (control) (×16); **(f)** Miscarriage (×16).

Table 1. VEGF, BCL-2, BCL-6 staining intensity in trophoblastic cells, n (%) from women with recurrent pregnancy loss (RPL) and those with programmed abortion (control).

Marker expression	VEGF		BCL-2		BCL-6	
	Control group	RPL group	Control group	RPL group	Control group	RPL group
Negative (–)	6 (30%)	8 (40%)	15 (75%)	4 (20%)	15 (75%)	4 (20%)
Weak (+)	14 (70%)	6 (30%)	5 (25%)	6 (30%)	5 (25%)	8 (40%)
Moderate (++)	0 (0%)	1 (5%)	0 (0%)	10 (50%)	0 (0%)	8 (40%)
Strong (+++)	0 (0%)	5 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Sum	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)

VEGF: Vascular endothelial growth factor, BCL-2: B-cell lymphoma-2 protein, BCL-6: B-cell lymphoma-6 protein

Table 2. VEGF, BCL-2, BCL-6 staining intensity in decidual cells from women with recurrent pregnancy loss (RPL) and those with programmed abortion (control), n (%).

Marker expression	VEGF		BCL-2		BCL-6	
	Control group	RPL group	Control group	RPL group	Control group	RPL group
Negative (–)	14 (70%)	4 (20%)	15 (75%)	2 (10%)	15 (75%)	6 (30%)
Weak (+)	6 (30%)	6 (30%)	5 (25%)	8 (40%)	5 (25%)	5 (25%)
Moderate (++)	0 (0%)	7 (35%)	0 (0%)	10 (50%)	0 (0%)	9 (45%)
Strong (+++)	0 (0%)	3 (15%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Sum	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)

VEGF: Vascular endothelial growth factor, BCL-2: B-cell lymphoma-2 protein, BCL-6: B-cell lymphoma-6 protein

significantly decreased, compared with those of women in normal early pregnancy.[10]

It has been indicated that placental ischemia/hypoxia and endothelial dysfunction are important etiological factors in miscarriages. A significant increase has been documented in VEGF soluble receptor-1 (sFlt1) and VEGF expression in women with recurrent spontaneous abortions compared with control subjects.[11] Transcription and translation of VEGF is regulated by hypoxia inducible factor-1 (HIF-1α) and the two factors are involved in placental trophoblast invasion and angiogenesis. It also appears that patients with unexplained recurrent spontaneous abortions demonstrate an increased expression of HIF-1α, VEGF and micro-lymphatic vessel density (MVD) in the placental villi, indicating that VEGF may be involved in vascular dysplasia in miscarriage.[12]

The endometrium is a highly angiogenic tissue and process of angiogenesis is likely to be altered in women with RPL. Immunostaining intensity for several angiogenic growth factors was shown to be significantly different in women with RPL, particularly in the vascular smooth muscle cells (VSMCs). VSMC expression of VEGF-R1 and VEGF-R2 was increased while expression of VEGF-A and VEGF-C was reduced.[13]

VEGF proteins with their receptors, along with the Tie receptors (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains), are crucial for the development of the embryo. The expression of these molecules was found altered in recurrent miscarriages, as VEGF and Tie-1 immunoreactivity was diminished in the placental trophoblast.[14]

A recent meta-analysis described the association between VEGF polymorphisms and RPL. The polymorphisms rs1570360 (especially in Caucasians) and rs3025039 (especially in East Asians) are potential risk factors for the manifestation of RPL.[15] Another recent study showed that gene-gene interactions of angiogenesis and endothelial dysfunction-related gene polymorphisms are also associated with RPL.[16]

Consistent with these findings, our study demonstrated that levels of VEGF were increased in the RPL group, and we can speculate that intensified expression of VEGF in patients with RPL contributes to abnormal vessel formation and may result in recurrent miscarriage.

BCL-2, BCL-6 and their association with RPL have been investigated in previous studies. The expression of BCL-2 and FAS was significantly higher in women with idiopathic infertility and lower in women suffering from RPL. Expression of cleaved caspase-3 has been found reduced in women with idiopathic infertility, compared to RPL groups. Expression of FASL was similar in both groups. Thus, alterations in the pathway of apoptosis are linked to RPL.[17]

Programmed cell death by apoptosis occurs in both fetal and maternal tissues during early pregnancy. The role of apoptosis in RPL was investigated in another study on the BCL-2 immunoreactivity in the villi syncytiotrophoblast of women with RPL, which showed that staining intensity was consistently greater in the group of women who underwent surgical termination of gestation.[18]

On the other hand, the expression of the apoptotic p53 protein has been found significantly increased in the placental tissues from women with RPL, and the expression of anti-apoptotic BCL-2 antibodies increased in the control subjects. Apoptosis in the placenta plays a vital role in pregnancy continuation.[19] Expressed miRNAs have been differentially identified in the placental decidua and villi in recurrent miscarriages. It was also found that the expression of BCL-2 and Pten, a predicated target gene of hsa-miR-1 or hsa-miR-372, respectively, was significantly up-regulated in the villi of recurrent miscarriages. The pathophysiology of miscarriages could be associated with the alternative expression of miRNAs profiles in the decidua and villi.[20]

Regarding BCL-6 and its relation to miscarriages, intense expression has been shown in the decidua of patients with unexplained recurrent spontaneous abortions. Similarly, B lymphocyte-induced maturation protein 1 (Blimp-1) also presented an increased expression in the decidua.[21] Evaluation of the endometrial BCL-6 expression as a prognostic biomarker in women with unexplained infertility showed that its aberration is associated with poor reproductive outcomes in subsequent *in-vitro* fertilization cycles.[22]

In accordance with these studies, we found increased expression of BCL-2 and BCL-6 in the specimens of our RPL group. This may lead to an abnormal pathway of apoptosis during embryonic implantation, and their dysregulation may have an important role in the pathogenesis of recurrent miscarriages.

In conclusion, in the final analysis, aberrant angiogenesis and apoptosis appear to be the main factors in the pathophysiology of RPL. This is the first study to document the expression of VEGF, BCL-2 and BCL-6 concurrently, by immunohistochemistry, in the endometrial tissue of patients with RPL. Future studies are needed to elucidate the role of VEGF, BCL-2 and BCL-6 in the pathological mechanisms of angiogenesis and apoptosis. These pathways could be the "key" for future development of therapies for recurrent miscarriage.

ΠΕΡΙΛΗΨΗ

Αξιολόγηση των δεικτών VEGF, BCL-2, BCL-6 με ανοσοϊστοχημεία στον ενδομήτριο ιστό ασθενών με επαναλαμβανόμενη απώλεια κύησης

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ΣΚΟΠΟΣ Η διερεύνηση του ρόλου των δεικτών VEGF, BCL-2 και BCL-6 στις επαναλαμβανόμενες αποβολές. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Η μια ομάδα μελέτης αποτελείτο από 20 γυναίκες, 35–42 ετών, οι οποίες απέβαλαν κατά τη διάρκεια του πρώτου τριμήνου της κύησης, και τη δεύτερη ομάδα, ομάδα ελέγχου, αποτελούσαν 20 γυναίκες, 27–39 ετών, οι οποίες είχαν εκούσια διακόψει τις εγκυμοσύνες τους κατά το πρώτο τρίμηνο της κύησης. Τα ιστοπαθολογικά δείγματα προήλθαν από τον βασικό φθαρτό, τον τοιχωματικό φθαρτό και την τροφοβλάστη και μελετήθηκαν ανοσοϊστοχημικά. Χρησιμοποιήθηκαν μονοκλωνικά αντισώματα έναντι των VEGF, BCL-2 και BCL-6. Τα αποτελέσματα αναλύθηκαν στατιστικά με τη δοκιμασία Mann-Whitney. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Ανιχνεύτηκε μια στατιστικά σημαντική διαφορά στην έκφραση των BCL-2, BCL-6 και VEGF μεταξύ της ομάδας αποβολών και της ομάδας ελέγχου στα φθαρτικά κύτταρα. Επί πλέον, παρατηρήθηκε στατιστικά σημαντική διαφορά τόσο στην έκφραση BCL-2 όσο και στην BCL-6 στα τροφοβλαστικά κύτταρα μεταξύ των δύο ομάδων, ενώ δεν υπήρχε διαφορά στην έκφραση του VEGF. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Πρόκειται για την πρώτη μελέτη που τεκμηριώνει ταυτόχρονα την έκφραση των VEGF, BCL-2 και BCL-6, με ανοσοϊστοχημεία, στον ενδομήτριο ιστό ασθενών με επαναλαμβανόμενη απώλεια κύησης. Η ομάδα μελέτης παρουσίασε αυξημένη ένταση των δεικτών σε σχέση με την ομάδα ελέγχου. Η διερεύνηση των εν λόγω βιοδεικτών ενδέχεται να οδηγήσει στην καλύτερη κατανόηση της παθοφυσιολογίας των επαναλαμβανόμενων αποβολών. Η γνώση αυτή είναι ιδιαίτερα σημαντική, καθ' ότι μπορεί να συνδράμει στον σχεδιασμό και στην αποτελεσματικότητα μελλοντικών θεραπευτικών μεθόδων.

Λέξεις ευρετηρίου: BCL-2, BCL-6, Επαναλαμβανόμενη απώλεια κύησης, VEGF

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