The role of selectins in the first trimester pregnancy loss

Rola selektyn we wczesnych utratach ciąż

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Abstract

Objective: *There are no well-defined findings about reasons for first trimester abortion in some pregnancy cases. Selectins are cell adhesion proteins which are important for blastocyst implantation in the decidua. The goal of the study was to investigate the role of selectins in first trimester pregnancy loss by immunohistochemistry.*

Study design: *Decidual and placental tissue samples have been obtained from the women with unwanted pregnancy as the control group (n=40) and missed abortion (n=40) as the study group. Immunohistochemistry technique has been used to compare P, L and E-selectin expression of the fibroblast and the decidual cells in uterine decidual stroma; and fibroblasts and mesenchymal cells in placental villous stroma. Immunostaining for P,L,E-Selectin has been evaluated semiquantitatively by HSCORE analysis.*

Results: *Decidual cells, for E and L-selectin showed stronger staining in the study group than controls, and the difference was statistically significant (p = 0.001, p = 0.001). P-selectin showed stronger staining in the control group, but the difference was not as significant as the E and L-selectins (p=0.04). In the placenta, cytotrophoblasts and syncytiotrophoblasts showed stronger staining for P,E,L-selectins for the control group (p<0.001, p=0.001 and p<0.001, respectively).*

Conclusion: *Strong expression of each of the three investigated selectins in healthy pregnancy villi shows their contribution to implantation and strong placentation. There is a need for better understanding of the functions of adhesive molecules in these events to reveal unknown causes for pregnancy loss.*

Key words: **selectins** / **pregnancy** / **missed abortion** /

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Streszczenie

Cel: *nie znane są dobrze udokumentowane przyczyny poronień w I trymestrze ciąży. Selektyny są białkami adhezyjnymi biorącymi udział w implantacji blastocysty w doczesną. Celem badania była immunohistochemiczna ocena roli selektyn we wczesnych (I trymestr) utratach ciąż.*

Materiał i metoda: *kosmówkę i doczesną uzyskano od kobiet, które poddały się aborcji z powodu niechcianej ciąży (grupa kontrolna, n = 40) oraz od kobiet z poronieniem zatrzymanym (grupa badana, n=40). Przy pomocy immunohistochemii porównano ekspresję P,L i E-selektyny w fibroblastach i komórkach doczesnej oraz w fibroblastach i komórkach mezenchymy w podścielisku kosmków łożyskowych. Barwienie na P,L i E-selektyny oceniono półilościowo metodą HSCORE.*

Wyniki: *komórki doczesnej w grupie badanej wykazały się silniejszym barwieniem na E i L-selektyny niż grupa kontrolna, a różnica ta była istotna statystycznie (p=0,001, p=0,001). Barwienie na P-selektyny było silniejsze w grupie kontrolnej, ale różnica była istotna nie tak istotna statystycznie jak dla E i L-selektyn (p=0,04). W łożysku, cytotrofoblaście i syncytiotrofoblaście barwienie na P,E i L-selektyny było silniejsze w grupie kontrolnej niż w grupie badanej (p<0,001; p=0,001; p<0,001).*

Wnioski: *Silna ekspresja każdej z trzech badanych selektyn w kosmkach prawidłowej ciąży wskazuje na ich związek z implantacją i tworzeniem łożyska. Potrzebne jest lepsze zrozumienie funkcji molekuł adhezyjnych w implantacji ciąży aby odkryć nieznane przyczyny wczesnej utraty ciąż.*

Słowa kluczowe: **selektyny** / **ciąża** / **poronienie zatrzymane** /

Introduction

Expulsion from the uterus of an embryo or a fetus and other pregnancy tissues, completely or partially, before 20 gestational weeks and less than 500 grams is accepted as an abortion [1]. Approximately 12%-15% of all pregnancies result in a miscarriage [2]. However, that represents the visible part of an iceberg; with the subclinical abortions the ratio reaches up to 60% of all pregnancies [3].

Recurrent pregnancy loss affects 1% of women in the reproductive age and the cause remains unknown in 50% of the cases [4]. These high rates might be decreased by a complete explanation of the factors affecting the implantation. The issue of implantation failures attracts attention to immunological events and selectins - adhesion molecules that regulate the interactions among leukocytes, platelets, and endothelial cells in these events [5].

Selectins, a family of single-chain transmembrane glycoproteins, are found on the endothelial cells, leukocytes and platelets [6, 7, 8]. Lymphocytic selectin (L-selectin), produced mostly by lymphocytes but also monocytes, granulocytes and Weibel-Palade bodies of the endothelial cells, and platelet selectin (P-selectin), situated in alpha granules, start the characteristic rolling movement of leukocytes on the endothelium [8, 9]. After these two interactions take place, endothelial selectin produced in the endothelial cells (E-selectin) displays a powerful effect, causing leukocyte extravasation, i.e. movement of leukocytes from blood vessels to lymphoid tissues and inflammation areas [8, 10]. The extravasations of leukocytes from the blood to the tissue of the pregnant uterus needs to be a well-controlled process, to ensure the recruitment of the appropriate cells to the correct location at the right time [11]. Consequently, selectins take part in the inflammatory and immune response by regulating leukocyte movement [8].

Selectins mediate adhesion by supplying the heterophilic interaction with endothelial cells and leukocytes [12]. An interaction between blastocysts and uterine surface epithelium must occur before the trophoblastic cells invade the epithelium on the uterine wall.

The expression of L-selectin ligands located in the endometrium increases in the luteal phase. Simultaneously, the expression of L-selectin located in trophoblastic cells increases as well [12]. P-selectin modulates the interaction of leukocytes and platelets with the endothelium [13]. Several experimental studies have shown a significant role of P-selectin in thrombus formation and induction of prothrombotic state [14], thus raising the question of a relationship between recurrent pregnancy loss and P-selectin [15].

These links demonstrate the importance of selectins, which take part in leukocyte extravasation, while investigating causes for pregnancy loss. Publications on all three of these selectins and their role in early pregnancy loss are scarce [16, 11]. Acar et al., [16] investigated plasma levels of selectins in humans and revealed the effect of all three selectins on missed abortions and Fernekorn et al., [11] studied pregnant mice with the use of immunohistochemical methods. We realized the scarcity of literature on the subject, therefore we aimed to investigate the role of selectins in human pregnancy loss at the maternal-fetal interface with immunohistochemical methods by comparing healthy term pregnancies with those ending with abortion.

Materials and methods

A total of 80 pregnant women who underwent dilation and curettage procedure at Celal Bayar University Department of Obstetrics and Gynecology were included into the study. Chorion and decidua samples from 40 women with unwanted pregnancy and 40 women with missed abortion have been obtained during curettage. Inclusion criteria into the study group were: CRL (crown-rump length) corresponding to gestational age of 6-11 weeks with transvaginal ultrasound and negative FHR (fetal heart rate). The control group consisted of unwanted pregnancies (pregnant women who wished curettage). In the control group, CRL was shorter than 10 weeks (curettage is not allowed if CRL is more than 10 weeks) and positive FHR was observed. Exclusion criteria for both groups were: chronic inflammatory disease, acute and chronic infections, diabetes mellitus, collagen tissue disease, recent history of anticoagulant and antiaggregant treatment.

Fetal death has been diagnosed by transvaginal ultrasound and confirmed by repeat ultrasound prior to the dilation and curettage procedure. Chorionic villi and maternal decidua have been separated and cleaned. Placental and decidual tissues have been fixed in 10% buffered formalin solution and embedded in paraffin. The blocks have been cut in 4-5 μm thick serial sections. The first tissue sections have been stained with Hematoxylin-Eosin of histochemical technique and the second sections have been stained by L- selectin, E- selectin and P-selectin primary antibodies by immunohistochemical technique.

Immunohistochemistry:

Formalin-fixed, paraffin-embedded sections have been used for immunohistochemical staining. The tissue samples have been stored at 60°C overnight and then have been dewaxed by xylene for 30 min. After dehydration with ethanol, the sections have been washed with distilled water. Then, they have been treated with 2% trypsin (ab970, Abcam, Cambridge, UK) at 37°C for 15 min. and incubated in 3% H2O2 solution for 15 min. to inhibit endogenous peroxidase activity. Then, the sections have been incubated with anti-L-selectin primary antibody (NCL-CD62L-489, Novocastra, UK), anti-E-selectin primary antibody (NCL-CD62L-382, Novocastra, UK) and anti-P-selectin primary antibody (NCL-CD62L-367, Novocastra, UK) in a 1/100 dilution for 18 h at +4°C. They have been given three additional 5-min washes in PBS, followed by incubation with biotinylated IgG and administration of streptavidin peroxidase (Histostain Plus kit Zymed 87-9999; Zymed, San Francisco, CA). After washing the secondary antibody with PBS three times for 5 min, the sections have been stained with DAB Substrate system containing diaminobenzidine (DAB, K007, DBS, Pleasanton, CA, USA) to detect the immunoreactivity and then have been stained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) for counterstaining. They have been covered with mounting medium (01730 Surgipath, Cambridge, UK) and have been observed with light microscopy (Olympus BX-40, Tokyo, Japan).

Immunostaining for P, E, L-selectin has been evaluated semiquantitatively by HSCORE analysis. Immunostaining intensity has been categorized into the following scores: 0 (no staining), 1 (weak but detectable staining), 2 (moderate staining), and 3 (intense staining). A HSCORE value has been derived for each specimen by calculating the sum of the percentage of cells for fibroblast and decidual cells in the uterine decidual stroma; and fibroblasts and mesenchymal cells in the placental villous stroma that stained at each intensity category multiplied by its respective score, by means of the formula H-score=∑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 epithelial cells for each intensity, varying from 0 to 100%. For each slide, ten different fields have been evaluated microscopically at 200x magnification. HSCORE evaluation has been 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 has been utilized.

Statistical analysis:

The statistical package SPSS for Windows 15.0 (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL) was used

Table I. HSCORE values of P, E, L-selectin in decidual stroma.

Group			P-selectin	E-selectin	L-selectin
Control		Mean± Std. Deviation	24.90±6.74	30.10±6.74	66.40±10.02
group	n=40	Min-max	10-33	16-54	54-81
Study		Mean± Std. Deviation	19.50±6.05	55.00±8.01	124.30±29.07
group	n=40	Min-max	6-28	43-71	60-157
		Mean Differences	5.4	24.9	57.9
		P	0.04	0.001	0.001

Table II. HSCORE values of P, E, L-selectin in villous stroma.

Group			P-selectin	E-selectin	L-selectin
Control		Mean± Std. Deviation	80.90±15.31	80.10±30.13	246.60±33.50
group	n=40	Min-max	56-107	52-152	204-296
Study		Mean± Std. Deviation	18.60±5.05	44.80±11.82	144.20±25.84
group	n=40	Min-max	10-25	24-61	116-190
		Mean Differences	62.3	35.3	102.4
		P	<0.001	0.001	<0.001

to analyze the data. Statistical comparisons between groups were performed using the Mann–Whitney U test. Mean and standard deviations were used to describe data. P values less than 0.05 were accepted as significant.

Results

The median of gestational week was 8 weeks (range 6-11) in the study group and 7 weeks (range 5-9) in controls. Five of the 40 women with missed abortion had experienced 2 or more abortions (12.5%), whereas the rest of the women had no history of abortion (87.5%).

P, E, L-selectin immunoreactivity has been evaluated in both, uterine decidual stroma and placental villous stroma of the control and study groups. The H-Score values of E and L-selectin staining fibroblast and decidual cell in the uterine decidual stoma were higher in the study group (55.00±8.01, 124.30±29.07, respectively) than controls (30.10±10.37, 66.40±10.02, respectively). (Table I), (Figure 2, b-a and Figure 3, b-a).

The differences between the two groups were statistically significant (p=0.001, p=0.001). P-selectin staining in the uterine decidual stroma was higher in the control group (24.90±6.74) than the study group (19.50±6.05) and the difference was statistically significant (p=0.04). (Table I), (Figure 1, a-b).

The staining for P, E and L-selectin of fibroblasts and mesenchymal cells has also been low in the placental villous stroma in the study group (18.60±5.05, 44.80±11.82 and 144.20±25.84, respectively) as compared to controls (80.90±15.31, 80.10±30.13 and 246.60±33.50, respectively).

The differences between the two groups were statistically significant ($p<0.001$, $p=0.001$ and $p<0.001$, respectively). (Table II), (Figure 1, d-c, Figure 2, d-c and Figure 3, d-c). Bars of H-SCORE, for each of the three selectins in the decidual and villous stroma, are presented in figures 4 and 5, respectively.

Discussion

First trimester pregnancy loss is a common and serious problem among women in the reproductive age. Approximately 12 to 15% of pregnancies result in abortion [2]. Recent evidence demonstrated that controlled immune cell access is an important component of the maternal-fetal interface and is likely to play a role in the immune regulation and protection of the fetus from maternal immune attack [11]. We conducted this study thinking of that the best way of understanding the pathological events in pregnancy losses would be to show the interactions inside the tissues where all these processes take place. To the best of our knowledge, our study has been the first to evaluate each of these three selectins by immunohistochemical methods in human pregnancy. We found that each of these three selectins were expressed stronger by placental villous stroma in the control group than in the study group. This demonstrates that all of these three selectins, particularly L-selectin, must be expressed in trophoblasts for a healthy pregnancy to occur. E and L-selectin expression was more powerful in decidual stroma of the study group. On the contrary, P-selectin expression was stronger in the control group. However, the difference of P-selectin between the two groups was not as significant as the differences of L and E-selectins.

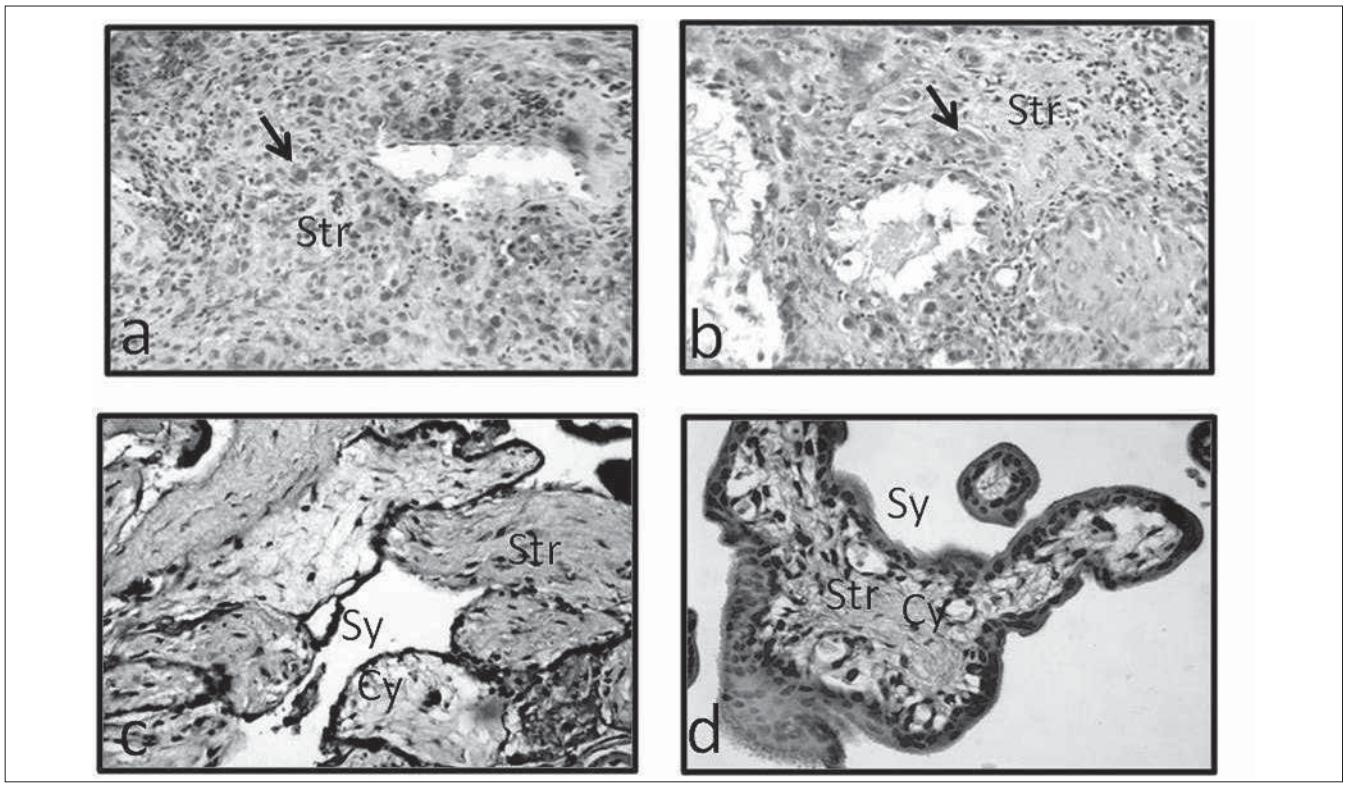

Figure 1. Immunohistochemical analysis revealed that P-selectin expression was seen in the decidual (a, b) and placental chorionic villi (c, d) of the control (a, c) and study (b, d) groups. Low in number decidual stroma especially decidual cells stained with P-selectin (arrows) were detected in both groups (a, b). Low P-selectin expression was seen in villous stroma, especially in syncytiotrophoblast (Sy) and cytotrophoblast (Cy) cell in controls (c) but more than in the study group (d). STR: Stroma (Original Magnification: 200×).

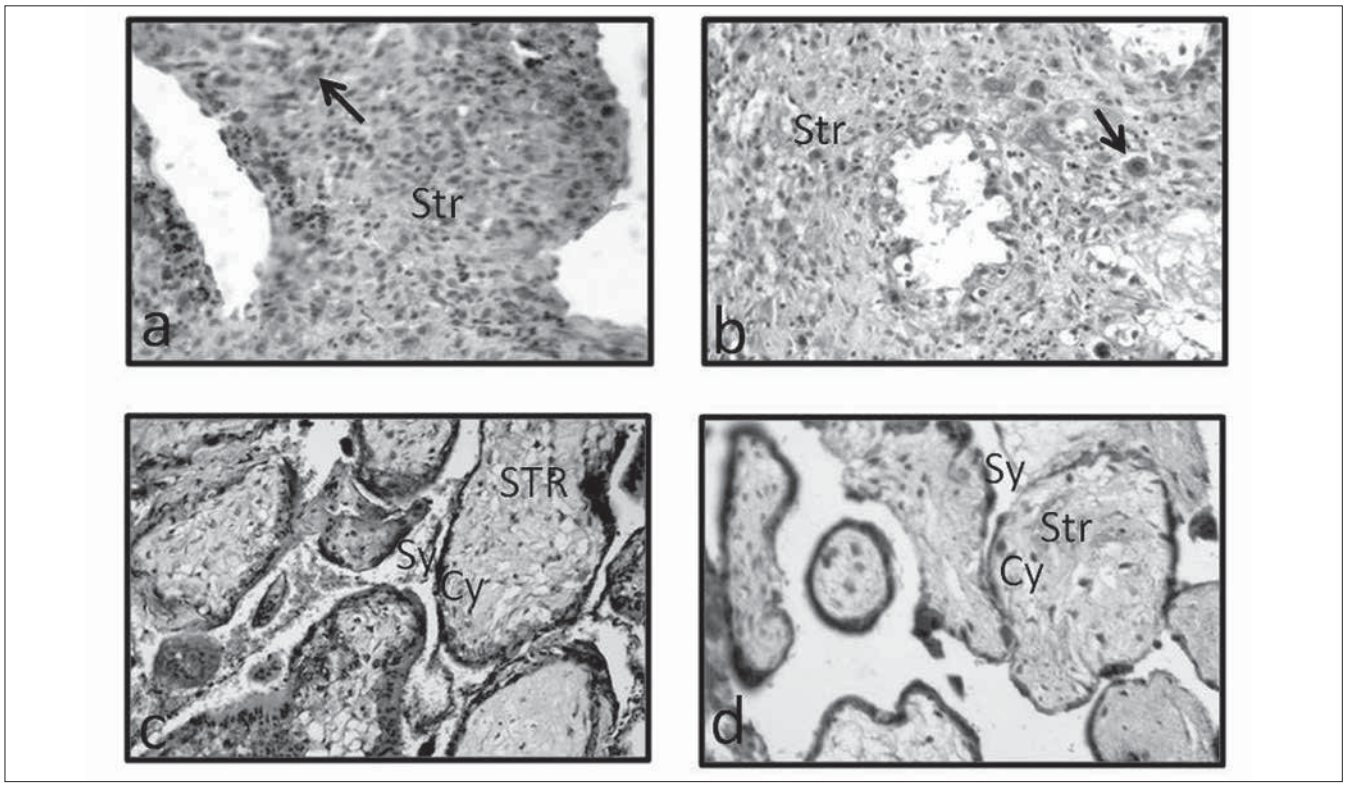

Figure 2. Immunohistochemical analysis revealed that E-selectin expression was seen in the decidual (a, b) and placental chorionic villi (c, d) of the control (a, c) and the study groups (b, d). The arrows indicate decidual stroma especially decidual cells stained with E-selectin which were moderate in number in the study group (b) as compared to controls (a). High E-selectin expression was seen in villous stroma especially in syncytiotrophoblast (Sy) and cytotrophoblast (Cy) in the control group (c) than the study group (d). STR: Stroma (Original Magnification: 200×).

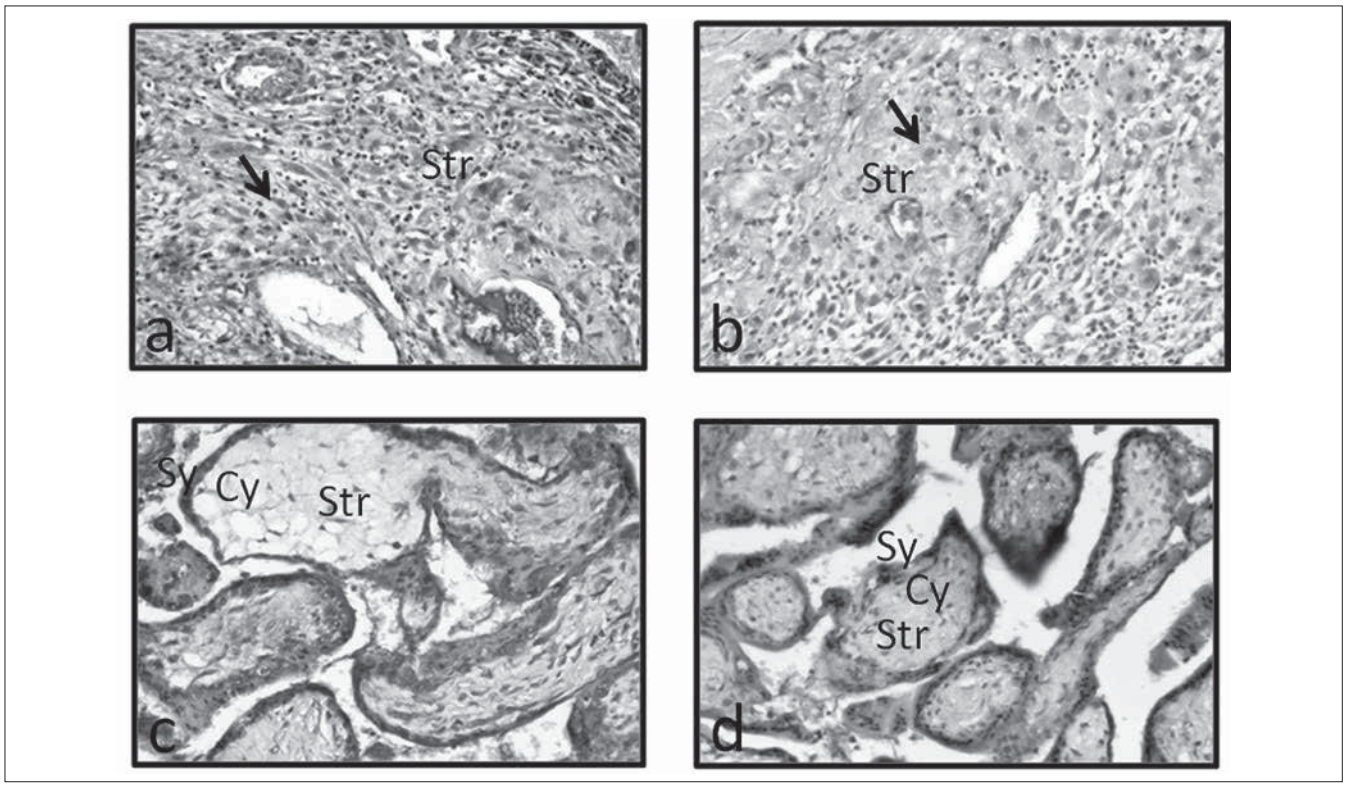

Figure 3. Immunohistochemical analysis revealed that L-selectin expression was seen in the decidual (a, b) and placental chorionic villi (c, d) of the control (a, c) and study (b, d) groups. The arrows indicate decidual stroma especially decidual cells stained with L-selectin which were higher in number in the study group (b) than controls (a). High L-selectin expression was seen in villous stroma especially in syncytiotrophoblast (Sy) and cytotrophoblast (Cy) in controls (c) as compared to the study group (d). STR: Stroma (Original Magnification: 200×).

Acar et al., [16] showed in their study including all three selectins that maternal plasma values were higher in healthy pregnancies than in the missed abortion group. Their results are consistent with our findings of strong expression of E and L-selectins in missed abortion from the decidua, the maternal side of the maternal-fetal interaction zone.

Trophoblast adhesion to the endometrium is the necessary first step of implantation and subsequently, placentation. On the maternal side of the maternal-fetal interface, human uterine epithelial cells up-regulate selectin ligands during the window receptivity. At the same time, on the fetal side, trophoblasts express L-selectin [17]. These interactions show that there is a dramatic parallel relationship between the free floating embryo and extravasation of leukocytes [12].

In our study, L-selectin expression in trophoblasts in the control group was significantly higher as compared to the study group. Moreover, it seems striking that L-selectin was the most expressed selectin in both, the study and control groups (Table 2). E-selectin is expressed by activated endothelial cells [18]. It is thought to play a role in leukocyte extravasation after L and P-selectins [8, 10]. Immunohistochemistry study of Zenclussen et al., [19] suggested that E-selectin was weakly expressed in decidual cells, with no difference between normal pregnancies and spontaneous abortions. In our study, we detected weaker expression in the decidual stroma than the placental villous stroma in both groups. E-selectin expression in every field showed parallel differences to the L-selectin but expression was weaker than L-selectin (Table I, Table II), (Figure 2 and Figure 3).

Pregnancy increases the risk of hypercoagulability and venous thromboembolism [20]. Previously the role of P-selectin in the formation of a thrombus was shown in experimental studies [14, 21]. In subsequent publications P-selectin has been identified as a risk factor for arterial and venous thromboembolic events [22, 23]. It was shown that pregnant women with a history of venous thromboembolism whose levels of soluble P-selectin elevated, were at an increased risk of stillbirth [24]. Another study revealed that cellular adhesion molecules play a key role in recurrent pregnancy loss. It was found that P-selectin gene polymorphism is effective in recurrent pregnancy loss [15]. It is claimed that P-selectin causes abortion by making Th1 abortive cells extravasating [25]. However, these results should not render the role of P-selectin on implantation in healthy pregnancies insignificant. P-selectin, which is situated in Weibel-Palade bodies of the endothelial cells and platelets in the alpha granules and is expressed by inflammatory stimulation on the surface of platelets and, starts the characteristic rolling motion of leukocytes on the endothelium together with L-selectin [8, 9]. Besides the role of P-selectin in the implantation, it is also known that maternal plasma levels increase during pregnancy [26, 27]. We were able to show a strong expression with other selectins on villous stroma in healthy pregnancies. We link the decidual weak expression to increased numbers of selectin binding ligands on the maternal side in response to increased expression of selectins on the fetal side. Olga et al., [17] showed increased expression of L-selectins on the fetal side at implantation, while on the maternal side there was an increase in L-selectin ligands.

However, we could not find the increased expression, either on the maternal or the fetal side in the study group, as it was claimed in some publications. Similarly, Ay et al., [24]

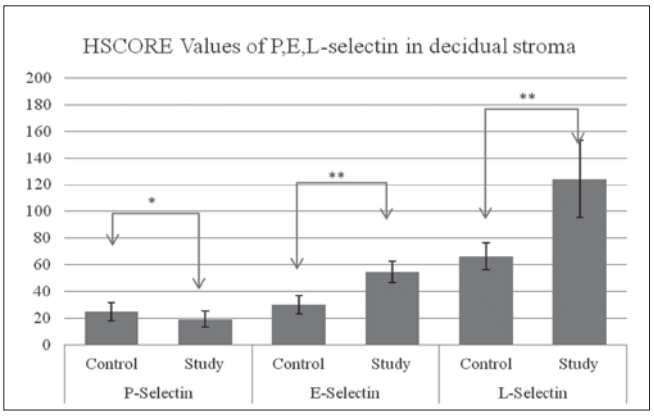

Figure 4. The HSCORE values of E and L-selectin staining fibroblast and decidual cell in the uterine decidual stoma were higher in the study group than in controls. P-selectin staining in the uterine decidual stroma was higher in the control group as compared to the study group. Bars represent Standard Deviations. *p 0.05, **p≤0.01

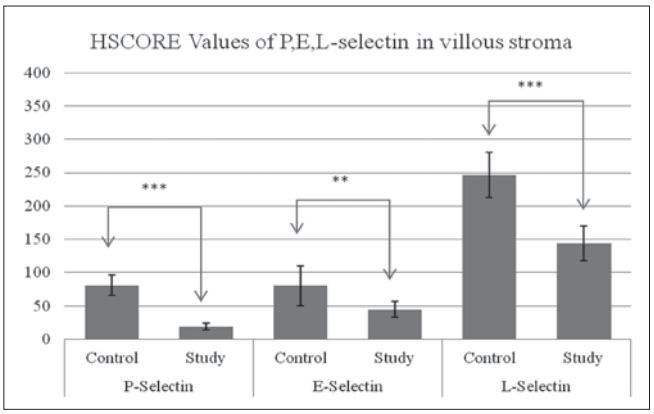

Figure 5. The HSCORE values of P, E and L-selectin staining in placental villous stroma were higher in the control group as compared to the study group. Bars represent Standard Deviations. **p≤0.01, ***p 0.0011.

demonstrated that elevated soluble P-selectin was related to the risk for stillbirth in pregnancy with a history of venous thromboembolism, but not to the risk of abortion. Kaptan et al., [28] could not find any correlation between elevated P-selectin levels and recurrent pregnancy loss. Abortion may occur due to many reasons such as anatomic abnormalities of the maternal uterus, karyotype, endocrine and coagulation abnormalities, maternal co-morbidities, etc. In addition, we usually obtain the materials of abortion not when the fetal loss occurs but after a certain period of time. A decrease in the expression of selectin in inevitable during that period of time so a larger sample study is needed to show an increased expression of P-selectin.

The implantation and the subsequent chain of events is a rather complex process. Nowadays it is known that various types of adhesion molecules take part in its different stages. Strong expression of each of the three investigated selectins in healthy pregnancy villi shows a strong role in implantation and placentation. They contribute to the endothelial damage and activation of leukocytes in pregnancy loss. However, more studies are needed to reveal the unknown facts of pregnancy loss and the role of adhesive molecules in these events.

Authors' Contribution

1. Fatma Eskicioglu – concept, study design, acquisition of data, analysis and interpretation of data, article draft, corresponding author, writing article.
2. Selman Laçin – concept, article draft, study design, acquisition of data, revised article critically.
3. Kemal Özbilgin – analysis and interpretation of data, writing article, revised article critically..
4. Can Köse – analysis and interpretation of data.

Authors' statement

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