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A comparison of the molecular distribution of proangiogenic factors in endometrium of missed abortions and of voluntary first trimester termination cases

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Summary

Objective: The authors aimed to evaluate the angiogenic changes that occur in the cases with missed abortions compared with the voluntary termination of pregnancy as control group, with this controlled clinical study. *Materials and Methods:* The study included fifteen healthy volunteer women with unwanted pregnancy less than 10th gestational week in an academic research environment. The patients were 19 women between 6th and 11th gestational weeks diagnosed with missed abortion as the patient group. Immunohistochemistry was utilized to examine temporal and spatial expression of vascular endothelial growth factor (VEGF) and their two receptors: VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), and Trombospondin-1, eNOS, iNOS, and HIF-1α in the both deciduas and placenta of the both groups. *Results:* This study discovered the significant difference ($p < 0.005$) between the groups of controlled and missed abortion in the decidual and placental cell components, and has put forward that thrombospondin and iNOS have an impact on abortion through antiangiogenic effect in cases of missed abortions. *Conclusions:* The potential role of molecules affecting angiogenesis in the etiology of missed abortion has been evaluated and the authors aimed for this to be a guide for studies on further treatments and on the prevention of the development of missed abortions.

Key words: Missed abortion; Angiogenesis; Immunohistochemistry.

Introduction

Missed abortion is the expulsion of an immature fetus that could not have the ability to sustain life in extrauterine environment from uterus due to any reason and the termination of pregnancy. The factors such as chromosomal anomalies, malformations, immunological factors, endocrine disorders, and environmental factors could be the reasons for missed abortion [1]. In the studies on the etiology of abortion, underlying pathophysiological mechanisms of the half of the cases have remained uncertain [1, 2].

Although the etiology of missed abortion has not been completely clear, recent studies have provided strong evidences that support the close relation between angiogenesis and embryonic developments. An adequate vascularization of chorionic villus is essential for the development of an uneventful pregnancy [3, 4]. The abnormal changes in the balance of utero-placental vascular development are the underlying causes of pregnancy loss, and an adequate and appropriate angiogenesis is a necessity for the successful continuation of a pregnancy [5].

Angiogenesis, the development of new blood vessels from existing blood vessels, is a natural process of the body and may be pathological in some cases. Angiogenesis is rather limited in organism except for physiological cases as the development of placenta after fertilization in which angiogenesis is strictly controlled, embryogenesis, wound healing, and the renewal of the inner layer of the uterus after menstruation [6]. Under normal conditions, angiogenesis is regulated by a balance among many growth factors enabling the proliferation and activation of endothelial cells and anti-angiogenic factors [7].

Vascular endothelial growth factor (VEGF) is the most important one of the factors having a role in angiogenesis. Many biological functions of endothelial cells, cytokine synthesis and release, the expression of molecules in thrombolytic and coagulation pathways, and the regulation of smooth muscle cell hyperplasia are carried out by means of VEGF [8]. The biological activity of VEGF occurs basically by means of its three receptors. These receptors with their structure of the tyrosine kinase are VEGF-R1 (Flt-1), VEGF-R2 (Flk-1/KDR) and VEGF-R3 (Flt-4). While

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VEGF-R1 and R2 of them are located in endothelial cells, VEGF-R3 is on lymph vessels [9-11].

Hypoxia, described as the case of the lack of adequate oxygen in environment, has a substantial role in the pathogenesis of many diseases [12]. Oxygen is largely necessary for the aerobic metabolism of many eukaryotic organisms including the development of embryo. A software agent HIF-1α has a role of critical importance in tissue cells such as pregnancy decidua in which vascularity is intense, by stimulating the expression of genes related to angiogenesis, glucose transportation, and anaerobic metabolism in hypoxic conditions [13]. The expression of VEGF is acknowledged to be controlled with HIF-1α during hypoxia [14].

Thrombospondin (TSP) -1 is a potential angiogenesis inhibitor with the ability of intercepting the cell migration in endothelial cells in response to the different types of angiogenic stimuli, stimulating apoptosis, and preventing the formation of new blood vessels [15, 16]. That TSP-1 has an impact on the regulation of the secretion of VEGF from extracellular stores is a significant point. VEGF and the receptor of VEGR are shown to increase in the absence of TSP-1 [17]. As a result of the increase in the level of tissue oxygen, the necessary genes have been observed to be stimulated in the adjustment of cell to hypoxia and TSP-1 has been seen to be inhibited. Consequently, the formation of new blood vessels has been observed [18].

With its role in the regulation of systemic blood pressure, nitric oxide (NO) has a critical and important role in angiogenesis and hyper permeability induced by VEGF [19]. VEGF induces the up-regulation of endothelial nitric oxide synthase (eNOS) enzyme and accordingly, the secretion of NO. Consequently, NO, which is produced as endogenous, increases the synthesis of VEGF. That eNOS undergoes a pharmacological blockade or a genetic disorder inhibits the angiogenesis and hyper permeability induced by VEGF [20].

This study has compared the distribution of VEGF and VEGF receptors which are angiogenic molecules; TSP-1 which is the inhibitor of eNOS and angiogenesis, and HIF-1α which stimulates the expression of genes related to angiogenesis in the iNOS and hypoxic conditions in the endometrium and placental tissue samples in the cases of missed abortions and voluntary termination of pregnancy. Thus, the authors have revealed the potential role of molecules affecting angiogenesis in the etiology of missed abortion, and have aimed for this to be a guide for the studies on further treatments and on the prevention of the development of missed abortions.

Materials and Methods

Fifteen unwanted pregnancies (5–10 weeks gestational age) and 19 missed abortions (6-11 weeks gestational age) endometrial tissue samples were obtained with informed consent and in accordance with the requirements of the Celal Bayar University Ethics Committee. The mean age of women was 27.53 years. The range was 21-37 years for normal pregnancy group and mean was 28.74 years old for range 18-41 years for missed abortion group.

The abortions were diagnosed by transvaginal ultrasound and confirmed by repeat ultrasound prior to the dilation and curettage procedure. Chorionic villi and maternal decidua were separated and cleaned. Placental and decidual tissues were fixed in 10% buffered formalin solution and embedded in paraffin. The blocks were cut in four to five μm thick serial sections. The first of the tissue sections were stained with primary antibodies (VEGF; VEGF-R1 (Flt-1), VEGF-R2 (Flk-1/KDR), Thrombospondin-1, eNOS, iNOS and HIF-1α) via immunohistochemical technique.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used for immunohistochemical staining. Tissue samples were stored at 60°C overnight and then were dewaxed by xylene for 30 minutes. After dehydration of the sections with ethanol, they were washed with distilled water. They were then treated with 2% trypsin (ab970) at 37°C for 15 minutes and incubated in 3% H2O2 solution for 15 minutes to inhibit endogenous peroxidase activity. Then, sections were incubated with Anti-VEGF Primer Antibody (251901), Anti-Flk Primer Antibody (25180), Anti-FLT Primer Antibody (25153), Anti-eNOS Primer Antibody (25078), Anti-iNOS Primer Antibody (25078), Anti-Thrombospondin Primer Antibody (ab93653) and Anti-HIF-1 Primer Antibody (ab463) in a 1/100 dilution for 18 hours at +4°C. They were then given an additional three 5-washes in PBS, followed by incubation with biotinylated IgG and administration of streptavidin peroxidase. After washing the secondary antibody with PBS three times for five minutes, the sections were stained with DAB substrate system containing diaminobenzidine to detect the immunoreactivity, and then stained with Mayer's hematoxylin (72804E) for counterstaining. They were covered with mounting medium (01730) and observed with light microscopy.

Immunostaining for VEGF, Flk, Flt, iNOS, eNOS, Thrombospondin, and HIF-1α were evaluated semiquantitatively using HSCORE analysis, Immunostaining intensity was categorized by 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 fibroblast and decidual cells in uterine decidual stroma; and fibroblasts and mesenchymal cells in placental villous stroma that stained at each intensity category multiplied by its respective score, using 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 decidual tissues (decidual cell, endothelial cells and endometrial glad cells) and placental tissues (cytotrophoblast, syncytiotrophoblast, stromal cells and endothelial cells) for each intensity, varying from 0 to 100%. For each slide, five different fields were evaluated microscopically at X200 magnification. HSCORE evaluation was performed independently by at least two investigators (KO, FS) blinded to the source of the samples as well as to each other's results; the average score of both was then used.

Results

Histochemistry

In the examination of the preparations stained with Hematoxylin-eosin of the normal pregnancy placentas, the chorionic villi, where mesenchymal tissue was located, were surrounded by trophoblastic cells. There were fusiform-shaped mesenchymal cells and dark-colored macrophages (Hofbauer cells) in these regions and various veins- the extensions of the umbilical arteries and veins inside the mes-

enchymal tissue (Figure 1a). The stromal cells in normal pregnancy endometrium were observed to turn into decidual cells with a quite large cytoplasm where there were various granules. Among the decidual cells, numerous uterine gland and blood vessels were determined (Figure 1b).

In the same examination, the enclosure of chorionic villi by trophoblastic cells and a thinner thickness of their syncytiotrophoblast and cytotrophoblast than the normal pregnancy were observed (Figure 1c). A decrease in the number in blood vessels in the chorionic and decidual regions of the missed abortion cases than the normal pregnancy was found remarkable while the endometrial glands had a slight increase (Figure 1d).

Immunohistochemistry

In the examination of placenta samples of the control group stained by means of immunohistochemical technique, it has been observed that VEGF, Flk, eNOS, and TSP immunoreactivities of syncytiotrophoblasts have been stained significantly stronger (Figures 2a, 3a, 5a, 7a) than the missed abortion group (Figures 2b, 3b, 5b 7b), while the immunoreactivities of Flt, iNOS, and HIF-1α (Figures 3a, 6a, 8a) have been significantly slightly stained in the control group than the missed abortion group (Figures 3b, 6b, 8b). The VEGF, eNOS and TSP-1 immunoreactivities of cytotrophoblast cells of the control group (Figures 2a, 5a, 7a) have been found to be stained stronger than the missed abortion group (Figures 2b, 5b, 7b); the Flt and HIF-1α antibodies have been stained slightly in the control group (Figures 4a, 8a) compared to the missed abortion group (Figures 4b, 8c). There was no significant difference observed in the Flk and HIF-1α immunoreactivity of cytotrophoblast cells of the both groups (Figures 3ab, 8ab). The placental stromal cells of the control group stained

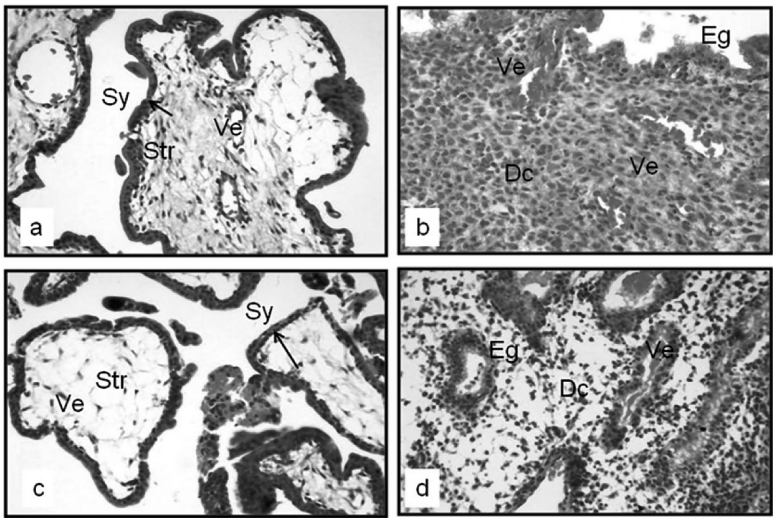

Figure 1. — Histochemical technique: control group (a, b), missed abortion (c, d); placental villi (a, c); decidua (b, d). Placental villi were surrounded by syncytiotrophoblast (Sy), while cytotrophoblasts (arrow) can be seen below. Mesenchymal stromal cells that were the center of villi stroma were composed of the stroma (Str). Many blood vessels (Ve) in the stroma were observed (a, c). Decidual cells of numerous large cytoplasm (Dc) in the deciduas, blood vessels (Ve), and endometrial glands (Eg) were observed. The missed abortion cases (c, d) in the region of chorionic syncytiotrophoblast (Sy) and cytotrophoblasts (arrow) have a finer structure and were observed to decrease the number of blood vessels (c). The parts of decidual endometrial glands (Eg) in the missed abortion cases (d) were increased whereas the arteries (Ve) were decreased (d). Original magnification (x200) Hematoksilen-Eosin.

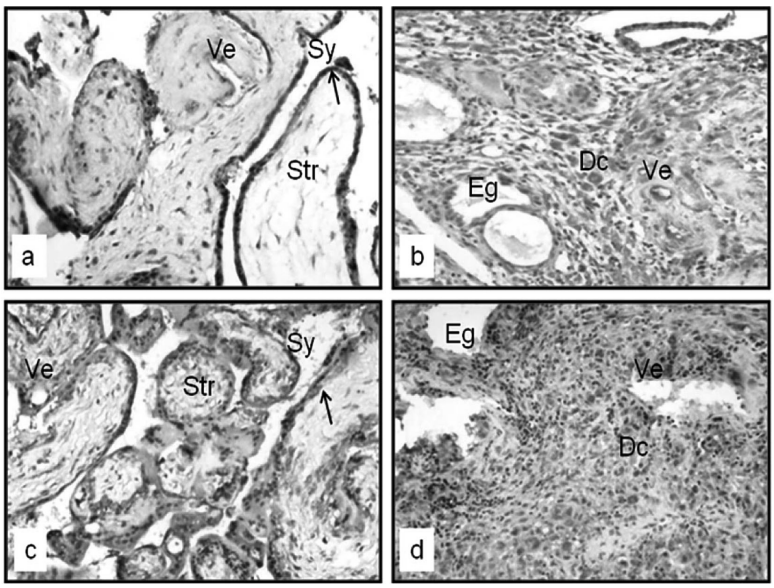

Figure 2. — Immunohistochemical analysis of VEGF: control group (a, b), missed abortion (c, d); placental villi (a, c); decidua (b, d). The VEGF immunoreactivity on syncytiotrophoblast (Sy), cytotrophoblasts (arrow), stromal cells (Str), and vascular endothelial cells (Ve) of the control group placenta were observed to be stronger (a) than the missed abortion group (c). In the control group, the decidual cells (Dc) stained strongly, vascular endothelial cells (Ve), and endometrial glands (Eg) stained quite weakly (b). Decidual VEGF immunoreactivity was rather weak in control group (b) than missed abortion group. (d). Original magnification (x200).

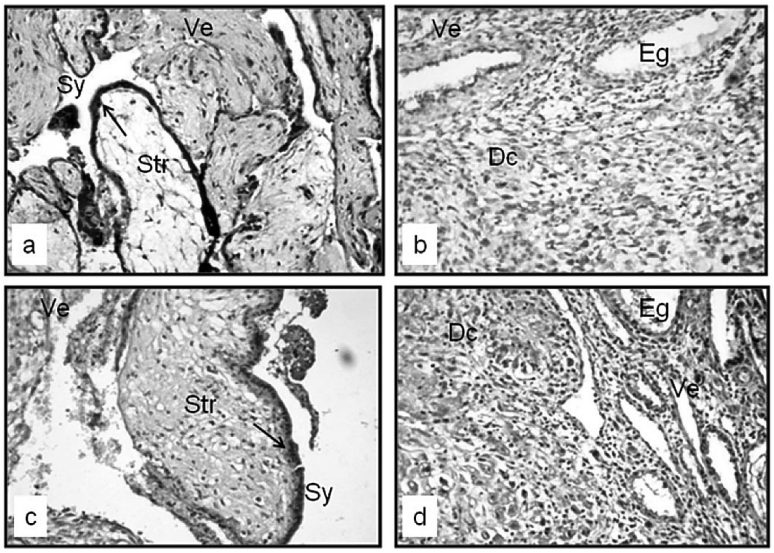

Figure 3. — Immunohistochemical analysis of Flk: control group (a, b); missed abortion (c, d); placental villi (a, c); decidua (b, d). Placental syncytiotrophoblast (Sy) and vascular endothelial cells (Ve) of the control group (a) stained strongly in control group (a) than missed abortion group (c). Decidual cells (Dc), vascular endothelial cells (Ve), and endometrial glands (Eg) of control group mildly stained (b) but strongly in missed abortion group (d). Original magnification (x200).

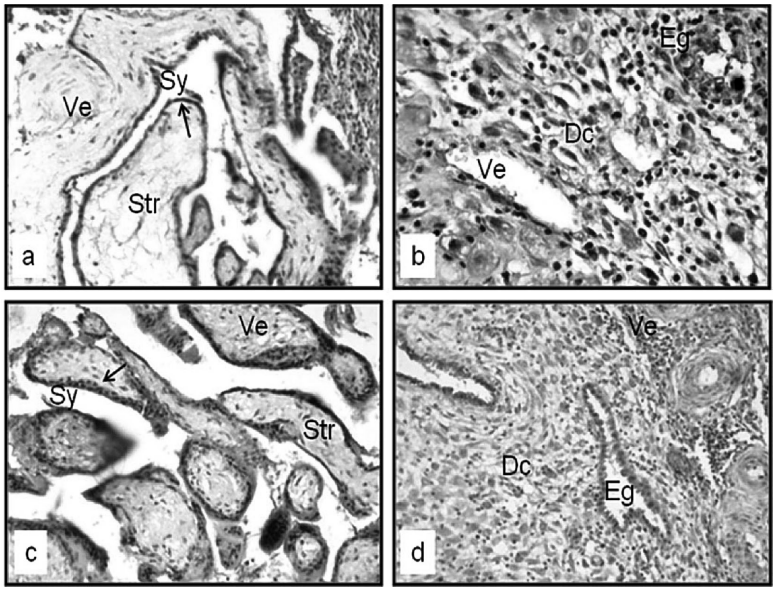

Figure 4. — Immunohistochemical analysis of Flt: control group (a, b); missed abortion (c, d); Placental villi (a, c); decidua (b, d). Placental syncytiotrophoblast (Sy), cytotrophoblasts (arrow), and vascular endothelial cells (Ve) of the control group was observed to be mildly stained (a) than missed abortion group (c). Decidual cells (Dc) and vascular endothelial cells (Ve) stained strongly and the endometrial glands (Eg) of control group (b) compared to missed abortion cases (d). Original magnification (x200).

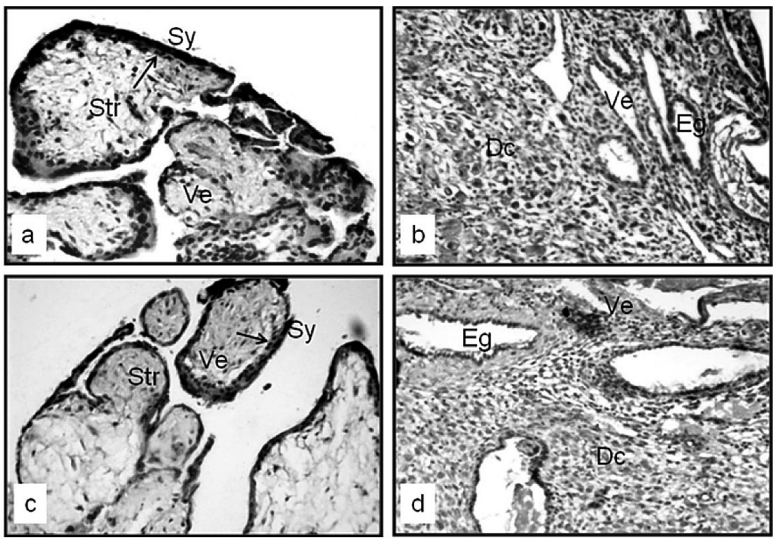

Figure 5. — Immunohistochemical analysis of eNOS: Control group (a, b), Missed abortion (c, d); Placental villi (a,c). Decidua (b, d). Placental syncytiotrophoblast (Sy), cytotrophoblasts (Arrow), and stromal cells (Str) was observed strong in control group (a) than the missed abortion group (c). We examined that the decidual cells (Dc) and endometrial glands (Eg) of control group were stained strongly (b) than missed abortion cases (d). Original magnification (x200).

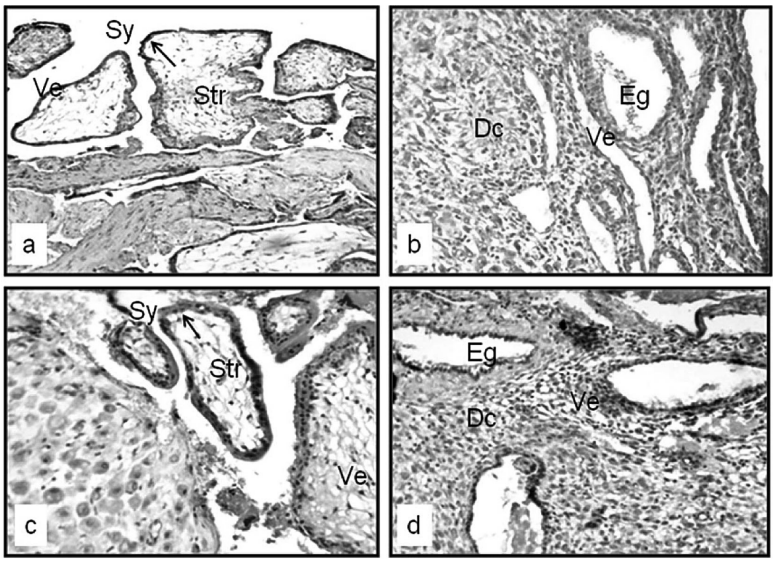

Figure 6. — Immunohistochemical analysis of iNOS: control group (a, b); missed abortion (c, d); placental villi (a, c); decidua (b, d). Placental syncytiotrophoblast (Sy), cytotrophoblasts (Arrow), stromal cells (Str), and vascular endothelial cells (Ve) weakly stained in number (a) in control group (a) than the missed abortion group (c). The decidual cells (Dc) and endometrial glands (Eg) of control group stained strongly (b) compared to missed abortion cases (d). Original magnification (x200).

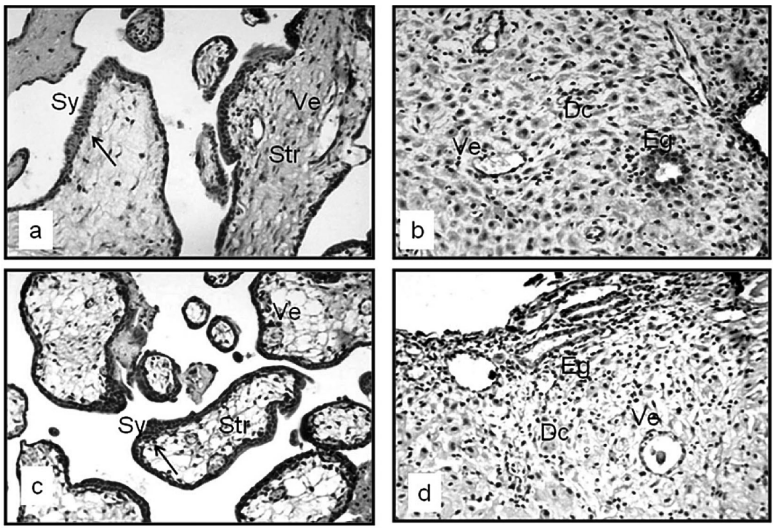

Figure 7. — Immunohistochemical analysis of Thrombospondin-1: control group (a, b); missed abortion (c, d); placental villi (a, c); decidua (b, d). Placental syncytiotrophoblast (Sy), cytotrophoblasts (arrow), stromal cells (Str), and vascular endothelial cells (Ve) were observed to stain rather slightly (a) compared to missed abortion group (c). The authors examined that the decidual cells (Dc) and endometrial glands (Eg) and vascular endothelial cells (Ve) of control group stained quite slightly (b), whereas the staining of missed abortion cases were increased (d). Original magnification (x200).

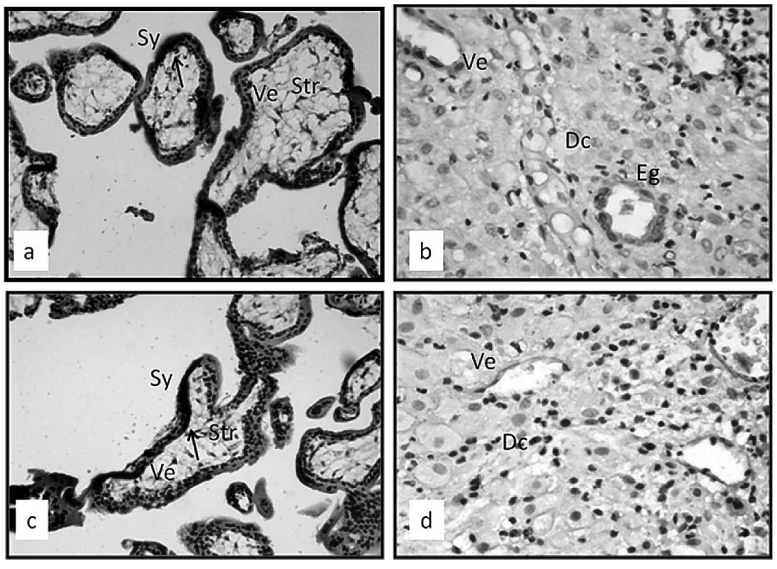

Figure 8: — Immunohistochemical analysis of HIF-1α: control group (a, b); missed abortion (c, d); placental villi (a, c); decidua (b, d). Placental syncytiotrophoblast (Sy), cytotrophoblasts (arrow), stromal cells (Str), and vascular endothelial cells (Ve) were observed to stain quite slightly in control group (a), whereas the immunoreactivity of syncytiotrophoblast (Sy) in missed abortion was increased (c). Decidual cells (Dc), endometrial glands (Eg), and vascular endothelial cells (Ve) with slight staining of both two groups (b, d). Original magnification (x200).

Table 1. — *Angiogenic factors expression in placenta.*

	Normal pregnancy / mean (max - min)				Missed abortion / mean (max - min)			
	Syncytiotrophoblasts	Cytotrophoblast	Stromal cell	Endothelial cell	Syncytiotrophoblasts	Cytotrophoblast	Stromal cell	Endothelial cell
VEGF	90 (80-115)	57.5 (45-76)	39 (25-60)	59.50 (25-80)	46 (36-65) **	37 (20-70) **	40.50 (23-75)	60 (40-75)
FLK	40 (30-76)	30 (20-50)	37.50 (12-49)	50 (20-90)	30 (10-40) **	30 (20-34)	30 (16-40)	30 (16-40) **
FLT	85 (70-100)	38 (30-50)	30 (20-40)	50 (40-70)	104.5 (65-125) **	107.5 (70-170)**	30 (16-40)	35 (20-40) **
eNOS	55 (20-90)	35 (20-75)	23 (15-40)	35 (25-51)	30 (20-40) *	16 (10-30) **	30 (20-34)	30 (20-40)
iNOS	105 (75-140)	102.5 (75-135)	70.5 (55-82)	137.5 (101-220)	135 (100-160) *	125 (100-140) *	120 (85-130) **	32 (30-40) **
TSP	20 (10-40)	20 (10-30)	10 (10-20)	35 (28-40)	10 (4-10) **	6 (4-10) **	10 (4-10) *	5 (4-10) **
TIA	10 (2-20)	4 (2-10)	4 (2-10)	6 (2-10)	30 (20-40) **	11 (6-16*)	11 (6-20) *	90 (20-120) **
HIF	8 (4-12)	6 (4-10)	9 (4-12)	9 (4-16)	10 (6-20) **	6 (4-16)	7 (4-10)	10 (4-16)

*$p < 0.05$; **$p < 0.005$

Table 2. — *Angiogenic Factors expression in decidua.*

	Normal pregnancy			Missed abortion		
Median	Endothelial cell	Decidual cell	Endometrial gland	Endothelial cell	Decidual cell	Endometrial gland
VEGF	75 (60-115)	42 (20-60)	22 (12-39)	37.5 (26-49) **	13 (10-20) **	13 (10-20) **
FLK	80 (30-115)	35 (20-50)	39.50 (16-50)	127.5 (110-150) **	145 (130-150) **	132.50 (105-170) **
FLT	115 (95-130)	85.5 (55-120)	109.5 (95-140)	165 (140-210) **	30 (20-39) **	152 (130-185) *
eNOS	62.5 (20-90)	55 (20-80)	60 (50-80)	34 (26-40) *	34 (30-80) *	90 (80-120) **
iNOS	117.5 (85-145)	102.5 (85-145)	92.5 (35-135)	127.5 (105-145)**	155 (130-185)	127.5 (95-150) *
TSP	20 (10-40)	30 (20-40)	30 (10-40)	10 (6-20) **	6 (2-10) *	10 (6-20) *
TIA	10 (6-20)	6 (4-10)	10 (4-20)	44.5 (26-70) **	30 (20-36) **	43 (30-66) **
HIF	10 (6-24)	11 (4-30)	10 (4-18)	10 (10-24)	10 (6-24)	12 (6-20)

*$p < 0.05$; **$p < 0.005$

stronger with eNOS antibody (Figure 5a) than missed abortion group (Figure 5b); Flt and iNOS antibodies stained significantly slighter in the missed abortion group (Figures 4b, 6b) than control group (Figures 4a, 6a). There was no significant immunoreactivity determined between VEGF, Flk, eNOS and HIF-1α of both groups (Figures 2ab, 3ab, 5ab, 8ab). Placental vascular endothelial, Flk, Flt, iNOS and TSP-1 antibodies showed a higher immunoreactivity in the control group (Figures 3a, 4a, 6a, 7a) than the missed abortion group (Figures 3b, 4b, 6b, 7b). There was no significant difference determined between the staining properties and placental endothelial cells, VEGF and HIF-1α antibody (Figures 2ab, 8ab; Table 1).

In the examination of decidual tissues, the immunoreactivities of VEGF, Flt, eNOS and TSP were found higher in the control group (Figures 2c, 4c, 5c,7c) than in the missed abortion group (Figures 2d, 4d, 5d,7d), while the immunoreactivity of Flk was determined higher in the missed abortion group (Figure 3d) than the control group (Figure 3c). There was no significant difference between the iNOS and HIF-1α immunoreactivities of decidual cells of the both group (Figures 6cd, 8cd). The VEGF, eNOS and TSP immunoreactivities of endothelial cells of the control group was found higher (Figures 2c, 5c, 7c) than in the missed abortion group (Figures 2d, 5d, 7d), while the Flk, Flt and iNOS activities were found significantly higher in the missed abortion group (Figures 3d,

4d, 6d) than in the control group (Figures 3c, 4c, 6c). There was no significant difference between the HIF-1α immunoreactivity of endothelial cells of both groups (Figure 8cd). The TSP immunoreactivity of endometrial glands in maternal decidual tissue of the control group (Figure 7c) was found higher than the missed abortion group (Figure 7d); Flk, Flt, eNOS and iNOS immunoreactivities were determined to be high in the missed abortion group (Figures 3d, 4d, 5d, 6d) than the control group (Figures 3c, 4c, 5c, 6c). There was no significant difference determined between the VEGF and HIF-1α immunoreactivity of endometrial glands of the both group (Figures 1cd, 8cd; Table 2).

Discussion

The aim of this study was to compare the distribution of factors having a role in angiogenesis in deciduas of missed abortion and voluntary termination cases, and to study the role of angiogenesis in the etiology of missed abortion.

Although the etiology of missed abortion has not been completely clear, recent studies have provided strong evidences that support the close relation between angiogenesis and embryonic developments [3, 5]. The authors have determined a low VEGF immunoreactivity in all decidual cell samples including decidual vascular endothelial, decidual stromal cells, and endometrial glands of missed abortion group. They have also found a relatively lower VEGF immunoreactivity than the control group in placental syncytiotrophoblast and cytotrophoblast cells. In the study of Coulam *et al.* in which the VEGF gene polymorphism was researched in the endometrium of fertile but non-pregnant women and women with recurrent pregnancy loss, abnormal gene polymorphism in VEGF could correlate with recurrent pregnancy losses was stated [21]. Nardo examined VEGF expression in the tissues of the normal endometrium, of endometrium in implantation window and of endometrium in early pregnancy period during the menstrual cycle [22]. He determined increased VEGF expression in the cells of endometrial glandular epithelium and endometrial stromal during the late secretory phase and peri-implantation period, and has emphasized the importance of angiogenesis in the development of endometrial tissue, as well as its importance for implantation and the continuation of the pregnancy. When assessing the findings of earlier studies, low VEGF expression in decidual and placental tissues results in insufficient angiogenesis, revealing the role of VEGF in pregnancy loss.

In the histochemical examination conducted, the thickness of trophoblast in the group of missed abortion is thinner than the control group has been observed, whereas the number of blood vessels both in chorionic and in decidual regions has been reduced compared to control group, has been found to be remarkable. Meegdes *et al.* compared the cases of intrauterine embryonic deaths to the cases of legal pregnancy terminations with normal villous architecture, weaker vessel development, and insufficient angiogenesis have been found in chorionic villi of the cases of intrauterine death [23].

VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1) are the receptors where VEGF with its role in angiogenesis basically carries out its biological activity. Many studies have shown that both receptors (Flt-1, Flk-1) have an obvious role in capillary formation and vascular development during embryogenesis [24-26]. When comparing the immunoreactivity of Flt-1 receptor with the control group, the present authors detected a lower immunoreactivity of Flt-1 in placental vascular endothelial cells, decidual vascular endothelial cells, and the missed abortion group. Muttukrishna *et al.* researched maternal serum levels in pregnant women with a threatened miscarriage of soluble endothelial growth factor-1 (sFlt-1) and placental growth factor (PIGF) which are angiogenic factors, in asymptomatic pregnant women and non-pregnant women with luteal phase [27]. The samples were reanalyzed on the basis of pregnancy outcome. The pregnancy of 19 women in lower threat group resulted in complete abortion, and relatively lower levels of flt-1 compared to pregnancies not resulting in abortion were determined. In the comparison between non-pregnant women and the group of asymptomatic pregnancy, higher levels of sFlt-1 were determined in the group of asymptomatic pregnancy, and the levels of sFlt-1 were also found to rise in early pregnancy. They also pointed out that these proteins could be more accurate projections in predicting subsequent pregnancy loss [27]. In the study of Fong *et al.* in which the mutation in VEGF-R1 locus was researched in early embryo development in mice, the decidua disorganization of endothelial and abnormal blood vessel formation was observed in cases of mutated VEGF-R1 [28].

When analyzing the immunoreactivity of Flk-1 which is another receptor of VEGF, the authors observed that the immunoreactivity in syncytiotrophoblast and placental vascular endothelial cells was rather low in the group of missed abortion compared to the control group in this study. Moreover, they have determined that the immunoreactivity of Flk-1 in the samples of decidua was higher in the group of missed abortion. In the study of Vuorela *et al.*, they found that there was no significant difference when comparing the immunoreactivity of flt-1 and flk-1 in placental vascular endothelial cells of the cases of missed abortion with the control group. However, they observed a much lower immunoreactivity in the group of missed abortion in decidual vascular endothelial cells, and supposed that the expression changes of vascular endothelial VEGF-R1 and VEGF-R2 in recurrent abortions could be associated with maternal decidua rather than placenta [29]. The present authors have found that the immunoreactivity of flt-1 and flk-1 in placental vascular endothelial cells of the group of missed abortion was lower when compared to healthy controls in this study. The immunoreactivity of flt-1 in decidual vascular endothelial cells was determined to be much higher in the group of control whereas the immunoreactiv-

ity of flk-1 was found to be higher in the group of missed abortion. These results have shown that VEGF has effect over both two receptors in the placental vascular development whereas they also suggested that the different expressions of two receptors in decidua vascular endothelium show an impact over the receptor of flt-1 during the decidual angiogenesis of VEGF.

During the first trimester, placenta develops in a hypoxic environment with the occlusion of extravillous trophoblast of uterine spiral arterioles. This physiological hypoxic environment in early pregnancy protects fetus from the harmful and teratogenic effects of oxygen radicals. All cells respond to hypoxia with a number of gene modifications. HIF-1α is an important mediator in this process. In the present study, the cytotrophoblast, syncytiotrophoblast, and stromal cells in the group of missed abortion hardly stained in the analysis of monoclonal antibody to HIF in decidua and placenta tissues whereas there was a mild staining in the placenta samples of the control group. The present authors did not observed any significant difference between the groups of missed abortion and control in terms of HIF immunoreactivity in the study of decidua cells. In the study of Patel *et al.*, the importance of HIF in the differentiation and development of the placenta was addressed [30]. Several studies have indicated that the existing physiological hypoxic environment during the development of the placenta in early pregnancy leads to induce HIF-1α, and HIF-1α expressed in the environment contributes to the increase of placental vascularization by enabling angiogenesis to be stimulated through angiogenic factors, such as the growth factors of VEGF, receptors of VEGF or insulin-like receptors [30-32]. Sun *et al.* stated that the abnormal level of HIF-1α in placenta may play a role in the pathogenesis of preeclampsia by affecting the cytotrophoblast invasion and placental vascular reconstruction through the modulation of VEGF and transcription of sFlt-1 [33].

NO has an important role in angiogenesis and hyperpermeability induced by VEGF. VEGF induces the up-regulation of endothelial nitric oxide synthase (eNOS) enzyme and accordingly, the secretion of NO. Consequently, NO which is produced endogenously increases the synthesis of VEGF. The expression of NOS isoforms in human endometrium have not yet been completely clarified. Recent immunohistochemical studies have revealed the presence of eNOS protein in the endometrial epithelial and endothelial cells [34-36]. The expression of eNOS mRNA in endometrium has been proved by in-situ hybridization [37]. In the examination of eNOS monoclonal antibody, the present authors have found that the eNOS immunoreactivity in stromal cells of the chorionic samples of the control group was quite lower than the group of missed abortion. When analyzing decidual eNOS immunoreactivity in the group of missed abortion, the eNOS immunoreactivity in all decidual components has been observed to be relatively lower than the control group. The important role of NO in the process of implantation and pregnancy has been proved in animal studies [38, 39]. Haddad *et al.* have shown the role of NO in early pregnancy loss in their studies on mice deciduas [40]. Fukurama *et al.* have indicated that the angiogenesis and hyperpermeability induced by VEGF would be inhibited in case that eNOS or its pharmacological blockade is genetically destroyed [20].

Inducible NOS (iNOS) can inhibit angiogenesis by means of the down-regulation of VEGF receptor. Brooks *et al.* have revealed that iNOS inhibits the neovascularization carried out by VEGF in a mouse model related to ischemic retinopathy [41]. In the examination of the decidual immunoreactivity of iNOS, the present authors have determined that there was a quite high immunoreactivity in the cytotrophoblast, syncytiotrophoblast, and stromal cells of the group of missed abortion. The immunoreactivity in the decidual vascular endothelium of the group of missed abortion was found to be relatively higher than the control group in this examination of the decidual immunoreactivity of iNOS. The study of Haddad *et al.* has indicated that NO in mice is produced by decidual macrophages and increases the synthesis of VEGF but iNOS inhibits this effect [40].

TSP-1 is a potential angiogenesis inhibitor with the ability of preventing new vessel formation in endothelial cells in response to the different types of angiogenic stimuli. In the examination of TSP-1 immunoreactivity of the group of missed abortion in the present study, trophoblast and stromal cells were found to be slightly stained whereas the chorionic stromal cells and vascular endothelium of the control group were found to have minor staining. In the evaluation of decidual components, decidual vascular endothelium, decidual cells, and endometrial glands in the control group were observed to have stained slightly while the thrombospondin immunoreactivity in the group of missed abortion was carried out in mild degrees. In the absence of TSP-1, VEGF, and the receptor VEGF-R1 have indicated to increase [17]. Jin *et al.* have stated that the abnormal expression of TSP-1 in decidua could cause unexplained recurrent pregnancy loss in some women [42].

In conclusion, this study has discovered the significant difference ($p < 0.005$) between the groups of controlled and missed abortion in the decidual and placental cell components, and has suggested that thrombospondin as iNOS has an impact on abortion through antiangiogenic effect in cases of missed abortions.

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