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The role of angiogenic factors in first trimester pregnancy losses

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Summary

Objective: A good blood supply towards the peri-implantation endometrium is an essential requirement for pregnancy. Intermedin (IMD), vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS), are angiogenic and vasoactive agents that play contributory roles in endometrial vascularity. The goal of this study was to immunohistochemically investigate the roles of various vasoactive factors in first trimester pregnancy losses. *Materials and Methods:* This was a prospective case-controlled study carried out on decidual and placental tissue samples obtained from women with unwanted pregnancies who served as the control group (n=10), and those with missed abortions who were the "missed abortion group"(n = 10). Immunohistochemistry techniques were used to compare IMD, receptor activity modifying protein (RAMP)1, RAMP2, RAMP3, VEGF, and eNOS expression of decidual and placental cells. Immunostaining for these factors was evaluated semiquantitatively by H-score analysis. *Results:* IMD and RAMPs in decidual cells exhibited higher expression in the control group. However, IMD and RAMP2 had a stronger expression in placental cells in the missed abortion group. In the control group, VEGF and eNOS had a higher expression in decidual cells and on the placental side, especially in syncytiotrophoblasts and cytotrophoblasts. *Conclusion:* Expressions of vasoactive agents, such as IMD, VEGF, and eNOS, decrease in first trimester pregnancy losses. Additionally, a compensatory mechanism against decreased endometrial and subendometrial vascularity results in the death of the embryo/fetus enhances in missed abortion cases. This mechanism characterized by increased expressions of IMD and RAMP2 initially begins in the syncytiotrophoblasts and cytotrophoblasts.

Key words: Intermedin; Vascular endothelial growth factor; Nitric oxide synthase type III; RAMPs; Spontaneous abortion.

Introduction

Expulsion of the embryo, fetus, and other pregnancy tissues from the uterus before 20 gestational weeks is considered an abortion [1]. Approximately 12–15% of all pregnancies result in a miscarriage, and with subclinical abortions, this ratio reaches 60% [2, 3]. However, according to abortion-related research, the reasons for abortions remain unknown in 50% of the cases [4]. Currently, it is a known fact that an unreceptive endometrium, leading to an abnormal implantation, is associated with a spontaneous miscarriage [5].

A close interaction between the blastocyst and the receptive endometrium results in a successful implantation. Endometrial and subendometrial vascularity is significantly higher in a pregnant woman's uterus. It has been thought that a good blood supply towards the peri-implantation endometrium is an essential requirement during a normal implantation [6]. However, an adequate blood flow through the uterine arteries throughout the course of a pregnancy is dependent on various angiogenic and vasoactive factors [7].

Intermedin (IMD) is a vasoactive peptide from the calcitonin gene-related peptide alpha/calcitonin gene-related peptide beta (CALCA/CALCB) family [8]. The actions of this peptide in support of the development of a pregnancy are mediated by the calcitonin receptor-like receptor (CALCLR) in association with one of three receptor activity-modifying proteins. These include CALCLR/receptor activity modifying protein (RAMP)1, CALCLR/ RAMP2, and RAMP3. RAMPs are required for the surface delivery of CALCLR and the determination of its phenotype [9]. IMD plays a decisive role in the remodeling of spiral arteries for trophoblast invasion into the uterine decidua during a successful implantation [10]. Blocking the endogenous effect of IMD causes feto-placental growth retardation, as well as a decrease in the expression of endothelial nitric oxide synthase (eNOS) in rats [11]. eNOS helps to produce nitric oxide (NO) in the blood vessels. NO is then used in the endothelium of uterine blood vessels to relax surrounding smooth muscles. Thus, vasodilatation occurs and this supports endometrial receptivity [12]. Another angiogenic factor is vascular endothelial growth factor (VEGF), which also increases during the implantation period [13]. Its role involves stimulating endothelial cell proliferation and increasing vascular permeability during implantation [14].

In this study the authors aimed to investigate the role of certain angiogenic and vasoactive agents in pregnancy losses at the maternal-fetal interface with immunohistochemical

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methods by comparing healthy pregnancies with missed abortions.

Materials and Methods

This prospective study was conducted on Merkez Efendi State Hospital, Department of Obstetrics and Gynecology. It was approved by Ethics Committee of Celal Bayar University. All the participants provided informed consent. Twenty pregnant women who underwent dilation and curettage procedures were included in the study. Chorion and decidua samples were obtained during curettage from ten women with unwanted pregnancies (control group) and ten women with missed abortions (missed abortion group). Inclusion criteria into the missed abortion group included: a crown-rump length (CRL) corresponding to a gestational age of six to 11 weeks determined with a transvaginal ultrasound and a negative fetal heart rate (FHR). The control group consisted of pregnant women wishing to have a curettage. In the control group, CRL was shorter than ten weeks (curettage is not allowed if the CRL is more than ten weeks) and a positive FHR was observed. Exclusion criteria for both groups included subjects having any gynecologic or systemic disorders and those using any type of medication in the previous three months.

Fetal death was 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-mm thick serial sections. The first tissue sections were stained with IMD, RAMP1, RAMP2, RAMP3, VEGF, and eNOS primary antibody by means of immunohistochemical technique.

Formalin-fixed, paraffin-embedded sections were used for immunohistochemical staining. The tissue samples were stored at 60°C overnight and then were dewaxed by xylene for 30 minutes. After the dehydration of the sections with ethanol, they were washed with distilled water. Then, they were treated with 2% trypsin at 37°C for 15 minutes and incubated in 3% H2O2 solution for 15 minutes to inhibit endogenous peroxidase activity. Then, the sections were incubated with anti-IMD antibody, Anti-RAMP1 antibody, Anti-RAMP2 antibody, anti-RAMP3 antibody, anti-VEGF antibody, and anti-eNOS antibody for 18 hours at +4°C. They were given an additional three five-minute 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 minute, the sections were stained with a substrate system containing diaminobenzidine to detect the immunoreactivity, and then were stained with Mayer's hematoxylin for counterstaining. They were covered with entellan and observed with light microscopy.

Immunostaining for IMD, RAMP1, RAMP2, RAMP3, VEGF, and eNOS were evaluated semi-quantitatively by means of IMD, RAMP1, RAMP2, RAMP3, VEGF, and eNOS analysis. Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak, but detectable, staining), 2 (moderate staining), and 3 (intense staining). A H-score value was derived for each specimen by calculating the sum of the percentage of cells for uterine epithelial cells, endometrial glandular cells, endothelial cells, fibroblast, and decidual cells in uterine decidual stroma, and syncytiotrophoblast, cytotrophoblasts, endothelial cells, and mesenchymal cells in placental villous stroma that stained at each intensity category multiplied by its respective score, by means of 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 epithelial cells for each intensity, varying from 0% to 100%. For each slide, five different fields were evaluated microscopically at ×200 magnification. H-score evaluation were 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 was utilized.

The statistical package SPSS 15.0 (Statistical Package for Social Sciences) was used 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 value < 0.05 was accepted as significant.

Results

The mean age of the control group was 31.9 (range: 26–43) years, while the mean age of the missed abortion group was 29.3 (range: 22–42) years. The median time for gestational weeks was eight (range: 6–11) weeks in the missed abortion group and seven (range: 5–9) weeks in the controls. Immunohistochemical values (mean ± standard deviation [SD]) were summarized in the decidua and placenta and are shown in Table 1. Error bars of the H-score for IMD, RAMP1, RAMP2, RAMP3, VEGF, and eNOS expression are shown in Figure 1 for the decidua and in Figure 2 for the placenta. A sample of the immunohistochemical analysis for IMD, RAMP1, RAMP2, RAMP3, VEGF, and eNOS are shown in Figure 1.

Decidua

IMD showed a higher expression in epithelial (p = 0.002), in glandular (p = 0.0004), and vascular endothelial cells (p = 0.0002) in the control group; however, there was a higher expression in stromal cells for the missed abortion group (p = 0.004). All RAMPs exhibited stronger expressions in the epithelial (p < 0.0001) and glandular cells (p < 0.0001) in the control group than in the missed abortion group. Only RAMP1 showed a higher expression in the stroma for the control group (p < 0.0001); however, RAMP3 showed a higher expression in the endothelium for the control group (p = 0.009). VEGF showed a higher expression in the control group in the stroma, glandular, and endothelial cells (p = 0.002, p = 0.0003, and p = 0.001, respectively); higher expressions were not observed in epithelial cells (p = 0.06). Additionally, eNOS had a stronger expression in the control group in epithelial, stromal, and endothelial cells (p < 0.0001, p = 0.0007, and p = 0.0003, respectively). However, eNOS had a higher expression in glandular cells of the missed abortion group than in those of the control group (p = 0.0001).

Placenta

IMD and RAMP2 exhibited stronger expression in the evaluated placental cells for the missed group; however, there was no statistically significant difference in the vascular endothelium for IMD. RAMP1 and RAMP3 showed a higher expression in the syncytiotrophoblasts for the

Table 1. — *H-score values of IMD, RAMP1, RAMP2, RAMP3, VEGF, and eNOS for uterine epithelial cells, endometrial glandular cells, endothelial cells, and fibroblast and decidual cells in uterine decidual stroma, and syncytiotrophoblast, cytotrophoblasts, endothelial cells, and mesenchymal cells in placental villous stroma.*

DECIDUA	Epithelial cells			Stromal cells			Endometrial glands			Vascular endothelium		
	Control	Missed	*p*	Control	Missed	*p*	Control	Missed	*p*	Control	Missed	*p*
IMD	151.0± 9.42	113.1± 4.11	0.002*	108.5± 4.89	136.5± 5.58	0.004*	97.50± 6.33	41.00± 3.14	0.0004*	36.60± 4.18	16.00± 1.63	0.0002*
RAMP1	288.0± 3.74	137.0± 4.23	< 0.0001*	171.0± 9.99	59.00± 2.33	< 0.0001*	228.5± 5.00	101.0± 2.33	< 0.0001*	19.20± 2.53	17.20± 1.12	0.315
RAMP2	166.0± 4.76	74.00± 3.39	< 0.000*1	84.00± 4.52	70.00± 4.50	0.089	180.0± 3.94	114.0± 3.05	< 0.0001*	10.00± 1.07	8.600± 0.5207	0.247
RAMP3	248.4± 5.34	149.0± 4.06	< 0.0001*	98.00± 4.16	115.0± 6.87	0.063	217.1± 10.28	142.0± 3.59	< 0.0001*	26.00± 2.98	15.60± 1.32	0.009
VEGF	130.6± 7.29	86.0± 18.97	0.06	54.00± 15.77	35.0± 7.07	0.0025*	52.4± 13.09	26.0± 6.99	0.0003*	80.00± 12.47	47.0± 6.7	0.001*
eNOS	145.5± 7.61	86.0± 13.49	< 0.0001*	61.0± 9.94	44.0± 5.16	0.0007	71.0± 7.3	97.00± 8.23	0.0001*	80.0± 8.167	60.00± 7.88	0.0003*

PLACENTA	Syncytiotrophoblasts			Cytotrophoblasts			Stromal cells			Vascular endothelium		
	Control	Missed	*p*	Control	Missed	*p*	Control	Missed	*p*	Control	Missed	*p*
IMD	48.0± 10.32	63.0± 6.74	0.003*	36.0± 12.63	56.0± 9.66	0.0017*	48.0± 19.88	68.40± 15.01	0.022*	27.0± 8.23	43.0± 11.59	0.063
RAMP1	269.5± 4.91	278.5± 19.89	0.007	207.7± 4.73	111.0± 4.58	< 0.0001*	172.6± 9.07	181.5± 6.50	0.481	34.50± 2.20	9.200± 0.61	< 0.0001*
RAMP2	37.2± 4.64	93.00± 2.60	< 0.0001*	17.60± 1.18	58.00± 2.49	< 0.0001*	15.20± 1.81	40.00± 2.10	< 0.0001*	5.600± 0.71	8.600± 0.52	0.007
RAMP3	195.4± 1.57	290.0± 3.16	< 0.0001*	164.0± 4.00	162.1± 7.29	0.739	142.0± 3.88	140.0± 7.60	1.000	17.80± 1.84	8.600± 0.52	0.0001*
VEGF	105.0± 13.54	56.00± 10.74	< 0.0001*	65.0± 10.8	49.0± 14.49	0.016	80.0± 20.65	60.0± 6.99	0.343	81.40± 18.52	84.40± 8.42	0.719
eNOS	90.0± 10.54	43.0± 8.23	0.0001	58.0± 9.18	40.0± 15.63	0.011	60.0± 9.94	70.0± 9.92	0.936	100.0± 11.00	90.0± 14.18	0.232

* $p < 0.05$. Data are presented as mean± SD. Missed: missed abortion group; control: control group.
$p < 0.05$ was considered statistically significant.

missed abortion group ($p = 0.007$ and $p < 0.0001$, respectively); however, these were higher in the vascular endothelium for the control group ($p < 0.0001$ and $p = 0.0001$, respectively). In the cytotrophoblasts, a higher expression in the control group was observed only in RAMP1 ($p < 0.0001$). The H-score values of VEGF and eNOS in the syncytiotrophoblasts ($p < 0.0001$ and $p = 0.0001$, respectively) and cytotrophoblasts ($p = 0.016$ and $p = 0.011$, respectively) were higher in the control group than in the missed abortion group.

Discussion

Human embryo implantation and development are very complex and still have unknown aspects. Pregnancy loss rates might be decreased by a complete explanation of the factors affecting implantation. In the present work, to understand the role of certain angiogenic and vasoactive agents in pregnancy losses, the authors determined that IMD and RAMPs in deciduas exhibited lower expressions in the missed abortions group. However, a compensatory expression increase in the placental side, especially for IMD and RAMP2, was observed in the missed abortions group. VEGF and eNOS, in those with healthy pregnancies, exhibited a higher expression in both decidual cells and the placental side, especially in syncytiotrophoblasts and cytotrophoblasts.

IMD is a 47-amino-acid peptide that was identified in 2004 by Roh *et al.*, [8] and Takei *et al.* [15]. It has approximately a 28% structural homology to adrenomedullin (ADM) and greater than 20% with CALCB. IMD is expressed in the rat ovary, uterus, placenta, and other tissues, such as the brain, heart, and pituitary gland [8, 15]. IMD has vasodilatory and hypotensive actions that are similar to, or more potent than, those of ADM and CALCB. According to Havemann *et al.*, [10] plasma levels of IMD in rats are elevated during pregnancy. They also reported that IMD is found not only in the serum, but also in placental and decidual tissues in the first trimester of pregnancy. At the end of their study, Havemann *et al.* [10] concluded that IMD plays a physiological role in

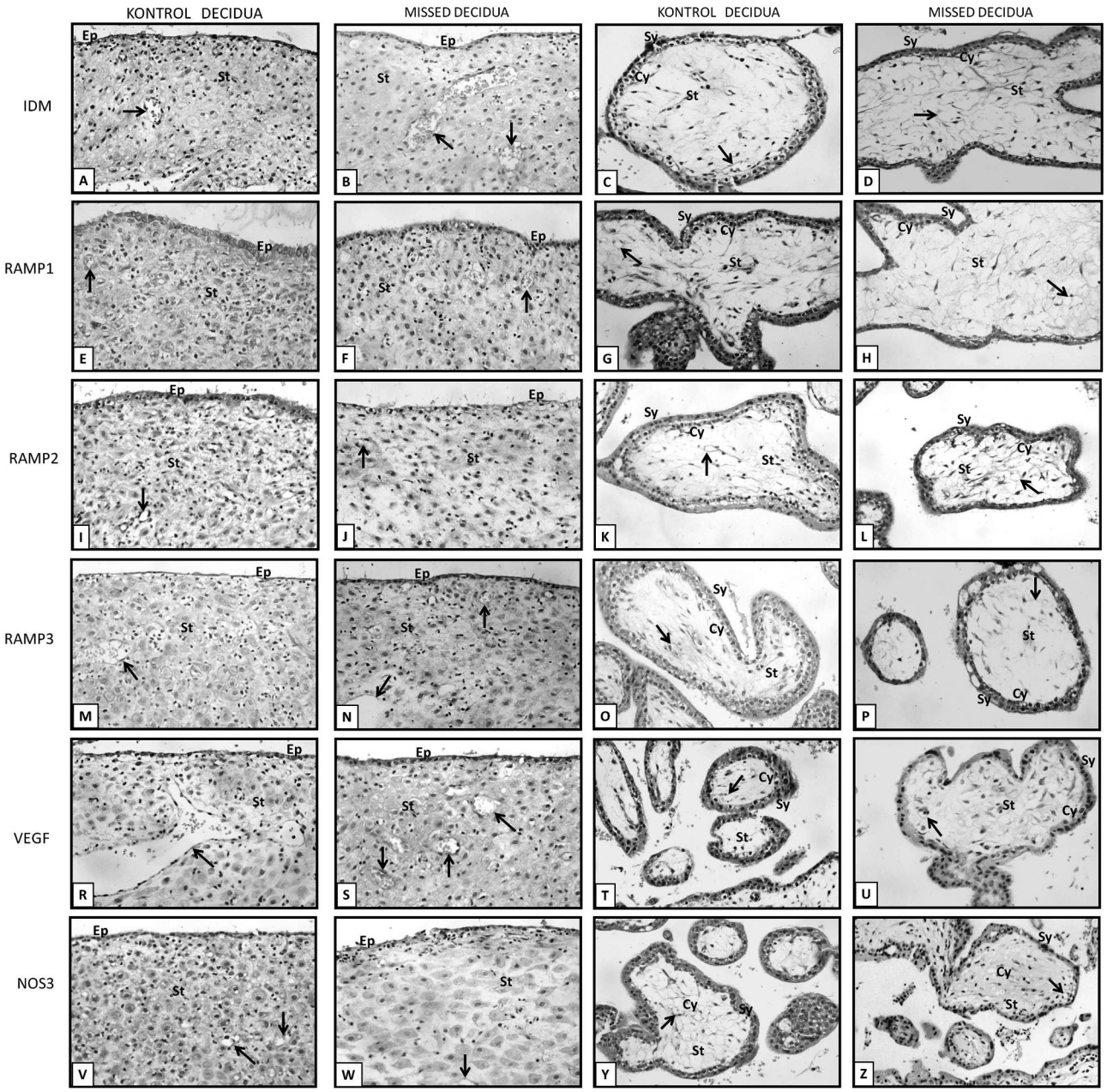

Figure 1. — Immunohistochemical analysis of IMD, RAMP1, RAMP2, RAMP3, VEGF, and NOS3 expression are seen in the decidual (A, B, E, F, I, J, M, N, R, S,V, W) and placental chorionic villi (C, D, G, H, K, L, O, P, T, U, Y, Z) of normal pregnancy and missed abortion groups. Uterine epithelium: Ep, Stroma: St; Arrows: blood vessels (original magnification: ×200).

trophoblast invasion during early placental development. Another study has shown that an infusion of an IMD antagonist (IMD_{17-47}) into pregnant rats results in a feto-placental growth restriction [11]. IMD_{17-47} downregulates certain factors that take part in implantation and placental development, such as VEGF [16]. Chauhan *et al.*, [17] showed that both human placenta and trophoblast cells are rich in terms of IMD immunoreactivity. However, they did not observe extensive expression of IMD in placental vascular endothelial cells. They claimed that a heterogeneic expression of IMD in placental vascular endothelial cells results from the presence of different phenotypes of endothelial cells [17, 18]. The present authors found that IMD in each of the three RAMPs had a stronger expression in healthy pregnancies than in pregnancy losses in the decidual side. However, the authors determined that all of these showed higher expressions in syncytiotrophoblasts of the missed abortions group. IMD and RAMPs, except for RAMP3, exhibited heterogeneic expres-

sions in other evaluated cells of the placenta between groups. Unlike in the present work, Havemann *et al.* [10] found that IMD expression was lower in spontaneous abortions. However, they subsequently identified that IMD levels in pregnancy tissue of spontaneous abortions were lower in the early weeks of the first trimester and increased while pregnancies proceeded toward the second trimester. They claimed that this increase was a compensatory mechanism. In the present study, the missed group was not composed of tissues obtained immediately after the fetal/embryo death. Furthermore, the durations after fetal/embryo deaths were different for all missed abortions. These tissue properties observed in the present study may explain the increased IMD in missed abortion cases. Therefore, the authors believe that a high expression in IMD, and partly in RAMPs, for missed abortions, may result from a compensatory response against a decline in IMD levels due to the loss of the pregnancy. Moreover, it seems that the compensatory response initially begins in the syncytiotrophoblasts and cytotrophoblasts.

IMD is a non-selective agonist for RAMPs, but it has greater potency with CALCRL/RAMP1 and CALCRL/RAMP3 [8]. CALCRL/RAMP2 appears to be more closely associated with reproductive functions and fetal development compared to the other two receptors. This receptor is important for the development of the fetal cardiovascular system and it can protect against a variety of vascular diseases [19]. The lack of RAMP2 expression results in severe reproductive defects characterized by fetal growth restrictions, intrauterine fetal death, and postnatal mortality in female mice [20]. RAMP2 showed lower expressions for both groups when compared to the other two RAMPs in the present study. However, the most parallel comparison between groups with IMD was observed for RAMP2. This relationship may indicate that IMD exhibits a greater potency with CALCRL/RAMP2 during pregnancy.

VEGF is an angiogenic factor and contributes to increases in vascular permeability [14]. VEGF plays an essential role in placental and fetal angiogenic development by proliferating endothelial cells and promoting the new formation of capillaries. VEGF is also responsible for oocyte maturation, decidualized endometrial vascularization, and embryo implantation and development in the early stages of pregnancy [21, 22]. Vuorela *et al.*, [23] found that VEGF immunoreactivity is diminished in the decidual vascular endothelium and placental trophoblasts for spontaneous miscarriages. In the present study, VEGF expression was higher in every evaluated cell group, except for the placental vascular endothelium in healthy pregnancies. The authors did not find any differences in the vascular endothelium of the placenta. The findings of Vuorela *et al.*, [23] also support the present study; they found that VEGF showed an equivalent level expression in the placental vascular endothelium for both missed abortions and healthy pregnancies. It seems that pregnancy losses are associated with alterations in VEGF expression when both decidual and placental areas are considered as a whole.

The eNOS enzyme is responsible for the production of NO in the vessel endothelium. Increased expression of endometrial eNOS during the mid-secretory phase shows its contribution on endometrial receptivity and blastocyst implantation [24]. The function of NO as a potent vasodilator plays a significant role in establishing and maintaining a pregnancy by being a myometrial smooth muscle relaxant and a participant in signal transduction pathways [25, 26]. A lack of NO leads to vasospasms, vascular infarctions, and subsequently miscarriages; in these women, eNOS gene polymorphism is responsible for the miscarriages [27]. The previous findings are supported by the present study's results, except for the endometrial glands. Unlike other cells, in endometrial glands, eNOS exhibited a stronger expression in the missed abortion group. Only one report supports the present finding in the endometrial glands, a study by Najafi *et al.*, [24] where higher eNOS expression in the endometrial glandular epithelium of miscarriages was observed. In their report, they claimed that excessive NO causes first trimester pregnancy losses. However, increased NO may be a result of compensatory mechanisms rather than a reason for the pregnancy losses. Studies with greater sample sizes are required to clarify this conflict.

In conclusion, this study has shown that expressions of vasoactive agents, such as VEGF, eNOS, and IMD, decrease in first trimester pregnancy losses. Additionally, this study has shown that a compensatory mechanism against decreased endometrial and subendometrial vascularity results in the death of the embryo/fetus enhances in missed abortion cases. This mechanism characterized by increased expressions of IMD and RAMP2 initially begins in the syncytiotrophoblasts and cytotrophoblasts.

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