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Investigation of FAS and IL-6 Expression in Placentas with HELLP Syndrome

Investigación de la Expresión de FAS e IL-6 en Placentas con Síndrome de HELLP

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SUMMARY: The aim of our study was to investigate the effect of inflammation in the placenta on the pro-apoptotic development after severe preeclampsia. Placenta tissue samples of 15 HELLP syndrome and 15 healthy 35-38th week-pregnant women were involved in the study. Tissue samples were taken only from the maternal side of the placenta and fixed in 10 % formaldehyde, then blocked in paraffin wax and 4-6 mm-thick sections were cut and stained with Harris Hematoxylene-Eosin. Antigen retrieval was performed for sections, incubated with FAS antibody and anti-IL-6 antibody. After the application of streptavidin peroxidase followed by AEC chromogen solution, sections were counterstained with Harris hematoxylin. Significant thickening of the fibrinoid layer, degeneration and apoptotic change in decidua cells, marked increase in the hyalinized area, degenerative changes in the syncytial regions of the chorionic villus and an increase in syncytial nodes and bridges and IL- expression were observed as positive. FAS expression was positive in the pycnotic nuclei of decidual cells in the maternal region and in the syncytial regions. It was observed that the proapoptotic process increased as a result of severe preeclampsia. It was concluded that the control of cytokine activity and reduction of pro-apoptotic signal during the inflammation process will slow down the development of HELLP syndrome.

KEY WORDS: HELLP syndrome; FAS expression; IL-6 Expression; Placenta.

INTRODUCTION

The placenta is a temporary organ that nourishes the fetus, regulates many metabolic activities and produces hormones necessary for the continuation of pregnancy, and ends its function at the end of pregnancy (Handwerger & Freemark, 2000). The placenta is implanted in any area of the uterine wall however in some clinical complications, it can prevent the fetal head from descending during delivery if it is too low in the uterus. Low-lying placentas can also cause unusual bleeding during pregnancy or delivery. As embryo develops, substances exchange between fetal and maternal blood circulation towards the end of pregnancy decreases due to some abnormal structural changes such as thickening of the basement membrane of fetal capillaries, increased fibrous tissue in the villous stroma and fibrinoid accumulation in chorionic plate and on root villi in the junction may be observed (Handwerger, 1991).

Preeclampsia (PE) is characterized by new onset of hypertension (blood pressure≥ 140/90 mmHg) proteinuria (≥ 300 mg in 24 h) and/or a feature indicative ofend-organ damage (cerebral symptoms, low platelet count, impaired renal function, impaired renal function, liver function, pulmonary edema or visual symptoms) after 20 weeks of gestation. Severe preeclampsia (HELLP syndrome) was defined as having one or more of the following ACOG (The American College of Obstetricians and Gynecologist) criteria: systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg; in addition, thrombocytopenia <100,000/mm^3, liver dysfunction (AST/ALT > twice normal, severe persistent right upper quadrant or epigastric pain unresponsive to medical therapy), new-onset headache, blurred vision, impaired mental status, pulmonary edema, or cyanosis , serum creatinine ≥ 1.2 mg/dL, or at least 2-fold increasing serum creatinine level. PE occurs in 3-5 % of pregnancies and is an important cause of maternal and perinatal morbidity and mortality (Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in

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Pregnancy, 2013). It is severe form of preeclampsia which is characterized by hemolysis, elevated liver enzymes, and decreased platelet count, is associated with high maternal and perinatal morbidity and mortality. HELLP is associated with high maternal and perinatal morbidity and mortality, characterized by hemolysis, elevated liver enzymes, and decreased platelet count. Incidence of HELLP in preeclamptic pregnancies is 4-20 %. HELLP syndrome was reported by Prichard in 1954 and named by Weinstein in 1982 (Weinstein, 1982). HELLP was generally accompanied by preeclampsia in the third trimester, however it may rarely be observed in early pregnancy or in the postpartum period and without hypertension (Vigil-De Gracia, 2001). Acute respiratory distress syndrome (ARDS), intracerebral hemorrhage, acute kidney failure, hepatic rupture, diffuse intravascular coagulation (DIC) and septic shock are common clinical symptoms observed in HELLP syndrome (Martin Jr. *et al.*, 1999). IL-6 is a pleiotropic immunomodulatory cytokine produced by many tissues in response to infection and tissue injury. It also plays a regulatory role in acute phase reactions, haematopoiesis, and immune cell differentiation and activation (Stinson *et al.*, 2019). FAS (APO-1 or CD95) is in the subgroup of the tumor necrosis factor receptor (TNF-R) family that contain an intra-cellular death domain and may able to trigger apoptosis. Its physiological ligand is FAS ligand (FASL) which is a peptide that takes a crucial role in host immune response (Strasser *et al.*, 2009).

The aim of our study was to investigate the effect of inflammation in the placenta on the pro-apoptotic development after severe preeclampsia.

MATERIAL AND METHOD

The study was approved by the Medical Committee of Diyarbakir Maternity and Child Health Hospital. All protocols were approved by local ethics committee. All patients were informed and signed consent form. All placenta tissues were provided from the Diyarbakır Maternity and Child Health Hospital (Department of Obstetrics and Gynecology). The study included 20 pregnant patients with Hellp syndrome placenta and 15 healthy pregnant patients between gestational age. Hellp syndrome criteria were defined by hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) and proteinuria (> 300 mg in 24 h). Clinical blood tests were gathered and experimental biochemical analysis were performed on blood samples of patients. The placental tissues were immersed in 10 % buffered formaldehyde. They were dehydrated in ascending alcohol series, cleaned in xylene and embedded in paraffin. Then 5 µm sections were cut and stained with H-E (Ermis & Deveci, 2021).

Immunohistochemical examination. All sections were bought to distilled water and for further immunohistochemical examination. Antigen retrieval process was performed in citrate buffer solution (pH: 6.0) for 12 minutes in a microwave oven at 700 W. Sections were permitted to cool down at room temperature for 30 minutes and washed in distilled water 3 × 5 minutes. 2 % hydrogen peroxide (H_2O_2) was used for endogen peroxidase blocking for 15 min. Samples were rinsed in distilled water and washed in PBS. The sections were then incubated with mouse monoclonal anti-FAS antibody (1: 100) and Mouse monoclonal anti-IL-6 antibody (1:100) overnight at + 4 °C. The next day, sections were cleaned with PBS and secondary antibody solution (Biotinylated Goat Anti-Mouse, Lab Vision) was applied for 20 min. Following PBS, streptavidin peroxidase solution (Streptavidin Peroxidase, Lab Vision) was performed for 20 min. Slides were washed 3 times in PBS and DAB chromogen solution were applied for 10 min. Sections were washed with distilled water and counter stained with 2 min Mayer hematoxylin (Yükselmis & Ermis, 2002). Slides were imaged with imager A2 Zeiss light microscope. Semiquantitative scaling of sinsitial knot, congestion in blood vessels, fibrinoid accumulation inflammation and degeneration in decidua were carried out. The intensity of these changes were graded from 0 to 4 (0: no change, 1: low, 2: moderate, 3: intense 4: most intense). Semiquantitative scaling of immunoreactivity was carried out. The intensity of staining was graded from 0 to 4 (0: no staining, 1: faint staining, 2: moderate staining, 3: intense staining, 4;most intense staining) (Ermis, 2021).

Statistical Analysis. Statistical analysis was performed by the IBM SPSS 25.0 software (IBM, Armonk, New York, US).The data were recorded as median (minimum – maximum) with mean rank value. Binary group comparisons were evaluated with Mann-Whitney U and. $P < 0.05$ was accepted as the significance level.

RESULTS

Statistical analysis of gynecological parameters (age, gravida, parity, systolic bp, diastolic bp, hemoglobin, platelet, glucose, urea, creatinine, ALT, AST, 2 h urine protein) were shown in Table I. Compared to control group, increase in systolic and diastolic BP, hemoglobin, urea and the decrease in hemoglobin values in HELLP group were significant. Graphical illustration of Table I was shown in Figures 1 to 4.

Table I. Gynecological parameters of patients with control and HELLP syndrome.

Parameter	Groups	n	Median (Min-Max)	Mean Rank	p value	Mann-Whitney U Test
Age	(1) Control	15	27.00 (20.00-40.00)	14.43	0.506	
	(2) HELLP	15	30.00 (19.00-60.00)	16.57		
Gravida	(1) Control	15	1.00 (1.00-8.00)	13.97	0.313	
	(2) HELLP	15	3.00 (1.00-10.00)	17.03		
Parity	(1) Control	15	0.00 (0.00-4.00)	13.40	0.155	
	(2) HELLP	15	2.00 (0.00-6.00)	17.60		
Systolic BP	(1) Control	15	115.00 (88.00-128.00)	8.00	<0.001	(2)
	(2) HELLP	15	150.00 (132.00-197.00)	23.00		(1)
Diastolic BP	(1) Control	15	70.00 (62.00-84.00)	8.00	<0.001	(2)
	(2) HELLP	15	95.00 (90.00-110.00)	23.00		(1)
Hemoglobin	(1) Control	15	12.00 (9.30-14.70)	18.77	0.042	(2)
	(2) HELLP	15	10.60 (8.00-14.10)	12.23		(1)
Platelet	(1) Control	15	245.00 (128.00-409.00)	14.83	0.678	
	(2) HELLP	15	269.00 (140.00-444.00)	16.17		
Glucose	(1) Control	15	78.00 (63.00-98.00)	16.57	0.506	
	(2) HELLP	15	72.00 (62.00-104.00)	14.43		
Urea	(1) Control	15	15.00 (10.00-26.00)	11.20	0.007	(2)
	(2) HELLP	15	21.00 (13.50-38.78)	19.80		(1)
Creatinine	(1) Control	15	0.60 (0.52-0.66)	5.55	0.803	
	(2) HELLP	15	0.58 (0.39-0.70)	15.45		
ALT	(1) Control	15	10.00 (6.00-23.00)	5.55	0.134	
	(2) HELLP	15	13.00 (7.00-80.00)	15.45		
AST	(1) Control	15	19.00 (12.00-49.00)	5.55	0.560	
	(2) HELLP	15	20.00 (10.00-121.00)	15.45		
2h urine protein	(1) Control	15	140.00 (98.00-195.00)	5.55	<0.001	(2)
	(2) HELLP	15	720.00 (360.00-18664)	15.45		(1)

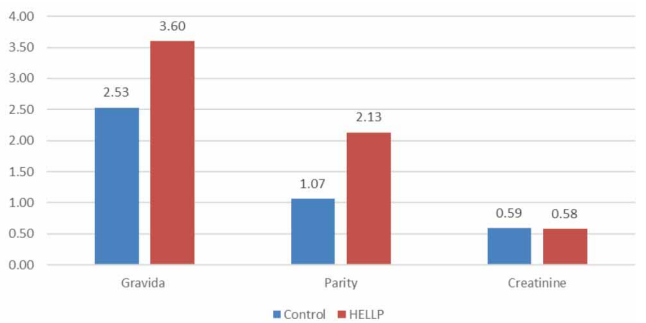

Fig. 1. Graphical illustration of gravida, parity and creatinine in control and HELLP patients.

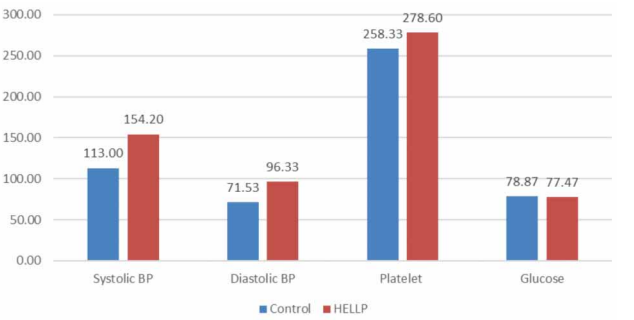

Fig. 2. Graphical illustration of systolic, diastolic, platelet, glucose in control and HELLP patients.

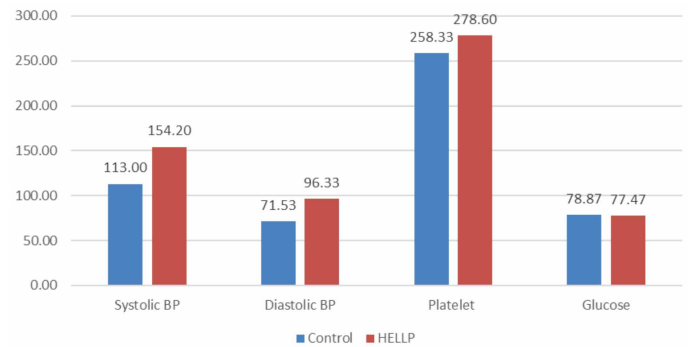

Fig. 3. Graphical illustration of age, hemoglobin, urea, ALT and AST in control and HELLP patients.

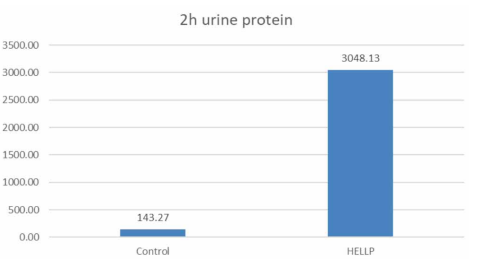

Fig. 4. Graphical illustration of 2h urine protein in control and HELLP patients.

Table II. Histological parameters of patients with control and HELLP syndrome.

Parameter	Groups	n	Median (Min-Max)	Mean Rank	p value	Mann-Whitney U Test
Syncytial knot	(1) Control	18	1.00 (1.00 – 2.00)	171.00	<0.001	(2)
	(2) HELLP	18	1.00 (0.00 – 2.00)	495.00		(1)
Congestion in blood vessels	(1) Control	18	1.00 (0.00 – 2.00)	172.00	<0.001	(2)
	(2) HELLP	18	1.00 (0.00 – 2.00)	494.00		(1)
Fibrinoid accumulation	(1) Control	18	1.00 (0.00 – 1.00)	172.00	<0.001	(2)
	(2) HELLP	18	1.00 (0.00 – 2.00)	494.00		(1)
Inflammation	(1) Control	18	1.00 (0.00 – 2.00)	172.00	<0.001	(2)
	(2) HELLP	18	3.50 (3.00 – 4.00)	494.00		(1)
Degeneration in decidua	(1) Control	18	4.00 (2.00 – 4.00)	171.00	<0.001	(2)
	(2) HELLP	18	4.00 (2.00 – 4.00)	495.00		(1)
FAS expression	(1) Control	18	4.00 (2.00 – 4.00)	171.00	<0.001	(2)
	(2) HELLP	18	3.00 (2.00 – 4.00)	495.00		(1)
IL-6 expression	(1) Control	18	3.00 (3.00 – 4.00)	171.50	<0.001	(2)
	(2) HELLP	18	4.00 (2.00 – 4.00)	494.50		(1)

Statistical analysis of histological parameters (syncytial knot, congestion in blood vessels, fibrinoid accumulation, inflammation, degeneration in decidua, FAS expression, IL-6 expression) were shown in Table II. Compared to control group, all values were significantly increased in HELLP group. Graphical illustration of Table II was shown in Figure 5.

In the control group sections, decidua cells in the maternal region consisted of nuclei rich in chromatin in a polygonal form, and it was observed that the chorionic villi towards the villous area consisted of a few syncytial bridges with regular syncytial structures (Fig. 6a).

In the histopathological examination of the sections belonging to the Hellp group, a significant thickening of the fibrinoid layer, degeneration and apoptotic change in the decidua cells, together with a significant increase in the hyalinized area, degenerative changes in the syncytial regions of the chorionic villi, and an increase in the syncytial nodes and bridges were observed (Fig. 6b).

In the control group IL-6 expression, IL-6 expression was positively observed in the syncytial region of the chorionic villi, cytotrophoblast cells and decidua cells. IL-6 expression was positive in some endothelial cells and Hoffbauer cells (Fig. 6c).

In sections belonging to the Hellp group, IL-6 expression was positively observed in the blood vessel endothelial cells in the maternal region, in some of the decidua cells, in the syncytial regions of the chorionic villi, in the syncytial bridge and knots (Fig. 6d).

In the control group, Fas expression was negative in the decidual cells in the maternal plaque region and in the cells in the syncytial region (Fig. 6e). In the placenta sections with Hellp syndrome, FAS expression was positively observed in the decidual cells in the maternal region, in the pycnotic nuclei and in the syncytial regions (Fig. 6f).

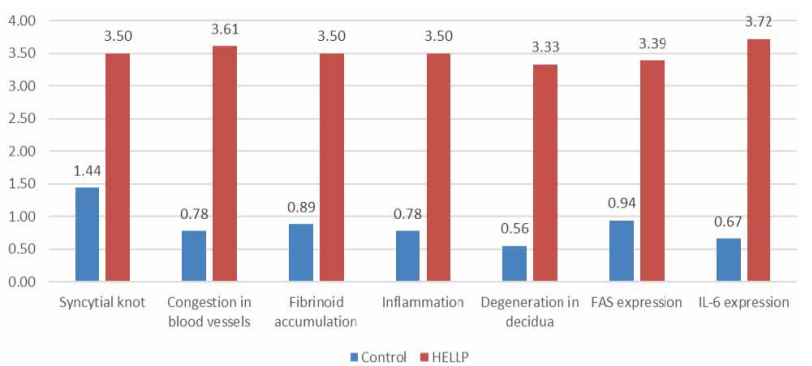

Fig. 5. Graphical illustration of histological parameters in control and HELLP patients.

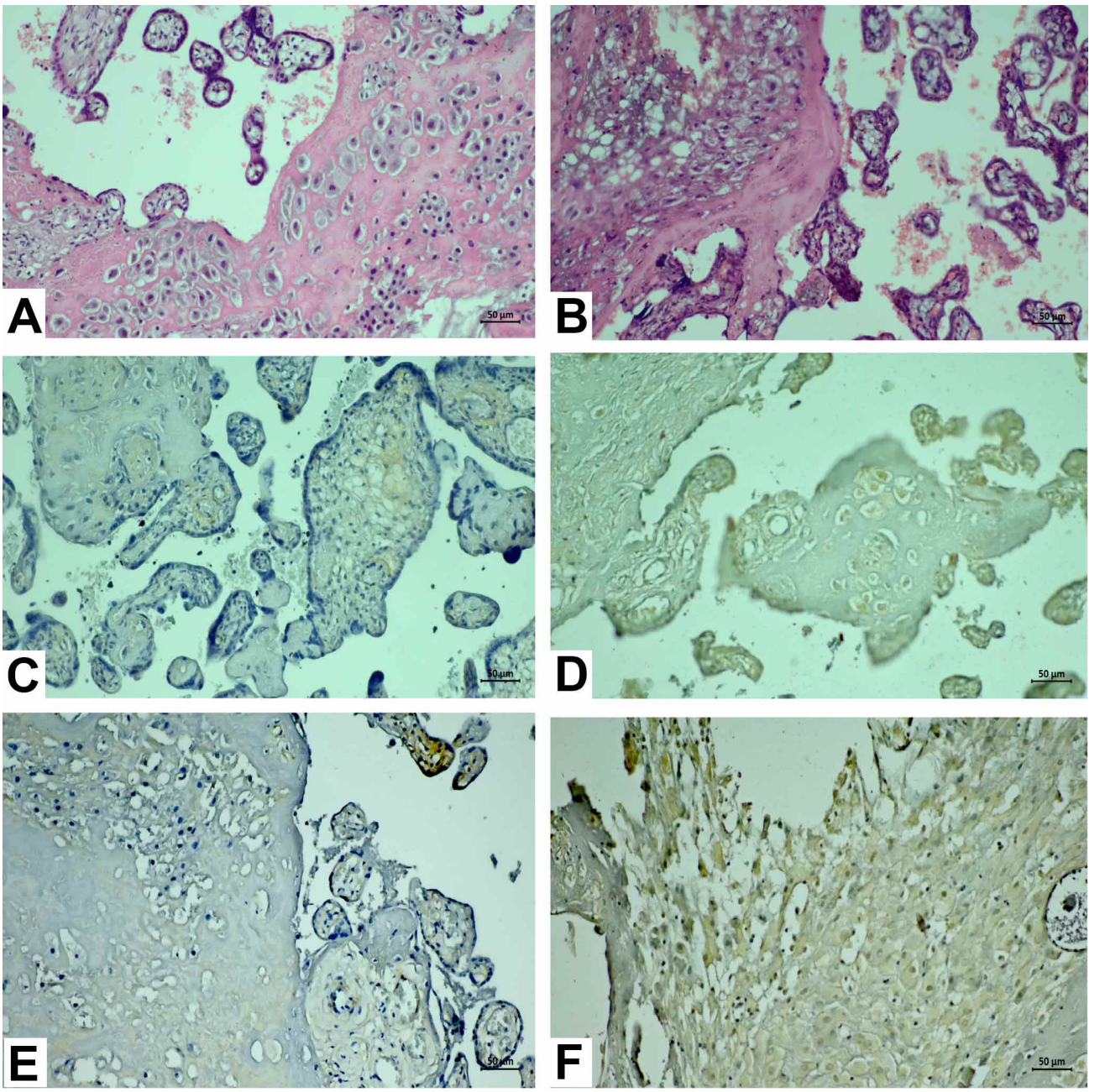

Fig. 6. Control group sections.

DISCUSSION

HELLP is severe form of preeclampsia which is characterized by hemolysis, elevated liver enzymes, and a low platelet count. Stevens *et al*. (2012) found significant changes in the pathology of vascularization in the decidual layer in the maternal region in preeclamptic patients. They showed fibrinoid necrosis in the vessels, significant loss of smooth muscle cells around the vessel and an increase in cell infiltrations. They thought that this developing negativity might develop depending on the severity of preeclampsia, and that it might be due to hypoxia that occurs during the passage of oxygen from the trophoblasts at the maternal and fetal interface (Stevens *et al*., 2012). Akhlaq *et al*. (2012), in their histopathological evaluation of preeclampsia patients, revealed that, depending on the severity of preeclampsia, smooth muscle

hypertrophy in the maternal-fetal region, hypertrophy of the distal villi, increase in syncytial nodes, hypertrophy in decidual cells, a significant increase in mononuclear cell infiltration in the embryonic connective tissue, and chorionic villi, depending on the severity of preeclampsia. They found that there was fibrin accumulation in the syncytial regions. Histopathological studies showed that HELLP caused periportal or focal parenchymal necrosis and fibrin deposition in hepatic sinusoids, leading to a decrease in portal venous flow (Ogge *et al*., 2011). Some studies found that infarction, intervillous thrombosis (villous lesions related to maternal malperfusion), and abruption, higher placental weight was observed in placentas of HELLP pregnancies (Tasin *et al.,* 2021). Another study showed that significant difference in the rate of decidual arteriopathy, vascular lesion related to maternal malperfusion (Barton *et al*., 1992). Mehrabian *et al*. (2012) investigate placental pathology in patients with severe pre-eclampsia (PE) and HELLP syndrome. They studied 32 severe pre-eclampsia and 25 HELLP syndrome patients and found that retroplacental hematoma, accelerated villous maturation and decidual arteriopathy in placentas of HELLP syndrome pregnancies (Mehrabian *et al*., 2012). Smulian *et al*. (2004), studied 31 women with HELLP syndrome and 56 with severe preeclampsia. They histopathologically showed that patients with HELLP syndrome had abruption lesions of the placenta, decidual vasculopathy, uteroplacental vascular lesions, chronic inflammation and coagulation. In the Hellp group of our study, significant thickening of the fibrinoid layer in the maternal region, degeneration and apoptotic changes in the decidua cells, significant increase in the hyalinized area, degenerative changes in the syncytial regions of the chorionic villus, and increase in the syncytial nodes and bridges were observed (Fig. 6b).

Fas (Apo-1/CD95/TNFRSF6) is a type I integral membrane protein and a member of the subgroup of the tumor necrosis factor receptor (TNF-R) family. FAS receptor involves in apoptotic pathways and have death receptor on its surface. Fas binds its counterpart Fas ligand (FasL) to iniate apoptosis (Strasser *et al*., 2009). FAS is an important molecule for immune system homestasis. Previous studies on mutant animals showed that FAS plays critical roles in removal of pathogen-infected cells and the death of obsolete and potentially dangerous lymphocytes (Lorenzo, 2013; Dag & Ermis, 2021). Scott *et al*. performed Fas and FasL immunostaining on term placentas at 37-40 weeks. In conclusion, the authors stated that FasL expression occurs on the trophoblast at the maternal-fetal interface, which induces apoptosis in activated lymphocytes (Kauma *et al*., 1999). Karara *et al*. performed Fas and FasL immunostaining on structures in the placenta of intrauterine growth retardation (IUGR) patients with or without preeclampsia (PE). FasL expression was found to be very low in villous trophoblasts and endothelial cells of villous vessels in IUGR placentas with PE. The authors suggested that FasL has a role in the etiology of IUGR with PE (Resic Karara *et al*., 2016). In a study, Fas ligand expression was investigated in 10 HELLP syndrome and 10 preeclamptic (PE) placentas. FasL expression was found to be higher in patients with HELLP syndrome than in the placenta of PE and normal patients. In the study, it was claimed that HELLP and PE have different mechanisms (Prusac *et al*., 2011). Gibbens *et al*. (2017) designed an animal HELLP model, claiming that placental Fas/FasL expression is increased in women with HELLP syndrome. The authors noted that placental FasL expression was higher and FAs expression was lower in rats with HELLP syndrome compared to rats with normal pregnancy. As a result, Fas/FasL expression was found to be irregular in pregnant women with HELLP syndrome (Gibbens *et al*., 2017). FAS expression was positively observed in decidual cells, pycnotic nuclei and syncytial regions in the maternal region in the sections of the placenta with Hellp syndrome. Due to the effect of severe preeclampsia, it was observed that the proapoptotic process accelerated significantly in decidual cells and syncytial region, and cellular organization and syncytial structure and basement membrane circulation were impaired (Fig. 6f).

Interleukin 6 (IL-6) is an interleukin secreted as an acute phase protein in the event of infection and tissue damage. It is secreted by macrophages in microbial infections and its expression is regulated by transcription factors. It has a pathological effect in chronic inflammation and autoimmune diseases (Tanaka *et al*., 2014). In a study of pregnant women with preeclampsia, serum IL-6 levels were compared between patients with preeclampsia and normotensive pregnant and non-pregnant women. At the end of the study, it was reported that high levels of the proinflammatory cytokine, IL-6, were associated with severe preeclamptic patients, which may increase the inflammation status in patients with severe preeclampsia. Anwer *et al*. (2017) measured maternal serum IL-6 levels in mild, severe preeclampsia and normal patients. The authors noted that the IL-6 variation between diseases was significant, the IL-6 level was higher in mild and severe preeclampsia than in control patients, and the highest level was in severe preeclampsia. The authors claimed that this condition contributes to systemic and endothelial dysfunction of preeclampsia. Due to the roles of IL-6 in oxidative stress and endothelial dysfunction, Ganap *et al*. (2018) investigated serum IL-6 levels in pregnant women with HELLP syndrome. The authors reported that serum IL-6 levels of pregnant women with HELLP syndrome were close to those of normotensive pregnant women and there was no significant difference (Ganap *et al*., 2018). Ozler *et al*. (2012) investigated the serum levels of IL-6 in pregnant women with mild, severe preeclampsia and HELLP syndrome and their relationship with the severity of the disease. It was noted that there was

no significant difference between the groups in terms of serum IL-6 level and it did not differ in diseases. In the Hellp group, IL-6 expression was positively observed in blood vessel endothelial cells, some decidua cells, syncytial regions of chorionic villi, Syncytial Bridge and nodes in the maternal region (Fig. 6d). It has been observed that IL-6, an inflammation marker, acts as a key in severe preeclampsia, inducing the apoptotic process and increasing the degeneration effect.

In Hellp syndrome, which occurs as a result of severe preeclampsia, increased decidual cell degeneration due to increased inflammation and trophoblastic invasion and increased pro-apoptotic effect together with changes in the basal membrane structure have been found to adversely affect fetal circulation and fetal development.

It was concluded that the control of cytokine activity and reduction of pro-apoptotic signal during the inflammation process will slow down the development of HELLP syndrome.

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RESUMEN: El objetivo de nuestro estudio fue investigar el efecto de la inflamación en la placenta sobre el desarrollo proapoptótico después de la preeclampsia severa. Se recogieron muestras de tejido de placenta de 15 mujeres con síndrome de HELLP y 15 mujeres sanas con un embarazo de 35 a 38 semanas. Se tomaron muestras de tejido solo del lado materno de la placenta y se fijaron en formaldehído al 10 %, luego se bloquearon en parafina y se cortaron secciones de 4-6 mm de espesor y se tiñeron con hematoxilena-eosina de Harris. La recuperación del antígeno se realizó para secciones, incubadas con anticuerpo FAS y anticuerpo anti-IL-6. Después de la aplicación de estreptavidina peroxidasa seguida de solución de cromógeno AEC, las secciones se contrastaron con hematoxilina de Harris. Se observó como positivo un engrosamiento significativo de la capa fibrinoide, degeneración y cambio apoptótico en las células de la decidua, aumento marcado en el área hialinizada, cambios degenerativos en las regiones sincitiales de la vellosidad coriónica y un aumento en los nódulos y puentes sincitiales y la expresión de IL-6. La expresión de FAS fue positiva en los núcleos picnóticos de las células deciduales en la región materna y en las regiones sincitiales. Se observó que el proceso proapoptótico se incrementó como consecuencia de la preeclampsia severa. Se concluyó que el control de la actividad de las citocinas y la reducción de la señal proapoptótica durante el proceso de inflamación ralentizarán el desarrollo del síndrome de HELLP.

PALABRAS CLAVE: Síndrome HELLP; FAS Expresión FAS; Expresión IL-6; Placenta.

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