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Evaluation of FAS and eNOS expression in COVID-19 placenta: histopathological and immunohistochemical study

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Abstract. – OBJECTIVE: In this study, the effects of cell adhesion, inflammation and apoptotic changes on fetal development in cases of COVID-19 placenta were investigated.

PATIENTS AND METHODS: Placenta tissue samples from 15 COVID-19 and 15 healthy pregnant women were taken after delivery. Tissue samples were fixed in formaldehyde, then blocked with paraffin wax and 4-6 μm thick sections were cut and stained with Harris Hematoxylene-Eosin. Sections were stained with FAS antibody and endothelial nitric oxide synthase (eNOS) antibody.

RESULTS: In COVID-19 placenta section, deterioration of the root villus basement membrane structure in the maternal region, decidua cells and syncytial cell degeneration, significant increase in fibrinoid tissue, endothelial dysfunction in free villi and intense congestion in blood vessels, increase in syncytial nodes and bridges were observed. In terms of inflammation, eNOS expression was increased in Hoffbauer cells, dilated blood vessels endothelial cells in chorionic villi, and surrounding inflammatory cells. Positive FAS expression was also increased in the basement membranes of root and free villi, syncytial bridge and nodes, and endothelial cells.

CONCLUSIONS: The effect of COVID-19 caused an increase in eNOS activity and acceleration of the proapoptotic process and the deterioration of cell-membrane adhesion.

Key Words:
Placenta, COVID-19, Fas, Enos.

Introduction

Coronavirus disease 2019 (COVID-19) is mainly transmitted from person to person through close contact and droplets, and its mechanism of action during pregnancy has not been fully elucidated[1]. Although there is not enough evidence for the vertical transmission of COVID-19 from infected pregnant mothers to their fetuses, it is known that an infected mother can transmit the COVID-19 virus through respiratory droplets during breastfeeding[2,3]. COVID-19 caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is known to have negative effects on organs that carry angiotensin converting enzyme 2 (ACE2) receptor. One of these organs is the placenta. The placenta has mechanisms to protect itself from SARS-CoV-2 so that most positive mothers had negative newborns that did not present virus-induced disease. However, some SARS-CoV-2 positive mothers have presented early-onset symptoms and pregnancy-related complications like miscarriage, restricted fetal growth, or still-birth[4,5].

FAS, cluster of differentiation 95 (CD95), is in the subgroup of the tumor necrosis factor receptor (TNF-R) family that contains an intra-cellular death domain and may be able to trigger apoptosis. Its physiological ligand is FAS ligand (FASL) which is a peptide that takes a crucial role in the host immune response[6]. The expression of FASL is made in organs with immunoprivilege such as the eye and the testis. Herein FASL binds to its receptor FAS, which is localized on activated immune cells, and as a result cell apoptosis. It is known that FASL is expressed in the human placental structures. Placental villi and early trophoblast cells have a role in maternal immune-tolerance to the fetus. FAS is observed in CD45 (leukocyte common antigen) positive cells that are found in maternal decidua[7]. The expression of FASL on the surfaces of placental cytotrophoblasts is vital both in the maintenance of pregnancy and in the normal development of fetus[8].

Endothelial NOS (eNOS) is an enzyme, one of the three isoforms synthesizing nitric oxide (NO),

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a gaseous and lipophilic molecule that takes role in several biological processes[9]. eNOS is primarily responsible for the generation of NO in the vascular endothelium regulating vascular tone, cellular proliferation, leukocyte adhesion, and platelet aggregation. NO produced by eNOS has antioxidant properties as it reduces superoxide anion formation. Impaired NO production results in the pathogenesis of several diseases such as hypertension and preeclampsia[10].

In this study, we aimed to investigate the effects of cell adhesion, inflammation and apoptotic changes on fetal development in cases of COVID-19 placenta.

Patients and Methods

This study was carried out with the approval of Clinical Trials Ethical Committee, Gazi Yaşargil Training and Research Hospital (2021/78). Placental tissues were taken from the patients who submitted to the Gynaecology and Obstetrics Department of Training and Research Hospital. 15 placentas of asymptomatic pregnant women with positive COVID-19 polymerease chain reaction (PCR) test and 15 placentas of pregnant women with COVID-19 negative test were included in the study. Patients with secondary disease or chronic disease in both groups (e.g., respiratory tract infections, pregnancy complications, or other systemic disorders) were not included in the study. Patients participating in the study were informed regarding the study and signed the informed consent form.

Histological Tissue Preparation

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[11-13].

Immunohistochemical Examination

All sections deparaffinized, passed through alcohol series, and washed in distilled water 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 minutes. Samples were rinsed in distilled water and washed in phosphate buffered saline (PBS). The sections were then incubated with mouse monoclonal anti-FAS antibody (1:100) and mouse monoclonal anti-eNOS 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, Fremont, CA, USA) was applied for 20 minutes. Following PBS, streptavidin peroxidase solution (Streptavidin Peroxidase, Lab Vision, Fremont, CA, USA) was performed for 20 minutes. Slides were washed 3 times in PBS and diaminobenzidine (DAB) chromogen solutions were applied for 10 min. Sections were washed with distilled water and counter stained with 2 min Mayer hematoxylin. Slides were imaged with imager A2 Zeiss light microscope (Zeiss, Oberkochen, Germany). Semi-quantitative scaling of syncytial knot, congestion in blood vessels, fibrinoid acumulation ınflamation and degeneration in decidua were carried out[14,15].

Statistical Analysis

Statistical analysis was performed by the SPSS 25.0 software (IBM Corp., Armonk, NY, USA). 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 blood parameters was shown in Table I. Compared to COVID-19 (-) group, the change in lactate dehydrogenase (LDH), white blood cell (WBC) and C-reactive protein (CRP) were significantly different. There was no statistical significance in red blood cell (RBC), monocytes (MONO), eosinophil (EOS), basophil (BASO), platelet (PLT), neutrophile (NEU), mean corpuscle volume (MCV), alanine aminotransferase (ALT), aspartate aminotransferase (AST), mean corpuscular hemoglobin (MCH), hematocrite (HCT), hemoglobin (HGB) and lymphocytes (LYM) between COVID-19 (-) and COVID-19 (+) patients. Statistical analysis of histological parameters (syncytial knot, congestion in blood vessels, fibrinoid accumulation, inflammation, degeneration in decidua, FAS expression, eNOS expression) were shown

Table I. Blood parameters of patients with COVID-19 (-) and COVID-19 (+).

Parameter	Groups	N	Mean ± S.D	*p*-value	Mann-Whitney U Test
RBC (10^12/L)	(1) COVID-19 (-)	15	4.1 ± 0.64	0.450	
	(2) COVID-19 (+)	15	4.98 ± 0.45		
MONO (%)	(1) COVID-19 (-)	15	5.44 ± 1.23	0.728	
	(2) COVID-19 (+)	15	4.82 ± 1.06		
EOS (%)	(1) COVID-19 (-)	15	0.73 ± 0.37	0.375	
	(2) COVID-19 (+)	15	0.98 ± 0.12		
BASO (%)	(1) COVID-19 (-)	15	0.19 ± 0.03	0.785	
	(2) COVID-19 (+)	15	0.28 ± 0.08		
PLT (103/mm3)	(1) COVID-19 (-)	15	287.72 ± 73.34	0.429	
	(2) COVID-19 (+)	15	292.12 ± 94.19		
LDH (U/I)	(1) COVID-19 (-)	15	182.32 ± 74.82	0.003	(2)
	(2) COVID-19 (+)	15	242.22 ± 82.03		(1)
NEU (%)	(1) COVID-19 (-)	15	79.12 ± 18.94	0.195	
	(2) COVID-19 (+)	15	72.34 ± 21.39		
MCV (fL)	(1) COVID-19 (-)	15	86.77 ± 6.93	0.549	
	(2) COVID-19 (+)	15	88.64 ± 5.18		
ALT (U/I)	(1) COVID-19 (-)	15	22.34 ± 1.84	0.160	
	(2) COVID-19 (+)	15	23.42 ± 1.32		
AST (U/I)	(1) COVID-19 (-)	15	21.66 ± 1.23	0.932	
	(2) COVID-19 (+)	15	23.98 ± 1.45		
MCH (pg)	(1) COVID-19 (-)	15	28.44 ± 2.40	0.433	
	(2) COVID-19 (+)	15	29.68 ± 3.72		
HCT (%)	(1) COVID-19 (-)	15	42.76 ± 2.65	0.269	
	(2) COVID-19 (+)	15	41.56 ± 3.36		
HGB (g/dL)	(1) COVID-19 (-)	15	12.34 ± 1.11	0.288	
	(2) COVID-19 (+)	15	13.72 ± 1.56		
WBC ($10^3/mm^3$)	(1) COVID-19 (-)	15	11.42 ± 1.73	0.012	(2)
	(2) COVID-19 (+)	15	8.64 ± 1.53		(1)
LYM (%)	(1) COVID-19 (-)	15	16.88 ± 3.28	0.791	
	(2) COVID-19 (+)	15	18.98 ± 3.78		
CRP (mg/dL)	(1) COVID-19 (-)	15	8.22 ± 5.92	0.014	(2)
	(2) COVID-19 (+)	15	28.12 ± 10.37		(1)

RBC: red blood cell, MONO: monocytes, EOS: eosinophil, BASO: basophil, plt: platelets, LDH: lactate dehydrogenase, NEU: neutrophil, MCV: mean corpuscle volume, ALT: Alanine transaminase, AST: Aspartate transaminase, MCH: mean corpuscular hemoglobin, hgb: hemoglobin, WBC: white blood cells, LYM: lymphocytes, CRP: C reactive protein.

Table II. Histological parameters in COVID-19 (-) and COVID-19 (+) patients.

Parameter	Groups	N	Median (min-max)	Mean rank	*p*-value	Mann-Whitney U Test
Syncytial knot	(1) COVID-19 (-)	15	1.00 (0.00-2.00)	9.50	< 0.001	(2)
	(2) COVID-19 (+)	15	1.00 (0.00-2.00)	27.50		(1)
Congestion in blood vessels	(1) COVID-19 (-)	15	1.00 (0.00-2.00)	9.56	< 0.001	(2)
	(2) COVID-19 (+)	15	1.00 (0.00-2.00)	27.44		(1)
Fibrinoid accumulation	(1) COVID-19 (-)	15	1.00 (0.00-1.00)	9.58	< 0.001	(2)
	(2) COVID-19 (+)	15	1.00 (0.00-2.00)	27.42		(1)
Inflammation	(1) COVID-19 (-)	15	1.00 (0.00-2.00)	9.67	< 0.001	(2)
	(2) COVID-19 (+)	15	3.00 (3.00-4.00)	27.33		(1)
Degeneration in decidua	(1) COVID-19 (-)	15	4.00 (2.00-4.00)	9.50	< 0.001	(2)
	(2) COVID-19 (+)	15	4.00 (2.00-4.00)	27.50		(1)
FAS expression	(1) COVID-19 (-)	15	3.50 (2.00-4.00)	9.58	< 0.001	(2)
	(2) COVID-19 (+)	15	3.00 (2.00-4.00)	27.42		(1)
eNOS expression	(1) COVID-19 (-)	15	3.00 (2.00-4.00)	9.56	< 0.001	(2)
	(2) COVID-19 (+)	15	4.00 (2.00-4.00)	27.44		(1)

eNOS: endothelial nitric oxide synthase.

in Table II. Compared to COVID19 (-) group, all values were significantly increased in COVID19 (+) group. Graphical illustration of Table I and II is shown in Figure 1.

Discussion

The effects of COVID-19 infection on the maternal and fetal regions during pregnancy

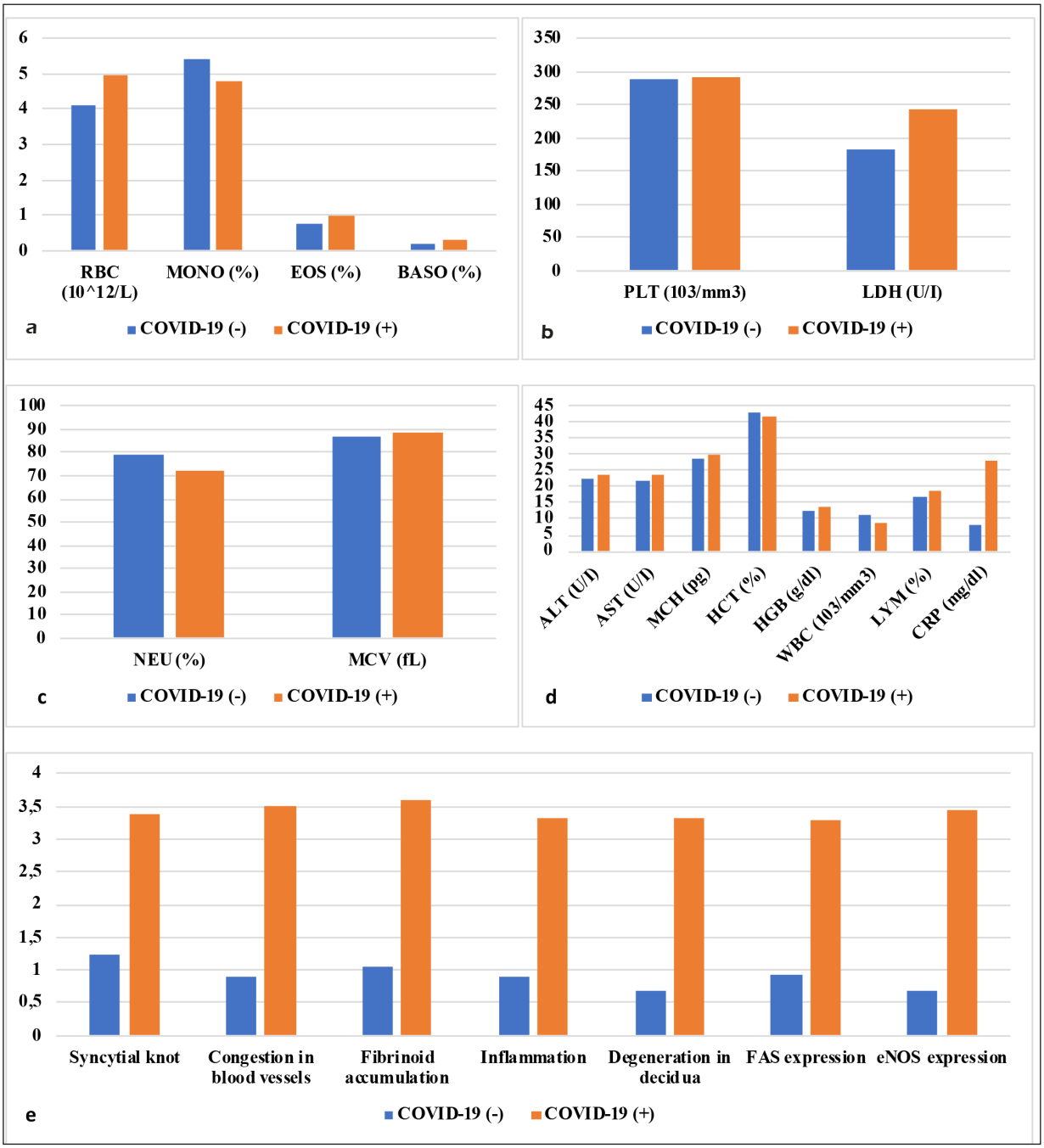

Figure 1. **a**, Graphical illustration of RBC, MONO, EOS and BASO in COVID-19 (-) and COVID-19 (+) patients. **b**, Graphical illustration of PLT and LDH in COVID-19 (-) and COVID-19 (+) patients. **c**, Graphical illustration of NEU and MCV in COVID-19 (-) and COVID-19 (+) patients. **d**, Graphical illustration of ALT, AST, MCH, HCT, HGB, WBC, LYM and CRP in COVID-19 (-) and COVID-19 (+) patients. **e**, Graphical illustration of histological parameters in COVID19 (-) and COVID19 (+) patients. RBC: red blood cell, MONO: monocytes, EOS: eosinophil, BASO: basophil, plt: platelets, LDH: lactate dehydrogenase, NEU: neutrophil, MCV: mean corpuscle volume, ALT: Alanine transaminase, AST: Aspartate transaminase, MCH: mean corpuscular hemoglobin, hgb: hemoglobin, WBC: white blood cells, LYM: lymphocytes, CRP: C reactive protein.

have not been fully clarified. Understanding the mechanisms of placental destruction resulting in placental malperfusion and insufficiency as well as the histopathology of the placenta may be helpful in SARS-CoV-2 infection during pregnancy. Valdespino-Vázquez et al[16] studied the placenta and fetal organs from an early pregnancy miscarriage in a COVID-19 maternal infection by immunohistochemical, reverse transcription quantitative real-time polymerase chain reaction (rPCR), immunofluorescence, and electron microscopy methods. Nnucleocapsid protein of SARS-CoV-2 and its RNA was detected in the placental and fetal tissues. In histological examination and immunohistochemical analysis, damages on the placenta and fetal organs due to hyperinflammation were evident. Sections of the placenta revealed severe inflammation with Hofbauer cells in the villous stroma and these cells positively immune reacted with cluster of differentiation 163 (CD163) antiboy. The study revealed that congenital SARS-CoV-2 infection is possible during the first trimester of pregnancy. According to Leal et al[17] the most common findings in placentas from infected women were fibrin deposition and intense recruitment of inflammatory infiltrates. Menter et al[18] observed inhomogeneous placental parenchyma and infiltration of infarcts, chronic villi and intervillocytes, intense CD8-expressing cytotoxic T

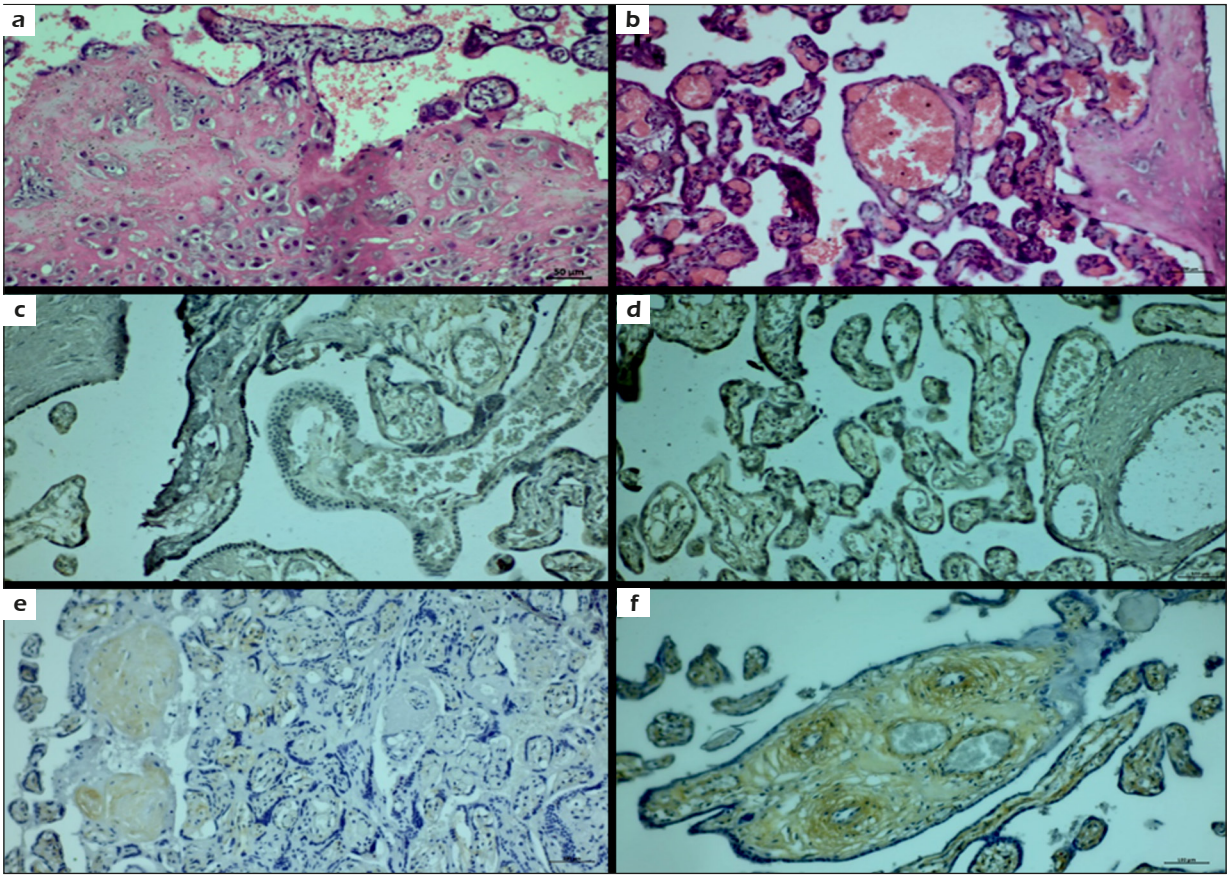

Figure 2. **a**, In the maternal placental part of the control group, decidua cells were oval, and connective tissue cells diffusely distributed among cells with dense chromatin structure; squamous cells in the syncytial region, and mild erythrocyte cell distribution in the inter villous area were observed. **b**, In the placental sections belonging to COVID-19 group, basement membrane in larger villi and decidual cells were degenerated with increased fibrinoid tissue. Endothelial dysfunction in the free villi and intense congestion in the blood vessels, an increase in the syncytial nodes and bridges were observed. **c**, In the control group, FAS expression was moderate in some cells in the connective tissue area with root villi, while FAS expression was negative in blood vessel endothelial cells in the syncytial region. **d**, In the COVID-19 placental section, positive FAS expression was observed in the basal membranes of roots and free villi, syncytial bridges and knots, and endothelial cells. **e**, In the control group, eNOS activity was positive in some of the decidua cells and some macrophage cells in the maternal area. **f**, In the COVID-19 group, eNOS expression was positively observed in Hoffbauer cells, in dilated blood vessels endothelial cells in chorionic villi and in surrounding inflammatory cells. Scale Bar: 50 μm, magnification: 20×.

cells and fewer CD68-expressing macrophages in the signs of placenta with COVID-19. Lymphohistiocytic, resulting in chorionic vasculitis and thrombosis, intervillous increase in fibrin as a result of maternal malperfusion, and the presence of SARS-CoV-2 in decidual cells were observed. We observed a deterioration in root villus basement membrane structure, decidua cells and syncytial cell degeneration, a significant increase in fibrinoid tissue in the maternal region due to the COVID-19 effect, while endothelial dysfunction in free villi, intense congestion in blood vessels, and an increase in syncytial nodes and bridges were observed (Figure 2b). Examinations of the placentas from SARS-CoV-2 positive mothers had different results in studies[19]. This confusion concluded with the result that there is a protective mechanism in the placenta provided by some receptors or signaling pathways. One of these is known to be the FAS. Guller and LaChapelle[20] mentioned a model on FASL production by human syncytiotrophoblasts and extravillous trophoblasts that may protect the fetus against the cytolytic actions of activated FAS + maternal lymphocytes in the intervillous space and in the placental bed. Researchers[6,21] showed that activation of FAS triggers apoptosis. In a study[22] with immunohistochemical methods, the expression of FASL has been determined in the human placenta.

Examining the placental villi and a human first-trimester trophoblast cell line, it was found with the immunoperoxidase staining that localization of FASL was observed on the surfaces of syncytiotrophoblasts and cytotrophoblasts in placental villi and chorionic extravillous trophoblast. FASL was also observed in placental villi and a human first-trimester trophoblast cell line (ED27) with Western blot analysis. FAS was colocalized to CD45 (leukocyte common antigen) positive cells found in the maternal decidua. These findings suggest that FASL expressed by fetal trophoblast cells can induce apoptosis in activated lymphocytes by providing a mechanism for maternal immune tolerance to the fetus[23]. FASL was expressed on the surfaces of placental cytotrophoblasts throughout normal pregnancy. It was showed[24] that the expression of FASL on the surfaces of placental cytotrophoblasts is necessary for the normal development of fetus as well. Maternal specific FAS+T cell apoptosis induced by FASL on the maternal-fetal interface was implicated to be one of the significant mechanisms of maternal-fetal immune tolerance[8]. In another study[25], the expression of FAS and FASL in placental trophoblast populations, in normal first trimester and term pregnancies using an avidin-biotin peroxidase technique on frozen and formalin-fixed paraffin-embedded placental tissues with both monoclonal and polyclonal antibodies, was performed.

The immunoreactivity for FAS and FASL was compared with monoclonal and polyclonal antibodies on frozen and paraffin-embedded sections. In normal early and molar pregnancy there were strong FASL expressions by villous cytotrophoblast and syncytiotrophoblast. However, there were significant differences in FASL expression by trophoblast subpopulations in both early and normal term pregnancy and between the same trophoblast subpopulation at different gestations, and FASL staining was weak[26,28]. Strong FASL staining in cytotrophoblast cells in the distal parts of cell columns contrasted with unstained cytotrophoblast in the proximal part of columns. FASL was expressed in distinct trophoblast subpopulations, and the expression was especially decreased in proliferating syncytiotrophoblast. However, there were no differences in FAS expression by trophoblast populations in normal early or term placental tissues. FAS expression was lower in villous cytotrophoblast of term placenta. TUNEL labelling apoptosis was rarely seen in placental trophoblast[29-31]. Differential FAS and FASL expression by trophoblast subpopulations in normal and pathological pregnancy does not appear to be related to the apoptosis of trophoblast[32].

As a result of the increased inflammatory effect of COVID-19, positive FAS expression was evident in the basal membranes of the root and free villi, syncytial bridge and nodes, and endothelial cells, with the increase of the pro-apoptotic process in the placental parts (Figure 2d).

Endothelial NOS-derived NO is known to be a physiological vasodilator, an inhibitor of platelet aggregation and functions as an intracellular antiviral defence mechanism[33]. The replication of various RNA and DNA viruses, such as SARS-CoV is inhibited by NO[34], and NO decreases viral replication and protein production in host cells by directly inactivating or modifying viral replicating proteins[35].

Guimarães et al[36] discussed in their study that the inflammatory process is triggered by SARS-CoV-2 infection and the intense inflammation leads to endothelium dysfunction in pulmonary

blood vessels, uncoupling eNOS activity, and lowering NO production. In the COVID-19 group of our study, it was observed that there was an increased inflammatory cell infiltration around the vessel and increased eNOS expression in macrophage activity due to degeneration in vascular structures (Figure 2e). We believe that this situation may cause developmental anomalies by negatively affecting fetal circulation.

Conclusions

The effect of COVID-19 caused an increase in eNOS activity, which is the precursor of the increased inflammation signal in the maternal region, and a decrease in the development of chorionic villi and blood flow in the fetal circulation, along with the acceleration of the proapoptotic process and the deterioration of cell-membrane adhesion.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

Ethics approval was obtained from Clinical Trials Ethical Committee, Gazi Yaşargil Training and Research Hospital (2021/78).

Informed Consent

All patients read the informed consent form and accepted to participate in this study.

Availability of Data and Materials

All generated data were presented in this study

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Authors' Contribution

Conceptualization and design: SO, ÇO; experiments: drafting and writing: SO, ÇO; editing: SO, ÇO.

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