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Determination of CTLA-4 Levels in Placenta Tissue of Pregnant Women with Preeclampsia and Smoking Pregnant Women with Preeclampsia

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Abstract

BACKGROUND/AIMS: In this study, the determination of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) levels in placenta tissue of pregnant women with preeclampsia and smoking pregnant women with preeclampsia was investigated using histological and immunohistochemical methods.

MATERIALS AND METHODS: Placenta tissues of 28 pregnant women were used in the study. The groups were formed into the categories of control, smoking, preeclampsia, and preeclampsia + smoking. Tissue samples taken at the end of delivery were fixed in 10% formalin, subjected to standard histological processing, and blocked in paraffin. Crossman's trichrome and haematoxylin-eosin staining was performed on sections taken from paraffin blocks. Immunohistochemical methods were applied to determine CTLA-4 immunoreactivity in placental tissues.

RESULTS: In the groups of smoking, preeclampsia, preeclampsia + smoking, changes such as: a decreased villous tree, congestion in the villi, and deposition of fibrin in the decidua were determined. In addition, different levels of CTLA-4 immunoreactivity were ascertained in the placental tissue and amniotic epithelium of all groups. The intensity of immunoreactivity in decidua cells and stem villi was identified to decrease in other groups compared to the control group.

CONCLUSION: It was thought that maternal immune system responses and histopathological changes in placenta tissue may cause decreased CTLA-4 immunoreactivity in smoking, preeclampsia and preeclampsia + smoking groups.

Keywords: CTLA-4, placenta, preeclampsia, pregnant, smoking, women

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INTRODUCTION

The placenta is an active and complex organ that contributes to the development and nutrition of the embryo, supports fetal and maternal immunotolerance, and has a wide variety of morphological variations among mammals.[1,2] Hypertensive disorders, one of the common complications in pregnancy, lead to serious negative consequences for both the mother and the fetus. Preeclampsia is one of the most important hypertensive disorders affecting 5-7% of all pregnancies, and preeclampsia causes an average of 70,000 maternal and 500,000 newborn deaths worldwide each year.[3] It is thought that immune system irregularities may play a role in the appearance of preeclampsia, regardless of the degree of placental abnormality or if it is early-onset or late-onset.[4,5] Smoking is the leading cause of preventable morbidity and mortality worldwide. More than five million premature deaths occur from smoking-related causes worldwide each year. This number is expected to reach eight million by 2030.[6] Smoking during pregnancy primarily affects the placenta, causing decreased blood flow to it and inhibiting the intrauterine growth of the fetus, thereby resulting in the birth of low birth weight babies. It also increases perinatal mortality.[7,8]

In general terms, the immune system, uses general and special defense mechanisms to resist foreign substances entering or given to the body, to protect itself and to destroy the harmful substance.[9] Negative costimulatory molecules are needed for the immune system to function in a balanced manner. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) plays a role in many immune control points, maintenance of tolerance of peripheral T-lymphocytes, prevention of autoimmunity, and the suppression of inflammation.[10,11] The *CTLA-4* gene is found in the chromosome 2q33 region. CTLA-4 encodes a protein that negatively regulates the T-cell response and is responsible for maintaining T-cell homeostasis.[12] When CTLA-4 is absent, peripheral T-cells can be overactive, causing fatal tissue damage.[13] In this study, CTLA-4 levels in placental tissue of pregnant women with preeclampsia and smoking pregnant women with preeclampsia were investigated using immunohistochemical methods. We think that our results will contribute to the enlightenment of the etiology of preeclampsia and to determining the effects of maternal smoking on placenta tissue.

MATERIALS AND METHODS

Material

Our study was designed prospectively and performed in compliance with the "Declaration of Helsinki". Tissue samples were obtained from Erzurum Nenehatun Obstetrics and Gynecology Hospital. In the study, placenta samples were used from pregnant women who were primigravida or multigravida, aged 20 and 40 years, who gave birth normally or via cesarean section, had no additional chronic diseases (e.g., diabetes, chronic renal insufficiency), and no early membrane rupture or chorioamnionitis, and who completed the 37th gestational week. Written consent from pregnant women was obtained, and they filled out demographic information forms developed by the researcher. Criteria for the diagnosis of preeclampsia were based on two blood pressure values of 140/90 mmHg or higher and 300 mg or more proteinuria in the urine collected over 24 hours, after the 20th week of pregnancy (the diagnosis of preeclampsia was made by the presence of at least two criteria listed below in the 2019 guideline of the "American Association of Obstetricians and Gynecologists".

Methods

Groups were designed as follows:

1. Control group (n=7): Pregnant women who did not have any health problems were included in this group.

2. Smoking group (n=7): Pregnant women who did not have any health problems and smoked during pregnancy were included in this group.

3. Preeclampsia group (n=7): Pregnant women who had been diagnosed with preeclampsia and did not smoke were included in this group.

4. Preeclampsia + smoking group (n=7): Pregnant women who were diagnosed with preeclampsia and smoked during pregnancy were included in this group.

A full-thickness section was taken from the middle part of the placenta from the fetal face to the maternal face and including the amnion and decidua for histopathological and immunohistochemical exeminations. Only one tissue sample was taken from each placenta. Tissue samples were obtained from the fetal and maternal parts of the placenta. The tissues were fixed in 10% formalin solution; a routine histological protocol was applied, and they were blocked in paraffin.

Histopathological Examinations

To examine the general structure of the placental tissue, 5 µm sections were taken from the blocks, and Crossman's triple staining and haematoxylin-eosin staining were performed. Six different areas were randomly evaluated from each tissue sample. Researchers made the evaluation independently of each other. The histopathological changes in placenta tissue were assessed according to their severity as none (0), weak (1), moderate (2), and strong (3).

Statistical Analysis

Data obtained from histopathological changes in placenta tissues, demographic characteristics, and blood pressure measurements were analyzed with SPSS version 22.00. Differences between the groups were determined by the Kruskal-Wallis test, and the Mann-Whitney U test was used to determine the group that made the difference. The results are presented with median, minimum, and maximum values. A $p<0.05$ was considered statistically significant. No corrections were made to the analysis using the SPSS software.

Immunohistochemical Examinations

The streptavidin-biotin peroxidase method was applied to the sections taken from placental tissue. During the immunohistochemistry procedure, all washing procedures were performed with PBS (0.1 M, pH 7.2) buffer. The sections were first soaked in 3% H_2O_2 for 15 minutes, then, citrate buffer solution was added and they were boiled in a microwave oven (600 watts for 10 minutes). Then large volume ultra V block solution was applied for 10 minutes. CTLA-4 (sc-376016) primary antibody was added to the sections and kept at room temperature in a humid environment for 1 hour (1/50 dilution). Then biotinylated goat anti B polyvalent and streptavidin peroxidase solutions were applied, respectively, for 30 minutes. Chromogen application was performed with diaminobenzidine hydrogen peroxide substrate solution. Contrast staining was performed with modified Gill III haematoxylin. In immunohistochemical evaluations, staining intensity and staining

characteristics of the cells were taken into account and semiquantitative scoring was performed as no staining (-), weak staining (+), moderate staining (++) and strong staining (+++) (evaluations were made by two independent observers). All sections were examined by light microscopy (Olympus BX51; Olympus Optical Co. Osaka, Japan) and photographed.

RESULTS

Statistical Results

The evaluation results of statistical and histopathological data of the women included in the study groups are presented in Table 1. When the demographic characteristics of the women in the study groups were examined (Table 1A), 60.7% had medium income, 67.9% of women gave birth by C-section, while the previous birth status of 46.4% was also C-section, 63.1% of women who gave birth by C-section were women in the preeclampsia and preeclampsia + smoking group and 85.7% of women did not working.

The age (χ^2=4.363, p>0.05) and weight (χ^2=7.057, p>0.05) were observed to not differ between the groups (Table 1C). Diastolic blood pressure (χ^2=19.572, p<0.001) and systolic blood pressure (χ^2=18.589, p<0.001) values showed statistically significant differences between the groups. In addition, the median values of the preeclampsia and preeclampsia + smoking groups were found to be higher for diastolic and systolic blood pressure than the median values of the control and smoking groups (Table 1D).

Histopathological Results

Serial sections were taken from placental tissue samples, and histopathological changes were found to be different between the groups (Table 1B). While the placenta samples in the control group had a normal histological structure (Figure 1), the reduction in the villous tree (VT), congestion in the villi, and fibrin deposition in the decidua were seen in the other groups. It was determined that these histopathological changes were weak in the smoking group, moderate in the preeclampsia group, and strong in the preeclampsia + smoking group (Figures 2-4).

Immunohistochemical Results

In the amniotic epithelium of the placenta tissue, moderate CTLA-4 immunoreactivity was determined in the control, smoking, and preeclampsia groups. Strong CTLA-4 immunoreactivity was determined in the preeclampsia + smoking group. Moderate immunoreactivity was detected in the chorionic plaque in all groups (Table 2, Figure 5). In decidua cells and stem villi, strong immunoreactivity in the control group, moderate immunoreactivity

Table 1. Statistical and histopathological results

A. Demographic characteristics of women included in the study groups

Variables		f	%	Variables		f	%
Income status	Low	11	39.3	Working status	Yes	4	14.3
	Middle	17	60.7		No	24	85.7
Type of birth	Normally	9	32.1	Number of children	0	5	17.9
	C-section	19	67.9		1	3	10.7
Previous form of birth	Normally	10	35.7		2	7	25.0
	C-section	13	46.4		3	6	21.4
	None	5	17.9		4	7	25.0

B. Histopathological changes in placenta tissue samples

Groups	Decrease of the VT	Congestion	Fibrin deposition	Diastolic blood pressure	Systolic blood pressure
C	0.33±0.51^{d}	0.33±0.51^{a}	0.16±0.40^{a}	70a (60-70)*	110^{a} (100-120)*
S	1.33±0.51^{c}	0.16±0.40^{a}	1.33±0.51^{b}	70a (60-90)*	110^{a} (100-140)*
P	2.16±0.40^{b}	1.83±0.40^{b}	2.16±0.40^{c}	100b (90-120)*	150^{b} (140-190)*
PS	2.83±0.40^{a}	2.66±0.51^{c}	2.83±0.40^{d}	90b (90-115)*	140^{b} (140-190)*

*Values are shown as median (minimum-maximum). $^{a-d}$: There is a statistically significant difference between the values indicated with different letters (p<0.05).

C. Age and weight values of the women included in the study groups

	C (n=7)	S (n=7)	P (n=7)	PS (n=7)
Age	27^{a} (24-30)*	32^{a} (28-37)*	30a (21-40)*	31a (23-41)*
Weight	67^{a} (43-80)*	80^{a} (67-93)*	72a (70-87)*	83a (57-95)*

*Values are shown as median (minimum-maximum). a: There is no statistical difference between the values indicated with the same letter.

D. Comparison of diastolic and systolic blood values between groups

	C (n=7)	S (n=7)	P (n=7)	PS (n=7)
Diastolic blood pressure	70^{a} (60-70)*	70^{a} (60-90)*	100^{b} (90-120)*	90^{b} (90-115)*
Systolic blood pressure	110^{a} (100-120)*	110^{a} (100-140)*	150^{b} (140-190)*	140^{b} (140-190)*

*Values are shown as median (minimum-maximum). ^{a,b}: There is a statistically significant difference between the values indicated with different letters. C: Control group, S: Smoking group, P: Preeclampsia group, PS: Preeclampsia + smoking group.

Table 2. Semiquantitative scoring of CTLA-4 immunoreactivity

Areas	Groups			
	C (n=7)	S (n=7)	P (n=7)	PS (n=7)
Amniotic epithelium	++	++	++	+++
Chorionic plaque	++	++	++	++
Decidua cells	+++	++	+	++
Stem villi	+++	++	+	++
Terminal villi	+	+	+	+

CTLA-4: Cytotoxic T-lymphocyte antigen-4, C: Control group, S: Smoking group, P: Preeclampsia group, PS: Preeclampsia + smoking group.

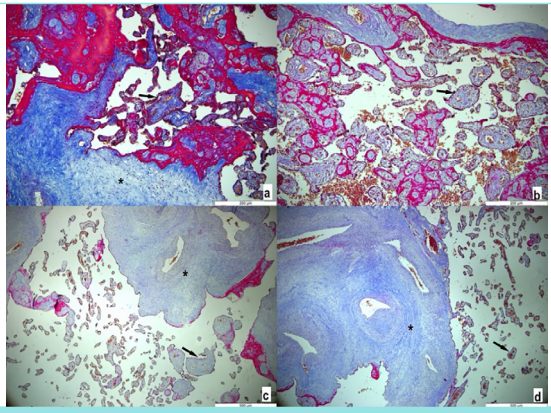

Figure 1. Placenta tissue. (a) control group, (b) smoking group, (c) preeclampsia group, (d) preeclampsia + smoking group. Crossman's trichrome staining. Asterisk: Decidua, arrow: Chorion villi.

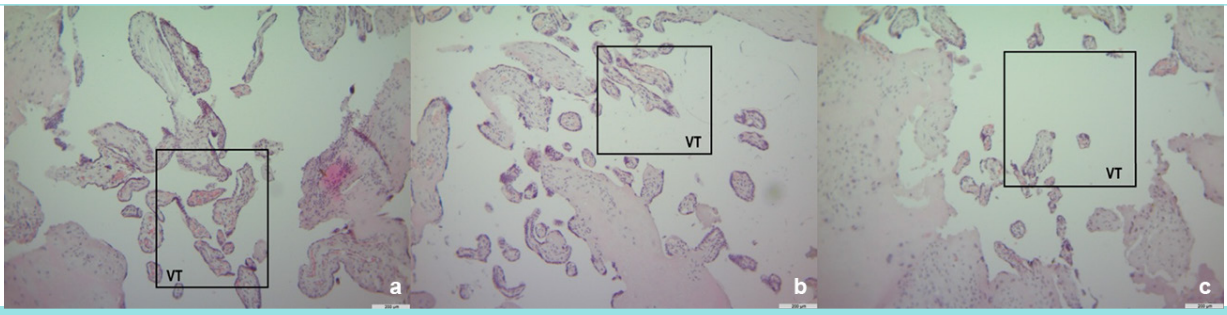

Figure 2. Placenta tissue. (a) cigarette group, weak decrease in VT; (b) preeclampsia group, moderate decrease in VT; (c) preeclampsia + smoking group, strong decrease in VT. H&E staining.

VT: Villous tree, H&E: Hematoxylin and eosin.

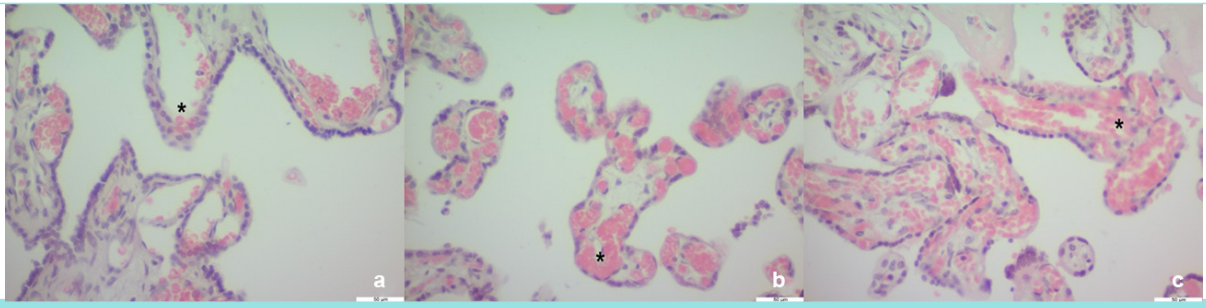

Figure 3. Placenta tissue. (a) cigarette group, weak congestion (asterisk); (b) preeclampsia group, moderate congestion (asterisks); (c) preeclampsia + smoking group. Strong congestion (asterisk), H&E staining.

H&E: Hematoxylin and eosin.

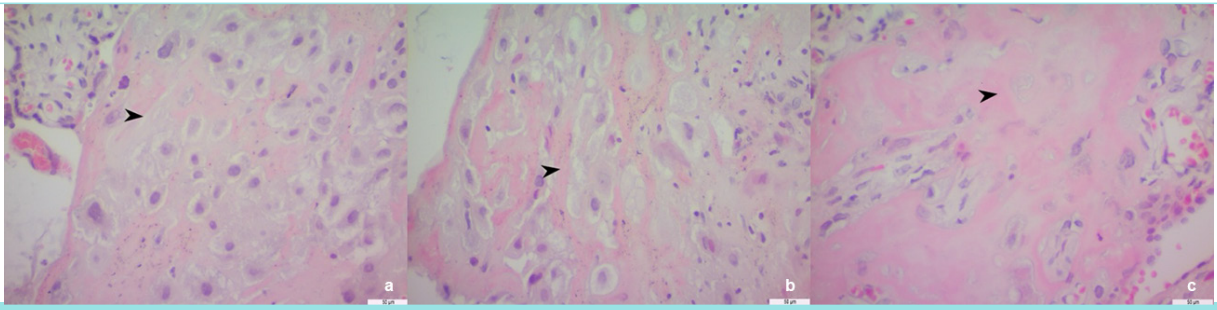

Figure 4. Placenta tissue. (a) cigarette group. Weak fibrin deposition in the decidua (arrowhead), (b) preeclampsia group. Moderate fibrin deposition in decidua (arrowhead). (c) preeclampsia + smoking group. Strong deposition of fibrin in the decidua (arrowhead). H&E staining.

H&E: Hematoxylin and eosin.

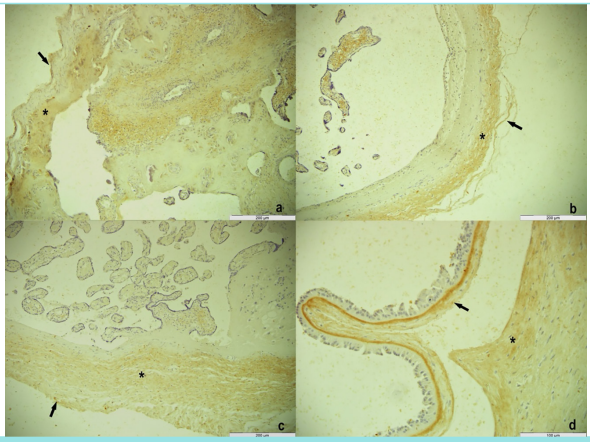

Figure 5. CTLA-4 immunoreactivity in amniotic epithelium and chorionic plate. (a) control group, (b) smoking group, (c) preeclampsia group, (d) preeclampsia + smoking group. Asterisk: Chorionic plaque, arrow: Amniotic epithelium.

CTLA-4: Cytotoxic T-lymphocyte antigen-4.

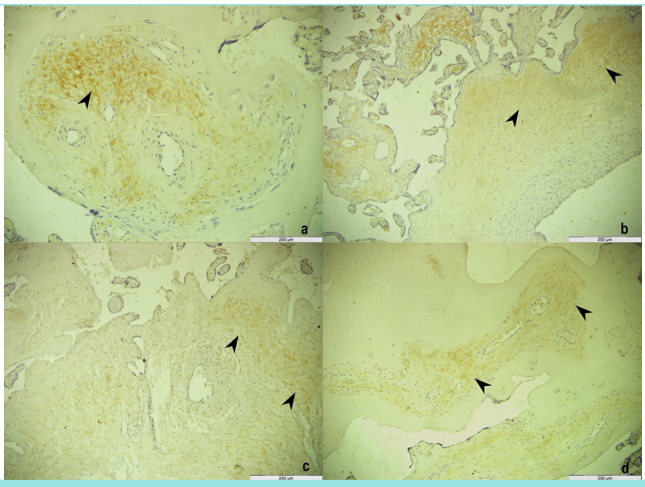

Figure 6. CTLA-4 immunoreactivity in decidua cells. (a) control group, (b) smoking group, (c) preeclampsia group, (d) preeclampsia + smoking group. Arrowhead: Decidua.

CTLA-4: Cytotoxic T-lymphocyte antigen-4.

in the smoking and preeclampsia + smoking group, and weak immunoreactivity in the preeclampsia group was observed. In terminal villi, weak immunoreactivity was detected in all groups (Table 2, Figures 6 and 7).

DISCUSSION

Although the factors that cause preeclampsia have not been completely known, the studies explain some points about the pathogenesis of preeclampsia. When the method of delivery of pregnant women with preeclampsia was examined, it was found that more than half of the patients had a cesarean delivery.[14,15] Considering whether there was a correlation between preeclampsia and age, it was determined that the average age of patients with severe preeclampsia symptoms was higher than that of the normotensive group. In addition, it was found that systolic and diastolic blood pressure levels were highest in the severe preeclampsia group, followed by the preeclampsia group, and lowest in the normotensive group.[16] In our study, the average age in the groups was homogeneous, the rate of cesarean sections was dominantly high at 67.9%, and systolic and diastolic blood pressure levels were higher in the preeclampsia and preeclampsia + smoking groups than in the

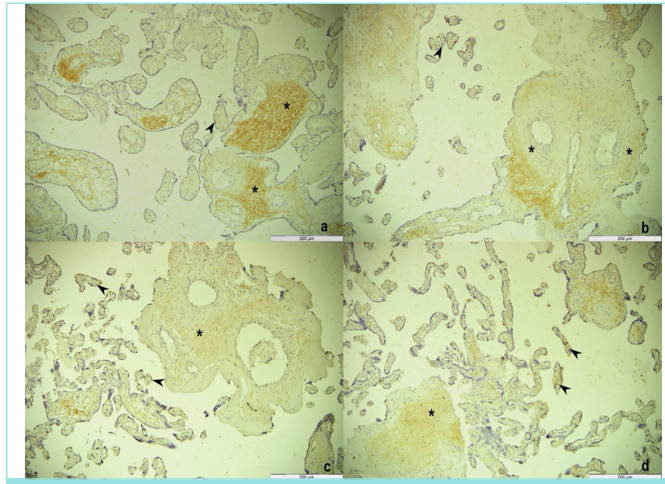

Figure 7. CTLA-4 immunoreactivity in chorionic and terminal villi. (a) control group, (b) smoking group, (c) preeclampsia group, (d) preeclampsia + smoking group. Asterisk: Stem villi, arrowhead: Terminal villi.

CTLA-4: Cytotoxic T-lymphocyte antigen-4.

other groups. These results suggested that demographic data may be meaningful for determining risk factors in patients with preeclampsia.

It has been suggested that there may be deficiencies in placental vascularization due to immunological problems in pregnant women with preeclampsia and that preeclampsia may be recessively inherited.[17-19] The storage of fibrin localized in the perivillous area in the placentas of normal pregnant women giving birth in time is not a pathological condition, but it has been reported in studies that the intensive deposition of fibrin in the placenta negatively affects fetal development.[20] It is noted that infarcts occurring in the placentas of women with preeclampsia are associated with a disorder in fetal blood flow; the thrombosis determined in the maternal vessels can cause a decrease in fetal blood flow.[21] Our study revealed the changes seen in the form of the declining VT, congestion in villi, and fibrin deposition in decidua among groups experiencing smoking, preeclampsia, and smoking + preeclampsia. These changes were found to be weak in the smoking group, moderate in the preeclampsia group, and strong in the preeclampsia + smoking group. It was thought that our results would contribute positively to the identification of the histopathological changes that smoking can produce in placental tissue, and to the illumination of the etiology of preeclampsia.

Also, 30-45% of the T-cells found in human deciduas are CD4+ T-cells and 45-75% are CD8+ T-cells, cytotoxic T lymphocytes cells.[22] CD8+ T-cells are less abundant in peripheral blood and more abundant in human decidua at term.[23,24] These cells are capable of recognizing allogeneic MHC molecules but do not attack fetal cells during pregnancy.[25] This condition is thought to be due to limited MHC class I expression in fetal trophoblast cells. CTLA-4 and CD28, which both interact with B7 belong to the immunoglobulin superfamily. It is responsible for the regulation of the immune system. It is also called CD152.[26] CTLA-4 is normally found at a low level on the surface of effector T-cells and Treg (regulatory T) cells, regulating the severity of early-stage T-cell activation.[27] When CTLA-4 is suppressed, cytotoxic T-cell activation increases and Treg cells are prevented from suppressing the immune system.[28] The successful continuation of pregnancy requires the establishment of maternal-fetal tolerance and the successful completion of placentation. When the immune balance is disturbed, spontaneous abortions, preeclampsia and intrauterine growth restriction of the fetus may occur due to inadequate placental perfusion. Extravillous trophoblasts instruct decidual immune cells to regulate fetal tolerance and promote placental development. CTLA-4 has important roles in the function of decidual immune cells. Blockade of CTLA-4 pathways results in abnormalities in the number and functionality of CD4+ T-cells, impairing the interaction of extravillous trophoblasts and decidual immune cells. It has been stated that this leads to poor placental development and increased fetal loss, and it has been emphasized that CTLA-4 plays important roles in maintaining normal pregnancy.[29,30] During pregnancy, CTLA-4 immunoreactivity was reported in numerous stromal cells in placental tissue, while immunoreactivity was not seen in trophoblast cells and endothelial cells.[31] In the placenta tissue of all groups in our study, CTLA-4 immunoreactivity was determined in the amnionic epithelium, decidual cells stem villi, chorionic plaque, and terminal villi. It was noted that CTLA-4 immunoreactivity in decidua cells and stem villi in placentas of the preeclampsia group decreased compared with the control group.

Smoking has negative effects on the immune system. Nicotine found in the composition of cigarettes is similar to acetylcholine in its chemical structure. It first stimulates transmission of stimuli in autonomic nervous system ganglia via acetylcholine, but then blocks it. Studies have determined that the nicotine metabolite "cotinine" crosses the placental barrier, as evidenced by its presence in amniotic fluid and cord blood. Although the mechanisms by which it negatively affects the fetus are not fully known, there are views that it can produce vasoconstriction in the uterine arteries, can exert direct toxic effects, or can cause placental damage.[32-34] Maternal smoking disrupts the balance between cytotrophoblast proliferation and differentiation and damages placental development.[35,36] Alkaline ribonuclease levels increase in the placentas of women who smoke, which is likely to result in impairment in protein synthesis. Moreover, there is villous hyperplasia in the placentas of these mothers.[37] The number of syncytial nodes-masses of multi-nucleated protoplasms that result from the fusion of single cells with the loss of cell membranes between them- and cytotrophoblastic cells, in pregnant smoking women, was reported to increase. Average birth weight and placental weight decreased as the number of cigarettes smoked daily increased in the third trimester.[38] It has been reported that cigarette smoking in pregnancy may lead to many adverse obstetric outcomes such as ectopic pregnancy and placental abruption, and may be a risk factor for gestational hypertension and preeclampsia.[39-42] On the other hand, some studies have suggested that the number of cigarettes smoked per day during pregnancy has a n inverse dose-response relationship to the likelihood of preeclampsia and that maternal cigarette smoking reduces the risk of pregnancy-induced hypertension and eclampsia.[43,44] The claim that smoking during pregnancy can have a protective effect against pre-eclampsia suggests that the mechanism called vascular placental pathology is a highly complex event.[45,46] It was thought that determining vigorous CTLA-4 immunoreactivity in the control group placenta tissue may result from suppression of the mother's immune responses so that the pregnancy could continue in its normal course. The decrease in CTLA-4 immunoreactivity in smoking, preeclampsia, and preeclampsia + smoking groups compared to the control group may be caused by histopathological changes in the placental tissue and deficiencies in villus development. It is also hypothesized that the increased immunoreactivity of CTLA-4 in both the smoking and the pre-eclampsia and smoking groups compared to the pre-eclampsia group could be the immune response by cells which have increased in the mother's body due to smoking.

CONCLUSION

It was thought that this research could make a positive impact on determining the impact of smoking in pregnancy on the health of the mother and fetus and illuminating the aspects of pre-eclampsia associated with the immune system. It also aims to determine the factors that may negatively affect the mother's immune system during pregnancy and the levels of immune cells and their role in pregnancy continuation.

MAIN POINTS

- Histopathologic changes occurred in placental tissue of smoking, preeclampsia and preeclampsia + smoking groups.
- Different levels of CTLA-4 immunoreactivity were detected in placental tissue and amniotic epithelium of all groups.
- Immunoreactivity intensity in decidua cells and stem villi decreased in smoking, preeclampsia, and preeclampsia + smoking groups.

ETHICS

Ethics Committee Approval: The study received approval from Atatürk University Faculty of Medicine Clinical Research Ethics Committee (approval number: 71, date: 16.01.2020).

Informed Consent: Written consent from pregnant women was obtained, and they filled out demographic information forms developed by the researcher.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Ş.Y.A., B.G.K., A.G., S.E.Y., Concept: Ş.Y.A., K.S., Design: Ş.Y.A., K.S., Data Collection and/or Processing: B.G.K., A.G., S.E.Y., G.F.A., Analysis and/or Interpretation: B.G.K., A.G., Literature Search: Ş.Y.A., Writing: S.E.Y., G.F.A., E.K.S.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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