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Research Article

INVESTIGATION OF PLACENTAL HOFBAUER CELLS BY IMMUNOHISTOCHEMISTRY METHODS IN COMPLICATED PREGNANCIES

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Abstract: *Development of the placenta without any complication is essential for normal pregnancy. The placenta is a multifunctional organ that plays a vital role in fetal development. Hofbauer cells are one of the most important groups of placental cells. These cells are placental macrophages and have a role in many placental events. The aim of this study is to investigate the placental distribution and density of Hofbauer cells and to contribute to the understanding of the causes and pathogenesis of complicated pregnancies. In this study, 60 full-term placentas were divided into 4 equal groups: control, preeclampsia, gestational diabetes (GDM), and (hemolysis, elevated liver enzymes, low platelet) HELLP group. The placenta was dissected and the samples were fixed 10% neutral buffered formalin. Following routine paraffin wax procedure, 5 µm sections were stained with CD68 for marking Hofbauer cells. In immunohistochemical evaluation, Hofbauer cells in villous stroma showed positive CD68 expression. Immunostaining Findings: CD68 showed a granular staining pattern in the cytoplasm of Hofbauer cells. The group with the highest CD68 positive cell number was HELLP group and the number of cells per cell (1.46 ± 0.25) was significantly different from all groups. CD68 positive cell count in the placental villus was the highest in HELLP group and the number of Hofbauer cells per villus was significantly different from the other groups.*

Keywords: *Hofbauer cells, CD68, preeclampsia, HELLP syndrome, gestational diabetes*

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1. Introduction

Preeclampsia (PE) is characterized by proteinuria >300 mg/24 hours, spot urine protein/creatinine >0.3≥1, high liver enzymes, thrombocytopenia, and hypertension >160/110 mmHg.The etiology of preeclampsia is still unknown. Vascular lesions in various organ systems, vasospasm, increased platelet activation, and platelet degeneration, and subsequent activation of the intravascular coagulation system are essential causes that induce preeclampsia. Preeclampsia is a quite popular topic since it causes complications at a rate of 6-8% after the 24th week of pregnancy and is responsible for 20-25% of all perinatal deaths [1].

Gestational diabetes mellitus (GDM) is a type of diabetes observed in the second or third trimester of pregnancy. Gestational diabetes developed due to the metabolic and hormonal changes in pregnancy, leading to carbohydrate intolerance; high blood sugar with insulin resistance after the 24th week of pregnancy. GDM is one of the prediabetes conditions that pose a risk for the development of type 2 diabetes in future life. GDM is one of the most common pregnancy problems with macrosomia,

birth injuries, cesarean section, hydramnios to various maternal and fetal complications. GDM may trigger preeclampsia, neonatal metabolic disorders, and Type 2 DM development after birth [2].

The formation of the placenta is highly complicated and mediated through fetal extraembryonic tissues and the endometrial tissues during pregnancy. During early pregnancy (approximately 10th day), numerous macrophages invade placental tissue. A full-term placenta without any complications is essential for a normal pregnancy. The placenta plays a vital role in fetal development. Hofbauer cells are one of the most important groups of placental cells. These cells are placental macrophages and have a role in many placental events [3].

There have been many studies indicating the presence of stromal large cells of the human chorionic villi since the 19th century. These villous stromal cells were identified by Kastchenko in 1884, while Virchow and later Chaletzky-Neumann were reporting large cells with clear cytoplasm isolated in the pregnancies with mole hydatid [4].

Previously, the term "Chalatezky-Neumann cells" has been used by many researchers. However, at the beginning of the 20th century, the morphological and functional definition of these cells in normal villi was described by Hofbauer [5].

After this definition, the term Hofbauer cells (HBCs) was widely used in the literature. Round, fusiform, or star-like Hofbauer cells are localized in the villous stroma and are pleomorphic. Their size depends on the length of their process with a diameter of 10-30 μm. In the first studies, the most striking feature of Hofbauer cells is that they have a granular cytoplasm with vacuoles [6].

In later studies, electron-lucent vacuoles of different volumes surrounded by numerous membranes were observed in the cytoplasm of these cells. In addition, lysosomes containing granules of varying density and short endoplasmic reticulum profiles were noted [7-11).

Many theories have been proposed about the origin of Hofbauer cells. Chaletzky suggested that these cells originate from the maternal decidua; Neumann stated they were from the syncytium, whereas other researchers have suggested that they originate from the endothelium [5]. The most important finding in this regard is that these cells, which Wynn claims based on sex chromatin staining, are of fetal origin [12]. Thus, most researchers believe that Hofbauer cells are of chorionic mesenchymal origin. These cells can be identified in the placental villi in early development (after the 18th day of pregnancy). In the placentas of uncomplicated pregnancies, Hofbauer cells either disappear or are found singlet after the fourth month of pregnancy. On the other hand, there is an increase in the density of these cells in pathological placental cases such as intrauterine growth retardation and gestational diabetes [8, 10, 13].

These cells are capable of both immune and non-immune phagocytosis in addition to their essential functions. They can capture maternal antibodies that pass into placental tissue. They are also an important source of thromboxane, prostaglandins, and cytokines in the placenta [14-16]. High levels of phagocytosis confirm the functions of these cells [17]. Recently, there have been reports that Hofbauer cells express "sprout" proteins and thus play an important role in the branching of the villous tree and the development of the placenta [18-19].

The aim of this study is to investigate the placental distribution and density of Hofbauer cells and to contribute to the understanding of the causes and pathogenesis of complicated pregnancies.

2. Materials and Methods

This study was approved by the Dicle University Medical School Non-interventional Ethical Committee with the record number of 2016/194. 60 full-term placentas were divided into 4 equal groups: control, preeclampsia, gestational diabetes, and HELLP group.

Group I: Control group

Group II: Preeclampsia group.

Group III: Gestational diabetic group.

Group IV: HELLP group.

a)Histological procedure: Placentas were dissected and the samples were fixed 10% neutral buffered formalin. Following routine paraffin wax procedure, 5 µm sections were stained with CD68 (Abcam, CAT No: ab133386) for marking Hofbauer cells. 5 µm sections from paraffin blocks were taken on positively charged slides. For Hofbauer cells, immunostaining was performed by streptavidin-biotin immunoperoxidase method using CD68 antibody (Neomarkers. prediluted).

CD68 Staining Protocol

Paraffin sections were kept in an oven at 56°C overnight, and then deparaffinized by keeping them in xylol for 2x15 minutes.

For cleaning, sections were kept in xylene, later in descending alcohol series (100%, 100%, 96%, 90%, 80%, 70%), and finally brought to distilled water for 5 minutes.

Sections were washed in PBS and confined in a circle with a hydrophobic pen.

Afterward, 3% hydrogen peroxide was dropped on the sections, and the endogenous peroxidase activity was blocked after waiting for 5 minutes.

The sections were washed in buffered phosphate saline (PBS) solution for 3x minutes.

The sections were placed in a special container containing citrate solution and boiled in a microwave oven for 10 minutes to release the antigen.

Sections were left to cool at room temperatures and later 100 microliters of CD68 primary antibody(1:750) were dropped on the sections for 45 minutes.

Sections were washed in PBS and then, the same amount of biotinylated secondary antibody (1:750) was added for 20 minutes.

Sections were incubated for enzyme binding with streptavidin peroxidase (Thermo, cat no: TS-125-HR) for 30 minutes at room temperature.

After incubation with streptavidin peroxidase, it was washed 3x5 minutes with PBS

Sections were reacted with 3,3'diaminobenzidine (DAB) chromogen (Thermo, cat no: TA-125-HD).

The reaction was observed under the microscope and then transferred to PBS to terminate the reaction.

Sections were counterstained with Mayer hematoxylin for 40 seconds.

Sections were washed under tap water for about 5 minutes.

Sections were kept in increasing alcohol series for 5 minutes.

Sections were kept in Xylol for 2x15 minutes and cleared.

The sections were then mounted with medium, and the sections were left to dry at room temperature.

Brown cytoplasmic staining was evaluated as positive in Hofbauer cells detected in villous stroma by CD68. The sections were examined with Zeiss Imager A2 light microscope and micrographs were taken.

b) Statistical analysis: Placenta sections in all groups were stained with CD68 in order to see placental Hofbauer cells accumulation. 10 placenta sections of each group were randomly selected and 100 villi were counted in each section in order to evaluate Hofbauer cells.

Syncytial layer, villus stroma, and /or Hofbauer cells were positively stained, and the mean of groups was calculated. The data were evaluated by One-Way Anova and Post-Hoc Tukey tests with SPSS 24 (IBM, USA). Results were analyzed by multiple comparisons and $p<0.05$ was considered significant.

Ethical statement:

This study was carried out in accordance with the rules of research and publication ethics. The study was approved by the Dicle University Medical School Non-Interventional Ethics Committee (5/5/2016/194.).

3. Results

a) CD 68 Immunostaining results

We would like to emphasize that the placental Hofbauer cells were stained quite intensively with CD68 and that this staining shows a gradual increase in the control, preeclampsia, gestational diabetes, and HELLP groups (Figure 1-4). CD68 showed a granular staining pattern in the cytoplasm of Hofbauer cells. The reason for this particular staining is that the CD68 antibody is activated by the lysosomal granules of Hofbauer cells.

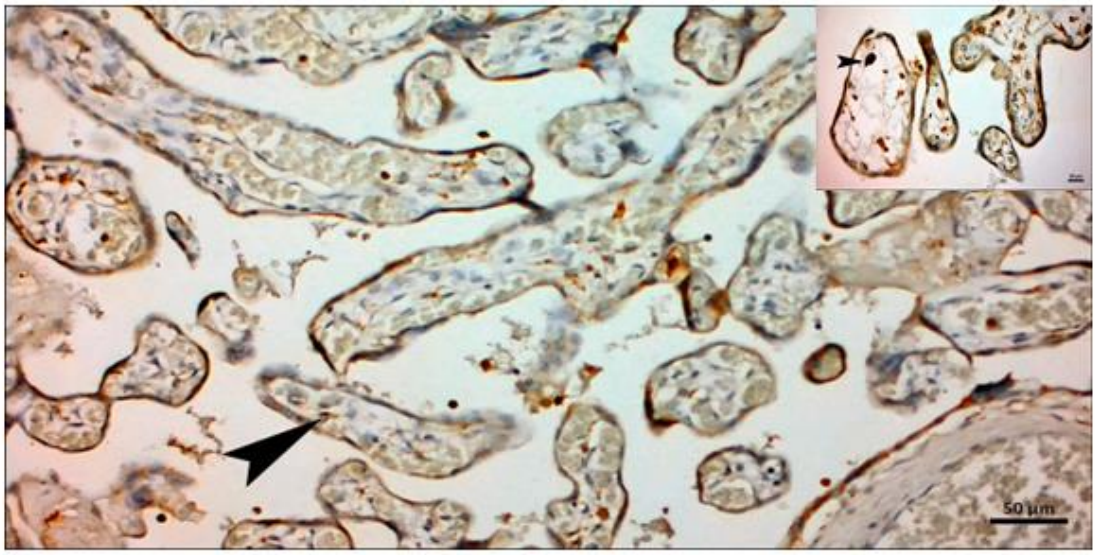

Figure 1. CD68 immunoreactivity in Hofbauer cells in placental tissue of the control group. Hofbauer cells show a small number of CD68 immunopositive reactions in mature villi. The inset shows some of Hofbauer cells (Black arrow) at higher magnification.

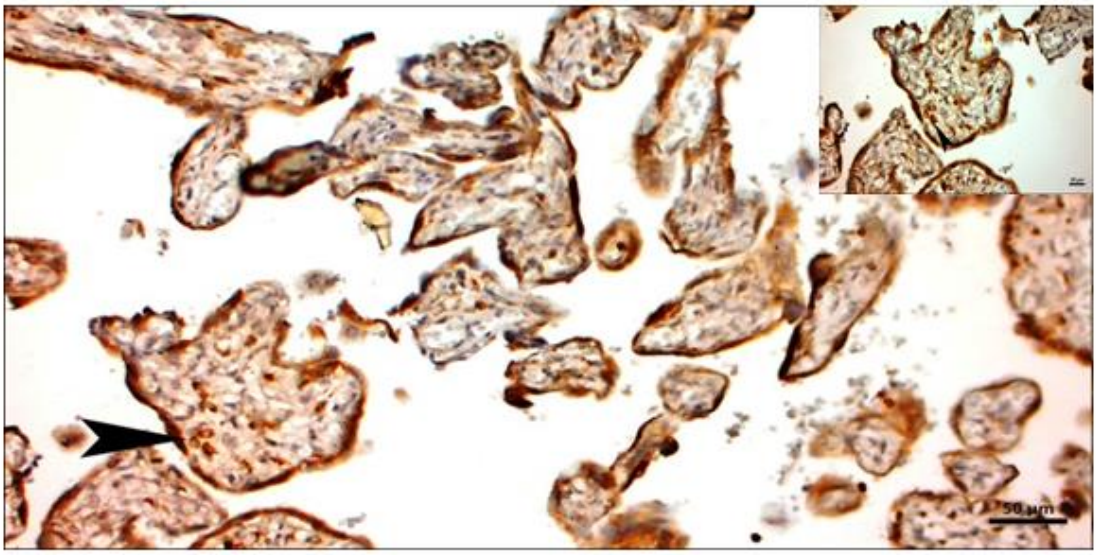

Figure 2. CD68 immunoreactivity in Hofbauer cells in placental tissue belonging to the group of gestational diabetes mellitus. CD68 immunopositive Hofbauer cells (black arrow) showed a relative increase in mature villi in the gestational diabetes mellitus group compared to the control group, (CD68, Bar: 50 μm). The inset shows a high magnification Hofbauer cells in high density.

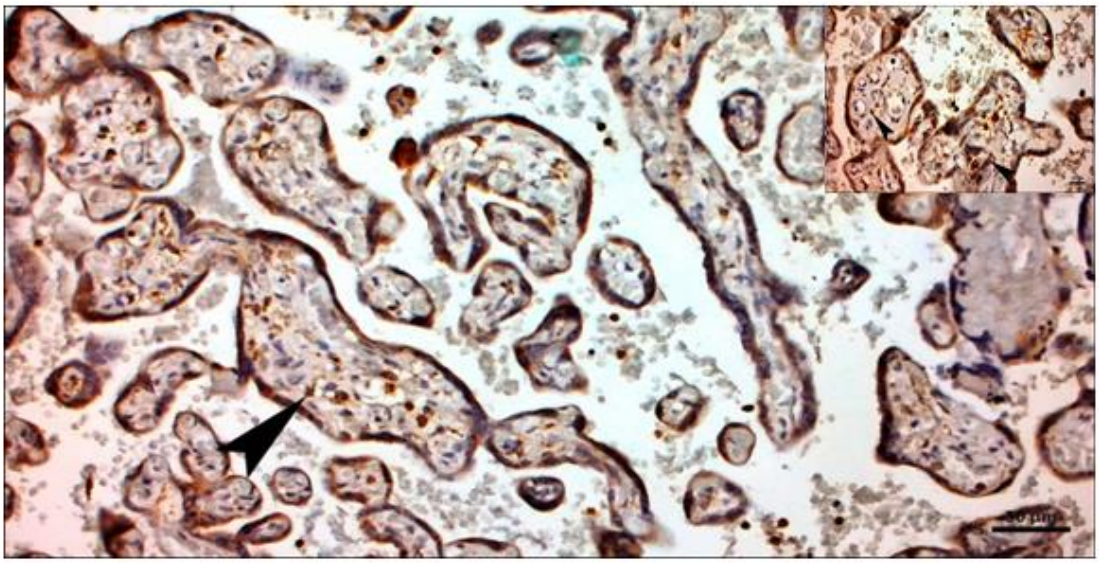

Figure 3. CD68 immunoreactivity in Hofbauer cells in placental tissue belonging to the preeclampsia group. CD68 immunopositive Hofbauer cells (black arrow) increased in the preeclamptic group compared to the control group (CD68, Bar: 50 μm). Hofbauer cells are seen in the histological section of the intermediate villi at high magnification.

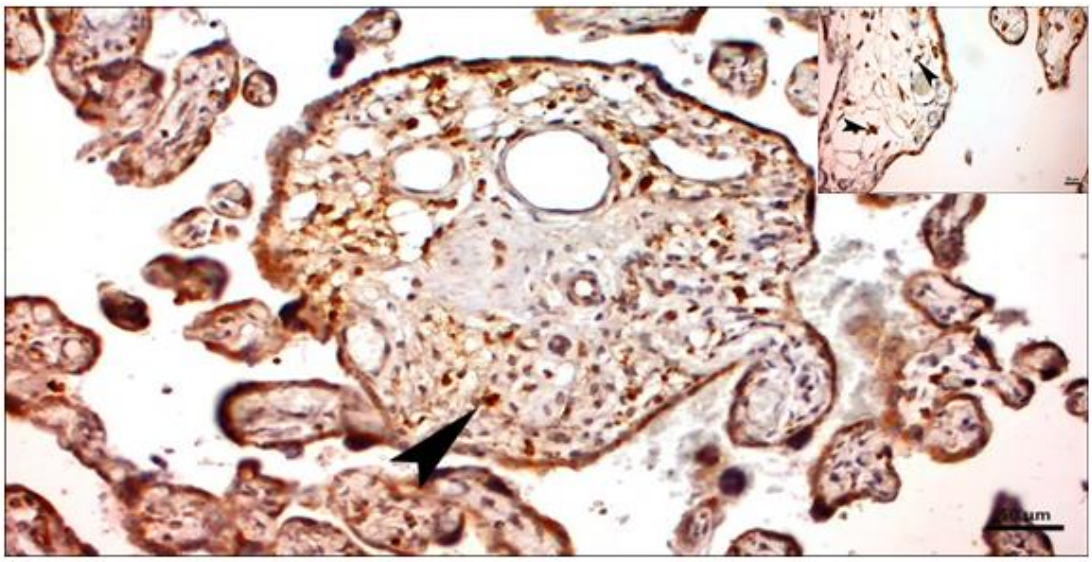

Figure 4. CD68 immunoreactivity in Hofbauer cells in placental tissue belonging to the HELLP group. In HELLP group placental sections, CD68 immunopositive Hofbauer cells (black arrow) showed a marked increase compared to the control group (CD68, Bar: 50 μm). The inset shows A large number of Hofbauer cells are seen in the stroma of the intermediate villus.

b) Statistical analysis results: It was found that the number of Hofbauer cells was 0,88 ± 0,21 in the preeclamptic group where the number of CD68 positive Hofbauer cells per villus was 0,46 ± 0,09 in the control group and this increase was significant compared to the control group ($p<0.05$). The number of cells per villus in the gestational diabetes group was 0.96 ± 0.15 and this increase was significant compared to the control group ($p <0.01$), but there was no significant difference between the preeclampsia group and the Gestational diabetes groups (0.05). The group with the highest CD68 positive cell number was HELLP group and the number of cells per villus (1.46 ± 0.25) was significantly different from all groups (Table 1).

Table 1. CD 68 positive Hofbauer Cells count in each villus

Groups	CD68+ cells (mean±sd)
Control group	0.46±0.09
Preeclampsia group	0.88±0.21
Gestational Diabetes group	0.96±0.15
HELLP group	1.46±0.25

4. Discussion

The placenta is a temporary organ necessary for the development of the embryo and fetus. There are many cells in the placenta. The most important of these are Hofbauer cells. There are limited studies on Hofbauer cells related to preterm birth. This is particularly the case in chorioamnionitis, which causes HELLP syndrome, severe preeclampsia, and spontaneous preterm delivery. In two independent studies, the number of CD68+ Hofbauer cells in the chorioamnionitis was found to be significantly lower [7, 20].

The mechanism of the low number of Hofbauer cells in chorioamnionitis is unknown, but it is estimated that these cells go to apoptosis. These cells especially transform into multinucleated giant cells in the placenta with chorioamnionitis. These cells have reduced immunological abilities when compared to Hofbauer cells in normal pregnancy [20].

Although HELLP is commonly observed with preeclampsia, it is actually a separate syndrome. The clinical presentation of preeclampsia and HELLP are different [21-22]. Preeclampsia is typically characterized by hypertension and proteinuria, while HELLP is mostly associated with the coagulation system.

HELLP and preeclamptic placentas show clinical differences in pathological aspects [22]. Although events such as infarction and intervillous thrombosis are very common in the placentas of patients with preeclampsia, this situation is less common in the placentas of patients with HELLP [21]. Generally, Hofbauer cell count and IL-10 are clearly low in patients with severe preeclampsia [23-24]. In preeclampsia, the scarcity of Hofbauer cells promotes inflammatory damage. On the other hand, the number of CD68+ Hofbauer cells is high in patients with HELLP. This excess of cells may be due to increased inflammation and the resulting response [22].

Hofbauer cells, one of the cell types in the placenta, are placental macrophages. Although there are many studies related to these cells, their possible roles in vasculogenesis and angiogenesis processes in the placenta have not been studied much. In a study conducted for this purpose, it was stated that the location and number of Hofbauer cells may be related to the vascular structures in the center of the villus, and thus these cells may play a role in vasculogenesis and angiogenesis. Curettage materials taken in the first trimester were used as placental tissue for the study.

Double immunohistochemical staining was performed with CD68 (macrophage marker) and CD31 (endothelial marker) to show the relationship of Hofbauer cells with primitive vessel formation. As a result, Hofbauer cells are thought to be involved in vasculogenesis and angiogenesis times, since they correlate with vascular structures [18]. In our study, we examined the change in the presence of gestational diabetes, preeclampsia, and HELLP syndrome in Hofbauer cells, which are thought to be involved in this process, which is very important for the development and functionality of the placenta, such as vascularization. We found that the placentas of pregnant women in the diabetic group and the preeclamptic group in our study, especially in pregnant placentas with HELLP syndrome, had more immunopositive Hofbauer cells in the villus stroma than in healthy pregnant women.

In another study, the number and functions of Hofbauer cells in the placenta were evaluated by examining pregnant placentas with complications of gestational diabetes. Tissue samples were taken for PCR and immunohistochemical evaluations from the placentas of 15 pregnant women with GDM and 10 healthy pregnant women for the study. CD68 and CD14 were selected as Hofbauer markers and IL-6 and TNF-alpha for inflammatory cytokines. As a result of immunohistochemistry staining, it was determined that CD68+ or CD14+ cells were significantly increased in the GDM group compared to the control group. According to these results, it is reported that Hofbauer cells increase in GDM pathology. In our study, we determined that CD68 immunopositive cells were higher in the GDM group than in healthy pregnant women.

As a result of our study, it was shown that there is macrophage accumulation in placentas with GDM and that the expression of pre-inflammatory mediators is higher than the control group, and it was concluded that these factors play an important role in the etiology of gestational diabetes. The data obtained as a result of this study and the findings obtained as a result of our study support each other [25]. It has been shown that Hofbauer cells may be important in the pathogenesis of early fetal losses.

5. Conclusion

In our study with diabetic, preeclamptic, and HELLP syndrome pregnant women, we found that the number of Hofbauer cells in the villus stroma increased in different pathological conditions.

Ethical statement

This study was carried out in accordance with the rules of research and publication ethics. The study was approved by the Dicle University Medical School Non-Interventional Ethics Committee (5/5/2016/194.).

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Conflict of interest

The authors declare no conflict of interest.

Authors' Contributions

All authors read and approved the final manuscript. All authors mentioned in the paper have significantly contributed to the research:

Y. N: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – review, and editing (%30)

Ş. N: Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft (%15)

F. A: Data curation, Software, Validation, Visualization, Writing – original draft, Writing – review and editing (%20)

A. Ş: Formal analysis, Funding acquisition, Methodology, Visualization, Writing – original draft, Writing – review and editing (%20)

E. A: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision (%15)

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