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### Highlights

**Phagocytic index of neutrophil granulocytes and monocytes in healthy and preeclamptic pregnancy**

Rudolf Lampé*, Ágnes Kövér, Sándor Szűcs, László Pál, Ervin Árnyas, Róza Ádány, Robert Póka

- The phagocytic function of granulocytes and monocytes was examined in normal and preeclamptic pregnancy.
- To our knowledge there are no scientific data on this topic.
- The phagocytic index decreased significantly in healthy pregnancy compared with the non-pregnant state.
- The phagocytic index decreased significantly in preeclampsia compared with the healthy pregnant state.
Phagocytic index of neutrophil granulocytes and monocytes in healthy and preeclamptic pregnancy

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A B S T R A C T
Neutrophil granulocytes and monocytes have been intensively studied, but there is no scientific data on one of their most important functions, namely the phagocyte function in pregnancy and preeclampsia. The aim of this study was to examine this function. Twenty-five healthy pregnant, 25 preeclamptic pregnant, and 20 healthy, non-pregnant women were enrolled into our study. Cells were isolated from peripheral blood samples, marked and evaluated for the phagocytic index with an immunofluorescent microscope after phagocytosing the zymosan molecules. The phagocytic function of monocytes and neutrophil granulocytes decreased significantly in healthy pregnancy compared with non-pregnant status, and this elevation is more pronounced in pathological pregnancies (Sacks et al., 1998; Calleja-Agius et al., 2012). Preeclampsia is one of the most severe complications of pregnancy that can be characterized as an increased systemic inflammatory response and endothelial dysfunction (Eastabrook et al., 2011). Clinical manifestation of preeclampsia is hypertension and proteinuria developing after the 20th week of pregnancy, and in untreated cases it can lead to multi-organ failure. The public health importance of preeclampsia is significant as it is the leading cause of maternal and fetal morbidity and mortality worldwide (Sibai et al., 2005). The etiology of preeclampsia is unknown, but placental origin is an accepted explanation of its pathophysiology. In normal pregnancy, placental trophoblast cells invade the myometrium and the uterine spiral arteries, and at the

1. Introduction
For an ongoing healthy pregnancy it is essential that the maternal immune system tolerates the ‘hemiallograft’ fetus (half of its genetic material is of paternal origin) and at the same time, protect the mother against infections. This seemingly contradictory behavior of the immune system has been intensively studied. Failure of the adaptation of the maternal immune system may lead to pathological pregnancies such as preeclampsia. It is known that in healthy pregnancy the level of biomarkers of systemic inflammation is increased compared with non-pregnant status, and this elevation is more pronounced in pathological pregnancies (Sacks et al., 1998; Calleja-Agius et al., 2012). Preeclampsia is one of the most severe complications of pregnancy that can be characterized as an increased systemic inflammatory response and endothelial dysfunction (Eastabrook et al., 2011). Clinical manifestation of preeclampsia is hypertension and proteinuria developing after the 20th week of pregnancy, and in untreated cases it can lead to multi-organ failure. The public health importance of preeclampsia is significant as it is the leading cause of maternal and fetal morbidity and mortality worldwide (Sibai et al., 2005). The etiology of preeclampsia is unknown, but placental origin is an accepted explanation of its pathophysiology. In normal pregnancy, placental trophoblast cells invade the myometrium and the uterine spiral arteries, and at the
end of the process, syncytiotrophoblast cells are in contact with the maternal circulation. In preeclampsia this process is damaged and the placentation will be abnormal (Khong et al., 1986). Because of this abnormality, syncytiotrophoblast microparticles (STBMs) are released from the hypoxic placenta in a significantly higher amount than in healthy pregnancy. STBMs have immunomodulatory effects and play a role in endothelial dysfunction (Redman et al., 2012). The maternal innate immune system is important in the elimination of these microparticles as well as the pathogenic microorganism. Two representatives of the innate immune system are the neutrophil granulocytes and monocytes, which are “professional phagocytes”. The phagocytic function of these cells is to take part in the destruction of apoptotic, necrotic, and tumor cells (Galli et al., 2011). The behavior of neutrophils and monocytes in healthy and preeclamptic pregnancy has been the subject of several experimental investigations (Gervasi et al., 2001; Kauma et al., 2002; Walsh, 2009). Based on this research it has been generally accepted that both neutrophils and monocytes are activated during healthy pregnancy and that this activated state is even more pronounced in preeclampsia. Transendothelial migration and the production of inflammatory cytokines of these cells are increased in preeclampsia. Previously, we have published that superoxide anion production of granulocytes is decreased in healthy pregnancy compared with non-pregnant controls, but in preeclampsia this decrease is absent (Lampé et al., 2011).

To our knowledge, the phagocytic function of neutrophil granulocytes and monocytes in healthy pregnancy and preeclampsia has not yet been examined. Our aim was to examine and describe the phagocytic function of granulocytes and monocytes for a better understanding of the physiology of healthy pregnancy and the pathophysiology of preeclampsia.

2. Materials and methods

2.1. Study population

After obtaining informed consent with the permission of the Institutional Ethics Committee, peripheral blood was collected in vacutainer test tubes containing EDTA (Becton-Dickinson, Le Pont de Claix, France) from 25 normal and 25 preeclamptic pregnant women in the third trimester of their pregnancy (gestational weeks 35–38). A group of 20 age-matched, non-pregnant, healthy women served as controls. Preeclampsia was defined as development of hypertension after the 20th week of pregnancy (≥140/90 mmHg measured on two consecutive occasions 6 h apart) and proteinuria of higher than 300 mg/day confirmed by 24-h urine collection (ACOG Committee on Obstetric Practice, 2002). Patients with active labor were excluded from the study. None of the patients with preeclampsia was on any medication, had a history of diabetes mellitus, or lacked major medical disease or prior surgical intervention. None of the control subjects was taking oral contraceptives or any other form of pharmaceuticals.

2.2. Preparation of FITC labeled and opsonized zymosan particles

Zymosan particles were labeled with fluorescein isothiocyanate (FITC), as described previously (Hed et al., 1987). Briefly, the particles (1 x 10⁶ ml⁻¹) were incubated in carbonate buffer, pH 9.6, containing FITC at a final concentration of 0.01 mg/ml for 60 min at 37 °C. Then they were washed three times and opsonized in Hanks’ solution containing 50% human AB serum at 37 °C for 30 min. The labeled and opsonized particles (FITC-OZ) were washed three times and stored at −20 °C in Hanks’ solution (3 x 10⁷ ml⁻¹) until the phagocytosis assay.

2.3. Separation and examination of the phagocytic function of granulocytes and monocytes

Mononuclear cells and granulocytes were separated as described previously (Öyum, 1968; English and Andersen, 1974). Blood samples were mixed with an equal volume of Hanks’ solution, pH 7.4, and layered on top of a discontinuous Ficoll gradient (1.077 and 1.119 g/ml) and centrifuged at 400 x g at 20 °C for 30 min. Mononuclear and polymorphonuclear cells were collected from the top and interface of the Ficoll layers, respectively. Then the cells were washed twice with Hanks’ solution. Their viability was determined using the trypan blue exclusion test and found to be 96–98%. The purity of granulocyte suspensions varied between 95% and 98%, as judged by morphology.

In the phagocytosis assay, phagocytosis of FITC-OZ was determined as described previously (Forslid and Hed, 1982; Vrsalovic et al., 2007). Briefly, 1 x 10⁵ granulocytes and mononuclear cells in 300-µl aliquots of Hanks’ solution containing 5% heat inactivated human AB serum were placed into the wells of chamber slides to allow the cells to adhere for 30 min at room temperature. Subsequently, the non-adherent cells were removed by washing, the adherent cells and FITC-OZ (3 x 10⁶ well⁻¹) were incubated in 300-µl aliquots of Hanks’ solution at 37 °C in 5% CO2/95% humidified air for 60 min. Following incubation, the fluorescence of non-ingested zymosan particles was quenched by trypan blue solution (Busetto et al., 2004; Chaka et al., 1995). Monocytes were identified using the indirect immunofluorescent method. The cells were labeled with anti-CD14 monoclonal antibody and then with immunoglobulin G-conjugated Dylight 594 fluoroscent dye. The nuclei of the granulocytes and monocytes were stained with 4,6-diamidino-2-phenylindole (DAPI) and the slide was then removed from the chamber for microscopic evaluation. The number of FITC-OZ/cell was determined using an Axiosplan fluorescent microscope (Zeiss Oberkochen, Germany) by examining 2 x 100 cells in randomly selected microscopic fields. Then the phagocytic index (PI), the average number of ingested particles/cell, was calculated and presented as mean ± SD.

2.4. Statistical analysis

The Shapiro–Wilks test was used to verify normal distribution of the data. For comparison of the average values of
3. Results

The clinical characteristics of the study population are presented in Table 1. As shown, there were significant differences in systolic and diastolic blood pressures ($p < 0.001$), body mass index (BMI, $p < 0.05$), gestational age at delivery ($p < 0.01$), proteinuria at the time of blood sampling ($p < 0.001$), and birth weight ($p < 0.01$) between normal and preeclamptic pregnant women.

3.1. Phagocytic function of neutrophil granulocytes

The PI of neutrophils from healthy non-pregnant, normal, and preeclamptic pregnant women is illustrated in Fig. 1. As shown, granulocytes from healthy pregnant women demonstrated a significantly decreased PI (3.76 ± 0.52; $p < 0.001$) compared with non-pregnant controls (4.67 ± 0.31). Neutrophils from preeclamptic pregnant women show a significantly decreased PI (2.73 ± 0.43) compared with healthy pregnant controls ($p < 0.001$) and non-pregnant controls ($p < 0.001$).

3.2. Phagocytic function of monocytes

The PI of monocytes from healthy non-pregnant, normal, and preeclamptic pregnant women is illustrated in Fig. 2. As shown, monocytes from healthy pregnant women demonstrated a significantly decreased PI (3.33 ± 0.64, $p < 0.001$) compared with non-pregnant controls (3.96 ± 0.33). Monocytes from preeclamptic pregnant women revealed a significantly decreased PI (2.32 ± 0.66) compared with healthy pregnant controls ($p < 0.001$) and non-pregnant patients ($p < 0.001$).

4. Discussion

The main representatives of the innate immune system are the monocytes (mononuclear phagocytes) and neutrophil granulocytes (polymorphonuclear phagocytes). These cells play an important role in the correspondence with the adaptive immune system, mainly with cytokine (interleukins, tumor necrosis factor, interferon) production and this relationship is true vice versa (Beutler, 2004). Clinical and experimental results confirm that cellular and humoral components of the innate immune system are activated during pregnancy (Sacks et al., 1999). As a part of the activation, white blood cell count is elevated significantly in normal pregnancy (Efrati et al., 1964).

Table 1

Clinical characteristics of non-pregnant (NP), healthy pregnant (HP), and preeclamptic pregnant (PE) women.

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>NP women (n = 20)</th>
<th>HP women (n = 25)</th>
<th>PE women (n = 25)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.1 ± 3.2</td>
<td>29 ± 5.2</td>
<td>29.9 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure at blood sampling (mmHg)</td>
<td>106.5 ± 6.1</td>
<td>108.9 ± 7.4</td>
<td>148.9 ± 7.9</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Diastolic blood pressure at blood sampling (mmHg)</td>
<td>66.9 ± 5.4</td>
<td>70.9 ± 7.3</td>
<td>100.7 ± 7.4</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Proteinuria at blood sampling, urine dipstick$^a$</td>
<td>N/A</td>
<td>0(0-0)</td>
<td>2(1-3)</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Gestational age at blood sampling (weeks)</td>
<td>N/A</td>
<td>36.4 ± 1.2</td>
<td>36.1 ± 0.9</td>
<td>NS</td>
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<tr>
<td>BMI at blood sampling$^b$ (kg/m²)</td>
<td>21.2 ± 2.5</td>
<td>24.9 ± 1.5</td>
<td>29.8 ± 1.3</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m²)</td>
<td>N/A</td>
<td>23.3 ± 1.2</td>
<td>24.2 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Parity$^c$</td>
<td>N/A</td>
<td>0(0-2)</td>
<td>0(0-2)</td>
<td>NS</td>
</tr>
<tr>
<td>Gravidity$^c$</td>
<td>N/A</td>
<td>2(1-3)</td>
<td>2(1-4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean values ± SD are presented. NA: not applicable. NS: not significant.

$^a$ Values are expressed as median (range).

$^b$ BMI: body mass index.

$^c$ Significant differences are indicated as follows: $p<0.05$ healthy pregnant versus preeclamptic pregnant women.

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In healthy pregnancy the maternal serum level of acute phase proteins (fibrinogen, factor VIII, several elements of complement system) is elevated (Comeglio et al., 1996). Nevertheless, pregnant women are more susceptible to infections than non-pregnant women, and these infections are usually more severe (Cunningham et al., 1984). Many aspects of the immunological background of preeclampsia have been examined. One of the generally accepted views is that because of placental hypoxia caused by abnormal placentation, STBMs break off from the placenta. These microparticles reach the maternal blood stream and play an important role in the creation of endothelial dysfunction and in the activation of maternal leukocytes (Laresgoiti-Servitje, 2013). It has been shown that some of the phagocytes play a role in the elimination of the STBMs (Abumaree et al., 2012). Syncytiotrophoblast does not express human leukocyte antigens (either class I or II), but does express several polymorph proteins and minor histocompatibility antigens that are responsible for the HLA-matched organ graft rejection (Holland et al., 2012).

Owing to these results, we speculate that the monocytes and neutrophil granulocytes might play a role in the elimination of STBMs. The aim of this study was to evaluate the phagocytic function of these leukocytes in pregnancy. We have found that in healthy pregnancy, the phagocytic function of neutrophil granulocytes and monocytes is significantly decreased compared with non-pregnant controls. We assume that this decrease may be part of the maternal immunosuppression for the protection of the fetus, a ‘hemallograft’; STBMs reach the maternal bloodstream even in healthy pregnancy, but in a much lower amount than in preeclampsia, and these microparticles can activate the maternal immune system. This may be one explanation for the elevated number of inflammatory cells and for the proinflammatory status in healthy pregnancy. Decreased phagocytic function in healthy pregnancy is still enough to eliminate enough STBMs that they do not cause endothelial dysfunction, but it is less effective against infections. In preeclampsia, the phagocytic function of neutrophil granulocytes and monocytes is significantly decreased compared with healthy pregnancy. This decrease can be part of an “immunoregulatory confusion” that may occur in preeclampsia. The numbers of STBMs are elevated in preeclampsia and phagocytes with remarkably decreased phagocytic function eliminate them with much lower efficiency. Thus, the decreased phagocytic function may contribute to the generation of endothelial dysfunction in preeclampsia. Decreased phagocytic function may even play a role in the defense against microorganisms. However, it is not known whether infection is partly a cause or a consequence of preeclampsia. Our results suggest that abnormal placentation, which is typical for preeclampsia, might happen in the very early phase of pregnancy. Although BMI was significantly elevated in our preeclamptic group, the pre-pregnancy BMI was normal for both the healthy pregnant and preeclamptic women. Higher average BMI found in the preeclamptic group was most likely due to maternal edema, which can be a sign of preeclampsia.

In summary, our findings revealed that in healthy pregnancies the phagocytic function of neutrophil granulocytes and monocytes is decreased compared with non-pregnant women. The decline in phagocytic function may play a role in natural maternal immunosuppression for the protection of the fetus. In preeclampsia phagocytic function declines further, which may be a part of the immunoregulatory abnormalities characteristic of preeclampsia. Further research is needed to define the factors that regulate phagocytic function. Our current results may contribute to a better understanding of the physiology of pregnancy and the pathophysiology of preeclampsia.

Conflict of interest statement

The author declares no conflict of interest.

Acknowledgments

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