

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**Superoxide-anion production of neutrophil granulocytes in  
healthy and preeclamptic pregnant women**

by **Rudolf Lampé, M.D.**

Supervisors:

Róbert Póka, M.D. Ph.D.

Sándor Szűcs, Ph.D.



UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF CLINICAL MEDICINE

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## INTRODUCTION

Preeclampsia is a pregnancy-specific multisystem disorder that develops after 20<sup>th</sup> week of pregnancy and characterized by newly developed hypertension and proteinuria. Although the exact etiology of the disease is still unknown, the placental hypoperfusion and the diffuse endothelial cell damage may play a central role. Preeclampsia can be divided into mild and severe forms and in suboptimally treated cases it can lead to kidney and liver failure, disseminated intravascular coagulation and central nervous system symptoms. As the definitive therapy of preeclampsia is delivery, it has a high maternal and fetal morbidity and mortality. In the United States this disease is accounted for 15% of preterm birth and 17.6% of maternal death. Worldwide, preeclampsia and eclampsia are accounting for about 14% of maternal death annually which affects 50.000 to 75.000 women. Many attempts have been made in the field of prevention because of the importance of the disease, but these have been completed with disappointing results.

Incidence of preeclampsia is 2-6% among primiparous women in the United States. The disease appears as mild in 75% of the cases, while severe in 25%. Ten percent of all cases of preeclampsia develop before 34<sup>th</sup> week of pregnancy. In the absence of magnesium prophylaxis in preeclampsia, in one case from 200 the disorder is so severe that eclampsia develops. Preeclampsia is more likely to develop in women with history of previous preeclampsia, multiple pregnancy, chronic hypertension and renal failure. Moreover, obesity, diabetes, thrombophilia and maternal age more than 40 are risk factors, too. In relation to family history of cardiovascular diseases it deserves special attention.

Hypertension occurring during pregnancy is divided into 4 groups, one of them is preeclampsia: hypertension and proteinuria after the 20<sup>th</sup> week of pregnancy. Diagnosis of mild preeclampsia may be set up if the blood pressure is greater than 140/90 mmHg,

measured at least twice a day for at least six hours apart, within a period of one week. The definition of proteinuria is greater than or equal to 1+ protein randomly examined by bedside test or the 24-hour urine collection for protein excretion is greater than 300 mg. Edema or hyperreflexia is not a diagnostic criterion for preeclampsia nowadays.

Clinical manifestations of preeclampsia may various and the diagnosis is not always easy. For example if the patient suffers from gestational hypertension, which diagnosis can only be set up retrospectively, the treatment is usually the same as the patient would be preeclamptic. If a pregnant has renal or cardiovascular disease, the diagnosis of preeclampsia is also difficult. The generally accepted consensus regarding the pathogenesis of preeclampsia is that it is an endothelial cell disorder which leads to mild and severe microangiopathy in the target organs such as brain, kidneys, liver and placenta. Although hypertension is the most common symptom of preeclampsia it can not be regarded that it is the primary pathogenic process. Sometimes other organs can be affected before hypertension becomes apparent. The level of circulating markers of endothelial cell damage usually increases before the onset of the symptoms of preeclampsia. These markers are endothelin, cellular fibronectin, plasminogen activator inhibitor-1 and the altered prostacyclin/thromboxane rate. Nowadays, the suspected backgrounds of the pathogenesis of preeclampsia are the oxidative stress, the immunological, humoral and metabolic abnormalities. All of these can lead to organ damage due to endothelial cell damage. The primary targets of superoxide anion ( $O_2^{\cdot-}$ ) produced by neutrophils granulocytes, are the cell membranes and lipoproteins. Reactive primary products forms the damaged polyunsaturated fatty acids and cholesterol which is known as lipid hydroperoxides, and the name of the process is peroxidation. Lipid hydroperoxides are necessary in the physiological processes of the body but uncontrolled production may lead to dysfunction and damage of cells.

Studies on  $O_2^-$  production by polymorphonuclear leukocytes (PMNLs) from normal pregnant and preeclamptic women have provided conflicting results. The data published by Tsukimori et al. demonstrated that N-formyl-methionyl-leucyl-phenylalanine- (FMLP) stimulated  $O_2^-$  generation by granulocytes was increased in women with preeclampsia compared with healthy pregnant women, and no significant difference was obtained between the  $O_2^-$  production by PMNLs from normal pregnant and healthy non-pregnant women. Selvaraj et al. showed a higher granulocyte  $O_2^-$  generation in normal pregnancy compared with non-pregnant controls. Sacks et al. measured an increased oxygen radical production by PMNLs in normal pregnancy and preeclampsia but they could not demonstrate a significant difference between these groups. In contrast, Crocker et al. published that the FMLP-induced granulocyte  $O_2^-$  generation was significantly reduced in healthy pregnant women compared with non-pregnant controls and they found no significant difference between  $O_2^-$  production by PMNLs from patients with preeclampsia and healthy non-pregnant women. Crouch et al., Miller and Russel also reported a significantly lower granulocyte  $O_2^-$  generation in healthy pregnant women compared with non pregnant controls. Several pregnancy-associated circulatory factors can suppress or enhance the  $O_2^-$  production of granulocytes in healthy pregnant and preeclamptic women.

The purpose of our current study was to test this hypothesis. Therefore, we aimed to examine reciprocally whether plasma samples from healthy non-pregnant, normal pregnant and preeclamptic women could influence the phorbol-12,13-dibutirate- (PDBu) and FMLP-induced  $O_2^-$  production by PMNLs from non-pregnant controls, healthy pregnant and preeclamptic women. Furthermore, the  $O_2^-$  generation by granulocytes from healthy non-pregnant, normal pregnant and preeclamptic women was also measured using the above mentioned stimulating agents.

## **AIMS OF EXPERIMENTS**

The work presented here deals with granulocyte-function in healthy pregnant, preeclamptic pregnant and non pregnant women. With our experiments we aimed to answer the following groups of questions:

### **I. Examine the $O_2^-$ production of granulocytes in healthy pregnant and preeclamptic pregnant women:**

I.1. Observe the change in FMLP and PDBu stimulated  $O_2^-$  production of granulocytes from healthy pregnant and preeclamptic pregnant women versus non pregnant women.

### **II. Justify the presence or lack of plasma factors in healthy pregnant and preeclamptic pregnant women which are influence granulocytes $O_2^-$ production.**

II.1. Examine the effect of healthy non pregnant women plasma samples on the  $O_2^-$  production of granulocytes from healthy pregnant and preeclamptic pregnant women.

II.2. Examine the effect of pregnant women plasma samples on the  $O_2^-$  production of granulocytes from healthy non pregnant and preeclamptic pregnant women.

II.3. Examine the effect of preeclamptic pregnant women plasma samples on the  $O_2^-$  production of granulocytes from healthy non pregnant and healthy pregnant women.

II.4. Examine the above mentioned changes with inactivated and non inactivated plasma samples.

II.5. Examine that does the foreign plasma from the same group affect granulocyte function?

## **PATIENTS AND METHODS**

### **Study population**

After informed consent and the approval of the Institutional Ethics Committee, peripheral blood was collected from 31 normal pregnant and 39 preeclamptic women in their third trimester of pregnancy. Blood samplings were performed in gestational weeks 26-38. A group of 35 age-matched non-pregnant women served as controls in this study. Preeclampsia was defined as development of hypertension after the 20<sup>th</sup> week of pregnancy ( $\geq 140/90$  mm Hg measured in two consecutive occasions 6 hours apart) and proteinuria of higher than 300 mg/day. The preeclamptic group included 9 mildly and 30 moderately ill patients with blood pressure of 140/90 – 149/99 mm Hg and 150/100 – 159/109 mm Hg, respectively. All of the patients with preeclampsia were not on any medication, without a history of diabetes mellitus and absence of major medical disease or surgical intervention. None of the women in the non-pregnant group used any kind of medication including hormonal contraceptives.

### **Separation of granulocytes from peripheral blood**

Peripheral blood was collected in vacutainer test tubes containing. Blood samples were layered on the top of a Ficoll solution (1.077 g/ml) and the supernatant containing the leukocytes was removed after sedimentation of erythrocytes at 1 g for 60 min at room temperature. The leukocyte rich plasma was layered on the top of a discontinuous Ficoll gradient (1.077 and 1.119 g/ml) and centrifuged at 350 g at 20 °C for 30 min. Granulocytes sedimented at the interface of the Ficoll layers were collected and washed twice with Hanks' solution, pH 7.4 at 20 °C. Then the cells were stained by May-Grünwald Giemsa and the preparations were examined microscopically. Granulocytes were indentified on the basis of

conventional morphological criteria. The purity of the granulocyte suspensions varied between 94-98 %. Cell viability checked by trypan blue exclusion test was found to be 98%. Red blood cells were not removed by hypotonic lysis since the erythrocyte contamination in the granulocyte suspensions was negligible.

### **Measurement of superoxide-anion production**

Superoxide-anion release was measured by SOD inhibitable reduction of ferricytochrome-c. Granulocytes ( $3 \times 10^5$ ) were incubated in Hanks' solution (pH 7.4) with PDBu or FMLP at 37 °C for 15 min. The total assay volume was 0.5 ml. The final concentrations of ferricytochrome-c, PDBu and FMLP were 50  $\mu\text{mol/l}$ , 100  $\text{nmol/l}$  and 1  $\mu\text{mol/l}$ , respectively. The control cell suspensions contained all of the above mentioned reagents plus 100 U/ml SOD. The change in absorbance was measured spectrophotometrically at 550 nm with a double beam Shimadzu UV-160A spectrophotometer at room temperature. The amount of superoxide-anion secreted into the medium was calculated on the basis of the molar extinction coefficient of reduced cytochrome-c  $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ .

### **Effect of plasma samples on superoxide-anion production by granulocytes from non-pregnant, healthy pregnant and preeclamptic women**

Plasma fractions were isolated from peripheral blood of healthy non-pregnant and normal pregnant women as well as patients with moderate preeclampsia by centrifugation at 800 g at 20 °C for 10 min and then the plasma samples were divided into two parts. Half of each fraction was heated to 56 °C for 30 min, while the other portion was not inactivated.

Subsequently the individual plasma preparations were not pooled. Every experiment was performed with inactivated and not-inactivated plasma samples simultaneously. Granulocytes ( $3 \times 10^6$ ) from healthy non-pregnant women were incubated with plasma samples (1.5 ml) from normal pregnant and preeclamptic women. PMNLs ( $3 \times 10^6$ ) from normal pregnant women were treated with plasma preparations (1.5 ml) from healthy non-pregnant and preeclamptic women. Granulocytes ( $3 \times 10^6$ ) from preeclamptic women were incubated with plasma fractions (1.5 ml) of non-pregnant and healthy pregnant women. In order to examine the possibility of stimulation or suppression of  $O_2^-$  production due to an immune reaction of the cells with foreign plasma factors, PMNLs ( $3 \times 10^6$ ) from non-pregnant, healthy pregnant and preeclamptic women were also treated with autologous and heterologous plasma samples (1.5 ml) of non-pregnant, normal pregnant and preeclamptic women, respectively. Following incubation of the cells at 37 °C for 1 hour, granulocytes were washed with Hanks' solution and  $O_2^-$  production was measured as described above.

### **Statistical analysis**

The distribution of data, checked by the Kolmogorov-Smirnov test, was normal. Differences between the clinical parameters of the study population, superoxide-anion production by granulocytes from non-pregnant controls, normal pregnant and preeclamptic women as well as  $O_2^-$  generation by PMNLs from non-pregnant, healthy pregnant and preeclamptic women treated with appropriate plasma samples were determined by one-way analysis of variance (ANOVA) using the Newman-Keuls post-hoc test. Values of  $p < 0.05$  were considered to be statistically significant.

## RESULTS

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 pregnant and preeclamptic women.

### **Superoxide-anion production by granulocytes**

Granulocytes from normal pregnant women demonstrated significantly decreased  $O_2^{\cdot-}$  generation compared with non-pregnant and preeclamptic women. There was no significant difference in  $O_2^{\cdot-}$  production by PMNLs from non-pregnant controls and patients with preeclampsia. Granulocytes from preeclamptic women released significantly greater amount of  $O_2^{\cdot-}$  compared with normal pregnant women. The FMLP-induced  $O_2^{\cdot-}$  production by granulocytes from normal pregnant women was significantly reduced compared with non pregnant and preeclamptic women. There was no significant difference in  $O_2^{\cdot-}$  generation by PMNLs isolated from non-pregnant controls and patients with preeclampsia. Granulocytes from preeclamptic women showed significantly increased  $O_2^{\cdot-}$  production compared with normal pregnant women.

### **Effects of plasma samples on superoxide-anion production**

Both inactivated plasma and not-inactivated plasma from healthy pregnant women significantly inhibited the PDBu-induced  $O_2^{\cdot-}$  generation by PMNLs from non-pregnant

controls compared with  $O_2^-$  generation by the cells treated with inactivated and not-inactivated autologous and heterologous plasma from healthy non-pregnant women as well as preeclamptic women. There were no significant differences in the PDBu-stimulated  $O_2^-$  generation by granulocytes from non-pregnant controls after incubation of the cells with autologous and heterologous plasma from non-pregnant women as well as plasma from preeclamptic subjects. The FMLP-stimulated  $O_2^-$  production by PMNLs from non pregnant controls was not influenced by inactivated healthy pregnant and preeclamptic plasma and heterologous non-pregnant plasma. However, incubation of the cells with not-inactivated healthy pregnant plasma resulted in a significant inhibition of  $O_2^-$  generation compared to  $O_2^-$  production by granulocytes treated with not-inactivated autologous and heterologous non-pregnant and preeclamptic plasma.

Both inactivated and not-inactivated healthy non-pregnant and preeclamptic plasma significantly increased the PDBu-induced  $O_2^-$  generation by granulocytes from normal pregnant women compared with  $O_2^-$  generation by PMNLs incubated with inactivated and not-inactivated autologous and heterologous healthy pregnant plasma. There were no significant differences in the PDBu-stimulated  $O_2^-$  generation following incubation of the cells with autologous and heterologous healthy pregnant plasma. Inactivated healthy non-pregnant and preeclamptic plasma as well as heterologous healthy pregnant plasma did not influence the FMLP-stimulated  $O_2^-$  production by granulocytes from normal pregnant women. In contrast, incubation of the cells with not-inactivated non pregnant and preeclamptic plasma resulted in a significant increase in  $O_2^-$  generation compared to  $O_2^-$  production by PMNLs treated with not-inactivated autologous and heterologous healthy pregnant plasma.

Treatment of the cells with inactivated and not-inactivated healthy pregnant plasma caused a significant decrease in the PDBu-induced  $O_2^-$  generation by PMNLs from patients with preeclampsia compared with  $O_2^-$  release by granulocytes incubated with inactivated and not-inactivated autologous and heterologous preeclamptic plasma. There were no significant differences in the PDBu-stimulated  $O_2^-$  production following incubation of the cells with autologous and heterologous preeclamptic plasma as well as non pregnant plasma. Exposure of granulocytes from preeclamptic women to inactivated non-pregnant plasma, autologous and heterologous preeclamptic plasma did not cause significant changes in the FMLP-stimulated  $O_2^-$  generation. However, treatment of the cells with not-inactivated healthy pregnant plasma resulted in a significant reduction in  $O_2^-$  generation compared to  $O_2^-$  production by granulocytes incubated with not-inactivated autologous and heterologous preeclamptic plasma.

## DISCUSSION

Data on respiratory burst activity of granulocytes from healthy pregnant and preeclamptic women have remained contradictory. Although it has been described in numerous studies that there is no significant difference between the stimulated  $O_2^{\cdot-}$  production by PMNLs from non-pregnant controls and healthy pregnant subjects, pregnancy-related depression of granulocyte reactive oxygen species (ROS) generation has also been reported by several researchers. In order to further investigate the involvement of granulocyte-derived free radicals in the endothelial injury in preeclampsia, we measured the  $O_2^{\cdot-}$  production by PMNLs from non-pregnant controls, healthy pregnant and preeclamptic women. Our results suggest that one of the most important granulocyte functions, the generation of reactive oxygen species, is suppressed in physiological pregnancy. Our finding is consistent with previous studies demonstrating depression of granulocytes' reactive oxygen species generation in healthy pregnant women. This reduction may be explained by a general suppression of cell-mediated immunity which has been demonstrated in normal pregnancy. The attenuation of  $O_2^{\cdot-}$  production may be required to protect foetal and maternal cells from the granulocyte-mediated oxidative damage in normal pregnancy. Similar decrease in ROS generation was not detected in patients with preeclampsia, which may be due to disturbances in the maternal immune system and may be partly responsible for endothelial injury in preeclampsia.

Apart from decreased  $O_2^{\cdot-}$  production, granulocytes from normal pregnant women have been found to show diminished chemotaxis, adherence, microbial killing and phagocytosis. Moreover, epidemiological studies have supported that the incidence of bacterial and viral infections is higher among pregnant women. As granulocyte-derived

reactive oxygen species play a pivotal role in the destruction of pathogenic microorganisms, our results and previous findings indicating decreased  $O_2^-$  generation in physiological pregnancy are in agreement with these epidemiological data. In addition, sera from pregnant women have been demonstrated to inhibit bacterial killing, phagocytosis and  $O_2^-$  production by control PMNLs. In accordance with the results of the aforementioned studies, both heat-inactivated and not-inactivated healthy pregnant plasma suppressed the PDBu-induced  $O_2^-$  generation by granulocytes from non-pregnant controls and patients with preeclampsia in our study. In contrast, depression of the FMLP-stimulated  $O_2^-$  production was not detected, when granulocytes from non-pregnant and preeclamptic women were incubated with inactivated healthy pregnant plasma. This suggests that the plasma factor responsible for the suppression of FMLP-induced respiratory burst may be heat-sensitive and thereby was degraded during heating. To exclude this possibility, control and preeclamptic granulocytes were also treated with not-inactivated normal pregnant plasma. Indeed, the results of these experiments indicated that not-inactivated healthy pregnant plasma was able to decrease the FMLP-stimulated  $O_2^-$  generation by granulocytes from non-pregnant and preeclamptic subjects. The inhibition was probably not associated with the effect of foreign plasma factors since there was no significant difference in the PDBu- and FMLP-stimulated  $O_2^-$  production by normal and preeclamptic granulocytes after incubation of the cells with autologous and heterologous plasma samples. Our investigations also indicated that preeclamptic plasma did not increase the PDBu- and FMLP-induced  $O_2^-$  generation by control granulocytes. Similarly, no activation of PMNLs by preeclamptic plasma was detected by Barden et al.

To show that the depression of  $O_2^-$  production was mediated by the healthy pregnant plasma, granulocytes from normal pregnant women were incubated with non-pregnant and preeclamptic plasma. The results of these experiments demonstrated that the decreased  $O_2^-$  generation by healthy pregnant granulocytes could be increased by treatment of the cells with

non-pregnant and preeclamptic plasma. In case of PDBu stimulation, both inactivated and not-inactivated plasma samples were able to enhance the  $O_2^-$  production by healthy pregnant granulocytes. The FMLP-stimulated  $O_2^-$  production by normal pregnant granulocytes exposed to not-inactivated autologous and heterologous plasma was also improvable by treatment of the cells with not-inactivated non-pregnant and preeclamptic plasma. In response to FMLP,  $O_2^-$  production by normal pregnant granulocytes treated with inactivated autologous and heterologous plasma samples showed a similar  $O_2^-$  release as healthy pregnant neutrophils treated with inactivated non-pregnant and preeclamptic plasma. This finding also confirms that the healthy pregnant plasma factor responsible for the inhibition of FMLP-induced respiratory burst was inactivated during heating, thereby was not able to suppress the  $O_2^-$  generation.

The mechanisms, by which a plasma factor of healthy pregnant women can inhibit the  $O_2^-$  production by non-pregnant and preeclamptic granulocytes have not been known. Our results suggest that the factor of question may affect the signal transduction pathways resulting in the activation and translocation of PKC from the cytosol to the plasma membrane. This have been reported to induce a sequence of events leading to the translocation of the cytosolic components of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to the cell membrane and subsequently the assembly of the active  $O_2^-$  generating enzyme complex. The agonists, used in our experiments, can activate PKC in different pathways. Following binding to formyl peptide receptors (FPRs), FMLP has been shown to initiate the hydrolysis of phosphatidylinositol-4,5-bisphosphate resulting in the production of inositol-1,4,5-trisphosphate and diacylglycerol (DAG) which activates PKC. PDBu mimics the action of DAG, bypasses receptor-mediated signalling events and directly activates PKC. We suppose that the not-inactivated plasma factor may interact with FPRs leading to a decrease in their ligand-binding capacity, thereby the receptors may be less efficient in

transducing signals required for the induction of  $O_2^-$  production. After heat-treatment, the plasma factor of question may undergo a conformational change that may abrogate its ability to influence receptor-mediated signalling

In conclusion,  $O_2^-$  production by PMNLs is decreased in normal pregnancy which may be due to a defence mechanism to protect the maternal and foetal cells against the granulocyte-mediated oxidative damage. The deficient  $O_2^-$  generation in normal pregnancy may be caused by maternal immunosuppressive circulatory factors. The failure of reduction in  $O_2^-$  production in preeclamptic women may be partly responsible for endothelial injury. Therefore, besides the proposed hypothesis of neutrophil-mediated oxidative stress a possible role of inefficient maternal immunosuppression should also be considered in the pathogenesis of preeclampsia.

## SUMMARY

Data on respiratory burst activity of granulocytes from healthy and preeclamptic pregnant women have remained contradictory. To further investigate a possible role of reactive oxygen species in the etiology of preeclampsia we measured the phorbol-12.13-dibutirate- and n-formyl-methionyl-leucyl-phenylalanine induced superoxide-anion generation by granulocytes from non-pregnant, healthy and preeclamptic pregnant women. We also examined the reciprocal effects of heat-inactivated and not-inactivated non-pregnant, normal and preeclamptic pregnant plasma on superoxide production by neutrophils from non-pregnant, healthy and preeclamptic pregnant subjects. Superoxide generation was measured by ferricytochrome c reduction. Both the phorbol-12.13-dibutirate- and N-formyl-methionyl-leucyl-phenylalanine-induced superoxide production was significantly decreased in normal pregnancy compared with non-pregnant and preeclamptic pregnant women. The phorbol-12.13-dibutirate-induced superoxide generation by non-pregnant and preeclamptic neutrophils was significantly inhibited by heat-inactivated and not-inactivated healthy pregnant plasma. The N-formyl-methionyl-leucyl-phenylalanine-stimulated superoxide production by non-pregnant and preeclamptic granulocytes was suppressed only by not-inactivated healthy pregnant plasma. The phorbol-12.13-dibutirate-induced superoxide generation by healthy pregnant neutrophils was significantly increased by inactivated and not-inactivated non-pregnant and preeclamptic plasma. The N-formyl-methionyl-leucyl-phenylalanine-stimulated superoxide production by healthy pregnant granulocytes was significantly enhanced following treatment of the cells with not-inactivated non-pregnant and preeclamptic pregnant plasma. The deficient superoxide generation in normal pregnancy may be caused by maternal immunosuppressive factors. The failure of reduction in superoxide production in preeclampsia

may be partly responsible for endothelial dysfunction. Apart from oxidative stress, a possible role of inefficient maternal immunosuppression should also be considered in the pathogenesis of preeclampsia.

In conclusion, superoxide production by neutrophils is decreased in normal pregnancy. This decrease may be due to a defense mechanism to protect the maternal and fetal cells against the neutrophil-mediated oxidative damage. The deficient superoxide generation in normal pregnancy may be caused by maternal immunosuppressive circulatory factors. The failure of reduction in superoxide production in preeclamptic pregnant women may be partly responsible for endothelial injury. Therefore, besides the proposed hypothesis of neutrophil-mediated oxidative stress a possible role of inefficient maternal immunosuppression should also be considered in the pathogenesis of preeclampsia.

## PUBLICATIONS

### Publications, lectures and posters used in this thesis:

#### PUBLICATIONS:

**Lampé R**, Ormos M, Szűcs S, Ádány R, Póka R (2008) Superoxide-anion production of granulocytes in healthy and preeclamptic pregnancy. *Magy Nőorv L* 71: 209-213.

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**Impact factor of publications used in this thesis: 5.297**

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**List of publications related to the dissertation**

1. **Lampé, R.**, Szűcs, S., Ádány, R., Póka, R.: Granulocyte superoxide anion production and regulation by plasma factors in normal and preeclamptic pregnancy.  
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