

CLINICAL RESEARCH

# NO Metabolism in Red Blood Cells and the Hemoglobin Oxygenation in Pregnant Women with the Cytomegalovirus Infection Exacerbation

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## Abstract

CMV infection during pregnancy induces a number of N-nitrosation reactions in red blood cells, which violate process of normal hemoglobin oxygenation. Abnormalities in the L-arginine-NO system cause a decrease in the antioxidant capacity of red blood cells, which is associated with reducing the amount of glutathione reductase, glutathione peroxidase and superoxide dismutase.

**Keywords:** *cytomegalovirus; pregnancy; red blood cells; hemoglobin oxygenation; L-arginine-NO system.*

## Introduction

Cytomegalovirus (CMV) is a virus found around the world. During pregnancy, CMV DNA is detected in 3% to 6% of women and the virus is present in 3% to 12% [1]. Though cervical viral shedding increases as gestation advances: < 5 % in the first trimester, 6 to 10% in the second trimester and 11 to 28% in the third trimester, the most likely pathway of vertical transmission to the fetus is probably hematogenous [2].

CMV is a member of the herpesviridae family and is an intranuclear virus whose core is assembled within the host's nuclei [2]. It shares with the herpes virus family the traits of persisting in human cells and alternating between latency and reactivation [3]. CMV infection induces a number of N-nitrosation reactions in red blood cells (RBCs), which violate process of normal hemoglobin oxygenation. Abnormalities in the L-arginine-NO system cause a decrease in the antioxidant capacity of RBCs, which is associated with reducing the amount of glutathione reductase (GR), glutathione peroxidase (GP) and superoxide dismutase (SOD).

Oxidative stress reflects an imbalance between the systemic manifestation of the reactive oxygen species (ROS) production and a biological system's ability to adequately remove ROS or to repair the resulting damage [4].

N-nitrosation reactions in the erythrocytes lead to the formation of various NO-containing compounds [5]. The reactions of nitric oxide (NO) with hemoglobin (Hb) have been investigated extensively for many years. The physiological importance of these reactions was established with the identification of NO as the endothelium-derived relaxation factor and product of L-arginin. As is known, oxyhemoglobin formation is associated with activity of L-arginine-NO system, in this regard, it is important to know the factors affecting this system [6].

It is known that viruses and bacteria contain a lipopolysaccharide in their envelopes, which can induce NO production in RBCs and create unfavorable conditions for hemoglobin oxygenation [7,8]. CMV envelopes, covering capsids, contain glycoprotein, which is an inducer of the initial stages of the lipid peroxidation formation and NO synthesis in erythrocytes. The presence of nitrite/nitrate in the erythrocyte and reduction of antioxidant enzymes leads to a decrease in activity of SOD, GP, GR and blocking of the hemoglobin oxygenation background of decreasing Fe<sup>2+</sup> content and oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup>. A threat to forming a hemic hypoxia

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and iron deficiency anemia are created [9,10].

The concentration of nitrite and nitrate increases in RBCs with an increase in levels of ROS and load of infectious factors [6,11]. Under physiological conditions, NO is synthesized from L-arginine by three isoforms of NO-synthase [12]. Two of them are constitutive: endothelial NOS (NOSIII) and neuronal NOS (NOSI). The third is inducible NOS (NOSII). NOSII is induced in various cells by products of Gram-negative and Gram-positive bacteria [13]. NOSII produces large quantities of NO upon stimulation, such as by pro-inflammatory cytokines. Induction of the high-output NOSII usually occurs in an oxidative environment, and thus high levels of NO have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity [14].

During exacerbation of various viral infections, endothelial cells release large amounts of NO, which can readily penetrate the erythrocyte membrane through the inside of the cell [15]. NO penetrating the erythrocytes influences their deformability [16]. NO is quantitatively and functionally different from the active oxygen. Metabolic functions involving oxygen occur in millimolar quantities, and with the participation of NO, at nanomolar quantities. It is believed that the synthesis of NO in erythrocytes, taking into account the accumulation of its end-products of metabolism (NO<sub>2</sub>- and NO<sub>3</sub>-), occurs independently [17].

**The aim** of this work was to study the metabolic processes of NO in the peripheral blood erythrocytes of pregnant women undergoing an exacerbation of CMV infection during gestation.

## Material and Methods

Investigations were conducted in the Clinic Hospital's Maternity Ward of the Far Eastern Scientific Center of Physiology and Pathology of Respiration SB RAMS. In all, 15 pregnant women, during III trimester of gestation with exacerbation of the chronic CMV infection (1:1600) and 15 healthy pregnant women (control group) were examined. CMV infection in pregnant women was manifested by acute respiratory disease accompanied rhinopharyngitis. CMV infection was diagnosed in a comprehensive study of the peripheral blood to check for the presence of IgM or a four-fold or more increase in the IgG antibody titer in paired serum in the dynamics after 10 days, an avidity index reading of more than 65%, and the presence of CMV DNA. The studies were conducted in line with the requirements of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" update 2008 and the Rules of Clinical Practice in the Russian Federation, approved by Order #266 of the Ministry of the Russian Federation of June 19, 2003. Written informed consent was obtained from all participants.

The verification of CMV, the definition of type-specific antibody, and the avidity index were determined by ELISA on the spectrophotometer "Stat Fax 2100" (USA) using the sets of CC "Vector-Best" (Novosibirsk, Russia) and by PCR on the machine DT-96 using sets of "DNA-Technology" (Moscow, Russia).

We proposed a histochemical method for determining the activity of arginine conversion to NO in the peripheral blood erythrocytes. The method is based on the reduction of tetrazolium salt to formazan by electrons accepted from L-arginine by reaction with the coenzyme NADPH. The number and distribution of the granules of the formazan formed in places of the enzyme localization indicate the enzyme activity in erythrocytes. A 0.1M solution of L-arginine sodium (SERVA, Germany) salt served as the substrate. The incubation solution was prepared on the basis of 0.1 M phosphate buffer (pH 7.4-7.6) with the addition of nitroblue tetrazolium (ICN Biomedicals, USA) in a final concentration of 5mM and 1mM NADP<sup>+</sup> (Applichem, Germany). 500 µl of incubation solution and 200µl of the whole blood taken from the cubital vein on an empty stomach in the morning in the standardized vacuum tubes PUTH (K3 EDTA) were added in the test tube. Incubation was for 30 min at 37°C; after that the monolayer smears were prepared by centrifugation («Diffe Spin-2»).

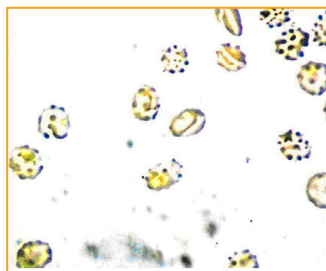
The obtained smears were dried at room temperature and examined using a digital microscope MT (Japan) associated with software-hardware complex «SCION Corporation», which allows the observer to allocate each cell separately on the image of the smear, and to determine its area and the content of the reaction product. For control, we performed the incubation in media containing the adequate amount of buffer instead of the arginine solution.

Oxyhemoglobin was determined by Evelyn/Malloy. The activity of GR and GP was determined using reagent kits «Sentinel Diagnostics». Superoxide dismutase was determined using kits «Randox Laboratories Ltd».

Specialized software was used for the automated processing of medical data (copyright holder - the Far Eastern Scientific Center of Physiology and Pathology of Respiration SB RAMS, Blagoveshchensk, Russia). For data with normal distribution, inter-group comparisons were performed using Student's t-test and F-test. The Mann-Whitney (U-test) was used to compare the differences between the two independent groups (for nonparametric data). P value less than 0.05 was considered significant.

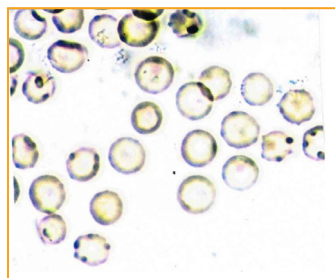
## Results and Discussion

Studies have shown that exacerbation of CMV infection in the third trimester of pregnancy is associated with an increase in the percentage of the erythrocytes with high activity of the L-arginine-NO system up to 45.0±0.95% in the peripheral blood. The reaction reaches 60.5±1.3CU under cytophotometric processing (Fig.1).



**Fig.1.** Peripheral RBCs of the pregnant women underwent an exacerbation of CMV infection during gestation with antibody titer 1:1600. L-arginine-NO activity is increased up to 60.5±1.3 CU. The number of RBCs with active reaction is 45.0±0.95%. (Magnification: 10 x 100).

In pregnant women without a history of CMV infection during gestation, the number of erythrocytes with high activity of the L-arginine-NO system did not exceed  $7.5 \pm 0.15\%$ . Intensity of the reaction was  $12.50 \pm 0.85\text{CU}$  (Fig.2).



**Fig.2.** Peripheral RBCs of the pregnant women without a history of CMV infection during gestation. The number of RBCs with high activity of the L-arginine-NO system does not exceed  $7.5 \pm 0.15\%$ . Intensity of the reaction is  $12.50 \pm 0.85\text{CU}$ . (Magnification:  $10 \times 100$ ).

Consequently, CMV infection, exerting a damaging effect on the erythrocyte membranes, facilitated an entry of NOS into erythrocytes and the activation of the L-arginine-NO system, i.e., promoted the formation of NO and its connection with hemoglobin that dramatically reduced the oxygenation of hemoglobin. In pregnant women undergoing an exacerbation of CMV infection, the amount of oxyhemoglobin was reduced to  $94.0 \pm 1.9\%$ . Enzymatic activity aimed at reducing the peroxidation products was significantly reduced in these conditions. Thus, using the cytochemical method, we found that under these conditions the activity of the reduced glutathione in the peripheral blood erythrocytes was reduced to  $18.90 \pm 0.85\text{CU}$ . This was also spectrophotometrically confirmed. With an increase of L-arginine-NO activity in erythrocytes, the content of GR was sharply reduced to  $4.00 \pm 0.15\text{U/gHb}$  (control:  $8.36 \pm 0.13\text{U/gHb}$ ). This indicates that the increased activity of the L-arginine-NO system in the erythrocytes suppresses the formation of the reduced glutathione due to a decrease of the GR activity. Decreasing the amount of the reduced glutathione in RBCs with increasing of the L-arginine-NO activity certainly affected the glutathione cycle, which performs the antioxidant function in erythrocytes under conditions of increased content of the fatty acid peroxides

GP activity, which contributes to the breakdown of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in RBCs and its transformation into the harmless substance  $\text{H}_2\text{O}$ , is also reduced. Increased activity of the L-arginine-NO system in RBCs reduces the GP activity up to  $5.30 \pm 0.20\text{U/gHb}$  (control:  $14.66 \pm 1.6\text{U/gHb}$ ). A parallel study of the content of  $\text{H}_2\text{O}_2$  in erythrocytes by ELISA showed that it increased up to  $115.0 \pm 3.0\ \mu\text{mol/l}$  (control:  $20.1 \pm 0.98\ \mu\text{mol/l}$ ).

Consequently, due to the suppression of GP activity in RBCs because of the NO accumulation, the antioxidant processes are significantly reduced. High activity of L-arginine-NO system in RBCs decreased the antioxidant potency of SOD up to  $240.0 \pm 5.8\text{U/gHb}$  (control:  $360.9 \pm 10.0\text{U/gHb}$ ).

Thus, the active forms of nitrogen promote the oxidation of amino acids in cells. NO is generated from L-arginine by the action of eNOS in the presence of several cofactors. Derivatives of NO ( $\text{NO}^-$ ,  $\text{NO}_2^-$ ,  $\text{ONOO}^-$ , etc.) mediate the damaging toxic effects in the body and the development of oxidative stress. The biochemistry of NO is two-faced. On the

one hand, NO may limit the oxidative damage (quench the radicals), and on the other hand, it may be a source of reactive nitrogen species [19].

There is a concept whereby the electron-donor systems involved in the formation of the reduced hemoglobin play an important role in NO formation [20]. The existence of erythrocytes' own mechanisms of NO synthesis is assumed, according to the accumulation of the end-products of its metabolism ( $\text{NO}_3^-/\text{NO}_2^-$ ) [21] and citrulline [22]. The globin radical formed during the oxidation of the methemoglobin causes the free-radical processes in the red blood cells and the covalent cross-linking of membrane proteins [23]. NO formed in the endothelium passes freely into erythrocytes and forms S-nitrosohemoglobin, which reduces the oxygenation of hemoglobin and contributes to the formation of methemoglobin.

### Competing interests

The authors declare that they have no competing interests.

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