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# HESTASİON POZUNTULARI VƏ ŞƏKƏRLİ DİABETİ OLAN HAMİLƏ QADINLARDA ERİPTOZ

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***Xülasə.*** *Məqalədə hestasion hipertenziyalı və şəkərli diabeti olan hamilə qadınların qanında dövr edən eritrositlərin hüceyrə membranlarında baş verən dəyişiklikləri öyrənmək məqsədilə aparılmış tədqiqat işi haqqında məlumat verilmişdir.*

*Tədqiqata cəlb edilmiş 57 nəfər hamilə qadın 4 qrupa bölünmüşdür. I qrup – hestasion şəkərli diabeti olan 15 qadın (HŞD), II qrup – prehestasion şəkərli diabeti olan 15 qadın (PŞD), III qrup – hipertenziv hamiləlik patologiyası (HHP) olan 15 qadın (HHD) və IV – kontrol qrupu – 12 qadın.*

*Eriptoz səviyyəsini öyrənmək üçün periferik qanın eritrositləri anneksin V-FİTC və 2,7- dixlordihidro-fluoresseindiasetatla boyadılmışdır. Fluoressensiyanın səviyyəsi BD FACS Canto™ II Cell Analyzer markalı analizatorda tədqiq edilmişdir. Eritrositar membranların vəziyyəti O1O (2-(2c-hidroksifenil)-5-fenil-1,3-oksazol) fluorescent zondunun köməyi ilə qiymətləndirilmişdir. Eritrosit suspenziyasında fluoressensiya "PerkinElmer FL8500" markalı fluoressent spektrometri vasitəsilə qiymətləndirilmişdir.*

*Tədqiqat göstərmişdir ki, HHP və PŞD zamanı eritroptoz prosesi aktivləşir. Bu, fosfatidilserinin eritro-sitar membranlardan xaric olmasının sürətlənməsi və oksigenin aktiv formalarının daha artıq əmələ gəlməsi ilə təzahür edir. Təsvir edilən dəyişikliklər hüceyrə membranlarında baş verən dəyişikliklərlə (məhz lipid spektrinin dəyişiklikləri ilə) təzahür edir. Hestasion şəkərli diabet isə nə membran dəyişikliklərilə, nə də eritroptozun aktivləşməsi ilə təzahür edir.*

***Açar sözlər:*** *eriptoz, hestasion şəkərli diabet, hipertenziv hamiləlik patologiyası, anneksin-V*

***Ключевые слова****: эриптоз, гестационный сахарный диабет, гипертензивные нарушения беременности, аннексин-V*

***Key words:*** *eryptosis, gestational diabetes mellitus, pregestation diabet mellitus, annexin-V, reactive oxygen species*

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# ERYPTOSIS IN PATIENTS WITH GESTATIONAL DIABETES MELLITUS AND HYPERTENSIVE DISORDERS OF PREGNANCY

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***Summary.*** *The study was conducted to analyze eryptosis indices and the presence of cell membrane alteration of circulating erythrocytes in patients with gestational hypertension and diabetes mellitus.*

*This study included 57 pregnant patients, which were divided into four distinct groups: 15 women with gestational diabetes mellitus (GDM), 15 women with pregestation diabetes mellitus (DM), 15 women with hypertensive disorders of pregnancy (HDP) and 12 pregnant women without extragenital and obstetric pathology.*

*Staining of erythrocytes with annexin V-FITC and 2',7'-dichlorodihydrofluorescein diacetate was used to assess the degree of eryptosis collected from the patients. Fluorescence was detected on a BD FACSCanto™*

*II Cell Analyzer. Cell membranes of erythrocytes were assessed using a fluorescent probe O1O (2-(2¢-hydroxy-phenyl)-5-phenyl-1,3-oxazole). Fluorescence in red blood cell suspensions was acquired on a "PerkinElmer FL8500" fluorescence spectrometer.*

*GDH and DM were associated with eryptosis activation, evidenced by an increased phosphatidylserine externalization and excessive reactive oxygen species (ROS) generation, against the background of cell membrane alterations, namely a decrease in the lipid order. GDM was accompanied by neither membrane changes, no eryptosis activation.*

*Thus, the results of the study show that ROS-dependent eryptosis and red blood cell membrane alterations are observed in gestational hypertension.*

## INTRODUCTION

Gestational diabetes mellitus (GDM) and hypertensive disorders of pregnancy (HDP) remain the most frequently observed complications in pregnancy [1]. The former is defined as hyperglycemia firstly registered during pregnancy [2], while the latter is associated with an increase in arterial blood pressure during pregnancy and can be classified into chronic hypertension, gestational hypertension, preeclampsia-eclampsia, and chronic hypertension with superimposed preeclampsia [3]. The prevalence is extremely variable worldwide and may reach up to 25 % in some regions [4]. Both GDM and HDP are associated with maternal and neonatal complications, such as pre-term birth, jaundice, respiratory failure, congenital defects, etc. [5].

There is compelling evidence that oxidative stress, which is defined as the imbalance between reactive oxygen species /reactive nitrogen species generation and antioxidant system capacities, plays an important role in the pathophysiology of GDM and HDP [6]. Oxidatively stressed circulating erythrocytes have been reported to undergo eryptosis, a type of programmed cell death typical for red blood cells only [7]. Accelerated eryptosis and damage to erythrocyte membranes has been reported in multiple diseases, including diabetes mellitus and hypertension [8].

Thus, the aim of this research was to study eryptosis indices and features of the physico-chemical state of phospholipid bilayer in cell membranes of circulating erythrocytes in patients with gestational hypertension and diabetes mellitus.

## MATERIALS AND METHODS

*Patients and study design*

This study included 57 pregnant women. According to the extragenital and obstetric pathology, patients were divided into three groups: 15 women (26,3%) with gestational diabetes mellitus (GDM), 15 women (26,3%) with pregestation diabet mellitus (DM), 15 women (26,3%) with hypertensive disorders of pregnancy (HDP) and 12 pregnant women (21,1%) without extragenital and obstetric pathology (control subjects). There were no differences between the groups regarding pregestational body mass index, age, parity, neonatal results and gestational weight gain.

The age of the examined pregnant women ranged from 18 to 47 years, the average age of pregnant women with gestational diabetes was 32.8±4.5 years, pregnant women with type 1 diabetes mellitus - 27.4±4.8 years, women with arterial hypertension - 30.0 ±3.6 years, pregnant women of the control group - 25.2±4.9 years.

*Erythrocyte suspension*

Erythrocyte suspensions were prepared from fresh whole blood, afterward, 50 µl of blood was diluted in 1950 µl phosphate-buffered saline. The diluted blood was centrifuged for 5 min at 500g. Thereafter, the supernatant was decanted and the procedure was repeated. Then 1 µl of erythrocyte mass from each sample was dissolved in phosphate-buffered saline to perform flow cytometric and spectrofluo-rimetric measurements.

*Annexin V staining*

The phosphatidylserine exposure, which is a major sign of both early and late eryptosis, was assessed by annexin V-FITC [9]. Initially, 1 µl of erythrocyte mass was diluted in 99 µl 1x annexin-binding buffer. Then 10 µl of this primary suspension was transferred to a new tube with 85 µl 1x annexin-binding buffer. To stain erythrocytes, 5 µl annexin V-FITC was added and the mixture was incubated for 15 minutes in the dark. To provide a volume required for the flow cytometric measurements, 400 µl 1x annexin-binding buffer was added to each sample. The excitation laser line was 488 nm, while the emitted light was detected at 525 nm.

*2′,7′-dichlorodihydrofluorescein diacetate staining*

A cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate dye was used to assess the redox status of circulating erythrocytes of control subjects and patients with DM, GDM, or HDP [10]. Erythrocyte masses were loaded with 2′,7′-dichlorodihydrofluorescein diacetate using a 10 mM stock solution in dimethyl sulfoxide stored at -20 °C. Briefly, 1 µl of erythrocyte mass was diluted in phosphate-buffered saline and the 2′,7′-dichlorodihydrofluorescein diacetate stock solution was used to stain cells in order to prepare 5 µM working solutions. The dye-loaded erythrocytes were stored for 30 minutes in the dark, washed with phosphate-buffered saline to remove excess dye and resuspended in phosphate-buffered saline to reach the volume of 500 µl. The fluorescence of dichlorofluorescein produced intracellularly from 2′,7′-dichlorodihydrofluorescein diacetate, which depends on ROS levels inside the cells, was detected using a BD FACSCanto™ II cytometer with the excitation performed by a 488 nm laser and the detection of emitted light at 525 nm.

*Fluorescent probe O1O*

The cells were stained with the fluorescent probe via addition of an aliquot of the probe stock solution in acetonitrile to the cell suspensions: the final probe concentration was $5x10^{-6}$ mol/L and lipid-to-probe molar ratio was 200:1. The cell suspensions were incubated with the probe at room temperature for 1 hour before fluorescence measurements. Fluorescence spectrometer "PerkinElmer FL8500" was used for the measurements of the probe emission in the range of 340-550 nm, with an increment of 0.1 nm.

Fluorescent probe O1O **(**2-(2¢-hydroxyphenyl)-5-phenyl-1,3-oxazole) was used in our research, because its fluorescence characteristics depend on the proton-donor ability and polarity of the probe environment [10].

The area of glycerol backbones of phospholipids closer to the center of the lipid bilayer, the area of carbonyl groups of phospholipids and the area of hydrocarbon chains of phospholipids near the area of the carbonyl groups of phospholipids are the regions that the probe O1O locates in lipid membranes (Figure) [10].

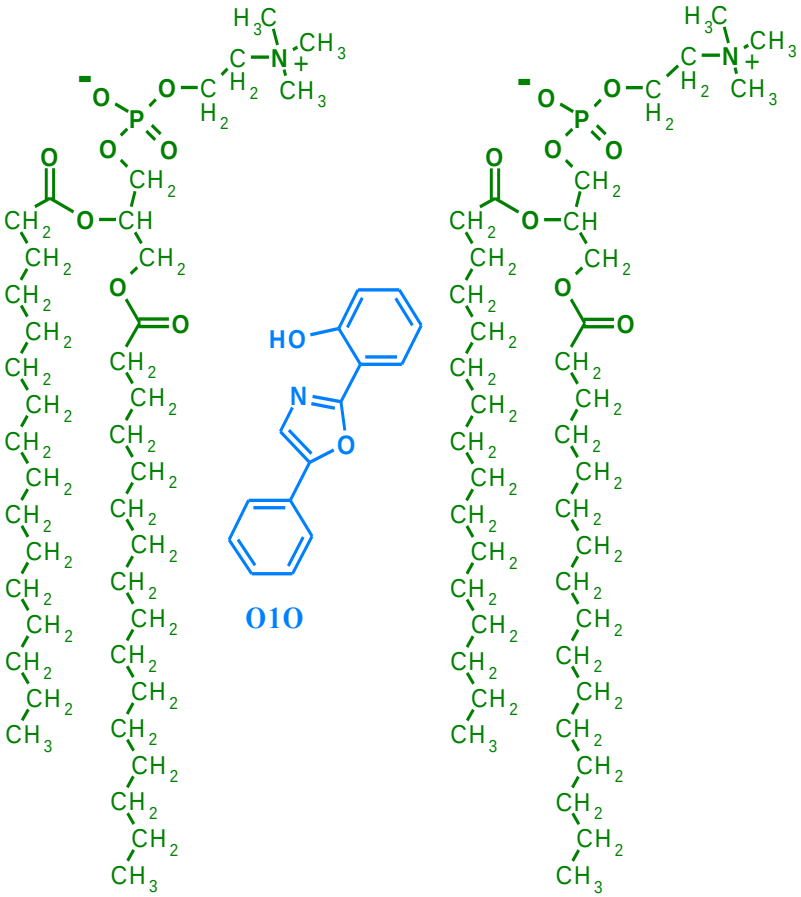

**Figure.** Localization and orientation of fluorescent probe O1O in the outer leaflet of the phospholipid membranes. Two molecules of 1,2-dipalmitoyl-phosphatidylcholine are displayed to show the location of the probe. (Adapted from [Posokhov Ye.O. 2018]).

In the excited electronic state, the initial (or "normal") form (N*) of probe O1O turns into the phototautomer form (T*). The latter emits in significantly longer wavelengths than the initial form of the probe. The amount of the photoproduct (T*) depends on the probe microenvironment [10].

Because the probe has two-band fluorescence, ratiometric measurements are possible: the phototautomer fluorescence intensity-to-the initial form fluorescence intensity ratio ($I_{T*}/I_{N*}$) can be used as a parameter to estimate the changes in chemical and physical properties of the microenvironment (e.g., with the increase in hydration of the media, the ratio $I_{T*}/I_{N*}$ decreases [10]).

*Statistical analysis*

Analysis of variance (ANOVA) with a Bonferroni post-hoc test was used to statistically process the data using *GraphPad Prism* 5.0 software. The data are represented as the mean and standard deviation. P values lower than 0.05 were regarded as statistically significant.

**RESULTS**

The results of flow cytometric measurements of eryptosis parameters are summarized in the Table below.

Two parameters were used to characterize phosphatidylserine externalization. HDP was associated with a statistically significant increase in the amount of annexin V-positive, i.e. phosphatidylserine -displaying eryptotic circulating erythrocytes ($p < 0.0001$), and the median fluorescence intensity of annexin V-FITC in erythrocytes ($p < 0.0001$). These findings indicate activation of eryptosis in pregnant women with HDP (Table). Moreover, these patients had higher median fluorescence intensity of dichlorofluorescein fluorescence ($p<0.001$) compared with the control subjects, indicating ROS overproduction in erythrocytes (Table).

Unexpectedly, all the parameters outlined above were unaffected in pregnant women with GDM ($p>0.05$). Thus, it can be assumed that GDM is not accompanied by the activation of eryptosis. In contrast to GDM, DM was accompanied by a statistically significant elevation of both the percentage of annexin V-positive cells and median fluorescence intensity of annexin V-FITC ($p<0.001$), suggesting a higher degree of eryptosis compared to healthy individuals. However, this increase in eryptosis parameters was less pronounced compared with the patients with HDP (see Table). It is important to note that median fluorescence intensity values of dichlorofluorescein in this group were the highest among all the groups involved in this study (see Table). The difference with the control group was statistically significant ($p < 0.0001$). This indicates that DM is associated with the highest degree of ROS generation.

**Table.** Eryptosis indices in control subjects and patients with diabetes mellitus (DM), gestational diabetes mellitus (GDM) or hypertensive disorders of pregnancy (HDP)

| Group of patients / Parameter | Control subjects (n = 12) | HDP (n = 15) | GDM (n = 15) | DM (n = 15) |
|---|---|---|---|---|
| Percentage of annexin V-positive eryptotic cells, % | 0.88 ± 0.29 | 2.10 ± 0.91, $p < 0.0001$ | 1.21 ± 0.45, $p > 0.05$ | 1.84 ± 0.91, $p < 0.001$ |
| The mean fluorescence intensity of annexin V-FITC, a.u. | 268 ± 29 | 352 ± 36, $p < 0.0001$ | 248 ± 36, $p > 0.05$ | 321 ± 30, $p < 0.001$ |
| The mean fluorescence intensity of dichlorofluorescein, a.u. | 266 ± 33 | 315 ± 24, $p < 0.001$ | 255 ± 31, $p > 0.05$ | 351 ± 37, $p < 0.0001$ |

**DISCUSSION**

Our findings suggest that the development of GDM is not associated with excessive phosphatidylserine exposure and ROS overproduction, suggesting that eryptosis is not activated in patients with GDM. However, DM was found to be associated with activetion of eryptosis. These data corroborate other reports on the impact of DM on eryptosis [8]. Surprisingly, HDP is accompanied by even more pronounced eryptosis intensification compared with DM. No data on eryptosis in patients with HDP are available, but this cell death mode is induced by hypertension [8].

The data indicate that ROS-dependent mechanisms are involved in HDP- and DM-induced eryptosis.

**CONCLUSIONS**

Thus, the data obtained indicate that erythrocytes in gestational hypertension are more prone to eryptosis and are characterized by cell membrane alterations, which is not observed in gestational diabetes mellitus. Gestational hypertension-induced eryptosis is ROS-dependent. Eryptosis indices are promising biomarkers in gestational hypertension.

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# ЭРИПТОЗ У БЕРЕМЕННЫХ С ГЕСТАЦИОННЫМИ ГИПЕРТЕНЗИВНЫМИ РАССТРОЙСТВАМИ И САХАРНЫМ ДИАБЕТОМ

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**Резюме.** Представлены результаты исследования, проведенного с целью изучения изменений клеточных мембран циркулирующих эритроцитов у пациенток с гестационной гипертензией и сахарным диабетом.

Исследованию были привлечены 57 беременных пациенток, которые были разделены на четыре группы: 15 женщин с гестационным сахарным диабетом (ГСД), 15 женщин с прегестационным сахарным диабетом (ПСД), 15 женщин с гипертензивными расстройствами беременности (ГРБ) и 12 беременных женщин без экстрагенитальной и акушерской патологии.

Для оценки степени эриптоза взятые у пациенток эритроциты окрашивали аннексином V-FITC и 2′, 7′-дихлордигирофлуоресцеиндиацетатом. Флуоресценцию определяли на анализаторе клеток BD FACSCanto™ II Ce ll Analyzer. Состояние клеточных мембран эритроцитов оценивали с помощью флуоресцентного зонда O1O (2-(2¢-гидроксифенил)-5-фенил-1,3-оксазол). Флуоресценцию в суспензиях эритроцитов изучали на флуоресцентном спектрометре «PerkinElmer FL8500».

Исследование показало, что ГРБ и ПСД были связаны с активацией эриптоза, о чем свидетельствует повышенная экстернализация фосфатидилсерина и избыточное образование активных форм кислорода (АФК) на фоне изменений клеточных мембран, а именно снижения липидного порядка. ГСД не сопровождался ни мембранными изменениями, ни активацией эриптоза.

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