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ERYPTOSIS AS A LINK BETWEEN BACTERIAL REPRODUCTIVE TRACT INFECTION AND ANEMIA IN PREGNANT WOMEN

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Summary

Genital tract infections of various etiologies and anemia remain common in pregnant women. At present, there is a paucity of data on the impact of reproductive tract infections on the development of anemia in pregnant women, with particular emphasis on the contribution of eryptosis.

The aim of the current study was to assess the ability of bacterial, viral and fungal reproductive tract infections to trigger eryptosis of circulating erythrocytes in pregnant women.

Methods. The study included 47 pregnant women. According to the pathology of the infection, the patients were divided into three groups: 14 women with bacterial, 12 women with viral, 11 women with fungal infections of the reproductive tract and 10 pregnant women without extragenital and obstetric pathology (control subjects). The following methods were used to determine infection: bacteriologic, microscopic, polymerase chain reaction, immunoenzymatic, etc. Flow cytometry-based detection of eryptosis parameters (cell morphology, membrane phospholipid scrambling, and oxidative stress) was performed in pregnant women with reproductive tract infections of bacterial, viral, or fungal origin and in healthy individuals. Annexin V-FITC staining and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) staining were used to quantify phosphatidylserine exposure and reactive oxygen species (ROS) generation, respectively. Fluorescence was acquired using a BD FACSCanto™ II flow cytometer.

ANOVA test was used to compare numerical data of eryptosis assays. Post-hoc Bonferroni test was applied afterwards. Data were processed by Graph Pad Prism 5.0 software (USA). The difference was considered statistically significant at $p < 0.05$.

The research was conducted in accordance with the fundamental principles of Good Clinical Practice (GCP, 1996), the Council of Europe Convention on Human Rights and Biomedicine (April 4, 1997), the Helsinki Declaration of the World Medical Association on Ethical Principles for Medical Research Involving Human Subjects (1964-2008), as well as the Ministry of Health of Ukraine Order No. 690 dated September 23, 2009 (amended by the Ministry of Health of Ukraine Order No. 523 dated July 12, 2012).

The research design was discussed and approved at the meeting of the Medical and Ethical Committee of the Kharkiv National Medical University (Protocol No. 19 dated May 19, 2024).

The paper is an excerpt from the scientific research project of the Department of Obstetrics and Gynecology No. 2 at Kharkiv National Medical University, entitled «Optimisation of diagnostics, treatment of diseases of the reproductive system and pregnancy complications in women with extragenital pathology» (state registration number 0121U110923).

Results. Erythropoiesis parameters were evaluated in pregnant women with bacterial, viral and fungal reproductive tract infections. Pregnancy-associated bacterial reproductive tract infections were associated with increased phosphatidylserine externalization in circulating erythrocytes, without ROS accumulation or cell shrinkage. Erythrocytes from patients with genital tract infections of viral or fungal origin show no signs of eryptosis, such as cell membrane scrambling, oxidative stress, or changes in cell morphology.

Conclusions. Reproductive tract infections of bacterial origin in pregnant women are associated with accelerated eryptosis, which may contribute to anemia in these women. Exclusively bacterial genital tract infection was shown to induce lipid membrane scrambling and hence eryptosis in erythrocytes from pregnant women. Phosphatidylserine externalization did not differ significantly between women with viral or fungal infections and healthy controls. Eryptosis induced by bacterial genital tract infection was not associated with cell shrinkage or ROS overproduction.

Key words: Reproductive Tract Infections; Erythrocyte; Anemia; Pregnancy; Eryptosis.

Introduction

Genital tract infections remain common in pregnant women, affecting up to 70 % of pregnancies, with widely varying prevalence rates reported in different studies [1-3]. Etiologically, lower tract infections can be caused by a variety of microorganisms [4]. Bacterial, viral, and fungal lower tract infections have been widely reported [5]. There is increasing evidence that candidiasis remains the most common genital tract infection in pregnant women [2, 6]. Lower tract infections have been associated with fetal infections and adverse pregnancy outcomes, including spontaneous abortion, preterm delivery, and perinatal complications [7, 8]. Thus, infections at the maternal-fetal interface remain of great clinical importance, and further

investigation of the molecular mechanisms and interplay governing host-pathogen interactions in genital tract infections in pregnant women should be encouraged [9]. In particular, anemia is considered to be common in pregnancy, being observed in up to 25 % of pregnancies [10, 11]. The relationship between anemia and UTIs in pregnancy has not been well studied. However, it has been reported that iron deficiency anemia is associated with susceptibility to UTI [12]. In this study, we hypothesized that eryptosis, a suicidal cell death mode of erythrocytes [13-15], may serve as a link between reproductive tract infections and anemia in pregnant women. Eryptosis has been shown to be a novel factor in the development of anemia in a variety of pathologies. In addition, it has recently been reported

to be a driver of anemia in chronic kidney disease [16]. In general, eryptosis is a cell death of mature erythrocytes that is morphologically similar to apoptosis but different from a signaling point of view [14, 17]. Eryptosis signaling is governed by intracellular Ca^{2+} elevation resulting in dysregulation of flippases and scramblases with subsequent phosphatidylserine externalization, activation of calpain resulting in cytoskeleton degradation and morphological changes in erythrocytes like cell shrinkage [13, 18]. Of note, ceramide and reactive oxygen stress (ROS) are considered important second messengers in eryptosis, which also contribute to the phospholipid membrane scrambling that is a hallmark of eryptosis [19-21]. It should be noted that phosphatidylserine externalization, an event that culminates eryptosis signaling, mediates the clearance of erythrocytic red blood cells from the circulation. This process, termed efferocytosis, which implies engulfment of erythrocytes by phosphatidylserine-associated mechanisms, underlies the pathogenesis of anemia in diseases associated with accelerated eryptosis.

The aim of the current study was to detect the impact of pregnancy-related genital tract infections in a causative agent-dependent manner on eryptosis of circulating mature erythrocytes, which has been hypothesized to contribute to anemia in pregnancy.

Materials and methods

Characteristics of patients.

The study included 47 pregnant women. According to the pathology of the infection, the patients were divided into three groups: 14 women (29.8 %) with bacterial, 12 women (25.5 %) with viral, 11 women (23.4 %) with fungal infections of the reproductive tract and 10 pregnant women (21.3 %) without extragenital and obstetric pathology (controls). There were no differences between the groups with regard to pregestational body mass index, age, parity, gestational weight gain and neonatal outcomes. The age of the studied pregnant women ranged from 20 to 37 years, the mean age of pregnant women with bacterial infection was 33.5 ± 4.1 years, pregnant women with viral infection – 28.2 ± 3.1 years, pregnant women with fungal infection – 24.7 ± 2.5 years, pregnant women in the control group – 25.1 ± 3.7 years. The following methods were used to determine infection: bacteriologic, microscopic, polymerase chain reaction, immunoenzymatic, etc. The diagnostics of infection agents was based on the result of the bacterioscopic research of the contents of the vagina, canal of the cervix, the urethra, bacteriological studies of vaginal secretion and identification of pathogens in the smears from the mucosa of the cervical canal by the method of polymerase chain reaction with the determination of the quantity infections agents.

Sample preparation.

Blood samples were collected in EDTA Vacutainers from pregnant women with genital tract infections and healthy volunteers. The samples were immediately used for flow cytometric detection of eryptosis markers. Briefly, samples were centrifuged and washed twice with phosphate-buffered saline (PBS; pH 7.4; BD, USA). Once red blood cell (RBC) suspensions were obtained,

a total of 2 μL of erythrocyte mass from each sample was resuspended in either 100 μL of PBS or annexin-binding buffer (Becton Dickinson) or PBS, depending on the staining procedure.

Membrane phospholipid scrambling detection.

RBCs resuspended in Annexin Binding Buffer as described above were stained with Annexin V-FITC (BD Pharmingen™ FITC Annexin V) for 15 minutes. After incubation, cells were resuspended in Annexin Binding Buffer. Fluorescence was acquired using a BD FACS Canto™ II flow cytometer (Becton Dickinson, USA): excitation wavelength was 488 nm; emission wavelength was 525 nm. To compare phospholipid scrambling, the percentage of cells displaying phosphatidylserine was analyzed [22-25].

ROS detection in erythrocytes.

Cells resuspended in PBS were stained with 5 μM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) provided by Invitrogen™ (USA). After incubation for 30 minutes, fluorescence was detected. The excitation and emission wavelengths were 488 nm and 525 nm, respectively. The mean fluorescence intensity of the ROS level-reflecting probe was analyzed to quantify ROS signaling [23-25].

Cell shrinkage analysis.

Forward scatter analysis was used to analyze cell morphology of erythrocytes from pregnant women with reproductive tract infections of bacterial, viral, or fungal origin. Since cell shrinkage is generally considered a hallmark of eryptosis, the degree of eryptosis was estimated based on the number of cells with low forward scatter (FSC) signal values. A comparison was made between the percentages of FSC-low erythrocytes [24].

Data analysis.

FlowJo™ (v10, BD Biosciences, USA) software was used to analyze data of eryptosis assays.

Statistical analysis.

ANOVA test was used to compare numerical data of eryptosis assays. Post-hoc Bonferroni test was applied afterwards. Data were processed by Graph Pad Prism 5.0 software (USA). The difference was considered statistically significant at $p < 0.05$.

The research was conducted in accordance with the fundamental principles of Good Clinical Practice (GCP, 1996), the Council of Europe Convention on Human Rights and Biomedicine (April 4, 1997), the Helsinki Declaration of the World Medical Association on Ethical Principles for Medical Research Involving Human Subjects (1964-2008), as well as the Ministry of Health of Ukraine Order No. 690 dated September 23, 2009 (amended by the Ministry of Health of Ukraine Order No. 523 dated July 12, 2012).

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women with extragenital pathology» (state registration number 0121U110923).

Results

The results of the study showed that during the examination of pregnant women met most common bacterial infections as *Escherichia coli* (45.5 %), *Staphylococcus epidermidis* (40.9 %), *Enterobacter aerogenes* (31.8 %), *Streptococcus agalactiae* (22.7 %), *Klebsiella pneumoniae* (13.6 %), *Ureaplasma urealiticum* (27.3 %), *Mycoplasma genitalium* (18.2 %); viral infections such as Cytomegalovirus (9.1 %), Herpes simplex virus (9.1 %), fungal infections *Candida albicans* – 59.1 %).

To determine whether genital tract infections affect the suicidal cell death modality of erythrocytes, key eryptotic parameters such as cellular morphology, asymmetry of phospholipid content, namely distribution of phosphatidylserine, in the plasma membrane of erythrocytes, and features of redox homeostasis were

evaluated. Detection was based on flow cytometry using commonly accepted biomarkers.

Importantly, phosphatidylserine externalization is considered a major biochemical sign of eryptosis that determines the clearance of circulating erythrocytes by phagocytic cells. In the current study, high Annexin V-FITC signaling indicated phosphatidylserine exposure and thus its location in the outer leaflet of the phospholipid bilayer. To quantify this process, we compared the percentage of cells displaying phosphatidylserine. The gating strategy is shown in Figure 1. Notably, fungal and viral reproductive tract infections during pregnancy are not associated with abnormal membrane phospholipid scrambling, as evidenced by the absence of statistically significant changes in the percentage of annexin V-positive cells (Figures 1 and 2a). Conversely, bacterial genital tract infections in pregnant women increased the percentage of circulating phosphatidylserine exposed cells, indicating activation of eryptosis (Figures 1 and 2a).

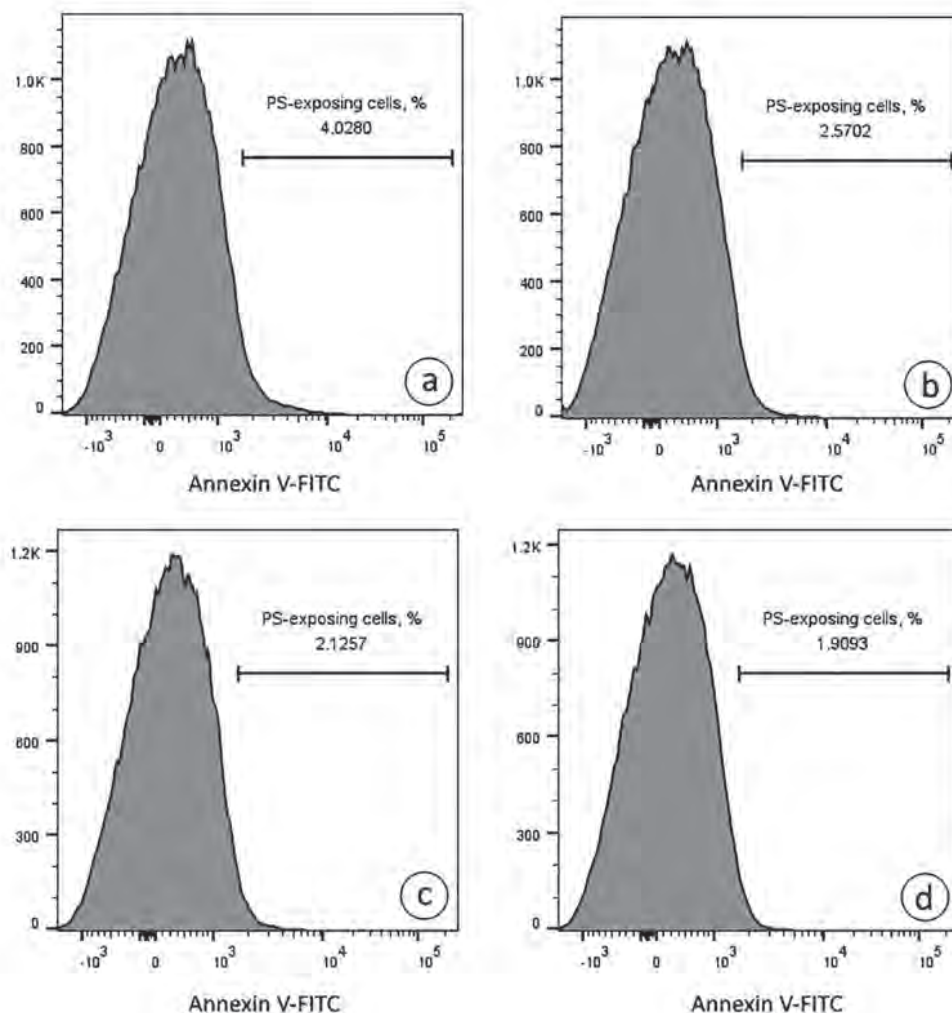


Fig. 1. Cell membrane phospholipid scrambling in circulating erythrocytes of pregnant women with genital tract infections. Representative histograms of pregnant women with bacterial (panel a), viral (panel b), fungal (panel c) reproductive tract infections and controls (panel d). The percentage of phosphatidylserine (PS)-exposing erythrocytes is statistically significantly higher in bacterial genital tract infections only. ANOVA and Bonferroni tests, Me and IQR (n = 10).

Note: * ($p < 0.05$) compared with the control group.

Oxidative stress is a common trigger for eryptosis. RBCs are known to be sensitive to ROS-mediated damage due to their inability to upregulate antioxidant enzymes. In this study, a ROS-sensitive H2DCDA probe was used to characterize the ROS-generating activity of red blood cells. Erythrocytes from pregnant patients with genital tract infections of bacterial, viral or fungal origin were found to

contain undetectable levels of intracellular ROS compared to control samples from healthy volunteers. As shown in Figures 2b and 3, the difference between the numerical values of the probe fluorescence, which characterizes the ROS content in erythrocytes, is negligible. Thus, oxidative stress is not observed in erythrocytes from patients with pregnancy-associated genital tract infections.

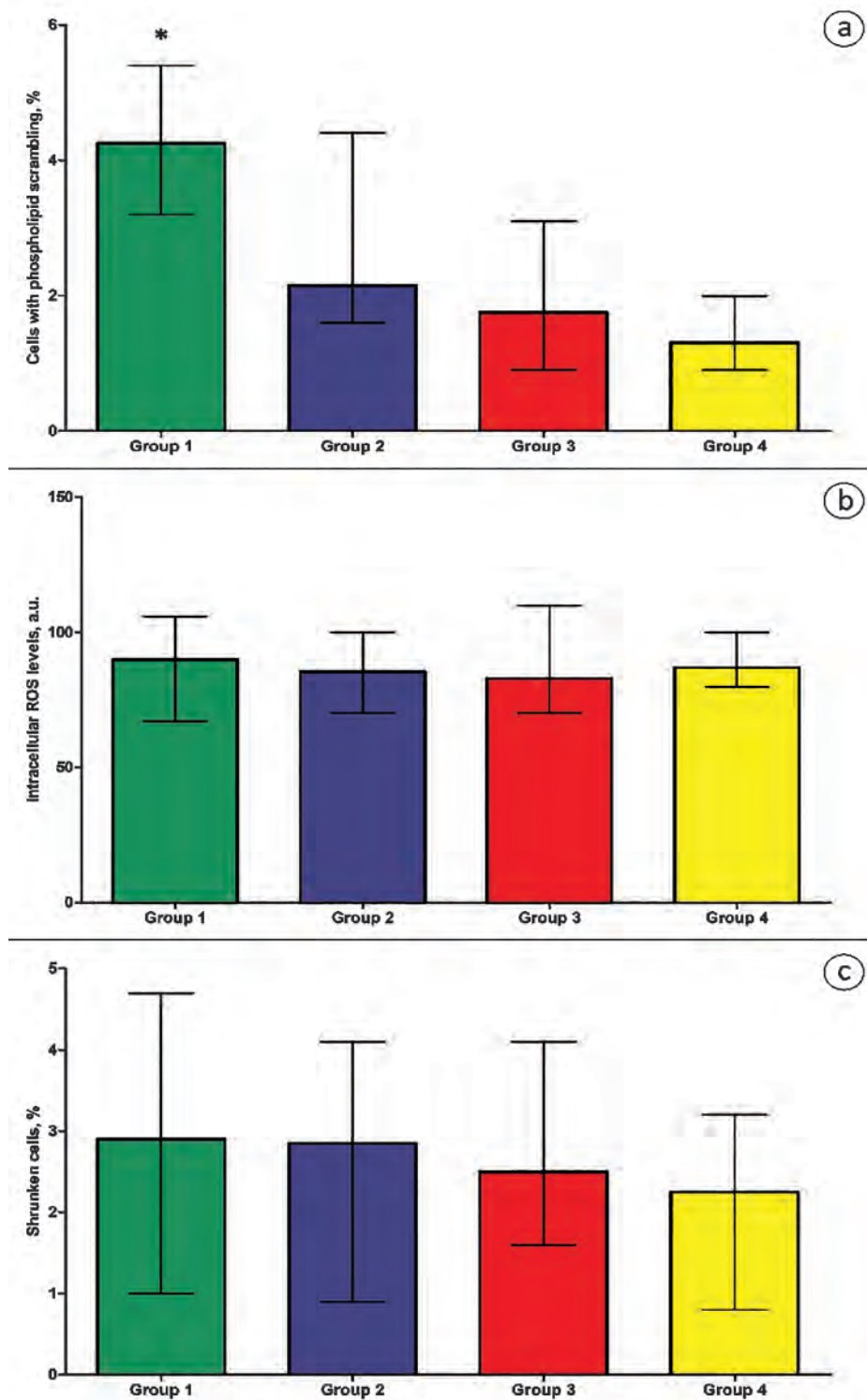


Fig. 2. Eryptosis-related parameters in pregnant women with bacterial (group 1), viral (group 2), fungal (group 3) reproductive tract infections and no pathology (group 4). Phosphatidylserine externalization (panel a), reactive oxygen species (ROS) production (panel b) and forward scatter (FSC) signaling (panel c) were quantified. ANOVA and Bonferroni tests, Me and IQR (n = 10). Note: * (p < 0.05) compared with the control group.

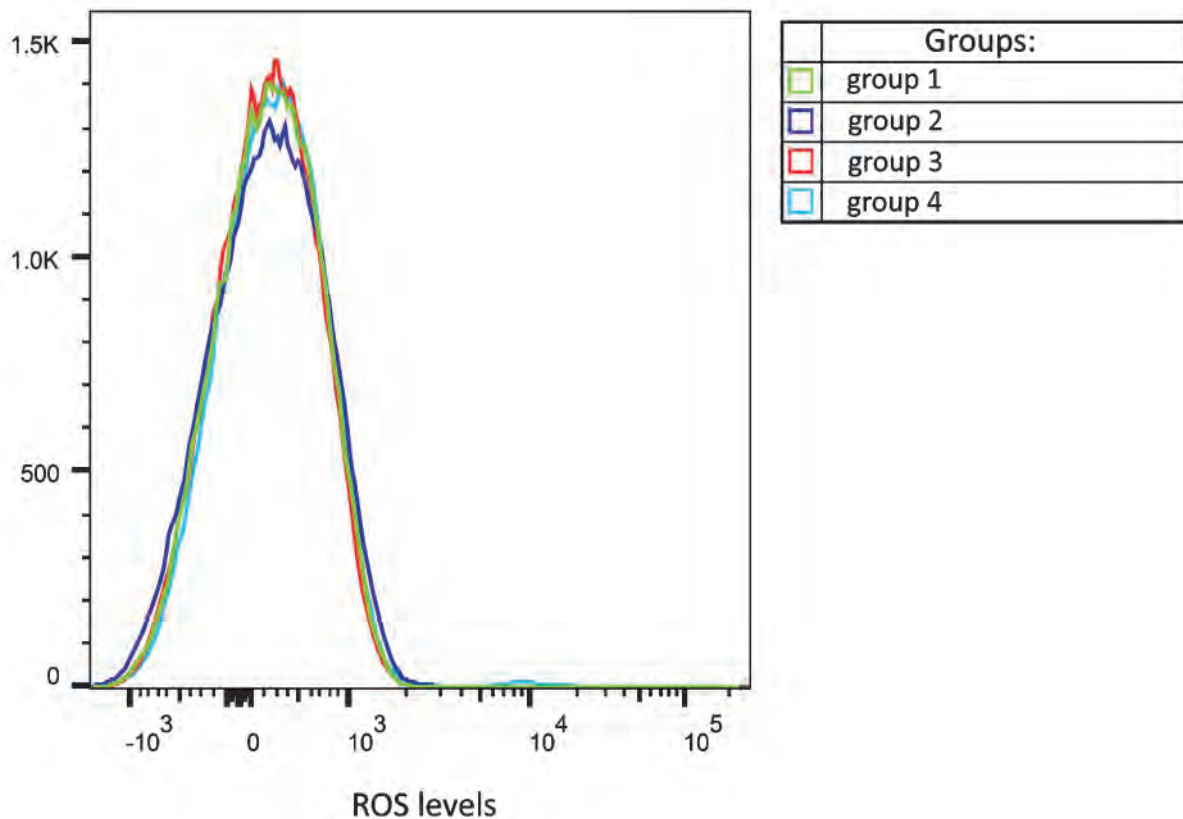


Fig. 3. Reactive oxygen species (ROS) production in red blood cells among pregnant females with bacterial (group 1), viral (group 2), fungal (group 3) reproductive tract infections and no genital tract infection (group 4). Representative histograms of ROS-sensitive probe's fluorescence demonstrate no changes in the redox homeostasis of circulating erythrocytes.

In addition to phospholipid bilayer asymmetry in cell membranes and oxidative stress, eryptosis is often associated with morphologic changes. Cell shrinkage is considered to be the most common abnormality in cell morphology observed in eryptosis. Flow cytometry-based detection of cell morphology focuses on the analysis of FSC signaling, which reflects cell shape. Our data show that reproductive tract infection in pregnant women is not associated with an increase in shrunken cells characterized by reduced FSC signaling (Figure 2c).

Discussion

In the current study, blood samples were collected from pregnant women with genital tract infections of bacterial, viral and fungal etiology to analyze key parameters of eryptosis. Adverse factors occurring in inflammatory conditions (circulating pro-inflammatory molecules or redox imbalance) are known to affect erythrocytes to induce their eryptosis, which may contribute to excessive erythrocyte loss, blood clotting, or abnormal rheological properties of blood [26]. However, eryptosis is generally considered to be protective because it occurs prior to hemolysis, preventing this pro-inflammatory accidental cell death mode of erythrocytes [23]. As has been widely reported, accelerated eryptosis may contribute to anemia in a variety of eryptosis-related diseases due to excessive erythrocyte clearance via externalized phosphatidylserine-

mediated efferocytosis [20, 27]. Indeed, our results clearly indicate that eryptosis may contribute to anemia in bacterial genital tract infections in pregnant women. Surprisingly, ROS-mediated pathways aren't involved in eryptosis associated with bacterial genital tract infections during pregnancy. However, bacterial urinary tract infections have been reported to be associated with impaired redox status [28]. Notably, oxidative stress is also observed at the systemic level in bacterial urinary tract infections in pregnant women and is manifested by elevation of circulating lipid peroxidation markers and depletion of the enzymatic linkage of the antioxidant system [29]. ROS overproduction has been suggested to be an important factor in the pathogenesis of pregnancy-associated urinary tract infections [30]. In eryptosis, ROS are known to mediate phosphatidylserine depletion by increasing scramblase activity and decreasing flippase activity, respectively [31]. However, this pathway was not activated in the present study. Thus, it is likely that other molecular mechanisms besides ROS are responsible for accelerated eryptosis in genital tract infections in pregnant women. In general, it is known that erythrocytes have a shorter lifespan and are more susceptible to destruction during pregnancy [32]. We believe that the pro-inflammatory environment associated with infections may facilitate erythrocyte elimination via eryptosis. Of note, viral and fungal infections have no effect on erythropoiesis

parameters in pregnant women, suggesting the pathogen-dependent nature of erythropoiesis stimulation.

Further exploration of the relationship between pregnancy-associated genital tract infections and eryptosis may provide a theoretical basis for targeting this cell death as a novel therapeutic approach to prevent anemia in pregnant women with this pathology.

Conclusions

This study demonstrates accelerated eryptosis in pregnancy-associated bacterial genital tract infections, which may provide a link between anemia and urinary tract infections. Eryptosis-

associated membrane phospholipid scrambling is observed exclusively in bacterial genital tract infections, whereas this hallmark of eryptosis is not seen in pregnant women with viral or fungal infections. Bacterial genital tract infection-induced eryptosis is ROS-independent and lacks cell shrinkage.

Conflict of Interests. The authors have no conflicts of interest to declare.

Founding source. This research received no specific grant from any funding agency in the public, commercial, or non-profit sectors.

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ЕРИПТОЗ ЯК ЗВ'ЯЗОК МІЖ БАКТЕРІАЛЬНОЮ ІНФЕКЦІЄЮ РЕПРОДУКТИВНОГО ТРАКТА ТА АНЕМІЄЮ ВАГІТНИХ

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Резюме.

Інфекції генітального тракту різної етіології і анемія залишаються поширеними серед вагітних жінок. На даний момент існують мізерні дані про вплив інфекцій репродуктивного тракту на анемію, яка розвивається у жінок під час вагітності, особливо фокусуючись на процесах ериптозу.

Метою даного дослідження було оцінити здатність бактеріальної, вірусної та грибкової інфекції репродуктивного тракту стати тригером процесів ериптозу циркулюючих еритроцитів у вагітних жінок.

Матеріали і методи. Було обстежено 47 вагітних, яких в залежності від наявності інфекційної патології було поділено на три групи: 14 вагітних з бактеріальним ураженням, 12 жінок з вірусним ураженням, 11 вагітних з грибковою інфекцією, 10 жінок без ознак інфікування склали контрольну групу. Для визначення інфікування використовували наступні методи діагностики: бактеріологічний, мікроскопічний, імуоферментний, полімеразну ланцюгову реакцію. За допомогою проточної цитометрії визначали параметри ериптозу (клітинну морфологію, скремблінг фосфоліпідної мембрани та оксидативний стрес) в крові вагітних з бактеріальною, вірусною, грибковою інфекцією репродуктивного тракту. Фарбування анексин V-FITC та 2',7'-діхлориддигідрофлуоресцина діацетата (H2DCFDA) використовувалося для кількісного визначення фосфатиділсерину і утворення активних форм кисню (АФК) відповідно Флуоресценція визначалася за допомогою наборів BD FACSCanto™ II для проточної цитометрії.

Тест ANOVA використовувався для порівняння числових даних аналізів ериптозу. Після цього був застосований тест Бонферроні. Дані обробляли програмою Graph Pad Prism 5.0 (США). Різницю вважали статистично значущою при $p < 0,05$.

Дослідження проводилися відповідно до основоположних принципів належної клінічної практики (GCP, 1996), Конвенції Ради Європи про права людини та біомедицину (4 квітня 1997 року), Гельсінської декларації Всесвітньої медичної асоціації про етичні принципи медичних Дослідження на людях (1964-2008), а також наказ МОЗ України від 23.09.2009 № 690 (зміни внесені наказом МОЗ України від 12.07.2012 № 523). Проект дослідження обговорено та схвалено на засіданні медико-етичної комісії ХНМУ (протокол № 19 від 19 травня 2024 р.).

Стаття є фрагментом наукового проекту кафедри акушерства та гінекології № 2 Харківського національного медичного університету «Оптимізація діагностики, лікування захворювань репродуктивної системи та ускладнень вагітності у жінок з екстрагенітальною патологією» (державний реєстраційний номер 0121U110923).

Результати. Параметри ериптозу були оцінені у вагітних жінок з бактеріальним, вірусним, грибковим інфікуванням репродуктивного тракту. Ураження репродуктивного тракту вагітних бактеріальною флорою супроводжувалося підсиленням екстерналізації фосфатидилсерину серед циркулюючих еритроцитів з/без накопичуванням АФК та зморщуванням клітин. Еритроцити від вагітних з вірусним або грибковим ураженням не продемонстрували ознак ериптозу, таких як скремблінг клітинних мембран, оксидативний стрес або зміни клітинної морфології.

Висновки. Інфекції репродуктивного тракту бактеріального походження у вагітних жінок пов'язані з прискоренням процесів ериптозу, який може призводити до анемії у цих вагітних. Виключно при бактеріальній інфекції генітального тракту було показано, що запускається скремблуння і з цього часу ериптоз в еритроцитах вагітних жінок. Екстерналізація фосфатиділсерину незначно відрізнялися серед вірусної або грибової інфекцій та здоровими вагітними групи контролю. Бактеріальне інфікування генітального тракту індукує процеси ериптозу, які не були пов'язані ні з усадкою клітин, ні з надлишковою продукцією АФК.

Ключові слова: інфекції репродуктивного тракту; еритроцити; анемія; вагітність; ериптоз.

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