ERYPTOSIS CONTRIBUTES TO GESTATIONAL DIABETES MELLITUS IN MATERNAL OBESITY

Lazurenko V.V., Zhelezniakov O.Yu., Prokopiuk V.Yu.

Kharkiv National Medical University, Kharkiv, Ukraine

https://doi.org/10.35339/ic.11.3.lzp

ABSTRACT

Background. Obesity is considered to be a risk factor for Gestational Diabetes Mellitus (GDM), which is characterised by hyperglycaemia in pregnant women. Recent studies have demonstrated that glycated erythrocytes are more prone to eryptosis, a unique regulated cell death observed only in mature erythrocytes.

The **aim** of the current study was to analyse how eryptosis might contribute to GSM in maternal obesity.

Materials & Methods. Eryptosis parameters were assessed in pregnant women enrolled for the study: group 1 consisted of 12 obese pregnant women without the signs of GDM, 15 pregnant women without obesity but with GDM were included in group 2, 14 obese pregnant women with GDM were in group 3, group 4 (control) consisted of 15 pregnant women without the signs of obstetric and extragenital pathology. Phosphatidylserine externalisation was assessed by flow cytometry following Annexin V-FITC staining of circulating erythrocytes isolated from the pregnant women. Additionally, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) staining was used to analyse oxidative stress parameters in circulating erythrocytes.

Results. Groups 1, 2 and 3 of pregnant women showed a higher degree of ROS-dependent eryptosis compared to the control group (IV). There was no statistically significant difference ($p>0.05$) in the eryptosis of circulating erythrocytes between pregnant women of groups 1 and 2. However, the percentage of phosphatidylserine-dispersed erythrocytes in eryptosis and the level of ROS was statistically significantly higher in pregnant women of group 3 compared to pregnant women of groups 1 and 2.

Conclusions. GSM associated with maternal obesity is accompanied by accelerated ROS-dependent eryptosis. Enhanced eryptosis might act as an additional factor contributing to thrombosis and endothelial dysfunction in obese pregnant women with GDM.

Keywords: *pregnancy, phosphatidylserine, flow cytometry, Annexin, erythrocytes.*

Introduction

Gestational Diabetes Mellitus (GDM) is a condition associated with hyperglycaemia developing in pregnant women [1]. Compelling evidence suggests that obesity, together with high maternal age, is a significant risk factor for GDM development [2]. Since overweight and obesity have become a global health concern with a clear increasing trend, including among women [3], it is important to focus on the search for novel links between obesity and GDM that might be of predictive value. In the current study, it has been hypothesised that eryptosis defined as a caspase-independent, calcium-dependent regulated cell death modality of erythrocytes [4] might be such a link. Notably, accelerated eryptosis has been demonstrated to be observed in diabetes mellitus and its enhancement has been primarily attributed to oxidative stress-mediated damage to erythrocytes [5]. In general, glycated erythrocytes have been shown to be more prone to oxidative damage and eryptosis [6]. At the same time, enhanced eryptosis is accompanied by intensified clearance of eryptotic red blood cells primarily related to recognition of eryptosis-associated externalised phosphatidylserine molecules by macrophages with their subsequent uptake referred to as efferocytosis, which might result in anaemia [7]. Moreover, the eleva-

Corresponding Author:

Lazurenko Viktoriya V. – MD, DMedSc, Professor, Head of the Department of Obstetrics and Gynecology No.2, Kharkiv National Medical University, Ukraine.

✉ Ukraine, 61022, Kharkiv, Nauki Ave., 4, KhNMU.

E-mail: vv.lazurenko@knmu.edu.ua

ted number of phosphatidylserine-exposing eryptotic erythrocytes might be associated with enhanced blood clotting and damage to endothelial cells leading to endothelial dysfunction [8]. It should be emphasised that platelet and endothelial dysfunction is a common issue in GDM, which still remains poorly investigated [9]. Given the thrombosis- and endothelial dysfunction-promoting consequences of eryptosis and its contribution to erythrocyte damage in diabetes mellitus, its activation seems to be a possible factor that might be of huge importance in the pathogenesis of GDM.

Aim

This study was designed to analyse oxidative stress-mediated eryptosis in GDM and investigate its possible contribution to the emergence of GDM in maternal obesity.

Materials & Methods

Patients and their groups

56 pregnant women included in the study were divided into four groups: group 1 consisted of 12 obese pregnant women without signs of GDM, 15 pregnant women without obesity but with GDM were included in group 2, 14 obese pregnant women with GDM were in group 3, group 4 (control) consisted of 15 pregnant women without signs of obstetric and extragenital pathology. The examination of women took into account their age, weight, height, Body Mass Index (BMI), results of clinical and laboratory tests (clinical and biochemical blood tests including blood glucose, C-reactive protein, coagulogram, lipidogram), instrumental examination (ultrasound examination of the fetoplacental complex, cardiotocography).

Sample preparation

Eryptosis parameters were assessed in pregnant women enrolled for the study: group 1 consisted of 12 obese pregnant women without signs of GDM, 15 pregnant women without obesity but with GDM were in group 2, 14 obese pregnant women with GDM were in group 3, group 4 (control) consisted of 15 pregnant women without the signs of obstetric and extragenital pathology. The samples were collected in anticoagulant-containing (K2EDTA-containing) vacutainers and analysed within 2 h of collection to ensure reliable results. Fresh blood samples were used to prepare erythrocyte solution. Briefly, 5 µl of blood was aliquoted and added to a Ringer solution containing 125mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 32mM HEPES, and 5mM glucose. The cells were centrifuged at 1500 g for 5 min. The cells were resuspended in the Ringer solution and the washing procedure was repeated twice to obtain erythrocyte suspensions for further staining [10; 11].

Detection of eryptosis by determining phosphatidylserine externalisation

The washed erythrocytes obtained from pregnant women were resuspended in 100 µl Annexin-binding buffer containing annexin V-FITC (BD Pharmingen™, FITC annexin V, Franklin Lakes, NJ, USA) for 30 min. This annexin-binding buffer contains calcium ions to ensure interactions of annexin V with the exposed phosphatidylserine molecules located on the surface of erythrocytes. After incubation, the cells were washed and resuspended in 100 µl Annexin-binding buffer [12]. The fluorescence was detected in the FL1 FITC channel (excitation 488 nm and emission 525 nm). The gating strategy included identification of the percentage of the erythrocytes with a high degree of translocated phosphatidylserine molecules indicating eryptotic cells.

Quantification of intracellular ROS levels in erythrocytes

In brief, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) supplied by Invitrogen™ (Waltham, MA, USA) was used to detect intracellular reactive oxygen species (ROS) levels characterising the state of the redox metabolism in erythrocytes. An aliquot of erythrocytes obtained as outlined above was stained with the stock solution of the dye in DMSO to obtain a final concentration of 10 µM. The cells were incubated for 30 min at 37°C in H2DCDA-containing phenol free medium. The fluorescence was detected in the FL1 FITC channel (excitation 488 nm and emission 525 nm). To quantify the data, the mean fluorescence intensity (MFI) was evaluated [11].

Data collection and post-acquisition analysis

A total of 100,000 events were collected per sample by a BD FACS Canto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The doublets were excluded. The fluorescence was analysed in the population of single cells. FlowJo™ (v10, BD Biosciences, USA) software was used to process the raw data.

Statistical analysis

Comparative analysis was performed by carrying out the ANOVA test followed by the post-hoc Tukey test. The difference was considered statistically significant at $p<0.05$. Data are shown in Figures as mean and standard deviation (SD). Graph Pad Prism 5.0 software (USA) was used to provide statistical analysis.

Results

Patient's characteristics

The age of pregnant women ranged from 18 to 39 years. The mean age of obese women was (32.4±3.2) years, women with GDM – (34.7±3.6) years, obese and gestational diabetes mellitus – (33.5±2.9) years, and control group women – (27.3±2.8) years. The BMI in obese pregnant women was (37.2±3.5) kg/m^2, in gestational diabetes mellitus – (28.4±2.9) kg/m^2; in gestational diabetes mellitus with obesity – (35.4±3.6) kg/m^2, in the control group – (24.9±2.1) kg/m^2.

In this study, the eryptosis of circulating erythrocytes was judged primarily by the evaluation of phosphatidylserine externalisation. To quantify the degree of eryptosis, the percentage of cells expressing the high content of phosphatidylserine molecules on the cell surface was identified and compared. Since this parameter is considered a hallmark of eryptosis, it was selected as the most reliable available marker. Expectedly, maternal obesity, GDM and their combination were found to be associated with enhanced phosphatidylserine externalization compared to healthy non-obese pregnant females (*Figure 1*), which suggests acceleration of eryptosis.

Comparative analysis revealed that non-obese women with GDM and obese women not diagnosed with GDM had a negligible statistically insignificant difference (p>0.05) in the number of cells with significantly externalised phosphatidylserine molecules (*Figure 1*) indicating that the degree of eryptosis was comparable between the above-mentioned groups. Representative histograms reflecting eryptosis-characterising phosphatidylserine externalisation are available in *Figure 2*.

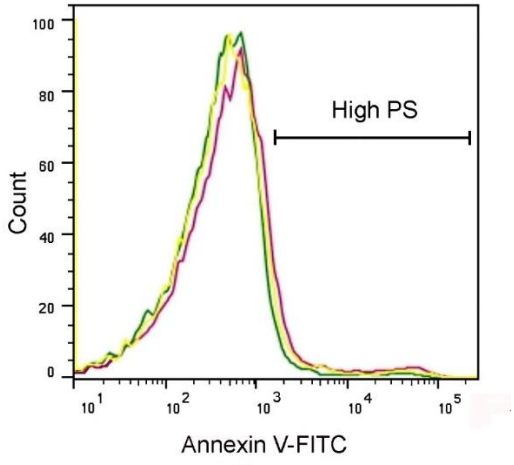

Fig. 2. Representative histograms demonstrate the population of the erythrocytes with the high degree of phosphatidylserine externalization (eryptotic cells) in obese pregnant women without GDM (green line), non-obese pregnant women with GDM (yellow line), and obese females with GDM (red line).

Note: GDM – gestational diabetes mellitus; PS – phosphatidylserine.

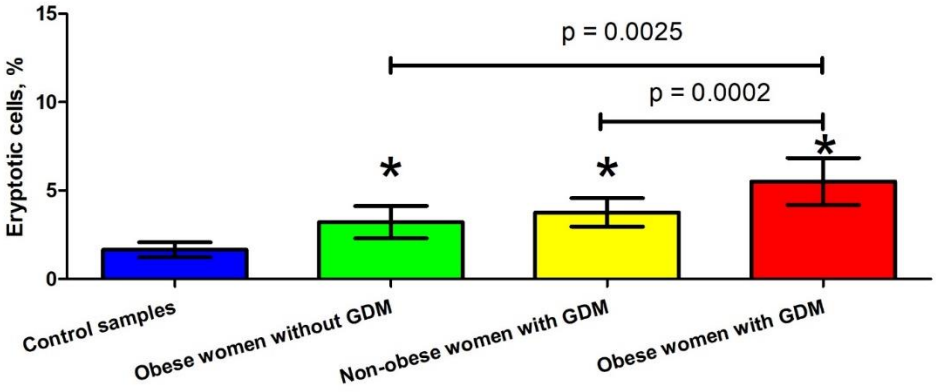

Fig. 1. Evaluation of eryptosis in circulating erythrocytes by assessing the percentage of cells with the high degree of phosphatidylserine externalization in healthy non-obese pregnant females (blue), obese pregnant females without GDM (green), non-obese pregnant women with GDM (yellow), and maternal obesity with GDM (red). ANOVA and Tukey tests, mean and standard deviation (SD).

Note: GDM – gestational diabetes mellitus;
* – indicates a statistical difference compared to the control samples (p<0.05).

On the contrary, GDM developed against a background of maternal obesity was found to be associated with accelerated eryptosis, evidenced by a statistically significant increase in the percentage of phosphatidylserine-expressing erythrocytes compared to both groups. Abundant evidence indicates that eryptosis is triggered by oxidative stress mediated by excessive production of ROS. Thus, our next step was to quantify ROS production in erythrocytes isolated from all three groups of pregnant women enrolled for the study. ROS quantification was performed by using the H2DCFDA staining. The data on the content of intraerythrocytic ROS were perfectly in line with the assessment of the phosphatidylserine externalisation. Maternal obesity, GDM and their combination resulted in elevation of intracellular ROS concentrations (*Figure 3*) compared to non-obese pregnant women with no GDM.

ROS levels were found to be comparable in non-obese women with GDM and non-GDM obese women. At the same time, obese pregnant women diagnosed with GDM had higher intracellular ROS levels, which was confirmed by higher values of the ROS-dependent fluorescence (Figure 3). Representative histograms demonstrating the ROS-dependent fluorescence in erythrocytes of pregnant women are shown in Figure 4.

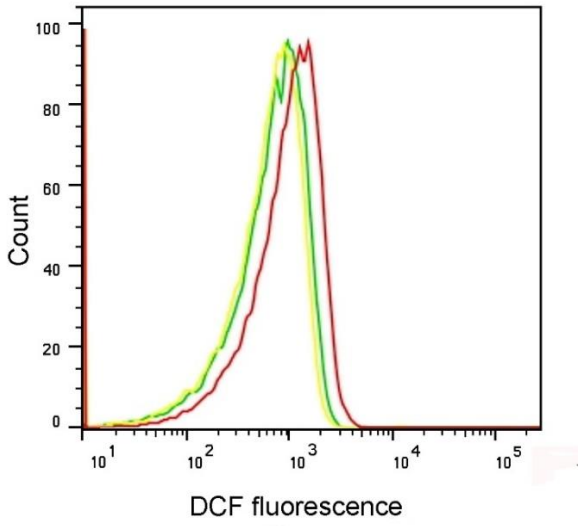

Fig. 4. Representative histograms reflect the intracellular ROS levels in circulating erythrocytes of obese pregnant women without GDM (green line), non-obese women with GDM (yellow line), and obese females with GDM (red line).

Note: DCF – dichlorofluorescein;
GDM – gestational diabetes mellitus;
ROS – reactive oxygen species.

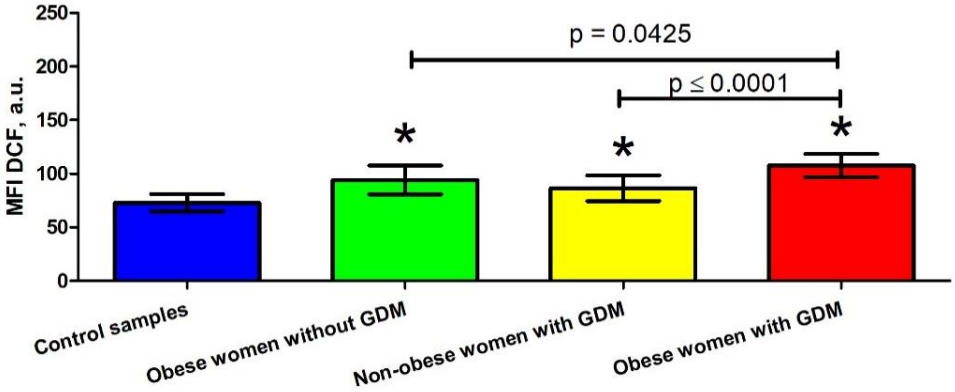

Fig. 3. Intracellular ROS levels in circulating erythrocytes of healthy non-obese pregnant females (blue), obese pregnant women without GDM (green), non-obese women with GDM (yellow), and obese females with GDM (red). ANOVA and Tukey tests, mean and standard deviation (SD).

Note: DCF – dichlorofluorescein;
GDM – gestational diabetes mellitus;
MFI – mean fluorescence intensity;
ROS – reactive oxygen species;
* – indicates a statistical difference compared to the control samples ($p<0.05$).

Discussion

In this study, eryptosis parameters were assessed in obese pregnant women without GDM, non-obese pregnant women with GDM, and obese pregnant females with GDM. We have hypothesized that eryptosis might be a factor influencing pathogenesis of GSM. In general, eryptosis is a controlled cell death of erythrocytes typically occurring in the injured or stressed cells to ensure their non-immunogenic clearance by phagocytic cells in the process called efferocytosis. Morphological changes characteristic of eryptosis are comparable to those observed in apoptosis and include cell shrinkage and membrane blebbing [13]. However, there are cell-specific signalling pathways making it unique in comparison with apoptosis [4]. A key role in eryptosis is played by Ca^{2+} signalling, which mediates structural changes typical for this cell death and phosphatidylserine externalization, a critical signal for macrophages that mediates clearance of eryptotic erythrocytes [7]. Phosphatidylserine externalization was used as a marker of eryptosis in this study and its analysis revealed that the obese pregnant women with GDM had enhanced eryptosis levels compared to other groups of the pregnant women investigated in this research. Notably, eryptosis can be enhanced when ROS get accumulated, ceramide is produced in the cells by sphingomyelinases or when prostaglandin E2 is overproduced [13]. As it turned out, phosphatidylserine externalization in the obese pregnant women with GDM is paralleled with ROS accumulation suggesting that eryptosis in this condition is ROS-dependent. Our findings are in line with other studies emphasizing the critical role of ROS in induction of eryptosis in hyperglycaemic conditions of diabetic patients [5]. Additionally, our study supports earlier remarks concerning the potential of evaluating eryptosis parameters in diagnostic purposes in diabetes mellitus [14].

Moreover, phosphatidylserine externalization revealed in the current study might contribute to pathological conditions associated with GDM in maternal obesity. Phosphatidylserine-expressing eryptotic erythrocytes interact with platelets and endothelial cells in a phosphatidylserine-dependent manner, which results in activation of platelet aggregation increasing blood coagulation and the development of endothelial dysfunction, respectively [8]. Notably, both obesity and GDM increased the risk of thromboembolic complication in pregnancy [15]. Similarly, endothelial dysfunction is common for maternal obesity and GDM [16]. Thus, our study sheds light on the new mechanism that might be involved in the development of thrombosis and endothelial dysfunction in GDM developed against a background of maternal obesity. It can be assumed therefore that eryptosis might be inhibited therapeutically in the obese pregnant women with GDM. However, more studies are necessary to clarify the diagnostic, prognostic and therapeutic potential of eryptosis in pregnant women.

Conclusions

Enhanced ROS-dependent eryptosis is revealed for maternal obesity, GDM, and their combination. Our findings indicate that maternal obesity with GDM is associated with enhanced eryptosis compared to maternal obesity with no GDM or GDM with no obesity suggesting that both conditions potentiate each other in promotion of eryptosis. Eryptosis in maternal obesity with GDM is dose-dependent. Eryptosis might contribute to thrombosis and endothelial dysfunction in maternal obesity with GDM.

DECLARATIONS:

Disclosure Statement

The authors have no potential conflicts of interest to disclosure, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

Statement of Ethics

The authors have no ethical conflicts to disclosure.

Data Transparency

The data can be requested from the authors.

Funding Sources

There are no external sources of funding.

Consent for publication

All authors give their consent to publication.

References

1. Quintanilla Rodriguez BS, Vadakekut ES, Mahdy H. Gestational Diabetes. 2024 Jul 14. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 31424780.

2. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. Int J Mol Sci. 2018;19(11):3342. DOI: 10.3390/ijms19113342. PMID: 30373146.

3. Quotah OF, Nishku G, Hunt J, Seed PT, Gill C, Brockbank A, et al. Prevention of gestational diabetes in pregnant women with obesity: protocol for a pilot randomised controlled trial. Pilot Feasibility Stud. 2022;8(1):70. DOI: 10.1186/s40814-022-01021-3. PMID: 35337389.

4. Tkachenko A. Apoptosis and eryptosis: similarities and differences. Apoptosis. 2024;29(3-4):482-502. DOI: 10.1007/s10495-023-01915-4. PMID: 38036865.

5. Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA, et al. Erythropoiesis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. Mol Cell Biochem. 2011;357(1-2):171-9. DOI: 10.1007/s11010-011-0887-1. PMID: 21625956.

6. Turpin C, Catan A, Guerin-Dubourg A, Debussche X, Bravo SB, Alvarez E, et al. Enhanced oxidative stress and damage in glycated erythrocytes. PLoS One. 2020;15(7):e0235335. DOI: 10.1371/journal.pone.0235335. PMID: 32628695.

7. Tkachenko A, Onishchenko A. Casein kinase 1α mediates eryptosis: a review. Apoptosis. 2023;28(1-2):1-19. DOI: 10.1007/s10495-022-01776-3. PMID: 36308624.

8. Restivo I, Attanzio A, Giardina IC, Di Gaudio F, Tesoriere L, Allegra M. Cigarette Smoke Extract Induces p38 MAPK-Initiated, Fas-Mediated Eruptosis. Int J Mol Sci. 2022;23(23):14730. DOI: 10.3390/ijms232314730. PMID: 36499060.

9. Valero P, Cornejo M, Fuentes G, Wehinger S, Toledo F, van der Beek EM, et al. Platelets and endothelial dysfunction in gestational diabetes mellitus. Acta Physiol (Oxf). 2023;237(4):e13940. DOI: 10.1111/apha.13940. PMID: 36700365.

10. Ewendt F, Schmitt M, Kluttig A, Kuhn J, Hirche F, Kraus FB, et al. Association between vitamin D status and eryptosis-results from the German National Cohort Study. Ann Hematol. 2023;102(6):1351-61. DOI: 10.1007/s00277-023-05239-w. PMID: 37121914.

11. Prokopiuk V, Onishchenko A, Tryfonyuk L, Posokhov Y, Gorbach T, Kot Y, et al. Marine Polysaccharides Carrageenans Enhance Eruptosis and Alter Lipid Order of Cell Membranes in Erythrocytes. Cell Biochem Biophys. 2024;82(2):747-66. DOI: 10.1007/s12013-024-01225-9. PMID: 38334853.

12. Onishchenko AI, Prokopiuk VY, Chumachenko VA, Virych PA, Tryfonyuk LY, Kutsevol NV, et al. Hemocompatibility of dextran-graft-polyacrylamide/zinc oxide nanosystems: hemolysis or eryptosis? Nanotechnology. 2023;35(3). DOI: 10.1088/1361-6528/ad02a3. PMID: 37827140.

13. Alghareeb SA, Alfhili MA, Fatima S. Molecular Mechanisms and Pathophysiological Significance of Eryptosis. Int J Mol Sci. 2023;24(6):5079. DOI: 10.3390/ijms24065079. PMID: 36982153.

14. Restivo I, Attanzio A, Tesoriere L, Allegra M. Suicidal Erythrocyte Death in Metabolic Syndrome. Antioxidants (Basel). 2021;10(2):154. DOI: 10.3390/antiox10020154. PMID: 33494379.

15. Yogev Y, Visser GH. Obesity, gestational diabetes and pregnancy outcome. Semin Fetal Neonatal Med. 2009;14(2):77-84. DOI: 10.1016/j.siny.2008.09.002. PMID: 18926784.

16. Lan Q, Zhou Y, Zhang J, Qi L, Dong Y, Zhou H, et al. Vascular endothelial dysfunction in gestational diabetes mellitus. Steroids. 2022;184:108993. DOI: 10.1016/j.steroids.2022.108993. PMID: 35219717.

Received: 27 Jul 2024
Accepted: 29 Sep 2024
Published: 30 Sep 2024

Cite in Vancouver style as: Lazurenko VV, Zhelezniakov OYu, Prokopiuk VYu. Eryptosis contributes to gestational diabetes mellitus in maternal obesity. Inter Collegas. 2024;11(3):23-8. https://doi.org/10.35339/ic.11.3.lzp

Archived: https://doi.org/10.5281/zenodo.14423811