European Review for Medical and Pharmacological Sciences 2023; 27: 476-482

Correlation between smoking and downregulation of red cell CD47 as eryptosis marker

M.M. ELTAYEB[1], H.A. WAGGIALLAH[2], N.Y. HAKAMI[3], Y.M. ELMOSAAD[4]

[1]Department of Nursing, [2]Department of Medical Laboratory Science, College of Applied Medical Science, Prince Sattam Bin Abdulaziz University, Alkharj, Saudi Arabia
[3]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[4]Department of Public Health, College of Applied Medical Sciences, King Faisal University, Saudi Arabia

Abstract. – OBJECTIVE: The purpose of this research is to discover a link between cigarette smoking and decreased red cell CD47 expression.

SUBJECTS AND METHODS: The current cross-sectional study included 72 smokers (who had smoked 20 cigarettes per day for at least two years) and 50 nonsmokers, as well as nonsmokers who had not been exposed to smokers on a regular basis and chose to participate as controls. Due to exclusion criteria, 11 participants were removed from the study; they had various genetic, immune, and metabolic disorders, leaving only 61 healthy people in the study. A flow cytometer was used to examine CD47.

RESULTS: There was a strong correlation between smoking and a decrease in CD47 markers in all types of smokers in the control samples (p-value = 0.000), as well as among cigarette smokers only (p-value = 0.000), cigarette and Shisha smokers (p-value = 0.024), and cigarette and e-cigarette smokers (p-value = 0.014). Furthermore, there is a strong correlation between the appearance of the CD47 marker in healthy smokers and smokers with non-hereditary blood diseases like iron deficiency anemia and polycythemia.

CONCLUSIONS: It can be concluded that smoking significantly reduces the expression of the CD47 marker.

Key Words:
Smoking, Cigarettes, Shisha, E-cigarettes, CD47, Eryptosis marker, Down regulation.

Introduction

Despite considerable epidemiological evidence connecting cigarette smoking to modifications in the red blood cell (RBC) structural system, the mechanisms causing abnormalities in red blood cell membrane components remain unknown. Since membrane constituents are thought to be a critical first step in RBC pathogenesis, it is reasonable to believe that cigarette smoke or some of its constituents could harm red blood cells (eryptosis) by down-regulating CD47. Phosphatidylserine (PS) exposure, like that of apoptotic cells, recognizes eryptotic RBCs and increases phagocytosis and clearance of these cells[1-3]. Surprisingly, a number of well-known apoptotic events raise PS levels in RBC[4].

CD47 is associated with various membrane proteins in mature red blood cells (RBCs), forming links with both cytoskeletal and non-cytoskeletal cellular organelles[5]. CD47 is critical in hindering RBC phagocytosis by binding to the signal regulatory protein (SIRP) on macrophages, which inhibits the phagocytosis of both non-opsonized and IgG or complement-opsonized RBCs[6,7]. It is thought that a decrease in cell surface CD47 expression throughout RBC aging *in vivo* encourages the removal of senescent RBCs[8]. Moreover, it has been reported[9] that micro particle release during RBC storage favors CD47 damage during RBC storage for transfusion. CD47 also mediates the interaction of fibrinogen with the RBC membrane[10], which may contribute to RBC hyperaggregation and altered hemorheology in inflammatory disorders[11,12]. CD47 promotes cell-cell contacts with SIRP-expressing splenic macrophages on adult RBCs lacking integrins. This link is thought to obstruct a phosphorylation pathway that restricts phagocytosis and RBC clearance from the bloodstream[13]. It is still unknown how cigarette smoking causes abnormalities in RBC membrane proteins because membrane components are considered a critical step in red cell eryptosis. The goal of this study is to find a link between ciga-

Corresponding Author: H.A. Waggiallah, MD; e-mail: hishamwagg30@hotmail.com

rette smoking and the downregulation of red cell CD47 expression as an eryptosis marker.

Subjects and Methods

The current cross-sectional study, which was guided by a questionnaire, included 72 smokers (who had smoked 20 cigarettes per day for at least two years) and 50 nonsmokers, as well as nonsmokers who had not been exposed to smokers on a regular basis and who chose to participate as controls. Participants will be healthy people aged 15 to 65 with no history of cardiovascular, endocrine, red blood cell genetic disorders, or immune problems. The subjects were also not given any drugs or nutritional supplements, nor have they been on any special diets. 11 participants were removed from the study due to exclusion criteria; they had various genetic, immune, and metabolic disorders, leaving only 61 healthy people in the study. CD47 was analyzed by flow cytometry.

Methodology

After obtaining informed consent, 61 healthy donors and 50 healthy controls had their venous blood drawn. Centrifugation at 270 g for 15 minutes separated erythrocytes from fresh heparinized whole blood. After removing the platelet-rich plasma and mononuclear cells from the peripheral blood, the erythrocytes were washed twice with saline-adenine-glucose-mannitol (150 mM NaCl, 1.25 mM adenine, 50 mM glucose, and 29 mM mannitol; Fresenius) and re-suspended in saline-adenine-glucose-mannitol. Advia 2120 was used to determine the final cell concentration (Siemens Medical Solutions Diagnostics, Erlangen, Germany). 50 liters of washed red cells and 50 liters of mouse anti-human CD47 (clone 2D3)-FITC were mixed together and incubated in a 37°C water bath for 30 minutes. To eliminate unbound anti-CD47, the tubes were fully washed with phosphate-buffered saline (PBS). Flow cytometry was then used to examine red cells and anti-CD47 binding (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

SPSS software version 21 (IBM Corp., Armonk, NY, USA) was used for data analysis. To tabulate and describe the data, descriptive statistics (frequency, proportion estimation, and mean) were used based on the nature of the variables. To determine the mean difference in CD47 among smokers, inferential statistics such as the independent sample *t*-test were used. The statistical significance level was set at $p < 0.05$.

Results

Table I describes the study's health status. Participants with genetic, immune, or metabolic disorders were excluded from the study, while participants with other blood diseases were included. Table II depicts the distribution of the socio-demographic data of the study, where the study included the type of occupation for the stu-

Table I. Participants with genetic blood, immune, and metabolism disorders were excluded from the study; participants with other blood diseases were included (n = 72).

	Count	%
Genetic Blood Disorders		
- No	70	97.2
- Yes	2	2.8
Genetics and Immune Disorders		
- No	71	98.6
- Yes	1	1.4
Metabolism		
- No	64	88.9
- Yes	8	11.1
Blood diseases		
- Iron deficiency anemia	6	9.8
- Polycythemia	4	6.6
- Healthy	51	83.6

dy's participants, where employees constituted the highest category in the study (59%), and the academic level, where the proportion of university graduates constituted the highest percentage among the participants, 73.8%. In terms of the age groups that took part in the study, we discovered that the 15-26-year and 27-35-year age groups had the highest percentage (39.3%). The unmarried group had the highest participation rate (63.9%) in the study. The majority of participants in the study were men, accounting for 96.7% of the total. Table III shows the different types of smoking among study participants, with cigarettes accounting for 68.9%, cigarettes and Shisha accounting for 14.8%, and cigarettes and electronic cigarettes accounting for about 16.4%. The vast majority of respondents (95.1%) smoked 1-20 cigarettes per day. We found that those who smoke shisha once a week with a regular cigarette made up the largest group (18%). We observed the largest group (11.5%) to be those who smoke electronic cigarettes more than once a day in addition to smoking regular cigarettes. Figure 1 depicts the flow cytometer result, which depicts the CD47 marker, which appears in various smoking patterns such as smoking cigarettes, cigarettes, and Shisha, cigarettes, and electronic cigarettes, as well as the clarity of the CD47 marker in control samples compared to test samples. Figure 2 represents the correlation between the CD47 marker in red blood cells in control samples and all types of smokers (p-value = 0.000), as well as among cigarette smokers only (p-value = 0.000), cigarette and Shisha smokers (p-value = 0.024), and cigarette and e-cigarette smokers (p-value = 0.014), where we noticed a strong correlation between smoking and a decrease in CD47 markers in all

Table II. Socio-demographic characteristics of the study participants (n = 61).

Variables	Count	%
Occupation		
- Students	18	29.5
- Employees	36	59.0
- Unemployed	7	11.5
Education level		
- High school	5	8.2
- University	45	73.8
- Postgraduate	11	18.0
Age group		
- 15-26 years	24	39.3
- 27-35 years	24	39.3
- 36-45 years	10	16.4
- 46-55 years	2	3.3
- 56-65 years	1	1.6
Marital status		
- Single	39	63.9
- Married	21	34.4
- Divorce	1	1.6
Gender		
- Male	59	96.7
- Female	2	3.3

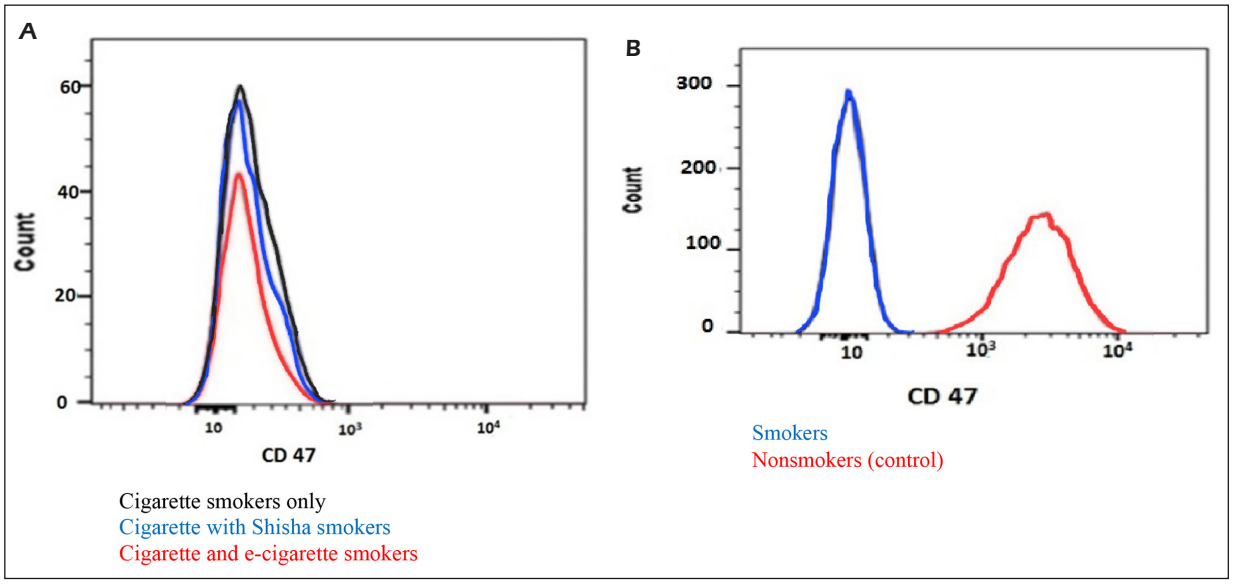

Figure 1. **A**, demonstrates the CD47 marker in all types of smokers. **B**, illustrates CD47 I test and control samples.

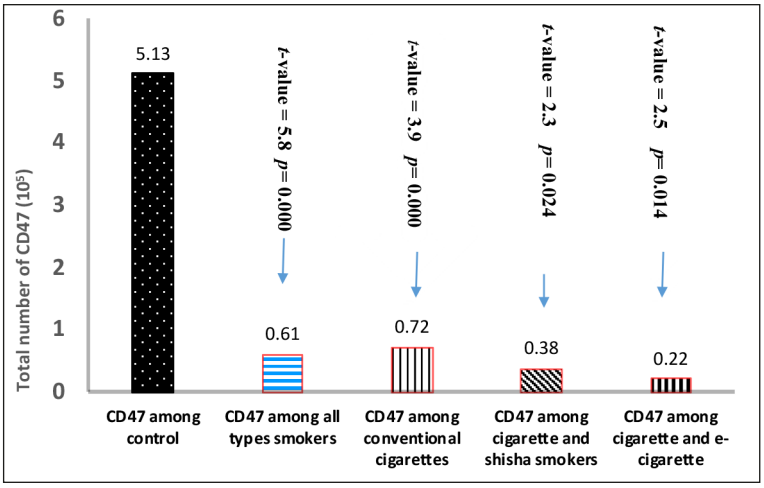

Figure 2. Association between cigarette smoking and the appearance of cell death receptors in red blood cells (CD 47).

types of smokers. Figures 3 and 4 demonstrate the strong relationship between the appearance of the CD47 marker in healthy smokers and smokers with non-hereditary blood diseases such as iron deficiency anemia and polycythemia.

Discussion

Human RBCs bind to the signal regulatory protein alpha (SIRP) on macrophages *via* the CD47 (integrin-associated protein) receptor. This interaction sends a "don't engulf me" signal, preventing these RBCs from being phagocytized and remaining in blood circulation[14]. To remove any interference from genetic problems, participants with genetic, immune, or metabolic disorders were excluded from this study, while participants with other blood diseases such as iron deficiency anemia and thalassemia were included. Many diseases, such as sickle cell anemia, cause eryptosis. Red cells may exhibit increased binding of the conformation-dependent CD47 antibodies 2D3 and thrombospondin-1 (TSP-1). This helps explain

Table III. Percentages of the behavior, type, and number of smokers (n = 61).

Variables	No	%	Average of CD47 mean ± SD
Smoking behavior			
- Regular smokers	61	100	7476.7 ± 3110.0
- Types smoking			7895.0 ± 3827.0
- Cigarettes	42	68.9	2201.1 ± 1411.5
- Cigarettes and Shisha	9	14.8	
- Cigarettes and e-cigarettes	10	16.4	6149.1 ± 3475.2
How many cigarettes do you smoke per day?			4610.0 ± 5014.4
- 1- 20 cigarettes	58	95.1	7476.7 ± 3110.0
- 21-40 cigarettes	3	4.9	7895.0 ± 3827.0
How many times do you smoke shisha with regular cigarettes?			
- One time per day	3	4.9	1574.7 ± 646.4
- More than one time per day	1	1.6	765.0 ± 0.0
- Once a week	11	18.0	3372.7 ± 1906.6
- I smoke conventional cigarettes only	46	75.4	7128.0 ± 3293.0
How many times do you smoke e-cigarettes with regular cigarettes?			
- One time per day	2	3.3	5779.0 ± 2657.3
- More than one time per day	7	11.5	1430.3 ± 546.9
- Once a week	3	4.9	2202.7 ± 975.0
- I'm don't smoke e-cigarettes	49	80.3	6985.7 ± 3220.6

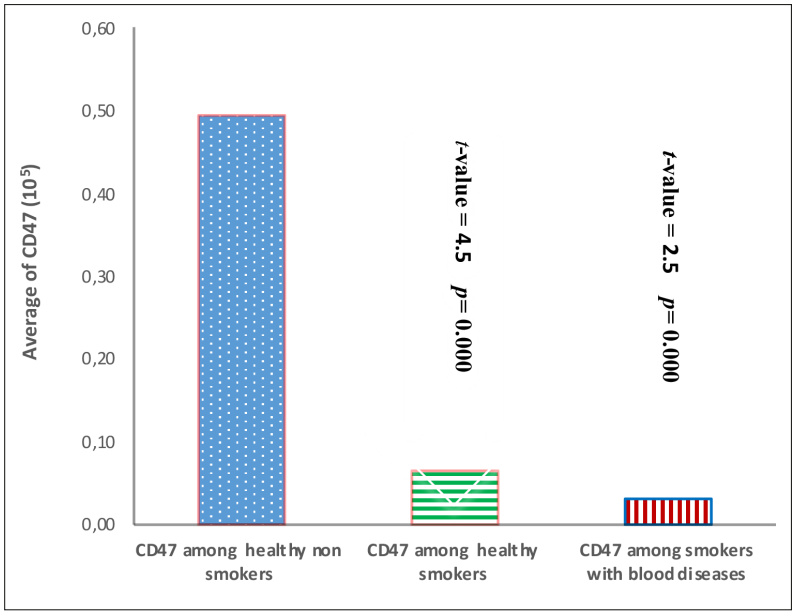

Figure 3. The association between cigarette smoking and the appearance of CD47 receptors in red blood cells among control smokers, healthy smokers, and smokers with blood diseases (iron deficiency anemia and polycythemia).

the increased uptake of sickle erythrocytes in the spleen[15].

The vast majority of respondents (95.1%) smoked 1-20 cigarettes per day. The largest group (18%) was found to be those who smoke Shisha with a regular cigarette once a week. We discovered that the largest group, 11.5%, smokes electronic cigarettes in addition to regular cigarettes more than once per day. Smoke from cigarettes includes nearly 5,000 chemicals, among them carbon monoxide (CO) and tar. Many of these ingredients are toxic to the human body. CO diffuses quickly and strongly binds to hemoglobin (Hb) in alveolar capillaries (with a binding ability 200-250 times greater than that of O_2), resulting in tissue hypoxia due to the formation of carboxyhemoglobin (HbCO) and elevated levels of Hb and RBCs[16].

It was first established that red blood cell lifespan is dependent on an adequate oxidative stress

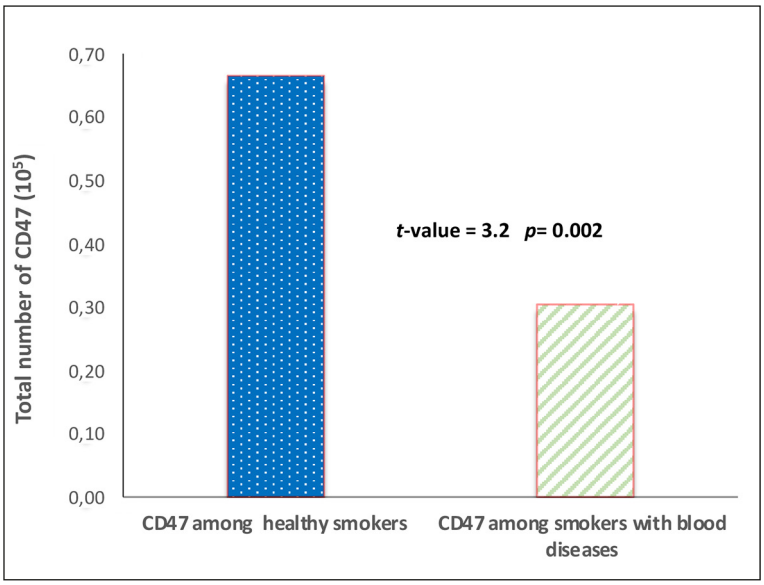

Figure 4. Comparison of CD47 marker appearance in healthy and non-healthy smokers, who suffer from iron deficiency anemia and polycythemia.

response induced by human diseases. The structure of protein-protein interaction networks in the RBC interaction confirmed that erythrocytes suffer from excessive oxidative stress and are constantly trying to repair damage to the cell, involving different pathways linked to protein repair, vesiculation, and apoptosis. Although it is still an active research topic, accumulating evidence suggests that oxidative stress plays an important role in RBC aging signaling. Aside from its effect on the formation of Band 3-derived neo-antigens and the activation of pro-apoptotic components, oxidative stress also has an effect on Hb and its interactions with membrane constituents[8]. Notably, red blood cell oxidation induced the conformational change in CD47 needed for TSP-1 binding, implying that oxidative damage may be a factor in the generation of this "engulf me" signal on red cells.

In the current study, there was a significant correlation between the CD47 marker in red blood cells in control samples and smoker samples in general, as well as among cigarette smokers only, cigarette and Shisha smokers, and cigarette and e-cigarette smokers, where we noticed a strong downregulation in the red blood cell CD47 marker in all different categories of smokers in comparison with control. Importantly, the oxidation of erythrocytes induced the conformational change in CD47 that was required for TSP-1 binding. We found evidence that CD47 undergoes a conformational change in response to oxidative stress during smoking. Because of this conformational change, CD47 is recognized as an "engulf me" signal by SIRP α after the binding of thrombospondin-1. This mechanism was used to phagocytize RBCs *in vivo* by primary human red pulp phagocytes, indicating that it is active *in vivo*[17,18].

In diseases such as sickle cell anemia, thalassemia, and glucose-6-phosphate dehydrogenase deficiency, susceptibility to eryptosis is enhanced. Increased phosphatidylserine exposure promotes RBC adhesion to endothelial cells[19]. In this study, however, smokers with non-hereditary blood diseases such as iron deficiency anemia and polycythemia had significantly lower levels of the CD47 marker than healthy non-smokers.

Conclusions

The data presented in this study showed significant down regulation in all categories of smokers due to oxidative stress that may be elevated in smoking, leading to early eryptosis and red cell clearance by spleen macrophages. To define the exact mechanisms of oxidative stress in smoking that decrease the expression of red cell CD47 and its association with eryptosis, an advanced, sophisticated approach would be used.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This research was approved by the Standing Committee on Bioethics Research (SCBR) of Prince Sattam Bin Abdulaziz University (SCBR-071-2022).

Informed Consent

Informed consent was obtained from all the participants.

Funding

This study was funded by Deanship of Scientific Research at Prince Sattam Bin Abdulaziz University under the research project (PSAU- 2022/03/20339).

Acknowledgments

This project was supported by the Deanship of Scientific Research at Prince Sattam Bin Abdulaziz University under the research project (PSAU- 2022/03/20339).

Authors' Contribution

All authors worked together to complete this project. The final manuscript was read and approved by all authors.

ORCID ID

Mudathir Mohamedahmed Eltaye: 0000-0002-5991-8903.
Hisham Ali Waggiallah: 0000-0001-6591-1831.
Nora Y. Hakami: 0000-0003-4956-0132.
Yousif Mohammed Elmosaad: 0000-0002-5219-0059.

References

1) Dreischer P, Duszenko M, Stein J, Wieder T. Eryptosis: Programmed Death of Nucleus-Free, Iron-Filled Blood Cells. Cells 2022; 11: 503-516.
2) Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. Antioxid Redox Signal 2006; 8: 1183-1192.
3) Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. Areceptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 2000; 405: 85-90.

4) Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Foller M. Eryptosis, a window to systemic disease. Cell Physiol Biochem 2008; 22: 373-380.
5) Oldenborg PA. CD47: a cell surface glycoprotein which regulates multiple functions of hematopoietic cells in health and disease. ISRN Hematol 2013; 2013: 614619.
6) Oldenborg PA, Gresham HD, Lindberg FP. Cd47-signal regulatory protein α (Sirpα) regulates Fcγ and complement receptor–mediated phagocytosis. J Exp Med 2001; 193: 855-862.
7) Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science 2000; 288: 2051-2054.
8) Antonelou MH, Kriebardis AG, Papassideri IS. Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus 2010; 8: 39-49.
9) Said AS, Doctor A. Influence of red blood cell-derived micro particles upon vaso-regulation. Blood Transfus 2017; 15: 522-534.
10) De Oliveira S, De Almeida VV, Calado A, Rosário HS, Saldanha C. Integrin-associated protein (CD47) is a putative mediator for soluble fibrinogen interaction with human red blood cells membrane. Biochim Biophys Acta 2012; 1818: 481-490.
11) Wiewiora M, Piecuch J, Sedek L, Mazur B, Sosada K. The effects of obesity on CD47 expression in erythrocytes. Cytometry B Clin Cytom 2017; 92: 485-491.
12) Grobler C, Maphumulo SC, Grobbelaar LM, Bredenkamp JC, Laubscher GJ, Lourens, PJ, Pretorius E. Covid-19: The rollercoaster of fibrin (ogen), d-dimer, von willebrand factor, p-selectin and their interactions with endothelial cells, platelets and erythrocytes. Int J Mol Sci 2020; 21: 5168.
13) Van Bruggen R. CD47 functions as a removal marker on aged erythrocytes. ISBT Science Series 2013; 8: 153-156.
14) Lutz HU. Comment concerning the role of CD47 and signal regulatory protein alpha in regulating the clearance of aged red blood cells. Transfus Med Hemother 2013; 40:140-141.
15) Burger P, Hilarius-Stokman P, De Korte D, Van Den Berg TK, Van Bruggen R. CD47 functions as a molecular switch for erythrocyte phagocytosis. Blood 2012; 119: 5512-5521.
16) Aldosari KH, Ahmad G, Al-Ghamdi S, Alsharif MHK, Elamin AY, Musthafa M, Al-Ghamdi H. The influence and impact of smoking on red blood cell morphology and buccal microflora: A case-control study. J Clin Lab Anal 2020; 34: e23212.
17) Bornstein P. Thrombospondins as matricellular modulators of cell function. J Clin Invest 2001; 107: 929-934.
18) Carlson CB, Lawler J, Mosher DF. Structures of thrombospondins. Cell Mol Life Sci 2008; 65: 672-686.
19) Lang E, Lang, F. Triggers, inhibitors, mechanisms, and significance of eryptosis: the suicidal erythrocyte death. Biomed Res Int 2015; 2015: 513518.