COMBINED EFFECT OF GAMMA RADIATION AND HYPERGLYCEMIA ON SOME BIOPHYSICAL PROPERTIES OF RED BLOOD CELLS

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Abstract. Hyperglycemia and exposure to radiation may have diverse effects on red blood cells (RBC). This work is intended to test and evaluate some biophysical parameters of RBC in order to assess the impact of combined hyperglycemia and gamma irradiation. Blood was collected from adult male Albino rats. The turbidity test and dielectric properties of RBC were determined. The membrane effective capacitance, AC conductivity, average membrane solubilization, and hemolysis showed to be correlated with the combined effect of hyperglycemia and gamma irradiation.

Key words: Hyperglycemia, gamma irradiation, dielectric properties, red blood cells.

INTRODUCTION

The concentration of blood glucose above the normal level, known in diabetic patients as hyperglycemia, results in elevation of free radicals with the subsequent increase of oxidative stress in the body. Free radicals attack RBC membrane through lipid peroxidation and damage of the integral proteins [1]. Inside the RBCs, the hemoglobin molecules are also affected. Glucose reacts with blood proteins through covalent bonding affecting cell structure resulting in membrane rigidity and, consequently, a decreased deformability of RBCs [4, 35]. It has been shown that the stiffening of RBCs and their decreased deformability have adverse effects on the blood vessels [17]. Shin *et al*. [30] reported a significant correlation between the reduction of RBC deformability and the increase in glycated hemoglobin.

The exposure of blood to gamma radiation results in adverse effects in the body. It was shown that gamma irradiation affects the blood dielectric properties, AC conductivity, permittivity, and dielectric loss [28]. These factors were correlated to change of cell membrane permeability, loss of ions, and decrease in

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the membrane surface charges [28]. The decrease in the membrane surface charge of RBCs leads to the increase in blood viscosity [36] and alteration of blood flow properties. It, also, affects RBC deformability and aggregation, and increases their adhesion to vessel walls [12]. Exposure to radiation increases lipid peroxidation [26], cross-linkages in the membrane lipids, and hemolysis [18].

Diabetic patients can be subjected to radiation exposure in the cases of X-ray diagnostic and radiotherapy. Hyperglycemia and exposure to radiation seem to induce different effects. For example, exposure to radiation results in decrease in AC conductivity and relative permittivity of RBCs [28], while hyperglycemia showed an increase of the same factors [9]. In this pilot study, it is intended to test *in vitro* both the effects of hyperglycemia and gamma radiation on RBCs, in order to evaluate the factors involved in their combined effect.

MATERIALS AND METHODS

SAMPLE PREPARATION

In the present study, 27 adult male Albino rats (150 ± 10 g) were housed in cages maintained in a light/dark cycle of 12 h and allowed to acclimatize for one week before starting the experiment. They were kept on standard food pellets containing all nutritive elements and water *ad libitum*. All animal procedures were carried out in accordance with the ethics committee for experimental studies at the Egyptian Atomic Energy Authority's National Center for Radiation Research and Technology, which is established and regulated in agreement with the CIOMS and ICLAS International Guiding Principles for Biomedical Research Involving Animals, 2012. Freshly drawn whole blood was collected in heparin tubes (20 μL heparin/5 mL blood). To avoid the variations between individuals of a group, each sample was first divided into four fractions:1) control, 2) hyperglycemic group, 3) irradiated group and 4) hyperglycemic-irradiated group. The whole blood containing glucose concentrations of 5 and 22 mM (equivalent to 90 and 400 g/dL) and 10 μM ampicillin [37], were incubated at 37 °C for 24 h. Since the natural blood glucose level in non-diabetic humans before a meal is about 90–120 g/dL, samples incubated in PBS-glucose (90 g/dL) were used as controls.

GAMMA IRRADIATION

After incubation, the samples were subjected to 10 Gy of gamma radiation at room temperature using a ^{137}Cs source with an absorbed dose rate 0.695 Gy/minute at the National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority.

NORMAL RED BLOOD CELLS HEMOLYSIS

The hemolysis of normal RBCs was monitored by measuring the absorbance of released hemoglobin from the cells in normal saline compared to the total cellular hemoglobin content. Ten μL of whole fresh blood was incubating in 5 mL normal saline. After 30 min, the samples were centrifuged at 3,000 rpm for 10 min, and the hemoglobin released was measured at 540 nm using UV-visible spectrophotometer CECIL 3041. The percentage of hemolysis was calculated by comparing it to the complete blood hemolysis [8].

DIELECTRIC MEASUREMENTS

The dielectric properties were determined using an HIOKI 3531 LCR (inductance, capacitance, and resistance) meter in the frequency range of 0.4 to 5 MHz. A parallel plate conductivity cell with platinum electrodes with a surface area of 4 cm^2 and a separating distance of 2 cm is used as the measuring cell. The RBCs were suspended in buffered saline (pH 7.4; conductivity 0.65 S/m), and the hematocrit was set at a value of 3 %. The samples were incubated in a water bath at 37 °C during the measurement [28]. The measured parameters were: capacitance *C*, resistance *R*, and conductance *G*, from which the permittivity ε' and AC conductivity σ, can be calculated as follows [19, 27]:

$$C = A\varepsilon' \varepsilon_o / d \tag{1}$$

$$\sigma = Gd/A \tag{2}$$

where *A* is the area of the electrode, *d* is the distance between the two electrodes, and ε_0 is the vacuum permittivity (F/m)**.** The permittivity can be formulated in complex form as:

$$\varepsilon^* = \varepsilon' - j\varepsilon'' \tag{3}$$

The permittivity real part represents the permittivity constant given by [21]:

$$\varepsilon' = \varepsilon_\infty + \frac{\varepsilon_S - \varepsilon_\infty}{1 + \omega^2\tau^2} \tag{4}$$

where ω is the angular frequency ($2\pi f$), ε_s is the limiting low frequency permittivity, and ε_∞ is the permittivity at the end of the dispersion (at f = 5 MHz). The permittivity is an indicator of the polar groups' polarizability in the electric field with associated time constant, τ*,* is called the relaxation time. This polarization does not occur instantaneously [16]. The permittivity imaginary part, ε" (the dielectric loss) is given by [21]:

$$\varepsilon''=\frac{(\varepsilon_S-\varepsilon_\infty)\omega\tau}{1+\omega^2\tau^2} \tag{5}$$

The membrane effective capacitance, C_{eff}, was calculated as previously discussed by Sezdi *et al.* [31] and Selim *et al*. [29].

MEMBRANE PERMEABILITY

The effects of the nonionic detergent Triton X-100 on the cell membrane were used to investigate membrane permeability. This detergent affects the RBCs' membrane in two ways: it changes ion permeability and membrane's molecular organization, which happen simultaneously but by two different mechanisms [5]. The membrane-detergent interaction was measured as the change in the turbidity of cell suspension as a function of detergent concentration. The principle of this test is based on the amount of scattered incident light when passes through cell suspension. The intensity of the scattered light depends on the number and size of the particles in the suspension. It can be defined in terms of the turbidity (T), which is the absorption coefficient due to scattering after subtracting the scattering due to solvent alone.

Different dilutions of Triton-X 100 solution ranging from 0.034 to 0.272 mM, equal to 0.002 to 0.016 percent v/v, were applied to diluted RBC suspension in PBS (pH 7.4). The transmittance of the samples was estimated at 600 nm after 20 minutes of incubation at 37 °C [30], and the turbidity, T, was determined using the following equation [7]:

$$I = I_0\, e^{-Tl} \tag{6}$$

where I_0 and I are intensities of the incident and transmitted light respectively, and l is the light pathlength through the sample [7].

The cell membrane permeability was measured by visible spectroscopy by recording the release of hemoglobin [13]. After incubation of the samples at 37 °C for 20 min, the samples were centrifuged at 3,000 rpm for 10 min, and the absorbance of the supernatant was measured at 540 nm [30]. The percentage of hemolysis, %H, was taken against complete hemolysis [2]:

$$\%H = \frac{A_{sample}}{A_{100\%lysis}} \times 100 \tag{7}$$

where A_{sample} and $A_{100\%lysis}$ are the absorbance of the hemoglobin released from RBCs in normal saline and, respectively, after complete hemolysis in distilled water.

STATISTICAL ANALYSIS

The experimental results were expressed as mean values ± standard deviation. The Student t-test was used to determine the significance of the variation between the values of the treated and control groups. Only the values with $p < 0.05$ were considered as statistically significant.

RESULTS

NORMAL HEMOLYSIS

Measuring the RBC hemolysis in normal saline represents a simple mechanical test which can give a rough indication about membrane damage. The normal hemolysis of the hyperglycemic group decreases (–20 %, $p = 0.05$) compared to control group, while the irradiated group showed significant increase by 22.6 % ($p = 0.04$). The hyperglycemic irradiated group showed a non-significant decrease (–2.22 %, $p = 0.09$) (Fig. 1).

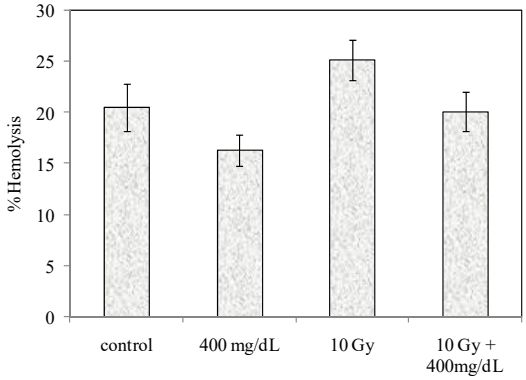

Fig. 1. Hemolysis of control, hyperglycemic, irradiated and hyperglycemic-irradiated red blood cells.

DIELECTRIC PROPERTIES

The relative permittivity, at low AC frequency region, depends on the surface charge distribution on the cell membrane [24]. The area under the dielectric loss curve depends on the total number of charges, irrespective to their relative positions [21]. The conductivity is related to the transport of ions through pores across the membrane [20]. Fig. 2 shows the relative permittivity, dielectric loss, and AC conductivity versus log frequency of AC in the case of RBC control. The relative permittivity is decreased non-significantly for the hyperglycemic group

and hyperglycemic irradiated group as compared to the control group, while it decreased significantly for the irradiated group. The area under loss peak decreased non-significantly for the hyperglycemic group while the hyperglycemic irradiated group showed non-significant increase as compared to the control group, being significantly decreased for the irradiated group.

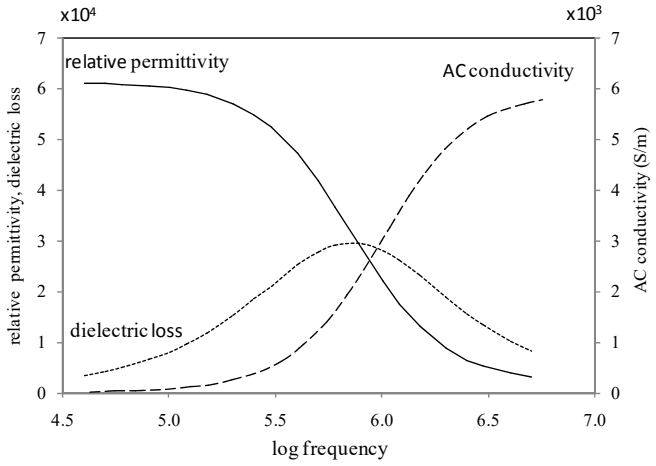

Fig. 2. Relative permittivity, dielectric loss and AC conductivity of control red blood cells.

Table 1

The relative permittivity (ε_s), area under loss curve, relaxation time (τ), AC conductivity at 5 MHz (σ_{5MHz}) and effective capacitance (C_{eff}) of control, hyperglycemic, irradiated and hyperglycemic-irradiated red blood cells

Groups	**Statistics**	**ε_s**	**Area under loss peak (dimensionless)**	**τ (s)**	**σ_{5MHz} (S/m)**	**C_{eff} (F)**
Control	Mean	6.59×10^{4}	4.92×10^{6}	2.32×10^{-7}	5.92×10^{3}	4.62×10^{-10}
	S. D.	2.95×10^{3}	4.36×10^{5}	9.80×10^{-9}	6.05×10^{2}	6.35×10^{-11}
400 mg/dL	Mean	6.05×10^{4}	4.78×10^{6}	2.27×10^{-7}	6.53×10^{3}	3.6710^{-10}
	S. D.	4.04×10^{3}	2.56×10^{5}	1.78×10^{-8}	7.59×10^{2}	2.54×10^{-11}
	p	0.163	0.391	0.130	0.043	0.007
10 Gy	Mean	4.63×10^{4}	3.41×10^{6}	1.93×10^{-7}	4.61×10^{3}	3.21×10^{-10}
	S. D.	1.48×10^{3}	1.09×10^{5}	1.59×10^{-8}	4.63×10^{2}	8.22×10^{-12}
	p	0.024	0.024	0.031	0.019	0.006
10 Gy + 400mg/dL	Mean	6.02×10^{4}	5.31×10^{6}	2.36×10^{-7}	6.25×10^{3}	3.19×10^{-10}
	S. D.	5.04×10^{3}	5.01×10^{5}	1.80×10^{-8}	2.61×10^{2}	3.60×10^{-11}
	p	0.256	0.257	0.408	0.180	0.002

The conductivity at 5 MHz is increased significantly for the hyperglycemic group while it decreased significantly for the irradiated group and hyperglycemic-irradiated group which showed a non-significant increase compared to the control group. The relaxation time, the time associated with the polarization process [16], decreased significantly for the irradiated group, and non-significantly for the hyperglycemic and hyperglycemic-irradiated groups. The total capacitance depends on the amount of charge stored across the membrane when the RBCs are exposed to an electric field. The effective membrane capacitance depends on the level of folding of the cell membrane [11]. It decreased significantly for all the studied groups (Table 1).

INTERACTION OF DETERGENT WITH CELL MEMBRANE

Triton-X is a non-ionic detergent that interacts with the cell membrane according to its spatial organization [23]. At higher concentration (0.01 %v/v), a complete solubilization of the membrane is achieved [8]. Turbidity is analyzed as a percentage of solubilization normalized to the turbidity of cell suspension without detergent. A plot of percentage turbidity against detergent concentration gives a sigmoidal curve, which can be characterized by three points:

1) Solubilizing detergent concentration (D_s): the point where the solubilization process begins (in this study, it was considered at 95 % turbidity).
2) Complete solubilization (D_c): the point at which all the membrane was dissolved giving a transparent solution (in this study it was considered at 5 % turbidity).
3) Average membrane solubilization ($D_{50\%}$): the concentration of detergent at which 50 % of the cell membrane is solubilized.

The membrane solubilization curves of Triton X-100 on RBCs for control, hyperglycemic, irradiated, and hyperglycemic-irradiated groups are shown in Fig. 3 (a, b, and c). The average membrane solubilization ($D_{50\%}$) decreased non-significantly for the hyperglycemic group, while the irradiated and hyperglycemic-irradiated groups decreased significantly compared to the control group (Table 2).

The solubilizing detergent concentration, D_s, showed significant increase for the hyperglycemic group, while for the irradiated group it decreased significantly. The hyperglycemic-irradiated group did not show significant change. The complete solubilization, D_c, for all treated groups decreased significantly (Table 2). The dispersion of the solubilization process can be obtained from the differentiation of this sigmoidal curve which yields a Gaussian distribution curve (Fig. 3d). It is the width at half maximum of the Gaussian curve (W %v/v). The dispersion of the hyperglycemic, irradiated and hyperglycemic-irradiated groups decreased significantly as compared to the control group (Table 2) which can be attributed to the decrease in membrane elasticity.

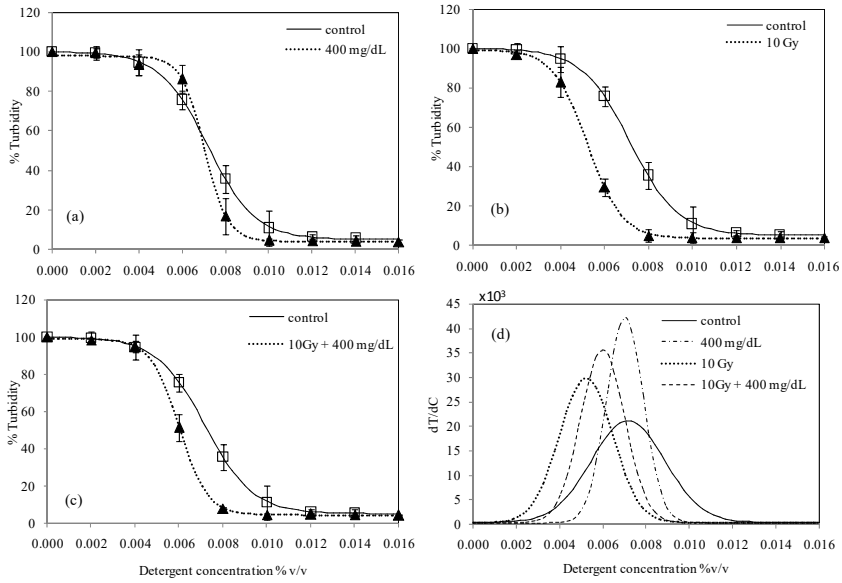

Fig. 3. The percentage turbidity versus detergent concentration for control, hyperglycemic (a), irradiated (b) and hyperglycemic-irradiated red blood cells (c) and the differentiation of the turbidity curves of the same groups (d).

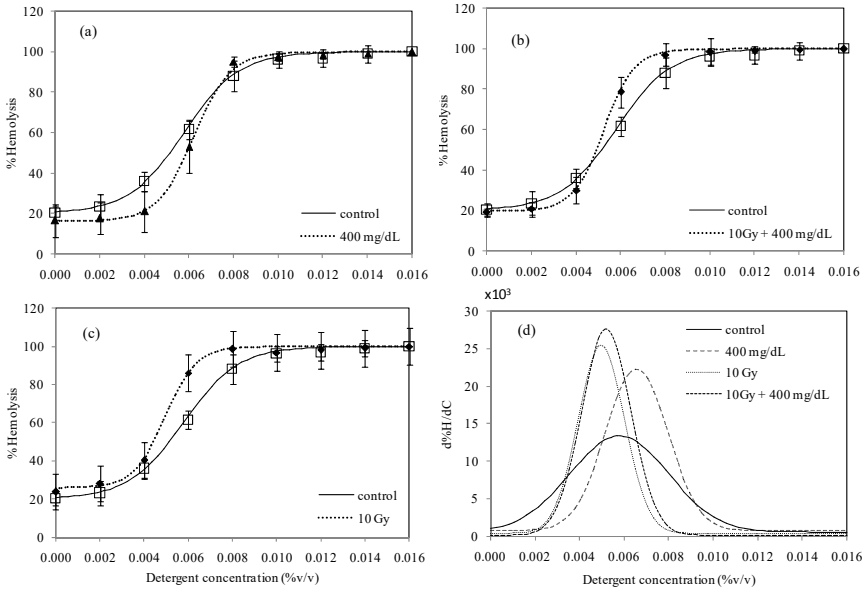

Fig. 4. The percentage of hemolysis versus detergent concentration for control, hyperglycemic (a), irradiated (b) and hyperglycemic-irradiated red blood cells (c) and the differentiation of the hemolysis curves of the same groups (d).

Table 2

The average membrane solubilization ($D_{50\%}$), solubilizing detergent concentration (D_s) and complete solubilization (D_c), dispersion of solubilization (W), dispersion of hemolysis (S) and average membrane hemolysis ($H_{50\%}$) for control, hyperglycemic, irradiated and hyperglycemic-irradiated red blood cells.

Groups	**Statistics**	$D_{50\%}$ **(%v/v)**	D_s **(%v/v)**	D_c **(%v/v)**	W **(%v/v)**	S **(g/L)**	$H_{50\%}$ **(g/L)**
Control	Mean	7.20×10^{-3}	5.64×10^{-3}	8.86×10^{-3}	3.30×10^{-3}	4.42×10^{-3}	5.73×10^{-3}
	S. D.	7.70×10^{-4}	7.84×10^{-4}	6.81×10^{-4}	2.18×10^{-4}	2.70×10^{-4}	6.55×10^{-4}
400 mg/dL	Mean	7.01×10^{-3}	6.39×10^{-3}	7.63×10^{-3}	2.17×10^{-3}	2.79×10^{-3}	6.46×10^{-3}
	S. D.	5.08×10^{-4}	4.81×10^{-4}	5.37×10^{-4}	2.29×10^{-4}	1.65×10^{-4}	5.43×10^{-4}
	p	0.371	0.035	0.002	0.027	0.040	0.030
10 Gy	Mean	5.26×10^{-3}	4.50×10^{-3}	6.19×10^{-3}	2.77×10^{-3}	2.10×10^{-3}	4.86×10^{-3}
	S. D.	4.83×10^{-4}	7.99×10^{-4}	7.24×10^{-4}	2.54×10^{-4}	1.41×10^{-4}	4.69×10^{-4}
	p	0.005	0.033	0.003	0.025	0.030	0.050
10 Gy + 400mg/dL	Mean	6.00×10^{-3}	5.27×10^{-3}	6.89×10^{-3}	2.50×10^{-3}	2.22×10^{-3}	5.26×10^{-3}
	S. D.	3.61×10^{-4}	6.12×10^{-4}	6.39×10^{-4}	2.88×10^{-4}	2.19×10^{-4}	3.33×10^{-4}
	p	0.036	0.238	0.015	0.005	0.020	0.012

CELL MEMBRANE PERMEABILITY

The hemolytic effect of the detergent depends on the osmotic fragility of RBCs [14], which depends on the cell shape, membrane flexibility, and cell area-to-volume ratio [33]. At low detergent concentration, it breaks the hydrophobic barrier of the cell membrane and increases its permeability to solutes [13]. Ions and associated water molecules enter into the cell and increase the internal osmotic pressure, resulting in swelling and subsequent rupture of the cell. This process is defined as colloid-osmotic lysis [22]. In this study, the percentage of hemolysis were normalized to 100 % hemolysis to facilitate the comparison between different samples without the interference of the hematocrit changes (Fig. 4 a, b, and c). The average membrane hemolysis ($H_{50\%}$) was considered as the detergent concentration producing 50 % hemolysis.

The differentiation of the hemolysis curve represents a Gaussian curve (the rate of hemolysis dH/dC versus detergent concentration) (Fig. 4 d), from which one can calculate the width at half maximum S (g/L). It reflects the dispersion of hemolysis process (lower dispersion indicates sudden rupture of the RBCs, while higher values of dispersion reflect the abnormal increase in the membrane elasticity). The average hemolysis for the hyperglycemic group increased significantly, while for the irradiated and hyperglycemic-irradiated groups it decreased significantly (Table 2).

DISCUSSION

EFFECT OF HYPERGLYCEMIA

The permittivity decreases with frequency increase in the range of 10 kHz – 10 MHz. This phenomenon is known as β-dispersion and occurs at the interface of cell membrane-electrolyte interfaces and it is related to cell membrane structure [16]. RBCs have negative surface charge, which create electrostatic repulsion that reduces their aggregation [12]. Hyperglycemia increases blood viscosity and aggregation rate of the RBCs [2]. Aggregation was shown to increase blood conductivity [6, 38] and lower the equivalent capacitance RBCs [3], which can explain the significant increase in conductivity and decrease in the effective capacitance reported in this study. The relative permittivity, the area under the loss peak, and relaxation time showed non-significant change. This can imply that the total number of dielectric dipoles did not change while their relative orientation changed significantly. Tura *et al*., [34] reported that the induced changes in blood impedance were not due to glucose concentration increase itself, but to the biochemical reactions triggered by hyperglycemia, which cause variations in the electrolyte balance across the membrane of RBCs. Also, the negative effect of glucose on RBCs can alter the phospholipid bilayer and result in modification of the integral proteins that can affect the physico-chemical properties of the blood [10]. The normal hemolysis was shown to be significantly decreased as compared to control which can be attributed to the change in membrane bilayer that can hinder the release of hemoglobin from RBCs. Hyperglycemia also appears to delay the membrane-detergent interaction with and significantly increase in the solubilizing detergent concentration (D_s). It was shown that the membrane detergent resistance increases with increasing of the rigidity of the acyl chain in the hydrophobic membrane region [25]. The glycosylation of membrane proteins was shown to affect the cell structure and function and create an unbalance resulting in cell destabilization [10]. These effects can explain the decrease in the dispersion of the solubilization and hemolysis processes and decrease in complete solubilization of the hyperglycemic group as compared to control.

EFFECT OF GAMMA RADIATION

The oxidative stress caused by the exposure to gamma radiation leads to alterations of cell membrane ionic permeability and increases in lipid peroxidation and proteolysis [15]. The exposure to gamma radiation was shown also to decrease the membrane surface charge and the number of dipoles in the lipid bilayer. The decrease in the effective capacitance implies that radiation affects the order of the lipid bilayer and the relative positions of the membrane dipole moments. It was

previously shown that radiation increases membrane osmotic fragility, a result of decrease in the membrane deformability [12]. In this study, the decrease in the average membrane solubilization ($D_{50\%}$), solubilizing detergent concentration (D_s), complete solubilization (D_c) and average membrane hemolysis ($H_{50\%}$) can be related to the loss of membrane integrity due to exposure to gamma radiation. The decrease in dispersion of both solubilization and hemolysis can be due to decrease of membrane elasticity and deformability.

COMBINED EFFECT OF HYPERGLYCEMIA AND GAMMA RADIATION

The obtained results of the hyperglycemic irradiated group seemed to be tricky. As shown in this work, the effects of hyperglycemia on some of the studied parameters are opposite to the effects of exposure to radiation. The non-significant change in the normal hemolysis of the hyperglycemic and irradiated group, the non-significant increase in the conductivity, relative permittivity, area under loss peak and relaxation time of the same group cannot be interpreted as no effect. The obtained results of the hyperglycemic and irradiated groups imply that both the effects of hyperglycemia and gamma radiation are superimposed. In the same time, some of the studied parameters showed significant changes from control. The effective capacitance (C_{eff}) showed significant decrease reflecting the decrease in the dipoles in the membrane and the change in their relative position due to the change in the spatial arrangement of the membrane structure. Also, the significant decrease in the average membrane solubilization ($D_{50\%}$), complete solubilization (D_c), the dispersion of both solubilization (W) and hemolysis (S) and the average membrane hemolysis ($H_{50\%}$) reflects the induced changes in the membrane constituents and organization as a result of hyperglycemia and exposure to gamma radiation.

CONCLUSIONS

This pilot *in vitro* study is intended to outline the importance of the investigation of the combined effects of hyperglycemia and gamma irradiation on RBCs. The choice of the suitable tests is crucial in the assessment of these effects.

The RBC membrane effective capacitance (C_{eff}), average membrane solubilization ($D_{50\%}$), dispersion of both solubilization (W) and hemolysis (S) and average membrane hemolysis ($H_{50\%}$) showed a significant correlation with hyperglycemia and gamma irradiation.

Although *in vitro* study of the effect of hyperglycemia and gamma irradiation has some limitations, it could be a guideline for the further *in vivo* studies.

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