EFFECT OF GAMMA RADIATION ON SOME BIOPHYSICAL PROPERTIES OF RED BLOOD CELL MEMBRANE

NABILA S. SELIM, O.S. DESOUKY*, SEHAM M. ALI*, I.H. IBRAHIM**, HODA A. ASHRY**

*Biophysics Laboratory, Radiation Physics Department, National Center for Radiation Research and Technology (NCRRT), EAEA, POB 29 Madinat Nasr Cairo, Egypt
**Physics Department, Faculty of Science, Ain Shams University, Cairo, Egypt

Abstract. The present work aims are to study the radiation effects on the red blood cell membrane from three different but correlated properties: electrical, mechanical and chemical, and to derive useful parameters for the evaluation of radiation effects. AC conductivity of cell suspension was measured in the frequency range 40 kHz to 5 MHz, the osmotic fragility of the membrane and solubilization of the membrane by detergent were also measured. Adult male rats were exposed to 1, 2.5, 3.5, 5, 7 and 9 Gy gamma radiation from Cs^{137} source. The results showed decrease in the AC conductivity, average osmotic fragility and average membrane solubilization. The effect of radiation on the red blood cell membrane was discussed.

Key words: Red blood cell membrane, radiation, osmotic fragility, conductivity, membrane solubilization.

INTRODUCTION

Biological membranes possess important roles in cells' life, which exceed being only an envelope for the cellular components. They rather regulate in and out transport of ions and metabolites, and govern intercellular communications. The general feature of the biological membranes is a phospholipid bilayer in which proteins and protein complexes are immersed. They are classified according to their position in the body and their functions. From the different types of body cells, the red blood cell possesses a unique structure. The mammalian red blood cells are anucleated, they are shaped like a disk with a biconcave cross-section, dumbbell resembling. The discoid shape provides maximum surface area for the same cell volume, to permit maximum gaseous change between tissues and the cells. During its existence, it must withstand great shearing forces as it travels many hundred miles through the circulatory system. During this circulation, it is

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forced through capillaries whose diameter, which is considerably narrower than the diameter of a resting cell. To pass these capillaries, it must be deformed. A too readily deformed cell would be easily injured under stress, resulting in hemolysis. A rigid cell would greatly increase the blood viscosity. Thus, the deformability of the red blood cell membrane is one of the conditions for its viability. This deformability is determined by the molecular and osmotic state of the cell. The red blood cell membrane is composed of a double layer asymmetrically organized lipid molecules acting as a boundary, in which integral proteins embedded. The structural and functional integrity of the lipid double layer and of the integral proteins depend on the association with a network of peripheral proteins (the cytoskeleton) attached to the inner membrane surface. The role of the cytoskeleton is to restore the shape of the red blood cell after mechanical deformation during its passage through the capillaries [9].

Red blood cell is not a very radiosensitive cell, thus choosing it is not a reflection of cellular radiation damage *in vivo* [10]. However, it is a suitable candidate for monitoring the radiation effect for many reasons. First of all, it is a representative sample for the whole body exposure, since it circulates all over the body, second its accessibility and ease in its separation to obtain cells with intact membrane. Also, being anucleated, it represents a useful model for measuring the membrane properties without the interference of intracellular membranes.

Gamma irradiation of red blood cells induces alterations at three different functional units of the membrane: lipid bilayer, protein components and cytoskeleton at the membrane surface [3]. In addition, radiation induces shortening in the lipid fatty acid chains by lipid peroxidation [18]. The production of hydroperoxides and cross-linkages in the membrane lipids can disorder the upper region of the bilayer favoring penetration of water and ending by hemolysis [16].

This work intends to study the radiation effects on the red blood cell membrane from three different but correlated properties: electrical, mechanical and chemical, using the following techniques: electrical conductivity of the cell suspension, AC conductivity, osmotic fragility and membrane solubilization of the membrane. The conductivity, in the frequency range 40 kHz to 10 MHz, takes into account the structural arrangement of the membrane, different transport processes occurring between the inner and outer media and permeability properties of the lipid bilayer [4]. The osmotic fragility of the membrane can be measured by placing the red blood cells in hypotonic salt solutions, the osmotic pressure exerted by the diffusion of water into the cells, makes them first swell and then hemolyse. The osmotic fragility measures the capacity of the cells to withstand hypotonicity and resist hemolysis, which is determined by their volume to surface area ratio [13]. The solubilization of the membrane by detergent is an induced transformation of the phospholipids bilayer and the proteins into mixed micelles of composed

detergent, phospholipids and membrane-bound proteins. This phase transformation depends on the molecular structure of the detergent and the composition of the membranes [12].

MATERIALS AND METHODS

GAMMA IRRADIATION

Adult male Wister rats weighing 200g were used. They were divided into 7 groups of 6 animals each. Rats were kept under standard conditions along the experimental period, 12/12 h light-dark regimen. Food and water were supplied daily *ad libitum.* All animals were housed according to the ethic rules in compliance with institutional guidelines. The irradiation process was carried out in the National Center for Radiation Research and Technology using Cs^{137} source for animals. The dose rate was (0.883 cGy/sec) at the beginning of the experiment. The animals were exposed to 1, 2.5, 3.5, 5, 7 and 9 Gy single doses. They were dissected 24 hours after exposure. The blood samples were withdrawn from the left ventricle of the heart using heparinized needles. The measurement was carried on blood extracted from each animal separated and afterwards average values were computed.

NORMAL RED BLOOD CELLS HEMOLYSIS

Normal red blood cells hemolysis was determined by measurement of hemoglobin released from the cells relative to the total cellular hemoglobin content. Ten μL of whole fresh blood was incubating in 5 mL normal saline for 30 min. The samples were centrifuged at 3000 rpm for 10 min, and the supernatant was measured spectrophotometrically at 540 nm. The percentage of hemolysis was taken against complete blood hemolysis [7].

$$\%H = \frac{A_{\text{sample}}}{A_{100\%\text{lysis}}} \times 100 \tag{1}$$

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

DETERMINATION OF HEMATOCRIT

The determination of the hematocrit was performed as follows: blood samples, in the hematocrit microcapillary tube (75 mm / 75 μL), were centrifuged for 5 minutes at 11,500 rpm. Then the hematocrit values were determined by means of the microcapillary tube reader [11].

MEAN CORPUSCULAR VOLUME (*MCV*), AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (*MCHC*)

The mean corpuscular volume (*MCV*) in femtoliter expresses the average size of the red blood cells. It is related to the hematocrit (*Hct*) by the following relations [11]:

$$MCV = \frac{Hct}{RBC\text{s count(million per litre)}} \tag{2}$$

while the mean corpuscular hemoglobin concentration (*MCHC*) in mg/mL is a measure of the concentration of hemoglobin in a given volume of packed red blood cell [11]. It can be calculated as follows:

$$MCHC = \frac{Hb\ \text{conc}\ (\text{mg/mL})}{Hct} \tag{3}$$

The hemoglobin concentration was evaluated using Drabkin's reagent and hemoglobin standard, obtained from EAGLE Diagnostics, USA.

CONDUCTIVITY MEASUREMENTS

The conductance was measured using LCR meter type HIOKI 3531, Japan, in the frequency range 40 KHz to 5 MHz. The measuring cell is a parallel plate conductivity cell with platinum black electrodes with area 4 cm^2 and separating distance 2 cm. The measured parameters were the capacitance *C* and resistance *R*, from which AC conductivity σ can be calculated as follows [17–19]:

$$C = A\varepsilon'\varepsilon_o / d \tag{4}$$

where *A* is the area of the electrode, *d* is the distance between the two electrodes, ε' is the sample permittivity and ε_o is the vacuum permittivity. The dissipation factor ($\tan\delta$) is given by:

$$\tan\delta = 2\pi fRC \tag{5}$$

where *f* is the frequency of applied voltage in cycles per second.
It is related to the dielectric loss ε'' by:

$$\varepsilon'' = \varepsilon' \tan\delta \tag{6}$$

$$\sigma_{AC} = \omega\varepsilon_o\varepsilon'' \tag{7}$$

where ω is $2\pi f$.

The blood samples were centrifuged at 3 000 rpm for 5 minutes. The plasma and buffy coat were removed by aspiration. They were washed twice in buffered saline and separated by centrifugation at 3000 rpm for 10 minutes. The red blood cells were re-suspended in isotonic buffered sucrose (0.3 M sucrose in phosphate buffer pH 7.4, and conductivity 0.223 S/m), and the hematocrit was adjusted at 3%. The samples were incubated in water bath at 37 ºC during measurement.

OSMOTIC FRAGILITY MEASUREMENTS

The degree of hemolysis can be quantitatively evaluated from the osmotic fragility test [6]. Whole blood samples were added to the hypotonic buffer saline in the proportion of 1:100 respectively. Hypotonic saline buffered to pH 7.4, with different concentrations (1, 2.5, 3.5, 5, 6.5, 7 and 9 g/L) was used. The samples were incubated for 30 minutes at 37 ºC, and centrifuged at 3000 rpm for 5 min., to precipitate the nonhemolyzed red blood cells. The osmotic lysis of red blood cells is detected by the release of hemoglobin into the extracellular fluid. The amount of hemoglobin appearing in media was determined colorimetrically according to the method reported by Dacie and Lewis 2006 [5]. The experimental curves were normalized to 100% hemolysis to facilitate the comparison between different samples without the interference of the hematocrit changes. The fragility curve can be evaluated by the average osmotic fragility (H_{50}) (the NaCl concentration producing 50% hemolysis). Other parameters can be obtained from the differentiation of the fragility curve, which represents a Gaussian curve (the rate of hemolysis $\mathrm{d}H/\mathrm{d}C$ versus NaCl concentration) (Fig. 4). These parameters are position, width, height and area of the peak. The position on the x-axis is equivalent to the average osmotic fragility (H_{50}). The width at half maximum reflects the dispersion of hemolysis process (lower dispersion than normal indicates sudden rupture of the *RBC*s, while higher values of dispersion reflect the abnormal increase in the membrane elasticity). The peak's height represents the maximum rate of hemolysis $(\mathrm{d}H/\mathrm{d}C)_{max}$ reached by the sample. The area under curve represents the rate of hemolysis of red blood cells.

MEMBRANE SOLUBILIZATION TEST

Triton X-100 is a non-ionic surfactant (commonly denoted detergent) most frequently used in wide applications to biomembranes. Its high solubilizing capacity is related to its ability to form mixed micelles with membrane lipids and proteins. The choice of Triton X-100 as detergent for the solubilization of red blood cells membranes lies on the fact that it protects the cells against hypo-osmotic lysis.

It is known that triton X-100 becomes hemolytic above a certain concentration range (about 0.01%, v/v), which provides the study of their interaction with membrane, in the low concentration ranges, without interference of the lysis effect [12]. The samples were prepared as for dielectric measurements. The turbidity (T) is given by:

$$I = I_0 e^{-Tl} \tag{8}$$

where I_0 and I are the incident and transmitted light, respectively, and l is the length of the light path through the scattering solution [13]. The transmittance was measured at (600 nm) using UV-visible spectrophotometer CECIL-3041. Turbidity measurement of the membrane as a function of the added detergent is analyzed in terms of percent solubilization normalized to the turbidity of cell suspension without detergent. The turbidity curve can be characterized by:

– solubilizing detergent concentration (D_s): the point at which the turbidity starts decreasing markedly (in this study it was considered at 95% turbidity);

– complete solubilization (D_c): the point at which all the membrane was transformed into mixed micelles yielding a transparent solution (in this study it was considered at 5% turbidity);

– average membrane solubilization (D_{50}): the concentration of detergent at which 50 % of the lipid bilayer is solubilized.

DATA FITTING

The fitting of the experimental data (osmotic fragility and membrane solubilization) was carried out by the Origin software. The applied two functions are:

– Gaussian function:

$$y = y_o + \frac{A}{S\sqrt{\pi/2}} e^{-2\frac{(x-C)^2}{S^2}} \tag{9}$$

where y_o is the offset, A, C and S are the area under the peak, the center and the width, respectively, and

– sigmoidal function:

$$y = \frac{x_i - x_f}{1 + \mathrm{e}^{(x-x_o)/\lambda}} + x_f \tag{10}$$

where x_i and x_f are are the initial and final values of hemolysis and turbidity (at 95% and 5% respectively), and x_o is the center (H_{50} and D_{50}, respectively) and λ is a constant.

RESULTS

NORMAL HEMOLYSIS

Normal red blood cells retain intracellular hemoglobin. The hemoglobin release from incubated cells in saline solution gives rough indication about membrane damage. The exposure to gamma radiation with different doses resulted in significant increase in the hemoglobin release from the red blood cells as the dose increases (Fig. 1). Meanwhile, the mean corpuscular hemoglobin concentration (*MCHC*) decreased significantly with dose, these results showing an increasing damage in the cell membrane as a function of dose increase.

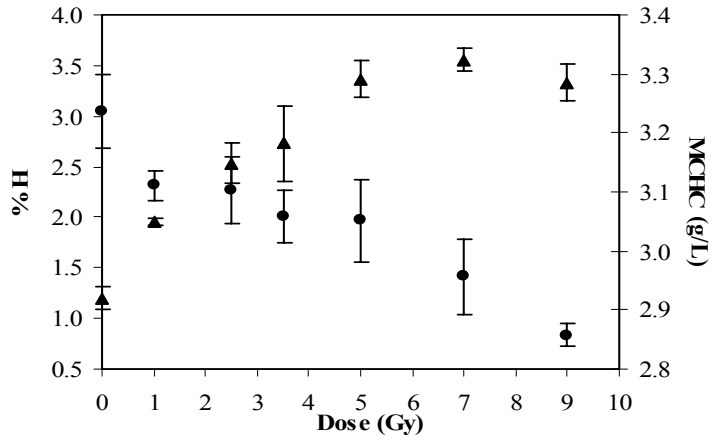

Fig. 1. The percentage of hemolysis (▲) and *MCHC* (●) versus dose for control and irradiated groups.

AC CONDUCTIVITY

The conductivity of red blood cells suspension showed a significant decrease as a result of exposure to gamma radiation. It decreased as the irradiation dose increased up to the lethal dose, 9 Gy (Fig. 2).

OSMOTIC FRAGILITY TEST

The present study shows a shift to the right of the hemolysis curve with the increase of the dose, indicating increase in the average osmotic fragility (H_{50}) as shown in Table 1. At the same time, the dispersion of hemolysis (S) decreased significantly with dose increase.

The results show an increase in the maximum rate of hemolysis $(dH/dC)_{max}$ with concomitant shift of the peaks center (H_{50}) toward higher values of NaCl concentration. Also, there is an increase in the rate of hemolysis (A) of red blood cells as the dose increases (Table 1).

Table 1

The width (S), height (P), area (A), and center (H_{50}) of the Gaussian peaks for control and irradiated groups

Dose (Gy)	S (g/L)	P (L/g)	A (g/L)	H_{50} (g/L)
Control	1.93±0.017	72.84±2.69	91.80±2.92	3.53±0.127
1.0	1.85±0.036	75.73±3.98	93.58±2.92	3.54±0.133
2.5	1.852±0.029	79.26±1.62	99.86±2.45	3.59±0.048
3.5	1.82±0.018	81.25±4.96	106.48±7.72	3.66±0.078
5.0	1.79±0.020	85.79±4.38	116.63±2.75	3.82±0.108
7.0	1.69±0.017	90.07±2.15	125.49±5.04	3.91±0.149
9.0	1.67±0.048	101.24±5.52	125.49±2.57	3.99±0.124

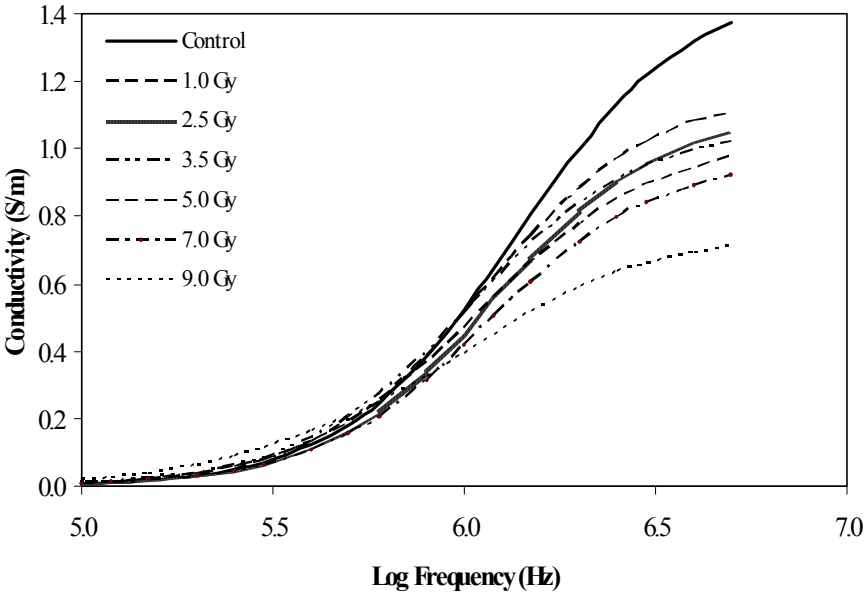

Fig. 2. AC conductivity for control and irradiated groups.

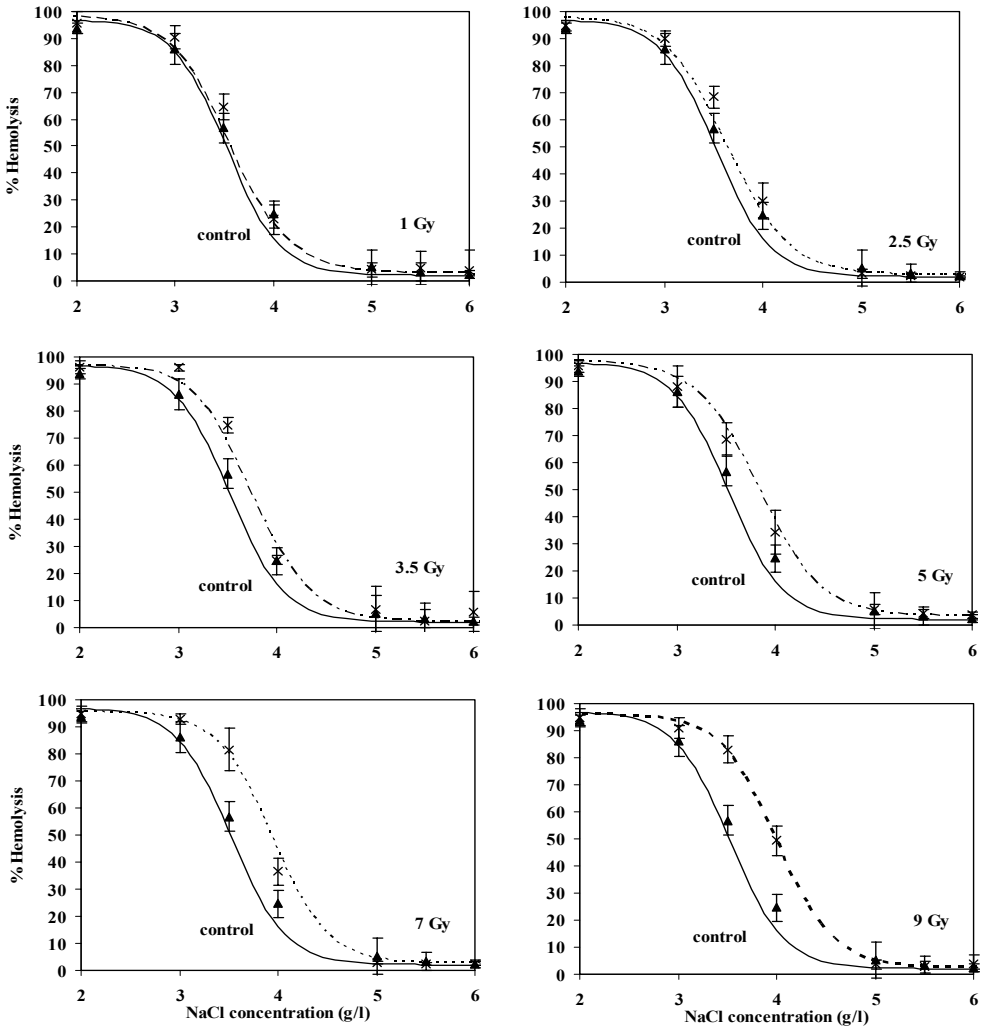

Fig. 3. Fragility curves for control (▲ and solid line) and irradiated (x and dashed line) groups. The dot with error bar is the experimental data and the solid and dotted line are the fitted data.

MEMBRANE SOLUBILIZATION

The changes in cell membrane were tested chemically by interaction with detergent. The solubilization process of membrane is an induced transformation of nearly flat phospholipid bilayers containing embedded proteins into mixed micelles of composed detergent, phospholipids and membrane-bound proteins. For the phase transformation (micellization) to occur, the added detergent distributes between the membrane bilayer and the aqueous medium. It induces spontaneous phase transformation when the ratio of the detergent to lipid bilayer exceeds a critical

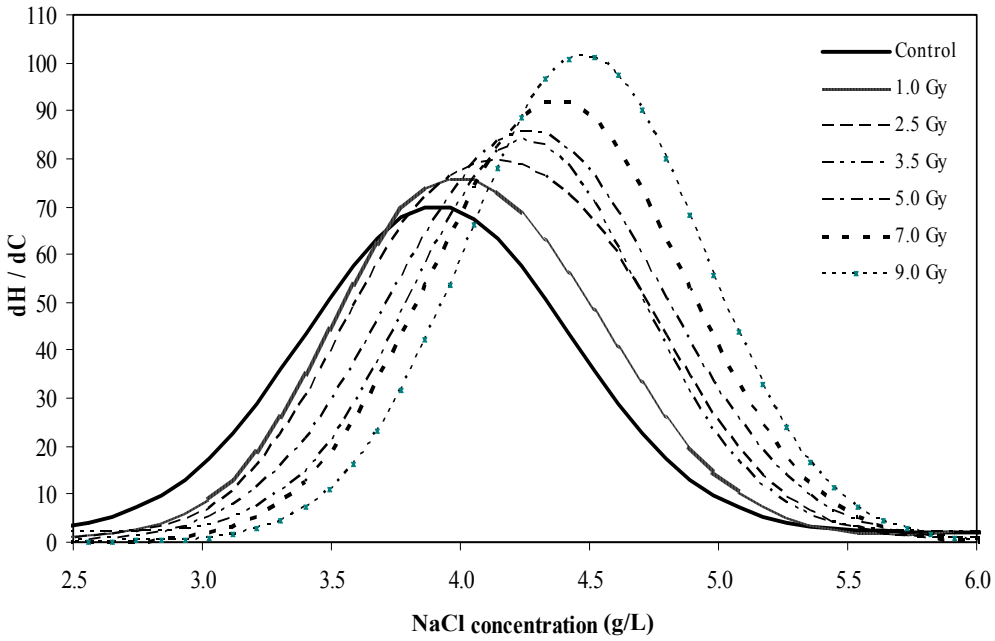

Fig. 4. The rate of hemolysis versus NaCl concentration for control and irradiated doses.

value [12]. In this study, this critical value will depend on the membrane structure (all other factors: temperature, type and concentrations of the detergent were kept constant). In the membrane solubilization curve (turbidity versus detergent concentration) (Fig. 5), the turbidity is initially affected only slightly by adding the detergent. Further detergent addition results in a large decrease of turbidity until complete solubilization is obtained (a point when additional detergent has no effect on the turbidity of the suspension). Throughout the range of detergent addition, which causes large decrease of turbidity, it may be assumed that lamellar and micellar structures co-exist.

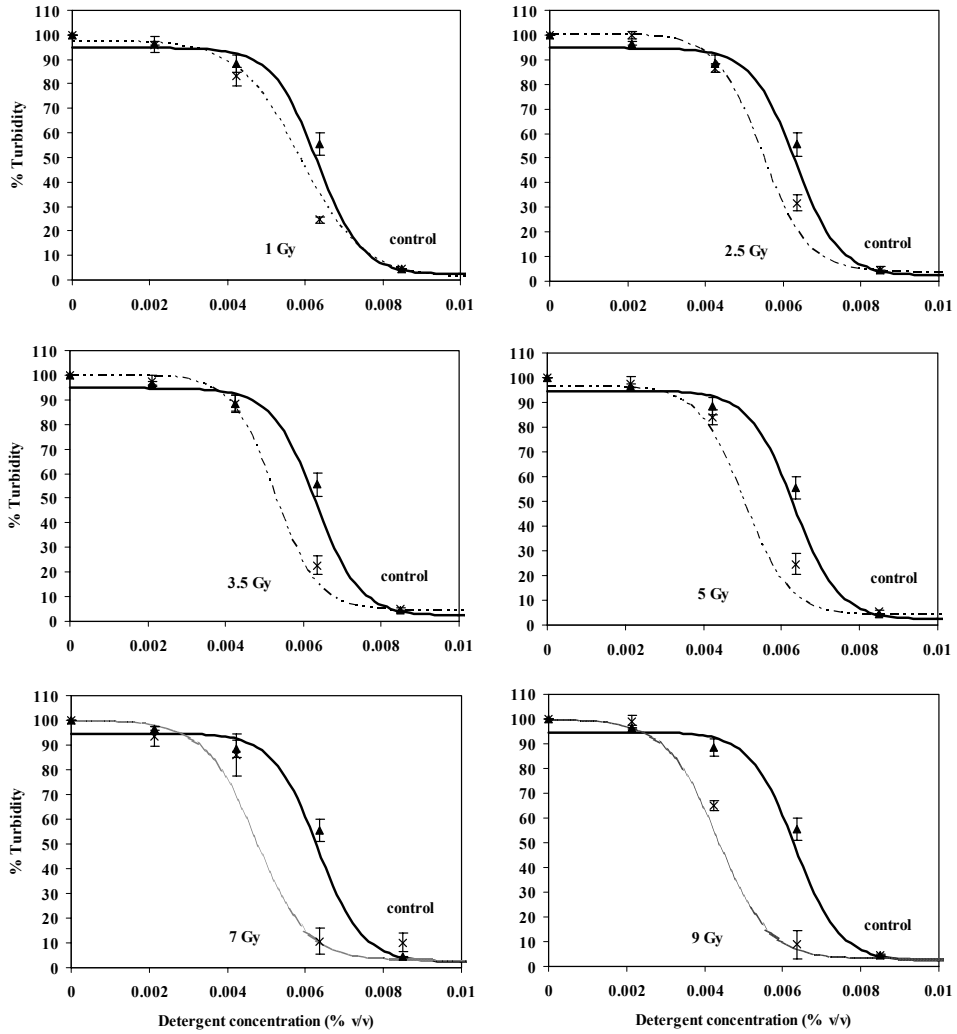

Fig. 5. The percentage of turbidity versus detergent concentration for control (▲ and solid line) and irradiated (x and dashed line) groups. The dot with error bar is the experimental data and the solid and dotted line are the fitted data.

Exposure to gamma radiation resulted in shift in a the membrane solubilization curve toward lower detergent concentration (Fig. 5). The detergent concentration (D_s), complete (D_c) and average membrane solubilization (D_{50}) exhibited a significant decrease as a function of dose increase (Table 3). Since the value of D_s depends to a large extent on the cell volume, an important conclusion can be derived from plotting the radiation induced change in both D_s with the mean corpuscular volume (*MCV*) (Fig. 6). The figure shows that as the *MCV* increased, D_s decreased.

Table 2

The average membrane solubilization (D_{50}), solubilizing detergent concentration (D_s) and complete solubilization (D_c) for control and irradiated groups

Dose (Gy)	D_{50} (% v/v)	D_s (% v/v)	D_c (% v/v)
Control	$6.34\times10^{-3}\pm1.90\times10^{-4}$	$4.64\times10^{-3}\pm3.45\times10^{-4}$	$8.38\times10^{-3}\pm1.59\times10^{-4}$
1	$5.85\times10^{-3}\pm3.25\times10^{-4}$	$3.66\times10^{-3}\pm3.44\times10^{-4}$	$8.24\times10^{-3}\pm5.60\times10^{-4}$
2.5	$5.38\times10^{-3}\pm2.24\times10^{-4}$	$3.44\times10^{-3}\pm2.20\times10^{-4}$	$7.46\times10^{-3}\pm2.21\times10^{-4}$
3.5	$5.36\times10^{-3}\pm5.42\times10^{-5}$	$3.35\times10^{-3}\pm1.05\times10^{-4}$	$7.42\times10^{-3}\pm2.05\times10^{-4}$
5	$5.11\times10^{-3}\pm3.81\times10^{-4}$	$3.33\times10^{-3}\pm1.67\times10^{-4}$	$7.08\times10^{-3}\pm1.41\times10^{-4}$
7	$4.59\times10^{-3}\pm3.01\times10^{-4}$	$3.23\times10^{-3}\pm1.67\times10^{-4}$	$6.67\times10^{-3}\pm3.34\times10^{-4}$
9	$4.47\times10^{-3}\pm2.66\times10^{-4}$	$2.91\times10^{-3}\pm1.21\times10^{-4}$	$6.35\times10^{-3}\pm1.71\times10^{-4}$

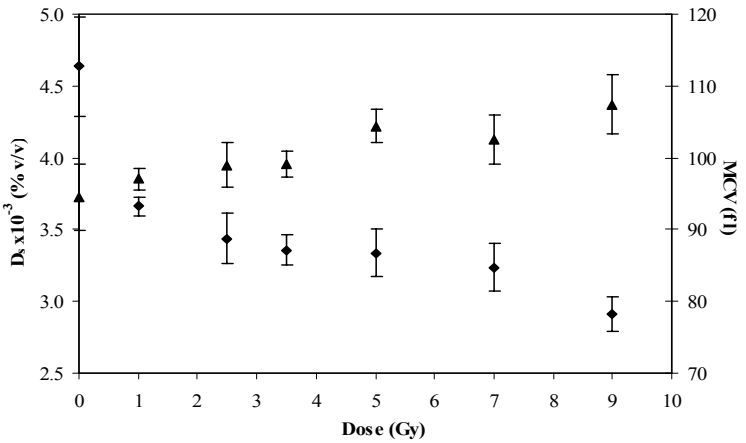

Fig. 6. The solubilizing detergent concentration (D_s) (♦) and mean corpuscular volume (*MCV*) (▲) for control and irradiated groups.

DISCUSSION

Gamma radiation affects biological membrane in different ways. To study and monitor the effects of radiation one needs a series of analyses to explore the different damaging events that may occur. Normal red blood cells retain intracellular hemoglobin. The hemoglobin release from incubated cells in saline solution gives rough indication about membrane damage. Free radicals formed during irradiation can cause a variety of membrane changes including lipid

peroxidation, hydrolysis of phospholipids head groups, lipid-lipid crosslinks, disulfide bridge formation and amino acid residue damage in membrane proteins and lipid-protein crosslinks [11]. Changes in membrane structures can also affect the cytoskeleton. The combined effects of free radicals on the red blood cell membrane and cytoskeleton may contribute to the leak of hemoglobin out of the cells. The hemolysis of the red blood cells reflects the loss of integrity of the cells which can lead to the liberation of intracellular hemoglobin [18]. In addition, ionizing radiation was reported to cause oxidation of the sulphydryl groups to the corresponding dithiols and induce conformational changes of membrane proteins [8].

The AC conductivity, in the β-dispersion region, gives information about the amount of freely movable ions inside cells, permeability properties of the lipid bilayer, protein-mediated transport processes, and existence of bound charges on the external and internal membrane surfaces. It describes the physico-chemical effects occurring not only within the membrane, but also near the membrane surface, where the protein organization appears to be one of the important factors in the functioning and in the chemical reactions with the ionic environment [4]. The conductivity of red blood cells was shown to be sensitive to gamma radiation, and this effect is enhanced by the presence of hemoglobin molecule [3]. The decrease in conductivity observed in our study reflects the permeability damage of the cell membrane with the subsequent loss of ions, electrolytes and intracellular components.

Radiation was, also, shown to affect the biochemical structure of the red blood cells membrane. It increases membrane cholesterol level, causes oxidation of membrane protein, thiol groups and lipid peroxidation, and impairment of membrane permeability barrier [20]. Radiation-induced changes in permeability and membrane elasticity were examined by the osmotic fragility test. Osmotic fragility is considered to be a function of the osmotic pressure gradient between intra and extracellular media, initial surface area to volume ratio, membrane tension of hemolysis and ionic content of the cell [1]. The observed decrease in the dispersion of hemolysis (S) can be attributed to the presence of unusually flattened red cells in which the surface area to volume ratio is increased [10].

It has been reported that alteration in the lipid composition of the red blood cell membrane has only minor effects on the mechanical behavior, whereas alterations in membrane skeletal proteins play a major role [14, 15]. Oxygen free radicals also alter cation permeability and reduce red blood cells deformability, disturbance in microrheological properties of red blood cell membrane (increase membrane rigidity) [2]. Hence, the change induced in the osmotic fragility reflects the damaging effects on the cytoskeleton. Our results show that the fragility of red blood cell membrane increased significantly as a function of dose increase. This appears from the increase in the rate of hemolysis (A), the maximum rate of hemolysis (P) reached by each group. Also, the average osmotic hemolysis (H_{50}) and the center of the Gaussian peak (C) which shifted toward higher NaCl concentration.

The radiation induced damage in the membrane permeability can facilitate the diffusion of detergent molecule within the cell membrane as we can see from the average membrane solubilization (D_{50}). The mean corpuscular volume (*MCV*) showed a significant increase as the dose increased (Fig. 6), and the decrease in the dispersion of hemolysis reflects the presence of unusually flattened cells (i.e. the well-defined discoid shape vanished) as we have reported previously. The change in the shape of red blood after exposure to 6 Gy gamma radiation altered cell permeability, and developed echinocytes and spherocytes with the progressive appearance of the regularly spaced spicules on cell surface [20]. The progressive appearance of the regularly spaced spicules on cell surface can increase the interaction of the detergent with membrane components and facilitate the transformation phase, thus decreasing the solubilizing detergent concentration with dose (D_s) as shown in Table 3. The decrease in the complete solubilization can be attributed to the radiation induced damage in the red blood cells membrane, which facilitate the membrane interaction and decrease the detergent concentration needed to solubilize the membrane completely as can be shown from the shift in the membrane solubilization curve toward lower detergent concentration (Fig. 5).

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