ROLE OF MELATONIN IN REDUCING MORPHOLOGICAL CHANGES IN RED BLOOD CELL INDUCED BY GAMMA IRRADIATION

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Abstract. Radioprotective effect of melatonin against morphological changes in red blood cell of female mice induced by ionizing radiation during cancer treatment was studied. This study was achieved by examining the osmotic fragility and blood film of RBCs membrane. The hemolysis percentage and osmotic fragility of RBCs were found to increase by injection of animals with Ehrlich tumor cells or exposing to γ-irradiation as compared to the control animals. RBCs membrane elasticity was found to decrease as its fragility increases. The images of RBCs indicated some irregularity in the shape of the cells in addition to the sticking of the cells together after injection with tumor cells or exposing to γ-irradiation or both. This shape irregularity in addition to the changes which occurred in RBCs membrane was found to be omitted by treating the animals with 10 and 30 mg/kg of melatonin.

Key words: Red blood cells, γ-irradiation, antioxidant, melatonin, osmotic fragility, blood film.

INTRODUCTION

Radiation therapy works by damaging the DNA of cells. The damage is caused by a photon, electron, proton, neutron, or ion beam directly or indirectly ionizing the atoms which form the DNA chain. Indirect ionization happens as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which damage the DNA, besides DNA, lipids and proteins are also attacked by free radical. Thus drug or antioxidant that scavenge or inhibit the formation of these radicals may have relevance to cancer patients by ameliorating damage of normal tissue exposed to ionizing irradiation therapy.

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Biophysical and biochemical changes in the characteristics of rat blood exposed to combined alternating and static magnetic fields were studied [15]. The results indicated that exposure of animals to magnetic field results in an increase in RBCs osmotic fragility, decrease in its membrane elasticity, partial change in Hb molecular structure.

The effect of both gamma rays and alpha particles on human erythrocytes to assess radiation induced membrane damage and hemoglobin oxidation and denaturation was studied [12]. The results indicated that alpha particles proved to be less efficient than the gamma rays. The time dependence of hemolysis showed clear differences with the gamma rays which are faster than alpha particles. Biophysical properties of RBCs cellular membrane were investigated. RBCs membrane was studied through the measurement of osmotic fragility and blood film. Results indicate that RBCs cellular membrane suffered pronounced injuries after injection with ^{99m}Tc for imaging with gamma camera [3].

The oxidative processes and antioxidative defense mechanisms in the aging brain were studied [13]. The results indicated that the vitamin antioxidants, vitamin E and vitamin C protect the brain from oxidative stress by directly scavenging toxic radicals. Also the pineal hormone melatonin is more effective in scavenging the highly toxic free radical (hydroxyl radical) and more efficient than vitamin E in neutralizing the peroxyl radical.

Osmotic fragility and lipid peroxidation of irradiated erythrocytes in the presence of radio protectors were studied [4]. The results indicated that osmotic fragility curves shifted toward higher NaCl concentration. In the presence of radio protectors significant protection occurs. Also the participation of free active form of oxygen in the damage of porcine erythrocytes was studied [8]. The result indicated a contribution of exogenous free radicals in radiation damage to porcine erythrocytes. In the presence of biological and chemical radioprotectors a protective effect was observed. The enhancement of radiation induced oxidative stress and cytotoxicity in tumor cells by ellagic acid (EA) was studied [16]. The results indicated that ellagic acid was found to generate (ROS) in tumor cells to increase its cytotoxicity when treated by radiation. Also EA was found to protect against radiation induced oxidative stress in splenic lymphocytes of tumor transplanted mice.

Melatonin and vitamin D3 increase TGF-β release and induce growth inhibition in breast cancer cell cultures [1]. The results indicated that both melatonin and vitamin D3 alone and in combination reduce growth of cancer cells but melatonin alone was ineffective for inhibiting cancer cell growth. So the combination therapy with melatonin and vitamin D3 is more effective in the treatment of breast cancer.

The effects of the potential radioprotective properties of pharmacological doses of melatonin against organ damage induced by whole body irradiation (IR) in rats have been reported [2]. The animals received two doses of 2 and 4 Gy (whole-body dose) and have been treated daily (four days prior irradiation) with melatonin dose 10 mg/kg. Tissue levels of malondialdehyde (MDA) – an index of lipid peroxidation and glutathione (GSH) – a molecular marker of antioxidant processes were estimated. The results indicated that after IR irradiation tissue levels of MDA were elevated, while GSH levels were reduced in all organs. On the other hand, melatonin reduces the levels of MDA and increased the GSH levels significantly.

The protective effects of melatonin on the ionizing radiation induced DNA damage in the rat brain were studied [17]. The results indicated that a significant increase in DNA damage was found in the radiation treated rat brain. Pre-treatment of rats with interaperitoneal dose of 100 mg/kg melatonin provided a significant decrease in the DNA strand breakage and lipid peroxidation. Treatment with melatonin can protect brain cells from oxidative damage induced by ionizing radiation.

The prophylactic role of melatonin against radiation induced damage in mouse cerebellum with special reference to Purkinje cells was studied [14]. The results indicated that the antioxidative properties of melatonin resulting in its prophylactic property against radiation induced biochemical and cellular alternation in the cerebellum. The findings support the idea that melatonin may be used as an anti irradiation drug due to its potent free radical scavenging and antioxidative efficacy.

The aim of the present study is to examine the protective effects of melatonin against the morphological changes which are expected to be formed in the red blood cells of female mice induced by ionizing radiation during cancer treatment. This study will be achieved through the osmotic fragility and blood film of RBCs membrane.

MATERIALS AND METHODS

ANIMALS

In this study 120 female mice having weight ranged between 20–25 g maintained at animal house laboratory, National Research Center under the normal conditions of water and diet supply. The animals were divided into five main groups with different subgroups:

A – Control group:

A1 – Normal animals.

A2 and A3 – Animals treated with 10 mg/kg and 30 mg/kg melatonin.

B – Cancer group:

B1 – Animals injected with Ehrlich tumor cells.

B2 and B3 – Animals injected with Ehrlich tumor cells treated with 10 mg/kg and 30 mg/kg melatonin.

C – Irradiated group:

C1 – Animals exposed to γ-irradiation.

C2 and C3 – Animals treated with 10 mg/kg and 30 mg/kg melatonin one day before exposing to γ-irradiation.

D – Irradiated cancer treated group:

D1 – Animals with solid tumor exposed to γ-irradiation.

D2, D3 – Animals with solid tumor treated with 10 mg/kg and 30 mg/kg melatonin one day before exposing to γ-irradiation.

After that all animals were sacrified and blood samples from all animals in a group were pooled.

EHRLICH TUMOUR CELLS INJECTION

A suspension of 2,106 cells/mL isolated from Ehrlich ascites carcinoma (Breast Cancer Cells) in mice was supplied and prepared in the virology unit, Cancer Biology Department in the National Cancer Institute, Cairo University. The animals were injected into shoulder with 2 mL of suspension. Two weeks post injection the solid tumor surface area was about 2–2.5 cm^2.

MELATONIN TREATMENT

Melatonin (N-acetyl-5-methoxytryptamine), from Mallinckrodt Inc., Paris, Kentucky, was used. It is a hormone of the pineal gland. The animals were injected with freshly prepared melatonin dose of 10 mg/kg and 30 mg/kg body weight one day before γ- irradiation or Ehrlich tumor cells injection. Melatonin was prepared in 0.9% NaCl/ethanol (vol/vol, 20/1) about 0.4 mg of melatonin dissolved in 1 mL of 0.9% NaCl/ethanol. All injections were administered intra-peritoneally in a volume of 0.025 mL/g body weight.

IRRADIATION FACILITIES

The animals were placed in a carton box W 20 × L 20 cm and depth of 5 cm many small holes were made in the box sides to enable the animals to live during the experiment of irradiation. The doses delivered to the animals were calculated and adjusted at the middle of the box width (2.5 cm from the surface) in order to be sure that all the animals receive a uniform and homogeneous field of irradiation.

The whole body animals were exposed to 6 Gy γ-irradiation emitted from linear accelerator (Elekta Precise Clinical Linear Accelerator, Elekta, Sweden) cited in the Radiotherapy and Nuclear Medicine Department of the National Cancer Institute, Cairo University. Dose rate was about 200 cGy per monitor unit at 100 cm SSD and 10×10 field area.

OSMOTIC FRAGILITY TEST

Osmotic fragility is the resistance of RBCs hemolysis to osmotic change that is used to evaluate RBCs fragility. Whole blood was added to varying concentrations of buffered sodium chloride solution and allowed to incubate at room temperature (25 °C). The amount of hemolysis is then determined by reading the absorbance of the supernatants of a mixture of the samples from all animals in a group at 540 nm on the spectrophotometer (Jasco UV/visible spectrometer type V-570). A normal control blood as well as the treated blood have been tested under the same condition [5].

The percent hemolysis was calculated for each supernatant as follows:

$$\%\text{Hemolysis} = 100 \times (\text{O.D. of supernatant}) / (\text{O.D. of supernatant no. 14}) \quad (1)$$

where: O.D. is the optical density, test tube No. 14 represents 100% hemolysis.

NaCl solution concentration (in %) ranged between 0.900, 0.765, 0.675, 0.585, 0.540, 0.500, 0.450, 0.400, 0.360, 0.315, 0.270, 0.180, 0.090 and 0.00.

BLOOD FILM

The blood film was done by a manual method to make wedge blood smears, a small drop of blood in the middle of the slide and spread the blood drop by using another glass slide. Blood smears stained with Lishman dye for about 5 minutes, then washed with water. Blood film was seen and scanned using light microscope through oil emersion lens. The image was picked up through digital camera (Yashica, EZ8032, 8.2 mega pixels).

RESULTS AND DISCUSSION

OSMOTIC FRAGILITY

Results of osmotic fragility measurements illustrate the variation of the percentage hemolysis as a function of the percentage of NaCl concentration in buffer solution for the red blood cells collected from animals of different groups A, B, C, and D as shown in Figures 1a to 4a.

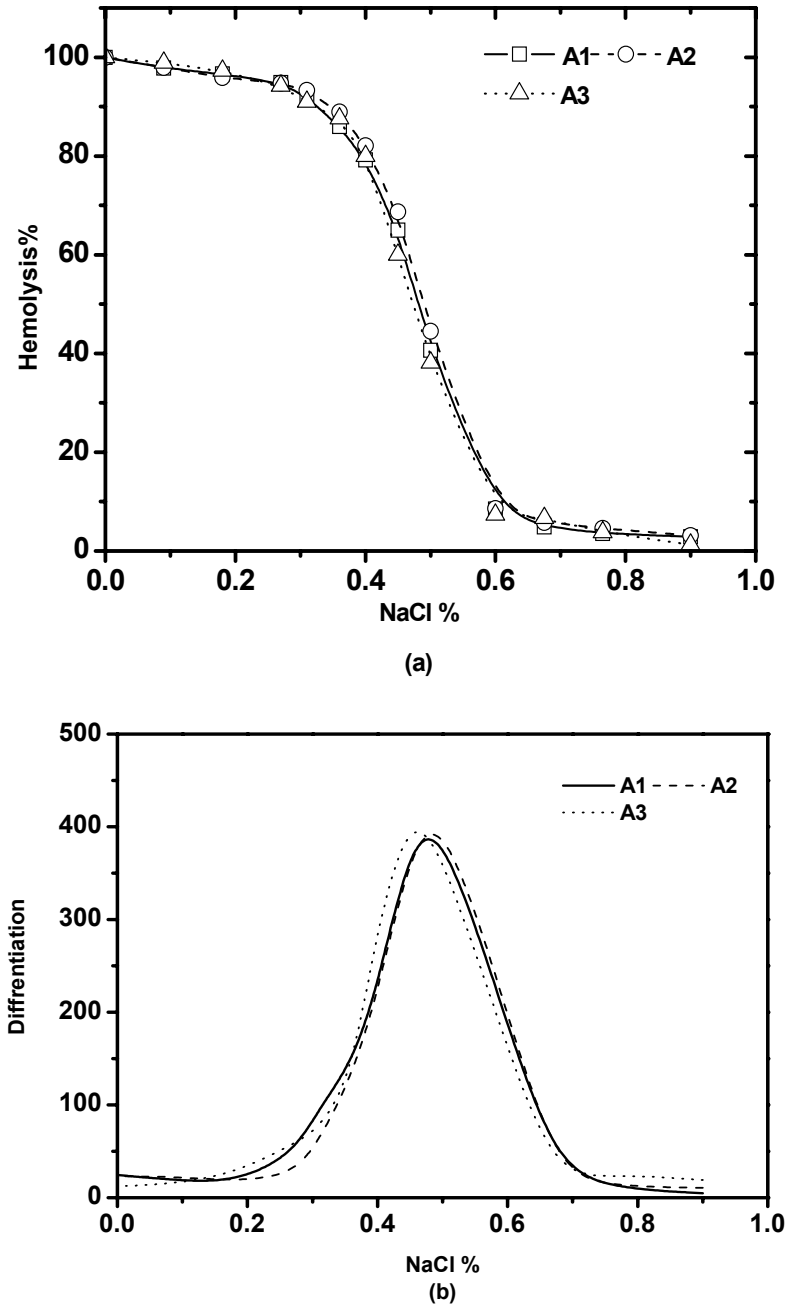
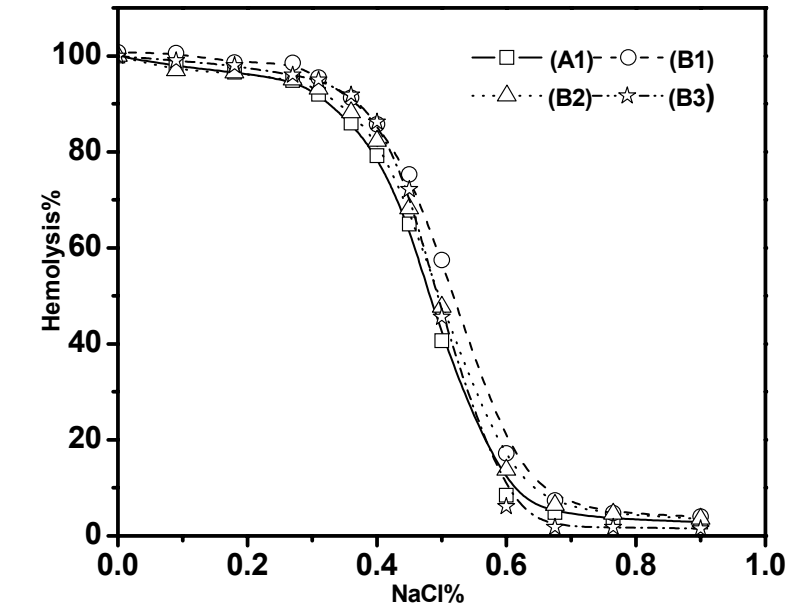
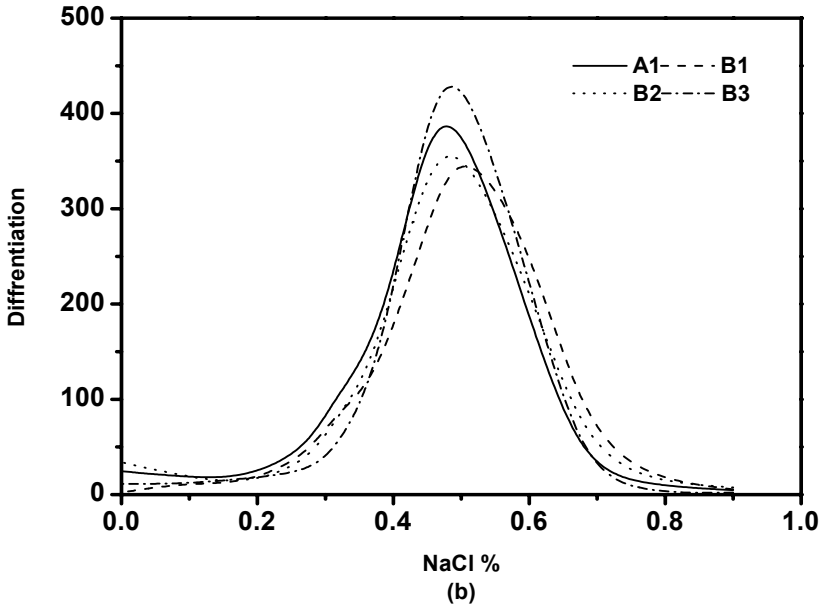


Fig. 1. a. Osmotic fragility curves; b. differentiation curves for the RBCs collected from animals of the control group (A).



(a)



(b)

Fig. 2. a. Osmotic fragility curves; b. differentiation curves for the RBCs collected from animals of the cancer group (B).

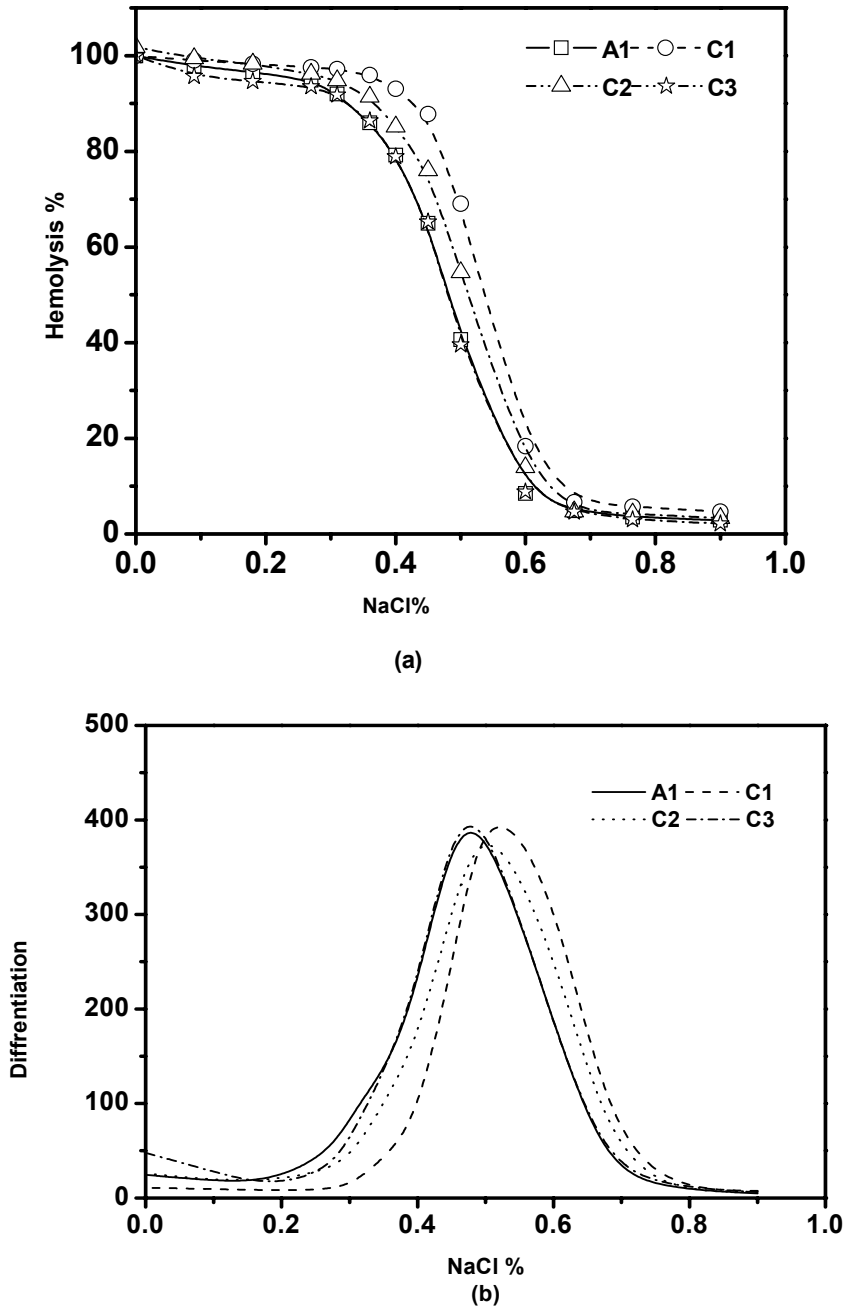
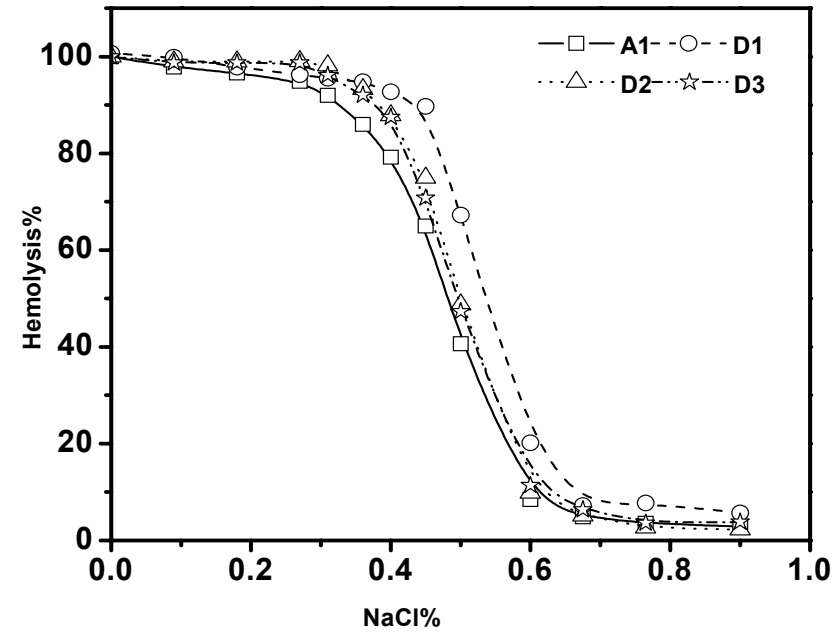


Fig. 3. a. Osmotic fragility curves; b. differentiation curves for the RBCs collected from animals of the irradiated group (C).



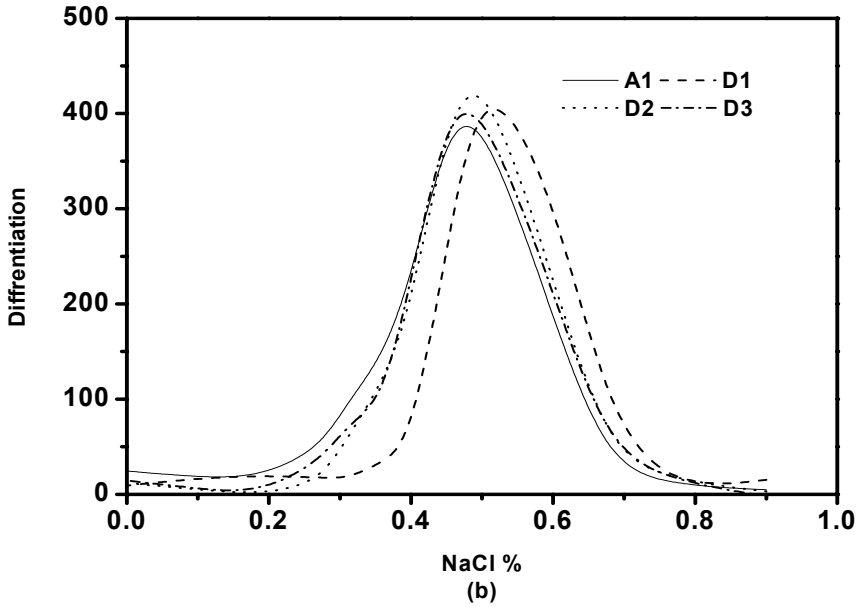


Fig. 4. a. Osmotic fragility curves; b. differentiation curves for the RBCs collected from animals of the irradiated cancer treated group (D).

To analyze these data, the graphs were differentiated and plotted as a function of the percentage of NaCl concentration as shown in Figures 1b to 4b. From osmotic fragility curves it is possible to calculate median corpuscular fragility (*MCF*); (the NaCl concentration at which 50% of RBCs are hemolysed). The differentiation curves were plotted from subtracting each point from previous one of the hemolysis curve. Then, the widths at half maximum (W_{hmax}) of differentiation curves were calculated and given in Table 1. Osmotic fragility studies for the RBCs membrane indicated some changes in the profile of the curves for blood samples from groups B, C, D as compared with the control group A. These changes can be expressed *MCF* and W_{hmax} values as compared with control group. Injection of animals with Ehrlich tumor cells slightly increased hemolysis percentage and osmotic fragility of RBCs as compared with those of normal animals as shown in Figure 2a and given in the table. Hemolysis percentage and osmotic fragility of RBCs are found to increase by exposing to γ-irradiation. This is detected through the shift which is observed in osmotic fragility curves towards higher NaCl concentration. The decrease in W_{hmax} noticed in the differential curves is considered to be an evidence for the decrease in flexibility of RBCs. It is interesting to find that this change is overcome by treating the animals with 10 and 30 mg/kg melatonin which are considered to be in the non-toxic range [7].

Table 1

Osmolarity curves parameters: median corpuscular fragility (*MCF*) and width at half maximum (W_{hmax}) for all the groups and exposure conditions

Group	*MCF* (g%NaCl)	W_{hmax} (arbitrary units)
Group A		
A1	0.47	0.212
A2	0.48	0.212
A3	0.47	0.2092
Group B		
B1	0.51	0.240
B2	0.49	0.2365
B3	0.49	0.2063
Group C		
C1	0.53	0.207
C2	0.51	0.226
C3	0.48	0.212
Group D		
D1	0.53	0.201
D2	0.50	0.207
D3	0.49	0.215

The increase in the RBCs osmotic fragility may be due to some changes in the properties of the RBCs membrane. Ionizing radiation causes disturbance in energy metabolism, disorganization of lipoprotein structure of biological membranes, peroxidation of membrane lipids and inactivation of various bound enzymes (Na^+,K^+) ATPase, Mg^+ATPase. It has also been suggested that radiation-

induced perturbations in Na^+ and K^+ transport system are connected with oxidation of membrane protein sulfhydryl groups [8, 11, 15]. Modification in the physical condition of the proteins on the cell membrane may lead to change in the permeability of the RBCs membrane. Some proteins on the cell membrane (ion channels) act as pores through which the liquid (water) bounds to ions carried inside the cell [3, 15].

The width at half maximum of differential curves (W_{hmax}) represents the relative decrease in the elastic range of RBCs membrane of irradiated animals or animals with cancer [15].

Since the blood capillaries diameter is smaller than diameter of the RBCs, RBCs have to be folded and squeezed to pass through blood capillaries in order to carry metabolic processes. The degree of squeezing depends on the elasticity of the RBCs membrane. Therefore, the decrease in membrane elasticity will lead to the increase of the blood capillaries resistance for RBCs passage to body cells for carrying normal metabolism, and hence toxicity in some organs may occur which is a phenomenon of anemic diseases.

It appears that radiation damage to RBCs is due to action of (superoxide O_2^- and hydrogen peroxide H_2O_2) radicals formed up on irradiation [10, 14]. In the presence of melatonin as free radical scavenger a protective effect against RBCs membrane damage is observed. Melatonin acts as a direct free radical scavenger and detoxifies the highly cytotoxic $OH^\bullet$ radical [9, 13, 14].

BLOOD FILM

Figure 5 illustrates blood film images for the samples collected from animals of different groups A, B, C, D. The results show normal shape of the RBCs collected from the control group (A) even after treating with melatonin as shown in Figure 5, as cells do not stick together due to coulomb repulsive forces between the positive electrostatic charges on the outer surface of the cell membrane. These electrostatic charges are formed on the surface of the normal healthy RBCs as a result of the K ion pump which forms the resting potential across cellular membranes [17]. Some sort of irregularity in the shape of the RBCs membrane and the sticking of several cells together are noticed after injection with Ehrlich cells or exposing to γ-irradiation as shown in Figures 7 and 8. This can be attributed to the changes in the packing properties of the phospholipid bilayer and macromolecules forming the cellular membrane which will cause changes in the membrane permeability to ion transport and the membrane will not be in the liquid crystalline phase. Therefore, one may state here that changes in the membrane permeability will result in the change of the membrane bioelectric potential and surface electrostatic charges [3, 9]. The loss or decrease of the surface electrostatic charges on the cellular membrane will deteriorate the repulsive forces between adjacent RBCs membranes and cause the sticking. Moreover, the loss or decrease of the intermolecular forces between the macromolecules forming the cellular membrane

will result in the noticed deformability in cellular shape. From the same figures it is also noticed that the irregularity in shape of the RBCs membrane and the sticking of cells decreased reaching mostly to normal shape by treating the animals with both doses of melatonin. Melatonin was efficient in lowering lipid peroxidation [11,18].

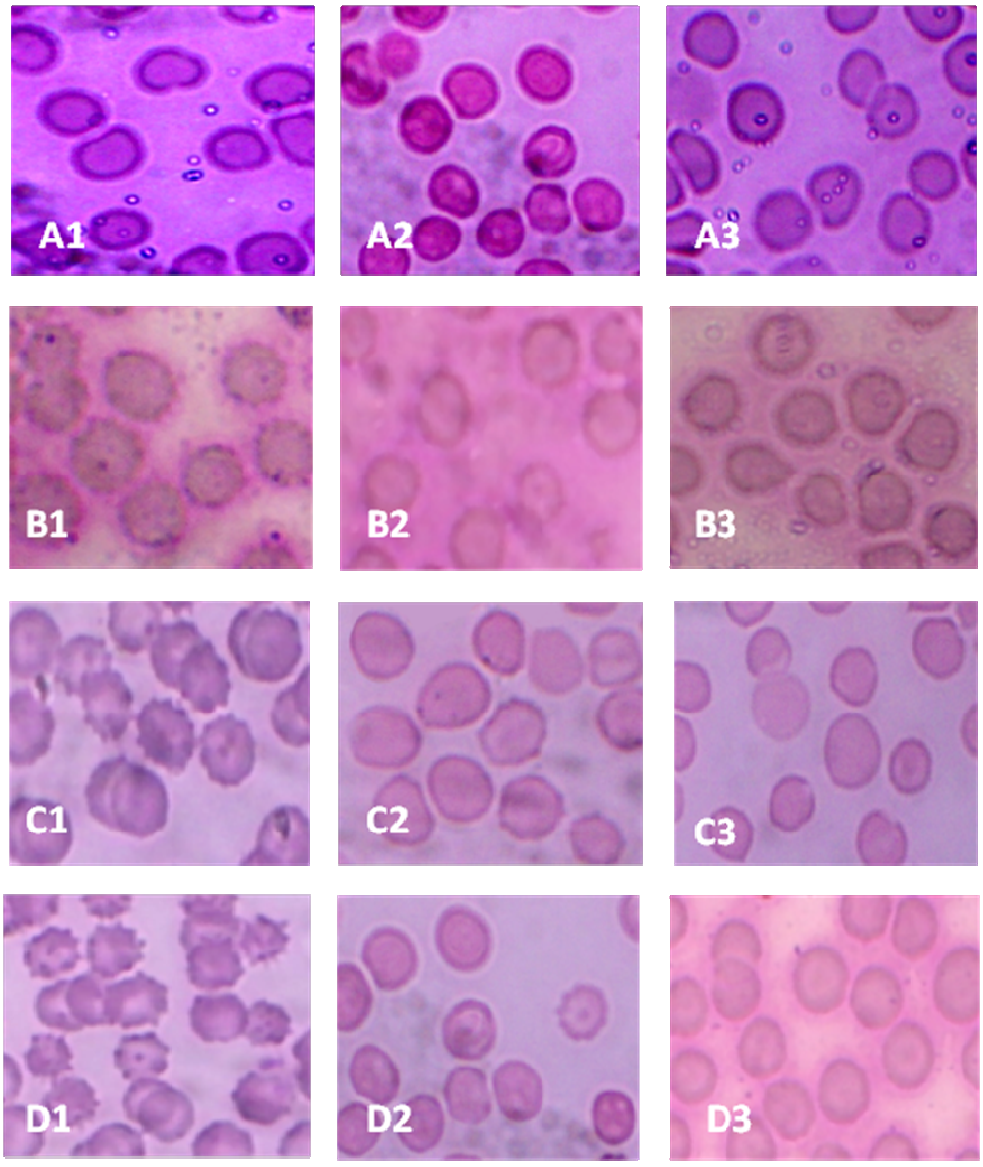


Fig. 5. Blood film images for the RBCs collected from animals of: control group (A1–A3), cancer groups (B1–B3), irradiated group (C1–C3) and irradiated cancer treated group (D1–D3).

CONCLUSIONS

Exposing RBCs to γ-irradiation rather than injection of the animals with Ehrlich tumor cells causes some changes in their membrane properties. These changes were also detected through the calculation of median corpuscular fragility (*MCF*) and the width at half maximum (W_{hmax}) obtained from the osmotic fragility curves as well as the differential curves. The change was also detected through the irregularity in the shape of the cells and sticking of several cells together. This result is comparable with that of normal cells as the cells do not stick together because of the Coulombian repulsive forces between the positive electrostatic charges on the outer surface of the cell membrane. Anyhow, all these studied changes were found to be eliminated by treating the animals with both doses of melatonin recommended. Melatonin has the ability to protect cells of mice from the damaging effects of acute whole-body irradiation.

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