EFFECTS OF EXPOSURE TO SINGLE ELECTRIC, FAST NEUTRONS FIELDS AND MIXED FIELDS ON RAT ERYTHROCYTE MEMBRANE FRAGILITY AND SOLUBILITY

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Abstract. This paper aims to study the biological effects of electric field, fast neutrons and mixed radiation exposures from electric field and fast neutrons on rat erythrocytes. The growing uses of nuclear radiation energy generated by electric power such as X-ray machines, linear accelerator, betatrons and electric power generated by nuclear power, etc., all involved exposures to high electric and magnetic fields mixed with ionizing radiation. Single field of Extremely Low Frequency (ELF) electric field 50 Hz, 6 kV/m and ^{252}Cf fast neutrons with doses up to 1 mSv at a dose rate of 8 μSv/h were used, in addition to mixed radiation exposures from electric field and fission fast neutrons. Forty male rats were equally divided into 4 groups, namely A, B, C and D. The erythrocytes membrane properties were tested through measurements of osmofragility and solubility with non-ionic detergent in addition to blood films for all animal groups. The results indicated changes in all measured parameters after irradiation and exposure to single and mixed radiation fields. It was concluded from the results that periodical medical examination of the radiation occupational workers should include the test of red blood cells morphology and functions. Counting of blood is unsatisfactory to inform about the radiation injury.

Key words: Rats' erythrocytes, electric field, fission neutrons, solubilization, osmofragility, blood films.

INTRODUCTION

The fast growing uses of nuclear accelerators in medicine, manufacture and research in addition to the use of nuclear reactors for generating electric power, all increased the health risk associated with mixed exposures to ionizing and non-ionizing radiation. There is long scientific history on the biological effects of ionizing radiation and the safe limits of exposures. The recommended dose limit for occupational workers is 20 mSv per year that was recommended by the International Commission on Radiological Protection (ICRP, publication 60) [19].

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On the other hand, great attention is focused on the biological effects of non-ionizing radiation and there is increasing interest about the possible health effects associated with exposures to extremely low frequency (ELF) electromagnetic fields (EMF). As a result, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) published guide lines in 1998 for the safe upper limits of public and occupational exposures [20]. The maximum allowable exposure limit to 50 Hz electric field, according to the rationale used by ICNIRP in its guidelines (ICNIRP 7/99) was 10 kV/m [10, 23]. So far, there is no legislation covering occupational exposure to ELF-EMF [24]. In the United States, a 5 years program of the National Institute of Environmental Health Sciences was designed to conduct mostly laboratory research to fill in data gaps by replicating isolated studies that had raised concern and to examine the effects of chronic, near life long exposures of rats and mice to multiple levels of electromagnetic fields [3].

The association between exposure to extremely ELF-EMF and cancer has been investigated in many epidemiological studies. A majority of the studies has focused on brain cancer and leukemia [23]. However, breast cancer has been of increasing interest in relation to magnetic field exposures [27]. Recently, several investigators have examined whether workers in particular occupations have a greater risk of developing breast cancer [29]. Exposures to ELF-EMF can alter the transcription and translation of genes [7], lead to generation of free radicals [19], influence cell proliferation rate and affect enzymes activities. Although other studies have provided that evaluations of the activity of 50 or 60 Hz magnetic fields in models of multistage mammary cancer in rodents have generally been negative; positive findings have been reported from only one laboratory and the mechanism of interaction between the non-ionizing radiation and the tissues is unclear until now [4, 5, 15].

Deformation and pores formation of red blood cells due to the exposure to high-frequency electric field (ν = 1 MHz, 0–18 kV/cm) had been reported [25]. Whole body exposures of rats to 50 Hz, 0.2 mT magnetic fields for a period of one month caused the decrease of erythrocytes membrane elasticity and permeability and caused changes in the molecular structure of the hemoglobin (Hb) in addition to heart injuries [11]. Observations support the hypothesis of 50 Hz electromagnetic fields modify cell morphology and interface in differentiation and cellular adhesion of normal cells [26]. Cells exposed to a 2 mT, 50 Hz, magnetic field, showed by scanning electron microscopy (SEM) modification in shape and morphology. These modifications were also in shape associated with different actin distribution as revealed by phalloidin fluorescence analysis [28].

Membrane solubilization of erythrocytes for rats irradiated with ^{252}Cf fast neutron fluences ranging from 10^6 to 5×10^7 n/cm^2 showed a decreasing trend of erythrocytes osmotic fragility with increasing radiation fluence [35].

Erythrocytes membrane solubilization and hemoglobin gelation of rats irradiated with γ-rays in the dose range of 0–5.6 Gy was found as a biomarker of radiation exposure to γ-rays [2]. Fadel *et al.* [12] found that solubilization,

osmofragility and ultrastructure of erythrocyte membrane for heart diseased patients injected with ^{99m}Tc for imaging with gamma camera were changed. Structural changes were also observed in the membrane lipid bilayer macromolecules exposed to fission neutrons which represented the damaging effects of fast neutrons to both the hydrophobic and hydrophilic regions of lipid bilayer [34].

One may conclude from the reported data that exposures to either neutrons or non-ionizing electromagnetic fields can alter the physical properties of the erythrocyte membrane.

There are no available personal dosimeters for exposures to non-ionizing radiation, similar to the ionizing. Moreover, radiation exposures from nuclear accelerators, reactors power plants and even diagnostic X-rays scanning facilities are all mixed fields of ionizing and non-ionizing radiations. Since the sources of non-ionizing radiation are numerous and the exposure field may be of different frequency components, the problem of personal non-ionizing radiation dosimetry seems complicated and cannot be easily solved. The query is that: do equal equivalent doses of ionizing radiation, one from a radio-isotope and the other from an electric nuclear plant, have the same biological effects?

Therefore, the aim of the present work is to study the effects of whole body exposure of rats to 50 Hz, 6 kV/m electric field, fast neutrons at a permissible dose (1 mSv) and mixed effects of both electric field and fast neutrons on the erythrocytes biophysical characteristics as a step forward to evaluate the risk associating exposures to mixed radiation fields [17].

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Forty male Albino rats of weights 200–250 g each and 2 to 2.5 months age were divided to four equal groups, namely A, B, C and D. Animals of group A were used as control group. Group B was whole body exposed to 50 Hz, 6 kV/m electric field for a period of 3 weeks (w), 5 days (d)/w, 8 hours (h)/d. Group C was whole body exposed to fission neutrons from ^{252}Cf source to a dose of 1 mSv distributed over a period of 3 w, 5 d/w, 8 h/d, at a constant dose rate of 8 μSv/h. Animals of group D were exposed to a mixed radiation field of fast neutrons and an electric field at the same exposure conditions as for groups B and C. The animals were kept in special plastic cages that permit normal ventilation and daylight. The cages with the animals were moved and fixed on supports inside the irradiation chamber during the period of exposure. Animals from all groups were kept under similar environmental conditions of temperature, illumination, acoustic noise, and ventilation, and received the same diet during the course of the experiment. Food

and water were kept in special open containers fixed on the walls inside the cages. Cleaning and changing water and food were done for all animals twice daily. There are no restrictions in Egypt for the use of experimental animals for scientific research.

At the end of the exposure period, animals of groups A, B, C and D each were then equally divided into two subgroups, namely A_1 & A_2, B_1 & B_2, C_1 & C_2, and D_1 & D_2. Animals of the groups B_1, C_1 and D_1 were used for direct effects studies and subjected to investigations while the groups B_2, C_2 and D_2 were used for delayed effects studies and left for a period of 45 days away of any radiation field (similar to the control group A_1). The 45 days period chosen for the delayed effect studies is based on the fact that the life span of the rat erythrocytes is 45 days and the investigations will be on the newly generated blood which will reflect bone marrow activity [12].

EXPOSURE FACILITY

The animals were exposed to a 50 Hz, 6 kV/m electric field generated between two parallel copper electrodes (fixed at the opposite ends of 1 m length plastic chamber) with a potential difference of 6 kV supplied from the mains through a transformer. The plates were covered by a sheet of electric insulating polymer, to prevent any electric shock to the animals during the course of the experiment. Fission neutrons from a 50 μg ^{252}Cf source were used to irradiate the experimental animals. The present neutron yield from the source is 0.89×10^6 n/s. The average neutron dose rate at the area of animal exposure in the cage was measured through the use of a neutron monitor type NM2 manufacture by Nuclear Enterprises UK and found to be 8 μSv/h. The neutron exposure facility is presented at the Egyptian National Institution for Standards Cairo, Egypt. Figure 1 shows a sketch diagram for the exposure system. Animals of the control group A (sham exposed) were housed for similar periods, as groups B, C and D in a similar cage away from any electric or neutron field.

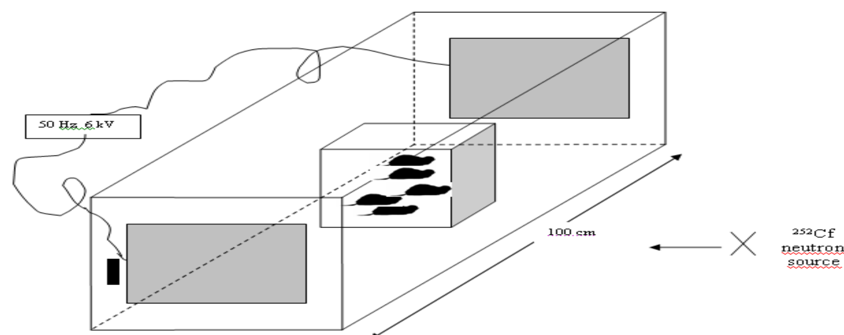


Fig. 1. Irradiation facility for mixed neutron and electric fields.

The following measurements were done on the animals of all groups:

BLOOD SAMPLES

Animals from different groups were anaesthetized with ether, and then blood samples were collected from the eye vein by heparinized capillary tubes for the following investigations:

Erythrocytes osmofragility

Normal red blood cells hemolysis was determined by measurement of hemoglobin released from the cells relative to the total cellular hemoglobin content. Twenty five μL of whole fresh blood were incubated in 5 mL normal saline for 30 min. The samples were centrifuged at 3000 rpm for 10 min at room temperature (25±1 ºC), and the absorbance of the supernatant was measured at 550 nm through the use of a spectrophotometer (model V_5, Jascow, Japan) at 550 nm. The percentage hemolysis was calculated by the following relation [1].

$$\%\text{Hemolysis} = \frac{A_{\text{sample}}}{A_{100\%\text{lysis}}} \times 100 \qquad (1)$$

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

Erythrocytes solubilization

The used nonionic detergent in this experiment was Octylglucoside (OG) which was purchased from Sigma Chemical Company (St Louis, U.S.A). This detergent has the property that it can solubilize membrane proteins without affecting important structural features [14, 29, 35]. After the blood samples were collected from the rats, erythrocytes were isolated by centrifugation at 3500 r.p.m., 4 ºC for 10 minutes then the plasma was removed. The red blood cells were then washed twice in buffered saline and separated by centrifugation at 3000 rpm for 10 minutes. The cells (25 μL) were re-suspended in isotonic buffered sucrose (0.3 M sucrose in phosphate buffer pH 7.4, and conductivity 0.223 S/m) [33]. The turbidity (T) was calculated as follows:

$$I = I_0 e^{-Tl} \qquad (2)$$

where I_0 and I are intensities of the incident and transmitted light, respectively, and l is the length of the light path through the scattering solution. The samples were incubated in water bath at 37 ºC during measurement [31]. It intercalates between lipid molecules and binds to integral proteins. The absorbance at 620 nm of the erythrocytes membrane as a function of the detergent concentration was measured

using the spectrophotometer (model V_5, Jascow, Japan). At this wavelength there is no characteristic absorption band for blood and the decrease in the measured absorbance of the sample will be only due to scattering and will reflect the decrease of the erythrocytes size.

Blood film

Blood films were prepared according to Brown [6]. The blood film was photographed by using an Automatic Image Contour Analysis system (SAMICA) (ELBEK GmbH, Germany). The SAMICA system is provided with an electronic camera connected to a computer through an interface built in card and the image can be magnified up to 1200 times and displayed by the computer. Detailed information about the morphology of the erythrocytes could be visualized and recorded by this system.

Statistical treatments

The statistical analyses of the data were used according to Harnet [16] by calculating arithmetic means and standard deviations for rbc's osmotic fragility and solubilization measurements. All these measurements were done for the animals from all groups and the average reading of 5 runs was used to calculate the mean and standard deviations for each group.

RESULTS

Figure 2 shows plots for the osmotic fragility curves for one animal from each group A_1, B_1, C_1 and D_1 where the % hemolysis is plotted as a function of the percentage of sodium chloride concentration (NaCl%). For analysis of these results, the curves were differentiated D (%hemolysis) and plotted as a function of NaCl concentration percentage as shown in Fig. 3. The osmofragility differential curves for erythrocytes collected from animals of groups A_1, B_1, C_1 and D_1 appeared in main peaks whose half maximum width ($W_{h,max}$) represents the elastic range of the rbc's cellular membrane [11]. The position of this peak (P_p) showed shifts for radiation exposed animals towards lower NaCl concentrations as compared with control A_1.

The average values with the statistical evaluation for $W_{h,max,}$ peak intensity $P_{I,}$ P_p and the concentration percentage of NaCl (C_s) at which hemolysis starts for the blood samples from the animals of groups A_1, B_1, C_1 and D_1 are given in Table 1. The increase of the value of $W_{h,max}$ is a function of the increase in the elastic range of the erythrocytes membranes. It is clear from the data that exposure to the electric, neutrons or mixed fields caused highly significant changes in the average values of $W_{h,max}$ and P_I. The profile of the osmofragility curve for samples from group B_1, C_1 and D_1 differ from control and several peaks appeared in the differential curves.

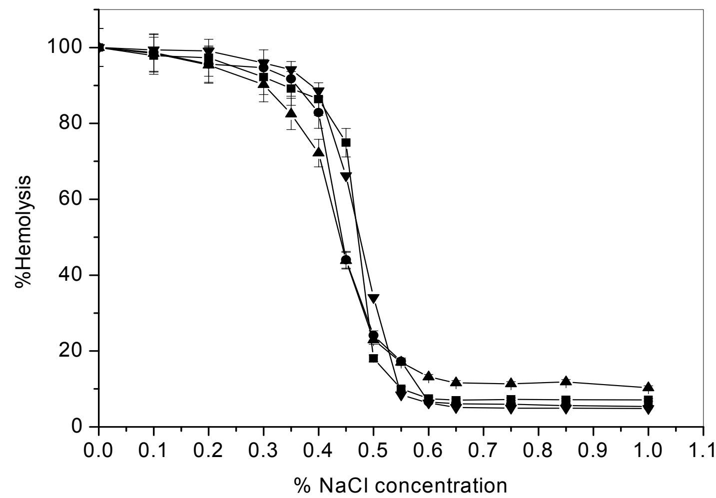


Fig. 2. The variation of the percentage hemolysis of erythrocytes membrane of one animal from the groups A_1, B_1, C_1 and D_1.

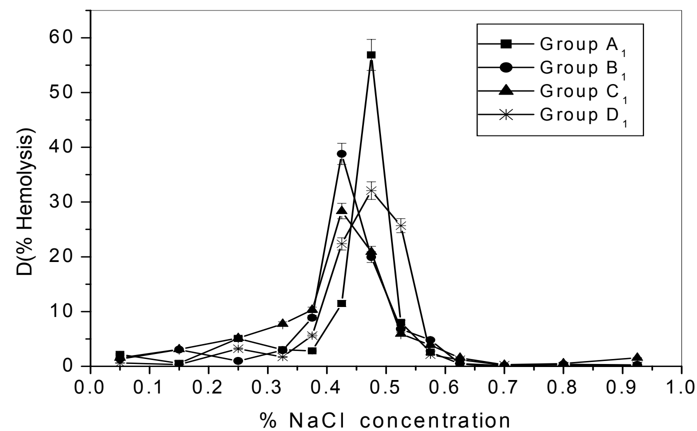


Fig. 3. Differentiation curves for samples for one animal from the groups A_1, B_1, C_1 and D_1 plotted as a function of NaCl concentration hemolysis.

Figures 4 and 5 illustrate the osmofragility curves and their differential plots for animals from the subgroups A_2, B_2, C_2 and D_2, respectively. The calculated average values for $W_{h,max}$, P_I, P_p and C_s for the samples from the animals of these subgroups are given in Table 1. The results indicate a pronounced increase in the values of $W_{h,max}$ for exposed groups as compared with the control A_2. There is remarkable increase in the average value of $W_{h,max}$ (about %300.0) together with the decrease of P_I (about %40.0) for the subgroup B_2 as compared with the control A_2.

Table 1

The average value of $W_{h,max}$, P_I, P_p and C_s for all blood samples from all animal groups

Group	$W_{h,max}$	P_I	P_p	C_s
A_1	0.06±0.007	58.7±2.16	0.47±0.030	0.39±0.040
A_2	0.05±0.004	56.8±2.20	0.44±0.020	0.37±0.020
B_1	0.09±0.008	34.2±2.62	0.43±0.024	0.33±0.036
B_2	0.15±0.014	24.0±2.50	0.38±0.020	0.20±0.016
C_1	0.11±0.010	28.4±1.75	0.43±0.029	0.33±0.032
C_2	0.12±0.130	34.0±3.00	0.45±0.032	0.33±0.003
D_1	0.13±0.012	32.2±0.25	0.45±0.025	0.32±0.003
D_2	0.10±0.011	52.5±7.50	0.41±0.018	0.30±0.025

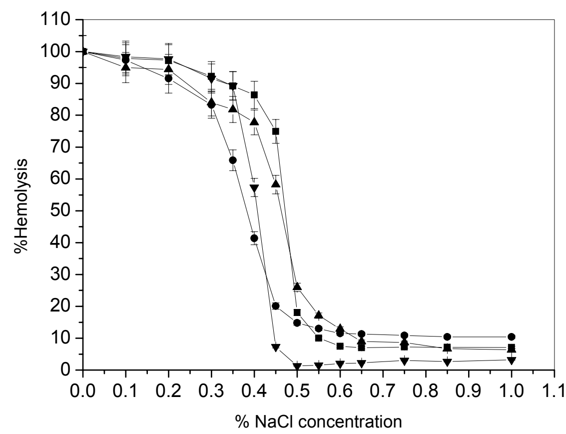


Fig. 4. The variation of the percentage hemolysis of erythrocytes membrane of one animal from the groups A_1, B_1, C_1 and D_1.

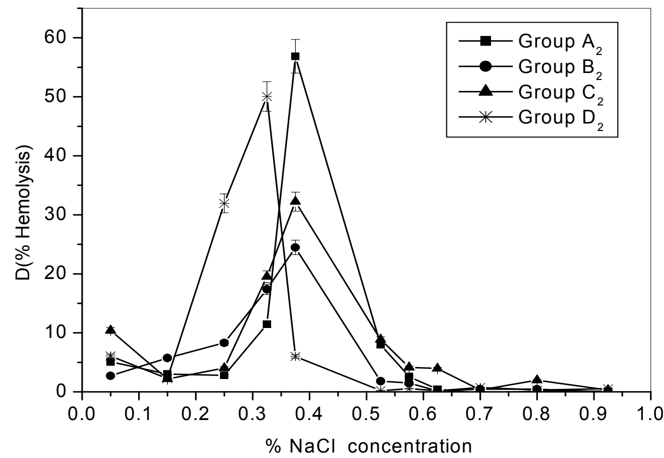


Fig. 5. Differential curves for samples for one animal from the groups A_2, B_2, C_2 and D_2 plotted as a function of %NaCl concentration hemolysis.

Figure 6 shows the solubilization curves for the erythrocytes collected from one animal from the subgroups A_1, B_1, C_1 and D_1, respectively. The differential curves are given in Fig. 7. The peaks presented in the differential curves reflect the stages of the interactions of the detergent at its different concentrations within the erythrocytes cellular membranes till complete micellization occurs. Fig. 8 and Fig. 9 show the solubilization curves and their differential for erythrocytes collected from groups B_2, C_2 and D_2 respectively.

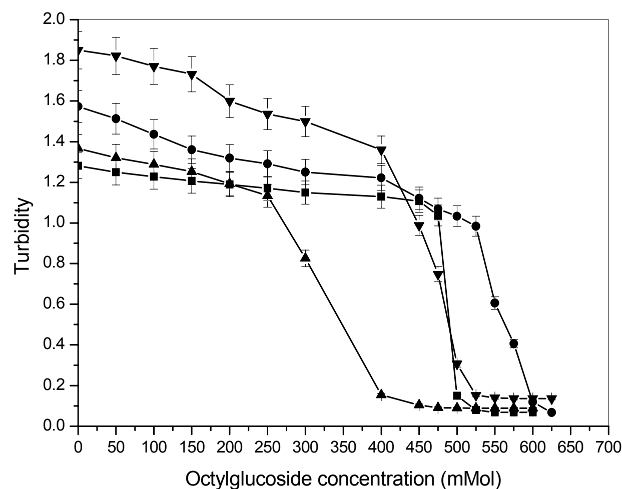


Fig. 6. The variation of the turbidity of erythrocytes membrane of one animal from the groups A_1, B_1, C_1 and D_1.

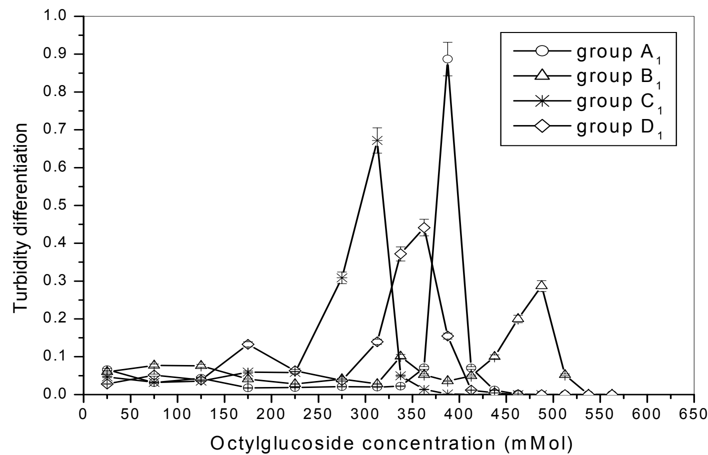


Fig. 7. Differentiation curves for samples for one animal from the groups A_1, B_1, C_1 and D_1 for direct effects as a function of OG in mMol.

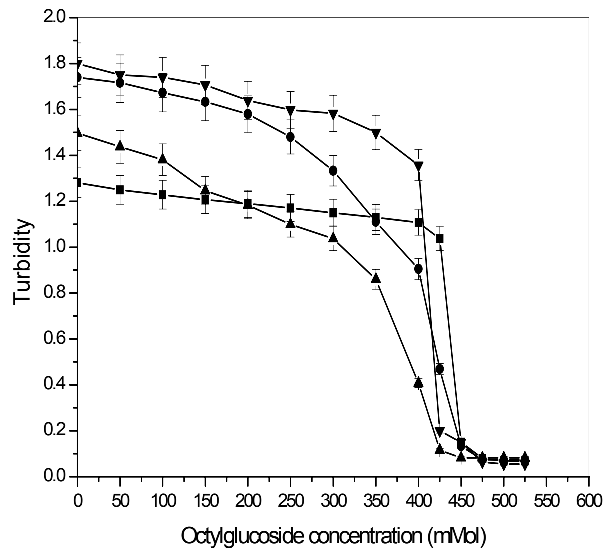


Fig. 8. The variation of the turbidity of erythrocytes membrane of one animal from the groups A_1, B_1, C_1 and D_1.

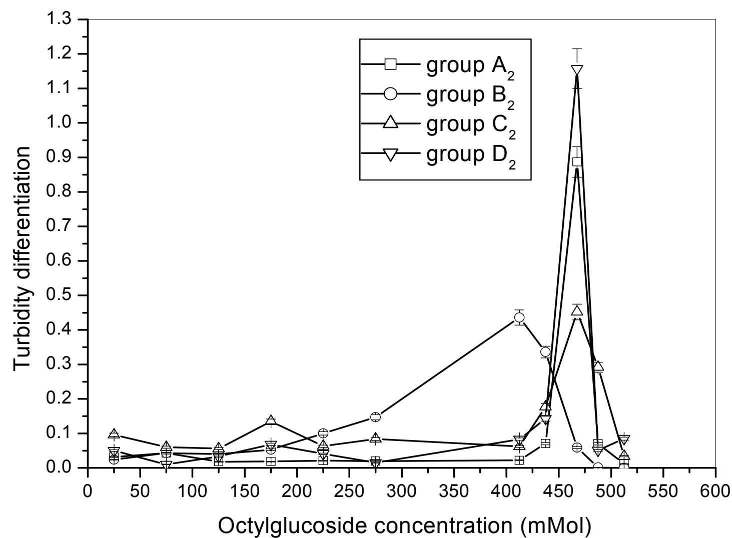


Fig. 9. Differentiation curves for samples from the groups A_2, B_2, C_2 and D_2 for delayed effect as a function of OG in mMol.

The solubilization curve passes through different stages, which indicates the modes of interaction of the detergent at its different concentrations with the molecules forming the erythrocytes membranes. At low concentrations of detergent, the extrinsic proteins are dissolved which causes the decrease in the

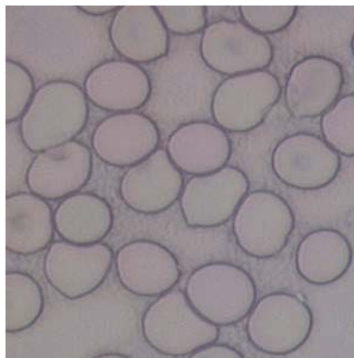
intensity of the scattered light. As can be noticed from the data, there are changes in the peak position and new additional peaks can be observed for all irradiated groups. These changes reflect the structural changes in the packing properties of the molecules forming the membranes bilayer.

Table 2

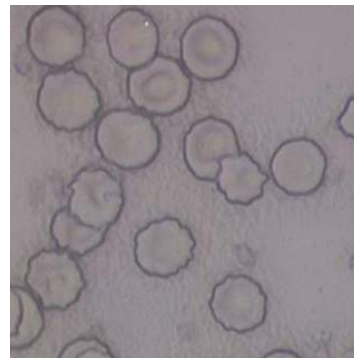
The average value of $W_{h,max}$, P_I, P_p and new peaks for all blood samples from all animal groups

Group	$\boldsymbol{W_{h,max}}$ (mMol)	$\boldsymbol{P_I}$ (mMol)	$\boldsymbol{P_p}$ (mMol)	**New peaks** (mMol)
A_1	28.3±3.2	0.87+0.07	380.5±68	–
A_2	30.2±0.6	0.88±0.06	469±54.0	–
B_1	65.0±4.0	0.41±0.04	380±32.6	410, 370, 100
B_2	97.0±9.5	0.68±0.07	320±29.0	–
C_1	70.0±6.5	0.66±0.05	310±25.0	175
C_2	90.0±9.1	0.45±0.03	430±38.4	275, 175, 75
D_1	80.0±6.3	0.44±0.04	400±35.2	175, 75
D_2	22.5±2.5	0.96±0.10	185±17.0	75

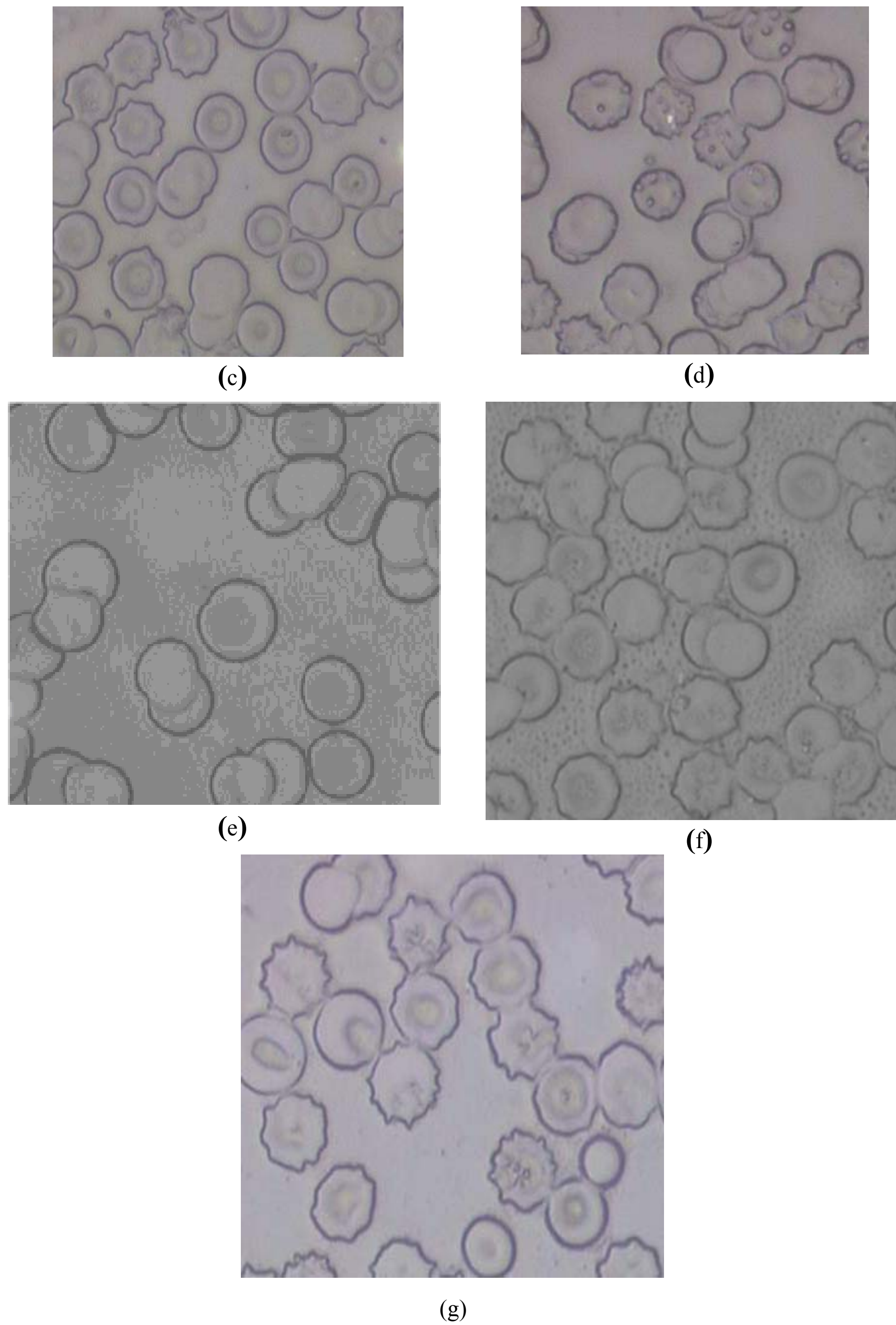
Figs 10 a, b ,c, d, e, f and g show the blood film images as recorded by the image analyzer (SAMICA) for the erythrocytes collected from animals of the studied groups. It can be noticed from the blood films the irregularity in the erythrocytes membranes and the sticking of several cells together forming one body in a single common membrane for all irradiated groups. This phenomenon is highly significant in the erythrocytes collected from neutrons and mixed field exposed groups. Erythrocytes were chosen as a good example for the changes that may occur in a single cell as a result of exposure to ELF magnetic field, since it is not easy to have single cells from the organs that fit all experimental measurements carried in this work.



(a)



(b)



(g)

Fig. 10. The blood film studying the direct effect on the erythrocytes for (a) group A, (b) group B_1 (c) group C_1 (d) group D_1 (e) group B_2 (f) group C_2 and (g) group D_2.

DISCUSSION

The results presented in this work indicated changes in the mechanical and morphological properties of the rbcs membrane of the rats blood after whole body exposure of the animals to 6 kV/m electric field, 1.0 mSv fast neutrons and combined electric and neutron fields. The schedule of animal exposures was deigned to be similar to the working hours of the radiation workers which is 8 h/d and 5 d/w. The dose rate of fast neutrons received by the animals was 8 μSv/h which is within the safe limits recommended by the ICRP-60. All blood films from the animals from groups A, B, C and D showed similar data as presented in Fig. 10 and none of the control group showed rouleaux or anemic forms.

The present data can be analyzed based on the basic interactions of radiation with biological systems. It may be presumed that the interaction of fast neutrons with biological systems is the formation of highly energetic nuclear recoils which are mainly the nuclei of hydrogen (protons), carbon, nitrogen and oxygen which result from neutron scattering, in addition to the neutron capture reactions especially with chlorine and nuclei of the electrolytes. Since hydrogen atoms play the major role in the structure of the phospholipids macromolecules forming the cellular membrane bilayer and the neutron scattering cross-section for hydrogen is relatively high (20.4 barns), the most neutron recoiled nuclei are protons. Because the logarithmic energy decrement per collision for hydrogen has the maximum value which is unity, a neutron can transfer all its full kinetic energy to the recoiled proton in the case of head on collision. These highly energetic nuclear recoils will migrate in the hydrocarbon network forming further damages and highly active species. These chemically active sites will carry random recombination between adjacent molecules leading to the disturbance of the intermolecular forces in the cellular membrane. All these possible changes in the cellular membrane structure will result in changes in the packing properties of the phospholipids bilayer macromolecule and hence the membrane permeability and morphology.

On the other hand, exposures to 6 kV/m electric field can result in the formation of induced electric dipoles and the disturbance of the regular free motions of the head group in the phospholipids bilayer macromolecules [8, 20]. Moreover, the recombination reactions of the highly energetic active species which resulted from the neutron interactions can be changed under the influence of the electric field.

The remarkable increase of the average values of $W_{h,max}$ given in Table 1 for radiation exposed groups compared with control are indicators for the loss of the binding intermolecular forces between the membrane phospholipids macromolecules which resulted from the changes in their packing properties.

Changes of the cellular membrane permeability will result in changes of the ionic pumps across the cellular membrane and hence disturb the normal electrostatic potentials and charges upon the surface of the cells. These surface electrostatic charges form coulomb repulsive forces between adjacent rbcs and prevent their sticking. The loss of these charges was the main reason of the sticking

of the rbcs and their fusion which was noticed in blood films for the irradiated groups. Since the diameter of the erythrocytes of rats is in the range of 12–16 μm and blood capillaries are in the range of 6–8 μm, the erythrocytes have to be folded to pass through blood capillaries in order to carry metabolic processes [18]. The sticking of adjacent cells together in a way to have a common membrane and/or the loss of the cellular membrane elasticity that deteriorates the folding mechanisms, all, will not permit erythrocytes to pass through blood capillaries and hence fail to carry metabolic processes. Measurement of the electrophoretic mobility of erythrocytes is now the most convenient method for estimating these charges both in experimental studies and in clinical practice [36].

The changes in cell membrane were tested chemically by interaction with detergent. The solubilization process of membrane is an induced transformation of nearly flat phospholipids bilayer 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 [33]. The effect of nonionic detergent at low concentrations on the erythrocytes membrane is the solubilization of extrinsic proteins which partially work as signal receptors to the cells. At higher concentration (plateau region in Fig. 6 and Fig.8) the detergent begins to incorporate within the bilayer macromolecules forming the membranes and dissolute of the intrinsic proteins. When the incorporation of the detergent molecules is completed (at relatively high concentrations) solubilization of the membranes macromolecules occurs and the membrane is ruptured which causes the pronounced decrease of the scattered light and the formation of the main peak (differential curves). As can be noticed from Fig. 7, the profile of the solubilization curves is changed and several additional peaks occurred in the differential curves. These changes indicate modifications in the phospholipids packing properties of the cell membranes molecules. The detergent concentrations at which the characteristic dissolution peaks occur were calculated for each animal from the studied groups then the average was considered (Table 2). As noticed from Table 2 marked changes of the values of $W_{\mathrm{h,max}}$ indicate the increase of the elastic range of the cells membranes. The noticed changes in the profile of the solubilization curves for erythrocytes collected from the exposed groups to radiation indicate that receptors on the surface of cells suffered structural changes upon irradiation which may lead to loss in their functions. The functional failure of the receptor may affect cell to cell communication and hence the run of the metabolic processes.

All the fore-mentioned interacting parameters will affect the metabolic functions of erythrocytes which may lead to anemic diseases. The study of the delayed effects of exposures of the animals to single or mixed type of radiation indicated the injuries of the blood generating system and unhealthy blood was generated. This may be due to two possible reasons; the first was the direct effect of radiation on the bone marrow cells membrane. The second reason is the possibility of growing toxicity in the bone marrow cells due to the failure of the erythrocytes to do their metabolism in the bone marrow.

CONCLUSION

It may be concluded from the present findings the following:

1. Exposure to 50 Hz, 6 kV/m electric field for occupational workers is risky and deteriorates the physiological functions of the red blood cells.

2. On the calculation of the radiation dose it is recommended to differentiate between doses received from radioactive isotopes and from nuclear generating facility.

3. Periodic medical examination of the radiation occupational workers should include the test of red blood cells morphology and functions. Counting of blood is unsatisfactory to inform about the radiation injury.

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