Protective effects of melatonin on hemoglobin damage induced by gamma irradiation

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Abstract: Ionizing radiation causes serious damage in biological system. Some drugs and antioxidants are used to prevent such damage. In the present study two doses of melatonin (10 mg/kgand30mg/kg) were selected to be used for such purpose. The radioprotective effects of melatonin on hemoglobin of red blood cells from female mice was studied through UV absorption spectrum, ESR spectroscopy, dielectric measurements and relative viscosity .The results of Hemoglobin absorption indicate that a pronounced increase in the average value of peak position and width at half maximum W_{hmax} followed by a decrease in the absorbance of sort band, decrease in absorption ratio A_{578} / A_{540} in addition to disappearance of globin band at 275 nm. The free radicals which are expected to be formed after exposing to γ-irradiation are detected by electron spin resonance spectroscopy (ESR). The results indicate that the intensity of ESR signal for hemoglobin extracted from animals exposed to γ-irradiation is greater as compared with normal hemoglobin. Dielectric measurements indicate that there is an increase in dielectric permittivity (ε'), the dielectric loss (ε") and the a.c conductivity (σ_{ac}) while some decrease is noticed in the viscosity measurements after exposing to irradiation. The data obtained from the whole studied parameters after treating animals with melatonin become closer to those for unirradiated samples.

***IndexTerms* – Melatonin, Radiation Protector, gamma radiation.**

I. INTRODUCTION

Radiation produces numerous biological perturbations in cells by direct ionization of DNA and other cellular targets such as proteins and lipid and by indirect effect through free radical production. Exposure to ionizing radiation produces oxygen derived free radical (ROS) in the tissue environment. These radicals include hydroxyl radicals (the most damaging radical), superoxide anion and other oxidants such as hydrogen peroxide. The intracellular generation and accumulation of these free radicals causes change in molecular structure of proteins such as hemoglobin. According to these induced radiation effect, numerous antioxidants could be used for biological and medical safety.

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 [(1)]. The result 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 the process was faster than alpha particles. Biological effect of short duration exposure to moderate and intense static magnetic field was studied by measuring absorption spectra of hemoglobin molecule and electric conductivity [(2)]. The result indicated that there is increase in the width at half the maximum of the sort band besides decrease in A_{578} /A_{542} ratio.

The effect of ionizing radiation on some bovine hemoglobin characteristics using electron paramagnetic resonance (EPR) spectroscopy and (IR) spectroscopy was studied [(3)]. It was found that the intensity of (EPR) signal which attributed to free radicals is greater in radiated sample than un irradiated, this means that ionizing radiation may lead to increase of free radical production and decrease in α helices contents, which reflect the degradation of hemoglobin molecular structure. Protective effect of ascorbic acid on molecular behavior changes of hemoglobin induced by magnetic field was observed, by measuring the relative permittivity, dielectric loss, relaxation time, conductivity, radius and diffusion coefficient of hemoglobin solution [(4)]. The result indicted that exposure to magnetic field resulted in changes in the molecular behavior of Hb molecule while treatment with ascorbic acid afforded comparatively more significant amelioration in these molecular changes.

The effect of gamma irradiation on the molecular properties of myogloin was studied [(5)]. the results indicated that irradiation caused initial fragmentation of the protein molecule and disrupted the hem group, result in decrease absorbance at (409 nm). Ascorbic acid protected against the degradation of Protein molecule by scavenging oxygen free radical that are Produced by irradiation. Protective effects of melatonin on the ionizing radiation induce DNA damage in the rat brain was studied [(6)]. The results indicated that significant increase in DNA damage was found in the radiation treated rat brain. Pre-treatment of rats with intraperitoneal dose of 100 mg/kg melatonin provided significant decrease in the DNA strand breakage and lipid peroxidation. Treatment with melatonin can protect brain cells from oxidative damage induced by ionizing radiation. Protective effect of melatonin on spinal cord damage after gamma irradiation was studied [(7)]. The results indicated that melatonin may be useful in preventing spinal cord damage against radiation toxicity due to its potential of free radical scavenging. Prophylactic role of melatonin against radiation induced damage in mouse cerebellum with special reference to purkinje cells was studied [(8)]. 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 this study is to examine radioprotective effects of melatonin against oxidative damage induced by γ-irradiation. This study will be achieved by investigating the change in biophysical properties of hemoglobin from female mice through UV absorption spectrum, ESR spectroscopy, dielectric measurements and relative viscosity.

II-Materials:

2.1-Animals:

In this study 60 female mice having weight ranged between 20-25g maintained at animal house laboratory, National Research Center under the normal conditions of water and diet supply. The animals were divided into two main groups each group divided into three subgroups:

1- Control group (A):

A_1: Normal animals.

A_2 and A_3 Animals treated with 10 mg/kg and 30 mg/kg melatonin.

2- Irradiated group (C):

C_1: Animals exposed to γ-irradiation.

C_2 and C_3 Animals treated with 10 mg/kg and 30 mg/kg melatonin one day before exposing to γ –irradiation. .

2.2-Melatonin treatment:

Melatonin (N-acetyl-5-methoxytryptamine) C_{13} H_{16} N_2 O_2, MW 232.3and M.P.117-120 ° C from Mallinckrodt Inc., Paris, Kentucky was used. The animals were injected with freshly prepared melatonin dose of 10 mg/kg and 30 mg/kg body weight one day before γ- irradiation. 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 administrated intraperitoneally in volume of 0.025 ml/g body weight.

2.3-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 alive during the experiment of irradiation. The dose delivered to the animals were calculated and adjusted in 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 homogenous field of irradiation the whole body animals were exposed to 6 Gy γ-irradiation emitted from linear accelerator cited in the radiotherapy and nuclear medicine department of the National Cancer Institute, Cairo University. The machine of irradiation was supplied from Elekta Company and equipped to 6 MV photon rays' production at different field areas at a 100 cm distance perpendicular to skin. Dose rate was about 400 MV per monitor unit.

2.4-Hemoglobin extraction:

The heparinized blood was centrifuged at 3500 r.p.m. for 10 minutes at 4oC.The packed red blood cells were washed with 5 volumes saline solution at 20oC. Recentrifuge to separate the washed red blood cells. Steps 2 and 3were repeated 3 times. The clean red blood cells were lysed with four volumes of deionized water finally; the mixture was centrifuged at 7000 r.p.m. for 40 minutes at 4oC to separate the hemoglobin.

III-Sample analysis;

3.1-Hemoglobin spectrum:

Hemoglobin spectrum was done through the use of the spectrophotometer (Jasco. UV/visible spectrometer typeV-570 (Germany) in the range from 250 nm to 700 nm.

3.2-ESR spectroscopy:

DMPO (5,5-dimethyle-1-pyrroline N-oxide) from sigma-Aldrich chemie -Jmbh, Steinhein, Germany. Was added to hemoglobin solution. About 1 μ l of DMPO to 0.5 ml of hemoglobin solution in a quartz ESR flat cell and placed into the T_m cavity of a Bruker electron paramagnetic resonance E_{500}, Elxsys using super-X-band 9 GHz ESR spectrometer Germany. The sample was scanned starting within 2 min of the addition of (DMPO) using the following instrument parameters: modulation amplitude 4.0 G; modulation frequency 100 k Hz; microwave power 20 mW ; gain 60; scan time 40 sec; time constant .02 sec.

3.3-Dielectric measurements:

Dielectric measurements were done in the frequency range from 100Hz to 1 MHz using computerized RLC HIOKI 3531 Z Hitester (E. E. Corporation, Japan)

The sample cell has two squared platinum black electrodes of area (1 x 1) cm^2 each with an interelectrode distance of 1 cm.

3.4- Measurement of viscosity:

The Measurements were performed at certain concentration (3.4×10^{-5}M) and constant temperature (25°C) with an AVS 350 automatic Ubbelohde-type capillary viscometer from schott-Geraete (Hofheim, Germany) which allows reproduction of the flow times with an accuracy of 0.03s. The instrument was also equipped with a model CT 1450 thermostat bath.

IV. RESULTS AND DISCUSSION

4.1-Absorption spectra:

Absorption spectra for hemoglobin (Hb) extracted from the animals of different subgroups are illustrated graphically in Figures (1) and (2) The obtained bands which characterize hemoglobin are as follows: 578 nm (hem-hem interaction band), 540 nm (Fe-N in porphyrine) nitrogen iron bonds in porphyrine, 414 nm (sort band), 340 nm (globin- hem interaction band) and 275 nm (protein band).These wave lengths are comparable with those found in literature[2,9 ,10].

The average values of peak height, peak position and the width at half maximum (W_{hmax}) of sort band and the absorption ratios of A_{578} / A_{540} in the absorption spectra for hemoglobin extracted from the animals of the two groups A and C are calculated and given in the following table .

Group	Peak hieght	Peak position	W_{hmax}	A_{578}/A_{540}
Group A				
A_1	3.3	414	40	1.007
A_2	3.33	414	40	1.010
A_3	3.36	414	40	1.020
Group C				
C_1	2.85	413	52	0.840
C_2	3.20	414	41	1.000
C_3	3.35	414	40	0.990

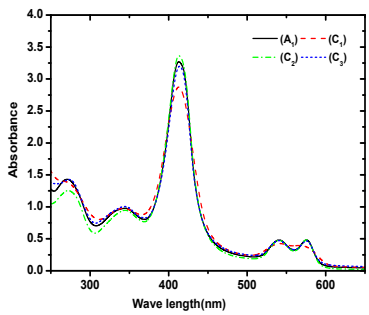

Figure (1): Absorption spectra of hemoglobin extracted from animals of control group A: (A1) normal animals. (A2) and (A3) animals treated with 10 mg/kg and 30 mg/kg melatonin.

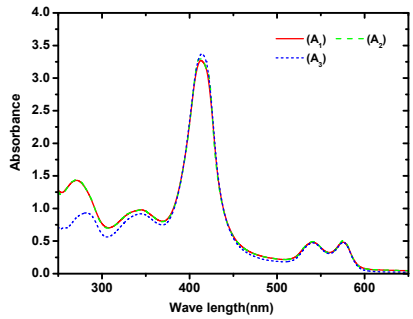

Figure (2): Absorption spectra for hemoglobin extracted from animals of whole body irradiated group C: (C1) animals with whole body exposed to γ-irradiation, (C2) and (C3) animals treated with 10 mg/kg and 30 mg/kg melatonin

No detectable change is observed in absorption spectra for hemoglobin extracted from the animals of control group A either before or after treating with melatonin .Two doses of melatonin were selected (10and30 mg/kg) which are considered to be in the non-toxic rang[11].

Great differences are detected in heme parts at visible wavelength for hemoglobin extracted from the animals exposed to γ-irradiation for the subgroups C_1, such as relatively disappearance of globin band at 280 nm. Moreover, there is an increase in the width at half maximum of the sort band beside decrease of the absorbance at sort band, decrease in heme–heme interaction band and decrease in A_{578} / A_{540}.These results indicate a partial loss of Hb molecule stability [10]. Irradiation disrupted the heme groups, resulting in decrease of the absorbance at sort band. It causes a slight breakdown of the polypeptide chain break covalent bonds and disrupts the ordered structure of proteins [5,12] as a result of the increase in the free radical production [3,8,14]. These free radicals contribute to hemoglobin denaturation and precipitation, leading to anemia [10, 13]. Also, these free radicals (reactive oxygen species) induce deteriorating effect on antioxidant defensive system, decreases antioxidant capacity of the organism and depletes levels of know antioxidant [14]. This promoted oxy hemoglobin to meet hemoglobin [2, 12]. In the presence of melatonin protective effect against γ-irradiation damage is observed. This finding is achieved by the studied parameters given in the table for the investigated animals treated with both doses of melatonin 10 mg/kg and 30 mg/kg before exposing to γ-irradiation (C_2, C_3). These parameters are found to become closer to those given of the control group A. Melatonin acts as direct free radical scavenger and detoxifies the highly cytotoxic $OH^{\bullet}$ and other radicals produced by ionizing radiation [8,14,15].Because of its rather small size and high lipophilicty, melatonin crosses biological membranes easily , thus reaching all compartments in the cell [16].

4.2-ESR spectra of DMPO spin adducts:

The free radicals which are expected to be formed by exposing the body to γ-irradiation is studied through the electron spin resonance ESR spectroscopy. It can detect spin adducts of spin-trapping agent 5,5-dimethyl 1-pyrroline n-oxide (DMPO) with reactive oxygen radicals which are expected to be generated in hemoglobin extracted from the animals of two groups A and C. As shown graphically in Figures (3) to (5).

As shown in Figure (3), no signal is detected for hemoglobin of control group except after the addition of DMPO to hemoglobin as shown in Figure (3(c)) in comparable with Figures (3- (a)) and (3- (b)).

When the spin-trapping agent (DMPO) is added to hemoglobin in the presence of hydrogen peroxide radical (H_2O_2), DMPO radical adduct has been detected which is assigned to peroxyl radical at tyrosin-103. This provides an evidence for unpaired electron density [17].

As shown in Figure ESR spectra consist of the three sharp, differentially broadened lines, which is characteristic of nitro oxide spin labels [18].

DMPO spin adducts signal intensity of oxygen radical in hemoglobin extracted from animals of control subgroups (A_1, A_2 and A_3) are not changed by treating with melatonin doses 10 mg/kg and 30 mg/kg as shown in Figure (4).

Figure (5) illustrates that the DMPO spin adducts signal intensity of oxygen radicals in hemoglobin extracted from animals of irradiated subgroups (C_1) is much greater than the control subgroup (A_1) and those for the subgroups (C_2andC_3) which are treated

with melatonin doses before exposing to γ-irradiation due to the increase in the free radical which are expected to be formed as a result of irradiation.

From Figure (5) it is also seen that treatment with melatonin decreases the signal intensity. So it could be conclude that melatonin is a power full scavenger for O_2 and H_2O_2 [8, 14, 15].

Finally, ESR is considered to be a sensitive tool to support the other investigated tools to detect the free radicals.

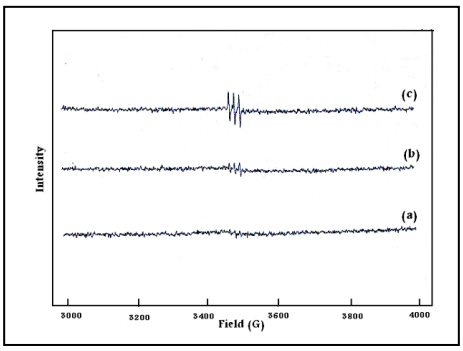

Figure (3): ESR spectra of hemoglobin extracted from animals of control group (a), DMPO (b) and DMPO- hemoglobin mixture (c).

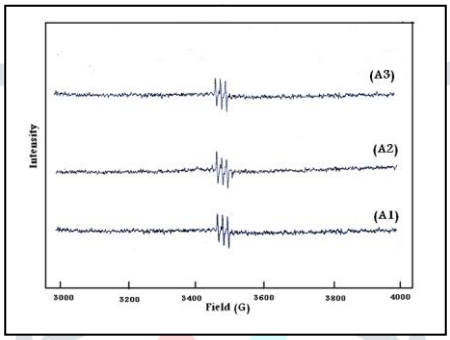

Figure (4): ESR spectra of hemoglobin extracted from animals of control group (A): (A1) normal animals, (A2) and (A3) animals treated with 10 mg/kg and 30 mg/kg melatonin.

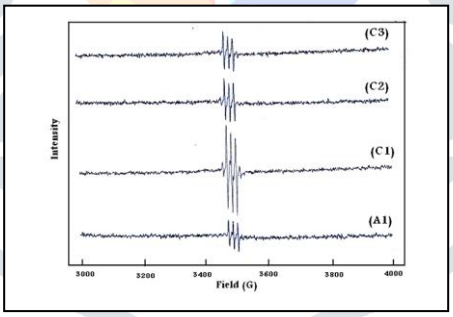

Figure (5): ESR spectra of hemoglobin extracted from animals of irradiated group (C). (C1) irradiated animals, (C2) and (C3) animals treated with 10 mg/kg and 30 mg/kg melatonin.

4.3-Dielectric measurements:

The dielectric permittivity ε' ,the dielectric loss ε'' as well as the electrical conductivity σ_{ac} for hemoglobin extracted from the animals of control group (A) and γ- irradiated group were measured in the frequency range 10^2 – 10^6 Hz and illustrated graphically in Figures (6) and (7). No detectable change is noticed when the animals from the control group (A) are treated with both doses of melatonin. On the other hand, it is noticed that there is some increase in ε', ε'' and σ_{ac} of sub group C_1 as compared with those of control sub group A_1. This could be a good evidence for the increase of free radicals which are expected to be formed by exposing to γ- irradiation. By treating with melatonin all these values become closer to those of control subgroup A_1.

So, it could be concluded that the dielectric spectroscopy is considered to be a good tool to support the trend given by UV spectra and confirmed by ESR spectroscopy.

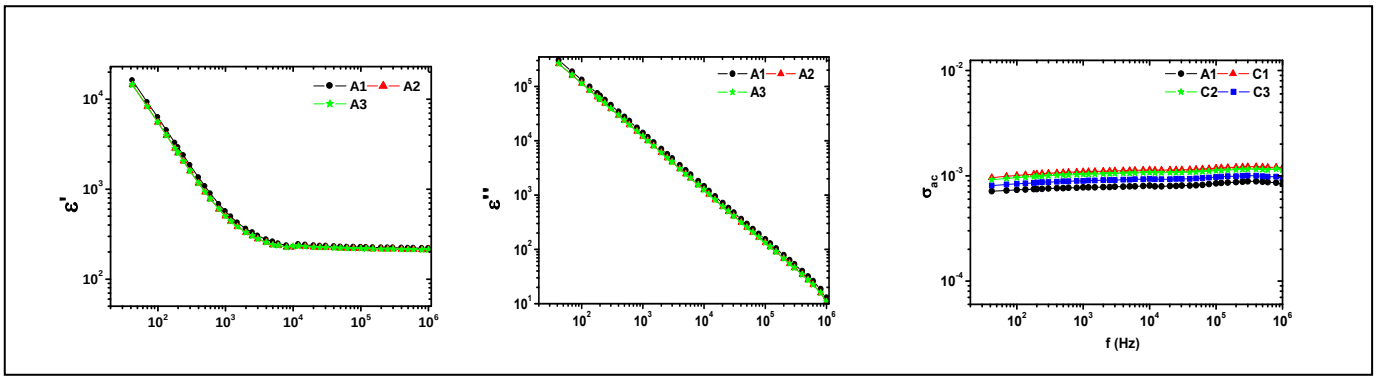

Figure (6): The variation of dielectric permittivity ε', dielectric loss ε'' and electrical conductivity σac as a function of the frequency for hemoglobin extracted from animals of control group A.

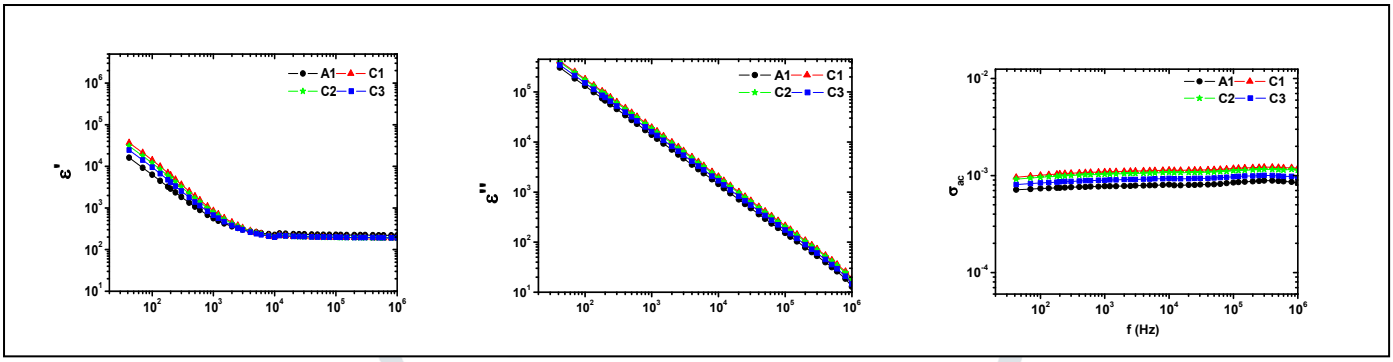

Figure (7): The variation of dielectric permittivity ε', dielectric loss ε'' and electrical conductivity σac as a function of the frequency

4.5-Viscosity measurements:

The dynamic viscosity (η) of hemoglobin extracted from animals of different subgroups is measured at certain concentration (3.4×10^{-5}M) and temperature (30°C). The data obtained are given in the following table.

Group	A_1	C_1	C_2	C_3
η (CP)	0.77	0.756	0.759	0.764

These values indicate that there is a slight decrease in the dynamic viscosity for hemoglobin of group exposed to γ–irradiation (C_1) as it compared with control group (A_1). By treatment with melatonin (C_2 and C_3) subgroups, the viscosity values become closer to those of control group (A_1). The decrease in viscosity by exposing to γ–irradiation (even it is very small) indicates some changes in dimensional and shape of hemoglobin molecule [(12)].

V- Conclusion:

Exposing hemoglobin to gamma irradiation causes disrupted in the heme groups, slight breakdown of the polypeptide chain and break covalent bond. The study is carried out on female mice depending on the change in the biophysical and biochemical properties which are expected to be happened under the effect of the ionizing radiation. These changes were studied through UV absorption spectra, ESR spectroscopy, dielectric and viscosity measurements. A pronounced increase in the average value of peak position and width at half maximum W_{hmax} followed absorption ratio A_{578} / A_{540} was noticed. An increase in the intensity of ESR signal was noticed which is considered to be an evidence for the formation of free radicals. These changes were also studied through the increase in the dielectric parameter and decrease in the viscosity measurements after exposing to irradiation. The studied changes which are obtained as a result of exposing to irradiation are found to be eliminated by treating with 10 mg/kg and 30 mg/kg melatonin.

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