EPR STUDY ON OXIDATIVE STRESS OF IRRADIATED AND β-THALASSEMIA HEMOGLOBIN. ROLE OF α-LIPOIC ACID

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Abstract. Lipoic acid is considered as an ideal therapeutic antioxidant because it is a naturally existing, low molecular weight compound with very powerful antioxidant effects in both aqueous and lipid domains. The aim of this work was to study *in vitro* the ability of α-lipoic acid to protect hemoglobin against the oxidative damage resulted from two different sources: exposure to gamma radiation and β-thalassemic disease. In β-thalassemia, the oxidative stress was found prior to the incubation with α-lipoic acid, while, the irradiated samples were incubated before the exposure to gamma radiation. EPR is used in this study, since it allows the determination of the concentration of unpaired electrons present in a sample even if the exact nature of the free radical is not known. In this study the EPR spectra had been recorded by means of a standard X-band spectrometer operating at 9.5 GHz. There was a significant increase in the EPR signal of the free radical (S4) at $g = 2.0000 \pm 0.00060$ in irradiated hemoglobin and β-thalassemia major. The percentage of increasing was 106% and 69% in the free radical signal intensity in irradiated and β-thalassemia major samples respectively comparing to the control value. In case of addition of α-lipoic acid to the irradiated blood and thalassemia major, the signal intensity of the free radical was diminished by factor 41% and 43% respectively. The obtained results confirmed that the exogenously supplied with α–lipoic acid had antioxidant properties and is effective in decreasing the damage caused by reactive oxygen species (ROS).

Key words: Lipoic acid, hemoglobin, thalassemia, gamma radiation.

INTRODUCTION

Oxidative stress is the potential biological damage caused by the harmful effect of oxygen free radicals. Oxygen free radicals or, more generally, reactive oxygen species (ROS) have been implicated in the pathogenesis of certain diseases, or the exposure to exogenous factors such as ionizing radiation. The biological effects of (ROS) are controlled *in vivo* by a wide spectrum of enzymatic

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and non-enzymatic defense mechanisms. An imbalance between the production and detoxification of ROS results in oxidative stress [22]. Introduction of exogenous antioxidants can be useful in the treatment of such cases. Antioxidant activity depends on the kind of oxidative stress and on oxidative substrate. According to Packer *et al*., 1995 [14], when we evaluate the antioxidant potential of a compound, criteria such as (a) specificity of free radical scavenging, (b) interaction with other antioxidants, (c) metal-chelating activity, (d) effects on gene expression, (e) bioavailability, (f) location (in aqueous or membrane domains, or in both), and (*g*) ability to repair oxidative damage have to be taken into consideration [12]. An antioxidant needs only to meet a few of the previously mentioned criteria to play an important role in the detoxification of oxidative stress. Among the extensively considered antioxidants it is α-lipoic acid. It is unique, among antioxidant molecules, because it retains protective functions in both its reduced and oxidized forms. It has been found that exogenously supplied α-lipoic acid has antioxidant properties and is effective in preventing or lessening damage caused by reactive oxygen species (ROS) [14]. It has been shown to be beneficial in various forms of oxidative stress and it is of interest as a therapeutic in ischemia-reperfusion injury, diabetic complications, cataract formation, HIV activation, neuro-degenerative disorders and radiation injury [14].

In vitro studies gained special interest since they can provide the basis for the detailed evaluation of the potential effects of antioxidants. Assay of free radicals, for example, can provide some leads regarding the antioxidant potential of the material tested [6]. The aim of this work was to study *in vitro* the ability of α-lipoic acid to protect hemoglobin against the oxidative damage resulted from two different sources: exposure to gamma radiation and β-thalassemic disease using electron paramagnetic resonance EPR technique.

Hemoglobin, or the breathing molecule, is more susceptible to oxidation by oxygen species. Hence, studying the effects of antioxidant on this molecule acquired special interest. Hemoglobin is a tetramer of two α- and two β-globin chains. Exquisitely coordinated expression of these α- and β-globin chains is required during erythropoiesis to generate high concentrations of hemoglobin, without production of either chain in excess; any disruption of normal globin gene expression patterns can lead to serious human diseases. One such disease is β-thalassemia, a common genetic disorder caused by mutations in one or more of the β-globin gene loci that result in reduced β-globin production. In addition to the direct effects of reduced β-globin synthesis, many of the symptoms of this disorder appear to be consequences of the resulting cytotoxic buildup of free α-globin. Free α-globin is highly unstable and readily precipitates, damaging membrane structures and triggering the apoptotic cell death of erythroid precursors [4]. It has been suggested that such an excess of α-chains is the source of oxidative stress. This is probably due to the fact that α-chains are more prone to release iron in reactive form [2, 18]. In α-hemoglobin chains loaded erythrocyte membrane

bound heme and iron were markedly elevated and the cells were more prone to oxidative stress. In β̃-thalassemic and sickle cells, elevated amounts of heme iron and especially of free iron were observed. This form of iron may thus represent the trigger for the oxidative damage seen in β̃-thalassemia cells [16]. Thalassemia is distributed widely in the Mediterranean area, the Middle East, Tropical Africa and the Caribbean. In Egypt, β-thalassemia is the commonest cause of chronic hemolytic anemia and it represents a major genetic disease and a public health problem [15]. The therapeutic treatment of β-thalassemic patients involves many aspects, among them blood transfusion. However, this continuous blood transfusion leads to an iron overload that induces a vicious circle resulting in chronic oxidative stress (COS). Indeed oxygen-free radicals and peroxidative tissue injury accompany the anemia and represent an unavoidable complication that accelerates the multi-organ abnormalities, especially organs that accumulate excess iron, including liver, pituitary gland, pancreas and heart. The latter, which has less developed antioxidant defenses, is particularly susceptible to the iron-induced peroxidative damage that, ultimately, can lead to congestive heart failure, which is the main cause of death in thalassemia patients [3, 20].

On the other hand, exposure to gamma radiation was known to induce changes either directly in the biological molecules or indirectly in their surrounding media, where water radiolysis and free radicals are the major products. The generated free radicals can react rapidly with a range of targets including the side chains of amino acids of the protein molecules. Hemoglobin molecule is very sensitive to any change in its environment, where it responds and changes its conformation to carry oxygen and release carbon dioxide or vice versa. Gamma radiation is shown to affect every part of the hemoglobin molecule. The damaging effect cannot be limited to molecular degradation or iron oxidation or even folding to the quaternary structure. The acute exposure of guinea pigs to 3 and 6 Gy gamma irradiation was investigated using IR spectroscopy. The results showed conformational transitions of a definite portion of the α-helix in the globules of irradiated hemoglobin in the β-form (stretched out portions of the chains). These observations are an indication of denaturation changes, i.e., changes in the three-dimensional structure, including the level of the quaternary, tertiary, and secondary structures of the hemoglobin macromolecules as a result of the influence of penetrating radiation, radiolysis products of water, and other radiotoxins, which induce breakage of hydrogen bonds, –S–S– bonds, lengthening of –C–N– bonds, the induction of Van der Waals contacts and ionic interactions within the globules of irradiated hemoglobins [23]. It was found that *in vitro* exposure of hemoglobin to ionizing radiation resulted in increase of free radicals production, the decrease in α-helices contents, which reflects the degradation of hemoglobin molecular structure, or at least its incomplete performance [10].

The oxidative stress resulting from β-thalassemia differs from that resulting from exposure to gamma radiation. The present work studies the effects of α-lipoic acid on the oxidative stress resulting from these two different conditions.

MATERIALS AND METHODS

CLINICAL SAMPLES

The β-thalassemia patients were previously diagnosed and were under the supervision of medical professionals during this period. The patients were transfusion dependent. Blood from twenty thalassemic patients was collected just before transfusion. Blood from healthy individuals was used as a normal control for this study. All individuals were aged between 6 and 10 years. The average hemoglobin concentration ranged between 3.4 and 7.7 g/dL. Patients were under chelation therapy with desferoxamine (DFO).

ALPHA LIPOIC ACID TREATMENT

α-lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid or thioctic acid) is a sulfur containing cofactor, and in its reduced form, the dihydrolipoic acid (DHLA, 6, 8-dimercaptooctanoic acid or 6, 8- thioctic acid), two thiol groups per molecule are present (Fig. 1). Alpha lipoic acid, a widely occurring coenzyme found in prokaryotic and eukaryotic microorganisms as well as in animals and plants. Heparinized blood samples were incubated with alpha lipoic acid at concentration 200 μM/L. The mixture was incubated at 37 ºC for one hour.

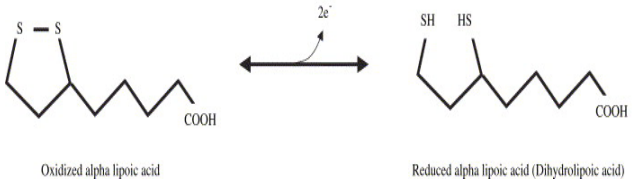

Fig. 1. Structure of the oxidized and reduced form of lipoic acid.

GAMMA IRRADIATION

Normal blood samples were received from adult healthy non-smoking volunteers. The blood samples were washed and diluted with Phosphate buffer saline (PBS) to make erythrocyte suspension with 45% hematocrit value. Erythrocyte suspension was incubated with α-lipoic acid at concentration 200 μM/L at 37 ºC for one hour before gamma irradiation. The suspension was

irradiated with ^{60}Co source belonging to the National Center for Radiation Research and Technology. The absorbed dose was 150 Gy and the dose rate of the source was 53.37 kGy/h.

HEMOGLOBIN EXTRACTION

Hemolysates of washed, packed erythrocytes were prepared by a modification of the method of Trivelli *et al.* [21]. The blood was centrifuged at 1500 rpm for 15 minutes at 4 ºC, then the plasma was removed and the packed cells were washed with 5 volumes phosphate buffered saline, centrifuged and the saline was removed. The procedure was repeated two additional times, and the saline was removed after the final wash. The packed cells were lysed with 2 volumes of deionized water, then the mixture was centrifuged at 10 000 rpm for 30 minutes at 4 ºC, and the supernatant was extracted. The extracted hemoglobin was lyophilized in a freeze drier, at –60 ºC and 70 mbar.

EPR SPECTROSCOPY

EPR spectra for hemoglobin were measured with an X-band ESR spectrometer (Bruker, EMX) at room temperature using standard rectangular cavity (4102 ST) operating at 9.7 GHz with a 100 kHz modulation frequency. The ESR parameters were chosen to provide the maximum signal-to-noise ratio. The microwave power and modulation amplitude were 8 mW and 1 G respectively. The response time constant was 40 ms with the field-sweeping rate of 100 G / 164 s. The Intensity of each sample has been measured 10 times as the peak-to-peak height. The average value of these measurements has been plotted. The standard deviation was about 5% from the mean value. Standard sample of MgO doped with Mn^{2+} was used to calibrate the ESR intensity and the *g*-factor of the signals.

RESULTS AND DISCUSSION

LA/DHLA are considered ideal therapeutic antioxidant because they are naturally existing, low molecular weight compounds with very powerful antioxidant effective in both aqueous and lipid domains. Their effects include free radical quenching [11], metal chelation [13] and regeneration of other antioxidant such as ascorbic acid, vitamin E and glutathione [8]. It has been found that exogenously supplied α-lipoic acid has antioxidant properties and is effective in preventing or lessening damage caused by reactive oxygen species (ROS) [14]. Because of its antioxidant properties, α-lipoic acid has been tested as a possible therapeutic agent in many common diseases like diabetes [1, 5]. This raises the possibility of using α-lipoic acid which can help against oxidative damage in other

cases such as β-thalassemia and exposure to ionizing radiation. The samples considered for this study were taken from patient receiving desferoxamine therapy as iron chelating agent. Quenching experiments in the presence or absence of desferoxamine have demonstrated that LA is an efficient hydroxyl radical quencher owing to its unique disulfide bond in the thiolane ring.

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR) and electron magnetic resonance (EMR), is the name given to the process of resonant absorption of microwave radiation by paramagnetic ions or molecules. Almost all electrons go around in pairs, but some are solitary. These "unpaired electrons" are found in free radicals (highly reactive molecules) and transition metals ions (e.g. iron, copper, cobalt). Therefore, EPR can be used to identify biological molecules that contain free radicals or transition metal ions in their structure. Even more usefully EPR is a quantitative technique, i.e. we can determine the concentration of unpaired electrons present in a sample even if one does not know the exact nature of the free radical being observed.

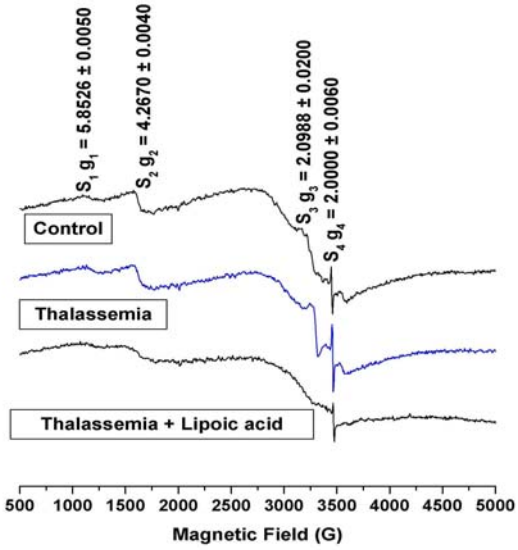

Fig. 2. EPR spectrum of the control, thalassemia and thalassemia with lipoic acid hemoglobin.

Fig. 2 represents an example of the X-band (9 GHz) EPR spectra of the human hemoglobin at room temperature. The first pronounced feature (S1) is the signal at g =5.8 which originates from heme protein in the high spin ferric state (Fe^{+3}). The EPR signal in hemoglobin spectrum with g-factor of 4.28 (S2) is characteristic of non-heme ferric ions Fe^{+3} in rhombic coordination. Third signal group (S3) is associated with low spin derivatives of ferri-hemoglobin called

"hemichrome", copper proteins and some transition-metal complexes. The third signal group (S3) is associated with low spin derivatives of ferri-hemoglobin called "4) that can be seen in the EPR spectrum of the hemoglobin is the signal at *g* = hemichrome", copper proteins and some transition-metal complexes. The last EPR signal (S4) that can be seen in the EPR spectrum of the hemoglobin is the signal at $g = 2 \pm 0.0006$ from free radicals. The free radicals responsible for this signal are tyrosyl radicals formed on hemoglobin following its interaction with peroxide [19].

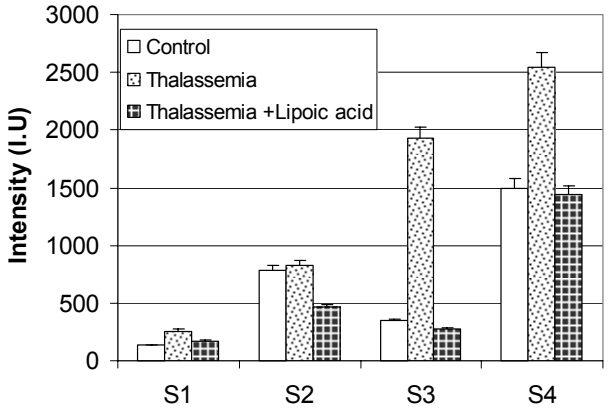

Fig. 3. Changes in the intensity of the EPR signals.

In spite of iron chelation therapy by desferoxamine (DFO), there was a pronounced increase in the intensity of the S1, S2, S3 and S4 by factors 90%, 5.4%, 456% and 69% respectively compared with the control samples. It is clear from the EPR spectra of thalassemic hemoglobin that the addition of α-lipoic acid diminishes the intensity of the first two signals (S1 and S2) and the last one (S4) by factors 42%, while it diminishes the S3 signal by factor 85.6% (Fig. 3). It is known that iron is a redox active element, which can seriously intensify oxidative stress by generating OH radicals *via* Fenton reaction. The obtained results revealed that the α-lipoic acid supplementation reduces the risk of Fe catalyzed oxidative damage. This is clear from the decrease of the intensity of free radical signal (S4) by 42%.

Fig. 4 shows the EPR spectra of normal and irradiated hemoglobin (150 Gy). Results showed that the most obvious radiation-induced change in irradiated hemoglobin EPR spectrum is the significant increase in S4. This may be due to the increase in the production of free radicals in hemoglobin protein (peroxyl and tyrosyl radicals) and reflects its high sensitivity to irradiation [10].

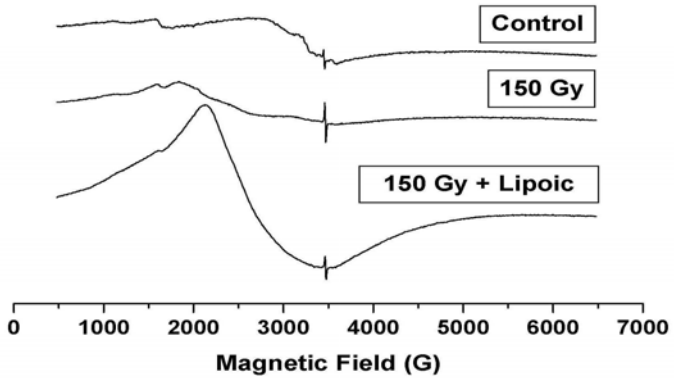

Fig. 4. EPR spectra of control, irradiated and irradiated hemoglobin with α-lipoic acid.

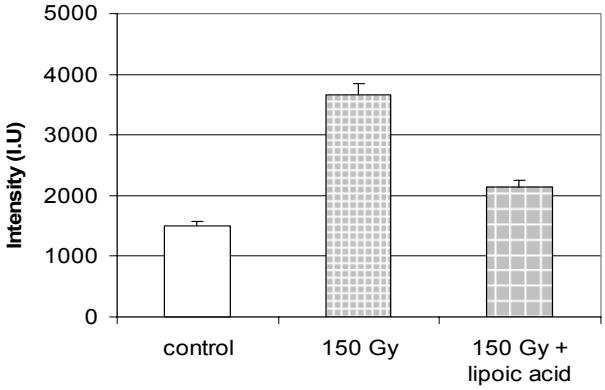

Fig. 5. Changes in the EPR (S4) signal intensity for control, irradiated and irradiated & α- lipoic acid hemoglobin.

Fig. 5 shows the change in the EPR S4 signal intensity (at $g = 2.0000 \pm 0.006$) for control, irradiated (150 Gy) and irradiated hemoglobin with lipoic acid. It clear that the gamma irradiation caused an increase in the free radical signal intensity by factor 106%. At the same time there was a decline in this signal after incubation with α-lipoic acid by factor 41% which reflects the protective role of α-lipoic acid against the oxidative stress of gamma radiation.

The obtained results demonstrated that the *in vitro* incubation of β-thalassemia and irradiated blood with α-lipoic acid lessened the EPR signals intensity. However, the two cases are completely different. In β-thalassemia, the oxidative stress was found prior to the incubation with α-lipoic acid, while, the irradiated samples were incubated before the exposure to gamma radiation. This effect can be explained by the ability of α-lipoic acid to exert multiple effects in order to diminish the oxidative stress. It can chelate the iron, scavenge the reactive oxygen species and repair oxidative damage. The advantage of using α-lipoic acid lies in

that it is capable of regenerating endogenous antioxidants in the body including vitamin C, vitamin E and intracellular reduced glutathione (GSH). Also, it is known that α-lipoid acid is converted normally to dihydroliopic acid (DHLA) in all cells and tissues [9].

In patients with thalassemia major, the regular program of transfusion leads to systemic iron overload that further potentiates the generation of reactive oxygen species (ROS), associated with decreased level of plasma antioxidants, low activities of enzyme antioxidant, reduced level of glutathione and increased lipid peroxidation of RBC membranes [17]. Thus, the ability of antioxidant to reduce the hazard and complications of iron overload is a paramount consideration. *In vitro* DHLA chelates both ferric and ferrous iron, therefore, preventing Fe oxidative damage [1]. Iron is a redox active element, which can seriously intensify oxidative stress by generating OH radicals *via* Fenton reaction. It was found that lipoic acid supplementation increases the cells ability to store Fe not by increasing the incorporation ratio, but by increasing the content of ferritin, thereby increasing cells' capacity for sequestering more Fe [7].

The obtained results showed that lipoic acid can be used as a safe therapeutic antioxidant and as a possible treatment in Fe overload conditions. Further *in vivo* studies are required.

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