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Assessment of the role of α-lipoic acid against the oxidative stress of induced iron overload

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ABSTRACT

This work was aimed to study the protective role of α-lipoic acid against the oxidative damage of induced iron overload. Iron (Fe) overload is a complication of the treatment, by chronic transfusion, of a number of genetic diseases associated with inadequate red cell production (anemias) and of other genetic diseases that lead to excessive iron absorption from the diet. Male rats were injected ip with 5 mg/kg body weight ferrous sulfate for 50 days. The animals were injected ip with α-lipoic acid 20 mg per kg body weight for 21 days. Serum iron, Total Iron Binding Capacity (TIBC), Malonyldialdehyde (MDA), Electron paramagnetic resonance (EPR) spectroscopy, UV-visible absorption spectrum of hemoglobin and osmotic fragility were studied. Results showed significant increase in serum iron, total iron binding capacity, and malonyldialdehyde levels in iron-loaded rats. Treatment with lipoic acid (LA) resulted in decreasing serum iron and TIBC levels by 47%and 29% respectively. At the same time the lipoic acid decreased the level of the MDA in liver, brain and plasma by 54%, 42% and 74% respectively. Also LA diminished the effect of iron-induced free radicals on erythrocyte membrane integrity; it decreased the elevated average osmotic fragility and decreased the elevated rate of hemolysis. Results from UV-visible spectrophotometric measurement of hemoglobin revealed that no oxidative changes of hemoglobin occurred in iron-loaded rats. EPR spectra showed increased in non-heme ferric ions Fe^{+3} and free radicals in iron-loaded rats. Whereas the injection of the lipoic acid leads to decreased in such toxic result. In conclusion, these observations suggested that lipoic acid might be a beneficial antioxidant that can be effective for limiting damage from oxidative stress of iron overload.

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1. Introduction

Iron (Fe) overload is a complication of the treatment, by chronic transfusion, of a number of genetic diseases associated with inadequate red cell production (anemias) and of other genetic diseases that lead to excessive iron absorption from the diet. Two relatively common anemic conditions that can result in iron overload from hypertransfusion are sickle cell disease and thalassemia. It can cause tissue damage and ultimately dysfunction of visceral organs (mainly in the heart, liver, and endocrine glands) (Britton, Leicester, & Bacon, 2002). Fe-related neurodegenerative disorders can result from both Fe accumulation or defects in its metabolism and/or homeostasis (Batista-Nascimento, Pimentel, Menezes, & Rodrigues-Pousada, 2012). Iron progressively accumulates in the brain during normal aging, however, iron accumulation in Alzheimer's disease (AD) occurs without the concomitant increase in ferritin normally observed with aging (Bartzokis et al., 2007; Quintana et al., 2006; Quintana & Gutierrez, 2010). Abnormal Fe metabolism can result in human neurologic disorders such as Alzheimer's, Parkinson's, and Huntigton disease (Gerlach, Ben-Shachar, Riederer, & Youdin, 1994).

A highly toxic form of iron, non-transferrin-bound iron (NTBI), is formed when the iron-binding capacity of transferrin has been exceeded. Uptake of the plasma NTBI into tissues contributes to increased intracellular labile iron pool (LIP) (Esposito et al., 2003; Livrea et al., 1996). Potentially, it can catalyze the formation of reactive oxygen species (ROS) through the Fenton reaction. Free Fe (II) can participate in the Fenton reaction, generating highly reactive hydroxyl radical.

$$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + OH^{\bullet} + OH^{-} \quad (1)$$

The superoxide radical, generated by mitochondria, participates in the Haber–Weiss reaction.

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-} \quad (2)$$

which combines a Fenton reaction and the reduction of Fe (III) by superoxide, yielding Fe (II) and oxygen.

$$Fe\ (III) + O_2^{\bullet -} \rightarrow Fe\ (II) + O_2 \text{ (Liochev \& Fridovich, 2002)} \quad (3)$$

The hydroxyl radical is highly reactive with a half-life in aqueous solution of less than 1 ns (Poster, Weinstein, Jamison, & Bernowitz, 2000). Thus when produced in vivo it reacts close to its site of formation. Patients with thalassemia have very high plasma levels of malonydialdehyde (MDA); a by-product of lipid peroxidation (Livrea et al., 1996).

Due to oxidative stress and iron overload conditions in transfusion-dependent β-thalassemia major patients, combination of effective iron-chelating agent with antioxidant can be very helpful for the patients.

Alpha-Lipoic acid (LA) is a naturally occurring compound that was shown to be synthesized by animals and humans (Carreau, 1979). It functions as a cofactor in several mitochondrial multienzyme complexes involved in energy production. In these reactions LA (Fig. 1) is reduced to dihydrolipoic acid (DHLA) that is reoxidized by lipoamide dehydrogenase accompanied with NADH formation (Biewenga, Haenen, & Bast, 1997) (Fig. 1).

Both LA and DHLA were shown to have antioxidant potential in vitro and in vivo systems (Goroca, Huk-Kolega, Kleniewska, Piechota-Polanczyk, & Skibska, 2013; Li, Liu, Shi, & Jia, 2013; Matsugo, Yan, Han, Trischler, & Packer, 1995; Suzuki, Tsuchiya, & Packer, 1991). This new view of an old cofactor, LA led scientists to explore the antioxidant properties and therapeutic implications of the compound in diseases associated with oxidative stress. Lipoic acid is very effective in the treatment of heavy metal poisoning. Gurer, Ozgunes, Oztezcan, & Ercal, 1999 demonstrated in vitro the effectiveness of LA in protecting cells against the cytotoxic effect of lead where Chinese hamster ovary (CHO) cells were used. LA is a potent chelator of divalent metal ions *in vitro*, so it was investigated whether feeding R-α-LA (0.2% [w/w]; 2 weeks) to aged rats could lower cortical iron levels and improve antioxidant status. Results show that cerebral iron levels in old animals fed LA were lower when compared to controls and were similar to levels seen in young rats (Suh, Moreau, Heath, & Hagen, 2005).

The aim of this study was to investigate the iron-chelating and free radicals scavenging capacities of lipoic acid in vivo. For this purpose, the animals were injected interaperitoneally (ip) with ferrous sulfate and levels of iron overload and oxidative stress parameters were measured in their blood and tissues.

2. Materials and methods

2.1. Chemicals

(±)-α-lipoic acid, ferrous sulfate, and dimethyl sulfoxide "DMSO" were purchased from sigma chemical co. (St. Louis, MO, USA).

2.2. Animals and treatment

Male albino rats (initial weight 90–110) were housed in plastic cages at ambient temperature, humidity, and controlled light (12-h day/night cycle), divided into four groups (8 animals each). Group 1 (control) was untreated, Group 2 was intraperitoneally injected with ferrous sulfate dissolved in sodium chloride 0.9% (ip 5 mg $FeSO_4$/kg body weight/day) for 50 days.

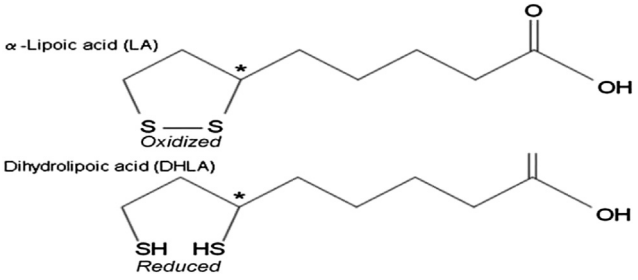

Fig. 1 – Lipoic acid and its reduced form, dihydrolipoic acid, (Cornille, Alkhallaf, Schwartz, Baronzio, & Delépine, 2012).

Group 3 was intraperitoneally injected with ferrous sulfate dissolved in sodium chloride 0.9% (ip 5 mg $FeSO_4$/kg body weight/day) for 50 days and injected intraperitoneally with lipoic acid dissolved in DMSO (ip 20 mg LA/kg body weight/day) for 21 days. Group 4 (positive control) was intraperitoneally injected with LA (ip 20 mg LA/kg body weight/day) for 21 days.

At the end of the treatment the rats were anesthetized under diethyl ether atmosphere, blood samples were collected from orbital sinus, and finally killed by rapid decapitation. Brains and livers were excised, washed, weight and quickly homogenized in sodium chloride solution 0.9% in a ratio of 10 ml saline to 1 gm of tissue. Portion of blood samples were collected in heparinized tubes for EPR spectroscopy, osmotic fragility test, UV-visible spectrophotometric measurement and to obtain plasma for MDA test after centrifugation at 3000 rpm for 15 min. The other portion of blood samples were collected in non-heparinized tubes to obtain serum for serum iron "SI" and total iron binding capacity "TIBC" tests.

2.3. *Measurement of serum iron "SI" and total iron binding capacity "TIBC"*

The method presented is a modification of that reported by Persijn, Van Der Slik, & Riethorst, 1971 using the chromogenic compound, Ferrozine, described by Stookey, 1970. In addition to iron, copper is the only other trace metal found in serum reported to form a colored complex with Ferrozine (Carter, 1971). Neocuproine is therefore used in the color reagent to prevent copper interference. All reagents are obtained from Stanbio laboratory, USA. Iron is released from its combination with transferrin in acid medium, reduced to the ferrous form by hydroxylamine and reacted with Ferrozine to form a violet colored complex which is measured at 560 nm. Since only about one-third of the serum iron is normally bound to transferrin, a separate technique for serum unsaturated iron-binding capacity (UIBC) which involves the addition of a known excess of ferrous ions, which saturate available transferrin iron binding sites. excess (unbound) iron is then quantitated as described above, with UIBC being the difference in iron concentration between that added and that determined in the remaining excess. It follows that serum total iron-binding capacity (TIBC) is the sum of iron and UIBC.

2.4. *Measurement of malonyldialdehyde "MDA"*

The formation of MDA is an indication of lipid peroxidation and one of the most commonly known tests used to quantify MDA formation is the Thiobarbituric acid reactive substances "TBARS" assay. Plasma or homogenized tissue mixed with 20% TCA "20 gm of Trichloroacetic acid in 100 ml distilled water". Thiobarbituric acid solution "0.67 gm in 100 ml distilled water" was added to the tubes containing unknown. All tubes were incubated at 100 °C for 30 min, cooled down rapidly to room temperature. Finally n-butylbutanol was added to tubes. The mixture was centrifuged at 3000 rpm for 10 min. Absorption was measured at 535 nm against reagent blank. The amount of TBARS was calculated as MDA equivalents using 1,1,3,3-tetramethoxypropane as the reference standard.

2.5. *Erythrocyte osmotic fragility*

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 (0, 2, 3, 3.5, 4, 5, 5.5, 6, 7.5 and 9 g/L) was used. The samples were incubated for 30 min at 37 °C, and centrifuged at 3000 rpm for 5 min to precepitate the nonhemolyzed red 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. The quantitative measurements (degree of hemolysis versus decreasing NaCl concentration) were plotted on a graph called the fragility curve (Mazeron, Diedelon, Muller, & Stoltz, 2000). The experimental curves were normalized to 100% hemolysis to facilitate the comparison between different samples without the interference of the hematocrit changes. The average osmotic fragility (H_{50}) (the NaCl concentration producing 50% hemolysis) can be evaluated by the fragility curve. Other parameters can be obtained from the differentiation of the fragility curve, which represents a Gaussian curve (the rate of hemolysis dH/dC versus NaCl concentration). These parameters are peak's position, peak's width, peak's area, and peak's height. The peak's position on the *x*-axis (C) is equivalent to the average osmotic fragility (H_{50}). The width at half maximum reflect the dispersion of the hemolysis process. The area under the curve (A) represents the rate of hemolysis of erythrocyte at certain interval NaCl concentration. The Gaussian peak (P) represents the maximum rate of hemolysis $(dH/dC)_{max}$ reached by the sample (Selim, Desouky, Ali, Ibrahim, & Ashry, 2009).

2.6. *Extraction of hemoglobin*

Hemolysates of washed, packed erythrocytes were prepared by a modification of the method of Trivelli, Ranny, & Lai (1971). The blood was centrifuged at 3000 rpm for 10 min at 4 °C, then the plasma was removed and the packed cells were washed with 5 volume saline at 20 °C, 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 distilled water, the mixture was centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was extracted for spectrophotometric measurements.

2.7. *Spectrophotometric measurements*

The absorption spectrum of hemoglobin was recorded in the wavelength range from 300 to 700 nm by means of UV–visible spectrophotometer Jenway type 6405 manufactured at UK. The hemoglobin concentration was adjusted to 0.2 mg/ml for spectrophotometric analysis, using Drabkin's reagent and hemoglobin standard, obtained from spectrum Diagnostics, Egypt.

2.8. *EPR spectroscopy*

EPR spectra for lyophilized blood were measured with an X-band ESR spectrometer (Bruker, EMX) at room temperature

Table 1 – The values of serum iron, TIBC, and % transferrin saturation for the four groups.

Groups	SI "μg/dL"	TIBC "μg/dL"	% Transferrin saturation
Control	231.5 ± 7.6	627 ± 11.3	36.9 ± 0.007
Iron overload	440.8[a] ± 38.7	858.6[a] ± 21.9	51.3[a] ± 0.040
Iron overload + lipoic acid	233.7[b] ± 6	607.7[b] ± 39.1	38.5[b] ± 0.014
Positive control	157.9 ± 5.6	467.5 ± 31.8	33.8 ± 0.015

[a] Statistically significant from control $P < 0.05$.
[b] Statistically significant from I. overload $P < 0.05$.

using high sensitivity cylindrical resonator (ER4119HS) 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.

2.9. *Data fitting*

The fitting of the experimental data (osmotic fragility) was carried out by the Origin software. The applied two functions are Gaussian function and sigmoidal function.

2.10. *Statistical analysis*

In this study, the values are expressed as mean ± standard deviation. The significance of the difference between each value presented by various groups was evaluated by the Student *t*-test and values with $P < 0.05$ were considered as statistically significant.

3. Results

3.1. *Serum iron "SI" and total iron binding capacity "TIBC"*

There is a significant increase in serum iron, TIBC, and percent of transferrin saturation in the iron overload group as a result of administration of ferrous sulfate $FeSO_4$ by factors 48%, 27% and 28% respectively. whereas in the third group the administration of ALA decrease the levels of the SI, TIBC, and percent of transferrin values by factors 47%, 29% and 33% respectively as shown in Table 1.

3.2. *Measurement of malonyldialdehyde "MDA"*

In this study, the levels of MDA in the plasma, liver and brain were significantly increased by factors 54%, 41% and 74% respectively in iron overload group comparing to the control group. After lipoic acid treatment the MDA significantly decreased to the control group levels (Table 2).

3.3. *Erythrocyte osmotic fragility*

In the present study, iron overload curve showed shift to the right of the control curve, indicating an increase in the average osmotic fragility (H_{50}). At the same time the dispersion of hemolysis (S) decreased significantly as shown in Table 3. The obtained results of iron overload curve showed an increase in the maximum rate of hemolysis (P) with concomitant shift of the peaks center (C) toward higher values of NaCl concentration (Table 3). Administration of lipoic acid diminished the iron-induced effect on RBCs structural integrity. (Table 3, Fig. 2, Fig. 3).

3.4. *Spectrophotometric measurements*

Fig. 4 showed the UV–visible spectra of hemoglobin for all groups. At first glance, the major observed effect for iron overload is the hypochromocity of the iron overload peaks. However, LA minimized the iron-induced effect as shown in Table 4.

The Soret band is the most characteristic intense band for porphyrine ring. It occurs at 410 nm. The porphyrine ring showed two peaks at 540 and 574 nm (α at 574 nm and β at 540 nm). The ratio between those two peaks (A_α/A_β) is larger than unity for oxygenated normal hemoglobin.

3.5. *EPR spectroscopy*

In the present work two features comprised EPR spectra of g-factor equal to: $g = 4.2664 \pm 0.0010$ and $g = 2.0015 \pm 0.0013$ as shown in Fig. 4.

The EPR signal recorded at $g = 4.26$ (S_1) is characteristic to the non-heme ferric ions Fe^{+3} in rhombic coordination. Other research (Gamarra et al., 2008; Maghraby & Ali, 2007) shows

Table 2 – The level of MDA in plasma, Liver, and brain for the four groups.

Groups	Liver MDA "nmol/gm"	Brain MDA "nmol/gm"	Plasma MDA "nmol/L"
Control	100.9 ± 17.1	67.1 ± 11.7	5.49 ± 1.07
Iron overload	**218.5**[a] ± 23.0	**115.8**[a] ± 24.0	**21.4**[a] ± 4.06
Iron overload + Lipoic acid	**91.4**[b] ± 13.8	**66.3**[b] ± 12.5	**7.8**[b] ± 1.99
Positive control	88.8 ± 15.3	43.2 ± 4.6	5.4 ± 0.50

[a] Statistically significant from control $P < 0.05$.
[b] Statistically significant from I. overload $P < 0.05$.

Table 3 – The center (H_{50}), the width (S), the area (A), and the height (P) of the Gaussian peaks for the four groups.

Groups	H_{50} (g/l)	S(g/l)	Area (A) mm^2	Height (P) %
Control	4.52 ± 0.005	0.98 ± 0.007	85.9 ± 0.640	69.62
Iron overload	**5.18**[a] ± 0.001	**0.87**[a] ± 0.006	85.3 ± 0.617	77.73
Iron overload + Lipoic acid	**4.37**[b] ± 0.003	**0.99**[b] ± 0.007	86.6 ± 0.647	69.39
Positive control	4.81 ± 0.0001	1.00 ± 0.007	89.5 ± 0.671	70.78

[a] Statistically significant from control $P < 0.05$.
[b] Statistically significant from I. overload $P < 0.05$.

that this signal is a single peak and in others (Moreira et al., 2008; Kolesar et al., 2008) it showed a complex. Most authors have interpreted this signal as originating from Fe^{+3} in transferrin (Gamarra et al., 2008; Kolesar et al., 2008; Krzyminiewski et al., 2011; Maghraby & Ali, 2007; Moreira et al., 2008). With regard to the second signal (S_2) it appeared as a singlet with no hyperfine structure as shown in Fig. 5. this signal is attributed to free radicals in hemoglobin formed by the degradation of blood constituents (Miki, Kai, & Ikeya, 1987).

In iron-induced group there was a pronounced increase in the intensity of the S_1 and S_2 by factors 23% and 40% respectively compared with the control samples. It is clear from the EPR spectra that the addition of α-lipoic acid diminished the intensity of the first two signals (S_1 and S_2) by factors 14% and 36% respectively.

4. Discussion

In this study, the iron overload was studied by administration of ferrous sulfate to animals. Organisms overloaded by iron (as a condition of hemochromatosis, β-thalassemia, sickle cell anemia) contain a higher amount of free available iron and this have deleterious effect. Free iron can participate in the Fenton reaction generating reactive oxygen species which can attack vital cell component like polyunsaturated fatty acid (lipid peroxidation), proteins and nucleic acid. Patients with thalassemia have very high plasma levels of malonydialdehyde (MDA); a by-product of lipid peroxidation (Carreau, 1979). Brain tissue has 60% lipid, and it has remarkable high energy consumption. In addition, these tissues are more susceptible to oxidative damage than other tissues (Foloyd, West, & Hensley, 2001). The obtained results, showed an increase in serum iron and TIBC levels in iron-loaded rats. This elevation is accompanied by an increase in lipid peroxidation in plasma, liver and brain.

Lipoic acid treatment decreased the SI and TIBC levels, this in agreement with Goralska, Dackor, Holley, & Christine McGahan, 2003 who provided that lipoic acid supplementation lowerd the concentration of free, chelatable Fe in the cytosolic, low-molecular-weight pool of Fe (LIP) of intact lens epithelial cells. It has been documented that extracellular lipoic acid taken up by the cells undergoes intracellular reduction into DHLA, which is subsequently released back into the media (Handelman, Han, Tritschler, & Packer, 1994). Hence, DHLA is a potent reductant and may mobilize and subsequently chelate transferrin-bound Fe making it unavailable for utilization by the cells. Also Suh et al. (2005) reported that LA is a potent chelator of divalent metal ions in vitro. Results showed that cerebral iron levels in old animals fed LA were lower when compared to controls and were similar to levels seen in young rats. These results showed that chronic LA supplementation may be a means to modulate the age-related accumulation of cortical iron content, thereby lowering oxidative stress associated with aging.

Lipid peroxidation (LPO) is one of the most important expressions of oxidative stress induced by ROS. Lipid peroxidation is a free radical-induced process that leads to oxidative deterioration of polyunsaturated fatty acids. Malonydialdehyde (MDA) is an indicator of lipid peroxidation induced by iron overload. On administration of lipoic acid, the level of lipid peroxidation in plasma, liver and brain was significantly decreased in LA-administrated rats. After ip administration of 20 mg LA/kg, the antioxidant is primarily accumulated in the liver, heart and skeletal muscle, after which it efficiently crosses the blood–brain barrier to accumulate in several brain regions (Arivazhagan, Thangaswamy, Ramanathan, Kumaran, & Pannerselvam, 2002), it reduced the amount of the hydroxyl radical that was generated by Fenton reaction, and it also scavenges the peroxide and superoxide radical (Sumathi, Jayanthi, & Varalakshmi, 1993). The palliative effect of LA on lipid peroxidation are in agreement with Çelik and Özkaya (2002) who demonstrated that the lipoic acid administration can be more effective than Vitamin E in preventing lipid peroxidation in brain tissue. This may be attributed to the bioactivity of lipoic acid to directly react with oxidation species, as well as its ability to interfere with the oxidation processes in the lipid and aqueous cellular compartments (Packer, Roy, & Sen, 1997). The dihydrolipoic acid, which formed by reduction of lipoic acid in cells, has two sulfhydryl groups that suggested a promising chelating effect on iron (Arivazhagan et al., 2002). This metal chelating property of lipoate may be responsible for the observed decrease in lipid peroxidation.

Erythrocyte osmotic fragility is an indirect method of assessing oxidative stress (Chihuailaf, Contreras, & Wittwer, 2000) as it gives information about the status of red blood cell metabolism and membrane stability (Sharma, Rai, Rai, Rizvi, & Watal, 2009). The hemolysis of the red blood cells reflected the loss of integrity of the cells which can lead to the liberation of intracellular hemoglobin (Schon, Ziegler, Gartner, & Kraft, 1994). Membrane lipids are vital for cellular integrity maintenance and survival (Jain, McVie, Duett, & Herbst, 1989). Elevated MDA, an indicator of lipid peroxidation, can disturb the biochemical and physiological functions of the red blood

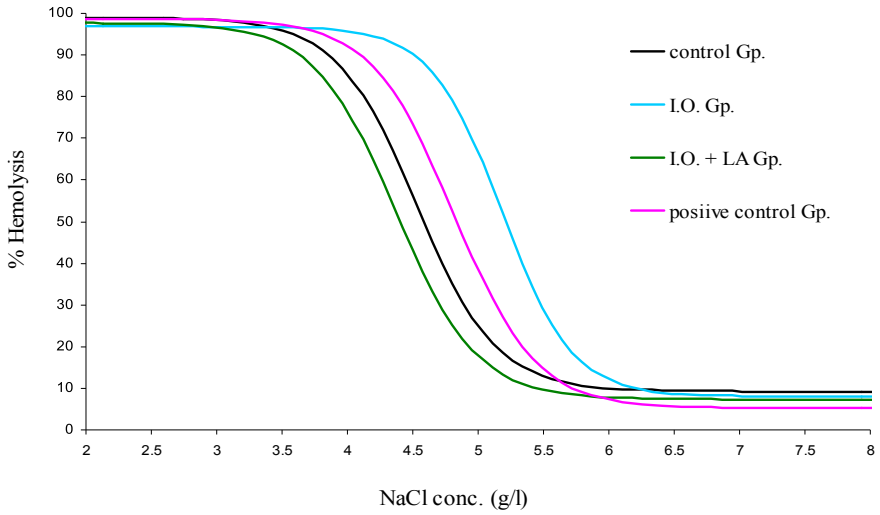

Fig. 2 – Fragility curves for the four groups.

cells. The lipid peroxidative alteration of erythrocyte membrane resulting in an increase in fragility of the red blood cell in iron overload group. This appears as an increase in the maximum rate of hemolysis (peak's height), and decrease in dispersion of hemolysis (lower dispersion than normal indicates sudden rupture of red blood cell). Also the center of the Gaussian peak (H_{50}) shifted toward higher NaCl concentration. 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 (Mohandas & Chasis, 1993; Mohandas, Chasis, & Shohet, 1983). Oxygen free radicals also alter cation permeability, reduce red blood cells deformability, and disturb microrheological properties of red blood cell membrane (membrane rigidity increase) (Baskurt & Meiselman, 1998). Hence, the change induced in the osmotic fragility reflectd the damaging effects on the cytoskeleton. Treatment with lipoic acid decreased the elevated osmotic fragility. This is due to the antioxidant ability of lipoic acid and its ability to regenerate other antioxidants such as vitamins E and C, and GSH from their radial or inactive forms (Uchendu, Ambali, Ayo, King, & Angela, 2014). Caylak, Aytekin, & Halifeoglu, 2008 demonstrated the ability of lipoic acid to offer protection against lead-induced oxidative stress in rat erythrocyte.

The absorption spectra of hemoglobin molecule can give some information about its conformational changes. In the range 300–700 nm, the absorption of light is associated with excitation of the porphyrin structure. The coordinated-covalent bond between iron and the proximal histidine exhibits transition at 340 nm (Zdravko and Panasyuk, 1986). The Soret band is the characteristic intense band for porphyrin ring. It occurs at 410 nm and it is a $\pi - \pi^*$ transition (Kenneth & Watson, 1992). Free radicals increased the energy of the $\pi -$ orbital thus decreased the activation energy "the minimal energy required to start transition" of the Soret band. Therefore free radicals affect porphyrine structure (Selim & El-Marakby, 2010), this appear in the hypochromocity of Soret band of iron overload rats. However, LA minimized iron-induced effect. The porphyrin ring showed two peaks at 540

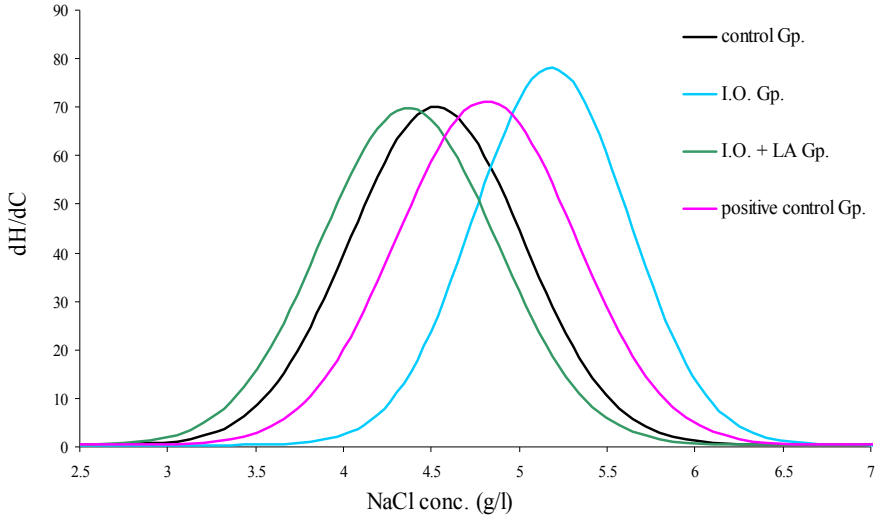

Fig. 3 – Gaussian curves (the rate of hemolysis versus NaCl concentration) for the four groups.

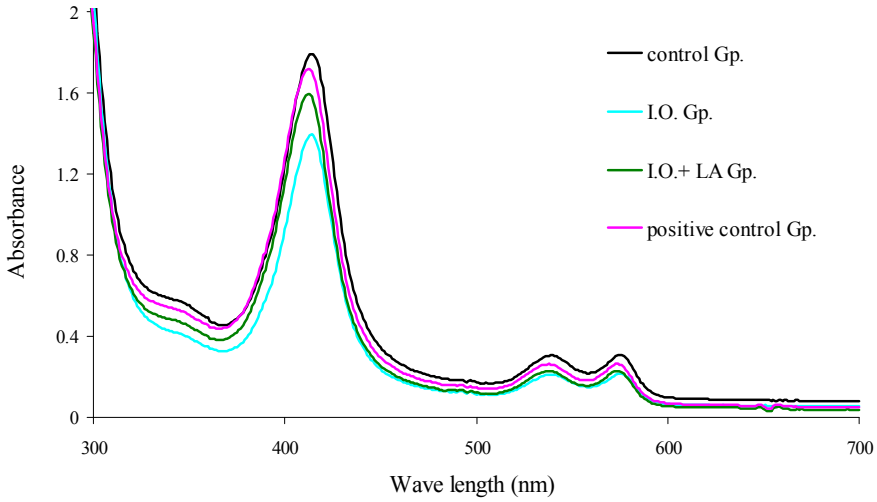

Fig. 4 – The UV-absorption spectra for the four groups.

and 574 nm (α at 574 nm and β at 540 nm). The α is considered to be purely electronic transition involving the porphyrin of the heme group. The β is considered to be composed of many vibronic transitions (Gouterman, 1978). The ratio between those two peaks (A_α/A_β) is larger than unity for oxygenated normal hemoglobin. Previous studies reported that the decrease in the ratio A_α/A_β together with the appearance of a peak at 630 nm, indicates the conversion of hemoglobin into its oxidized form "methemoglobin" (Abd El-Baset, 1986; Artyukhov & El-Baset, 1980). In this study no oxidative changes occurred in iron overload rats.

Electron paramagnetic resonance (EPR) is one of the most frequently applied methods in structural investigations of paramagnetic particles, e.g., free radicals. The EPR method makes possible the recording of the interaction between electromagnetic radiations and investigated paramagnetic samples placed into the magnetic field (Weil & Bolton, 2007). 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. it 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 (Desouky, Hassan, & Selim, 2008). The position and structure of resonance lines, apart from the content of paramagnetic complexes in blood, are affected also by many factors such as the sample storage conditions, pH or measurement temperature (Krzyminiewski et al., 2011). Comparing the data collected in Table 1 and Fig. 6. It was noticed that the signal at g = 4.26 is highly correlated with the transferrin saturation level. As mentioned above, S_2 signal is attributed to free radicals in hemoglobin formed by the degradation of blood constituents (Miki et al., 1987). Several investigators agreed that at least two different kinds of radicals are formed on the protein (Gunther, Kelman, Corbett, & Mason, 1995; Kelman, DeGraz, & Mason, 1994). Although the formation of peroxyl radicals is well proven (Ikeya, 1993; Miki et al., 1987), this kind of radical constitutes only a fraction of the total concentration of radicals (Svistunenko, Nathan, et al., 1997 and Svistunenko, Rakesh, Voloshchenko, & Wilson, 1997). The globin-based free radicals were suggested to be major contributor for S_2 (Svistunenko, Nathan, et al., 1997 and Svistunenko, Rakesh, et al., 1997). Many investigations revealed that it is the tyrosin radical (Krzyminiewski et al., 2011; Maghraby & Ali, 2007;

Table 4 – The values of Hb conc., Soret band and A_α/A_β ratio of the absorption spectra for the four groups.

Groups	Hb conc. "g/dl"	Soret band	A_α/A_β
Control	11.1 ± 0.90	1.79 ± 0.08	1.004 ± 0.006
Iron overload	**16.3**[a] ± 4.12	1.39 ± 0.22	1.004 ± 0.006
Iron overload + Lipoic acid	**10.9**[b] ± 0.15	1.59 ± 0.04	1.019 ± 0.002
Positive control	5.5 ± 0.336	1.71 ± 006	1.017 ± 0.010

[a] Statistically significant from control $P < 0.05$.
[b] statistically significant from I. overload $P < 0.05$.

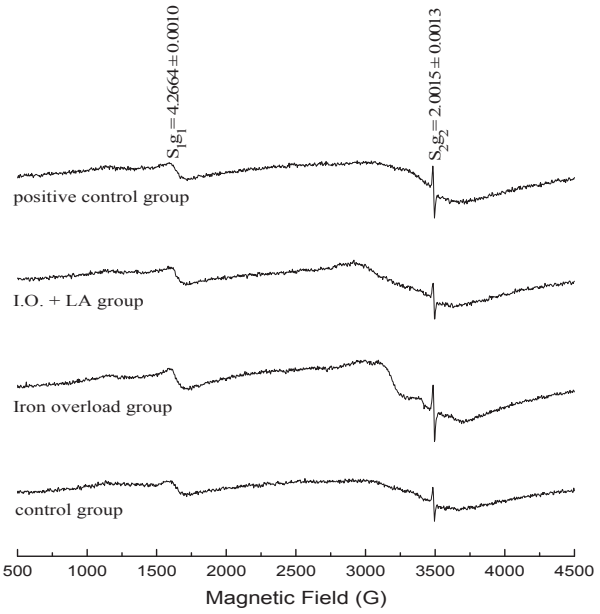

Fig. 5 – EPR spectra of lyophilized blood for the four groups recorded at room temperature.

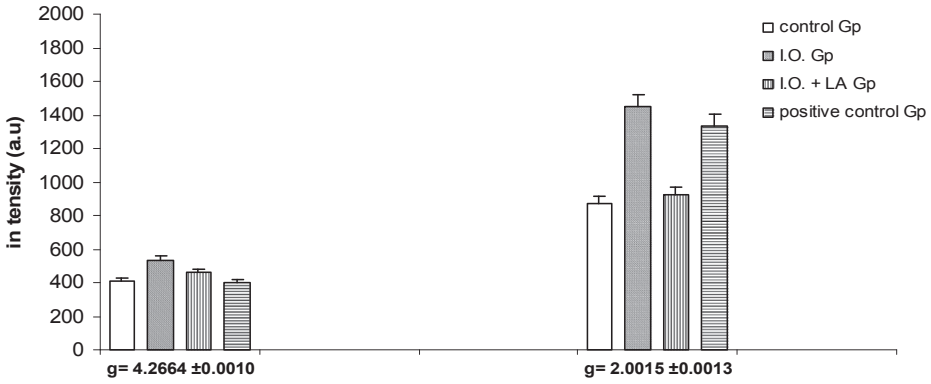

Fig. 6 – Changes in the intensity of the EPR signals.

Svistunenko, Nathan, et al., 1997 and Svistunenko, Rakesh, et al., 1997). Lipoic acid administration prevented the cellular damage caused by iron-induced free radicals, this is clear from the decrease of the intensity of free radical signal (S_2) by 36% (Fig. 6). The obtained results are in agreement with pervious report (Desouky et al., 2008).

Evidence from this study and previous work revealed that LA/DHLA are considered ideal therapeutic antioxidants because they are naturally existing, low molecular weight compounds with very powerful antioxidant properties in both aqueous and lipid domains. Their effects include free radical quenching (Matsugo et al., 1995), metal chelation (Ou, Tritschler, & Wolff, 1995) and regeneration of other antioxidants such as ascorbic acid, vitamin E and glutathione (Guo & Packer, 2000; Han, Tritschler, & Packer, 1995; Li et al., 2013; Xu & Wells, 1996). Although antioxidant properties of DHLA have been proven there is some evidence of their prooxidant properties.

5. Conclusion

Iron toxicity affects the function of RBCs and organs as liver and brain. The effects of lipoic acid on iron status and oxidative parameters appear very promising in animal models. These observations suggest that LA may be a possible treatment in Fe-overload condition. Although antioxidant properties of DHLA, there are some evidence of their pro-oxidant properties, more extensive studies is needed concerning both the safety and optimal dose of LA.

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