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Original Research Article

Di-Mercapto-Succinic Acid (DMSA) Combination with L-Carnitine Oral Supplementation Enhance the Functional Hemoglobin Derivatives in Heavy Metal Chelation Therapy

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ABSTRACT

The aim of this work was to study to what extend application of L-carnitine can enhance the hemoglobin different derivatives concentration in heavy metal intoxication status. Forty-eight New Zealand albino rabbits weighing between 2.5 and 3 kg were conducted to the study. Study was approved from the NRC, Ethical Committee. Animals were subdivided into four groups. Control group: animals did not receive neither lead ions in water nor L-carnitine administration. Group 1: animals received lead ions for 21 days as lead acetate in drinking water. Group 2: animals received lead ions for 21 days as lead acetate in drinking water and then received DMSA as a chelator of lead ions for 15 days. Group 3: animals received lead ions for 21 days as lead acetate in drinking water and then received DMSA as a chelator of lead ions for 15 days concomitant with L-carnitine powder (1000 mg) for two weeks. Hemoglobin derivatives concentration were evaluated in both groups spectrophotometrically and then by solution of linear equations. Results revealed a statistical improve in oxygenated hemoglobin derivative-oxyhemoglobin, and also showed a dramatic decrease in oxidized form of hemoglobin that is called methemoglobin after usage of L-carnitine as a supplement.

Keywords: L-Carnitine, Lead, Hemoglobin, Derivatives, Linear Equations.

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INTRODUCTION

Carnitine is an amino acid derivative and nutrient involved in lipid (fat) metabolism in mammals and other eukaryotes. It is in the chemical compound classes of β-hydroxy acids and quaternary ammonium compounds, and because of the hydroxyl-substituent, it exists in two stereoisomers, the biologically active enantiomer L-carnitine, and the essentially biologically inactive D-carnitine.[1] Both are available through chemical synthesis, and the L-form is continuously biosynthesized in eukaryotic organisms from the proteinogenic amino acids lysine and methionine. In such eukaryotic cells, it is specifically required for the transport of fatty acids from the intermembraneous space in the mitochondria into the mitochondrial matrix during the catabolism of lipids[2], in the generation of metabolic energy. Carnitine was originally found as a growth factor for mealworms and labeled vitamin B_T, although carnitine is not by biochemical definition a true vitamin.[3,4] It is used efficaciously, clinically, in the treatment of some conditions[5], e.g. systemic primary carnitine deficiency[6], and it is available over the counter as a nutritional supplement, though its efficacy for most conditions for which it is advertised is controversial or not yet established.[7]

In animals, the biosynthesis of carnitine occurs primarily in the liver and kidneys from the amino acids lysine (via trimethyllysine) and methionine.[8] Carnitine transports long-chain acyl groups from fatty acids into the mitochondrial matrix, so they can be broken down through β-oxidation to acetyl CoA to obtain usable energy via the citric acid cycle. In some organisms, such as fungi, the acetate is used in the glyoxylate cycle for gluconeogenesis and formation of carbohydrates.[9] Fatty acids must be activated before being covalently linked to the carnitine molecule to form acylcarnitine for transport. The free fatty acid in the cytosol is first adenylated by reaction with ATP, then attached with a thioester bond to coenzyme A (CoA), with expulsion of AMP. This reaction is catalyzed by the enzyme fatty acyl-CoA synthetase and driven to completion by inorganic pyrophosphatase.[10]

The acyl group on CoA can now be transferred to carnitine and the resulting acylcarnitine transported into the mitochondrial matrix. This occurs via a series of similar steps:

(1) Acyl CoA is transferred to the hydroxyl group of carnitine by carnitine acyltransferase I (palmitoyltransferase) located on the outer mitochondrial membrane.

(2) Acylcarnitine is shuttled inside by a carnitine-acylcarnitine translocase.

(3) Acylcarnitine is converted to acyl CoA by carnitine acyltransferase II (palmitoyltransferase) located on the inner mitochondrial membrane.[11] The liberated carnitine returns to the cytosol. Carnitine acyltransferase I and peroxisomal carnitine octanoyl transferase (CROT) undergo allosteric inhibition by malonyl-CoA, an intermediate in fatty acid biosynthesis, to prevent futile cycling between β-oxidation and fatty acid synthesis.[10]

Human genetic disorders, such as primary carnitine deficiency, carnitine palmitoyl transferase I deficiency, carnitine palmitoyl transferase II deficiency and carnitine-acylcarnitine translocase deficiency, affect different steps of this process.[12]

Lead poisoning is a medical condition caused by increased levels of the heavy metal lead in the body. Lead interferes with a variety of body processes and is toxic to many organs and tissues including the heart, bones, intestines, kidneys, and reproductive and nervous systems.[13] It interferes with the development of the nervous system and is therefore particularly toxic to children, causing potentially permanent learning and behavior disorders. Symptoms include abdominal pain, confusion, headache, anemia, irritability, and in severe cases seizures, coma, and death.[14]

Elevated lead in the body can be detected by the presence of changes in blood cells visible with a microscope and dense lines in the bones of children seen on X-ray.[15] However, the main tool for diagnosis is measurement of the blood lead level or a urine test. When blood lead levels are recorded, the results indicate how much lead is circulating within the blood stream, not the amount being stored in the body.[16] There are two units for reporting blood lead level, either micrograms per deciliter (µg/dl), or micrograms per 100 grams (µg/100 g) of whole blood, which are both numerically equivalent. The Centers for Disease Control has set the standard elevated blood lead level for adults to be 25 (µg/dl) of the whole blood. For children however, the number is set much lower at 5 (µg/dl) of blood as of 2012 down from a previous 10 (µg/dl) Children are especially prone to the health effects of lead and as a result, blood lead levels must be set lower and closely monitored if contamination is possible. The major treatments are removal of the source of lead and chelation therapy.[17] Chelation therapy is the administration of chelating agents to remove heavy metals from the body. Chelation therapy has a long history of use in clinical toxicology. Poison centers around the world are using this form of metal detoxification. For the most common forms of heavy metal intoxication those involving lead[18], arsenic or mercury the standard of care several chelating agents are available.[19] DMSA dimercaptosuccinic acid has been recommended for the treatment of lead poisoning in children by Poison Centers around the world.[20] Other chelating agents, such as 2,3-dimercapto-1-propanesulfonic acid (DMPS) and alpha lipoic acid (ALA), are used in conventional and alternative medicine.[21]

The aim of this work was to evaluate the hypothesis if L-carnitine as an oral supplement has an effect to improve the functional hemoglobin derivatives and reduce the non-functions ones in lead intoxicated subjects.

MATERIALS AND METHODS

Forty-eight New Zealand albino rabbits weighing between 2.5 and 3 kg, aged two months, of both sexes were used for this study. All animals were subjected to distilled water as drinking water for 30 days before starting the experimental design. All through the experiment duration, rabbits were housed in separate cages, fed standard laboratory food and allowed free access to water in room lightening with a 12-hour light-dark cycle in animal house of National Research Center (NRC). Experimental animals design was priory approved from the National Research Center ethical committee

Animals were subdivided into four groups. Control group: animals did not receive neither lead ions in water nor L-carnitine administration (N=12).

Group 1: animals received lead ions for 21 days as lead acetate [$Pb(CH3COO)_2$. $3H_2O$] in drinking water (N=12).

Group 2: animals received lead ions for 21 days as lead acetate [$Pb(CH3COO)_2$. $3H_2O$] in drinking water and then received DMSA as a chelator of lead ions for 15 days (N=12).

Group 3: animals received lead ions for 21 days as lead acetate in drinking water and then received DMSA as a chelator of lead ions for 15 days concomitant with L-carnitine powder (1000 mg) for two weeks (N=12).

The millimolar extinction coefficients were put into four linear equations with the four unknown concentrations of hemoglobin pigments (C_{HbO2}, C_{HbCO}, C_{MetHb} and C_{SHb}).

$$A^{500} = 5.05\,C_{HbO_2} + 5.35\,C_{HbCO} + 9.04\,C_{Met.Hb} + 7.2\,C_{SHb} \quad \textbf{(1)}$$

$$A^{569} = 11.27\,C_{HbO_2} + 14.27\,C_{HbCO} + 4.1\,C_{Met.Hb} + 8.1\,C_{SHb} \quad \textbf{(2)}$$

$$A^{577} = 15.37\,C_{HbO_2} + 10.0\,C_{HbCO} + 4.1\,C_{Met.Hb} + 8.1\,C_{SHb} \quad \textbf{(3)}$$

$$A^{620} = 0.24\,C_{HbO_2} + 0.33\,C_{HbCO} + 3.35\,C_{Met.Hb} + 20.8\,C_{SHb} \quad \textbf{(4)}$$

Where the absorption bands at wavelengths 500, 569, 577 and 620 nm represent the absorption maxima of Met-Hb, HbCO, HbO_2 and SHb, respectively. The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 5.05 & 5.35 & 9.04 & 7.2 \\ 11.27 & 14.27 & 4.10 & 8.1 \\ 15.37 & 10.0 & 4.10 & 8.1 \\ 0.24 & 0.33 & 3.35 & 20.8 \end{bmatrix} \cdot \begin{bmatrix} C_{HbO_2} \\ C_{HbCO} \\ C_{Met.Hb} \\ C_{SHb} \end{bmatrix} = \begin{bmatrix} A^{500} \\ A^{569} \\ A^{577} \\ A_{620} \end{bmatrix} \quad \textbf{(5)}$$

This linear system of equations was solved by mathematical manipulation, using the Gaussian elimination method. For matrix calculation, to yield the following equations :

$$C_{SHb} = \frac{A^{620} - 0.442293A^{500} + 0.1065519A^{569} + 0.0515769\ A^{577}}{18.895404} \quad (6)$$

$$C_{Met.Hb} = \frac{9.0602343A^{500} - A^{577} - 2.6960235A^{569} - 35.295898\ C_{SHb}}{66.750821} \quad (7)$$

$$C_{HbCO} = \frac{A^{569} - 2.2316831A^{500} + 16.074415\ C_{Met.Hb} + 7.9681188\ C_{SHb}}{2.330495} \quad (8)$$

$$C_{HbO_2} = \frac{A^{500} - 5.35\ C_{HbCO} - 9.04\ C_{Met.Hb} - 7.2\ C_{SHb}}{5.05} \quad (9)$$

Where A^{500}, A^{569}, A^{577} and A^{620} are the absorbances of hemoglobin solution at the wavelengths 500, 569, 577 and 620 nm, respectively.

The results calculated using the last four equations were found to be identical to those obtained by using a computer program for matrix calculation. During sample preparation, care was taken to avoid contamination, therefore, every step from the moment of sample until analysis was regarded as potential source of contamination and was checked not to contain or leach detectable amount of any contaminant.

Dosages

Lead acetate was given to animals in drinking water with concentration 1000 ppm. Acidification of water is essential for dissolving lead acetate so 1 ml of conc. hydrochloric acid was added per liter of deionized water. This concentration (1000 ppm lead acetate) is known to induce the desired level of toxicity in blood and other soft tissues. DMSA (50 mg/kg body weight) was orally administrated to rabbits three times per week. Di-Mercapto Succinic Acid (DMSA) dosage were calculated as 1000 ppm.

Statistical analysis

The data was expressed as mean + standard deviation. All analyses were made using the SPSS statistical software package. A one-way ANOVA test was applied to data to detect significant differences between different groups. Differences were considered significant at $p < 0.05$.

Table 1: Met-Hemoglobin derivative concentration in animals received lead ions as lead acetate treated with DMSA and those treated with DMSA concomitant with L-carnitine supplementation as compared to control (mean ± SD)

Group	Met-Hemoglobin concentration (gm\dl)
Control (n = 10)	0.313 ± 0.025 [a]
G1 (n = 13)	0.847 ± 0.051 [C]
G2 (n = 13)	0.605 ± 0.044 [b]
G3 (n = 12)	0.451 ± 0.036 [C]

[a]Mean + SD; [b]Significant difference compared to G_1; [c]Highly significant difference compared to G_1.

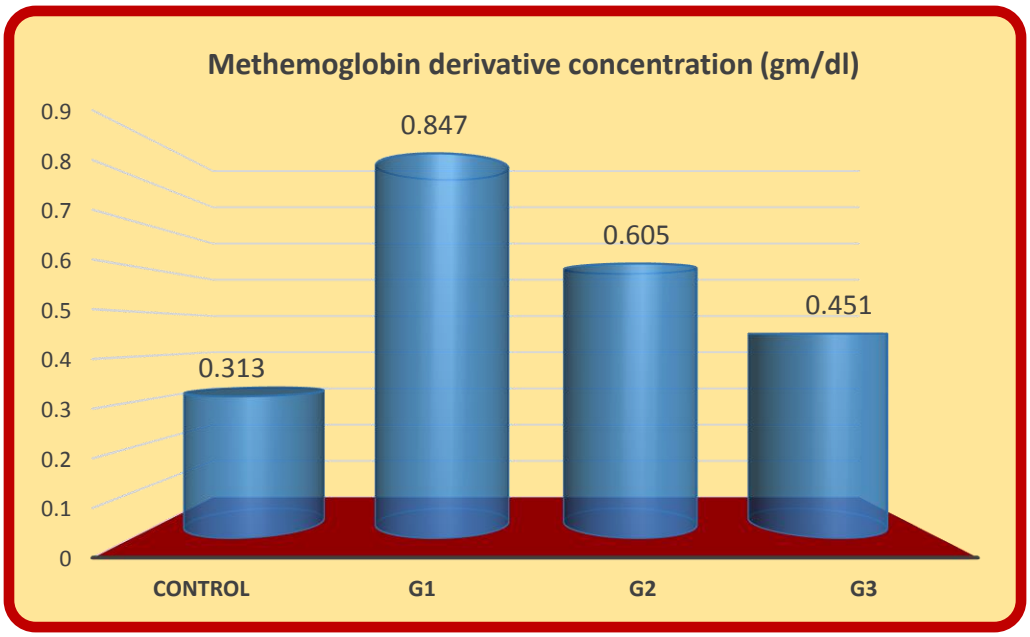

Figure 1: Met-Hemoglobin derivative concentration in animals received lead ions in drinking water as lead acetate treated with DMSA with and without application of L-carnitine as compared to control.

RESULTS

Table 1, shows the main non-functional hemoglobin derivative concentration which is met-hemoglobin. Data revealed a highly significant increment in the second group in which animals received lead acetate in the drinking water with no treatment application. In both treated groups, there is a significant improve in methemglobin concentration and the maximum enhacement was detected in the group in which animals received DMSA as a chemical chelator of elevated lead ions that concomitant with L-carnitine administration.

Table 2, shows the other hemoglobin derivatives concentration in all groups. Sulpho-hemoglobin and carboxy-hemoglobin revealed the same pattern of results as those obtained in the methemoglobin concentration. The best improvement was found in animals received L-carnitine continuously with the application of the chemical chelator.

The functional oxyhemoglobin derivative concentration comes in opposite direction as it revealed a highly significant decrease in animals received lead ions only with no treatment application. While, both groups received DMSA as a chelator of lead ions revealed significant increased levels. The maximum improvement, approximately normal, was detected in the animals received DMSA with L-carnitine.

Table 2: Sulf-Hemoglobin, Carboxy-Hemoglobin and Oxyhemoglobin derivatives concentration in animals received lead ions as lead acetate treated with DMSA and those treated with DMSA concomitant with L-carnitine supplementation as compared to control (mean ± SD)

Group	S-Hb (gm/dl)	CO-Hb (gm/dl)	O_2-Hb (gm/dl)
Control (n = 10)	0.034 ± 0.005 [a]	0.334 ± 0.005 [a]	13.567 ± 1.23 [a]
G1 (n = 13)	0.098 ± 0.003 [b]	0.982 ± 0.003 [b]	11.643 ± 0.903 [C]
G2 (n = 13)	0.078 ± 0.004 [C]	0.681 ± 0.004 [C]	12.045 ± 1.567 [C]
G3 (n = 12)	0.044 ± 0.006 [b]	0.386 ± 0.006 [b]	13.098 ± 1.098 [b]

[a]Mean + SD; [b]Significant difference compared to G_1; [c]Highly significant difference compared to G_1.

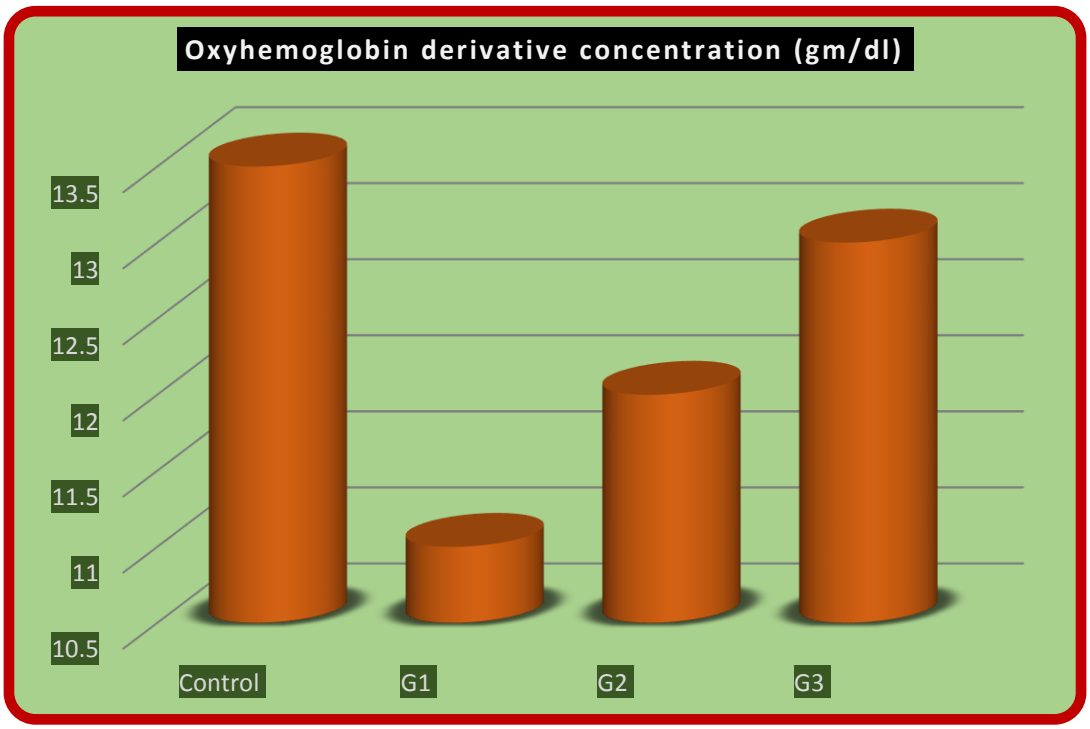

Figure 2: Sulpho, Carboxy and Oxy-Hemoglobin derivative concentration in animals received lead ions in drinking water as lead acetate treated with DMSA with and without application of L-carnitine as compared to control.

DISCUSSION

Lead poisoning is a type of metal poisoning and a medical condition in humans and other vertebrates caused by increased levels of the heavy metal lead in the body.[22] Lead interferes with a variety of body processes and is toxic to many organs and tissues including the heart, bones, intestines, kidneys, and reproductive and nervous systems.[23] It interferes with the development of the nervous system and is therefore particularly toxic to children, causing potentially permanent learning and behavior disorders. Symptoms include abdominal pain, confusion, headache, anemia, irritability, and in severe cases seizures, coma, and death.[24]

Elevated lead in the body can be detected by the presence of changes in blood cells visible with a microscope and dense lines in the bones of children seen on X-ray, but the main tool for diagnosis is measurement of the blood lead level. When blood lead levels are recorded, the results indicate how much lead is circulating within the blood stream, not the amount stored in the

body.[25] There are two units for reporting blood lead level, either micrograms per deciliter (µg/dl), or micrograms per 100 grams (µg/100 g) of whole blood, which are numerically equivalent. The Centers for Disease Control (US) has set the standard elevated blood lead level for adults to be 10 µg/dl of the whole blood. For children, the number is set much lower at 5 µg/dl of blood as of 2012[26] down from a previous 10 µg/dl.[27] Children are especially prone to the health effects of lead. As a result, blood lead levels must be set lower and closely monitored if contamination is possible. The major treatments are removal of the source of lead and chelation therapy.[15]

Lead poisoning can cause a variety of symptoms and signs which vary depending on the individual and the duration of lead exposure.[28] Symptoms are nonspecific and may be subtle, and someone with elevated lead levels may have no symptoms.[29] Symptoms usually develop over weeks to months as lead builds up in the body during a chronic exposure, but acute symptoms from brief, intense exposures also occur. Symptoms from exposure to organic lead, which is probably more toxic than inorganic lead due to its lipid solubility, occur rapidly. Poisoning by organic lead compounds has symptoms predominantly in the central nervous system, such as insomnia, delirium, cognitive deficits, tremor, hallucinations, and convulsions.[30]

Symptoms may be different in adults and children; the main symptoms in adults are headache, abdominal pain, memory loss, kidney failure, male reproductive problems, and weakness, pain, or tingling in the extremities.[31]

Early symptoms of lead poisoning in adults are commonly nonspecific and include depression, loss of appetite, intermittent abdominal pain, nausea, diarrhea, constipation, and muscle pain.[32] Other early signs in adults include malaise, fatigue, decreased libido, and problems with sleep. An unusual taste in the mouth and personality changes are also early signs.[33]

In adults, symptoms can occur at levels above 40 µg/dL, but are more likely to occur only above 50–60 µg/dL. Symptoms begin to appear in children generally at around 60 µg/dL. However, the lead levels at which symptoms appear vary widely depending on unknown characteristics of everyone.[34] At blood lead levels between 25 and 60 µg/dL, neuropsychiatric effects such as delayed reaction times, irritability, and difficulty concentrating, as well as slowed motor nerve conduction and headache can occur. Anemia may appear at blood lead levels higher than 50 µg/dL. In adults, abdominal colic, involving paroxysms of pain, may appear at blood lead levels greater than 80 µg/dL.[35] Signs that occur in adults at blood lead levels exceeding 100 µg/dL include wrist drop and foot drop, and signs of encephalopathy (a condition characterized by brain swelling), such as those that accompany increased pressure within the skull, delirium, coma, seizures, and headache. In children, signs of encephalopathy such as bizarre behavior, dis-coordination, and apathy occur at lead levels exceeding 70 µg/dL.[36] For both adults and children, it is rare to be asymptomatic if blood lead levels exceed 100 µg/dL.[37]

L-carnitine is an amino acid (a building block for proteins) that is naturally produced in the body. L-carnitine supplements are used to increase L-carnitine levels in people whose natural level of L-carnitine is too low because they have a genetic disorder, are taking certain drugs (valproic acid for seizures), or because they are undergoing a medical procedure (hemodialysis for kidney disease) that uses up the body's L-carnitine.[38] It is also used as a replacement supplement in strict vegetarians, dieters, and low-weight or premature infants.[39] L-carnitine is used for conditions of the heart and blood vessels including heart-related chest pain, congestive heart failure (CHF), heart complications of a disease called diphtheria, heart attack, leg pain caused by circulation problems (intermittent claudication), and high cholesterol.[40]

Some people use L-carnitine for muscle disorders associated with certain AIDS medications, difficulty fathering a child (male infertility), a brain development disorder called Rett syndrome, anorexia, chronic fatigue syndrome, diabetes, overactive thyroid, attention deficit-hyperactivity disorder (ADHD), leg ulcers, Lyme disease, and to improve athletic performance and endurance.[41]

The body can convert L-carnitine to other amino acids called acetyl-L-carnitine and propionyl-L-carnitine. But, no one knows whether the benefits of carnitines are interchangeable. Until more is known, don't substitute one form of carnitine for another.[42]

The aim of this work is to test the hypothesis states if application of L-carnitine may enhance the chelation process takes place by a chemical chelator like DMSA in lead intoxicated subjects. The work was conducted to animals those subdivided into groups according to the exposure to lead ions as lead acetate in drinking water and also based on the way those animals were treated. The experiment was designed four a main group in which animals subjected to lead acetate in the drinking water for 21 days and then ended with 15 days of treatment with both DMSA concomitant with L-carnitine oral administration.

Data shown in table 1 revealed a significant finding when the non-functional hemoglobin derivative was measured. In this derivative the entire heme atom of the hemoglobin was oxidized from the reduced form (ferrous state) to the higher one (ferric state). This oxidation process may play a role in reducing the oxygen affinity of the hemoglobin makes it unable to bind with oxygen neither carbon dioxide. So, it is called the afunction form of hemoglobin in which no dramatic changes were recorded regarding the shape and size of the molecule but the changes were in the electrical environment of the molecule. A highly significant increase in this afunction form of hemoglobin was recorded in the second group in which animals were exposed to lead with no application of any treatment. On the other hand, the rest two groups in which animals were exposed to lead and then followed by administration of treatments showed a significant decrement of the methemolgobin derivative concentration.

The maximum enhancement of the methemoglobin concentration was recorded in the last group in which animals received both DMSA concomitant with L-carnitine as oral supplement. This may reflect a role of L-carnitine to control the oxidative stress resulted from the high formation of ROS as a role of lead ions concentration. This situation can be described as L-carnitine may has a role as antioxidant. These findings are clearly shown in figure 1. Supho- hemoglobin and carboxy- hemoglobin concentrations those shown in table 2 also revealed the same pattern of results as that obtained from met-hemoglobin concentration.

Another interesting finding was obtained when the other derivatives were measured (Table 2) as a highly significant increase in the functional hemoglobin was detected in animals received both treatment DMSA and L-carnitine. The level of oxyhemoglobin approximately become near the normal level. This finding also comes to verify the antioxidant role of L-carnitine.

In conclusion, it safe to say that administration of L-carnitine as an oral supplement played a positive role beside the chelation therapy process of lead intoxicated subjects. This role may be explained as antioxidant role of L-carnitine or in other words by enhancing the filtration rate of the heavy metals ions done by the chemical chelator.

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