The role of vitamin C in relieving lead toxicity on some of hematological parameters and reproductive performance in mice (*Mus musculus*).

A Thesis Submitted to the Council of the College of Veterinary Medicine, University of Basrah in Partial Fulfillment of the Requirements for the Degree of Master of Science

in

Veterinary Physiology.

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DEDICATION

To that man….Who devoted his soul and sacrificed his life to saving a good future life for me, and who implanted the seeds of propensity and stamina inside me to serve others…My deceased father.

To the woman….Who was the blooming daffodil in my thorny way and the balm of my wounds…My darling mother.

To those….Who were the safe shelter to which I resort, to deposit my burdens…My dear brothers Salaam, Hussam and Ali.

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IN THE NAME OF ALLAH THE MOST GRACIOUS THE MOST MERCIFUL

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Abstract

The present study was conducted at Veterinary Medicine College– University of Basra to investigate the effect of vitamin C on the relief of the toxic effect of lead exposure on some hematological parameters, biochemical parameters and reproductive parameters. The experiment was divided into two parts. The first was dealing with hematological parameters and biochemical parameters, whereas the second was dealing with the reproductive efficiency.

In the first part, 56 male mice were used. They were divided into four groups. The first two groups (T1 and T2) consisted of 16 male mice each. They were dosed via intraperitoneal injection with 60, and 80 mg/kg lead acetates in the form of $Pb(CH_3COO)_2$ for 15 days, then 8 mice of each group were terminated for the hematological and biochemical tests. The remaining 8 mice of each group were dosed via intraperitoneal injection with 400 mg/kg of vitamin C in the form of C6H10O6 for additionally 15 days. The third group (T3) consisted of 8 male mice. They were dosed via intraperitoneal injection with 60 mg/kg of lead acetates and then with 400 mg/kg of vitamin C after one hour of lead acetates injection for 15 days. The fourth group which was set as a control group, consisted of 16 male mice. They were dosed via intraperitoneal injection with (0.9 % NaCl) for 15 days, then 8 mice of this group were terminated for the hematological, and biochemical tests. The remaining 8 mice of this group were dosed via intraperitoneal injection with (0.9 % NaCl) for additionally 15 days. The investigation of blood parameters included Red Blood Cells count (RBC), Haemoglobin concentration (Hb), Packed cells volume (P.C.V.), total and differential White Blood Cells count (W.B.C.); biochemical parameters included

Total Serum Cholesterol (TSCH), alanine transferase (ALT) and aspartate transferase (AST). In the second part of the experiment, 28 male and 56 female mice were divided into seven similar groups, six of them were dosed intraperitoneally with 80 mg/kg of lead acetates with/without 400 mg/kg of vitamin C daily, whereas the seventh was dosed with (0.9 % NaCl) for the four weeks as a control group. Mice were allowed to mate, then separated after 16 days to let females deliver. Semen aspects were reported for all male mice. Female mice reproductive abilities were estimated after delivery. When the animals were treated with lead acetates, the results showed a significant decrease ($P \leq 0.05$) of R.B.C. count, Hb concentration, P.C.V. value, neutrophil count, serum ALT and AST activities, and a decrease in massive and individual motility of sperms viabilities of treated males. Fertility rate of treated females and the weight of litters of treated females were also declined. In contrast, there was a significant increase ($P \leq 0.05$) of the total W.B.C. count, lymphocytes account, monocytes account, acidophils account, and total serum cholesterol, abnormal and dead sperms of treated males. No basophiles were seen in all treated groups and the control as well. Furthermore, when the lead-poisoned animals were treated with vitamin C either for 15 days or after one hour of lead acetates injection, there was an increase of R.B.C. count, Hb concentration, and P.C.V. value, neutrophil account, but still significantly less than those of the control group on ($P \leq 0.05$). Serum ALT and AST activities increased significantly ($P \leq 0.05$). There was a decrease in total serum cholesterol but still significantly higher than that of the control group ($P \leq 0.05$). Massive and individual motility of sperms viabilities of treated males, and fertility rate of treated females increased but still significantly less than those of the control group ($P \leq 0.05$). There was a significant increase ($P \leq 0.05$) in the total W.B.C. count, lymphocytes account, and a significant decrease ($P \leq 0.05$) in the

monocytes account. Also there was a decrease in acidophils, abnormal and dead sperms of treated males but still significantly higher than those of the control group ($P \leq 0.05$). No basophiles were seen in all treated groups and the control one. Lead causes the sex ratio to increase when the males are treated rather than the females being treated.

1- Introduction

Lead is a ubiquitous element in all environmental media (Elias, 1985) . The majority of lead in the environment arises from the burning of fossil fuels in automobiles, industrial emissions, and from the use of lead containing solder paints . Most lead in the air is inorganic and the predominant source is the combustion of tetraethyl and tetramethyl lead which is used as a fuel additives (USEPA.,1986) . Lead is present in tobacco, it has been reported that cigarettes contain 2.4 mg of lead and 5% of this occurs in ash and side stream smoke (Maussalo *et al*.,1986) . Lead also found to impair learning, memory and audio-visual functions in rats and man (Bushnell and Levin, 1983; Cohn *et al.*, 1993), haematopoiesis (Baloh,1974) and produces nephrotoxicity (Nolan and Shaikh,1992), and cardiovascular damage (Stophen,1974) . Toxicological properties of lead have been extensively studied (Needleman,1996), and its carcinogenic effect has been receiving increasing attention (IARC.,1993). It is known to cause oxidative stress in the body by inducing the generation of free radicals and reducing the antioxidant defense system of the cells (Gurrer and Ercal,2000) .

In a recent study, lead intoxication caused an increase in the contents of brain thiobarbituric acid reactive substances accompanied by altering the antioxidant defense system (Adanylo and Oteiza,1999) .

Drugs of natural origin have been found to be quite protective to mammalian tissues at optimum dose level (Sunita *et al*.,2006) .

Vitamins are known as organic substances of nutritional nature, present in low concentration as a natural component of an enzyme system and catalyze required reactions and may be derived externally or by extrinsic biosynthesis. They act as co-factors in enzyme systems, antioxidants or antagonist (Englard and Seifter ,1986) .

The properties of ascorbic acid (vitamin C) are well documented and have generated a great deal of interest in recent years for a wide range of protective effects in biological systems (Henson *et al.*,1991) .

Ascorbic acid is a powerful antioxidant that can neutralize harmful free radicals, and it helps make collagen, a tissue needed for healthy bones, teeth, gums and blood vessels (Carr and Frei,1991) . Excellent food sources of vitamin C are citrus fruits or citrus juices, berries, green and red peppers, tomatoes, broccoli, and spinach. The present study was designed to focus on the effect of vitamin C on hematophysiological effect and tissues damage resulting from lead toxicity , therefore ; the aim was to study the following items :

1- The effect of lead on blood parameters.
2- The effect of lead on reproductive parameters.
3- The effect of lead on serum ALT and AST enzymes activity.
4- The effect of vitamin C on relieving lead poisoning at the short and long periods.

2- Literature Review

2–2–Vitamin C

2–2–1–Historical perspective

Although vitamin C was not known till the advent of the thirties of the twentieth century, the disease caused by this vitamin difficiency in human food was known since the sixteenth century as scurvy (Goodhart and Shils, 1973).

The disease was reported first among sailors in 1700, where it was found that the drinking of lemon juice by the sailors prevented them from being afflicted with scurvy, while approximately half of the sailors who did not drink the juice were afflicted with scurvy (Harper *et al*., 1979).

Scurvy was first described by the scientist James Lind on 1753. In 1923, the scientist Zilva described anti-scurvy substances found in lemon juice (Nobile and Woodhill, 1981). Frolich and Holst notice that guinea pigs are afflicted with scurvy if they are fed only on grains and thrashed wheat due to lack of vitamin C in these foods (Aurand and Woods, 1973). In 1928, the Russian scientist Szent Gorgyi isolated the Hexuronic acid compound from orange juice and adrenal cortex (Neuman, 1977). In 1932, the scientists Waugh and King considered the Hexuronic acid compound as an anti scorbutic agent. In 1933, the scientists Haworth and Szent Gorgyi changed the name of Hexuronic acid into Ascorbic acid (Kutsky, 1973).

The clinical signs of scurvy include joints pain, teeth flaccidity, gingivitis, decrease of the resistance to cold illnesses, wounds healing delay, gastric disturbances, weight loss, and anaemia (Wilson *et al*., 1975). It is found also that the clinical signs of vitamin C difficiency do not appear till after 90 days after sustenance on vitamin C free food (FAO/ WHO Expert Group, 1970). Gilman *et al.*(1985) reports that the scurvy occurs most in older people, drugs

addicts and dipsomaniac, and also those who sustain on vitamin C lack foods. The scurvy seldom inflicts six months to two years old children as the mother's milk is fluent with vitamin C (Meyers *et al*., 1976).

For the biosynthesis of this vitamin inside the body, the studies report that the plants and all mammals except the human, monkies, and guinea pigs have the ability for the synthesis of this vitamin in the liver from the glucose. The cellular vitamin synthesis sites are the Microsomes, Mitochondria, and Golgi complex (Kutsky, 1973).

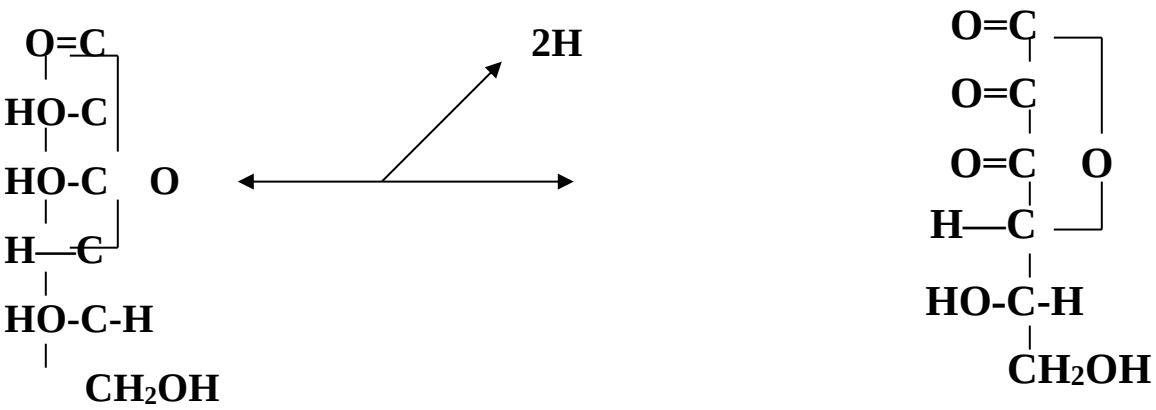
The inability of some living organisms to synthesize the vitamin is due to the lack of L-gulonolactone oxidase which is produced by the hepatic cells, and responsible for the transformation of L-gulonolactone acid into Ascorbic acid (Gilman *et al*., 1985).

The half life time of vitamin C in human is (16) days, (4days) in guinea pigs, and (3days) in rats (Ginter *et al*., 1971), due to the lack of the decarboxylase enzyme in humans (Kagawa, 1962), where this enzyme oxidizes vitamin C into CO_2 (Hornig *et al*., 1973); therefore, the period of scurvy emission in human is (3-4months), and (3weeks) in guinea pigs when sustained on vitamin C lacking foods (Kip *et al*., 1996).

Citrus fruits such as orange, lemon, and grape fruit are rich sources for vitamin C, where the vitamin amount in one segment is (50-60mg), but the richest sources for the vitamin is the guava where the one segment of which has about 200mg of the vitamin, also some vegetables are good source for the vitamin such as tomatoes, green pepper, cabbages, cauliflowers, broccoli, lettuce, pineapple, water melon, potatoes, green beans, and apple (Aurand and Woods, 1973).

Vitamin C is one of the glycoacids that have a great biological importance, and it is called also L-Xyloascorbic acid and is white, amorphous,

water soluble, less ethyl alcohol soluble, not soluble in fat solvents, and rather stable in acid solutions compound. Yet, it is sensitive to oxidation where it is oxidised rapidly by oxygen in the alkaline solutions in the presence of little metal ions forming the Dehydroascorbic acid which has the same activity of the ascorbic acid (McDonald *et al*., 1984). Ascorbic acid is optically active, thus there are two forms of the vitamin, L-Ascorbic acid which is the active form, and D-Ascorbic acid which is the inactive form.



L-Ascorbic acid Dehydro-L-Ascorbic acid

The chemical formulas of Ascorbic acid and Dehydroascorbic acid (Murray *et al*., 1999).

2–2–2–Vitamin C Kinetics.

The absorbtion of vitamin C occurs along the small intestine(Mglo and Wilson, 2000). The ratio of absorbtion in the distal parts is three times more than the proximal parts, and the absorbtion of the reduced form of the vitamin (L-ascorbic acid) is dependent on sodium, while that of the oxidized form (Dehydro-L-ascorbic acid) is of a facilitated diffusion type, also it is found that the acidic medium is inhibitive for the vitamin absorption (Mglo and Wilson,

2000). Misner (1998) mentioned also that vitamin C is absorbed from the intestine in the form of (L-ascorbic acid), while the reduced form (Dehydro-L-ascorbic acid) is reduced into (L-ascorbic acid) then it is absorbed by the small intestine. The normal plasma level of vitamin C in the human is 1mg/dl (Lehninger, 1975). Harper *et al*. (1979) mention that providing the fasting leads to a plasma vitamin level of about 1-1.4 mg/dl, and the increase in plasma level of the vitamin above this leads to increase excretion in urine. Usually the vitamin is excreted with urine as Ascorbate, Dehydro-ascorbate and Oxalate (Gowenlock *et al*.,1988).

The best percentage of absorption of vitamin C in the intestine is optimum on the dose 200mg, but this percentage decreases when the dose increases (Lawson, 1998). Misner (1998) and Benzie *et al*.(1998) report that the daily dose division gives better results than the one dose where the best daily vitamin absorption takes place when the daily doses are taken about 500mg/day divided into two or three doses within 24 hours.

Gilman *et al*.(1985) notice that the absorption vitamin percentage by the elimentary tract does not exceed 80-90 % of the taken dose. Malins *et al*.(1996) mention also that some diseases have a relationship to the plasma levels of vitamin C where it would be very little in those people who suffer from Asthma, Arthritis, Diabetes, and Heart diseases, and the low levels of the vitamin are correlated with the death caused by malignancy (Ramberg,2000). Ames *et al*.(1993) report that vitamin C is stored in different body tissues if it is taken in high doses because of the high water solubility of the vitamin.

2–2–3–Physiological effects of vitamin C

Recent studies have been published on the vitamin C (ascorbic acid) on different physiological parameters . Recent publications have

supported earlier work in that vitamin C plays an important role in determining response to erythropoietic agents .

These investigations suggest that vitamin C deficiency may be associated with decreased availability of stored iron and that administration of supplemental vitamin C may improve the availability of iron found in tissue storage sites (NPA, 2003) .

Vitamin C also improves the hemoglobin and hematocrotic in hemodialisis patients with iron – overload (Vanrenterghem *et al.* , 2003) . In case of sepsis , vitamin C has been shown to reduce the expression of the inducible nitric oxide synthase (iNOS) which is induced by lipopolysaccharides (LPS) (Wu *et al.*, 2003) . Vitamin C also completely prevents the endothelial dysfunction caused by LPS without altering the responsiveness of the vascular smooth muscle and it prevents endotoxin translocation from the gut (Archie *et al.* , 2005) .

In an in vitro study on mouse macrophages by Victor *et al* . (2000) , it was reported that macrophages were challenged with E.coli endotoxin and then treated with various concentrations of vitamin C , "the increased adherence ingestion and superoxide anion production by macrophages from animals with endotoxic shock were lower in the presence of vitamin C , reaching similar values to those of the control animals". Vitamin C prevents the programmed cell death (PCD) in some systems (Buttke and Sandstrom , 1994) , and reduces greatly the liver damage caused by ochratoxin A (OTA) in mice (Faik *et al.*, 2000) .

A study by Armour *et al.* ,(2001) revealed the effects of vitamin C on microvascular dysfunction in the skeletal muscle of the septic rat . Rats were given acaecal ligation and perforation (CLP) to introduce bacteria into there general circulation . 24 hours after the surgery , plasma

ascorbate levels dropped by 50% and urinary ascorbate concentrations increased by 1000% in rats that were not given vitamin C ; arterial pressure dropped by 20% and there was a 30% decreased in the density of perfused capillaries in those rats . Intravenous ascorbate (7.6mg/100g body weight-approx 5-6 grams for a 70kg human) given , as a single bolus after surgery , restored all of those parameters to near control levels .

The intense or endurance exercise produces a concentration of endotoxin in the blood similar to that found in patients with sepsis . The oral ascorbate pretreatment of as little as 1gm has been found to completely block the increasing in circulating endotoxin and nitrate typically found during and after intense exercise (Ashton ,et al., 2003) . Vitamin C and other antioxidants preserve the adequate function of immune cells against homeostatic disturbances caused by oxidative stress (De la Fuente , 2002) . It is clear that a diet supplemented with vitamin C seems to reduce the risk of coronary heart disease , a disease in which apoptosis is demonstrated in the destruction of cardiac cells (Riemersma *et al.*, 1991) . Also the antioxidants including vitamin C have been shown to have beneficial effects in diabetes (Bloomgarden, 1997) .

Vitamin C is reported to decrease the total serum cholesterol in adults (Joel et al, 1998) . A number of studies have reported that vitamin C administration lowers blood cholesterol levels in the guinea pig , an animal that like, humans, lacks the enzyme required for the hepatic synthesis of vitamin C (Nambisan and Kurup , 1975; Hanck and Weiser ,1977; Sharma *et al*., 1988) . Experiments on the guinea pig show that vitamin C is necessary in the rate – limiting steps in the conversion of cholesterol to bile acids. Vitamin C may reduce the incidence of infection and thus lower plasma fibrinogen concentrations , and there is experimental evidence that a large

dose of vitamin C increases fibrinolytic activity (Bordia , 1980) . Vitamin C is recently found to decrease the blood pressure (Ghosh *et al.* , 1994) . Experimental data suggest that vitamin C at daily dose of 2gm lowers serum cholesterol concentration and prevents the oxidation of low density lipoproteins (Bordia , 1980 ; Dobson *et al.* , 1984 ; Witzum , 1994) . The hepatic concentration of vitamin C affects the rate of cholesterol catabolism (Harris *et al.*,1979) . Vitamin C also helps prevent the "post marathon sniffle "-colds that develop after heavy exercise (Peters *et al*.,1993; Hemila ,1996). Some studies suggest that the use of vitamin C combined with vitmin E might slightly reduce the risk of developing preclampsia , a complication of pregnancy (Chappell *et al*.,1999; Beazeley *et al*.,2005). Another study suggests that vitamin C at a daily dose of 500 mg may help prevent reflex sympathetic dystrophy (RSD) , a poorly understood condition that can follow injuries such as fractures (Zollinger *et al*.,1999).

Overtime , the body develops tolerance to drugs in the nitrate family (such as nitroglycerin) , several evidences suggest that the use of vitamin C can help maintain the effectiveness of these medications (Watanabe *et al.* , 1998 ; Bassenge *et al;* 1998 ; Mc Veigh *et al.*, 2002) . Studies suggest that vitamin C might be helpful for minor injuries (Miller, 1960), and speed recovery from bedsores , easy bruising , and autism (Dolske *et al.* , 1993), anterior uveitis when taken in combination with vitamin E (Van Rooij *et al.*, 1999) , It also protects the liver in non alcoholic steatohepatitis (Harrison *et al.*, 2003) , treats female infertility (specially a condition called " luteal phase defect ") (Henmi *et al.*, 2003) , and preventing the early rupture of the chorioaminiotic membranes (the water breaking) in pregnancy (Casanueva *et al.*, 2005) . Vitamin C may improve the appearance of aging or sun damage skin (Traikovich , 1999;

Humbert *et al.,* 2003) . Vitamin C might also improve the effectiveness of antibiotic treatment for Helicobacter pylori , the cause of most peptic ulcer (Chuang *et al.,* 2007) . Khaw and Woodhouse (1995) examine the association between a low vitamin C concentration in elderly people and a high fibrinogen concentration . They speculate that vitamin C may protect against cardiovascular disease through an effect on haemostatic factors at least partly through the response to infection (Woodhouse *et al.* , 1993) . There is a positive relationship between vitamin C and endothelium dependent vasodilatation , where in case of increasing vitamin C supplementation , brachial and radial artery dilations are increased ; while coronary artery vasoconstriction and epicardial artery vasoconstriction are decreased (Timimi *et al.,* 1998) . Studies on the role of vitamin C for the following conditions have yielded positive results : asthma (Tecklenburg *et al.* ,2007) , male infertility (Rolf *et al.,* 1999) , reducing the muscle soreness that typically develops after exercise (Connolly *et al.* , 2006) , and hypertension (Kim *et al.,* 2002 ; Mulan *et al.* , 2002) .

Al-Katib (2001), reports that vitamin C is important in the fats metabolism in the blood and different body tissues of the humans.

Studies show that the regular use of vitamin C reduces the symptoms of colds and shortens the length of the illness (Hemila, 1997; Doglas *et al.*,2000) .

3. Materials and methods

3.1. Experimental animals

The experiment was conducted at the animal house of the Veterinary Medicine College–University of Basrah, where 56 females and 84 males mice (*Mus musculus*) sexually mature, 12 weeks old, and of 20–25 grams weights were used. The experiment conditions were unified for all animals, where the room temperature was set between 20–25 C by the use of an air conditioner, and the daily light period was 12 hours by the use of two fluorescent lamps, and the humidity rate was about 50 %. Food and water were provided daily (*ad libitum*).

3.2. Experimental design

The experiment was divided into two parts.

The first part was divided into four groups, three groups consisted of 16 male mice each, and the fourth group consisted of 8 male mice. The hemato–biochemical tests and Histopathological changes studies were done after 15 and 30 days of injection. The groups were :

- **Control group:** In this group, 16 male mice were injected I.P. with 0.9 % normal saline (N.S) daily for 15 days then 8 mice of them were terminated for the necessary tests and the remaining were injected I.P. with 0.9 % (N.S) for additional 15 days.
- **The first treated group (T1) group:** This group consisted of 16 male mice which were injected intraperitoneally (I.P) with 60 mg/kg of lead acetates daily for 15 days, then 8 mice of them were terminated for the necessary tests and the rest were injected I.P. with 400 mg/kg of vitamin C daily for additional 15 days.

❖ **The second treated group (T2) group:** This group consisted of 16 male mice which were injected I.P. with 80 mg/kg of lead acetates daily for 15 days, then 8 mice of them were terminated for the necessary tests and the remaining mice were injected I.P. with 400 mg/kg of vitamin C daily for additionally 15 days.

❖ **The third treated group (T3) group:** This group consisted of 8 male mice only, which were injected I.P. with 400 mg/kg of vitamin C after one hour of I.P. injection with 60 mg/kg of lead acetates daily for 15 days.

The second part of the experiment was related with the reproductive parameters and it was done after the first part had been accomplished. It consisted of 28 male mice and 56 female mice which were separated from each other during the 30 days of I.P. injection. The animals were divided into seven groups of 4 males and 8 females each.

The groups of the second part were as follows :

❖ **Control group :** 4 males and 8 females were injected with 0.9 % N.S for 30 days daily.

❖ **Treatment 2 (T2) group :** In this group, the 4 males only were injected I.P. with 80 mg/kg of lead acetates for 30 days daily.

❖ **Treatment 3 (T3) group :** In this group, the 4 males only were injected I.P. with 400 mg/kg of vitamin C after one hour of injection with 80 mg/kg of lead acetates for 30 days daily.

❖ **Treatment 4 (T4) group :** In this group, 4 males and 8 females were injected I.P. with 80 mg/kg of lead acetates for 30 days daily.

❖ **Treatment 5 (T5) group :** In this group, 4 males and 8 females were injected I.P. with 400 mg/kg vitamin of C after one hour of injection with 80 mg/kg of lead acetates for 30 days daily.

❖ **Treatment 6 (T6) group :** In this group, the 8 females only were injected I.P. with 80 mg/kg of lead acetates for 30 days daily.

❖ **Treatment 7 (T7) group :** In this group, the 8 females only were injected I.P. with 400 mg/kg vitamin of C after one hour of injection with 80 mg/kg of lead acetates for 30 days daily.

3.3. Specimens collection

Once the mice were anaesthetized, blood samples were collected directly from the myocardium by the use of a disposable syringes of 1 cc capacity at the end of the injection period, and the blood samples were divided into :

- ♦ 0.4 ml was poured into ethylene diamine tetra acetic acid (EDTA) containing tubes as an anticoagulant to accomplish the blood parameters.
- ♦ 0.6 ml was poured into test tubes free from anticoagulant to isolate blood serum to estimate the biochemical parameters.

3.4. Study parameters

3.4.1. Physiological parameters

3.4.1.1. Hematological parameters

A. Red blood cells count (R.B.C.) (cell/mm^3)

The red blood cells count was obtained by the use of haematocytometer (Neubauer improved double) and (Hayme's solution) and a special pipette for dilution (Sood, 1996).

1. Dilution and counting

The blood is sucked by the haemocytometer R.B.C. specified pipette to the mark 0.5, then it's diluted by (Hayme's solution) sucking to the mark 101, and the pipette is stirred horizontally to mix the solution. Then some of the liquid drops are spilt to elevate the non diluted solution. After that the special slide of the appliance is filled and covered then left for few minutes to permit the cells to settle over the counting square area.

The cells are counted in five middle squares of 25 squares (4 squares in the 4 angles and one square in the middle) by the use of a high microscopic amplification, then the following equation of corpuscles counting (cell/mm^3) is applied :

$$\text{No. of corpuscles (cell/mm}^3\text{)} = \frac{\text{No. of cells counted in five middle squares} \times \text{dilution factor}}{\text{Volume of dilution solution in which the cells are counted}}$$

$$= \frac{\text{No. of cells counted in five middle squares} \times 200}{1/50\ mm^3}$$

2. Dilution solution preparation (Hayme's solution)

This solution was prepared by mixing the following materials :

No.	Material	Quantity
1	Sodium chloride	0.5 g
2	Sodium sulphate	2.5 g
3	Mercuric chloride	0.25 g
4	Distilled water	100 ml

B. Hemoglobin concentration (Hb) (g/dl)

Hb concentration is estimated by Sahli apparatus, where an amount of 10 ml_s of Hcl of 0.1 normality is poured into Sahli tube and 10 µl of blood specimen is added and mixed together, then the mixture is let for 10 minutes, and later on distilled water is added gradually till the color of the blood is rendered similar to that of the standard tube color.

C. Packed cells volume (P.C.V.)

The microhematocrit method is used to calculate the percentage of P.C.V. by the use of capillary tubes which contain heparin, where one end of which was closed by an elastoplasts after being filled to 3/4 of its length with blood, and it is put in micro centrifuge on a velocity 1200 rotation/minute for five minutes, then the hematocrit value is obtained by Service device (Schalm *et al.*,1975).

D. White blood cells count (W.B.C.) (cell/mm3)

The W.B.C. was obtained by the use of Haemocytometer (Neubaur improved double) and Thoma's solution and special pipette for dilution (Dacie and Lewis, 1984).

A. Counting and dilution

The blood is sucked to the mark 0.5 by the service device then it is flooded to the mark 11 by the dilution solution (Thoma's solution), then the pipette is stirred to get the blood mixed. The non diluted solution is elevated by spilling some drops, then the glass slide is filled and covered and left for few minutes to permit the cells to settle over their squares. The cells are counted in four large

squares found in the four angles of the large squares of the R.B.C. counting, then the following equation is applied to the total W.B.C. count (cell/mm3):

$$\text{Cells number (cell/mm}^3\text{)} = \frac{\text{Cells counted in four large squares} \times \text{dilution factor}}{\text{Volume of dilution solution in which the cells are counted}}$$

$$= \frac{\text{Cells counted in four large squares} \times 20}{4/10}$$

B. Thoma's solution preparation

It is prepared by mixing the following materials :

No.	Material	Quantity
1	Glacial acetic acid	1.5 ml
2	Gention Violet 1 %	1.0 ml
3	Distilled water	97.5 ml

E. Differential W.B.C. count

After the blood smear is done, the slide is stained with Leishman's stain for 10 minutes then it is washed with water to eliminate the over stain, and left to

dry , then examined under oil immersion power to count the percentage of each type of W.B.C. (Schalm, 1975).

3.4.1.2. Biochemical parameters

A. Total serum cholesterol estimation

Total serum cholesterol (TSCH) is enzymatically measured by using a chemical kit (BIOCON/CHOD – PAP, Germany).

Test principle

The principle of this measurement is presented in the following equation (Siedel *et al.*, 1983).

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestin–3–on} + H_2O_2$$

$$2H_2O_2 + \text{phenol} + 4\ \text{aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinineimine dye} + 4H_2O$$

- The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically.

Testing procedure

Solution	Blank	Standard	Sample

Standard	—	µl 10	—
Sample	—	—	µl 10
Working reagent	µl 1000	µl 1000	µl 1000

♦ The tubes were incubated 5 minutes at 37 C. Within 60 minutes, the absorbance of calibrator and sample against reagent blank at 546 nm wave length is read.

♦ Total cholesterol concentration was calculated by following form

$$\frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times \text{Standard concentration} = \text{TC concentration}$$

♦ Standard concentration: 200 mg/dl.

B. Serum transaminases activity determination

Serum transaminases activity GOT or GPT is determined by using a special kit (BIOMÉRIEUX® sa Transaminases-Kit, France) (Reitman and Frankel,1957).

Test principle

Colorimetric determination of GOT or GPT activity is obtained according to the following reactions :

GOT

GOT : Aspartate + α keto glutarate $\rightleftharpoons$ oxaloacetate + glutamate

GPT : Alanine + α keto glutarate $\overset{\text{GPT}}{\rightleftharpoons}$ pyrovate + glutamate

The pyrovate or oxaloacetate formed is measured in its derivative form, 2.4-dinitrophenylhydrazone.

Test procedure

Wave length :——————————— 505 nm (490 – 520 nm)

1. Standard curve

Pipette into test tubes (ml) :

Tube no.	1	2	3	4	5	6
Distilled water	0.2	0.2	0.2	0.2	0.2	0.2
Reagent 1 or R2	1	0.9	0.8	0.7	0.6	0.5
Reagent 4	— 1	0.1 1	0.2	0.3	0.4	0.5

Reagent 3			1	1	1	1
Mix. Let stand for 20 minutes at room temperature						
NaOH 0.4 N	10	10	10	10	10	10
Mix. Wait 5 minutes. Measure.						
GOT units/ml	0	22	55	95	150	215
GPT units/ml	0	25	50	83	126	—

Plot the standard curve :

— abscissa : number of units/ml

— ordinate : OD

2. Measurement

The following tubes are set up for each serum :

	GOT	**GPT**
Reagent 1 Reagent 2	1 ml —	— 1 ml
Incubate for 5 minutes at 37 C		
Serum	0.2 ml	0.2 ml
Mix and incubate at 37 C for :	Exactly 1 hour	Exactly 30 minutes
Reagent 3	1 ml	1 ml
Mix. Let stand for 20 minutes at room temperature		
NaOH 0.4 N	10 ml	10 ml
Mix. Wait 5 minutes. Measure under conditions identical to those used for the standard curve.		

The color intensity is stable : ——————— 1 hour

The number of GOT and GPT units/ml of serum are calculated using the standard curve.

3.4.1.3. Reproductive parameters

These parameters were done for the animals of the second part of the experiment. Once the females mice give birth, the males are killed. Testes are cut and the epididymi are removed for the sperms viability measurements, and the mice babies are weighed by a sensitive balance once a week for 5 weeks. The fertility and sex ratio are documented and the corpora lutea were counted. The parents of each group were mixed after the injection period diminished, and they were left together for 16 days (Al–Zobaidy, 2003). Then the parents were separated again and females were left alone to deliver. The necessary tests such as massive and individual sperms movement, total sperms concentration, dead and alive sperms, and the sperms malformations were done after that.

A. Massive sperms motility measurement.

This test is done according to Evans and Maxwell (1987) method.

1. Raw epididymis is dropped on a warm clean slide.

2. The slide is examined under the light microscope using 4 X power. 3. The swirling motion is observed from the density and speed of the wave motion then the estimation is converted into the percentage as follow :

Type of movement	***Degree***	***Percentage***
Dense semen, very fast moving waves, no individual movement.	5	90-100
Strong motion but the waves and swirls are not like the previous.	4	75-85
Little motion, slow waves, individual movement is seen.	3	45-65
No waves, but some sperms move.	2	20-40

Very few sperms to be alive, weak movement.	1	10
No movement.	0	0

Evans and Maxwell (1987)

B. Individual sperms motility measurement.

The individual motility of the epididimical sperms is measured depending upon the graduation basis suggested by Chemineau *et al*., (1991) as follows :

1. Drops of the diluted semen are dropped on a warm clean slide at 37 c and covered with a cover slide.
2. The sperms are examined under the light microscope using 40 X power.
3. Depending upon the progressive forward sperms movement and the strength and speed of their motion are converted into a percentage.

Type of movement	***Degree***	***Percentage***
Sperms move rapidly and straightly.	5	90-100
Sperms are rapid, moving fast and some of them move in a circle.	4	75-85
Sperms go on a straight slope, without a shivering motion.	3	45-65
Simple irregular shivering motion, some of them move.	2	20-40
Movement is very slow, sperms shiver with swinging tail.	1	10
No movement.	0	0

3.4.2. Statistical Analysis

In this study, ANOVA Analysis and LSD tests are used according to (SPSS version) programme at the ($P \leq 0.05$) to find the means for all treatments (SPSS , 1989).

4- Results

4.1.4. Total and differential white blood cells count (W.B.C.)

The results in table (4) show that the lead caused the total W.B.C. to increase significantly on ($P \leq 0.05$) where T1 (17.38) and T2 (17.57) in comparison with the control (3.69). It seems also a significant difference between T1 and T2 is observed.

Considering the differential number of W.B.C., the lead also causes the lymphocytes to increase significantly on ($P \leq 0.05$) where T1 (48.37) and T2 (51.87) comparing with the control (37.00). A significant difference between T1 and T2 is obvious. Lead also increases the acidophils significantly T1 (3.25) and T2 (4.25) compared with the control (1.25) , with a significant difference between T1 and T2 . The monocytes are also increased by lead injection significantly T1(14.87) and T2 (15.00) compared with the control (4.00). No significant difference between the T1 and T2 is observed.

Considering the neutrophils, lead causes a significant decrease in the neutrophils (neutropenia) T1 (27.75) and T2 (25.50) compared with the control (57.00). It seems also a significant difference between T1 and T2 is observed. Considering the basophiles, no cells are seen in all studied groups T1, T2 and the control.

Table (5) shows that when the animals lead-poisoned are treated with vitamin C for 15 days , the total W.B.C. decreases, where T1 and T2 are (16.68) and (16.62) respectively but it is significantly higher than that of the control (3.72) , and there is no significant difference between T1 and T2 .

Considering the differential number of total W.B.C., it is found that vitamin C also causes the lymphocytes to increase significantly on ($P \leq 0.05$)

where T1 and T2 are (60.50) and (59.62) respectively compared with the control (37.20) , and there is no significant difference between T1 and T2 . Vitamin C also decreases the acidophils in treated animals (T2) is (1.75) , T1 (1.25) and control (1.21) , but there is no significant difference between T1 and the control on ($P \leq 0.05$) . The monocytes are also decreased by vitamin C significantly T1(1.75) , T2 (2.87) compared with the control (4.15) , and there is a significant difference between the T1 and T2 .

Considering the neutrophils, vitamin C causes an increase in the neutrophils T1 (34.50) and T2 (31.62) but still it does not reach that of the control significantly (57.12). It seems also a significant difference between T1 and T2 is observed. Considering the basophiles, no cells are observed in all treated groups, T1, T2 and the control. It seems from table (6), that when the vitamin C is offered after one hour of lead injection as a curing drug, it causes the total W.B.C. to decrease significantly ($P \leq 0.05$) in T3 (14.86) compared with T1(17.38) but still it does not reach that of the control which differs significantly from it (14.86 and 3.69 respectively). Considering the differential number of the total W.B.C., it seems that vitamin C is able to increase the neutrophil in lead injected animals T3 which is (27.75) compared with T1 (25.50) but it is still less significant than that of the control one (57.00).

Table 6 shows also that vitamin C is capable of decreasing the acidophils in lead-treated animals T1 which is (3.25) significantly on ($P \leq 0.05$) to (2.12) but it is still higher than that of the control (1.25). The lymphocytes of lead treated animals is also affected by vitamin C. It seems that the lymphocytes in T1 is decreased significantly by vitamin C in T3 (48.37 and 41.50 respectively) but it is still significantly higher than that of the control one (41.50 and 37.00 respectively).

Vitamin C is also able to decline the ratio of monocytes in T1 (14.87) to be (10.50) in T3. It seems also that monocytes in T3 are still significantly higher than in the control (10.50) and (4.00) respectively. No basophiles are observed in all groups of animals T1, T2 and the control.

Table (4) The effect of lead acetates on total and differential white blood cells count of male mice (n=8) for 15 days.

Parameters / Groups	W.B.C. (Cell/mm^3)×10^3	Neutrophil %	Acidophil %	Lymphocyte %	Monocyte %	Basophil %
Control (0.9 % N.S.)	3.69 c ± 0.29	57.00 a ± 1.69	1.25 c ± 0.46	37.00 c ± 1.69	4.00 b ± 0.75	0
T1 (60mg/kg lead acetate)	17.38 b ± 0.18	27.75 b ± 0.88	3.25 b ± 0.46	48.37 b ± 2.13	14.87 a ± 0.64	0
T2 (80mg/kg lead acetate)	17.57 a ± 0.10	25.50 c ± 0.75	4.25 a ± 0.70	51.87 a ± 2.58	15.00 a ± 1.06	0
LSD	0.18	1.06	0.49	1.94	0.75	

The numbers represent the mean ± Standard Deviation .

The different letters refer to significant differences among groups ($P \leq 0.05$).

Table (5) The effect of vitamin C on total and differential white blood cells count of lead poisoned male mice (n=8) for 15 days.

Parameters / Groups	W.B.C. (Cell/mm^3)×10^3	Neutrophil %	Acidophil %	Lymphocyte %	Monocyte %	Basophil %
Control (0.9 % N.S.)	3.72 b ± 0.29	57.12 a ± 1.69	1.21 b ± 0.46	37.20 b ± 1.69	4.15 a ± 0.75	0
T1 (60mg/kg lead acetate + 400 mg/kg vitamin C)	a 16.68 ± 0.37	b 34.50 ± 0.92	b 1.25 ± 0.46	a 60.50 ± 0.92	c 1.75 ± 0.88	0
T2 (80mg/kg lead acetate + 400 mg/kg vitamin C)	a 16.62 ± 0.44	c 31.62 ± 2.32	a 1.75 ± 0.70	a 59.62 ± 2.77	b 2.87 ± 0.83	0
LSD	0.33	1.56	0.49	1.74	0.74	

The numbers represent the mean ± Standard Deviation .

The different letters refer to significant differences among groups ($P \leq 0.05$).

Table (6) The effect of vitamin C on total and differential white blood cells count of male mice (n=8) after one hour of lead acetates injection for 15 days.

Parameters / Groups	W.B.C. (Cell/mm^3)×10^3	Neutrophil %	Acidophil %	Lymphocyte %	Monocyte %	Basophil %
Control (0.9 % N.S.)	3.69 c ± 0.29	57.00 a ± 1.69	1.25 c ± 0.46	37.00 c ± 1.69	4.00 c ± 0.75	0
T1 (60mg/kg lead acetate)	17.38 a ± 0.18	25.50 b ± 0.75	3.25 a ± 0.46	48.37 a ± 2.13	14.87 a ± 0.64	0
T3 (60mg/kg lead acetate + 400 mg/kg vitamin C)	14.86 b ± 0.48	21.75 c ± 0.88	2.12 b ± 0.64	41.50 b ± 1.51	10.50 b ± 0.92	0
LSD	0.31	1.06	0.47	1.60	0.70	

The numbers represent the mean ± Standard Deviation .

The different letters refer to significant differences among groups (P≤0.05).

Table (7) The effect of lead on biochemical parameters of male mice (n=8) for 15 days.

Parameters / ***Groups***	Alanin aminotransferase (ALT) IU	Aspartate aminotransferase (AST) IU	Total serum cholesterol (TSCH) mg/dl
Control (0.9 % N.S.)	17.06 ^{a} ± 0.07	22.20 ^{a} ± 0.14	37.46 ^{c} ± 0.02
T1 (60 mg/kg lead acetates)	5.25 ^{b} ± 0.46	17.50 ^{b} ± 0.46	100.57 ^{b} ± 0.63
T2 (80 mg/kg lead acetates)	4.50 ^{c} ± 0.53	17.00 ^{c} ± 0.46	113.65 ^{a} ± 0.27
LSD	0.36	0.34	0.35

The numbers represent the mean ± Standard Deviation .
The different letters refer to significant differences among groups ($P \leq 0.05$).

Table (8) The effect of vitamin C on biochemical parameters of lead-poisoned male mice (n=8) for 15 days.

Parameters / *Groups*	**Alanin aminotransferase (ALT) IU**	**Aspartate aminotransferase (AST) IU**	**Total serum cholesterol (TSCH) mg/dl**
Control (0.9 % N.S.)	17.09 ± 0.07 c	22.15 ± 0.14 c	37.40 ± 0.02 c
T1 (60 mg/kg lead acetates + 400 mg/kg vitamin C)	69.37 ± 0.51 a	79.87 ± 0.64 a	80.38 ± 1.31 b
T2 (80 mg/kg lead acetates + 400 mg/kg vitamin C)	56.25 ± 0.88 b	56.25 ± 0.88 b	88.91 ± 0.85 a
LSD	0.53	0.56	0.81

The numbers represent the mean ± Standard Deviation .
The different letters refer to significant differences among groups ($P \leq 0.05$).

Table (9) The effect of vitamin C on biochemical parameters of male mice (n=8) after one hour of lead acetates injection for 15 days .

Parameters / *Groups*	**Alanin aminotransferase (ALT) IU**	**Aspartate aminotransferase (AST) IU**	**Total serum cholesterol (TSCH) mg/dl**
Control (0.9 % N.S.)	17.06 b ± 0.07	22.20 b ± 0.14	37.46 c ± 0.02
T1 (60 mg/kg lead acetates)	5.25 c ± 0.46	17.50 c ± 0.46	100.57 a ± 0.63
T3(60 mg/kg lead acetates + 400 mg/kg vitamin C)	29.87 a ± 0.83	64.25 a ± 1.03	90.64 b ± 0.36
LSD	0.49	0.59	0.37

The numbers represent the mean ± Standard Deviation .
The different letters refer to significant differences among groups ($P \leq 0.05$).

4.3. Reproductive Parameters

4.3.1. Sperms viability

Table (10) shows the effect of lead on sperms viability and the role of vitamin C in relieving the lead toxicity. It is obvious that the injection of male mice with (80m/kg) of lead acetates in T2 leads to a sharp significant decrease ($P \leq 0.05$) in all sperms viabilities with an increase in sperms abnormalities compared with the control group . Once vitamin C is offered and male mice are injected with it (400mg/kg) in T3 , all the sperms viabilities measurements rise significantly compared with T2 group (treated male) .

Furthermore, the lead exposure causes a sever grossly testicular damage as it is seen in (picture: 1), and different microscopic sperms abnormalities appear such as curved sperm (picture: 3), headless sperm (picture: 4), immature sperm (picture: 5), abnormal shaped head sperm (picture, 6), and hookless head sperm (picture: 7) compared with the control group normal sperms (picture: 2).

TABLE (10) The effect of vitamin C on semen examinations of lead_poisonned male mice.

Treatment	Individual movement %	Mass movement %	Sperms count ($\times 10^6$ / ml)	Abnormal sperms %	Dead sperms %	Alive sperms %
control N.M	a 85.25 ± 0.50	a 81.25 ± 2.50	a 183.25 ± 3.94	c 8.00 ± 1.63	c 17.50 ± 1.00	a 74.50 ± 1.91
T2 Pb T.M + N.F	c 20.00 ± 0.00	c 10.00 ± 0.00	c 55.50 ± 4.20	a 29.25 ± 2.98	a 54.75 ± 3.30	c 16.00 ± 1.15
T3 Pb & vit.C T.M + N.F	b 65.00 ± 0.00	b 63.25 ± 2.36	b 148.25 ± 2.36	b 14.00 ± 1.41	b 23.75 ± 2.21	b 62.25 ± 2.06
LSD	0.07	0.04	3.80	2.06	2.42	1.56

The numbers represent the mean ± Standard Deviation .
The different letters refer to significant differences among groups ($P \leq 0.05$).

T.M	Treated male
Pb	Lead acetates
Vit.C	vitamin C
N.F	Normal female

4.3.2. Reproductive ability

Table 11 shows the effects of vitamin C on reproduction physiology of lead-treated animals. It seems that the fertility reaches zero when males animals are treated with lead compared with the control one, whereas when the males are treated with vitamin C, 1 female only out of 8 gave birth to 5 litters out of 24 mature ova are released from the ovary forming 24 corpora lutea. The sex ratio (% percent of males) is 16.66 %. The table indicates also that when both parents are treated with lead, the fertility is zero. But when vitamin C is offered to this group of animals, the results show that 2 females conceived out of 8 treated females. The mice pregnant females gave birth to 10 mice litters out of 33 mature ova are released from the ovary forming 33 corpora lutea. The sex ratio in this group is 40 %. Table 11 shows also when the females only are treated with lead, the fertility is zero. When vitamin C is given to this group, it improves the fertility and 4 out of 8 females are pregnant and 19 mice litters are born. The results indicate also that 31 mature ova are released out forming 31 corpora lutea . The sex ratio is 42.10 %.

4.3.3. Body weight of mice litters .

Table (12) represents the role of vitamin C on the weight of litters of lead-treated parents. As it is seen in table (12), when one of the parents either male or female or both of them are treated with lead, the females are not able to give birth at all. But when vitamin C is offered in a dose of 400mg/kg after one hour of lead injection, the results in table 12 indicate the ability of females in the groups (T3, T5 and T7) to conceive and give birth to litters whose weights are less significant than those of the control, from day one till day twenty one of delivery. The litters body weights in 7 days, 14 days and 21 days are significantly less than that of the control in all groups and there are significant differences among the groups T3, T5, and T7. On the 28^{th} day old, there are no significant differences in the body weights of all groups comparing with the control group and among them at ($P \leq 0.05$).