

# Effect of L-carnitine on Lead-induced Infertility in Female Rats

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**Abstract:** *The correlation between lead toxicity and infertility was confirmed in many studies. The present study aimed to explore the effect of L-carnitine on the adverse effects caused by lead metal on female rat fertility. Animals were organized into four groups (negative control group, lead acetate positive control group (50mg/L), L-carnitine low dose group (50mg/Kg Bwt/day) and L-carnitine high dose group (100mg/Kg Bwt/day). After 21 day period of treatment blood samples were collected and female rat ovaries were excised for Estradiol quantitative estimation and CYP19a1 gene expression respectively, samples were collected related to the four phases of the estrous cycle. The results of the present study revealed that lead metal in a dose of (50mg/L) causes significant decreases ( $p < 0.05$ ) of CYP19a1 gene expression and estradiol hormone level. L-carnitine have an outstanding beneficial effects on the previous two parameters which increased by increasing the dose. Therefore, the potential of using L-carnitine to combat lead metal adverse effects on female infertility is very promising.*

**Keywords:** Lead toxicity; female rat fertility; Estrous cycle; CYP19a1 gene; Estradiol; L-carnitine.

## 1. Introduction

Lead is a heavy metal presents in earth crust [1], hasn't biological function [2] and is non-biodegradable [3]. In spite of its toxicity problems lead is still heavily used in developing countries [4, 5]. Storage batteries, cable covers [6], paints, ceramics, glazes, dyes [7], manufactories products, cigarette, house wares and kitchen tools [8] are an examples of its endless usage. Pharmacokinetics of lead facilitate occurrence of toxicity, it enters the body through several routes as digestive absorption, respiration and through the skin [9, 10], it is slowly excreted and easily accumulates and stored in various tissues [11, 12] especially in bones (90%) [13]. The main route for excretion is through urinary system [14].

Lead induces oxidative damage in different tissues including liver, kidney, heart, Gastro intestinal tract (GIT) and reproductive system [15], it causes deleterious effects on various biological functions [16, 17]. The underlying basis of lead toxicity is mostly due to production of large amount of reactive oxygen species (ROS) [16] and inhibition of antioxidant enzymes as catalase (CAT), superoxide dismutase (SOD) and enzymes responsible for glutathione reactions [11, 16].

Reproductive system in female undergoes several periodical and physiological changes [18], these physiological changes undergo great pathological disruption by any toxicant [19]. The estrous cycle divides into four phases (proestrous, estrous, metestrous, and diestrous). Three types of cells appearing during the estrous cycle phases [20-22], cornified cells, epithelial cells and leukocytes are observed in the vaginal smear [23].

Estradiol (E2) is the most effective estrogen in female fertility [24], it produced from aromatization of testosterone during reaction catalyzed by aromatase which originates from CYP19a1 gene [25, 26]. There are three isomers of CYP19 gene CYP19a1(gonads), CYP19a2(brain) [27, 28] and CYP19a3 (Sus scrofa (Pig)) [27, 28].

Levo-carnitine (L-carnitine) is a biologically active water soluble antioxidant molecule [29, 30] which is derived from dietary source (75%) and internally synthesized from essential amino acids (Lysine and Methionine)(25%)[31]. It is present in all mammals, localized on the inner mitochondrial membrane [32, 33] and facilitates the transport of long chain fatty acids (LCFAs) through mitochondrial membrane for utilization in energy production ( $\beta$ -Oxidation). L-carnitine has a negligible side effects [32], thereby it is preferred as a treatment of oxidative stress in comparison to artificial antioxidant supplementation which causes hepatocellular damage and carcinogenesis [34, 35].

L-carnitine has a potent antioxidant property and is used for prevention and treatment of oxidative stress [36, 37]. It protects the cells from apoptosis and improves much of the mechanisms of physiological antioxidants [38, 39]. Antioxidant properties of L-carnitine appears clearly in more than one mechanism, for example decreasing the production of ROS [40, 41], maintenance and protection of antioxidant enzymes (SOD, CAT and glutathione peroxidase)[42] L-carnitine has a potent scavenger capacity for free radicals [43, 44].

## 2. Materials and Methods

### A. Chemicals

Lead acetate, Mouse/Rat Estradiol ELISA kits and gene specific primers (2X Maxima SYBR Green/ROX qPCR Master Mix, thermo scientific, USA, # K0221) were purchased from Sigma Company, Egypt). L-carnitine ampoule (1gm/5ml) produced by Medical union pharmaceuticals company and purchased from medical pharmacy.

### B. Animals

80 female albino rats weighting about (150-200 gm.) were purchased from the laboratory animal house research center Kafr Elsheikh University. Animals were housed in separate wire mesh cages and exposed to good ventilation, humidity and 12 hr. light/dark cycle. They were provided with a constant supply of standard pellet diet and fresh drinking water -ad libitum. All animals were organized into four groups (n=20), negative control group (GI), positive control group (GII) (oral lead acetate solution (50mg/liter)(Ronis et al., 1996), low dose L-carnitine group (GIII) (oral lead acetate solution (50mg/liter) plus treated by low dose L-carnitine (50mg/kg Bwt) (Dokmeci et al., 2006, Evans and Fornasini, 2003) and high dose L-carnitine group (GIV) (oral lead acetate solution (50mg/liter) plus treated by high dose L-carnitine (100mg/kg Bwt) (Dokmeci et al., 2006, Evans and Fornasini, 2003) for 21 day. The experiments were performed in accordance with the guidelines for animal care of the Faculty of veterinary Medicine, Kafr-Elsheikh University, Egypt.

### C. Vaginal smear

Vaginal fluids collected from animals on the morning (8:00-9:00 a.m.) in eppendorf, vaginal smears were prepared to differentiate between the four phases of the estrous cycle and stained by methylene blue. Examination the smears by using Light microscope (10X then 40X) and digital microscope (The Leica DFC295) (40X) was used to capture coloured picture. See figure (4)

### D. Blood and tissue

Blood samples were collected from animals at the end of experiment (21days) after overnight fasting. The animals were anesthetized by diethyl ether and blood collected through retro-orbital venous plexus in eppendorf, centrifuged (5000 rpm for 10 min) for separation of serum samples, serum samples were stored in (-20°C) refrigerator for further examination.

Animal killed by decapitation, then an ovarian tissues were dissected immediately in eppendorf and stored at (-80°C) until RNA extraction and CYP19a1 gene expression.

### E. Real Time – Polymerase Chain Reaction (RT- PCR)

Total RNA was extracted by Trizol kits following the manufacturer protocol (Thermo Scientific, Fermentas, #K0731). Samples are homogenized in Lysis Buffer. The lysate is then mixed with ethanol and loaded on a purification column. Impurities are effectively removed from the membrane subsequently by washing the column with

wash buffers. Pure RNA is then eluted under low ionic strength conditions with nuclease-free water.

Complementary DNA (cDNA) synthesis was prepared by using Reverse transcription kits (Thermo Scientific, Fermentas, #EP0451) and the technique was done using Reverse Transcriptase which is a genetically modified to convert RNA into cDNA. Real-time PCR with SYBR Green was used to measure expression of mRNAs of target genes in the ovaries with  $\beta$ -actin as an internal reference. The isolated cDNA were amplified using a kit named 2X Maxima SYBR Green/ROX qPCR Master Mix and according to the manufacturer protocol Thermo scientific, USA, # K0221 and gene specific primers. These primers based on published rat and mouse sequences (accession no NM\_031543 for P450 2E1)(Singh et al., 1988).

### F. Estradiol quantitative determination

Enzyme-linked immune sorbent assay (ELISA) technique was used to measure Estradiol (E2) hormone in serum samples. Mouse/Rat Estradiol ELISA kits were used for the quantitative determination of E2 concentration in Mouse/Rat serum and plasma. It is based on the principle of competitive binding between E2 in the test specimen and E2 Enzyme Conjugate for a constant amount of anti-Estradiol polyclonal antibody.

During the incubation a fixed amount of Horseradish peroxidase (HRP)-labeled E2 competes with the endogenous E2 in the standard sample or quality control serum for a fixed number of binding sites of the specific E2 antibody. E2 Peroxidase Conjugate progressively decreases as the concentration of E2 in the specimen increases. Tetramethylbenzidine (TMB) Reagent is added and incubated at room temperature resulting in the development of blue color. The color development is stopped with the addition of Stop Solution and the absorbance is measured spectrophotometrically at 450 nm.

### G. Statistics analysis

Data of CYP19a1 gene expression were expressed as mean fold change  $\pm$ S.E. The statistical significance was evaluated by One-way analysis of variance test (ANOVA) using Statistical Package for the Social Sciences (SPSS) and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when ( $p < 0.05$ ).

The data of Estradiol (E2) was expressed as means  $\pm$ S.E. The statistical significance was evaluated by One-way Analysis Of Variance test (ANOVA) using Graph Pad prism 5. Values were considered statistically significant when  $p < 0.05$  and Statistical significance determined using Tukey option which compares all pairs of columns.

## 3. Results

### a) CYP19a1 gene expression

#### 1) Relative changes of CYP19a1 gene expression in normal rat ovaries during estrous cycle

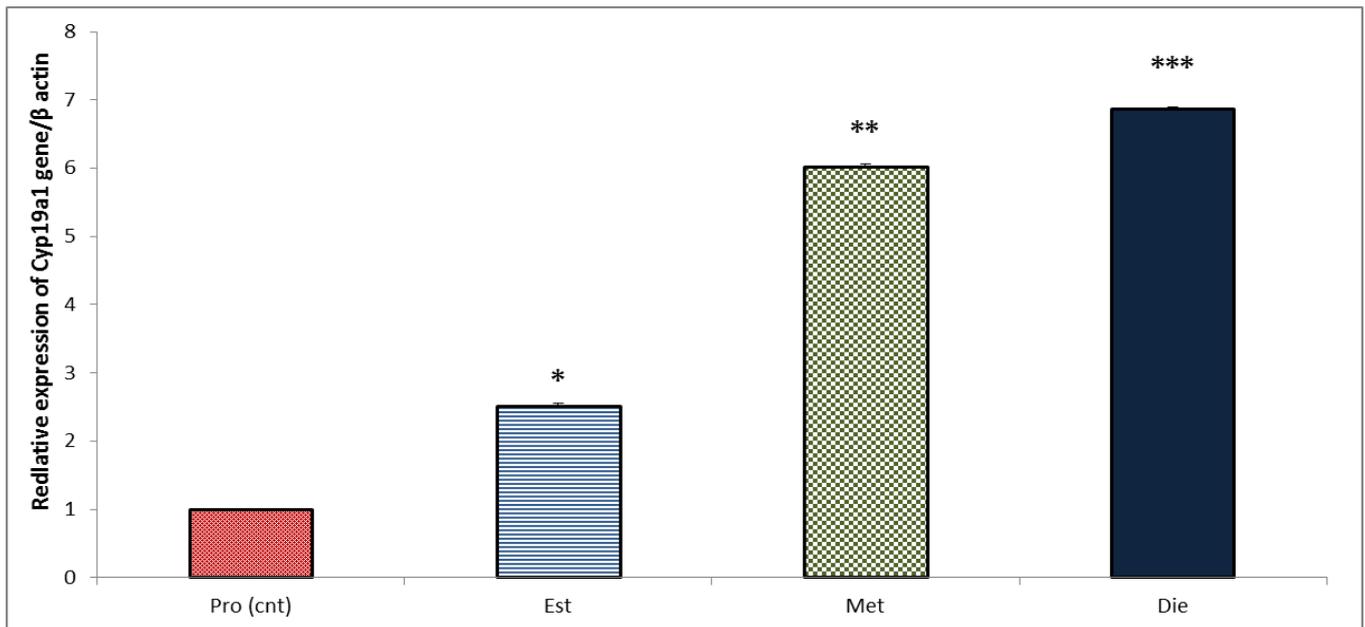
The results of the present study revealed a significant ( $P < 0.05$ ) gradual increase of CYP19a1 gene expression level

during the four phases of estrous cycle with the highest expression in diestrous phase and the lowest in proestrous phase in negative control rats. There is a significant variation ( $P < 0.05$ ) of CYP19a1 gene expression between the four phases of the estrous cycle. Data are expressed as fold change versus proestrous group. The gene expression reaches its peak at diestrous phase and decreased to the lowest value at proestrous phase (Table: 1, Figure: 1).

**Table 1:** Showing relative changes of CYP19a1 gene expression in normal rat ovaries during the four phases of the estrous cycle in female rat

Estrous phase	Mean fold change $\pm$ SEM
Proestrous	1.00 $\pm$ 0.01
Estrous	2.51 $\pm$ 0.05*
Metestrous	6.02 $\pm$ 0.04**
Diestrous	6.87 $\pm$ 0.03***
LSD	0.101

Data expressed as fold change versus proestrous. Data within the same column having different superscript symbols are significantly different based on Fisher's LSD. (\* $P < 0.05$  means significant, \*\* $P < 0.01$  means highly significant and \*\*\* $P < 0.001$  means very highly significant)



**Figure 1** showing the relative changes of CYP19a1 gene expression in normal rat ovaries during the four phases of the estrous cycle in female rat in negative control group (GI); Pro= proestrous; Est. = estrous; Met= metestrous; Die=diestrous. (\* $P < 0.05$  means significant, \*\* $P < 0.01$  means highly significant and \*\*\* $P < 0.001$  means very highly significant)

**2) Comparison between the results of each phase in all groups**

The results of CYP19a1 gene expression showed a significant decrease ( $P < 0.05$ ) in all treated groups (GII-IV) as compared to the negative control groups (GI) in all estrous cycle phases. Treatment by L-carnitine (50mg/kg Bwt. and 100mg/kg Bwt.) produced a significant increase

( $P < 0.05$ ) of CYP19a1 gene expression as compare to lead-treated groups of the four phases of estrous cycle with no significant difference between the increases of CYP19a1 gene expression following the two doses except for the diestrous phase. (Table: 2, figure: 2)

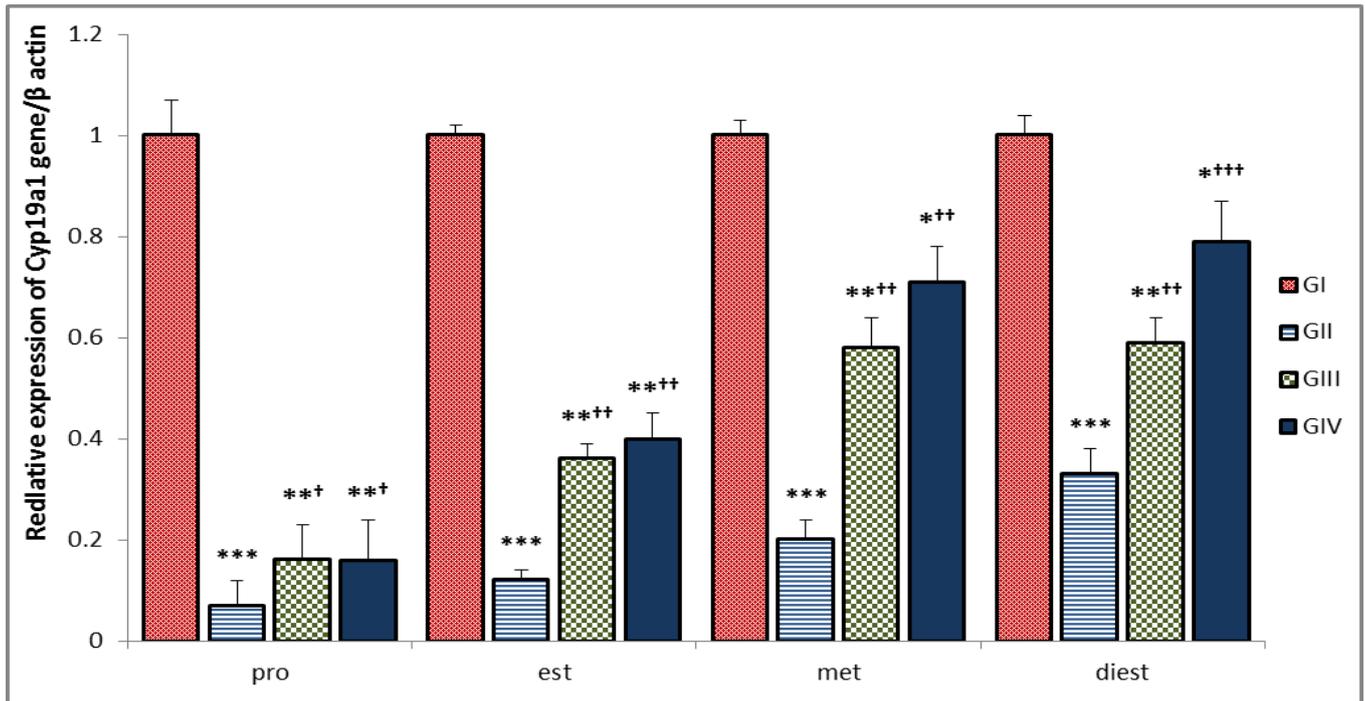
**Table 2** Showing relative changes of CYP19a1 gene expression of each phase of the four phases of the estrous cycle in comparison with the corresponding phase in the other groups

Groups	Mean Fold change $\pm$ SEM			
	Proestrous	Estrous	Metestrous	Diestrous
GI	1.00 $\pm$ 0.07	1.00 $\pm$ 0.05	1.00 $\pm$ 0.07	1.00 $\pm$ 0.08
GII	0.07 $\pm$ 0.02***	0.12 $\pm$ 0.02***	0.20 $\pm$ 0.03***	0.33 $\pm$ 0.05***
GIII	0.16 $\pm$ 0.03**†	0.36 $\pm$ 0.04**††	0.58 $\pm$ 0.06**††	0.59 $\pm$ 0.07**††
GIV	0.16 $\pm$ 0.03**†	0.40 $\pm$ 0.05**††	0.71 $\pm$ 0.05*††	0.79 $\pm$ 0.08*†††
LSD	0.005	0.009	0.006	0.009

Data expressed as fold change versus normal control group (GI). Data within the same column carrying different superscript symbols are significantly different based on Fisher's LSD). (\* $P < 0.05$  means significant, \*\* $P < 0.01$  means

highly significant and \*\*\*  $P < 0.001$  means very high significant versus GI) († $P < 0.05$  means significant, †† $P < 0.01$

means highly significant and  $^{\dagger\dagger\dagger}P < 0.001$  means very highly significant versus GII)



**Figure 2** Showing graphical presentation of the relative changes of CYP19a1 gene expression of each phase of the four phases of the estrous cycle in comparison with the corresponding phase in the other groups in negative control group (GI), lead acetate-treated group (GII), 50mg/kg Bwt. L-carnitine-treated group (GIII), and 100mg/kg Bwt. L-carnitine-treated group (GIV). Data expressed as fold change versus normal control group (GI). (\* $P < 0.05$  means significant, \*\* $P < 0.01$  means highly significant and \*\*\*  $P < 0.001$  means very high significant versus GI) ( $^{\dagger}P < 0.05$  means significant,  $^{\dagger\dagger}P < 0.01$  means highly significant and  $^{\dagger\dagger\dagger}P < 0.001$  means very highly significant versus GII)

**a) Estradiol (E2) level (ng/dl):**

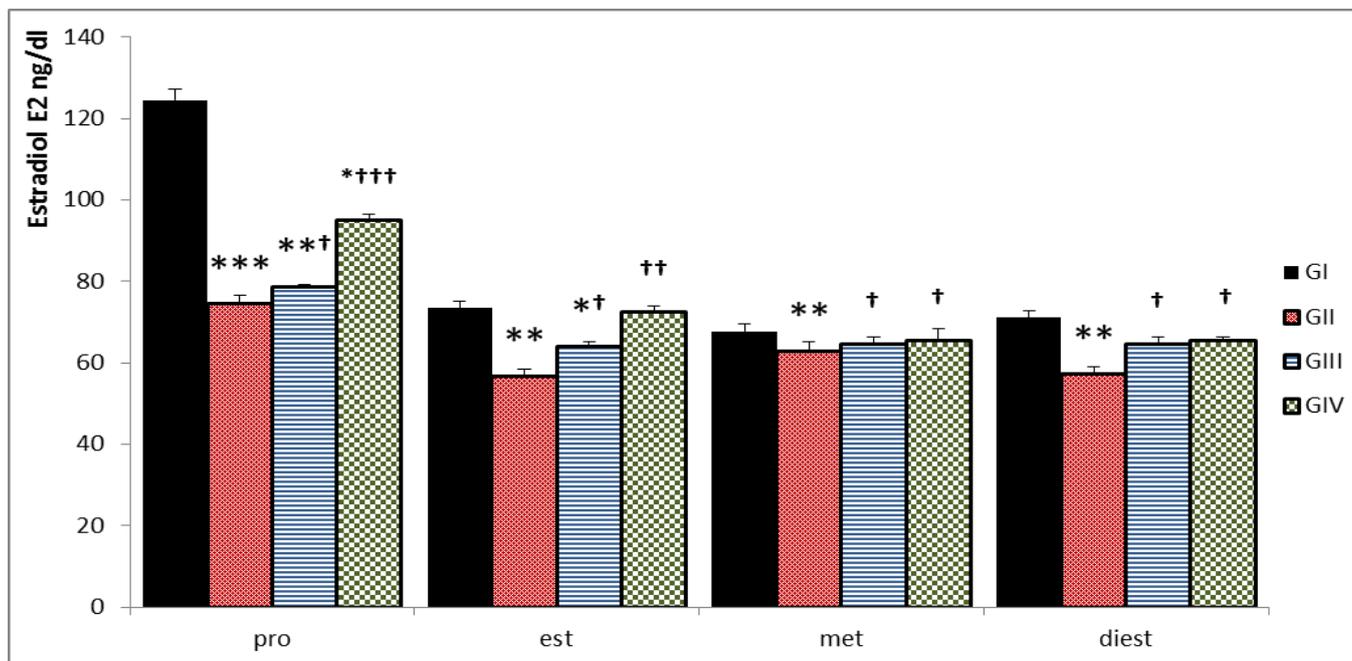
The results of Estradiol (E2) showed a significant decrease ( $P < 0.05$ ) in lead acetate-treated group (GII) as compared to the negative control groups (GI) in all phases of the estrous cycle (Figure: 3). using of Low dose of L-carnitine supplementation showed slight increases in E2 levels of all phases of the estrous cycle. Remarkable significant increase

of E2 levels occurred by increasing the dose of L-carnitine especially in proestrous and estrous phases, on the other hand the peak Estradiol (E2) levels appeared in proestrous phase then significantly decreased ( $P < 0.05$ ) during estrous, metestrous and diestrous phase. (Table: 3, figure: 3)

**Table 3** Showing estradiol (E2) levels in each phase of the four phases of the estrous cycle in comparison with the corresponding phase in the other groups

Groups	Mean ± SEM ng/dl			
	Proestrous	Estrous	Metestrous	Diestrous
GI	124.45±2.89	73.45±2.03	67.67±0.39	71.15±1.24
GII	74.47±1.54***	56.77±1.59**	62.95±1.29**	57.32±1.46**
GIII	78.80±1.86**†	63.97±2.20**†	64.65±1.74 $^{\dagger}$	64.67±3.10 $^{\dagger}$
GIV	95.12±1.63*†††	72.52±1.75 $^{\dagger\dagger}$	65.40±1.59 $^{\dagger}$	65.55±0.68 $^{\dagger}$

Data expressed as Mean ± SEM. Data within the same column carrying different superscript symbols are significantly different using Graph Pad prism (5) and based on Tukey option which compares all pairs of columns. (\* $P < 0.05$  means significant, \*\* $P < 0.01$  means highly significant and \*\*\*  $P < 0.001$  means very high significant versus GI) ( $^{\dagger}P < 0.05$  means significant,  $^{\dagger\dagger}P < 0.01$  means highly significant and  $^{\dagger\dagger\dagger}P < 0.001$  means very highly significant versus GII)



**Figure 3** Graphical presentation of estradiol (E2) levels in each phase of the four phases of the estrous cycle in comparison with the corresponding phase in the other groups in negative control group (GI), lead acetate-treated group (GII), 50mg/kgm Bwt./day L-carnitine-treated group (GIII) and 100mg/kg Bwt./day L-carnitine-treated group (GIV). (\*P<0.05 means significant, \*\*P<0.01 means highly significant and \*\*\*P<0.001 means very high significant versus GI) (†P<0.05 means significant, ††P<0.01 means highly significant and †††P<0.001 means very high significant versus GII)

#### 4. Discussion

Lead metal toxicity on reproductive organs is a popular research topic. Lead metal causes reproductive toxicity in both sexes, but only a few researches on female rats are available. Tomoum et al reported that about 50% of Egyptian schools children suffer from delayed puberty of both sexes and confirmed that the problem is correlated to reproductive toxicity of lead metal (Tomoum et al., 2010) which gives an alarm for a dangerous public health problem in our country. Others studies confirmed that lead metal causes disruption in reproductive hormones FSH, LH and steroids, thereby disturbance in spermatogenesis, folliculogenesis, ovulation and steroidogenesis (Wirth and Mijal, 2010) and causes infertility problem in both sexes (Luszczek-Trojnar et al., 2014, Hosni et al., 2013, Qureshi and Sharma, 2012).

On the other hand many studies confirmed the genotoxic effect of lead metal which occurred via direct and/or indirect reasons (Ahmed et al., 2012, Kryston et al., 2011). The direct mechanisms of DNA damage caused by direct interaction of lead with DNA (Kumar et al., 2013). Lead ion ( $Pb^{2+}$ ) binds to specific binding sites on DNA such as oxygen atoms of the phosphate groups of DNA backbone.  $Pb^{2+}$  enters into the minor grooves of DNA double helix and binds to guanine bases, oxygen atom in deoxyribose and imidazole ring in adenine (Zhang et al., 2014). Lead metal reduces Messenger RNA Levels of Cytochrome P450 Aromatase in Human Ovarian Granulosa Cells through reducing the mRNA of CYP19a1 gene (Taupeau et al., 2003). Indirect mechanism occurs through production of large quantities of ROS which causes oxidative stress and oxidative damage to DNA (Ziech et al., 2011, Nampoothiri and Gupta, 2006), thereby

transcription of gene prevented and translation into a protein altered and produces genomic disturbances, carcinogenesis and cell death (Best, 2009, Marnett, 2000).

The results of the present study revealed that the relative expression of CYP19a1 gene in the ovaries of normal rat (GI) during the estrous cycle significantly ( $P<0.05$ ) varied during the four phases of estrous cycle. Transcripts were measured by real-time RT-PCR using appropriate primers and normalized to  $\beta$ -actin mRNA levels. Data are expressed as fold changes versus proestrous group. The results showed significant variation between the four phases of the estrous cycle with highest gene expression (peak) in diestrous phase while the lowest expression appeared in proestrous phase (Table:1, Figure:1). These findings are in agreement with the results of (Carretero et al., 1999, Galmiche et al., 2006b) who reported that an expression of CYP19a1 gene varied during the four phases of the estrous cycle in female rats where it showed lowest expression at proestrous phase and reaches its peak at diestrous phase.

The results of the present study revealed that orally administered lead acetate (50mg/L) produced a significant decrease ( $P<0.05$ ) of CYP19a1 gene expression during the four phases of the estrous cycle. Data expressed as fold change versus normal control group (GI) (Table: 2, Figure: 2). these results are consistent with taupeau et al. who reported that lead metal has an adverse effect on the expression of CYP19a1 gene in granulosa cells of an ovaries (Taupeau et al., 2003). The work of many researchers confirmed the genotoxic effect of lead metal (Schneider et al., 2013, Hirsch et al., 2010, Makhoul et al., 2008).

L-carnitine in a dose of (50 mg/kg B wt. /day and 100mg/kg B wt. /day) produced a significant ( $P<0.05$ ) increase of CYP19a1 gene expression during the four phases of the estrous cycle as compared to gene expression level after oral lead administration (50mg/L). The increase in gene expression is not significantly different between the low dose and high dose of L-carnitine in all phases except the diestrous phase. The significant difference between the effect of the low and high doses of L-carnitine on gene expression is explained after Stocco who reported that the physiological expression of CYP19a1 gene during the first three phases compared to diestrous phase is low so the difference in response to the high and low doses of L-carnitine is not significant except in diestrous phase (Stocco, 2009, Galmiche et al., 2006a). See (Table: 2, Figure: 2)

Lead metal toxicity on CYP19a1 gene expression was reported to be due to Oxidative stress through increasing the production of reactive oxygen species (ROS) and decreasing the activity of physiological antioxidant CAT and SOD (Pandya et al., 2012, El-Nekeety et al., 2009). The use of antioxidant supplementation such as vitamin C and E as an effective treatment for the adverse effects of lead metal emphasized the antioxidant theory effect of lead toxicity on gene expression (Eshginia and Marjani, 2013, Khordad et al., 2013). The use of L-carnitine as a natural antioxidant for the same purpose is another strong prove for the theory (El-Masry et al., 2014, Zambrano et al., 2013, Yildirim et al., 2013).

The changes in the concentration of Estradiol (E2) are useful to confirm hormonal disruption effect of lead metal on steroidogenesis. As an end product of aromatase enzyme (end product of the central dogma pathway of CYP19a1 gene) any change in E2 gives not only a picture on CYP19a1 gene expression changes but also on the fertility status (Simpson, 2004, Boon et al., 2010, Wirth and Mijal, 2010).

The present study demonstrated a significant ( $P<0.05$ ) decreases of E2 level during the four phases of estrous cycle after oral lead administration (50mg/l) (Table: 3, Figure: 3). The disrupted effect of lead metal on estradiol level was supposed to be due to its effect on hypothalamus pituitary gonadal axis (HPG-axis) (Dumitrescu et al., 2008, Pillai et al., 2010, Hamadouche et al., 2013) and due to its genotoxic effect on CYP19a1 gene expression (Taupeau et al., 2003, Teijon et al., 2006).

L-carnitine in a dose of (50 mg/kg B wt. /day and 100mg/kg B wt. /day) produced a significant ( $P<0.05$ ) increase of E2 during the four phases of the estrous cycle as compared to its level after oral lead administration (50mg/L). The increase in E2 level is not significantly different between the low dose and high dose of L-carnitine in all phases except the proestrous phase. The significant difference between the effect of the low and high doses of L-carnitine on E2 level is can be explained on the same scientific basis of CYP19a1 gene expression changes. See (Table: 3, Figure: 3)

The results of the present study regarding the effect of L-carnitine on estradiol level during the phases of estrous cycle

are compatible with the results of L-carnitine on CYP19a1 gene expression during the stages of estrous cycle.

## 5. Conclusion

Lead toxicity causes adverse effects on all biological processes including reproduction in both sexes directly by interfering with the function of enzymes and binding to DNA or indirectly through causing an oxidative stress. The later occurs either by increasing ROS production or by decreasing the activity of physiological antioxidant.

Oxidative stress causes alteration in the biological molecules including DNA, lipids and proteins which causes damage in Oocytes and disruption in reproductive hormones which leads to infertility. The present study reported that L-carnitine supplementation is able to improve and repair the deleterious effects caused by lead metal through decreasing the production of ROS, increasing the capacity of physiological antioxidant systems and maintaining the reproductive systems in a healthy state. Therefore we conclude that L-carnitine can sweep the adverse effects caused by lead metal on fertility of female rats.

Further investigation should be done to explore more of the beneficial effects of L-carnitine as a promising treatment for the hazardous effects of lead toxicity on the female reproductive system.

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## 7. Declaration of interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

## 8. Abbreviations

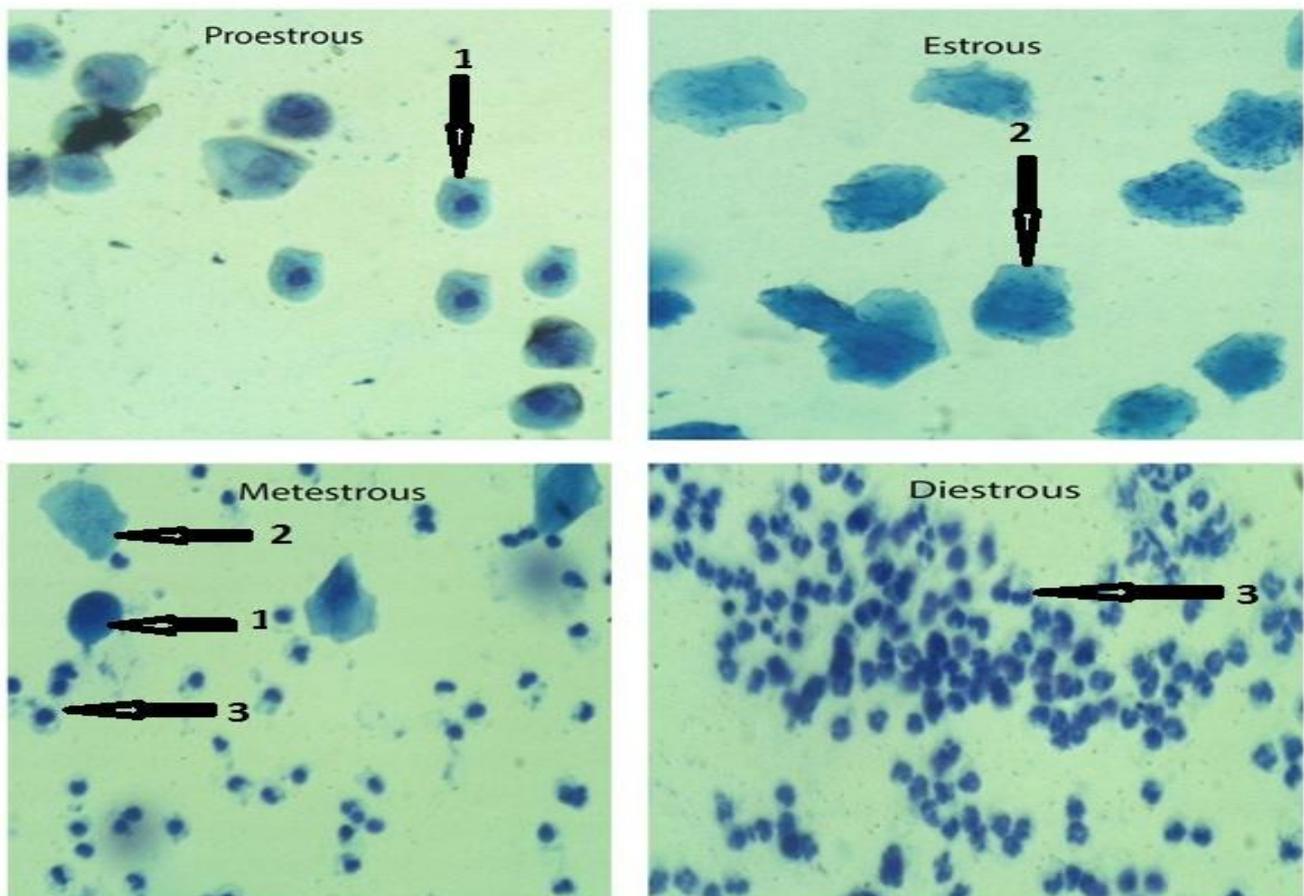
ROS: Reactive Oxygen Species  
CAT: Catalase  
SOD: Super Oxide Dismutase  
GIT: Gastro Intestinal Tract  
E2: Estradiol  
LCFAs: Long Chain Fatty Acids  
HPG-axis: Hypothalamus Pituitary Gonadal –axis  
PCR: Polymerase Chain Reaction  
ELISA: Enzyme Linked Immune Sorbent Assay

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**Figure 4** Showing vaginal smear of female rats (methylene blue stain) to differentiate between the four phases of estrous cycle. Proestrous phase has oval well-formed nucleated epithelial cells (1). Estrous phase has cornified squamous anucleated epithelial cells (2). Metestrous phase has different cell types with predominance of Leukocytes. Diestrous phase characterized by predominance of Leukocytes (3).