Original Research Article

L-Citrulline Supplementation Enhances Reproductive Functions Of Lead Acetate Induced Testicular Toxicity In Male Sprague Dawley Rats

ABSTRACT

INTRODUCTION: Lead (Pb) is a transition metal and a known male reproductive toxicant that induces its effects mainly through oxidative stress. This study investigated the effects of L-Citrulline (Cit) supplement on reproductive functions and antioxidant activities in Lead acetate treated male rats. **MATERIALS AND METHOD:** Twenty male rats (180-200 g) were grouped into four and treated as follows; Group 1 (Control), given distilled water, Group 2 was given Pb acetate (2.25mg/kg), Group 3 was co-treated with Pb acetate (2.25mg/kg) and Cit (900mg/kg) and Group 4 was given Cit (900mg/kg) only. All administrations were done orally for thirty days. Caudal sperm, serum hormone levels and testicular antioxidant activities and Nitric oxide levels were evaluated at the end administrations.

RESULTS: Results showed decrease ($p<0.05$) in sperm morphology, count, viability, motility, FSH, LH, Testosterone, Catalase, Nitric Oxide and Super oxide dismutase in Group 2(Pb treated rats), when compared to Group 1(Control).However, in Group 3 (Pb acetate co-administered with Cit) the effect was significantly reversed *($p<0.05$)* compared with Group 2 and significant increase was observed in Group 4 (Cit only group) compared with Group 2.

There was an increase *($p<0.05$)* in Malondialdehyde level in Group 2 compared with (Group 1) Control while a significant decrease *($p<0.05$)* was observed in Groups 3 and 4 compared with Group 2.**CONCLUSION:**The results of this study suggest that L- Citrulline supplement has ameliorating capacity on the toxic effect of Lead acetate on sperm parameters.

Keywords: L- Citrulline, Lead acetate, *sperm parameters, reproductive hormones, antioxidants, rats*

1.0 INTRODUCTION

L-Citrulline (L-Cit) ($C_6H_{13}N_3O$) is a non-protein amino acid, and an organic compound that is water-soluble. It is derived from *Citrullus vulgaris* (Watermelon) where it was first isolated from in the 1930s [1]. Until recently, Citrulline has been considered as only an intermediate chemical in the urea cycle. However, recent studies have indicated the importance of this amino acid in various cellular metabolism and organs function [2].

L-Cit has been shown to have the potential to increase the plasma nitrite and nitric oxide (NO) level, reduce the tonicity of the muscle in blood vessels, retinal arterioles dilation, beneficial impact on either SBP (systolic blood pressure) or DBP (diastolic blood pressure), protection against overweight and obesity in men, attenuated cardiovascular risk, reduced nephron number and renal dysfunction in rat's adult offspring due to maternal calorie restriction was reversed by L-cit and maternal L-cit supplementation exacerbates the elevation of blood pressure [3-15]. Lead is a naturally occurring bluish-gray metal available in small amounts in the earth's crust which is found throughout our environment. An increased amount of lead in the environment comes from human activities that include fuel, mining, burning fossil and manufacturing [16]. Lead has been shown to have deleterious effects in several organs and organ systems which include the hematopoietic, nervous, renal, cardiovascular, reproductive, and immune system and it is also mutagenic[17]. The routes of Lead exposure are mainly ingestion and inhalation due to its presence in food, air, and tobacco leaves [18-20]. (The World Health Organization (WHO) has published a list of 10 chemicals or groups of chemicals of concern for human health and this includes Lead [21]. Additionally, the US Agency for Toxic Substances and Disease Registry (ATSDR) ranked Lead in second place on the priority list of dangerous substances [22].

Many studies have indicated that the human male reproductive capacity has deteriorated considerably during the past few decades [23-25]. This decreasing trend in male fertility has led to speculation that recent environmental, dietary and/or lifestyle changes are interfering with man's ability to produce spermatozoa [26]. Transition metals like Lead constitute an important group of environmental factors that can adversely affect or impair male reproductive function. Studies have shown a considerable increase in transition metal contamination in relation to worldwide distribution, anthropogenic activity, and extensive use of transition metals [27]. Increased levels of transition metal ions in blood plasma or semen appear to be significantly and positively correlated with male infertility [28-31].

2.0 MATERIALS AND METHODS

2.1 Experimental Animals

Twenty male Wistar rats weighing between 180 to 200g were used. They were kept in the animal house of Madonna University, Elele campus, Nigeria under standard laboratory conditions with 12 hours light and 12 hours dark cycle. They were fed with standard laboratory animal chow and had access to water *ad libitum.* The animals were acclimatized for one week. The animal grouping is as follow:

Table 1: Study design

Groups	Treatments
1	Distilled water
2	Pb acetate (2.25mg/kg)
3	Pb acetate (2.25mg/kg) + Cit (900mg/kg)
4	Cit (900mg/kg)

2.2 Chemicals:

Lead acetate and L-Citrulline and all other chemicals used for this study were of analytical grade and products of Sigma Aldrich, United Kingdom

2.3 Experimental Procedure

At the end of the 30 days administration blood samples were collected from the anesthetized animals through cardiac puncture, serum was obtained and used for FSH, LH, and testosterone assays.

2.3.1 Tissue Collection:

The epididymis was collected and used for sperm analysis. The testes were collected, homogenized, and used for determination of testicular protein, Cholesterol, Malondialdehyde (MDA), Catalase, Nitric Oxide and Super oxide dismutase

2.3.2 Sperm motility

As described previously by [32], the caudal epididymis was identified, and its content squeezed into 1ml of normal saline at room temperature. One drop of the semen suspension was charged

into a Makler counting chamber and the number of motile and non-motile spermatocytes was counted in ten random fields. The number of motile spermatocytes was then expressed as a percentage of the total number of the counted spermatocytes [33].

2.3.3 Sperm count

Sperm count was performed as reported earlier [34] with minor modifications. Briefly, caudal epididymis was minced in 2 ml of normal saline to obtain sperm suspension, which was then stained with 2% eosin and sperm heads were counted using an improved Neubauer counting chamber. The sperm were counted and expressed as million per ml.

2.3.4 Sperm Viability

The caudal epididymis sperm was dropped on the slide and mixed with a drop of 0.5% eosin solution. After 2 minutes, the slide was examined under microscopy with 40X objective lens to count the percentage of viable (unstained) and non-viable sperm (stain red) [35].

2.3.5 Sperm morphology

This was determined by smearing a drop of the stained semen suspension obtained during determination of sperm count on a glass slide; the smear could dry and subsequently examined under the light microscope at X40 magnification. For each sample, 200 spermatocytes were carefully observed, and the percentage of total abnormalities of the spermatocyte head and total abnormalities of the spermatocyte tails were determined as described by [34].

2.4 Reproductive hormones measurement

Testosterone, Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) in rat serum were measured by enzyme linked immune-assay (ELISA) using commercially available kits from Endocrine Technologies, USA. Samples were run in the same assay to avoid inter-assay variations.

2.5 Nitrite analysis

Nitrite accumulation in the testicular supernatant was determined by a diazotisation reaction using the standard Griess reagent [36, 37]

2.6. Antioxidant Assays:

The testicular supernatant was used for Lipid peroxidation and antioxidant assays.

2.6.1. Malondialdehyde (MDA) Assay: This was done according to the method of [38]. The principle is based on the reaction of Malondialdehyde, a product of lipid peroxidation with thiobarbituric acid to give a red species that can be detected at 535 nm.

2.6.2. Superoxide dismutase (SOD) Assay: This was estimated according to the methodof [39]. The principle is based on rapid auto-oxidation of adrenaline in aqueous solution to adrenochrome due to the presence of superoxide anions. The concentration was determined with a spectrophotometer at 420nm.

2.6.3. Catalase (CAT) Assay: This was determined according to the method of [40]. Upon the addition of 30 mM H_2O_2 in 50 mM of phosphate buffer (pH 7.4) to sample, it is converted to oxygen and water. This action was stopped after three minutes by the addition of 1 mL of H_2SO_4 to the mixture, followed by 7.0 mL of $KMnO_4$. Catalase (CAT) activity was estimated by adecrease in absorbance of H_2O_2 at 520 nm

2.7. Statistical Analysis

All statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the LSD post hoc tests for pair-wise comparisons performed using IBM®SPSS 17.0 version. All data were expressed as Mean ± Standard Error of mean (SEM) and $p<0.05$ was considered significant.

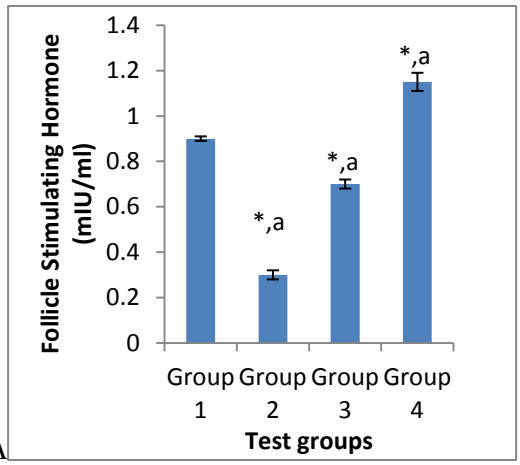
3.0 RESULTS

Table 2: Effect of L-Citrulline on some sperm parameters of male Wistar rats following exposure to lead acetate

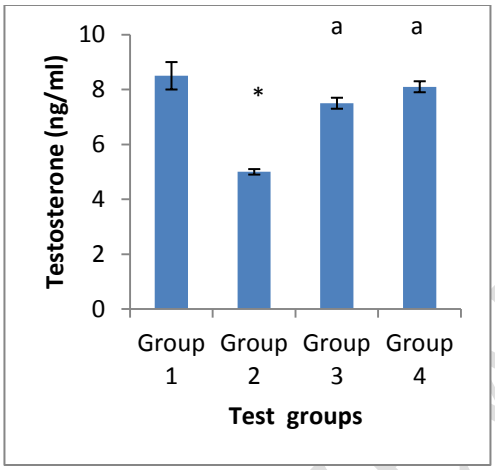
Parameters	Control	Lead only	Lead+900mg/kg Citrulline	900mg/kg Citrulline
Motile cells (%)	88.00±1.22[a]	48.00±4.89*	64.00±9.79[a]	90±5.50*,[a]
Active motile cells (%)	79.00±3.67	34.00±2.44*	51±8.57*,[a]	85±7.3[a]
Sluggish motile cells (%)	9.00±2.45	14.00±2.45	13±1.22	5±0.20*[a]
Dead cells (%)	12.00±1.22[a]	52.00±4.89*	36±9.79*,[a]	10±1.5[a]
Head defect (%)	0.40±0.24a	2.00±0.01*	2.8±0.06*	2.4±0.54*
Tail defect (%)	0.6±0.24a	4.80±0.48*	0.94±0.02*	2.5±0.46*
Mid-piece defect	0.60±0.24[a]	6.00±0.01*	1.96±0.02*,[a]	0.2±0.20*,[a]
Viable cells (%)	96.00±1.22[a]	58.00±0.01*	90±0.84a	94.6±0.24[a]
Non-viable cells (%)	4.00±1.22[a]	42.00±0.01*	10±0.24*,[a]	5.4±0.24[a]
Sperm count(million/ml)	56.00±0.01[a]	16.40±0.40*[a]	22.6±2.69*	73.00±1.46*,[a]

Values are expressed as mean± Standard error of mean, [a]$p<0.05$ were considered significant relative to control and lead groups respectively*

A



C



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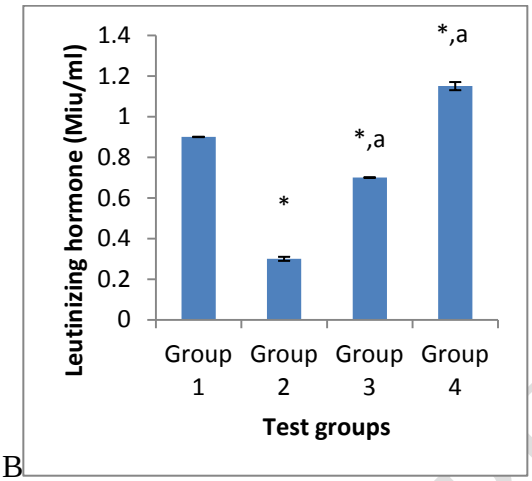

Figure 1. E**ffect of L-Citrulline** supplement on (A) Follicle stimulating hormone (B)Leutinizing hormone (C) Testosterone levels in lead acetate treated male Wistar rats
Values are expressed as mean± Standard error of mean, [a]p<0.05 were considered significant relative to control and lead groups respectively*

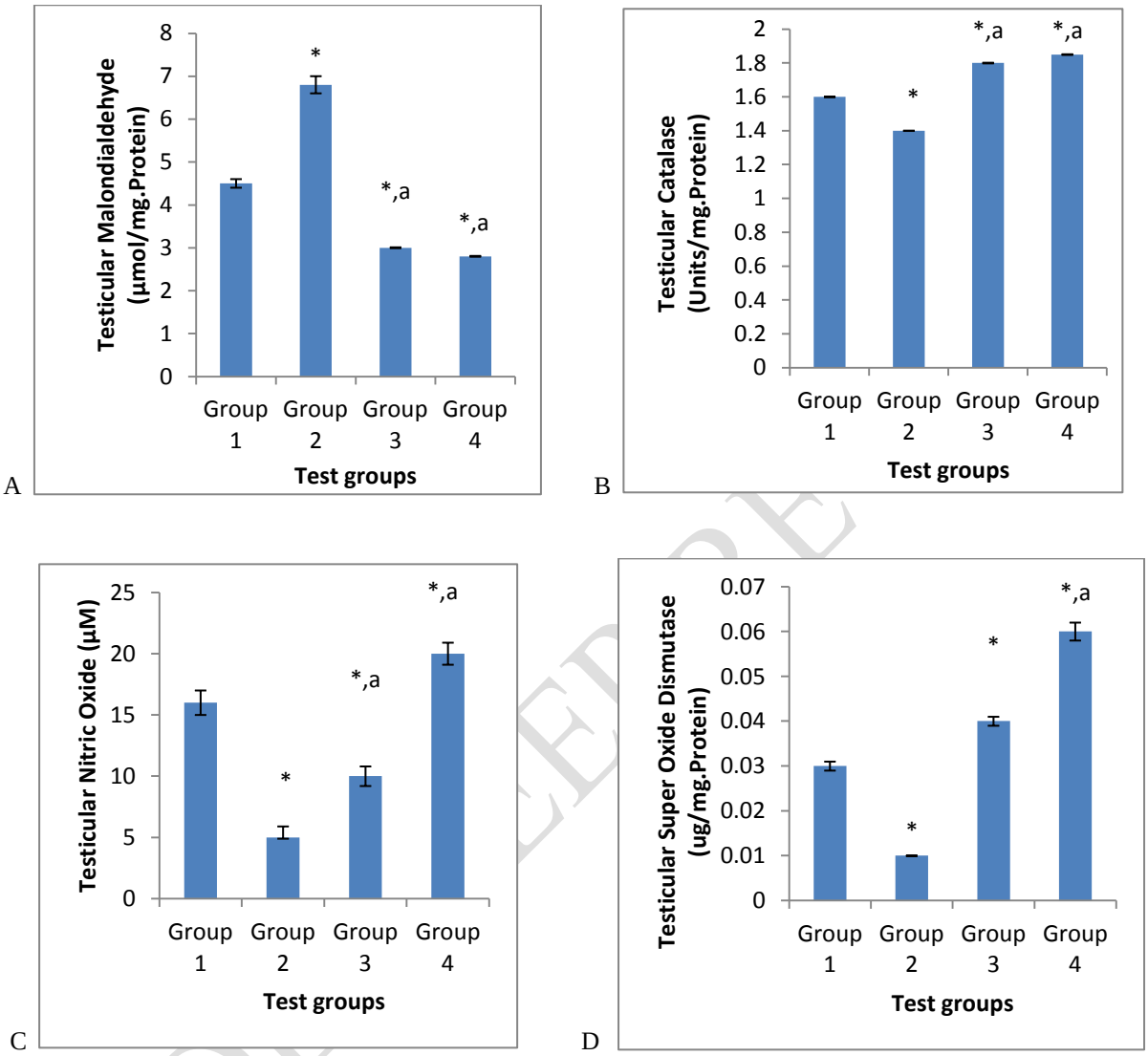

Figure 2. Effect of L-Citrulline supplement on Testicular (A) Malondialdehyde (B) catalase activity (C) Superoxide Dismutase activity (D) Nitric oxide activity levels in lead acetate treated male Wistar rats

Values are expressed as mean± Standard error of mean $^{*a}p^{<}0.05$ *were considered significant relative to control and lead groups respectively*

4.0 **DISCUSSION**

The present study describes the effects of oral L- Citrulline supplement administration on some sperm parameters, reproductive hormones, some testicular antioxidant enzymes activities, testicular cholesterol, protein and nitric oxide in lead acetate treated male Wistar rats. In this study, a decrease in sperm parameters such as count, motility, viability and morphology in the groups treated with Lead acetate was observed and this is in agreement with the previous report of [41].

It has been previously shown that there is a number of mechanisms by which lead may affect male reproductive health.

It has been shown that transition metals adversely affect spermatogenesis, sperm cells and can cause testicular necrosis through a direct effect on the testicular vasculature [42, 43, 44, 45] thereby reducing their motility, viability and/or affecting their morphology [46, 47, 48, 49, 50]. Both animal experiments and human studies suggest that the sperm chromatin structure is altered already even at low exposure to lead.

A biological rationale for this finding is that lead may cause a partial replacement of zinc which is essential for sperm head chromatin stabilization. Failure of or delay in sperm chromatin decondensation may lead to decreased fertility or different kinds of DNA damage in the fertilization process [51]. Zinc is essential for the maintenance of germ cells, the progression of spermatogenesis, stabilization of the cell membrane and regulation of capacitation, acrosome reaction and sperm motility [52]. Its deficiency leads to gonadal dysfunction, decreases testicular weight, and causes shrinkage of seminiferous tubules [53].

There was an ameliorating effect of L-Citrulline on the detrimental effects of lead acetate on the sperm parameters. L – citrulline is an amino acid which is converted to L- arginine by the body. L – Arginine improves blood flow by creating nitric oxide (NO). Increase in L- Arginine by supplementation of L- Citruline must have led to increase generation of NO which in turn decreases lipid peroxidation in the spermatozoa [54, 55].

CIT is a major precursor of Arginine (through renal conversion) [1] and this has led several authors to suggest that CIT might be particularly useful for patients with impaired Arginine metabolism. It actively participates in sperm formation. Administration of l-arginine to oligospermic and asthenospermic patients results in an improvement in both the sperm count and motility without any side effects [56, 57]. L-arginine plays an important role in stimulating

sperm motility in humans, rabbits, and goats under in vitro conditions [57, 58, 59]. In earlier publications, it was shown that l-arginine enhances the rate of glycolysis, resulting in higher rates of ATP and lactate generation in spermatozoa [59]. The influence of arginine in reversing impairment caused by glycolytic inhibitors (potential contraceptives) [60] has also been studied. Also it was noticed that some reproductive hormones that were accessed during the study were negatively affected in Lead treated group. This also is in agreement with the work of [41].

The observed decrease in testosterone, luteinizing hormone, prolactin and follicle stimulating hormone in the groups treated with lead acetate in this study is in consonance with the reports [41].

It has been demonstrated in toxicological studies that many transition metals which include Lead can accumulate in testes and/or epididymis thereby impairing their endocrine and reproductive functions [61, 62, 63].

Several studies reported a significant decline in serum testosterone level in exposed experimental animals to lead [30, 64, 65]. This may be due to inhibition of the action of the steroidogenic enzymes in Leydig cells [66].

Lead acetate caused a reduction in gonadotropin secretion and this may be on account of a possible depressive effect on the hypothalamic neural mechanisms essential for the release of Gonadotropin Releasing Hormone [67, 68]. This eventually will lead to disturbances in the secretion of pituitary gonadotropins an essential for both spermatogenesis and steroidogenesis [69].

Also, significant increase in serum testosterone, FSH and LH levels were observed in nicotine treated rats in addition to Citrulline supplementation. This may be due to the ability of Citrulline as an antioxidant to mop up reactive oxygen species and prevent lipid peroxidation of the sperm cells in the hypothalamic-pituitary-testicular axis.

Increased plasma levels of L-Cit have been associated with a protective role in oxidative damage and supporting the maintenance of ‘normal’ functioning of the cardiovascular system due to its role in NO metabolism [30, 31].

An increase was observed in the level of MDA, while a decrease was observed in the levels of Catalase and SOD of lead acetate treated rats. This is in support of earlier study [41]. It has been shown that exposure to lead increase the production of reactive oxygen species (ROS) and,

consequently, induce lipid peroxidation and alteration of antioxidant defense systems in mice [70] resulting in oxidative stress [71].

The membrane lipids of spermatozoa (which are mainly phospholipids) are highly susceptible to the action of peroxidizing agents, which may be natural or inadvertently present due to extraneous factors [54].

Natural lipid peroxidation is commonly observed in all living cells including spermatozoa. ROS are the bye products of numerous degenerative reactions in various tissues, which affect the regular metabolism by damaging the cellular components [72]

Decreasing the possibility of lead interacting with critical biomolecules and stimulating oxidative damage or bolstering the cell's antioxidant defense might be attributed to the beneficial role of antioxidant nutrients through exogenous supplementation of antioxidant molecules regulation and signaling.

The possible ameliorative effects of L-Citrulline supplementation in Lead treated rats may be due to the fact that CIT which is a major precursor of Arginine (through renal conversion) [1] and has the ability to prevent membrane lipid peroxidation in spermatozoa under different peroxidation conditions [73, 74] .

Citrulline is used in the nitric oxide system in humans and has potential antioxidant effect. Increased plasma levels of L-Cit has been associated with a protective role in oxidative damage and supporting the maintenance of 'normal' functioning of the cardiovascular system due to its role in NO metabolism [3,75]

It has been proposed that the beneficial effects of l-arginine are linked to nitric oxide (NO) [76]; NO is a short-lived free radical, synthesized in many mammalian cell types by a class of NADPH dependent enzymes called nitric oxide synthases (NOS). These enzymes catalyze the conversion of l-arginine to l-citrulline and NO [77]). The reaction is inhibited by l-arginine analogs.

L-arginine has been shown to increase generation of NO. Based on this, it can also be postulated that l-arginine protects spermatozoa against lipid peroxidation through increased NO production.

Nitric oxide has been shown to inactivate superoxide (O_2^-) anions [78]. These anions are regularly released by mammalian cells during oxygen consumption and cause peroxidative damage to membrane phospholipids. Sperm are known to be particularly susceptible to such lipid peroxidation [79]. Sperm with damaged membranes are impaired functionally, which suggests

that the presence of a free radical scavenging system would be beneficial to sperm-producing tissues. Kisa et al. have shown a correlation between sperm motility and the levels of NO and TBARS present in rat testicular tissue [55].

5. 0. CONCLUSION

L-Citrulline supplementation from this study has shown to be a beneficial treatment option against lead-acetate induced oxidative stress and toxicity in testicular tissue. Among the most beneficial results are increased sperm motility, vitality, increased sperm viability, decreased sperm morphological alterations, increased sperm count, increased FSH, LH, testosterone levels and increased SOD,CAT and decreased MDA level.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that principle of laboratory animal care (ARRIVE guidelines) was dully followed, as well as specific national laws where applicable in this study

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