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Protective Role of Vitamin C on Sperm Morphology and Biochemical Enzymes in Lead Nitrate Treated Albino Rats

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ABSTRACT
Lead is a well known heavy metal and is found in various components of earth environment and is a major industrial byproduct. Manipulation of lead from these sources causes toxicity in human beings by oxidative stress, which results in decrease fertility due to increase in the production of abnormal sperm population. Vitamin C is an antioxidant and acts by scavenging the free radicals, thus ameliorating the oxidative stress. The present study was carried out to evaluate the effect of lead nitrate and Vitamin C on testis. The study was conducted on 30 male albino rats of Wistar strain, weighing 150-250 gm. The rats were divided into 5 groups (6 rats in each group). Group 1 served as control and rest 4 groups were experimental. Except the control group, the animals of other 4 groups received lead nitrate and vitamin C orally for 6 days a week for 2 months. All the animals were sacrificed at the end of experiment and the result showed abnormal sperm count 5% in control group while it was 66% in group 4 and 17% in group 5. Sperm abnormalities observed were double head, banana head, amorphous head, defective head, headless, bent neck, bent tail, double tail, defective tail and looped tail. Malondialdehyde, a marker of lipid peroxidation was increased in experimental group while glutathione reductase (GR) enzyme was decreased in testicular tissue but this change in enzyme showed some improvement in vitamin C treated groups.
Key words: *Lead nitrate, biochemical enzymes, sperm morphology, vitamin C.*

INTRODUCTION

Various environmental factors may decrease the reproductive capability of males. A hazardous effect on male fertility may be manifested by decrease in the number of sperms, disordered motility or abnormal structure. Lead is a male reproductive toxicant "Winder [(1)]". The primary mechanism of the toxic action of lead appears to be disruption of hypothalamic control of pituitary hormone secretion and in turn, spermatogenesis "Sokol [(2)]". Occupational exposure of lead to men decreases their fertility "Thomas et al [(3)]". "Rodamilans et al [(4)]" suggested that prolonged lead exposure initially produces a direct testicular toxicity followed by hypothalamic or pituitary disturbances. "Saxena et al [(5)]" concluded that lead exposure during growing period when spermatogenesis is proceeding might result in reproductive impairment during adulthood. Lead causes oxidative stress by inducing the generation

of reactive oxygen species (ROS) and weakening the antioxidant defense system of cells "Flora [6]". Research is in progress to identify the mechanisms that are involved in the etiology of reproductive diseases caused by ROS, and to create effective strategies that can counteract oxidative stress "Agarwal and Allamaneni [7]". Several antioxidant molecules such as reduced glutathione (GSH), glutathione disulphide (GSSG) and antioxidant enzymes such as super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) are the most common parameters used to evaluate lead induced oxidative damage "Ding et al [8]".

Antioxidants are the substances that are capable of counteract the damage of oxidation that occurs in the physiological processes of animal tissue. They have protective mechanism in the form of nutrients, vitamins and several enzymes "Mudipalli A et al [9]". Antioxidants have beneficial effect on lead induced toxicity in various tissues including kidney, brain, liver, sperm and blood cells "Patra et al [10]".

Ascorbic acid is found in various vegetables, fruits and milk and plays protective role against metal toxicity. It acts mainly as an antioxidant molecule and its beneficial effects could be attributed to its ability to complex with lead "Flora and Tandon[11]". Early reports found that vitamin C might act as a possible chelator of lead, with similar potency to that of EDTA "Goyer and Cherion [12]". Vitamin C could protect somatic cells of reproductive system against toxins that act through oxygen radical mechanism "Anderson et al [13]". Vitamin C, acting as a potent antioxidant and free radical scavenger, further helps reconstitution of the depleted GSH as it ameliorates the oxidative stress and enhance, as an important enzyme co-factor, the activities of GSH detoxification enzyme system hence promotes normal sperm cell production "Grajeda-Cota et al [14]".

MATERIAL AND METHODS

In the present study thirty male albino rats weighing 150-250 gm were taken. Animals were obtained from animal house of Indian Institute of Toxicology & Research, Lucknow. The rats were maintained under standard laboratory conditions in an air conditioned room and housed in polyethylene cages at temperature 22±3°C and relative humidity 30–70%. They were fed with standard pellet diet and water ad libitum. Animal care was as per Indian National Science Academy (INSA) guidelines for Care and Use of Animals in Scientific Research. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC). After acclimatization for 2 weeks in laboratory conditions, animals were divided into 5 groups of 6 rats each. Group 1 was control, group 2 received low dose lead nitrate (40 mg/kg bodyweight), group 3 received low dose lead nitrate (40 mg/kg body weight) and vitamin C (200mg/kg body weight), group 4 received high dose of lead nitrate (80 mg/kg body weight), group 5 received high dose of lead nitrate (80 mg/kg body weight) and vitamin C (200mg/kg body weight) 6 days a week for 2 months. After 2 months, animals of all the five groups were anaesthetized by intraperitoneal administration of Nembutol (30 mg/kg body weight). Rats were sacrificed and testes were taken out along with epididymis. Epididymis was separated from the testis and testis was washed with normal saline and weighed and was kept at -80ºC for biochemical analysis. Sperms were obtained by mincing the epididymis in normal saline and filtering the suspension through nylon mesh. 2-3 drops of this suspension was spread over the slide to fix the material. Slides were fixed with ethanol and screened for the sperm morphological abnormalities under a high power microscope. In each slide, hundred fields were studied for counting number of total sperms and abnormal sperms. Sperms presenting the defect in shape and structure of either head or tail or both were considered as abnormal and percent incidence of the total abnormalities was calculated. For enzyme

estimation, ten percent (w/v) homogenate of testis was prepared with the aid of York's homogenizer fitted with Teflon plunger in KCl (0.15 M) or 0.1 M phosphate buffer (pH 7.1), as per requirement.

The whole homogenate was first centrifuged at 2500 × g for 10 minutes in a refrigerated centrifuge. The pellet consisting of nuclear fraction and cell debris was discarded. The supernatant was further centrifuged at 11,000 × g for 15 minutes and mitochondrial fraction was separated. The clear supernatant was further centrifuged at 105,000 × g for 90 minutes and the resultant supernatant was used for enzyme activities. Statistical analysis was done using Analysis of variance (ANOVA) & Post-Hoc test (Tukey's HSD test).

RESULTS

Sperm morphology

It was observed that mean percentage of abnormal sperm count in control (group 1) was 5.00 ± 2.10 % while 29.00 ±3.16 % in group 2, 20.00 ±3.90 in group 3, 66.0±4.43 in group 4, 17.00±3.52 in group 5 (Table 1, Fig.1). The percent abnormality was found to be increased in treated groups as compared to control group but showed some improvement in recovery group and statistically, there were significant differences ($p<0.001$) in mean % sperm count with abnormal morphology in different groups (Table 2). Different types of abnormalities observed in the present study were double head, banana head, amorphous head, defective head, headless, bent neck, double tail, looped tail, bent tail and defective tail (Fig.2).

Table 1: Mean % of abnormal sperm morphology in different groups

S.No.	Group	Mean	SD	Minimum	Maximum
1.	Group-1 C	5.00	2.10	3	8
2.	Group-2 L1	29.00	3.16	24	33
3.	Group-3 L2	20.00	3.90	15	25
4.	Group-4 H1	66.00	4.43	60	72
5.	Group-5 H2	17.00	3.52	14	22

SD= standard deviation

[C- control group, L1-low dose lead nitrate treated group, L2-low dose lead nitrate and vitamin C treated group, H1-high dose lead nitrate treated group, H2- high dose lead nitrate and vitamin C treated group]

Table 2: Group Comparison of Mean % of abnormal sperm count (Tukey's HSD)

SN	Comparison	Mean Difference	SE	"p"
1.	Group I vs Group II	-24.00	1.94	<0.001
2.	Group I vs Group III	-15.00	1.94	<0.001
3.	Group I vs Group IV	-61.00	1.94	<0.001
4.	Group I vs Group V	-12.00	1.94	< 0.001
5.	Group II vs Group III	9.00	1.94	0.001
6.	Group IV vs Group V	49.00	1.94	<0.001
7.	Group II vs Group IV	-37.00	1.94	< 0.001
8.	Group III vs Group V	3.00	1.94	0.829

SE= standard error

Biochemical enzymes:

Lipid peroxide

It was observed that mean percentage of lipid peroxide level in control (group 1) was 5.14 ± 0.42 % while 7.85 ±0.72 % in group 2, 6.03 ±0.63 in group 3, 13.92±2.04 in group 4, 10.48±0.54 in group 5 (Table 3, Fig.3). Analysis of variance showed a significant intergroup differences in mean LPO levels ($p<0.001$).

Intergroup differences between group 1and 2, group 1and 4, group 1and 5, group 4 and 5, group 2 and 4 were statistically significant, whereas inter group differences between group 1 and group 3, group 2 and group 3, group 3 and group 5 were not statistically significant (Table 4).

Table 3: Lipid Peroxide (LPO) level in different groups (n mole MDA/g tissue)

S.No.	Group	Mean	SD	Minimum	Maximum
1.	Group-1 C	5.14	0.42	4.57	5.64
2.	Group-2 L1	7.85	0.72	6.91	8.97
3.	Group-3 L2	6.03	0.63	5.33	6.96
4.	Group-4 H1	13.92	2.04	11.11	17.29
5.	Group-5 H2	10.48	0.54	9.89	11.25

SD= standard deviation

[C- control group, L1-low dose lead nitrate treated group, L2-low dose lead nitrate and vitamin C treated group, H1-high dose lead nitrate treated group, H2- high dose lead nitrate and vitamin C treated group]

Table 4: Intergroup Comparison of Lipid Peroxide (LPO) levels

S.No.	Comparison	Mean Difference	SE	"p"
1.	Group I vs Group II	-2.72	0.60	0.001
2.	Group I vs Group III	-0.46	0.60	0.997
3.	Group I vs Group IV	-8.78	0.60	<0.001
4.	Group I vs Group V	-3.03	0.60	< 0.001
5.	Group II vs Group III	-2.26	0.60	0.013
6.	Group IV vs Group V	5.75	0.60	<0.001
7.	Group II vs Group IV	-6.06	0.60	< 0.001
8.	Group III vs Group V	-2.57	0.60	0.003

SE= standard error

Glutathione reductase

It was observed that mean percentage of glutathione reductase level in control (group 1) was 75.41 ± 4.64 % while 56.51 ±4.87 % in group 2, 63.42 ±5.06 in group 3, 33.44±3.60 in group 4, 44.93±4.40 in group 5 (Table 5, Fig.4). Analysis of variance showed a significant intergroup difference in mean GR levels ($p<0.001$).

Intergroup differences between group 1and group 2, group 1and group 4, group 1and group 5, group 2 and group 4, group 3 and group 5 were statistically significant, where as inter group differences between group 1 and group 3, group 2 and group 3, group 4 and group 5 were not statistically significant (Table 6).

Table 5: Glutathione reductase (GR) level in different groups (NADPD) oxidized/min/mg protein

S.No.	Group	Mean	SD	Minimum	Maximum
1.	Group-1 C	75.41	4.64	70.03	81.65
2.	Group-2 L1	56.51	4.87	50.89	62.38
3.	Group-3 L2	63.42	5.06	55.81	69.31
4.	Group-4 H1	33.44	3.60	28.17	38.21
5.	Group-5 H2	44.93	4.40	39.48	49.83

SD= standard deviation

[C- control group, L1-low dose lead nitrate treated group, L2-low dose lead nitrate and vitamin C treated group, H1-high dose lead nitrate treated group, H2- high dose lead nitrate and vitamin C treated group]

Table 6: Intergroup Comparison of Glutathione Reductase levels

SN	Comparison	Mean Difference	SE	"p"
1.	Group I vs Group II	18.90	2.90	<0.001
2.	Group I vs Group III	11.99	2.90	0.004
3.	Group I vs Group IV	41.97	2.90	<0.001
4.	Group I vs Group V	30.49	2.90	< 0.001
5.	Group II vs Group III	-6.91	2.90	0.316
6.	Group IV vs Group V	11.49	2.90	0.007
7.	Group II vs Group IV	23.08	2.90	< 0.001
8.	Group III vs Group V	18.50	2.90	< 0.001

SE= standard error

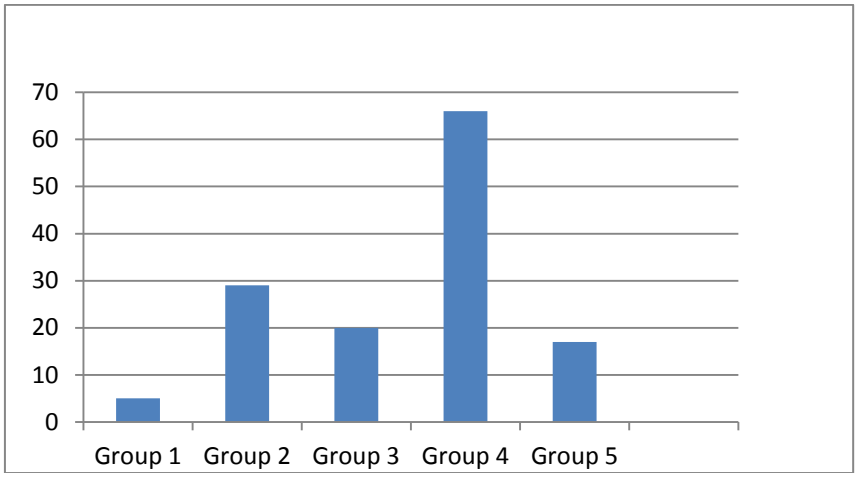

Fig. 1: Bar diagram showing abnormal sperm count in control and experimental groups

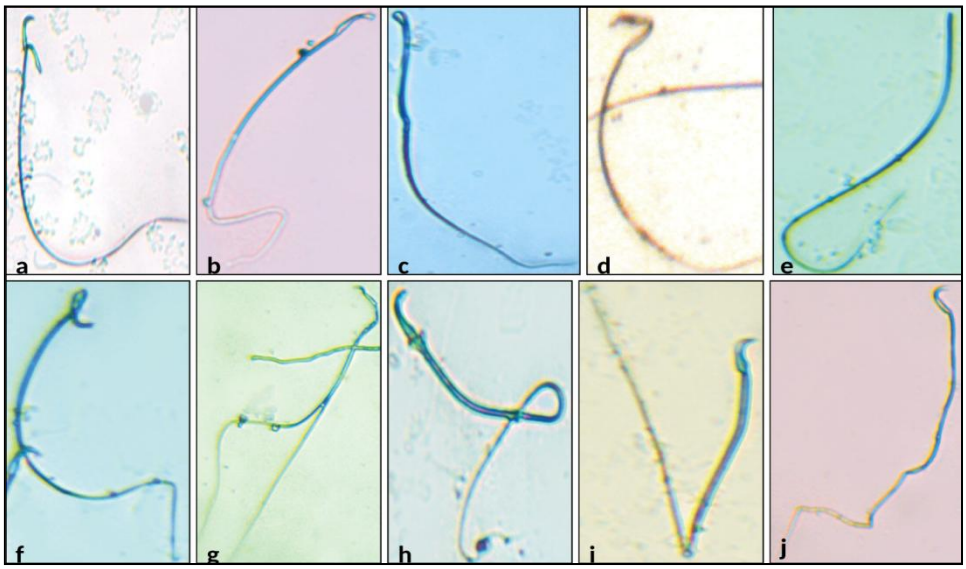

Fig.2: Photomicrograph showing abnormal sperms
a. double head, b. banana head, c. amorphous head, d. defective head, e. headless, f. bent neck, g. double tail, h. looped tail, i. bent tail j. defective tail

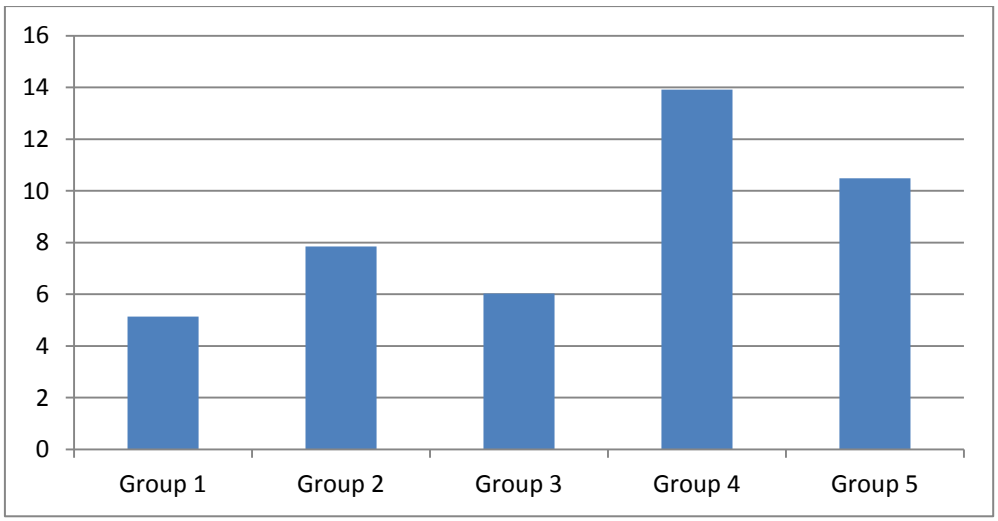

Fig. 3: Bar diagram showing lipid peroxide level in control and experimental groups

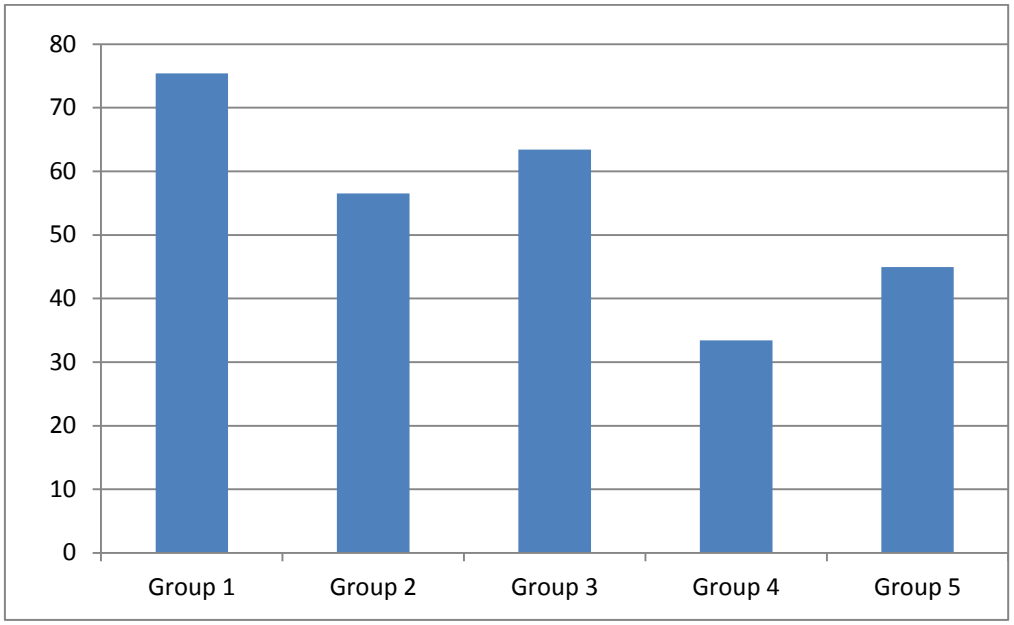

Fig. 4: Bar diagram showing glutathione reductase level in control and experimental Groups

DISCUSSION

A highly significant rise in the abnormal sperm count was observed after treatment of lead nitrate in the present study. It was 5.00±2.10 in control group which increased to 66.00±4.43 after high dose treatment which was highly significant ($p<0.001$). The findings are in accordance with "Ahmed et al [15]" who also studied the effect of lead acetate on the testis of rabbit. Different type of abnormal sperm morphology was seen as double head, banana head, amorphous head, headless sperm, defective head, bent neck, double tail, looped tail, bent tail and defective tail. Similarly, "Ahmed et al [15]" reported that mean percentage of sperm shape abnormalities for animals treated with lead acetate significantly ($p<0.01$) increased than control animals in which the value was 1.46±0.49 and the effect was dose dependent. The mean percentage of sperm abnormalities reached 17.96±0.48 after treatment with highest dose of lead acetate and the different type of abnormalities were amorphous, triangle, small, big and coiled tail sperms.

The possible cause behind this may be due to indirect damage to DNA affecting the stabilization of chromatin or interacting with repair processes or lead induced reactive oxygen species (ROS) involved in damaging and gene alteration in the germ cells that lead to varieties of abnormal sperm."Antnio et al [16]"also noticed detached sperm head after lead chloride treatment for three days but in the recovery group i.e. group treated with lead chloride for three days followed by 32 day recovery period, no significant improvement was observed. "Gautam et al [17]", "Lohiya et al [18]", "Sarkar et al [19]" observed sperm head abnormality after lead exposure. The present study is very similar to "Naha et al [20]" who worked on humans working in lead acid battery factory. He reported significant gross sperm abnormality in high dose group which was ameliorated by giving Vitamin C. The abnormalities seen were amorphous head, small head, double head, coiled tail and broken tail. "Wadi et al [21]" found that high dose of lead causes increased percentage of abnormal sperm within the epididymis. "Acharya et al [22]" found increased incidence of abnormal sperm population which was in agreement with the present study. "Tohamy et al [23]" found increased percentage (34.31± 1.42) of primary sperm abnormality after high dose of lead treatment than control (15.33± 0.55) which was decreased after giving Vitamin C along with lead (19.35± 0.78) which shows that Vitamin C has ameliorating effect. Vitamin C supplementation reduced reactive oxygen species (ROS) generation and improved semen quality. The beneficial effect of Vitamin C in improving fertilization rate was

possibly due to reduction in lipid peroxidation potential. Similarly, in our experiment percentage of abnormal sperm count increased after low and high dose lead treatment than control. The value of abnormal sperm count in control rats was 5.00±2.10 which increased to 29.00± 3.16 in low dose group and 66.00± 4.43 in high dose group. The values decreased to 17.00± 3.52 after Vitamin C administration in high dose group.

Lead is well known to produce oxidative damage by enhancing lipid peroxidation. Lipid peroxidation inactivates cell constituents by oxidation and causes oxidative stress by undergoing radical chain reaction, ultimately leading to loss of membrane integrity. Higher membrane lipid content of testis is presumed to make them more vulnerable to oxidative stress.

In the current study, treatment with lead resulted in a significant increase in lipid peroxidation as indicated by the significant increase in malondialdehyde (MDA) levels, which is the marker of lipid peroxidation. We found significant increase in malondialdehyde in group II (low dose lead treated) and in group IV (high dose lead treated) as compared to controls. "Shabani and Rabbani [24]", "Acharya et al [22]", "Abdel-Wahhab and Aly [25]", "Abdel-Wahhab et al [26]", "Abdel-Wahhab et al [27]", "Moniem et al [28]", also reported significant increase in lipid peroxide levels. "Tohamy et al [23]" studied significant increase in malondialdehyde after low and high dose of lead acetate treatment in the semen of male rabbits. He also noticed decrease in MDA level when he administered Vitamin C along with low and high dose of lead acetate but this decrease in the level of MDA was significant in high dose group. We also observed that this increase in MDA level was partially reversible in group III and in group V in which we gave antioxidant vitamin i.e. Vitamin C along with low and high dose of lead nitrate and this improvement was significant in group V similar to the study of "Tohamy et al [23]". In another study, "Turguta et al [29]" reported that administration of the antioxidant agent Vitamin E together with metal resulted in the recovery of malondialdehyde. "Beytut et al [30]" and "Ognjanovic et al [31]" demonstrated reduction in increased lipid peroxidation due to heavy metal toxicity by Vitamin E."Moneim et al [28]" found that synchronous treatment of flaxseed oil with lead acetate caused increase in the level of malondialdehyde, which is in contrast with above findings.

We also assessed antioxidant enzyme, glutathione reductase (GR) at the end of two months in testicular tissue of animals of different groups and found that the level of this enzyme was decreased in lead nitrate treated group in comparison to control group because of persistent oxidative stress. Moreover our results are in concurrence with the earlier report of "Asayana et al [32]". In the recovery group i.e. group III and group V we found significant improvement in the activity of glutathione reductase after Vitamin C treatment.

"Al-Attar [33]" reported decrease in the activity of superoxide dismutase after heavy metal treatment and improvement with Vitamin E treatment given along with heavy metal. "Murray [34]" observed decrease in the activity of catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. He proposed that a powerful antioxidant can reverse the oxidative damage.

CONCLUSION

From the present study it has been concluded that lead nitrate toxicity causes changes in sperm morphology and in biochemical enzymes level, which were more marked with high dose of lead nitrate but the changes were recovered after giving vitamin C. So it is proved that vitamin C has protective effect on infertility in lead nitrate exposed individual.

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