Protective Effects of flaxseed & zinc against lead induced reproductive toxicity in male mice

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Abstract:

The toxic effect of drugs and environmental chemicals on the human reproductive system has become a major health concern. Lead is recognized as the most dangerous of the toxic metals and a major threat to the biological systems of both human and animals. In this study we detect the effect of two types of antioxidants: flaxseed and zinc, on the toxicity of low dose of lead acetate on reproductive organs of male mice, this study was conduct.Two groups of mature male mice,exposed to low dose of lead, were administered either zinc sulphate (0. 5mg/kg body weight/day) or flaxseed oil (2.5% of total daily food intake), for 6 weeks. The results showed that lead caused a significant reduction in the total body weight, testicular weights & adverse effects on sperm parameters, while the concomitant administration of antioxidants caused elevation in these weights with significant improvement in sperm quality as compared to the only lead treated group. It is concluded that lead, like all other heavy metals, could induce oxidative stress and the concomitant administration of antioxidants (zinc or flaxseed) can ameliorate the toxic effect of lead on reproduction.

Key words: Sperm parameters, Male reproduction, Lead acetate, Antioxidants, Zinc, Flaxseed, Mice

Introduction

Lead (Pb) is recognized as the most dangerous of the toxic metals for both human and animals; it apparently exerts an oxidative stress on the cell, thereby causing its accelerated destruction, by the disruption of normal cell membrane architecture, leading to oxidative damage 1. Nutritional status has profound effect on the response of organism to a given dose of a poisonous substance; this is especially true in the case of Pb poisoning the severity of which is influenced by a wide variety of nutrients2. Antioxidants (AO) are the main defense mechanism against oxidative stress induced by free radicals, which include enzymes3, and non enzymatic substances such as vit. C, zinc, vit. E, taurine, hypotaurine, and micronutrients 4.Zinc is an integral component of nearly 300 enzymes in different species. It plays an important role in the physiology of spermatozoa and may have a role in sperm production, prevention of spermatozoa degradation and in sperm membrane stabilization5. Flaxseed oil contains substances that promote good health 6. A balance between the risks and benefits of reactive oxygen species (ROS) and AO, both in vivo and in vitro, appears to be necessary for the survival and normal reproductive functioning 7. So in order to detect the effect of different AO (zinc or flaxseed) on the toxicity of low dose of Pb on the reproductive organs of mature male mice, as a model for human representative, this study was conducted.

Materials and Methods:

Laboratory animals: Seventy five mature male Swiss-Webster mice; their ages ranged between 6-8 weeks with BW of 28-30 gm, were used. They were obtained from the colony of the animal house of the High Institute of Assisted Reproductive Technology & Infertility Diagnosis, Al-Nahrain University. Animals were kept in a controlled temperature and light room, housed in plastic cages (3mice/cage), food and water were presented to them ad libitum 8. All animals were divided into 4 groups, (15 mice / group) after weighing them, as follows:

1. Control group (G1): was orally administrated with distilled water and regarded as negative control.

2. Experimental groups include: (G2) was orally administrated with lead acetate (5 mg/kg.BW/day). The other 2 experimental groups were given, in addition to the previous dose of Pb, either zinc sulphate (0.5mg/kg BW./day) for G3, or flaxseed oil (2.5% of total daily food intake) ,for G4, orally administrated during the 6 weeks period of the experiment. Pb and Zn stock solutions were made separately by dissolving Pb (300mg/l distilled water) and zinc sulphate (30mg/l distilled water), 9, 10, 11. On completion of treatments, BWs were recorded, then mice were sacrificed by cervical dislocation,

reproductive organs (testis & epididymis) were quickly excised and immersed in few drops of normal saline to be cleared from surrounding adipose tissue under dissecting microscope, then weighed with electrical balance, while both right and left testes were fixed with 10% formal saline for a subsequent histological study, the right and left epididymis were put in a small Petri dish contained 1ml of global culture media, minced by microsurgical scissor about 200 times until a homogeneous solution containing the spermatozoa were obtained.

Assessment of mouse spermatozoa: one drop (about 10 microliters) of spermatozoal suspension was mounted on a warm glass slide and covered with cover slip (22×22 mm), then left for one minute in an incubator to stand before microscopical examination. Assessment of sperm parameters in about 10 of randomly selected microscopical fields under a high power magnification of 400X was done 12. Parameters of mouse spermatozoa include:

a- Sperm concentration: was determined by recording the mean numbers of spermatozoa in 10 random microscopical fields multiplied by a factor of one million 12.

b- Sperm motility: at least one hundred spermatozoa were calculated 13. The prepared slide was examined for determination of sperm motility percentage, which was assessed by categorization into four groups of motility according to the WHO category corresponding velocity 14:

1-Linear and rapid progressive (A)>25micrometer/sec.
2-Linear non rapid or rapid non linear progressive (B) 5-24micrometer/sec.
3-Non progressive (C) < 5micrometer/sec.
4-Immotile (D).

Grade motility% = (No. of sperms in specific motility/ Total No. of sperms) ×100

c- Sperm morphology: at least 100 spermatozoa were counted and percent of normal sperm morphology was reported according to following formula:

Normal sperm morphology%= (No. of normal sperm/ Total number of sperm)x 100

Preparation of histological sections: The fixed testes were washed with running tap water for 30 minutes, then dehydrated with graded ethyl alcohols (70%, 80%, 90%,and absolute alcohol), cleared with xylene followed by infiltration and embedding in paraffin to prepare paraffin blocks. Serial sections with thickness of 5 microns were stained with hematoxylene-eosin stain 15. Slides were examined with a light microscope to measure diameters of seminiferous tubules (ST), using an ocular micrometer 16. Fifty STs were examined for each animal and a total number of 750 tubules for each group were measured. Also, the presence or absence of any abnormal cells or any vacuoles, the peri tubular tissues, Leydig cells, blood vessels, edema, all are observed in comparison to control.

Statistical analysis _ Data were expressed as mean ± standard error (SEM) and analyzed using Student's t-test to compare values from experimental and control groups at individual time points. ANOVA test was applied as well, to compare between mean groups of different antioxidants doses, used in the experimental groups. Differences between values were considered significant at P? 0.05 and highly significant at P?0.001 17.

Results

1-Results of administration of Pb and AOs (zinc or flaxseed) on body and reproductive organs weights

a-Body weight: after 6 weeks of treatment, results showed that there was a highly significant (P? 0.001) reduction in the BW of treated mice in G2 (Pb only), and G4 (Pb +flaxseed) in comparison with that of G1 (control), while the reduction was only significant (P?0.05) in G3 (Pb +zinc), as compared to G1.On comparing weights of animals treated with AOs plus Pb (G3 and G4) with the group administrated with Pb only (G2), a highly significant increase (P?0.001) was noticed. In addition, animals of (G3) showed a significant (P?0.05) increase in their BWs, as compared to that of G4 (table-1).

b - Reproductive organs weights: Testicular weights decreased with a highly significant (P?0.001) difference in Pb treated animals (G2) in comparison with control group (G1) for both the right and left side while the reduction was significant (P?0.05) in G4 in comparison to G1. There was no significant difference in G3 as compared to G1 .Testicular weight increased with a highly significant (P?0.001) difference in animals receiving zinc with Pb(G3) as compared to Pb only group (G2), while the increase was only significant (P?0.05) in G4 as compared to G2. There was significant reduction in testicular weight in G4 as compared to G3. The above results were similar for both the right and left sides (Table-1). Epididymal weight decreased with a highly significant (P?0.001) difference in all the treated groups as compared to control; while animals treated with AOs (G3 and G4) showed a highly significant (P?0.001) increase as compared to those treated with Pb only (G2). A significant (P?0.05) increase in epididymal weight was recorded in G3 in comparison to G4. However, the above results for both right and left epididymis were similar (Table-1).

2. Results of administration of Pb and AOs on sperm parameters

a- Sperm concentration: The effects of administration of Pb and Pb with different AOs showed a highly significant (P?0.001) decrease in sperm concentration in G2 and G4, and a significant decrease (P?0.05) in G3 as compared to G1. In G3and G4, results showed

Highly significant (P?0.001) increase in sperm concentration in comparison to the group treated with Pb only (G2), and no significant difference was recorded between any Pb plus AO treated groups (G3, G4) as compared with each others (Table -2).

B- Sperm motility: The results of progressive sperm motility percentage revealed a highly significant (P?0.001) decrease in treated groups (G2, G3, G4) as compared to control (G1). Moreover,

administration of (zinc or flaxseed) with Pb in G3and G4 showed a highly significant (P?0.001) increase in percentage of progressively motile sperms as compared to G2. No significant difference was noticed between G3and G4. Percentage of non progressive sperm motility showed no significant difference in all treated groups (G2, G3 and G4), in comparison to control (G1). Also a significant (P?0.05) increase in non progressive sperm motility was noticed in G3 and G4 as compared to G2. There is no significant difference in non progressive sperm motility percentage in G3 and G4 when compared with each other. A highly significant (p?0.001) increase in percentage of non motile sperm was noticed in all treated groups as compared to control (G1). But as G3 and G4 compared to G2, a highly significant (p?0.001) reduction in non motile sperm percentage was noticed. No significant difference was recorded among the groups (G3and G4) as compared with each other (Table 2).

C- Sperm morphology: Results showed a highly significant (P?0.001) reduction in normal sperm morphology percentage in all treated groups (G2, G3 and G4) as compared to control (G1), while a highly significant (P?0.001) increase in normal sperm morphology percent was noticed for animals treated with zinc plus Pb (G3) as compared to those treated with Pb only (G2), while the increase was significant (P?0.05) in animals treated with flaxseed plus lead (G4) in comparison to G2. Moreover, a significant (P?0.05) increase in normal morphology percent was noted in G3 as compared to G4, (Table -2).

3- Histological study for the effect of Pb and AOs (zinc or flaxseed) on testis

A- Diameters of ST: The diameters of ST showed a highly significant (P?0.001) decrease in Pb only treated group (G2) in comparison to control (G1), while the reduction was not significant in G3 and G4, in comparison to control (G1). Animals treated with AO, (G3 and G4) , showed a highly significant (P?0.001) increase in their tubular diameters as compared to (G2). No significant differences were recorded between G3and G4 , (Figure-1).

B- Histological observations: Testicular sections of the negative control group showed ST with normal germ cell population layer thickness and a normal orderly arranged pattern up to mature spermatid. No, malignant or abnormal cell was seen within the germinal epithelium; also no vacuoles were present in the tubules. There were adequate Sertoli cells populations. The interstitial spaces contained normal Leydig interstitial cells, blood vessels and no edema was present. The peritubular surrounding tissues were normal (Figure-2a and figure -3).In Pb treated group (G2), sections showed a decrease in thickness of germ cell layer, widening of the central ST lumen and prominent germ cell population necrosis. Multiple rounded vacuoles were seen within the tubules. Sertoli cells were normal in shape as compared to control. Widening of the interstitial spaces was detected and edema within it was markedly present, Leydig cells were not affected. (Figure-2 b & figure -4). In (G3), tubular sections showed recovery of germ cell population, normal germinal epithelium thickness with order arrangement of the germ cells up to spermatid, adequate Sertoli and Leydig interstitial cells populations. Mild edema was seen at the periphery of the testicular section in this group (Figure-2 c & figure 5).The testis of the animals in (G4),showed STs with normally arranged germ cells, no vacuoles was seen within the germinal epithelium, adequate Sertoli and interstitial cells population, less edema was seen at the periphery (Figure-2 d & figure -6).

Table -1: Effects of lead acetate and antioxidants on body and reproductive organs weights.

Weights(gms)	G 1/control	G 2/Pb	G 3/Pb+Zn	G 4/Pb+flaxseed
Weight of animal before treatment	28.355 ±0.480	28.385 ±0.356	28.140 ±0.419	27.808 ±0.458
Weight of animal after treatment	34.509 ±0.638	28.049 ±0.503^{a**}	32.847 ±0.470^{b*,d**}	31.072 ±0.380^{c**,e**,f*}
Weight of right testis	0.095 ±0.010	0.064 ±0.001^{a**}	0.089 ±0.002^{d**}	0.079 ±0.002^{c*,e*,f*}
Weight of left testis	0.094 ±0.002	0.063 ±0.001^{a**}	0.088 ±0.009^{d**}	0.078 ±0.002^{c*,e*,f*}
Weight of right epididymis	0.042 ±0.001	0.023 ±0.002^{a**}	0.037 ±0.001^{b**,d**}	0.031 ±0.001^{c**,e**,f*}
Weight of left epididymis	0.042 ±0.001	0.023 ±0.002^{a**}	0.036 ±0.001^{b**,d**}	0.032 ±0.001^{c**,e**,f*}

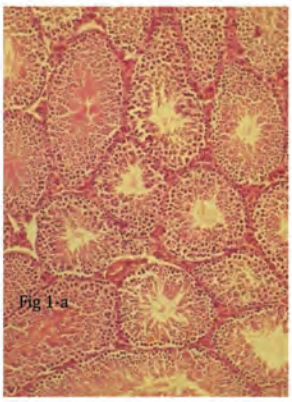
Values are mean ± (S.E.M), Number of animals per group=15.
** Highly significant difference (P<0.001). *Significant difference (P<0.05).

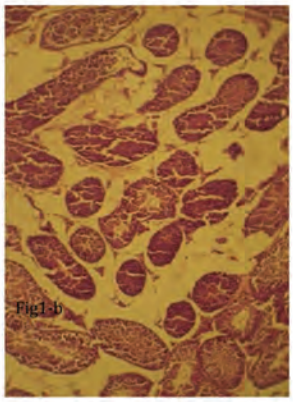
Table -2: Effects of lead acetate and antioxidants on mice sperm Parameters.

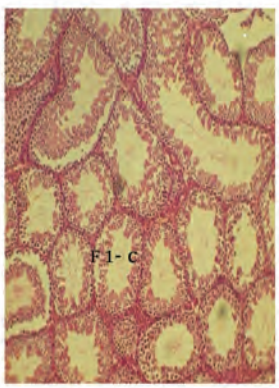
	G I/control	G 2/Pb	G3/Pb+Zn	G 4/Pb+flaxseed
Sperm concentration Million/ml	50.000 ±1.944	27.900 ±1.386^{a**}	42.200 ±2.159^{b*,d**}	38.200 ±1.413^{c**,e**}
Progressive sperm motility % A+B	56.500 ±2.349	21.800 ±1.162^{a**}	38.900 ±1.464^{b**,d**}	42.800 ±2.657^{c**,e**}
Non-progressive sperm motility%	30.800 ±2.032	28.200 ±1.604	33.600 ±1.310^{d*}	34.200 ±1.590^{e*}
Non-motile Sperm% D	12.700 ±0.730	50.000 ±1.667^{a**}	26.700 ±1.432^{b**,d**}	23.000 ±2.134^{c**e**}
Normal sperm morphology%	70.600 ±2.099	39.100 ±1.602^{a**}	57.000 ±1.994^{b**,d**}	47.500 ±1.593^{c**,e*}

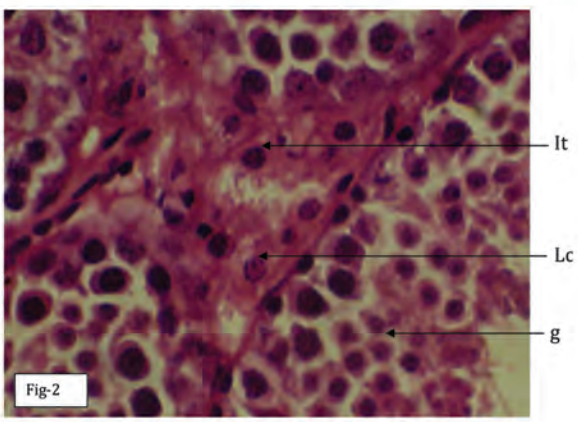
Values are mean ± (S.E.M.), Number of male mice per group=15.
** Highly significant difference (p<0.001).
* Significant difference (p<0.05).

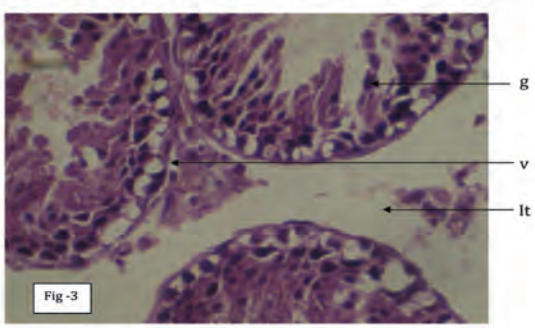
The significant reduction in animal's BWs in Pb treated group (G2) compared to that in control group (G1), may be attributed to decrease in feeding habit which indicates that Pb has a general inhibitory action on metabolism 18. Pb most likely interferes with the functions performed by essential minerals such as calcium, iron, copper, and zinc. Moreover Pb does interrupt several red blood cell enzymes systems, including delta-aminolevulinic dehydratase and ferrochelatase which are considered as the key enzymes in heme synthesis 19, resulting in

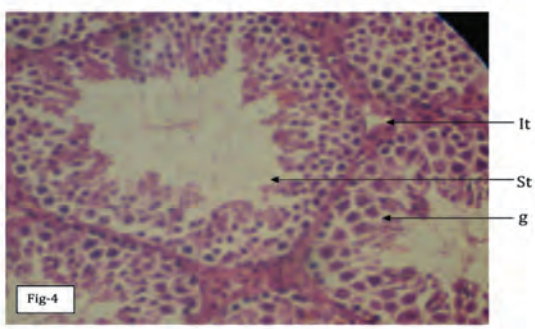


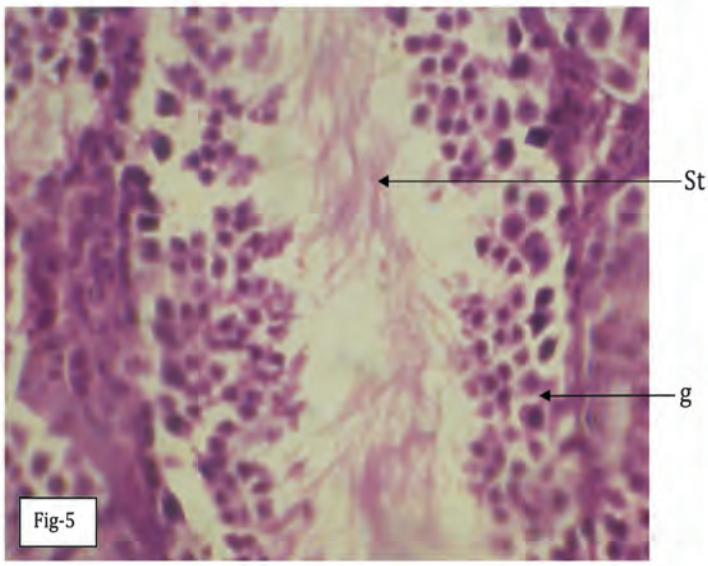












Legends to the figures:

Figure -1: Effects of administration of lead acetate and lead acetate with different types of antioxidants on the diameter of seminiferous tubules.
Values are mean ± S.E.M. Number of male mice per group =15.
G1: control group. G 2: administrated with lead only.
G 3: administrated with lead + zinc.
G 4: administrated with lead +flaxseed

Figure -2: Effects of administration of lead acetate and lead acetate with different types of antioxidants on Testicular histology. (H&E,20X)
a. Negative Control b. Positive Control (Pb)
c. Pb +Zinc d. Pb +flaxseed

Figure -3: Testicular section of male mice (negative control group). Note the normal arranged germ cells layer(g) with extensive interstitial tissues (It) contained normal Leydig cells (Lc) population (H&E, 40x)

Figure -4: Testicular section of male mice treated with Pb only 5mg/kg BW. (positive control group). Note the germ cells necrosis (g), the edema in the interstitial tissues (It) and vaculation (v) (H&E, 40x)

Figure -5: Testicular section of male mice treated with Pb plus zinc. Note the well organized seminiferous tubules (st) with normal arranged germ cells layer(g) and normal interstitial tissues (It), (H&E, 40x).

Figure -6: Testicular section of male mice treated with Pb plus flaxseed. Note the well organized seminiferous tubules (St) with normal arranged germ cells (g) up to the spermatid, (H&E, 40x).

diminished hemoglobin synthesis in haemopoietic tissues and anemia, thereby, reduce oxygen carrying capacity to the cells. The highly significant reduction in testicular and epididymal weights recorded in G2, may also be related to decrease feeding, as even mild food restriction resulted in serum testosterone concentration reduction in the weight of androgen dependant organs, and in serum LH concentration 20.These results coincide with the findings of Mariola *et al.*21, that Pb exposure and toxicity cause testicular and epididymal damage and degeneration of epithelial and interstitial tissues.

The highly significant ($P<0.001$) decrease in sperm concentration in group 2 may be attributed to testicular germ cell destruction caused either by membrane damage or macromolecular degradation incurred by ROS, leading to a significant decline in spermatogenesis and ultimately testicular weight loss22. This is because Pb, like all other heavy metals is well known to induce oxidative stress in animals indicated by the sharp rise in lipid peroxidation (LPP) 23. The decrease in sperm count in G2 supports the finding of Faisal, *et al.* 24, that the testicular sperm count is an important indicator of the adverse effect of Pb on spermatogenesis. Also the highly significant decrease in normal sperm morphology recorded in G2 compared to G1, confirms the findings of 25, 26. This could be accounted for, by the possibility of gene alternation in germ cells induced by ROS generated through Pb catalysis which can apparently cause DNA breaks 27. On the other hand, testicular germ cells carrying minor gene mutation are not eliminated but are manifested as morphologically deformed sperms 28.

The highly significant reduction in progressive and non progressive sperm motility recorded in G2as compared to G1, may have been brought about by the known displacement activity of lead with calcium, which is essential for sperm functions including motility 29, 30. Also, Pb may accumulates in the mitochondria leading to both structural and functional alteration 31. So, Pb may have a detrimental effect on mitochondria in the tail of the spermatozoa, leading to reduction in its motility. These results support the findings of 25, and 26 that administration of lead acetate resulted in decline in sperm motility.

The histopathological changes seen in the testes of Pb intoxicated animals, including germ cell destruction, might have been resulted from membrane damage or macromolecular degradation, as mentioned previously, leading either to arrest of germ cells multiplication and/ or differentiation 32. The presence of edema in the STs of G2 might be due to the destruction of lining epithelium of blood vessels which lead to prevalence of plasma to the interstitial tissues of the testes. Similar results were recorded by33. Reduction in the diameter of STs recorded in this study could be due to accumulation of Pb in the testicular tissues 34, causing testicular damage with degenerative changes 35. It may also cause dysfunction of the Sertoli cells, which are responsible for the germ cells proliferation and maturation 36. In addition, Pb may cause dysfunction of Leydig cells, which may affect indirectly the spermatogenesis in these tubules. So, the germ layer showed a reduction in their thickness and consequently a reduction in the diameters of STs 37.

In G 3, the concomitant administration of zinc was able to prevent most of the adverse effects of Pb on body growth and reproduction. Since, zinc plays an important role in body metabolism which is reflected by an increase in BW and reproductive organs weights. Also the highly significant improvement of sperm parameters, confirms the importance of zinc as a potent AO and free radicals scavenger which ameliorated the potential increase in free radicals generation from Pb toxicity 38. This finding is in agreement with others 39, who suggested that seminal plasma has a high content of zinc; which exerts protective antioxidant-like activity, sufficient to cope with the presence of excessive amount of reactive oxygen radicals. Zinc has been reported to have a membrane stabilizing AO activity and maintains sperm viability by inhibiting DNA ase 5. It is also an integral part of many metalloenzymes that appears to be a potent scavenger of excessive superoxide anion produced by defective spermatozoa 40, 41.

Testicular sections of G3 had normal histology in comparison with G1, zinc was able to protect most of lesions produced by Pb. These results are in agreement with other studies which have shown that zinc is important for normal testicular development, maintenance of the germinal epithelium and sperm morphology in mice 42.

Co-administration of flaxseed with Pb in G4, causes a highly significant increase in body, testicular and epididymal weights. This could be attributed to the constituent of flaxseed, which include amino acids, folic acid; vit.B6, vit. E as well as minerals, like magnesium, phosphorous, and copper 43. Folic acid helps in the formation of the heme part of hemoglobin, copper in the form of copper-\protein complex called ceruloplasmin and vit. B 6 helps in hemoglobin synthesis 44. Also results showed improvement of sperm parameters in (G4) in comparison to (G2). Flaxseed oil contains omega-3fatty acids, especially alpha-linolenic acids. These polyunsaturated fatty acids control the composition of membranes, hence their fluidity, and consequently, the enzyme activities 45. The importance of polyunsaturated fatty acids, in normal cell growth, was proved previously and correlation between the insufficient intake of these fatty acids and impaired sperm motility and abnormal morphology among sub-fertile men was found 46.

The histological testicular sections of G4 showed recovery from the pathological changes caused by Pb. Flaxseed oil protects against oxidative stress induced by free radicals. It may scavenge free radicals produced by mutagens and thus inhibit damage to cellular DNA 47.The protection offered by flaxseed oil might be due to the anti oxidative action of its important constituents, the lignans, like secoisolariciresinol and pinoresinol which were reported to have strong AO nature 48. In spite of

the observed cyto-protective effect of zinc and flaxseed, and the significant improvement of the sperm parameters in G3and G4 as compared to G2 (Pb only treated group), but they don't reach the controls values .Thus, although zinc and flaxseed did not totally prevent the adverse effect of Pb on sperm parameters, they significantly reduced its adverse effects. This may be due to the fact that spermatozoa leaving the testis are not fully developed and they continue their maturation and acquire motility during their passage through the epididymis 49. These results agree with the findings of Mariola et al. 21 who reported that the chemiluminescence emitted by the Pb treated tissues is significantly higher in the caput of epididymis than in the testes, so Pb accumulate in the epididymis more than that in the testes and it can affect the sperms during their transport through it.

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