

**Protection against lead-induced hepatic lesions
in Swiss albino mice by ascorbic acid**

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Summary

The objective of this study was to screen for the hepatoprotective role of ascorbic acid (vitamin C) against lead acetate intoxication in mice. For this purpose, animals were randomized into control and experiment groups. Mice of the experimental group were administered ascorbic acid (400 mg/kg of body weight/ animal) orally once in a day for 7 consecutive days. Animals were then treated with lead acetate (intraperitoneally) on 7th day, one hour after ascorbic acid administration. These were then autopsied at various post-treatment intervals (6 hrs to 20 days) to examine quantitative as well as qualitative alterations in the liver. Results from the present investigation reveal a certain degree of histopathological and biochemical alterations caused by lead due to impaired oxidant-antioxidant balance and enhanced oxidative stress. It was observed that ascorbic acid treatment prior to lead intoxication reduced the depletion in the normal hepatocytes count and the elevation in binucleate as well as abnormal hepatocytes in comparison to their respective control. Also, supplementation of ascorbic acid ameliorated the biochemical alterations i.e., glycogen, cholesterol and protein, in the liver of mice. A fall in glutathione level was noted in blood and liver in mice; whereas administration of Vitamin C significantly ($p < 0.05$) reduced the level of glutathione in the Vitamin C treated experimental group as compared to the control.

Key words: Antioxidants, Ascorbic acid, Glutathione, Lead acetate, Liver, Swiss albino mice.

Introduction

Lead is a ubiquitous element detected in all environmental media. Adult human beings and older children receive lead the most from food, air and water intake (1). The majority of lead in the environment arises from burning of fossil fuels in automobiles, industrial emissions, and from the use of lead containing solder paints. Most lead in the air is inorganic lead and the predominant source is from the combustion of tetraethyl and tetramethylated lead used as fuel additives (2,3). Lead occurs widely in the biosphere and is found to be a potent hepato-toxicant. It is present in tobacco; cigarettes contain 2.4µg of lead and 5 % of this occurs in ash and side stream smoke (4). Lead impairs learning, memory and audio-visual functions in rats (5) and man (6), hematopoiesis (7) and produces nephrotoxicity (8) and cardio-vascular damage (9). Toxicological properties of lead have been extensively studied (10), and its carcinogenic effect has been receiving increasing attention (11). It is known to cause oxidative stress in the body by inducing the generation of free radicals and reducing the antioxidant defense system of the cells (12).

Dietary ingredients may be useful if they are found to protect against the deleterious effects of heavy metals, as they will be widely acceptable, would not add an extra foreign substance into the body, and can be safely manipulated without toxic manifestations. Drugs of natural origin have found to be quite protective to mammalian tissues at optimum dose levels (13-16). Several micronutrients in the diet are known to modify free-radical reaction occurring within the cells (17). Vitamins are known as organic substances of nutritional nature, present in low concentration as a natural component of an enzyme system, catalyze required reactions and may be derived externally or by extrinsic biosynthesis. They act as co-factors in enzyme systems, antioxidants or antagonists (18).

The properties of ascorbic acid (vitamin C) are well documented and have generated a great deal of interest in recent years for a wide range of protective effects in biological systems (19). Ascorbic acid is a powerful antioxidant that can neutralize harmful free radicals, and it helps make collagen, a tissue needed for healthy bones, teeth, gums, and blood vessels (20). Excellent food sources of vitamin C are citrus fruits or citrus juices, berries, green and red peppers, tomatoes, broccoli, and spinach. Many breakfast cereals are also fortified with vitamin C. A study of the influence of ascorbic acid on the tissue deposition of lead in rats suggests its usefulness as a prophylactic agent for lead poisoning (21). Furthermore, ascorbic acid is believed to decrease lipid peroxidation, either directly or indirectly by regenerating Vitamin E, the major lipid soluble antioxidant (22). Based on these properties and significance, the present study was undertaken to investigate the modulatory effect of ascorbic acid on lead induced hepato-lesions in mice.

Materials and Methods

Animal care

Animal care and handling were performed according to the guidelines set by the World Health Organization (WHO), Geneva, Switzerland and the Indian National Science Academy (INSA), New Delhi, India. Swiss albino male mice (*Mus musculus*), 6-8 weeks

old and 25 -28 gm body weight, were selected from an inbred colony. These were maintained under controlled conditions of temperature ($25 \pm 2^\circ\text{C}$) and light (light: dark, 10h: 14 h). Four to six animals were housed in a polypropylene cage containing saw dust (procured locally) as bedding material. They were maintained on standard mice feed (procured from Hindustan Lever Ltd., India) and water *ad libitum*. Tetracycline hydrochloride water was given for prevention against infection once a fortnight. The Departmental Animal Ethical Committee approved the present study.

Lead acetate

In the present study, lead acetate in the form of powder ($\text{C}_4\text{H}_6\text{O}_4\text{Pb}$) was procured from Ranbaxy Laboratories Ltd., India. It was injected intraperitoneally in mice at a dose of 20 mg/kg body weight with a concentration of 4 mg/ml in double distilled water as vehicle.

Ascorbic acid

Vitamin C (L-ascorbic acid; $\text{C}_6\text{H}_8\text{O}_6$) powder, procured from Glaxo Laboratories Ltd. Mumbai, was used in the present study. It was dissolved in double distilled water to give a concentration of 50 mg/ml and introduced orally once daily for 7 consecutive days in Swiss albino mice, at a dose rate of 400 mg/kg b. wt.

Experimental protocol

To assess the possible protective role of ascorbic acid on liver of mice against lead acetate intoxication, animals selected from above mentioned close bred colony, were divided into following four groups of 6 animals each, according to the mode of treatment:

Group I (n= 42): Animals of this group received double distilled water (DDW), volume equal to ascorbic acid (i.e., 400 mg/kg b. wt. / animal).

Group II (n= 42): Animals of this group were given ascorbic acid (400 mg/kg b. wt. / animal) orally once in a day for 7 consecutive days dissolved in 100 μl of DDW/ animal.

Group III (n= 42): Animals of this group were injected 20 mg/kg b. wt. of lead acetate solution intraperitoneally.

Group IV (n= 42): Animals of this group were given ascorbic acid (400 mg/kg b. wt. / animal) by oral gavage once in a day for 7 consecutive days, before lead acetate administration. The latter was given intraperitoneally on the 7th day, one hour after ascorbic acid administration.

Animals from each group were autopsied by cervical dislocation at various post-treatment intervals, *viz.*, 6 hrs., 12 hrs., 1 day, 2 days, 5 days, 10 days and 20 days, and their liver were removed and processed for routine histological and biochemical alterations.

Histopathological study

Liver was excised out and five microns thick sections were prepared after routine processing for the evaluation of quantitative variations in different hepatocytes (i.e., binucleate, abnormal and normal cells). Histopathological alterations and variations in the number of different hepatocytes were observed in definite areas of liver measured with planimeter.

Biochemical study

Some part of liver was used for biochemical study after homogenate preparation. Glycogen, protein and cholesterol levels were measured by Montgomery (23), Lowry

(24) and Leibermann methods (25), respectively, and their concentrations were measured in the tissue by using a (UV-VIS) Systronic Spectrophotometer.

Statistical analysis

For the comparison of data between different groups, all results were expressed as mean \pm Standard Error (S.E.). Degree of significance was determined by the Student's 't' test for comparison between the groups.

Results

No signs of sickness and mortality were observed in all the four groups, and their food and water intake was found to be quite normal throughout the experiment. Body weight and tissue weight increased constantly from the beginning till the end of experimentation.

The present study did not show any significant variation in the number of different types of hepatocytes in the animals treated with ascorbic acid alone (Group II) as compared to double distilled water treated mice (Group I). In lead acetate treated group (Group III), a sharp decline was noted on day 2, followed by an elevation from day 5 reaching to near normal on day 20. Ascorbic acid pretreated animals (Group IV) showed a higher frequency of normal cells than control from day 2 to 10. A noticeable recovery was observed on day 5 onwards reaching to normal on day 20 (Table-I).

Table-I: Variation in hepatic cell types (Mean \pm S.E.) in mice after administration of lead acetate with (Experimental) or without (control) Ascorbic acid

Post treatment Autopsy Intervals	Group	Types of hepatocytes (%)		
		Normal	Binucleate	Abnormal
6 hrs	Control	89.35 \pm 0.33 ^a	7.75 \pm 0.29	2.90 \pm 0.22
	Experimental	89.61 \pm 0.34	7.68 \pm 0.34	2.71 \pm 0.22
12 hrs	Control	88.09 \pm 0.53 ^c	7.99 \pm 0.25 ^a	3.92 \pm 0.27 ^c
	Experimental	88.53 \pm 0.34	7.85 \pm 0.27	3.62 \pm 0.21
Day 1	Control	86.37 \pm 0.43 ^c	8.88 \pm 0.22 ^c	4.75 \pm 0.36 ^c
	Experimental	87.29 \pm 0.27	8.29 \pm 0.16 ^a	4.42 \pm 0.21
Day 2	Control	83.88 \pm 0.31 ^c	9.53 \pm 0.20 ^c	6.59 \pm 0.25 ^c
	Experimental	85.08 \pm 0.39 ^b	8.91 \pm 0.18 ^b	6.01 \pm 0.11 ^a
Day 5	Control	84.22 \pm 0.37 ^c	8.50 \pm 0.32 ^c	7.28 \pm 0.23 ^c
	Experimental	85.52 \pm 0.27 ^b	7.71 \pm 0.16 ^a	6.77 \pm 0.31
Day 10	Control	86.62 \pm 0.18 ^c	7.71 \pm 0.17	5.67 \pm 0.10 ^c
	Experimental	87.41 \pm 0.33 ^a	7.59 \pm 0.23	5.00 \pm 0.23 ^b
Day 20	Control	89.04 \pm 0.33 ^c	7.55 \pm 0.21	3.41 \pm 0.20 ^c
	Experimental	89.79 \pm 0.29	7.45 \pm 0.29	2.76 \pm 0.21 ^b
	Normal	90.18 \pm 0.19	7.36 \pm 0.16	2.38 \pm 0.19
	Vitamin C alone	89.99 \pm 0.12	7.38 \pm 0.13	2.37 \pm 0.161

Significance level

^ap < 0.05

^bp < 0.01

^cp < 0.001

Statistical comparison

Normal v/s Control

Control v/s Experimental

In group III, the frequency of binucleate hepatocytes exhibited a significant increase over the normal from 12 hrs. to day 5. After this, it started declining without returning to normal even till the last autopsy interval (i.e., day 20). In group IV, the frequency of these cells increased continuously soon after the initiation of treatment (i.e., 6 hrs.), exhibiting a significant difference on days 1, 2 and 5. On day 10th onwards, no significant alterations could be seen between control and experimental values. The percentage of abnormal hepatocytes increased after lead treatment from day 5 but could not be restored to normal level till last day of post-treatment. In ascorbic acid administered group (Group IV), number of these cells regained normal value by day 20 (Table-I).

A biphasic mode of elevation over normal in liver glycogen content was noted first at day 1 and a sharp rise (almost 2 folds than day 1) later on day 5, but it decreased gradually till the end of experimentation without reaching to normal level (Table-II). In group IV animals, the glycogen level increased till day 1 (as similar to control) but the elevation was of lower degree. After this, it declined significantly on day 2 but further increased soon at the subsequent intervals. On day 5 onwards, the value decreased significantly, though normal level could not be attained even till day 20. Protein content increased slightly at 6 hrs., thereafter showing a subsequent decline at 12 hrs., and day 1 autopsy intervals. On day 2 onwards, protein level started elevating till day 5, but later decreased and a normal value was restored by day 20 post-treatment. In the ascorbic acid treated (Group IV), protein content was marked almost constant throughout the experiment and a non-significant difference in comparison to control was noted at all autopsy intervals.

Table-II: Variation in biochemical constituents (Mean \pm S.E.) in mice after administration of lead acetate with (Experimental) or without (control) Ascorbic acid

Post-treatment autopsy interval	Group	Biochemical parameters		
		Glycogen	Cholesterol	Protein
6 hrs	Control	6.53 \pm 0.16 ^c	4.71 \pm 0.05 ^a	154.99 \pm 3.74
	Experimental	6.20 \pm 0.32	4.68 \pm 0.03	150.07 \pm 3.12
12 hrs	Control	6.73 \pm 0.25 ^c	4.83 \pm 0.02 ^c	145.44 \pm 5.26
	Experimental	6.48 \pm 0.31	4.74 \pm 0.04 ^a	147.67 \pm 5.95
Day 1	Control	6.91 \pm 0.27 ^c	4.90 \pm 0.07 ^c	140.46 \pm 3.89
	Experimental	6.52 \pm 0.26	4.85 \pm 0.03	145.14 \pm 5.43
Day 2	Control	4.05 \pm 0.33	5.15 \pm 0.04 ^c	155.29 \pm 4.67
	Experimental	5.23 \pm 0.13 ^b	5.03 \pm 0.04 ^a	151.74 \pm 4.03
Day 5	Control	8.00 \pm 0.23 ^c	4.85 \pm 0.10 ^b	158.29 \pm 5.67
	Experimental	7.77 \pm 0.30	4.77 \pm 0.01	160.03 \pm 5.90
Day 10	Control	7.41 \pm 0.24 ^c	4.71 \pm 0.02 ^c	153.60 \pm 3.82
	Experimental	6.60 \pm 0.23 ^a	4.65 \pm 0.04	154.00 \pm 6.01
Day 20	Control	6.00 \pm 0.26 ^c	4.50 \pm 0.04	152.34 \pm 3.18
	Experimental	4.79 \pm 0.19 ^b	4.47 \pm 0.05	150.60 \pm 4.75
	Normal	4.23 \pm 0.13	4.40 \pm 0.03	149.39 \pm 3.23
	Vitamin C alone	4.50 \pm 0.69	4.45 \pm 0.02	149.40 \pm 2.71

Significance level

^ap < 0.05

^bp < 0.01

^cp < 0.001

Statistical comparison

Normal v/s Control

Control v/s Experimental

Cholesterol level exhibited an increase soon after lead administration and the highest peak was observed on day 2, which decreased further gradually till day 20, without retaining the normal level. A pattern quite similar to Group III was observed in Group IV animals, however, the values noted were comparatively lower. A significant decrease was measured at 12 hrs., and day 2 post-treatment intervals (Table-II).

Discussion

The natural antioxidants (Vitamins and glutathione) temper the negative influence of free radicals and associated reactions. Ascorbic acid (vitamin C) is an important water soluble antioxidant in biological fluids and an essential micronutrient required for normal metabolic functioning of the body that prevents oxidative stress in tissues. Ascorbic acid can also interact with tocopherol radical to regenerate reduced tocopherol that can directly react with superoxide, hydroxyl radical and singlet oxygen (26). Shastri (1999) (13) observed that *Spirulina*, a very rich source of antioxidants like provitamin A (β -carotene), vitamin C and vitamin E, is more effective in terms of heavy metal (lead/cadmium) protection at a dose of 800 mg/kg body weight.

In the present study, normal hepatocytes counts declined significantly after lead acetate intoxication, whereas the frequency of binucleate hepatocytes showed a considerable rise. The membranous unsaturated fatty acids are the obvious targets of lipid peroxidation (27), which is caused by heavy metals and results in the loss of affected organelles (hepatocytic membrane). In addition to this localized damage, the breakdown products of lipid peroxides, such as aldehydes migrate far from their production site and may cause damage at distant loci; this may be the reason for dissolution of cell membrane of two adjacent cells which results in an increase in the binucleate hepatocytes count. The results obtained by Banu and Sharma (2005) (28) revealed severe histopathological alterations in liver after toxic exposure to 160 mg lead/kg/day for three months and the ameliorative changes with the administration of antioxidant vitamins.

Ascorbic acid pre-treated animals (group IV) showed a similar pattern of alteration in normal and binucleate hepatocytes number, but to a lesser extent indicating that the vitamin rendered a significant level of protection at later intervals. Vitamin C is a powerful scavenger which breaks the autocatalytic process of lipid peroxidation of membrane fatty acids, thereby preserving membrane integrity. The histopathological observations revealed that the reason for an early increase of the binucleate cells before degeneration is due to the fusion of liver cells. Observations on day 5 exhibit that lead intoxication caused the death and removal of binucleate cells which result in the depletion of such cells and some of these even form mononucleate giant cells. In the present study, two types of binucleate cells could be distinguished morphologically; first the two nuclei remained adhered to each other in binucleate cells resulting from an incomplete cell division, and second, the nuclei remained separate in binucleate cells resulting from the fusion of mononucleate cells.

The histopathological alterations exhibited a correlation with the number of abnormal cells in the present study. The elevation in their number is associated with an increase in lead induced lesions and these cells declined during the recovery phase. Similar observations was made by Matsuda (1956) (29) who suggested that the percentage of dead and abnormal cells serve as good indicators of teratogenic sensitivity of liver cells.

Lead acetate induces lipid peroxidation, which can damage membrane of cell organelles (30). Once this cell membrane is disturbed, it leads to various structural and functional alterations in the cell. Previous studies from our laboratory have also demonstrated similar results of ascorbic acid on lead induced hepatic toxicity in mice (15).

The variations in the number of abnormal hepatocytes in group IV was evident quite similar to that of group III, but their magnitude was of lower degree (Table-I). It is possible that ascorbic acid reduces DNA damage by antiradical action (31). Similar finding of Berthold (1988) (32) shows the increase in DNA synthesis by β -carotene present in *Spirulina* by enhancing the recovery process and protection against heavy metal intoxication. A similar mechanism may be postulated for the action of ascorbic acid against lead used in the present investigation.

The mechanism of action of lead toxicity is controversial; lead is believed to covalently interact with tertiary phosphate ions in nucleic acids and proteins (33). Lead is reported to affect the fidelity of DNA synthesis *in vitro* (34). Lead acetate treated group III animals showed an increasing pattern in the percentage of abnormal cells in liver. It induces lipid peroxidation, which can damage cell membrane of various cell organelles leading to various structural and functional changes in the cell. Quinlan *et al.*, (1988) (27) stated that lead is able to accelerate iron dependent lipid peroxidation *in vitro* which has physiological significance in lead poisoning leading to cell death.

An increase in glycogen content by day 2 in lead intoxicated group may be due to destructive action of lead on tissue and enzymatic activities. The enhanced glycogen level reported at the later intervals may be due to the fact that more substrate for these metabolic processes is made available as a result of tissue breakdown. When ascorbic acid were given before lead treatment, the change in glycogen content remained similar to that of control group, but the values were found to be significantly lower. Mice protected with ascorbic acid showed an early recovery from glycogen alteration as compared to intoxicated ones. Gajawat *et. al*, (2005) (15) also noted lower level of glycogen in ascorbic acid pretreated lead intoxicated mice.

A marked variation in the liver protein content of group III animals was found. It has been reported that lead forms mercaptides with the -SH (thiol) groups of cysteine and less stable complexes with other amino-acid side chains (35) and inhibits most enzymes, bearing a single functional -SH group. This may be a reason for change in protein content. In group IV, protein level was marked almost constant throughout the experiment and with a non-significant difference with respect to control. Lead attacks on -SH group of proteins and it may be possible that vitamins exhibit some protection by providing additional -SH groups and by increasing glutathione level in tissue and blood against the destructive action of lead. Dreyfus (1985) (36) stated that the vitamins have been reported to prevent the oxidation of -SH groups during lipid peroxidation. The maintenance of protein in ascorbic acid treated (Group IV) is attributed to this protective action against -SH group oxidation.

Cholesterol level was found to increase in lead-intoxicated mice (Group III). Since, cholesterol is an important constitute of the cell membrane and a precursor for steroid hormone, the toxicant induced changes in this parameter may be related to either a disruption of plasma membrane and/or altered steroidogenesis. Increased rate of triglyceroid input or impairment of fatty acids oxidation causes accumulation of lipids

and inhibit the biosynthesis of lipo-proteins. In group IV, pattern of cholesterol change was found to be similar to group III, however, the values noted were comparatively lower.

Manimegalai (1993) (37) reported that vitamins reduce lipid peroxide levels but elevate the level of reduced glutathione in hyperlipidemic rats. The depletion of glutathione level is one of the main causes of hepatic toxicity; vitamin C helps in restoring glutathione level, thus offering a good protection against lead generated toxicity. Lead binds with the thiol groups (-SH) of the cellular components, responsible for protecting repair system against damage caused by free radicals (38), thus making free -SH groups not available for protection which in turn contributes to an increased risk during exposure to lead. Glutathione is a versatile protector and executes its function through free radical scavenging, reduction of peroxides and maintenance of protein thiols in the reduced state. The depletion of glutathione in both blood and liver as observed in the present study is one of the main causes of lead induced hepatic toxicity. Ascorbic acid protects the biological systems by increasing sulphhydryl groups as well as endogenous glutathione level of blood and liver (36). A similar mechanism may be postulated in the present study for the lower value of cholesterol in AA treated mice (Table -III).

Table-III: Glutathione (GSH) level in Swiss albino mice after lead intoxication (20 mg/kg b. wt.) in the presence (experimental) or absence (control) of ascorbic acid.

Treatment	Glutathione level	
	Blood ($\mu\text{g/ml}$)	Liver ($\mu\text{mole/gm}$)
Normal (Group-1)	3.94 \pm 0.19	6.89 \pm 0.19
Vitamin C alone (Group-2)	4.61 \pm 0.11 ^b	7.65 \pm 0.16 ^b
Lead acetate alone (Group-3)	3.11 \pm 0.15 ^a	6.00 \pm 0.14 ^a
Ascorbic acid+ lead acetate (Group-4)	3.54 \pm 0.11 ^b	5.54 \pm 0.11 ^c

Significance level

^ap< 0.05

^bp< 0.01

^cp< 0.001

Statistical comparison

Normal v/s Control

Control v/s Experimental

Results from the present investigation indicate that the transition metal, lead causes severe histopathological and biochemical alterations in the liver of mice due to impaired oxidant-antioxidant balance and enhanced oxidative stress. Supplementation of ascorbic acid, an antioxidant vitamin, prior to lead intoxication ameliorates the hepatic alterations in Swiss albino mice.

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