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A histometric study to assess preventive action of ascorbic acid and garlic on cadmium induced hepatotoxicity in albino mice

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Abstract

The present work was planned to investigate the effect of ascorbic acid (AA) and garlic against cadmium (Cd) induced toxicity in liver of albino mice by considering histometric studies. The thirty adult male albino mice were divided into following five groups (n=6/group). Group 1: served as control group. Group 2: received cadmium chloride ($CdCl_2$; 0.05mg/kg/day, i.p.). Group 3: treated with AA (200mg/kg/day, i.p.) + $CdCl_2$ (0.05mg/kg/day, i.p.). Group 4: administered with ethanolic garlic extract (EGE; 100mg/kg/day, i.p.) + $CdCl_2$ (0.05mg/kg/day, i.p.). Group 5: administered with AA+EGE+$CdCl_2$. The respective treatments were given to each group for 30 days. On the day of autopsy, liver from each mouse was removed and slides were prepared for histometric analysis. It was observed that the Cd treated mice exhibited significant increase in percentage of abnormal cells such as pyknotic nuclei and giant cells, whereas enucleated and binucleated cells were increased non significantly in comparison to control mice. However, these changes were restored partially by administration of AA and EGE separately, but their composite action (AA+EGE) provided more efficient protection against Cd induced toxicity in liver.

1. INTRODUCTION

Heavy metals are natural components of the earth's crust and considered as persistent environmental contaminants since they cannot be degraded or destroyed easily[1-5]. Some heavy metals (copper, zinc, iron, magnesium) are necessary for biological functioning of the living organisms in small amounts, while others (arsenic, mercury, nickel, lead, cadmium) cause poisoning or death[2,6-9].

Cadmium (Cd), the 48th element in the periodic table, is considered as one of the most hazardous substances. It is ubiquitous and can be found in all components of our environment viz air, water and soil due to extensive use of Cd based products. The Agency for Toxic Substances and Disease Registry (ATSDR) has listed Cd as number 7 in its list of top 20 hazardous substances and International Agency for Research on Cancer (IARC) has classified Cd as "category I" human carcinogen[1,3,10-14]. Besides it, Cd has been reported to cause injury in various organs during chronic or acute exposures[4,15-20]. The first reports of severe health problems due to Cd intoxication were reported in 1940s in Japan, where "itai itai"

disease was endemic and was mainly characterized by bone and renal damage, which was caused by eating Cd polluted rice[5]. Exposure to Cd also occurs as a result of atmospheric emission during Cd production and processing from combustion of fossil energy sources, phosphate fertilizers and deposition of waste and sledges at disposal sites[6,21-26]. Moreover, in many countries, contamination of rivers and adjoining seas by Cd and other heavy metals have been reported because of excessive discharge of the waste liquid matter from industrial sites and residual sledges of fertilizers and pesticides[7,27-32].

Liver is the major organ of Cd accumulation and intoxication[8,33-37]. Approximately half of the Cd absorbed systematically accumulates rapidly in the liver, resulting in reduced availability of the Cd to the other organs such as kidneys and testes, which are more sensitive to its toxicity[9]. Apart

from it, Cd can also cause osteoporosis, anemia, non-hypertrophic emphysema, irreversible renal tubular injury, eosinophilia, anosmia and chronic rhinitis[1,38].

It is reported that the mechanism of Cd induced cytotoxicity is mainly via (i) induction of oxidative stress[10, 39 - 43] and (ii) by interference with cellular antioxidant system[11,44-46] which in turn cause chronic and permanent damages in liver and other organs[12,47-50]. To counteract the damaging effects of reactive oxygen species, aerobic cells are provided with extensive antioxidant enzymes and other molecules e.g. Vitamins. The balance between the production of free radicals and antioxidant defense is important for proper functioning of the body, but when this balance gets disturbed by toxicants, it causes an oxidative stress which leads to various clinical dysfunctions in the body[10,51-54].

Nowadays, lot of research has been conducted on the use of natural product as antioxidant because of their fewer side effects. Among them, Vitamin C ($C_6H_8O_6$; L-Ascorbic Acid) is a water soluble vitamin required to accomplish various biological functions and also aids in cell defense by scavenging oxygen free radicals[13,55]. It is an unstable, easily oxidized acid and can be destroyed by oxygen, alkali and high temperature[14]. The main sources of ascorbic acid (AA) are citrus fruits, green peppers, red peppers, strawberries, tomatoes, broccoli, brussels sprouts, turnip and other leafy vegetables. Fish and milk also contain small amounts of vit C, which declines in amount as food ages[15,56-60].

Garlic (*Allium sativum* L.*;* Family: Liliaceae) is one of the widely used food stuff since antiquity and has received greater attention in pharmacy and medicine. It is a member of lilly family cultivated worldwide. Garlic contains unique sulfur-containing chemical constitute such as alliin (2*R*)-

2-amino-3-[(*S*)-prop-2-enylsulfinyl] propanoic acid), which is converted to the active form allicin ($C_6H_{10}OS_2$; thio-2-propene-1-sulfonic acid S-2- propenyl ester) by alliinase enzyme[16,61-64]. Allicin is an odorous and extremely unstable compound that decomposes to some active sulfides, including ajoene and dithiins, which are the main antioxidants of garlic[17]. Moreover, garlic is also one of the most effective antimicrobial herbs, as it possesses antibacterial, antifungal, antiviral, antihelmintic, antiseptic, anticancer, antiaging and antihypertensive properties[17,65-69].

These two antioxidants, ascorbic acid and garlic were chosen for the present work because they are quite affordable and easily available and the relevant studies regarding their synergistic action against heavy metal toxicity have not been reported yet. Therefore, the main aim of the present research was to evaluate the composite protective action of ascorbic acid and garlic against cadmium induced damages in liver of albino mice through histometric analysis.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Cadmium chloride ($CdCl_2$) was bought from S.D. Fine Chemical Limited, Mumbai. Ascorbic acid (AA) was obtained from Loba Chemie Private Limited, Mumbai. Both chemicals were separately dissolved in distilled water and administered intraperitoneally (i.p.) to mice.

2.2 Collection and extraction of garlic

Garlic (*Allium sativum* L.) was obtained from the local market. Ethanolic garlic extract was prepared by following the method described earlier[18,70]. Fresh garlic was ground to a fine paste using a mechanical grinder. 50g of this paste was immersed with 100ml of 80% ethanol and allowed to stand for 48 hours. The ethanolic garlic extract (EGE) was concentrated under reduced pressure using rotary evaporator. About, 15.47g of ethanolic garlic extract was obtained from 50g of garlic paste. The extract stock was then stored in an air tight container and refrigerated until required.

2.3 Experimental animal

Albino mice weighing 23-25g were procured from Guru Angad Veterinary and Animal Sciences University, Ludhiana. They were kept and acclimatized to laboratory conditions for 15 days with a 12h dark/light cycle, temperature of 25 ± 5ºC and a mean relative humidity of 50± 5%. They were fed standard mice feed (Ashirwad Industries, Chandigarh) and water *ad libitum*. The animals were handled in accordance with the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Institutional animal ethical committee (Reg no. 107/1999/CPCSEA/2009-03) approved the present study.

2.4 Acute toxicity study

It was determined by following the guideline suggested by CPCSEA. The mice were observed by housing them individually in polypropylene cages for toxic symptoms and mortality if any for 96 hours after treatment of different doses (250, 500, 1000, 1500mg/kg, body weight, i.p.) of AA and EGE.

In the case of $CdCl_2$, LD_{50} of Cd in mice was considered to be 3.2mg/kg, body weight in previous studies[19, 71-74]. Thus, a chronic dose of

0.05mg/kg, body weight, i.p. for Cd ($1/64^{th}$ LD_{50}) was selected in the present research.

2.5 Experimental procedure

Animals were randomly divided into five groups (n=6/group) as follow: Group 1 - Animals were treated with distilled water and kept as control.

Group 2 - Albino mice were treated with 0.05mg/kg body weight, i.p. of $CdCl_2$ for 30 days.

Group 3 - Mice were administered ascorbic acid (AA) at a dose of 200mg/kg body weight, i.p., 1 hour prior to the exposure of $CdCl_2$ (0.05mg/kg body weight, i.p.) for 30 days.

Group 4 - Animals were injected with 100mg/kg body weight, i.p. of fresh ethanolic garlic extract (EGE), 1 hour prior to injection of $CdCl_2$ (0.05mg/kg, body weight, i.p.) for 30 days.

Group 5 - Mice were treated with AA (200mg/kg, body weight, i.p.) and EGE (100mg/kg, body weight, i.p.) simultaneously, 1 hour prior to $CdCl_2$ (0.05mg/kg, body weight, i.p.) injection for 30 days.

2.6 Histometric studies

On the day of autopsy, liver from each mouse was removed and blotted dry. The tissue was cut into small pieces and fixed in alcoholic Bouin's fixative for 24 hours. After fixation, tissue was washed, dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax (58-60ºC) and sections were cut (5-7µm thickness) by rotary microtome (Leica RM2235, India). Then permanent slides of liver tissue were made by employing double staining technique using haematoxylin and eosin stains. Quantitative study of each slide was done by counting normal, binucleated and abnormal cell populations in 10 randomly selected fields (objective X 400) by research microscope (Kyowa Getner, N- 800M,Tokyo). A minimum of 500 counts were taken from each slide and the results were presented in the form of percentage following the modified procedure of Gajawat et al.[20]. Pyknotic nuclei, giant cells and enucleated cells were also counted and compared in all groups.

2.7 Statistical analysis

The data was expressed as Mean±S.E.M and analysed statistically (SPSS program) by using one way ANOVA. Statistical significance was considered at $p<0.05$.

3. RESULTS

3.1 Acute toxic study

Administration of different doses of ascorbic acid (AA) and ethanolic garlic extract (EGE) upto 1500mg/kg, body weight, i.p. did not elicit any toxic symptoms and mortality either immediately or during 96 hours observation period. Hence, the results indicate that AA and EGE are non- toxic upto 1500mg/kg, body weight to mice. Therefore, doses of 200mg/kg and 100 mg/kg, body weight, i.p. for AA and EGE respectively, were selected.

3.2 Histometric studies

In the present work, administration of Cd to mice resulted in significantly increase ($p<0.0001$) in the percentage of abnormal cells such as pyknotic cells ($p<0.0001$) and giant cells ($p<0.001$), while binucleated and enucleated cells were increased non significantly in comparison to control mice. However, in group 3, mice co-administration of ascorbic acid (AA) and Cd depicted moderate signs of regeneration as many normal hepatocytes (61.50±1.96%) were observed (Table 1; Fig.2). Few pyknotic cells ($p<0.01$) were observed at many loci. Plenty of giant cells ($p>0.05$) and enucleated cells ($p>0.05$) were still reported, which were significantly higher than group 1. Similar results were recorded in EGE treated mice (group 4), where, the number of normal cells was increased significantly ($p<0.01$) to 64.17±2.74% (Table 1). Also, very few pyknotic nuclei ($p<0.001$), giant cells ($p<0.01$) and enucleated cells ($p>0.05$) were observed in comparison to group 2 (Fig. 2).

Table 1: Histometric observations of liver in control, cadmium, AA+Cd and EGE+Cd and AA+EGE+Cd treated groups

Groups	Normal cells (%)	Binucleated cells (%)	Total abnormal cells (%)
Control	75.83±1.40	8.17±0.87	16.00±1.06
Cd	50.17±1.74**	10.17±0.79	39.67±1.45**
AA+Cd	61.50±1.96º	6.50±0.56	32.00±2.29º
EGE+Cd	64.17±2.74º	7.67±1.36	28.16±2.19º
AA+EGE+Cd	70.67±3.36*	5.33±0.95*	24.00±3.86*

Values are expressed as mean±SEM (Standard Error Mean);

*Level of significance values are **p< 0.0001, * p<0.001, º p<0.01 analyzed by one-way ANOVA followed by Post hoc test ; n = 6 mice in each group.*

Cd: Cadmium, AA: Ascorbic acid, EGE: Ethanolic garlic extract.

During present study, mice treated with combination of AA+EGE and Cd showed prominent recovery in the liver, as a plenty of normal cells (p<0.001) was counted. In parallel, very few binucleated cells (p<0.001), less pyknotic nuclei (p<0.001), some giant cells (p<0.01) and enucleated cells (p>0.05) were observed relative to group 2 (Table 1; Fig. 2) Thus, cumulative effect of AA and EGE showed marked protection.

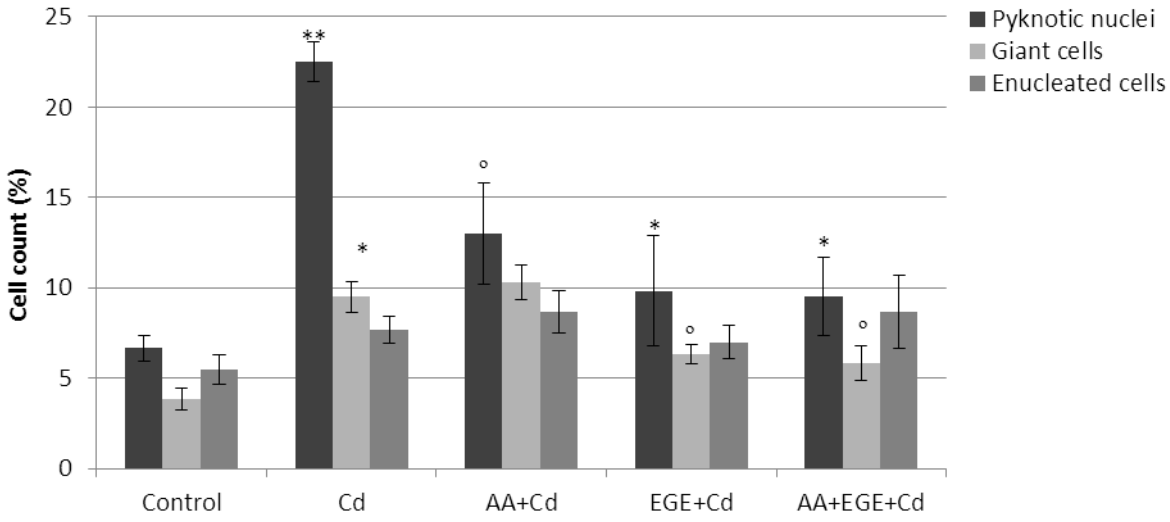

Fig. 1: Histometric observation of abnormal hepatocytes in control, Cd, AA+Cd, EGE+Cd and AA+EGE+Cd treated groups.

** Significant differences at p<0.0001 (Control vs Cd), *p<0.001 & º p<0.01 (Cd vs protection provided groups)

4. DISCUSSION

Cadmium (Cd) is known to be one of the most toxic environmental and occupational pollutant. Thus, high level exposure to this metal causes severe injuries and histological damages to various organs in experimental animals as well as in human beings[20,21]. The present work also supports these results as the mice exposed to even a low dose of cadmium (Cd) for 30 days exhibited significant rise in the percentage of abnormal hepatocytes including giant cells, enucleated cells, and pyknotic nuclei in comparison with control group. Giant cell formation is an irreversible phenomenon. These cells multiply for one to five generations after which multiplication ceases and a proportion of these cells continue to grow and form giant cells which ultimately undergo degeneration or necrosis. There are two types of giant cells (i) containing a single large nucleus, resulting presumably from continued DNA synthesis causing polyploidy in the cells that are unable to enter mitosis and (ii) those with two or more nuclei[22]. Pyknotic nuclei refers to the irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis[23] clearly indicating the genotoxic effect of cadmium. Binucleated cells were also observed, which may possibly be due to the fusion of liver cells, failure of complete telophase or inhibition of cell division[20]. A perusal of literature reported that the percentage of dead and abnormal cells serves as good indicator of the teratogenic sensitivity in liver cells[24]. Occurrence of such abnormal cells was also reported in other heavy metal treated liver and intestines in rodents[20,25].

The histometric changes observed in liver of treated mice may be attributed to Cd induced hepatotoxicity. The mechanism of Cd mediated hepatotoxicty has been the subject of numerous investigations and although some uncertainties persist, sufficient evidence has emerged to

provide a reasonable account of the toxic process. It is proposed that hepatotoxicity involved two pathways, one for initial injury produced by direct effects of Cd and other for subsequent injury produced by inflammation[26]. Primary injury appears to be caused by the binding of Cd^{2+} to sulphydryl groups on critical molecules in mitochondria which may lead to thiol group inactivation causing oxidative stress, mitochondrial permeability transition and mitochondrial dysfunction. It is also suggested that Cd suppresses functional activity of tRNA, which in turn decreases the activity of whole translation process resulting in apoptosis of liver cells[27]. Though, cadmium may injure hepatocytes directly, hepatocellular injury can be produced by ischemia caused by damage to endothelial cells[28].

It is hypothesized that chronic exposure to Cd releases inflammatory pro-apoptotic cytokines which predominately function to eliminate damaged cells[29]. Hence, the increased number of apoptotic cells and pyknotic nuclei in liver can also be considered as an

attempt of harmless elimination of the damaged hepatocytes formed in liver due to the toxic action of heavy metals[27].

The present study has demonstrated that treatment with ascorbic acid (AA) moderately ameliorated the Cd induced histometric alterations in liver. Although, the protective action of ascorbic acid against other heavy metals was also reported in earlier studies[30,31,32] , no relevant literature is available regarding histometric study. Ascorbic acid is a very important hydrophilic antioxidant. It can work both inside and outside of the cells to trap free radicals in the aqueous medium and thus protects biomembrane from peroxidation[33]. Further, the beneficial effects of AA may also be due to its ability to enhance iron absorption from gastrointestinal tract and it also reduces Fe^{+3} to Fe^{+2} to form stable chelates. The positive effects of AA on iron absorption are very desirable since Cd is known to decrease dietary iron adsorption. Therefore, AA forms the first line of antioxidant defense system of the body[34].

Similarly, very encouraging results were found in ethanolic garlic extract (EGE) treated mice, which enlighten the protective role of garlic against heavy metal toxicity in liver. The hepatoprotective property of garlic may be due to the presence of organosulfur compounds such as diallyldisulfide (DDS) and diallyltrisulfides (DTS) which have antioxidant and detoxifying properties[35]. These organosulfur compounds have

ability to attenuate Cd induced cytolethality or apoptosis by following ways: it (i) reduces Cd induced oxidative stress below threshold level[36]. (ii)

produces preconditioning effect by stimulating survival signals[37]. (iii) restores the deactivated DNA repair system by reducing the binding of Cd with DNA and protects the cell. (iv) reduces the accumulation of heavy metals such as Cd within the tissue[38]. This detoxifying effect can also be explained by induction of phase II antioxidant enzymes such as GSH and *p*-nitrophenol UDP-glucuronosyl transferase (UGT) activities[39]. The results also provided direct evidence that combined treatment of Cd-exposed animals with AA and EGE was more effective in reversing Cd- induced histometric changes indicating their syngeristic effects, convincingly making it a suitable antidote for Cd toxicity in rodents and possibly in human subjects.

5. CONCLUSION

It is evident in the study that exposure to cadmium elicited an increase in number of various abnormal hepatocytes indicating severe injury to liver. However, ascorbic acid and garlic administration might have provided partial protection against cadmium-induced damage in liver but the composite action of garlic with ascorbic acid assured even more satisfactory and encouraging results which may be due to the free radical scavenger effects of these antioxidants or their enhancing effects on the antioxidant capacity of the body. Thus, regular intake of ascorbic acid and garlic can be beneficial in reducing the toxic effects of the heavy metals in the exposed populations.

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