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Role of Vitamin C in Attenuation of Carbon Tetrachloride Induced Toxicity in Heart of Mice

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Abstract: ***The present study is aimed at evaluating the protective effect of vitamin C on carbon tetrachloride induced toxicity in Swiss albino mice. Vitamin C is a water soluble micronutrient required for multiple metabolic functions including the activation of B vitamin, folic acid, conversion of cholesterol to bile acids and the conversion of amino acid tryptophan to the neurotransmitter serotonin. It is an antioxidant that protects body from free radicals damages. CCl_4 is regarded as highly toxic. It is a known animal carcinogen and a potential human carcinogen. It is a clear, colourless, volatile heavy and nonflammable liquid which produce chemical tissue toxicity by generation of free radicals in many tissues. Mice were divided into three groups of six mice each i.e. (1) Control group (II) Mice given 1ml/kg body weight of CCl_4orally (III) Mice treated with CCl_4 (1ml/kg body weight) plus vitamin C (150 mg/kg body weight). Significantly increased levels of lipid peroxidation and lowered level of reduced glutathione and catalase enzymes were observed in heart of mice exposed to CCl_4 when compared with normal mice heart. Histopathological changes in mice heart has been investigated under normal, CCl_4 treated and CCl_4 plus vitamin C treated groups. There was significantly lesser degree of damage to CCl_4 plus vitamin C treated mice heart. But there was higher degree of damage to CCl_4treated heart architecture.***

Keywords: Carbon tetrachloride; antioxidant; lipid peroxidation; vitamin C and catalase.

1. Introduction

Animal tissues have to constantly cope with highly reactive oxygen species (ROS), such as the superoxide anion, hydroxyl radicals, hydrogen peroxide and other radicals which are generated during various metabolic reactions[1,2]. ROS are implicated in the pathogenesis of several diseases including Alzheimer's disease, multiple sclerosis, diabetes mellitus and liver cirrhosis. Excessive production of free radicals and oxidative stress can be induced by a variety of factors such as exposure to drugs and xenobiotics e.g. carbon tetrachloride. Carbon tetrachloride (CCl_4) is a well known hepato and nephrotoxicant[3,4]. It has been used as a dry-cleaning agent, fabric-spotting fluid, reagent in chemical synthesis, fire extinguisher fluid, and grain fumigant[5]. It is also used in the manufacture of paints and plastics, as a solvent in metal cleaning, and in fumigants. Carbon tetrachloride (CCl_4) is one of the xenobiotics that have been reported to induce acute and chronic tissue injuries and is a well established hepatotoxin[6]. It has been used extensively to study hepatotoxicity in animal models by initiating lipid peroxidation, thereby causing injuries to kidney, heart, testis and brain, in addition to liver pathogenesis[7].

Carbon tetrachloride was released into the environment predominantly through direct emissions to air, with lower amounts discharged to soil and water. In air, carbon tetrachloride will exist as a vapour, as indicated by its vapour pressure. Carbon tetrachloride does not undergo photodegradation[8] or absorb light at wavelengths found in the troposphere and hence does not undergo direct photolysis in that region of the atmosphere. Carbon tetrachloride that remains in the troposphere eventually rises into the stratosphere, where it is photolyzed by the shorter wavelength of light[9]. When carbontetrachloride (CCl_4) photolyzes in the stratosphere, the chlorine radicals responsible for the destruction of atmospheric ozone are released. A fraction of carbon tetrachloride in soil may adsorb to the soil organic matter, the adsorption of carbon tetrachloride will be affected by the composition of the soil organic matter and water content of the soil[10].

Carbon tetrachloride (CCl_4) causes cirrhosis in heart tissue. Cirrhosis is known to be associated with the activation of angiotensin II[11], which is also a factor in inducing cardiac hypertrophy. It is one of the chlorinated hydrocarbons that have a wide spread use in various industries as a solvent and also used in medicine as a vermifuge in treatment of hookworm diseases[12,13]. Free radicals are produced through biological processes and in response to exogenous stimuli, and controlled by various enzymes and antioxidants in the body. Vitamin E, vitamin C, and B-carotene often referred to as "antioxidant vitamins" have been suggested to limit oxidative damage in humans.

Vitamin C is available in reduced form (L-ascorbic acid) and oxidised form (L-dehydroascorbic acid). It is found in citrus fruits, green pebbers, red pebbers, strawberries, tomatoes, broccoli, brussels sprouts, turnip and other leafy vegetables. Fish and milk also contain small amount of vitamin C as foods age[14]. Vitamin C helps to prevent atherosclerosis by strengthening the artery walls through its participation in the synthesis of collagen and by preventing the undesirable adhesion of white blood cells to damaged arteries[15,16,17]. It contributes in maintaining serum cholesterol levels, blood pressure levels and protects the organism from many different oxidative challenges[18,19]. Vitamin C enhances endothelium-dependent vasodilation, thereby preventing endothelial dysfunction associated with atherosclerosis, hypercholesterolemia, hypertension, diabetes and smoking. This process seems to involve the ability of vitamin C to increase the atheroprotective nitric oxide[20].

Viamin C helps in the formation and maintainance of collagen, the connective tissue, which is found in skin, ligaments, cartilages, vertebral discs, joint linings, capillary walls, bones and teethes. Collagen protein requires vitamin C for "hydroxylation", a process that allows the molecule to achieve the best configuration and prevent collagen from

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becoming weak and susceptible to damage. Evidence indicates that vitamin C increases the level ofprocollagen messenger RNA[21]. It protects small blood vessels from damage, this may help to prevent excessive menstrual blood loss. It also helps in the metabolism of cholesterol, increasing its elimination and thereby assisting lower blood cholesterol. Heart is a pulsatile two- chamber pump composed of an atrium and a ventricle. Each atrium is a week primer pump for the ventricle, helping to move blood into the ventricles. The ventricles then supply the main pumping force that propels the blood either through the pulmonary circulation by the left ventricle. The heart rate of an adult human is about 60–70 beats/min[22], whereas the rates in the mouse and the rat are higher, between 500 and 600 beats/min in mice and between 260 and 450 beats/min in rats[23].

2. Materials and Methods

Adult sexually mature Swiss albino mice weighing between 25-28 g were procured from Central Research Institute (CRI), Kausauli (H.P.). They were maintained in polypropylene cages in the animal house of Department of Biosciences, Himachal Pradesh University, under hygiesnic conditions with proper temperature and light. Mice were fed upon Hindustan lever pellets diet and water *ad libitum.* All experimental procedures were conducted, after approval of Institutional Animals Ethics Committee (IAEC/Bio/HPU/2016/02) of H.P. University, Shimla. Experimental mice were randomly divided into 3 groups of 6 animals each. First group served as control group. It contains the normal mice as control for each experimental stage. Second group served as carbontetrachloride (CCl_4) treated group. Mice of this group were maintained under identical conditions and received oral administration of carbontetrachloride (CCl_4; 1ml/kg body weight). Third group served as carbon tetrachloride plus vitamin C treated group. Mice of this group received oral administration of carbontetrachloride (1ml/kg body weight) and vitamin C (150mg/kg body weight).

Body weight of normal and treated mice were recorded. The mice were sacrificed by cervical dislocation at 1day, 11th and 21st days. Heart was excised, weighed and employed for biochemical and histological changes. The histological studies were intended to understand histopathological alternations induced by CCl_4 and CCl_4 plus vitamin C. Tissues sections were cut and stretched on albuminized coated slides. These were subjected to dewaxing in xylene at 37 °C over night followed by dehydration in descending grades of alcohol (100%, 90%, 70%, 50% and 30% for 30 minutes each). Sections were then finally kept in distilled water and subjected to haematoxylin-eosin stain for 20-30 minutes. These were passed through acid water (0.1% HCl in distilled water) and the alkali water (1% ammonia in distilled water). Tissues were again washed in distilled water and dehydrated in ascending grades of alcohol (30%, 50%, 70%, 90% and 100%) for 30 minutes each. Counterstaining was done in 1% alcoholic eosin for 2-3 minutes. Excess of stain was removed in 90% alcohol. Sections of 4-5 µ thickness were then dehydrated completely in absolute alcohol, cleared in xylene and mounted in DPX. The permanent slides were dried, tissue sections examined, important observations noted and sections were photographed.

Level of malondialdehyde index of lipid peroxidation was estimated according to the method of Dhindsa *et al*., (1981) using thiobarbituric acid. Tissue was homogenized in 2ml of 0.1% TCA in pestle and mortar. Homogenate was then centrifuged at 6000rpm for 15 minutes. To 1ml of supernatant, 2ml of 0.5% TBA prepared in 10% TCA was added. The test tubes containing above solution were kept in boiling water bath for 30 minutes. Tubes were then cooled in ice cold water and then again centrifuged. Absorbance of supernatant was taken at 532nm and 600nm. Difference of two absorbances was taken as actual value used for calculating TBA reactive substance malondialdehyde formed. The MDA contents were calculated in n moles/mg of fresh tissue weight.

Estimation of glutathione (GSH) content was done by the method of Moron *et al.*, (1979). Tissues was homogenized in 100ml phosphate buffer (PBS). A portion of tissue homogenate (500µl) was precipitated by adding 125µl of 25% TCA (Tri chloroacetic acid) and the tubes were cooled for 5 minutes on ice. The mixture was further diluted with 600µl of 5% TCA, centrifuged at 1000 rpm for 10 minutes and supernatant (100µl) was made upto 1ml with 0.2 M sodium phosphate buffer (pH8.0). Freshly prepared DTNB solution (2ml) in 0.2M sodium phosphate buffer was added to the tubes and yellow colour formed after 10 minutes was spectrophotometrically measured.

The catalase enzyme assay was done as per the method of Aebi, (1984). Known amount of tissue was homogenized in 50mM phosphate buffer (pH-7.0). The homogenate was centrifuged at 9168 X g for 15 minutes and supernatant was collected. Reaction mixture contained 1ml of phosphate buffer. 50µl of supernatant sample (supernatant: phosphate buffer, 1:20), 500µl of 30 Mm H_2O_2 (i.e. substrate) with which reaction was started.The enzyme activity was determined by monitoring the decomposition of H_2O_2 by measuring the change in absorbance at 240nm against blank. Change in absorbance (ΔA) per unit time was calculated and taken as a measure of catalase activity. The enzyme activity was finally calculated in units/mg protein.

3. Results and Discussion

The results obtained for biochemical and histopathological studies on heart of mice were presented in tables 1-3 and figures 1-6 discussed as follows:

Normal mice heart revealed normal structure of cardiac muscle fibre with rounded nuclei, some spindle shaped nuclei and less interfascicular spaces (Fig. 1).Normal mice heart depicted many long and branched cardiac muscle fibres with centrally placed oval or rounded nuclei at 21 days stage. Some spindle shaped nuclei in muscle fibres along with small interfibrillar and interfascicular spaces were also noticed in this section (Fig. 2).

The treated mice heart showed inflammation of blood cells, interfibrillar spaces of varying sizes and clumped nuclei. Some neeedle shaped nuclei were also seen. (Fig. 3). CCl_4

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treated mice heart demonstrated large number of enucleated muscle fibres. Nuclei in varying number, different size, pyknotic and hypertrophied were also observed. Some lysed fibres with extrusion of nuclei were also seen. Maximum clumping of nuclei was observed. Normal outline of muscle fibre was lost and the fibres appeared disfigured. Certain fibres revealed splitting and swelling along with dissolution of nuclei. Polymorphonuclear leucocyte infiltration along with degenerative lesions were also observed (Fig. 4). These results are in accordance with CCl_4 treated rat heart which showed inflammatory infilterate with edema, cellular damage, necrotic changes and disrupted blood capillaries with respect to control healthy rat heart[24].

CCl_4+Vit. C treated mice heart revealed interfibrillar spaces of varying sizes. No prominent changes in muscle architecture were observed. Degeneration of muscle fibres was less and mild clumping of nuclei was visualised. However, some hypertrophied nuclei and collagen proliferation was also evident (Fig. 5). CCl_4+Vit. C treatedmice heart demonstrated mild clumping of nuclei. Little swelling of muscle fibres and splitting of fibres with large number of oval or rounded nuclei is also observed. Muscle fibres were fused together and some of them were enucleated. Collagen proliferation between the fibrillar spaces and some spindle shaped and hypertrophied nuclei were also seen with very little degenerative lesions (Fig. 6).

Enhanced lipid peroxidation associated with depletion of antioxidants in the tissues is a characteristic observation in CCl_4 intoxicated mice. Malondialdehyde, a secondary product of lipid peroxidation is used as an indicator of tissue damage. In the present study disruption of fatty acids possibly accounted for the observed increase in MDA levels in heart of CCl_4 administered mice when compared to normal mice. Elevated levels of MDA following CCl_4 administration have been well documented in various organs such as liver, kidney and heart[25,26]. The CCl_4+Vit. C treated mice group showed decrease in MDA level as compared to CCl_4 treated mice which suggests the tissue protective property of vitamin C.

The GSH level in CCl_4 treated mice significantly decreased as compared to control mice in the present study. The lowered GSH levels in CCl_4 treated mice heart might reflect to increased oxidative damage[27,28]. Interestingly, in the present study, CCl_4+Vit. C treated mice showed increased levels of GSH as compared to control mice. During the present investigation, decrease in catalase activity was observed in CCl_4 treated mice heart as compared to control. CCl_4 treated rats kidney, heart and brain showed decrease in catalase activity[29]. Significant increase in catalase activity was observed in CCl_4+Vit. C treated mice strongly suggests the antioxidant and tissue protective potential of vitamin C.

4. Conclusion

In conclusion, the present study demonstrated that the cardioprotective beneficial effect of the antioxidant vitamin C may be related to their ability to attenuate the extent of peroxidation of membrane lipids, recover the enzymatic antioxidant defence system, up-modulate the bioenergetic state of cardiac tissue and may be used as prophylactic and curative agents against cardio-toxic agents. The present study suggests that vitamin C has a potent cardioprotective activity in CCl_4 induced heart injury in mice. This preventive effect of vitamin C is due to its free radical scavenging, antioxidative, immuno-modulatory and anti-inflammatory properties.

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Table 1: Change in lipid peroxidation activity (n moles of TBARS formed/g of fresh tissue weight) in heart of Swiss albino mice in different groups.
Values are mean ± SEM; n = 3 (p*< 0.05)

Groups	Days		
	1	**11**	**21**
Control	12.517 ± 1.187	13.854 ± 1.185	14.196 ± 0.942
CCl_4	18.187 ± 0.943	18.855 ± 0.980*	19.861 ± 0.979*
% Increase or decrease	45.29%	36.09%	39.90%
CCl_4 + Vit.C	15.196 ± 1.700*	16.516 ± 1.656*	17.113 ± 1.707*
% Increase or decrease	-16.44%	-12.40%	-13.83%

Table 2: Changes in glutathione (GSH) levels (µg/mg protein/min) in heart of Swiss albino mice in different periods
Values are mean±SEM; n=3 (P *<0.05)

Groups	Days		
	1	11	21
Control	0.166 ± 0.004	0.168 ± 0.002	0.170 ± 0.002
CCl_4	0.152 ± 0.009*	0.143 ± 0.002*	0.138 ± 0.001*
% Increase or Decrease	-8.44%	-14.89%	-18.82%
CCl_4 + Vit. C	0.155 ± 0.002*	0.160 ± 0.002*	0.163 ± 0.005
% Increase or Decrease	1.97%	11.88%	18.11%

Table 3: Changes in catalase specific activity (units/mg protein) in heart of Swiss albino mice in different periods
Values are mean±SEM; n=3 (P *<0.05)

Groups	Days		
	1	11	21
Control	9.347 ± 0.005	9.409 ± 0.002	9.452 ± 0.004
CCl_4	5.019 ± 0.005	5.000 ± 0.002*	4.451 ±0.003*
% Increase or Decrease	-46.31%	-46.86%	-52.91%
CCl_4 + Vit.C	6.320 ± 0.002	6.388 ± 0.001	6.418 ± 0.001*
% Increase or Decrease	25.92%	27.76%	44.19%

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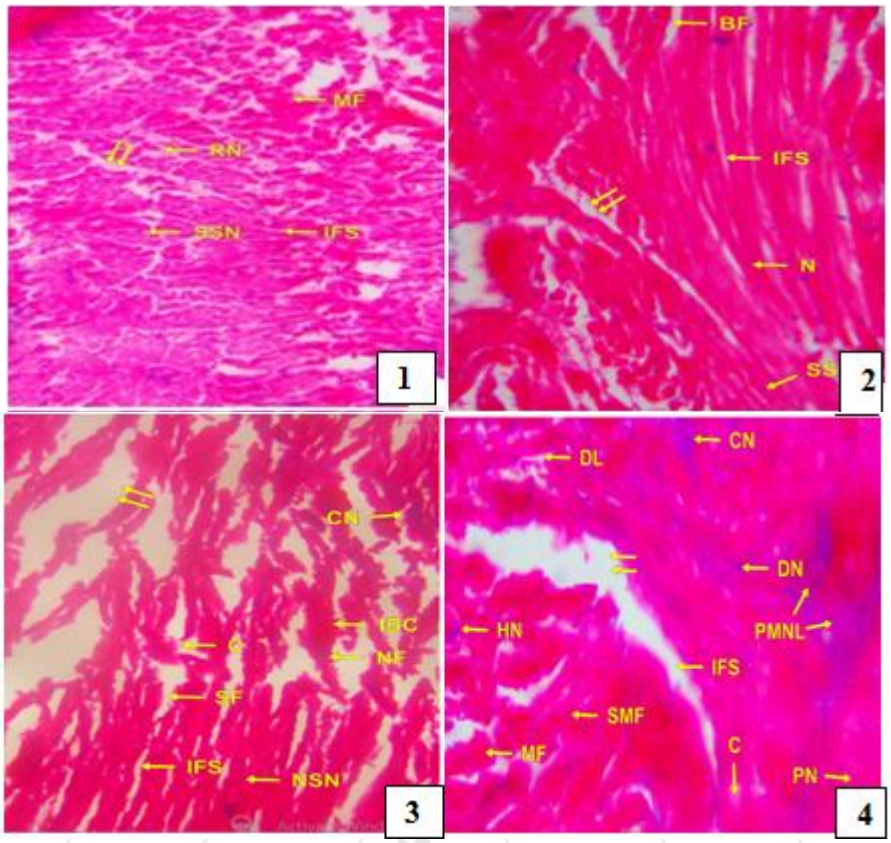

Fig. 1: T.S. of normal mice heart after 1 day showing normal architecture of cardiac muscle fibre (MF) with centrally placed rounded (N) and spindle shaped (SSN) nuclei. Section showed small interfibrillar (IFS) and interfascicular (↑↑) spaces X 200.

Fig. 2: T.S. of normal mice heart after 21 days showing long branched muscle fibres (BF) with centrally placed oval or rounded nuclei. Some spindle shaped nuclei were also observed (SSN). Muscle fibres showed less interfibrillar (IFS) and interfascicular (↑↑) space X 200.

Fig. 3: T.S. of CCl_4 treated mice heart after 11 days showing large splitting of muscle fibres (SF) with clumping of nuclei (CN)**,** necrosis of fibres (NF) and inflammation of blood cells (IBC). Some needle shaped nuclei (NSN) were also present. Widening of interfibrillar space (IFS) with streaks of collagen (C) in between them were seen in the section X 200.

Fig. 4: T.S. of CCl_4 treated mice heart after 21 days revealing swellen muscle fibres, degenerating nuclei (DN) and increase in interfascicular spaces (↑↑). Polymorphonuclear leucocyte (PMNL) infiltration between muscle fibres was noticed. Pyknotic (PN) and hypertrophied nuclei (HN), clumping of nuclei (CN) and degenerative lesions (DL) were also seen X 400.

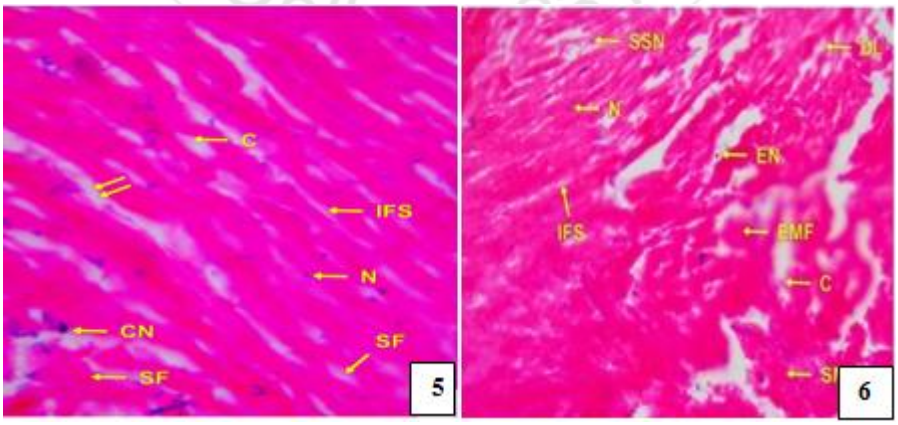

Fig. 5: T.S. of CCl_4+Vit. C treated mice heart after 11 days demonstrating some oval or rounded nuclei (N) along with reduction in splitting of cardiac muscle fibres (SF). Thin streaks of collagen (C) were also observed along with mild clumping of nuclei (CN). However, section showed normal interfibrillar (IFS) and interfascicular spaces (↑↑) X 400.

Fig. 6: T.S. of CCl_4+Vit. C treated mice heart after 21 days demonstrating some spindle shaped nuclei (SSN) and interfibrillar spaces (IFS). Few degenerative lesions (DL), collagen proliferation (C) between the fibrillar space, some enucleated muscle fibre (EMF) due to extrusion of nuclei (EN) and swollen muscle fibres () were also seen in this section X 200.

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