

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

Sushma Sharma, Babita Kumari

Department of Biosciences, H.P. University, Summer Hill, Shimla-171005; India

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. CCl₄ 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. (I) Control group (II) Mice given 1ml/kg body weight of CCl₄ orally (III) Mice treated with CCl₄ (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 CCl₄ when compared with normal mice heart. Histopathological changes in mice heart has been investigated under normal, CCl₄ treated and CCl₄ plus vitamin C treated groups. There was significantly lesser degree of damage to CCl₄ plus vitamin C treated mice heart. But there was higher degree of damage to CCl₄ treated 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 (CCl₄) 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 fumigant⁵. It is also used in the manufacture of paints and plastics, as a solvent in metal cleaning, and in fumigants. Carbon tetrachloride (CCl₄) is one of the xenobiotics that have been reported to induce acute and chronic tissue injuries and is a well established hepatotoxin⁶. 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 pathogenesis⁷.

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 photodegradation⁸ 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 light⁹. When carbon tetrachloride (CCl₄) 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 soil¹⁰.

Carbon tetrachloride (CCl₄) causes cirrhosis in heart tissue. Cirrhosis is known to be associated with the activation of angiotensin II¹¹, 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 pebbles, red pebbles, strawberries, tomatoes, broccoli, brussels sprouts, turnip and other leafy vegetables. Fish and milk also contain small amount of vitamin C as foods age¹⁴. 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 oxide²⁰.

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

becoming weak and susceptible to damage. Evidence indicates that vitamin C increases the level of procollagen messenger RNA²¹. 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 weak 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/min²², 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 rats²³.

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 hygienic 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 carbon tetrachloride (CCl₄) treated group. Mice of this group were maintained under identical conditions and received oral administration of carbon tetrachloride (CCl₄; 1ml/kg body weight). Third group served as carbon tetrachloride plus vitamin C treated group. Mice of this group received oral administration of carbon tetrachloride (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 1 day, 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 CCl₄ and CCl₄ 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 were 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 up to 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 H₂O₂ (i.e. substrate) with which reaction was started. The enzyme activity was determined by monitoring the decomposition of H₂O₂ 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 needle shaped nuclei were also seen. (Fig. 3). CCl₄

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 CCl₄ treated rat heart which showed inflammatory infiltrate with edema, cellular damage, necrotic changes and disrupted blood capillaries with respect to control healthy rat heart²⁴.

CCl₄+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). CCl₄+Vit. C treated mice 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 CCl₄ 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 CCl₄ administered mice when compared to normal mice. Elevated levels of MDA following CCl₄ administration have been well documented in various organs such as liver, kidney and heart^{25,26}. The CCl₄+Vit. C treated mice group showed decrease in MDA level as compared to CCl₄ treated mice which suggests the tissue protective property of vitamin C.

The GSH level in CCl₄ treated mice significantly decreased as compared to control mice in the present study. The lowered GSH levels in CCl₄ treated mice heart might reflect to increased oxidative damage^{27,28}. Interestingly, in the present study, CCl₄+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 CCl₄ treated mice heart as compared to control. CCl₄ treated rats kidney, heart and brain showed decrease in catalase activity²⁹. Significant increase in catalase activity was observed in CCl₄+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 CCl₄ 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.

References

- [1] Castillo, T., Koop, D. R. and Kamimura, S. 1992. Role of cytochrome P-4502E1 in ethanol, carbontetrachloride and iron dependent microsomal lipid peroxidation. *Hepatology*. 16: 992-996.
- [2] Hartley, D.P., Kolaja, K.L., Reinhold, J. and Peterson, D.R. 1999. 4 hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: immunochemical detection and lobular localization. *Toxicol. Appl. Pharmacol.* 161: 23-33.
- [3] Thrall, K. D., Vucelick, M. E., Gies, R. A., Zanger, R. C., Wietz, K. K., Poet, T. S., Springer, D. L., Grant, D. M. and Benson, G. M. 2000. Comparative metabolism of carbontetrachloride in rats, mice and hamsters using gas uptake and PBPK modelling. *J. Toxicol. Environ. Health.* 60(8): 531-48.
- [4] Ogeturk, M., Kusi, Colakoglu, N., Zararsiz, I., Ithan, N. and Sarsilmaz, M. 2005. Caffeic acid phenethyl ester protects kidneys against carbontetrachloride. *J. Ethnopharmacol.* 97: 273-280.
- [5] Holbrook, M. T. 1993. Carbon tetrachloride. In: *Encyclopedia of chemical technology*. Eds. J.I., Kroschwitz, M., Howe-Grant, and Kirk-Othmer. 4th edition. New York, NY: John Wiley and Sons. 5: 1062-1072.
- [6] Xu, J. Y., Su, Y. Y., Cheng, J. S., Li, S.X., Liu, R. and Li, W.X. 2010. Protective effects of fullerene on carbon tetrachloride-induced acute hepatotoxicity and nephrotoxicity in rats. *Carbon*. 1388-1396.
- [7] Khan, R. A., Khan, M. R. and Sahreen, S. 2012. CCl₄-induced hepatotoxicity: protective effect of rutin on p53, CYP2E1 and the antioxidative status in rat. *BMC Complement. Altern. Med.* 8: 172-178.
- [8] Holbrook, M. T. 1993. Carbon tetrachloride. In: *Encyclopedia of chemical technology*. Eds. J.I., Kroschwitz, M., Howe-Grant, and Kirk-Othmer. 4th edition. New York, NY: John Wiley and Sons. 5: 1062-1072.
- [9] Molina, M.D. and Rowland, F.S. 1974. Predicted present stratospheric abundances of chlorine species from photodissociation of carbontetrachloride. *Geophys. Res. Lett.* 1: 309-312.
- [10] ATSDR (Agency for Toxic Substances and Disease Registry) 2005. Toxicological profile for carbon tetrachloride. *Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.* PB2006-100002.
- [11] Helmy, A., Jalan, R. and Newby D. E. 2000. Role of angiotensin II in regulation of basal and sympathetically stimulated vascular tone in early and advanced cirrhosis. *Gastroenterol.* 118: 565-572.
- [12] Preis, S. and Kallas, J. 2004. Gas-phase degradation of CCl₄, CHCl₃ and CH₂Cl₂ over metallic Fe. *Env. Chem. Lett.* 2(1): 9-13.

- [13] Das, R. K., Ugir Hossam, S. K. and Bhattacharya, S. 2007. Protective effect of diphenylmethyl selenocyanate against CCl₄ induced hepatic injury. *J. Appl. Toxicol.* 27: 527-537.
- [14] Platt, B. S., Eddy, T. P. and Pellet, P. L. 1963. Food in Hospitals. Anonymous. Nuffield Provincial Hospitals Trust and Oxford University Press London.
- [15] Rath, M. 1993. Eradicating heart diseases. Health Now San Francisco, CA.
- [16] Lehr, H. A. 1995. Protection from oxidised LDL induced leukocyte adhesion to microvascular endothelium in vivo by vitamin C but not by vitamin E. *Circulation* 91: 1525-1532.
- [17] Weber, C. 1996. Increased adhesiveness of isolated monocytes to endothelium is prevented by vitamin C intake in smokers. *Circulation* 93: 1485-1492.
- [18] Whitaker, M.J., 1985. Reversing Heart Disease. Warner Books, NY.
- [19] Trout, D. L. 1991. Vitamin C and cardiovascular risks factors. *Am. J. Clin. Nutr.* 53: 322S-325S.
- [20] May, J. M. 2000. How does ascorbic acid prevent endothelial dysfunction? *Free Rad. Biol. Med.* 28: 1421-1429.
- [21] Gaby, S. K. and Singh, V.N. 1991. Vitamin C- Vitamin intake and health: *A Scientific Review*, Gaby, S.K., Bendich, A., Singh, V. and Machlin, L.(eds.). Marcel Dekker, N.Y.
- [22] Wessels, A. and Sedmera, D. 2003. Developmental anatomy of the heart: a tale of mice and man. *Physiol. Genomics.* 15: 165-76.
- [23] Orr, H.E. 2002. Rats and mice. In: *BSAVA Manual of Exotic Pets* (A. Meredith, S. Redrobe, eds). 4th edn. Barcelona, Spain: Grafos, 14.
- [24] Jayakumar, T., Sakthivel, M., Thomasb, P. A. and Geraldine, P. 2008. Pleurotus ostreatus an oyster mushroom decreases the oxidative stress induced by carbontetrachloride in rat kidneys, heart and brain. *Chem. Biol. Interacs.* 176: 108-120.
- [25] Eshaghi, M., Zare, S., Banihabib, N., Nejati, V., Farokhi, F. and Mikaili, P. 2012. Cardioprotective effect of cornus mas fruit extract against carbon tetrachloride induced-cardiotoxicity in Albino rats. *J. Basic Appl. Sci. Res.* 2 (11): 11106-11114.
- [26] Tirkey, N., Pilkhwai, S., Kuhad, A, and Chopra, K. 2005. Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. *BMCPharmacol.* 52: 1-8.
- [27] Thieophile, D., Emery, T. D., Paul Desire, P. B., Veronique, P. B. and Njika, N. 2006. Effects of *Alafia multiflorastapf* on lipid peroxidation and antioxidant enzyme status in carbon tetrachloride treated rats. *Pharmacol.* 2: 76-89.
- [28] Srinivasan, M. Rukkumani, R., Ram Sudheer, A. and Menon, V. P. 2005. Ferulic acid, a natural protector against carbon tetrachloride-induced toxicity. *Fund.Clin.Pharmacol.* 19: 491-496.
- [29] Azizi, H., Chung, S. L., Tanaka, T. and Asahara, Y. 2010. Isotopic dating of the khoy metamorphic complex (kmc), north western Iran: A significant revision of the formation age and margin source. *Precambrian Res.* 15: 1-20.

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%

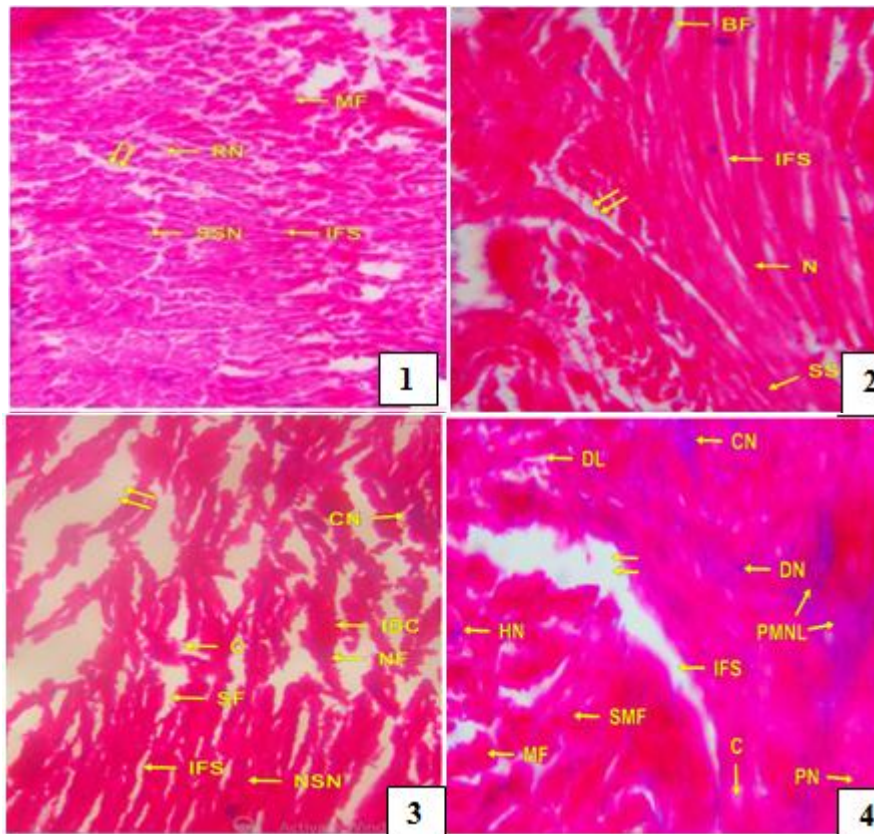


Fig. 1: T.S. of normal mice heart after 1 day showing normal architecture of cardiac muscle fibre (MF) with centrally placed rounded (N) and spindle shaped (SSN) nuclei. Section showed small inter-fibrillar (IFS) and inter-fascicular (↑↑) spaces X 200.

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 inter-fibrillar (IFS) and inter-fascicular (↑↑) space X 200.

Fig. 3: T.S. of CCl₄ 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 inter-fibrillar space (IFS) with streaks of collagen (C) in between them were seen in the section X 200.

Fig. 4: T.S. of CCl₄ treated mice heart after 21 days revealing swollen muscle fibres, degenerating nuclei (DN) and increase in inter-fascicular 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.

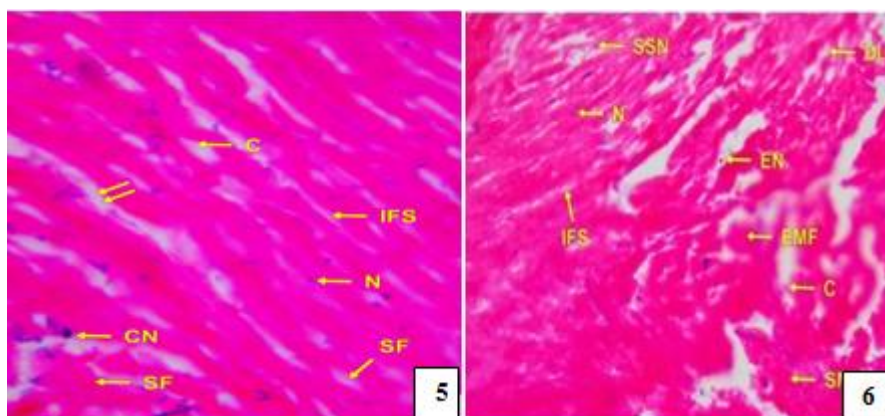


Fig. 5: T.S. of CCl₄+Vit. C treated mice heart after 11 days demonstrating some oval or rounded nuclei (N) along with reduction in splitting of cardiac muscle fibres (SF). Thin streaks of collagen (C) were also observed along with mild clumping of nuclei (CN). However, section showed normal inter-fibrillar (IFS) and inter-fascicular spaces (↑↑) X 400.

Fig. 6: T.S. of CCl₄+Vit. C treated mice heart after 21 days demonstrating some spindle shaped nuclei (SSN) and inter-fibrillar 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 (S) were also seen in this section X 200.