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Protective Effect of Vitamin C on Carbon Tetrachloride Administered Toxicity in Lungs of Mice

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Abstract: ***The present study is undertaken to examine the protective mechanism of vitamin C in lung tissue in the intoxicated mice. Here, lung toxicity was induced by administering dose of CCl_4 in experimental adult balb-c mice and radical scavenging activity of the vitamin C was evaluated by measuring the levels of GSH and lipid peroxidation in lung tissue homogenates via GPx. Mice were divided into three groups. Mice of first group served as control (group 1) received saline solution, CCl_4 group (Group 2) received CCl_4 orally at a dose level of 1ml/kg body weight mixed in olive oil and vitamin C + CCl_4 group (group 3) received vitamin C (150mg/kg body weight) with CCl_4 for different experimental stages. After each experimental stage, lung tissue were employed for histological and biochemical studies. Results showed that Vitamin C prevented the lung against the oxidizing effects of CCl_4 by decrease in glutathione peroxidase activity and levels of reduced glutathione. The histopathological damage of the lung and peroxidation of membrane lipids was significantly prevented by vitamin C treatment as compared to CCl_4 alone treatment.***

Keywords: Antioxidant enzymes, Carbon tetrachloride, Histological studies, Lungs and Vitamin C.

1. Introduction

Carbon tetrachloride(CCl_4) is a xenobiotic producing hepatotoxicity in human beings and animals [1,2]. CCl_4 administration has been demonstrated to cause injury to the lungs [3,4]. It has been established that trichloromethyl (CCl_3) radical and chloride (Cl) are formed as a result of the metabolic conversion of CCl_4 by cytochrome P-450 [5].These free radicals react with polyunsaturated fatty acids (PUFA) of lung membranes and enhance lipid peroxidation (TBARS), DNA fragmentation [6], deplete activities of antioxidant enzymes such as catalase(CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), GSR and amount of tissue soluble proteins [7].

Tissues have developed an antioxidant defense system that includes nonenzymatic antioxidants (eg., glutathione, uric acid, bilirubin, vitamin C and E) and enzymatic activities(SOD, CAT, and GSH-Px) to prevent the damage caused by oxygen-free radicals. SOD catalyzes dismutation of the superoxide anion (O^{-2}) into H_2O_2 and GSH-Px and CAT both detoxify H_2O_2 and convert lipid hydroperoxides to nontoxic alcohols [8]. Vitamin C is a well known cell protective natural antioxidant. The protective effects of vitamin C are observed in oxygen-dependent pathophysiological conditions [9]. It was observed that vitamin C exhibited a reputable hepatoprotective effect in humans and animals.It was observed that dietary vitamin C supplementation increases global antioxidant capacity of guinea pig heart tissues [10].The presence of vitamins and other nutrients with antioxidant activity, acting in conjunction with antioxidant enzymes has been shown to have beneficial effects against free radicals produced under normal physiological and pathological conditions [11]. Vitamin C is a water-soluble antioxidant which decreases lipid peroxidation either directly or indirectly by regenerating vitamin E, the major lipid soluble antioxidant [12]. Vitamin C was also reported to scavenge aqueous reactive oxygen species (ROS) by rapid electron transfer that inhibits lipid peroxidation [13].Thus, present study is designed to investigate the protective role of vitamin C against free radical mediated damages induced by CCl_4 on lungs of mice.

2. Materials and Methods

Swiss albino mice weighing between 24-28 g were procured from Central Research Institute (CRI), Kasauli, Himachal Pradesh and were maintained in the animal house of department of Biosciences, Himachal Pradesh University, under hygienic conditions with proper light and temperature. Mice were fed upon Hindustan lever pellets and water ad libitum. The experimental procedures were carried out in strict compliance with Institutional Animals Ethics Committee (IAEC/Bio/HPU/2016/01) of H.P. University.Mice were divided into three groups. Mice of first group served as control (group 1) received saline solution, CCl_4 group (Group 2) received CCl_4 orally at a dose level of 1ml/kg body weight mixed witholive oil and vitamin C + CCl_4 group (group 3) received vitamin C (150mg/kg body weight) with CCl_4 for different experimental stages.

Administration and dose rationale of CCl_4

Mice belonging to second group were given CCl_4 (1ml/kg.b.w.) orally daily till the end stage of single oral dose for 24 hours and 11-21 days. The mice of group 3rd were given CCl_4 and vitamin C (synthetic vitamin) for different experimental stages. The mice (control, CCl_4 treated and CCl_4 + vit C) were maintained and sacrificed after 24 hrs, 11 days and 21 days respectively along with control mice. The animals were sacrificed by cervical dislocation and lung tissue was excised and processed for further investigations.

Histopathological studies

Lung tissue was fixed in Bouin's fixative for 24 hrs. Thorough washing in running tap water was done so as to remove excess of fixative. Tissues were dehydrated finally in different grades of alcohol (30%, 50%, 70%, 90% and 100%) and embedded in paraffin wax (55˚-60°C). Sections were cut in microtome and employed for haematoxylin eosin staining. Then different sections were examined.

Biochemical parameters

Level of malondialdehyde, index of lipid peroxidation was estimated according to method of Dhindsa*et al.*, (1981) using thiobarbituric acid (TBA). The protein concentration was determined using the method described by Lowry *et al.*, (1951) using Folin-ciocalteu's reagent and bovine serum albumin as the standard. Estimation of glutathione (GSH) content was done by the method of Moron *et al.*, (1979). It was measured by its reaction with Di-thio-Bis-nitrobenzoic acid (DTNB) to give a compound that was absorbed at 412nm. and the activity glutathione peroxidase was measured by the method described by Rotruck*et al.*, (1973).

Statistical analysis

Statistical difference between means of various groups were evaluated using one-way analysis of variance (ANOVA) followed by Turkey's test. Data were considered statistically significant at P values<0.05.

3. Results and Discussion

The results obtained for histopathologicaland biochemical studies on lungs of mice were presented in figures A-L and tables 1-3 were discussed as follows:

Histopathological studies

Histopathological studies of the control lung depicted healthy lung parenchyma, numerous oval shaped alveoli appeared as small outpockets, open alveolar spaces underlined by interconnected interalveolar septum, pulmonary vein, blood vessel and bronchus. An extensive capillary network filled with RBCs was seen within the alveolar wall. There was no evidence of inflammation, destruction or other distinct pathological changes in the lungs of control mice.The histopathological examination of CCl_4 treated mice demonstrated loss of normal alveolar architecture, severe destruction of alveolar ducts which resulted in cytoplasmic vacuolization, parenchymal congestion accompanied by intense inflammatory infiltrates and reduced air spaces with thickened alveolar wall. There was mild to moderate congestion of blood vessels resulting in acute hemorrhage at some points. Distorted bronchiole, excessive vacuolization and parenchymal infiltration was common in CCl_4 treated group at different experimental stages. These changes in lung architecture strongly suggest the presence of noticeable fibrotic lesions in the lung of CCl_4 induced cirrhotic mice. Vitamin C + CCl_4 treated mice showed normal blood vessel and numerous oval shaped alveoli with well preserved lung parenchyma. However, initiation of vacuolization and parenchymal infiltration was also visible at some places. Destruction and widening of alveolar wall at some areas was also evident. But, the severity of alterations in lung parenchyma was less in vitamin C treated group when compared with that of CCl_4 treated group.

4. Biochemical Measurements

Lung TBARS

Carbon tetrachloride administration increased lung TBARS significantly for different experimental periods as shown in (Table.1).1. In the vitamin C + CCl_4 treated group, the lung TBARS were lower as compared to CCl_4 treated group. Vitamin C treated group TBARS level was near to non-exposed groups (control).

Lung glutathione and GPx

The activity of glutathione and glutathione peroxidase was observed to be decreased on CCl_4 intoxication, whereas treatment with vitamin C appeared to exert a beneficial effects on the lung GSH and GPx level compared to CCl_4groups. GSH and GPx were depleted to0.252 ± 0.018 µg/mg and 0.446 ± 0.009 µg/mg protein/min. respectively. The reduced levels were ameliorated to values as0.283 ± 0.023* µ/mg and0.537 ± 0.001µg/mg protein/min. in the vitamin C treated groups. (Table.2 and Table.3).

5. Discussion

Histopathological studies of the control lung depicted healthy lung parenchyma, numerous oval shaped alveoli appeared as small outpockets, open alveolar spaces underlined by interconnected interalveolar septum, pulmonary vein, blood vessel and bronchus. In CCl_4 treated mice loss of normal alveolar architecture, severe destruction of alveolar ducts which resulted in cytoplasmic vacuolization, parenchymal congestion accompanied by intense inflammatory infiltrates, reduced air spaces with thickened alveolar wall were observed. Distorted bronchiole, excessive vacuolization and parenchymal infiltration was common in CCl_4 treated group. These changes in lung architecture strongly suggest the presence of noticeable fibrotic lesions in the lung of CCl_4 induced cirrhotic mice. These variations are in accordance with the findings of [14]. Vitamin C + CCl_4 treated mice showed normal blood vessel and numerous oval shaped alveoli with well preserved lung parenchyma. However, initiation of vacuolization and parenchymal infiltration was also visible at some places. Destruction and widening of alveolar wall at some areas was also evident. But, the severity of alterations in lung parenchyma is less in vitamin C treated group when compared with that of CCl_4 treated group suggesting the protective role of vitamin C against CCl_4 induced damge in lung tissue.

CCl_4 when administered is distributed and deposited in organs such as liver, kidney, lung and heart [15]. The reactive metabolite trichloromethyl radical (*CCl_3) and trichloromethyl peroxide radical (CCl_3O_2*) has been formed from the metabolic conversion of CCl_4 by cytochrome $P\text{-}_{450}$. As oxygen tension rises, a greater fraction of *CCl_3 present in the system reacts very rapidly with O_2 and more reactive free radicals, like CCl_3OO* are

generated from *CCl_3. These free radicals initiate the peroxidation of membrane poly unsaturated fatty acids (PUFA), cell necrosis, GSH depletion, membrane damage and loss of antioxidant enzyme activity.Our study has revealed increased lipid peroxidation in lungs after exposure to CCl_4 which is supported by [3,4]. CCl_4 is converted into trichloromethyl radical, a toxic metabolite[16], which attacks the cellular components and produces oxidative injury [17].

Glutathione (GSH) is a vital intra and extra cellular protective antioxidant in the lungs. GSH is often referred to as the body's master antioxidant. It is a very simple molecule and is produced naturally all the time in our body. It is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals [18]. It's deficiency causes cells to be more vulnerable to oxidative stress, which contributes to cancer development. Vitamin C helps to raise glutathione in red blood cells and lymphocytes.In our study lungs GSH level in CCl_4 treated group was significantly decreased as compared to control group which is similar to the works of [19]. The antioxidant enzymes in the lungs include glutathione peroxidase and superoxide dismutase. These enzymes are known to protect the lungs against oxidative damages. Superoxide dismutase protects cell by converting superoxide anions (O_2*-) to H_2O_2. These molecules, which are toxic to cells, can be further broken down to release hydroxyl radical (*OH), a reactive species which is more damaging to the cells. The enzymes responsible for detoxifying H_2O_2 are glutathione peroxidase and catalase, which prevents the formation of *OH by converting H_2O_2 to non-harmful products, which are oxygen and water [20,21]. A significant increase in the lung glutathione peroxidase enzyme activity was observed in our study when mice exposed to CCl_4 in comparison to the non-exposed control. This endogenous enzyme is important in preventing damages in the lung tissue [22,23], where it had been shown to detoxify reactive oxygen species in the lungs[2]. The increased activity of glutathione peroxidase can be explained by the findings of [24]. Our results concluded that vitamin C + CCl_4 treated group showed less elevations in the lungs GSH and GP-x activity when compared with CCl_4 treated group which may be due to its abundant antioxidant properties.

6. Conclusion

The finding of above studies indicated that CCl_4 had many adverse effects on body. It is a xenobiotic producing hepatotoxicity in human beings and animalsand also causes injury to lungs[3,4]. It is well evidenced by our biochemical and histopathological studies leading to lung dysfunction. It is proven that CCl_4 promotes injuries in these organs via oxidative stress by increasing the lipid peroxidation and lowering the endogenous antioxidant [25,26]. Therefore, the various chelating agents with antioxidant features are needed. Vitamin C is an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxide damage. These properties of vitamin C are well evident from our work where vitamin C compensates the effect of CCl_4 on lungs of mice. Our study also suggests that the vitamin C has the potential to protect the lung tissue against oxidative damages and could be used as a protector against CCl_4 induced lung damages. Further works are needed to fully characterize the different antioxidant properties of vitamin C present in different vegetables and fruits.

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Table 1: Lipid peroxide (n moles of TBARS formed/g fresh tissue weight) in lungs of control, CCl_4 treated and CCl_4 + vitamin C treated mice after 1-21 days stages. Values are mean ± SEM; n=3 (P*<0.05).

GROUPS	DAYS		
	1	11	21
Control	10.58 ± 0.47	12.23 ± 0.67	13.65 ± 0.35
CCl_4	11.51 ± 0.49*	19.99 ± 0.69	24.02 ± 1.71
%increase or decrease	8.76%	63.45%	75.97%
CCl_4 + vitamin C	10.98 ± 1.11*	16.11 ± 0.59*	19.67 ± 0.72
%increase or decrease	-4.61%	-19.41%	-18.11%

Table 2: Changes in glutathione (GSH) levels (µg/mg protein/min) of normal, CCl_4 and CCl_4 + vit C treated mice lungs after 1-21 days period. Values are mean ± SEM; n=3 (p*<0.05).

GROUPS	DAYS		
	1	11	21
Normal	0.866 ± 0.001	0.859 ± 0.004	0.857 ± 0.002
CCl_4	0.736 ± 0.020*	0.434 ± 0.017*	0.252 ± 0.018
%increase or decrease	-15.01%	-49.48%	-70.59%
CCl_4 + vitamin C	0.754 ± 0.011*	0.468 ± 0.017*	0.283 ± 0.023*
%increase or decrease	2.45%	7.83%	12.30%

Table 3: Changes in activity of glutathione peroxidase (µg/mg protein/min) of normal, CCl_4 treated and vitamin C + CCl_4 treated lungs of mice after 1-21 days period. Values are mean ± SEM; n=3 (p*<0.05).

GROUPS	DAYS		
	1	11	21
Control	2.538 ± 0.002	2.450 ± 0.001	2.366 ± 0.008
CCl_4	1.313 ± 0.002*	0.839 ± 0.001	0.446 ± 0.009*
%increase or decrease	48.25%	65.76%	81.15%
CCl_4 + vitamin C	1.524± 0.003*	0.929 ± 0.001	0.537 ± 0.001
%increase or decrease	39.95%	10.73%	20.40%

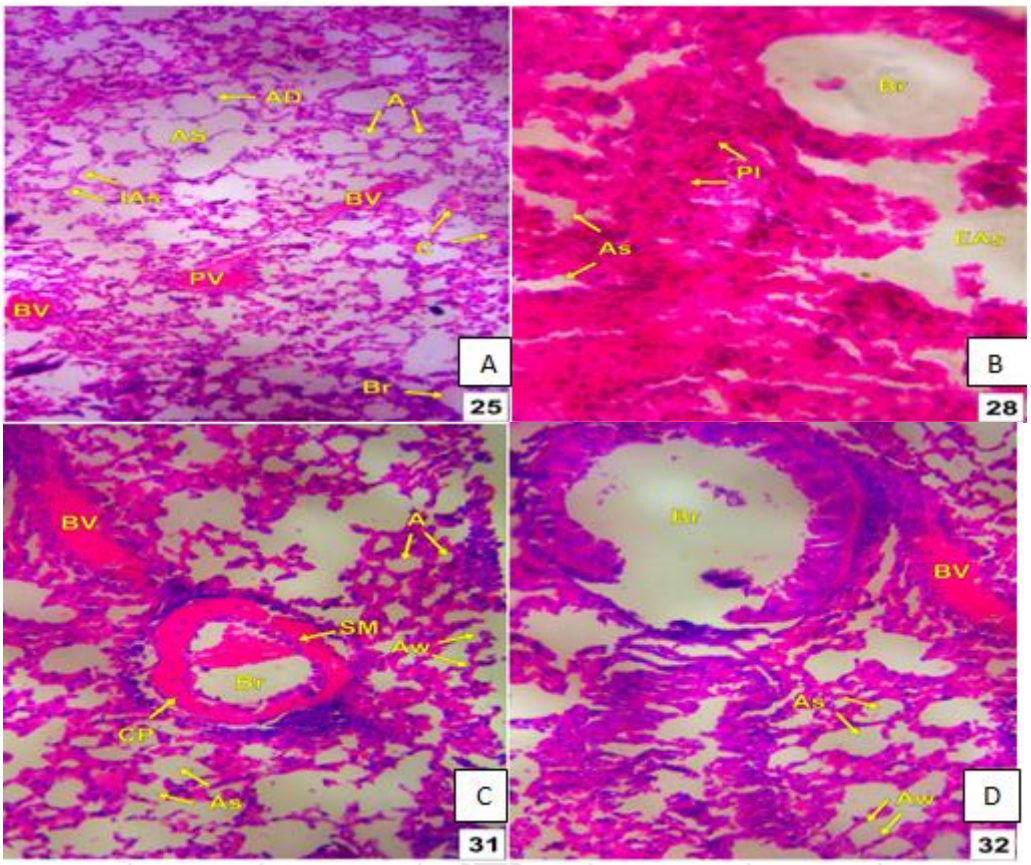
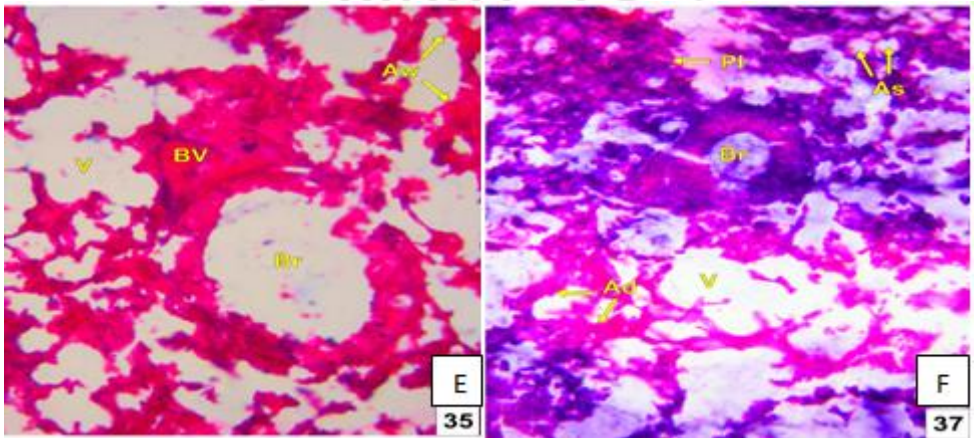


Fig.A: T. S. of control mice lungs exhibiting open alveolar spaces in lung parenchyma showing alveolar sacs (As), alveolar ducts (AD), and numerous oval shaped alveoli (A) with a common interalveolar septum (IAs) between them and capillary (C) plexuses. Bronchial vessels (BV) including pulmonary vein (PV) can be seen in the section X 200.
Fig.B: T. S. of CCl_4 treated mice lungs after 24 hour exhibiting open alveolar spaces (As), enlargement of airspaces (EAs) distal to bronchiole (Br) due to destruction of alveolar wall and parenchymal infiltration (PI) X 400.

Fig.C: T. S. of vitamin C + CCl_4 treated mice lungs after 24 hour exhibiting an interpulmonary bronchus (Br) with hyaline cartilage plate (CP) in its wall. A thin layer of pink smooth muscle (SM) lies between cartilage plate and bronchiolar epithelium. Numerous alveoli (A) with open alveolar spaces (As), thin alveolar wall (Aw) and bronchial vessel (BV) close to a bronchus can be seen in the section X 400.

Fig.D: T. S. of vitamin C + CCl_4 treated mice depicting open alveolar spaces (As), bronchial vessel (BV) close to a bronchus (Br) can be seen. Thin alveolar wall (Aw) withcapillary plexus is seen in the section. No inflammation around bronchus and blood vessel is observed X 400.



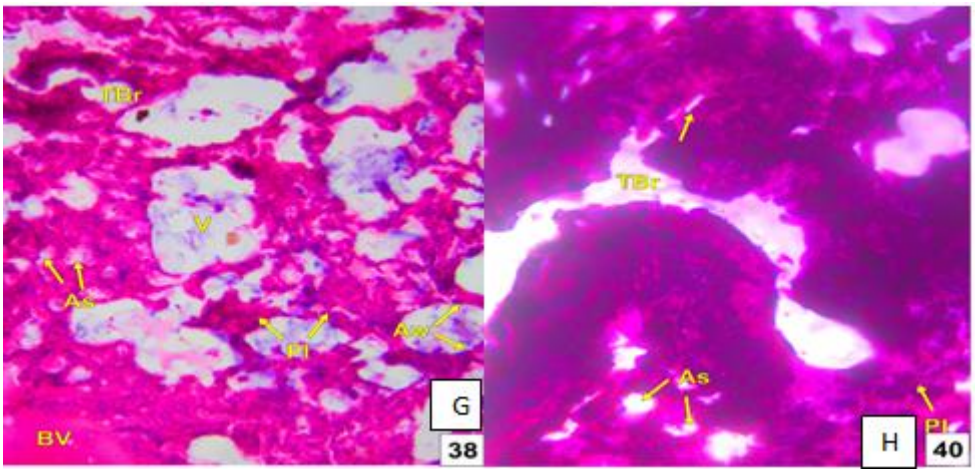


Fig.E: T. S. of CCl_4 treated mice lung at day 11 showing ruptured bronchus (Br) and congestion of blood vessel (BV), vacuolization (V), severe destruction of lung parenchyma, thickening of alveolar wall (Aw) and narrow alveolar spaces were seen in the section X 400.

Fig.F: T. S. of CCl_4 treated mice lung at day 11 depicting severe peribronchial (Br) and perivascular inflammation, parenchymal infiltration (PI), reduced air spaces (As), thickening of alveolar duct (Ad) and cytoplasmic vacuolization (V) X 400.

Fig.G: T. S. of vitamin C + CCl_4 treated mice lung at day 11 exhibiting oval shaped alveoli and terminal bronchiole (TBr) is seen in the section. However, vacuolization (V), parenchymal infiltration (PI) and widening of alveolar duct is also evident at some places X 400.

Fig.H: T. S. of vitamin C + CCl_4 treated mice lung exhibiting terminal bronchiole (TBr), normal oval shaped alveoli with alveolar spaces (As) without any evidence of inflammation. However, at some places little parenchymal infiltration (PI) is also evident X 400.

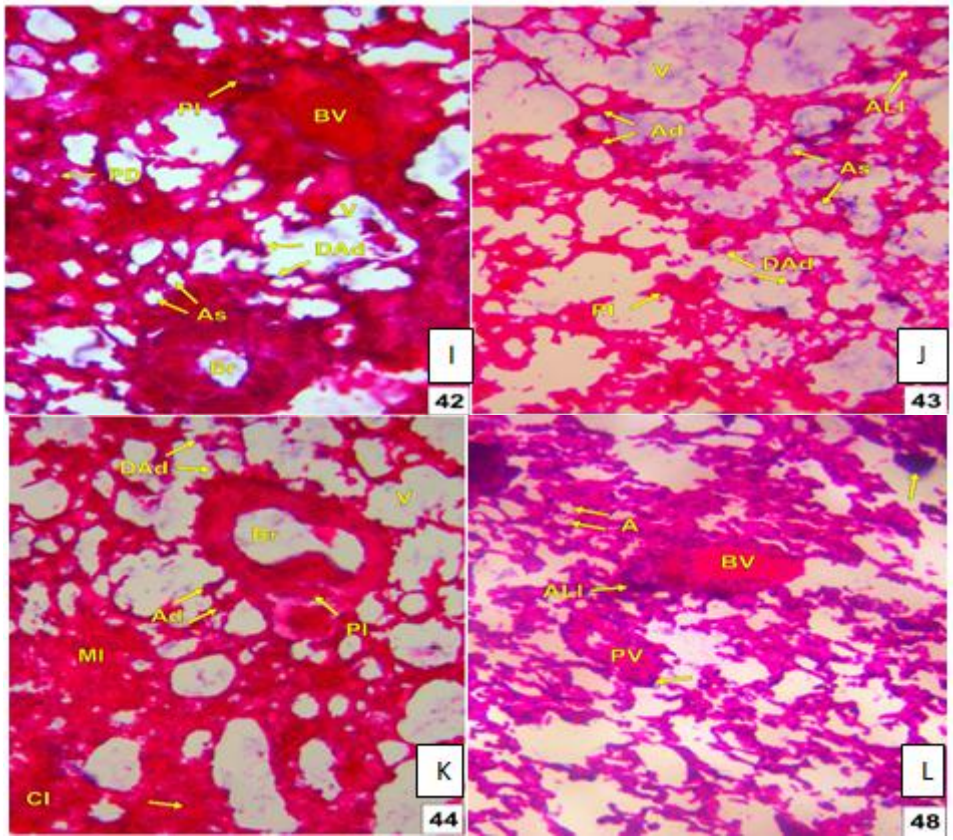


Fig.I: T. S. of CCl_4 treated mice lung at day 21 demonstrating parenchymal infiltration (PI) around bronchiole blood vessel (BV), destruction of large amount of alveolar duct (DAd), reduced air spaces (As), vacuolization (V), parenchymal destruction (PD), inflammatory cells invasion around bronchus (Br) and blood vessel (BV) X 400.

Fig.J: T. S. of CCl_4 treated mice lung at day 21 revealing alveolar lymphocytic infiltration (ALI), reduced air spaces (As), widening and destruction of alveolar duct (Ad), parenchymal infiltration (PI) with mixed neutrophilic and eosinophilic inflammation in parenchyma and cytoplasmic vacuolization (V) due to destruction of alveolar septum X 400.

Fig.K: T. S. of CCl_4 treated mice lung at day 21 exhibiting bronchus (Br) surrounded by parenchymal infiltration (PI), destruction of alveolar ducts (DAd) at many places, vacuolization (V) and infiltration of abundant neutrophils and macrophages (MI) in lung parenchyma. Activated cell infiltrates (CI) including alveolar macrophages associated with inflammatory infiltrations (→) are seen in the alveolar area X 400.

Fig.L: T. S. of vitamin C + CCl_4 treated mice lung exhibiting normal oval shaped alveoli (A) with open alveolar spaces, alveolar lymphocytic infiltration (ALI) around blood vessel (BV), inflammatory infiltrates in the pulmonary vein (PV) are noticed X 200.