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Clinico-patho-physiological changes associated with carbon tetrachloride (CCl4) induced sub-acute hepatotoxicity in rats

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Abstract
CCl4 is widely used to induce hepatotoxicity in animal models although its mechanism of action in relation to changes associated with apoptosis, oxidative stress and APPs are not clearly understood. In present study sub acute hepatotoxicity was induced by CCl4 along with olive oil 1:1 v/v @ 2 ml/kg b wt intra-gastrically twice a week to evaluate changes associated with oxidative, histopathological, apoptotic and relative quantification of haptoglobin (Hp), C-reactive protein (CRP) and alanine aminotransferase (ALT) in liver tissue apart from biochemical changes rat serum. Eighteen randomly selected rats were divided into three groups consisting of healthy control (Gr I), disease control receiving CCl4 for 17 days (Gr II), and CCl4 receiving for 35 days (Gr III). Biochemically, Reduced values of serum total protein, albumin and globulin and increased level of serum ALT, AST, ALP, total and direct bilirubin and glucose were observed in disease control groups. However, decreased level of oxidative stress indices except LPO were noticed in rat liver tissue receiving CCl4. Histopathologically, hepatotoxic degenerative changes along with moderate fatty changes in the hepatocytes, mostly in the centrilobular region in Gr II while cytoplasmic vacuolations of variable size with marginal nucleus in hepatocytes and severe fat globules in Gr III were observed. Increased level of apoptotic and necrotic hepatocytes along with relatively increased level of Hp, CRP and ALT in rat liver tissue exposed to CCl4 till day 18 followed by remarkable decrease in relative level of Hp and CRP by day 36 of study.

Keywords: apoptosis, acute phase protein, carbon tetrachloride, hepatotoxicity, oxidative stress, rats

Introduction
Liver is the major organ for detoxification process (Tamburro, 1979) [41] of xenobiotic compounds such as carbon tetrachloride (CCl4), cadmium, arsenic etc. These compounds predominantly oxidized by microsomal mixed function oxidase (MFO) system along with nicotinamide adenine dinucleotide phosphate-cytochrome phosphate (NADPH-CYP) reductase enzyme system in liver (Zimmerman, 1999) [47]. All liver injury involves co-lateral oxidative/ per oxidative damage of cell. Death of liver cell interrupts mitochondrial electron transport promoting secondary oxidative stress. Increasing evidences (Campos *et al.,* 1989) indicate that reactive oxygen species (ROS) are important mediators in liver injury. A deficiency of hepatic L-glutathione (GSH) and its antioxidant partners and or free radicals species may contribute to the progression of liver disease. CCl4 is widely used to induce hapatotoxicity in animal models (Kujawaski *et al.,* 2009; Pooranaperundevi *et al.,* 2010) [30]. CCl4 metabolism begins with the formation of trichloromethyl free radical (CCl3•) that in the presence of oxygen converted into trichloromethyl peroxy radical (CCl3OO). This radical is more reactive and is capable of abstracting hydrogen from polyunsaturated fatty acids (PUFA) which initiate the process of lipid peroxidation. Role of free radicals in the causation of liver diseases has been well established. Several substances have been known to produce excessive free radicals and thereby produce tissue damage. Since liver is the main organ involved in detoxification of xenobiotics, it is the main target for tissue injury produced by these chemicals and their metabolites. ROS produce deleterious effect on membrane lipids of the cellular components thereby producing peroxidation of lipids, which leads to cell death (Ryter *et al.,* 2007) [33]. Scavengers of free radicals can reduce side effect of these drugs. Therefore, a comparative study with well established methods for diagnosis with serum biochemistry and serum markers of hepatic damage is the need of present scenario.

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Material and Methods
Animals
Male albino wistar rats (200-250 gm) obtained from Laboratory Animal Resource Section of IVRI Izatnagar were kept in experimental animal shed of Division of Medicine with standard managemental condition after approval from the Institutional Animal Ethics Committee of IVRI. Rats were housed in polypropylene cages equipped with feeder and water bottles along with a balanced ration obtained from feed technology unit, IVRI @ 15 gm/rat/day. Fresh drinking water was offered *ad libitum*. The room temperature was maintained at 25 °C ± 2°C and the relative humidity at 55% ±10%, with a 12 hr light/dark cycle.

Study Design
Rats were divided randomly into three groups (n=6) viz. Gr I (healthy control), Gr II (disease control) for 18 days and Gr III (disease control) for 35 days. Sub acute hepatotoxicity was induced in rats by giving carbon tetrachloride (CCl4) analar grade along with olive oil (1:1 v/v) @ 2 ml CCl4 + 2 ml olive oil/kg b wt intra-gastrically twice a week for 35 days (Doi *et al.*, 1991) [10]. Disease control rats of Gr II were humanized on day 18 whereas rats of Gr III on day 36 of experiment as per CPCSE norms to evaluate the changes associated with various diagnostic markers.

Sample Collection
Samples for laboratory examination were collected after sacrificing animals on day 18 and 36 of CCl4 exposer. Blood samples from anesthetized rat were collected by cardiac puncture for haematological study. Serum samples were separated for various biochemical parameters estimation. Liver samples were collected for oxidative stress indices, apoptosis, expression of Hp, CRP and ALT, and histopathological study.

Laboratory Analysis
Serum Biochemistry
Serum alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, total protein, albumin, globulin and glucose were estimated using standard test kit (Span diagnostic Ltd.). Serum globulin level was estimated by subtracting albumin from total protein, and serum albumin globulin (A/G) ratio was estimated by dividing albumin value to globulin value. γ-Glutamyl-transferase (γ-GGT) activity was estimated by Carboxy substrate method using standard test kit (Crest Biosynthesis).

Assays of oxidative stress markers in the liver tissue sample
Estimation of various oxidative stress marker parameters in liver tissue was executed. A double beam UV-VIS spectrophotometer (UV 5704 SS, ECIL, India) was used for recording the absorbance of the test samples.

Preparation of liver homogenates
For oxidative stress indices, 500 mg of liver tissue was taken in 5 ml of ice-cold PBS (pH 7.4). Another 200 mg liver tissue was taken in 2 ml of 20 mM EDTA in distilled water and used for the estimation of reduced glutathione (GSH). The homogenate prepared with homogenizer (IKA, Germany) under ice-cold conditions were centrifuged for 10 min at 3000 rpm and finally supernatant was stored at -20^0C until assayed.

Lipid peroxidation (LPO) assay
The concentration of malondialdehyde (MDA), a reliable marker for lipid peroxidation, was estimated in tissue homogenate following the method suggested by Placer *et al.* (1966) [29].

Reduced glutathione (GSH) assay
The concentration of GSH in tissue homogenates was estimated by 5, 5-dithiobis-(2-nitro- benzoic acid) (DTNB) method as per the procedure of Sedlak and Lindsay (1968) [36].

Glutathione peroxidase
GSH-Px activity in liver homogenate was determined by the method of Paglia and Valentine (1967) [25].

Glutathione -S-Transferase (GST)
The GST activity in liver homogenate was determined according to the standard procedure of Habig *et al.* (1974).

Superoxide dismutase (SOD) assay
SOD activity in liver homogenate was measured by using nitro blue tetrazolium as a substrate after suitable dilution as per the method of Menami and Yoshikawa (1979) [24].

Catalase (CAT) assay
Catalase activity in liver homogenate was estimated by using H_2O_2 as a substrate as per the method of Bergmayer (1983).

Estimation of protein in tissue homogenates
Protein content in liver and kidney homogenates was determined by Lowry *et al.* (1951) [23]. A volume of 980 μl of distilled water and 2.5 ml of alkaline copper sulphate solution (D) were added to 20 μl of tissue homogenate. The reaction mixture was incubated for 10 min at room temperature. Folin-Ciocalteau reagent (0.25 ml) was added and shaken immediately. Whole mixture was again incubated for 30 min at room temperature and finally absorbance was recorded at 750 nm. Standard curve was prepared by using 0.1 mg, 0.2 mg, 0.3 mg to 1mg of bovine serum albumin (BSA).

Apoptosis and necrosis analysis
Preparation of single cell suspension of hepatocytes
Single cell suspension was prepared from freshly isolated rat liver tissue. Briefly, a piece of liver tissue was collected in ice-cold PBS (pH-7.4) and minced into 3 to 4 mm pieces with sterile scalpel. Tissue pieces were re-suspended in PBS and allowed to settle down. Supernatant was removed and washing was done for three times. Tissue pellet was placed on ice and to that 0.25% trypsin in PBS @1 ml of trypsin per 100 mg of tissue was added and incubated at 4^0C for 12 hours for maximum penetration of the enzyme. Trypsin solution was decanted from the tissue pieces and the tissue pieces were incubated with residual trypsin at 37^0C for another 20 minutes. Tissue was re-suspended in PBS and sieved through muslin cloth and centrifuged at 8000 rpm for 10 minutes. The supernatant was discarded and the pellet was collected in micro-centrifuge tube and re-suspended in PBS. Cell density was adjusted to 1.5×10^6 cells/ml (Freshney, 1987) [14].

Flow cytometric analysis of cell death by apoptosis and necrosis
For quantification of apoptotic and dead cells, dual parameter analysis of Annexin V-EGFP detection kit (GenScript,

Centennail Ave., Piscataway, USA) was used (Vermes *et al.*, 1995) [44].

Relative quantification of CRP, Hp and ALT mRNA expression in liver samples
Isolation of total RNA from liver sample
As per the standard protocol of MRC Inc, liver sample was homogenized in TRIzol and transferred to a new DEPC treated microfuge tube and incubated at room temperature for 5 minutes. 200 µl of chloroform was added to the tube, shaken vigorously upside down for 30 seconds, and allowed to stand at room temperature for 15 minutes. The samples were centrifuged at 11000 rpm for 15 minutes at 4^0C. After centrifugation, aqueous phase was transferred to another DEPC treated tube with due care and 0.5 ml of isopropyl alcohol was added to the tube to precipitate the RNA. Tube was placed at room temperature for 10 minutes and again centrifuged at 11000 rpm for 10 minutes at 4^0C. Supernatant was removed immediately by inverting the tube and the pellet was washed with 75% ethyl alcohol. After centrifugation at 6000 rpm for 5 min, the supernatant was discarded very carefully and the RNA pellet was air-dried. The air-dried RNA pellet was reconstituted in nuclease-free water, incubated at 55-60^0C for 10 minutes and stored at -80^0C until

Reverse transcription and synthesis of first strand cDNA from total RNA
Total RNA isolated from liver sample was used for the synthesis of cDNA. The 20 µl reaction mixture containing 5 µg of total RNA, 1 µl of oligo dT primer (0.5 µg/µl) and DEPC treated water to 11 µl. This reaction mixture was incubated at 70^0C for 5 minutes, then 1µl of RNasin Ribonuclease inhibitor (20 units/µl), 2µl of 10 mM dNTP mix, 4 µl of 5X reverse transcriptase buffer were added and incubated for 37ºC for 5 min and finally 2 µl M-MuLV reverse transcriptase enzyme (20unit/µl) was added. Synthesis of cDNA was carried out at 37^0C for one hour in thermocycler. The reaction was stopped by heating the mixture at 70ºC for 10 min and chilling on ice. cDNA was stored at -20ºC till use.

Polymerase chain reaction (PCR) analysis
PCR was performed with the primers as mentioned in table 1 using Thermo Cycler (Techne, U.K. Model 312).

Self designed primers for ALT, CRP, Hp and B Actin

Gene	Primers	Product (bp)
Rat ALT	Forward Primer : 5' GATGCTGAGGTGCAGAAACA 3' Reverse Primer : 5' GCCTCATTGAAGACCTGCTC 3'	203
Rat CRP	Forward Primer : 5' GTCTCTATGCCCACGCTGAT 3' Reverse Primer : 5' CCGTCAAGCCAAAGCTCTAC 3'	226
Rat Hp	Forward Primer : 5' GAAAGGCGCTGTAAGTCCTG 3' Reverse Primer : 5' CCATACTCAGCTACGGCACA 3'	198
Rat B Actin	Forward Primer : 5' AGCCATGTACGTAGCCATCC 3' Reverse Primer : 5' TCTCAGCTGTGGTGGTGAAG 3'	227

Quantitative real-time PCR assay with SYBR Green Mastermix
Quantitative real-time PCR assay was performed with Brilliant® SYBR® Green Master Mix (Stratagene, USA) and Mx3000P spectrofluoremetric thermal cycler operated by MxPro™ QPCR software.

Determination of relative quantification of mRNA of CRP, Hp and ALT gene
Relative quantification of real time PCR product was performed using the comparative $\Delta\Delta C_T$ method and SYBR green florescent labeling (Pfaffl, 2001). The results of Real time PCR were depicted as fold change of CRP, Hp and ALT mRNA level in liver sample of disease control and treatment, with healthy rats.

Histopathological study
Liver tissues of rats were fixed with 10% neutral buffer formalin for histopathological examination. The lesions in different sites of liver were graded by developing histopathological score system (HPS) (Culling, 1963). The liver sections were looked for pathological changes and graded on scale at 0-3 (0 = No change, 1 = mild change, 2 = moderate change, 3 = severe change). The maximum score was taken as most severe inflammatory changes.

Statistical analysis
Results were expressed as mean (±) standard deviation. Thereafter, one-way ANOVA was applied, followed by Tukey's test using the SPSS (Statistical Package for the Social Sciences) version 19. A difference between means with $P<0.05$ was considered significant.

Results
Biochemical parameters *viz* serum ALP, AST and ALT showed significant ($P<0.05$) difference among Gr. I, II and III. Significantly ($P<0.05$) increased level of ALP, AST, ALT, total and direct bilirubin and glucose were observed in groups II and III as compared to group I indicating sub acute hepatotoxicity. Significantly ($P<0.05$) decreased level of serum total protein, albumin, direct bilirubin and glucose were observed in group II and group III confirming sub acute hepatotoxicity as compared to healthy control group (I) (Table 1).

Table 1: Biochemical profile in rat serum

Parameters/Groups	Group I	Group II	Group III
ALT (U/L)	39.62±4.04 [a]	286.91±33.96 [b]	201±20.72 [c]
AST (U/L)	158.90±11.09 [a]	499.67±18.05 [b]	356.88±39.98 [c]
ALP (U/L)	84.92±24.58 [a]	462.43±17.19 [b]	238.11±20.15 [c]
Total protein (g/dl)	7.33 ±0.62 [b]	5.96±0.58 [a]	5.98±0.58 [a]
Albumin (g/dl)	4.46±0.12 [b]	3.75±0.51 [a]	3.67±0.33 [a]
Globulin (g/dl)	2.87±0.23 [b]	2.29±0.20 [a]	2.31±0.24 [a]
A G ratio	1.55±0.21 [b]	1.63±0.24 [b]	1.08±0.13 [a]
Total bilirubin (mg/dl)	0.34±0.02 [a]	0.52±0.05 [b]	0.46±0.02 [b]
Direct bilirubin (mg/dl)	0.20±0.01 [a]	0.37±0.09 [b]	0.34±0.02 [b]
Glucose (mg/dl)	63.69±3.88 [a]	99.93±10.54 [b]	95.45±13.52 [b]

Values (mean ± SE of 06 rats) bearing no common superscript vary significantly at $P<0.05$.

Oxidative stress indices like LPO revealed significant (P<0.05) variation among groups. LPO was found significantly (P<0.05) higher in groups II and III as compared to group I. SOD, Catalase, GSH and GPx showed significantly (P<0.05) decreased level in group II and III as compared to group I. However, between the group II and III no significant variation was observed in SOD, Catalase and GSH activity (Table 2).

Table 2: Effect of CCl4 on oxidative stress indices in rat liver tissue

Parameters/Groups	Group I	Group II	Group III
LPO (nmol/mg protein)	4.18±0.40[a]	7.65±0.55[b]	9.56±1.43[c]
SOD (U/mg protein)	3.30±0.04[b]	2.09±0.04[a]	1.76±0.15[a]
Catalase (U/mg protein)	12.04±0.28[b]	6.91±0.87[a]	5.62±0.65[a]
GSH (µmoles/mg protein)	3.25±0.71[b]	1.98±0.15[a]	1.69±0.60[a]
GST (µmoles/mg protein)	3.37±0.21	2.45±0.21	2.70±0.68
GP_X (U/mg protein)	80.02±1.70[c]	64.59±2.20[b]	49.80±1.31[a]

Values (mean ± SE of 06 rats) bearing no common superscript vary significantly at P<0.05.

Significantly (P<0.05) increased levels of apoptotic hepatocytes were present in CCl_4 exposed Gr. II (41.98±2.07) and Gr. III (49.54±1.64) as compared to healthy Gr. I (3.98±0.17) and highest significant increase was observed in the liver of Gr III.

Fold expression of Hp, CRP and ALT in rat liver tissue calculated by $\Delta\Delta C_t$ (Pfaffl, 2001) method revealed remarkable higher level in CCl_4 exposed rats on day 18 (Gr. II), however, results on day 36 revealed remarkable decreased expression of Hp and CRP while increase expression of ALT gene. Study depicts gradual decrease in Hp and CRP with increase in ALT mRNA expression during sub-acute chronic hepatotoxicity of rat induced by CCl_4 confirming establishment of hepatotoxicity (Table 3).

Table 3: Relative quantification of Hp, CRP and ALT in rat liver tissue

Parameters/Groups	Group I	Group II	Group III
Hp	X	27.38	0.65
CRP	X	3.18	0.21
ALT	X	3.83	46.56

On gross examination of livers of rats treated with CCl_4 revealed congested appearance with scattered yellow and whitey areas attributed to fatty and necrotic changes as compared to healthy rats liver although the gross changes were mild in CCl_4 receiving rats for 17 days. Healthy section of rat liver (group I) showed normalcy of hepatic cord and failed to reveal any specific pathological changes. Section of rat liver in Gr II revealed hepatotoxic degenerative changes as well as moderate fatty changes in hepatocytes
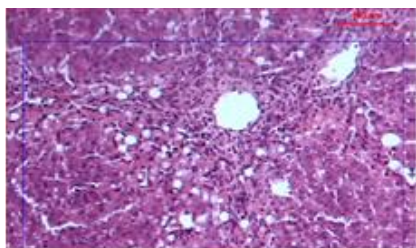

Fig 1a: CCl_4 receiving group for 17 days (Gr II)

showing mild to moderate fatty changes, cytoplasmic vacuolation, marginal nucleus in hepatocytes, marked thickening of central vein with infiltration mononuclear cells. H & E x100 characterized by cytoplasmic vacuolations of variable sizes pushing the nucleus to marginal sites. The changes were mostly confined to centrilobular regions (Fig 1a). Liver section in Gr. III revealed cytoplasmic vacuolations of variable sizes with marginal nucleus in hepatocytes. Affected cells contained severe fat globules either in the form of small droplets or mixture of small and large droplets (Fig 2b). Histopathological study clearly indicates establishment of sub-acute hepatotoxicity in both the CCl_4 induced groups of rat.
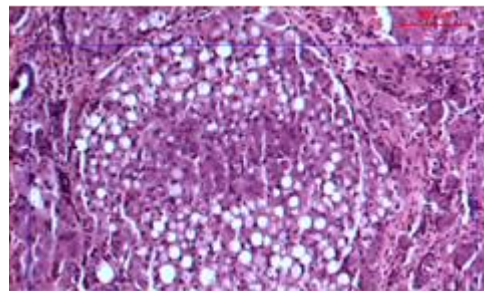

Fig 2b: Gr III showing severe fatty changes, cytoplasmic vacuolation marginal nucleus, hepatocytes with infiltration of, mononuclear cells and degenerative changes. H & E x1000.

Discussion

Carbon tetrachloride (CCl_4) is one of the oldest and most widely used chemical agent for experimental induction of hepatotoxicity in laboratory animals (Tsukamoto et al., 1990). A number of investigators utilized this chemical to produce liver cirrhosis in experimental animals (Parola et al., 1992; Shahani, 1999; Soja, 2003). The hepatotoxicity induced by CCl_4 is due to its metabolite CCl_3, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Bishayee et al., 1995). CCl_4-induced reactive free radicals initiate cell damage through two different mechanisms of covalent binding to the membrane proteins and cause lipid peroxidation (Parola et al., 1992). Hepatocellular necrosis leads to elevation of the serum marker enzymes, released from the liver into blood (Shenoy et al., 2002). The increased levels of ALT, AST, ALP and serum bilirubin are conventional indicators of liver injury (Achliya et al., 2003).

Serum ALT, AST, ALP and direct bilirubin level in CCl_4 receiving rats (Gr. II and Gr. II) were significantly (P<0.05) higher as compared to healthy groups owing to release of cytosolic enzyme into blood stream due to liver cell plasma membrane damage and loss of functional integrity of cell membrane in liver (Kazeem, et al., 2011). The increased levels of serum ALT and AST are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman and Lowhorn 1978). Hepatocellular necrosis leads to elevation of the serum marker enzymes, released from the liver into blood (Shenoy et al., 2002). Increased levels of serum ALT, AST, ALP and bilirubin are conventional indicators of liver injury (Achliya et al., 2003). Rao and Mishra (1997) reported the increased level of serum total and direct bilirubin in acute hepatic injury due to CCl_4 toxicity, supporting the present findings. Significantly (P<0.05) lower level of serum albumin, and increased level of

glucose were observed in CCl_4 receiving groups (Gr II and Gr III) as compared to healthy group in the present study. Hypoalbuminemia and hypoproteinemia are the useful index for severity of hepatocellular damage (Aniya *et al.*, 2005) as noticed in present study.

Biological effects of oxidative stress indices are based on a highly regulated equilibrium between the production of reactive oxygen species (ROS) and the counteracting intrinsic cellular defense mechanism of antioxidant scavenging system (Hollan, 1995). These reactive oxygen species can induce degenerative changes by oxidation of protein, peroxidation of lipids and damage to nucleic acids (Freeman and Grapo, 1982; Doelman and Bast, 1990). One of the principle causes of CCl_4-induced liver injury is formation of lipid peroxides by free radical derivatives of CCl_4 (CCl_3). Carbon tetrachloride induced adverse changes are evident from the decreased hepatic antioxidant enzyme ability *viz.*, SOD, GP_X followed by GSH of the present study. The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, catalase and GP_X. These enzymes constitute a mutually supportive team of defense against ROS (Bebe and Panemangalore, 2003). In CCl_4-induced hepatotoxicity, the balance between reactive oxygen species (ROS) production and these antioxidant defenses may be lost resulting in 'oxidative stress', which through a series of events deregulates the cellular functions leading to hepatic necrosis. In our study, significant ($P<0.05$) elevation of lipid peroxidation in the liver of rats receiving CCl_4 (Gr. II and Gr. III) reflects membrane damage and alterations in structure and function of cellular membranes. The increase in malondialdehyde levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals (Shenoy *et al.*, 2002). The significantly ($P<0.05$) reduced activities of SOD, catalase, GSH, GST and GPX in group receiving CCl_4 (Gr. II and Gr. III) point out the hepatic damage (Kazeem *et al.,* 2011). Regarding non-enzymic antioxidants, GSH is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals, including CCl_4 (Hewawasam *et al.*, 2003).

Apoptosis describes an intrinsic cell suicide program that may be activated by both endogenous and exogenous stimuli. Specific morphological features including chromatin condensation, nuclear fragmentation, cell shrinkage, membrane blabbing characterize this method of cell death and the formation of membrane-bound vesicles termed apoptotic bodies. In our study, significant ($P<0.05$) increment in hepatic apoptosis were present in CCl_4 exposed groups as compared to healthy group. Apoptosis occurs naturally in the liver and increases in specific pathogenic processes (Ward *et al.,* 1998). Alcohol feeding resulted in a 4.5-fold increase in apoptosis of hepatocytes compared to pair-fed control rats (Carmody, *et al.,* 2000). Active oxidants produced during ethanol metabolism mediate fragmentation of DNA in hepatocytes, and that intracellular antioxidants such as glutathione play a critical role in the cytoprotective mechanisms of hepatocyte against lethal cell death, *i.e.*, apoptosis, induced by ethanol (Kurose *et al.,* 1996) [21] as in present study with CCl_4. Pagliara *et al.* (2003) showed *in vivo* hepatic apoptosis induced by $Pb(NO_3)_2$ administration and recorded reduced glutathione in hepatocytes.

Acute phase protein is part of the non-specific first line of defense against microbes and plays a critical role in the host defense mechanisms, promoting the clearance of invading particles and modulating the immune response against them (Fey and Gauldie, 1990; Gaby and Kushner, 1999). The proteins acting as acute phase proteins differ from humans to animals and from one species to another. In rat, α1-acid glycoprotein (α1AGP), α2-macroglobulin (α2M), haptoglobin and thiostatin (Fey and Gauldie, 1990) are among the major acute phase proteins, whereas C-reactive protein, predominant in humans, does not partake (Giffen *et al.,* 2003). Gene expression for Hp and CRP in rat liver tissue exposed with CCl_4 revealed remarkable higher level on day 18 (Gr. II) depicting clear indication of inflammatory changes due to induction of sub acute hepatotoxicity. However, results on day 36 revealed remarkable decrease in the values of Hp and CRP. Some initiate or sustain the response while others have tissue-protective or anti-inflammatory actions (Gaby and Kushner, 1999). The animals with high levels of APR at the start of CCl_4 treatment developed a more severe degree of fibrosis and cirrhosis than the control group in which no acute phase reaction was induced. C-reactive protein is a strongly positive acute phase protein in the mouse, but is not found in the rat. The changes in the synthesis rates of acute phase proteins during inflammation are closely reflected by corresponding changes in intracellular mRNA levels. In the liver, the capacity to induce the acute phase pattern of synthesis and secretion of plasma proteins probably develops around birth. Changes in mRNA levels are brought about by changes in transcription rates or by changes in mRNA stability. Kinetics of mRNA changes during the acute phase response differ for individual proteins (Schreiber *et al.,* 1989). Changes in the levels of APR largely reflect altered production by hepatocytes (Kushner *et al.,* 2010).

The elevation of ALT, regarded as an indicator of liver damage based on the presumption that ALT protein is specially and abundantly expressed in the liver. ALT has been used as a marker for liver injury in humans and in preclinical toxicity studies. Serum ALT activity is significantly elevated in a variety of liver conditions including viral infection, cirrhosis, non-alcoholic steatohepatitis, and drug toxicity. ALT serves as a major marker for liver damage attributable to its abundant expression in liver (Doufur *et al.,* 2000; Sherman *et al.,* 1991). In present study, consistent increase in ALT expression during sub-acute to chronic hepatotoxicity (from day 18 to 35) of rat induced by CCl_4 suggests establishment of liver damage. Yang *et al.* (2007) reported the elevated level of ALT expression in rat liver tissue by CCl_4 induced hepatotoxicity.

Gross examination of CCl_4 treated liver of rat revealed congested appearance with white area attributed to fatty and necrotic changes (Ramdas, 2010). Administration of CCl_4 in rats for 17 days (Gr. II) produced variable degree of hepatotoxic degenerative changes as well as moderate fatty changes in hepatocytes characterized by cytoplasmic vacuolations of variable sizes, pushing the nucleus to marginal sites. Liver section in rats given CCl4 for 35 days (Gr. III) revealed severe hepatotoxic degenerative changes, cytoplasmic vacuolations of variable sizes with marginal nucleus in hepatocytes and mixture of small and large droplets. Doi *et al*. (1991) [10] has postulated post necrotic fibrosis and fatty changes in rats on the 4th week itself treated by CCl_4 along with olive oil (1:1 v/v) @ 4ml/kg.b.wt. p.o twice a week for 12 weeks. Hepatic damage produced by CCl_4 is characterized by fatty changes, centrilobular necrosis,

hydropic degeneration and cirrhosis (Shirwaikar *et al.,* 2002; Venukumar and Latha, 2002). The above findings are in concurrent with present findings. In conclusion apoptotic and oxidative changes along with acute phase protein play a vital role in the pathogenesis of CCl₄-induced sub-acute hepatotoxicity as evidenced by the present study and further investigation can be conducted for therapeutic measure considering the changes involved in the pathophysiology in liver diseases.

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