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Research Article

INHIBITION OF NADPH OXIDASE ATTENUATES INFLAMMATION AND CELLULAR SENESCENCE AT EARLY PHASE OF LIVER INJURY IN MICE

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The aim of this study is to evaluate differential expression of different isoforms of NOX in early phase of liver injury and its mechanism to develop inflammation and cellular senescence.

C57BL/6 mice were treated with carbone tetra chloride (CCl_4) (0.2 and 0.5 ml/kg) thrice in a week by ip route to develop a acute liver injury models and Di phenyl indinium chloride (DPI) is injected 30 min prior to CCl_4 injection. Batches of mice were sacrificed after 3rd injection to evaluate the role of isoforms of NADPH oxidase. Liver histology, immunohistochemistry, b-galactosidase activity, Western blotting, Real Time PCR were performed.

Among different isoforms of NOX in the liver NOX2 is expressed in significantly ($p<0.001$) high amount in response to CCl_4 treatment mainly in kupffer cells resulting in inflammation and subsequently cellular senescence. Administration of a NOX inhibitor (DPI) resulted in marked reduction the NOX expression, liver injury and inflammation which ultimately reduce cellular senescence.

As mainly the expression of phagocytic NOX2 is high in early phase of liver injury resulting inflammation and senescence NOX2 may be a therapeutic target to reduce early phase of liver injury.

INTRODUCTION

Liver is the most important organ of our body which plays important role in metabolic homeostasis. Taub R *et; al* (2004) and regenerative capability which can protect against liver injury and mass loss. Higgins GM *et al*.; (1931) Due to the potential ethical issues in conducting liver disease research directly in human beings, people are trying to conduct a study that is difficult to perform in humans using appropriate animal models which helps to develop new treatment modalities. Blumberg BS *et al*.; (1985) and Mullen KD *et al*.; (1989). Carbon tetrachloride (CCl_4) is a well known hepatotoxin that is injected to the different laboratory animal including mice to induce acute liver injury Rao PS *et al*.; (1987). The acute hepatotoxicity of CCl_4 occurs through its biotransformation by the cytochrome P450 oxygenase system of the endoplasmic reticulum, which ultimately causes oxidative stress and membrane damage.

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Masuda Y *et al*.; (2006) and Basu S *et al*.; (2011) CCl_4 intoxication results necrosis of hepatocytes and development of fatty liver which mimics the acute hepatitis and subsequently followed by infiltration of different inflammatory cells in the injured zone. Novobrantseva T *et al*.; (2005)

When ROS levels exceed oxidative stress is generated which leads to injury and death by binding with DNA, proteins, and lipids and also regulate signal transduction pathways directly or indirectly by altering the cellular redox state. Czaja MJ *et al*.; (2010) In additional to the exogenous source of ROS there are two major sources of endogenous ROS-the multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzyme complexes and the mitochondrial respiratory pathway. Block K *et al*.; (2012) Study showed that another isoforms of NOX (DUOX2), mainly expressed in type II alveolar epithelial cells (AECs) in the lungs was critical in hyperoxia-induced acute lung injury in mice and cell death by activation of ERK and JNK mediated signalling pathways in mouse lung during hyperoxia. Kim MJ *et al*.; (2014). Moreover elevated expression NOX2 results in activation of nuclear factor-kappa B (NF-kB) predominantly in microglia/macrophages which results reduction in reactive

oxygen species production of myeloid cells and protected neurons from oxidative damage. NOX2 knockout mice also showed suppression in the M1 "pro-inflammatory" profile of microglia/macrophages and increased the M2 "anti-inflammatory" profile in the injured brain and down-regulation of the NF-kB pathway. Wang J *et al*.; (2017). In response to acute liver injury there is high expression of several proinflammatory cytokines which in turn results infiltration of inflammatory cells. Chemokine expression by dead hepatic parencymal cell generates a chemoattractant gradient which promotes infiltration of different inflammatory cell such as monocytes, macrophages, Natural killer (NK cells) cells, Natural killer T cells (NKT cells), neutrophils, B cells, and T cells. The activities of the cells are highly regulated by the specific chemokine profiles within the liver. Saiman Y *et al*.; (2012). NADPH oxidase plays an important role in radiation-induced oxidative stress in brain with a significant up-regulation of mRNA and protein expression levels of Tumour necrosis factor α (TNF-α) and Monocyte chemoattractant protein 1 (MCP-1). Cho HJ *et al*.; (2017).

NOX1 and NOX2 are expressed in Hepatic stellate cells (HSCs) and deficiencies of NOX1 or NOX2 decrease liver inflammation in the carbon tetrachloride (CCl_4) and bile duct ligation (BDL) models. Cui W *et al*.; (2015) and Paik YH *et al*.; (2017). On the contrary, NOX4, a nonphagocytic NOX homolog is expressed in hepatocytes, stellate cells, and endothelial cells of the liver, and is different from the other NOX isoforms as it does not require any cytosolic structural subunits to form the active enzyme to produce ROS. Reiner R *et al*.; (2005).

Cellular senescence is a stable form of cell-cycle arrest that may limit the proliferative potential of premalignant cell. Campisi J *et al*.; (2007) Studies show that in response to liver injury, some amounts of senescent cells were found which are detected by senescent associated β-galactosidase (SA-β-gal) activity. Krizhanovsky V *et al*.; (2008). It has been also found that Diphenyleneiodonium Chloride (DPI), NADPH oxidase inhibitor prevents early alcohol-induced liver injury in the rat. Kono H *et al*.; (2001)

However, the role of different NOX isoforms expressed in different cell types in the liver in acute phase of hepatic injury and inflammation and its role to develop the cellular senescence is still lacking. In this study, we report for the first time the role of NOX in the initiation of liver injury, inflammation and senescence in acute models induced by CCl_4, which is a well-known hepatotoxic industial solvent

MATERIALS AND METHODS

Animal and treatment

Adult (date 8 - 10 week) male wild type C57BL/6 mice having body weight between 26-30 gm were purchased from National Centre of Laboratory Animal Sciences (NCLAS, Hyderabad, India) and were housed in a temperature and light controlled animal house facility at Institute of Post Graduate Medical Education and Research, Kolkata. The mice were maintained on commercial mouse chow containing 20% protein (NCLAS) and water *ad libitum*. The study was approved by institutional animal ethics committee (Approved No -IAEC/AC-10/2010/UCM-71). All procedures were performed as per the guidelines of the animal ethics committee. To develop liver injury, mice were treated thrice a week with intraperitoneal (i.p.) injection of CCl_4. Mice were given the first injection at a dose of 0.2ml/kg to sensitize the drug followed by two injection at a dose of 0.5ml/kg body weight of CCl_4 (Merck, India; diluted 1:5) in corn oil (Sigma, U.S.A). The control mice received equal volume of corn oil as per the schedule of the CCl_4 exposed mice. All the mice belonging to both control and CCl_4 treatment groups were sacrificed 72 hours after the last dose of CCl_4 injection.

In some selected experiments, the mice were treated with DPI (Sigma, U.S.A) at a dose of 10 mg/kg body weight 30 minutes prior to CCl_4 administration thrice a week.

Blood and liver tissues samples were collected during sacrifice. Blood was obtained by cardiac puncture and the serum samples were stored at -20°C for assessment of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The mice liver was removed, rinsed with phosphate buffered saline (PBS), and divided into four portions: (a) fixed in 10% formalin and embedded in paraffin; (b) homogenized in appropriate buffers and aliquots frozen at -80°C for biochemical assays and protein measurement; (c) placed in RNAlater (Sigma) solution for RNA expression study and (d) snap frozen at -80°C for cryosection.

Assessment of Hepatic Injury

Serum ALT and AST activities were measured using a semi auto autoanalyser (Robonic, India) with a commercial kit (Siemens, India) according to the manufacturer's instruction. Formalin-fixed liver tissues were processed and stained with Hematoxylin and Eosin (H & E; Sigma, U.S.A) for the determination of degree of liver injury Nanji AA *et al*.; 1994 and Goodman ZD *et al*.; (2007). To detect fat deposition, frozen sections from the livers were further processed for Oil O-Red staining and evaluated. Liver pathologic characteristics were scored in a blind manner as follows: steatosis (the percentage of liver cell containing fat), <25% = 1+ ;<50% = 2+; <75% = 3+, >75% = 4+, inflammation, <2 foci per 40x = 1+; 2 to 4 foci per 40x = 2+; >4 foci per 40x = 3+.

Hepatic biochemical assays

A 10% liver homogenate was used for determination of protein content using Bradford reagent (Sigma, U.S.A) spectrophotometrically Bradford MM *et al*.; (1976). Cellular glutathione (GSH) and oxidised glutathione (GSSG) content in liver were measured enzymatically at 412 nm according to the method as described Tietze F *et al*.; (1969) and Griffith OW *et al*.; (1980) respectively. Lipid peroxidation was assessed by their content of Thiobarbituric Acid Reactive Substances (TBARs) Slater T *et al*.; (1971). Activities of hepatic Catalase, Glutathione peroxidise (GPx), Superoxide dismutase (SOD) and NADPH oxidase (NOX) were also determined according to the method as described respectively Beers RF *et al*.; (1952), Paglia DE *et al*.; (1969), Nandi A *et al*.; (1988), and Herrera B *et al*.; (2004).. Cytochrome P450 2E1 (CYP2E1) activity was measured from the microsomal fraction prepared by differential centrifugation of the liver homogenates by assaying the oxidation of p-nitrophenol to p-catechol Reinke LA *et al*.; (1931).

Assesment of Senescence

β-gal Activity: The detection of senescence associated-β-galactosidase (SA-β-gal) activity was performed as described by previous study. Yun Lee BY *et al*.; (2006) The frozen liver

tissue sections were fixed with 0.5% gluteraldehyde in PBS for 15 min, washed with PBS supplemented with 1 mM MgCl2, and then stained for 5-6 hours in PBS containing 1 mM MgCl2, 1mg/ml X-Gal, and 5 mM of each potassium ferricyanide and potassium ferrocyanide. The sections were counterstained with Eosin and examined under light microscope.

Immunohistochemistry

Immunohistochemistry in the liver sections was performed as previously described. Heiss EH *et al*.; (2011) using fluorescent tagged antibodies against P21 (sc-397; Santa Cruz biotechnology, U.S.A), P53 (sc-6243; Santa Cruz biotechnology, U.S.A) and C-C motif chemokine ligand 5 (CCl5) (sc-373984; Santa Cruz biotechnology, U.S.A) from the deparaffinised liver sections and slide were captured by Leica TCS SPE confocal microscope.

Western blot analysis

Western blots were performed as previously described method. Xue W *et al*.; (2007) Forty micrograms of protein was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5% SDS-PAGE) gel and transferred onto PVDF membranes (Thermo Fisher Scientific, U.S.A). The blots were then probed with the following antibodies: mouse monoclonal anti NOX1 (sc-25545; Santa Cruz Biotechnology, U.S.A), NOX2 (sc-74514; Santa Cruz Biotechnology, U.S.A), NOX4 (sc-30141; Santa Cruz Biotechnology, U.S.A), IL-6 (sc-1265; Santa Cruz Biotechnology, U.S.A), 1L-12 (sc-7926; Santa Cruz Biotechnology, U.S.A), CCl5 (sc-373984; Santa Cruz Biotechnology, U.S.A) and mouse monoclonal anti beta actin (sc-47778; Santa Cruz Biotechnology, U.S.A). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:1000) (Thermo scientific) was used as secondary antibody. Blots were developed using the enhanced chemiluminescence immunoblot detecting reagent (Thermo Scientific, U.S.A).

Isolation of mouse hepatocytes, stellate cells and kupffer cells

Hepatocytes were isolated from overnight-fasted male C57BL/6 mice by collagenase perfusion as described previously. Pertoft H *et al*.; (1987). Hepatocytes were resuspended in DMEM containing 2 mM L-glutamine, 10% fetal bovine serum, 100 nM insulin, 100 nM dexamethasone, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell viability was greater than 97%, as determined by trypan blue exclusion test. Cells were seeded at a density of 1×10^6 cells in DMEM.

Hepatic stellate cells (HSCs) and Kupffer cells (KCs) were isolated from C57BL/6 mice by in situ liver perfusion with collagenase and pronase, followed by density gradient centrifugation with Nycodenz according to published protocol. Bataller R *et al*.; (2003) Cell viability (95%) was assessed by trypan blue exclusion test. Nieto N *et al*.; (2001) HSCs and KCs were incubated overnight in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Reverse-transcription PCR (RT-PCR) and real-time quantitative PCR (qRT-PCR)

RNA was extracted from liver tissues as well as from hepatocytes, KCs and HSCs using TRIzol® Reagent (Invitrogen). A high-capacity cDNA reverse transcription kit (Applied Biosystems) was used to generate cDNA from extracted RNA. qRT-PCR was carried out on cDNA using primer sets (Table-1) and SYBR® green PCR master mix (Applied Biosystems) on an ABI prism 7500 sequence detection system according to the manufacturer's instructions. Data were normalized against the expression of β-actin.

Table 1 List of Primers used in this study

Genes	Primers sequence
NOX1	Forward- 5'-GTGACAAGTACTATTACACGAGA -3'
	Reverse- 5'-GATATATGCCACCAGGTTATGGA -3'
NOX2	Forward- 5'-AACTGTATGCTGACTCCTGTGC-3'
	Reverse-5'-TTCTCATTGTCACCGATGTCAG-3'
NOX4	Forward- 5'-TTAGGAGTCACTGAACTA-3'
	Reverse-5'-TGACTGAGGTACAGCTGGA-3'
Il-6	Forward- 5'-CCATCTGGCTAGGTAACAGA-3'
	Reverse-5'-CCAATGCTCTCCTAACAGAT-3'
IL-12	Forward- 5'- TGGAAGCACGGCAGCAGAA-3'
	Reverse-5-TGCGCTGGATTCGAACAAAG-3'
CCl5	Forward- 5'-GGTACCATGAAGATCCTCTGCA-3'
	Reverse-5'-AAACCCTCTATCCTAGCTCAT-3'
IL-17	Forward- 5'-CCTCCCGAAGCCCTCAGA-3'
	Reverse- 5'-TTCCGGCTGGAGAAGCA-3'
MCP1	Forward-5'-GCTCAGCCAGATGCAGTTAA-3'
	Reverse- 5'-GCTCAGCTTGGTGACAAAAACT-3'
CCl3	Forward-5'-TCTCCAGCGCCATATGGAGCT-3'
	Reverse- 5'-TTCCGGCTGGAGAAGCA-3'
β-actin	Forward 5'-TGGAATCCTGTGGCATCCATGAAAC-3'
	Reverse 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS software (SPSS 16 for windows; SPSS Inc., Chicago, IL). Inter group differences were analyzed using student's t test. p value of < 0.05 was considered statistically significant.

RESULTS

Liver injury associated with oxidative stress and inflammation

Liver sections were stained with H&E to determine levels of fatty infiltration and inflammation in the parenchyma and portal ducts after 3 doses of CCl_4 treatment in a week. Histopathological examinations of liver specimen's revealed massive necrosis and infiltration by inflammatory cells in mice treated with CCl_4 as evidenced by H&E stain (Fig 1Aib) mainly in the periportal region of the hepatic lobule. Accumulation of cytoplasmic lipid droplets was also found in CCl_4 treated mice liver. Steatosis was further confirmed by Oil Red O staining (Fig 1Aiib). Histopathological examination of control mice however, revealed normal architecture (Fig 1Aiia)

Liver injury due to CCl_4 intoxication was further supported by a marked increase in the levels of serum liver enzymes (AST and ALT) in mice treated with CCl_4 where serum ALT showed a 75-fold increase and AST showed a 60-fold increase in their level in comparison to the control group (Fig 1Bi).

Inflammation is a common outcome of early phase of liver injury. Liver injury may activate the KCs, the resident macrophages resulting in release of ROS and secretion of an array of cytokines (chemokines) that recruit other potentially cytotoxic inflammatory cells, therefore we checked expression of various chemokines such as MCP1, interlukin -17 (IL-17) and C-C motif chemokine ligand (CCl3) at mRNA level from CCl_4 treated liver because these chemokines play an important

role in the recruitment of several inflammatory cells and up regulation of the different proinflammatory cytokines. Real time PCR revealed significant increase of the expression of all the chemokines after 1 week of CCl_4 treatment in the liver tissue (Fig 1B ii). Apart from the above mentioned chemokines, real time PCR and western blot analysis also revealed high expression of IL-6, IL-12 and CCl5 in the whole tissue at 1st week CCl_4 treatment group as compared to the control group (Fig 1C i and ii).

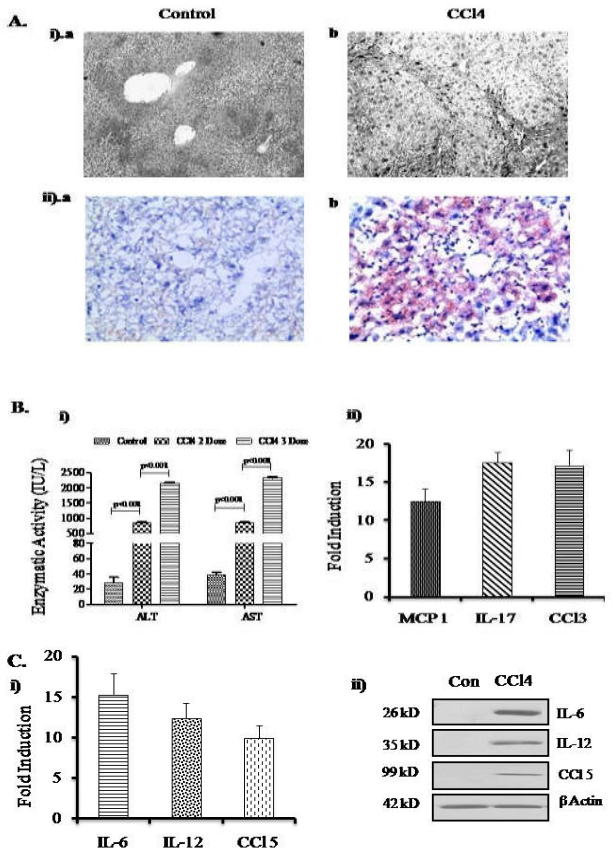


Figure 1 Early liver injury is associated with oxidative stress and inflammation: Ai) Hematoxylene & Eosin stain was performed in the paraffin embedded liver section and (Aii) Oil O Red stain was performed from frozen liver section of both control and CCl_4 treated mice to evaluate inflammation and steatosis. Original magnification 10X for Ai and 40X for Aii. B) (i) Biochemical markars of liver injury by means of ALT and AST value in control as well as two different doses of CCl_4 injected mice to confirm acute liver injury. (ii) mRNA expression of different chemokines level in liver tissues after 1 weeks CCl4 exposure. C) (i) mRNA expression by RT PCR and (ii) protein expression by western blot of different cytokine and chemokines level in liver tissues post CCl_4 treatment. β-actin was used as in house control.

GSH is the most abundant non protein antioxidant in cells and is important in maintaining proper cellular redox balance and protection of cells against stress and injury. We further assessed hepatic GSH and GSSG content in mice exposed to CCl_4. Results exhibited 30% GSH depletion and near about 2 folds increase of GSSG in CCl_4 treated mice as compared to the control group (Table-2).

Table 2 Several anti-oxidant emzymes were measured biochemically in control as well as in the CCl4 treated group. #p<0.001 vs Control

Parameter	Control	CCl_4 treated
GSH (umol/mg of protein).	106.84 ± 4.23	72.28 # ± 7.18
GSSG (nmol/mg of protein).	47.56 ± 2.57	87.42 # ± 6.47
LPX (nmol/mg of protein).	0.52 ± 0.01	1.13 # ± 0.12
SOD (unit/mg of protein).	247.97 ± 7.98	172.55 # ± 3.1
GPX (unit/mg of protein).	91.65 ± 9.11	214.44 # ± 12.34
Catalase (unit/mg of protein).	4.4 ± 0.21	9.4 # ± 0.33

Administration of CCl_4 further resulted significant elevation of malondialdehyde (MDA), a product of lipid peroxidation in liver of mice treated with CCl_4 which is indicative of development of oxidative stress in the liver due to CCl_4 intoxication. Following CCl_4 administration, a marked reduction in the SOD enzyme activity was observed, whereas level of GPX and catalase enzyme were found to be significantly increased indicating an attempt to protect the liver from oxidative stress induced injury (p<0.001) [Table-2].

Taken together, the data demonstrated that, administration of CCl_4 caused significant liver injury and inflammation through development of oxidative stress.

CCl4 induced oxidative stress is via NADPH Oxidase activation

There are numerous sources of ROS generated during oxidative stress in our body and NOX is a major oxidant generating enzyme. So, we tested whether NOX was involved in CCl_4 mediated oxidative stress in the early phase of liver injury. NOX activity exhibited a significant rise in CCl_4 treated mice in comparison to the control group (p<0.001) (Fig.2A). Parallel to increase in the NOX activity, CYP2E1 activity, which in turn is an effective generator of ROS was also found to be increased (Fig 2B) because CYP2E1 is a loosely coupled enzyme that displays high NADPH oxidase activity Cederbaum AI *et al*.; (2001).

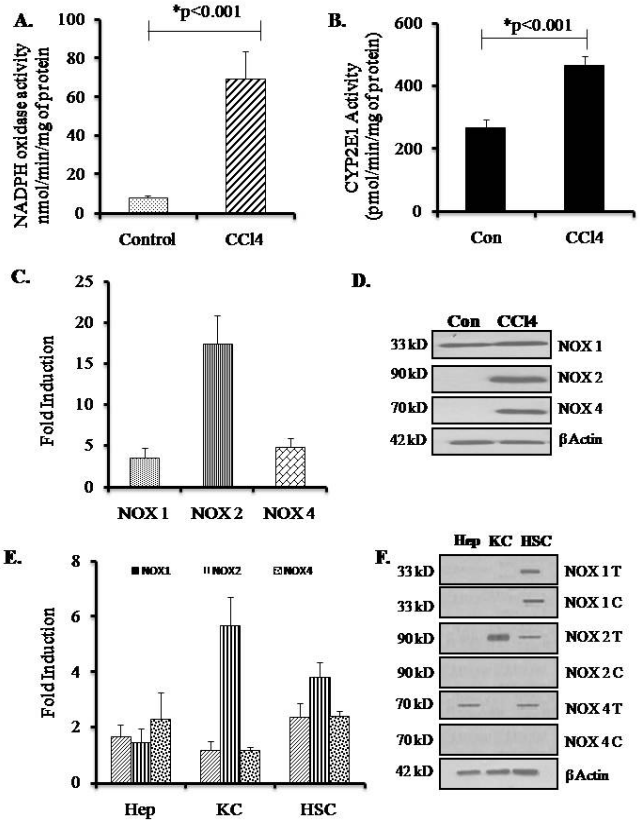


Figure 2 CCl4 induced acute liver injury is via NADPH Oxidase activation. A) Enzymatic activity of NADPH oxidase and B) Evaluation of CYP2E1 activity in control as well as in the CCl_4 treated group. C) mRNA expression of different isoforms of NADPH oxidase. Expression was by means of fold induction in respect to their control group. D) Protein expression of different NOX catalytic components in control liver tissues as well as in CCl_4 treated tissues. β-actin was used as in house control. E) Messenger RNA expression of different NOX isoforms in different cell population of liver and F) protein expression of different NOX isoforms in different liver cells like hepatocytes, Kupffer cells (KC) and hepatic stellate cells (HSCs) isolated from liver tissues after 1 weeks exposure of CCl_4 treatment and control groups.

We next checked the expression of different NOX isoforms in the early phase of liver injury both at mRNA level by real time PCR method and protein expression by western blot analysis and found that expression of NOX2 was significantly elevated in the CCl_4 treated group in comparison to the other NOX isoforms both in real time PCR and in western blot analysis. (Fig 2C, D). The mRNA expression as well as protein expression of different NOX isoforms in different cell population of the liver in the early phase of liver injury (after 1 week of CCl_4 treatment) revealed significant high NOX2 expression in the KCs in comparison to NOX2 expression in other cell population. Hepatic stellate cells also exhibited high NOX2 expression whereas insignificant amount of NOX2 expression was found in hepatocytes (Fig. 2E, F). Among these three cell types, only hepatic stellate cell exhibited significant amount of NOX1 and NOX4, whereas insignificant expression of NOX1 and NOX4 was observed in the hepatocytes and Kupffer cells (Fig 2E, F). Taken together, the data demonstrated that, in response to CCl_4 induced liver injury, NADPH oxidase enzyme activity was increased mainly with the expression of NOX2 in the early phase of CCl_4 intoxicated liver tissue.

Inhibition of NOX attenuates cellular inflammation which in turn reduced CCl_4 induced cellular senescence

To confirm the role of NOX in liver injury, inflammation and senescence, we used DPI, an inhibitor of NOX before CCl_4 intoxication. Results revealed that DPI has a potential role to reduce NOX activity significantly ($p<0.001$) in the DPI plus CCl_4 treated group in comparison to the only CCl_4 treated group (Fig 3A). As depicted in Fig 3B, a marked reduction of NOX1, NOX2 and NOX4 expression as evidenced by western blot was observed in the CCl_4 plus DPI treated group as compared to only CCl_4 treated group. β-actin was used as an internal control.

Histologically also there was improvement in the liver architecture as the number of infiltrating cells were reduced significantly in CCl_4 plus DPI treated group as compared to the only CCl_4 treated group (Fig.3C). This finding was confirmed by significantly reduced serum ALT & AST level in DPI plus CCl_4 treated group in comparison to only CCl_4 treated group ($p<0.001$) as depicted in Fig 3Di.

To further confirm the role of DPI to reduce inflammation, we assessed the levels of various chemokines (MCP1, IL-17 and CCl3) by RT PCR. As noted in Fig 3Dii, significant reduction of MCP1, IL-7and CCl3 mRNA expressions was observed in DPI plus CCl_4 treated group. We also determined the effect of DPI on and mRNA and protein expression of IL-6, IL12, and CCl5. Pre-treatment with DPI significantly reduced expression of all these chemokines both at mRNA and protein level in comparison to the only CCl_4 group as seen in Fig 3E i, ii. DPI control was used here as vehicle control.

Cellular senescence is a state of irreversible cell-cycle arrest, which can be induced in response to diverse cellular damage. SA-β-gal activity, marker of cellular senescence [31] confirmed senescence as evidenced by enhanced amount β-gal positive cells at the injured zone in CCl_4 treated mice as compared to the control group. The number of β gal positive cells got markedly reduced by pre-treatment with DPI in comparison to the only CCl_4 treated group as noted in Fig 3F.

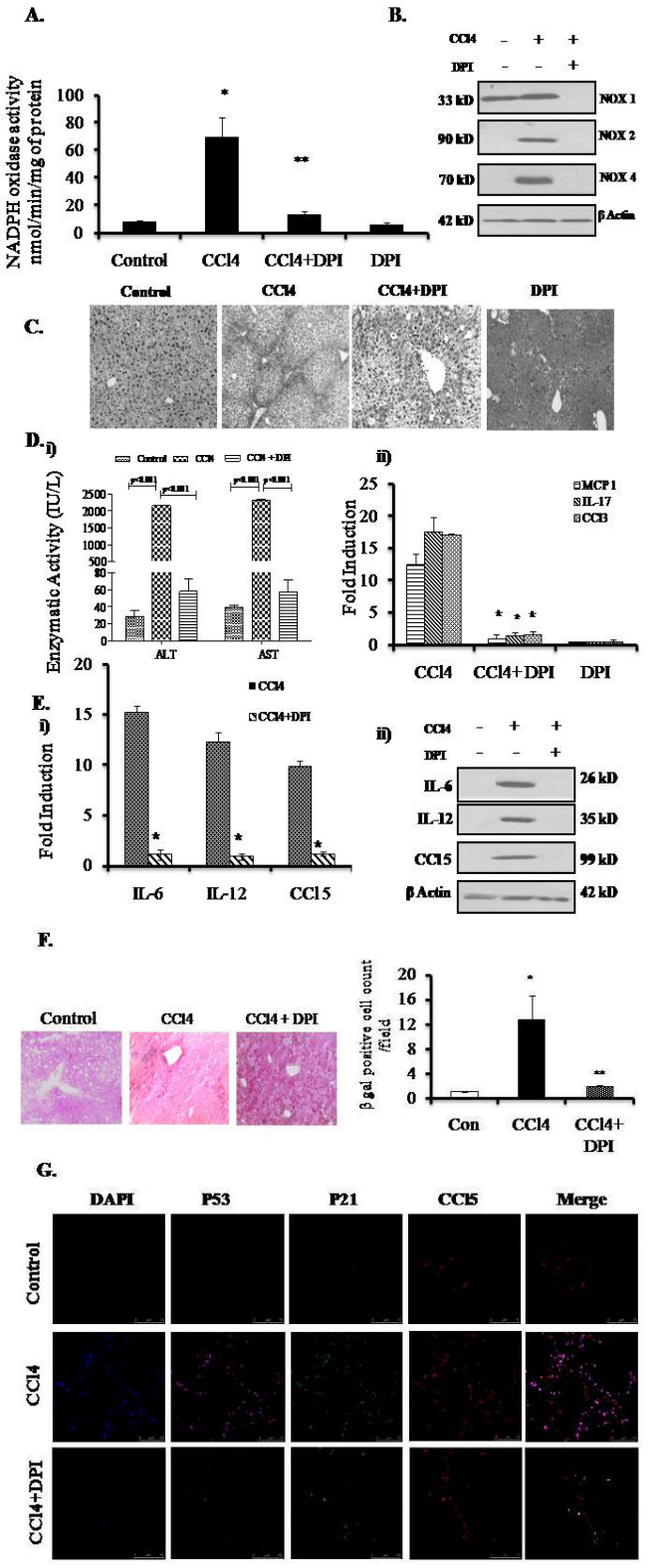
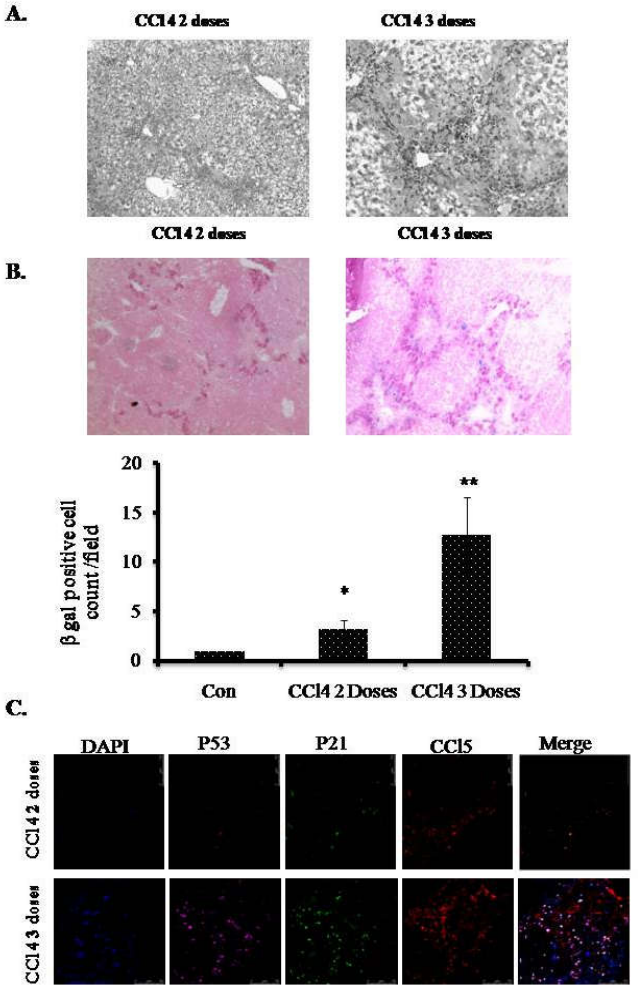


Figure 3 Inhibition of NOX attenuates cellular inflammation which in turn reduced CCl4 induced cellular senescence. A) NADPH Oxidase was measured after DPI administration 30 minutes prior to CCl_4 injection.(*$p<0.001$ vs. control; **$p<0.001$ vs. CCl_4). B) Protein expression of different isoforms of NOX was evaluated by western blot after DPI administration. C) Acute liver injury was significantly reduced after administration of DPI histopathologically. D) (i) Liver injury was also reduced after DPI administration which was evident in reduced ALT and AST value. (ii) mRNA expression of several chemokines which were secreted from liver macrophages as well as from infiltrating cells (*$p<0.001$ vs. CCl_4). E) (i) mRNA and (ii) Protein expression of IL-6, IL-12 and CCl5 was also get reduced as evidenced by western blot. F) Cellular senescence was evaluated by β-gal stain. Positive cells were counted in high power view (40X) in five random fields and plotted graphically. *$p<0.001$ vs control, **$p<0.001$ vs CCl4 and (G) immunohistochemical staining of p53, p21 and CCl5 in different groups of mice.

Senescent cells express p21 as well as by p53, universal cell cycle inhibitors. Senescence was further confirmed by co localization of p53 and p21 protein by immunohistochemistry in CCl_4 treated liver tissue whereas the control mice did not show any expression of these markers. Further CCl5 was also localized in CCl_4 treated liver tissue indicating the fact that cellular senescence is related to inflammation (Fig 3G). Expression of all these markers was significantly reduced in the CCl_4 plus DPI treated group (Fig.3G).

Cellular senescence is progressively increased with inflammation

We compared liver histology in two different groups; mice injected with 2 dose of CCl_4 and mice injected with 3 dose of CCl_4 in a week. Gradual increase in the number of infiltrating cells along with increase in number of senescent positive cell was observed in a dose dependent manner as evidenced by H&E staining and SA-β-gal staining (Fig 4A,B). The expression of other surrogate markers of senescence as well as CCl5 was also found to be increased in mice injected with 3 doses of CCl_4 in comparison to mice injected with 2 doses of CCl_4 (Fig 4C). Next we investigate the relation of NOX with inflammation and senescence. The increased NOX activity was found to be positively related with the increased inflammation as evident from Fig.4D. A positive correlation was also observed as we checked the relation of inflammation score with the senescence (Fig 4E).



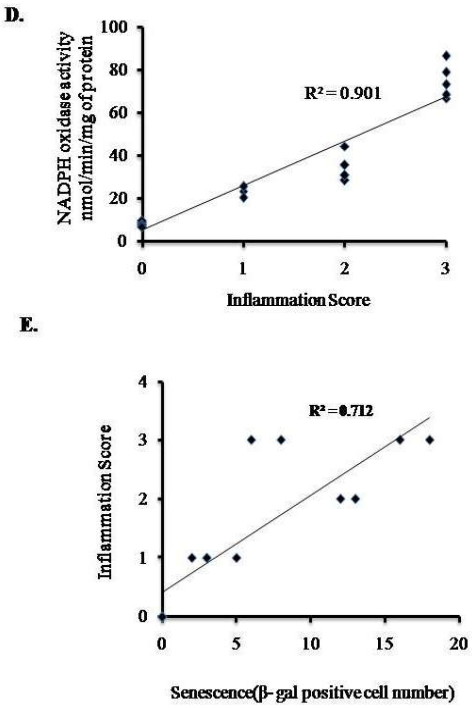


Figure-4: Cellular senescence is progressively increased with inflammation. A) Hematoxylene and Eosin stain of paraffin embedded liver section and B) β gal stain of CCl4 treated mice after 2 dose and 3 dose of CCl_4. Positive cells were counted in high power view (40X) in five random field and plotted graphically. *p<0.01 vs control, **p<0.001 vs CCl_4 2 does. C) Immunohistochemical comparison of different markars of senescence between 2 dose and 3 doses of CCl_4. D) Correlation of NADPH oxidase activity along with inflammation score and E) correlation of inflammation score with senescence at different doses of CCl_4 treatment.

DISCUSSION

The model of liver injury with CCl_4 has been extensively used to study acute liver injury as damage caused by this hepatotoxin mimics human liver diseases of different etiologies. Morio LA *et al*.; (2001) and Yang J *et al*.; (2010). In this study, we used a smaller dose of CCl_4 (0.2-0.5ml/kg) to study the early phase of liver injury than a larger dose (1ml/kg) used by most of the workers. Santra A *et al*.; (1998) Using this model, we have identified the protective effect of DPI against NOX mediated CCl_4 induced early liver injury and inflammation. Serum aminotransferase activities including AST and ALT are widely used as biochemical markars of liver injury as these enzymes are released from damaged hepatocytes. Chen CH *et al*.; (2003) Our current data demonstrated that CCl_4 treatment (both 2 doses and 3 doses) significantly elevated serum ALT and AST level compared to control mice. Abnormal histologic changes were noticed in liver specimen of all CCl_4 treated mice accompanied with gross hepatic necrosis and infiltration of inflammatory cells, mostly involving in the periportal region. Evidence of both macro and micro vesicular steatosis was observed due to metabolism of CCl_4 in the hepatocytes and activation of CYP2E1 which may stimulate several lipogenic genes.

Oxidative stress is a well known phenomenon in almost all etiologies of liver disease induced by different etiologies in both human and experimental animals. Parola M *et al*.; (1994) Oxidative stress plays a important role in mediating the pathogenesis of CCl_4 induced liver injury. The conversion of trichloromethyl ($CCl_3^{\cdot}$) free radical by NADPH oxidase dependent cytochrome 450 mediated mono-oxygenase system is the most Santra A *et al*.; (1998) important step in the development of CCl_4 induced hepatotoxicity. $CCl_3^{\cdot}$ further

reacts with O_2 to form trichloromethyl free radical (CCl_3O_2). Santra A *et al*.; (1998). These highly toxic and potent free radical intermediates initiate liver damage through GSH depletion and lipid peroxidation. CCl_4 is detoxified by formation of GSH conjugate which was evidenced by significant reduction in the hepatic GSH level after administration of CCl_4. Reduced cellular GSH level and increased level of MDA, which is a product of lipid peroxidation are considered to be important indicators of oxidative stress. SOD also act as primary defences which in turn attenuate the oxidative stress and inhibit the activation of different inflammatory mediators. Bureau C *et al*.; (2001) Our data showed that CCl_4 treatment decreased SOD activities in mice significantly. Enzyme activity of catalase and GPX were significantly up regulated for prevention against oxidative stress.

To investigate the underlying mechanism, we evaluated the effects of CCl_4 administration on the mRNA levels of certain key chemokines and cytokines tightly related with inflammation. MCP1/ CCl2 is among the most extensively studied chemokines in liver injury. It results in monocyte, neutrophil and macrophage accumulation in the injured zone. Apart from MCP1, CCl5 (RANTES) and CCl3 have significant roles in the early phase of liver injury by recruiting T cells, dendritic cells, eosinophils, NK cells, mast cells, and basophils to sites of inflammation by interacting with 3 specific G protein-coupled receptors: CCR1, CCR3, and CCR5. Saiman Y *et al*.; (2012) In the present study, we observed that CCl_4 administrated mice demonstrated significantly up-regulated expression of MCP1, CCl3 and CCl5. IL-12, IL-17 and IL-6 are considered to be the special biomarkers that reflect inflammatory status. Reyes-Gordillo K *et al*.; (2017) We found that IL-6, IL-12 and IL-17 levels were enhanced in the similar pattern as the levels of CCl2, CCl3 and CCl5.

Among different NOX catalytic subunits mainly NOX1, NOX2 and NOX4 are expressed in the liver. The phagocytic NOX or NOX2 induces the oxidative burst required by neutrophils to kill pathogenic bacteria. Genetic deficiency of NOX2 in patients causes chronic granulomatous disease with life threatening infections Paik YH *et al*.; (2111). NOX1 is catalytically activated by several pro-fibrogenic agonists such as Ang II, LPS, and PDGF that induce HSC activation and proliferation. Bataller R *et al*.; (2013) NOX4 is a direct TGF-responsive gene which is regulated at the level of transcription. These two NOX isoforms are mainly responsible for initiation and progression of liver fibrosis. But the phagocyte NADPH oxidase (NOX2) is very important for antimicrobial host defence and it has a significant role in the inflammation at the early phase of liver injury. Study showed that there was high expression of the different NOX isoforms along with the inflammation at the acetaminophen induced liver injury. Lin YP *et al*.; (2017)

A dramatic increase in NADPH oxidase as well as CYP2E1 activity was observed in mice after CCl_4 treatment, suggesting that NADPH oxidase is the major factor involved in the CCl_4-induced hepatotoxicity in mice. This study showed the differential expression of different NOX isoforms in mice liver. NOX2 was found to be highly expressed in the early phase of liver injury induced by CCl_4 mainly by kupffer cells and infiltrating cells at the injured zone. There are several studies which shows that inflammatory cell is also a source of NADPH oxidase. Luo M *et al*.; (2015)

Several studies demonstrated that DPI attenuated liver fibrosis and ROS production as well as fibrotic genes in both CCl_4 and BDL models of liver fibrosis, suggesting that NOX may be a new therapy for liver fibrosis. Kono H *et al*.; (2001) But the mechanism by which DPI reduce acute liver injury is not clear. Chronic elevation of ROS derived from NOX1 and NOX4 is mainly responsible for a premature senescent growth arrest in primary human endothelial cells, with the growth arrest occurring in S phase of the cell cycle. Schilder YDC *et al*.; (2009) They further confirmed that this ROS is mainly from NADPH oxidase because DPI and knock down of NOX can reduce the ROS and cellular senescence. In this study we have showed that NADPH oxidase mediated liver injury was reduced to the basal level when we injected DPI to the animal prior to CCl_4 injection. DPI has a significant role in the reduction of liver injury or inflammation at the early phase of injury which ultimately reduced cellular senescence. So, our current study demonstrated that NOX signaling mediates hepatic injury through inflammation and senescence.

CONCLUSION

Here we reported for the first time a direct relation of NOX activity with the cellular inflammation and senescence in acute liver injury induced by CCl_4. Our results supported the concept that NOX2 was involved in CCl_4 induced liver injury resulting in inflammation and cellular senescence in a dose dependent way which can be prevented by direct inhibition of NOX by DPI.

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References

Basu S. (2011) Carbon Tetrachloride-Induced Hepatotoxicity: A Classic Model of Lipid Peroxidation and Oxidative Stress. In: Basu S, Wiklund L, editors. Studies on Experimental Models. Totowa: Humana Press: 467-480.

Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, Qian T, Schoonhoven R, Hagedorn CH, Lemasters JJ, Brenner DA. (2003). NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J Clin Invest*; 112:1383-1394

Beers RF, Jr Sizer IW. (1952)A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* ; 195:133-140.

Block K, Gorin Y. (2012). Aiding and abetting roles of NOX oxidases in cellular transformation. *Nat Rev Cancer;* 12(9):627-37.

Blumberg BS, Fox RC. (1985) The Daedalus effect: changes in ethical questions relating to hepatitis B virus. *Ann Intern Med;* 102:390-394.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*; 72: 248-254.

Cem Gabay. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research & Therapy*; 8:(Suppl 2):S3.

Chen CH, Wallace CG, Chen KH, Chang CL, Chen HH, Sung PH, Lin KC, Ko SF, Sun CK. (2003). The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol*; 98: 960-967.

Cederbaum AI. (2006). CYP2E1-Biochemical and Toxicological Aspects and Role in Alcohol-Induced Liver Injury. The Mount Sinai School of medicine; 73. 4

Campisi J, d'Adda di Fagagna F. (2007). Cellular senescence: When bad things happen to goodcells. *Nat Rev Mol Cell Biol*; 8: 729.

Cui W, Matsuno *K*, *Iwata K*, Matsumoto MM , Zhang J, Zhu K. (2011). NOX1/nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase promotes proliferation of stellate cells and aggravates liver fibrosis induced by bile duct ligation. *Hepatology*; 54: (3):949-58.

Cho HJ, *Lee* WH, Hwang OMH, Sonntag WE, Lee YW. (2017). Role of NADPH Oxidase in Radiation-induced Pro-oxidative and Pro-inflammatory Pathways in Mouse Brain. *International Journal of Radiation Biology;* 10: 1080.

Czaja MJ. (2010). Another Way for NADPH Oxidase to Send a Signal for Liver Injury. Gastroenterology; 129-6.

Goodman ZD. (2007). Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *Journal of Hepatology*; 47: 598-607.

Griffith OW. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem; 106: 207-212.

Higgins GM. Anderson RM. (1931). Experimental pathology of liver: restoration of liver in white rat following partial surgical removal. *Arch Pathol*; 12: 186-202.

Heiss EH, Schilder YD, Dirsch VM. (2011). Chronic treatment with resveratrol induces redox stress- and ataxia telangiectasia-mutated (ATM)-dependent senescence in p53-positive cancer cells. *J. Biol. Chem* ;26759-26766; 2007

Herrera B, Murillo MM, Alvarez-Barrientos A. (2004). Source of early reactive oxygen species in the apoptosis induced by transforming growth factor-β in fetal rat hepatocytes. *Free Radical Biol. Med*; 36:16-26.

Kim MJ, Ryu JC, Kwon Y, *Lee S, Bae YS, Yoon JH,* Ryu JH. (2014). Dual Oxidase 2 in Lung Epithelia Is Essential for Hyperoxia-Induced Acute Lung Injury in Mice. *Antioxidant & redox Signaling*; 21: (13) 5677.

Krizhanovsky V, Yon M, Ross DA. (2008). Senescence of Activated Stellate Cells Limits Liver Fibrosis. *Cell*; 134: 657-667.

Kono H, Rusyn *I,* Uesugi T, Yamashina *S,* Connor *HD*, Dikalova *A,* Mason *RP,* Thurman *RG.* (2001). Diphenyleneiodonium sulfate, an NADPH oxidase inhibitor, prevents early alcohol-induced liver injury in the rat. *Am J Physiol Gastrointest Liver Physiol* ; 80: G1005-G1012

Luo M, Dong L, Li J. (2015). Protective effects of pentoxifylline on acute liver injury induced by thioacetamide in rats. *Int J Clin Exp Pathol*; 8(8):8990-8996.

Lin YP, Chen CH, Wallace CG. (2017). Therapeutic effect of rosuvastatin and propylthiouracil on ameliorating high-cholesterol diet-induced fatty liver disease, fibrosis and inflammation in rabbit. *Am J Transl Res;* 9(8):3827-3841.

Mullen KD, McCullough AJ. (1989). Problems with animal models of chronic liver disease: suggestions for improvement in standardization. *Hepatology*; 9:500-503.

Masuda Y. (2006). Learning toxicology from carbon tetrachloride induced hepatotoxicity. Yakugaku Zasshi; 126: 885-899.

Morio LA, Chiu H, Sprowles *KA*, Zhou P, Heck DE, Gordon MK, Laskin DL.(2001). Distinct roles of tumor necrosis factor-alpha and nitric oxide in acute liver injury induced by carbon tetrachloride in mice. *Toxicol Appl Pharmacol;* 172: 44-51.

Novobrantseva *T*, Majeau *GR*, Amatucci A, Kogan S, Brenner I, Casola S, Shlomchik MJ, Koteliansky V, Hochman PS, Ibraghimov A. (2005). Attenuated liver fibrosis in the absence of B cells. *J Clin Invest*; 115: 3072-3082 .

Nanji A A, Khettry U, Sadrzadeh SM. (1994). Lactobacillus feeding reduces endotoxemia and severity of experimental alcoholic liver disease. *Proc. Soc Exp Biol Med;* 205: 243 - 247.

Nandi A, Chatterjee IB. (1988).Assay of superoxide dismutase activity in animal tissues. *J Biosci*; 13: 305-315.

Nieto N, Jose A, Dominguez-Rosales D, Fontana L, Salazar A, Armendariz J Borunda, 3 Greenwell P, Rojkind M. (2001). Contribute to the acute-phase response with increased expression of alpha1(I) and alpha1(IV) collagens, tissue inhibitor of metalloproteinase-1, and matrix-metalloproteinase-2 messenger RNAs. *Hepatology*; 33:597-607.

Parola M, Leonarduzzi G, Albano E.(1994) Oxidative damage in pathogenesis of liver cirrhosis.In:Daanzani MU, gentilin P,Eds. Liver Pathology. Amsterdam:Excerpta medica; 3-12.

Pertoft H. Smedsrød B. Cell Separation. Pretlow TG, Pretlow TB, (1987) editors. Vol. 4. Academic Press; New York; p. 1-24.

Paglia DE, Valentine WN.(1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med*; 70: 158-169.

Paik YH, Iwaisako K, Seki E, Inokuchi S, Schnabl B, Osterreicher CH. (2011). The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues

NOX1 and NOX2/gp91(phox) mediate hepatic fibrosis in mice. *Hepatology*; 53(5):1730-41.

Rao PS, Mangipudy RS, and Mehendale HM. (1997) Tissue injury and repair as parallel and opposing responses to CCl4 hepatotoxicity: a novel dose-response. *Toxicology*;118: 181-93 .

Reinehr R, *Becker S*, *Eberle* A, Grether-Beck S, Haussinger D.(2005). Involvement of NADPH oxidase isoforms and Src family kinases in CD95-dependent hepatocyte apoptosis. *The Journal of biological chemistry*; 280(29):27179-94.

Reinke LA, Moyer MJ.(1985) q-nitrophenol hydroxylation. A microsomal oxidation which is highly inducible by ethanol. *Drug Metab Dispos*; 13:548-552

Reyes-Gordillo K, *Segovia J*, *Shibayama M*, Vergara P, Moreno MG, Muriel P. (2007). Curcumin protects against acute liver damage in the rat by inhibiting NF-kappaB, proinflammatory cytokines production and oxidative stress. *Biochim Biophys Acta;* 1770: 989-996.

Schilder YDC, Heiss EH , Schachner D, Ziegler J. (2009). NADPH oxidases 1 and 4 mediate cellular senescence induced by resveratrol in human endothelial cells. *Free Radical Biology & Medicine*; 46: 1598-1606.

Santra A, Das S, Maity A, Rao SB , Guha- Mazumder DN.(1998). Prevention of carbone tetrachloride -induced hepatic injury in mice by Pichorhi Kurrooa.Indian *journal of Gastroenterol*; 7:7.

Slater T, Sawyer B. (1971) The stimulatory effect of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions in vitro. *Biochem J*; 123: 805-814.

Saiman Y, Friedman SL. (2012). The role of chemokines in acute liver injury. Frontiersin Physiology. *Gastrointestinal Sciences*; 3: 213 - 1

Taub R.(2004). Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol*; 5: 836- 847.

Tietze F.(1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem*; 27: 502-522.

Wang J*,* Ma MW, Dhandapani KM, Brann DW. (2017) Regulatory Role of NADPH Oxidase 2 in the Polarization Dynamics and Neurotoxicity of Microglia/Macrophages after Traumatic Brain Injury. *Free Radical Biology and Medicine*; 17;0891-5849-30770-0.

Xue W*,* Zender L*,* Miething *C*, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo *C*, Lowe SW. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*; 4458.

Yun Lee BY, Han AJ, Im JS. (2006) Senescence-associated β-galactosidase is lysosomal β -galactosidase. *Aging Cell;* 5:187-195.

Yang J*,* Li Y*,* Wang F*,* Wu C.(2010). Hepatoprotective effects of apple polyphenols on CCl4-induced acute liver damage in mice. *J Agric Food Chem*; 58: 6525-6531.

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