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Pentoxiffiline Prevents Oxidative Stress Mediated Acute Liver Injury in Carbon Tetrachloride Treated Mice

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Abstract: Pentoxifylline (PTX) is a non-selective phosphodiesterase inhibitor which have role in prevention of oxidative stress, inflammation and fibrosis which ultimately affect the liver. The major goal of the study was to evaluate the role of PTX on carbon tetrachloride (CCl_4) induced acute liver injury model and its possible mechanisms in mice. Male C57BL/6 mice were divided into four groups: control, PTX, CCl_4 and PTX+ CCl_4 treated groups. Mice were administrated CCl_4 together with or without PTX for a week and sacrificed 72 hour after the last injection of CCl_4 and PTX. Histopathological evaluation was performed. The liver function test, indices of oxidative stress including NADPH oxidase (NOX) and cytochrome P450 2E1 (CYP2E1) enzyme activity, malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) levels in liver tissues were measured. mRNA expression of different pro inflammatory cytokines and protein expression of nuclear factor-κB (NF-Kb) were checked. PTX significantly attenuated CCl_4-induced liver injury histopathologically and improves the liver function. PTX also remarkably suppressed the secretions of pro-inflammatory cytokines and translocation of the p65 subunits of NF-κB to nucleus induced by CCl_4. In addition PTX can also reduce the generation of oxidative stress by decreasing the enzyme activity of NOX, CYP2E1 and the levels of MDA and also by increasing the cellular anti-oxidant GSH and SOD. In conclusion, PTX ameliorated the effects of CCl_4 induced acute liver injury in mice by inhibiting oxidative stress, expression of pro-inflammatory cytokines and NF-κB activation.

Keywords: Acute Liver Injury, Oxidative Stress, Pentoxifilline

1. Introduction

Liver is considered as one of the major vital organ of our body as it is responsible for regulation of the metabolism of body [1]. Acute liver injury may be caused by a variety of etiologies which ultimately induce inflammation, oxidative stress, and necrosis of liver cells [2]. Carbon tetrachloride (CCl_4) is widely used to generate the model of acute liver injury in experimental animal. This well known hepatotoxin is widely used because it causes specially membrane damage, oxidative stress and accumulation of lipid droplets in the hepatocyte cytoplasm to enhance inflammation and liver injury [3]. The hepatotoxin can generate a model of acute liver injury within few hours as hepatitis is generated within a short time after giving of the injection. CCl_4 intoxication results necrosis of the hepatic parenchyma and subsequently results development of fatty liver which mimics acute hepatitis. These acute hepatitis then followed by infiltration of different inflammatory cells in the injured zone [4].

In body when reactive oxygen species (ROS) levels exceed its physiological limit 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 [5]. In

addition to the exogenous source of ROS there are different sources of endogenous ROS - the multicomponent nicotinamide adenine dinucleotide phosphate NADPH oxidase (NOX) enzyme complexes, peroxisomal cytochrome P450 2E1 (CYP2E1) enzyme system, xanthin oxidase system and the mitochondrial respiratory pathway. Correlation between inflammation and oxidative stress at the time of liver injury plays a crucial role for mediating liver injury. The inflammatory response is mediated by activated pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), different interleukins (IL-1β and IL-6) and oxygen radicals which are responsible for sensitization and damage of the hepatocytes [6, 7].

Pentoxifylline (PTX), 1-[5-oxohexyl]-3, 7-dimethylxanthine] is a non-specific phosphodiesterase inhibitor that has been routinely used for treatments of different circulatory diseases for more than 20 years [8]. There are several studies in recent few years which gives us the information about the therapeutic effects of PTX on alcoholic hepatitis, non-alcoholic fatty liver and liver fibrosis, but its effect on acute liver injury remains unclear [9-11]. Therefore, the aim of the study was to evaluate the protective role of PTX on CCl_4 induced acute liver injury model and to investigate its possible mechanisms.

2. Materials and Methods

2.1. 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 given commercial chow containing 20% protein (NCLAS) and water *ad libitum*. The present study was approved by institutional animal ethics committee. To develop Acute 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 PTX (Sigma, U.S.A) at a dose of 40 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 RNA later (Sigma) solution for RNA expression study and (d) snap frozen at -80°C for cryosection.

2.2. Assessment of Hepatic Injury

Serum ALT and AST activities were measured using a semi auto auto-analyser (Robonic, India) with a commercial kit (Siemens, India) according to the manufacturer's instruction. Formalin-fixed liver tissues were processed and stained with Haematoxylin and Eosin (H & E; Sigma, U.S.A) for the determination of degree of liver injury [21, 22]. Liver pathological characteristics were scored in a blind manner as follows: steatosis (the percentage of liver cell containing fat), <25% = 1+;< 50% = 2+; <75% = 3+ and >75%=4+ and inflammation, <2 foci per 40x = 1; 2 to 4 foci per 40x = 2; >4 foci per 40x = 3. [12, 13].

2.3. Reverse-Transcription PCR (RT-PCR) and Real-Time Quantitative PCR (qRT-PCR)

RNA was extracted from liver tissues as well as from hepatocytes, kupffer cells (KCs) and hepatic stellate cells (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.

2.4. Western Blot Analysis

Western blots were performed as previously described method [14]. Forty micrograms of protein was separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (12.5% SDS-PAGE) gel and transferred onto Polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, U.S.A). The blots were then probed with the following antibodies: mouse monoclonal anti p65 antibody of NFkB (Sc-8414; Santa Cruz Biotechnology, USA) 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).

2.5. Assessment of Oxidative Stress by Hepatic Biochemical Assays

A 10% liver homogenate was used for determination of protein content using Bradford reagent (Sigma, U.S.A) spectrophotometrically [15]. Cellular glutathione (GSH) content in liver were measured enzymatically at 412 nm according to the method as described [16]. Lipid peroxidation was assessed by their content of Thiobarbituric

Acid Reactive Substances (TBARs) [17]. Activities of hepatic Superoxide dismutase (SOD) and NADPH oxidase (NOX) were also determined according to the method as described respectively [18, 19]. 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-nitrocatechol [20]

2.6. 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.

Table 1. *List of Primers used in this study.*

Genes	Primers sequence
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'

3. Results

3.1. Effects of PTX on Liver Histopathological Changes

To evaluate the effects of PTX on CCl_4 induced hepatocellular inflammation, the liver sections were stained with H & E stain. Liver sections from the control and PTX control groups, exhibited a normal lobular architecture with radiating hepatic cords and clear central veins, without inflammation, necrosis and steatosis. In contrast, liver section from CCl_4 treated mice showed significant changes in normal liver architecture with severe infiltration of inflammatory cells in the periportal regions. Accumulation of cytoplasmic lipid droplets was also found in CCl_4 treated mice liver. However, treatment with PTX and CCl_4 showed a reduced number of inflammatory cell infiltration in the injured areas (Figure 1A).

Inflammation score was found to be maximum ($p<0.001$) in the CCl_4 treated mice as compared to the control mice and was significantly ($p<0.001$) reduced in the PTX and CCl_4 treated groups of mice in comparison to only CCl_4 administered mice. Steatosis was also reduced significantly ($p<0.001$) in the PTX and CCl_4 treated groups in comparison to the only CCl_4 treated groups (Figure 1B). These data clearly suggested that PTX significantly reduced the degree of liver injury.

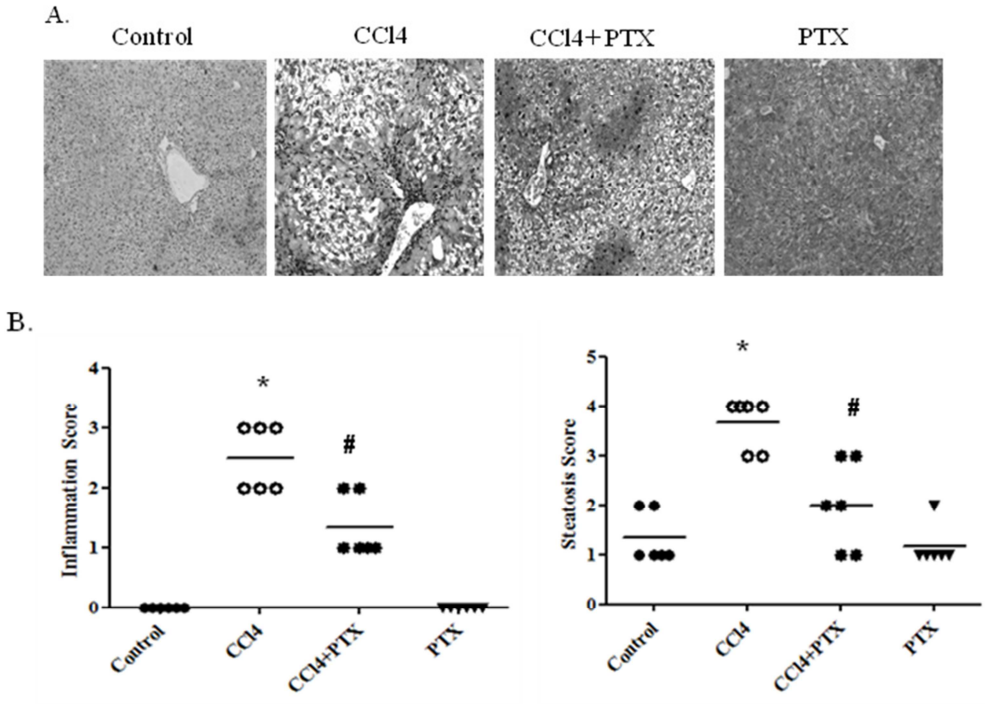


Figure 1. *Effects of PTX on liver histopathological changes. (A) H&E stain was performed in the paraffin embedded liver Sections of four different groups of mice. (B) Inflammation and steatosis score from H&E Stained liver section of four different groups of mice.*p<0.001 vs. Control, #p<0.001 vs. CCl_4 groups.*

3.2. Effects of PTX on Liver Function

For assessment of the liver damage, serum was obtained to measure serum markers of liver injury (serum ALT and AST). There was no such statistical difference observed between PTX group and control group. In CCl_4 treated groups, levels of liver enzyme activities were significantly increased compared with controls ($p<0.001$), whereas the levels of the enzymes were in significantly lower amount ($p<0.001$) in PTX pre-treated CCl_4 group (Figure 2). Thus, these data suggested that PTX attenuates CCl_4 induced liver injury.

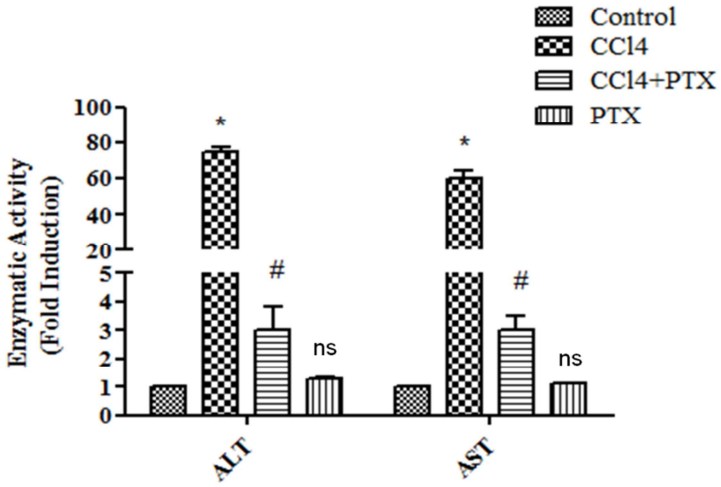
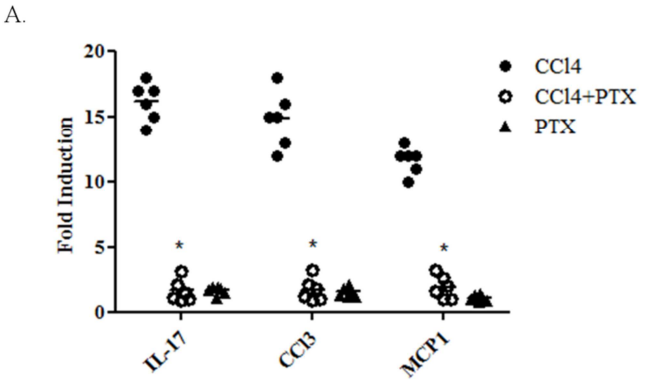


Figure 2. *Effect of PTX on liver Function. Serum markers of liver injury in terms of ALT and AST,*p<0.001 vs. control, #p<0.001 vs. CCl_4, ns: non-significant vs. Control.*

3.3. Effects of PTX on Pro-inflammatory Cytokine Response and Activation of NF-κB

To determine whether PTX can reduce inflammation caused by CCl_4, we examine the mRNA expression of different pro-inflammatory cytokines and chemokines such as interlukin-17 (IL-17), CC motif chemokine ligand 3 (CCl3) and monocyte chemoattractant protein 1 (MCP 1). As shown in results, the mRNA expression of these different cytokines and chemokines were elevated in the CCl_4 treated groups. In contrast PTX has an effect to reduce the expression of these chemokines and cytokines significantly ($p<0.001$). However, PTX per se had no effect on the mRNA expression of these pro-inflammatory cytokines (Figure 3A).

It has been documented that NF-κB is a key player in the progression of liver inflammation and its activation is essential for pro-inflammatory cytokine production. The translocation of p65 subunits of NF-κB to the nucleus is the results of its activation. To elucidate whether PTX could modulate NF-κB, we detected the expression of NF-κB subunit p65 in protein level. Compared to the control group, protein level of NF-κB was significantly increased in the nuclear fraction after CCl_4 treatment. In the PTX and CCl_4 co-treated group, the expression of NF-κB showed an effective decrease at the protein level (Figure 3B).



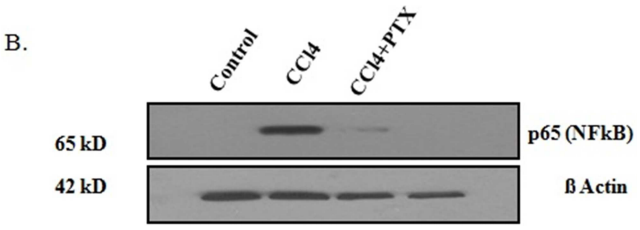


Figure 3. *Pentoxifilline reduced Inflammation. (A) mRNA expression of different pro-inflammatory cytokine and chemokines *p<0.001 vs. CCl_4 (B) Western blot analysis of translocation of p65 subunits of NF-κB to the nucleus.*

3.4. Effects of PTX on Oxidative Stress

In order to evaluate liver oxidative stress mediated liver injury, we measured the activity of two major ROS producing enzymes NOX and CYP2E1 and levels of malondialdehyde (MDA), superoxide SOD, and GSH in liver tissues. Results showed that the activity of both NOX and CYP2E1 enzymes were increased ($p<0.001$) drastically in the CCl_4 treated groups in comparison to the control groups whereas PTX pre-treatment significantly reduced ($p<0.001$) the activity of these two enzymes. The level of MDA was significantly elevated in CCl_4 treated group while contents of SOD and GSH were decreased compared with the control group. In the group receiving PTX and CCl_4, SOD and GSH were increased almost at a normal level ($p<0.001$) and the level of MDA was preserved which suggests that PTX can prevent the generation of oxidative stress (Figure 4).

4. Discussion

Our present study demonstrated that PTX effectively reduced CCl_4 induced histopathological changes and the serum transaminase levels were improved. Moreover, the generation of oxidative stress, activation and NF-κB and up regulation of several pro-inflammatory cytokines and chemokines (IL-17, CCl3 and MCP1) were reduced. Taken

together, our study revealed that PTX could protect against the oxidative stress mediated acute liver injury in mice model induced by CCl_4.

CCl_4 is a useful hepatotoxin to study the experimental liver injury model. It has been used for several years because this hepatotoxin mimics the situation of most cases of liver diseases in human and makes it be an appropriate model to study the mechanism in vivo. Previous study showed that due to CCl_4 intoxication, there was development of acute liver injury and up regulation of different cytokines [21].

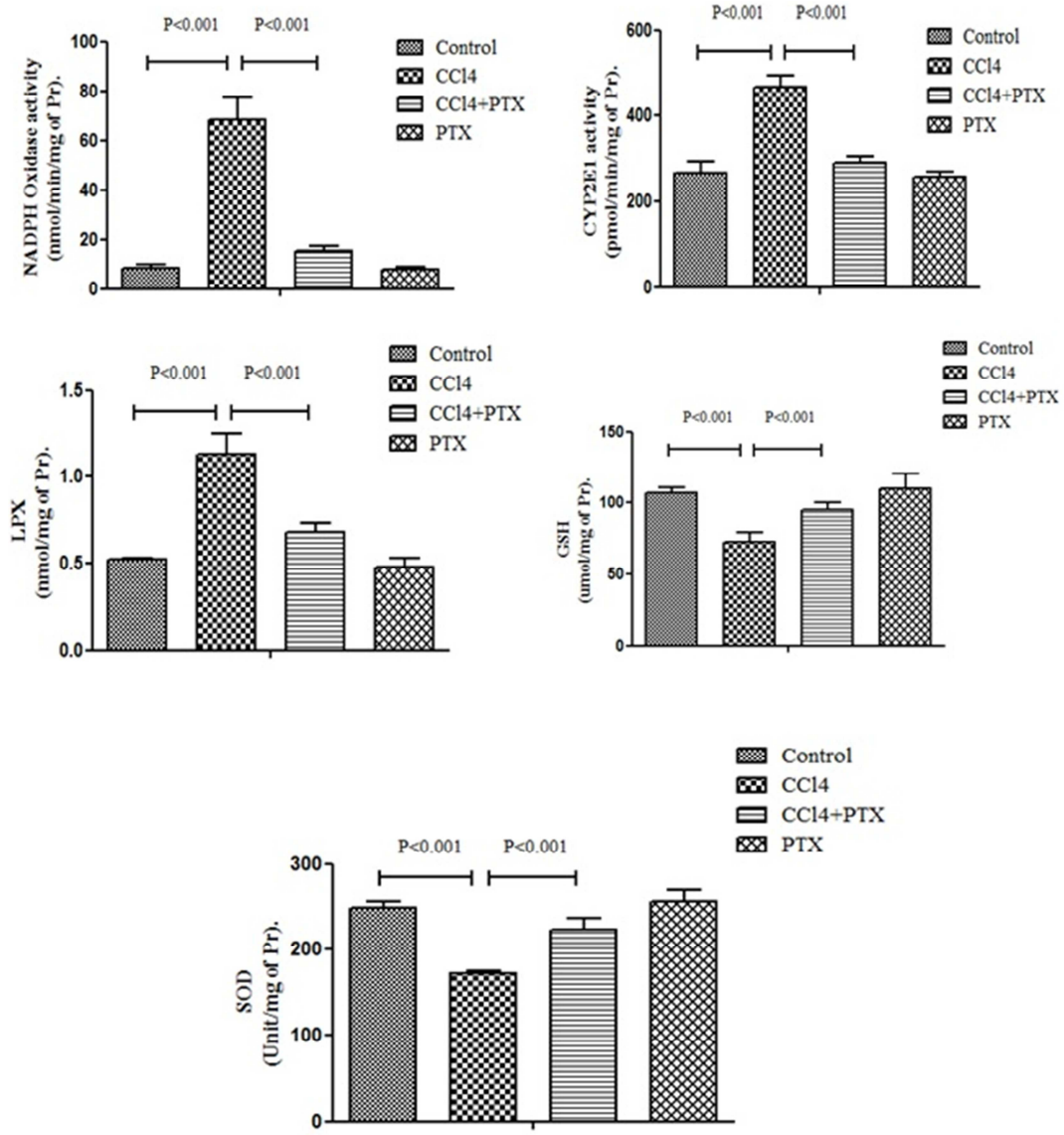


Figure 4. *Pentoxifilline reduced CCl_4 mediated Oxidative stress. Several markers of oxidative stress were measured biochemically in four different groups to assess the oxidative stress mediated liver injury and the role of PTX to prevent the liver injury.*

PTX is a phosphodiesterase inhibitor that is widely used for the treatment of peripheral vascular disease. The immunomodulatory, antioxidant and anti-inflammation activities of PTX have been described in the previous studies [22]. Recent studies showed the protective effect of PTX in rat liver injury where PTX administration reduced serum aminotransferase activities including ALT and AST [23]. These two enzymes which released from damaged hepatocytes into the blood have been used as important markers to determine the degree of liver injury [24]. Our study showed that intra-peritoneal (i.p) administration of PTX attenuated the acute surge of ALT and AST in serum induced by CCl_4.

We also used histologic scoring methods to reveal the cell inflammation and steatosis as supportive data to show the degree of liver injury and found that inflammation and steatosis both were increased due to CCl_4 intoxication and which is the features of acute liver injury. Both these two features were decreased after PTX treatment in the CCl_4 treated groups.

The liver resident macrophages or Kupffer cells are the major source of various cytokines [25]. Hepatotoxins such as CCl_4 can induce pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 by the activity of Kupffer cells [21, 26]. These cytokines serves important role in the initiation of the inflammatory response. Our data revealed that due to CCl_4 administration we have found high expression of these pro-

inflammatory cytokines and chemokines (IL-17, CCl3, and MCP1) and PTX has an inhibitory effect upon the expression of these cytokines and chemokines. There are also studies which tell us that NF-κB plays an important role in the transcriptional control of expressions of pro-inflammatory genes in various cells [27]. There is also evidence which suggest that the induction of NF-κB dependent gene expression in kupffer cells contributes thioacetamide induced acute liver injury [28]. Although PTX is known to suppress the activation of NF-κB and its translocation to nucleus and inhibit inflammation [29, 30], we found that compared with the control group, CCl_4 intoxication led to increased NF-κB expression in protein level, however, PTX inhibited the activation of NF-κB in CCl_4 treated mice.

Oxidative stress plays a pivotal 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 crucial step in the pathogenesis of CCl_4 induced hepatotoxicity. CCl_3 further reacts with O_2 to form trichloromethyl free radical (CCl_3O_2) [31]. The activity of NOX and CYP2E1 enzymes are increased in the CCl_4 treated mice because these two enzymes are the indicator of oxidative stress whereas PTX ameliorated the activity of these two main ROS producing enzymes to almost basal level. The level of MDA which is a product of membrane lipid peroxidation processes is known to be an important indicator of oxidative stress [32]. This present study showed that the oxygen-derived free radicals play the major role in the pathogenesis of CCl_4 induced acute liver injury in mice. CCl_4 treated mice were found to exhibit decreased liver SOD, GSH and increased MDA level. By PTX administration, their levels were transferred to normal level as control groups. These data suggests that CCl_4 induced liver injury is mediated through oxidative stress.

5. Conclusions

In conclusion, the present study demonstrates that administration of PTX can reduce CCl_4 induced liver injury in mice. The protective effect of PTX may be due to the inhibition of oxidative stress, the expression of pro-inflammatory cytokines, and the activation of NF-κB.

Recommendations

Our studies revealed that antioxidant and anti-inflammatory effect of PTX are possible mechanisms to improve the oxidative stress mediated CCl_4 induced liver injury. Since the pro-inflammatory cytokines exists in Kupffer cell, which is the target of PTX is still unclear from this study. So another in vitro study is needed in the future to address this issue.

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