Preventive Effects of Different Doses of Pentoxyfilline Against CCl_4-Induced Liver Toxicity in Rats

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Abstract

The liver protective effects of pentoxifylline were studied through pre-treatment of rats with various intraperitoneal (IP) doses (25, 50 and 100mg/kg/day) 14 days before induction of liver toxicity by carbon tetrachloride (CCl_4). The parameters of oxidative stress, malondialdehyde (MDA) and reduced glutathione (GSH) were measured in liver homogenate in addition to histopathological examinations. Analysis of data revealed significant amelioration of oxidative stress in groups of animals pre-treated with different doses of pentoxifylline (PTX) compared to group of animals intoxicated by CCl_4 as evidenced by lowering MDA contents and elevation of GSH levels in liver tissue homogenate but the levels still significantly different compared to controls. Additionally, increasing doses of PTX produce a dose-dependent improvement in liver tissue damage induced by CCl_4, as evident histologically by the stained liver sections. In conclusion, these findings suggest that, the hepatoprotective effects of pentoxifylline are dose dependent.

Key words: pentoxifylline, CCl_4, liver toxicity.

الخلاصة

تم دراسة تقييم التأثير الوقائي لجرع متعددة من البنتوكسيفيلين (٢٥، ٥٠، ١٠٠ ملغم لكل كيلوغرام من وزن الجسم) داخل الصفاق في الجرذان ١٤ يوم قبل أستحداث التسمم الكبدي بواسطة رباعي كلوريد الكربون حيث تم قياس الأدلة الخاصة بظاهرة فرط الأكسدة مثل تراكيز MDA , GSH في نسيج الكبد هذا بالاضافة الى دراسة نسيجية بفحص مقاطع من نسيج الكبد المصاب تحت المجهر الضوئي لمتابعة المتغيرات الحاصلة فيه. أظهر تحليل النتائج وجود تحسن ظاهر في معايير فرط الأكسدة من خلال انخفاض معنوي في مستوى MDA وأرتفاع مستوى GSH في نسيج الكبد مقارنة بالمجموعة المصابة الا ان مستويات الأدلة الخاصة بفرط الأكسدة بقيت مختلفة عن مستوياتها في مجموعة السيطرة ، كما أظهرت النتائج التأثير الوقائي للبنتوكسيفيلين من خلال منعه حدوث التأثيرات التلفية والألتهابية التي سببتها الجرعة السامة لرباعي كلوريد الكربون لدى الجرذان. ومن خلال النتائج التي تم الحصول عليها، يمكننا الاستنتاج بوجود علاقة وثيقة بين الجرعة المستخدمة من البنتوكسيفيلين وفعاليته الوقائية ضد التسمم في الكبد.

Introduction

Carbon tetrachloride (CCl_4) is a vehicle for organic compounds, formerly used as fire extinguisher, dry cleaner; however, its use for these purposes has now been abandoned because safer alternatives are available, but it is still used in fumigation of grain and insecticides [(1)]. It has been suggested that, free radical-mediated damage to the hepatocytes play an important role in the development of liver toxicity with CCl_4 [(2)] . The injury seems to be mediated by a reactive metabolites tri-chloromethyl free radical ($\cdot CCl_3$) formed by homolytic cleavage of CCl_4 or by an even the more reactive species, tri-chloromethyl peroxy free radical ($Cl_3COO^{\cdot}$), formed by the reaction of $^{\cdot}CCl_3$ with O_2 [(3)]. The reaction of the above mentioned free radicals with lipids and proteins causes the peroxidation of polyenoic lipids of the endoplasmic reticulum and the generation of secondary free radicals derived from these lipids (a chain reaction) [(4)]. Destructive lipid peroxidation leads to the breakdown of membrane structure and function. Furthermore, CCl_4 causes damage to the mitochondria and this consequently lead to decrease ATP synthesis and the hepatocytes accumulate large droplets of triglycerides in its cytosol as a result of membrane damage [(5)]. Pentoxifylline (PTX) is a synthetic dimethyl xanthine derivative designated chemically as 3, 7dimethyl-l-(5-oxohexyl) xanthine ($C_{13}H_{18}N_4O_3$) [(6)]. It is nonselective phosphodiesterase enzyme (PDE) inhibitor with rheologic property,

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as it inhibits erythrocytes PDE which results in an increase in erythrocytes cyclic adenosine monophosphate (c-AMP) activity, which in turn allows the erythrocytes membrane to maintain its integrity, with increasing red blood cell flexibility, promoting enhancement in tissue perfusion and improvement in regional microcirculation [7], thus, the drug is used in patients with venous leg ulcers or peripheral vascular disease to improve blood flow [8]. In addition, PTX reduces plasma fibrinogen concentration and increases the fibrinolytic activity, thus decreasing blood viscosity [9]. Apart from its effect on blood cell rheology, PTX has, in addition, anti-inflammatory properties through inhibition of cytokine production. It inhibits lipopolysaccharide-induced production of tumor necrosis factor alpha (TNF-α) by monocytes and T-cells as well as interleukin two (IL_2)-induced adherence of leukocytes [10, 11]. Moreover, the anti-inflammatory properties of pentoxifylline are probably related to its ability to suppress oxygen radical production and scavenge reactive oxygen species (ROS) *in vitro* from activated neutrophils [12] or in human erythrocyte membrane [13]. In addition, pentoxifylline and its metabolites modulate polymorphnuclear (PMN)adherence, superoxide production, deregulation, and migration altered by mononuclear leukocyte-produced inflammatory cytokines [14].This study was designed to evaluate the possible hepatoprotective effect of different doses of pentoxifylline against CCl_4-induced liver toxicity in rats.

Methods

Sixty white Albino male rats weighing 200-250g were used in this study; they were obtained from and maintained in the animal house of the College of Pharmacy, University of Baghdad under conditions of controlled temperature. Animals were fed commercial pellet and tap water *ad libitum*. Animal groups are treated as follows:

Group I – Ten rats received single daily dose of IP injection of 2 ml /kg /day of D.W for 14days. The animals were killed by anesthetic ether on the day 15.The group served as control.

Group II- Ten rats received single IP injection of pentoxifylline 100 mg/kg/day alone for 14 days to examine the effect of pentoxifylline on the liver function. The animals were killed by anesthetic ether on the day 15.

Group III – Ten rats received single daily dose of IP injection of 2 ml /kg /day of D.W. for 14 days. At the day 15, the animals received single dose of CCl_4 (99%) (2 ml of a mixture of 1:1 V/ V in a corn oil /kg /day) orally by gavages tube to induce liver damage in rats. The animals were killed by anesthetic ether 24 hr after CCl_4 administration [15]. The group served as positive control of hepatotoxicity.

Group IV- Thirty rats were utilized to study the possible protective effects of different doses of pentoxifylline against CCl_4-induced liver damage and allocated as follows:

- Ten rats received single daily dose of IP injection of pentoxifylline 25 mg/kg/day started 14 days prior treatment with CCl_4. The animals were killed by anesthetic ether on the day 16.
- Ten rats received single daily dose of IP injection of pentoxifylline 50 mg / kg / day started 14 days prior to treatment with CCl_4. The animals were killed by anesthetic ether on the day 16.
- Ten rats received single daily dose of IP injection of pentoxifylline (100 mg/kg/day) started 14 days prior to treatment with CCl_4. The animals were killed by anesthetic ether on the day 16.

Tissue homogenate was prepared by standard procedure [16] and the levels of malondialdehyde (MDA) [17] and reduced glutathione (GSH) [18] were analyzed in liver tissue homogenate. Small pieces of hepatic tissues were prepared for histopathological examination according to standard procedure and evaluated by ordinary microscope after staining with hematoxyline and eosin [19]. Statistical analysis of data was performed utilizing Student's t-test and ANOVA. 95% confidence of data was considered for significance.

Results

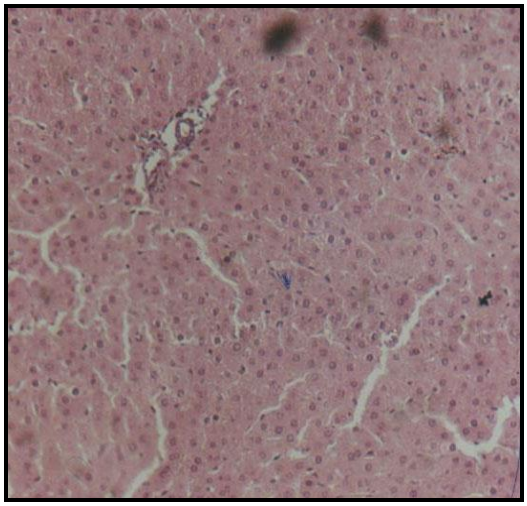
The data presented in (table 1) showed that, CCl_4 produces a highly significant increase in MDA contents of liver tissue homogenate compared to control group. ($P < 0.05$); while it produces a significant decrease in the level of GSH in rat's liver homogenate compared to control group. ($P < 0.05$). Single I.P. injection of $100mg.kg^{-1}$ pentoxifylline given to rats for 14 days, showed a non-significant difference ($P > 0.05$) on either the contents of lipid peroxidation product (MDA) in rat's liver homogenate or on the level of reduced glutathione (GSH) compared to control group as shown in (Table 1). Pre-treatment of rats with $25mg.kg^{-1}.day^{-1}$, $50mg.kg^{-1}.day^{-1}$ and $100mg.kg^{-1}.day^{-1}$ pentoxifylline IP 14-days before orally-administered CCl_4, resulted in 29.63%, 50.5% and 68.16% decline in hepatic MDA contents

compared to CCl_4-treated animals, respectively ($P<0.05$); but still significantly higher (167.29%, 88% and 20.91% respectively) compared to control group ($P < 0.05$). (Table1). Moreover, table 1, showed a significant difference between different doses of pentoxifylline on MDA contents of rat's liver homogenate ($P < 0.05$). Pre-treatment of rats with single I.P. injection of 25mg.kg^{-1}, 50mg.kg^{-1} and 100mg.kg^{-1} pentoxifylline for 14 days before orally-administered CCl_4, resulted in significant increase in hepatic GSH levels 106%, 183.98% and 344.13% ,respectively ($P < 0.05$); but still significantly lower 65.74%, 53.39% and 27.11% respectively, compared to the control group ($P < 0.05$). (Table 1). Concerning the effect of different doses of pentoxifylline on hepatic GSH levels, the results showed that, there were significant differences between 25mg.kg^{-1}.day^{-1}, 50mg.kg^{-1}.day^{-1} and 100mg.kg^{-1}.day^{1} pentoxifylline. ($P < 0.05$). (Table 1).The histological examination of liver sections from each animal treated with CCl_4 showed wide areas of severe ballooning degeneration, necrosis of hepatocytes, severe cholestasis especially around central vein (zone 3) with severe inflammatory cells reaction and severe steatosis (Figure 2 IandII) as compare with normal architect liver in control group (Figure 1).Increasing doses of pentoxifylline, produces a dose-dependent improvement in the degree of fatty degeneration in the scattered hepatocytes, previously induced by CCl_4 administration, associated with the attenuation of the degree of congestion and hemorrhage. The extent of the inflammatory cell infiltration was decreased as seen in (Figures 3, 4 and 5). Sections of the rat's liver treated with 100mg.kg^{-1}.day^{-1} pentoxifylline alone showed normal looking appearance of livers with mild congestion of blood vessels and mild dilatation of sinusoid in continuation with central vein (Figure 6).

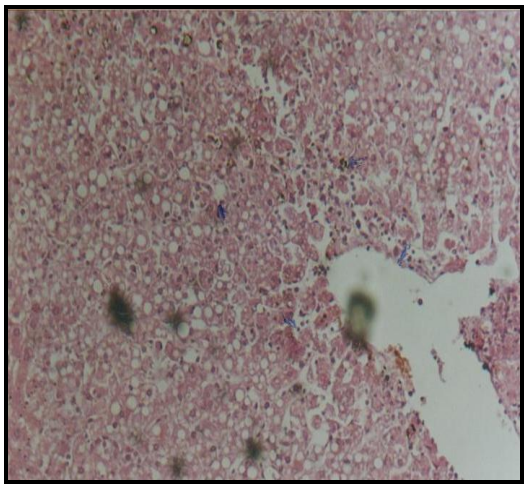
Table (1)The effects of pre-treatment with different doses of pentoxifylline and 100mg.kg^{-1} pentoxifylline alone on (MDA) contents and reduced glutathione (GSH) levels in rat's liver homogenate compared to CCl_4 and control groups.

	N	MDA nmol/g tissue	GSH µg/g tissue
Group I	10	108.912 ± 10.19^{a}	12.76 ± 2.488^{a}
Group II	10	117.024 ± 15.78^{a}	11.878 ± 2.96^{a}
Group III	10	413.686 ± 7.317^{b}	2.094 ± 0.634^{b}
Group IV-A	10	291.116 ± 12.65 $^{c\,A}$	4.371 ± 0.559 $^{c\,A}$
Group IV-B	10	204.76 ± 18.086 $^{d\,B}$	5.947 ± 0.466 $^{d\,B}$
Group IV-C	10	131.68 ± 9.05 $^{e\,C}$	9.3002 ± 1.186 $^{e\,C}$

-Each value represents mean ± SD.
-Values with non-identical superscripts (a, b, c, d and e) within each parameter are considered significantly different ($p <0.05$). -N = number of animals.
-Values with non-identical superscripts (A, B and C) within each parameter of pre-treated groups with different doses of PTX for 14 days are considered significantly different ($p <0.05$).
Group I **:** Control group.
Group II **:** Animals treated with 100mg.kg^{-1}.day^{-1} PTX alone.
Group III **:** CCl_4-treated group.
Group IV-A: Animals pre-treated with 25mg.kg^{-1}.day^{-1} PTX prior to CCl_4 intoxication.
Group IV-B: Animals pre-treated with 50mg.kg^{-1}.day^{-1} PTX prior to CCl_4 intoxication.
Group IV-C: Animals pre-treated with 100mg.kg^{-1}.day^{-1}PTX prior to CCl_4 intoxication.



Figure(1):section showing normal rat's liver
Magnification: (10*10) ; staining: Haematoxylline and Eosin.



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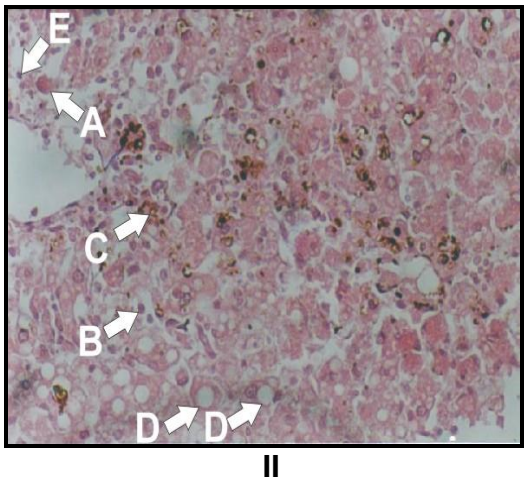
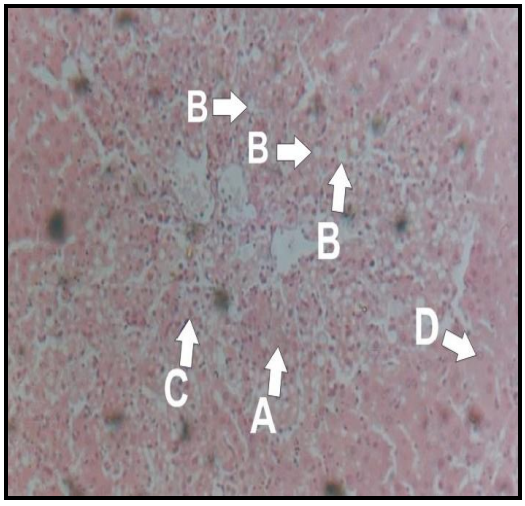


II

Figure (2): Sections showing morphological alteration of liver from CCl_4-treated rats
-**A**: Necrotic damage. -**B**: Ballooning degeneration. -**C**: Cholestasis. -**D**: Steatosis .-**E**: Inflammatory cell infiltration.
Magnification: I (10*10), II (10*20); staining: Haematoxylline and Eosin.



Figure(3): Section of rat's liver showing the effect of pre-treatment with 25mg.kg^{-1}.day^{1} PTX for 14 days prior to CCl_4.
-A: Necrotic damage.
-B: Steatosis.
-C: Inflammatory cell infiltration
-D: Normal hepatocyte.
Magnification: (10*10); staining: Haematoxylline and Eosin.

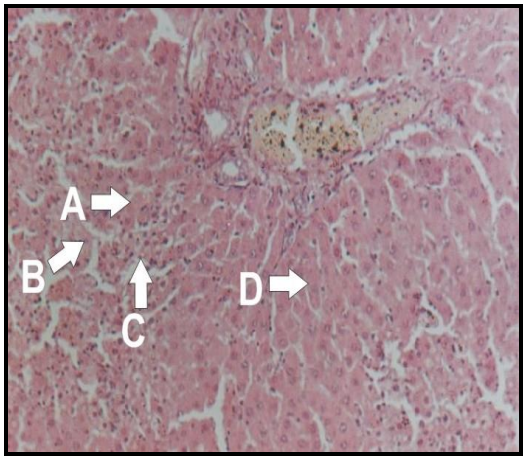


Figure (4): Section of rat's liver showing the effect of pre-treatment with 50mg.kg^{-1}.day^{1}PTX for 14 days prior to CCl_4.
-A: Necrotic damage.
-B: Inflammatory cell infilteration
-C: Kupffer cell no hyperplasia.
-D: Normal hepatocyte.
Magnification: (10*10); staining: Haematoxylline and Eosin.

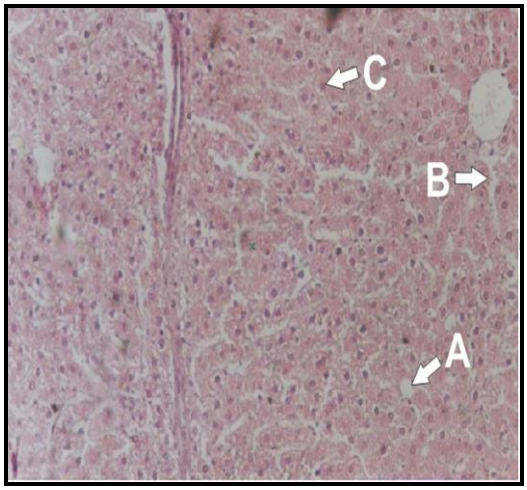


Figure (5): Section of rat's liver showing the effect of pre-treatment with 100mg.kg^{-1}.day^{1}PTX for 14 days prior to CCl_4.
-A: Mild steatosis
-B: Mild sinusoid dilatation.
-C: Normal hepatocyte.
Magnification: (10*10); staining: Haematoxylline and Eosin.

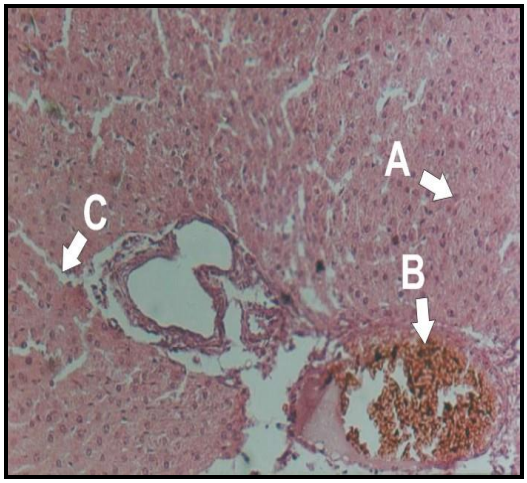


Figure (6): Section of rat's liver treated with 100mg.kg^{-1}.day^{-1} PTX alone for 14 days.
-A: Normal hepatocyte
-B: Congestion**.**
-C: Mild sinusoid dilatation.
Magnification: (10*10); staining: Haematoxylline and Eosin.

Discussion

Carbon tetrachloride (CCl_4) exerts its toxicity through its metabolites, including the free radicals ($\cdot CCl_3$) and ($Cl_3COO^{\cdot}$), both of which injure the hepatocytes by causing lipid peroxidation and binding covalently to cell systems associated with the formation of protein-lipid cross linkage, and among these also the binding of ($\cdot CCl_3$) adducts with DNA proteins and lipids [20]. The most serio consequence of free radical-induced liver injury is lipid peroxidation, and it has been found that, free radical can cause oxidative damage to cellular proteins and alter cellular function [21].The biochemical and histological evidences presented in this study clearly demonstrated the state of oxidative stress-induced hepatic tissue damage by CCl_4 treatment, manifested by the elevation of MDA contents in liver tissue homogenate, which is associated with sever depletion of GSH levels as shown in Table 1. These results are consistent with those observed by others [22]. The present work showed that, treatment of rats with different doses of PTX I.P., 14 days before orally-administrated CCl_4, resulted in a significant decrease in the MDA contents in liver tissue homogenate (Table 1). Many *in vivo* and *in vitro* studies concerning the antioxidant activity of PTX are conflicting. Reports relating to its antioxidant property are associated with leukocyte-driven radicals by inhibiting phagocytosis and superoxide production *in vitro* from activated neutrophils and monocytes [23]. Pentoxifylline may act as agonist for both adenosine receptors, A1 and A2; in addition to its property for inhibiting phosphodiesterase enzymes both effects leading to inhibition polymorphneuclear cells response [24]. The reduction of the activated neutrophils by PTX might be important, because increased oxidative stress is a feature of the CCl_4-induced liver injury and significant protection was obtained with the use of antioxidants [25]. Noyan, T. *et al* in 2006 [26] reported that, pre-treatment of mice with 50mg.kg^{-1} PTX for 7 days prior to CCl_4 the present work decreases MDA contents in liver tissue homogenate. The results of our work showed a similar data to that reported by previous authors [26]. Moreover, PTX has been shown to inhibit superoxide anion production by kupffer cells in rat liver preservation and transplantation models [27]. The reduction of hepatic lipid peroxidation induced by CCl_4 after treatment with different doses of PTX may be associated the inhibition of phosphodiesterase enzyme activity and increase of cAMP and cGMP levels [28]. In the contrary, our results are inconsistent with the results of the *in vitro* studies done by Oytun, P. *et al* in 1999 [29]; and by Horvath, B. *et al* in 2002 [30], where 50mg.kg^{-1} PTX neither preventing oxidative damage after short-term hepatic ischemia / reperfusion, nor have significant antioxidant capacity at therapeutic concentration against phenazine methosulphate, a free radical-generation agent, respectively. The present work showed that, pre-treatment of rats with different doses of

PTX (25mg.kg^{-1}.day^{-1}, 50mg.kg^{-1}.day^{-1} and 100mg.kg^{-1}.day^{-1}) for 14 days prior to orally-administrated CCl_4, significantly increases GSH levels in liver tissue homogenate (106%, 183.98% and 344.13%), respectively compared to CCl_4-treated animals (Table 3-1). Glutathione has powerful antioxidant properties through direct and indirect action. The main action of GSH is direct effect through the reduction of hydroperoxides, a reaction that is catalyzed by the enzyme glutathione peroxidase, while the indirect effect is through the reduction of other antioxidants like recycling vitamins (C and E) [31]. These reactions run nonenzymatically and depend on the presence of reducing agents [34]. Pentoxifylline has been shown to increase the activities of glutathione peroxidase (GSH-PX) and catalase enzymes, which found to be decreased in serum of mice intoxicated by CCl_4 [26].Pentoxifylline has largely been used in peripheral venous and cerebral circulatory disorders characterized by defective regional microcirculation [32]. It increases red blood cell flexibility, reduces blood viscosity, and decreases platelets aggregation, thereby improving microcirculation [33] and supporting the tissue with more oxygen, which in turn has been showed to potentiate the activity of GSH [21]. The effect of pentoxifylline in elevating GSH levels observed in the present study (Table 1) may be attributed to the vasodilator effect of pentoxifylline through the release of NO^* [34] and prostacycline [35], in addition to its properties for improvement the circulation and increased tissue oxygenation [36] , which in turn as similarly observed in previous report [37] may potentiate GSH activity for decreasing lipid peroxidation caused by CCl_3^* and CCl_3OO^*, a free radicals of CCl_4 and preventing the covalent binding of CCl_3OO^* radical but not CCl_3^* to cellular macromolecules [21]. The vasodilator effect of PTX is clearly evident histologically in section of rat's liver pre-treated with 100mg.kg^{-1}.day^{-1} prior to CCl_4 and in livers of animals treated with 100mg.kg^{-1}.day^{-1} PTX alone, figures (5 and 6), in which PTX promotes dilatation of sinusoids in continuation with central vein and congestion of blood vessels. The decline in the severity of degeneration and necrosis in rats pre-treated with 25mg.kg^{-1}.day^{-1} and 50mg.kg^{-1}.day^{-1} pentoxifylline and the disappearance of the degeneration and necrosis seen in rats pre-treated with 100mg.kg^{-1}.day^{-1} pentoxifylline may be due to its inhibitory effects on lipid peroxidation as evidence by decrease in MDA content and the ability for preserving GSH levels in liver tissue homogenate (Table 3-1). The data are consistent with others [26]. The inflammatory features observed by histological examination of rat's liver pre-treated with different doses of pentoxifylline 14 days prior to CCl_4 treatment was decreased from wide dispersed inflammatory cells to very few or no hyperplasia of kupffer cells with few polymorphneuclear cells. (Figures 3, 4 and 5), this indicates that, pentoxifylline may have anti-inflammatory effects against CCl_4-indued hepatic damage and the degrees of inflammation were gradually decreased upon increasing the dose of pentoxifylline. The data are comparable with the results obtained by the work of Abdel-Salam and his colleagues, in 2003 [38] where pentoxifylline was given at doses comparable to those used in man for the treatment of circulatory disorder (36 or 72mg.kg^{-1}) before the inflammatory agent carrageenan, significantly reduces carrageenan-induced paw edema response. Similarly, pentoxifylline was shown to suppress the activation of kupffer cells and thereby decreasing liver injury after transplantation [39]. Rockey *et al* in 1997 [40], suggested that, acute CCl_4 intoxication can results in a state of necro-inflammation through enhanced production of cytokines that could activate inducible nitric oxide synthase in the Kupffer cells. Nitric oxide (NO^*) and superoxide anion (O_2^{*-}) can form peroxynitrites with the subsequent release of highly reactive sulfhydryl oxidation and lipid peroxidation [41]. Pentoxifylline has a property for suppression the inducible nitric oxide synthase at mRNA levels [42]; and this may be one of the reasons that PTX attenuates liver damage and necro-inflammation. Moreover, pentoxifylline may inhibit the generation of mRNA for TNF-α *in vitro* and decrease the *in vivo* production of TNF-α in human and in experimental animals [43]. These inhibitory effects of PTX on TNF-α could be one of the reasons for its attenuating effects against CCl_4-induced liver injury and this supported by histological examination observed in the present work. (Figures 3, 4 and 5).In conclusion, the results of this study demonstrated the protective effect of PTX, by inhibiting the oxidative stress state, reversing the histopathological changes, improving the inflammatory changes and lowering the incidence of hepatic tissue necrosis induced by CCl_4 in a dose-dependant manner, were increasing the dose of PTX consequently reflected in better protection.

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