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Involvement of Kupffer Cell in CCl_4 Induced Liver Injury: The Role of Calcium

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ABSTRACT

The hypothesis that calcium provoke O_2^- formation by Kupffer cells and may contribute to carbon tetrachloride (CCl_4) induced liver injury was studied in SD rats. In CCl_4-treated animals, hepatic malonaldehyde (nmole/gm liver) and plasma ALT (IU/ml) levels elevated significantly from 119.63±13.00 to 268.97±14.82 and from 17.3±0.18 to 806.08±37.63, respectively, compared to those in controls. Activation of Kupffer cells with high dose of retinol (250,000 IU/kg/day, po, for 7 day) significantly enhanced ALT levels, while inactivation of Kupffer cells with gadolinium chloride (7.5 mg/kg/day, ip, for 2 day) attenuated the increase of serum ALT level following CCl_4 treatment. Diltiazem (10 mg/kg/day, ip for 2 day) given in combination with retinol led to a marked decrease in ALT levels compare to the level in rats treated only with retinol against CCl_4 treatment. In order to determine any alterations in cytochrome P450 activities, the P450 content and the CYP2E1 activity were measured and all CCl_4-treated rats showed significantly lower levels compared to those in controls and vehicle-treated animals. There were significant increases in glutathione peroxidase in all CCl_4-treated rats except diltiazem treated groups. No difference was found among untreated and vehicle-treated rats. It is concluded that Kupffer cells contribute to CCl_4-induced liver injury and that calcium antagonist attenuated the increased CCl_4-induced liver injury due to activation of Kupffer cells.

Key Words: CCl_4, Kupffer cell, Calcium, Lipid peroxidation

INTRODUCTION

The liver is particularly susceptible to chemically induced injury due to its extensive metabolic capacity and cellular heterogenicity. Carbon tetrachloride (CCl_4) has been extensively investigated as a model for inducing hepatotoxicity. CCl_4 is metabolized by cytochrome P450 2E1 (CYP2E1) to the highly reactive trichlormethyl radical, which induces lipid peroxidation and cytotoxicity (Hall *et al.*, 1991; Coleman *et al.*, 1986). Thus, it has been known that injury to the liver by CCl_4 is due to reactive radical metabolites. It, however, is unknown whether other cell types present in the liver also contribute to CCl_4-induced hepatotoxicity.

Acute liver damage is characterized by inflammation, hepatocellular necrosis and insufficient hepatocytes regeneration, which also has been shown after liver injury by CCl_4. Local production of cytokines by inflammatory cells at the sites of CCl_4 injury must play a key roles in modulating the sequence of reactions leading to the fibrotic response. Therefore, we studied a particular attention paid to the role of Kupffer cell in CCl_4-induced hepatotoxicity.

Kupffer cells, the resident macrophages in

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the liver are located at the lining of sinusoid and participate in the clearance of foreign bodies and endotoxines (Jaeschke and Farhood, 1991; Edwards *et al.*, 1993). Recent studies revealed that Kupffer cells contains a large amount of lysosomal enzymes and the cells activated with lipopolysacharide have an increased capacity to produce toxic form of oxygen (Hashimoto *et al.*, 1985). The oxygen derived radicals peroxidize lipid, disturb cell membrane function, and are implicated in tissue injury. Following hepatocyte damage by a toxicant, Kupffer cells are activated to release reactive oxygen species, immune mediators, increased inflammatory response then results in increased injury to the already toxicant-damaged hepatocytes (Sauer *et al.*, 1995).

Of particular interest has been the finding that metabolism of CCl_4 causes damage to the plasma membrane, and an increase increased Ca^{2+} content prior to cell death (Tsokos-Kunt, 1989). Calcium sequestration by the endoplasmic reticulum is especially sensitive to CCl_4-induced lipid peroxidation as well as to binding of metabolic products of CCl_4 to lipids and proteins of the endoplasmic reticulum (Glende *et al.*, 1986). In addition, Ozaki and Masuda (1993) reported that entry of extracellular Ca^{2+} into hepatocytes is a prerequisite for CCl_4-induced hepatocyte death and that association of Ca^{2+} with a CCl_4-derived radical-mediated process may be necessary for early and irreversible plasma membrane damages. It is possible that the metabolism of CCl_4-induced hepatotoxicity involves an activation of Kupffer cells by, if not all, increasing intracellular Ca^{2+} content, thereby may contribute to the death of hepatocytes. Advantages were taken of the ability of $GdCl_3$ and retinol to greatly diminish and activate, respectively, the function of Kupffer cells in the liver (Prazybocki *et al.*, 1992) Therefore, the major objective of this study was to evaluate the role of Kupffer cells in the response of Ca^{2+} to CCl_4 exposure and in the CCl_4-induced hepatotoxicity. The protective effect of calcium channel blocker on the CCl_4-induced hepatotoxicity was also studied.

MATERIALS AND METHODS

Animals and treatment

Male Sprague-Dawley rats (National Institute of Safety Research, Seoul), 200~300 g, were allowed free access to water and standard rat chow (Sam Yang). Comparison of the action of CCl_4 on the liver *in vivo* was made using control, $GdCl_3$, retinol-treated animals. The protective effects of diltiazem on CCl_4-induced liver injury were also studied. Groups of animals were pretreated as follows. (1) Retinol (250,000 IU/kg/day) was dissolved in corn oil and administered orally for 7 days. (2) $GdCl_3$ (7.5 mg/kg/day) was dissolved in saline and injected intraperitoneally 24 hr prior to and simultaneously with the experiment (3) Diltiazem (10 mg/kg/day) was dissolved in saline and injected intraperitoneally 24 hr prior to and simultaneously with the experiment. (4) Combination of retinol and diltiazem was administered as (1) and (3). After each pretreatment, CCl_4 (1 gm/kg, 30 w/w%) was dissolved in corn oil and administered orally 24 hr prior to sacrifice. All animals were fasted 24 hr prior to CCl_4 treatment. Blood were collected by heart puncture and liver were removed and processed for analytical determination.

Tissue preparation

Animals were killed between 10:00 and 12:00 a.m. Subcellular fractions were prepared by homogenizing the liver in 0.1 M Tris buffer containing 1.15% KCl (pH 7.5). The homogenate was centrifuged at 10,000 g for 20 min to remove nuclei, cell debris and mitochondria. The supernatant was further centrifuged at 105,000 g for 60 min. The resulting soluble supernatant (cytosolic) fraction was collected, and the microsomal pellet was resuspended in 1 M phosphate buffer (pH 7.25) containing 10 mM EDTA, 20% glycerin, 0.25 mM phenylmethylsulfonyl fluoride and 0.1 mM dithiothreitol. The subcellular preparations were stored at −70℃ until used.

Analytical procedures

Lipid peroxidation in the liver homogenate

was ascertained by the formation of malondialdehyde (MDA) and measured by the thiobarbituric acid methods as described by Uchiyama and Mihara (1978). Glutathione (GSH) content was assayed using 5,5'-dithiobis-(2-nitrobenzoic acid) according to the method of Spiesky *et al.* (1988). GSH peroxidase activity was assayed by the method of Lawrence and Burk (1976) wherein the oxidation of NADPH by cumene hydroperoxide was followed at 340 nm. GSH reductase was assayed by the method of Worthington and Rosemeyer (1974) by measuring the reduction of GSSG (oxidized GSH) to GSH by NADPH. Cytochrome P450 in liver was determined by the method of Omura and Sato (1964), using an extinction coefficient of 91 mM^{-1} for the 490~450 nm wavelength pair. The catalytic activity of CYP2E1 was measured by p-nitrophenol hydroxylation (Tierney *et al.* 1992). Biochemical liver function was determined by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

The activities of ALT and AST were determined by the method of Reitman and Frankel (1957) with a commercially available kit (Asan Pharmaceutical Co.) Protein concentrations were determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistical analysis

Data are expressed as mean±SEM. Statistical analysis was carried out using the one-way analysis of variance (ANOVA), and Duncan's multiple range finding test (1955) was used to evaluate differences among the group means.

RESULTS

The effects of $GdCl_3$, retinol and diltiazem on AST and ALT release following CCl_4 exposure

As expected, loss of plasma membrane integrity was evident as dramatically elevated level of serum AST and ALT activities 24 hr after a single oral dose of CCl_4 (1 gm/kg) as compared with vehicle treated rats (Fig. 1). This increase was more pronounced in the retinol-pretreated

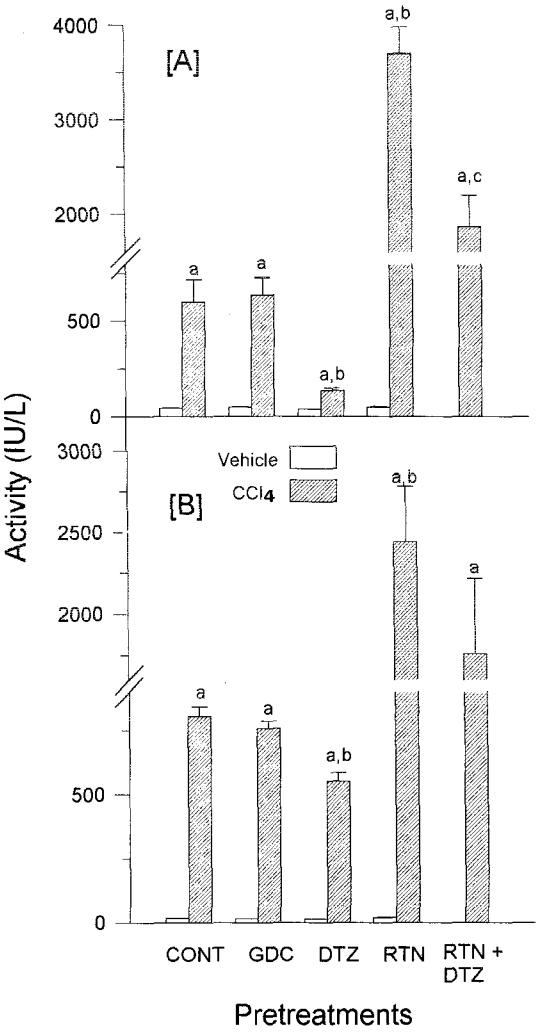

Fig. 1. The effects of GdCl3, retinol and diltiazem on AST and ALT release following CCl_4 exposure.

Rats were given 1 gm/kg (ig) in corn oil 24 hr prior to sacrifice. Some rats were pretreted with $GdCl_3$ (GDC), retinol (RTN), diltiazem (DTZ), or retinol and diltiazem (RTN+DTZ) as described under Methods. Serum samples were collected and analyzed [A] AST and [B] ALT activities.

[a] Significantly different from similarly pretreated controls, $p<0.001$

[b] Significantly different from CCl_4 controls, $p<0.001$

[c] Significant different from RTN and RTN+DTZ, $p<0.001$

Mean±SEM.

animals. The serum aminotransferase levels were not significantly different among $GdCl_3$, retinol and diltiazem pretreated rats following vehicle treateatment. When diltiazem was administered 24 hr prior to and simultaneously with CCl_4, there was significant reestablishment of these parameters. Further, there was considerable reduction in serum aminotransferase activities in retinol along with diltiazem pretreated group.

The effects of $GdCl_3$, retinol and diltiazem on AST and ALT release following CCl_4 exposure

Since lipid peroxidation mediated by CCl_4 contributes significantly to the hepatotoxicity of this substrate, we determined malondialdehyde (MDA) formation as an index for lipid peroxidation (Fig. 2). As expected, significant increase in MDA formation was detected in the CCl_4-treated rats as compared with vehicle-treated group. As compared with CCl_4-treated controls, MDA levels in liver from retinol pretreated rats were markedly enhanced. Importantly, the pretreatment with diltiazem followed by retinol also significantly inhibited the enhanced MDA formation observed in retinol alone pretreated rats administered CCl_4

The effects of CCl_4 intoxication on hepatic GSH peroxidase and GSH reductase activities in animals pretreated with $GdCl_3$, retinol and diltiazem

As illustrated in Table 1, there was no alteration in either GSH peroxidase nor GSH reductase activities in liver following $GdCl_3$, retinol and diltiazem. These results suggest that reduction on GSH-dependent lipid peroxidation inhibiting factors was not involved in the enhanced hepatotoxicity by CCl_4 administration following retinol pretreatment. A small increase in GSH peroxidase was observed after CCl_4 treatment. The increased activity of GSH peroxidase was reversed by pretreatment with diltiazem.

The effects of CCl_4 intoxication on hepatic microsomal cytochrome P450 in animals pretreated with $GdCl_3$, retinol and diltiazem

Pretreatment with retinol and diltiazem resulted no observed changes in either cytochrome P450 content nor CYP2E1 activity measured as *p*-nitrophenol hydroxylation (Table 2). In contrast, small but statistically insignificant decrease in those values was noted in $GdCl_3$-treated microsomes (Table 2). The specific content of cytochrome P450 (pmoles/mg protein) in microsomes from animals pretreated as described above and then challenged with CCl_4 24 hr prior to sacrifice was significantly reduced. The CYP2E1 activity also decreased, to a lesser extent, in all the pretreated animals following CCl_4 exposure. The total microsomal protein recovered was approximately 1.5 fold decreased by CCl_4 treatment (data not shown).

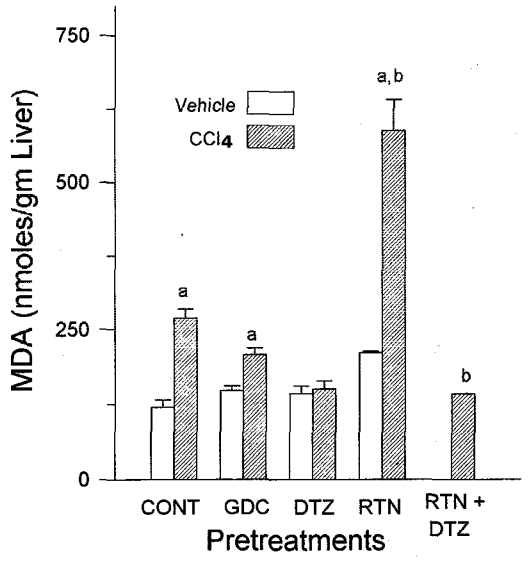

Fig. 2. The effects of $GdCl_3$, retinol and diltiazem on lipid peroxidation following CCl_4 exposure. Rats were given 1 gm/kg (ig) in corn oil 24 hr prior to sacrifice. Some rats were pretreted with $GdCl_3$ (GDC), retinol (RTN), diltiazem (DTZ), or retinol and diltiazem (RTN+DTZ) as described under Methods. Liver was homogenized and analyzed malondialdehyde (MDA) production.
[a] Significantly different from similarly pretreated controls, $p<0.001$
[b] Significantly different from CCl_4 controls, $p<0.001$
Mean±SEM.

Table 1. The effects of $GdCl_3$, retinol and diltiazem on cytochrome p450 contents and CYP2E1 activities following CCl_4 exposure

	GRx activity(umol/min/mg protein		GPX activity(umol/min/mg protein	
	Vehicle	CCl_4	Vehicle	CCl_4
Controls	241.41±1.57	213.59±11.22	26.54±0.77	43.31±3.25[a]
$GdCl_3$	239.31±8.96	212.89± 5.86	31.52±0.99	49.47±6.01[a]
Diltiazem	256.56±6.31	187.26±13.12[a,b]	31.41±2.56	33.31±3.75
Retinol	238.23±26.41	261.51±22.64	26.81±3.27	47.47±3.11[a]
Retinol+Diltiazem	n.d	177.59± 6.55	n.d	32.65±2.00

Rats were given 1 gm/kg (ig) in corn oil 24 hr prior to sacrifice. Some rats were pretreted with $GdCl_3$ (GDC), retinol (RTN), diltiazem (DTZ), or retinol and diltiazem (RTN+DTZ) as described under Methods.

[a]Significantly different from similarly pretreated controls, $p<0.001$.

[b]Significantly different from CCl_4 controls, $p<0.001$.

Mean±SEM.

Table 2. The effects of $GdCl_3$, retinol and diltiazem on cytochrome p450 contents and CYP2E1 activities following CCl_4 exposure

	Cytochrome P450(pmoles/mg protein		CYP2E1 activity(nmol/min/mg protein)	
	Vehicle	CCl_4	Vehicle	CCl_4
Controls	502.66±17.01	80.01±18.67[a]	2.46±0.05	1.45±0.06[a]
$GdCl_3$	372.33±83.21	68.33±10.33[a]	1.98±0.14	1.39±0.08[a]
Diltiazem	502.67±73.98	59.67± 3.67[a]	2.45±0.18	1.34±0.04[a]
Retinol	491.33±23.67	38.56±12.33[a,b]	2.59±0.36	1.39±0.05[a]
Retinol+Diltiazem	n.d	46.33±10.00	n.d	1.50±0.08

Rats were given 1 gm/kg (ig) in corn oil 24 hr prior to sacrifice. Some rats were pretreted with $GdCl_3$ (GDC), retinol (RTN), diltiazem (DTZ), or retinol and diltiazem (RTN+DTZ) as described under Methods.

[a]Significantly different from similarly pretreated controls, $p<0.001$.

[b]Significantly different from CCl_4 controls, $p<0.001$.

Mean±SEM.

DISCUSSION

The objectives of this study was to elucidate the relationship between Kupffer cell and calcium on CCl_4-induced liver injury. The potentiation of MDA production and serum aminotransferases activities by retinol pretreatment and reestablishment of those parameters by diltiazem were significant. These findings lead to the hypothesis that reactive species of oxygens released from activated Kupffer cells are responsible for the increase in CCl_4-induced lipid peroxidation in retinol treated rats, thus for the increase in CCl_4-induced liver injury. It was possible that the mechanisms of potentiation of retinol on CCl_4-induced liver injury were by the alterations in any of GSH-dependent system, cytochrome P450, and Kupffer cell activation.

Connor *et al.* (1990) showed that hepatic GSH reacted with the trichlormethyl radical from CCl_4 metabolism to form a radical adduct. Pro-

tection of cellular membranes is largely afforded by the a variety of scavenging compounds and antioxidant enzymes oppose the initiation and propagation of lipid peroxidation. Several of these enzymes depend on soluble thiol compounds, particularly GSH (Murphy *et al.* 1992; Shull *et al.*, 1991). Conceivably, oxidant damages results when antioxidant enzyme defence are overwhelmed. Therefore, it is possible that the effects of $GdCl_3$, retinol or diltiazem on CCl_4-induced liver injury are due to alterations in GSH dependent systems, such as GSH, GSH peroxidase, and GSH reductase. However, neither of those parameters in liver of rats treated in vivo with $GdCl_3$, retinol or diltiazem were different from values in untreated livers, making this hypothesis unlikely. The increased GSH peroxidase activity was in accordance with the hypothesis reported by Lundqvist and Morgenstern (1992) that oxidative stress up-regulate the enzyme which could thereby afford increased protection against the deleterious effects of oxidative stress (e.g. lipid peroxidation).

Another possibility is that the CYP2E1 metabolic pathway is altered by either of $GdCl_3$, retinol or diltiazem pretreatment and therefore the toxic trichlormethyl radical formation is affected. The assumption that retinol was modulating the response of the liver to CCl_4 directed the type of studies performed to determine the mechanism of this potentiation. The alcohol inducible CYP2E1 has been well characterized (Howell et al., 1986) It has been suggested that induction of this isozymes by various alcohol provides an explanation on a molecular level for the potentiating effect of aliphatic alcohol and ethanol on CCl_4 hepatotoxicity (Hall *et al.* 1991). However, specific cytochrome P450 content and CYP2E1 activity were not altered following retinol nor diltiazem pretreatment. $GdCl_3$ treated animals showed decreased in those values but the reduction was not statistically significant. These results make the possibility that alteration in cytochrome P450 following pretreatment affect CCl_4 toxicity also unlikely.

Since CCl_4 is a suicide substrate of cytochrome P450, increased radical production enhances enzyme loss (Azri *et al.*, 1992; Tierney *et al.*, 1992). The residual specific contents of microsomal cytochrome P450 in animals were markedly diminished following CCl_4 treatment. The loss in cytochrome P450 content was far greater than the changes in CYP2E1 activity.

Kupffer cells, normally protect hepatocyte by incoming particle. When activated, however, Kupffer cells release large amount of lysozyme enzymes which have the potential to kill the hepatocyte. Pretreatment of rats with large dose (250,000 IU) of retinol dramatically increased the hepatotoxicity of CCl_4. which support the mechanism of this potentiation appears to involve of reactive species of oxygen from Kupffer cells activated by retinol. Towner *et al.* (1994) have reported that CCl_4 caused a localized region of hepatic edema within in 1 hr by in in vivo proton magnetic resonance imaging. However, if rat were pretreated with gadolinium chloride, and inhibitor of Kupffer cell function, the CCl_4-induced edema was greatly decreased. Electron micrographs of samples that were taken from regions of the liver where the edema was localized indicated formation of vacuoles and lipid droplets in parenchymal cells and enlargement of Kupffer cells. This electron micrograph changes were also attenuated by pretreatment of the rats with $GdCl_3$. These observations support present results the Kupffer cells may involved in CCl_4-induced hepatotoxicity.

The role of Ca^{2+} in the regulation of physiological processes and their role in a variety of pathological and toxicological alterations is well established. Alterations in Ca^{2+} homeostasis induce important functional and morphological changes in cells prior to cell death. This Ca^{2+}-dependent cell damage is not restricted to a specific cell system and the liver is very sensitive to this alteration (Farber, 1981; Romero *et al.*, 1994) Since the metabolism of CCl_4 causes damage to the plasma membrane and an increase intracellular Ca^{2+} content prior to cell death as shown by Romero *et al.* (1994), it is possible that the metabolism of CCl_4 activates Kupffer cells by increasing intracellular Ca^{2+} contents. Calcium ions has been implicated in responses of phagocytosing cells to external stimuli. Rat Kupffer cells as resident macrophages of the liver react in a manner similar to other phagocytosing cells. Additional mononuclear phagocytes are also recruited from

blood. Once localized in the injured area, the macrophage become activated and release mediators that contribute to damage initiated by CCl_4. This eventually leads to cell death and necrosis. Therefore, we assume that diltiazem, a calcium channel blocker, can reduce CCl_4-induced hepatotoxicity especially that with retinol pretreatment.

Taken together, the results suggest a novel mechanism of interaction between Kupffer cells and Ca^{2+} in CCl_4-induced liver injury. It is recognized that the Ca^{2+} njury, is in part, a consequence of the metabolism by the liver of the CCl_4 into highly reactive trichlormethyl radical (Danni *et al.*, 1991). The subsequent reaction of these radicals within the endoplasmic reticulum results in the initiation of peroxidative degradation of phospholipids and the binding of electrophilic radicals to membrane lipids and protein, which results in disruption of calcium homeosis. In addition, large doses of retinol can cause a n activation of the resident macrophage of the liver, Kupffer cells. The activated Kupffer cells are stimulated to release reactive species of oxygen. These reactive oxygen species again can stimulate lipid peroxidation. Following hepatocyte damage by CCl_4 exposure, Kupffer cells activation and increased intracellular calcium might contribute to an increased injury to the already toxicant-damaged hepatocytes. The net results of Kupffer cell activation and increased Ca^{2+} levels contribute a dramatic potentiations of CCl_4-induced hepatotoxicity. Therefore, we conclude thar Kupffer cells participate in the mechanism of CCl_4 toxicity in vivo, partly activated by intracellular calcium and diltiazem, calcium channel blocker, had inhibitory effects on the lipid peroxidation and liver injury after CCl_4 exposure

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=국문초록=

사염화 탄소에 의한 간손상에 있어 Kupffer cell 칼슘의 역할

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양 미 라

사염화 탄소에 의한 과산화 지질 증가 및 간 손상에 calcium 및 Kupffer cell의 역할 및 calcium channel blocker의 간 손상에 대한 방어 효과를 연구하였다. 사염화 탄소는 (1 gm/kg, ig) 간의 malondialdehyde (nmole/gm liver) 및 혈중 AST와 ALT (IU/ml) 활성도의 현저한 증가를 나타내었다. 고 농도의 Retinol (250,000 U/kg/day)로 인한 Kupffer cell의 활성 증가는 사염화 탄소에 의한 간 과산화 지질 증가 및 간 손상에 상승 작용을 나타낸 반면, $GdCl_3$ 전처리는 CCl_4로 인한 ALT의 증가를 감소시켰다. 한편 Retinol 처치군에 Diltiazem (10 mg/kg/day)을 병행하여 처치한 결과, 사염화 탄소에 의한 혈중 AST 및 ALT의 증가를 Retinol 단독 처치군에 비하여 현저하게 억제시킬 수 있었다. 이 결과들이 Retinol 혹은 Diltiazem의 투여에 의한 사염화 탄소가 cytochrome P450에 의한 대사 활성 또는 GSH와 관련된 항산화 기전에 미치는 영향에 기인한 것인가를 규명하기 위하여 cytochrome P450, cytochrome P4502E1 활성도, GSH reductase 및 GSH peroxidase 활성도를 측정하였다. 그 결과, Retinol 및 Diltiazem의 전처리는 이들 효소의 활성도에 미치는 영향은 대조군에 비하여 유의한 차이가 없었다. 이상의 실험 결과를 종합하여 보면, 사염화 탄소의 투여에 의한 간 손상은 세포내 calcium의 증가를 가져오며, 이는 이차적으로 Kupffer cell을 활성화 시켜 이미 손상된 간세포의 독성을 증가시켰으며, calcium channel blocker인 Diltiazem의 투여는 사염화 탄소의 간독성을 현저하게 감소시키는 효과를 나타내었다.