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# The Effect of Amlodipine, Diltiazem and Enalapril on Hepatic Injury Caused in Rats by the Administration of CCl₄

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**Abstract:** The present study compared the effect of the calcium channel blockers amlodipine and diltiazem with the ACE inhibitor enalapril on $CCl_4$-induced acute hepatic injury in rats. Amlodipine (0.9 or 1.8 mg kg$^{-1}$), diltiazem (10.8 or 21.6 mg kg$^{-1}$) and enalapril (0.9 or 1.8 mg kg$^{-1}$) were administered per os daily for 7 days, then acute hepatic injury was induced by treating rats using a gavage with a single dose of $CCl_4$-olive oil (1:1, 0.2 mL/100 g). Drug administration continued after $CCl_4$ and the treated animals were killed on day 3 after $CCl_4$ administration. Results indicated that whereas a reduction in serum enzyme levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was obtained with amlodipine and enalapril, no protective effect was observed for diltiazem in this model of hepatic injury. Thus, compared with the $CCl_4$ control group, serum ALT decreased by 56.2-61.4 and AST by 16-25.1%, after the administration of amlodipine at 0.9 and 1.8 mg kg$^{-1}$, respectively. Serum ALP was significantly reduced by 40% by 1.8 mg kg$^{-1}$ amlodipine. Enalapril administered at 0.9 and 1.8 mg kg$^{-1}$ significantly decreased ALT by 22.8-61.4%, while AST and ALP were significantly reduced by 37.3 and 54.5%, respectively by 1.8 mg kg$^{-1}$ enalapril. In contrast, diltiazem administered at 20.4 mg kg$^{-1}$ increased ALT and AST levels by 43 and 16%, respectively. Histologic examination of haematoxylin and eosin stained sections from the livers of rats treated with $CCl_4$ and amlodipine showed prominent improvement in liver architecture, a decrease in inflammatory cells and necrotic area. Electron microscopic examination of hepatocytes of $CCl_4$-treated rat showed disorganization of the cytoplasmic structure and degeneration of cytoplasmic organelles. Electron microscopy of hepatocytes from the rats treated with $CCl_4$ + amlodipine revealed healthy cytoplasmic content with healthy nuclei and normal mitochondria. Similar findings were observed after enalapril treatment. In contrast, intensive necrosis and degeneration was seen in livers of rats treated with diltiazem. On electron microscopy in most of hepatocytes, the cytoplasm became lytic with distorted mitochondrial cristae and highly dilated endoplasmic reticulum. Thus two calcium channel blockers behaved differently as regards to hepatic injury in the model of $CCl_4$-induced hepatic injury. It is suggested that profound haemodynamic effects of diltiazem which undergoes extensive hepatic metabolism and the resultant decrease in hepatic blood flow account for the observed effect of the drug.

**Key words:** Diltiazem, amlodipine, enalapril, carbon tetrachloride, hepatoprotective

## INTRODUCTION

In the search for drugs that can lessen hepatic injury and prevent fibrosis, experimental evidence suggest that calcium channel antagonists and Angiotensin Converting Enzyme (ACE) inhibitors would prove of value (Isozaki *et al.*, 2000; Rimola *et al.*, 2004). Calcium channel blockers limit the entry of

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calcium into hepatocytes and cytosol. Increased calcium entry has been linked to hepatic cell necrosis by many toxicants and it is widely accepted that these agents protect the liver by limiting the influx of extracellular calcium in liver cells. The calcium channel blockers verapamil, nifedipine and diltiazem were reported to exert protective effects against hepatic necrosis caused by a number of toxicants including thioacetamide, acetaminophen, dimethylnitrosamine (Landon *et al.*, 1986), ethanol, $CCl_4$ (Romero *et al.*, 1994), diamidinothionaphthene (Sippel *et al.*, 1993), lipopolysaccharide (Mustafa and Olson, 1999), tert-butyl hydroperoxide (Farghali *et al.*, 2000), atractyloside (Obatomi *et al.*, 2001) as well as from liver injury resulting from ischaemia-reperfusion (Konrad *et al.*, 1995, 1997; Isozaki *et al.*, 2000).

Angiotensin II is a peptide autacoid and a potent vasoconstrictor formed by the action of the enzyme renin secreted from the juxtaglomerular cells of the kidneys on angiotensin I. The latter is derived from its precursor angiotensinogen, synthesized and released into the blood by the liver. The blockade of angiotensin II synthesis by ACE-inhibitors or blockade of its action by angiotensin I receptor (AT-1) antagonists are currently the medications of choice in management of hypertension and cardiac failure (Katovich *et al.*, 2005). Circulating angiotensin II is dispersed to the target tissues of the body, where it exerts a multiplicity of physiological functions via their interactions with specific angiotensin receptors (De Gasparo *et al.*, 2000; Mehta and Griendling, 2007). Angiotensin II is pro-fibrotic and has been implicated in the stimulation of fibroblast proliferation and collagen synthesis by non-parenchymal cells. Its inhibition of its synthesis by ACE inhibitors attenuates fibrogenic response in different organs such as the kidney (Border and Noble, 2001), colon (Wengrower *et al.*, 2004), pancreas (Uesugi *et al.*, 2004) and liver (Rimola *et al.*, 2004).

In light of the above, the present study was designed to investigate and compare the effects of two calcium channel blockers diltiazem and amlodipine and an ACE inhibitor enalapril on the development of hepatic injury induced in rats by the administration of the hepatotoxin $CCl_4$. The effect of these agents was evaluated both on biochemical markers as well as by histological techniques and electron microscopy.

## MATERIALS AND METHODS

### Animals

Adult Sprague-Dawley rats of either sex, weighing 150-180 g of body weight were used throughout the experiments and fed with standard laboratory chow and water *ad libitum*. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

### Drugs and Chemicals

Carbon tetrachloride (BDH Chemicals, England), amlodipine (Alkan Pharma. Co., Egypt), Diltiazem (Global Napi Pharmaceutical, Egypt) and enalapril (Kahira Pharm and Chem. IND Co., Egypt) were used in the experiments.

### Drug Administration

The drugs used in the study were diltiazem, amlodipine and enalapril. Doses of 10.8 or 21.6 mg $kg^{-1}$ diltiazem, 0.9 or 1.8 mg $kg^{-1}$ amlodipine and 0.9 or 1.8 mg $kg^{-1}$ enalapril were administered orally in a volume of 0.5 mL. Controls received the appropriate volume of vehicle. The choice of the doses was based on the human daily doses of the drugs (10 and 20 mg for both amlodipine and enalapril and 120 mg for diltiazem) after being converted to those of rat/kg of body weight according to Paget and Barens (1964). Drug were freshly dissolved in physiologic saline immediately before administration.

**CCl₄-induced Hepatic Injury**

Different groups of rats (n = 7-8 per group) received two doses of each of the drugs in the study once daily for 7 days, then acute hepatic injury was induced by treating rats by gavage with a single dose of $CCl_4$ in olive oil (1:1, 0.2 mL/100 g). Control rats were treated with a single dose of olive oil (0.2 mL/100 g). Drug administration continued after $CCl_4$. The treated animals were killed on day 3 after $CCl_4$ or olive oil administration. Rats had free access to food and drinking water during the study.

**Biochemical Assessment**

Blood samples were obtained from the retro-orbital vein plexuses 72 h after $CCl_4$ administration. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were measured according to Reitman-Frankel colorimetric transaminase procedure (Crowley, 1967), whereas colorimetric determination of ALP activity was done according to the method of Belfield and Goldberg (1971), using commercially available kits (BioMérieux, France).

**Histological Methods**

After the end of the treatment period, rats were killed by decapitation, livers were excised and fixed in 10% formalin saline for the histological investigations. After trimming, the tissues were processed through graded alcohol for dehydration and cleared in xylene and embedded in paraffin. Paraffin sections were made at 5μ and stained with haematoxylin and eosin. Sections were examined under light microscope. Liver sections (1 mm each), were removed and pre-fixed for 2 h in 2.5% glutaraldehyde. The samples were then rinsed in the same buffer and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3). Post-fixation was followed by dehydration in ethanol, embedding in Epon 812 and polymerization. Tissues were cut using an LKB 2088 ultra-microtome, stained with 1% uranyl acetate and lead citrate and examined with a transmission SEO electron microscope.

**Statistical Analysis**

All results are expressed as means±SE. Multiple group comparisons were performed by ANOVA followed by Duncan multiple range test. $p < 0.05$ was considered statistically significant.

## RESULTS

**Biochemical Observations**

The administration of $CCl_4$ resulted in marked rise in levels of AST, ALT and ALP in plasma compared with the control group that received only olive oil, indicating the severity of hepatic injury caused by $CCl_4$. Seventy-two hours after $CCl_4$, the serum ALT and AST levels were significantly elevated from 49.2±1.4 and 87.0±5 (olive oil) to 188.4±12.2 and 254±9.2 IU $L^{-1}$, respectively ($p < 0.01$). Rats given $CCl_4$ exhibited in addition elevated levels of ALP in serum (54.9±5.1 vs 146.3±11.3; $p < 0.01$) indicative of liver cell injury and release of membrane enzymes or congestion and cholestasis. The administration of amlodipine at 0.9 and 1.8 mg $kg^{-1}$ caused a significant reduction in the levels of the serum enzymes ALT and AST by 56.2-61.4 and 16-25.1%, respectively. Serum ALP was significantly reduced by 39% by 1.8 mg $kg^{-1}$ amlodipine. In contrast, diltiazem given at 10.2 mg $kg^{-1}$ to $CCl_4$-treated rats did not significantly affect the indices of hepatic injury and stasis. Diltiazem administered at 20.4 mg $kg^{-1}$ increased ALT and AST levels by 43 and 16%, respectively. Enalapril administered at 0.9 and 1.8 mg $kg^{-1}$ resulted in a significant decrease in ALT by 22.8-61.4%, while AST and ALP were significantly reduced by 37.3 and 54.5%, respectively by 1.8 mg $kg^{-1}$ enalapril (Table 1).

Table 1   Effect of amlodipine, diltiazem and enalapril on serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in $CCl_4$-treated rats

| Treatments | ALT (IU $L^{-1}$) | AST (IU $L^{-1}$) | ALP (IU $L^{-1}$) |
|---|---|---|---|
| Saline (normal) | 49 2±1 4 | 87 0±5 0 | 54 9±5 1 |
| $CCl_4$ (control) | 188 4±12 2 | 254 0±12 0 | 146 3±11 3 |
| $CCl_4$ + amlodipine 0 9 (mg $kg^{-1}$) | 82 6±6 9* | 213 5±10 2* | 129 1±13 6 |
| $CCl_4$ + amlodipine 1 8 (mg $kg^{-1}$) | 72 8±5 1* | 190 2±8 0* | 89 3±6 2* |
| $CCl_4$ + diltiazem 10 2 (mg $kg^{-1}$) | 195 8±20 2 | 244 6±6 7 | 153 9±6 2 |
| $CCl_4$ + diltiazem 20 4 (mg $kg^{-1}$) | 270 2±10 7* | 294 8±11 0* | 157 7±10 0 |
| $CCl_4$ + enalapril 0 9 (mg $kg^{-1}$) | 145 4±11 2* | 234 0±13 2 | 127 4±11 4 |
| $CCl_4$ + enalapril 1 8 (mg $kg^{-1}$) | 72 8±6 1* | 159 2±11 0* | 66 5±6 1* |

Amlodipine (0 9 or 1 8 mg $kg^{-1}$), diltiazem (10 8 or 21 6 mg $kg^{-1}$) and enalapril (0 9 or 1 8 mg $kg^{-1}$) were administered per dose daily for 7 days, then acute hepatic injury was induced by treating rats using a gavage with a single dose of $CCl_4$ in olive oil (1 1, 0 2 mL/100 g)  Control rats were treated with a single dose of olive oil (0 2 mL/100 g)  Drug administration continued after $CCl_4$ and the treated animals were killed on day 3 after $CCl_4$ administration  Results are mean±SE  * $p<0.05$ (ANOVA) vs control group  One way ANOVA and Duncan's multiple range test
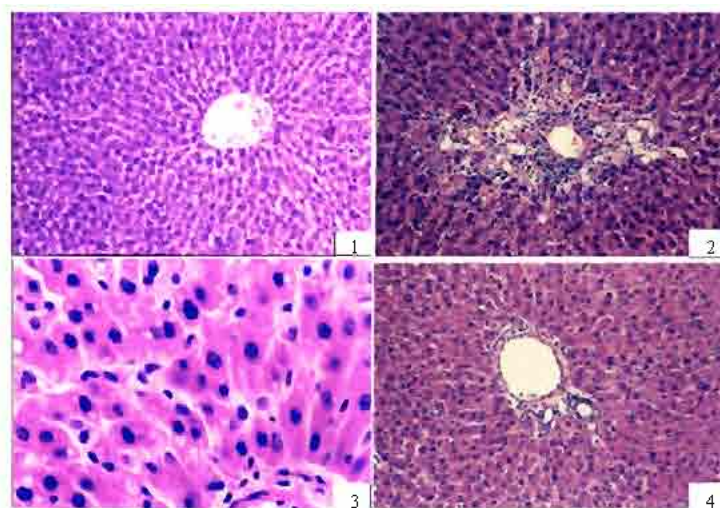


Fig. 1-4:  Hematoxylin and eosin stained sections from
1:  Normal rat liver showing normal liver architecture (Hx and E X 150)
2:  Rat liver treated with $CCl_4$-olive oil showing perivascular necrosis, mononuclear cell infiltration and fatty degeneration; (Hx and E X 150)
3:  Rat liver treated with $CCl_4$-olive oil showing kupffer cell hypertrophy, increase in size of many hepatocyte nuclei, together with a number of pleomorphic giant cells are also prominent. Some nuclei are hyperchromatic, others showing different degrees of degeneration (goast, karyolysis, karyorhexis or fragmented) (Hx and E X 500) and
4:  Rat liver treated with $CCl_4$ and amlodipine (1.8 mg $kg^{-1}$) showing prominent improvement in hepatocytes and a decrease in inflammatory cells and necrotic area (Hx and E X 150)

**Histological Observations**
**Light Microscopy**

Haematoxylin and eosin stained sections from control rats showed the characteristic hepatic architecture with central veins and radiating hepatic cords (Fig. 1). Examination of liver sections from $CCl_4$-olive oil showed perivascular necrosis, mononuclear cell infiltration and fatty degeneration,

Kupffer cell hypertrophy, increase in size of many hepatocyte nuclei (Fig. 2 and 3). Examination of sections from the liver of rats treated with $CCl_4$ and amlodipine showed prominent improvement in liver architecture, a decrease in inflammatory cells and necrotic area (Fig. 4 and 5). In contrast, intensive necrosis and degeneration were seen in liver of rats treated with diltiazem (Fig. 6 and 7). Pretreatment with enalapril resulted in the hepatocytes regaining their normal pattern (Fig. 8).
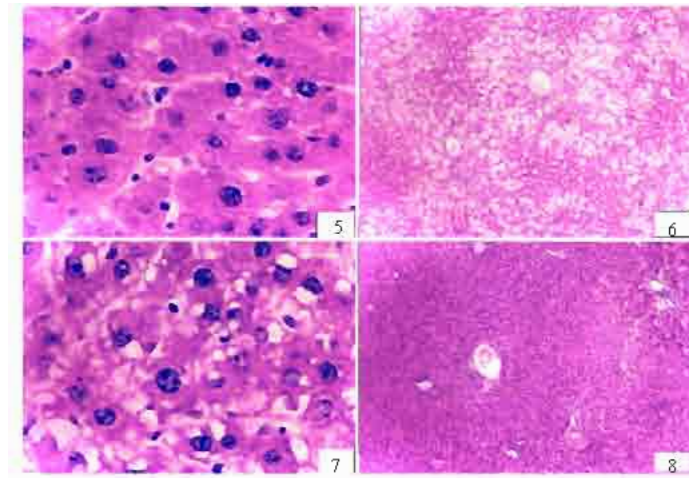


Fig. 5-8: Hematoxylin and eosin stained sections from

5: Rat liver treated with $CCl_4$ and amlodipine (1.8 mg kg$^{-1}$) showing remarkable regenerative capacity. Most cells are in mitosis and nuclei were activated with many nucleoli. Granular cytoplasm and dilated sinusoids are also seen (Hx and E X 500)

6: Rat liver treated with $CCl_4$ and delitiazem showing intensive necrosis and degeneration. Most hepatocytes were apoptotic as well (Hx and E X 50)

7: Rat liver treated with $CCl_4$ and delitiazem showing an increase in nectrotic sites in parenchymal cells and a decrease in the number of nuclei. The nuclei are showing abnormal size and shape (Hx and E X 500) and

8: Rat liver treated with $CCl_4$ and enalapril showing the hepatocytes regaining their normal pattern (Hx and E X 50)
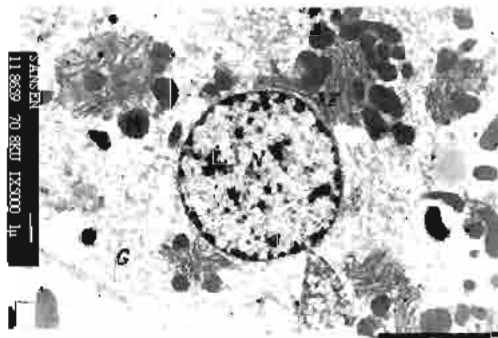


Fig. 9: An electron micrograph of hepatic cell of normal rat. M: Mitochondria, RE: Rough endoplasmic reticulum, G: glycogen granules (X 5000)

**Electron Microscopy**

Figure 9 shows an electron micrograph of hepatic cell of normal rat. After treatment with $CCl_4$, disorganization of the cytoplasmic structure and degeneration of the cytoplasmic organelles were evident. Most mitochondria were small, round and with electron dense matrix. The endoplasmic reticulum profiles and the glycogen granules disappeared (Fig. 10). Electron microscopy of hepatocytes from rats treated with $CCl_4$ + amlodipine revealed a healthy cytoplasmic content with healthy nuclei and normal mitochondria (Fig. 11). In contrast, in hepatocytes of rats treated with diltiazem, in most of hepatocytes, the cytoplasm became lytic with distorted mitochondrial cristae and highly dilated endoplasmic reticulum (Fig. 12). After enalapril treatment, hepatocytes showed normally arranged cisternae of rough endoplasmic reticulum and condensed mitochondria with normal cristae (Fig. 13).
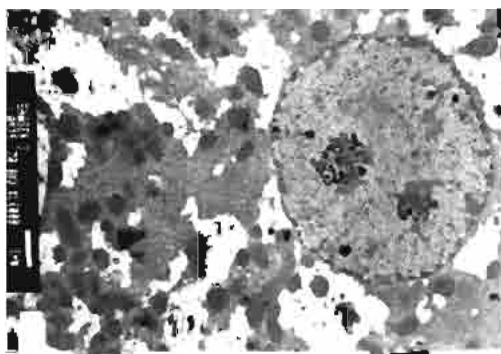


Fig. 10: An electron micrograph of hepatic cell of $CCl_4$-treated rat, showing disorganization of the cytoplasmic structure, the cytoplasmic organelles are degenerated, most mitochondria are small, rounded and with electron dense matrix. The endoplasmic reticulum profiles and the gylcogen granules disappeared (X 6000)
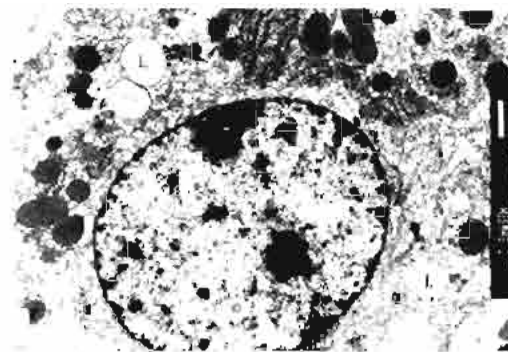


Fig. 11: An electron micrograph of hepatocytes of $CCl_4$-and amlodipine (1.8 mg kg$^{-1}$)-treated rat, showing healthy cytoplasmic content with healthy nuclei, rounded and had condensed chromatin. The mitochondria are in normal size and their membrane seems to be normal but still with electron dense matrix. The endoplasmic reticulum is well defined. The rough endoplasmic reticulum has ribosomes. Glycogen particles (g) are also observed. Few fat droplets (L) are also present (X 6000)
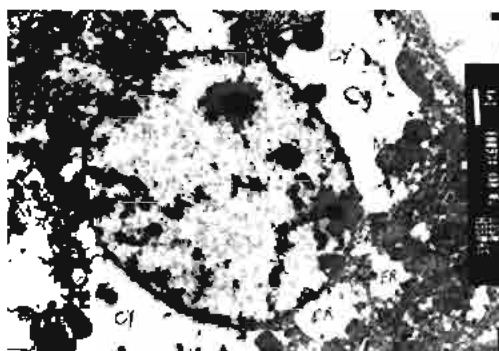
Fig. 12: An electron micrograph of hepatocyte of $CCl_4$-and dilitazem (21.6 mg $kg^{-1}$)-treated rat, showing that in most of hepatocytes, the cytoplasm (CY) became lytic and some mitochondria appeared as fixed observation (H), distorted mitochondrial cristae (m). highly dilated endoplasmic reticulum (ER). The nucleus appeared with condensed clumped chromatin and located on the outer margin in most instances. The gylcogen particles are absent (X 6000)
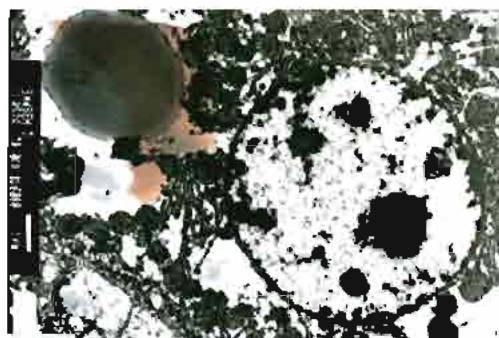


Fig. 13: An electron micrograph of hepatocytes of $CCl_4$-and enalapril (1.8 mg $kg^{-1}$)-treated rat, showing normally arranged cisternae of rough ER, condensed mitochondria with normal cristae and depletion in sites of glycogen (X 6000)

## DISCUSSION

The present study indicates that the calcium channel blocker amlodipine and the ACE inhibitor enalapril administered for one week prior to induction of hepatic injury by the hepatotoxin $CCl_4$ provided significant hepatoprotective effect. These results are in accordance with the beneficial effects for amlodipine or enalapril that have also been reported in hepatic injury caused by ischemic/reperfusion in rats (Piratvisuth *et al.*, 1995; Anthuber *et al.*, 1997). In other studies, a number of calcium antagonists were shown to protect the liver against injury evoked by a variety of toxicants (Romero *et al.*, 1994; Sippel *et al.*, 1993; Mustafa and Olson, 1999; Farghali *et al.*, 2000).

The calcium antagonists are a heterogeneous class of drugs which block the inward movement of calcium into cells through 'slow channels' from extracellular sites and it is widely accepted that these agents protect the liver by limiting the influx of extracellular calcium in liver cells (Konrad *et al.*, 1997). Other mechanisms might be also involved e.g., reduced potassium efflux (Konrad *et al.*, 1995),

preservation of membrane microviscosity in plasma membranes and liver microsomes (Landon *et al.*, 1984), improved energetic situation of the hepatocytes (Brecht *et al.*, 1993), restoration of ATP synthesis in hepatocytes (Salducci *et al.*, 1995; Obatomi *et al.*, 2001). Inhibition of toxin-induced inducible nitric oxide synthase (iNOS) expression in rat liver Kupffer cells (Mustafa and Olson, 1999), increasing gluconeogenesis, preventing glutathione depletion (Obatomi *et al.*, 2001) may be other means by which calcium channel antagonists limit hepatocellular injury. Calcium antagonists also showed antioxidant activity and inhibited lipid peroxidation (Mason *et al.*, 1999; Farghali *et al.*, 2000; Obatomi *et al.*, 2001). Amlodipine had the most potent antioxidant activity than either verapamil or diltiazem as a result of the compounds' relative affinity for the membrane lipid bilayer and ability to modulate membrane thermodynamic properties (Mason *et al.*, 1999).

In the present study, diltiazem, another calcium channel blocker did not confer protection and in fact increased hepatic injury. Calcium channel blockers possess important vascular effects. These drugs by depressing calcium ion flux in vascular smooth muscle cells, reduces the active tone of blood vessels and produces vasodilatation. This pharmacological action has been the basis for the use of these agents for the management of hypertension (Godfraind, 2006). Intravenous nimodipine increased blood flow in the rat liver (McCann *et al.*, 1986), while in man, administration of verapamil caused a rapid and intense increase in liver blood flow (Bauer *et al.*, 2000). The hepatic microcirculation plays an important role in the development of $CCl_4$-induced liver injury (Tanaka *et al.*, 1999) and protection from such injury has been obtained with increased hepatic arterial blood flow (Tanaka *et al.*, 1999; Hsu *et al.*, 1993). Improvement of hepatic microcirculation by such drugs as nimodipine (Chavez-Cartaya, 1996), diltiazem (Chin *et al.*, 2005) and pentoxifylline (Aslana *et al.*, 2001) might also be responsible for protecting the liver from ischaemia-reperfusion injury. An improvement in hepatic tissue perfusion may thus be involved in the protective effect of calcium antagonists. Conversely, compromising hepatic perfusion will have profound effects on the capacity of hepatocytes to withstand chemical or hypoxic injury. Diltiazem also reduces systemic blood pressure by decreasing the vascular smooth muscle tone and in this way might aggravate hepatic injury. In portal vein-ligated and sham-operated rats, intra-arterial diltiazem and nicardipine reduced mean arterial pressure, but only in portal vein-ligated rats, did diltiazem and nicardipine increased portal tributary blood flow, while portal tributary vascular resistance decreased (Nagasawa *et al.*, 1996). Furthermore, Bracht *et al.* (1999) reported that diltiazem causes vasoconstriction of the great vessels in the liver. With high concentration of diltiazem, the effects of were pronounced to the point of excluding completely about 2/3 of the liver parenchyma from the microcirculation (Bracht *et al.*, 1999). The calcium antagonists verapamil, diltiazem and nifedipine (and their analogs) are all eliminated by hepatic metabolism and the rate of disposition is also dependent on the rate of liver blood flow. During long-term administration, the profound haemodynamic effects of these agents result in changes in hepatic blood flow in association with decreases in arterial pressure and either increases or decreases in measured cardiac output (McAllister *et al.*, 1986).

Thus, it is likely that significant haemodynamic changes caused by diltiazem might have accounted for the lack of a protective effect observed in the present study *in vivo*. This might have resulted from high diltiazem levels due to impaired metabolism by the liver damaged by $CCl_4$.

In addition to being implicated in fibrogenesis, the auacoid angiotensin II is involved in the pathogenesis of liver injury under different experimental circumstances, where the administration of ACE inhibitors or A TII receptor antagonists have been shown to exert beneficial effects (Miyoshi *et al.*, 2003; Nascimento *et al.*, 2005; Freise *et al.*, 2006). The effect of these agents may be by maintaining sinusoidal blood flow and inhibition of inflammation. AII haemodynamic actions are mainly presinusoidal and can be inhibited by the receptor antagonist losartan (Nascimento *et al.*, 2005). Thus, in rats subjected to hepatic ischaemia and reperfusion injury, the expression of angiotensinogen in liver increased fivefold 3 h after reperfusion, while the administration of captopril or

losartan reduced the indices of liver damage and inflammation (e.g., alanine aminotransferase levels, pathological features, tumor necrosis factor-alpha levels and intercellular adhesion molecule-1 expression) (Guo *et al.*, 2004). The administration of ramipril, another ACE inhibitor, restored the reduction in microvascular blood flow and inhibited. Leukocyte adherence caused by liver ischaemia. This effect is mediated by bradykinin-2-receptor stimulation (Freise *et al.*, 2006). In the present study, the ACE inhibitor enalapril displayed marked protective effect against the CCl$_4$-induced liver toxicity. In a model of ischaemia/reperfusion injury, enalapril treatment significantly improved the sinusoidal perfusion rate and reduced leukocyte-sticking in both liver sinusoids and postsinusoidal venules (Anthuber *et al.*, 1997). Other researchers have shown that enalapril exerted a protective effect on hepatic steatosis and its inflammatory reaction in an experimental model of nephrotic syndrome in rats (Toblli *et al.*, 2002). Enalapril in addition displayed antioxidant properties (de Cavanagh *et al.*, 2001) that might contribute, at least partly, to its hepatic protective effect observed in the present study. Enalapril, can be given to patients with severe liver dysfunction as it is also not a prodrug (White, 1998).

In summary, the present study indicates that the calcium channel blocker amlodipine and the ACE inhibitor enalapril administered for one week prior to induction of hepatic injury by the hepatotoxin CCl$_4$ provided significant hepatoprotection. In contrast, diltiazem, another calcium channel blocker did not confer protection and in fact increased hepatic injury. It is suggested that profound haemodynamic effects of diltiazem which undergoes extensive hepatic metabolism and the resultant decrease in hepatic blood flow is likely to account for the observed effect of the drug.

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