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Anti-fibrogenic effects of captopril and candesartan cilexetil on the hepatic fibrosis development in rat

The effect of AT1-R blocker on the hepatic fibrosis

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With 12 figures and 2 tables

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Summary

Background/Aim: Angiotensin converting enzyme (ACE) and angiotensin II (AT-II) have been suggested to play an important role in liver fibrogenesis. There is a significant relationship between inheritance of hightened expression of transforming growth factor β1 (TGF-β1) and AT-II and the development of progressive hepatic fibrosis. The purpose of this study was to investigate the effects of captopril, an ACE inhibitor and candesartan cilexetil, an AT-II type 1 receptor (AT1-R) blocker, on liver fibrosis induced in rats by carbon tetrachloride (CCl_4) administration.

Methods: rats were divided into 4 experimental groups: The first group was given CCl_4 alone; the second was given both CCl_4 and captopril (100 $mg \cdot kg^{-1} \cdot day^{-1}$); the third was given both CCl_4 and candesartan cilexetil (8 $mg \cdot kg^{-1} \cdot day^{-1}$); fourth group was given 0.9% NaCl only. Seven weeks after initiating the treatment, indices of fibrosis were assessed.

Results: Candesartan cilexetil treatment significantly reduced the fibrosis development. These inhibitory effects were not observed in the captopril-treated group. The mean fibrosis score was significantly lower in the CCl_4/candesartan group compared with the group applied to CCl_4 alone and the group applied to CCl_4/captopril. Similarly, the number of α-smooth muscle actin positive cells was markedly suppressed by candesartan treatment.

Conclusions: The results suggest that AT-II plays a pivotal role in hepatic fibrogenesis and candesartan significantly attenuates the progression of liver fibrosis. This drug may provide an effective new strategy for prevention of liver fibrosis. Its effectiveness should be investigated in chronic liver disease associated with progressive fibrosis.

Introduction

Hepatic fibrosis is a common pathological feature of progressive chronic liver diseases, and is characterized by abnormal increase in extracellular matrices. It can lead to cirrhosis, and ultimately, end-stage liver failure and increased risk for hepatocellular carcinoma (FRIEDMAN 1999; JONSSON et al. 2001; OHISHI et al. 2001). The most important cells directly related to hepatic fibrosis are hepatic stellate cells (HSCs, Ito cells) distributed in Disse's cavity. When hepatic parenchymal cells are disturbed, HSCs enlarge, proliferate, and transform into myofibroblasts. These transformed HSCs synthesize and locally excrete a variety of extracellular matrices and promote hepatic fibrosis (FRIEDMAN 1999; OHISHI et al. 2001; IREDALE et al. 1998).

Transforming growth factor β1 plays a dominant role in the development of fibrosis (JONSSON et al. 2001; SCHUPPAN et al. 2001). TGF-β1 production may be enhanced by AT-II, the principal effective molecule of the renin-angiotensin system (RAS) (JONSSON et al. 2001;YOSHIJI et al. 2001). Tissue RAS is reported to be activated in patients with chronic liver disease, such as cirrhosis (YOSHIJI et al. 2001; HELMY et al. 2000). AT-II, which is octapeptid produced mainly by proteolytic cleavage of its precursor AT-I by ACE, has many physiological effects, including vascular hormonal secretion, tissue growth and neuronal activities (YOSHIJI et al. 2001). Recently, a variety of physiological roles of AT-II

have been clarified not only in the pathogenesis and maintenance of high blood pressure but also in the stimulation of fibroblast proliferation and collagen synthesis by non-parencimal cells (OHISHI et al. 2001). AT-II is also considered a potential mediator of intrahepatic portal hypertension, because its plasma levels were increased in patients with cirrhosis, and its administration induced elevation of portal pressure (YOSHIJI et al. 2001). Several types of AT-II receptors have been identified. The AT-II type 1 receptor (AT1-R) mediates most of the biological effects of AT-II, including increase in the intracellular Ca^{2+} concentration, cell contraction, and proliferation (YOSHIJI et al. 2001; ARDAILLOU 1999).

Captopril, an ACE inhibitor, has been well studied and reported to suppress rat hepatic fibrosis induced by pig serum recently (OHISHI et al. 2001). Similarly AT1-R antagonists have been shown to reduce the portal pressure in hepatic cirrhosis (SCHNEIDER et al. 1999).

The aim of present study was to compare the hepatoprotective effects of candesartan cilexetil, an AT1-R blocker, and captopril, an ACE inhibitor, on hepatic fibrosis caused by CCl_4 in rat model.

Material and methods

Animals and experimental design: Male Sprague Dawley rats weighing 240–310 g (11–12 weeks old, n = 38) were used in our experiments. They were obtained from the Yüzüncü Yil Research Hospital Animal Resources Center (Van, Turkey) and maintained under temperature-controlled conditions (22 °C ± 3 °C) with an artificial 12-hour light/dark cycle. The animals were given commercial diet in pellet form and water *ad libitum* throughout the acclimatization and experimental periods. All animal experiments were approved by the Animal Experimentation Committee of the Faculty of Medicine, Yüzüncü Yil University.

Experimental groups: Thirty-eight rats were divided into 4 experimental groups: The first group was given CCl_4 only (n = 10, CCl_4 alone group); the second was given both CCl_4 and captopril simultaneously (n = 10, CCl_4/captopril group); the third was given both CCl_4 and candesartan cilexetil simultaneously (n = 10, CCl_4/candesartan group); fourth was given isotonic sodium chloride (0.9% NaCl) only (n = 8, control group).

Administration of CCl_4, captopril, candesartan and 0.9%NaCl: NaCl was administrated at the dose of 1.5 ml/kg of body weight three times a week intraperitoneally (ip). CCl_4 (KgaA 64271, Merck, Darmstadt, Germany) was dissolved in olive oil at (1:7) and administered at a dose of 1.5 ml/kg of body weight three times a week ip as described previously (ROJKIND 1973). The same amount of CCl_4 was used in all experiments. Captopril (100 $mg \cdot kg^{-1} \cdot day^{-1}$; Kapril, Mustafa Nevzat, Turkey) was dissolved in the drinking water and was given *ad libitum* by free drinking throughout the experiments. The captopril dose chosen for this study was based on previous studies that determined the dose, which was effectively attenuating the progression of hepatic fibrosis in rats (JONSSON et al. 2001). The animals were weighed three times a week, and the volume of captopril consumed over the previous 2 days was recorded. A fresh captopril solution was prepared, the concentration of which was dependent on the average body weight and drinking volume for each cage in every 2 days. Candesartan cilexetil (8 $mg \cdot kg^{-1} \cdot day^{-1}$; Atacand, Astra, Sweden) was given by gavage (YOSHIJI et al. 2001; ISHIGAI et al. 1997). The volume of supplied drinking water was measured and was found to be almost equal between the groups. On average, a rat drank water for about 20–25 ml/day.

At the end of 7th week, all animals were sacrificed under ether anesthesia, and terminally bled via cardiac puncture. The blood was centrifuged and serum was stored at –20 °C for use. The liver, kidney and heart of each animal were removed. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin for histological examination.

Histological and immunohistochemical analysis: Formalin fixed, paraffin-embedded sections of liver were cut at 4 µm and stained with hematoxylin-eosin (H&E), and reticulin. The sections were coded and histology was evaluated by a single pathologist (S.U.) without knowledge of treatment groups or laboratory data. Fibrosis was staged as 0–4, based on Scheuer's (SCHEUER 1991) scoring system as follows: stage 0, no fibrosis; stage 1, expansion of the portal tracts without linkage; stage 2, portal expansion with portal to portal linkage; stage 3, extensive portal to portal and focal portal to central linkage; and stage 4, cirrhosis.

Immunohistochemical staining of smooth muscle actin (α-SMA) was performed on sections of formalin-fixed, parafin-embedded liver to detect activated myofibroblasts using a α-SMA antibody (clone 1A4; Dako, Copenhagen, Denmark) by previously described methods (YOSHIJI et al. 2001; SAKAIDA et al. 1998). The liver sections of the all groups were stained with α-SMA antibody including 0.9% NaCl – only group which was served as the control. We did not count α-SMA positive vessels in the portal area, which were assumed to be hepatic arteries. We only counted α-SMA-positive cells in the sinusoidal lining. Positive cells were counted at 40× magnification using an ocular micrometer. For each specimen, 10 randomly selected areas were counted and mean numbers of the cells were calculated.

At the end of the experiment, total protein, albumin, direct/indirect bilirubin, gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and prothrombin time (PT) were analyzed using a Hitachi Modular P-880 Analyzer (Roche, Germany).

Statistical analysis: Data were represented as the mean ± SD. Both parametric and nonparametric techniques were used for analysis. Analysis of variance (ANOVA) with Tukey's post-hoc comparison was used to test for differences in medians between treatment groups. Chi-square test was used to test histopathological changes between each group. A p value < 0.05 was considered statistically significant.

Results

Three animals (two CCl_4/captopril group, one CCl_4/candesartan group) died during experimental peri-

od. In the CCl_4 alone and CCl_4/captopril group body weights decreased more compared to CCl_4/candesartan and control group but these changes were not statistically significant ($p > 0.05$). Candesartan and captopril have a beneficial effect on serum levels of creatinine, AST, and ALT. Candesartan treatment also had a beneficial effect on serum levels of ALP (see table 1).

Histological findings

Histopathological evaluation and comparisons of liver specimens was made between the experimental groups. Histopathological changes were indicated in the figures (fig. 1A, B, C, D). Histological examination of liver obtained 7 weeks after the start of treatment showed that CCl_4-alone group and CCl_4/captopril group had developed severe (stage 3 or 4) fibrosis, characterized by ballooning degeneration, acidophilic bodies, extensive portal-portal and portal-central fibrous linkage, distortion of liver architecture, marked regeneration nodule.

In contrast, severe hepatic fibrosis was not detected in rats treated by CCl_4/candesartan (fig. 2A, C) and 0.9% NaCl (fig. 2B). Candesartan markedly inhibited progression of hepatic fibrosis and mononuclear cell infiltration, whereas captopril did not show significant preventive effects. The mean fibrosis score was significantly lower in the CCl_4/candesartan group compared with CCl_4-alone group and CCl_4/captopril group ($p < 0.001$). Inflammation was generally mild in CCl_4/candesartan group compared with CCl_4-alone group and CCl_4/captopril group ($p < 0.01$) and although there was a trend for inflammation to be lower in the candesartan-treated rats (table 2).

Immunohistochemistry

Immunohistochemical analysis of α-SMA was performed to examine the effect of these RAS inhibitory agents on HSC activation. In control group α-SMA stainig was mainly confined to the portal area and only very few α-SMA-positive cells were present in the parenchyme. The activated HSCs in liver sections from CCl_4-alone group and CCl_4/captopril group, which express α-SMA and are therefore named myofibroblast-like cells, were significantly increased (fig. 3C, D) compared with CCl_4/candesartan group and 0.9% NaCl group (fig. 3A, B) ($p < 0.001$). Candesartan treatment significantly reduced the number of α-SMA-positive cells (fig. 4). These results suggested that prevention of fibrogenesis by candesartan appeared to be related to the suppression of HSC activation and proliferation.

Table 1. Body weights and biochemical parameters.

Parameters	CCl_4 alone group *(n=10)*	CCl_4/Captopril group *(n=8)*	CCl_4/Candesartan group *(n=9)*	0.9% NaCl group *(n=8)*
Body weight (g)	248.5 ± 36.7	292 ± 32.1	273 ± 11.7	256.2 ± 18.3
Creatinine (mg/dl)	0.31 ± 0.06	0.24 ± 0.05[a]	0.26 ± 0.05[a]	0.23 ± 0.03[b]
ALP (U/L)	1200 ± 420	1121 ± 198	876 ± 334[a]	695 ± 175[cd]
AST (U/L)	1334 ± 778	687 ± 436[b]	597 ± 461[b]	116 ± 18[cd]
ALT (U/L)	1041 ± 546	528 ± 271[d]	397 ± 246[d]	55 ± 11[cd]

Values are mean ± SD. ALP – alkaline phosphatase; AST – aspartate aminotransferase; ALT – alanine aminotransferase.
[a] $p < 0.05$ vs. CCl_4
[b] $p < 0.01$ vs. CCl_4
[c] $p < 0.01$ vs. CCl_4/captopril
[d] $p < 0.001$ vs. CCl_4

Table 2. Histopathological changes in the liver of rats in each group.

Microscopic observation	CCl_4 alone group	CCl_4/Captopril group	CCl_4/Candesartan group	0.9% NaCl group
Regeneration nodule	++++[a]	++++[a]	–	–
Balooning degeneration	++++[b]	++[c]	+	–
Acidophilic bodies	+++[c]	++[c]	+	–
Mitosis	+++[c]	+++[c]	+	+

(–), Absent; (+), mild; (+++), moderate; (++++), extremely severe.
[a] $p < 0.001$ CCl_4 alone and captopril groups vs. control and candesartan groups.
[b] $p < 0.01$ CCl_4 alone vs. candesartan group.
[c] $p < 0.05$ CCl_4 alone and captopril groups vs. control and candesartan groups.

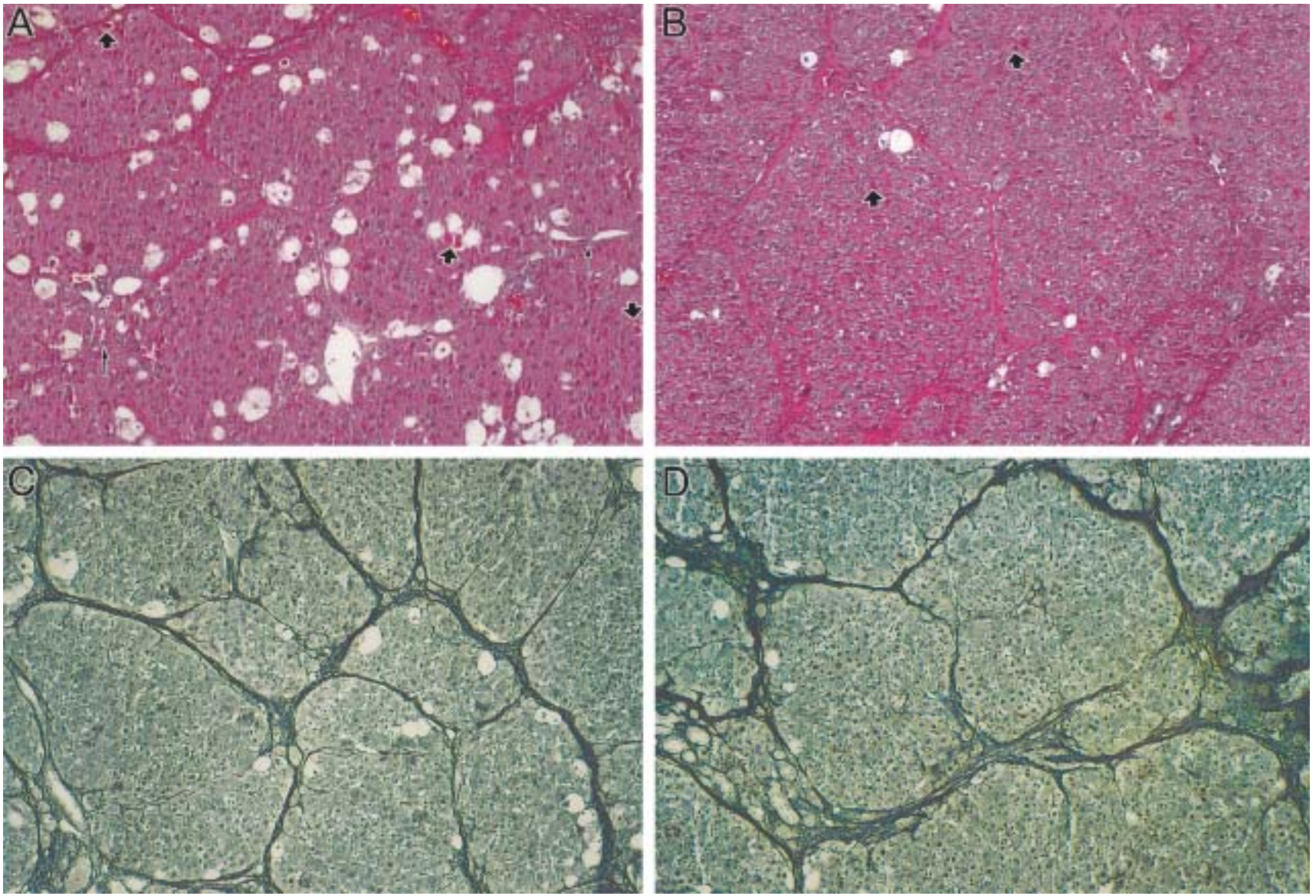

Fig. 1. Typical histological analysis of liver section from CCl_4 induced liver fibrosis at 7 week. In CCl_4 alone group (**A**) and CCl_4/captopril group (**B**): there was an obvious nodular appearance with deposition of fibrosis septa, asidophilic bodies (short arrows) and mononuclear cells (long arrow). In addition, moderate steatosis and diffuse balooning degeneration were observed in CCl_4 alone group (H&E, ×25). In CCl_4-alone group (**C**) and CCl_4/captopril group (**D**): extensive portal-portal and portal-central fibrous linkage, distortion of liver architecture, marked regeneration nodules (reticulin, ×25).

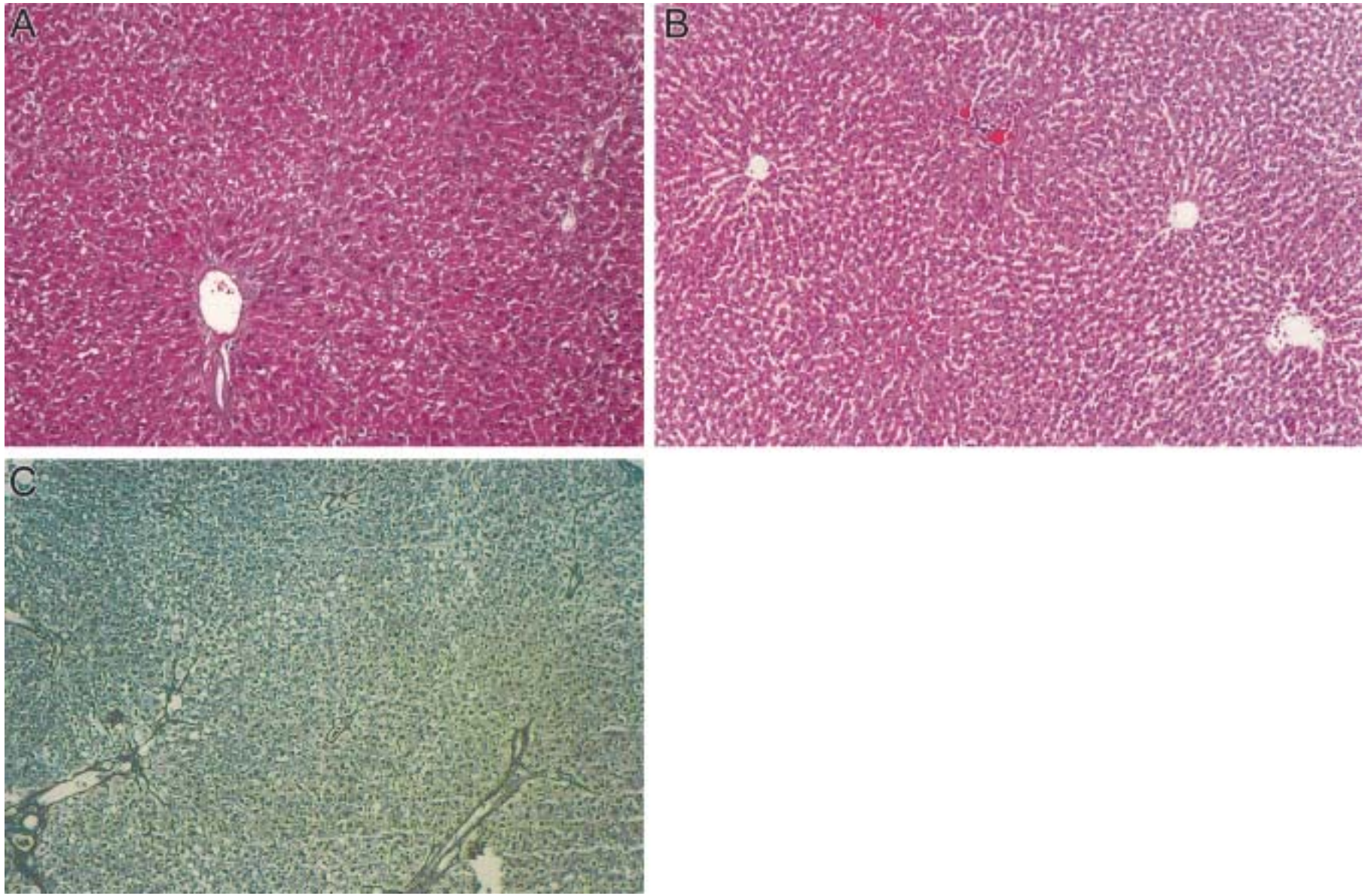

Fig. 2. In CCl_4/candesartan (**A**) and 0.9% NaCl group (**B**): no fibrotic changes, no balooning degeneration or regeneration nodules were observed in these groups. The liver appeared to be histologically fairly normal (H&E, ×25). Candesartan markedly inhibited progression of hepatic fibrosis (**C**) (reticulin, ×25).

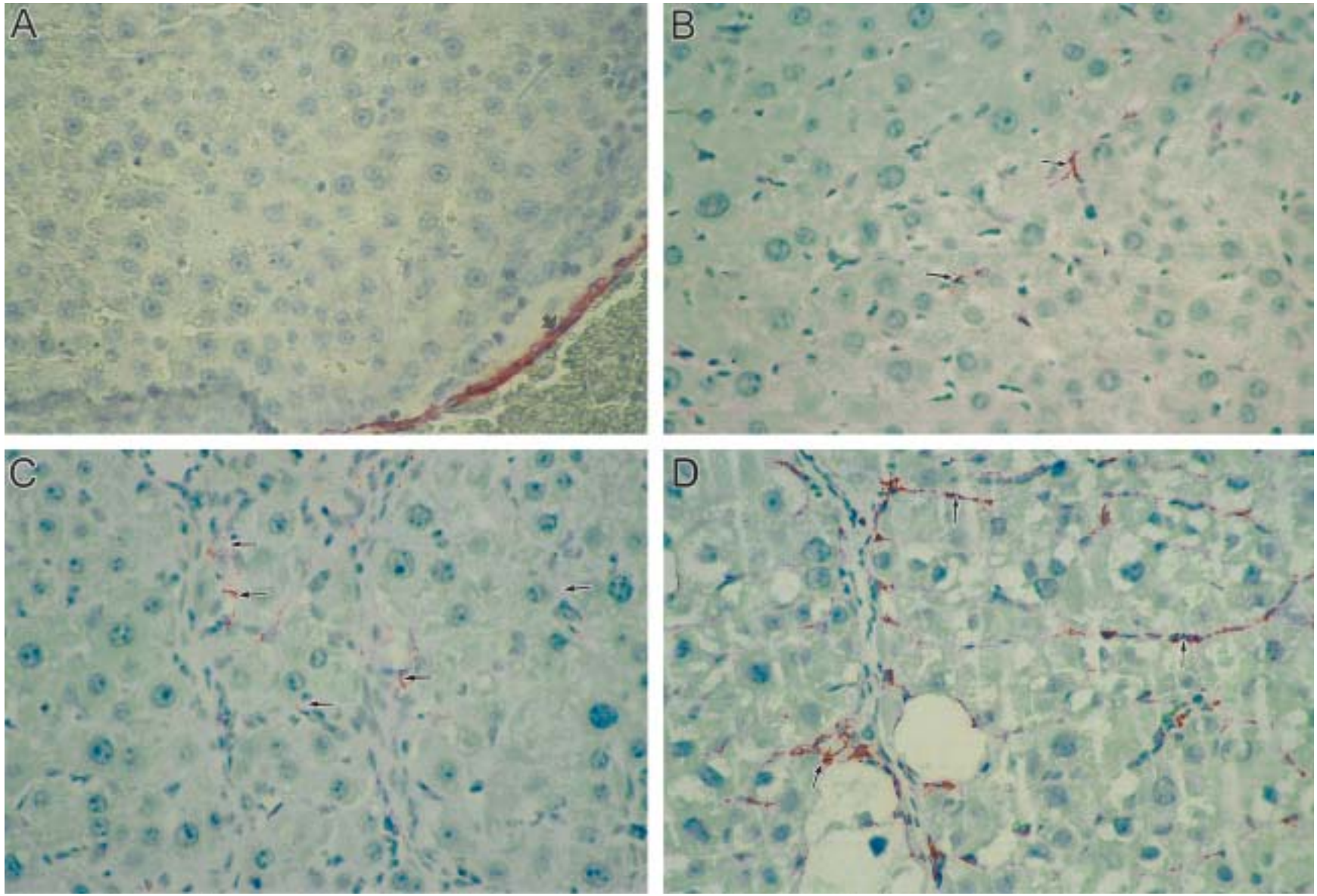

Fig. 3. Immunohistochemical investigation of α-SMA. Immunopositive cells of α-SMA were stained in brown-red colors (arrows). α-SMA staining was significantly reduced in the liver of the 0.9% NaCl group (A) and in CCl_4/candesartan group (B) compared with the CCl_4/captopril group (C) and CCl_4-alone group (D). Short arrow indicates vessel wall in portal space and long arrows indicate myofibroblast-like cells in liver parenchyme stained by anti-α-SMA in varying degrees (Original magnification ×40).

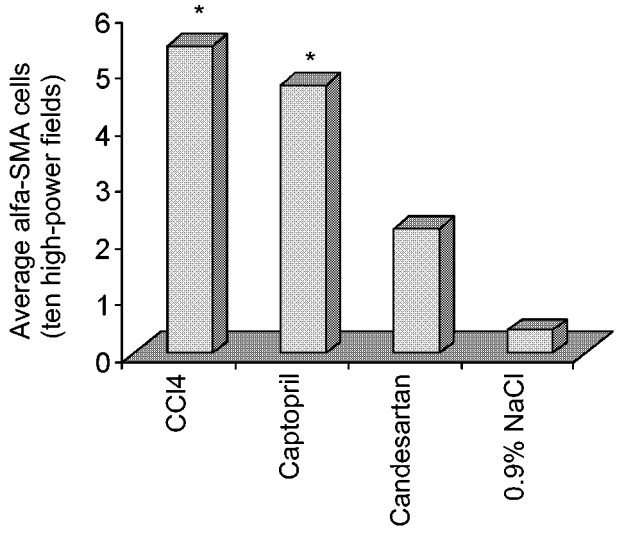

Fig. 4. Average α-SMA positive cells within the liver in all groups. *$p < 0.001$ CCl_4-alone and CCl_4/captopril group vs CCl_4/candesartan and 0.9% NaCl group.

Discussion

Liver fibrosis is characterized by increased deposition and altered composition of extracellular matrix, in such way as there is an excess of collagens I, III, IV. Hepatic stellate cells (HSC) are central to the process of fibrosis as the major source of fibrillar and nonfibrillar matrix proteins. The inhibition of hepatic fibrosis is beneficial in preventing progression of chronic liver disease (JONSSON et al. 2001; OHISHI et al. 2001; KNITELL et al. 1999; BATALLER et al. 2000).

Drugs modulating the action of vasoactive substances are currently used in the treatment of different types of human fibrosis (KLAHR and MORRISSEY 2000). These vasoactive substances include vasoconstrictors (AT-II, aldosterone, and endothelin-1) and vasodilators (prostaglandins and nitric oxide). Among these factors, AT-II appears to play an important role (OHISHI et al. 2001; YOSHIJI et al. 2001; MATSUSAKA et al. 1999; NODA et al. 1999). Recently, several studies have demonstrated that ACE inhibitors attenuate progression of hepatic fibrosis. One of the ACE inhibitors, captopril has been well studied and reported to suppress rat hepatic fibrosis (JONSSON et al. 2001; OHISHI et al. 2001; RAMOS et al. 1994).

In our study, we showed that candesartan cilexetil, AT1-R blocker, significantly reduced hepatic fibrosis induced by CCl_4. Our findings demonstrated that there were considerable differences between the hepatoprotective effects of candesartan and captopril *in vivo*. Although, candesartan administration significantly ameliorated the progression of hepatic fibrosis, captopril did not show similar effects. These results suggest that candesartan directly inhibits hepatic fibrogenesis induced by CCl_4. In addition to antifibrotic effects, candesartan treatment had a beneficial effect on serum liver enzymes. Our results showed coherence with those reported by Yoshiji (YOSHIJI et al. 2001).

The difference between candesartan cilexetil and captopril with regard to hepatoprotective effects may be explained as follows; first, the inhibition of ACE by an ACE inhibitor results in the accumulation of bradikinin. Bradikinin plays an important role in the progression of fibrosis, stimulating the proliferation of mesangial cells. Bradikinin also activated TGF-β1, which plays a pivotal role in the accumulation of extracellular matrix proteins (NODA et al. 1999; EL-DAHR et al. 1996). Second, AT1-R antagonists can completely inhibit AT-II's effects at the level of AT1-R, whereas captopril cannot inhibit the effect of AT-II. Third, AT1-R antagonists are reported to activate AT2-receptors via the elevation of plasma AT-II (NODA et al. 1999).

In the current study, we have shown that the number of α-SMA-positive cells in the livers from CCl_4/candesartan-treated rats dramatically reduced compared with CCl_4-alone group and CCl_4/captopril group, suggesting that AT1-R blocker suppresses activation and proliferation of hepatic stellate cells in response to CCl_4 (fig. 3B).

In this study, the hepatoprotective effects of candesartan cilexetil and captopril were investigated in hepatic fibrosis. We have shown that candesartan cilexetil effectively delayed the progression of hepatic fibrosis, whereas captopril did not show similary beneficial effect on hepatic fibrosis. In conclusion, significant differences were documented between the beneficial effects of candesartan cilexetil and captopril on hepatic fibrosis. Our results suggest that AT1-R antagonist candesartan cilexetil may be useful agent for trials in human chronic liver diseases associated with fibrosis. More prolonged follow-up studies should be performed to clarify this results.

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