Beneficial Effect of Angiotensin-Blocking Agent Candesartan on Compensated Alcoholic Liver Fibrosis: A Randomized Controlled Trial

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A dissertation submitted to the Department of Medical Science and the Graduate School of Yonsei University in fulfillment of the requirements for the degree of Doctor of Philosophy

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July 2010

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Phil Ho Jeong, July 2010

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ABSTRACT

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Recent studies have shown that the renin-angiotensin system is implicated in hepatic fibrogenesis in vitro and in vivo. However no study was done via histology in humans with alcoholic liver disease. We prospectively studied the antifibrotic effect of angiotensin II blocking agents (ARB) in patients with alcoholic liver disease.

Patients with compensated alcoholic liver fibrosis (≥ Fibrosis stage 2, F2) were randomized to receive either the ARB, candesartan (8 mg/day) with ursodeoxycholic acid(UDCA)(600 mg/day)(n = 37), or UDCA alone(n = 36) as control for 6 months. All enrolled patients underwent liver biopsies twice at baseline and 6 months later for measurement of fibrosis score, area of fibrosis and α-smooth muscle actin (SMA) positive and hydroxyproline. Transforming growth factor-β1(TGF-β1), collagen-1, angiotensin II type I receptor (AT1-R), tissue inhibitor of metalloproteinase-1(TIMP-

1), Rac1 and p22phox which represent oxidant stress were also measured by real-time RT–PCR before and after 6 months of therapy.

Candesartan reduced the fibrosis score according to the Laennec fibrosis system from 3.4±1.4 to 3.0±1.0 ($P < 0.05$). Candesartan also reduced the area of fibrosis and α-smooth muscle actin (SMA) positive from 11.2±6.0 to 8.2±5.1 and 27.4±10.6 to 22.3±9.4, and hydroxyproline levels (μg/g liver tissue) from 7.8±2.6 to 5.9±1.9, respectively ($P < 0.05$). In addition, the relative expression of TGF-β1, collagen-1, AT1-R, TIMP-1, Rac1 and p22phox by real-time RT-PCR were decreased in the candesartan group ($P < 0.05$). No significant complication and side effect was observed during the present study.

In conclusion, administration of ARB in compensated alcoholic liver disease induces decrease of fibrosis in both histological and quantitative measurements. These results provide an evidence for a beneficial role of ARB in compensated alcohol-related liver fibrosis.

Key words: renin-angiotensin system; hepatic fibrosis; angiotensin II; alcoholic liver disease

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I. Introduction

Alcohol is one of principal causes of hepatic fibrosis. Although the most effective treatment for alcoholic hepatic fibrosis is abstinence of alcohol consumption, additive treatment to reduce the accumulation of scar tissue can accelerate the improvement of hepatic fibrosis in alcoholic liver disease.

The renin-angiotensin system can be an attractive antifibrotic target in liver. Several lines of evidence indicate that overproduction of angiotensin II (ANG II) in chronic liver injury stimulates the activation of hepatic stellate cells (HSCs) attributed to fibrogenesis (Friedman SL, Roll FJ, et al. 1985; Baik SK, Jo HS, et al. 2003). Additionally, the antifibrotic effect of ANG II blocking agent has been shown in various animal models and hepatitis C patients (Kim MY, Baik SK, et al. 2008; Park DH, Baik SK, et al. 2007; Sookoian S, Fernandez MA, et al. 2005; Corey KE, Shah N, et al. 2009; Debernardi-Venon W, Martini S, et al. 2007; Terui Y, Saito T, et al. 2002). Hence, drugs that inhibit the renin-angiotensin system have promise in ameliorating hepatic fibrosis in chronic liver injury. However, no study has been conducted in patients with alcoholic liver disease to evaluate the effect ANG II type I receptor (AT1-R) blocking agent on hepatic fibrosis.

Because the action of ANG II mediated via AT1-R is related to its therapeutic

interventions, we conducted a novel study with the candesartan, an AT1-R blocking agent, administered for 6 months in alcoholic liver disease patients. This study aimed to investigate the safety and the efficacy of chronic administration of candesartan to hepatic fibrosis patients with alcoholic liver disease.

II. Patients and Methods

1. Study Design

This study was done prospectively as a double-blind randomized controlled trial involving one center in Korea. The ethics committee of the hospital and Korea Food and Drug administration (KFDA) approved the protocol, and all patients were provided written informed consent to participate in the study. The study was conducted following the principles of the Declaration of Helsinki (revised in Edinburgh 2000).

2. Patients

Patients aged between 18 and 70 years with chronic alcoholic liver disease who visited the Wonju College of Medicine Wonju Christian Hospital between September 2007 and December 2008 and who discontinued alcohol intake for at least 6 months were considered eligible for the study. To exclude other causes of liver disease and

hepatocellular carcinoma or other malignancy, and to estimate the stage of liver disease, serologic tests including analysis of hepatitis B and hepatitis C infection, upper gastrointestinal endoscopy, imaging studies including ultrasonography and computed tomography (CT) scan were performed. Patients with decompensated cirrhosis were excluded because angiotensin II type 1 receptor (AT1-R) blocking agent could develop adverse effect in patients with advanced cirrhosis such as renal insufficiency (Gonzalez-Abraldes J, Albillos A, et al. 2001; Schneider AW, Kalk JF, et al. 1999). According to these criteria, 95 patients were considered eligible for the study from a group of 137 consecutive patients initially considered for the study. All 95 patients underwent a liver biopsy to screen for patients with clinically significant fibrosis (METAVIR fibrosis score and Laennec fibrosis score ≥ Fibrosis stage 2, F2). As a result, 10 patients were excluded and 85 patients were enrolled.

3. Randomization and treatment

Patients were chronologically randomized into two groups by the pharmacy of Wonju Christian Hospital using serially numbered sealed envelopes in batches of 90 that designated one of two treatments. One was given oral candesartan at a daily dose of 8 mg (candesartan group, 42 patients) in addition to ursodeoxycholic acid (UDCA,

600 mg/day) and the other was given no treatment except UDCA administration (control group, 43 patients) for 6 months. Patients and investigators were blinded, and the result of randomization was disclosed at the end of the study.

4. Follow-up

All patients underwent paired liver biopsy before entry into the study and again at the completion of the study period to determine the histological effect of candesartan on hepatic fibrosis. During the study, patients did not receive any other medication; clinical and biochemical test follow ups, blood pressure measurements, and assessment for the presence of side effects were conducted every month. Plasma rennin activity (supine) was also estimated twice at baseline and after 6 months treatment. Alcohol intake monitoring was done twice a week by phone calls to patients and during their monthly visits to the hospital with serologic testing of carbohydrate deficient transferrin (CDT (%)) and liver function (LFT); each visit, the remaining medications were also counted. Patients who ingested alcohol (alcohol intake more than 60 gm/week continuously or a CDT > 2.5% with/without compatible LFT profile) or showed lack of compliance for medication intake were dropped mid-way through this study. Double-blindness was maintained until the study was

complete and the data were entered into a final database. Ultimately, 37 (candesartan group) and 36 (control group) patients were analyzed to assess the safety and efficacy at the end of study (Figure 1). Patients' general characteristics are summarized in Table 1.

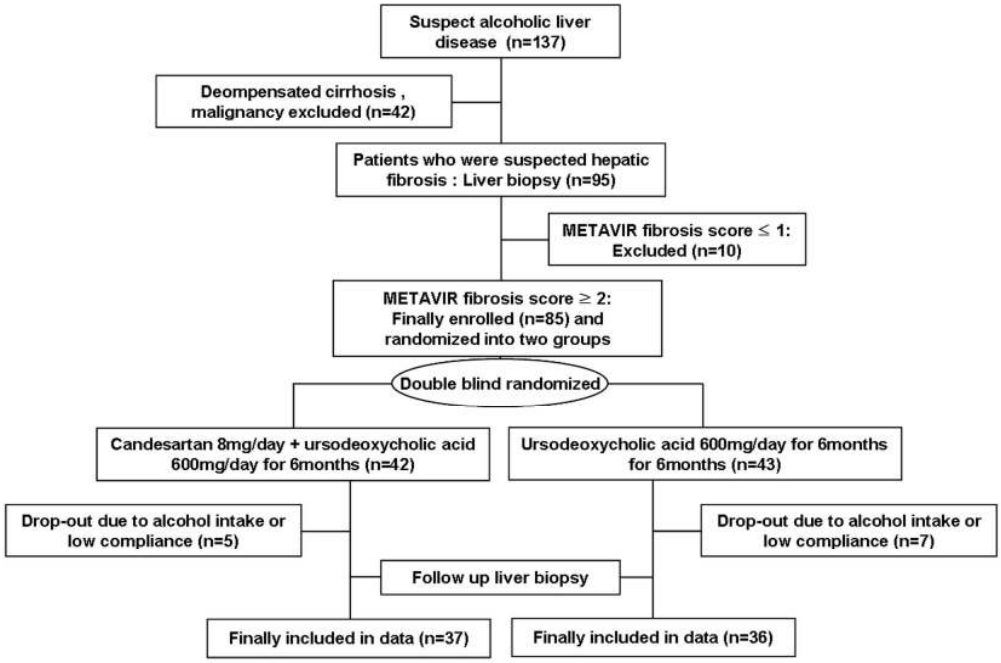

Figure 1. Flow chart of the disposition of patients and the study design.

Table 1. General Characteristics

		Candesartan group (n = 37)	Control group (n = 36)	P-value
Age		51.7 ± 9.2 (34–69)	49.2 ± 9.3 (28–67)	> 0.05
Sex (M:F)		31 (83.8%): 6 (16.2%)	30 (83.3%): 6 (16.7%)	> 0.05
Albumin(g/dL)		3.8 ± 0.5	3.7 ± 0.5	> 0.05
AST(U/L)		37.6 ± 12.7	38.3 ± 13.2	> 0.05
ALT(U/L)		36.4 ± 11.9	36.8 ± 12.2	> 0.05
rGT(U/L)		54.8 ± 18.5	56.2 ± 17.9	> 0.05
Total bilirubin(mg/dL)		1.2 ± 0.8	1.3 ± 0.7	> 0.05
Prothrombin time(INR)		1.1 ± 0.1	1.1 ± 0.2	> 0.05
Platelet count(/mm^3)		187.3 ± 114.7	210.3 ± 116.1	> 0.05
Laennec fibrosis score		3.4 ± 1.4	3.2 ± 1.2	> 0.05
Laennec fibrosis stage				
F2(n)		13	11	
F3(n)		13	14	
F4A(n)		1	6	
F4B(n)		5	2	
F4C(n)		5	3	
Mean BP	baseline	115.3 ± 21.5	117.5 ± 24.3	> 0.05
	6 month later	103.4 ± 19.7	118.2 ± 23.9	< 0.05
Plasma renin activity (ng/mL/hr)	baseline	1.1 ± 0.7	1.0 ± 0.4	> 0.05
	6 month later	1.1 ± 0.6	1.0 ± 0.3	> 0.05
CDT (%)		1.9 ± 0.8 (0.8-3.4)	1.8 ± 0.8 (0.8-3.3)	> 0.05

AST, aspartate aminotransferase; ALT, alanine aminotransferase; rGT, gamma glutamyl transferase; BP, blood pressure; CDT, Carbohydrate deficient transferring

5. End points and outcomes

The primary end point was the improvement of histological findings and direct markers related with hepatic fibrosis. The second end point was the development of side effects such as shock or hypotension resulting in treatment discontinuation.

6. Calculation of Sample Size

According to a previous study (Sookoian S, Fernandez MA, et al. 2005), patients with hepatic fibrosis treated with AT1-R blocking agent have a 30% chance of improving after 6 months of follow up. The sample size needed to detect a 25% reduction in the risk of hepatic fibrosis was calculated to be 35 patients in each treatment group, using a 2-sided test with 80% power at a significance level of 0.05.

7. Fibrosis assessment

(1) Histomorphological and immunohistochemical analysis

Ultrasound-assisted liver biopsy was performed using a needle biopsy gun (Acecut, TSK Laboratory, Japan) with a 14 gauge x 11.5 cm needle and a 15 mm biopsy specimen notch. After 5 μm sections were prepared, they were stained with hematoxylin and eosin (H&E) and Masson-trichrome (MTC). Fibrosis was evaluated

by two liver pathologist(M. Y. Cho and M. S. Eom) who were blinded to clinical data of patients, using the Laennec fibrosis scoring system (Table 2, Figure 2)(Kutami R, Girgrah N, et al. 2000; Wanless IR, Sweeney G, et al. 2002; Robert D. Odze M, et al. 2009). To estimate the chance adjusted agreement, kappa value were calculated for inter-observer agreement and which was 0.89. When two pathologists disagreed, they discussed and reached consensus on the fibrosis score. In addition, to estimate the change of liver fibrosis, fibrosis area (%) expressed as the percentage of the total area that was positive for MTC stain in the digital photomicrographs, quantified by computerized image analysis system (GmbH, Münster, Germany). For quantification of fibrosis area, microscopic areas were selected randomly at an original magnification of X200. For immunohistochemical analysis, tissue sections were incubated with primary antibody against α-smooth muscle actin (α-SMA) (diluted 1:800, Neomarkers, Fremont, CA, USA) for 90 min at room temperature after washing with buffer. Tissue sections were incubated with 3-amino-9-ethylcarbazole chromogen (BioGenex, San Ramon, CA, USA) for 5–7 min. Prior to mounting, sections were counterstained with hematoxylin and then dehydrated. UltraVision LP Large Volume Detection System (Lab Vision, Runcorn, UK) was used as the detection system. Morphological analysis of immunopositive cells was also performed with a computerized image analysis system. Activated HSC were

quantified by determining the areas of α-SMA positive cells.

Table 2. Laennec Scoring System for Staging Fibrosis in Liver Biopsies

Stage	Name	Septa (Thickness and Number)	Criteria	score
0	No definite fibrosis			0
1	Minimal fibrosis	+/−	No septa or rare thin septum; may have portal expansion or mild sinusoidal fibrosis	1
2	Mild fibrosis	+	Occasional thin septa; may have portal expansion or mild sinusoidal fibrosis	2
3	Moderate fibrosis	++	Moderate thin septa; up to incomplete cirrhosis	3
4A	Cirrhosis, mild, definite, or probable	+++	Marked septation with rounded contours or visible nodules. Most septa are thin (one broad septum allowed)	4
4B	Moderate cirrhosis	++++	At least two broad septa, but no very broad septa and less than half of biopsy length composed of minute nodules	5
4C	Severe cirrhosis	+++++	At least one very broad septum or more than half of biopsy length composed of minute nodules (micronodular cirrhosis)	6

Adopted by reference Robert D. Odze M, et al. 2009

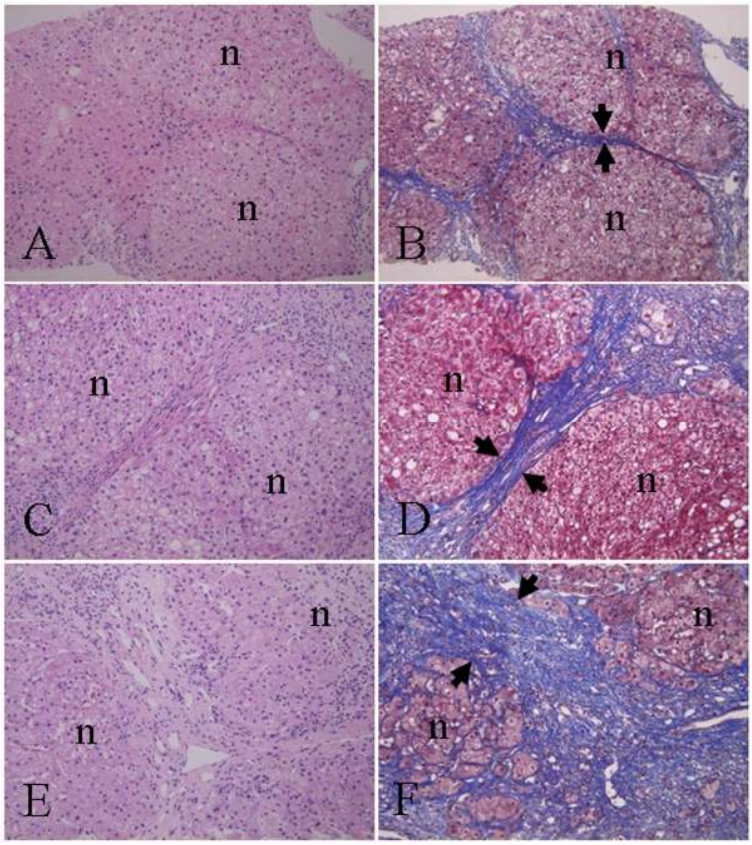

Figure 2. The histological sub-classification of cirrhosis (F4) according to the Laennec fibrosis scoring system. (A) and (B) show mild cirrhosis with marked septation with rounded contours or visible large nodules. Most septa are thin (F4a) (H&E and MTC stain, respectively, X 100), (C) and (D) show moderate cirrhosis with at least two broad septa, but no very broad septa and minute nodules (F4b)(H&E and MTC stain, respectively, X 200), (E) and (F) show severe cirrhosis with at least one very broad septum with minute nodules (F4c) (H&E and MTC stain, respectively, X 200). The widths between two arrows show the significant difference among subclass of cirrhosis. n, regenerating nodule; MTC, Masson trichrome stain.

(2)Hepatic hydroxyproline contents

Hepatic tissues were hydrolyzed in 300 μl 6 N HCl at 120°C overnight, and hydrolysates were evaporated under vacuum four times. The hydrolysate was centrifuged at 8000*g* for 10 min, and 500 μl of the supernatant was evaporated under vacuum. Supernatants were titrated to pH 5–6 with HCl and 6 N NaOH. These solutions were then supplemented with 7% chloramine T, acetate/citrate buffer (5.7 g sodium acetate 3H2O, 3.75 g trisodium citrate $2H_2O$, 0.55 g citric acid H_2O, and 38.5 ml isopropanol) and 150 μl of Ehrlich's solution, consisting of *p*-dimethylaminobenzaldehyde dissolved in perchloric acid, and isopropanol. After cooling, absorbance was measured at 560 nm with an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Hydroxyproline levels were expressed in micrograms of hydroxyproline per gram of liver tissue.

(3) Real-time reverse transcriptase–polymerase chain reaction (PCR) of transforming growth factor β1 (TGF- β1), collagen-1, angiotensin II type I receptor (AT1-R), tissue inhibitor of metalloproteinase-1(TIMP-1), Rac1 and p22phox

Total RNA was isolated from liver tissue homogenates. RNA was extracted using a

TRIZOL reagent (Invitrogen, San Diego, CA, USA). To each sample, 0.2 ml chloroform was added per 1 ml TRIZOL reagent and centrifuged for 15 min at 4°C. RNA was precipitated by mixing 0.5 ml isopropyl alcohol per 1 ml TRIZOL reagent and then incubated at 15–30°C for 10 min. RNA extracts were washed with 1 ml 75% ethanol and centrifuged for 5 min. RNA purity and concentration were determined using a Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience, Freiburg, Germany). Two-step PCR was performed using a Rotor-Gene 3000 (Applied Biosystems, Foster City, CA, USA). For cDNA synthesis, 1 μg RNA, 50 units Moloney murine leukemia virus RT, 20 units RNase inhibitor, 2.5 μM oligo d(T) primer, 2 μl RT buffer, 1 mM dNTP mixture, and 0.1% diethylpyrocarbonate solution were added to make a total volume of 20 μl. Samples were incubated at 42°C for 60 min, 99°C for 5 min, and 4°C for 5 min. PCR conditions were as follows: 2 μl cDNA solution, 25 μl 2× QuantiTect SYBR Green PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA), 2 μl forward primer, and 2 μl reverse primer were added to make a total volume of 50 μl. Thermal cycler conditions were 15 min at 95°C, and 35 cycles of 30 s at 94°C, followed by 30 s at 60°C and 60 s at 72°C. Data were analyzed by the ΔΔCt method using Rotor-gene Analysis software 6.1 (Applied Biosystems, Foster City, CA, USA). The forward and reverse primer sequences used for this study are shown in Table 3.

Table 3. Primer sequences used to detect TGF-β1, collagen 1, AT1-R, TIMP-1 and Rac-1, p^{22phox}

GAPDH	
Forward primer	5`-CGA CCA CTT TGT CAA GCT CA-3`
Reverse primer	5`-AGG GGT CTA CAT GGC AAC TG-3`
TGF-beta1	
Forward primer	5`-GGC AGT GGT TGA GCC GTG GA-3`
Reverse primer	5`-TGT TGG ACA GCT GCT CCA CCT-3`
Collagen 1	
Forward primer	5`-CCC AGA ACA TCA CAT ATC AC-3`
Reverse primer	5`-CAA GAG GAA CAC ATA TGG AG-3`
AT1-R	
Forward primer	5`-CAC CAT GTT TTG AGG TTG AGT GAC-3`
Reverse primer	5`-CAG GCT AGG GAG ATT GCA TTT CTG-3`
TIMP-1	
Forward primer	5`-GGG GCT TCA CCA AGA CCT ACA C-3`
Reverse primer	5`-AAG AAA GAT GGG AGT GGG AAC A-3`
Rac-1	
Forward primer	5`-AGC CTT CGC ACT CAA TGC CAA C-3`
Reverse primer	5`-TCG GCA CAA CAA TGC AGT GTA G-3`
p22phox	
Forward primer	5`-CGC TGG CGT CCG GCC TGA TCC TCA-3`
Reverse primer	5`-ACG CAC AGC CGC CAG TAG GTA GAT-3`

TGF-β1, Transforming growth factor-beta 1; collagen 1, type 1 collagen; AT1-R, angiotensin II type 1 receptor; TIMP-1, tissue inhibitor of metalloproteinase 1

8. Statistical analysis

Results were expressed as mean ± SDs. A $P < .05$ was considered to demonstrate statistical significance. Differences and hemodynamic effects between groups and pre – post comparisons were compared by paired or unpaired *t* test, as appropriate. Medical statistician (SJC) supported the study design and analysis of data.

III. Results

No difference in general characteristics between candesartan and control group was seen (Table1). During this study, no significant change in clinical or biochemical parameters between responders and non-responders to candesartan or between candesartan and control group was found.

(1) Histomorphological and immunohistochemical analysis

Candesartan reduced the fibrosis score according to the Laennec system from 3.4 ± 1.4 to 3.0 ± 1.0 ($P < 0.05$) however, in the control group, no change in fibrosis score was observed (from 3.2 ± 1.2 to 3.3 ± 1.3; $P > 0.05$) (Table 4, Figure 3). The percentage of fibrosis area which was calculated by the computerized image analysis system also showed significant decrease from 11.2 ± 6.0 to 8.2 ± 5.1 ($P < 0.05$)

after 6 months of candesartan administration, whereas controls did not showed change in fibrosis area (8.7 ± 4.8 to 9.6 ± 6.5, $P > 0.05$) (Table 4, Figure 4).

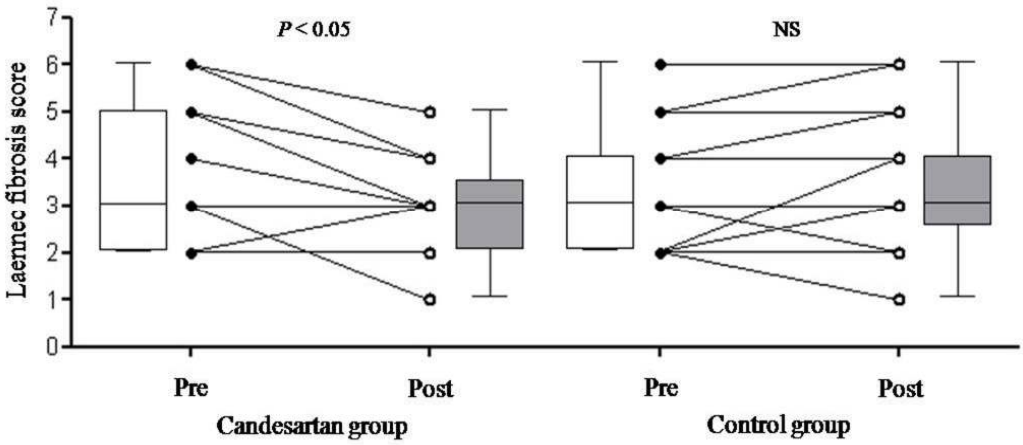

Figure 3. Comparison of the overall and individual changes of fibrosis score between pre- and post-treatment according to the Laennec system. The candesartan group showed a significant decrease in fibrosis score after 6 months of treatment ($P < 0.05$) however, the control group did not show change in fibrosis score ($P > 0.05$). White boxes, before treatment; dark boxes, after treatment.

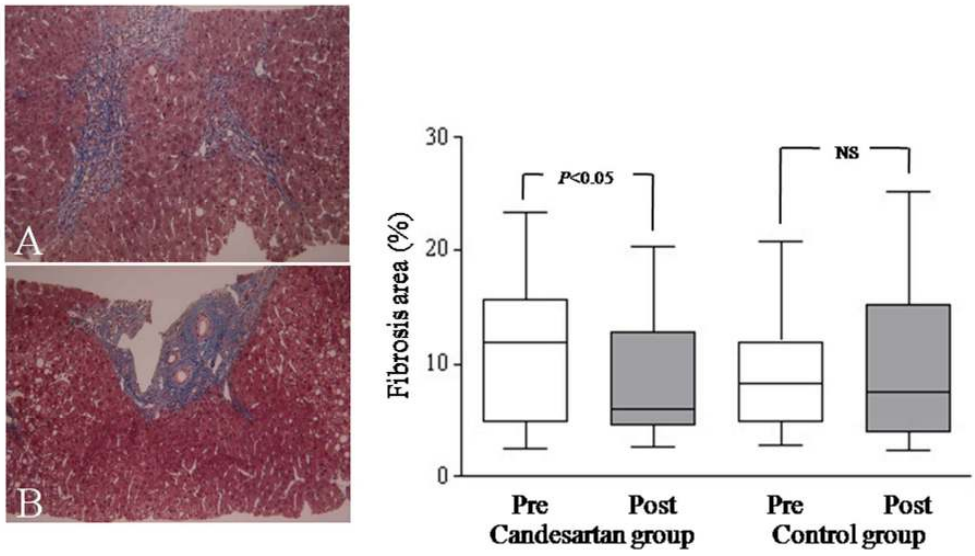

Figure 4. The photograph shows improvement of fibrosis expression by Masson-trichrome (original magnification X 200) between pre- (A) and post-treatment (B). The graph shows comparison of the change in the percentage of fibrosis area stained by MTC which was calculated with computerized image analysis system between pre- and post-treatment. The candesartan group showed a significant decrease in MTC stained area after 6 months of treatment ($P < 0.05$) however, the control group did not show change ($P > 0.05$). White boxes, before treatment; dark boxes, after treatment.

Immunohistochemical stain revealed α-SMA expression on activated HSCs as a brown color, and the percent area occupied by activated HSCs calculated by the image analysis system also decreased significantly from 27.4 ± 10.6 to 22.3 ± 9.4 ($P < 0.05$) after 6 months of candesartan administration. In the control group, no significant change was seen (from 26.3 ± 9.9 to 27.6 ± 12.5, $P > 0.05$) (Table 4, Figure 5).

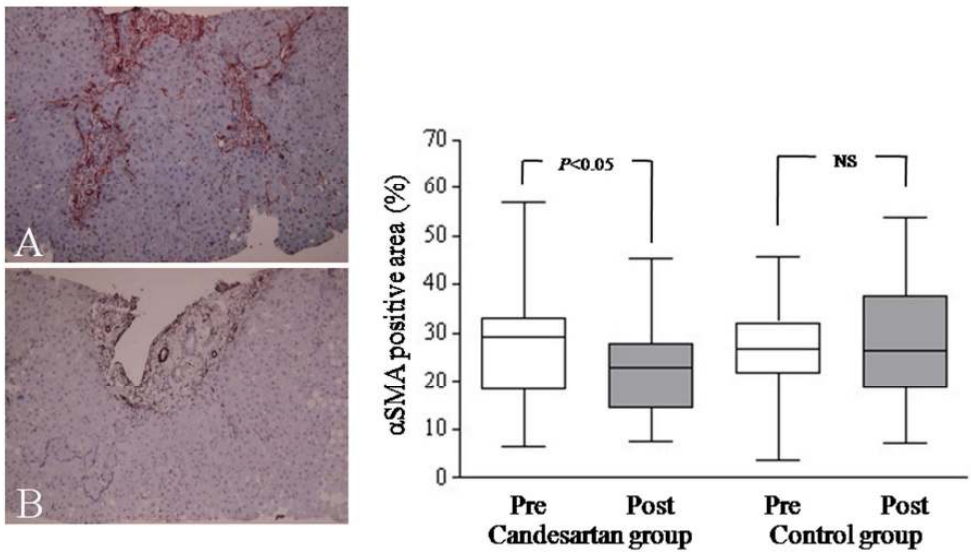

Figure 5. The photograph shows improvement of α-SMA expression on activated hepatic stellate cells by immunohistochemical stain between pre- (A) and post-treatment (B) (original magnification X 200). The graph shows comparison of the change in α-SMA expression which was calculated with computerized image analysis systembetween pre- and post-treatment. The candesartan group showed a significant decrease in stained area after 6 months of treatment ($P < 0.05$)however, the control

group did not show change in α-SMA expression ($P > 0.05$). α-SMA, alpha-smooth muscle actin. White boxes, before treatment; dark boxed, after treatment.

(2) Measurement of hepatic hydroxyproline content

Quantitative analysis showed that the content of hepatic hydroxyproline (μg/g liver tissue) was significantly decreased in the candesartan group from 7.8 ± 2.6 to 5.9 ± 1.9, whereas no change was seen in the control group (from 7.5 ± 2.9 to 8.1 ± 3.5, $P > 0.05$) (Table 4, Figure 6).

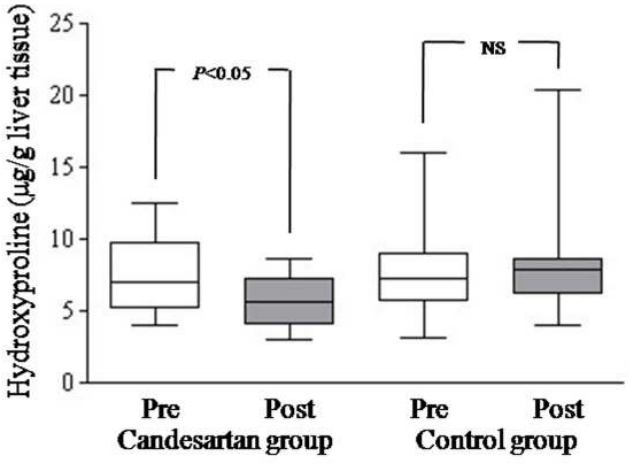

Figure 6. Quantitative analysis of the change of hepatic hydroxyproline content in liver between pre- and post-treatment. After 6 months of treatment, hepatic hydroxyproline content in the candesartan group decreased significantly ($P < 0.05$) however, no change was observed in the control group ($P > 0.05$). White boxes, before treatment; dark boxes, after treatment.

(3) Gene expression associated with fibrosis

The relative expressions of TGF-β1, a mediator of hepatic fibrogenesis; collagen-1; AT1-R; and TIMP-1 were significantly decreased in the candesartan group as measured by real-time RT–PCR($P < 0.05$) however, in the control group, TGF- β1, collagen-1, and AT1-R inversely increased significantly ($P < 0.05$) (Table 4, Figure 7). Rac1 and p22phox which represent oxidant stress, also showed a significant decreased expression after candesartan administration ($P < 0.05$), whereas no changes were seen in the control group as measured by real-time RT–PCR ($P > 0.05$) (Table 4, Figure 8).

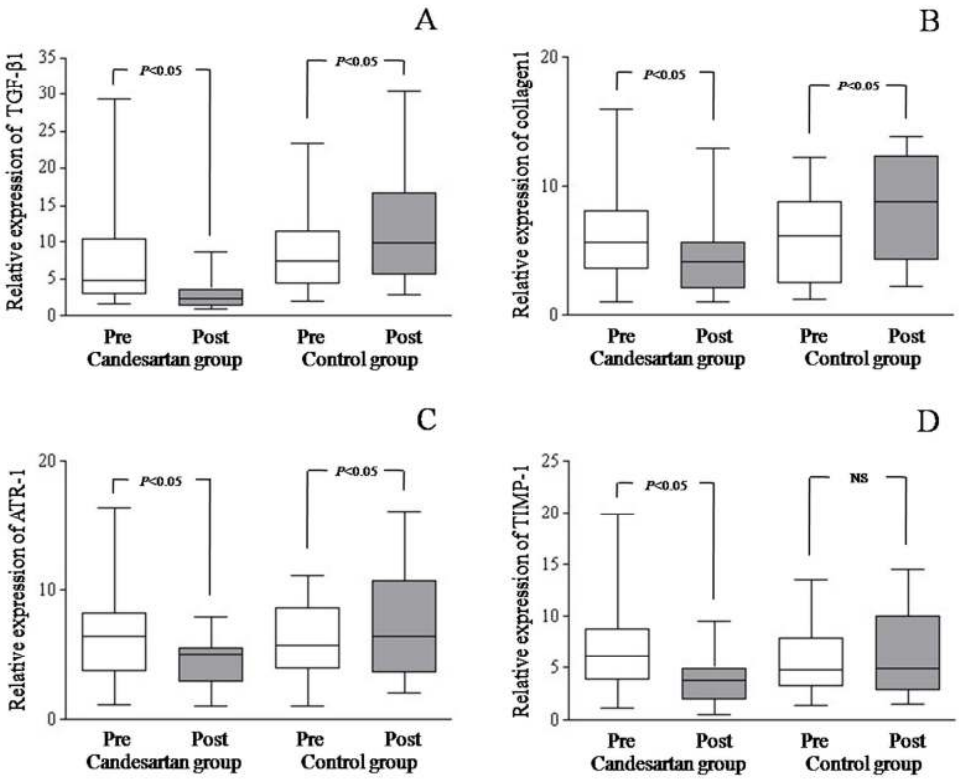

Figure 7. Comparison of the direct markers related with hepatic fibrosis by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) between pre- and post-treatment. (A) TGF-β1, (B) collagen 1, (C) AT1-R, (D) TIMP-1. All markers were decreased significantly in candesartan group ($P < 0.05$) inversely, in the control group, TGF-β1, collagen 1, AT1-R were significantly increased ($P < 0.05$). TIMP-1 did not show change in the control group ($P > 0.05$). TGF-β1, transforming growth factor-β1; collagen 1, type 1 collagen; AT1-R, angiotensin II type 1 receptor; TIMP-1, tissue inhibitor of metalloproteinase. White boxes, before treatment; dark boxes, after treatment.

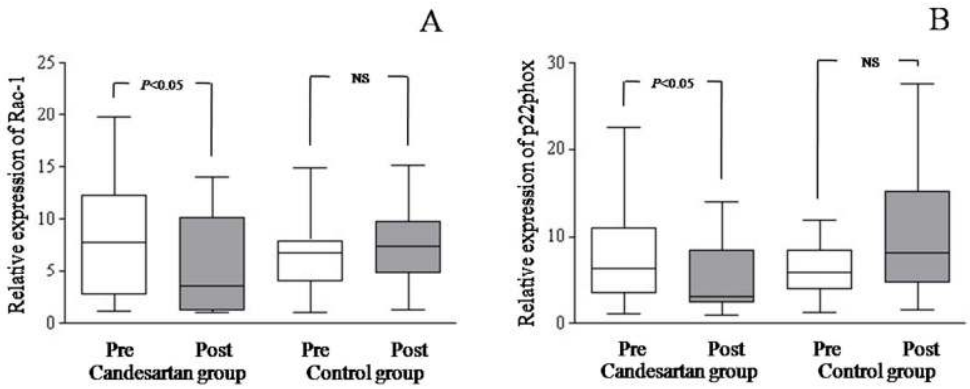

Figure 8. Comparison of the markers related with oxidative stress by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) between pre- and post-treatment. (A) Rac-1, (B) p22phox. Both markers were decreased significantly in candesartan group ($P < 0.05$) however, no change was observed in the control group ($P > 0.05$).

Table 4. Comparison of the change of direct fibrosis markers after 6-month treatment between candesartan group and control group.

	Candesartan group (n = 37)				Control group (n = 36)				P^*
	pre	post	change(Δ)	$P^\dagger$	pre	post	change(Δ)	$P^\dagger$	
Laennec fibrosis score‡	3.4 ± 1.4	3.0 ± 1.0	-0.4 ± 0.8	< 0.05	3.2 ± 1.2	3.3 ± 1.3	0.1 ± 0.7	NS	< 0.05
Fibrosis area (%)	11.2 ± 6.0	8.2 ± 5.1	-3.0 ± 3.4	< 0.05	8.7 ± 4.8	9.6 ± 6.5	0.9 ± 4.4	NS	< 0.05
αSMA (%)	27.4 ± 10.6	22.3 ± 9.4	-5.1 ± 7.2	< 0.05	26.3 ± 9.9	27.6 ± 12.5	1.3 ± 11.27	NS	< 0.05
Hydroxyproline (μg/g liver tissue)	7.8 ± 2.6	5.9 ± 1.9	-1.9 ± 3.1	< 0.05	7.5 ± 2.9	8.1 ± 3.5	0.7 ± 1.5	NS	< 0.05
TGF-β	7.4 ± 6.6	3.1 ± 2.3	-4.2 ± 5.8	< 0.05	8.5 ± 5.4	11.5 ± 7.2	3.0 ± 5.6	< 0.05	< 0.001
Collagen 1	6.4 ± 4.0	4.4 ± 2.9	-2.0 ± 2.0	< 0.05	6.0 ± 3.6	8.5 ± 4.1	2.5 ± 3.2	< 0.05	< 0.001
AT1-R	6.5 ± 3.6	4.4 ± 2.0	-2.1 ± 2.4	< 0.05	5.8 ± 3.0	7.5 ± 4.3	1.7 ± 2.4	< 0.05	< 0.001
TIMP-1	7.1 ± 4.4	3.8 ± 2.3	-3.3 ± 2.7	< 0.05	5.7 ± 3.3	6.4 ± 4.3	0.8 ± 1.8	NS	< 0.001
Rac-1	8.1 ± 5.7	5.5 ± 4.8	-2.6 ± 2.1	< 0.05	6.7 ± 3.3	7.5 ± 4.0	0.8 ± 3.1	NS	< 0.001
p22phox	7.9 ± 5.5	5.1 ± 3.7	-2.8 ± 3.9	< 0.05	6.4 ± 3.2	10.4 ± 7.5	4.0 ± 5.5	NS	< 0.001

* Comparison of change(Δ) between candesartan and control group. † Comparison between pre- and post-treatment in each group.

‡ Estimated according to Laennec scoring system. αSMA, alfa-smooth muscle actin; TGF-β1, Transforming growth factor-beta 1; collagen 1, type 1 collagen; AT1-R, angiotensin II type 1 receptor; TIMP-1, tissue inhibitor of metalloproteinase 1

(4) Safety

Mean systolic and diastolic blood pressure in the candesartan group was mildly but significantly decreased ($P < 0.05$, Table 1). However, no patient complained of dizziness or other symptoms related with blood pressure reduction. Plasma rennin activity also showed no significant change in both group after 6 months treatment (Table 1). In addition, no other severe side effect related with candesartan administration was observed.

IV. Discussion

Multiple pieces of evidence from experimental and clinical studies suggest that the rennin–angiotensin system is involved in hepatic fibrosis (Friedman SL, Roll FJ, et al. 1985; Baik SK, Jo HS, et al. 2003; Kim MY, Baik SK, et al. 2008; Park DH, Baik SK, et al. 2007). HSCs, which play a key role in the pathogenesis of liver fibrosis, express AT1-R. ANG II induces the proliferation and contraction of activated HSCs through AT1-R (Baik SK, Park DH, et al. 2003; Lee KI, Kong ID, et al. 2004). In several previous studies, the administration of ANG II blocking agents reduced hepatic fibrosis in the bile-duct ligation rat model and in patients with hepatitis C (Kim MY, Baik SK, et al. 2008; Park DH, Baik SK, et al. 2007; Sookoian S, Fernandez MA, et

al. 2005; Corey KE, Shah N, et al. 2009; Debernardi-Venon W, Martini S, et al. 2007; Terui Y, Saito T, et al. 2002). However, no study has examined the effect of ANG II blocking agents on hepatic fibrosis in patients with alcoholic liver disease.

Alcohol is fibrogenic *per se* in liver, and oxidative stress is a key element of the ethanol-induced fibrogenic cascade. Acetaldehyde, a metabolite of ethanol, upregulates the expression of type I collagen genes in HSCs via increased formation of hydrogen peroxide (H_2O_2), which induces an oxidative stress response. Furthermore, H_2O_2 is associated with the activation of HSCs and enhances their production of TGF-β1. Therefore, reactive oxygen species (ROS) are second messengers of ethanol-induced hepatic fibrogenesis.

AGN II has a similar action on the ethanol-induced ROS in HSCs. Similarly, angiotensin stimulates the proliferation of HSCs and their increased production of extracellular matrix via induction of TGF-β1 expression both in vivo and in vitro (Pueyo ME, Arnal JF, et al. 1998). Moreover, in vascular endothelium, AGN II induces hypertension mediated by increased nitric oxide degradation, secondary to the stimulatory effects of vascular superoxide production (Laursen JB, Rajagopalan S, et al. 1997; Heitzer T, Schlinzig T, et al. 2001). Expression of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH) subunit p22phox is increased and superoxide dismutase treatment inhibits expression of p22phox, indicating that

oxidative stress may be crucial for enzyme expression (Fukui T, Ishizaka N, et al. 1997). Consequently, both AGN II and alcohol exert a powerful influence on hepatic fibrosis via a similar mechanism. Hence, we can suggest that AT1-R blocking agents may ameliorate hepatic fibrosis in alcoholic liver disease through the inhibition of the ethanol-induced overproduction of ROSs. The present study indicates that a decrease in the expression of p22phox occurs after candesartan treatment, suggesting that inhibition of oxidative stress by AT1-R blocking agents is associated with the prevention of hepatic fibrosis in alcoholic liver disease. In addition, the significant increase and decrease of TGF- β1, collagen-1, and AT1-R in control group and candesartan group respectively after 6 months suggests the beneficial effect of ARB in stopping of on-going inflammation and fibrosis which could be seen in more than half of alcoholic hepatitis and fibrosis in spite of alcohol abstinence.

We previously reported the beneficial effects of AGN II blockade by AT1-R blocking agents or ACEIs on hepatic fibrosis in a rat model (Kim MY, Baik SK, et al. 2008; Park DH, Baik SK, et al. 2007). Particularly, AT1-R blocking agents were superior to ACEIs in the suppression of hepatic fibrosis (Kim MY, Baik SK, et al. 2008). Of the AT1-R blocking agents, candesartan is a potent long-lasting selective AT1-R blocking agent, which can produce insurmountable antagonism due to its tight binding and slow dissociation from AT1-Rs. Therefore, its starting and maintenance

doses (8–16 mg once a day) are lower than those of other antagonists (McClellan KJ, Goa KL. 1998; Ojima M, Inada Y, et al. 1997; Burnier M, Brunner HR. 2000). Furthermore, candesartan is known to reduce scar tissue in patients with pulmonary, renal, and cardiac failure, resulting in the prevention of disease progression (Otsuka M, Takahashi H, et al. 2004; Kim S, Iwao H. 1997). In this regard, we chose candesartan among various AGN II blocking agents. To determine the safety and anti-fibrosis effect of candesartan on alcohol-related hepatic fibrosis in human, candesartan was given to patients with alcoholic liver disease.

With regard to the biochemical examination, liver, and renal function, electrolyte balance as well as blood cell contents did not change in either the candesartan or the control group. In addition, no serious side effects, including renal failure or hepatic decompensation, were noted. Mean blood pressure was mildly but significantly changed between study entry and the completion of the study period. However, no patients had a hypotensive reaction that required cessation of medication during treatment, which is indicative of good tolerance. Patients with an early stage of alcoholic liver cirrhosis and a Child-Pugh score of less than 10 were chosen because the adverse effects of AT1-R blocking agents in advanced cirrhosis is controversial (Gonzalez-Abraldes J, Albillos A, et al. 2001; Schneider AW, Kalk JF, et al. 1999). Consequently, candesartan administration was safe in terms of circulatory

hemodynamics as well as liver function in patients with alcoholic liver disease.

The fibrosis score measured by the METAVIR scoring system, which consists of only four grades of fibrosis, was unable to provide any information about the anti-fibrotic effect of any drugs before and after treatment, because it lacks discriminating power. Among cirrhosis patients classified as F4 by the METAVIR scoring system, the degree of fibrosis was variable. Therefore, several pathologists suggested that a subclassification of F4 is needed for an exact diagnosis to discriminate the level of fibrosis, particularly to assess the anti-fibrotic effects of therapeutic agents. Therefore the present study applied new fibrosis scoring system, the Laennec system which was suggested by some pathologist recently (Kutami R, Girgrah N, et al. 2000; Wanless IR, Sweeney G, et al. 2002; Robert D. Odze M, et al. 2009), and measured fibrosis area(%) which was estimated as trichrome positive area/ the total area. In both estimations about fibrosis, the treatment of candesartan showed significant decrease of fibrosis compared to control.

We incorporated other methods to assess hepatic fibrosis in this study because the validity of morphometrical analysis remains controversial. Hydroxyproline is a major component of collagen fiber that makes helical changes of collagen. Because it quantitatively represents fibrosis, hydroxyproline was measured from liver tissue obtained by liver biopsy. Additionally, TGF-β1, collagen 1, AT1-R, TIMP-1, Rac1,

and $p22^{phox}$ in liver parenchyma were measure by RT–PCR to assess the fibrosis grade from biopsied liver tissues.

This report is the first to determine the effect of AT1-R blocking agents on hepatic fibrosis in patients with compensated alcoholic liver disease. Our results support the approval of this class of agents for the treatment of hepatic fibrosis. Candesartan may provide a new strategy for anti-fibrosis treatment of alcoholic liver disease. However, further studies are required to more fully demonstrate their roles.

V. Conclusion

In conclusion, we suggest that the chronic administration of candesartan is safe and can be beneficial for hepatic fibrosis in patients with compensated alcoholic liver disease.

Abbreviations: ARB, angiotensin receptor blocker; αSMA, alfa-smooth muscle actin; TGF-β1, Transforming growth factor-beta 1; collagen 1, type 1 collagen; AT1-R, angiotensin II type 1 receptor; TIMP-1, tissue inhibitor of metalloproteinase 1

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국문요약

Beneficial Effect of Angiotensin-Blocking Agent Candesartan on Compensated Alcoholic Liver Fibrosis: A Randomized Controlled Trial

알콜성 간경변 환자에서 안지오텐신 길항제 (candesartan)의 간섬유화에 대한 효과

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정필호

간경변증은 광범위한 간세포괴사가 장기간 진행된 결과 섬유화가 초래되고, 이 광범위한 섬유화가 정상적인 간소엽을 둘러쌈으로써 정상 구조가 파괴된 대신 재생 결절이 생기는 간 질환이다. 간섬유화 및 이의 진행형인 간경변증의 원인은 여러 가지이지만 세계적으로 가장 흔한 것은 알코올의 과다섭취와 B 형 및 C 형 간염 바이러스의 만성적인 감염이다.

우리나라 간경변증 환자에서는 만성 B 형간염이 63-73%로 가장 흔한 원인이지만, 최근 다양한 B 형 간염 바이러스 치료제가 개발되면서 이의 치료 및 관리에 많은 진전이 있어 왔다. 그러나 알코올성 간경변증의 경우 오히려 그 수가 세계적으로 증가하는 추세에 있으나 아직까지 이렇다 할 간섬유화 치료제가 개발되지 못하고 있다. 간성상세포(hepatic stellate cell)는 섬유 생성에 관여하는 주된 세포로, 최근의 연구에서 레닌-안지오텐신 시스템 (rennin-angiotensin system, 이하 RAS)의 중요 구성요소인 angiotensinⅡ가 간의 angiotensinⅡ type1 receptor 에 반응하여 간성상세포의 수축과 활성화를 유발함이 밝혀졌다. 또한 angiotensinⅡ는 간성상세포 외에도 kupffer cell 와 NF-κB 의 활성화를 통해서 간 섬유화를 일으킨다고 알려져 있다. 그러나 이러한 RAS 의 알코올성 간질환 환자의 간섬유화에 대한 영향에 대한 연구는 아직까지 매우 부족한 상태이며, 특히 이들 RAS 의 조절에 따른 간섬유화의 변화에 대한 연구는 전무한 상태이다. 이에 본 연구에서는 알코올 간질환 환자에서 안지오텐신 수용체 차단제(angiotensinⅡ type1 receptor blocker, ARB)의 항섬유화 효과에 대해 전향적 방법으로 연구하였다.

대상성 알코올성 간섬유화증((≥ F2)을 가진 환자 73 명을 두 군으로 이중맹검 무작위 추출하여 치료군에는 6 개월동안 candesartan(ARB) (8mg/day)과 ursodeoxycholic acid(UDCA) (600mg/day) (n=37)를 투약하였고, 대조군(n=36)에는 UDCA 만을 투약하였다. 모든 환자는 치료 전과 치료 6 개월 후에 두 번에 걸친 간조직 검사를 통해서 Laennec fibrosis scoring system 에 의한 간섬유화 점수(fibrosis score), 활성화된 간성상세포를 나타내는 α-smooth muscle actin(SMA) 양성 정도 및 hydroxyproline 치를 측정하였다. 또한 간섬유화와 직접적으로 관련 있는 인자인 Transforming growth factor-β1(TGF-β1), collagen-1, angiotensin II type I receptor(AT1-R), tissue inhibitor of metalloproteinase-1(TIMP-1)들과 산화 스트레스 (Oxidant stress)를 나타내는 Rac1 과 p22phox 를 치료 전 과 치료 6 개월 후에 real-time RT-PCR 로 측정하였다.

Candesartan 치료군은 Laennec fibrosis scoring system 에 따라서 섬유화 점수가 3.4±1.4 에서 3.0±1.0 으로 유의하게 감소하였으며($P < 0.05$), imaging analysis system 에 의해 계산된 간섬유화 면적에 있어서도 Candesartan 치료군은 11.2±6.0 에서 8.2±5.1 로 유의하게

감소하였다($P < 0.05$). 면역화학 검사상 α-smooth muscle actin(SMA) 양성을 나타내는 면적 또한 27.4±10.6 에서 22.3±9.4 로 유의하게 감소하였고($P < 0.05$), hydroxyproline 치 (μg/g liver tissue) 또한 7.8±2.6 에서 5.9±1.9 로 유의하게 감소하였다($P < 0.05$). Real-time RT-PCR 로 측정한 TGF-β1, collagen-1, AT1-R, TIMP-1, Rac1 and p22phox 의 상대적 발현은 candesartan 치료군에서 유의하게 감소하였다($P < 0.05$). 본 연구에서 본 치료와 관련하여 저혈압 및 기타 부작용은 발생하지 않았다.

정리하면, 대상성 알코올 간질환에서 안지오텐신 수용체 차단제의 투약은 조직학적 소견과 간섬유화 관련 직접적 인자들에서 유의한 호전을 보여, 향후 간섬유화에 대한 치료 및 새로운 항섬유화제 개발에 있어 유용한 자료가 될 것으로 생각된다.

핵심단어: 알콜성 간경변, 안지오텐신 길항제, 항섬유화