Original Article

Nifedipine Enhances the Cardioprotective Effect of an Angiotensin-II Receptor Blocker in an Experimental Animal Model of Heart Failure

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This study was designed to examine the hypothesis that a calcium channel blocker nifedipine (CCB) could enhance the cardioprotective effect of an angiotensin-II receptor blocker candesartan (ARB) in the treatment for heart failure. Isoproterenol (ISP) was injected into male rats at 300 mg/kg to produce progressive heart failure. Three months later, the rats were divided into 4 groups and treated for 4 weeks with 1) vehicle (n=20), 2) ARB at 0.2 mg/kg/day (n=6), 3) CCB at 10 mg/kg/day (n=6), or 4) both drugs (n=8). Rats injected with saline served as controls (n=13). ISP caused severe myocardial degeneration and decreased the capillary density (D_{cap}) of the left ventricular (LV) myocardium (mean±SD: 2,197±627 *vs.* 2,847±298 N/mm^2 for normal controls), while increasing plasma thiobarbituric acid-reactive substances (TBARS; 3.6±1.1 *vs.* 1.9±0.5 nmol/ml). Although ARB therapy preserved cardiac morphology, it had little effect on D_{cap} or oxidative stress. On the other hand, CCB decreased plasma TBARS and 4-hydroxy-2-nonenal protein expression in LV myocardium. Furthermore, the combination of CCB and ARB increased D_{cap} and preserved the ultrastructure of LV myocardium, so this combination may be a useful option for the treatment of heart failure. (*Hypertens Res* 2005; 28: 431–438)

***Key Words*: nifedipine, heart failure, cardioprotection, angiotensin-II receptor blocker, oxidative stress**

Introduction

Heart failure is a major and escalating public health problem in many countries. Despite advances in various medical treatments, the number of patients with chronic heart failure is increasing (*1*, *2*), and their background has become more diverse. Angiotensin-II receptor blockers (ARBs) are known to improve the prognosis of patients with heart failure, and are often prescribed for such patients (*3*, *4*).

Calcium channel blockers (CCBs) are widely used in the cardiovascular field for the control of angina, hypertension, and other vascular conditions (*5*–*7*). The CCB nifedipine was recently reported to upregulate endothelial superoxide dismutase expression by stimulating vascular endothelial growth factor (VEGF) production from adjacent vascular smooth muscle cells and reducing oxidative stress (*8*, *9*). In the recent clinical trial, nifedipine reduced the new onset of overt heart failure and the need for coronary angioplasty in patients with stable angina (*10*).

To treat patients with heart failure, addition of a β-blocker to standard therapy with an angiotensin-converting enzyme (ACE) inhibitor or ARB has been recommended (*11*). We have previously reported that combination therapy consisting of an ARB and a β-blocker led to marked improvement of both systolic and diastolic dysfunction in an animal model of congestive heart failure (CHF) (*12*). When a β-blocker is contraindicated or when it causes side effects, a CCB might be considered instead.

In this study, we evaluated the hypothesis that a CCB could

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Table 1. Haemodynamic Data, TBARS, and Cardiomyocyte Diameter

	Control (n=13)	Vehicle (n=20)	ARB (n=6)	CCB (n=6)	ARB+CCB (n=8)
Hw/Bw (mg/g)	2.5±0.3	2.8±0.2*	2.4±0.3	2.5±0.2	2.3±0.1$^{\#}$
LV_{sys} (mmHg)	95±5	102±8	90±4$^{\#}$	96±8	88±6$^{\#}$
LV_{ed} (mmHg)	5±2	18±6*	5±3$^{\#}$	7±3$^{\#}$	4±2$^{\#}$
min dP/dt (mmHg/s)	3,600±220	2,350±450*	3,420±420$^{\#}$	3,380±360$^{\#}$	3,400±250$^{\#}$
TBARS (nmol/ml)	1.9±0.5	3.6±1.1*	3.2±1.0	2.2±1.2$^{\#}$	2.3±0.3$^{\#}$
Diameter (μm)	16.4±1.5	23.2±3.3*	17.5±2.1$^{\#}$	18.6±2.4	16.9±1.9$^{\#}$

Values are mean±SD. Hw/Bw, heart/body weight ratio; LV_{sys} and LV_{ed}, left ventricular end-systolic and end-diastolic pressure; min dP/dt, peak negative dP/dt; TBARS, thiobarbituric acid-reactive substances; Diameter, mean cardiomyocyte diameter; ARB, angiotensin-II receptor blocker candesartan; CCB, calcium channel blocker nifedipine; ARB+CCB, combination of candesartan and nifedipine. *$p<0.05$ *vs.* control, $^{\#}p<0.05$ *vs.* vehicle.

enhance the cardioprotective effect of an ARB and that the combination of these drugs might be preferable to the use of ARB monotherapy for heart failure.

Methods

Subjects

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, USA) (n=76) were used. All animals were housed in a specific pathogen-free facility under controlled temperature (20–24°C) and humidity (40–70%), with a 12-h lighting cycle, and were given free access to standard laboratory rat chow (MF, Oriental Yeast, Tokyo, Japan) and tap water. All procedures were performed in accordance with our institutional guidelines for animal research.

Experimental Protocol

At the age of 5 weeks, animals were assigned to receive subcutaneous injection of isoproterenol (ISP) at a dose of 300 mg/kg or saline on 2 consecutive days. Three months after injection, the rats were divided into 4 groups that were treated with 1) the vehicle, 2) nifedipine at 10 mg/kg/day (CCB), 3) candesartan at 0.2 mg/kg/day (ARB), or 4) the combination of candesartan and nifedipine (ARB+CCB) using osmotic minipumps (Alzet model 2ML2 for CCB and Alzet model 2004 for ARB; Alza Co., Palo Alto, USA) placed in the peritoneal cavity for 4 weeks. Rats injected with saline served as controls.

After left ventricular (LV) pressure tracings were obtained and blood was sampled for the measurement of plasma thiobarbituric acid-reactive substances (TBARS), the heart was excised under general anesthesia (pentobarbital at 50 mg/kg i.p.), and part of the LV myocardium was used for morphological and immunohistochemical studies.

Light and Electron Microscopy

For light microscopy, specimens were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm thick sections. The tissue sections were stained with haematoxylin and eosin and Mallory-azan stains, and were examined under a microscope.

For the semi-quantitative analysis of histological changes, each finding of 1) disarray of myofibers, 2) scarcity of myofibrils, 3) nuclear changes, 4) vacuolization, and 5) proliferation of collagen fibers was graded 1+ to 4+ by the method previously published (*13*). Grade – signifies no apparent change, 1+ a minimal degree, 2+ a moderate degree, 3+ a marked degree, and 4+ an excessively marked degree of change. The sum of graded scores in each sample was defined as the semi-quantitative score of histological changes.

For transmission electron microscopy, specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose, after which ultrathin sections were cut from the embedded blocks and examined with a Hitachi H-7000 electron microscope (Hitachi, Tokyo, Japan) (*14*).

Immunohistochemistry of 4-Hydroxy-2-Nonenal (HNE) Protein

For immunohistochemical light microscopy, additional sections were obtained from the paraffin block. After incubation with normal serum for blocking, the sections were incubated overnight at 4°C with a monoclonal antibody targeting 4-HNE (No. MHN-020, Japan Institute for the Control of Aging, Shizuoka, Japan). After serial washing with phosphate-buffered saline, the slides were incubated with a biotinylated secondary antibody for 45 min at room temperature. Then the sections were reacted with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, USA) for 30 min. After incubation with the peroxidase substrate solution (Vectastain 3′,3′-diaminobenzidine substrate kit, Vector Laboratories), the slides were counterstained with Mayer-hematoxylin, cleaned, and mounted for light microscopy (*15*).

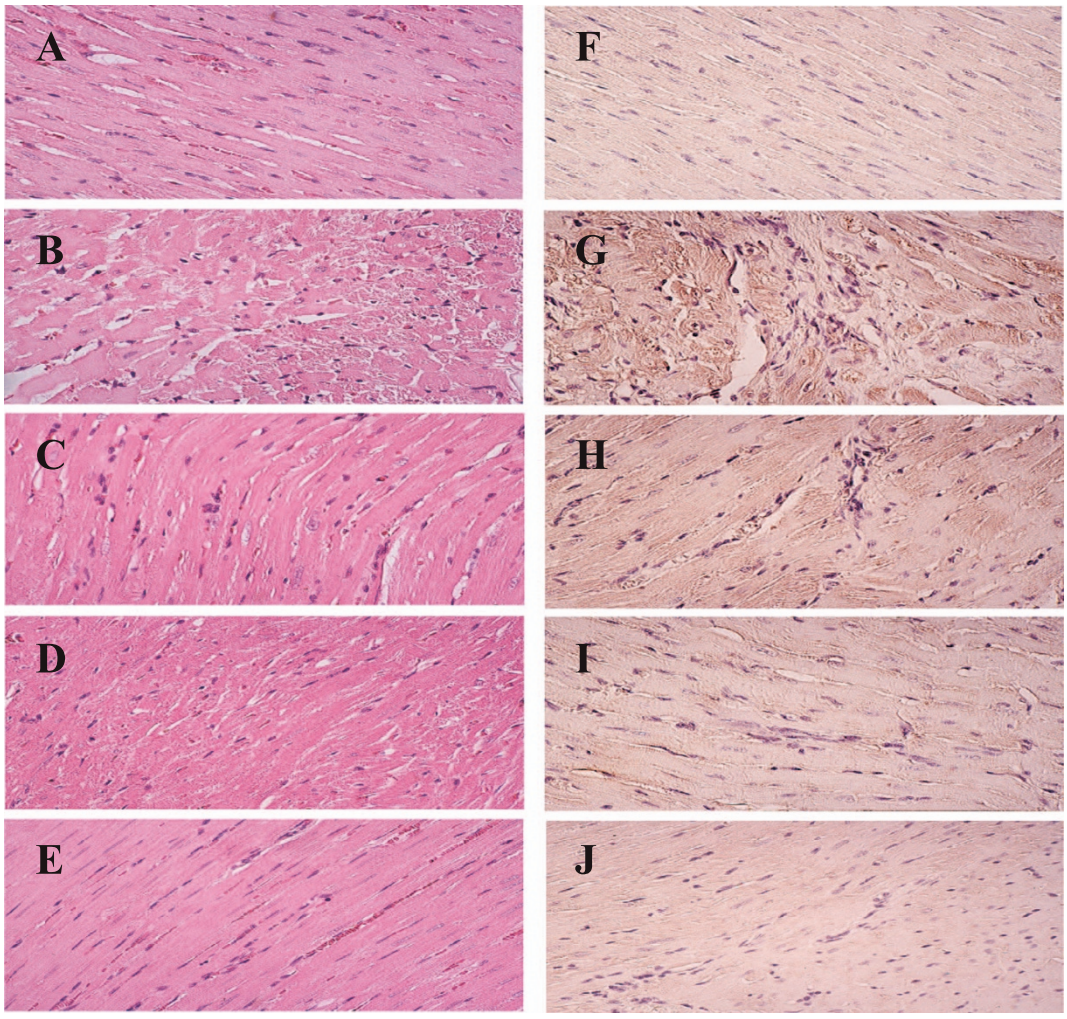

Fig. 1. *Light micrographs. Left panel (A, B, C, D, E): haematoxylin-eosin staining. Right panel (F, G, H, I, J): immunohistochemistry for 4-HNE. Control rats showed a normal morphology (A) and immunostaining for 4-HNE was not detected (F). In ISP rats, hypertrophy of cardiomyocytes, disarray of myofibers, and perivascular fibrosis were observed (B). These changes were suppressed by ARB therapy (C) and CCB therapy (D), and were more markedly suppressed by combination therapy (E). Positivity for 4-HNE was markedly increased in the cytoplasm of cardiocytes in ISP rats (G). Although ARB therapy (H) showed little effect on 4-HNE expression, CCB therapy (I) and combination therapy (J) suppressed the increase. Magnification ×100.*

Quantitative Analysis of 4-HNE Protein

The myocardial HNE-modified protein content was measured by quantitative analysis of HNE-modified protein-stained myocardial tissue. After immunostaining, digital images of each section were obtained with a digital camera (Fujix Digital Camera HC-300Z; Fuji Photo Film, Tokyo Japan) mounted on a Nikon Microphot-FXA (Nikon, Tokyo Japan). Color images were obtained from 5 randomly selected separate high-power fields (×200) in 5 sections per rat. Each image covered an area of 780402 pixels and was analyzed using NIH Image 1.56 software. Both intensity and area were analyzed by the method of Matsuo *et al*. (*16*). The data from all the examined sections were used to calculate the % area of positive pixels relative to the total examined pixel area.

Measurement of Capillary Density

Using an electron microscope, more than 15 negatives at ×1,000 magnification were taken of each block by systematic random sampling. The number of capillaries was measured using a morphometric method and the myocyte density (N/mm^2) was calculated (*17*).

All morphometric analyses were done by a single examiner who was blinded to the experimental group of each sample.

Table 2. Semi-Quantitative Score of the Histological Changes

Histological findings	Control	Vehicle	ARB	CCB	ARB+CCB
Disarray of myofibers	0.3±0.1	2.8±0.3*	1.6±0.2	2.4±0.2*	0.9±0.1$^{\#}$
Scarcity of myofibrils	0.8±0.2	3.1±0.4*	1.8±0.2	2.0±0.3	1.6±0.3$^{\#}$
Nuclear changes	1.0±0.1	2.5±0.2*	2.1±0.3*	1.7±0.2	1.5±0.2
Vacuolization	0.8±0.1	2.3±0.2*	1.8±0.2	1.8±0.3	1.7±0.3
Proliferation of collagen fibers	0.3±0.1	2.3±0.2*	1.5±0.4*	2.3±0.2*	1.4±0.3*

ARB, angiotensin-II receptor blocker candesartan; CCB, calcium channel blocker nifedipine; ARB+CCB, combination of candesartan and nifedipine. *$p<0.05$ *vs.* control, $^{\#}p<0.05$ *vs.* vehicle.

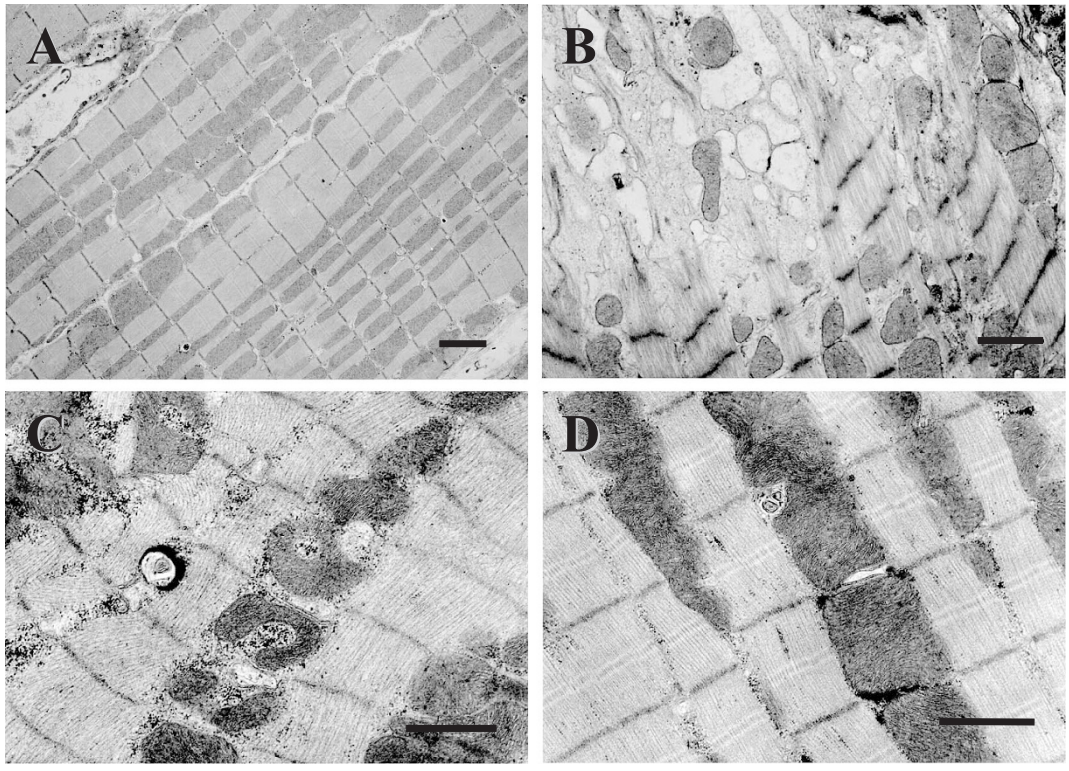

Fig. 2. *Electron micrographs. Control rats showed normal myocardium (A). ISP rats showed severe degeneration, including myofibrillar lysis and vacuolization (B). ARB therapy helped to preserve the ultrastructure (C), while combined therapy with a CCB better preserved the ultrastructure, especially that of the mitochondria (D). Bar = 1 μm.*

Statistical Analysis

All values are expressed as mean±SD, and statistical analysis was performed by 1-way ANOVA. When significant differences were identified, Scheffe's multiple range test was applied to determine the level of significance. The results were considered significant at a probability value of $p<0.05$.

Results

Heart Weight, Body Weight, and TBARS

In the ISP rats treated with vehicle, the heart weight to body weight ratio increased significantly. This increase was ameliorated by ARB, CCB, or ARB+CCB treatment. ISP increased the plasma TBARS level, and this increase was significantly ameliorated by CCB therapy or combination therapy (Table 1).

Haemodynamic Data

The LV end-diastolic pressure was significantly increased in ISP rats, and the increase was attenuated by ARB, CCB, or ARB+CCB treatment. LV systolic pressure tended to be increased in ISP rats, and was decreased by all of these treatments (Table 1).

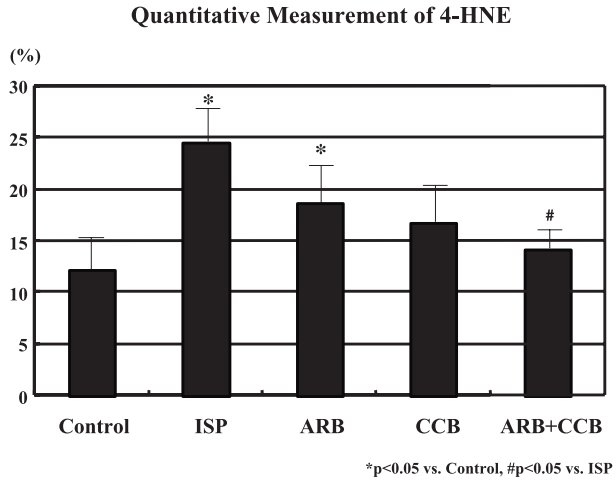

Fig. 3. *Percent area of immunostaining for 4-HNE protein. The mean % area of 4-HNE staining was increased in ISP rats. In response to ARB or CCB therapy, the % area decreased, and combination therapy significantly decreased the % area in the left ventricular myocardium.*

Light and Electron Microscopy Findings

ISP caused a variety of degenerative changes in the LV myocardium, including hypertrophy of cardiomyocytes, disarray of myofibers and interstitial fibrosis (Fig. 1B). The diameter of the cardiomyocytes was significantly increased in ISP rats compared with control rats (Table 1). Treatment with the ARB (Fig. 1C), CCB (Fig. 1D), and both drugs tended to prevent such changes (Fig. 1E). Both the ARB and ARB+CCB significantly suppressed ISP-induced hypertrophy of cardiomyocytes.

The semi-quantitative analysis clarified the histological changes after each treatment: the ARB+CCB combination significantly reduced the score of disarray and scarcity (Table 2). Although ARB and ARB+CCB tended to reduce the interstitial fibrosis, there was no significant improvement.

Electron microscopy revealed myofibrillar lysis, vacuole formation, and mitochondrial degeneration in ISP rats (Fig. 2B). ARB therapy tended to exert a cardioprotective effect (Fig. 2C), and additional treatment with a CCB preserved the ultrastructure, especially that of the mitochondria (Fig. 2D).

Immunohistochemistry of 4-HNE Protein

To determine whether oxidative stress was increased in the failing hearts, we examined the level of HNE-modified protein in the LV myocardium of rats with CHF. Immunohistochemical staining for 4-HNE protein was increased in all ISP rats, and was observed in the cytoplasm of cardiomyocytes (Fig. 1G). The standard deviation of the stained area in each rat was relatively large, so considerable heterogeneity in the extent of oxidative stress was suggested. The mean % area was significantly increased in ISP rats compared with control rats (24.5±4.2 *vs.* 12.1±3.2%, $p<0.05$), but was reduced to 18.7±4.3% in ARB rats, 16.9±3.7% in CCB rats, and 14.2±2.6% in ARB+CCB rats (Fig. 3). These findings indicate that CCB therapy might reduce oxidative stress on the myocardium and enhanced the modest antioxidant effect of the ARB.

Capillary Density

The capillary density of the LV myocardium was evaluated by electron microscopy. ISP rats had a significantly lower capillary density than the controls. The capillary density tended to be increased by CCB or ARB monotherapy, but only the ARB+CCB combination significantly increased the capillary density compared with that of ISP rats (Fig. 4).

Discussion

Subcutaneous injection of the β-adrenoreceptor agonist ISP produces infarct-like lesions in the myocardium of rats and causes diffuse myocardial necrosis resulting in progressive LV enlargement (*12*, *18*, *19*). In our study, the heart to body weight ratio and oxidative stress were increased significantly in the ISP-treated rats. Various histological changes of the LV myocardium also occurred in these rats, including hypertrophy of cardiomyocytes, disorganization of myofibers, and interstitial fibrosis, all consistent with the findings in congestive heart failure (*20*, *21*).

We previously reported that treatment with the combination of an ARB and a β-blocker improved both systolic and diastolic function in the present animal model of heart failure (*12*). In this study, we showed the usefulness of treatment with an ARB plus a CCB. Several studies have shown the additive beneficial effects of the combination of CCB and ARB at a variety dose (*22*, *23*). The doses of ARB and CCB in this study were determined from previous studies using osmotic mini-pumps, and were considered to be sufficient to elicit a cardioprotective effect (*12*, *24*, *25*). However, blood pressure-lowering effect observed in this study should be taken into consideration, and the experiments would be repeated using smaller amounts of each treatment.

Berkels *et al*. reported that exposure of endothelial cells to a CCB led to increased nitric oxide (NO) release and enhanced the availability of NO *via* antioxidant mechanisms (*26*). Such increased availability of NO may contribute to the beneficial effects of CCBs that have been found by others, including antiplatelet (*27*, *28*), antiproliferative (*29*, *30*), and antiatherosclerotic effects (*31–33*). In our study, treatment with nifedipine decreased plasma TBARS and decreased 4-HNE in the LV myocardium, which means that the oxidative stress in heart failure was decreased by CCB therapy (Table 1, Figs. 1, 3).

Taniyama *et al*. reported a decrease of capillary density in a hamster model of heart failure (*34*), and we also found that

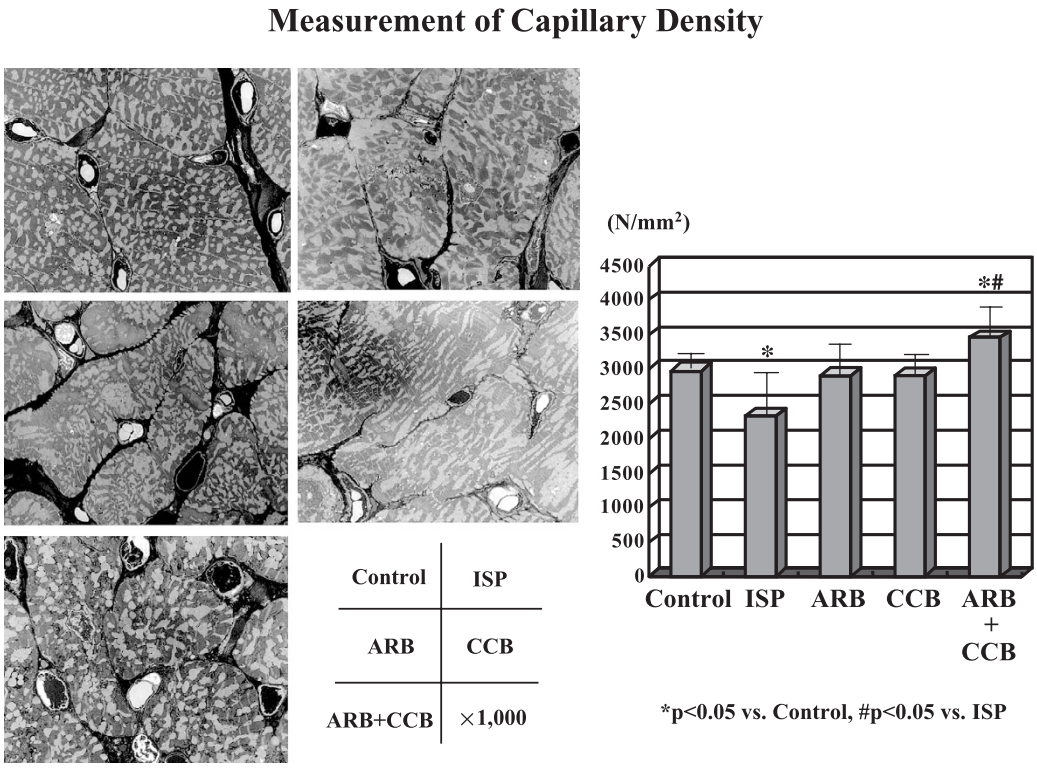

Fig. 4. *Capillary density. Electron micrographs of the left ventricular myocardium were used for morphometric determination of capillary density (D_{cap}). In ISP rats, D_{cap} was significantly lower than in control rats. The ARB + CCB combination significantly increased D_{cap} in comparison with that in control or ISP rats.*

the LV capillary density was decreased in ISP rats. Treatment with an ARB might increase the capillary density of the LV myocardium in diabetic heart (*24*). Haider *et al*. observed the increased capillary density and regional blood flow after transplantation of myoblast carrying VEGF in porcine heart (*35*). In this study, both ARB therapy and CCB therapy tended to increase the LV capillary density, and a synergistic effect was obtained with combination therapy (Fig. 4). Such an increase of capillary density might have improved the oxygen supply to the cardiomyocytes.

The dose of ARB prescribed in this study was relatively low compared with that of CCB, which might have contributed to the negative findings in ARB monotherapy. Thus, higher dose of ARB should be evaluated in the same experimental model of heart failure. For all that, it might be possible that nifedipine stimulated the release of VEGF from vascular smooth muscle cells (*36*). Our preliminary immunohistochemical study showed that CCB therapy tended to increase VEGF expression in the LV myocardium (data not shown). Further examination by Western blot analysis should be done to evaluate VEGF protein expression in the LV myocardium after each treatment.

In ISP rats, evidence of severe degeneration was observed, including myofibrillar lysis, vacuolization, and mitochondrial dgeneration. ARB therapy exerted a cardioprotective effect and tended to reduce the interstitial fibrosis (Table 2). Additional treatment with a CCB reduced the semi-quantitaive score of disarray and scarcity, and preserved the ultrastructure of the LV myocardium. Kim-Mitsuyama *et al*. reported the reduction of cardiac fibrosis by the combination with olmesartan and azelnidipine in hypertensive-rat heart failure model (*23*). It might be possible that high dose of candesartan could reduce the fibrosis. Combination with another ARB and CCB should be examined to evaluate their class effect as well.

In summary, this study showed that candesartan monotherapy improved cardiac function, but had small effect on oxidative stress and LV myocardial capillary density. Addition of nifedipine had an antioxidant effect, increased capillary density, and improved the ultrastructure of the LV myocardium. Accordingly, nifedipine enhanced the cardioprotective effect of candesartan, and this combination may be a useful option for the treatment of heart failure.

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