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Original Articles

Recovery Effects from Oxidative Cell Damage by *So-Hap-Hyang-Won* on Bovine Aortic Endothelial Cells (BAEC)

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So-Hap-Hyang-Won, a traditional oriental medicine used in the treatment of stroke patients, was examined for its ability to reverse the cell damage caused by lipid peroxidation products and oxidative stress in bovine aortic endothelial cells (BAEC).

The effects of herbal medicine on cell proliferation and recovery of oxidative damaged situation were studied in BAEC, which was considered an appropriate *in vitro* model for stroke resulting from various vascular diseases prevalent in advanced age. In a clinical study of stroke patients, *So-Hap-Hyang-Won* appeared to improve considerably arm and leg movements as well as consciousness disturbance condition, compared with other traditional medicines used for stroke. When BAEC were treated with extracts of the lyophilized herbal medicines, only that of *So-Hap-Hyang-Won* stimulated cell proliferation and showed no toxicity even at high concentrations. In studies of BAEC treated with extracts of the lyophilized material of the 14 components of *So-Hap-Hyang-Won*, only the extract of *Foeniculi Fructus* stimulated cell growth at all concentrations tested. Moreover, when cells were treated with *Foeniculi Fructus* (10 and 100 mg/ml) extract after prior exposure to t-BHP (10μM) or HNE (0.2μM), lipid peroxidation products which are known to be involved in aging and vascular diseases, or after the exposure to SIN-1 (500μM), which generates nitric oxide (NO) and other reactive oxygen species, there was substantial recovery from the oxidative damage, presumably due to the radical-scavenging effect of *Foeniculi Fructus* extract. *Foeniculi Fructus* not only showed stimulatory effects on cell growth and cell damage repair in BAEC, but also appeared to show the most anti-aging activity among all the herbal components of *So-Hap-Hyang-Won*. *(Korean J of Oriental Med 2003;24(4):71-81)*

Key Words: *Foeniculi Fructus* extract, Stroke, Radical scavenging, Cell Damage Repair, Anti-aging activity

Introduction

Stroke is an infarction of a portion of the brain caused by the sudden disturbance of blood supply to that area. This obstruction in circulation can be caused by arterial occlusions, most of which are due to atherosclerosis (Anderson *et al.*, 1990; Chun *et al.*, 1992; Lee and Kang, 1995). Atherosclerotic changes in arteries increase with age, (Kang and Choi, 2000) and can be promoted by oxidative stress (Lee *et al.*, 2000). Several studies have reported that stroke appears most frequently in the Korean population during the sixth decade (Feigensen, 1995; Malet *et al.*, 1998; Suh, 1999; Kim, 1992).

So-Hap-Hyang-Won is one of the herbal medicines used in the traditional treatment of stroke patients. It is

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known to induce recovery from several types of brain damage, and reverse the effects of obstruction of the blood circulation in the body, such as weak consciousness and hypothermic limbs. This oriental medicine is composed of equal amounts of *Atractylodis Rhizoma Alba, Saussurea Radix, Aquilariae Lignum, Caryophylli Flos, Santali albi Lignam, Benzoinum, Chebulae Fructus, Cyperi Rhizoma, Piperis longi Fructus, Agastachis Herba, Cinnamomi Cortex, Pteropi Faeces, Corydalis Tuber and Foeniculi Fructus* (Nho and Kim, 1998). Dried *Foeniculi Fructus* is used in other traditional medicines as well. *Foeniculi Fructus* has been used for promoting digestion and early paralysis (Choi *et al.*, 1998). 3-Morpholinosydnonimine (SIN-1) is a well-known NO generator, which increases cGMP and intracellular Ca^{2+}. Therefore, it stimulates vasorelaxation and decreases platelet aggregation through inhibition of thromboxane release (Forman *et al.*, 1987; Fraga and Tappel, 1988; Esterbauer *et al.*, 1991). NO and superoxide (O_2^-), produced by SIN-1 degradation, forms peroxynitrite (NO_3) (Fraga and Tappel, 1988).

Superoxide anion can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase, and hydrogen peroxide is degraded by catalase or produces hydroxyl groups (OH-) (Beneditti *et al.*, 1980). The reactive oxygen species such as hydrogen peroxide, superoxide, and hydroxyl groups are capable of damaging cellular biochemical constituents including DNA, lipids and proteins, and are linked to many common human diseases such as cancer, heart attack, stroke and emphysema (Bakson *et al.*, 1993; Hahan *et al.*, 1994; Riley, 1994; Foresti *et al.*, 1999; Warren *et al.*, 2000).

4-Hydroxy-2-nonenal (HNE), an unsaturated fatty acid, is formed via oxidation or peroxidation of arachidonic acid, linoleic acid or other related lipids and is a particularly reactive aldehydic species. HNE can be detected in cells under physiological conditions and its level can be elevated under oxidative conditions. HNE exerts a wide variety of biological effects, depending on its concentration and the target cell, and especially contributes to the cytotoxic effects of oxidative stress (Sandoval *et al.*, 1997). One of the HNE-producing substances is the lipid component of low-density lipoprotein (LDL), which is usually retained in the LDL particle. HNE enhances the mobility of LDL probably by oxidizing basic sites in the apoprotein (Mark *et al.*, 1977; Esterbauer *et al.*, 1991), resulting in structural changes that are believed to be responsible for the increased atherogenicity of oxidized LDL (Fuecker *et al.*, 1989).

LDL reacted with HNE is found to be accumulated in human atherosclerotic lesions. Tert-butyl hydroperoxide (t-BHP) is a typical organic oxidant that is widely used to produce a more physiological model of the oxidative stress imposed by hydrogen peroxide (Kim *et al.*, 1998). t-BHP treatment is known to cause the peroxidation of cellular lipids, oxidation of glutathione, loss of membrane thiols, release of Ca^{2+} from the endoplasmic reticulum, and an increased permeability of the mitochondrial inner membrane. t-BHP also causes programmed cell-death involving an increase in cytosolic free Ca^{2+} (Forman *et al.*, 1987; Lovell *et al.*, 1997; Esterbauer *et al.*, 1991; Mark *et al.*, 1997).

According to the literature and the results of our clinical research, the effects of *So-Hap-Hyang-Won* and *Foeniculi Fructus* extract suggest that these herbal products may have a certain effect on promoting the formation of new blood vessels and inducing recovery from cellular damage due to oxidative stress or aging. To examine this hypothesis, *Foeniculi Fructus* extract was examined at various concentrations for its restorative effect on bovine aortic endothelial cells (BAEC) previously treated with SIN-1, HNE or t-BHP, all known to exert oxidative stress on vascular

endothelial cells and to contribute to cellular aging. Light microscopy results indicated that:

1) *So-Hap-Hyang-Won* stimulated BAEC proliferation,
2) *Foeniculi Fructus* was the sole source of this growth-stimulatory effect even when other plant constituents were tested at high concentrations, and
3) *Foeniculi Fructus* extract induces recovery from cell damage caused by the above oxidants.

Materials and Methods

1. Reagents and extracts of Oriental medicinal herbs

3-Morpholinosydnonimine (SIN-1) was purchased from Molecular Probes(Eugene, OR) and tert-butyl hydroperoxide (t-BHP) and 4-hydroxy-2,3-nonenal (HNE) were generously donated by Dr. Jung, College of Pharmacology, Pusan National University, Pusan, Korea. Ethidium bromide and acridine orange were purchased from Sigma. *So-Hap-Hyang-Won, O-Yak-Sun-Ki-San, Sun-Ki-Hwal-Hyul-Tang, Seong-Hyang-Jeong-Ki-San, Bo-Yang-Hwan-O-Tang* and all individual herb samples were supplied by Dong-eui Oriental Hospital. The samples were lyophilized in a vacuum freeze dryer (Samwon, Korea) and mixed with serum-free medium. The medium containing herb-extracts were then passed through 0.45 mm filter system (Corning).

2. Cell Culture

Bovine aortic endothelial cells were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10%(v/v) fetal bovine serum (Gibco BRL). Cells were incubated in tissue culture flasks (Orange Scientific) at 3- or 4-day intervals and incubated at 37 °C in a 95% *humidified, 5%* CO_2 incubator (Forma Scientific, Marietta, OH).

3. Light Microscopy

Cells were harvested by trypsin-EDTA (Gibco BRL) treatment and split into 60 ml tissue culture dishes. After incubation for 24 hr in complete medium or serum-free medium, cells of the two groups were treated with the extracts of the 5 herbal medicines at concentrations of 10 and 100 $\mu g/ml$ in serum-free medium. After incubation for 24 hr in the presence of extract, cells were examined under a Nikon inverted microscope.

4. Fluorescence microscopy

Cells were distributed in Lab-Tek 8-well chamber slides, and incubated in complete medium overnight. After incubation, cells were treated for 6 hr with 200 μl /well of HNE, tBHP, and SIN-1 at concentrations of 0.2, 10, and 500 M, respectively.

The medium was then removed and the cells were incubated further in medium supplemented with Foeniculi Fructus extract (100 $\mu g/ml$) for 6, 12, and 24 hr. After the extract-supplemented medium was aspirated off, cells were stained with 2 μl/well of an equal volume mixture of acridine orange (AO) and ethidium bromide (EtBr) in phosphate buffered saline, and observed under a reflected fluorescence microscope (Olympus Optical, Tokyo, Japan).

5. Laser cytometry ACAS 570

Cells grown in Mat-Tek 35㎜ ACAS dishes were treated with HNE, tBHP and SIN-1 for 6 hr, and then incubated in Foeniculi Fructus extract-supplemented medium for 6, 12 and 24 hr. Cells were stained with acridine orange and ethidium bromide as described above, and then examined by laser cytometry ACAS 570 (Packard Instrument Co., Meridien, CT).

6. Clinical experiments

For 5 months, stroke patients who had suffered a

cerebral infarction and required hospitalization, were entered in a 4-week clinical study to investigate the effects of five traditional oriental medicines used for stroke. The patients were given *Seong-Hyang-Jeong-Ki-San* for the first 2 or 3 days after their admission because it was generally used as a first-aid for stroke patients. The patients were then divided into 5 groups, one for each herbal medicine. The first group of 21 patients took *Sun-Ki-Hwal-Hyul-Tang*, the second group of 20 took *Bo-Yang-Hwan-O-Tang*, the third group of 23 took *Seong-Hyang-Jeong-Ki-San*, the fourth group of 22 took *So-Hap-Hyang-Won*, and the last group of 17 took *O-Yak-Sun-Ki-San*. The patients were given their herbal medicine in a pre-warmed 120 *ml*-package 30 minutes after each meal. During the 4-week trial, blood pressure, extent of arm and leg movements, speech disturbance, and consciousness disturbance of the patients were assessed once a week, and scored arbitrarily according to improvement in each case (data not shown, note: see Lee *et al.*, 2000).

7. Statistics

The results of the clinical study were analyzed with the SAS program.

According to the herbal medicine administered to each patient group, the clinical characteristics (blood pressure, extent of arm and leg movements, speech disturbance and consciousness disturbance) of the

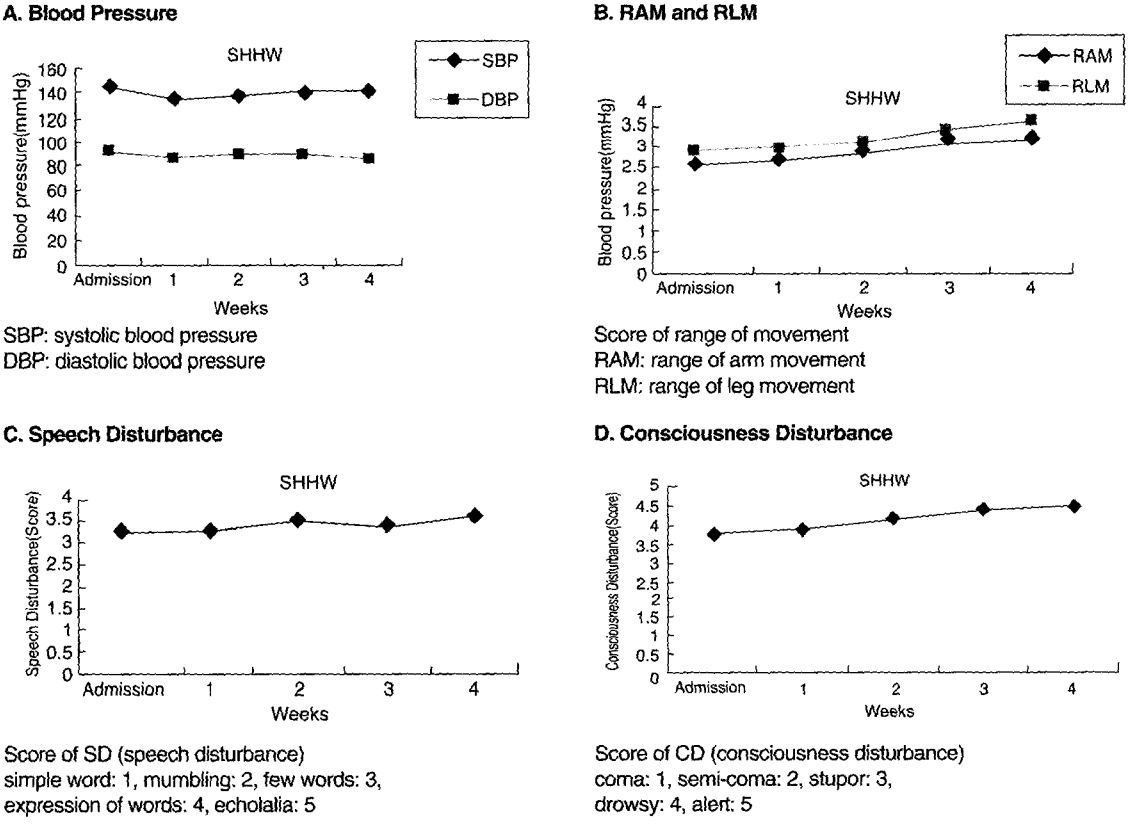

Fig. 1. Graphical analyses of changes in the clinical characteristics of each herbal treated-patient group.
A. Blood Pressure, B. Range of Arm Movement (RAM) and Range of Leg Movement (RLM), C. Speech Disturbance, D. Consciousness Disturbance.

groups are shown in Fig.1. The analysis of the changes between the time the patients were first hospitalized and end of the 4-week clinical study was determined using a paired *t*-test.

Results

1. Clinical study

The patients who had different clinical symptoms were shown in Table 1 and were statistically evaluated (N=103). Most of the patients were over 50 years old and examined according to the methods of Lee *et al.*[5].

Treatment with *Sun-Ki-Hwal-Hyul-Tang* (SKHHT), *Bo-Yang-Hwan-O-Tang* (BYHOT), *Seong-Hyang-Jeong-Ki-San* (SHJKS), and *O-Yak-Soon-Ki-San* (OYSKS) had statistically more differences to normal situation in systolic blood pressure *(t=4.22, P=0.0004; t=3.44, P=0.0028; t=2.11, P=0.0463; t=3.23, P=0.0052)*. Diastolic blood pressure of patients taking herbal medicine (SKHHT, SHJKS, and OYSKS) decreased significantly *(t=2.13, P=0.0459; t=2.68, P=0.0136; t=3.12, P=0.0066)*. The ranges of movement of patient' s arm and leg increased statistically after taking SKHHT, BYHOT, and So-Hap-Hyang-Won (SHHW) *(t=4.74/4.95, P=0.0002/0.0001; t=2.25/2.44, P=0.0368/0.0248; t=5.85/6.76, P=0.0001/0.0001)*. Speech disturbances of patients were not recovered after taking SKHHT and BYHOT *(t=4.50, P=0.0002; t=3.32, P=0.0036)*. Also, consciousness disturbances of patients were not significantly recovered after taking SKHHT, BYHOT, SHJKS, and SHHW *(t=6.32, P=0.0001; t=8.32, P=0.0001; t=3.74, P=0.0012; t=5.14, P=0.0001)*.

Blood pressure and speech disturbance of the patient group taking the *So-Hap-Hyang-Won* (SHHW) did not show any statistically significant changes compared with the normal group (Fig. 1 A, C). However, physical motions of patient' s arms and legs improved to recovery (Fig. 1 B, D). In general, all five kinds of herbal medicines were found to exert a reversal effect

Table 1. Clinical Characteristics of Case Study (n=103)

Variation	Classification	N	Percentage(%)
Main Symptom	SD	4	3.88
	H	8	7.77
	SD + H	77	74.76
	SD + H + CD	7	6.80
	SD + H + FP	4	3.88
	SD + FP	3	2.91
Hemiplegia	Right	51	49.51
	Left	45	43.69
	none	7	6.80
Past medical history	hypertension	52	50.49
	diabetes mellitus	7	6.80
	heart disease	1	0.97
	hypertension + diabetes mellitus	11	10.68
	hypertension + heart disease	2	1.94
	none	30	29.13
Medication	hypertension	17	16.51
	diabetes mellitus	5	4.85
	hypertension + diabetes mellitus	5	4.85
	none	76	73.79

SD = speech disturbance; CD = consciousness disturbance
H = hemiplegia; FP = facial palsy

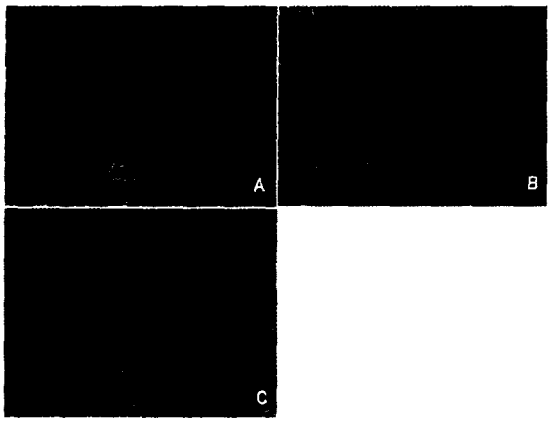

Fig. 2. SHHW extract was treated onto bovine aortic endothelial cells (BAEC) for 24 hr.
A. control, B. 10 $\mu g/ml$, C. 100 $\mu g/ml$

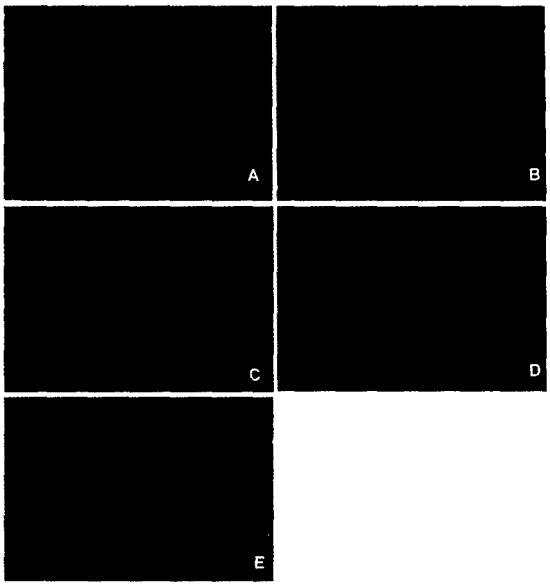

Fig. 3. The effects of bovine aortic endothelial cells treated with 100 $\mu g/ml$ of each component-plant extract of SHHW for 24 hr.
A. control, B. Foeniculi Fructus, C. Cinnamomi Cortex, D. Piperis longi Fructus, E. Corydalis Tuber

on consciousness disturbances. SHHW improved limb movements, as did the four other herbal medicines, but to a greater degree. Those on SHHW had more recovery than those on the other 4 kinds of herbal medicines. The endothelial cells were more rapidly grown and performed more syncytium than other herbal medicines.

2. Microscopic examination of BAEC treated with the stroke medicines

BAEC were treated with extracts of the five traditional herbal medicines studied. The cells treated with 10 and 100$\mu g/ml$ of SHHW for 24 hr showed cell-cell adherence and a growth rate comparable to the control (Fig. 2 A). SHHW induced syncytium between adjacent cells, indicating that it could have some effect on the recovery of damaged blood vessels, possibly by promoting angiogenesis.

The OYSKS-treated group showed a significant recovery in cell growth at a concentration of 10 $\mu g/ml$, but the 100 $\mu g/ml$ concentration appeared to be toxic, resulting in dead cells. The other 3 herbal medicines caused cell death at both concentrations tested (data not shown because dead cells were not anchored on the surface of culture dishes or slides). The results, however, suggest that SHHW may help the recovery effect of stroke patients more than the other herbal medicines tested.

3. Microscopic examination of BAEC treated with extracts of the individual components of SHHW

SHHW consists of 14 different herbal plants. BAEC were treated with an aqueous extract of each herb component, at concentrations of 10 and 100 $\mu g/ml$. Only the *Foeniculi Fructus* extract was non-toxic at all the concentrations studied (Fig. 3 B).

The *Pteropi Faeces* extract at 100 $\mu g/ml$ did not hinder cell growth but at the higher concentration cell growth was completely blocked. The cells were especially swelled and rounded in shape after treatment with the extract of Cinnamomi Cortex (Fig. 3 C).

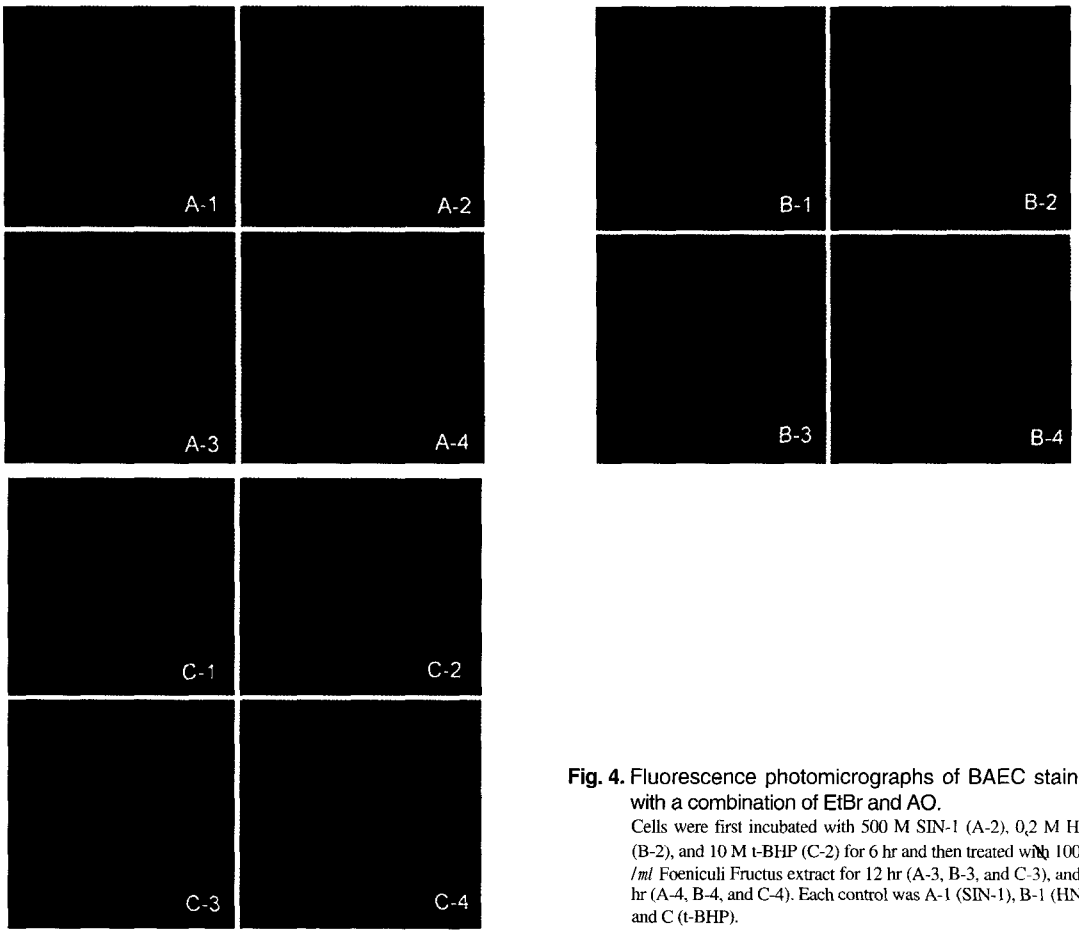

Fig. 4. Fluorescence photomicrographs of BAEC stained with a combination of EtBr and AO.
Cells were first incubated with 500 M SIN-1 (A-2), 0.2 M HNE (B-2), and 10 M t-BHP (C-2) for 6 hr and then treated with 100 *μg/ml* Foeniculi Fructus extract for 12 hr (A-3, B-3, and C-3), and 24 hr (A-4, B-4, and C-4). Each control was A-1 (SIN-1), B-1 (HNE), and C (t-BHP).

In this case, none of the cells have growth or cell-cell adherence. The other plant extracts at 100 *μg/ml* were all toxic to the cells and caused cell death (Fig. 3 C, E). Most plant extracts at 100 *μg/ml* concentrations showed no effect on BAEC growth except Piperis longi Fructus.

4. Fluorescence microscopy of EtBr- and AO-stained BAEC treated with Foeniculi Fructus extract after 6-hr exposure to oxidants

With fluorescence microscopy, damaged cells appeared red and normal cells green. BAEC exposed to SIN-1 (500 M), HNE (0.2 M), and t-BHP (10 M) for 6 hr were severely damaged and all cells appeared red (Fig. 4 A-2, B-2, C-2). Twelve hours after treatment with 100 *μg/ml Foeniculi Fructus* extract, the oxidant-exposed BAEC still appeared damaged, however, at 12 hr the cells appeared partially green or some of the cells began to be appear green (Fig. 4 A-3, B-3, C-3). In SIN-1 treatment cells, they began to be recover more rapidly to normal condition than HNE- and t-BHP- treatment cells. A 24-hr incubation with the herb (Foeniculi Fructus) extract allowed further recovery of the oxidant-exposed BAEC, with most of the cells showing a green color (Fig. 4 A-4, B-4, C-4) comparable to the control

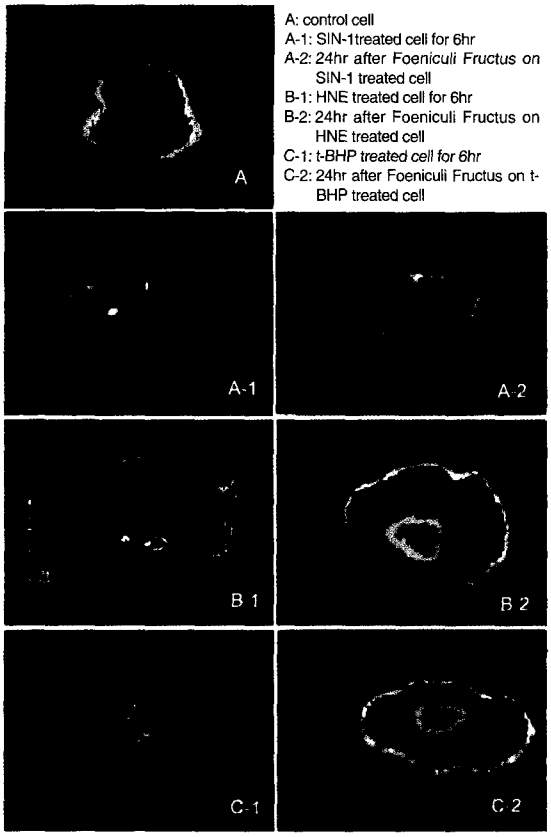

Fig. 5. Laser scanning cytometry of BAEC performed with ACAS 570.
BAEC were first treated with 500 M SIN-1 (A), 0.2 M HNE (B), or 10 M t-BHP (C) for 6 hr, and then incubated with 100 $\mu g/ml$ Foeniculi Fructus extract (A-2, B-2, C-2). The extract-treated cells appear normal comparable to the control (A).

groups (Fig. 4 A).

5. Laser cytometric observation of EtBr- and AO-stained BAEC treated with Foeniculi Fructus extract for 24 hr after 6-hr exposure to oxidant-chemicals

BAEC treated with SIN-1 (500 M), HNE (0.2 M), and t-BHP (10 M) for 6 hr had red spots in their nuclei (Fig. 5 A-1, B-1, C-1), showing apparent damage. A 24-hr treatment with 100 $\mu g/ml$ *Foeniculi Fructus* extract resulted in the recovery of damaged cells (Fig. 5 A-2, B-2, C-2), with cells appearing normal comparable to the control (Fig. 5 A).

Discussion

Angiogenesis, the continued expansion of the vascular system, is the most important step in the recovery of damaged tissues. It is mediated through the action of fibroblast growth factor (FGF), vascular endothelial cell growth factor (VEGF), VEGF receptor-1 (VEGFR-1), VEGF receptor-2 (VEGFR-2), and tie-2 (tek), a tyrosine kinase receptor that binds to angiopoietin 1 and 2 (Ang1 and Ang2). The combination of the above factors results in the maintenance of mature vessels, the development of new vessels, and the regression of formed vessels. This regression is accompanied by the loss of vessel structure and matrix contacts as well as the absence of growth and survival signals, probably leading to cell death. Angiogenesis involves the loosening of matrix contacts and decreased support of cell interactions. The maintenance of mature vessels needs the recruitment of mesenchymal cells and the inhibition of endothelial cell proliferation. The most conspicuous phenomenon is the accumulation of extracellular matrix (Anderson *et al.*, 1990). Under microscopic observations, BAEC treated for 24 hr with 10 $\mu g/ml$ and 100 $\mu g/ml$ SHHW showed an almost normal growth pattern with cell-cell adherence, and with 100 mg/ml SHHW some BAEC were enlarged and spread out. *Foeniculi Fructus* was found to be the only component of SHHW that affected BAEC proliferation as did the whole herbal product. BAEC treated with SHHW and those treated with *Foeniculi Fructus* only in SHHW appeared to have a morphology very similar to those of cells in the stages of regression, angiogenesis and eventual mature vessel formation, suggesting that the recovery of ruptured vessels and tissue damage by *Foeniculi Fructus* in SHHW may be accomplished

through all the above steps. Fig. 2-C showed some BAEC that are enlarged and spread out, a general feature corresponding to cells involved in the maintenance of mature vessels. All the results strongly suggest that SHHW promotes angiogenesis by stimulating the growth of vascular endothelial cells in damaged brain tissue, and that the angiogenic effect of *Foeniculi Fructus* may be mediated through an as yet unknown mechanism involving the expression of some growth factors and their receptors.

The stimulatory effect of *Foeniculi Fructus* on the growth of BAEC may not be enough to prove its anti-aging effects. Aging is believed to be due to free radicals mainly generated by oxidative stress including reactive oxygen species (ROS) and reactive nitrogen species (RNS), all of which are known to induce cell damage (Foresti *et al.*, 1999). These reactive compounds can be produced during normal metabolism as well as by external stimuli. In this study, SIN-1, HNE and t-BHP were used to induce oxidative stress conditions. SIN-1 degrades and produces NO. NO is necessary for the regulation of vascular tone, but it reacts with O_2^-, generating the RNS $ONOO^-$. HNE and t-BHP are products of lipid oxidation that exert harmful effects on cells. Fig. 4 shows that *Foeniculi Fructus*-treated cells recovered successfully from cell damage caused by the above reactive agents. After 24 hr *Foeniculi Fructus* treatment, BAEC previously exposed to SIN-1, HNE or t-BHP appeared to regain cell membrane integrity and resume normal cell division, indicating that *Foeniculi Fructus* promotes cell recovery from oxidative stress. A possible mechanism is that it may prevent harmful radicals from causing cell damage. However, such a mechanism does not explain how *Foeniculi Fructus* helps the recovery of oxidant-exposed BAEC. The recovery process requires almost one day, suggesting that the action of *Foeniculi Fructus* may involve effects on gene expression. For example, oxidized low-density lipoproteins (OxLDL) produced during lipid peroxidation by oxidative stress, exogeneous or endogeneous, play a key role in the generation of atherosclerotic lesions (Li *et al.*, 1996; Kang and Choi, 2000) by transforming the endothelium to a dysfunctional state in which cell surface adhesion molecules are expressed. The initial step in the formation of atherosclerotic lesions is likely to be the adherence of circulating monocytes to the "dysfunctional" endothelium. *In vitro* studies have shown that OxLDL induces a gene expression profile in endothelial cells similar to those seen in early lesions (Benedetti *et al.*, 1979; Benedetti *et al.*, 1980; Benedetti *et al.*, 1987). *Foeniculi Fructus* may block the perturbation of gene expression by OxLDL or may inhibit DNA damage due to oxidative stress. It is also possible that *Foeniculi Fructus* induces the expression of genes whose products have critical roles in the recovery of BAEC damage due to oxidative conditions.

As mentioned above, *Foeniculi Fructus* stimulates cell proliferation in BAEC and promotes cell recovery from oxidative cell damage possibly through the modulation of gene expression, suggesting that this herb may have an anti-aging effect. Although *Foeniculi Fructus* contains volatile oils including trans-anethole and fenchone, little is known about the effects of these phytochemicals (Choi *et al.*, 1997 & 1998). Further studies are warranted on the anti-aging effect of *Foeniculi Fructus* at the molecular level including the isolation, identification and characterization of the properties of the active principles.

Conclusion

The present study showed that the traditional oriental medicine *So-Hap-Hyang-Won* improved arm and leg movement and diminished consciousness disturbance in stroke patients, and stimulated *in vitro* cell proliferation

in BAEC, indicating that it may promote the recovery of damaged brain tissue. *Foeniculi Fructus*, a component of SHHW, reversed BAEC damage caused by HNE, t-BHP, and SIN-1, all of which induce oxidative stress. A possible mechanism for this biological activity may involve the radical-scavenging effect of *Foeniculi Fructus*. Other possible mechanisms of action may involve a *Foeniculi Fructus*-induced expression of genes whose products are necessary for cellular defense against oxidative stress.

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