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신경세포에서 天竺黃과 페놀성 물질의 항산화 및 신경보호 효과

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The antioxidative and neuroprotective effects of Bombusae concretio Salicea and phenolic compounds on neuronal cells

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산화적인 스트레스가 여러가지 신경 및 비신경계에서의 병리원인으로 알려져 있다. 퇴행성 뇌질환에 대한 예방과 치료에는 항산화 방어기술이 주요대상이며 스테로이드 분자중에서 estrogen만이 산화적인 원인에 의한 신경세포사를 방어하는데 특이적인 효과를 가지고 있다. 본 연구는 天竺黃의 항산화적 뇌신경 보호기전을 연구하는 것으로 신경세포주, 뇌세포막, 이의 산화적 정량실험법을 사용하여 天竺黃이 갖는 항산화 및 신경보호활성이 소수성 페놀 (phenolic molecules)성 물질과 유사함을 밝히게 되었다. 즉, 페놀성 물질로서 2,4,6-trimethylphenol, N-acetylserotonin, 및 5-hydroxyindole와 유사한 뇌신경 보호활성을 나타내었으며 天竺黃은 생쥐의 N2a cell과 사람 SK-N-MC neuroblastoma cell에서 산화적인 글루탐산 독성에 대하여 보호를 하였다. 天竺黃의 산화적 글루탐산 독성에 대한 보호활성은 과산화수소에 대한 것과 유사하였다. 이러한 항산화 활성은 20 ㎍/ml에서, LDL의 산화적 보호 활성은 5 ㎍/ml농도에서 발휘되었다 (최대활성은16㎍/ml). 이러한 결과는 天竺黃이 노인성 치매에 보호효과가 있음을 시사하였다.

Key Word : Bombusae concretio Salicea, phenolic compounds, antioxidative effect,neuroprotection, alzheimer's disease

I. Introduction

Recently, special interest has been attracted to estrogen's and phytoestrogen's properties as a neurotrophic and neuroprotective effector[1,2].

There are two lines of argument that assign to estrogen a special function in relation to neurological and neurode generative disorders. First, a number of epidemiological and clinical data exist on the beneficial effects of estrogens in Alzheimer's disease[3,4], in Parkinson's disease[5], or on mentyal performance in general[6,7]. Second, estrogen has been shown to have beneficial effects in cellular and molecular systems relevant to neurodegenerative disorders[2,8-15].

Apart from the steroidal estrogens, it has been long known[16,17] that a large variety of exogenous compounds, the xenoestrogens and the phytoestrogens, mimic the actions of endogenous estrogen to different extents. Xenoestrogens comprise plastic-material monomers [e.g., bisphenol A][18], certain polymer plasticizers[19], and detergent-related chemicals[20,21] as well as special pharmacological molecules such as diethylstilbestrol[22,23]. Major phytoe strogens[24,25] are the flavonoids, such as quercetin and catechin, and the stilbens, such as resveratrol. Besides steroidal estrogen, a number of these environ mental estrogens also have been shown to exhibit antioxidative properties[26,27] or have been suggested to exert beneficial pharmacological effects on neurological disorders on the basis of in vitro observations[28,29].

The idea of a novel, nonhormonal neuroprotective function of Bombusae concretio Salicea (BC) had arisen because BC showed comparable neuroprotection[30]. To delineate the mechanisms of action of BC in experimental systems of oxidative neuronal cell death, author have investigated the neuroprotective effects exhibited by a variety of concentrations.

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To this purpose, author have employed mouse N2a cells and human SK-N-MC neuroblastoma cells, two well established systems for the study of oxidative neuronal cell death. Two experimental systems measuring antioxidative activity relevant to the nervous system [brain lipid peroxidation and low density lipoprotein (LDL) oxidation] were designed and employed.

II. Materials and Methods

1. Chemicals

All chemicals were purchased from Sigma unless otherwise indicated. Phenolic compounds to be rested were ordered in the highest grade available and were assayed for purity by analytical TLC. Compounds presumed to be subject to oxidation by air were checked for contaminating quinones by UV/visible spectrophotometer. Where appropriate, compounds were recrys tallized from ethanol/water in degassed solvents. Stock solutions of the phenolic compounds were prepared in ethanol and stored at -20C.

2. Cell cultures of mouse neuro blastoma (N2a) cells, human SK-N-MC neurob lastoma cells, and human breast carcinoma cells (MCF7)

Murine N2a cells, human SK-N-MC neuroblastoma cells, and human breast carcinoma cells (MCF7) were from the American Type Culture Collection (Manassas, VA) and maintained in DMEM medium supplemented with 5% FBS and antibiotics (GIBCO/BRL), under standard cell culture conditions with the absence or presence of BC.

3. Cell viability assays

Cellular viability was quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide tests measuring metabolic activity as described[31,32]. The colorimetric tests generally were accompanied by microscopic examination and ionic dye exclusion assays using trypan blue as indicators of intact cellular membrane structure[8].

4. Rat brain membrane oxidation

Dissected cerebral cortex of adult Sprague-Dawley rats were homoge nized (Charles River Breeding Laboratories and KCTC, Genetic Resoutces Center, Korea Research Institute of Bioscience and Biotechnology) in 3 vol of degassed nonreducing lipid buffer (20 mM Tris, pH 7.4/ 1 mM MgCl2/ 5 5mM KCl) with a Kontes glass homogenizer (all steps were supplemented with degassed liquids). After centrifugation (3,000 x g, 5 min) the pellet was solubilized in liquid buffer supplemented with 0.5 M NaCl by sonication, incubated for 20 min, and dentrifuged again (100,000 xg, 20 min). This step was repeated and followed by three analogous washings (without incubation) using water insteada of lipid buffer. The pellet was resuspended in water at a concentration of 5 mg/ml protein and frozen at -80C.

Low-level chemiluminescence occuring during the course of lipid peroxidation was measured as described[33]. The ratbrain membrane preparation was diluted with PBS to a concentration of 0.6 mg/ml protein and sonicated. BC and phenolic compounds (final ethanol concentration, 0.4%) were added to the 1-ml aliquots, starting the oxidative chain reaction by the reaction of 50 μM ascorbic acid and transfer to 37C. Six hours later, single photon counting was done for 1 min (Beckman Scintillation Counter) after decay of static electricity. Data were corrected for the baseline photocurrent and normalized to control values.

5. LDL oxidation

Fresh human blood plasma LDL was purchased from Sigma. The quality of the lots was tested by measuring their endogenous resistance to oxidation; only lots resistancet for at least 20 min at 37℃ were used. The copper-cat alyzed oxidation of LDL was measured essentially as described[34]; the 4C preparations of LDL were matched to a ceoncentration of 100 μg.ml protein in PBS with 0.5 mM MgCl2. Oxidation was initiated by the addition of 10 mM CuSO4 at 37℃As indicators of LDL peroxidation, conjugated dienes were measured at 234 nm. Phenolic compounds were added as 100-fold ethanolic solutions, and the increase in absorption after 1 h was plotted vs. concentration.

6. Statistics

Student's t-tests were performed to

quantify statistical significance.

III. Results

1. Neuroprotective effect of Bombusae concretio Salicea and various phenolic com pounds

All the phenolic compounds and BC were effective protectants against oxidative glutamate toxicity in mouse neuronal cells of Murine N2a cells and human SK-N-MC neuroblastoma cells when employed at a concentration of 20 μM (Fig. 1). But non of the phenolic componds was effective at a concen tration of 1 μM.

The dose-dependent curves of the neuroprotective effect appear highly cooperative in the murine N2a cellular system as exemplified for BC and selected compounds in (Fig. 2). The compound's protective effect on the survival of human neuronal SK-N-MC neuroblastoma cells exposed to toxic doses of hydrogen peroxde (H2O2), a mediator of various neurotoxins35), is completely analogous.

2. The neuroprotective activity of Bombusae concretio Salicea and phenolic com pounds against oxidative stress corresponds to their direct antioxidant activity.

The concentration-dependent neuro-protective activities of the BC and selected phenolic compounds against glutamate-induced oxidative murine N2a cell death are shown in Fig. 2, and the protective effects against H_2O_2 toxicity in SK-N-MC cells are presented in Fig. 3. BC and all com-pounds exhibited similar protective properties against oxidative glutamate toxicity as well as H_2O_2 toxicity, with

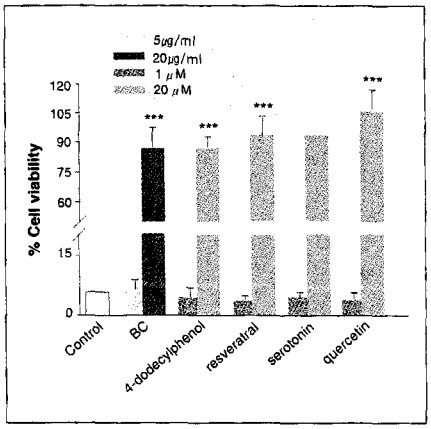

Fig 1. Effect of Bombusae concretio Salicea and the phenolic compounds on glutamate-treated murine N2a cells.For each compound, the left column indicates the relative viability of the cells after treatment with 1 μM of the respective phenolic compound 3 h before the addition of 3 mM glutamate overnight. The reight column indicates the incubation with 20 μM of the respective compound given 3 h before glutamate. A quadroplicate determination is shown. The dashed line indicates the viability of nonpretreated cells.Results are represented as means±SD, n=3 (statistically different from untreated controls by Student's t-test; ***, P<0.001)

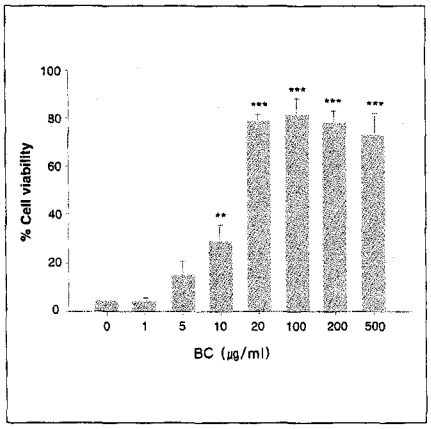

Fig 2-1. Dose-responsive cell viability of Bombusae concretio Salicea on 3 mM glutamate-treated murine N2a cells. The indicated concen trations of the phenolic compounds were added concomitantly with the oxidative stressor. Triplicate deter mination are shown. The dashed line indicates the viability of solely glutamate-treated cells.
Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; *, P<0.05; **, P<0.01)

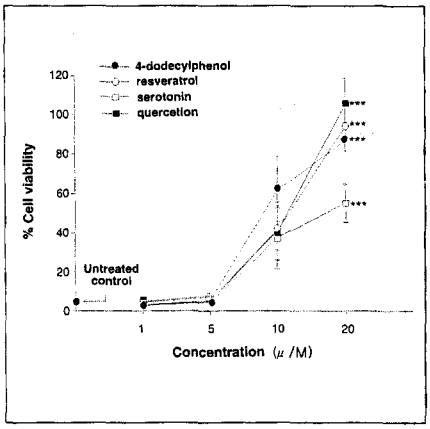

Fig 2-2. Dose-responsive cell viability of 4-dodecylphenol,resveratrol, serotonin, 4,4'-biphenol com pounds on 3 mM glutamate-treated murine N2a cells.The indicated concentrations of the phenolic compounds were added con comitantly with the oxidative stressor.Triplicate determination are shown. The dashed line indicates the viability of solely glutamate-treated cells.Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; *, P<0.05; **, P<0.01)

half-maximal effective concentrations ranging from 5 to 20 μM for phenolic compounds and 20-30 μg/ml for BC.

Apart from the different maximum viabilities, there is no significant difference between the results of the two experimental cellular systems of murine N2a cells and SK-N-MC cells. BC did not show any significant protective effect at a concentration of 5 μg/ml or less in either system. Also, none of the compounds shows any significant protective effect at a concentration of 500 nM μg/ml or less.

3. Protective activity of Bombusae concretio Salicea and phenolic compounds against membrane perox idation

Fig 4. shows the peroxidation of cell-free brain membranes induced by low indicator of ascorbate. In this system, measuring low level chemilumines cence as an indictor of actual peroxid ation processes[33], BC and all of the compounds exhibited a very similar antioxidant activity with nearly identical half-maximal effective concentrations of abour 3 μM those phenolic compo unds and about 20 μg/ml in BC.

4. Protective activity of Bombusae concretio Salicea and phenolic compounds against LDL oxidation

Another biologically relevant system to measure antioxidant activity is LDL oxidation[34]. Again, concentrat ions of the BC of 5 μg/ml or less and four compounds of 500 nM or less are

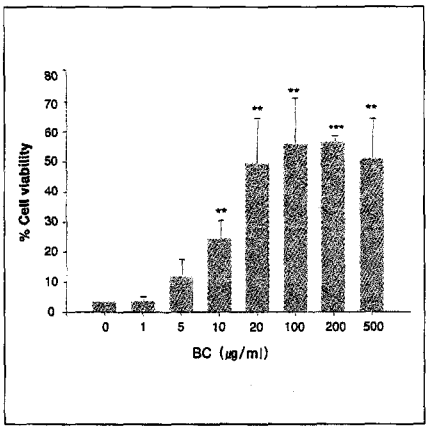

Fig 3-1. Dose-responsive cell viability of Bombusae concretio Salicea on 150 μM H_2O_2-treated human SK-N-MC neuroblastoma cells. Phenolic compounds were added 3 h before the 160 μM H_2O_2 toxin. Triplicate determination are shown. The dashed line indicates the via bility of solely glutamate-treated cells.Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; **, P<0.01; ***, P,0.001)

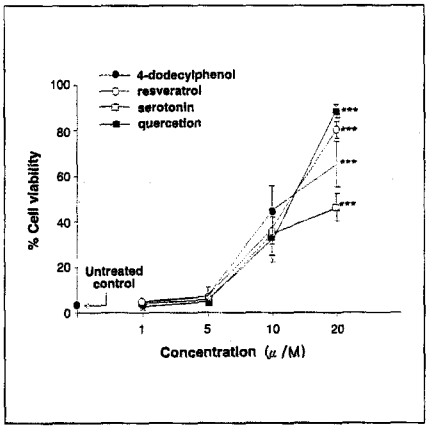

Fig 3-2. Dose-responsive cell viability of 4-dodecylphenol, resveratrol, sero tonin, 4,4'-biphenol com pounds on 150 μM H2O2-treated human SK-N-MC neuroblastoma cells. Phenolic compounds were added 3 h before the 160 μM H_2O_2 toxin. Triplicate determination are shown. The dashed line indicates the viability of solely glutamate-treat ed cells. Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; **, P<0.01; ***, P,0.001)

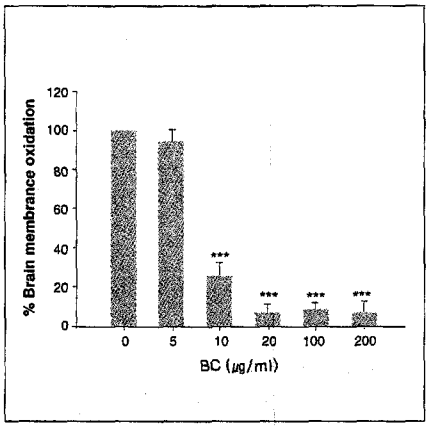

Fig 4-1. Rat brain membrane oxidation as measured by low-level chemilu minecence. Six hours after addi tion of Bombusae concretioSalicea, light emission was quantified by single photon counting. Phenolic com pounds were added 3 h before the 160 μM H_2O_2 toxin. Duplicate deter minations, normalized to control val ues, are shown. Statistical signif icance of the results vs. the respec tive controls (dashed lines) is indicat ed by Student's t-test; ***, P<0.001)

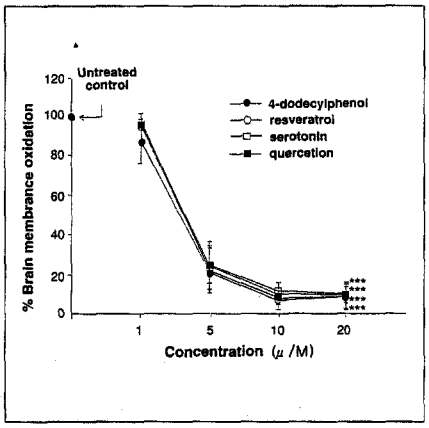

Fig 4-2. Rat brain membrane oxidation as measured by low-level chemilu minecence. Six hours after addi tion of phenolic compounds, light emis sion was quantified by single photon counting. Phenolic com pounds were added 3 h before the 160 μM H_2O_2 toxin. Duplicate determinations, normalized to control values, are shown. Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; ***, P<0.001)

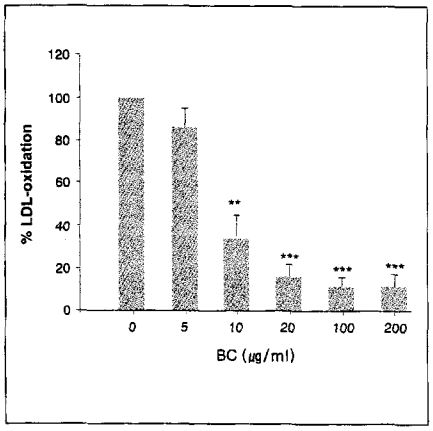

Fig 5-1. Cu^{2+}-catalyzed oxidation of human LDL after 1 h at 37C with or without Bombusae concretio Salicea. Conjugated diene formation was monitored photometrically at 234 nm; triplicate determinations relative to the control values are shown. Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; **, P<0.01; ***, P<0.001)

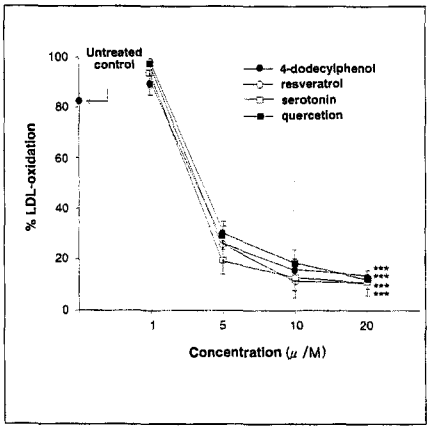

Fig 5-2. Cu^{2+}-catalyzed oxidation of human LDL after 1 h at 37C with or without Bombusae concretio Salicea. Conjugated diene formation was monitored photometrically at 234 nm; triplicate determinations relative to the control values are shown. Statistical significance of the results vs. the respective controls (dashed lines) is indicated by Student's t-test; **, P<0.01; ***, P<0.001)

ineffective in preventing theoxidative modification of LDL (Fig. 5). The half-maximal effective concentration for the best antioxidant in this experiment,4,4'-biphenol, was 2.0 μM. The half-maximal effective concentration for BC was 16 μg/ml.

IV. Discussion

A pathological role of oxidative stress has been described for various non neuronal and neuronal disorders[36,37]. Many approaches toward neuro-protection against neurodegenerative events currently are focussing on antioxidative defense systems and antioxidants. These efforts have been fueled further by a recent first success of a multicenter trial in moderately severe Alzheimer's disease patients, using vitamin E as an antioxidant[38]. There fore, the aim of this study was to elucidate the antioxidant neuro-protective mechanism of BC.

Comparing the activities in phenolic compounds and BC with their protec-tive characteristics against oxidat-iveglutamate toxicity in murine N2a cells shown in Fig. 1 and 2, it is remarkable that (i) BC and all these molecules are protective against glutamate and (ii) they all behave very similarly with respect to the con-centration needed to afford the pro-tective effect. A neuroprotective antioxidant effect in murine N2a cells thereby is shared by the oriental herbal BC, the neurotransmitter serotonin, the red wine phenol resveratrol, and also Ginko biloba flavonoid quercetin.

The phenolic compounds and BC show significant cytoprotective (Fig. 2,3) or biochemically antioxidant (Fig. 4,5) effects. The neuroprotective properties of these compounds and BC and their effects in cell- free assays of antioxidant acitivty show almost identical dose-responsive curves.

The exact nature of the experimental system used for determining the neu-roprotective or antioxidant potential of the phenolic compounds and BC did not influence the EC50 values founds, e.g., disturbance of intracellular antioxidant metabolism in murine N2a cells, exogenous reactive oxygen species overload in SK-N-MC neuroblastoma cells, one-line quan-tification of oxidation reactions in brain membranes, and monitoring of per-oxidation end products in lipopro teins (Fig 2-5). Therefore, this stepwise transition from a biologically relevant system (N2a) toward a biochemically clearly defined system (LDL) allows us to hypothesize that the similar EC50 values of the compounds and BC indeed may result from a similar mechanism of action in all of these systems.

Compounds that are to be considered candidates for neuroprotective antiox-idants in vivo should be able to cross the blood-brain barrier (BBB) readily. Another critical feature concerning neuroprotective antioxidants is the BC's potential toxicity, because their required concentrations may be high. BC was tested up to 500 μg/ml and found to be nontoxic to cultivated cells, but there are also in vivo examples of phenolic

antioxidants that were nontoxic to mice when given as 0.5% of the total diet[39]. Neverthless, phenolic compounds in high concentrations or doses may be hepatotoxic, but toxicity seems to arise from hydroxylation and conjugation reactions performed by cytochrome P450 enzymes, and these reactions can be minimized by an appropriate molecular design. Flavonoids probably are not optimal structure for use as antioxidants because of their pronounced estrogenic properties. Higher concentrations of flavonoids usually are required to observe antioxidant effects[26]. Possibly, our BC contains some phenolic compounds such as flavonoids or their derivatives to exert their pharmacological activity in antioxidative effect.

Ultimately, our results may have important implications for the prevention and therapy of oxidative stress-related disorders such as Alzheimer's disease.

V. Conclusion

Oxidative stress has been attributed to pathological role for various non neuronal and neuronal disorders. Neuroprotection against neurodegenerative events are subjectes on antioxidative defense systems and antioxidants. The aim of this study was to elucidate the antioxidant neuroprotective mechanism of Bombusae concretio Salicea (BC). Among the family of steroidal molecules, only estrogens have the capability of preventing neuronal cell death caused by increased oxidative burden. Employing neuronal cell lines, brain membrane, and low density lipoprotein oxidation assays, author shows that the antioxidant and neuroprotective effects of BC's constituents and estrogens are dependent on their basic chemical properties as hydrophobic phenolic mo lecules. BC and phenolic compounds such as 2,4,6-trimethylphenol, N-acetylserotonin, and 5-hydroxyindole exhibit neuroprotective effects. BC was effective protectants against oxidative glutamate toxicity in mouse neuronal cells of Murine N2a cells and human SK-N-MC neuroblastoma cells. BC exhibited similar protective properties against oxidative glutamate toxicity as well as H2O2 toxicity. BC exhibited an antioxidant activity with about 20 μg/ml in BC. BC of 5 μg/ml was ineffective in preventing the oxidative modification of LDL. The half-maximal effective concentration for BC was 16 μg/ml. These results suggested that BC supplementation in elderly men may be protective in the treatment of alzheimer`s disease (AD).

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