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과산화수소로 유발된 사람 신경아세포종의 아폽토시스에서 호도 약침액의 신경보호효과

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Neuroprotective effect by Juglandis semen-herbal acupuncture against H_2O_2-induced apoptosis in human neuroblastoma, SH-SY5Y cells

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Abstract

목 적 : 과산화수소는 산화적 스트레스를 통해 아폽토시스를 유도하는 것으로 알려져 있다. 본 논문 에서는 과산화수소로 유발된 신경아세포종 아폽토시스 과정에서 호도약침액의 효과를 관찰하였다.

방 법 : 과산화수소로 인한 신경아세포종의 아폽토시스에서 호도약침액 의 효과를 알아보기 위해 배양 중인 신경아세포종에 과산화수소를 처리하고, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 분석법, 4,6-diamidino-2-phenylindole (DAPI) 염색법, reverse transcription-polymerase chain reaction (RT-PCR), western blotting의 방법으로 확인하였다.

결 과 : 과산화수소로 인한 신경아세포종의 아폽토시스에서 호도약침액을 처리한 결과, 약침액을 처리한 세포의 생존이 약 30% 정도 증가하고, 핵 응축과 단편화를 막아주며, CASP3와 BAX단백질의 발현이 감소되었다.

결 론 : 이러한 결과로 호도약침액이 과산화수소로 인한 신경아세포종의 아폽토시스과정에서 보호효과를 나타내는 것으로 사료된다.

Key words : herbal acupuncture, JSD(Juglandis semen), apoptosis, neuroprotection, SH-SY5Y

1. Introduction

Oxidative stress is the imbalance between cellular production of free radical species and the ability of the cells to eliminate them employing endogenous antioxidant defence mechanisms. Oxidative stress causes cellular damage and subsequent cell death, especially in organs such as the brain, which have high metabolic rate and are constantly exposed to excitatory signals. In this manner,

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oxidative stress has been implicated in the etiology and progression of many neurodegenerative diseases[1-5]. The reactive oxygen species (ROS), superoxide anions and hydrogen peroxide, trigger apoptosis or programmed neuronal cell death[6,7]. Hydrogen peroxide is a causative to major neurodegenerative diseases. For instance, the neurotoxic effect of the β -amyloid protein, the major constituent of the senile plaque in Alzheimer's disease (AD), is mediated by free radicals. The neuropathological hallmark of amyotrophic lateral sclerosis (ALS) is the deregulation of the endogenous antioxidant enzyme superoxide dismutase (SOD). In the case of Parkinson's disease (PD), brains of patients exhibit high concentrations of iron (available iron in the cells promotes free radical generation through the Fenton's reaction) and decreased levels of glutathione, which is the major brain antioxidant. Oxidative damage to DNA, lipids, and protein is a characteristic of PD[8-10]. The herbal acupuncture has been developed and attracted much attention due to their therapeutic effects on neuronal injury such as stroke. Futhermore, several studies have shown neuroprotective effects of herbal acupuncture in various neuronal cells and animal models[11,12].

Herbal acupuncture is one of the newly developed acupuncture methods and also, is combined acupuncture therapy and medicinal herbs intensifying the therapeutic effects. In herbal acupuncture treatments, which have been used to treat specific disorders, such as stroke, osteoarthritis, and rheumatoid arthritis, a fixed dose of herbal extracts is injected at specific acupoints into the body[13-16]. The main stream of herbal acupuncture therapies are divided according to the types of injecting materials: injection of distilled herbal extract (*Palgangyakchim* or Eight Diagnostic Herbal Acupuncture); injection of highly purified herbal extract (*Gyungrakyakchim* or Meridian herbal acupuncture); and injection of highly purified bee venom into acupoints (*Bongdokyakchim* or bee venom acupuncture)[23]. Juglandis semen, the fruit of the *Juglandis semesis* DODE (JSD), has been used intensify yang energy in the body[24]. JSD herbal acupuncture has been developed to combine the actions of JSD and acupuncture synergistically, and suggested to be able to apply neurological disorders[13]

In this study, we investigated the protective effect of JSD in human neuroblastoma cells, SH-SY5Y on H_2O_2 -induced neuronal cell death.

2. Materials and Methods

2.1. Effect of JSD-herbal acupuncture against H2O2-induced apoptosis in human neuroblastoma cell line, SH-SY5Y

2.1.1 Preparation of JSD herbal acupuncture

The materials for JSD herbal acupuncture were obtained from Korea Institute of Herbal Acupuncture (KIHA) and were prepared according to the standards of the Korean Food and Drug Administration (KFDA) for clinical use.

2.1.2 Cell culture

The SH-SY5Y cells were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Cells were maintained in a humidified incubator with 5% CO_2 - 95% O_2 air at 37°C, and the medium was changed every 2 days.

2.1.3 MTT assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit. In order to detect the protective effect of JSD, cells were treated with 100 μ M H_2O_2 for 3 hours. The control group was treated with the same amount of vehicle. After the MTT labeling reagent (5mg/ml) was added to each group and incubated for 4 hours at 37°C, they were incubated for 12 hours with the solubilization solution in which the formazan crystals formed by MTT were dissolved. The absorbance was measured with a microtiter plate reader (Molecular Device, USA) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the reference wavelength and the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample / O.D. of none-treated control) × 100.

2.1.4 DAPI staining

The nucleic condensation of apoptosis was determined by 4,6-diamidino-2-phenylindole (DAPI) staining. SH-SY5Y cells treated with 100 μ M H_2O_2, or 100 μ M H_2O_2 and 0.1% JSD for 3 hours were cultured on four-chamber slides (Nalgene Nunc International, Rochester, NY, USA). The cells were fixed in 4% paraformaldehyde for 30 minutes, and were incubated for 30 minutes in the dark, including 1μg/ml DAPI solution. The cells

were observed through a fluorescence microscope (Zeiss, Oberköchen, Germany).

2.1.4 RT - PCR analysis

Total RNA was isolated from SH-SY5Y cells with RNAzol^{TM}B (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's instruction. The cDNA was produced using random hexamer primers and reverse transcriptase (Promega). The corresponding cDNA was amplified in PCR reactions with following primers for *BCL2-associated X protein (BAX)* (5′ -AAC ATG GAG CTG CAG AGG ATG ATT-3′ , 5′ -CTG GTC TTG GAT CCA GCC AGC CCA ACA G-3′), or for and *caspase 3 apoptosis-related cysteine peptidase (CASP3)* (5′ -CTT GGT AGA TCG GCC ATC TGA AAC-3′ ; 5′ -GGT CCC GTA CAG GTG TGC TTC GAC-3′). *CYCLOPHILIN* (5′ -ACC CCA CCG TGT TCT TCG AC-3′ , 5′ -CAT TTG CCA TGG ACA AGA TG-3′) was used as an internal standard. The annealing temperatures were 50°C for *BAX*, 57°C for *CASP3*, and 56°C for *CYCLOPHILIN*. The amplified fragment sizes were 249 bp (*BAX*), 405 bp (*CASP3*), and 300 bp (*CYCLOPHILIN*). The PCR products were electrophoresed on a 1.2% agarose gel, and stained with ethidium bromide. The polymerase chain reaction (PCR) amplifications were performed 30 cycles for *BAX*, and *CASP3*, and 24 cycles for *CYCLOPHILIN*.

2.1.4 Western blot analysis

Protein was isolated, using the Propprep® protein extraction solution (Intron Biotech, Seoul, Korea), and protein content was measured, using a Bio-Rad colorimetric protein assay kit (Bio-Rad, CA, USA). Fifty μ g protein sample was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Postfach, Germany). Mouse anti-human CASP3 antibody (Santa Cruz Biotechnology, CA, USA) and anti-human BAX antibody (Santa Cruz Biotechnology, CA, USA) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibody (Serotec, Oxford, UK) was used as secondary antibody. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, IL, USA).

2.2 Statistical analysis

Results were expressed as mean ± SEM. All experiments were done in triplicate. The data were analyzed by one-way ANOVA followed by Newman Keul's *post-hoc* analysis using SPSS. Differences were considered

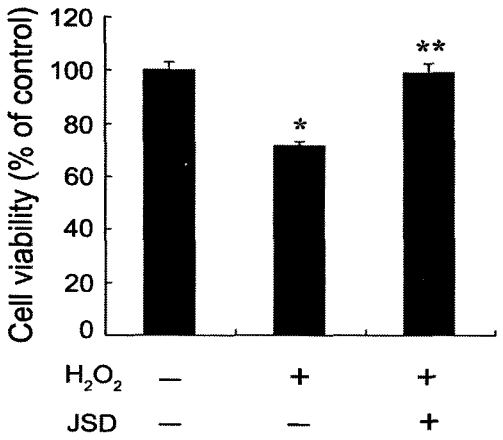

Fig. 1. Protective effects of JSD herbal acupuncture treatment on hydrogen peroxide - induced cytotoxicity in SH-SY5Y cells. Cell viability was estimated by MTT assay. Results are presented as mean ± standard error mean (SME). *Represents $p<0.001$ *vs.* control group. **$p<0.001$ *vs.* H_2O_2- treated group.

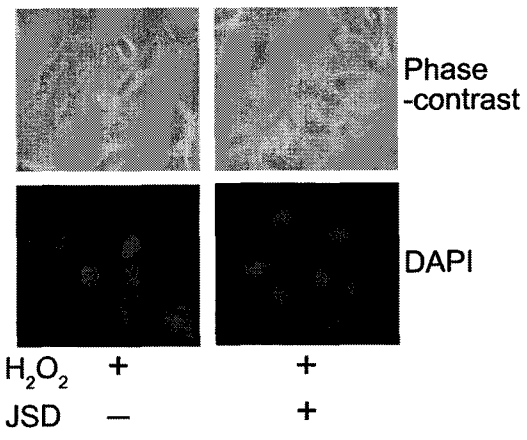

Fig. 2. H_2O_2-induced apoptosis and its blockage by the treatment with 0.1% JSD in SH-SY5Y cells. Microscopic observation of untreated control cells (A), cells treated with H_2O_2 alone (B) or H_2O_2 plus JSD (C) in DAPI staining.

significant at $p<0.05$.

3. Results

3.1 Effect of JSD on H2O2 - induced cytotoxicity

We studied the effect of JSD on H_2O_2-induced cell death by MTT assay. Initially, we confirmed the concentration of H_2O_2 to induce approximately 50% of cell death by incubating neuroblastoma cells with various concentrations of H_2O_2 for 6 hours. The concentration of 100 μ M was identified for this whole experiment (data not shown). Then, we assessed the effect of JSD by adding 0.1% JSD to H_2O_2-treated cells and incubating for 3 hours. As shown in Fig. 1, 0.1% JSD exhibited the protective effect on the H_2O_2-induced cytotoxicity (100.7 ± 0.7 % in JSD group *vs.* 69.8 ± 1.2 % in H_2O_2-treated group, $p<0.001$).

3.2 Protective effect of JSD on the H2O2 - induced apoptosis

In Fig. 2, fragmentation and nuclear condensation that are characteristic features of apoptosis, were found in 100 μ M H_2O_2-treated neuroblastoma cells. JSD treatment inhibited H_2O_2-induced fragmentation and nuclear condensation as evidenced in Fig. 2, indicating protective effect of JSD on H_2O_2-induced apoptosis in human neuroblastoma

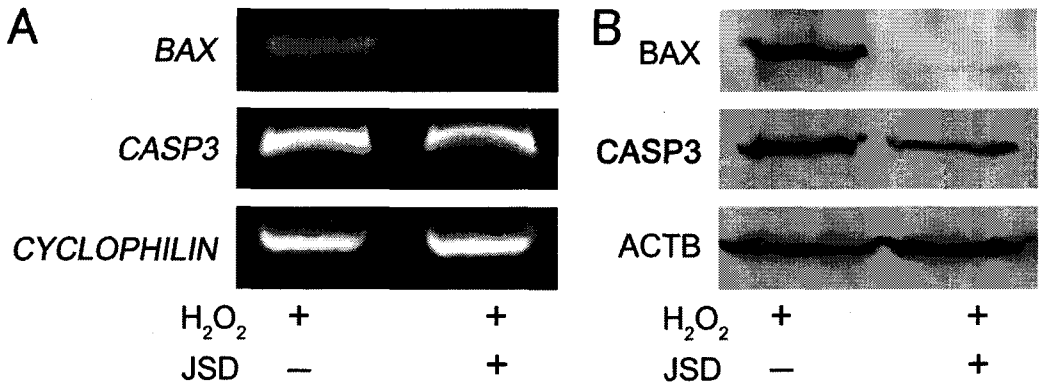

Fig. 3. RT-PCR (A) and western blot (B) analysis of BAX and CASP3. CYCLOPHILIN and ACTB were used as an internal control. BAX: BCL2-associated X protein, CASP3: caspase 3 apoptosis-related cysteine peptidase, ACTB: β -actin, JSD: Juglandis semen.

cells.

3.3 Effect of JSD on the protein and mRNA expressions of BAX and CASP3

To evaluate the underlying mechanism by which JSD exerts protective effect on H_2O_2-induced apoptosis in SH-SY5Y, we performed RT-PCR analysis of BAX and CASP3 mRNA. In the present study, BAX mRNA level treated with 0.1% JSD was greatly decreased compared to the H_2O_2-treated control group. BAX mRNA level was nearly undetectable in 0.1% JSD treated group. CASP3 mRNA level was also markedly decreased by 0.1% JSD treatment (Fig. 3).

In the cells treated with 0.1% JSD, BAX protein level was markedly suppressed. As is the case with CASP3 mRNA, CASP3 protein level was also decreased in JSD -treated cells compared to the control (Fig. 3).

4. Discussion

In the current work, we have used the human neuroblastoma SH-SY5Y cell line as a tool for the effects of JSD during oxidative stress-induced cell death. The hydrogen peroxide, one of the major causes of oxidative stress, destroys neurons via the process of apoptosis and necrosis. The levels of H_2O_2 were increased in pathological conditions including ischemia and neurodegenerative diseases[5,17,18]. Some studies showed that herbal extract or sublimate, injection of herbal acupuncture, possess protective effects against neuronal damage induced by oxidative stress[19]. Furthermore, these effects can be

increased synergistically by injecting a fixed dose of herbal extract or sublimate at specific acupoints on the body[13].

As shown in Figure 1, the protective effect of JSD against the H_2O_2-induced cytotoxicity in SH-SY5Y neuronal cell. JSD treatment reduced H_2O_2-induced apoptotic morphological changes such as nuclear shrinkage, chromatin condensation, irregularity in shape and retraction (Fig. 3).

In several studies, it has been demostrated that the progress of apoptosis is regulated by the expression of several genes. One of such genes is BAX, a pro-apoptotic gene of the family that is expressed abundantly and selectively during apoptosis, promoting cell death[20]. At the execution phase of apoptosis, a series of morphological and biochemical changes appear to be resulted from the action of caspases[21]. Particularly, Casp3 is believed to be one of the most commonly involved proteins in the process of apoptosis in various cell types[22]. Our results showed that JSD inhibits H_2O_2-stimulated protein and mRNA expressions of BAX and Casp3 (Fig. 3).

As a result, these results showed the protective effect of JSD against H_2O_2-induced cell death in SH-SY5Y cell through inhibition of both mRNA and protein expression levels of BAX and Casp3. These findings suggested the therapeutic potential of JSD in the treatment of neuronal apoptosis related disorders.

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