ABSTRACT: Herbal medicines with antioxidant properties are believed to have potential therapeutic effect against oxidative stress in neurodegenerative diseases. In this study, we evaluated the antioxidant and neuroprotective effects of JP05, a polyherbal medicine, on 

\[ \text{H}_2\text{O}_2 \text{-induced oxidative injury in Neuro-2a (N2a) cells.} \]

The cells were treated with or without JP05, and then stimulated with 500 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). Cell viability was determined by MTT assay, and the releases of NO and LDH and intracellular ROS were measured. The levels of MDA and PCO, the activities of SOD, CAT and GPx, and contents of GSH and GSSG were assayed with respect to expressions of iNOS, nNOS, Bax and Bcl-2 via the NF-\( \kappa \)B and MAPK pathways. JP05 potently inhibited the \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), \( \text{OH}^- \), NO, and possessed metal chelating properties. JP05 improved cell survival, decreased the levels of NO, LDH and intracellular ROS, modulated antioxidants by suppressing the expression of iNOS, nNOS and Bax, and increased Bcl-2 expression by modulating the NF-\( \kappa \)B and MAPK pathways. Our results indicate that JP05 protects neuronal cells against \( \text{H}_2\text{O}_2 \)-induced oxidative injury by preventing cell apoptotic cascades and modulating NF-\( \kappa \)B and MAPK pathways, suggesting that JP05 has therapeutic potential for the treatment of neurodegenerative diseases.

KEY WORDS: Antioxidant, Apoptosis, MAPK, Neuro-2a, Neuroprotection, NF-\( \kappa \)B

INTRODUCTION

Oxidative stress is a fundamental pathological insult faced by cells. In the central nervous system, oxidative stress is a critical pathological factor in neuronal death and is heavily involved in the pathogenesis for various neurological disorders (Moreira et al., 2005). When neurons are under oxidative stress, excess reactive oxygen species (ROS) are produced (Chan, 2005). The balance between the generation of ROS and the antioxidative processes can become disturbed as reported for aging and several neurological disorders (Satoh et al., 1996). Therefore, antioxidants have been shown to protect neuronal cells by preventing excess ROS generation in oxidative cell death, and may aid in the development of drugs for various neurodegenerative diseases (Ji and Gao, 2008).

The discovery of new drugs from herbal medicines is not a new phenomenon. Herbal medicines exist various prescriptions or formulae designed to contain a combination of medicinal plants to improve therapeutic efficacies for the treatment of many human diseases (Wang et al., 2008; Zhu et al., 2010). The herbal constituents in the formulae can enhance the activity of the component compounds or to reduce the toxicity or side effects of compounds from other herbs in the formulae (Bansky and Narolet, 1990). Korean Traditional Medicine (TKM) is based on the philosophy of ancient medical science and originated in 11 oriental books recording details of the ancient remedies. Among these books, Dongui Bogam by Heo Jun (AD1610) is the greatest masterpiece of Korean traditional medicines (Choi et al., 2002). As described, Bo-Yang-Hwan-O-Tang (BHT) is a decoction of seven herbs (Choi, 2986; Jin, 1993; Choi et al., 2003 S.S. Choi, K.J. Han, H.K. Lee, E.J. Han and H.W. Suh, Antinociceptive profiles of crude extract from roots of Angelica gigas NAKAI in various pain models, Biological and Pharmaceutical Bulletin 26 (2003), pp. 1283–1288. View Record in Scopus | Cited By in Scopus (11). BHT has commonly been used as a prescription for treatment of senility, stroke, vascular dementia, ischemic brain and heart damage (Jin, 1993; Kim et al., 2002). It also was shown to have immune modulating and
neuroprotective effects (Qu et al., 2002; Irie and Keung, 2003; Lee et al., 2003). For the synergistic enhancement of BHT, we modified the BHT prescription by adding five herbs that have long been used as a prescription for stroke, senility and vascular dementia, ischemic brain, and heart damages that have also been reported to have neuroprotective effects (Robak and Gryglewski, 1988; Kang et al., 2006; Kim et al., 2006; Jeong et al., 2008; Choi et al., 2011). This herbal composition is named JP05. Although JP05 appears to be effective for the treatment of neurodegenerative diseases, its mechanisms are poorly understood and have not yet been determined.

In the present study, we determine the antioxidant and neuroprotective effects of JP05 and its mechanism of action in H2O2-induced oxidative injury in mouse Neuro-2a (N2a) neuroblastoma cells.

MATERIALS AND METHODS

Reagents

Nitro blue tetrazolium salt (NBT), phenazine methosulfate (PMS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium nitroprusside (SNP), sulphanilic acid, N- (1-Naphthyl) (PMS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), Reagents.

MATERIALS AND METHODS

neuroblastoma cells.

TABLE 1. Composition of JP05

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th>Latin Name</th>
<th>Family Name</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astragalus membranaceus</td>
<td>Bunge Atragali Radix</td>
<td>Fabaceae</td>
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<tr>
<td>Salvia miltiorhiza</td>
<td>Bunge Salviae Miltiorhizeae</td>
<td>Lamiaceae</td>
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<tr>
<td>Angelica gigas</td>
<td>Angelicae gigantis</td>
<td>Apiaceae</td>
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<td>Paeonia lactiflora</td>
<td>Paeonia Radix Rubra</td>
<td>Paeoniaceae</td>
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</tr>
<tr>
<td>Achyranthes fauriei</td>
<td>Achyranthis Radix</td>
<td>Amaranthaceae</td>
<td>1.5</td>
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<tr>
<td>Cnidium officinale</td>
<td>Cnidi Rhizoma</td>
<td>Umbelliferae</td>
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<tr>
<td>Pheretima aspergillum</td>
<td>Lumbriculus</td>
<td>Megascoleidiae</td>
<td>1</td>
</tr>
<tr>
<td>Primus persica (L.) Batsch</td>
<td>Perciae Semen</td>
<td>Rosaceae</td>
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<tr>
<td>Carthamus tinctorius</td>
<td>Carthami Flos</td>
<td>Asteraceae</td>
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<tr>
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<td>Cinnamomi Ramulus</td>
<td>Lauraceae</td>
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<tr>
<td>Polygala tenuifolia Wildl</td>
<td>Polygalae Radix</td>
<td>Polygalaeeae</td>
<td>1</td>
</tr>
<tr>
<td>Acorus calamus L.</td>
<td>Acori Graminei Rhizoma</td>
<td>Acoraceae</td>
<td>1</td>
</tr>
</tbody>
</table>

HPLC analysis

For quality control of JP05, chromatographic analysis was carried out using a Shimadzu LC-10AD series HPLC system (Japan), with a column oven and a diode-array detector. A Phenomenex-C18 column (2.1 x 150 mm, 5 μm) from Agilent (CA, USA) was used for separation at 25 °C. The mobile phase consisted of 10% methanol in water containing 0.05% formic acid (A) and 90% methanol (B) with a flow rate of 0.4 ml/min. The gradient program was used according to the following procedure: 0 to 30 min, linear increase of 0% to 40% B; to 60 min, linear increase to 75% B, and 65 min, maintained to 75% B. The UV spectra were recorded from 190 to 400 nm, and the monitoring wavelength was set at 250 nm. Phenomenex-C18 mobile phase: A: 10% methanol (0.05% formic acid), B: 90% methanol, gradient program; 0 min, 0%B; 30 min, 40%B; 60 min, 75% B; 65 min, 75%B, flow rate; 0.4 ml/min, detection; 250 nm (Fig. 1).

FIGURE 1. JP05 was analyzed by HPLC fingerprinting analysis. HPLC analysis was performed on a Waters 510 system equipped with Waters 486 UV detector (Waters Corp., MA, USA). Chromatographic separation was carried out on a Phenomenex C18 column (4.6 mm x 150 mm, 5 μm) heated to room temperature with an injection volume of 10 ml using a gradient elution of solvent (A) 10% methanol in water containing 0.05% formic acid and solvent (B) 90% methanol in water at a flow rate of 1 ml/min as follows: 0-40% (30 min), 40-75% (30 min), 75% (5 min), 75-100% (10 min) and 100% (5 min). Peaks were detected at 250 nm of UV detection.

Preparation of JP05

Herbs used in the present study were purchased from Medicinal Materials Company (Kwangmyungdang Medicinal Herbs, Ulsan, Republic of Korea). Professor J-H. Lee, a medical botanist in the Department of Herbology, College of Oriental Medicine, Dongguk University (DUCOM), Republic of Korea, authenticated all herbs. Voucher specimens were deposited in the Herbarium of the DUCOM under registration number 08001C. The mixing ratio of each herb is shown in Table 1. The JP05 herbal mixture (50 kg) was extracted with boiling water for 3 h filtered through a two-layer mesh and concentrated under vacuum at 700 mmHg for 15 h. The concentrated extracts were lyophilized by freeze-drying and the powder extract (yield: 34.8%) was used for in vitro experiments.

TABLE 1. Composition of JP05

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Amygdalin standard was purchased from Sigma and used without further purification. Decursin, paeoniflorin, salvianolic acid B and calycosin-7-O-β-D-glycoside were isolated from Angelica gigas, Paeonia lactiflora, Salvia miltiorrhiza and Astragalus membranaceus, respectively, in laboratory of Pharmacognosy in Pharmacy School, Chungnam National University and their structures were confirmed on the basis of spectroscopic analysis and were compared with published data. The quantity of major compounds contained in JP05 was calculated by the standardized formula. The major compounds from JP05 constituent herbs included 0.12% amygdalin from Prunus persica (peak 1), 0.27% paeoniflorin from Paeonia lactiflora (peak 2), 0.32% calycosin-7-O-β-D-glucoside from Astragalus membranaceus (peak 3), 0.37% salvianolic acid B from Salvia miltiorrhiza (peak 4), and 0.28% decursin from Angelica gigas (peak 5).

**In vitro free radical scavenging assays and 50% inhibition concentration (IC_{50})**

In vitro free radical scavenging activities were assayed by superoxide radical (O_2^-), hydrogen peroxide decomposition, hydroxyl radical (OH^·), nitric oxide radical (NO^·) inhibition, and metal chelation (Shnha, 1972). All experiments were conducted in triplicate. The concentration of the JP05 that was required to scavenge 50% of radicals was calculated by using the percent scavenging activities of five different JP05 (0-150 μg) concentrations. Percent scavenging activity was calculated as [1- (Ai-Aj)/Ac] x100, Where. Ai is the absorbance measured with JP05 in the particular assay with a ROS source; Aj is the absorbance measured with JP05 in the particular assay but without a ROS source; Ac is the absorbance of control with particular solvent.

**Cell culture and treatments**

Neuro-2a (N2a) cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, and kept at 37 °C in humidified 5% CO_2/95% air. The cells were passaged every 3 days when grown to 75% confluence. For experiments, the cells were plated and allowed to settle for 24 h before replacement with serum free media. Cells were treated with JP05 (0.5, 0.75 or 1.0 mg/ml) for 1 h prior to H_2O_2 (500 μM) stimulation, with vehicle, and each treatment lasted for 12 h.

**Cell viability assay**

Cell viability was measured by MTT assay. The assay is based on the ability of living cells to convert soluble MTT into insoluble formazan by mitochondrial dehydrogenases in viable cells. The amount of formazan produced is proportional to the number of living cells. The optical density of the formazan formed in the normal cells was taken as 100% viability. Data are mean percentages of viable cells versus the respective normal.

**Quantification of nitrite and LDH assay**

The nitric oxide (NO) production was measured as the nitrite (NO_2^-) concentration in the medium using the Griess reagent. Sodium nitrate was used as a standard. N2a cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) leakage in culture medium by using an LDH diagnostic kit (Promega, Madison, WI) according to the manufacturer’s instructions. The activity was calculated by measuring absorbance at 492 nm. Results are expressed as percentage of LDH release in normal cells.

**Measurement of intracellular ROS generation**

Formation of ROS was evaluated using DCFH-DA, a membrane-permeable probe that is deesterified intracellularly. The non-fluorescent dye freely penetrates cells, then hydrolyzed by intracellular esterases to DCFH, and trapped inside the cells. Upon oxidation by ROS, DCFH yields the highly fluorescent product DCF. Cells with different concentrations of JP05 (0.5, 0.75 or 1.0 mg/ml) for 1 h prior to 12 h treatment with H_2O_2 were loaded with DCFH-DA (50 μM final concentration) in DMEM media for 60 min in the dark. After rinsing cells twice with phosphate-buffered saline solution, fluorescence was read at the excitation wavelength of 485 nm and the emission wavelength of 530 nm.

**Intracellular ROS and Hoechst staining**

To detect intracellular ROS, cells were incubated with 100 μM DCFH-DA in culture medium for 30 min in the CO_2 incubator. Cells were then washed 3 times in phosphate-buffered saline (PBS) for 5 min and further incubated with Hoechst 33342 solution (stock diluted 1:200 in culture medium) for 15 min at 37 °C. The cells were washed in PBS (2 × 5 min), and mounted onto glass slides using Permafluor aqueous mounting media. DCF and Hoechst fluorescence was observed under an Olympus BX50 microscope and images were acquired using an Olympus DP71.

**Measurement of oxidants and antioxidants**

Cells were seeded at 0.5 × 10^4/ml for each experiment and allowed to grow for 24 h. All treatments were performed in DMEM with 1% BSA for 12 h after 24 h incubation in serum-free media. Twelve hours following H_2O_2-induction, cells were washed twice with PBS and homogenized at 0 °C in 50 mM phosphate buffer (pH 7.4) for further assays. The extent of lipid peroxidation in N2a cell homogenates was determined by measuring the release of thiobarbituric acid reactive substance (TBARS) in terms of malondialdehyde (MDA) as described by Garrat et al. (1964). The results are expressed as nmol MDA formed per milligram of protein. The protein carbonyl (PCO) content of N2a cell homogenates was evaluated by the method of Levine et al. (1990). The results were presented in nmol of DNPH incorporated per milligram of protein. Superoxide dismutase (SOD) was assayed according to the technique of Kakkar et al. (1984) based on inhibition of NADH-PMS-NBT formation. A single unit of enzyme was expressed as 50% inhibition of NBT reduction per minute per milligram of protein. Catalase
(CAT) was assayed colorimetrically at 620 nm and expressed as micromoles of H$_2$O$_2$ consumed per minute per milligram of protein, as described by Sinha (1972). Glutathione peroxidase (GPx) activity was measured by the method described by Rotruck et al. and expressed as micromoles of GSH consumed per minute per milligram of protein. Reduced GSH was determined by the method of Rotruck et al (1973). Oxidized glutathione (GSSG) was measured by masking GSH with 2-vinylpyridine by the same. Protein concentration was determined using Bio-Rad (Hercules, CA) protein assay reagent.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

A RT-PCR assay was used to determine the mRNA levels of iNOS in relation to GAPDH. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA). The RT-PCR assays were performed with ImProm II™ RT-PCR System kit (Promega, Madison, WI). Briefly, 0.5 mg of total RNA from each sample was added to 20 mL of a reaction mixture. GAPDH expression was included as an internal, housekeeping gene control. Ethidium bromide-stained reaction products were separated by electrophoresis on 1% agarose gel in TAE and visualized by UV transillumination. Images were captured by a Kodak EDAS 290 camera system (Kodak, Rochester, NY). The primers used in these experiments were designed to span introns, thereby allowing differentiation between amplified genomic DNA and cDNA PCR products. Primer sequences were as follows; GAPDH (XM_994067) as an internal control for PCR, 52 -GAC ATC ATA CTT GGC AGG-32 (sense), 52 -CTC GTG GAG TCT ACT GGT-3’ (anti-sense) and iNOS (NM_010927), 52 -CCT CCT CCA CCC TAC CAA GT-32 (sense), 52 -CAC CCA AAG TGC TTC AGT CA -32 (anti-sense). These genes were then amplified using a PCR system consisting of denaturation at 94 °C for 45 s, primer annealing at 59 °C (for GAPDH) or 61 °C (for iNOS) for 30 s, and extension at 70 °C for 1 min. The number of cycles was determined from samples not reaching the amplification plateau (27 cycles for GAPDH and 36 cycles for iNOS). The amplified conditions for GAPDH were the same to those for others. The band intensity was quantified by densitometric analysis (Biorad QuantityOne, Hercules, CA).

**Immunocytochemistry**

N2a cells treated with JP05 and H$_2$O$_2$-induction were fixed in 4% paraformaldehyde (in PBS pH 7.4) for 15 min at room temperature. Cells were then washed three times with PBS and quenched in 3% H$_2$O$_2$, for 5 min at room temperature. After three more washes in PBS, the cells were blocked in 10% normal goat serum and 0.1% Triton X-100 in TBS (0.1 M Tris-HCl [pH 7.4], 0.9% sodium chloride, 0.1% Tween-20) to block non-specific binding, and then incubated with primary antibodies that recognized iNOS (1:1000, BD Transduction, Santa Cruz, CA), nNOS, Bax, Bcl-2 (1:1000, Santa Cruz Biotech, Santa Cruz, CA), β-actin (1:5000, Sigma-Aldrich, St. Louis, MO), and the phospho- or total forms of mitogen activated protein kinases (MAPks), nuclear factor NF-κB p65 and IκB antibody (Cell Signaling), diluted in 2% normal goat serum and 0.1% Triton X-100 for 24 h at 4 °C. The cells were then washed three times with PBS and incubated with biotinylated secondary antibody (diluted 1:1000 in 3% bovine serum albumin) for 1 h at room temperature. After further washes with PBS, the cells were incubated with “Vectastain” ABC reagent (Vector, Burlingame, CA) at room temperature and washed three times with PBS followed by incubation for 10 min with DAB reagent. The cells were washed with water and examined under bright field in Olympus BX50 microscope images were acquired using an Olympus DP71.

**Statistical analysis**

All data analysis was completed using the Graphpad PRISM 5.0 software. Data are expressed as mean ± S.D. The significance level of treatment effects was determined by one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests between groups. Values of p < 0.05 were considered statistically significant.

**RESULTS**

**Effects of JP05 on free radical scavenging and metal chelation**

The effect of JP05 on in vitro free radical scavenging of O$_2^•$, H$_2$O$_2$, OH$,NO^*$, and metal chelation were determined. JP05 effectively scavenged O$_2^•$, H$_2$O$_2$, OH$,NO^*$, and
chelated metal in a concentration-dependent manner, where it is compared with ascorbic acid as standard. The IC_{50} of JP05 (0-150 mg/ml) was IC_{50} of 14.41, 329.23, 607.51, 79.14, and 650.75 μg/ml, respectively, while that of ascorbic acid was 8.76, 8.05, 3.03, 9.89, and 43.24 μg/ml for O_2•-, H_2O_2, OH•, NO• scavenging, and metal chelation.

Effects of JP05 on cell viability
To examine the effect of JP05 on cell viability, we carried out MTT assay in N2a cells (Fig. 2A). JP05 alone did not significantly affect viability. H_2O_2-induction (500 μM) reduced cell viability up to 72.48±1.63% and JP05 treatment increased cell viability in a dose-dependent manner at concentrations ranging from 0.5 to 1.0 mg/ml reaching 85.61 ± 0.93, 90.66 ± 2.04, and 92.44 ± 2.08% of control levels, respectively. Moreover, the release of NO_2• (Fig. 2B) and LDH (Fig. 2C), and the generation of intracellular ROS (Fig. 2D) were determined after incubation in the presence of JP05 (0.5, 0.75 or 1.0 mg/ml) with or without 500 μM H_2O_2-induction.

The generations of NO, LDH, and ROS were increased to 111.6%, 136.4 and 138.3%, respectively after incubation with H_2O_2 for 12 h compared with normal group. Pretreatment with JP05 at 1.0 mg/ml significantly reduced the release of NO_2•, as did 0.75 mg/ml. Pretreatment with JP05 at 0.5, 0.75 or 1.0 mg/ml inhibited the H_2O_2-induced LDH release (120.2, 109.8, and 102.3%), and ROS production (101.8, 91.33, and 81.03%) from N2a cells, respectively.

On the other hand, the normal cells treated with JP05 show marked effect on release of NO_2• (92.6%; Fig. 2B) and ROS production (83.40%; Fig. 2D) at 1.0 mg/ml. In the fluorescent microscopic study (Fig. 3), cells with H_2O_2-induction were reactive to DCFH-DA and the nuclei of the cells were stained with Hoechst 33342 compared to normal cells. On the other hand, JP05 treatment at 0.5, 0.75 or 1.0 mg/ml in conjunction with H_2O_2-induction had significant scavenging effect on ROS and apoptosis.

Effects of JP05 on oxidants and antioxidants
The effect of JP05 on oxidative stress, the MDA and PCO levels, the end products of lipid peroxidation (LPO) and protein oxidation, respectively, were studied. Exposure to H_2O_2-induction increased of the intracellular MDA and PCO levels, while pretreatment of cells with 0.5, 0.75 or 1.0 mg/ml JP05 significantly attenuated the increase (Table 2). In addition, the effects of JP05 on the enzymatic and non-enzymatic antioxidants were analyzed (Table 3).
TABLE 3. Effects of JP05 on enzymatic and non-enzymatic antioxidants. Values are mean ± SD. A difference as considered statistically significant when "P < 0.01 and ""P < 0.001 vs. normal (↑) or H₂O₂ group (↓). SOD, 50% reduction of NBT per min per mg protein; CAT, micromoles of H₂O₂ consumed per min per mg protein; GPx, micromoles of GSH utilized per min per mg protein; GSH and GSSG, micrograms per mg protein. Group I, normal; group II, normal+JP05 (0.5 mg/ml); group III, normal+JP05 (0.75 mg/ml); group IV, normal+JP05 (1.0 mg/ml); group V, H₂O₂ (500 μM, 12 h); group VI, JP05 (0.5 mg/ml)+H₂O₂; group VII, JP05 (0.75 mg/ml)+H₂O₂; group VIII, JP05 (1.0 mg/ml)+H₂O₂.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GSH</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>9.350 ± 0.06</td>
<td>81.212 ± 4.571</td>
<td>51.047 ± 1.085</td>
<td>17.395 ± 0.586</td>
<td>0.148 ± 0.001</td>
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<tr>
<td>Group II</td>
<td>9.825 ± 0.29</td>
<td>77.600 ± 1.081</td>
<td>51.68 ± 1.085</td>
<td>19.535 ± 4.712</td>
<td>0.147 ± 0.005</td>
</tr>
<tr>
<td>Group III</td>
<td>9.216 ± 0.39</td>
<td>78.916 ± 3.887</td>
<td>51.68 ± 1.085</td>
<td>19.535 ± 4.712</td>
<td>0.147 ± 0.005</td>
</tr>
<tr>
<td>Group IV</td>
<td>8.967 ± 0.18</td>
<td>77.501 ± 2.796</td>
<td>53.79 ± 0.374</td>
<td>14.735 ± 0.467</td>
<td>0.128 ± 0.002</td>
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<tr>
<td>Group V</td>
<td>12.920 ± 0.49</td>
<td>94.295 ± 3.334</td>
<td>69.25 ± 0.096</td>
<td>26.717 ± 4.054</td>
<td>0.212 ± 0.012</td>
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<td>Group VI</td>
<td>9.067 ± 1.17</td>
<td>69.784 ± 7.514</td>
<td>48.38 ± 0.886</td>
<td>14.599 ± 1.341</td>
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<tr>
<td>Group VII</td>
<td>8.129 ± 0.35</td>
<td>71.136 ± 1.580</td>
<td>47.18 ± 1.495</td>
<td>12.361 ± 0.262</td>
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<td>Group VIII</td>
<td>8.966 ± 0.38</td>
<td>79.355 ± 3.849</td>
<td>51.76 ± 0.221</td>
<td>12.412 ± 0.833</td>
<td>0.105 ± 0.001</td>
</tr>
</tbody>
</table>

The SOD, CAT and GPx activities were increased sharply upon exposure to H₂O₂ alone (500 μM) for 12 h, and there was a statistically significant decrement by pretreatment with JP05 (0.5, 0.75 or 1.0 mg/ml). Moreover, H₂O₂-induction in N2a cells increased in the GSH and GSSG contents. Pretreatment with JP05 significantly attenuated the changes of GSH and GSSG contents markedly. JP05 (0.1 mg/ml) treatment to normal cells significantly lowered the GSSG content in N2a cells.

Effects of JP05 on the expressions of iNOS and nNOS

The immunoblot assays showed that iNOS and nNOS proteins were highly induced in the presence of H₂O₂. Consistent with the results for nitrite production, JP05 (0.5, 0.75 or 1.0 mg/ml) significantly suppressed the H₂O₂-induced iNOS and nNOS protein expressions. As noted for the changes in iNOS protein, the RT-PCR further showed that H₂O₂-induced iNOS mRNA expression was dose-dependently suppressed by JP05 (Fig. 4). At a concentration of 1 mg/ml, JP05 produced no significant changes in iNOS and nNOS proteins and iNOS mRNA expression in normal cells.

Effects of JP05 on the expression of Bax and Bcl-2

The levels of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were examined in N2a cells by Western blot. As shown in Figure 5A, the cells induced with 500 μM H₂O₂ for 12 h exhibited a significant increase of Bax protein and a significant decrease of Bcl-2 (P < 0.05). Treatment with JP05 (0.5, 0.75 or 1.0 mg/ml) significantly decreased the expression of Bax protein, while increasing the expression of Bcl-2 protein. Figure 5B shows that H₂O₂-induced elevation of the Bax/Bcl-2 ratio was substantially reduced in cells pre-treated with JP05.
FIGURE 5. Effects of JP05 on H$_2$O$_2$-induced expression of Bax and Bcl-2. N2a cells were pretreated with JP05 (0.5, 0.75 or 1.0 mg/ml), and then harvested 12 h after H$_2$O$_2$ (500 μM)-induction. Proteins separated by SDS-PAGE were immunoblotted and probed to Bax and Bcl-2 (A). β-actin served as a loading control. The relative density was estimated by Quantity One 1-D analysis software and expressed as Bax/Bcl-2 ratio (B). All assays were performed in at least three independent experiments. A difference was considered statistically significant when \( ^{**}P \leq 0.001 \) vs. normal (a) or H$_2$O$_2$ group (b).

Effects of JP05 on NF-κB pathway

Western blot analyses of cytosolic and nuclear fractions for NF-κB and Iκ-Bα proteins are shown in Figure 6. In nuclear fractions, the expression level of NF-κB was evaluated, while the expression levels of NF-κB and Iκ-Bα were evaluated in cytosolic fractions. H$_2$O$_2$-induction reduced the cytosolic NF-κB p65 and increased nuclear NF-κB p65, indicating that H$_2$O$_2$ induced the translocation of NF-κB p65 from the cytosol into the nucleus. Furthermore, Iκ-Bα degradation in H$_2$O$_2$-induction was also increased (Fig. 6). The translocation of NF-κB p65 to the nuclei was also evident by immunocytochemistry (Fig. 7). Incubation with anti-NF-κB p65 showed cytoplasmic staining within unstimulated cells, whereas cells induced with H$_2$O$_2$ showed marked nuclear staining. In addition, H$_2$O$_2$-induction to neurons induced cytoplasmic staining, indicative of Iκ-Bα phosphorylation, while those unstimulated neurons exhibited only background staining (Fig. 7). JP05 treatment (0.5, 0.75 or 1.0 mg/ml), reduced nuclear NF-κB p65 and increased cytosolic NFκB p65, indicating that JP05 induced the translocation of NF-κB p65 from the nucleus into the cytosol. Furthermore, Iκ-Bα degradation in H$_2$O$_2$-induction was also decreased by JP05 treatment in neuronal cells.

FIGURE 6. Effects of JP05 on H$_2$O$_2$-induced NF-κB/Iκ-Bα pathway. N2a cells were pretreated with JP05 (0.5, 0.75 or 1.0 mg/ml), and then harvested 12 h after H$_2$O$_2$ (500 μM)-induction. Nuclear and cytosolic proteins separated by SDS-PAGE were immunoblotted and probed for NF-κB and Iκ-Bα. β-actin served as a loading control. The relative density was estimated by Quantity One 1-D analysis software. All assays were performed in at least three independent experiments. A difference was considered statistically significant when \( ^*P \leq 0.05, ^{**}P \leq 0.01 \text{ and } ^{***}P \leq 0.001 \) vs. normal (a) or H$_2$O$_2$ group (b).

FIGURE 7. Effects of JP05 on H$_2$O$_2$-induced subcellular localization of NF-κB/Iκ-Bα. N2a cells were pretreated with JP05 (0.5, 0.75 or 1.0 mg/ml), and then induced for 12 h with H$_2$O$_2$ (500 μM). The cells were then hybridized with anti-NF-κB p65 (A) and p-Iκ-Bα (B) antibodies. (a) Normal, (b) H$_2$O$_2$ only, (c) JP05 (0.5 mg/ml) + H$_2$O$_2$, (d) JP05 (0.75 mg/ml) + H$_2$O$_2$, (e) JP05 (1.0 mg/ml)+H$_2$O$_2$. Scale bars represent 100 μm.
Effects of JP05 on MAPK pathway

As shown in Figure 8, neurons were induced with 500 µM of H_2O_2 for 12 h and the protein phosphorylation levels of p38 and c-jun N-terminal kinase (JNK) were significantly increased after H_2O_2 incubation. In contrast, no apparent change in the activation state of extracellular signal-regulated kinase (ERK1/2) signaling was observed, suggesting that H_2O_2 stimulated the activation of p38 and JNK pathways in the process of N2a cell apoptosis. Furthermore, H_2O_2-induced activation of p38 and JNK signaling pathways was markedly inhibited by pre-treatment with JP05 at 1.0 mg/ml. The results suggest that JP05 directly regulates the MAPK signaling pathway to inhibit H_2O_2-induced neuronal apoptosis.

FIGURE 8. Effects of JP05 on H_2O_2-induced MAPK pathway. N2a cells were pretreated with JP05 (0.5, 0.75 or 1.0 mg/ml), and then harvested 12 h after H_2O_2 (500 µM)-induction. Proteins separated by SDS-PAGE were immunoblotted and probed for total and phosphorylated forms of MAPK. β-actin served as a loading control. The relative density was estimated by Quantity One 1-D analysis software. All assays were performed in at least three independent experiments. A difference was considered statistically significant when ^P< 0.05 and ^P< 0.01 vs. normal (a) or H_2O_2 group (b).

DISCUSSION

Oxidative stress, occurring as a consequence of increased intracellular levels of ROS, such as H_2O_2, forms a common pathway leading to neuronal death (Ames et al., 1993). The balance between the generation of ROS and the antioxidative processes can become disturbed as reported for aging and several neurological disorders (Wang et al., 2008). As the major component of ROS, hydrogen peroxide has been extensively used as an inducer of oxidative stress in vitro (Ji and Gao, 2008). In vitro models represent an effective system for determining the mechanisms involved in H_2O_2-induced neurodegeneration. In the present study, exposure of N2a cells to H_2O_2 significantly decreased cell viability and its cytotoxic effects could be attenuated by JP05. This indicates that JP05 has the ability to protect neurons against H_2O_2, and it might be a potential protective agent.

Further experiments demonstrated that JP05 decreased H_2O_2-induced NO production when administered prior to H_2O_2. Significant increases of NO synthesis by iNOS have been reported in a variety of pathological processes including various forms of inflammation by inflammatory mediators (Reed et al., 1996). Our results show that JP05 inhibits H_2O_2-induced NO production in N2a cells. This suppression was correlated with the action of JP05 as an anti-inflammatory agent. Also, we found that JP05 significantly inhibited neuronal apoptosis by reducing LDH release and decreasing excessive ROS generation induced by H_2O_2. Previous studies showed that LDH was released from apoptotic cells and that release began within 2 h of the initiating stimulus (Johnson et al., 2005; Jemmerson et al., 2002). The findings of this study confirmed that LDH is released from apoptotic cells, since inhibition of apoptosis with JP05 reduced LDH release in the media.

The above results clearly show that apoptotic cell death is induced by H_2O_2, and that apoptosis also plays a role in the neurotoxicity. Normal cells exhibited regular, round-shaped nuclei and no ROS fluorescence. In H_2O_2-induction, the characteristic of apoptotic cells was evident with ROS fluorescence. Several enzymes contribute to the production of ROS, including NADPH oxidase, cyclooxygenases, lipoxygenases, myeloperoxidase, nitric oxide synthase, and xanthine oxidase (Manuchair et al., 1996). H_2O_2 can generate the more detrimental hydroxyl radicals and increase the ROS levels in neuronal cells. JP05 effectively scavenged ROS as supported by the decreased DCF fluorescence. A possible mechanism of neuroprotective effects of JP05 might be its direct scavenging of ROS such as superoxide anion, hydroxyl radicals, and nitric oxide radicals. These results suggest that the ROS scavenging effects of JP05 might be an important factor in reducing the level of cell death and apoptosis induced by H_2O_2 and JP05 exerts its antioxidant effects in the intracellular compartment.

The excess formation of ROS may lead to peroxidative impairment of membrane lipids consequently disrupting neuronal functions and causing cell death (Ames et al., 1993; Manushair et al., 1996). In this study, the contents of MDA and PCO, commonly used indicators of oxidative stress in N2a cells were increased after 12 h H_2O_2-induction. These results supported the hypothesis that apoptosis seen in vitro following induction with H_2O_2 could contribute to neurodegeneration by accumulation of lipids and proteins oxidized by ROS. Moreover, it suggests that the protein modifications elicited by direct oxidative attack on amino acid side chains or by the modification of side chains with lipid peroxidation products or with reducing sugars can all lead to the formation of protein carbonyl derivatives. JP05 reduced the MDA and PCO contents in N2a cells subjected to exposure in H_2O_2-induction.
Its mechanism of action appears to be primarily that of preventing the effects of hydroxyl radical, peroxynitrite- and nitric oxide-mediated oxidation.

To counteract the assault of the ROS/RNS, living cells have a biological defense system composed of enzymatic antioxidants that convert ROS/RNS to harmless species. For example, $O_2^{•−}$ is converted to $O_3$ and $H_2O_2$ by SOD. The $H_2O_2$ in the cells is removed by CAT, which is active only against $H_2O_2$ and by GPx, which can also act on GSH (Chan, 2001). In the present study, the activities of SOD, CAT, and GPx were increased in N2a cells after $H_2O_2$-induction. This may indicate enhanced hydrogen peroxide and superoxide radical production. Previous studies showed that over expression of SOD is neuroprotective against stroke, and nitric oxide (Ames et al., 2003; Reed et al., 1996). When increased SOD activity is coupled with increased activity of CAT and/or GPx, the excess hydrogen peroxide is readily detoxified. Pretreatment with JP05 to N2a cells exposed to $H_2O_2$, reduced the activities of enzymatic antioxidants to normal levels. This can be understood as an adaptive response to decreased hydrogen peroxide levels in cells after treatment with JP05. The oxidative stress not only changes the intracellular levels of antioxidant enzymes, but also modulates the substrates, GSH levels (Rotruck et al., 1973). In this study, the contents of GSH and GSSG were increased in N2a cells exposed to 12 h $H_2O_2$-induction. In the presence of free radicals, mainly $H_2O_2$, GSH acts as an electron donor and is oxidized into GSSG by GPx. Surviving cells developed an adaptive response that included increased synthesis of GSH and the maintenance of a glutathione-based reduction potential. Aguirre et al. (2006) reported that surviving neurons were able to adapt to oxidative stress by increasing GSH synthesis, thus controlling their reductive environment. JP05 depleted the GSH and GSSG contents to normal levels in N2a cells exposed to $H_2O_2$.

Still, nitric oxide is implicated in numerous physiological and pathological processes in the brain. However, under chronic stimulation and/or stimulation with a high amount of a toxin, it might initiate transcription of pro-apoptotic genes. Inducible NOS (iNOS), is rapidly transcribed (Jemmerson et al., 2002) and up-regulated by a rise in intracellular calcium due to ROS accumulation (Panet et al., 2001) and generates a sustained and elevated NO release. In the present study, the iNOS and nNOS protein levels as well as iNOS mRNA levels were increased after $H_2O_2$-induction in N2a cells. JP05 was found to significantly reduce $H_2O_2$-induced NO production, implying that JP05 had an inhibitory activity on iNOS or suppressive effects on the expression of iNOS and nNOS proteins in $H_2O_2$-induced N2a cells. It also suggested that the modulation of NO and NOS levels were involved in the cytoprotective effects of JP05.

The Bcl-2 family consists of a series of homologous genes that include Bcl-2, Bax, Bcl-xL, Bcl-xS, and Bad, all of which are important intracellular regulators of programmed cell death (Yang and Korsmever, 1996). Usually bcl-2 inhibits apoptosis, while Bax induces apoptotic cell death. In accordance with previous studies, we found that a significant increase in pro-apoptotic Bax expression was induced after $H_2O_2$ stimulation. In contrast, a significant down-regulation of anti-apoptotic Bcl-2 protein levels was found after the neurons were treated with $H_2O_2$. From these results, the level of apoptosis correlated better with the Bax/Bcl-2 ratio. Interestingly, JP05 dramatically attenuated Bax expression and increased Bcl-2 expression. These results together with a decreased Bax/Bcl-2 ratio support a critical role of JP05 in protecting cells from oxidative injury.

To investigate the mechanism of neuroprotection by JP05, its link to the NF-κB explored. NF-κB is a critical regulator of the expression of numerous genes implicated in immune and inflammatory responses. It plays an important role during the inflammation reaction of stroke (Zhang and Stanimirovic, 2002). NF-κB is normally located in the cytoplasm bound to its endogenous inhibitor protein, IκB. The upstream IκB kinase (IKK) phosphorylates IκB, leading to its ubiquitination and degradation in the 26S proteasome. This degradation liberates NF-κB and allows it to translocate to the nucleus, and bind to κB sites, specific domains within the promoters of downstream genes, thus activating their transcription. As expected, the present results indicated that $H_2O_2$-induction stimulated the translocation of NF-κB p65 from the cytosol into the nucleus and increased the cytosolic IκBα degradation in N2a cells. The immunocytochemistry studies also revealed the translocation of the NF-κB p65 protein to the nuclei and IκB phosphorylation in following $H_2O_2$-induction. This was prevented by JP05 treatment, which blocked phosphorylation and degradation of IκB, thus preventing nuclear translocation of NF-κB, NO production and expression of NOS, and thereby reducing inflammation. This finding demonstrated that JP05 is protective under oxidative stress indicating that JP05 might be useful in the pathogenesis of neurological diseases and injuries, such as stroke.

In addition to the $H_2O_2$-induced activation of NF-κB, three members of the MAPK family, i.e., ERK1/2, JNK, and p38 MAPK, also play essential roles in the induction of iNOS and neuronal apoptosis. Our results indicate that $H_2O_2$-induction to N2a cells enhanced the activities of p-P38 and pJNK, but not p-ERK1/2. Treatment of the cells with JP05 at 1 mg/ml significantly reduced the levels of phosphorylated P38, JNK, and ERK1/2, suggesting that the anti-inflammatory reaction of JP05 in N2a cells may also be involved in inhibiting the MAPK pathways.

In conclusion, the present investigation showed that JP05 markedly inhibits $H_2O_2$-induced NO, LDH, and ROS production and iNOS, nNOS, and Bax expressions and enhances Bcl-2 expression by blocking NF-κB activation and inhibiting the phosphorylation of MAP kinases in N2a cells. These results suggest that understanding the transcription factors and gene products involved in oxidative stress induced in neurodegenerative diseases will enable to the development of target drugs from functional foods to treat those diseases.
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SUPPORTING INFORMATION AVAILABLE
An HPLC spectrum of JP05. This material is available free of charge via the Internet at http://pubs.acs.org.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

REFERENCES


