Protection of NMDA-Induced Neuronal Cell Damage by Methanol Extract of Myristica Fragrans Seeds in Cultured Rat Cerebellar Granule Cells

Ju Yeon Ban, Soon Ock Cho, Ji Ye Kim, Kyong-Hwan Bang, Nak Sul Seong, Kyung-Silk Song, KiWhan Bae, and Yeon Hee Seong

College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea
1
National Institute of Crop Science, RDA, Savon, Kyunggi, 441-100, Korea
2 College of Agriculture and Life-Sciences, Kyungpook National University, Daegu 702-701, Korea
3 College of Pharmacy, Chungnam National University, Taejon 305-764, Korea

Abstract – Myristica fragrans seed from Myristica fragrans Houtt. (Myristicaceae) has various pharmacological activities peripherally and centrally. The present study aims to investigate the effect of the methanol extract of Myristica fragrans seed (MF) on N-methyl-D-aspartate (NMDA)-induced neurotoxicity in primary cultured rat cerebellar granule neurons. MF, over a concentration range of 0.05 to 5 μg/ml, inhibited NMDA (1 mM)-induced neuronal cell death, which was measured by trypan blue exclusion test and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. MF (0.5 μg/ml) inhibited glutamate release into medium induced by NMDA (1 mM), which was measured by HPLC. Pretreatment of MF (0.5 μg/ml) inhibited NMDA (1 mM)-induced elevation of cytosolic calcium concentration ([Ca2+]i), which was measured by a fluorescent dye, Fura 2-AM, and generation of reactive oxygen species (ROS). These results suggest that MF prevents NMDA-induced neuronal cell damage in vitro.

Keywords – Myristica fragrans seed, Neuroprotection, NMDA, Neurotoxicity, Cerebellar granule cells

Introduction

Glutamate is the major excitatory transmitter as well as an important neurotoxin in the CNS (Choi, 1988). Elevated extracellular glutamate levels have been shown to affect neuronal activity profoundly by activating specific ionotropic and metabotropic receptors and have been implicated in neurodegenerative processes associated with ischemia and other neuropathological conditions (Rothman and Olney, 1986). Numerous studies have related ionotropic glutamate receptors to the regulation of cell survival, in vivo as well as in vitro. In most cases, exposure to agonists of glutamate receptors has been reported to lead to increased cell death, whereas antagonists were found to be protective (Choi, 1985; Regan and Choi, 1994; Lesort et al., 1997; Solum et al., 1997; Drian et al., 1999). While it is presently clear that both kainate and amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors are also involved (Larn et al., 1997; Solum et al., 1997; Bardoul et al., 1998; Jensen et al., 1998), N-methyl-D-aspartate (NMDA) receptors had been initially considered as possible actors in this domain (Tecoma et al., 1989; Lesort et al., 1997; Drian et al., 1999). Neurotoxicity initiated by overstimulation of NMDA receptors and the subsequent influx of free Ca2+ leads to an intracellular cascade of cytotoxic events (Choi, 1985). Ca2+-dependent depolarization of mitochondria has been suggested to contribute to oxidative stress in neuronal injury, through the production of reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion or nitric oxide (NO) (Choi, 1992; Dykens, 1994; Whit and Reynolds, 1996).

Myristica fragrans Houtt. (Myristicaceae) is an evergreen aromatic tree cultivated in many tropical countries (Ram et al., 1996). It is also mentioned in modern scientific literature as a medicinal plant (Merck Index, 1989). Seeds of Myristica fragrans Houtt. (MF), commonly known as nutmeg, have been reported to contain pectin which has anti-oxidant property and niacin which has hypolipidaemic action. It also contains 25-35% fixed oils and 5-15% volatile oils (myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid and lauric acid) and chemical
substances such as myricitin, elminic and myristic acid (Nadkarni, 1976; Merck Index, 1989). The plant material possesses carminative, astringent, hypolipidemic, antitrombotic, antiplatelet aggregation, and anti-inflammatory activities (Evans, 1996; Nadkarni, 1976). Centrally it has been reported to have anxiogenic and psychoactive or hallucinogenic properties (Brenner et al., 1993; Van Gils & Cox, 1994; Sonavane et al., 2002). It, however, also has sedative effect and has been used for insomnia (Van Gils & Cox, 1994; Huang, 1999).

To extend the knowledge on the pharmacological actions of MF in the CNS, the present study examined whether MF has the neuroprotective action against NMDA-induced cell death in primarily cultured rat cerebellar granule neurons. The methanol extract of MF exhibited significant protection against the excitotoxicity induced by NMDA. It was also examined the effect of MF on the NMDA-induced elevation of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)], glutamate release and ROS generation.

**Materials and Methods**

**Materials** – MF was purchased from an oriental drug store in Taegu, Korea, and identified by Professor K.-S. Song, Kyungpook National University. Glutamate, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), O-phthalaldehyde (OPA), 2-mercaptoethanol, trypsin (from bovine pancreas), Dulbecco’s modified Eagle’s medium (DMEM), Joklik-modified Eagle’s medium, poly-L-lysine, amino acids for HPLC standard, cytosine 1-β-D-arabinofuranoside hydrochloride (cytosine arabinoside), 0.4% trypsin blue solution (pH 7.4), and Fura 2-AM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NMDA and (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine(MK-801) were purchased from RBI (Natick, MA, USA). 2,7-Dichlorodihydrofluorescein diacetate (H\(_2\)-DCF-DA) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from Gibco (Logan, Utah, USA). All other chemicals used were of the highest grade available.

**Preparation of methanol extract of MF** – MF (300 g) was extracted three times in a reflux condenser for 24 h each with 2L of 70% methanol. The solution was combined, filtered through Whatman NO. 1 filter paper, and concentrated using a rotary vacuum evaporator followed by lyophilization. The yield was about 10% (w/w).

**Primary culture of cerebellar granule neurons** – Cerebellar granule cells were cultured as described previously (Koh et al., 2003). Briefly, 7 to 8-day-old rat pups (Sprague-Dawley) were decapitated, and the heads were partially sterilized by dipping in 95% ethanol. The cerebellum was dissected from the tissue and placed in Joklik-modified Eagle’s medium containing trypsin (0.25 µg/ml). After slight trituration through a 5 ml pipette 5-6 times the cells were incubated for 10 min at 37°C. Dissociated cells were collected by centrifugation (1,500 rpm, 5 min) and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM), penicillin (40 µg/ml), gentamicin (50 µg/ml) and 10% fetal bovine serum. The cells were seeded at a density of about 2×10⁶ cells/ml into poly-L-lysine coated 12 well-plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, glass cover slips for the measurements of [Ca\(^{2+}\)\(_{cyt}\)], and coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of ROS. After 2 days incubation, growth medium was aspirated from the cultures and new growth medium containing 25 mM KCl and 20 µM cytosine arabinoside, to prevent proliferation of nonneuronal cells, was added. Cultures were kept at 37°C in a 7% CO\(_2\) atmosphere.

**Neurotoxicity experiment** – NMDA and MK-801 were solubilized in the incubation buffer described below. MF was dissolved in absolute ethanol with the concentration of 5 mg/ml and further diluted with the buffer. The final concentration of ethanol was 0.1%, and did not affect cell viability (data not shown). Neurotoxicity experiments were performed on neurons grown for 8-10 days in vitro on either 12-well culture plates or glass coverslips placed in culture dishes. The culture medium was removed and neurons were washed with a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM CaCl\(_2\), at pH 7.4. They were then incubated for 30 min in the same medium, and incubated for a further 3 h (unless otherwise indicated) in the presence of NMDA at 37°C. To confirm the NMDA receptors activation with the tight environment, glucose and Mg\(^{2+}\) were omitted from the incubation buffer. For every experiment, MF or MK-801 was added 15 min prior to the exposure of cells to NMDA and was present in the incubation buffer during the NMDA exposure.

**Analysis of cell viability**

**Trypan blue exclusion assay** – After completion of incubation with NMDA (1 mM), the cells were stained with 0.4% (w/v) trypan blue solution (400 µl/well, prepared in 0.81% NaCl and 0.06% K\(_2\)HPO\(_4\)) at room temperature for 10 min. Only dead cells with a damaged cell membrane
are permeable to trypan blue. The numbers of trypan blue-permeable blue cells and viable white cells were counted in 6 randomly chosen fields per well under a phase-contrast microscope (Olympus IX70, Tokyo, Japan). MF and MK-801 (10 μM) were pretreated 15 min prior to the NMDA treatment.

**MTT colorimetric assay** – This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductases (Berridge and Tan, 1993). Therefore, the amount of formazan produced is proportional to the number of viable cells. After completion of incubation with NMDA (1 mM), the culture medium was replaced by a solution of MTT (0.5 μg/ml) in serum-free growth medium. After a 4 h incubation at 37°C, this solution was removed, and the resulting blue formazan was solubilized in 0.4 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm using microplate reader (Bio-Tek ELX808, Vermont, USA). Serum-free growth medium was used as blank solution. MF (0.5 mg/ml) and MK-801 (10 μM) were pretreated 15 min prior to the MTT treatment.

**Measurement of [Ca²⁺]** – Cells grown on glass cover slides were loaded with 5 μM Fura 2-AM [dissolved in dimethyl sulfoxide (DMSO)] for 40 min in serum-free DMEM at 37°C in the CO2 incubator, and washed with the incubation buffer. Cell culture slides were mounted into spectrophotometer cuvette containing 3 ml incubation buffer. Fluorescence was measured with a ratio fluorescence system (Photon Technology International, RatioMaster™, NJ, USA) by exciting cells at 340 and 380 nm and measuring light emission at 510 nm. Baseline of [Ca²⁺] was measured for 120 sec prior to the addition of NMDA (1 mM). In order to test the effects of MF (0.5 μg/ml) and MK-801 (10 μM) on NMDA-induced [Ca²⁺], change, the cells were exposed to the compounds in the incubation buffer for 15 min, after being loaded with Fura 2-AM and washed. The compounds were also present in the cuvette during the measurement of NMDA-induced [Ca²⁺] change. NMDA was applied into the cuvette through a hole using a micropipette and mixed by an attached magnetic stirring system. The increase of [Ca²⁺], was expressed as the fluorescence intensity ratio measured at 340 nm and 380 nm excitation wavelength (F340/F380). This experiment was carried out in the dark.

**Measurement of glutamate concentration** – After completion of incubation with NMDA (1 mM) for 3 h, glutamate secreted into the medium from the treated cells was quantified by high performance liquid chromatography with an electrochemical detector (ECD) (BAS MF series, Indiana, USA) (Ellison et al., 1987). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 μm; 4.6×100 mm) after pre-derivatization with OP A2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ml/min. MF (0.5 μg/ml) and MK-801 (10 μM) were pretreated 15 min prior to the NMDA treatment.

**Measurement of ROS generation** – The microfluorescence assay of 2’,7’-dichlorofluorescin (DCF), the fluorescent product of H2DCF-DA, was used to monitor the generation of ROS (Gunasekar et al., 1996). Cells, grown on coverslips, were washed with phenol red-free DMEM 3 times and incubated with the buffer at 37°C for 30 min. Then, the buffer was changed into the incubation buffer containing 1 mM NMDA, and the cells were incubated for a further 1 h. In order to test the effects of MF (0.5 μg/ml) and MK-801 (10 μM) on NMDA-induced generation of ROS, the compounds were added 15 min prior to the treatment with NMDA. The uptake of H2DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with NMDA. After washing, coverslips containing granule cells loaded with H2DCF-DA were mounted on the confocal microscope stage, and the cells were observed by confocal scanning laser microscopy (Bio-rad, MRC 1021ES, Maylands, England) using 488 nm excitation and 510 nm emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. The challenge of H2DCF-DA and measurement of fluorescence intensity was performed in the dark.

**Statistical analysis** – Data were expressed as mean±SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) with subsequent Turkey’s tests. P values of <0.05 were considered to be significant.

**Results**

MF protects neurons against toxicity induced by NMDA – Cell death after plasma membrane damage was assessed with the ability of cerebellar granule neurons to take up trypan blue. The trypan blue assay that detects multiple forms of cell death, including apoptosis or
necrosis, has been used as an initial non-specific indicator of cell death. The number of cells stained by trypan blue with plasma membrane damage significantly increased with exposure of the cells to NMDA. In control cultures, the number of trypan blue-negative cells reached 95.9±0.5%, while the value decreased to 33.7±3.0% with the treatment with 1 mM NMDA. MF showed a significant inhibition on the increase of neuronal cell death induced by NMDA (1 mM) over a concentration range of 0.05 to 5 μg/ml, showing 86.0±3.3% with 5 μg/ml (Fig. 1). MK-801 (10 μM), an NMDA receptor antagonist, also caused a marked inhibition of the NMDA-induced neuronal cell death. For the following experiments, the concentration of 0.5 μg/ml for MF was used for the determination of the protective effects on the NMDA-induced neuronal cell damage.

As an additional experiment to assess NMDA-induced neuronal cell death, the MTT assay was performed. The MTT assay is extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. When cerebellar granule neurons are exposed to 1 mM NMDA, the MTT reduction rate decreased to 49.5±2.6%. MF (0.5 μg/ml) significantly reduced the decrease of cell viability induced by NMDA, showing 76.0±2.1% (Fig. 2). Similarly, MK-801 (10 μM) significantly inhibited the decrease of the MTT reduction rate caused by 1 mM NMDA.

**MF inhibits NMDA-induced elevation of [Ca²⁺]c** – The increase of [Ca²⁺]c has been postulated to be associated with cell death in many studies. The fluorescence intensity ratio of 340 nm excitation to 380 nm excitation (F340/F380) from Fura 2-AM loaded cells is proportional to [Ca²⁺]c. As shown in Fig. 3, [Ca²⁺]c started to elevate immediately after the treatment with 1 mM NMDA and reached maximal fluorescence intensity after 3-4 min. In contrast, NMDA application in the presence of MK-801 (10 μM) failed to produce the increase of [Ca²⁺]c throughout the measurement period. MF (0.5 μg/ml) significantly, but not completely, inhibited the NMDA-induced [Ca²⁺]c elevation. MF or MK-801 did not affect basal [Ca²⁺]c (data not shown).

**MF inhibits NMDA-induced elevation of glutamate release** – Glutamate released into the extracellular medium was measured using a ratio fluorescence system. In the plots shown, each line represents F340/F380 ratio from a representative cell population.
was quantified after the incubation of cells with 1 mM NMDA for 3 h. As shown in Fig. 4, 1 mM NMDA markedly elevated the basal glutamate level from 0.50±0.04 to 1.19±0.11 µM and MF (0.5 µg/ml) strongly blocked the NMDA-induced elevation of glutamate release showing 0.62±0.06 µM. In addition, MK-801 (10 µM) markedly inhibited NMDA-induced elevation of glutamate.

**MF inhibits NMDA-induced ROS generation** – NMDA increased glutamate release and the cytosolic concentration of free Ca\(^{2+}\). Furthermore, the pathological condition induced by glutamate is associated with accelerated formation of ROS. In H\(_2\)DCF-DA-loaded cerebellar granule cells, NMDA increased the fluorescence intensity, indicating the generation of ROS. The fluorescence intensity in 1 mM NMDA-treated cells was increased about six fold to 125.73±1 compared to control cells of 21.6±3.3. MF (0.5 µg/ml) and MK-801 (10 µM) significantly blocked NMDA-induced increase in fluorescence intensity (Fig. 5). MF did not show direct reaction with H\(_2\)DCF-DA to generate fluorescence.

**Discussion**

Most of the previous hypotheses dealing with neurodegenerative diseases have invoked abnormal release and/or decreased uptake of the excitatory amino acid glutamate as playing a key role in the process of excitotoxicity. The neuronal death in such conditions as ischemia, hypoglycemic coma, cerebral trauma or action of neurotoxins appears to be mediated at least in part by the extensive release of glutamate and its interaction with receptors (Rothman and Oiney, 1986). The released glutamate, acting on glutamate receptors, secondarily triggers Na\(^+\) influx and neuronal depolarization. This leads to Cl\(^-\) influx down its electrochemical gradient, further cationic influx and osmotic lysis of the neuron, resulting in neuronal cell death (Van Vliet et al., 1989). There is a great deal of data which shows that activation of the NMDA receptors elevates the influx of Ca\(^{2+}\) and non-NMDA receptors promote the influx of Na\(^+\), which can lead to membrane depolarization. In turn, depolarization can activate membrane voltage-sensitive Ca\(^{2+}\) channels, leading to additional Ca\(^{2+}\) influx. Many studies have shown that NMDA-induced elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) plays a fundamental role in the process of excitotoxicity (Choi, 1985; Mody and MacDonald, 1995). Transient increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) induced by NMDA can result from the entry of Ca\(^{2+}\) from receptor-operated channels, voltage-activated channels, or from the release of Ca\(^{2+}\) from internal stores. A sustained increase in [Ca\(^{2+}\)], triggers a series of events including the elevation of cGMP, the glutamate release and the activation of NOS (Mei et al., 1996; Baltrons et al., 1997). Released glutamate secondly acts on glutamate receptors and therefore potentiates the neurotoxicity. Using Ca\(^{2+}\) channel antagonists and NMDA receptor antagonists such as MK-801 reverses these conditions (Tecoma et al., 1989; Weiss et al., 1990).

In the present study, long-term treatment with NMDA produced neuronal cell death in cultured rat cerebellar granule cells, in accordance with many previous reports. NMDA caused significant elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\), glutamate release and ROS generation. This neurotoxicity induced by NMDA was completely reversed by MK-801, indicating that the neurotoxicity was mediated by the activation of these receptors. Although a clear concentration-response
relationship was not found. MF significantly protected neurons against the NMDA-induced cell death in used concentration range. MF also blocked the NMDA-induced increase of $[\text{Ca}^{2+}]_c$, glutamate release and ROS generation.

The elucidation of the variety of events occurring downstream of neuronal Ca$^{2+}$ overloading is still a matter for further research. ROS generation undoubtedly takes place in glutamate neurotoxicity and is likely due to Ca$^{2+}$ influx in the cytosol (Pereira and Oliveira, 2000). Ionotropic glutamate receptor agonists have been reported to increase the rate of ROS formation in an isolated synaptoneurosomal fraction derived from rat cerebral cortex (Bondy and Lee, 1993; Giusti et al., 1996). Long glutamate treatment results in permanent damage of mitochondria and large uncoupling, which occurs simultaneously with high mitochondrial ROS production. In this case, cytosolic Ca$^{2+}$ deregulation is followed by membrane permeability transition (Nicholls and Budd; 2000). In contrast with many reports that Ca$^{2+}$ signals activate enzymes which are associated in ROS generation (e.g. xanthine oxidase, nitric oxide synthase, phospholipase A2) leading to lipid peroxidation and neuronal damage, it has been demonstrated that ROS generation can facilitate $[\text{Ca}^{2+}]_i$ increase by damaging the $[\text{Ca}^{2+}]_i$ regulatory mechanism and activating Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores (Duffy and MacViar, 1996). It was not elucidated whether MF suppressed ROS generation through the inhibition of $[\text{Ca}^{2+}]_i$ increase, or vice versa, in the present study. It was assumed, however, that the neuroprotective effects of MF were mainly due to the inhibition on NMDA-induced elevation of $[\text{Ca}^{2+}]_c$, as shown in many compounds having the CNS inhibitory activities due to their inhibition on neuronal depolarization, and then this effect was followed by the inhibition on glutamate release and ROS generation. We also carried out the $[^{3}H]$MK-801 binding assay in cultured cerebellar granule cells, MF did not affect the specific $[^{3}H]$MK-801 binding to the cells (data not shown).

Elevated extracellular glutamate levels have been implicated in convulsion through NMDA receptor stimulation (Meldrum, 1992). NMDA receptor antagonists such as MK-801 have been shown to have anticonvulsant actions (Wong et al., 1986; McNamara et al., 1988). MK-801 acts by preventing Ca$^{2+}$ and Na$^{+}$ flow through NMDA receptor-coupled ionic channel (Chavko et al., 1998), as proved in the present study. It also has been reported that inhibition of NMDA receptor prevents oxygen free radical formation (Tecoma et al., 1989; Bondy and Lee, 1993), which is deeply involved in convulsive pathophysiology (Musch et al., 1994; Bashkatova et al., 2003). MF blocked NMDA-induced increase of $[\text{Ca}^{2+}]_c$ and ROS generation, and in result, prevented neuronal cell death. These MF-induced neuroprotection might be related to anticonvulsant effect of it. However, it must be made further studies to clarify the active components against the NMDA-induced neurotoxicity.

In conclusion, we demonstrated in the present study a novel pharmacological action of MF and its mechanism. The NMDA receptor-mediated neuroprotection by MF may help to explain at least some of its central inhibitory actions like anticonvulsant effect, and furthermore provide the pharmacological basis of its clinical usage in treatment of neurodegenerative condition such as stroke.

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References


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