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The Tuyama and Gastrodin Attenuate Excitatory Damage in Kainate Stressed Neuronal Cells

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Abstract

The tuyama is a precious Chinese medicine contains gastrodin and other constituents and commonly used for the treatment of neurodegenerative disorders. The protective effects of the tuyama and gastrodin, against Kainic Acid (KA) induced seizure have not been studied clearly. We investigated the effect of the tuyama extract and gastrodin on KA-induced neuronal injury in vitro and the tuyama extract-assisted sedative function by human HRV. The results confirmed that they provided effective protection against the excitatory damage of PC12 and BV-2 neuronal cells subjected to kainate. They could remove the ROS/NO, reduce MDA, PGE2 production, and caspase-3 protein. Western results revealed that p-ERK MAPK and COX-2 expression were significantly reduced by the tuyama and gastrodin. Summary, this study demonstrated that the tuyama and gastrodin provided a neuroprotective effect against excitotoxin and in part of involves with inhibiting p-ERK MAPK cell signal.

Keywords: Tuyama; Gastrodin; MAPKs; Kainic acid; Reactive oxygen species; Epilepsy; ROS; COX-2

Introduction

The Tuyama (*Gastrodia elata*) is a saprophytic herb and traditional Chinese plant [1]. The Tuyama is commonly used as a traditional herbal medicine for hypertension, the treatment of neurodegenerative disorders and memory improvement [2,3]. The Gastrodin is one of predominant bioactive constituents of the Tuyama. Many authors had reported that there are beneficial effects of the Gastrodin on diverse diseases of the CNS, including epilepsy, and cerebral ischemia [4,5]. Due to neuroprotective and anti-neuroinflammatory effects of tuyama and gastrodin, it can be considered as a promising candidate for status epilepticus therapy and the tuyama reduces oxygen free radicals and protects against neuronal damage [1,2,6,7].

There were anxiolytic-like property of the tuyama by way of the GABA-ergic nervous system and protective effect against global ischemia and apoptosis [8,9]. The gastrodin is one of bioactive components of the tuyama which plays an important role in the neuroprotection [6,10]. The other bioactive constituents of tuyama including vanillin and benzyl alcohol that mediated anti-inflammatory effects, inhibited ROS and the activity of Cyclooxygenase-2 (COX-2) apoptotic protein [11]. The tuyama is known for its neuroprotective effect against oxidative neuronal injury.

Status epilepticus is defined as a period of continuous seizure activity and cause recurrent spontaneous seizures, and releases of free radicals in experimental models of kainate [12]. Kainic acid (KA), a glutamate-related chemical, increases nerve excitability and is widely known that the systemic administration of KA induces neuronal damage [13]. KA causes neuron epilepticus and excitotoxicity with the increased production of Reactive Oxygen Species (ROS) and lipid peroxidation [14]. Mitogen-Activated Protein Kinases (MAPKs) and COX-2 are associated with seizures, inflammation, and apoptosis in excitotoxic neuronal cell [15-17]. The excitotoxin-induced ROS and cell apoptosis can be scavenged and reduced by antioxidants in neuron [18]. Rat Pheochromocytoma (PC12) cells and murine microglia BV-2 cells have been used as neuronal stress models [19,20].

Specifically, the Extracellular Signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK) and the p38 Mitogen-Activated Protein Kinase (MAPK) signaling pathways can be activated by ROS in PC12 cell and BV-2 cells on oxidative neuronal injury [21,22]. The kainate triggers neurons apoptosis by the release of ROS, which are involved in nerve mitochondrial dysfunction, but the ascorbic acid and dehydroascorbic acid can reduce apoptosis in the hippocampal cells [23]. Another

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extract of plant with an antioxidant effect, white rose petal extract, can also reduce the neuronal damage and provide neuroprotection in mice [24]. The resveratrol, trans-resveratrol, red wine antioxidant, and polyphenols present in olive seeds may induce a neuroprotective effect against the neurotoxin kainic acid [25-28]. Because the gastrodin is one of most important bioactive constituents of the tuyama and plays neuroprotective and anti-neuroinflammatory effects [6]. Therefore, the aim of this study was to investigate the protective mechanism of the gastrodin and tuyama extract against KA-induced injury in neuronal cells *in vitro*.

Materials and Methods

Chemicals

The dry tuyama (*Gastrodia elata*) sticks were purchased from local Chinese medicine clinic (Da-An-Tang Chinese Medicine Clinic, Miaoli, Taiwan). The gastrodin and Kainic Acid (KA, kainate) were obtained from Sigma-Aldrich (Steinem, Germany) and Cayman Chemical (Ann Arbor, MI), and 2′,7′-Dichlorodihydrofluorescein Diacetate (H2DCF-DA) was obtained from molecular probes (Eugene, OR). The dry Tuyama, were dried for 24 h at 50°C in a hot-air oven, ground to a fine powder, stored, and the extraction 0.5 g sample of Tuyama powder was mixed with 5 mL of Phosphate-Buffered Saline (1xPBS), ground in mortar bowl with pestle vigorously for 10 min, and then centrifuged at 2000 g for 10 min. The tuyama extracts were sterilized by filtration through a 0.22 μm Millipore membrane filter. Working standard solutions of the gastrodin were freshly prepared by diluting the stock solution in purified water to concentrations of 1 mg/mL, and 10 mg/mL respectively.

Determination of the gastrodin content

The quantity of gastrodin in the extracts of tuyama stick was determined using the method described by Chen et al. [29]. The gastrodin contents of tuyama stick were characterized by HPLC using a Shimadzu SCL-LC 10A HPLC fitted with a SIL 10AD auto sampler. Chromatography was performed with an ODS HYPERSIL (Thermo Scientific) reverse phase column (25 cm × 0.46 cm i.d., 5 μ) and the UV−Vis detector (ShimazuSystems Co., Foster City, CA). The mobile phase consisted of a mixture of 0.02% phosphoric acid in water (solvent A) and acetonitrile (solvent B) pumped in the gradient mode was prepared, freshly, the peak of gastrodin was detected at 220 nm and the flow rate was set at 1.0 mL/min.

Human Studies, the Tuyama-assisted Sedative function by HRV Assay

Heart Rate Variability (HRV) is the physiological phenomenon of variation in the time interval between heartbeats, recordings obtained under resting conditions can discriminate two main spectral components: A high frequency one (ranging from 0.15 Hz to 0.40 Hz) and a low frequency one (ranging from 0.04 Hz to 0.15 Hz), considered to be markers of parasympathetic and sympathetic control, respectively. It have been largely studied and found to be involved in the role of calming associated with slow down the heart rhythm. A human-research study (YPU-IRB-1071213 of Human Research Ethics Committee, Yuanpei University of Medical Technology, Taiwan) was conducted with 10 young and middle-aged healthy men (N=6) and women (N=4). Volunteer after the lunch and two hour later, carry out the first HRV test. One gram of the Tuyama powder was soaked in 10 ml hot water, after cooling, and then the experimental subject drank the cool solution after HRV test. All subjects were studied in the resting condition tests in the seated position. To investigate the effect of the tuyama on sedative function, 10 people whose HRV were normal were randomly divided into two groups: A controlled group with placebo treatment and an experimental group treated with tuyama. The second HRV test was conducted after one gram of placebo or tuyama treatment. Human Research Ethics Committee guidelines for good clinical practice are based on the declaration of Helsinki policy for the protection of human subjects. All persons had been given their informed consent prior to their inclusion in the study.

Cell culture

Rat Pheochromocytoma (PC12) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, at 37°C in a humidified incubator under 5% CO_2. Murine BV-2 microglial cell line was maintained in DMEM supplemented with 10% (v/v) FBS and under 5% CO_2 at 37°C. Confluent cultures were passaged by trypsinization. For experiments, cells were washed twice with warm DMEM (without phenol red) and then treated in serum-free medium. In all experiments, cells were treated in the presence of the Gastrodin or Tuyama extract and with KA stress for the indicated times.

Preparation of Cell Extracts

The test medium was removed from culture dishes, and cells were washed twice with ice-cold PBS, scraped off with a rubber policeman, and centrifuged at 200 g for 10 min at 4°C. The cell pellets were re-suspended in an appropriate volume (4×10^7 cells/mL) of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10 μg/mL aprotinin, and 5 μg/mL pepstain A. The suspension was then sonicated. The protein concentration of samples was determined by Bradford assay (Bio-Rad, Hemel, Hempstead, United Kingdom), and samples were equilibrated to 2 mg/mL with lysis buffer western blotting. Protein samples containing 50 μg of protein were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred to immobile polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated for 1 h with 5% dry skim milk in TBST buffer (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, and 0.1% Tween-20) to block nonspecific binding and then incubated with rabbit anti-COX-2, and antiphospho-MAPKs (Abcam, Cambridge, UK). Subsequently, membranes were incubated with secondary antibody streptavidin-horseradish peroxidase conjugated affinity goat antirabbit IgG (Jackson, West Grove, PA).

ROS generation

Intracellular accumulation of ROS was determined with H2DCF-DA. This non-fluorescent compound accumulates within cells upon deacetylation. H2DCF then reacts with ROS to form fluorescent Dichlorofluorescein (DCF). The BV-2 cells were plated in 96-well plates and grown for 24 h before the addition of DMEM plus 10 μM H2DCF-DA, incubation for 60 min at 37°C, and were carried out in the gastrodin and tuyama extract for various concentrations. The cells were subjected to 100 μM H_2O_2 for 60 min, and washed twice with room temperature Hank's balanced salt solution (HBSS without phenol red). Cellular fluorescence was monitored on a Fluoroskan Ascent fluorometer (Labsystems Oy, Helsinki, Finland) using an excitation wavelength of 485 nm and emission wavelength of 538 nm.

MTT reduction assay for cell viability

Cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial

Table 1: The gastrodin contents in samples obtained by different species.

Relative content			
Sample	**Gastrodin**	**Unit**	**Ref**
Gastrodiaelata f. flavida (Dry powder in water extraction)	1.13 ± 0.22	mg/g	Present study
Gastrodiaelata Blume (Water extraction after baking)	0.00149 ± 0.00085	g/g	Liu et al. [46]
Anoectochilus formosanus Hayata (Dry tissue in 50ºC water extraction)	300.8 ± 43.6	ug/g	Shiau et al. [47]

Values represent the mean from three independent experiments. Data are expressed as the mean ± SD

dehydrogenases, which are active only in live cells. The PC12/ BV-2 cells were pre-incubated in 24 well plates at a density of 5×10^5 cells per well for 24 h. The cells with various concentrations of the gastrodin and tuyama extract were carried out in the presence of 150 μM KA for 24 h and grown in 0.5 mg/mL MTT at 37ºC. One hour later, 200 μL of solubilization solution, Dimethylsulfoxide (DMSO) was added to each well, and absorption values were read at 540 nm on micro titer plate readers (Molecular Devices, Sunnyvale, CA). Data were expressed as the mean percent of viable cells from the control.

Lactate dehydrogenase release assay

Cytotoxicity was determined by measuring the release of LDH. The PC12/BV-2 cells with various concentrations of the gastrodin and tuyama extract were treated in the presence of 150 μM KA for 24 h, and the supernatant was used to assay LDH activity. The rate of absorbance was read at 490/630 nm on micro titer plate reader. Data were expressed as the mean percent of LDH release from the KA control.

NO production in LPS-activated BV-2 cells

BV-2 microglia cells were stimulated with 150 μM KA in the presence or absence of samples for 24 h and NO in the culture was measured by the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). The absorbance at 540 nm was measured using a micro plate reader.

Assay of PGE2 concentration and caspase-3 activation

PGE2 release and caspase-3 activity were measured by ELISA assay. The PC12/BV-2 cells with various concentrations of the gastrodin and tuyama extract were treated in the presence of 150 μM KA for 24 h, the PC12 cells supernatant was used to assay Caspase-3 activity and the BV-2 cells supernatant was used to assay PGE2 generation. The PGE2 concentration and caspase-3 activity were determined by the PGE2 and caspase-3 ELISA Kits (R&D Systems, Minneapolis, MN, USA). The absorbance at 450 nm was determined using a microplate reader (spectraMAX 340, molecular devices). The Data of caspase-3 activation were expressed as the mean percent of the KA control.

Measurement of lipid peroxidation

Lipid peroxidation was quantified by measuring MDA level of the PC12 cells by lipid peroxidation assay kit (Cayman Chemical). The cells (5×10^5) were added to 0.5 mL of homogenization buffer (0.1 M phosphate, 1 mM EDTA) and homogenized. The lysate was then centrifuged at 12000 g for 15 min at 4ºC. The supernatant was transferred to a new tube, and its total protein content was analyzed using the advanced protein assay. This kit works on the principle of condensation of one molecule of either MDA or 4-hydroxyalkenals with two molecules of N-methyl-2-phenylindole to yield a stable chromophore, and the absorbance at 500 nm was determined using an ELISA reader (spectraMAX 340, molecular devices).

Statistical analysis

All data were expressed as the mean ± Standard Errors of the Mean (SEMs). For single variable comparisons, Student's test was used. For multiple variable comparisons, data were analyzed by one-way Analysis of Variance (ANOVA) followed by Scheffe's test. P values less than 0.05 were considered significant.

Results and Discussion

The gastrodin, bioactive component from the dry Tuyama stick was 1.13 ± 0.22 mg/g. The results showed that the content of gastrodin was much higher from than A. formosanus in the sample obtained by extraction with 50ºC water (Table 1).

Effect on sedative function

In the HRV Assay, It has been known that Heart Rate Variability (HRV) is related to autonomic nerves, Low Frequency (LF) is related to sympathetic nerves, and High Frequency (HF) and parasympathetic nerves are concerning. The present results show that the effect of experimental group was markedly superior to that of placebo group in LF and HF ($P<0.05$). The tuyama may improve significantly HRV in sedative function (Table 2). The tuyama treatment phase-advanced LF, HF (Table 2) and LF/HF (data not show), and these shifts were significantly greater than after controlled treatment. We conclude that the tuyama influences sedative function by assisted rest and associated changes in HRV.

Protection from KA Toxicity

We evaluated the protective mechanism, KA-induced injury in neuronal PC12/BV-2 cells were investigated with LDH released and MTT assay. As shown in Figure 1, PC12/BV-2 cells were protected from the injury by the tuyama extract (1, and 10 mg/mL) and gastrodin

Table 2: The Tuyama could improve significantly HRV in sedative function.

Subjects	Condition	Placebo		Tuyama	
		LF	HF	LF	HF
1	Before	1.85	1.8	1.52	1.43
	Treated	1.82	1.77	1.57	2.12
2	Before	1.57	1.39	1.96	2.32
	Treated	1.52	1.43	1.7	2.11
3	Before	2.73	2.29	2.27	1.56
	Treated	2.99	2.22	1.89	2.27
4	Before	1.34	2.16	2.04	2.87
	Treated	1.41	2.21	1.85	2.48
5	Before	1.73	3.55	1.9	1.43
	Treated	1.67	3.61	2.23	2.2
Mean	Before	1.84 ± 0.53	2.24 ± 0.81	1.94 ± 0.27	1.92 ± 0.64
	Treated	1.88 ± 0.64	2.25 ± 0.83	1.85 ± 0.25	2.24 ± 0.15

Data are reported as values. Healthy men, N=6; Healthy women, N=4
Before: Before taking drug; Treated: Taking one gram of the Tuyama drug; LF: Low Frequency; HF: High Frequency

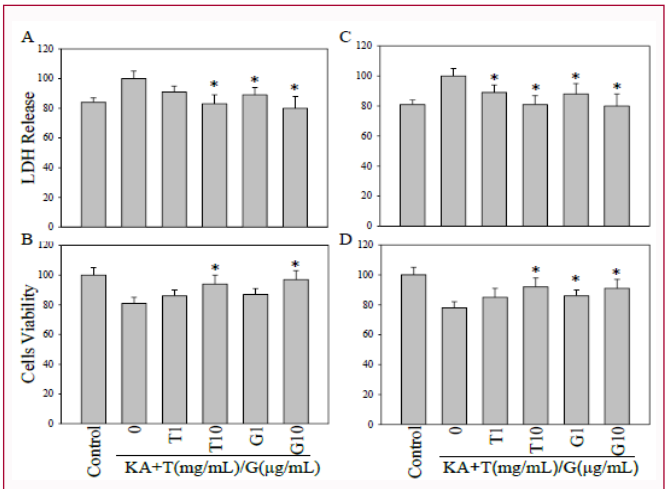

Figure 1: Protective effect of the Tuyama extract and gastrodin on the KA-treated neuronal cells. The MTT and LDH assay in (A) cytotoxicity of PC12 cells; (B) viability of PC12 cells; (C) cytotoxicity of BV-2 cells; (D) viability of BV-2 cells, and the KA treatment supplemented with 1, 10 mg/mL Tuyama extractor 1, 10 μg/mL gastrodin. The neuronal cells pre-treated with the Tuyama extract or gastrodin, reduced KA-induced excitatory injury as compared with the KA control. Values represent the mean from three independent experiments. Data are expressed as the mean ± SD. *P<0.05 as compared to the KA treated group.
T: Tuyama; G: Gastrodin

(1, and 10 μg/mL). The tuyama extract and gastrodin reduced LDH released and increased cell viability (P<0.05).

Kainic acid-induced nitrite production

We know that the nitric oxide is one of the major conductive substances in the neuron system. Studies have shown that large amounts of NO produce neuronal damage and it is related to the cause of seizures induced by kainic acid [30,31]. We further evaluated whether the tuyama or gastrodin protected BV-2 cells against KA by ameliorating the KA-induced NO accumulation. BV-2 cells were treated with KA (150 μM) alone or with various concentrations of the tuyama extract and gastrodin for 24 h. KA-induced nitrite production was dose-dependently decreased the treatment with the tuyama extract and gastrodin (Figure 2).

ROS and lipid peroxidation

Studies reported that there is a large amount of unsaturated

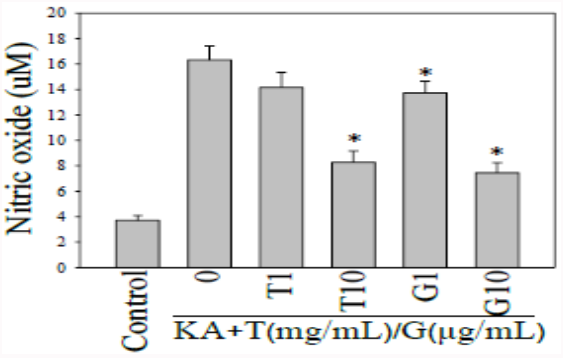

Figure 2: The effect of the Tuyama extract and gastrodin on the nitric oxide level under KA stress. Subjecting with KA 24 h. NO was increased four times in BV-2 cells in compared with the control cells. The Tuyama extract and gastrodin reduced KA-induced NO production in the serum of KA treated SD rats, dose dependently. Values represent the mean from three independent experiments. Data are expressed as the mean ± SD. *P<0.05 as compared to the KA treated group.

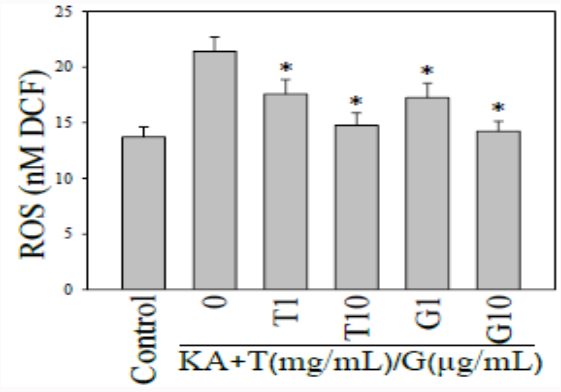

Figure 3: The effect of the Tuyama extract and gastrodin of ROS level in KA-treated BV-2 cells. The ROS of BV-2 cells were increased by 60 min KA stress significantly. The Tuyama extract and gastrodin scavenged KA-induced ROS production in the KA-treated BV-2 cells, dose dependently. Values represent the mean from three independent experiments. Data are expressed as the mean ± SD. *P<0.05 as compared to the KA group.

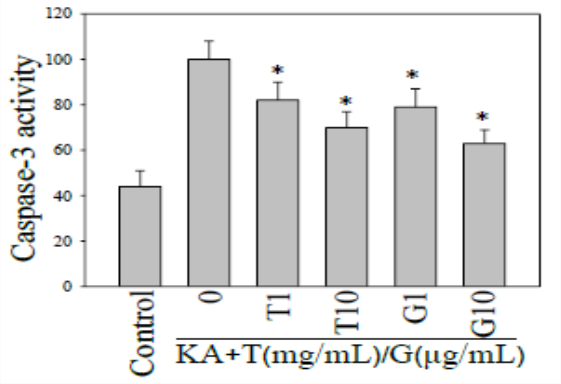

Figure 4: Effects of the Tuyama extract and gastrodin on caspase-3 levels of PC12 cells under KA-stress. The caspase 3 activity was determined by ELISA method after KA treatment. Values represent the mean from three independent experiments. Data are presented as the mean ± SD. *P<0.05 as compared to the KA group.

fatty acids in neuron, nerve cells are easily denatured by free radical attack and the ROS could increase neuronal cell cytotoxicity. DCF fluorescence of KA-treated cells increased sharply by nearly 50% to 60%, after 60 min stress, as compared with the control cells. Treatment with the tuyama or gastrodin protected cells against 150 μ MKA by neutralizing the KA-induced ROS accumulation, and this was consistent with the data of cell cytotoxicity and viability in Figure 1. Dose-dependently, of the tuyama extract and gastrodin effectively reduced the ROS production of KA-stressed BV-2 cells at 60 min (Figure 3). A forty percent increasing of MDA levels was observed in PC12 cells subjected to kainate, as compared with the control cells. The tuyama or gastrodin protected cells against KA toxicity by lowering MDA levels dose-dependently (p<0.05, as compared to the KA-treated cells).

Inhibition of caspase-3 activation

We further evaluated whether the apoptotic signaling pathways was involved in the apoptosis of KA-treated PC12 cells. The results showed that the tuyama extract and gastrodin affected caspase-3 activation (Figure 4). Cells were treated with KA (150 μM) alone or with the tuyama extract and gastrodin in various concentrations for 24 h. Both the tuyama extract and gastrodin decreased thecaspase-3

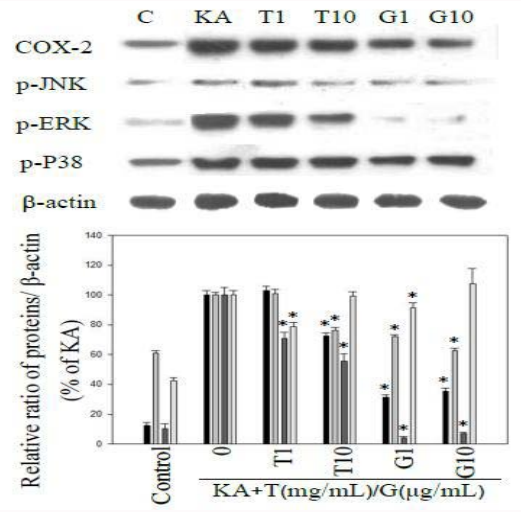

Figure 5: Effect of the Tuyama extract and Gastrodin on KA-activated signaling pathway. The p-JNK, p-ERK, p-P38 MAP kinases, and COX-2 proteins expression in PC12 cells subjected to KA stimulated were determined by Western blot assay. The protein expression of the neuronal apoptosis and inflammatory protein were all significantly increased in the KA treated group compared with the control group. Values represent the mean from three independent experiments. *P<0.05 as compared to the KA control.

activity significantly in KA-treated PC12 cells. The tuyama extract and gastrodin reduced caspase-3 activation and increased cell viability, which was consistent with the in vitro data of LDH released and MTT assay.

COX-2 and MAPKs cell signal expression

The effect of the tuyama extract and gastrodin on KA-induced activation of signaling pathways in PC12cells was evaluated by western blot assay. KA induced the activation of phosphate-MAP kinases (JNK, ERK, and P38MAP kinase) and COX-2 in PC12 cells at 30 min, was suppressed by the tuyama extract and gastrodin as compared to KA controls. The tuyama extract suppressed 30% COX-2 expression and 30% to 40% p-ERK MAPK, whereas the gastrodin suppressed 70% COX-2 and 90% p-ERK MAPK expression as compared to KA controls (Figure 5).

PGE2 Production in BV-2 Cells

Since COX-2 control PGE2 production, we then examined whether the KA-induced COX-2 expression would affect PGE2 production. The result showed that the tuyama extract and gastrodin significantly reduced the PGE2 production in KA-stressed BV-2 cells as predicted (Figure 6).

The present results showed that the tuyama extract and gastrodin protected PC12 or BV-2 cells from KA-induced excitatory damage. MDA and apoptosis were significantly reduced in the tuyama extract and gastrodin-treated neuronal cells as compared with the control. We confirmed that the tuyama extract and gastrodin decreased in LDH release, ROS generation, lipid peroxidation, caspase-3 activation, but increase in cell viability of KA-stimulated PC12 or BV-2 cells.

Cytotoxic activity

We know that the gastrodin is one of leading bioactive constituents from the tuyama. The tuyama and gastrodin has been long advocated for protection against tumour, oxidative stress, and neuroinflammation [32-34], but few studies have evaluated its efficacy and possible mode of action under KA-stimulated excitatory

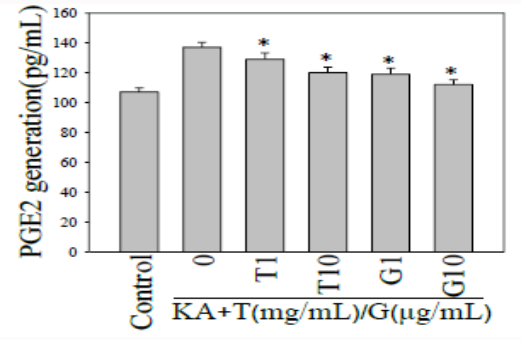

Figure 6: The effect of the Tuyama extract and gastrodin on serum PGE2 production in KA stimulated BV-2 cells. The Tuyama extract and gastrodin could reduce PGE2 generation in KA stimulated BV-2 cells dose dependently. Values represent the mean from three independent experiments. Data are expressed as the mean ± SD. *P<0.05 as compared to the KA group.

stress. The present study demonstrates that the gastrodin effectively protected PC12 or BV-2 cells from KA-induced injury dose-dependently. The tuyama extract and gastrodin decreased the KA-induced ROS releases and lipid peroxidation from PC12 or BV-2 cells.

Cell signaling

Western blot analysis revealed that MAPKs and COX-2 expression were increased in PC12 cells subjected to150 μM kainic acid. Specially, COX-2 and p-ERK MAPK, expression were significantly reduced by the tuyama extract (10 mg/mL) and Gastrodin (10 μg/mL). Furthermore, the Tuyama extract and Gastrodin were able to reduce PGE2 production of BV-2 cells exposed to KA. PC12 cells derived from rat pheochromocytoma and murine microglia BV-2 have been widely used for neurological studies [19,20].

Antioxidant capacity

The increase of ROS accumulation in KA-stimulated BV-2 cells and the lipid peroxidation of MDA were observed in KA-treated PC12 cells significantly. KA-induced NO and ROS accumulation in KA-stimulated BV-2 cells were significantly reduced by the tuyama extract or gastrodin (Figure 2, 3). This result is consistent with those of earlier studies that kainate induces lipid peroxidation in rat neurons [35,36]. Lipid peroxidation triggers highly reactive and unstable ROS of saturated or unsaturated lipids. There are growing reports that free radical generation could play the important role in the neuronal damage induced by prolonged convulsions and free radical scavengers are known to inhibit neuronal death induced by exposure to excitotoxins [24,35]. The tuyama extract and gastrodin significantly lessened the KA-exposed increase of NO and ROS accumulation, they could exert its neuroprotective effect by inhibiting lipid peroxidation.

Anti-inflammation and excitotoxicity

We found that KA induced the activation of MAP kinases (JNK, ERK, and p38) and COX-2 in PC12 cells. However, it is surprising that the expression of KA-activated COX-2 and p-ERK MAPKs was reduced by the gastrodin and tuyama extract. Especially, the gastrodin and tuyama extract significantly suppressed KA-activated COX-2 and p-ERK MAPK expression. This result is supported by the studies, indicating that administration of Pu-Erh tea or black tea extract to mice/rats with status epilepticus or cerebral ischemia-reperfusion reduced signal protein expression of COX-2, iNOS, and NF-κB activation *in vivo* [37,38]. We know that the excitotoxicity is one of pivotal mechanisms of neuronal death. Prostaglandins generated

during excitotoxicity play important roles in neurodegenerative conditions [39]. Previously, we demonstrated that antioxidant suppressed lipid peroxidation and PGE2 production and COX-2 activity in kainic acid-induced status epilepticus *in vivo* and *in vitro* [40]. The present results are consistent with those of previous reports that show KA-induced neuronal death is prevented either by inhibition of the enzyme xanthine oxidase, a cellular source of superoxide anions, or by the addition of free radical scavengers to the culture medium [41]. Thus, our data showing the suppression of ROS was consistent with the advocated protective role of the gastrodin and tuyama extract. The reports that the role of cerebral COX-2 mRNA and protein in KA toxicity parallels the appearance with neuronal apoptosis and free radicals formation [42,43]. The gastrodin and tuyama extract, as predicted, reduced the PGE2 production dose dependently and COX-2 activation in KA-induced PC12/BV-2 cells. Present data also showed that the tuyama could improve the sedative behavior in the HRV Assay of human.

However, further studies are needed to confirm whether the gastrodin and tuyama extract have direct effects on seizure behavior and the related molecular mechanisms. The present results are consistent with those of previous reports that antioxidants such as trans-resveratrol and vitamin E provide protection against excitatory damage in terms of the oxidative stress or convulsions [44-47]. Therefore, intervention by antioxidants can be a potential beneficial approach in the treatment of excitotoxin. Summing-up, these results indicate that antioxidant and anti-inflammatory effects might account for the protective mechanisms of the gastrodin and tuyama extract on KA-induced PC12/BV-2 cell injury. This study demonstrated that the tuyama and gastrodin provided a neuroprotective effect against excitotoxin and in part of involves an increase in the antioxidant capacity and inhibition with p-ERK MAPK cell signal of the cells.

Conclusion

In conclusion, the Gastrodin and the tuyama extract attenuate oxidative stress in KA-induced excitatory damage. We found that tuyama had abundant gastrodin content, and metabolites of gastrodin are potent antioxidants and anti-inflammatory agents. This suggests that natural antioxidants play an important role in neuroprotection against excitotoxins, and gastrodin in the tuyama were responsible for this protection. The molecular mechanisms of Tuyama extract and gastrodin on SE-induced excitotoxicity warrant further study for their therapeutic potential.

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