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Rhus verniciflua Stokes Attenuates Glutamate-induced Neurotoxicity in Primary Cultures of Rat Cortical Cells

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Abstract – The methanolic extract of *Rhus verniciflua* Stokes (RVS-T) and its fractions (RVS-H, RVS-C, RVS-E and RVS-B) showed significant neuroprotective activity against glutamate-induced toxicity in primary cultures of rat cortical cells. RVS-B, which showed the most potent neuroprotective activity, was further fractionated to yield RVS-B5. Treatment of cortical cells with the RVS-T, RVS-B and RVS-B5 reduced the cellular ROS level and restored the reduced activities of glutathione reductase and SOD induced by glutamate. Although, the activity of glutathione peroxidase was not virtually changed by glutamate, RVS-B5 increased the glutathione peroxidase activity. In addition, these three tested fractions significantly restored the content of GSH which was decreased by glutamate insult in our cultures. Taken together, it could be postulated that RVS extract, in particular its fraction RVS-B5, protected neuronal cells against glutamate-induced neurotoxicity through acting on the antioxidative defense system.

Keywords – *Rhus verniciflua* Stokes, neuroprotection, primary cultures of rat cortical cells, glutamate, antioxidant activity

Abbreviations – ROS; reactive oxygen species, SOD; superoxide dismutase

Introduction

Glutamate is found naturally in millimolar levels in the brain and plays a dominant role in central excitatory neurotransmission. This transmission is involved in such activities as neuronal survival, synaptogenesis, neuronal plasticity, learning and memory processes (Albright *el al*., 2000). However, excessive amount of glutamate is also recognized as causing neuronal cell loss (Choi, 1988; Coyle and Puttfarcken, 1993). Abnormalities in glutamate neurotransmitter systems may be involved in neurological disorders such as seizures (Lipton and Rosenberg, 1994), ischemia and spinal cord trauma (Chase and Oh, 2000; Heintz and Zoghbi, 2000) and neurodegenerative disorders including Alzheimer's disease (Choi and Rothman, 1990) and Parkinson's disease (Lee *et al*., 1999). In view of this, neuroprotection against glutamate-induced neurotoxicity has been a therapeutic strategy for preventing and treating neurodegeneration (Muir and Lees, 1995; Trist, 2000; Meldrum, 2002).

In the course of searching natural product with protective activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells, we found that an extract of the stem bark of *Rhus verniciflua* Stokes (RVS) exhibited significant neuroprotective activity. RVS has been traditionally used as an ingredient in Korean and Chinese medicines to treat gastritis, stomach cancer, arteriosclerosis (Kim, 1996). A variety of flavonoids such as fustin, fisetin, butein and sulfuretin have been isolated from this plant (Lee *et al*., 2002; Park *et al*., 2004). Recent studies have reported the antioxidant activity of RVS extract by scavenging reactive oxygen species (ROS) (Jung *et al*., 2006; Park *et al*., 2007). In addition, anti-apoptotic, anti-rhematoid arthritis and antimutagenic activities of RVS were found to be mediated via antioxidant activity (Lim *et al*., 2000; Choi *et al*., 2003; Park *et al*., 2004).

Oxidative stress is well-known mechanism responsible for glutamate-induced neuronal degeneration (Coyle and Puttfarcken, 1993). Hence, we assessed effects of RVS extract and its fractions against toxicity induced by glutamate in primary cultures of rat cortical cells. To elucidate the mechanism, we assessed the effects of the extract and its fractions on cellular ROS level and the activities of glutathione peroxidase, glutathione reductase and superoxide dismutase (SOD), and the content of reduced glutathione (GSH) in glutamate-injured cells.

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Experimental

Preparation of RVS samples – The stem bark of RVS was purchased from Kyungdong Oriental Herbal Market (Seoul, Korea). Dried stem bark of RVS (12 kg) was extracted with 80% MeOH in an ultrasonic apparatus. The methanolic extract (RVS-T) was concentrated *in vacuo* to give a crude extract (1.13 kg). This methanolic extract was suspended in H_2O and fractionated successively with *n*-hexane, $CHCl_3$, ethylacetate (EtOAc) and *n*-butanol (*n*-BuOH) to yield RVS-H (36 g), RVS-C (91.6 g), RVS-E (101.3 g) and RVS-B (102 g), respectively. RVS-B which showed the most potent neuroprotective activity was subjected to column chromatography on HP column eluting with a gradient of MeOH-water (0 : 100 → 1 : 4 → 2 : 3 → 3 : 2 → 4 : 1 → 100 : 0) to give six subfractions; RVS-B1 (66.6 g), RVS-B2 (9.5 g), RVS-B3 (8.2 g), RVS-B4 (7.2 g), RVS-B5 (6.5 g), RVS-B6 (1.4 g).

Chemicals – All chemicals for rat cortical cell cultures and biochemical assays were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, U.S.A.). Fetal bovine serum was obtained from Hyclone Co. (Logan, UT, U.S.A.).

Cell culture – Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared from 17 to 19-day-old fetal Sprague-Dawley rats as described previously (Kim *et al*., 1998). In brief, the trypsin-dissociated cortical cells were plated on multiwell culture plates (Corning, NY) coated with collagen at a density of 1×10^6 cells per well. The cortical cells were grown in DMEM containing 10% heat-inactivated FBS with penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 95% air-5% CO_2. All experiments were performed with Ethical Approval from the Seoul National University.

Neurotoxicity and cell viability – Samples for the test were dissolved in DMSO (final culture concentration, 0.1 %); preliminary studies indicated that the solvent had no effect on cell viabilities of control cells and glutamate-treated cells at the concentration used (Kim *et al*., 1998). Seventeen-day-old cortical cell cultures were pretreated with the samples for 1 h and then exposed to 100 μM L-glutamate. After 24 h incubation, the cultures were assessed for neurotoxicity. Neuronal cell viability was quantified by MTT assay, which reflects mitochondrial succinate dehydrogenase function (Kim *et al*., 1998).

Measurement of cellular peroxide – The relative level of free radicals, that is peroxide, in cultured cells was measured with the oxidation-sensitive compound, 2,7-dichlorofluoresein diacetate (DCF-DA) by the method of Goodman and Mattson (1994). Cells were loaded with DCF-DA (50 μM, 50 min incubation) followed by three times washes in HBSS. DCF fluorescence was then determined after 3 h incubation by measuring light emitted at 530 nm of exciting cells with light at 485 nm. Values shown are the mean ± S.D.

Assay for the activities of antioxidant enzymes – Cells from three culture plates were pooled in 2 mL of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at 3000 g at 4°C and the supernatant was used for the measurements of antioxidant enzyme activity and GSH contents. The activity of SOD was determined according to the method of McCord and Fridovich (1969) by the xanthine-xanthine oxidase reaction. Glutathione reductase activity was measured according to the method of Carlberg and Mannervik (1975) based on the reduction of GSSG by glutathione reductase in the presence of NADPH. The activity of glutathione peroxidase was determined by quantifying the rate of oxidation of GSH to GSSG by cumene hydroperoxide (Flohe and Gunzler, 1984). Values shown are the mean ± S.D.

Determination of total GSH content – Total GSH in the supernatant was determined spectrophotometrically using the enzymatic cycling method (Tietz, 1969). Values shown are the mean ± S.D.

Determination of protein concentrations – Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Sigma, MO, USA) with bovine serum albumin as a standard (Smith *et al*., 1985).

Statistical analysis – The levels of cell viability and antioxidant values were expressed as the mean ± S.D. and analyzed by one-way ANOVA. The data was considered to be statistically significant if the probability had a value of 0.05 or less.

Results

The effect of RVS on the neurotoxicity induced by glutamate – Neuroprotective activities of the RVS extract and its fractions were quantified by MTT assay in primary cultures of rat cortical cells (Table 1). Under phase-contrast microscope, over 50% of neurons died in cortical cultures after exposure to 100 μM glutamate for 24 h. At a concentration of 100 μg/mL, all of the RVS fractions (RVS-T, RVS-H, RVS-C, RVS-E, RVS-B) significantly increased the viability of glutamate-treated cells. Among the fractions tested, RVS-B showed the most potent neuroprotective activity against glutamate-induced damage. Increment of the concentrations of the treated fractions over 100 μg/mL did not improve their

Table 1. Neuroprotective activities of RVS extract and its fractions in glutamate-treated cortical cell cultures

	Protection (%)
Control	100.0 ± 0.2
Glutamate-injured	0.0 ± 0.1
RVS-T	40.2 ± 0.6**
RVS-H	11.4 ± 0.2
RVS-C	32.4 ± 0.3*
RVS-E	33.1 ± 0.6*
RVS-B	62.8 ± 0.6***

Cortical cell cultures were incubated with test samples for 1 h before exposure to 100 μM glutamate. After 24 h incubation, cultures were assessed for the extent of neuronal damage (throughout the experiment). Values shown are the mean ± S.D. of three experiments. Data are expressed as the percentage of cell viability relative to control culture; 100 × [optimal density (OD) of glutamate + test samples-treated cultures − OD of glutamate-injured cultures] / [OD of control cultures − OD of glutamate-injured cultures]. RVS-T; methanolic extract of RVS, RVS-H; subfraction from RVS extract eluted with *n*-hexane, RVS-C; subfraction from RVS extract eluted with $CHCl_3$, RVS-E; subfraction from RVS extract eluted with EtOAc, RVS-B; subfraction from RVS extract eluted with *n*-BuOH. Glutamate-injured cultures significantly differ from the control at a level of $P < 0.001$. Results significantly differ from the values of glutamate-injured cultures: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

neuroprotective activity (data not shown).

The effects of subfractions obtained from RVS-B fraction on glutamate-induced neurotoxicity were further evaluated (Table 2). RVS-B4, RVS-B5 and RVS-B6 showed significant protective activities against glutamate-induced neurotoxicity. At the concentration of 100 μg/mL, the relative cell viability was 38.2 ± 1.3%, 84.2 ± 1.0% and 25.2 ± 0.8%, respectively.

The antioxidant effect of RVS on the glutamate-induced oxidative stress – Glutamate-induced neurotoxicity is known to be mediated by oxidative stress (Coyle and Puttfarcken, 1993). Thus the effects of RVS-T, RVS-B and RVS-B5, which showed potent protective activity in cultured cortical cells, on cellular peroxide and the activities of antioxidant enzymes and the cellular GSH content in glutamate-injured cells were evaluated to elucidate the biochemical mechanisms of these fractions. Under our experimental condition, the exposure of cortical cells to glutamate increased the content of cellular peroxide compared to control cells. Treatment of RVS-T, RVS-B and RVS-B5 significantly attenuated the formation of cellular peroxide, respectively (Fig. 1).

Exposure of the cultures to glutamate resulted in a significant reduction in the activities of glutathione reductase and SOD (Table 3). The reduction in the activity of glutathione reductase induced by glutamate was

Table 2. Neuroprotective activities of the subfraction from RVS-B in glutamate-treated cortical cell cultures

	Protection (%)
Control	100.0 ± 0.1
Glutamate-injured	0.0 ± 1.5
RVS-B1	−15.4 ± 0.3
RVS-B2	−6.8 ± 0.9
RVS-B3	18.4 ± 0.4
RVS-B4	38.2 ± 1.3*
RVS-B5	84.1 ± 1.0**
RVS-B6	25.2 ± 0.8*

Cortical cell cultures were incubated with test samples for 1 h before exposure to 100 μM glutamate. After 24 h incubation, cultures were assessed for the extent of neuronal damage (throughout the experiment). Values shown are the mean ± S.D. of three experiments. Data are expressed as the percentage of cell viability relative to control culture; 100 × [optimal density (OD) of glutamate + test samples-treated cultures − OD of glutamate-injured cultures] / [OD of control cultures − OD of glutamate-injured cultures]. Glutamate-injured cultures significantly differ from the control at a level of $P < 0.001$. Results significantly differ from the values of glutamate-injured cultures: $^{*}P < 0.01$, $^{**}P < 0.001$

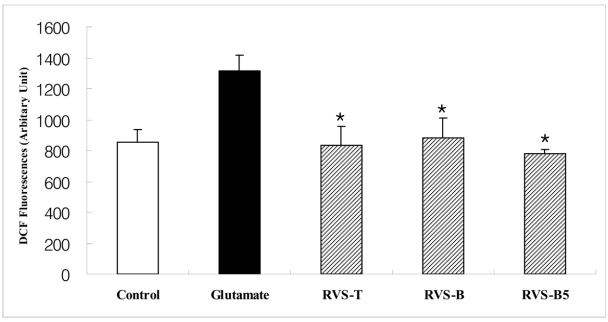

Fig. 1. The effects of RVS extract and its fractions on cellular peroxide in glutamate-treated cortical cell cultures. Cortical cell cultures were incubated with test samples for 1 h before exposure to 100 μM glutamate. The relative contents of intracellular peroxide was determined using the fluorescent dye 2,7-DCF-DA. Results significantly differ from the values of glutamate-injured cultures: $^{*}P < 0.001$.

significantly reversed by the treatment of cortical cells with RVS-B or RVS-B5. The activity of glutathione peroxidase was virtually unchanged by the glutamate insult, however, it was increased to some extent by RVS-B and RVS-B5 treatment, respectively. Treatment of cultures with RVS-T, RVS-B or RVS-B5 significantly restored the activity of SOD. The reduced SOD activity by the glutamate insult was elevated up to the control level with RVS-B treatment. Glutamate insult on the cultures also resulted in a depletion of GSH content. RVS-T, RVS-B and RVS-B5 significantly restored the GSH content reduced by glutamate insult to a level of 38%, 36% and 51% of control, respectively.

Table 3. The effects of RVS extract and its fractions on the activities of glutathione peroxidase, glutathione reductase and SOD, and the contents of GSH in glutamate-treated cortical cell cultures

Groups	Glutathione peroxidase (μmol NADPH oxidized/min/mg protein)	Glutathione reductase (μmol NADPH oxidized/min/mg protein)	SOD (μmol NADPH oxidized/ min/mg protein)	Reduced GSH (nmol/mg protein)
Control	0.444 ± 0.085	4.306 ± 0.099	11.788 ± 1.825	29.633 ± 0.514
Glutamate	0.423 ± 0.005	0.736 ± 0.073$^{\#\#}$	7.999 ± 1.069$^{\#}$	4.936 ± 0.125$^{\#\#}$
RVS-T	0.384 ± 0.037	2.842 ± 0.066*	11.471 ± 0.122**	11.344 ± 0.130**
RVS-B	0.480 ± 0.024	3.120 ± 0.124**	10.799 ± 0.220**	10.763 ± 0.859**
RVS-B5	0.528 ± 0.016*	3.546 ± 0.131***	11.532 ± 1.877**	15.375 ± 1.151***

The activity of each enzyme was measured as described in Materials and Methods. Each value represents the mean ± S.D. Results significantly differ from the values of control: $^{\#}P < 0.001$, $^{\#\#}P < 0.0001$ and glutamate-injured cultures: $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$

Discussion

The preventive and therapeutic measures to deal with the diseases caused by oxidative stress become more common, as the opportunities for biochemical and clinical application of natural antioxidant reagents increase. Consequently, numerous reagents particularly from plant-derived antioxidants have been used to treat diseases by reducing oxidative stress (Mates and Sanchez-Jimenez, 2000; Middleton and Kandaswani, 1992).

RVS has been traditionally used as an ingredient in Korean and Chinese medicine and much attention has been paid because of its potent antioxidant activity. Several studies have demonstrated that RVS extract has significant cytoprotective activity against oxidative damage by radical scavenging activity and by enhancing the detoxificant enzyme activities (Lee *et al*., 2002; Lim *et al*., 2000; Jung *et al*., 2006).

During our search for natural sources with protective effects against glutamate-induced neurotoxicity in primary cultures of rat cortical cells, we found that an extract from the stem bark of RVS exhibited significant neuroprotective activity. The subfraction (RVS-B5) obtained from butanolic fraction of RVS extract showed the most potent activity in this in vitro assay system. RVS-T, RVS-B and RVS-B5 reduced the cellular ROS levels and restored the reduced activities of glutathione reductase and SOD in glutamate-injured cells. Although, the activity of glutathione peroxidase was not changed by the glutamate insult, treatment of cortical cells with RVS-B5 significantly elevated the activity of glutathione peroxidase to a level higher than that of control. Glutathione peroxidase reduces toxic radicals using GSH as a substrate, subsequent to the oxidation of GSH to GSSG. GSSG is in turn reduced again to GSH by glutathione reductase at the expense of NADPH, forming a redox cycle (Lu, 1999). It can be suggested that the restoration of the activities of glutathione peroxidase and glutathione reductase by these fractions might promote scavenging free radicals using recycled GSH from GSSG. Consistent with this hypothesis regarding the activity of glutathione peroxidase and glutathione reductase, we showed that the depletion of brain GSH which normally accompanies glutamate insult was restored significantly by the treatment with RVS-T, RVS-B and RVS-B5, respectively. The recovery of the activities of antioxidant enzymes and the GSH content reduced by glutamate insult was the most significant with the RVS-B5 treatment.

These results demonstrated that RVS, in particular RVS-B and RVS-B5, scavenges ROS and exerts a protective effect against oxidative damage induced by glutamate by diminishing the reduction in the activities of glutathione reductase and SOD. As such, RVS might offer a useful therapeutic choice in either the prevention or the treatment of various neurodegenerative disorders.

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