

Water Extract of Rhei Rhizoma Prevent Production of Reactive Oxygen Species and Loss of Mitochondrial Membrane Potential in a Hypoxia Model of Cultured Neurons

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Rhei Rhizoma (RR; 大黃) consists of the underground parts (rhizome and root) of *Rheum officinale* Baill. and *Rheum palmatum* L. (Polygonaceae), and is widely used in Southeast Asian folk medicine to alleviate liver and kidney damages. In this study, we investigated into the efficacy and mechanism of RR water extract in supporting neuronal survival in a hypoxia model of cultured rat hippocampal neurons. RR exhibited no cytotoxicity up to 10 µg/ml and exhibited neurosupportive effects at 2.5 µg/ml in normoxia. When RR was added to the culture media on 10 days *in vitro* (DIV10) and given a hypoxic shock (2% O₂/5% CO₂, 3 hr, 37°C) on DIV13, RR exhibited neuroprotective effects on 5 days post-shock. H₂DCF stainings indicated that RR effectively prevents ROS production in both normoxia and hypoxia. JC-1 stainings showed that RR prevents dissipation of MMP in hypoxia. These results indicate that RR protects neurons by suppressing ROS production and MMP loss.

Key words : Cell culture, hippocampal neuron, hypoxia, MMP, Rhei Rhizoma, ROS

Introduction

Rhei Rhizoma (RR; 大黃) consists of the underground parts (rhizome and root) of *Rheum officinale* Baill. and *Rheum palmatum* L. (Polygonaceae). RR is widely used in Southeast Asian folk medicine to alleviate liver and kidney damage. Major constituents of RR are anthranoids, rheinosides, rhein, and stilben such as rhaponticin and rhapontigenin [9]. By bacterial enzymes in the large intestine, the primary anthranoids sennoside A and B, and rheinosides are transformed into rhein which lowers serum cholesterol [12]. Rhaponticin in the rhizome of RR has extensive anti-allergic and anti-thrombotic properties [9].

Beneficial effects of RR on human health have been reported. For example, RR extracts lower serum cholesterol and improve diabetic nephropathy [7,9]. Long-term treatment with RR extract decreased serum creatinine levels in diabetic patients with neuropathy and retinopathy [4]. RR has also been reported to improve memory ability. By comparing the effects of the Compound Tong Jiang Oral Liquid with Da Huang added (TJ) and Qi Yin Oral Liquid (QY) without Da Huang on senile persons' memory ability, Tian *et al.* [13] discovered that TJ shortens the interval and dura-

tion of defecation and improves senile persons' memory ability. Thus, it is indicated that RR exhibits anti-senility effects and can improve mentality. However, there have been few studies on the effects of RR at the cellular level. In the present study, we investigated into the efficacy of RR in the protection of neurons in a hypoxic model of cultured rat hippocampal neurons. Furthermore, to get an insight into the mechanism for neuroprotective effects, we measured reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). Here, we report that RR protects neurons in both normoxia and hypoxia by suppressing ROS production and MMP loss.

Materials and Methods

Preparation of water-extract of RR

Rhei Rhizoma (RR; 大黃) was selected according to the Korean Pharmacopoeia and obtained from Dongguk University Oriental Hospital (Gyeongju, Korea). Distilled water was added to the RR powder, agitated initially for 4 hrs at room temperature (RT) followed by overnight at 4°C. After centrifugation (15,000 rpm, 15 min, RT), the supernatant was filter-sterilized (pore size 0.2 µm) and stored at -20°C in small aliquots.

Neuronal culture

Hippocampi from time-pregnant rats (Sprague-Dawley)

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at embryonic day 18 (E₁₈) were dissected, dissociated by trypsin treatment and mechanical trituration, and plated onto polylysine/laminin-coated glass coverslips (12 mm in diameter) at a density of ~500 cells/mm² as described [2]. Cells were plated initially in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA), 25 μM glutamate, and 500 μM glutamine, and fed 5 days after plating and weekly thereafter with the same media (without glutamate).

Hypoxia

Hypoxic shock was given to cells by transferring culture plates to a humidified CO₂ Water Jacketed Incubator (Forma Scientific Inc., Marettta, OH, USA) which was equilibrated at 2% O₂/5% CO₂ (37°C). After 3 hr incubation, plates were returned to normoxic incubator (5% CO₂, 37°C).

Lactate dehydrogenase (LDH) assay

Cell viability was quantified by measurement of LDH released by injured cells. RR water extracts was added to culture media on day 10 in vitro (DIV10). A hypoxic shock was given on DIV13. Eight 20 μl aliquots from duplicate 24-well plates were transferred to 96-well plates. To each well containing 20 μl of culture media, 20 μl of pyruvate substrate (NADH, 1.0 mg/ml) was added. The plates were agitated for 2 min at RT, followed by 30 min at 37°C. Color reagents (Sigma 505-2; 20 μl) were added, agitated for 20 min at RT. NaOH (0.4 N, 100 μl) was added and agitated for 15 min at RT. OD₄₅₀ was measured using a ELISA reader.

Detection of ROS-producing cells

After rinsing cells in fresh culture media, 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCF; Molecular Probes, Eugene, OR, USA) was added at 10 nM. Cells were incubated in a CO₂ incubator for 10 min, rinsed in culture media. A stock 5 mM H₂DCF-DA was prepared in dimethylsulfoxide (DMSO).

Measurement of mitochondrial membrane potential (MMP)

MMP was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbo cyanine iodide (JC-1), a fluorescent cationic dye, which accumulates in mitochondria and changes its emission from wavelength 527 nm (monomer) to 590 nm (aggregates) depending on the mitochondrial membrane potential. RR was added to the culture medium

on DIV10 and stained with JC-1 as follows at the indicated times. Coverslips with cells were briefly rinsed with fresh culture media. JC-1 (200 μg/ml stock solution in DMSO) was added to a final concentration of 1.0 μg/ml and the plates were incubated for 20 min in the CO₂ incubator.

Image capture, surface plot, and density measurement

After washing cells twice with culture media, fluorescent digital images were captured with a CCD camera (Photometrics Inc., Germany) connected to a fluorescence microscope (Leica Research Microscope DM IRE, Germany) using FW4000 software (Leica). Using an Adobe PhotoShop software (version 5.0), fluorescence images were converted into a gray scale mode (value from 0 to 255), and inverted so that positive signals become dark. Surface plots were obtained using a Scion Image software (Beta 4.03; Scion Corp., Frederick, MD, USA) after removing backgrounds through the 2D rolling ball mode. Densities of each signal from the inverted images were measured. A small circular area including the signals was selected and the mean density of the selected area was measured using a Scion Image software.

Statistics

Cell viability was expressed in % of total (mean±SD). Multiple pairwise comparisons for statistical significance were evaluated by Kruskal-Wallis one-way ANOVA, and the differences between groups by Mann-Whitney *U*-test. The *p* values less than 0.05 and 0.01 were considered to be significant and very significant, respectively.

Results and Discussion

Effect of RR on the cell viability in normoxia

RR water extract was added in the culture media at various concentrations (0-50 μg/ml) on DIV10. After 3 days, cell viabilities were measured by LDH assays. As shown in Fig. 1A, RR increased cell viability by 112.8±2.0% over control culture at 2.5 μg/ml concentration. This increase was statistically very significant (*p*<0.01). There was neither neuro-supportive nor cytotoxic effects at 5 and 10 μg/ml concentrations. On the other hand, significant cytotoxicity was evident at 25 μg/ml concentration (*p*<0.05). The median lethal dose (LD₅₀) was approx. 50 μg/ml. These results show that RR water extract has beneficial effects on neuronal health in normal culture conditions.

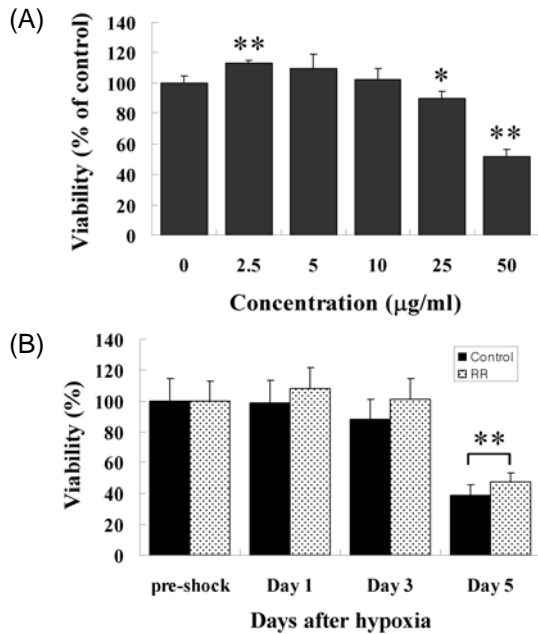


Fig. 1. Effect of Rhei Rhizoma (RR) on the cell viability. RR water extract was added to culture media on DIV10. Cell viability was measured on DIV13 by LDH assay maintained in normoxia (A) or at the indicated times after given a hypoxic shock on DIV10 (B). Viability was expressed as percentages of controls. *, $p < 0.05$; **, $p < 0.01$.

Effect of RR on the cell viability in hypoxia

To investigate whether RR also supports neuronal survival in hypoxia, RR was added to culture media (2.5 µg/ml) on DIV10. On DIV13 cells were given a hypoxic shock (2% O₂/5% CO₂) for 3 hrs. LDH assays just before hypoxic shock, and 1, 3, and 5 days after shock showed that survival rates were higher in RR(+) groups on day 1, 3, and 5 after shock (Fig. 1B). However, the effect was very significant only on day 5 ($p < 0.01$; 47.3±5.6% viability in the RR group compared to 39.0±6.5% in the control group). These results indicate that RR water extract has neuroprotective effects in hypoxia.

Effect of RR water extracts on ROS production in normoxia

H₂DCF-DA is cell-permeant and converted by esterase into 2',7'-dichlorodihydrofluorescein (H₂DCF). H₂DCF in turn is readily oxidated by ROS and becomes the highly green fluorescent 2',7'-dichlorofluorescein. RR water extract (2.5 µg/ml) was added to culture media on DIV10. Green fluorescence images just before hypoxic shock on DIV13 cells were shown in Fig. 2A. When no RR was present in the medium (RR-), about 20% of total neurons were ROS-positive. In many instances, the ROS signals were punctate

representing mitochondria (Fig. 1A, *c* and *d*, arrowheads). In contrast, about 8% of total neurons were ROS-positive when RR was added to the medium. The surface plots (Fig. 1A, *d*) and statistics (Fig. 1C, pre-shock) of the ROS signals clearly show that the signal intensities were much higher in the control cultures (RR-). Strongly ROS-producing cells (>50 in 0-255 scale) were frequently encountered in the control cultures while such cells were very rare in RR-treated cultures (Fig. 1C, *b*, pre-shock). These results indicate that RR water extracts suppress the production of ROS very effectively in normoxia.

Effect of RR water extract on ROS production in hypoxia

To investigate if RR water extract is also effective in suppressing ROS in hypoxia, RR-treated (for 3 days) DIV13 neurons were given a hypoxic shock and ROS production was measured by H₂DCF-DA staining at 1 hr, 3, and 5 days after shock. Representative images taken at 3 days after shock were shown in Fig. 2B. When RR water extracts were not added to the media (RR-), strong ROS(+) cells were frequently encountered. Examples of these ROS(+) cells were shown enlarged in the inset and its surface plot clearly show high intensity of ROS signals (arrow in *c* and *d* of Fig. 2B, RR-). In high contrasts, there appeared almost no strong ROS(+) cells in RR(+) cultures (Fig. 2B, RR+), although very weak ROS-producing cells were present. Surface plots of these cells show weak intensity for ROS (panel *d* of Fig. 2B, RR+). Statistic analysis of the relative signal intensities (0-255 scale) for ROS(+) cells were shown in Fig. 2C. ROS signals were strongest at 1 hr after shock, and control cultures (RR-) showed very significantly ($p < 0.01$; pre-, 1 hr, and 3 days post-shock) or significantly ($p < 0.05$; 5 days post-shock) higher signal intensities than RR(+) cultures (Fig. 2C, *a*). When only strong ROS(+) cells (> intensity 50) were considered, there were almost no such neurons until 3 days post-shock in RR(+) cultures. However, there were many strong ROS(+) neurons in the control (RR-) cultures throughout the culture (Fig. 2C, *b*). These results clearly show that RR water extracts effectively suppressed ROS production.

Effect of RR water extract on the prevention of MMP loss

Mitochondria play a central role in determination of cell fate [5] by regulating the mitochondrial permeability transition pore and the release of a number of apoptotic

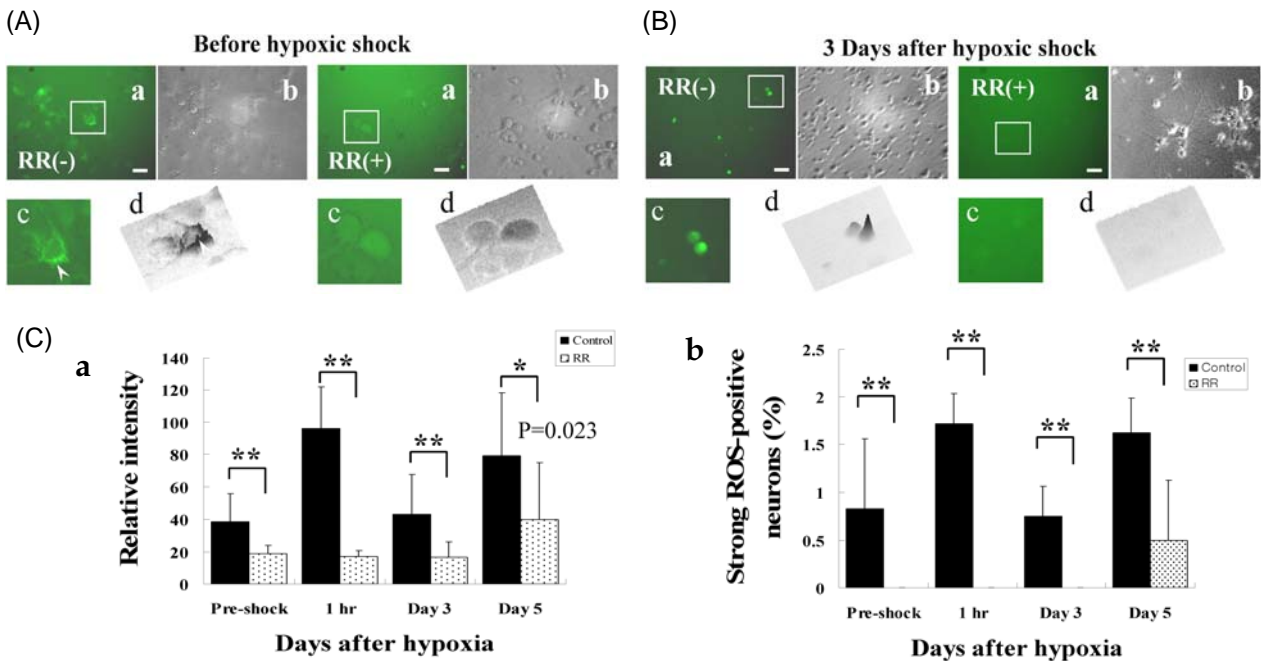


Fig. 2. Suppression of ROS by Rhei Rhizoma (RR) water extract in a hypoxia model. RR 2.5 μ g/ml was added to cultures on DIV10. Cells were given a hypoxic shock on DIV13, and stained with H₂DCF-DA (10 nM) on 1 hr before (pre-shock), 1 hr, 3, and 5 3 days after hypoxia. **A**, Normoxic culture. Typical fluorescence images (panel *a*) of H₂DCF-DA stained neurons, which were maintained with (RR+) or without (RR-) RR extracts on 3 days after shock, along with phase-contrast images (panel *b*), were shown. Boxed areas were shown enlarged in panel *c* and their surface plots in panel *d*. **B**, Hypoxia. Annotations are same as in **A**. **C**, Statistics. *a*, relative intensity of ROS(+) cells in general. *b*, percentage (greater intensity than 50 in 0-255 scale) of strong ROS(+) neurons. *, $p < 0.05$; **, $p < 0.01$. Scale bar, 25 μ m.

factors. Mitochondrial permeability transition (MPT), the collapse of electrochemical gradient across the mitochondrial membrane, is one of the early events during cellular apoptosis. The measurement of mitochondrial membrane potential is one of the methods used to study signaling mechanisms involved in the initiation of the apoptotic cascade. The 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is a unique fluorescent cationic dye to measure the collapse of the electrochemical gradient across the mitochondrial membrane. The JC-1 dye accumulates in the mitochondria of healthy cells as aggregates, which are fluorescent red in color. This dye has been extensively used as a semiquantitative indicator of neuronal mitochondrial membrane potential [1,3,14]. Upon the onset of apoptosis, the mitochondrial potential collapses and the JC-1 dye can no longer accumulate in the mitochondria and remains in the cytoplasm in a monomeric form which fluoresces green.

RR water extract was added into culture media on DIV10 and the cultures were stained before hypoxic shock, 1 hr, 3, and 5 days after shock (Fig. 3). Typical merge images of

green and red channels of JC-1 stainings on day 3 post-hypoxia were shown in Fig. 3A. Generally, green fluorescence was dominant over red one in RR(-) cultures, while reverse was true of RR(+) cultures. Images of single green or red channel (panel *a* of Fig. 3) of a portion of neuropil area (box) from each culture were shown at the bottom. The surface plots of red channels (panel *b* of Fig. 3) clearly show stronger red intensities in RR(+) cultures. Statistics analysis revealed that the intensities for red channels are very significantly stronger in RR(+) cultures than control one, except 1 hr after hypoxia. These results indicate that RR water extracts are effective in preventing MMP dissipation in hypoxia.

RR is widely used in Southeast Asian folk medicine to alleviate liver and kidney damage. Efficacy of RR has been manifested scientifically. For example, RR extract lowers serum cholesterol, improve diabetic nephropathy, and protect pro-oxidation [7,9]. Sennoside A and B and rheinosides, the primary constituents of RR, are transformed into rhein that increases motor locomotion in the large intestine [9]. Rhein also lowers serum cholesterol [12]. The present study have shown that RR water extract is very effective in suppressing

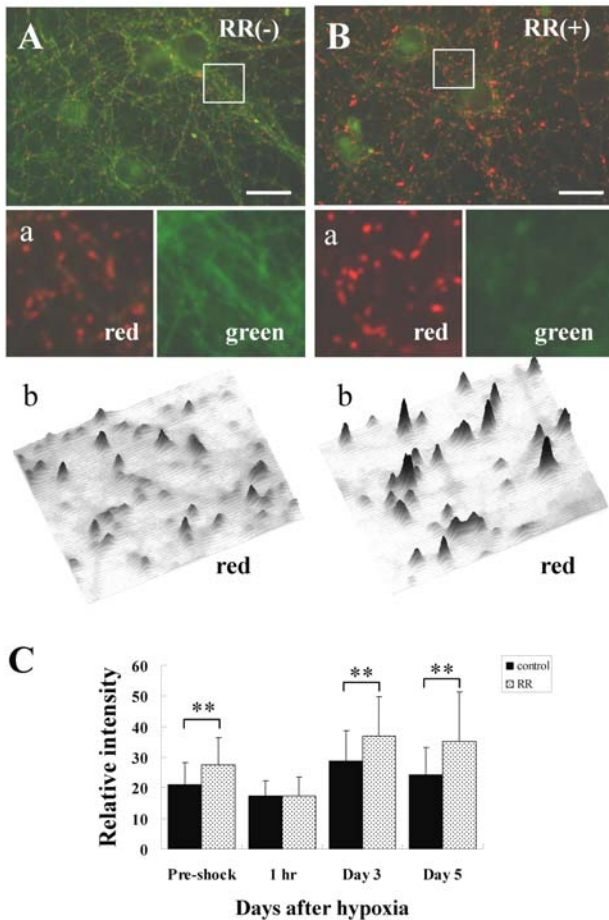


Fig. 3. Prevention of MMP loss by Rhei Rhizoma (RR) water extract in a hypoxia model. Cells were grown, treated with RR water extract, and give a hypoxic shock as in Fig. 2. *A & B*, Typical merge fluorescence images of JC-1 stained neurons, which were maintained with (RR+) or without (RR-) RR extracts on 3 days after shock, were shown. Single green and red channel images of boxed areas were shown enlarged at the bottom panel *a* and their surface plots in *b*. *C*, Statistics. Intensities of red spots were measured in 0-255 scale after converting to black and white mode. **, $p < 0.01$. Scale bar, 25 μm .

ROS production. Effective prevention of ROS production was previously reported. Rhyu *et al.* [10] showed that boiled-water extracts from RR effectively protected oxidative injury, and further showed that two of its tannin compounds, (-)-epicatechin 3-*O*-gallate and procyanidin B-2 3,3'-di-*O*-gallate, inhibited ROS significantly. ROS is a major causative for cell death in hypoxia. Therefore, effective prevention of ROS production would ameliorate potential damages from neurons in hypoxia. Efficacy of RR in ROS suppression was also true for normoxia. This suggests that RR can be applied for improving neuronal health not only in

hypoxia but in normal environment. The collapse of electrochemical gradient across the mitochondrial membrane, i.e., MMP, is one of the early events during cellular apoptosis. The present study has proven that RR water extract efficiently prevent MMP dissipation. Prevention of MMP loss, together with ROS suppression, may have reduced neuronal damages.

Experimental data on the efficacy of RR on human behavior are rare. Nevertheless, RR has been reported to improve memory ability [13]. Although the exact mechanism for this memory improvement was not addressed, the present study provides a good rationale for the explanation. Since this study have shown evidence for neuroprotective roles for RR, it can be possible that the brain neurons were healthier in the RR-treated group than controls. The neuroprotective role for RR would have been significant because the memory test was performed with the senile persons. In conclusion, the present study provides evidence for the neuroprotective effect of RR, and presents usefulness of RR in improvement of brain health.

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초록 : 배양 신경세포의 저산소증모델에서 대황 물추출액의 항산화 및 사립체막전위 소실 억제 효능

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대황(Rhei Rhizoma; RR, 大黃)은 *Rheum officinale* Baill.와 *Rheum palmatum* L. (Polygonaceae)의 땅속부분(rhizome 및 root)으로 남아시아의 민속의학에서 간 및 신장의 손상을 치료하는데 널리 이용되고 있다. 본 연구에서는 배양한 흰쥐 해마신경세포의 저산소증모델을 이용하여 대황의 물추출물이 신경세포사를 억제하는 효능에 대하여 조사하였다. 배양 10일(DIV10)에 RR을 배양액에 첨가하고 DIV13일에 생존율을 조사한 결과 10 µg/ml 농도까지는 세포독성이 없었으며, 정상산소 환경에서 2.5 µg/ml의 농도에서 세포생존율을 높이는 것으로 나타났다. 또한 배지에 대황을 첨가한 경우 DIV13일에 저산소증을 유도한 후 5일째에 세포생존율을 조사한 결과 대조군에 비하여 매우 유의하게 생존율을 증가시켰다. H₂DCF 염색 결과 대황은 활성산소(ROS)의 생성을 유의하게 감소시켰고, JC-1 염색 결과 사립체 막전위의 소실을 유의하게 억제함을 알 수 있었다. 이러한 결과들은 대황 물추출물이 활성산소를 효과적으로 제거하고, 세포의 에너지 생성을 보전함으로써 세포사를 억제할 수 있음을 보여주며, 향후 뇌신경세포의 건강에 유용하게 이용될 수 있음을 시사한다.