

# Ratanasampil Suppresses the Hypoxia-Related Inflammatory Responses by Inhibiting Oxidative Stress and NF- $\kappa$ B Activation in Microglia

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## Abstract

**Objective:** Hypoxia, as an important environment factor, is associated with many neurodegenerative diseases caused by microglia-mediated neuroinflammation, especially in Alzheimer's Disease (AD). Ratanasampil (RNSP) is a traditional Tibetan medicine and reported that improves the cognitive function of mild-to moderate AD patients, who are living at high altitude. *In vivo*, RNSP can improve memory and learning impairment caused by A $\beta$  accumulation in the AD mouse model (Tg2576). To illuminate the molecular mechanism of anti-inflammatory properties of RNSP, investigating the effects of RNSP on hypoxia-reoxygenation-related inflammation in MG6 microglia cells.

**Methods:** MG6 Cells were exposed to hypoxia (1% O<sub>2</sub>) for 6 h with or without pre-treatment with RNSP (10  $\mu$ g/mL), and returned to normoxia (20% O<sub>2</sub>) at different time points. RNSP cytotoxicity was examined by cell counting kit-8. Production of inflammatory mediators were examined by real-time PCR. ROS production and oxidative damage were examined by immunofluorescence images of MitoSOX Red and 8-oxo-2'-deoxyguanosine (8-oxo-dG, an oxidative DNA marker). The activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) was examined by western blotting and immunofluorescent staining of p65.

**Results:** Hypoxia significantly reduced the viability of MG6 cells from 6 h. RNSP pretreatment significantly ameliorated the hypoxia-reoxygenation (1% O<sub>2</sub> for 6 h, normoxia for 12 h, H6/R12) induced cytotoxicity in MG6 cells. RNSP pretreatment significantly suppressed the pro-inflammatory cytokines induced by H6/R24, including IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 and increased the anti-inflammatory cytokines, including TGF- $\beta$ 1 and Arginase. The expressions of p-I $\kappa$ B $\alpha$  were significantly inhibited by pretreated with RNSP, and the nuclear localizations of p65 were significantly inhibited by RNSP, respectively.

**Conclusion:** These observations suggest that the anti-inflammatory effects of RNSP on H/R-induced inflammatory responses through inhibition of the activation of NF $\kappa$ B in MG6 cells. Therefore, RNSP may be a new strategy in preventing H/R-induced neuroinflammation of AD.

**Keywords:** RNSP; A Tibetan medicine; Alzheimer's disease; Microglia; Neuroinflammation; Oxidative stress; NF- $\kappa$ B activation

## Introduction

Neuroinflammation has been generally accepted as a hallmark in Alzheimer's Disease (AD), and microglia, the resident innate immune cells in the brain, closely mediate neuroinflammation [1,2]. Recently, two research groups have provided evidence that neuroinflammation is a key driver of AD [3,4].

Hypoxia is a known neuroinflammation in the brain, as the brain requires highly oxygen supply [5]. Hypoxic conditions activate microglia to produce and secrete pro-inflammatory mediators, including interleukin-1 $\beta$  (IL-1 $\beta$ ) and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) [6,7], which may promote cognitive decline in aged people and AD patients [8,9]. Previous studies, including our own, have found that hypoxia can drive microglia to generate Reactive Oxygen Species (ROS) [10-13], which further activates nuclear factor- $\kappa$ B (NF- $\kappa$ B), resultant in the expression of pro-inflammatory mediators [11,14]. Therefore, targeting regulating neuroinflammation is a major approach for the prevention and early intervention of AD.

Ratanasampil (RNSP), Tibetan for "70-taste pearl-balls," clinically used in the treatment of hypoxia-related diseases, such as stroke. RNSP comprises around 32 kinds of plants ingredients (Saffron, Lignum Dalbergiae Odoriferae, Lagotis brachystachya, Licorice, Myrobalan, Nepeta hemsleyana etc.) 8 kinds of animal ingredients (musk, bezoar et al.), 20 kinds of mineral ingredients (Pearl and Opal etc.) and 10

kinds of mental ingredients (Gold, Silver and Copper etc.). The clinical effects of RNSP were focused on the components from plants including crocin from saffron and glycyrrhetic acid from *glycyrrhiza uralensis* [15,16]. The possible mechanisms of RNSP are considered by the antioxidant effects of crocin (Figure 1A) [17,18] and anti-inflammatory effects of glycyrrhetic acid (Figure 1B) [19,20]. In previous studies, we have shown that RNSP improves the learning and memory and reduces  $\beta$ -amyloid (A $\beta$ ) protein levels in mouse AD models [21,22]. Furthermore, RNSP was also found to improve the cognitive function and decrease the serum levels of A $\beta$ 42 and pro-inflammatory mediators in mild-to-moderate AD patients, who living at high altitude. These

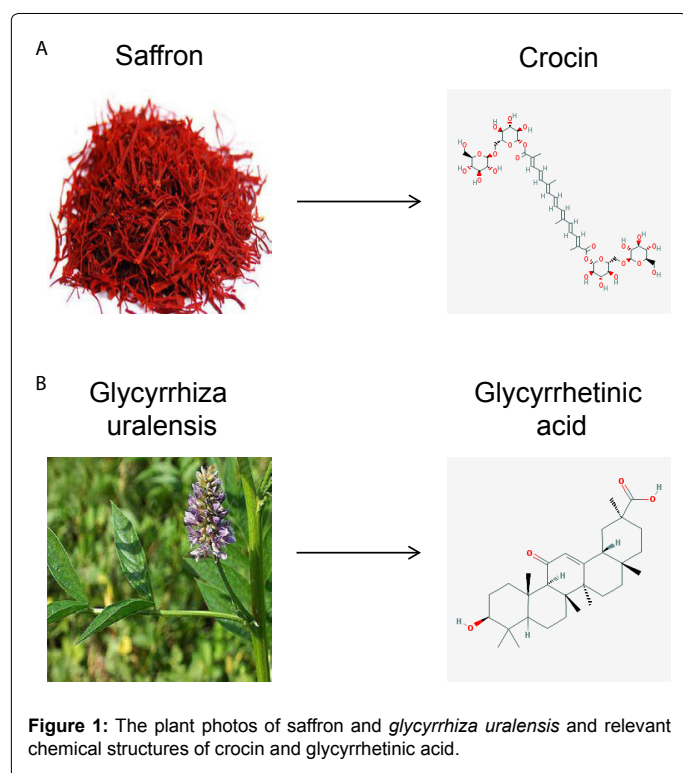
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Received: October 10, 2018; Accepted: October 22, 2018; Published: October 29, 2018

Citation: Meng J, Wu Z, Ni J, Zhong X, Hayashi Y, et al. (2018) Ratanasampil Suppresses the Hypoxia-Related Inflammatory Responses by Inhibiting Oxidative Stress and NF- $\kappa$ B Activation in Microglia. J Alzheimers Dis Parkinsonism 8: 451. doi: 10.4172/2161-0460.1000451

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**Figure 1:** The plant photos of saffron and *glycyrrhiza uralensis* and relevant chemical structures of crocin and glycyrrhetic acid.

findings suggest the utility of RNSP in clinical applications for AD [23]. However, the molecular mechanisms of the effects need to be clarified.

Our current findings concerning the neuroprotective effects of RNSP encouraged us to investigate further targets of RNSP [24]. In this study, we examined the effects of RNSP on microglia and its molecular mechanisms using MG6 microglia under Hypoxia/Reoxygenation (H/R) conditions.

## Materials and Methods

### Reagents

RNSP (Zhunzi Z63020062) was purchased from Qinghai Jinke Tibetan Medicine Pharmaceutical Co., Ltd. (Xining, China). In order to eliminate the interference caused by the methanol solvent, a suitable methanol concentration for cell culture was titrated. Antibodies against mouse anti-phospho-I $\kappa$ B $\alpha$ , rabbit anti-I $\kappa$ B $\alpha$ , mouse antiphospho-p65, P65 antibody and 8-oxo-dG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Microglia cell culture

The MG6 cell line (Riken Cell Bank, Tsukuba, Japan) was cultured in DMEM supplemented with 1% penicillin-streptomycin (Invitrogen, Grand Island, NY, USA), 100  $\mu$ mol/L  $\beta$ -mercaptoethanol, 4500 mg/L glucose (Invitrogen), 10  $\mu$ g/mL of insulin, and 10% FBS according to previously described methods.

### Assays for cell viability

MG6 cells were seeded in 96-well plates at density of  $5 \times 10^3$  cells/well overnight and then exposed to hypoxic conditions (1% O<sub>2</sub>) for 6 h, after which they were returned to normoxic conditions (20% O<sub>2</sub>) for various durations. The Cell-Counting Kit (Dojindo, Kumamoto, Japan) was used to examine relative cell viability. The cell viability was analyzed

by dividing the optical density of the H/R group by the control group. MG6 cells were treated with of RNSP ranging from 1 to 500  $\mu$ g/mL for 24 h. The cell viability was analyzed by dividing the optical density of the RNSP-treated group by that of the no-treatment group. RNSP upto 10  $\mu$ g/mL had no significant toxic effect on MG6 cells, so 10  $\mu$ g/mL RNSP was used in subsequent experiments.

### Real-time quantitative polymerase chain reaction (qRT-PCR)

RNA extraction, cDNA synthesis and qRT-PCR were performed as previously described [14]. The sequences of primer pairs were as follows: IL-1 $\beta$ : 5'-CAACCAACAAGTGATATTCTC-CATG-3' and 5'-GATCCACACTCTCAGCTGCA-3'; inducible nitric oxide synthase (iNOS): 5'-GCCACCAACAATGGCAAC-3' and 5'-CGTACCGGATGAGCTGTGAATT-3'; TNF- $\alpha$ : 5'-ATG-GCCTCCCTCTCAGTTC-3' and 5'-TTGGTGGTTTGCTAC-GACGTG-3'; IL-6: 5'-CCTCTGGTCTTCTGGAGTACC-3' and 5'-ACTCCTCTGTGACTCCAGC-3'; arginase1: 5'-CGCCTT TCT-CAAA AGGACAG-3' and 5'-CCAGCTCTTCATTGGCTTTC-3'; IL-4: 5'-TGGGTCTCAACCC CCAGTAGT-3' and 5'-TGCATGGC-GTCCCTTCTC CTGT-3'; IL-10: 5'-GACCAGCT GGACAACATACT-GCTAA-3' and 5'-GATAAGGATTGGCAACCAAGTAA-3'; TGF- $\beta$ : 5'-CCTGCAAGACCATCGATG-3' and 5'-TGTTGTACAAAGC-GAGACC-3'. Expression for all experiments was normalized to Actin.

### Detection of mitochondrial ROS

Mitochondrial ROS was examined using Mito SOX Red (Invitrogen), which is alive-cell permeant and rapidly and selectively targets mitochondria. The MG6 microglia cells were exposed to normoxia and H/R in the presence or absence of RNSP (10  $\mu$ g/mL) pre-treatment. The cells were incubated in Hank's Balanced Salt Solution (HBSS) containing 5 mM Mito SOX Red for 10 min at 37°C after H/R. After washing twice by PBS, the cells were mounted in warm buffer for imaging.

### NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> assay

MG6 were cultured at a density of  $5 \times 10^5$  cells/mL. After treatment with RNSP (10  $\mu$ g/L) for 24 h, microglia were exposed to hypoxic conditions (1% O<sub>2</sub>) for 6 h, after which they were returned to normoxic conditions (20% O<sub>2</sub>) for various durations and then collected. The amounts of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> were measured by NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> assay kits (R&D Systems, Minneapolis, USA).

### Western blotting

24 h after treatment with RNSP (10  $\mu$ g/ml), MG6 microglia cells were exposed to hypoxic conditions (1% O<sub>2</sub>) for 6 h, after which they were returned to normoxic conditions (20% O<sub>2</sub>) for various durations. The cell lysates were collected at various time points. Western blotting was performed using an SDS-PAGE electrophoresis system. Protein samples (30  $\mu$ g) were re-suspended in sample buffer and then electrophoresed on a 15% or 12% Tris gel, followed by blotting to a PVDF membrane. After blocking by 5% nonfat dry milk, the membranes were incubated at 4°C overnight with first antibody: mouse anti-phospho-I $\kappa$ B $\alpha$  (1:1000) and rabbit anti-I $\kappa$ B $\alpha$  (1:1000) antibodies. After washing, the membranes were incubated with corresponding second antibodies for 2 h at room temperature followed by ECL (Amersham Pharmacia Biotech). Immunoreactive bands were visualized using ImageQuant (LAS-4000; Fuji Photo Film, Tokyo, Japan).

### Immunofluorescence imaging

The MG6 cells were fixed with 4% paraformaldehyde after

H 6 h/R 1 h stimulation in presence or absence of RNSP pretreatment. They were then incubated with mouse anti-p65 (1:500) or mouse anti-8-oxo-dG (1:500) overnight at 4°C. The sections were incubated with donkey anti-mouse Alexus 488 (1:500; Jackson ImmunoResearch, West Grove, PA, USA) after washing by PBS followed by Hoechst (1:200) and mounted in Vectashield anti-fading medium (Vector Laboratories, Burlingame, CA, USA). Images were obtained using a confocal laser-scanning microscope (CLSM; 2si Confocal Laser Microscope, Nikon, Tokyo, Japan). The line plot profile and fluorescence intensity were analyzed using the Image J software program.

### Statistical analyses

The independent experiments and statistical analysis used (One-way ANOVA with a post hoc Tukey's test and a two-tailed unpaired Student's t-test) were all indicated in the figure legends. All values are presented as the mean  $\pm$  SEM with p values using the GraphPad Prism 7 Software (GraphPad Software Inc., San Diego, CA, USA). A value of  $p < 0.05$  was considered to indicate statistical significance.

## Results

### The effects of RNSP on the H/R-induced toxicity in microglia

We first examined the MG6 microglia viability under H/R condition using a Cell Counting Kit-8 wherein the cells were exposed to hypoxic conditions (1% O<sub>2</sub>, H) for 6 h, after which they were returned to normoxic conditions (20% O<sub>2</sub>, reoxygenation, R) for various durations. The MG6 cell viability was markedly reduced from H 6 h/R 6 h to H 6 h/R 24 h (Figure 2A). To determine the most suitable concentration of RNSP for the treatment of MG6 microglia, cells were treated by RNSP at concentrations from 1 to 500  $\mu$ g/mL for 72 h. The cell viability was not significantly changed after treatment with 1-10  $\mu$ g/mL RNSP for 72 h (Figure 2B). However, it was significantly decreased after pretreatment with over 50  $\mu$ g/mL RNSP (89% of viable cells). We therefore used RNSP at 10  $\mu$ g/mL and H 6 h/R 24 h to examine the effects of RNSP on the H/R-induced cytotoxicity of MG6 microglia in the subsequent experiments.

Pretreatment with RNSP for 24 h significantly inhibited the H/R-induced cytotoxicity in MG6 microglia (Figure 2C). It strongly demonstrates that pretreatment with RNSP protects MG6 microglia

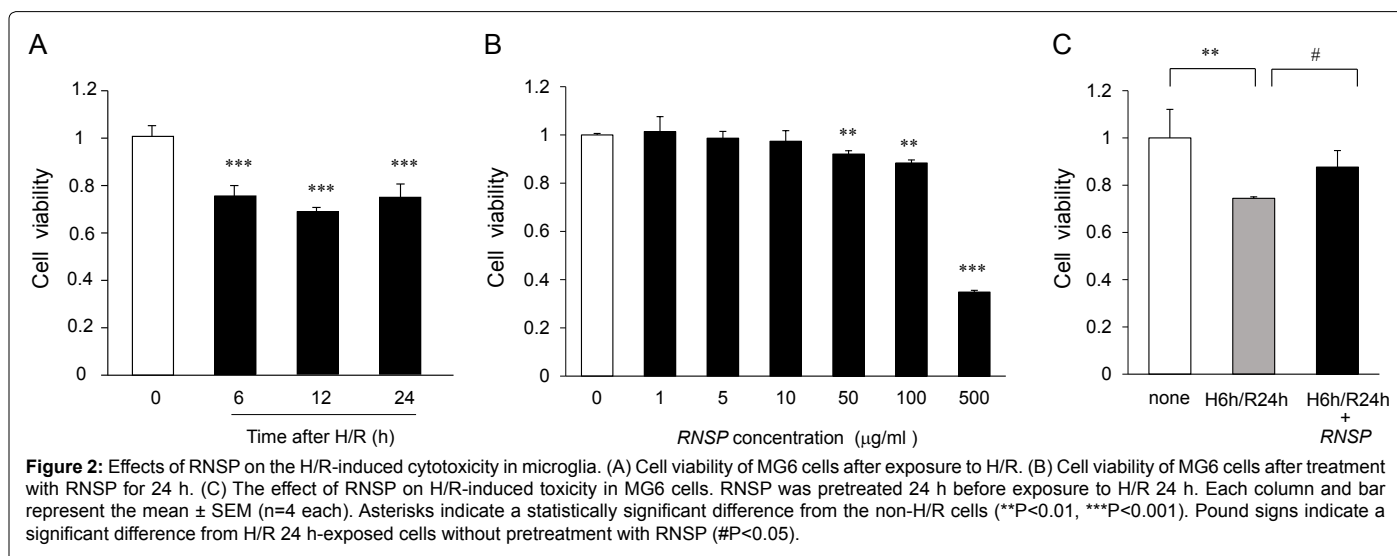
from H/R-induced cytotoxicity. Therefore, pretreatment with RNSP (10  $\mu$ g/mL) for 24 h was used in the subsequent experiments.

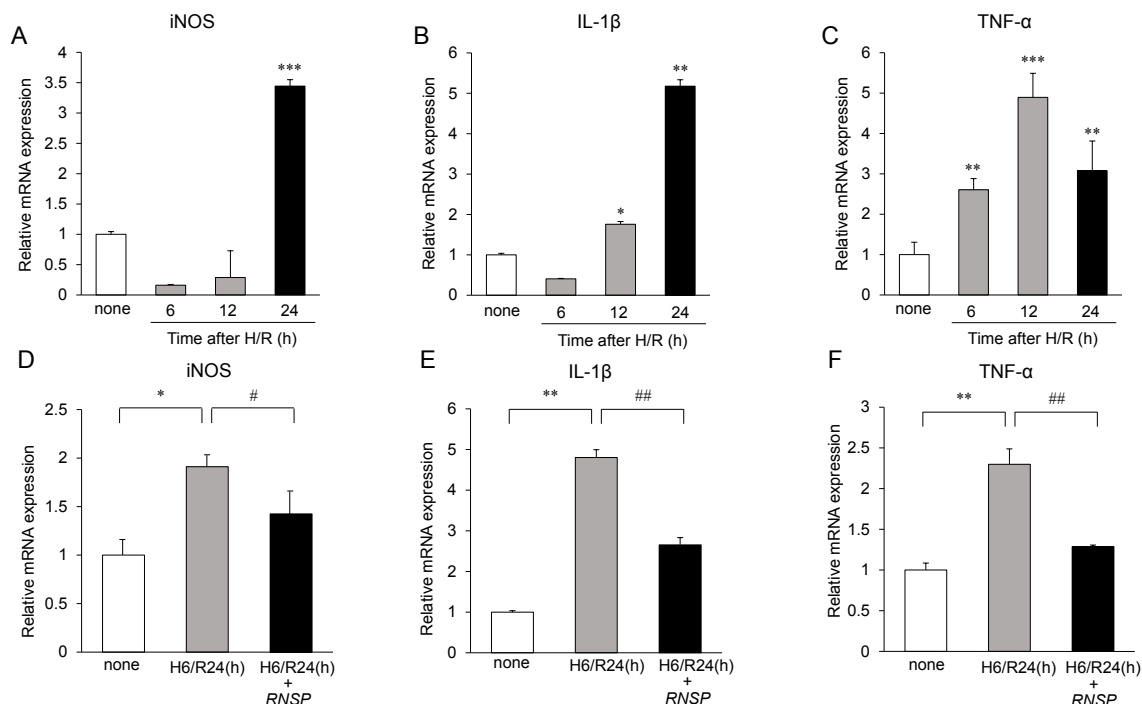
### The effects of RNSP on the H/R-induced expression of pro-inflammatory mediators in microglia

Microglia-related neuroinflammation is involved in the cognitive deficits seen in AD patients [2-4]. Therefore, we next examined the effects of RNSP (10  $\mu$ g/mL) on the H/R-induced expression of pro-inflammatory mediators by microglia. The mean mRNA expression of iNOS, IL-1 $\beta$  and TNF- $\alpha$  during H/R exposure were assessed using quantitative RT-PCR. The mean mRNA expression of iNOS was slightly decreased from H/R 6 h to H/R 12 h but significantly increased at H 6 h/R 24 h (Figure 3A). The mean mRNA expression of IL-1 $\beta$  were slightly decreased at H/R 6 h but significantly increased from H/R 12 h to H/R 24 h (Figure 3B). The mean mRNA expression of TNF- $\alpha$  were significantly increased from H 6/R 6 h to H 6/R 24 h (Figure 3C). These observations indicate that the pro-inflammatory mediators are significantly increased at the lately times (H/R 24 h) during H/R exposure. It was noted that RNSP (10  $\mu$ g/mL) significantly inhibited the mean mRNA expression of iNOS, IL-1 $\beta$  and TNF- $\alpha$  in MG6 microglia at H 6/R 24 h (Figures 3D-3F).

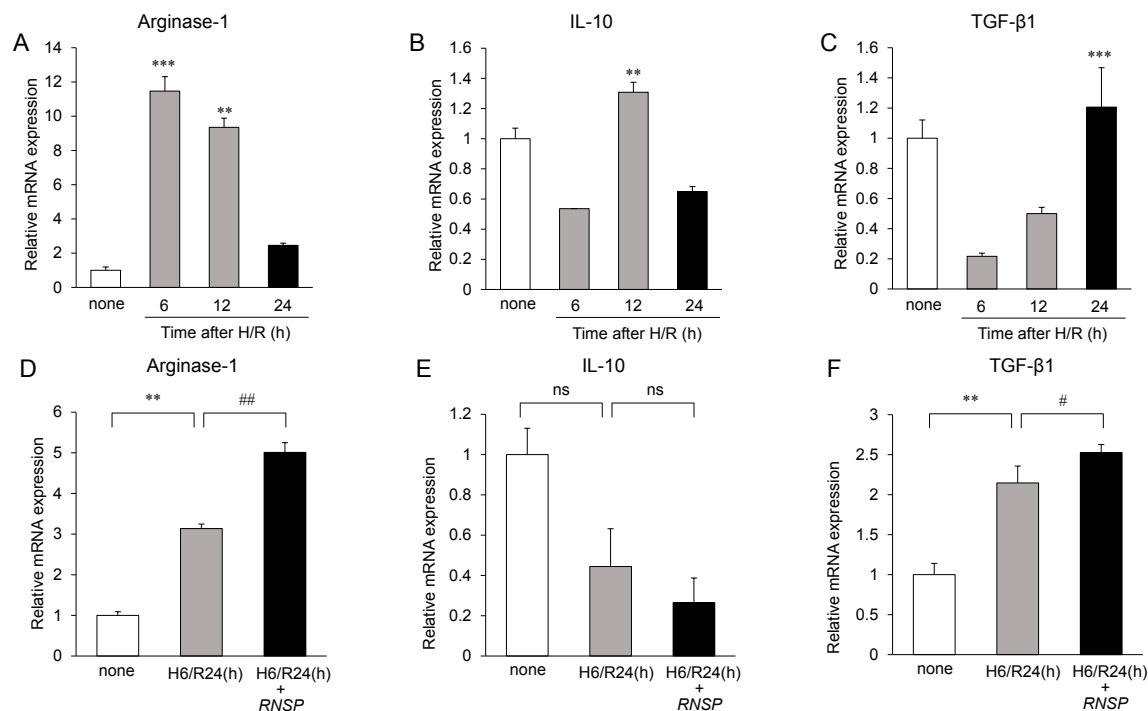
### The effects of RNSP on the H/R induced expression of anti-inflammatory mediators in microglia

To further explore the effects of RNSP on microglia-related neuroinflammation, the effects of RNSP (10  $\mu$ g/mL) on the H/R-induced expression of anti-inflammatory mediators by MG6 microglia were examined under H/R exposure. The mean mRNA expression of arginase-1 was significantly increased from H/R 6 h to H/R 12 h but sharply decreased at H/R 24 h (Figure 4A). The mean mRNA expression of IL-10 was significantly increased at H/R 12 h and decreased at H/R 24 h (Figure 4B). However, the mean mRNA expression of TGF- $\beta$ 1 was slightly decreased from H/R 6 h to H/R 12 h but significantly increased at H/R 24 h (Figure 4C). These observations indicate that the anti-inflammatory mediators were significantly decreased at later times during H/R exposure. It was noted that RNSP (10  $\mu$ g/mL) significantly increased the mean mRNA expression of arginase-1 and TGF- $\beta$ 1 in MG6 microglia at H 6 h/R 24 h; however, the effect of RNSP on IL-10 has not been determined (Figures 4D-4F).





**Figure 3:** Inhibitory effects of RNSP on the H/R-induced expression of pro-inflammatory mediators in microglia. The relative mRNA expression of iNOS (A), IL-1 $\beta$  (B) and TNF- $\alpha$  (C) in MG6 cells after exposure to H 6 h/R 6 h, H 6 h/R 12 h and H 6 h/R 24 h. The mRNA expression of iNOS (D), IL-1 $\beta$  (E) and TNF- $\alpha$  (F) in MG6 cells after exposure to H 6 h/R 24 h with or without pretreatment with RNSP (10  $\mu$ g/mL) for 24 h. Each column and bar represent the mean  $\pm$  SEM (n=4 each). An asterisk indicates a significant difference from the normoxic conditions (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001). Pound signs indicate a significant difference from H/R-exposed cells without pretreatment with RNSP (# $P$ <0.05, ## $P$ <0.01).



**Figure 4:** Effects of RNSP on the H/R-induced expression of anti-inflammatory mediators in microglia. The expression of anti-inflammatory cytokines induced by H/R with time-dependence. The relative mRNA expression of arginase-1 (A), IL-10 (B) and TGF- $\beta$ 1 (C) in MG6 cells after exposure to H 6 h/R 6 h, H 6 h/R 12 h and H 6 h/R 24 h. The mRNA expression of arginase-1 (D), IL-10 (E) and TGF- $\beta$ 1 (F) in MG6 cells after exposure to H 6 h/R 24 h with or without pretreatment with RNSP (10  $\mu$ g/mL) for 24 h. Each column and bar represent the mean  $\pm$  SEM (n=4 each). An asterisk indicates a significant difference from the normoxic and H/R cells (\*\* $P$ <0.01, \*\*\* $P$ <0.001). Pound signs indicate a significant difference from the H/R-exposed cells without pretreatment with RNSP (# $P$ <0.05, ## $P$ <0.01).

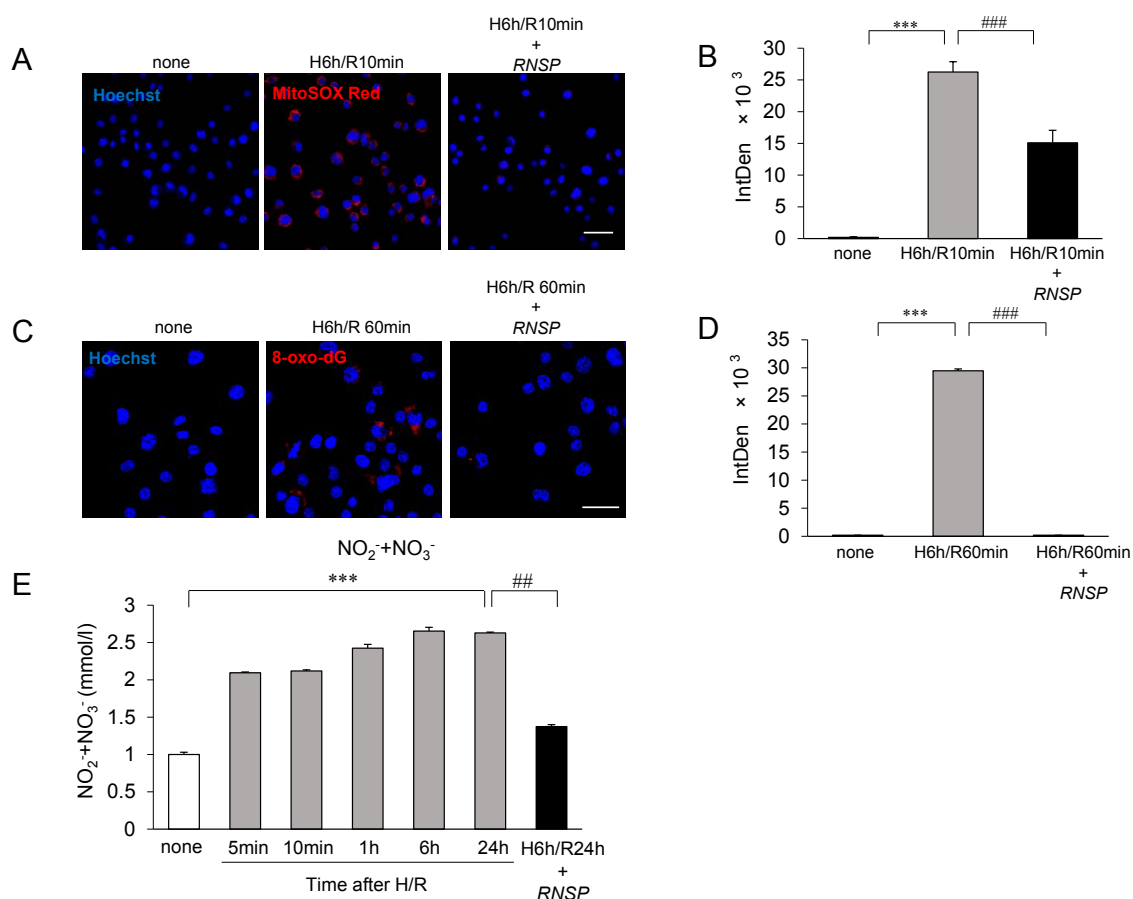
## The effects of RNSP on the H/R-induced oxidative stress in microglia

Our previous study showed that hypoxia-induced mitochondrial oxidant generation was involved in oxidative stress in microglia [14]. These observations prompted us to examine the effects of RNSP on H/R-induced oxidative stress in microglia using two approaches: MitoSOX Red probe was used to examine mitochondria-derived ROS generation [25] and immunofluorescence imaging for 8-oxo-dG was to examine the DNA oxidation [26]. Compared to the untreated MG6 microglia, the immunofluorescence intensity of MitoSOX Red was significantly increased in MG6 microglia at H/R10 min (Figures 5A and 5B), suggesting the ROS under H/R conditions were derived from mitochondria. Pretreatment with RNSP (10  $\mu$ g/mL) significantly inhibited the mean fluorescent intensity of MitoSOX Red in microglia at H/R 10 min (Figures 5A and 5B), thus suggesting the early antioxidant effects of RNSP on microglia. Immunofluorescence imaging showed 8-oxo-dG was significantly increased comparing to the cells without exposure to H/R 1 h ( $***p<0.001$ ). The H/R-induced NO production in microglia was further examined. Compared to the untreated MG6

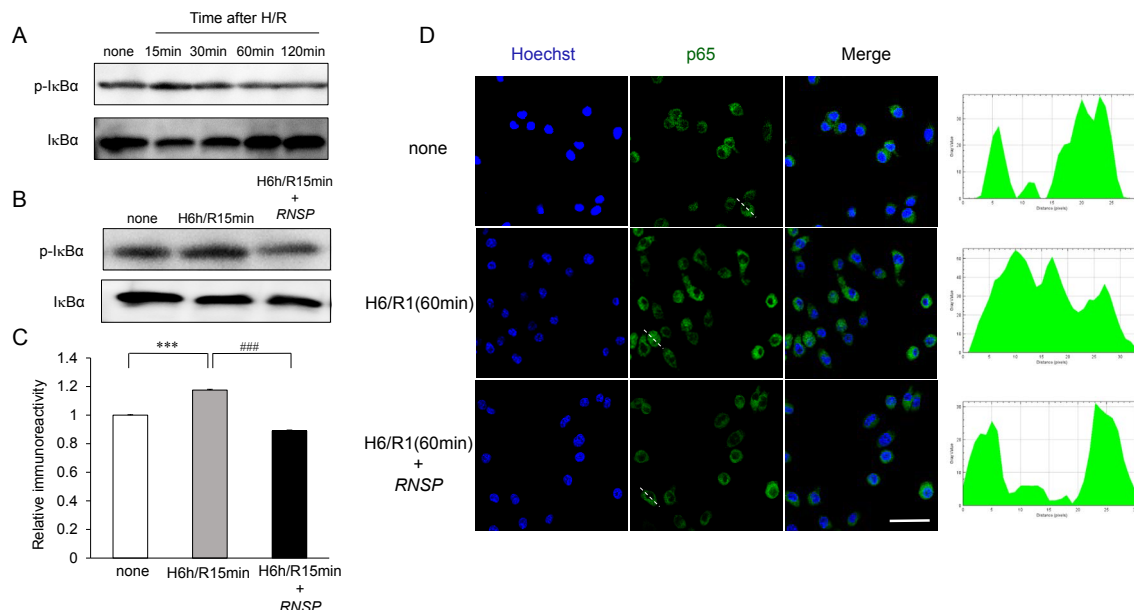
cells, the mean levels of  $\text{NO}_2^-/\text{NO}_3^-$  were significantly increased from H/R 5 min to H/R 24 h in the in the culture medium of MG6 cells, and pretreatment with RNSP (10  $\mu$ g/mL) markedly inhibited the mean levels of  $\text{NO}_2^-/\text{NO}_3^-$  at H/R 24 h in MG6 cells (Figures 5C-5E). These findings confirm that RNSP inhibits the H/R- induced oxidative stress in microglia.

## The effects of RNSP on the H/R-induced NF- $\kappa$ B activation in microglia

Finally, the effects of RNSP on the activation of NF- $\kappa$ B during H/R exposure were examined, as NF- $\kappa$ B regulates most of inflammatory molecules. Compared with the untreated cells, the phosphorylation of I $\kappa$ B $\alpha$  in MG6 microglia was significantly increased from H/R 15 min to H/R 30 min and gradually recovered to the base levels at H/R 2 h (Figure 6A). Pretreatment with RNSP (10  $\mu$ g/mL) significantly inhibited the H/R- induced phosphorylation of I $\kappa$ B $\alpha$  in microglia (Figure 6B and 6C). Furthermore, p65 nuclear translocation was induced in MG6 microglia at H/R 60 min, and pretreatment with RNSP (10  $\mu$ g/mL) significantly inhibited the H/R-induced p65 nuclear translocation in



**Figure 5:** Inhibitory effect of RNSP on H/R-induced mitochondria ROS, DNA damage and NO production by microglia. (A) Fluorescent images of MitoSOX Red fluorescence signals in MG6 cells after exposure to H 6 h/R 10 min in the presence or absence of pretreatment with RNSP (10  $\mu$ g/mL) for 24 h. Scale bar=30  $\mu$ m. (B) The quantitative analyses of MitoSOX Red fluorescence intensity in (A). Each column and bar represent the mean  $\pm$  SEM (n=4 each). An asterisk indicates a significant difference from the normoxia and H/R cells ( $***P<0.001$ ). Pound signs indicate a significant difference from the H/R-exposed cells without RNSP ( $###P<0.001$ ). (C) CLMS images of 8-oxo-dG (red) with nuclei (blue) in the H/R 1 h-exposed cells, with or without pretreatment with RNSP (10  $\mu$ g/mL) for 24 h. Scale bar=30  $\mu$ m. (D) The quantitative analyses of the 8-oxo-dG immunofluorescence intensity in (C). Each column and bar represent the mean  $\pm$  SEM (n=4 each). Asterisks indicate a significant difference from the untreated cells ( $***P<0.001$ ). Pound signs indicate a significant difference from the H/R- exposed cells without RNSP ( $###P<0.001$ ). (E) Examination of NO metabolites in the MG6 cells at different time points under normoxic or H/R conditions with or without pre-treatment of RNSP (10  $\mu$ g/mL) by an  $\text{NO}_2^-/\text{NO}_3^-$  assay. Each column and bar represent the mean  $\pm$  SEM (n=4 each). An asterisk indicates a significant difference from the normoxia and H/R cells ( $***P<0.001$ ). Pound signs indicate a significant difference from the H/R- exposed cells without RNSP ( $###P<0.01$ ,  $####P<0.001$ ).



**Figure 6:** Effects of RNSP on the H/R-induced NF- $\kappa$ B activation. (A) Immunoblots showing expression of phospho-I $\kappa$ B $\alpha$  at different time points under H/R conditions. (B) Immunoblots showing expression of phospho-I $\kappa$ B $\alpha$  in MG6 cells at 15 min under H/R conditions with or without pretreatment with RNSP (10  $\mu$ g/mL) for 24 h. (C) The quantitative analyses of immunoblotting for phospho-I $\kappa$ B $\alpha$  (B). An asterisk indicates a significant difference from the H/R and H/R + RNSP-treated cells (\*\*\*\* $P$ <0.001). (D) CLMS images of p65 (green) in MG6 cells with nuclei (blue) under H 6 h/R 1 h in the presence or absence of RNSP (10  $\mu$ g/mL). Scale bar=30  $\mu$ m. The typical cells in (D) were analyzed by a line plot profile to indicate the cytosol and nuclear location of p65.

microglia (Figure 6D). These findings confirm that RNSP suppresses the H/R-induced NF- $\kappa$ B activation in microglia.

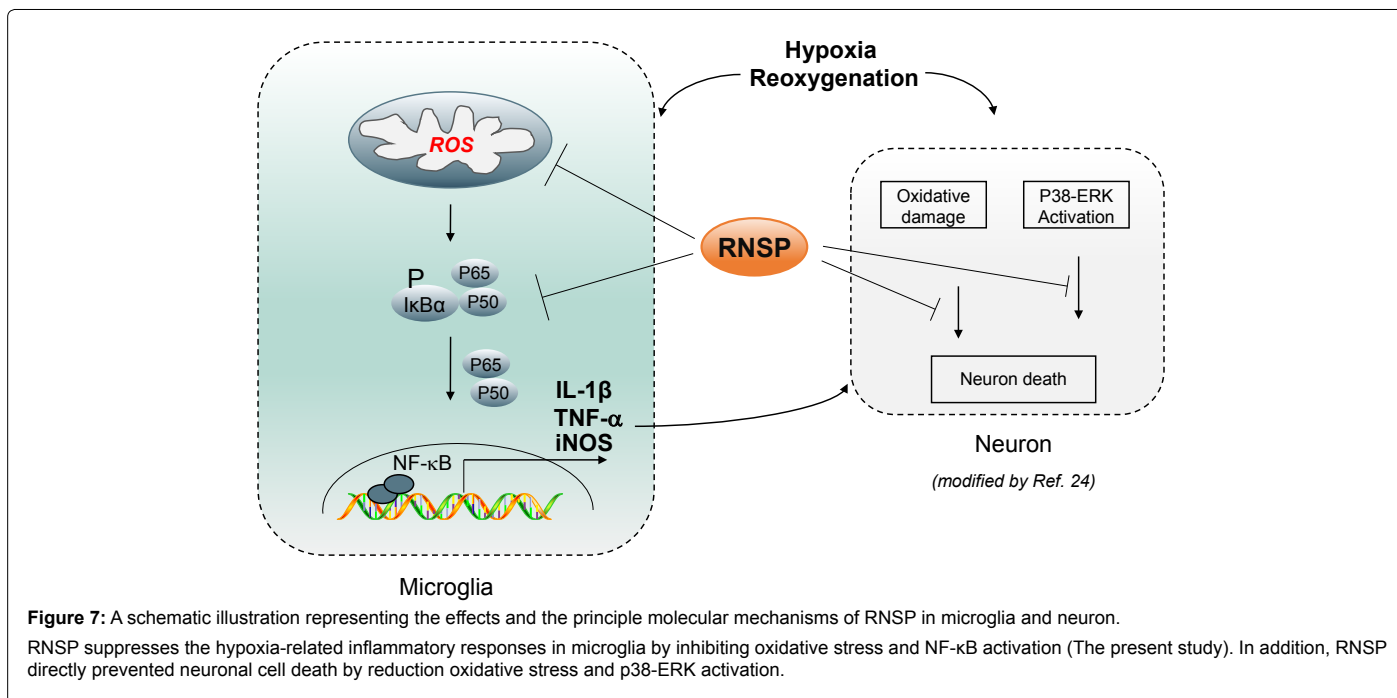
## Discussion

The present study indicates that RNSP protects against H/R-induced cytotoxicity and regulates the H/R-induced inflammatory responses in MG6 microglia by reducing the oxidative stress and NF- $\kappa$ B activation (summarized in Figure 6). This is first to describe the principle molecular mechanisms underlying the clinical benefits of RNSP in AD patients. Oxidative stress was proved to damage the cellular components, including DNA, resulting in subsequent cell death [27]. Present observations found the intensity of MitoSOX Red, a marker for mitochondria-derived ROS generation, increased as quickly as at H/R 10 min, which involved 10 min of reoxygenation after hypoxia, and the fluorescent intensity of 8-oxo-dG, a biomarker for oxidative stress-damaged DNA [26] was significantly increased from H/R 1 h, which involved 1 h of reoxygenation after hypoxia in the MG6 microglia. These findings indicated that mitochondria are the origin of ROS generation, thus inducing oxidative stress in microglia. In addition, the increased levels of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>, which are metabolic agents for NO production, persisted through H/R 24 h, indicating the continuative ROS overproduction due to oxidative stress in microglia under H/R conditions. Of note, pretreatment with RNSP significantly inhibited the H/R-induced mitochondrial ROS generation, 8-oxo-dG expression and NO production (Figure 5), resulting in the protection against subsequent cell death in microglia (Figure 2). This indicated that RNSP was able to reduce oxidative stress in microglia (Figure 7).

Therefore, the clinical effects of RNSP on improving the cognitive functions in mild-to- moderate AD patients living at high altitude may due to a reduction in oxidative stress [23]. NF- $\kappa$ B activation is rapidly and transiently induced by oxidative stress [28]. In the present study,

phosphorylation of I $\kappa$ B $\alpha$  in MG6 was detected after H/R 15 min, and p65 nuclear translocation was induced at H/R 60 min, which suggests that NF- $\kappa$ B activation is associated with an increased intracellular redox state during H/R 60 min [29]. NF- $\kappa$ B activation polarizes microglia into the neurotoxic phenotype, as NF- $\kappa$ B is a transcription factor that encodes the genes of the pro-inflammatory (neurotoxic) mediators, such as IL-1 $\beta$ , TNF- $\alpha$  and iNOS [30]. In the present study, the increased expression of neurotoxic mediators (IL-1 $\beta$ , TNF- $\alpha$  and iNOS) paralleled the decreased expression of neuroprotective (anti-inflammatory) mediators (arginase-1 and IL-10) at H/R 24 h in MG6 microglia, indicating that microglia are shifted to the neurotoxic phenotype at the later phases under H/R conditions. Indeed, the lasting expression of neurotoxic mediators establishes a feedforward loop for NF- $\kappa$ B activation, as pro-inflammatory mediators such as IL-1 $\beta$  promote NF- $\kappa$ B activation [31]. Pretreatment with RNSP significantly decreases the H/R-induced NF- $\kappa$ B activation and the expression of pro-inflammatory mediators but reverses the H/R-decreased expression of the anti-inflammatory mediator TGF $\beta$ 1 in microglia (Figures 3-6), suggesting that RNSP may be able to ameliorate the microglia-mediated neuroinflammation and shift activated microglia to neuroprotective phenotypes. The effects of RNSP on ameliorating microglia-mediated neuroinflammation will guide its clinical usage in the early intervention of AD, as microglia-mediated neuroinflammation is widely accepted as an early hallmark in AD [2-4,32]. The beneficial effects of RNSP on microglia-mediated neuroinflammation may depend on its anti-pro-inflammatory components, such as *glycyrrhiza uralensis* [19, 20].

Hypoxia is known to worsen the cognitive function, as evidenced by hypoxia-suffering mountaineers having a deteriorated memory [33,34] and healthy individuals living in high altitude having greater cognitive dysfunctions than those at lower altitudes [35]. The brain



requires oxygen, which is largely dependent on the cerebral blood flow [36,37], and the cerebral blood flow is slowed with aging and further decreased in AD patients [38,39]. This low cerebral oxygen availability results in more cognitive defects [40]. Therefore, chronic hypoxia may contribute to the cognitive decline in aging individuals as well as AD patients [40,41]. The microglial proliferation and activation associated with neuronal loss could be histologically observed in human AD brain [9,32]. It is well known that hypoxia activated microglia induce neuronal death by producing IL-1 $\beta$ , TNF- $\alpha$  as well as and IL-6 [2,6,9]. And hypoxia shifts microglia into neurotoxic phenotype in ROS-dependent [14]. In the present cultured cell study, we give the first evidence that RNSP inhibiting the H/R induced productions of ROS and neurotoxic mediators in microglia, thus demonstrate the anti-inflammation and antioxidant effects of RNSP on microglia. Taking together with the effects on mitigating microglia-related neuroinflammation and the directly neuroprotective effects on neurons [24], RNSP could be used to prevent or treat for delaying pathophysiology of AD and other neurodegenerative diseases. These findings along with the observation of the direct roles of RNSP in neuroprotection and microglia regulation prove the clinical benefits of RNSP in the prevention and management of AD [23].

## Conclusion

The present study provides the first evidence of the potential protective effects of RNSP on the hypoxia-related neuroinflammatory responses in microglia. The effects were dependent on reducing the oxidative stress and NF- $\kappa$ B activation, highlighting a new molecular target for RNSP in the clinical intervention of AD.

## Acknowledgement

This work was supported by funding from the National Natural Science Foundation of China (No. 81560711), Qinghai Province Natural Science Foundation (No.2012-Z913) and the China Scholarship Council (No. 201608635002) to Aiqin Zhu and Grants-in-Aid for Scientific Research (16K11478, 16H0848) from MEXT (Japan) to Zhou Wu.

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