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kinds of mental ingredients (Gold, Silver and Copper etc.). e clinical e ects of RNSP were focused on the components from plants including crocin from sa ron and glycyrrhetinic acid from *glycyrrhiza uralensis* [15,16]. e possible mechanisms of RNSP are considered by the antioxidant e ects of crocin (Figure 1A) [17,18] and anti-in ammatory e ects of glycyrrhetinic acid (Figure 1B) [19,20]. In previous studies, we have shown that RNSP improves the learning and memory and reduces β-amyloid (Aβ) protein levels in mouse AD models [21,22]. Furthermore, RNSP was also found to improve the cognitive function and decrease the serum levels of Aβ42 and pro-in ammatory mediators in mild-to-moderate AD patients, who living at high altitude. ese

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ndings suggest the utility of RNSP in clinical applications for AD [23]. However, the molecular mechanisms of the e ects need to be clari ed.

Our current ndings concerning the neuroprotective e ects of RNSP encouraged us to investigate further targets of RNSP [24]. In this study, we examined the e ects 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-IκBα, rabbit anti-IκBα, mouse antiphospho-p65, P65 antibody and 8-oxo-dG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Microglia cell culture

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

Assays for cell viability

H 6 h/R 1 h stimulation in presence or absence of RNSP pre-treatment. 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

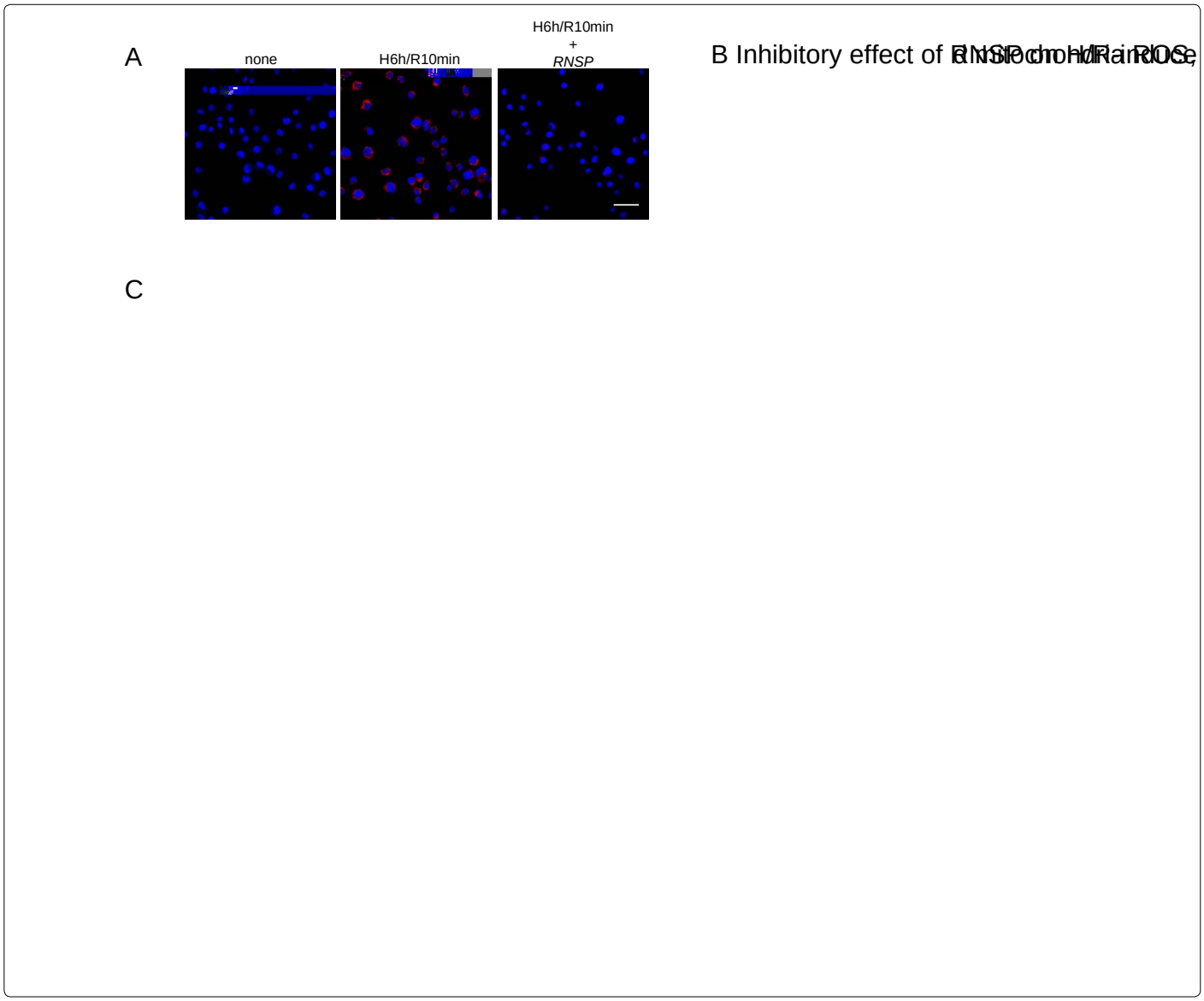
e independent experiments and statistical analysis used (One-way ANOVA with a post hoc Tukey's test and a two-tailed unpaired

e e ects 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]. ese observations prompted us to examine the e ects 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 immuno uorescence 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 signi cantly 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 ug/mL) signi cantly inhibited the mean fluorescent intensity of MitoSOX Red in microglia at H/R 10 min (Figures 5A and 5B), thus suggesting the early antioxidant e ects of RNSP on microglia. Immuno uorescence imaging showed 8-oxo- dG was signi cantly increased comparing to the cells without exposure to H/R 1 h (***p<0.001). e H/R-induced NO production in microglia was further examined. Compared to the untreated MG6 cells, the mean levels of NO_2-/NO_3- were signi cantly 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 μg/mL) markly inhibited the mean levels of NO_2-/NO_3- at H/R 24 h in MG6 cells (Figures 5C-5E). ese ndings con rm that RNSP inhibits the H/R- induced oxidative stress in microglia.

e e ects of RNSP on the H/R-induced NF-κB activation in microglia

Finally, the e ects of RNSP on the activation of NF-κB during H/R exposure were examined, as NF-κB regulates most of in ammatory molecules. Compared with the untreated cells, the phosphorylation of IκBα in MG6 microglia was signi cantly 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 μg/mL) signi cantly inhibited the H/R- induced phosphorylation of IκBα 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 μg/mL) signi cantly inhibited the H/R-induced p65 nuclear translocation in



microglia (Figure 6D). ese ndings con rm that RNSP suppresses the H/R-induced NF-κB activation in microglia.

Discussion

e present study indicates that RNSP protects against H/R-induced cytotoxicity and regulates the H/R-induced in ammatory responses in MG6 microglia by reducing the oxidative stress and NF-κB activation (summarized in Figure 6). is is rst to describe the principle molecular mechanisms underlying the clinical bene ts 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 a er hypoxia, and the uorescent intensity of 8-oxo-dG, a biomarker for oxidative stress-damaged DNA [26] was signi cantly increased from H/R 1 h, which involved 1 h of reoxygenation a er hypoxia in the MG6 microglia. ese ndings indicated that mitochondria are the origin of ROS generation, thus inducing oxidative stress in microglia. In addition, the increased levels of NO_2^-/NO_3^-, 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 signi cantly 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). is indicated that RNSP was able to reduce oxidative stress in microglia (Figure 7).

erefore, the clinical e ects 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-κB activation is rapidly and transiently induced by oxidative stress [28]. In the present study, phosphorylation of IκBα in MG6 was detected a er H/R 15 min, and p65 nuclear translocation was induced at H/R 60 min, which suggests that NF-κB activation is associated with an increased intracellular redox state during H/R 60 min [29]. NF-κB activation polarizes microglia into the neurotoxic phenotype, as NF-κB is a transcription factor that encodes the genes of the pro-in ammatory (neurotoxic) mediators, such as IL-1β, TNF-α and iNOS [30]. In the present study, the increased expression of neurotoxic mediators (IL-1β, TNF-α and iNOS) paralleled the decreased expression of neuroprotective (anti-in ammatory) mediators (arginase-1 and IL-10) at H/R 24 h in MG6 microglia, indicating that microglia are shi ed 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-κB activation, as pro- in ammatory mediators such as IL-1β promote NF-κB activation [31]. Pretreatment with RNSP signi cantly decreases the H/R-induced NF-κB activation and the expression of pro-in ammatory mediators but reverses the H/R-decreased expression of the anti- in ammatory mediator TGFβ1 in microglia (Figures 3-6), suggesting that RNSP may be able to ameliorate the microglia-mediated neuroin ammation and shi activated microglia to neuroprotective phenotypes. e e ects of RNSP on ameliorating microglia-mediated neuromedi(og)-77elia-medihe c

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]. is low cerebral oxygen availability results in more cognitive defects [40]. erefore, chronic hypoxia may contribute to the cognitive decline in aging individuals as well as AD patients [40,41]. e 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-1β, TNF- α 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. ese 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

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

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