Target engagement of ginsenosides in mild cognitive impairment using mass spectrometry-based drug affinity responsive target stability

Zhu Zhu, Ruimei Li, Wei Qin, Hantao Zhang, Yao Cheng, Feiyan Chen, Cuihua Chen, Lin Chen, Yunan Zhao

Background: Mild cognitive impairment (MCI) is a transitional condition between normality and dementia. Ginseng is known to have effects on attenuating cognitive deficits in neurogenerative diseases. Ginsenosides are the main bioactive component of ginseng, and their protein targets have not been fully understood. Furthermore, no thorough analysis is reported in ginsenoside-related protein targets in MCI.

Methods: The candidate protein targets of ginsenosides in brain tissues were identified by drug affinity responsive target stability (DARTS) coupled with label-free liquid chromatography-mass spectrometry (LC–MS) analysis. Network pharmacology approach was used to collect the therapeutic targets for MCI. Based on the above-mentioned overlapping targets, we built up a protein–protein interaction (PPI) network in STRING database and conducted gene ontology (GO) enrichment analysis. Finally, we assessed the effects of ginseng total saponins (GTS) and different ginsenosides on mitochondrial function by measuring the activity of the mitochondrial respiratory chain complex and performing molecular docking.

Results: We screened 2526 MCI-related protein targets by databases and 349 ginsenoside-related protein targets by DARTS. On the basis of these 81 overlapping genes, enrichment analysis showed the mitochondria played an important role in GTS-mediated MCI pharmacological process. Mitochondrial function analysis showed GTS, protopanaxatriol (PPT), and Rd increased the activities of complex I in a dose-dependent manner. Molecular docking also predicted the docking pockets between PPT or Rd and mitochondrial respiratory chain complex I.

Conclusion: This study indicated that ginsenosides might alleviate MCI by targeting respiratory chain complex I and regulating mitochondrial function, supporting ginseng’s therapeutic application in cognitive deficits.

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1. Introduction

Mild cognitive impairment (MCI) refers to a newly acquired cognitive deficits that is more severe than expected, and the memory function of these individuals is abnormal for the same age and educational background. However, general cognitive function of daily living is preserved [1]. Neurocognitive disorders (NCD) may develop insidiously and progress gradually. MCI may present an incipient stage of NCD, and the initial symptom is a decline in memory [2]. Pathological changes such as amyloid plaques and agminated tau protein in the olfactory system can be found in MCI patients [3]. The prevalence of MCI increases with age (approximately 6.7%–25.2% in adults older than 60) [4]. Moreover, MCI patients tend to have high risk at a rate of 10%–15% per year of...
developing Alzheimer’s disease (AD). By contrast, the annual rate of AD in normal older subjects is 1%–2% [5]. Therefore, exploring the effective strategies to prevent and delay the progress of MCI is urgent.

Ginseng, the root of Panax ginseng Meyer, which belongs to the family Araliaceae, is used as a traditional herbal medicine for more than two thousand years. The early Chinese empirically uses ginseng in stews and soups in medicinal diets to improve cognition and mood [6]. A great deal of clinical and preclinical studies have reported the effects of ginseng extracts and ginsenosides (main bioactive components in ginseng) are considered related to memory, attention, and other features of cognitive function, particularly those associated with aging and memory deterioration [7]. Furthermore, previous studies demonstrated that ginseng and ginsenosides had neuroprotective effects and could improve cognitive impairments via promoting synaptic plasticity, increasing the levels of neurotransmitter, anti-oxidation, and inhibiting apoptosis in neurons [8–11]. Zhao et al. found that long-term administration of ginseng total saponins (GTS) for 8 months could improve the performance of 12-month-old C57BL/6J mice in Morris water maze and step-down test by upregulating brain-derived nerve growth factor (BDNF) and synaptic-plasticity-related proteins [12]. These findings indicates that ginsenosides produce positive effects on MCI in clinical and preclinical studies. However, the protein targets of ginsenosides intervening the progress of MCI remain unknown.

Identification of the small molecules target proteins is crucial to understanding the mechanisms of action as molecular probes and potential therapeutic drugs. A variety of methods have been developed for target identification of small molecules and natural products. Drug affinity responsive target stability (DARTS) is a novel method for the direct identification of target proteins without modification of drugs. It takes advantage of the reduction in the protease susceptibility of the target proteins upon drug binding. Binding of a drug to a target protein may result in local or global stabilization of protein conformation and/or alter the availability of protein hydrolysis. The binding of compounds may mask the sites of proteolysis, leading to differences in the conjugated and free proteolytic patterns [13]. Therefore, DARTS is accomplished by simply processing aliquots of cell or tissue lysate with the compound of interest and either vehicle control or an inactive analog, followed by using proteases to digest the proteins in cell or tissue lysates in a limited way. The potential target proteins are then further validated by the proteomics techniques, such as mass spectrometry (MS). To date, DARTS is a valuable research paradigm that investigates the protein targets for drugs to treat a certain disease, which facilitates the understanding of the therapeutic effects of drugs.

In this study, we aimed to identify the protein targets of ginsenosides in brain tissues via MS-based DARTS. We then used network-based analysis to determine the protein targets of ginsenosides in MCI. Finally, we verified major protein targets of ginsenosides using enzyme activity assay and computational analysis to provide a basis for the study of ginsenoside pharmacological mechanisms in MCI (Fig. 1).

2. Materials and methods

2.1. Preparation of high-purity GTS

Chinese ginseng (particularly the root of Panax ginseng) was obtained from Beijing Tong Ren Tang Group Co., Ltd. (Beijing, China). Following our previous methodology, high-purity GTS was purified and powdered as described [14]. The ginsenosides Rg1, Rg2, Re, Rb2, Rb3, Rh1, Rc, Rd, F2, and Rg3 were detected in high-purity GTS. For quality control, the estimated contents of total saponins were 107% by the colorimetric method with a vanillin vitriol system and 90% by the ultrahigh-performance liquid chromatography with a charged aerosol detector [15]. Rg1, Rb1, Rd, Rh1, Rh2, 20(S)-protopanaxadiol (PPD), and 20(S)-protopanaxatriol (PPT) standards (purity >98%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).
2.2. Animals

Male adult Sprague-Dawley rats weighing 180–220 g were purchased from Laboratory Animal Center of Nanjing University of Chinese Medicine. Rats were kept at 18 °C–22 °C with a 12 h/12 h light/dark cycle and given free access to water and food. Rats needed for brain tissue were sacrificed under deep anesthesia with pentobarbital sodium. The samples in the present study were conducted in accordance with the Guidelines of Accommodation and Care for Animals formulated by the Chinese Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (Permit Number: 201909A030).

2.3. DARTS

The protocol in performing DARTS was modified from Pai et al. [16]. Three 12-month-old rats were decapitated under deep anesthesia with pentobarbital sodium. Brain sections were quickly isolated, and the hippocampus was homogenized in lysis buffer (pH – 7.4). The homogenate (2 mg/μL) was then separated in the same aliquots. Each aliquot was treated with GTS (10 μg/mL) or pure water (0.6%). After incubation for 20 min at room temperature, the GTS- or vehicle-treated solutions were divided into 40 μL in each PCR tube and digested individually for 20 min at 25 °C by adding 4 μL of pronase solution (pronase:protein ratio = 1:800) in a Biosafser PCR machine (Safer Biotech Co., Nanjing, China). For the non-digested sample, 4 μL of TNC buffer (50 mM of Tris–HCl, 50 mM of NaCl, 10 mM of CaCl2, and pH 8.0) was added instead of protease. Digestion reaction was stopped by adding 4 μL of 20 × protease inhibitor cocktail (Roche) and incubated for 10 min on ice. Finally, the samples were analyzed by a label-free liquid chromatography tandem mass spectrometry (LC–MS–MS).

2.4. Label-free LC–MS and data analysis

Quantitative proteomic analysis was performed using label-free LC–MS analysis, which was carried out through the technical services provided by AIMS (Shanghai, China; http://www.aims.mass.cn/index.jsp). The MS data were retrieved from the UniProt human database and were analyzed using MaxQuant software (version 1.6.10).

2.5. Identification of disease-related targets for MCI

In this study, the target genes for MCI were collected from two databases, including Gene Cards (https://www.genecards.org/) [17] and Online Mendelian Inheritance in Man (OMIM) database (http://www.omim.org/) [18].

2.6. Protein–protein interaction (PPI) network construction

STRING online database (https://string-db.org/) was used to obtain PPI data of overlapping targets [19]. Then, using Cytoscape 3.6.1 software, the key genes were screened by the Matthews correlation coefficient (MCC) algorithm; the interactions among genes were analyzed; the number of nodes of each gene was counted, and the PPI network of the core genes was constructed.

2.7. Gene ontology (GO) enrichment analysis

GO analysis was performed by DAVID (https://david.ncifcrf.gov/). There are three types of literature-derived or experimentally validated evidence, including biological processes (BP), cellular component (CC) and molecular function (MF). The GO enrichment analysis results were selected using p value < 0.05 as the critical criterion.

2.8. Mitochondrial extraction and mitochondrial respiratory chain complex assay

The rats were decapitated under deep anesthesia with pentobarbital sodium. Brain sections were quickly isolated, and the mitochondria was extracted using a Mitochondrial Extraction Assay Kit (Beyotime, Jiangsu, China) following the manufacturer’s instructions. In brief, the brain was homogenized in a lysis buffer and then centrifuged at 600 g for 10 min at 4 °C. The supernatant was collected and centrifuged again at 11,000 g for 15 min at 4 °C. After adding the extraction solution in each sample, the mitochondrial pellet was disrupted by an ultrasonic machine. Samples were treated with different concentrations (4, 8, 16, 32, and 64 μg/mL) of GTS or water to examine different mitochondrial respiratory chain complex activities in rat brains. Samples were treated with Rg1, Rh1, Rd, Rh2, PPD and PPT, or DMSO to detect respiratory chain complex I activity in rat brains. After incubation for 30 min at room temperature, the activities of the respiratory chain complex were determined in a microplate reader (Allsheng, Hangzhou, China) using different wave lengths according to the manufacturer’s protocol. The mitochondrial respiratory chain complex I, II, and V kits were purchased from Solarbio (Beijing, China). The mitochondrial respiratory chain complex IV kit was obtained from Cayman Chemical (USA).

2.9. Molecular docking

The structure of mitochondrial respiratory chain complex I (PDB ID: 6g2j) was downloaded from research collaboratory for structure bioinformatics (RCSB) Protein Data Bank (http://www.rcsb.org/pdb). The structure of PPT and ginsenoside Rd was obtained from the national center for biotechnology information (NCBI) PubChem compound database (http://www.ncbi.nlm.nih.gov/compound). Next, we predicted the possible binding pockets and performed molecular docking calculation using Yinfo Cloud Computing Platform (http://cloud.yinfotek.com). The docking result was measured, and the best conformation was recorded.

2.10. Statistical analysis

Data were represented as mean ± SD. All statistical comparisons were performed on data from at least three independent samples. Statistical analysis was performed by one-way ANOVA. p < 0.05 was considered a significant difference.

3. Results

3.1. Protein target identification of ginsenosides in brain tissues

The protein targets of ginsenosides were identified by the DARTS-based proteomics approach. DARTS can directly compare the proteomics profiles of GTS– and vehicle-treated brain homogenates after proteolytic digestion and identify drug-stabilized proteins as targets. In our study, DARTS was performed by simply treating the aliquots of rat brain homogenate with GTS or pure water, followed by digestion of the proteins with proteases. Subsequently, label-free LC–MS was used to identify the potential protein targets that interacted with ginsenosides. Based on LC–MS analysis, we profiled the total number of differential proteins in the GTS-treated group normalized to the control group (Supplementary Table 1). Fig. 2A showed the volcano plot for
differential proteins, and these proteins (upregulated $>$ log2 (0.5)-fold or downregulated $<$ -log2 (0.5)-fold, $p$ $<$ 0.05) were highlighted in red and blue. With regard to the following data analysis, 349 upregulated (upregulated $>$ log2 (0.5)-fold) protein targets were used (Fig. 2A).

3.2. Identification of known therapeutic targets for MCI

A total of 7547 MCI therapeutic targets were acquired from the GeneCards database. Relevance greater than 10 was used as a criterion to simplify and improve the accuracy of our data. Then, 2524 candidate targets were collected. In addition, 36 reported therapeutic targets for MCI were obtained from the OMIM database. Finally, a total of 2526 therapeutic targets for MCI were shortlisted after removal of duplicated gene symbols.

3.3. Construction and analysis of the PPI network in ginsenoside-related MCI targets

Among the 2526 therapeutic targets for MCI and 349 candidate protein targets for GTS, 81 overlapped (Fig. 2B). This result indicated that ginsenosides might mediate MCI through these 81 genes.

In investigating the mechanism of the overlapping of these genes, we inputted these 81 genes to the STRING database and obtained a PPI network, including 80 nodes and 177 edges (Fig. 3A). The nodes with higher degree may be more important in the pharmacological process in the PPI network. After the MCC algorithm processed the core gene data analyzed by the STRING database, we collected 10 key genes, including NDUF13, NDUF8, NDUF51, NDUF7, NDUF6, COX5A, GABRA1, SYN1, GRM5, and NTRK2 (Fig. 3B and C) (Supplementary Table 2).

3.4. GO analysis

GO enrichment analysis of protein targets was performed by DAVID service to verify the potential biological features involved in the 81 targets of ginsenosides in MCI. The top 10 highly enriched GO terms under BP, CC, and MF are displayed in Fig. 4. The results indicated that a number of BP were involved in the MCI effects of ginsenosides, including mitochondrial respiratory chain complex I assembly (GO:0032981), mitochondrial electron transport, NADH to ubiquinone (GO:0006120), and neurotransmitter secretion (GO:0007269, Fig. 4A). In the CC classification, extracellular exosome is the most important CC of target proteins. Moreover, the mitochondria is one of the main classifications of the target proteins (Fig. 4B). As shown in Fig. 4C, GO analysis revealed that these target proteins were associated with MF, including electron carrier activity (GO:0009055) and NADH dehydrogenase (ubiquinone) activity (GO:0008137).

3.5. Impact of ginsenosides on activities of the mitochondrial respiratory chain complex

Based on the analysis of the PPI network and GO in these candidate protein targets, we found that the mitochondria played an important role in ginsenoside mediated-MCI pharmacological process. The mitochondria is the main source of ATP, the energy-producing structures and the main sites of oxidative phosphorylation in the cells. We then assessed the effects of different ginsenosides and GTS on mitochondrial function by measuring the activities of the mitochondrial respiratory chain complex. As shown in Fig. 5A, GTS increased the activity of complex I in a dose-dependent manner (4, 8, 16, 32, and 64 μg/mL), particularly at the concentration of 32 and 64 μg/mL. However, the effects of GTS were minimal or inconsistent with the other mitochondrial complexes (Supplementary Figure 1). Subsequently, we examined the mitochondrial respiratory chain complex I activities of different ginsenosides, including Rg1, Rb1, Rd, Rh1, Rh2, PPD, and PPT (10 μM). PPT, Rh2, and Rd significantly increased the activities of complex I (Fig. 5B). We then detected the mitochondrial respiratory chain complex I activities of PPT and Rd in a dose-dependent manner (0.625, 1.25, 2.5, 5, and 10 μM). PPT, Rh2, and Rd significantly increased complex I activities at the concentration of 5 and 10 μM, which were in accordance with the GTS results (Fig. 5C and D).
Fig. 3. (A) The protein–protein interaction (PPI) network of 81 overlapping genes. (B, C) The interaction of 10 key genes in the PPI network.

Fig. 4. Gene Ontology enrichment analysis of 81 overlapping genes. (A) Top 10 significantly enriched biological process. (B) Top 10 significantly enriched cellular component. (C) Top 10 significantly enriched molecular function.
3.6. Molecular docking

Then, we performed molecular docking analysis to validate the interaction patterns between mitochondrial respiratory chain complex I and PPT or Rd. The best affinity between mitochondrial respiratory chain complex I and PPT was $-9.6$ kcal/mol, and the best conformation was shown in Fig. 6A and B. Meanwhile, the best affinity between mitochondrial respiratory chain complex I and Rd was $-9.4$ kcal/mol, and the best conformation was shown in Fig. 6C. Negative values indicated the binding of the receptor to the ligand. The smaller the score value (the greater the absolute value of the negative value), the stronger the binding force. Affinity greater than $-4$ kcal/mol indicated poor binding force; $-4$ to $-7$ kcal/mol indicated moderate binding force, and Affinity $<-7$ kcal/mol indicated good binding force.

4. Discussion

Recently, systematic investigations on the protein targets of natural products are the key to understand the mechanisms of action and to develop natural products as molecular probes and potential therapeutic approaches. DARTS is a rapid and direct approach to identify protein targets of small molecule ligands [20]. It relies on the stabilization of the target protein, which can increase resistance to proteolysis through direct binding of small molecule compounds. This approach can detect potential protein targets without any chemical alteration of the drug components [21]. With the development of mass spectrometer-based proteomics instruments and techniques, various chemical proteomics methods for DARTS-based target engagement have emerged. Label-free proteomics, which does not involve chemical modification of
proteins, has attracted increasing attention in the scientific community [22]. In this study, we unraveled the neuropharmacological mechanisms of ginsenosides against MCI using DARTS-based target engagement in combination with network pharmacology approach. Initially, we utilized DARTS followed by label-free LC–MS to identify candidate protein targets of ginsenosides. We used high-purity GTS as ligands to ensure that the protein targets of different ginsenosides can be found while using DARTS-based target engagement. Moreover, we acquired the known therapeutic targets for MCI in the databases and took the intersection 81 genes in total, which may be the protein targets of ginsenosides in the treatment of MCI.

The network pharmacology data indicated that the overlapping of 81 genes showed a close association with many crucial BP at the molecular and cellular levels. The PPI network analysis highlighted 10 core genes, including NDUFA13, NDUFB8, NDUFS1, NDUFS7, NDUFA6, COXSA, GABRA1, SYN1, GRM5, and NTRK2 (Fig. 3). As shown in Fig. 3B, NDUFA13, NDUFB8, NDUFS1, NDUFS7, and NDUFA6 are the core or accessory subunits of the mitochondrial respiratory chain NADH dehydrogenase (complex I). COXSA is the cytochrome c oxidase chain containing heme A, which is the terminal oxidase in mitochondrial electron transport. The mitochondria, particularly mitochondrial respiratory chain complex I, played an important role in the ginsenoside-mediated MCI pharmacological process. GABRA1, GRM5, and NTRK2 are the receptors for different neurotransmitters. Based on previous reports, SYN1 played a role in the regulation of neurotransmitter release (Fig. 3C). Thus, ginsenoside contributed to the functional regulation of neurotransmitter in the treatment of MCI.

GO-based bioinformatics analysis identified several enriched BP, including mitochondrial respiratory chain complex I assembly, mitochondrial electron transport, neurotransmitter secretion, chemical synaptic transmission, glutamate secretion, and oxidation–reduction process, which were linked with the pathology of MCI (Fig. 4). Combined with our PPI network results, we found that mitochondria-related BP and protein targets were particularly enriched, indicating that the function of the mitochondrial GTS could play a significant role in the improvement of ginsenosides on MCI. Therefore, we evaluated the effects of ginsenosides on the activities of the mitochondrial respiratory chain complex in vitro to validate whether ginsenosides affected the mitochondrial function. Our data suggested that GTS significantly increased the activities of mitochondrial respiratory chain complex I at the concentration of 32 and 64 μg/mL (Fig. 5A). The experimental result was strongly consistent with previous network analysis.

It is reported that the high energy demand of brain results in it is sensitive to changes of energy and mitochondria dysfunction [23]. The mitochondria is responsible for a variety of functions, such as energy production, metabolism regulation, cellular signaling modulation, calcium buffering, and neuronal function [24]. ATP production in mitochondria begins at complex I and continues through complex II-V of the electron transport chain. Complex I is a large multimeric enzyme complex located in the inner membrane of mitochondria, consisting of at least 45 protein subunits. Defects in complex I, such as reduced enzyme activity and/or subunit concentration, are a classic marker of cellular dysfunction associated with aging and neurodegeneration [25]. Recent studies have shown that partial complex I defects caused by neural ablation of complex I subunit, NDUFA5, cause mild chronic encephalopathy in mice [26]. These results suggested that complex I and/or its subunits may contribute to the MCI.

Although no studies have evaluated the efficacy of ginseng on MCI mitochondrial dysfunction, the beneficial effects of ginseng and its related components on the mitochondria have been reported in several studies. The administration of ginsenoside Rg1 in primary neurons prevented Aβ-mediated mitochondrial dysfunction by regulating mitochondrial membrane potential, ATP production, and cytochrome c oxidase (COX) activity [27]. The protective effects of Rg1 in Aβ-induced neuronal death were observed through the suppression of mitochondrial oxidative stress, which may protect the neurons in AD [27]. Ginsenoside Rg3
regulated mitochondrial dynamic remodeling, thereby enhancing the quantity and quality of the mitochondria [28]. Ginsenoside Rd inhibited the release of cytochrome C and the permeability transition of mitochondria in the isolated spinal cord mitochondria through protein kinase-dependent mechanism [29]. These findings suggested that various components of ginseng may have positive effects on mitochondrial dysfunction. Ginsenosides are the main bioactive ingredients of ginseng, and various ginsenosides have been identified and isolated. Ginsenosides can be primarily grouped into two categories according to the chemical structure, namely, the protopanaxadiol (e.g., Rd2, Rd1, Rd, Rc, Rh2, and Rg3) and propanaxatriol groups (e.g., Re, Rg1, Rf, and Rh1). We assessed the mitochondrial respiratory chain complex I activities of different ginsenosides (Rg1, Rd1, Rd, Rh1, Rh2, PPD, and PPT) in brain tissues to determine the ginsenosides involved in the mitochondrial function, PPT, Rh2, and Rd significantly increased the activities of mitochondrial respiratory chain complex I (Fig. 5B). Next, the mitochondrial respiratory chain complex I activities of PPT and Rd were examined in a dose-dependent manner. We found that PPT and Rd could enhance the mitochondrial function in brain tissue (Fig. 5C and D). Molecular docking between small molecules and proteins involved placing the small molecules on the active site of the target protein. We simulated the molecular docking pockets of mitochondrial respiratory chain complex I and PPT or Rd. Fig. 6 showed the highest affinity and best conformation between mitochondrial respiratory chain complex I and PPT or Rd. These results indicated that PPT and Rd may modulate the MCI process through interacting with mitochondrial respiratory chain complex I. So far, no study has shown a correlation between PPT and mitochondrial function. However, some researchers reported that Rd has mitochondria-related protective effects in neurological diseases. The combination of Rd with Re attenuates the extent of depolarization of MMP and restores calcium levels in rotenone-induced oxidative stress and mitochondrial impairment in SH-SYSY neuroblastoma cells [30]. In middle cerebral artery occlusion (MCAO) rat models, Rd administration reduces mtDNA damage and the cleavage of caspase-3, improving the survival rate and neurological function 7 days after MCAO [31]. Rd also reduced mitochondrial hydrogen peroxide production and depolarizing mitochondrial membrane potential in the aged MCAO mice, that prevented mitochondrial damage after reperfusion by preserving mitochondrial electron transport chain activity and aconitate activity [32]. These results indicated that Ginsenoside monomers may regulate mito-apoptosis and mito-biogenesis to exert pharmacological effects in neurological diseases.

Given the limitations of experimental research methods, the effective targets and mechanisms of ginsenosides in MCI have not been reported yet. Our study focuses on major protein targets of ginsenosides by DARTS-based proteomics combined with network pharmacology to study the therapeutic mechanisms of ginseng in MCI. It is a key contributor for deepening the understanding of the related mechanisms and interpreting protein targets. For the first time, multi-target action on MCI was used to explain the molecular mechanisms of the therapeutic effect of ginsenosides. Furthermore, we verified the correlation of ginsenosides with mitochondrial respiratory chain complex I in vitro. In the future, modulating mitochondrial function can be examined in vivo to verify whether or not ginseng can serve as a potential therapeutic approach to alleviate MCI by targeting respiratory chain complex I.

Contributors

Zhu Zhu designed and wrote the draft of the manuscript; Ruimei Li undertook in vitro enzyme activity analysis; Yao Cheng designed and conducted molecular docking; Hantao Zhang prepared the figures and supplementary material; Feiyun Chen designed and conducted DARTS; Cuihua Chen assisted in the data analysis; Lin Chen and Yunan Zhao conceived the idea, supervised, and supported the project; and all authors discussed the results and commented on the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.12.003.

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