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The expression of bradykinin and its receptors in spinal cord ischemia-reperfusion injury rat model

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ABSTRACT

Objective: To investigate the expression and time-dependent manner of bradykinin (BK) as well as its receptors (Bradykinin receptors 1 and 2, B1R and B2R) in spinal cord ischemia-reperfusion injury (SCII) in rat model.
Methods: Sprague-Dawley (SD) rats were subjected to 1 h of infra-renal abdominal aorta occlusion and reperfused for 3 h to 5 d to induce SCII. The concentration of BK in serum was detected by enzyme linked immunosorbent assay (ELISA). In situ expression of BK receptors was evaluated by immunochemistry and their mRNA level was evaluated by Real time quantitative-PCR (RTq-PCR).
Results: The concentration of BK in serum was increased right after following SCII. Both of the BK receptors were detected and up-regulated in 24 h and 48 h after injury. The levels of B1R and B2R mRNA were up-regulated after SCII, and the B1R mRNA dropped to basal level after 6 h, but B2R mRNA dropped to lower level right after injury, peaked at 3 h, then remained a lower level from 6 h till 5 day.
Conclusion: This study provides the evidence of the expression of BK and its receptors in SCII in rat model, and suggests that BK and its receptors may have some physiological or pathological significance in SCII.

1. Introduction

Spinal cord ischemia-reperfusion injury (SCII) is a common secondary spinal cord injury in clinical practice, which has very complicate mechanisms and lead to severe spinal cord disorders. The blood flow of spinal cord could be dropped during spinal or thoracoabdominal aorta surgeries, although it would be restored after surgery, but that still might lead to ischemia-reperfusion injuries [1–3]. Several biological reactions are involved in SCII, including recruitment of leucocytes, cascade of cytokines, microvessel endothelia damage, and apoptosis [3]. But the complete mechanical of SCII still remains to be discovered.

Kallikrein-kinin System (KKS), including bradykinin (BK), bradykinin receptors (B1R and B2R), etc., is an intricate endogenous pathway involved in several pathophysiological processes. In central nervous system, these components are located in cerebral cortex, cerebellum, brain stem, hypothalamus, hippocampus, and pineal gland, among others [4–8]. KKS has been related to several central nervous system diseases, such as stroke, Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis [7,9]. Especially, the bradykinin receptors (B1R and B2R) have been found to be up-regulated in cerebral ischemia-reperfusion injury [10–12] and involved in blood-brain-barrier(BBB) damage, tissue edema, neurotoxicity, apoptosis, inflammatory activation, etc. [10–21].

In a spinal cord study, Yan-Feng, et al. [22] found that BK preconditioning can improve the neurological function, and decrease blood-spinal cord barrier (BSCB) permeability damage in SCII rats. Xu, et al. [23] demonstrated that BK preconditioning can attenuate the expression of aquaporin-4 (AQP4) which reduces the tissue edema in SCII. What's more, Mechirova, et al. [24] suggested that the BK preconditioning against SCII effect is through mitochondrial protection and decreasing the synthesis of Mn-SOD as well as by promoting the survival of neurons.

Although BK preconditioning was well studied in SCII, the expression of BK as well as its receptors in SCII is still little reported. In this study, we investigated the time-course of BK production and the expression of its receptors by using SCII rat model.

2. Materials and methods

2.1. Experimental animals and grouping

A total of 72 male Sprague-Dawley (SD) rats weighing 250–300 g from the Animal Center of Xi'an Jiaotong University were purchased and housed in the specific pathogen free (SPF) animal care center of Xi'an Jiaotong University. The rats were randomly divided into sham (n = 12), ischemia (I, n = 6) and ischemia-reperfusion groups (IR,

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n = 54). According to different reperfusion time points, the IR group was divided into 7 subgroups (IR3h, IR6h, IR12h, IR24h, IR48h, IR72h and IR5d), with 12 rats in IR24h group and IR48h group, and 6 rats in each other groups. In Sham, IR24h and IR48h groups, 6 rats were used to perform Hematoxylin and Eosin (H&E) staining and immunohistochemistry (IHC), while remained 6 rats were used for Enzyme-linked immunosorbent assay (ELISA) and RTq-PCR.

2.2. Materials and methods

According to Zivin et al. [25] and others [16,26]. and our previous studies [1,27,28], the SCII model was built by using infra-renal abdominal aorta clamping. Rats were fasted for 8–12 h and anesthetized with 0.75% amobarbital sodium (4 ml/kg) before surgery. Ischemia of lower lumbar spinal cord was induced by clamping the abdominal aorta just under the branch of left renal artery and last for 60 min, then the clamp was removed to induce reperfusion. The neural function of lower limbs was evaluated using the Basso, Beattie and Bresnahan (BBB) Locomotor Rating Scale [29]. Serum and the L4–L6 segments of spinal cord were collected for further analysis.

2.3. Hematoxylin and eosin (H&E) staining

In order to confirm the successful establishment of the rat model of SCII, H&E staining was performed in the L4–L6 segments of spinal cord from Sham, IR24h and IR48h groups. (Result show in Supplementary material).

2.4. Immunohistochemistry of B1R and B2R in spinal cord tissue

Immunohistochemical staining was performed in the L4–L6 segments of spinal cord from Sham, IR24h and IR48h groups. The samples were harvested and fixed in 4% paraformaldehyde (PFA) then embedded in paraffin. Rabbit anti-rat B1R (1:100 dilution, sc-25484, Santa Cruz Biotechnology, Inc., USA), rabbit anti-rat B2R (1:100 dilution, sc-25671, Santa Cruz Biotechnology, Inc., USA), Biotin-Streptavidin HRP Detection Kit (SP-9001, ZSGB-BIO, Bingjing, China) and DAB kit (ZLI-9031, ZSGB-BIO, Bingjing, China) were used in the procedure. After deparaffinization, the tissue sections were placed inside a microwave (Midea EG823EC2-NS) with pre-boiled Sodium Citrate Buffer (0.1 mol/L Sodium Citrate, pH 6.0) and heated for 15 min at middle power then cooled down in room temperature. After washing with dH2O and 0.01 mol/L phosphate-buffered saline (PBS), the sections were blocked by 10% goat serum for 1 h at 37 °C and incubated overnight at 4 °C with primary antibodies (B1R or B2R). Next, the sections were washed with PBS and incubated with 3% H2O2 for 10 min at 37 °C. After further washing, the sections were incubated with biotin-conjugated secondary antibodies (goat anti-rabbit IgG antibodies) for 1 h at 37 °C. The sections were rinsed with PBS again and incubated with Streptavidin-Peroxidase compound for 1 h at 37 °C. Then the sections were rinsed with PBS and reacted with DAB solution. Finally, the sections were stained using Mayer's Hematoxylin staining solution. The sections were dehydrated, cleared and cover slipped for analysis. 0.01 mol/L PBS was used to dilute primary antibodies and for negative control.

2.5. Enzyme-linked immunosorbent assay for BK in serum

Serum from all the groups was collected and used to detect the concentration of BK by an ELISA kit for rat(Elabscience Biotechnology Co., Ltd., Cat. #: *E*-EL-R0124c) according to the product description. Results were expressed as nanogram per milliliter (ng/ml).

2.6. Real time quantitative-PCR for mRNA of B1R and B2R

The L4–L6 segments of spinal cord were harvested and used to detect the mRNA of B1R and B2R. The mRNA was extracted by TRIzol® Reagent (Life technologies, USA) and reverse transcribed to cDNA by MyCycler™ Thermal Cycler PCR system (Bio-Rad Laboratories, Inc. USA) in accordance with PrimeScript™ RT reagent Kit (Takara Bio Inc., Cat. #: RR047A). cDNA was amplified with corresponding primers using 2 μl solution of the reverse transcription. Real time quantitative-PCR was performed with Step-One™ Real-Time PCR system (Applied Biosystems, Foster, CA, USA) with SYBR® Premix Ex Taq™IIkit (Takara Bio Inc., Cat. #: RR047A), in a 20 μl reaction system, and following the Manufacturer's product description. Parallel wells were used for all samples. The primers were designed according to GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine, USA) and synthesized by Aoke Biotechnology, Beijing, China. The sequences of primers are as follows: β-actin (utilized as internal reference): forward, 5′-CTATCGGCAATGAGCGGTTCC-3′, reverse, 5′-TGTGTTGGCATAGAGGTCTTTACG-3′; B1R: forward, 5′-GGTGGCAG CAACGACAGAG-3′, reverse, 5′-GCAGAGGTCAGTTCCGAAGG-3′; B2R: forward, 5′-GCCTGCGTCATTGTCTAC-3′, reverse, 5′-ACTTCTTCATCTC GTTGTTCC-3′. The results of Real Time qPCR were evaluated using the 2-Ct method.

2.7. Statistical analysis

The data was analyzed with IBM SPSS Statistics 23.0 (International Business Machines Corp., USA). Kolmogorov-Smirnov test was performed for each group. Then, groups were compared by *t*-test if the data was in line with normal distribution. Otherwise they were compared by Mann-Whitney test. Results were described as mean ± SD. Statistical significance was set to $P < 0.05$.

All applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

3. Results

3.1. The concentration of BK in serum increased following SCII

Compared with sham group, BK concentration in serum was increased at 3 h post-injury and significantly decreased at 48 h post-injury (Fig. 1). (sham group: 6.7683 ± 1.11647 ng/ml; I group: 15.695 ± 0.99438 ng/ml, $P < 0.05$; IR3h group: 18.2917 ± 2.13969 ng/ml, $P < 0.05$; IR6h group: 12.3617 ± 1.49955 ng/ml, $P < 0.05$; IR12h group: 15.6733 ± 1.29747 ng/ml, $P < 0.05$; IR24h group: 16.9267 ± 0.46224 ng/ml, $P < 0.05$; IR48h group: 5.8100 ± 0.82910 ng/ml, $P > 0.05$; IR 72 h group: 5.5900 ± 1.02666 ng/ml, $P > 0.05$; IR5d group: 4.7183 ± 1.25560 ng/ml, $P < 0.05$).

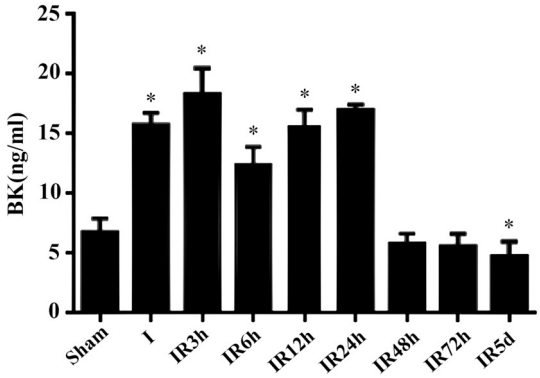

Fig. 1. BK concentration in serum post-spinal cord ischemia. BK concentration was increased at 3 h post-injury and significantly decreased at 48 h post-injury. *$P < 0.05$ vs the sham group. Data are presented as mean ± SD.

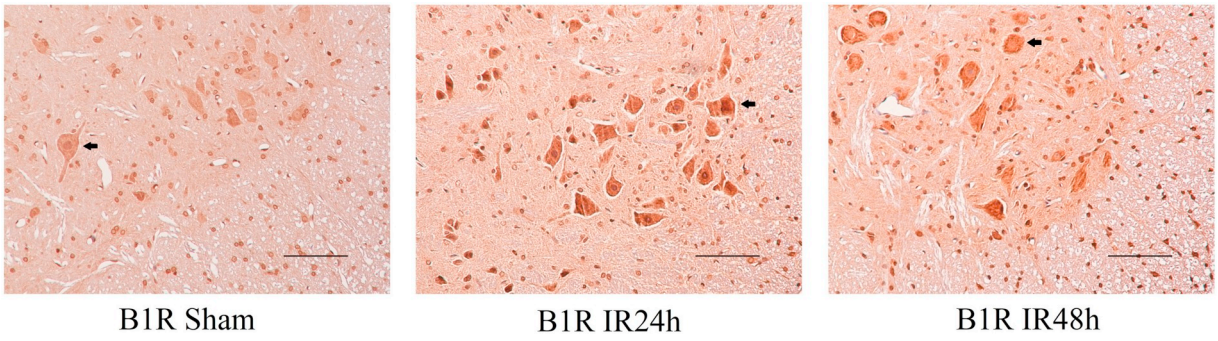

Fig. 2. Immunohistochemical staining of B1R following SCII: The expression of B1R in Sham, IR24h and IR48h groups indicated B1R expression on neurons and other different types of cells. Compared to sham group, B1R expression in IR24h and IR48h groups were increased. (Scale bar = 100 μm. Arrow: Neuron).

3.2. Immunohistochemistry indicated that the expression of B1R and B2R changed after SCII

In immunohistochemical staining in L4–L6 segments of spinal cord from Sham, IR24h and IR48h groups, we found that the BK receptors was expressed in both normal (sham group) and injured (IR24hand IR48h group) spinal cord tissue. Due to the large and complex cell morphology of neurons, B1R expression was identified on neurons as well as on diverse types of cells. Compared with sham group, B1R expression was increased at 24 h and remains increased at 48 h post-injury (Fig. 2). Also, B2R was found to be expressed in neurons and other cells in normal spinal cord tissue. Compared with sham group, B2R expression was increased at 24 h, but decreased at 48 h post injury (Fig. 3).

3.3. The mRNA expression of B1R and B2R in spinal cord was up regulated following SCII

The mRNA expression of B1R in spinal cord was up-regulated and peaked at 3 h after reperfusion, then quickly fell back to the basal level (Fig. 4). The mRNA expression of B2R was decreased significantly right after spinal cord ischemia was induced. Similar to B1R, B2R mRNA was increased and peaked at 3 h after reperfusion then decreased rapidly. While B2R mRNA remained a lower level than B1R or B2R sham till 5 days after reperfusion (Fig. 5).

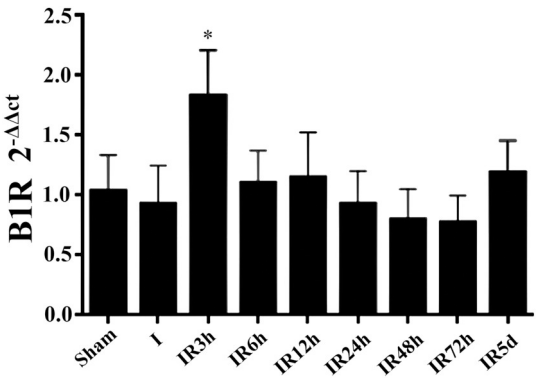

Fig. 4. the mRNA expression of B1R: B1R mRNA expression was increased and reached its maximum at 3 h after reperfusion, then quickly dropped to its basal normal level. *P < 0.05 vs the sham group. Data are presented as mean ± SD.

4. Discussion

The function of BK-B1R/B2R pathway in ischemia-reperfusion injuries is quite complicate. In cerebral ischemia associated studies, BK level in tissue and plasma was found to be related to ischemic cerebral edema [9,11,30,31]. And cerebral BK level was found to rise three times after being subjected to 45 min of middle cerebral artery occlusion (MCAO) in mice model [11]. And similar research about ischemic stroke suggested that blocking of plasma kallikrein, which cleaves high molecular weight kininogen to release bradykinin, ameliorates stroke by reducing thromboinflammation [32]. In a MCAO mouse model, the ischemic lesion was reduced through the combination of urodilatin and BK, and this protect effect was thought to be activated G-Protein-Signaling Protein Type 4 at the atrocities and neurons cellular as well as the endothelial cells and isolated MCAO blood vessel [33] The BK level in plasma and tissue was discovered to corresponded to the progression of ischemic cerebral edema and suppression of BK reduced edema formation in rat [30,31].

Similar to cerebral studies, BK-B1R/B2R were found protective effect in spinal cord ischemia reperfusion injury. In a SCII model, rabbits were preconditioned with BK and then underwent 20 min of abdominal

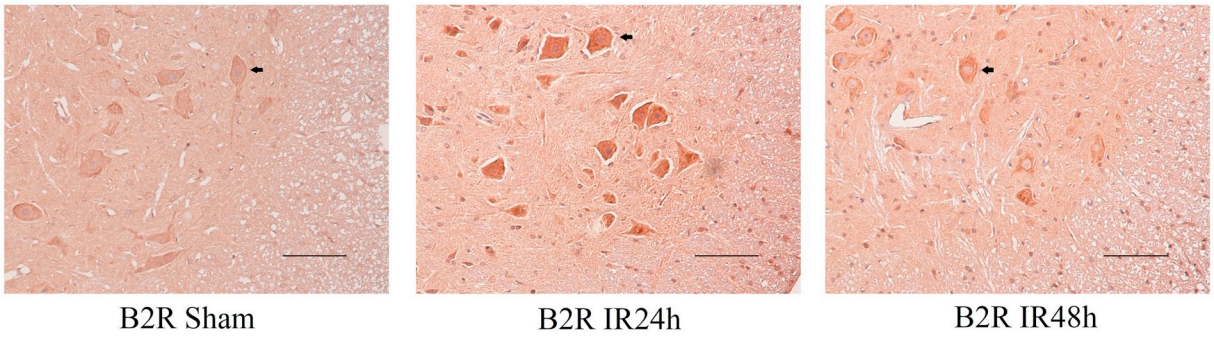

Fig. 3. Immunohistochemical staining of B2R following SCII: The expression of B2R in Sham, IR24h and IR48h groups indicated B2R expression on neurons and other different types of cells. Compared to sham group, B2R expression in IR24h group was increased. (Scale bar = 100 μm. Arrow: Neuron).

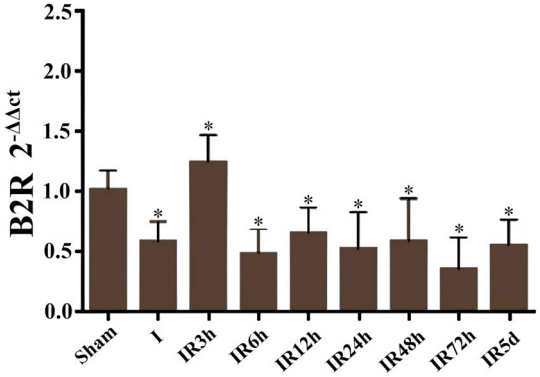

Fig. 5. the mRNA expression of B2R: B2R mRNA expression decreased significantly after spinal cord ischemia was induced, and increased 3 h after reperfusion. *P < 0.05 vs the sham group. Data are presented as mean ± SD.

aorta ligation followed by 24 and 48 h of reperfusion, and suggested that BK precondition could promotion spinal neuron survival through mitochondrial protection and decreased synthesis of Mn-SOD [24]. In rat model, bradykinin preconditioning could modulate aquaporin-4 expression after spinal cord ischemic injury [23]. And these researches were also demonstrated that BK preconditioning could decrease blood-spinal cord barrier (BSCB) permeability damage, reduced tissue edema, promoted the survival of neurons and improved the neurological function after SCII in rat.

As for BK receptors, they were detected to be up-regulated in stroke [11–13]. Austinat et al. [13] blocked B1R and B2R expression and comparing them by using B1R and B2R knockout and wild-type mice after MCAO, found that blocking B1R can protect brain from edema and infarction, while blocking B2R didn't show these protective effects. However, specific inhibition of B2R showed beneficial effects in other aspects, such as reduced infarct volume, attenuated post-ischemic brain swelling and inflammatory responses, decreased the number of necrotic neurons, and improved the functional neurological recovery after MCAO. Similar results were obtained by Groger [11], Relton [17], Ding-Zhoua [14], and Zausinger [19–21]. It was also demonstrated that blockage of B2R effectively reduced BBB permeability damage, cytokines release and neurological disorders than B1R blocking after reperfusion [12]. Moreover, the therapeutic window for B2R inhibition extends up to 6.5 h after MCAO [15], and low dose treatment can still give rise to a better outcome [20,21]. A cerebral ischaemia-reperfusion study showed that inhibition of B1R could reduce infarct volume, neurological deficits, cell apoptosis, and neuron degeneration, but B2R antagonist had opposite effects, and exacerbated BBB penetrability and tissue inflammation [34]. Some other studies demonstrated that blockage or knockout of B2R aggravates infarction formation, cellular apoptosis, inflammatory responses, and mortality rate after MCAO [18], suggesting that B2R expression has also a positive influence in ischemia-reperfusion injury. All of these studies indicate that both BK receptors may play important roles in the cerebral ischemia-reperfusion injury, including the BBB damage, brain edema formation, cellular necrosis, cerebral infarction, and inflammatory response. But somehow, there were some conflicts in these studies, and we conjecture that BK-B1R/B2R in different pathways or biological process might have different functions.

As for the reported pathways of BK-B1R/B2R, it was mainly studied in cardiovascular diseases. In ischemic preconditioning, bradykinin exerts cardioprotective effect via activation of PI3K/Akt/eNOS signaling pathway, mitochondrial anti-apoptotic pathway as well as the signaling cascade through the activation of B2R [35]. It was also reported that B2R activation appeared to be a common step to promote eNOS expression when using several vasodilator drugs [36]. In an in vivo study, deep vein thrombosis was inhibited after exogenous bradykinin was performed, and that effect was activated through eNOS/phosphoinositide 3-kinase/Akt signaling pathway [37]. A study reported that bradykinin could regulate redox state through inhibited oxidative stress-induced cardiomyocytes senescence and this effect was acting through B2R receptor induced NO release, upregulated antioxidant Cu/Zn-SOD and Mn-SOD activity and expression while down-regulating NADPH oxidase activity and subsequently inhibited ROS production [38]. In Duchenne muscular dystrophy, bradykinin restored vascular endothelial dysfunction through upregulation of eNOS and nNOS [39]. And this pathway even could promote circulatory nitric oxide metabolite availability during hypoxia [40]. Angiogenesis and stimulates the EGF-R signaling pathway are associated with NO, and related study indicated that BK could mediate angiogenesis in endothelial cells, through induct the expression of VEGF associated with the activation of the NO/EGF-R/p21Ras/ERK1/2 MAP kinases signaling pathway [41]. All above studies were focus on the pathway in cardiovascular diseases, while BK-B1R/B2R pathway in neural cells still needs to be uncovered.

In our study, B2R expression was decreased at 48 h, while B1R was not. And B2R mRNA level was also lower than sham group after injury except a short time up-regulation. This different might result from different function of B1R and B2R [7,42]. B2R is associated with Ca^{2+} channels, which are mainly distributed on the endoplasmic reticulum, and B2R can achieve a variety of physiological functions through Ca^{2+} channels [43,44], such as hormone secretion [45], cell proliferation [46], hypoxia/reoxygenation injury [47], and modulation of cardiac vagal tone[48]. While B1R is latent in healthy tissues but induced by tissue insult, and its function more associates with inflammatory disease [49], acute gout attacks [50], memory impairment [51], and glioblastoma migration [52]. B1R also shows deleterious effects in insulin resistance and peripheral inflammation [53]. The function of B1R and B2R might exchange in different pathophysiological conditions, and their protection effect or deleteriousness still need to be investigated.

Additionally, it should be noted that B1R protein level is significantly suppressed when treated with B2R inhibitor 24 h after reperfusion [12], suggesting that there might be a potentially interacting mechanism between B1R and B2R. In other words, B1R activation may be related to B2R expression, and the neuroprotection function of B2R might be achieved by up-regulating B1R expression. All of these biological functions still need to be discovered in spinal cord ischemia reperfusion injury.

5. Conclusion

To conclude, the kallikrein-kinin system was shown to be activated in SCII. As a part of this system, the serum bradykinin level was increased after spinal cord ischemia in rat model. The B1R mRNA level increased but B2R mRNA level decreased, and both of these receptors were found in normal and injured spinal cord tissue, but have different expression and different time-depend manner. These foundings indicated that BK-B1R/B2R pathway may have important physiological or pathological functions in SCII. Our research can be used as a theoretical basis for the general understanding of BK and BK receptors expression in SCII, further studies are needed to discover the exact mechanism and function of these factors.

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CRediT authorship contribution statement

Zheng Ma: Conceptualization, Data curation, Formal analysis, Methodology, Software, Visualization, Writing - original draft. **Quan Dong:** Data curation, Funding acquisition, Methodology. **Boqiang Lyu:** Data curation, Formal analysis, Software, Visualization, Writing - review & editing. **Jubo Wang:** Data curation, Methodology. **Yu Quan:** Data curation, Methodology. **Shouping Gong:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation.

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