Mongolian Herbal Medicine Garidi-13 Upregulates Igf2 Expression in Cerebral Cortex and Attenuates Neuronal Damage in MCAO Rat Model

Lu Chen1*, Saren Gaowa2*, Qiburi Qiburi1, Meng He1, Tsogzolmaa Ganbold1, Mingming Bao1, Qingming Bao1, Man Da2, Temuqile Temuqile2,*, Huricha Baigude1,*

1Inner Mongolia Key Laboratory of Mongolian Medicinal Chemistry, School of Chemistry & Chemical Engineering, Inner Mongolia University, Hohhot, Inner Mongolia, P.R.China
2Inner Mongolia International Mongolian Hospital, Hohhot, Inner Mongolia, P.R.China
*These authors contributed equally to this work.

ABSTRACT
Garidi-13 (GRD-13) is one of the most important Mongolian medicines with long history of clinical application in Inner Mongolia of China as well as the Republic of Mongolia. Clinical application demonstrated that GRD-13 has remarkably high neuroprotective effect in stroke patients. However, the mechanism of action of GRD-13 is unknown. Herein, we extracted the active compounds of GRD-13, analyzed and identified neuroactive components of GRD-13 by using UPLC-qTOF mass spectrum. Rat MCAO models were treated with either positive control (nimodipine), or GRD-13, and the neurological impairment was assessed by Bederson scale. Next, we observed the infarct volume of all MCAO groups using TTC staining. Finally, we analyzed the gene expression patterns in the cerebral lesion of rat MCAO models with or without GRD-13 treatment by using RNA-seq technology. Six previously reported neuroactive compounds (isoliquiritigenin, myristicin, dehydrocostus lactone, costunolide, α-asarone, eudesmin) were identified in the extract of GRD-13. Rat MCAO model treated with GRD-13 showed significantly improved neurorecovery compared to the non-treated control animals, demonstrated by reduced stroke lesion area and edema, higher neuron survival rate as well as reduced neurological impairment assessed by Bederson scaling. Analysis of transcriptome further elucidated that the mechanism of action of GRD-13 possibly mediated by up-regulating the expression of Igf2 gene in the ischemic area of MCAO model rats. These findings would provide theoretical guidance for the further development of GRD-13 and related Mongolian medicines.

Keywords: Mongolian medicine; Garidi-13; Stroke; MCAO model; Gene regulation; RNA-seq; Insulin-like growth factor 2

Published on: 19/06/2018
Author for Correspondence: * Temuqile Temuqile: International Mongolian Hospital, Hohhot, 010021, P.R.China. Tel: +86 471 5182005; Fax: +86 471 5185378; E-mail: tmqyx01@163.com
* Huricha Baigude: Institute of Mongolian Medicinal Chemistry, School of Chemistry & Chemical Engineering, Inner Mongolia University, Hohhot, 010020, P.R.China. Tel.: +86 471 4993165; Fax: +86 471 4992511; E-mail: hbaigude@imu.edu.cn


INTRODUCTION
Stroke happens when blood supply to brain is restricted, resulting in the hypoxia and subsequent neuronal necrosis in the affected brain tissues [1-8]. Bleeding from the burst vessels causes hemorrhagic stroke, and lack of blood induces ischemic stroke which has a higher incidence rate (70%) in stroke patients. The main factor for stroke incidence is high blood pressure, followed by hyperlipidemia and obesity. In 2017, there were 2.4 million strokes happening in China, with a very high stroke related mortality of 1.1 million and 11.1 million survivors with varying degree of recovery [9]. Worldwide, 6.3 million deaths resulted from stroke has been reported, which accounts for 11% total deaths in 2015 [10]. Therefore, there is an urgent need for development of efficient preventive as well as therapeutic drugs against stroke.

The cause of brain blood supply restriction that potentially leads to stroke could be either a thrombus or an embolus. Therefore, after acute stroke, thrombolysis (such as recombinant tissue plasminogen activator, rtPA) within hours shows significant benefits in terms of living without disability [11], although the chance of survival is not improved. Surgically removing troublesome blood clot can also improve outcome [12]. Clinical intervention of stroke involves sustained stroke rehabilitation after first aid treatment of thrombolysis and thrombectomy. A number of Mongolia traditional medicines including Garidi-13 and Eerdun Wurile (EW) have remarkable therapeutic effect during stroke recovery [2]. We discovered that EW can effectively up-regulate the expression of growth factor genes as well as markers for anti-inflammatory microglia in the affected brain region of MCAO rat models [13].
Ethnic Mongolian medical practice is an independent medical system that is greatly influenced by Tibetan medicine. Mongolian medicine is also related to Asian medical tradition but has been uniquely developed based on the environment and culture on Mongolian plateau through Mongolian history [14]. GRD-13 is a prescription mainly consisting of medicinal plants, and such combination of the ingredients is specific to ethnic Mongolian medicine [15]. As one of the most regularly used herbal medicines for the treatment of neurological disorders including stroke, the herbal medicine GRD-13 has the distinct prescription that is composed of 13 ingredients including 8 medicinal herbs (Table 1) [3]. Previous studies have suggested that GRD-13 may have anti-thrombotic effect [16]. Although the neuro-recovery activity of GRD-13 has long been proved in clinic, the detailed underlying neuroprotective mechanism and potential neurogenesis pathways have never been elucidated.

In this report, we first confirmed the neuro-recovery effect of GRD-13 in rat MCAO model; then we analyzed the transcriptome of stroke legion by RNA-seq and found a number of significantly up-regulated genes including a key growth factor that may involve in neurogenesis during stroke recovery.

**METHODS**

**Ethics statement**

All experimental procedures involving animals were approved by the Animal Care and Use Committee of Inner Mongolia University. We made all efforts to minimize the number of animals used and their suffering.

**Chemicals and instruments**

Garidi-13 (internal medicine number M14010082, batch number 20170405) was prepared by National Mongolian Pharmaceutical Preparation Center, Inner Mongolia International Mongolian Hospital, China. Voucher specimens were deposited in the Virtual Herbarium of Inner Mongolia Medical University, Hohhot, China. Formic acid solution is purchased from Sigma-Aldrich, China. Leucine enkephalin is purchased from Waters, USA. Chloral hydrate was purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China). Monofilament nylon suture was purchased from Beijing Cinontech Co. Ltd. (Beijing, China). Ethanol absolute, petroleum, ethyl acetate and n-butanol were purchased from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd (Tianjin, China).

For analysis of active components in GRD-13, Waters Acquity UPLC/qTOF (Waters, USA) equipped with quaternary solvent manager, online degasser, sample manager, column manager, Acquity PDA detector, ESI source, Lockspray source, Xevo G2-XS QToF four-pole flight time tandem mass spectrometer and Masslynx V4.1 Workstation was used. Acetonitrile is purchased from Fisher Scientific, USA.

**Analysis of UPLC-QToF-MS**

To prepare samples for UPLC-qTOF mass measurement, GRD-13 powder was weighed into 3 round bottom flasks (0.2 g per flask), and added 20 mL of anhydrous ethanol (I), ethyl acetate (II) and petroleum ether (III), respectively. Then, the flasks were heated in oil bath to reflux at 35°C while stirring. After 12 hours the suspension was filtered through a sand funnel with diatomite. Subsequently, the solvents were removed under reduced pressure. The solid samples were stored at 4°C for further analysis. The following chromatographic conditions were applied for analysis of the active small molecular components of GRD-13: Waters ACQUITY UPLC® BEH Shield RP18, 2.1 x 100 mm Column (1.7 μm) was connected to a Vanguard HSS T3 guard column. Column temperature was 40°C; the mobile phase was as following: A, water; B, acetonitrile, both contains 0.1% formic acid; the mobile phase gradient elution was: 50% → 90% B, 10 → 15min: 100% B, 15 → 20min: 50% B; the flow rate was 0.4 mL/min; the injection volume was: 2 μL. The following conditions were used for mass spectrometry: electrospray ionization (ESI) positive ion mode; mass detection range 100-1200 Da; capillary voltage 3 kV; sample cone 40 V; extraction cone 4 V; source temperature 100°C; desolvation temperature 400°C; desolvation gas 800 L/h, lockmass 556.2771 (positive ion mode). The accuracy error threshold was fixed at 5 mDa. Data acquisition is controlled by Mass Lynx 4.1 software. The information on the chemical constituents of GRD-13 were collected and that chemical compositions structure were drawn using ChemDraw software, and the molecular formula and theoretical relative molecular mass database was built.

**Experimental animals and MCAO model**

Wistar rats (8-week-old, male, body weight 240 g) were purchased from Experimental Animal Center of Inner Mongolia University, Hohhot, Inner Mongolia Autonomous Region. Ethanol absolute, petroleum, ethyl acetate and n-butanol were purchased from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd (Tianjin, China).

### Table 1. Components of GRD-13 prescription*

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>Component</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminalia chebula Retz</td>
<td>18</td>
<td>Syzygium aromaticum (L.) Merr. EtPerry</td>
<td>6</td>
</tr>
<tr>
<td>Aconitum nagarum Stapf</td>
<td>18</td>
<td>Aquilaria sinensis (Lour.) Gilg</td>
<td>6</td>
</tr>
<tr>
<td>Acorus tatarinowii</td>
<td>6</td>
<td>Myristica fragrans Houtt.</td>
<td>6</td>
</tr>
<tr>
<td>Sauussurea costus</td>
<td>12</td>
<td>Glycyrrhiza uralensis Fisch</td>
<td>6</td>
</tr>
</tbody>
</table>

* Other components include musk (0.7%), coral (3.3%), pearl (6%), red claystone (6%), magnetite (6%).
Region, China. The rats were housed in a controlled environment (4 animals per cages, 55 ± 5% relative humidity, 22°C, 12 h:12 h light/dark cycle), provided with free access to food and water, and were acclimated for at least 7 days before the start of any experiments. The rat model of middle cerebral artery occlusion/reperfusion (MCAO/R) was established according to a previously published method [17].

Treatment groups
Rats were randomly divided into five groups and were continuously injected saline, nimodipine (positive control) and GRD-13 by intragastric administration for 2 weeks. The doses for each group were as following: NT, non-treated group (n=11, saline); IS, model group (MCAO/R, n=10, saline); NI, positive control group (MCAO/R+Nimodipine, n=7, nimodipine, 4 mg/100 g body weight); GRDlow, low dose test group (MCAO/R+GRD-13, n=16; GRD-13, 61.7 mg /100 g body weight), and GRD-13high , high dose test group (MCAO/R+GRD-13, n=11, GRD-13, 123.4 mg /100 g body weight).

TTC and H&E staining
TTC staining and H&E staining was performed according to the literature [18,19]. To analyze the liver toxicity effect of GRD-13, the liver enzyme ALT and AST in the serum of GRD-13 treated rats was analyzed by biochemistry analyzer (Pronto Evolution, Italy). Alanine Aminotransferase Activity Assay Kit (ALT, lot number: 172611) and Aspartate Aminotransferase Activity Assay Kit (AST, lot number: 171331) were purchased from Biosino Bio-technology and Science Inc., Beijing, China). Automated biochemistry analyzer (PRONTO Evolution Biochemistry Analyzer, Italy).

Sample collection for RNA-seq
Rat brain tissue (cerebral cortex area) was collected, immediately frozen in liquid nitrogen and submitted for RNA isolation and subsequent RNA-seq to BGI (Shenzhen, China).

Statistical analysis
Statistical significance was determined using unpaired t test or one-way analysis of variance (ANOVA) with a Dunnett's multiple comparisons test. P values of <0.05 were considered statistical significance. All the results were expressed as mean ± SEM.

Table 2. Identification of neuroactive compounds in GRD-13 by UPLC-qTOF analysis.

RESULTS
Analysis of neuroactive compounds in GRD-13
To identify the neuroactive molecules in GRD-13, we first built a ChemDraw structural library of compounds that exist in each individual herb of GRD-13 and have been previously reported to exhibit neuroprotective effects. Analysis of the water, ethanol, and petroleum extraction from GRD-13 by UPLC-qTOF mass spectrometry, which gives the exact mass of the compounds four digit after the decimal point, confirmed the existence of 6 bioactive molecules in GRD-13 extracts, i.e. costunolide, dehydrocostus lactone, myristicin, isoliquiritigenin, a-asarone and eudesmin [5-8] (Table 2, Figure 1 and Figure 2).

Functional assessment of rat MCAO/R model after GRD-13 treatment
Bederson scale is widely used to assess the impairments after neurological injury including ischemic stroke [20]. Therefore, we assessed the neuro-recovery effect of rat MCAO/R model treated with either GRD-13, or non-treated, or positive control (nimodipine) by Bederson scaling. Noticeably lower scores were obtained in GRD-13low  and GRD-13 high  treated MCAO model (Table 3). The successful establishment of MCAO/R model was indicated by a score of 2.10 in IS group, which showed some extent of self-recovery, scaling 1.62 after 2 weeks. Significantly, GRD-13high  treated MCAO/R model showed reduced impairment after 2 weeks: the scale dropped from 2.75 before treatment to 0.64 after treatment.

Morphological assessment of infarction
Effective recovery from stroke can be assessed by examination of the infarct volume in experimental animals. To examine the infarction in model animals, we conducted TTC staining after 2 weeks of continuous treatment of rat MCAO models with the control substance or GRD-13. Clear infarction observed in IS group indicates the successful MCAO modeling. Drug treated groups showed different degrees of reduction in infarction. However, the GRD-13 high  treated group showed most significantly reduced infarct area (Figure 3). To assess the therapeutic effect of GRD-13 at cellular level, we performed the H&E staining of brain tissues of rats. In
Figure 1. UPLC-QTOF-MS BPI chromatogram of GRD-13 extracted in ethanol (A), ethyl acetate (B), and petroleum ether (C).

<table>
<thead>
<tr>
<th>groups</th>
<th>no. of animals</th>
<th>Bederson scale*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before treatment</td>
</tr>
<tr>
<td>Non-treated (NT)</td>
<td>11</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>MCAO/R model (IS)</td>
<td>10</td>
<td>2.10 ± 0.39*</td>
</tr>
<tr>
<td>MCAO/R model + nimodipine (NI)</td>
<td>7</td>
<td>2.13 ± 0.41</td>
</tr>
<tr>
<td>MCAO/R model + GRD-13 low</td>
<td>16</td>
<td>2.75 ± 0.57</td>
</tr>
<tr>
<td>MCAO/R model + GRD-13 high</td>
<td>11</td>
<td>2.75 ± 0.45</td>
</tr>
</tbody>
</table>

* Average scale of animals in each group. * P < 0.05, ** P < 0.01

Table 3. Bederson scale scores of MCAO/R rat models before and after treatment. Each value represents the mean ±SEM. The symbol “*” and “**” indicate significant difference p<0.05 and p<0.01, respectively.
Figure 2. Plot of the identified neuroactive compounds extracted from EW in ethanol (A), ethyl acetate (B), and petroleum ether (C) phase using UPLC-QTOF-MS and UNIFI data processing.

Figure 3. Infarction area of the MCAO model animals were visualized by TTC staining. NT, non-treated group; IS, MCAO model; NI, nimodipine treated group; GRD-13\textsubscript{low} and GRD-13\textsubscript{high} represent low dose and high dose GRD-13 treated animals.
the infarction region of MCAO model cells shrank due to karyopyknosis, and apparent encephalomalacia was also observed in the extracellular matrix. The cells density was decreased in the infarction region, with apparent cellular swelling and edema formation (Figure 4A and Figure 4B). Reduced encephalomalacia as well as karyopyknosis was observed in the control group (NI) to some extent, although apparently less neurons were also observed compared to NT group (Figure 4C). In GRD-13 treated groups (GRD-13_{low} and GRD-13_{high}), encephalomalacia, edema formation as well as karyopyknosis were apparently reduced compared to MCAO model group. Increased infiltration of astrocytes and microglia was also observed in GRD-13 treated group (Figure 4D and Figure 4E). These data demonstrated the neuroprotective therapeutic effect of GRD-13.

Liver toxicity assessment

High dose of herbal medicines may have toxicity and induce damage to the liver. To test whether GRD-13 has liver toxicity, we examined the liver histopathology as well as the serum level of liver enzymes in rats treated with different doses of GRD-13, and compared to the NT, IS and NI groups. Liver histology of all groups showed normal cell morphology and regular tissue arrangement (Figure 5). Moreover, marker enzymes (AST, ALT) in serum (of 6 animals randomly chosen for analysis) were not elevated in GRD-13 treated animals, indicating the structural integrity of the hepatocellular membrane (Table 4). Therefore, the cytotoxicity of GRD-13 in the liver is negligible.

Differential gene expression after GRD-13 treatment

To investigate whether or not treatment with GRD-13 regulates gene expression in the brain after stroke, we extracted total RNA from infarction area (cerebral cortex) and analyzed the difference in the group of animals with or without GRD-13 treatment. RNA-seq analysis revealed that gene expression differed between different groups (Figure 6A, Supplementary Table 1). The most enriched KEGG pathways that potentially involved in neurotransmission, neuroprotection and neurogenesis include GABAergic synapse [21], cholinergic synapse, retrograde endocannabinoid signaling, glutamatergic synapse, and axon guidance (Figure 6B).

One of the most prominently upregulated and most abundantly expressed genes in GRD-13 treated group was Igf2 (Insulin-like growth factor 2) (Figure 7A). RNA-seq analysis revealed that the Log2 fold change of Igf2 expression in GRD-13 treated group compared to non-treated MCAO model group was 5.96,
which equals a fold change of 62. A subsequent validation of RNA-seq result by qPCR analysis confirmed that the expression of Igf2 was indeed significantly upregulated in the GRD-13 treated rats (Figure 7B). The treatment of MCAO model rats with nimodipine also induced upregulation of Igf2 expression, with a moderate fold change (Figure 7B).

To identify the Igf2 positive brain cells, and assess the Igf2 expression in all groups at protein level, we performed immunohistochemical analysis of the infarct lesion by staining with antibody against Igf2, as well as Iba-1 (microglia marker) and NeuN (neuron marker), respectively. Induction of stroke increased Igf2 protein level to some extent, both in Iba-1 and NeuN positive cells. Treatment with either nimodipine or GRD-13 accelerated protein synthesis, with slightly increased Igf2 protein detected in GRD-13 treated group (Figure 8A). In Iba-1+ cells, both nimodipine and GRD-13 increased Igf2 protein level; however, in NeuN+ cells, only GRD-13 treatment enhanced Igf2 protein content (Figure 8B).

Table 4. Liver enzyme analysis*. Each value represents the mean ±SEM (n=6). The symbol “*” indicate significant difference p<0.05.

<table>
<thead>
<tr>
<th>groups</th>
<th>no. of animals</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated</td>
<td>6</td>
<td>160.8 ± 28.9</td>
<td>77.7 ± 13.2</td>
</tr>
<tr>
<td>MCAO/R model</td>
<td>15</td>
<td>140.2 ± 25.3</td>
<td>60.7 ± 25.3</td>
</tr>
<tr>
<td>MCAO/R model + nimodipine</td>
<td>6</td>
<td>116.8 ± 25.2</td>
<td>76.6 ± 12.5*</td>
</tr>
<tr>
<td>MCAO/R model + GRD-13low</td>
<td>8</td>
<td>106.5 ± 8.14</td>
<td>71.7 ± 22.1</td>
</tr>
<tr>
<td>MCAO/R model + GRD-13high</td>
<td>7</td>
<td>113.8 ± 34.1</td>
<td>50.7 ± 11.1</td>
</tr>
</tbody>
</table>

*a The doses used for liver toxicity test: nimodipine was 4.0 mg per 100 g body weight; GRD-13low: 61.7 mg per 100 g body weight; GRD-13high: 123.4 mg per 100 g body weight. AST, aspartate aminotransferase; ALT, alanine aminotransferase. * P < 0.05

Figure 5. Representative liver histopathology in different group of rat by hematoxylin-eosin staining (H&E). A, non-treated; B, MCAO model; C, Nimodipine treated; D, GRD-13low treated; E, GRD-13high treated. Original magnification: X 400.
Figure 6. Treatment of rat MCAO model induced differential expression of genes in the infract area. (A) Heapmap of gene expression in the brain of 3 non-treated MCAO rats (IS_LA) versus 3 GRD-13 treated MCAO rats (GA_LA). (B) Enriched KEGG pathway showing the most significantly enriched pathways.

Figure 7. Treatment of rat MCAO model significantly upregulates Igf2 expression. (A) Scatter plot of IS group (MCAO model group, IS_LA) versus GRD-13 group (GA_LA). (B) Validation of Igf2 expression level in all treatment groups by qPCR. Each value represents the mean ± SEM (n=3). *** indicates p<0.05, **** indicates p<0.01.
DISCUSSION

Immediate medication of either thrombolysis or thrombectomy is crucial to increase the overall recovery and lowering mortality after stroke hit. However, herbal medicines including traditional Chinese medicine and Mongolian medicine have remarkable and irreplaceable advantages of neuroprotective and neuro-regenerative functions during post-stroke recovery [22]. The shortage of oxygen and nutrition in the brain during stroke severely damages brain cells and induce toxic substances such as reactive oxygen species (ROS). Many herbal medicine prescriptions contain anti-oxidant active molecules that decrease ROS level in the brain after stroke. Moreover, herbal prescriptions are well known for induction of angiogenesis and boosting blood flow in the injured tissues.

We have detected six previously reported neuroactive compounds (α-asarone, eudesmin, dehydrocostus lactone, costunolide, myristicin, isoliquiritigenin) in GRD-13. α-Asarone is neuroprotective and anti-oxidative, and suppresses secretion of pro-inflammatory cytokines [5]. Eudesmin may up-regulate GABA_A in the brain and induce anti-apoptosis of neuron [6]. Dehydrocostus lactone and costunolide have neuroleptic effect similar to chlorpromazine [7]. Isoliquiritigenin protects against cerebral ischemia injury through amelioration of energy metabolism in cerebral, as well as the anti-oxidative effect [8]. The combined neuroactivity of the aforementioned active molecules may attribute to the overall ameliorating effect of GRD-13 in rat model of ischemic stroke.

Gene expression in CNS undergoes tremendous fluctuation during and after injury such as stroke. For example, resident microglia turn into an anti-inflammatory phenotype (M2) soon after the happening of ischemic stroke, and a set of significantly up-regulated gene expression (such as TGFβ, G-CSF, IL-10, CCL-22 and Arginase-1) can be detected. However, during the recovery process, microglia subtype switches to a pro-inflammatory M1 phenotype, which expresses high level of IL-1β, IFNγ, TNFα, and iNOS etc [23-25]. Therefore, modulation of gene expression may be beneficial for stroke recovery.

Igf2 is a mitogenic growth factor that normally signals via Igf type-1 receptor (IGF1R) during fetal development. Igfs are structurally homologous to pro-insulin with the affinity to IGF1R in the order of Igf1>Igf2>insulin [26]. In adults, expression of Igf2 is restricted to the brain but the exact functions of adult Igf2 have not been elucidated. Rat choroid plexus express high level of Igf2, which is secreted into the cerebrospinal fluid (CSF) and distributed to subventricular zone (SVZ), where it signals via IGF1R and promotes proliferation of progenitor cells [27]. Recent study confirmed that Igf2 plays important regulatory role in mouse adult neurogenesis in the SVZ and the sub granular zone (SGZ) of the hippocampus [28].

We discovered that the mRNA level of Igf2 in cerebral cortex of rat MCAO models administered with GRD-13 was remarkably upregulated (Figure 7, Figure 8). The Igf2 protein level was also elevated in GRD-13 treated group, both in microglia (Iba-1+cells) and mature neurons (NeuN+cells), although
the changes were not as prominent as the changes of mRNA level. This is because Igf2 is a small secretory polypeptide that undergoes either paracrine or autocrine signaling. Therefore, the upregulated Igf2 protein level in the cells may not be accurately confirmed by IHC analysis due to the secretion. Our previous study demonstrated that another famous Mongolian medicine Eerdun Wurile (EW) also regulates Igf2 expression in rat MCAO model [13], although in a milder extent compared to GRD-13. Both EW and GRD-13 are regularly used for stroke recovery in clinic throughout Inner Mongolia Autonomous Region of China as well as Mongolia. EW and GRD-13 share multiple components which may responsible for the gene regulatory role of both medicines. Previous studies revealed that administration of Igf1 to rat model of ischemic acute kidney injury induced angiogenesis [29,30]; intramuscular injection of Igf1 decreased the neuronal apoptosis and improved motor function in brain ischemic rats [31]. Because Igf2 is predominantly signaled through IGF1R receptor, it may also induce neuroprotection and angiogenesis in rat MCAO models treated with GRD-13.

GRD-13 also upregulated the expression of genes reportedly involved in neuroprotection and neuro-growth (Supplementary Table 1). For example, the chemokine CXCL12 (stromal cell-derived factor 1a) is a key element in neuro-immune interface. Stimulation of CXCR4 receptor by CXCL12 regulates the synaptic release of glutamate and y-aminobutyric acid (GABA) [32]. CXCL12-CXCR4 axis also served as an urgent signal for initiating stem cell-based tissue repair [33]. GRD-13 treatment of MCAO rats resulted in significant up-regulation of CXCL12 in the infarct area, suggesting the neurorecovery effect of GRD-13 may have resulted from the elevated expression of CXCL12. Meanwhile, potential growth factor Psap (prosaposin) was also significantly up-regulated. Psap expressed in nervous tissue promotes neurite outgrowth, prevents programmed cell death, facilitates nerve regeneration and rescues neurons in ischemic stroke [34]. Treatment of MCAO rat models with GRD-13 further enriched SPARC expression in the brain. Recent study has confirmed that the expression of SPARC (Secreted Protein, Acidic and Rich in Cysteine) is up-regulated in microglia and astrocytes after MCAO, leading to improved cell survival of neurons [35].

CONCLUSION

The traditional Mongolian medicine GRD-13 contains multiple neuroactive small molecular components. Administration of GRD-13 to rat MCAO model significantly reduced neurological impairment, improved neuron survival, and reduced edema in the infarction. GRD-13 prescription showed negligible liver toxicity in experimental rats. The gene expression in the infarction of MCAO rat significantly altered following GRD-13 administration, with significantly enrichment to Igf2, and the potent neurotrophic factors as well as anti-inflammatory cytokines. Collectively, our data suggested that the upregulation of Igf2 may contribute to the remarkable neurorecovery effect of GRD-13.

DATA AVAILABILITY

A Table containing the list of significantly altered genes upon GRD-13 treatment used to support the findings of this study are included within the supplementary information file.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

FUNDING STATEMENT

This research was kindly supported by Natural Science Foundation of Inner Mongolia Autonomous Region (2017MS0821), Internal Funding from Research Institute of Mongolian Medicine of Inner Mongolia Autonomous Region (2016YJS31), Inner Mongolia Plan of Science and Technology (201701013). All the materials (chemicals and bio-reagents) used in the study were covered by the above funding bodies.

REFERENCES


SUPPLEMENTARY INFORMATION:

Supplementary Table 1. Significantly altered gene upon GRD-13 treatment

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene ID</th>
<th>Regulation</th>
<th>log2 Fold Change (GRD-13/IS)</th>
<th>P-value</th>
<th>Molecular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Igf2</td>
<td>84352</td>
<td>Up</td>
<td>5.36</td>
<td>1.92E-33</td>
<td>Growth factor [1]</td>
</tr>
<tr>
<td>Gjb2</td>
<td>84032</td>
<td>Up</td>
<td>5.45</td>
<td>1.87E-40</td>
<td>Cell-cell communication [2]</td>
</tr>
<tr>
<td>*CXCL12</td>
<td>24772</td>
<td>Up</td>
<td>2.1</td>
<td>2.59E-22</td>
<td>Chemokine: Key players of the neuro-immune interface; signal for initiating endogenous stem cell-based tissue repair [3,4]</td>
</tr>
<tr>
<td>*Psap</td>
<td>25524</td>
<td>Up</td>
<td>1.7</td>
<td>3.48E-09</td>
<td>Potent neurotrophic factor [5,6]</td>
</tr>
<tr>
<td>Sparc</td>
<td>24791</td>
<td>Up</td>
<td>1.7</td>
<td>0.002148</td>
<td>Promote neuronal health following CNS injury [7]</td>
</tr>
<tr>
<td>Thbs2</td>
<td>292406</td>
<td>Up</td>
<td>4.79</td>
<td>9.94E-42</td>
<td>Modulate angiogenesis [8]</td>
</tr>
<tr>
<td>Slc22a2</td>
<td>29503</td>
<td>Up</td>
<td>4.1</td>
<td>1.38E-17</td>
<td>Choline transporter [9]</td>
</tr>
<tr>
<td>Aqp1</td>
<td>29509</td>
<td>Up</td>
<td>4.87</td>
<td>2.18E-15</td>
<td>Edema after TBI [10]</td>
</tr>
<tr>
<td>Aplp</td>
<td>502317</td>
<td>Down</td>
<td>-1.27</td>
<td>5.21E-13</td>
<td>Essential modulators of glucose and insulin homeostasis and growth [11]</td>
</tr>
<tr>
<td>Sep-05</td>
<td>116728</td>
<td>Down</td>
<td>-2.16</td>
<td>6.68E-08</td>
<td>Axon growth [12]</td>
</tr>
<tr>
<td>Nrgn</td>
<td>64356</td>
<td>Down</td>
<td>-3.17</td>
<td>1.23E-16</td>
<td>Reflects infarct volume [13,14]</td>
</tr>
</tbody>
</table>

REFERENCES


