Huatanmaitong tablet alleviate cerebral ischemic reperfusion injury with hyperlipidaemia in rats by regulating OATPs/VEGF axis

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ABSTRACT
Stroke is the top priority pathogenesis of disability and death globally, affecting people worldwide. The presence of high levels of lipids in the blood has been confirmed as a vital factor of ischemic stroke. We aim to examine the effectiveness of Huatanmaitong tablet in hyperlipidaemia rats that have experienced an ischemic stroke. We created a rat model of middle cerebral artery occlusion (MCAO) with hyperlipidemia as a basis. Following 8 weeks of high-fat diet, the model rats underwent MCAO surgery. Subsequently, the rats were administered huatanmaitong tablets and lipitor tablets as treatments. Therefore there are five groups, CONTROL, MCAO, hyperlipidemia (HLP), Huatanmaitong tablet (HTMTT) and Lipitor (LIPITOR) groups respectively. To assess the efficacy of the medication, the serum lipid levels of rats were measured both prior to and following administration. Hematoxylin eosin staining was used to observe the alterations in the brain and liver structures within each group. VEGF and OATPs-related factors were detected in brain, and liver by using immunohistochemistry, Western blotting, and Quantitative PCR. After the model was established successfully, the infarct volume and behavioral scores of the model group, hyperlipidemia group, Huatan Maitong tablet group and Lipitor group had statistical differences (P<0.05). Blood lipid levels of rats were measured before and after treatment, and it was found that Huatanmaitong tablets effectively reduced these levels. Hematoxylin and eosin staining of the brain and liver showed that huatanmaitong tablets maintained the microstructure stability. Western blotting and real-time PCR revealed that Huatanmaitong tablets improved the expression level of organic anion transport (OATP1B1, OATP2B1) in rat tissues with ischemic stroke, enhancing the transmembrane transport of exogenous substances and maintaining homeostatic balance. Additionally, it down-regulated the expression of VEGF in various organs such as the brain, and liver, demonstrating the ability of Huatanmaitong tablets to remove phlegm, blood stasis, and promote circulation by regulating serum lipid levels, organic anion transport peptide, and VEGF in rats. The behavioral score of ischemic stroke rats can be improved and the neurological impairment symptoms of rats can be alleviated by Huatanmaitong tablet through the regulation of OATPS/VEGF axis.

Introduction
Stroke, which is leading to the highest number of disability and mortality in China, has an annual incidence of over 2 million cases (1). Ischemic stroke can result in severe neurological impairments, including consciousness disorders, limb paralysis, and cognitive decline, imposing significant economic and social burdens on affected regions (2). Hyperlipidemia has been established as a vital risk factor for ischemic stroke (3). Relevant studies have confirmed an increase in serum cholesterol levels and following administration. Hematoxylin eosin staining was used to observe the alterations in the brain and liver structures within each group. VEGF and OATPs-related factors were detected in brain, and liver by using immunohistochemistry, Western blotting, and Quantitative PCR. After the model was established successfully, the infarct volume and behavioral scores of the model group, hyperlipidemia group, Huatan Maitong tablet group and Lipitor group had statistical differences (P<0.05). Blood lipid levels of rats were measured before and after treatment, and it was found that Huatanmaitong tablets effectively reduced these levels. Hematoxylin and eosin staining of the brain and liver showed that huatanmaitong tablets maintained the microstructure stability. Western blotting and real-time PCR revealed that Huatanmaitong tablets improved the expression level of organic anion transport (OATP1B1, OATP2B1) in rat tissues with ischemic stroke, enhancing the transmembrane transport of exogenous substances and maintaining homeostatic balance. Additionally, it down-regulated the expression of VEGF in various organs such as the brain, and liver, demonstrating the ability of Huatanmaitong tablets to remove phlegm, blood stasis, and promote circulation by regulating serum lipid levels, organic anion transport peptide, and VEGF in rats. The behavioral score of ischemic stroke rats can be improved and the neurological impairment symptoms of rats can be alleviated by Huatanmaitong tablet through the regulation of OATPS/VEGF axis.

Materials and Methods
Animals and feeding
We acquired 50 male Sprague-Dawley rats from the Me-
Subsequently, the percentage of Infarct volume in relation was utilized to assess the size of the cerebral infarction. 15 minutes in the absence of light. Afterward, the stained in thickness. Next, the cerebral portions were placed in a machine, various sets of animals underwent behavioral as sterilized and stitched up.

Following 2 hours of cerebral ischemia, the clot made of sterilizing and blocking ICA to stop the blood flowing into the beginning of the middle cerebral artery. The thread plug was inserted 20-22 mm, which was exactly the depth to block the MCA opening, and the silk thread at the root of ECA was ligated. Following 2 hours of cerebral ischemia, the clot made of thread was extracted and the external carotid artery was tied off to avoid hemorrhaging. Subsequently, the cut was sterilized and stitched up.

Changes in blood lipid levels
Blood samples were obtained from the orbit of rats after eight weeks on a high-fat diet to assess their blood lipid levels. Additionally, blood samples were collected again 7 days after the MCAO procedure and drug administration. The samples were analyzed using an automatic biochemical analyzer (CX-7, Beckman, Franklin Lakes, NJ, USA) to detect the blood lipid-related indexes, for example, TC, TG, HDL-C, NHDL-C and LDL-C.

Hematoxylin and eosin staining
Xylene I and Xylene II were used sequentially for 15 minutes. They were then immersed in 100% ethanol I and 100% ethanol II for 5 minutes respectively, 95% ethanol for 3 minutes, 95% ethanol again for 3 minutes, 90% ethanol for 2 minutes, then after 2 minutes in 85% ethanol, 2 minutes in 75% ethanol, and 5 minutes in deionized water, the samples are placed in deionized water for 5 minutes. Following two minutes in 85% ethanol, two minutes in 75% ethanol, and five minutes in deionized water, the samples are placed in deionized water. Following that, the sections were rinsed with water for 1 minute and differentiated using 0.3% alcohol hydrochloride for approximately 1 to 2 seconds. In the end, the sections were washed with tap water for 30 seconds. The sections were treated with eosin dye solution for a duration of 1 minute and 30 seconds, followed by a 30-second rinse with tap water. The liver tissue sections were soaked in 70% ethanol for 20 seconds, followed by the specific steps for dehydration transparency to dehydrate and make transparent. Afterwards, neutral gum was applied to the slide. Carefully position the cover glass to eliminate bubbles and secure the sheet.

Immunohistochemical assay
Paraffin sections were dewaxed in water. The cells were exposed to a 3% hydrogen peroxide solution for 5-10 minutes at normal temperature. It was then rinsed twice with distilled water and cleaned with PBS for 5 minutes each time. To prevent any non-specific binding, 5% normal goat serum diluted in PBS was applied at 37°C for 5-10 minutes. The serum was then discarded without washing. We applied the targeted antibody working solution dropwise and hatched it overnight at 4°C. Subsequently, a dropwise application of the second antibody working solution was performed for 30 minutes. DAB color development was performed, and hematoxylin was counterstained, transparent and sealed.

Analysis of Western blotting
Measure the weight of the infarcted brain area, which should be 100 mg, and place the samples in a radiomunoprecipitation assay (RIPA) lysis buffer that is ice-cold. Utilize a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA) to determine the protein content according to the instructions in the manual. Separate equal amounts (20–40 μg) of protein using 10–15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer them onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA, Cat# ISEQ00010). To block the PVDF membranes, they were washed with TBS (pH 7.4) and blocked for 90 minutes with 5% skim milk powder. Then, they were hatched with target antibodies overnight at 4°C:
Table 1. The Primers of OATP1B1, OATP2B1, VEGF and GAPDH.

<table>
<thead>
<tr>
<th>Targets genes</th>
<th>Sense primer (5′–3′)</th>
<th>Antisense primer (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>CAGTGGCAGGCTAAACACC</td>
<td>GGATCCCAGTGTTCGTTGAG</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>TTCCAGTCGGCAGAAGCCA</td>
<td>AGGAGATCCCAAGGGCTGA</td>
</tr>
<tr>
<td>VEGF</td>
<td>TGTACCTCACCACCGCAAAG</td>
<td>CTGGCGTGGTAGACGTCCAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTATGACTCTACCGGGGAAGT</td>
<td>TCTCGCTCCTCGGAGATG</td>
</tr>
</tbody>
</table>

Changes in blood lipid levels

As shown in Figure 2, after the intervention, there were some changes in total cholesterol (TC), total triglyceride (TG), non-high-density lipoprotein (NHDL-C), high-density lipoprotein (HDLC) and low-density lipoprotein (VLDL-C) among the groups. Although some changes were not statistically significant between groups. In the Figure 2A, Figure 2B, Figure 2C, and Figure 2D, the changes of TC, TG, NHDL-C and LDL-C were significant in HTMTT and LIPITOR groups.

HE staining of rat brain and liver

The results of HE staining of the liver and brain tissue of rats in each group can be seen in Figure 3. According to the results of HE staining, the liver cells in the CONTROL group were arranged neatly, the liver lobules were regular, the cells were polygonal, and the cytoplasm was uniform. There were lipid droplet vacuoles of varying sizes in the HLP group the nuclei were located on the edge, and the cell boundaries were unclear. We saw improvements in these changes in the HTMTT group and LIPITOR group.

In the CONTROL group, the nerve cells in the brain tissue of rats were arranged neatly, the shape was normal, the cell color was uniform, the cell membrane structure was complete, and the axon was not broken, and the white matter part was intact. In the MCAO group and HLP group, nerve cells are thinned, structural integrity is damaged, and axons are broken.

After drug intervention, these pathological changes were improved to varying degrees.

Results

Huatanmaitong tablet improved behavioral scores and reduced the infarct volume of ischemia

After a week of drug administration via the stomach, we assessed the behavioral scores of the respective rats, there was the control group (0), MCAO group (3.50±0.54), HLP group (3.16±0.75), HTMTT group (1.83±0.75), LIPITOR group (2.00±0.63). There is a significant difference in neurological function compared with the CONTROL group. Also it’s obvious to conclude that the behavioral scores of the HTMTT group and LIPITOR group alleviate neurological damage and improve behavioral scores. However, there is no significant difference between the HTMTT group and the LIPITOR group (P<0.05). The results are shown in Figures 1A and 1B. In the CONTROL group, there were no ischemic areas, and the infarct volume of the HLP group was significantly different from the other four groups. The HTMTT group can significantly reduce the volume of cerebral infarction, and the effect is also statistically significant compared with the LIPITOR group (Figures 1C and 1D).

Figure 1. Behavioral scores (A,B) and TTC staining (C,D). The study measured behavioral scores (A) and quantitative results of post-surgery score levels (B). Additionally, the rat brain was stained using TTC after drug intervention (C) The results show an apparent difference (P<0.001) when compared with the control group (B, D). This suggests that the administration of huatanmaitong tablets can enhance the behavioral score and alleviate the neurological deficit in rats.
HTMTT weakens the positive staining of VEGF and increases the positive staining of OATP2B1 in the brain

The expressions of OATP2B1 and VEGF were examined in brain tissue by immunohistochemistry. The MCAO group showed an increased level of VEGF expression compared to the CONTROL group and HLP group ($P<0.001$), which decreased in the HTMTT group and LIPITOR group ($P<0.001$). In contrast, the MCAO group showed a decreased level of OATP2B1 expression compared to the CONTROL group ($P<0.05$), which increased in the HTMTT group and LIPITOR group. The HTMTT group and LIPITOR group exhibited an increased protein expression level compared to the HLP group ($P<0.05$) (Figure 4).

HTMTT up-regulated the relative protein expression of OATP2B1, and OATP1B1 and decreased the mRNA levels of VEGF

The relative expression level of OATP2B1, OATP1B1 and VEGF in liver tissues in Figure 5A-D and brain tissues in Figure 5E-H by western blot. The protein expression of VEGF in the MCAO and HLP group was significantly higher in the model group compared to the CONTROL group, HTMTT group and LIPITOR group ($P<0.05$). This proves that the HTMTT group and LIPITOR group can play a role by down-regulating the expression of VEGF. As to the OATP2B1, OATP1B1, it showed that when compared with the MCAO group and HLP group, the expression of OATP2B1, OATP1B1 can be upregulated and restored to the normal level (Figure 5B, C). In the brain tissue, the protein expression levels of OATP2B1 and OATP1B1 were down-regulated in the MCAO group and HLP group compared to the CONTROL group. The protein expression levels of OATP2B1 and OATP1B1 could be enhanced in the HTMTT group and LIPITOR group, with statistical significance ($P<0.05$) in Figure 5F, 5G. Both the HTMTT group and the LIPITOR group were able to decrease the level of VEGF protein expression.
HTMTT up-regulated the mRNA levels of OATP2B1, OATP1B1 and decrease the mRNA levels of VEGF

q-PCR results revealed the relative expression level of OATP2B1, OATP1B1 and VEGF in liver tissues (A-C) and brain tissues (D-F). In Figure 6A-C, in the liver tissue, the expression levels of OATP2B1 and OATP1B1 were found to be down-regulated expression in the MCAO group compared to the CONTROL group (P<0.05), but increased in the HTMTT group and LIPITOR group. VEGF mRNA levels increased in the MCAO and HLP group, while significantly decreasing in the HTMTT group and LIPITOR group (P<0.001). In the Figure 6D-F, The mRNA expression levels of OATP2B1 and OATP1B1 in brain tissue were reduced in the MCAO group and HLP group compared to the CONTROL group (P<0.001), but increased after HTMTT and Lipitor. (P<0.01). Both the Huatan Maitong tablet group and Lipitor group were able to decrease the expression level of VEGF mRNA compared to the MCAO group and HLP group, with statistical significance (P<0.001).

Discussion

Establishing a stable and consistent animal model is of utmost importance. After three days of adaptive feeding, they were then given a high-fat diet to simulate the corresponding symptoms in traditional Chinese medicine (11). By assessing the rats’ serum lipid levels before and after

the intervention, we confirmed that the high-fat diet causes changes in blood lipid levels in rats (Figure 2). Both Huatanmaitong tablet and Lipitor can reduce the level of blood lipid in rats to some degree, and the effect of Huatanmaitong tablet is not inferior to Lipitor. Additionally, we examined the changes in the rats’ brain and liver structures through using hematoxylin and eosin staining. We found that Huatanmaitong tablet effectively lessen lipid droplets associated with ischemic stroke in rats with hyperlipidemia, while also maintaining microstructural stability. Through Western blotting (Figure 5) and qPCR (Figure 6), we further demonstrated that Huatanmaitong tablet enhanced the expression of OATP2B1 and OATP1B1 in the rat tissues affected by ischemic stroke. This improvement facilitated the transmembrane transport of exogenous substances within the body, thereby playing a crucial role in maintaining homeostatic balance between the cellular environment and the external surroundings.

Among the potential brain repair regulators, VEGF is famous for its functional roles in post-stroke neuroprotection, neurogenesis and angiogenesis (12). Simultaneously, it has the ability to decrease the expression of VEGF in various organs like the brain, and liver. This demonstrates that Huatanmaitong tablet can effectively perform the function of promoting blood circulation by modulating serum lipid levels, organic anion transport peptide, and vascular endothelial growth factor in rats. The difference may be due to variations in expression levels, distribution across tissues, specificity for substrates, or affinity for substrates between different species (13,14).

After reviewing specific blood-brain barrier uptake
transports, the human organic anion transport polypeptides (OATP) that can be targeted for improved neuroprotective drug delivery is an important transporter, including OATP2B1 and OATP1B1 and so on (15). This finding was further utilized to evaluate the involvement of various locations in the recognition of transporter substrates. The recognition and transportation of sulfated steroids is complex, that relies on several amino acid residues in the C-terminal portion of the transporter, leading us to our conclusion (16).

Following cerebral ischemia and reperfusion, VEGF engages in diverse pathological processes, including the promotion of vascular permeability and initiation of a cascade of inflammatory responses (12,17). OATPs, a crucial component of the SLC superfamily, facilitate the sodium ion-independent transportation of numerous amphipathic substances (including various medications) across cell membranes (18,19). Research has demonstrated that OATPs hold promise as targets for delivering drugs to the central nervous system. Additionally, substrates of OATPs, like statins, exhibit favorable neuroprotective properties in conditions such as hypoxia, inflammatory diseases, and multiple sclerosis (20).

Ethical approval
This study was approved by the Experimental Animal Ethics Committee of the Guangdong Provincial Hospital of Chinese Medicine.(NO.20200005).

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Author declaration
All authors contributed to the writing of the manuscript. The final version of the manuscript has received approval from all authors.

Conflict of interest
No conflicts of interest are declared by any of the authors.

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