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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 hyperlipidemia 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 downregulated 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.

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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 within a population could lead to an estimated 9.2 million additional cardiovascular events between 2010 and 2030 (4,5). Research has demonstrated that Oatps can serve as potential targets for delivering drugs to the central nervous system, and their substrates, such as statins, exhibit favorable neuroprotective effects in hypoxia, inflammatory diseases, and multiple sclerosis (6,7). Traditional Chinese medicine operates on multiple levels, targets, and aspects. Traditional Chinese medicine (TCM) is usually a mixture of herbal plants or extracts that contain hundreds of different ingredients with widely different physical and chemical properties (8). It has been substantiated that OATPs actively participate in the transportation process of statins, making them potential targets for these medications (9). Huatanmaitong Tablet, developed by Professor Shen Baofan, a renowned Chinese medicine expert, based on clinical experience, effectively reduces blood lipid levels and demonstrates positive outcomes in stroke patients with hyperlipidemia. Clinical observation experiments conducted by Liang Keyi have confirmed the efficacy of Huatan Maitong tablets in patients with carotid atherosclerosis. To delve deeper into the specific mechanism of action of Huatanmaitong tablets, we have designed and executed this experiment.

Materials and Methods

Animals and feeding

We acquired 50 male Sprague-Dawley rats from the Me-

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dical Laboratory Animal Center of Guangdong in Guangzhou, China, which were about 8 weeks old and weighed 180-200 grams. These rats were kept in the animal room under controlled conditions of 22-24°C temperature, normal circadian rhythm and free to eat and drink. All animal experiments and relevant procedures were checked and approved by the Ethics Committee of the Guangdong Academy of Chinese Medicine (Guangzhou, China). The rats were fed ad libitum for 3 days before the experiments. The rats, following 3 days of adaptive feeding, were fed with a high-fat diet consisting of a high-fat diet fed to the rats for 8 weeks. Among the groups, the control group was used as the control group, and the MCAO group received surgical intervention. HLP was performed on rats fed a high-fat diet before MCAO surgery, HTMTT rats received Huatan Maitong tablet intervention after MCAO operation, and the LIPITOR group received LIPITOR intervention

The precedure of MACO

According to the method reported by Zea Longa et al, we take the conventional surgical techniques (10). Following the administration of anesthesia, a vertical incision was performed on the front of the neck to uncover the left cervical triangle. First of all, we carefully separated the common carotid artery to avoid damaging the blood vessels and stimulating the vagus nerve. Then we carefully separate the external carotid artery and the internal carotid artery from the distal end. The pterygopalatine artery of ECA branch was electrocoagulated with a bipolar electrocoagulator, the main ECA trunk was ligated, cut off and free, and the distal end was ligated to prevent bleeding. The blood flow of CCA and ICA was temporarily blocked with sutures. A cut was made at the stump of ECA, approximately half the size of its diameter, and the thrombus that had been prepared was inserted into ICA. A slipknot was then tied at the base of ECA to block the blood flow in ICA, and the suture that was temporarily blocking ICA blood flow was removed. To prevent entering the pterygopalatine artery, the threaded plug was carefully and slowly inserted into the intracranial part of the internal carotid artery through the initial part of the external carotid artery 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.

Behavioral score and TTC staining

Following a week of drug administration via the stomach, various sets of animals underwent behavioral assessments and TTC staining. In summary, the brains were extracted and cut into coronal slices measuring 2-3 mm in thickness. Next, the cerebral portions were placed in a 2% TTC solution and incubated in a dark box at 37°C for 15 minutes in the absence of light. Afterward, the stained sections were captured on camera and the ImageJ program was utilized to assess the size of the cerebral infarction. Subsequently, the percentage of Infarct volume in relation to the total area was computed.

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 are 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 radioimmunoprecipitation 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.

Targets genes	Sense primer (5'–3')	Antisense primer (5'–3')
OATP1B1	CAGTGGCAGGCTTAACAACCT	GGATCCCATGTGTTCGTTGAG
OATP2B1	TTCCAGTCGCACAGAAACCA	AGGAGATCCCAAAGGGCTGTA
VEGF	TGTACCTCCACCATGCCAAGT	CTGCGCTGGTAGACGTCCAT
GAPDH	GTATGACTCTACCCACGGCAAGT	TCTCGCTCCTGGAAGATGGT

rabbit OATP1B1 (AB3572P, Millopore, Billerica, MA, USA), rabbit VEGF (AF5131, Affinity, Melbourne, Australian), rabbit OATP2B1 (Bs-3913R, BIOSS, Woburn, MA, USA), and rabbit anti-GAPDH (1:1000, Ab181602, Abcam, Cambridge, MA, USA). Following three washes using tris buffered saline-tween (TBST), the membranes underwent incubation with a secondary antibody, specifically goat anti-mouse IgG-HRP (1:5000, BS13278, bioworld.at), at room temperature for 2 hours. By using a Bio-Rad chemiluminescence system (Hercules, CA, USA), we were able to visualize target proteins.

Quantitative PCR

After a period of ischemia, the cortex tissues of rats were collected days following reperfusion. Total mRNA was extracted from the liver and brain with TRIzol reagent. Next, the mRNA was converted into cDNA by employing the TransStart® Green qPCR SuperMix. For the Quantitative -PCR experiments, the primers used were as follows: The relative expression of OATP1B1, OATP2B1, VEGF, and GAPDH mRNA were calculated by using the $2^{-\Delta\Delta ct}$ method (Table 1).

Statistical Analysis

The GraphPad Prism© software (GraphPadSoftware, Inc., La Jolla, USA) was utilized for conducting the statistical analysis. The mean values are reported based on a minimum of three separate experiments. The experiments were performed in sets of three, using biological replicates. We set P-value < 0.05 as the criterion to determine whether there is statistical significance. One-way ANOVA analysis and paired sample t-test were applied to analyze.

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 HTMTP 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).

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 (HDL-C) and low-density lipoprotein (VDL-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, 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.

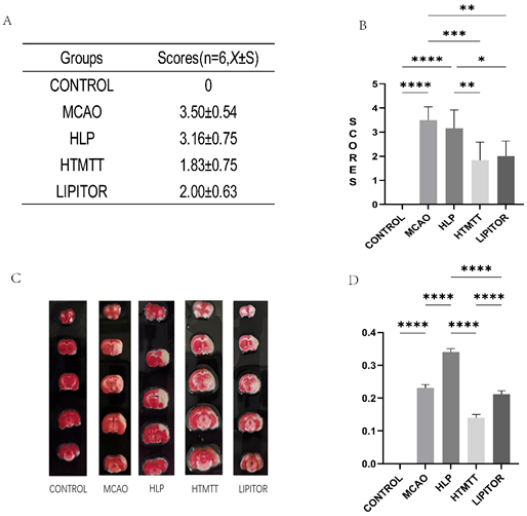

Figure 1. Behavioral scores (A,B) and TTC staining (C,D). The study measured behavioral scores (A) and quantitative results of post-surgery score levels (B). Additionally, the rat brain was stained using TTC after drug intervention (C) The results show an apparent difference (P<0.001) when compared with 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.

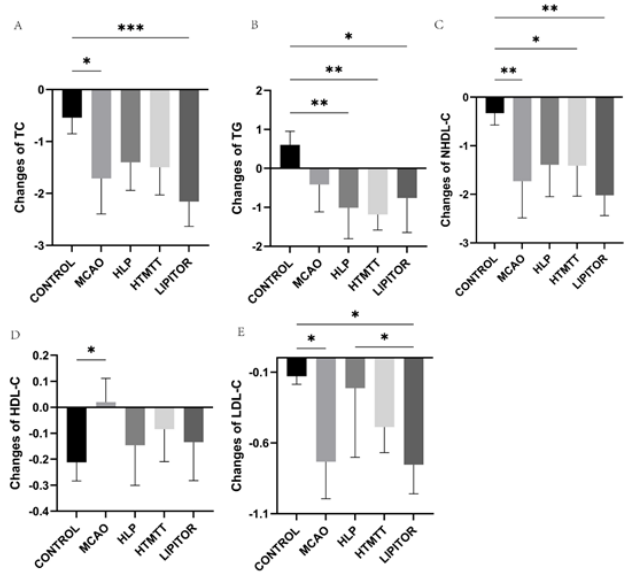

Figure 2. Changes in blood lipid levels (n=3). A is for total cholesterol (TC), B is for total triglyceride (TG), C is for non-high-density lipoprotein (NHDL-C), D is for high-density lipoprotein (HDL-C), E is for low-density lipoprotein (VDL-C). The serum lipid levels of each group were measured before and after. We statistically analyze the significance of the value of the change between them. Following high-fat feeding, there was an obvious up-regulation in TG, TC and LDL-C, which differed statistically from the levels observed in the control group. The rats had a large body size and thick subcutaneous fat, which decreased after treatment, while the high-density lipoprotein remained relatively unchanged. Huatanmaitong Tablet could reduce the blood lipid level of the rats.

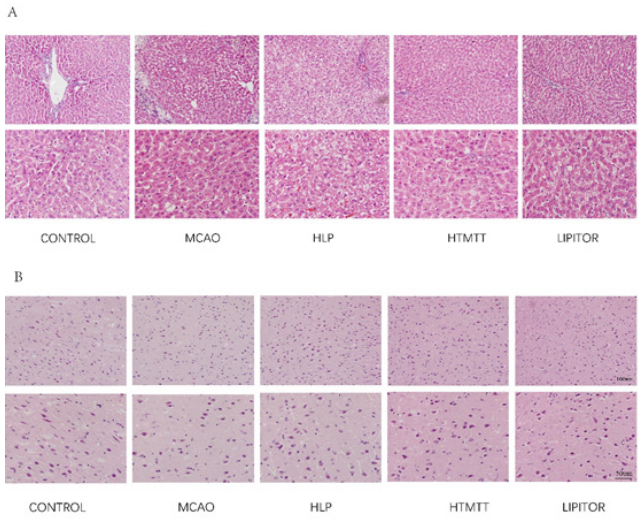

Figure 3. HE staining in the liver and brain (A is for liver, B is for brain, n=3). The irregularity of brain tissue in the infarct area, disintegration of the shaft, and improvement in the drug group are indicated by A. The liver leaf rule states that liver cells are arranged in groups, with each cell having a polygonal shape and uniform cytoplasm. The liver of the damp phlegm blood-stasis group exhibits unclear liver size and cell limit. However, the condition has improved following the administration of huatanmaitong tablets and Lipitor.

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.

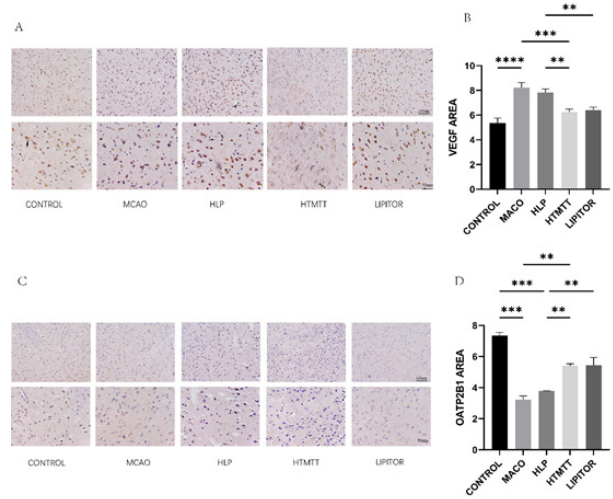

Figure 4. OATP2B1 and VEGF expression in brain tissue by immunohistochemistry (n=3). Immunohistochemistry was used to examine the expression of VEGF and OATP2B1 in the brain. The model group showed an increased level of VEGF expression compared to the CONTROL group (P<0.001), which decreased after drug intervention. The HTMTT group and LIPITOR group exhibited a decreased protein expression level compared to the MCAO group and HLP group (P<0.001). In contrast, the model group showed a decreased level of OATP2B1 expression compared to the control group (P<0.05), which increased after drug intervention. The HTMTT group and LIPITOR group exhibited an increased protein expression level compared to the MCAO group and the HLP group (P <0.05).

HTMTT up-regulated the mRNA levels of OATP2B1, OATP1B1 and decresse 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

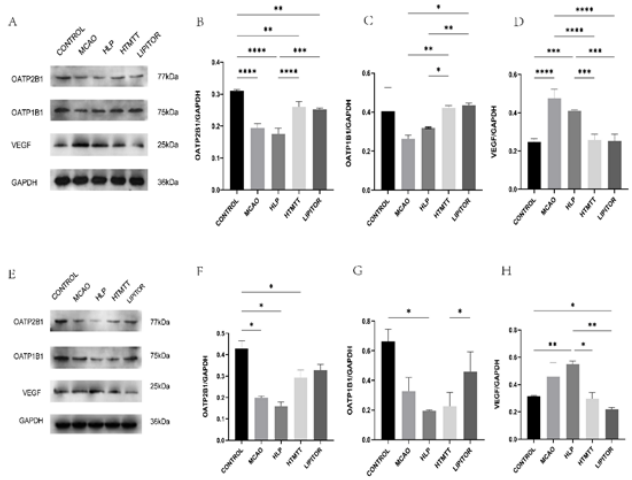

Figure 5. Relative expression level of OATP2B1, OATP1B1 and VEGF in liver tissues (A-D) and brain tissues (E-H) by Western blot (n=3). In liver tissue, the protein expression levels of OATP2B1 were lower in the MCAO and HLP groups compared to the CONTROL group. The protein expression levels of OATP2B1 and OATP1B1 could be enhanced by Huatanmaitong Tablets and Lipitor with statistical significance (P<0.05). Simultaneously, the protein manifestation of VEGF in the Huatan Maitong tablet and Lipitor groups exhibited a notable reduction, with a statistically significant distinction (P<0.05). In brain tissue, the expression of OATP2B1 and OATP1B1 were down-regulated in each group as to the normal group (P<0.05). The protein expression of VEGF in the MCAO and HLP groups was significantly higher in the model group compared to the other groups. Additionally, the protein expression levels of VEGF in the HTMTT group and the LIPITOR group showed a decrease (P<0.05). These findings indicate that the Huatan Maitong tablet can reduce the expression level of VEGF protein in the brain tissue of rats with ischemic stroke.

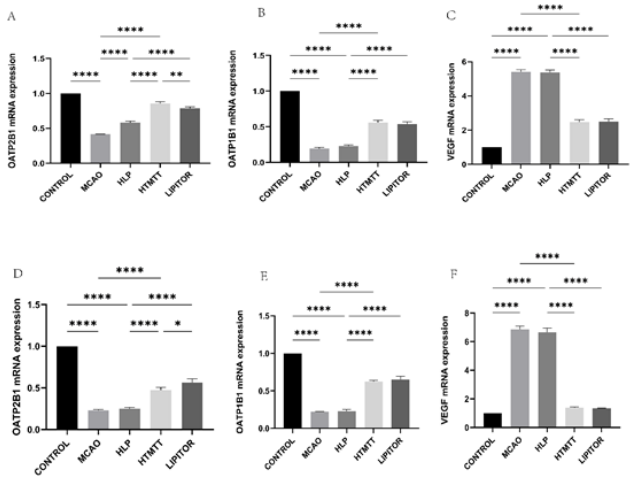

Figure 6. Relative expression level of OATP2B1, OATP1B1 and VEGF in liver tissues (A-C) and brain tissues (D-F) by q-PCR (n=3). (A-C) In liver tissue, the levels of OATP1B1, OATP2B1, and VEGF mRNA expression were examined. In the liver tissue, the expression levels of OATP2B1 and OATP1B1 were found to be down-regulated expression in the MCAO group compared to the control group (P<0.05), but increased after drug intervention. VEGF mRNA levels increased in the MCAO and HLP group, while significantly decreasing in the Huatanmaitong tablet group and Lipitor group, showing statistical significance when compared to the MCAO and HLP group (P<0.001). in the (D-E). 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 drug intervention. VEGF mRNA showed a significant increase in the MCAO and HLP group (P<0.01).

transporters, 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.2020005).

Acknowledgments

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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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