Research Article

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Salidroside Attenuate Neurological Impairment By Reducing Blood-Brain Barrier Permeability After Diabetic Cerebral Hemorrhage Mice

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Authors' contributions
The data were extracted and analyzed by YW,LL. The rough manuscript was drafted by CQ. JQ and YS designed the study and reviewed the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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1. Abstract

Diabetes mellitus [DM] is a high-risk factor for cerebral hemorrhage [ICH], which can damage the structure and function of the body's macrovessels and microvessels, leading to cerebral atrophy, neuropathy, and inflammatory response, which as a major mechanism of secondary brain tissue damage after cerebral hemorrhage, neuroinflammation can exacerbate the brain damage. As an herbal remedy, sal can exert its ability to reduce inflammation, anti-apoptosis, and anti-toxicity responses in a number of diseases. We examined the blood-brain barrier permeability and the degree of endothelial cell damage in vivo and in vitro using electron microscopy, westernblot, NO content assay, CCK8 and other experimental methods, In conclusion,we found that sal could reduce the blood-brain barrier permeability after diabetic cerebral hemorrhage by alleviating the endothelial cell damage, and ultimately improve the secondary neurological impairment.Thus, it may be a candidate medicine for further study of molecular or therapeutic targets.

2. Introduction

Cerebral hemorrhage is a serious life-threatening disease that accounts for 15-20% of all strokes, and most survivors suffer lifelong disability [1]. The pathophysiological mechanism of cerebral hemorrhage is very complex. Early hematoma expansion after cerebral hemorrhage, reduced blood flow around the hematoma, activation of blood clots and blood and release of large amounts of thrombin, activation of microglia, leukocyte infiltration, activation of the complement system, resulting in inflammatory response, oxidative stress, apoptosis, etc., resulting in a series of secondary injuries to the brain tissue locally and causing an increase in the permeability of the blood-brain barrier [2]. The increased permeability of the blood-brain barrier further promotes the infiltration of leukocytes and inflammatory mediators into the periphery of the hematoma, forming a malignant cycle, aggravating the patient's condition and worsening the prognosis [3].

Blood glucose is an important contributor to cerebral hemorrhage, and elevated blood glucose is associated with an increased risk of cerebral hemorrhage and worse outcomes after cerebral hemorrhage. This may be related to the microcirculation disorder after elevated blood glucose, resulting in inflammation, oxidative stress, apoptosis, and nerve growth factor deficiency [4]. Long-term disorders of glucose metabolism cause brain tissue edema and increased pressure, which promotes the development of cerebral hemorrhage disease and leads to disruption of the blood-brain barrier, worsening the condition after cerebral hemorrhage [5]. Mild blood-brain barrier disruption is associated with cellular pathways that allow only some small molecules to pass, while severe blood-brain barrier disruption is mediated by disruption of tightly connected sealed cells that allow larger molecules to pass and can lead to severe cerebral edema. Therefore mitigating blood-brain barrier damage after diabetic cerebral hemorrhage is urgent.

Salidroside [sal] is mainly isolated from the Sedum family [6] and other plants such as Lignaceae [6,7], Labiatae [8,9]and Loganaceae [10,11]. Previous studies have shown that sal has a wide range of biological activities such as anti-inflammatory, anti-apoptotic, anti-hypoxic, antidepressant and antioxidant effects [8,11]for the treatment of Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, cancer, diabetes mellitus, liver injury, and addictions, and that sal has a significant role in the prevention of neuronal cell damage following cerebral ischemia in vitro

and vivo, which is worthy of promotion[6,10,12]. Increasing evidence further demonstrates that the neuroprotective effect of sal involves multiple mechanisms of action, especially antioxidant, anti-inflammatory, anti-apoptotic and attenuating blood-brain barrier damage, significantly reduces cerebral infarction, relieves cerebral edema in advance and improves neurological function. In diabetic cerebral hemorrhage whether salin diabetic cerebral hemorrhage can reduce the blood-brain barrier damage and thus reduce the secondary neurological impairment after diabetic cerebral hemorrhage still needs to be explored.

3. Materials and methods

3.1. Experimental Animals

Laboratory C57BL/6 mice[20–22g, male]were purchased from Changsheng Biology [Licence No.: SCXK(Liao)2020-0001], and raised day and night alternately. Animal group: control group; Diabetic cerebral hemorrhage group; Sal group[2.5mg/kg 5mg/kg 10mg/kg].They were raised according to the guidelines of the Institutional Animal Care and Use Committee [IACUC] of Harbin Medical University [2022016]. All animal experiments were carried out with the aim of reducing the number and severity of injuries to the experimental animals.

3.2. Induction of Diabetes

C57BL/6 mice were intraperitoneally injected with streptozotoleutin [STZ, SolarBio]. They were fasted without water the night before injection and fed with grains 2 hours after injection and glucose solution 4–6 hours after injection. Blood glucose levels were measured on days, 3, 7, and 14, and blood glucose ≥16.7 was considered as successful model.

3.3. Induction of ICH and Salidroside Treatment

A collagenase-induced cerebral hemorrhage model was prepared using well-built diabetic mice, which were fasted for 12 hours and dehydrated for 2-4 hours before modelling. The mice were anesthetised with isoflurane at 22-24°C. The isoflurane concentration was adjusted to 2%-5% for 3-5 minutes for induction anaesthesia, and then to 5% for 5-7 minutes for deep anaesthesia. After the mice were fully anaesthetized, the mice were placed in the prone position in the stereotaxic operating table with ear clips, and the isoflurane concentration was adjusted to 1.5% to maintain the anaesthesia. A 5 ml micro syringe was fitted to the stereotaxic device and an appropriate amount of collagenase was withdrawn. The needle position was adjusted to 2.2 mm to the left and 1.0 mm to the front, and the needle was inserted slowly until a depth of 2.7 mm was reached. The needle was held for 30 seconds, then the needle was withdrawn upward to a depth of 2.6 mm, and the drug was injected at a rate of 0.25 μl/min. After injection, the needle is held for more than 10 minutes to prevent reflux of the drug. The needle was slowly lifted to a depth of 2.0mm and held for a further 5 minutes before being slowly withdrawn. The round hole was closed with melted paraffin wax, and then the skin incision was sutured and sterilised with iodophor. After the operation, the mice were placed on a thermostatic heating pad and their tails were marked or ear-marked, and the anaesthetized mice were placed back into the corresponding marked cages for rearing after awakening.

At 1 day after diabetic cerebral hemorrhage, sal was injected into mice by intraperitoneal injection at 10 mg/kg in the high-dose group, 5 mg/kg in the medium-dose group and 2.5 mg/kg in the low-dose group.

3.4. Cell Culture

In this study, Human cerebral microvascular endothelial cells [HCMEC] was used for in vitro experimental research, purchased from Saibaikang [Shanghai] Biotechnology Company. The cell line was cultured in DMEM with high glucose[30 μM]+10% fetal bovine serum+1% penicillin and streptomycin at 37°C,CO2concentration 5%.Cell grouping: high glucose [HG] group [HCMEC+30 μM glucose high glucose medium]; HG+hemin group [HCMEC/D+30μM glucose high glucose medium + 30μMhemin]; sal group [HCMEC+high glucose medium + hemin + sal] .

3.5. Western Blot Analysis

Proteins were extracted from brain tissue using pyrolysis liquid with PMSF and were separated by SDS-PAGE. Then, the separated proteins were transferred onto PVDF membranes. After blocking with 5% BSA, the PVDF membranes were incubated with the primary antibodies, including MMP9 [1:1000, Affinity], ZO-1 [1:1000, Affinity], occludin [1:1000, Affinity], claudin-1 [1:10000, Affinity], at 4 °C overnight. The next day, the membranes were incubated with the corresponding secondary antibodies [1:2500, Affinity] at 37°C for1h.The proteins were visualized using the enhanced chemiluminescence substrate [Sweden/LAS500] and analyzed using Image J. β-tubulin [1:10000, Affinity] served as the internal control.

3.6. Calculation of Hematoma Area

After the brain was extracted by perfusion, the brain tissue was sliced coronally in successive layers of thickness 1 mm in a stainless-steel mouse brain sectioning mold, placed sequentially on smooth photographic paper, and analyzed using Image-J software. The area of each section with hemorrhagic foci was measured and multiplied by the thickness of 1 mm to determine the volume of the hemorrhagic foci in that section.

3.7. Neurological Tests

The neurological function of the mice was evaluated using a 24-point neural score system. The evaluation included body symmetry, gait, climbing behavior, circling behavior, forelimb symmetry, and forced circling behavior, each of which was rated on a 0–4 scale out of 24 points (Table 1). Next, the mice were allowed to walk on a crutch of80 × 2.5 × 2.5 cm^3 × 10 cm above the ground, and the balance beam score was evaluated. The scores were as follows: 0: the mice jumped on the balance beam and walked without falling down; 1: the mice could jump on the balance beam and walk, and the chance of falling down was less than 50%; 2: the mice could jump on the balance beam and walk, and the probability of falling was over 50%; 3: the mice could jump on the balance beam with the help of the ipsilateral hind limb but could not move forward because of paralysis of the contralateral hind limb; 4: the mice could not walk on the balance beam but could sit on the balance beam; and 5: the mice were placed on the balance beam after falling. The researchers were blinded.

Journal of Clinical Cases

Table 1

	0	1	2	3	4
body symmetry	normal	sight asymmetry	moderate asymmetry	significant asymmetry	extreme asymmetry
gait	normal	siffness and rigidity	limping	trembling,stumbling falling	no walking
climbing	normal	stressful climbing and weakness of limbs	grasping the sslope with out sliding or climbing	sliding down a slope, falling uncontrollably	immediately slides and falls uncontrollably
turning behavior	no appearance	tendency to turn to one side	occasional tendency to turn to one side	constant tendency to turn to turn to one side	rotation,swaying, immobility
anterior symmetry	normal	mild asymmetry	significant asymmetry	clearly asymmetry	slight asymmetry, immobility of body limbs
forced circling	no appearance	tendency to turn on one side	——	——	——

3.8. Cerebral Edema Was Measured Using The Wet And Dry Weight Method

The water and blood stains on the surface of the brains were blotted with filter paper. The whole brain was weighed on an electronic scale, and the weight of M1 was considered as the wet weight of the mouse brain, then, the brain was placed in a dryer for 16-24 hours and subsequently removed. The weight was measured again [M2], which was the dry weight of the mouse brain. The amount of cerebral edema was defined as [M1-M2]/M1×100%.

3.9. CCK8

The experimental design of the control group and Salidroside different dose [100 50 25 12.5umol/ml] group, after modeling, cell viability was detected using the cck8 method. 10ul of cck8 working solution was added to each well and mixed thoroughly to ensure that there were no bubbles, followed by incubating the culture class in the incubator for 2h-4h, at the end of the incubation, the plates were removed and the 96-well plates were placed in an enzyme meter to measure the OD value at a wavelength of 480nm.

3.10. NO Content Measuring

After HGwith hemin induced HCMEC, to measure NO content, each group of 50ul supernatant per well was aspirated into 96-well plate, NO detection working solution A and B were added sequentially, and the reaction was carried out at room temperature for 5min. The 96-well plate was placed in the enzyme calibrator for absorbance measurement, the wavelength was set to 540nm, the od value was read, the standard curve was plotted, and the NO content in the cell supernatant was calculated according to the standard curve.

3.11. FITC-Dextron

HCMECcells were inoculated into 24-well transwell chambers according to $5*10^4$ cells/ml, placed in the incubator for 24h and removed. The culture medium in the original chamber was discarded, DMEM mixed with 7.5mg/ml FITC-dextran [4kDa] and hemin was added, and the lower chamber was replaced with PBS solution. 50ul of PBS was withdrawn from the lower chamber at 0h, 30min, 60min, 120min, 180min and 360min respectively [note that 50ul of PBS should be added after each withdrawal to make up the total volume], and 50ul of PBS was used as a fluorescent enzyme marker. 50ul PBS to make up the total volume], use a fluorescence enzyme marker to read the pbs in the 96-well plate, and detect the fluorescence intensity at the excitation wavelength of 480nm and emission wavelength of 520nm.

3.12. Statistical Analysis

Data were expressed as the mean ± standard deviation [SD] and were compared using one-way ANOVA. GraphPad Prism 8.0.1 software [GraphPad Software Inc, La Jolla, CA, USA] was used for statistical analysis. A *P* value of less than 0.05 was considered statistically significant.

3.13. Ethics Approval

Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

4. Results

4.1. Salidroside Attenuate Neurological Impairment In Diabetic Cerebral Hemorrhagemice

Compared with the sham group, the neurological function scores as well as the balance beam scores of mice after diabetic cerebral hemorrhage were significantly elevated, indicating that the neurological impairment was aggravated after diabetic cerebral hemorrhage in mice. Compared with the mice in the diabetic cerebral hemorrhage group, the neurological function scores as well as the balance beam scores of the mice were reduced after the administration of different concentrations of sal, suggesting that sal can reduce the neurological function damage [Figure 1AB].

Journal of Clinical Cases

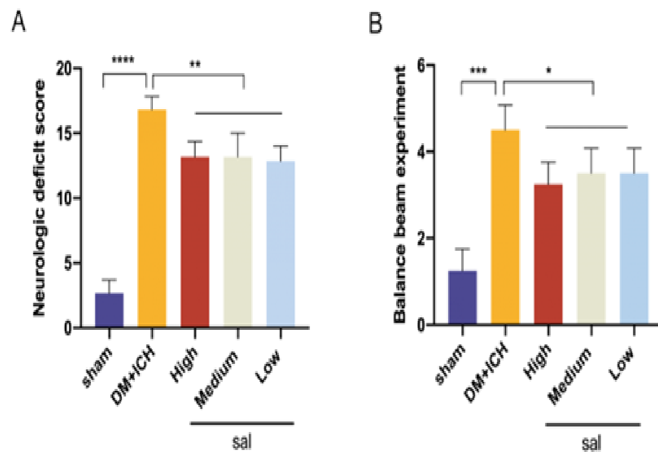


Figure 1: Salidroside attenuate neurological impairment in diabetic cerebral hemorrhage mice

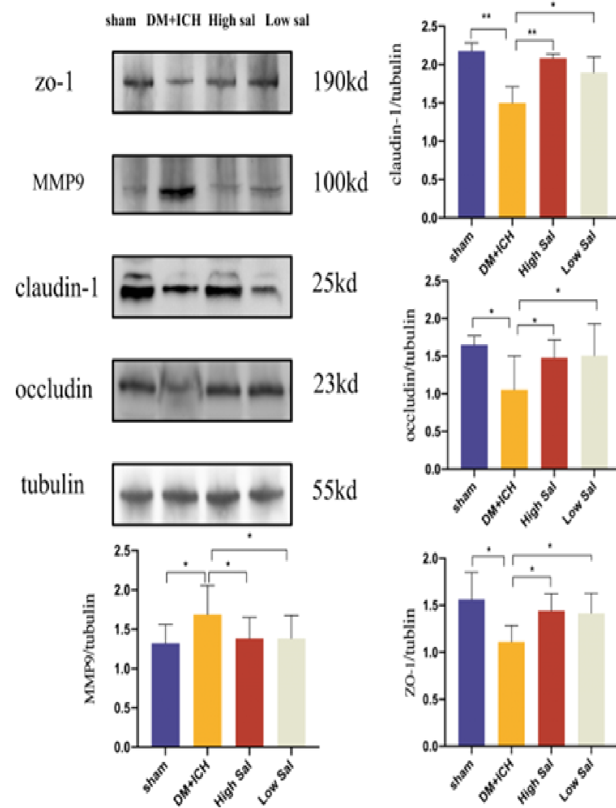
(A) Neurologic deficlt score of mice. (B) Balance beam experiment of mice. the experimental data were expressed as mean ± standard deviation. * p<0.05, ** p<0.01, *** p<0.001, t test.

4.2. Salidroside Attenuate Blood-Brain Barrier Permeability After Diabetic Cerebral Hemorrhage

western blot detected the expression of the tight junction proteins zo-1, claudin 1 and occludin expression, and it was found that tight junction proteins zo-1,claudin 1,occludin were significantly decreased after diabetic cerebral hemorrhage [Figure 2A], indicating that intercellular tight junctions were reduced, and their expression was significantly elevated after administration of sal treatment. In contrast to tight junction proteins, matrix metalloproteinase MMP9 expression was significantly elevated after diabetic cerebral hemorrhage [Figure 2A], and was decreased after administration of sal. The blood-brain barrier consists of endothelial cells, basement membrane and astrocytes, we observed the structure of blood-brain barrier with electron microscope, and found that endothelial cells were tightly connected in the normal group, and after diabetic cerebral haemorrhage the endothelial cell structure was loosened, and the tight connection between the cells was disrupted, and the mitochondria were swollen obviously, and most of the endothelial cell tight connection was restored after the administration of sal [Figure 2B].

4.3. Salidroside Attenuate Hematoma Volume And Brain Water Content After Diabetic Cerebral Hemorrhage Mice

Wet and dry gravimetric methods were used to detect the brain water content after diabetic cerebral hemorrhage mice, which was significantly increased in mice after diabetic cerebral hemorrhage compared with the sham group , and the cerebral edema was improved after the administration of sal [Figure 3A]. Next, the volume of cerebral hematoma after cerebral hemorrhage was measured, and it was found that sal significantly reduced the volume of cerebral hematoma after diabetic cerebral hemorrhage in mice [Figure 3B].



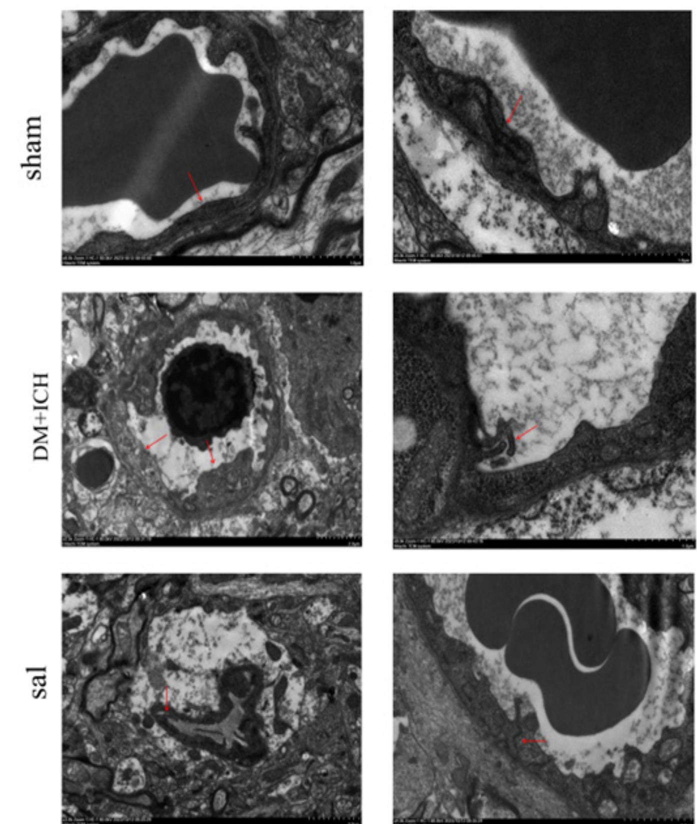


Figure 2: Salidroside attenuate blood-brain barrier permeability after diabetic cerebral hemorrhage

Journal of Clinical Cases

(A) western blot to detect the expression of claudin occludin ZO-1 and MMP9. the experimental data were expressed as mean ± standard deviation. * $p<0.05$, t test. (B) Electron microscopic observation of blood-brain barrier structure and endothelial cell damage.

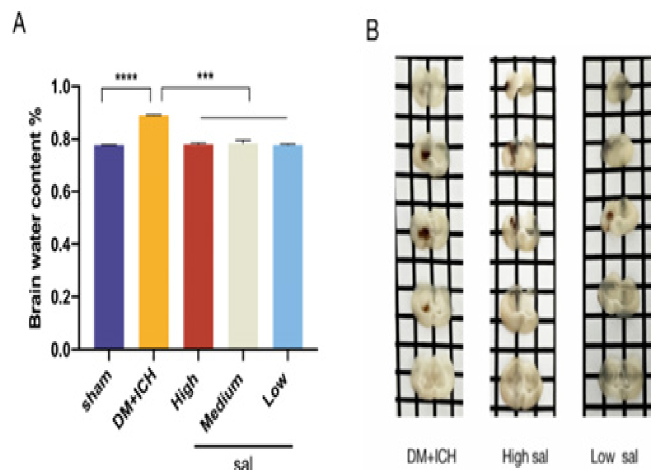


Figure 3: Salidroside attenuate hematoma volume and brain water content after diabetic cerebral hemorrhage mice

(A) Brain water content of mice in different groups. (B) the volume of cerebral hematoma, the experimental data were expressed as mean ± standard deviation. * $p<0.05$, t test.

4.4. Effect of Salidroside On Endothelial Cell Viability In Vitro

Sal had no significant effect on the viability of normally growing HCMEC cells in the dose range of 12.5-100um [Figure 4A], and thus subsequent efficacy and mechanism validation experiments could be performed in this dose range. Compared with the control group, HCMEC cell viability was significantly reduced in the HG+hemin group, and the sal 100um and 50um dose groups significantly protected the damaged HCMEC cells after HG+hemin and improved cell viability [Figure 4B].

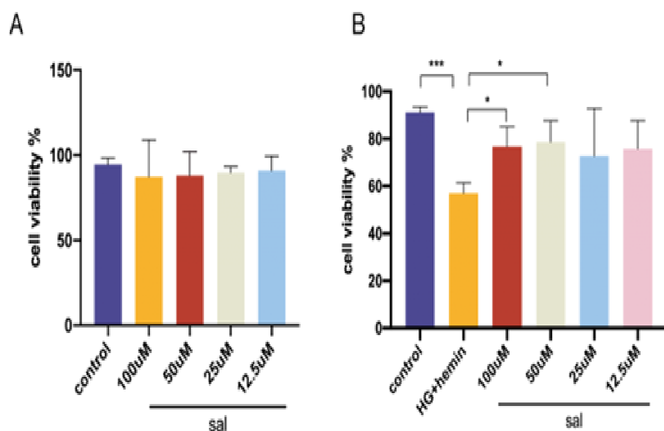


Figure 4: Effect of Salidroside on endothelial cell viability in vitro

(A B) CCK8 to detect the viability of cells. the experimental data were expressed as mean ± standard deviation. * $p<0.05$, *$p<0.001$, t test.

4.5. Salidroside Increase NO Release From Endothelial Cells In Vitro

The release of NO from HG+hemin-treated HCMEC cells was significantly reduced compared to the control group, and was significantly increased in sal-treated groups [Figure 5].

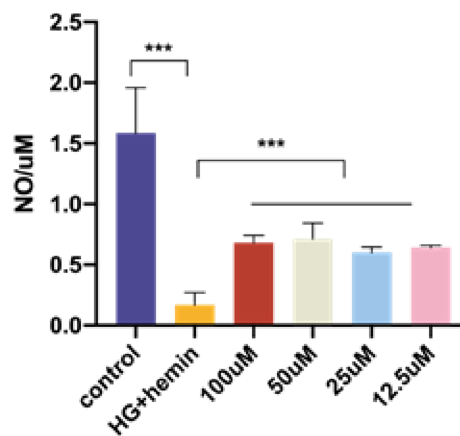


Figure 5: Salidroside increase NO release from endothelial cells in vitro

the release content of NO. the experimental data were expressed as mean ± standard deviation. ***$p<0.001$, t test.

4.6. Salidroside Attenuate Endothelial Cell FITC Permeability

We also examined the permeability of FITC at different time points at different sal concentrations and found that FITC permeability was significantly higher in the HG+hemin group and decreased after administration of sal, suggesting that sal attenuates endothelial cell permeability[Figure 6].

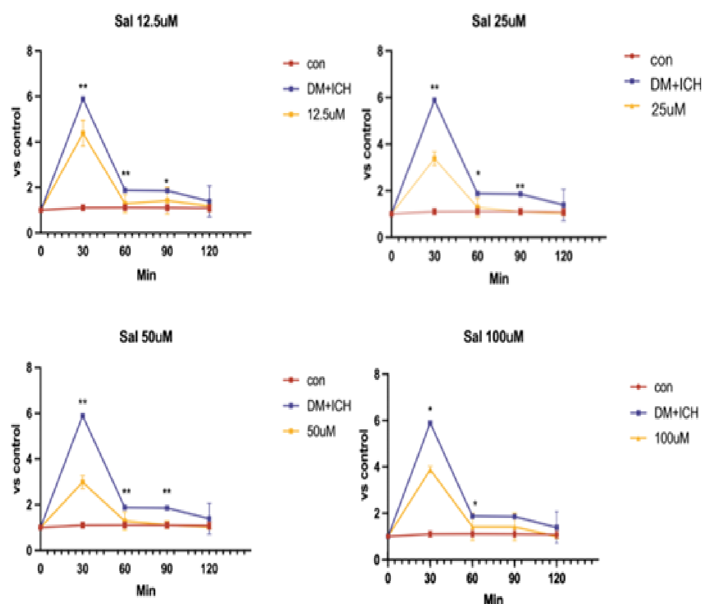


Figure 6: Salidroside attenuate endothelial cell FITC permeability

FITC to detect the permeability of HCMEC. the experimental data were expressed as mean ± standard deviation. ** $p<0.01$, t test.

Journal of Clinical Cases

5. Discussion

Diabetes mellitus is a serious metabolic disorder, persistent elevation of blood glucose levels can damage the function of the body's large blood vessels and microvessels, which in turn leads to cerebral atrophy, neuropathy and neuroinflammation, of which neuroinflammation is the main factor causing diabetic cerebral haemorrhage secondary to brain damage, When neuroinflammation occurs, microglial cells release a large number of inflammatory factors, leading to cerebral endothelial cell damage, destroying the blood-brain barrier and exacerbating neurological function damage, so in-depth study of treatment methods in this process is of non-negligible importance. Salidrosid is a perennial herbaceous plant that grows mainly in high altitude areas with severe cold, dryness, lack of oxygen, strong ultraviolet radiation and large temperature differences between day and night, and it has a strong ability to adapt to the environment and vitality. Salidrosid has anti-fatigue, anti-aging, immunomodulation, anti-fibrosis, anti-tumour and free radical scavenging functions. Salidrosid [sal] is the main active constituent of salidrosid. It has been confirmed that salidrosid can reduce blood glucose and alleviate the pathophysiological processes such as inflammation, oxidative stress, apoptosis, and other damage to tissues of the body caused by hyperglycaemia[13]. Sal has an important role in preventing neuronal cell damage after cerebral ischaemia in vitro and in vivo, and it has been found that sal attenuates secondary neurological damage after cerebral ischaemia. Therefore, we investigated whether salidrosid could attenuate blood-brain barrier [BBB] damage by reducing blood-brain barrier permeability after diabetic cerebral hemorrhage. Our experimental results showed that compared with the ICH+DM group, the neurological function scores and balance beam scores of mice in the sal group were significantly reduced, indicating that sal could reduce neurological damage after diabetic cerebral hemorrhage.

Next, we used western blot to detect the expression of bloodbrainbarrier-related proteins. These tight junction proteins control the transport of large and small molecules between the blood and the brain parenchyma and regulate the leakage of cells and proteins from the blood into the brain parenchyma.Our results found that in diabetic cerebral hemorrhage group, tight junction proteins zo-1, claudin 1, occludin expression was significantly reduced, tight junction proteins are involved in the barrier function of the constitutive tight junction structure between adjacent cells, and the reduced expression indicates that there is a disruption of the tight junction structure between cells, when sal was treated, zo-1, claudin 1, occludin expression was significantly increased, indicating that sal can attenuate the blood-brain barrier damage and reduce its permeability. We also found that the expression of metalloproteinase MMP9 was increased in the diabetic cerebral haemorrhage group and decreased after sal administration. The function of metalloproteinase is to degrade and remodel the dynamic balance of extracellular matrix, and the elevated expression of MMP-9 indicates that the degradation of extracellular matrix is obvious, and MMP-9 can also disintegrate the basal lamina of endothelial cells to a certain extent, directly destroying the barrier integrity of endothelial cells.In summary, our results obtained that sal could attenuate BBB damage and reduce its permeability. The blood-brain barrier consists of endothelial cells, basement membrane and perivascular foot of astrocytes with loose junctions. We observed the morphology of the endothelial cells by electron microscopy, and found that the endothelial cells in the normal group were tightly junctional, but after diabetic cerebral hemorrhage, the endothelial cells became loose and the tight junctions were disrupted, resulting in a greater permeability of the blood-brain barrier, and the degree of disruption of tight junctions was ameliorated by the administration of sal treatment. In addition, we also found that sal could reduce brain water content after diabetic cerebral hemorrhage and reduce the volume of cerebral hematoma. This suggests that sal may reduce secondary neurological damage after diabetic cerebral hemorrhage. The blood-brain barrier is an important physical and metabolic barrier between the brain and the blood that restricts the entry of macromolecules from the blood into the brain parenchyma, and plays an important role in maintaining homeostasis in the brain[14].

Endothelial cells are both a component of the BBB and a fundamental part of blood vessels, participating in the formation of the inner wall of blood vessels and providing an anticoagulant barrier between the vessel wall and blood. The normal functioning of nerve cells in the brain requires a tightly controlled environment that protects them from blood-borne toxicants and pathogens, so an intact endothelial barrier is extremely important for safeguarding microenvironmental homeostasis in the brain. Endothelial cells are located on the luminal surface of cerebral capillaries, which is an important site for signalling and injury effects in post-ischemic ROS, inflammation and other pathological responses. After ischemia, endothelial cells are the first to be stimulated by ROS and inflammatory factors in the blood after ischemia, and there is a disruption of the barrier function. After the endothelial cell barrier is disrupted, toxic substances in the blood can enter the brain parenchyma through the incomplete barrier, altering the microenvironment in the brain and leading to neurological damage, e.g., leukocytes and neutrophils are two common cells that enter the brain parenchyma through the endothelial cells, and leukocyte and neutrophil infiltration induces inflammatory responses and exacerbates neuroinflammation[15]. At the same time, due to the special location of endothelial cells, although the amount of salin the blood into the brain parenchyma is extremely limited, but it may be on the blood side to protect the endothelial cells from ischemic damage, improve the function of the damaged endothelial barrier, so as to indirectly play a cerebroprotective role, under normal physiological state, the endothelial cells overlap each other and are closely connected, and the endothelium is the main place of the endothelium and endothelial cells to the brain parenchyma transport of the substances in the blood is the main place .

Therefore, after we verified the function of sal in vivo, we explored the effect of sal on vascular endothelial cells, and found that sal had no significant effect on the viability of normal-growing HCMEC cells in the dose range of 12.5-100um, so subsequent experiments could be carried out in this dose range. HCMEC cell viability was significantly reduced in the HG+hemin group compared with the control group, whereas sal 100um and 50um dose groups significantly protected damaged HCMEC cells

Journal of Clinical Cases

after HG+hemin and increased cell viability. As an important endogenous cytokine, nitric oxide [NO] plays an important role in the process of inflammation and tissue cell injury. NO has the role of regulating vascular tension, regulating coagulation process, mediating inflammatory response, and participating in oxidation[16]. In the central nervous system, NO mainly promotes the release of transmitters and participates in synaptic reversal process[16]. In the central nervous system, NO mainly promotes the release of transmitters, participates in the process of synaptic reversibility, regulates the permeability of the blood-brain barrier, and participates in the higher functional activities of the brain[17]. Under high glucose conditions, vascular wall damage, destruction of vascular endothelial cells, and reduction of NO synthase production lead to a decrease in NO synthesis, which ultimately exacerbates the damage to the blood-brain barrier after cerebral haemorrhage[18]. Some studies have confirmed that sal can promote the production of NO in diabetic mice, thereby reducing tissue inflammation, apoptosis and a series of pathophysiological processes[19].

In this experiment we found that NO synthesis was significantly reduced in the HG+Hemin group and significantly increased in the sal group, indicating that sal can promote NO synthesis, regulate endothelial cells permeability, and attenuate BBB damage. We also examined the permeability of FITC and found that the permeability of FITC was significantly increased in the HG+hemin group and decreased after administration of sal, indicating that sal could attenuate the permeability of endothelial cells. In summary we can conclude that sal can reduce blood-brain barrier permeability and thus secondary neurological impairment after diabetic cerebral hemorrhage by attenuating endothelial cell injury.

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