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Systematic Comparison Suggesting Intranasal Transplantation was the Best Route of Administration of Human Umbilical Cord Mesenchymal Stem Cells (hUC-MSCs) in Hypoxic-Ischaemic Brain Damage (HIBD) Rat Model

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Abstract

Aims: Hypoxic-Ischaemic Brain Damage (HIBD) remains a common sequelae of various nervous system diseases. Human Umbilical Cord derived Mesenchymal Stem Cells (hUC-MSCs) transplantation was considered to be promising in treating HIBD. However, the best route of administration to transplant hUC-MSCs remains open. In this study, we systematically compared the three routes of administration. The Intravenous (IV), Intracerebral (IC) and Intranasal (IN) administration for the first time to guide the best clinical practice.

Methods: The HIBD rat models were built on the 7th (PN7) day after birth of rats. The three routes of administration of hUC-MSCs were conducted on the 14th day (PN14) after birth of rats. And these three groups (HIBD+IV, HIBD+IN, HIBD+IC) were compared with HIBD and sham group on motor function learning and memory function improvement by hanging wire, vertical pole test and Morris Water Maze (MWM) test on 10th (PN10) and 21th (PN21) day after birth of rats. Moreover, the pathological tests were used to compare the pathological repair effects of three routes of administration: The morphological changes of brain tissue were tested by Haematoxylin and Eosin staining (HE staining); the proliferation of reactive astrocytes were compared by detecting the expression of Glial Fibrillar Acidic Protein (GFAP) by immunohistochemistry; and the number of neuronal apoptosis in cortex and hippocampus were compared by TUNEL staining.

Results: The motor function of rats in HIBD group was significantly lower than that in sham group on the PN10, both in hanging wire and vertical pole tests ($p<0.0001$). This shows the effectiveness of our HIBD model. All of the three routes of administration groups showed significant improvement of motor and learning function, reducing the liquefaction necrosis, GFAP expression and apoptosis rate of nerve cells in cerebral cortex and hippocampus of HIBD rats. Among the three routes of administration groups, the functional improvement and pathological repair effect of Intracerebral (IC) and Intranasal (IN) administration were better than those of Intravenous (IV) administration stem cells. And no significant difference between intracerebral and intranasal administration. As Intranasal (IN) administration is more compliant and convenient in clinical practice than Intracerebral (IC) administration, thus we suggest that Intranasal (IN) administration is the best route of administration of hUC-MSCs on HIBD treatment.

Keywords: HIBD; hUC-MSCs; Brain damage; Cell therapy; Administration routes

Abbreviations: HIBD: Hypoxic-Ischaemic Brain Damage; IC: Intracerebral; IN: Intranasal; FBS: Fetal Bovineserum; CP: Cerebral Palsy; EEG: Electroencephalogram; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovineserum; MWM: Morris Water Maze.

Introduction

HIBD is a non-progressive injury, which caused by insufficient oxygenation/perfusion of fetal and neonatal brain tissue due to various perioperative infections, placental abnormalities, metabolic disorders or abnormal coagulation function [1]. Because of the high demand for energy in the brains of fetuses and newborns, they are more vulnerable to hypoxia-ischaemia. When hypoxia and ischemia occur, microglia and astrocytes are activated and the release of various harmful substances and inflammatory factors increases, resulting in the destruction of the blood-brain barrier and the dysfunction of mitochondrial function, which leads to neuronal necrosis and apoptosis [2]. The death of nerve cells is widely known as non-renewable. Therefore, if this kind of injury is not effectively repaired, HIBD will be often the sequelae of various nervous systems, such as CP, seizures, mental retardation and so on, which have a great impact on the growth and development of children themselves, their social activities and family members.

With the progress of medical technology, the identification of HIBD can be realized by a variety of auxiliary examinations, such as bedside EEG, craniocerebral ultrasound, craniocerebral CT and MRI, etc., but only therapeutic hypothermia has been effectively approved.

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Hypothermia treatment can reduce child mortality and the risk of moderate to severe injury [3], but its functional recovery is limited and must begin within 6 hours of birth [4]. What's more, it cannot repair and replace the lost neurons. In addition, the adverse complications caused by hypothermia itself also need to be vigilant. For example, the prolongation of hypothermia treatment time or the decrease of temperature will increase the mortality of children [5] and increase the death of brain cells in animals [6]. Even a recent multicenter randomized controlled study showed that hypothermia not only did not reduce 18-month-old infant mortality or moderate to severe injuries, but also significantly increased the number of deaths in developing countries [7]. Therefore, we urgently need to find a new treatment for HIBD.

In recent years, the repair effect of stem cells on nervous system has also been recognized by many experts [8-11]. In particular, hUC-MSCs are relatively easy to obtain, and there are nearly no ethical problems, the immunogenicity is also low. HUC-MSCs also have all the characteristics of other stem cells, such as multi-differentiation potential, strong value-added ability and so on, so it is considered to be promising cells [12,13]. HUC-MSCs have also been proved to be effective by many preclinical studies and initial clinical trials in the treatment of HIBD and sequelae such as CP [14-17]. However, if the specific treatment plan is to be implemented on a large scale in children, there are still many problems to be solved, such as which way of transplantation can restore the neurological function of children better, have less side effects and be stable for a long time. The IV transplantation is the most widely used approach in early clinical trials [18], which is relatively easy to operate and has a high acceptability of family members and patients. Although it is still unknown whether it can pass through the blood-brain barrier, many experts believe that the IV transplantation can play a therapeutic role by regulating the secretion of peripheral related immune factors [19,20]. However, it should be noted that even if IV-transplanted cells cannot enter the central nervous system and play a protective role, its first-pass effect in lung, liver and other peripheral organs needs to be considered [21] and whether there are adverse effects on peripheral organs and tissues is still unknown. The IC transplantation is considered to be a better way in animal experiments because it can directly ignore the blood-brain barrier to reach the brain [22]. It not only makes the number of stem cells reach the brain injury area more, but also reduces the risk of damage to the peripheral tissue. Many studies [23,24] believe that IC transplantation cannot only regulate the expression of neurotrophic factors in the brain, promote the differentiation of endogenous neural stem cells, but also replace the dead nerve cells. However, it is highly invasive and risk and the maneuverability of repeated transplantation is small. It is also necessary to consider the acceptance of family members and patients in clinical practice, so the clinical application is limited. And although IN administration of pharmaceuticals has a long history, the administration of stem cells from the nose is also a new treatment which has only been implemented in recent ten years. It has been known that cells can bypass the blood-brain barrier directly through the nasal mucosa, migrate along the olfactory nerve pathway to the olfactory bulb and other parts of the brain, or move along the surface of the cortex into cerebrospinal fluid and then into the brain parenchyma [25]. Many preclinical studies have also confirmed the feasibility of transplantation of stem cells by IN transplantation in the treatment of HIBD [26,27]. But it remains question that how to get more stem cells through the nasal mucosa, the appropriate volume of transplantation fluid and it is unknown what the effect of administration is to compare the other two ways. There are no clinical reports of IN transplantation of stem cells in the treatment of HIBD yet.

Therefore, in this study, we compared for the first time the most widely used the IV transplantation, the IC transplantation, which had been considered to be effective in recovery and the new IN transplantation in recent years, in order to pave the way for the clinical application of stem cells in the treatment of HIBD in children.

Materials and Methods

Cell culture

Were hUC-MSCs obtained from stem cell research center of Taihe hospital were cultured in DMEM containing 10% FBS (Gibco Invitrogen, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL). Upon attaining about 80% cell confluence, the cells were trypsinized and passaged. In the following experiments, we used the 3rd to 5th passages cells.

HIBD model

Healthy PN7 clean rats (SPF grade, Sprague-Dawley), weighing (15+5) g, a total of 78 rats (regardless of sex), provided by the Experimental Animal Center of Hubei University of Medicine. The construction of HIBD model based on the modified RICE as previously described [28]. In simple terms, PN7 rats were anesthetized and placed on an operating table with a 37°C constant temperature heating pad. The right common carotid artery of rats was exposed surgically, the sheath was opened, the nerves and veins were separated. We ligated the distal and proximal ends of the right common carotid artery with 6-0 surgical silk thread and severed in the middle. The operation time is less than 15 minutes. The rats after the operation were placed on a heating pad (37°C) and put back to the female when they woke up. After 2 hours of recovery, the rats were transferred to an anoxic chamber with a heating pad (37°C) and exposed to a mixture (containing 8% oxygen+92% nitrogen) of hypoxia for 2 hours. In Sham group, the right common carotid artery was isolated only after anesthesia, no ligation and no cut, then put back to the female rats after anaesthesia woke up.

Study design

Previous studies showed that there was no significant difference in behavior and pathology among the HIBD group, the HIBD with PBS administration group and the HIBD with fibroblasts administration group, so HIBD group was directly used as injury control group in our experiments. In order to evaluate the effect of hUC-MSCs transplantation in different ways on HIBD rats, PN7 rats were randomly divided into five groups: 1) a Sham group (n=14); 2) a HIBD group (n=16); 3) a HIBD+IV group (Intravenous injection hUC-MSCs after rats suffered from HIBD) (n=16); 4) a HIBD+IN group (Intranasal administration of hUC-MSCs after rats suffered from HIBD) (n=16); 5) a HIBD+IC group (Intracerebral administration of hUC-MSCs after rats suffered from HIBD) (n=16). The brief timeline of the experiment is shown in the Figure 1A.

Similar to Rosenblum et al., hUC-MSCs transplantation was performed on the 7th day (PN14) after HIBD in rats, and the number of hUC-MSCs was 0.5 × 106/head [29]. In HIBD+IV group, 0.5 ml hUC-MSCs suspension was injected intravenously through tail vein and the puncture point was pressed with clean cotton swabs for 2 minutes to prevent leakage. In HIBD+IN group, 6 ul hUC-MSCs suspension was dripped into the nasal cavity of the anesthetized rats by the Hamilton syringe a total of 12 ul/rat and only one side of the nasal cavity was

dripped into one side at a time to prevent asphyxia. After drip, the rats were placed on their backs for at least 10 minutes until they were completely absorbed and replaced with the other side. In HIBD+IC group, 5 ul hUC-MSCs suspension was injected from the left lateral ventricle with brain stereotactic apparatus and Hamilton syringe at a speed of 1 ul/min. Stop injection for 2 minutes at each injection of 2 ul, stop injection for 5 minutes after injection and slowly pull out the Hamilton syringe, seal skull with bone wax, suture wound and disinfect.

Functional tests

Preliminary evaluation of the success of the model through the head rotation behavior of rats on the same day after hypoxia and ischemia. On the 3rd day after injury (PN10), in order to further determine whether the model was successful or not, we evaluated the neurobehavior of rats in each group by hanging wire test and vertical pole test [8]. We evaluated the neuromuscular development ability of rats by hanging wire test. First, take a wire cage cover of the right size, place the rat on it, gently shake the rat three times to make the rat grasp the wire and then reverse it. The rat will hold on to the wire with his limbs to prevent falling. We need to place a box full of sawdust under the wire to protect the falling rat. The distance between the box and the wire should be greater than 20 cm to prevent the rats from falling intentionally. Finally, use the stopwatch to record the falling time of each rat. The vertical pole test was used to experiment the motor balance ability of rats. First of all, take a horizontal wooden pole, put the rat in the center. And then we gently move the wooden rod from the horizontal position to the vertical position, the rat falls to stop moving and record the angle between the wooden rod and the ground when the rat falls.

On PN21, as before, we carried out hanging wire test and vertical pole test to see if the motor function of rats improved after transplantation of stem cells through different ways. And then began to prepare the MWM test to evaluate the learning and memory function of rats in each group, as previously described [30]. Briefly, on the first day of the test, rats were exposed to a visible platform, using the time and distance they reached the platform to assess their movement and vision while swimming. From day 2 to day 5, the invisible platform was used to train to enhance their learning and memory ability and the time when the rats arrived at the platform was recorded. The platform was removed on the 6th day and the memory function was evaluated by recording the target quadrant wandering time within 60 s.

Histopathological staining

At the end of behavioral experiment, the rats in each group were killed after anesthesia and the brain was taken after perfusion with 4°C saline and 4% paraformaldehyde. The brain tissue was fixed in 10% paraformaldehyde for 24 hours and embedded in paraffin. The coronal section of brain was made with embedded wax block, the thickness of which was 5 μm, dewaxed in xylene in turn, hydrated with gradient alcohol and then stained with Haematoxylin and eosin. The histologic changes of the cortex and hippocampus of the five groups were observed under light microscope.

Immunohistochemistry

In each group, 5 slices of brain tissue were dewaxed and rehydrated and blocked with 5% bovine serum albumin. The brain tissue slices were incubated with the primary antibody to glial fibrillar acidic protein (anti-GFAP antibody, Abcam, ab7260, 1:3000, UK) overnight at 4°C, then incubated with the secondary antibody (Rabbit two-step kit, ZSGB-BIO, PV-6001, China) at room temperature for 1 hour and then DAB was added for 5 minutes to develop the color. After conventional dehydration and sealing, the tablets were observed under light microscope (Olympus BX53+DP74, Japan). The Cells and Standard software was used to collect images under the same exposure conditions, and FIJI Image-J v1.52p software was used to process the images. 8-10 visual fields were selected to quantify the positive cells in each part of the coronal section. The cell density was measured by the number of positive cells per HPF (400x). Then the positive staining area was statistically analyzed to reconfirm the results of cell density.

TUNEL apoptosis

According to the instructions of TUNEL kit (One Step TUNEL Apoptosis Assay Kit, Beyotime, C1088, China), 3 or more sections in each group were stained with TUNEL. The brain tissue slices were dewaxed and hydrated and soaked in distilled water for 3 minutes, then reacted with protease K at 37°C for 30 minutes. After cleaning with PBS for 3 times, each slice was treated with 50 ul TUNEL reaction solution and reacted in a wet box at 37°C for 1 hour. The nucleus was stained with DAPI and sealed with sealant containing anti-fluorescent quenching agent. We selected 3-5 random visual fields of cortex and hippocampus from each sample to count the number of TUNEL-positive cells and calculated the apoptosis rate, which was equal to the number of TUNEL-positive cells/total number of cells × 100%.

Statistical analysis

Statistical analysis and chart drawing of the data are carried out by using Prism v8.0.2 software (GraphPad Software, San Diego, CA, USA). A two-tailed student's t-test was used for comparisons of two groups of samples with normal distributions. When there are two sets of data for comparison, we choose a one-way Analysis of Variance (ANOVA) with Tukey's multiple comparison post-hoc test, and when we need to evaluate two independent variables, we use two-way ANOVA with Tukey's post hoc test. The measured data are represented by mean+standard deviation. Only when $p<0.05$ was considered to have statistical difference.

Results

Behavioral dysfunction of HIBD

PN7 rats had irritability and cyanosis after hypoxia and ischemia for half an hour. With the prolongation of time, fecal incontinence, even rotation to the left and reversal of angular arch appeared gradually, while some rats showed inhibition of activity. Rats who automatically rotate to the left when lifting their tails after attacked were selected to continue the following steps. Individuals who rotate poorly or die in the process were excluded. A total of 72 rats were considered qualified and selected for following experiments.

Comparison of motor and cognitive function improvement of HIBD by three hUC-MSCs transplantation ways

In order to investigate the effects of hUC-MSCs transplantation on growth and motor function of HIBD rats by different ways, the weight of rats had been monitored dynamically and hanging wire and vertical pole tests were carried out in PN10 and PN21.

As shown in Figure 1B, the weight gain of the three hUC-MSCs treatment groups was significantly faster than that of the HIBD group,

and the weight gain of the HIBD+IC group was more obvious than that of the HIBD+IV and HIBD+IN groups and the weight gain of the HIBD+IC group was even faster than that of the sham group on PN21. The motor function of rats in each HIBD experimental group was significantly lower than that in sham group on the PN10, both in hanging wire and vertical pole tests. And on the PN21, the transplantation of hUC-MSCs through three routes distinctly improved the behavioral function of hypoxia-ischemic rats. The behavioral function of the HIBD+IV group was still lower than that of sham group, and the improvement of HIBD+IC group was also better than HIBD+IV group in hanging wire test on the PN21, but no significant difference between HIBD+IN group and HIBD+IC group (Figures 1A-1D).

To investigate the improvement of learning and memory function in HIBD rats by hUC-MSCs, we carried out the MWM test. It shows the representative trajectory of swimming in each group of rats in the MWM test. There was no significant difference in escape latency and path length among the five experimental groups during the visible platform. This suggests that neither HIBD nor hUC-MSCs transplantation groups damaged the visual acuity and activity of rats while swimming. We also found that as the number of training sessions increases, the time for subjects to reach the platform gradually decreased. And the escape latency of HIBD+IN and HIBD+IC group from 3rd to 5th day of learning was significantly lower than that of HIBD group, but IV injections did not show statistical differences. In addition, the escape latency of HIBD+IN and HIBD+IC group was similar to that of Sham group on the 5th day, which also means that IN and IC transplantation can obviously improve the learning and memory function of HIBD rats, almost catch up with the normal level. However, in evaluating memory retention ability, transplantation through the three routes showed statistical differences of time in target quadrant compared with the HIBD group, which the HIBD+IC group seemed better (Figures 2A-2D).

The pathological changes of HIBD by three hUC-MSCs transplantation ways

By observing the overall specimens of brain tissue, we found that the brain tissue on the right of HIBD rats was atropd HIBD and obvious liquefaction necrosis was observed and white infarction foci were found around them, while the reduction or even disappearance of local necrotic foci and infarction foci could be observed in three stem cell transplantation groups. We observed the pathological changes of cerebral cortex and hippocampus by HE staining. The results showed that the atrophy of cortex and hippocampus could be seen obviously in HIBD group than that in sham group under lower magnification of injured lateral brain tissue. Under high magnification microscope, the normal cells of cortex and hippocampus were disordered and more inflammatory cells were infiltrated, as well as necrotic nerve cells with nuclear pyknosis, nuclear fragmentation and deep staining of cytoplasm. However, the transplantation of stem cells in three ways showed significant improvement (Figure 3)

In order to compare the effect of stem cell transplantation on astrocytes in brain tissue of HIBD rats, we compared the expression of GFAP in cortex and hippocampus of each group. When brain tissue is injured by ischemia and hypoxia, the main changes of astrocytes are the increase of cell body area and cell number, so we compared the area of GFAP positive expression and the number of GFAP positive cells to make the results more reliable. We found that the positive expression of GFAP in cortex and hippocampus increased significantly after HIBD, while stem cell transplantation could significantly decrease the expression of GFAP. The decrease of GFAP expression in hippocampal DG region of HIBD+IN group was more obvious than that of HIBD+IV group, but there was no significant difference between HIBD+IC group and HIBD+IN group. Even, no significant difference was shown in the improvement of GFAP expression in the cortex between the three treatment groups (Figures 4A-4C).

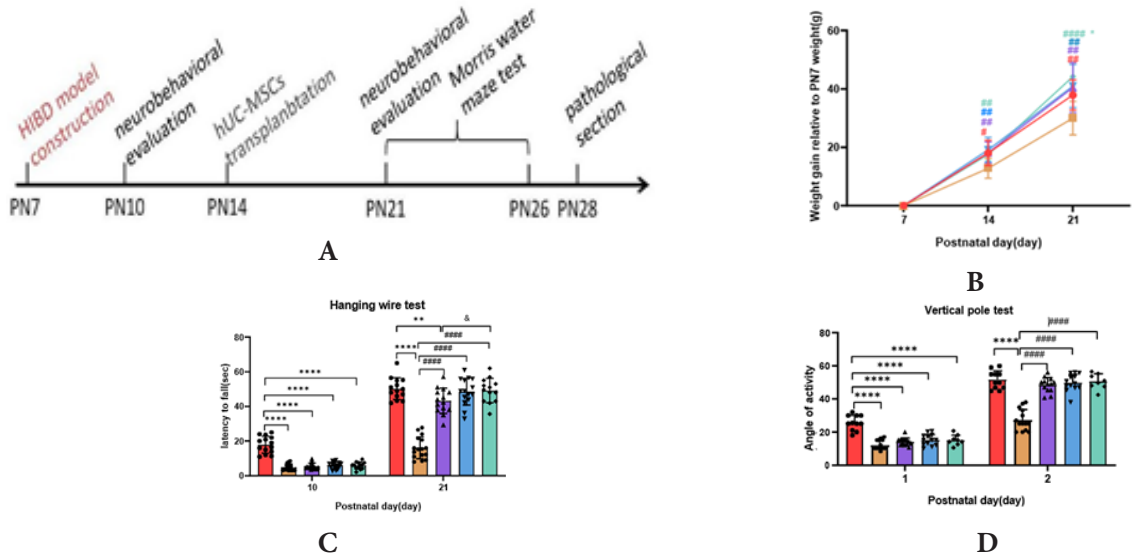


Figure 1: hUC-MSCs transplantation promote cognitive function in rats with HIBD. (A) The time line of hUC-MSCs transplantation in rats with HIBD; (B) Weight gain of rats in each experimental group after operation and hUC-MSCs treatment. hUC-MSCs therapy could increase the body weight of HIBD rats and even on PN21 day, the weight of HIBD+IC group was faster than that of sham group; (C) Hanging wire test. After ischemia and hypoxia injury, the retention time on the wire was significantly shortened, while the residence time on the wire was significantly prolonged after hUC-MSCs treatment. The retention time on the wire in the HIBD+IC group was longer than that in the HIBD+IV group; (D) Vertical pole test. hUC-MSCs treatment improved the duration on pole of the rats after ischemia and hypoxia injury. **Note:** (●) Sham; (■) HIBD; (▲) HIBD+IV; (▼) HIBD+IN; (◆) HIBD+IC; (■) sham; (■) HIBD; (■) HIBD+IV; (■) HIBD+IN; (■) HIBD+IC. **Significance:** n=8-14 rats per group; *, **, **** represent p<0.05, 0.01 and 0.0001 respectively compared to the sham group; #, ##, #### represent p<0.05, 0.01 and 0.0001 compared to the HIBD group and "&" represent p<0.05 compared to the HIBD+IV group.

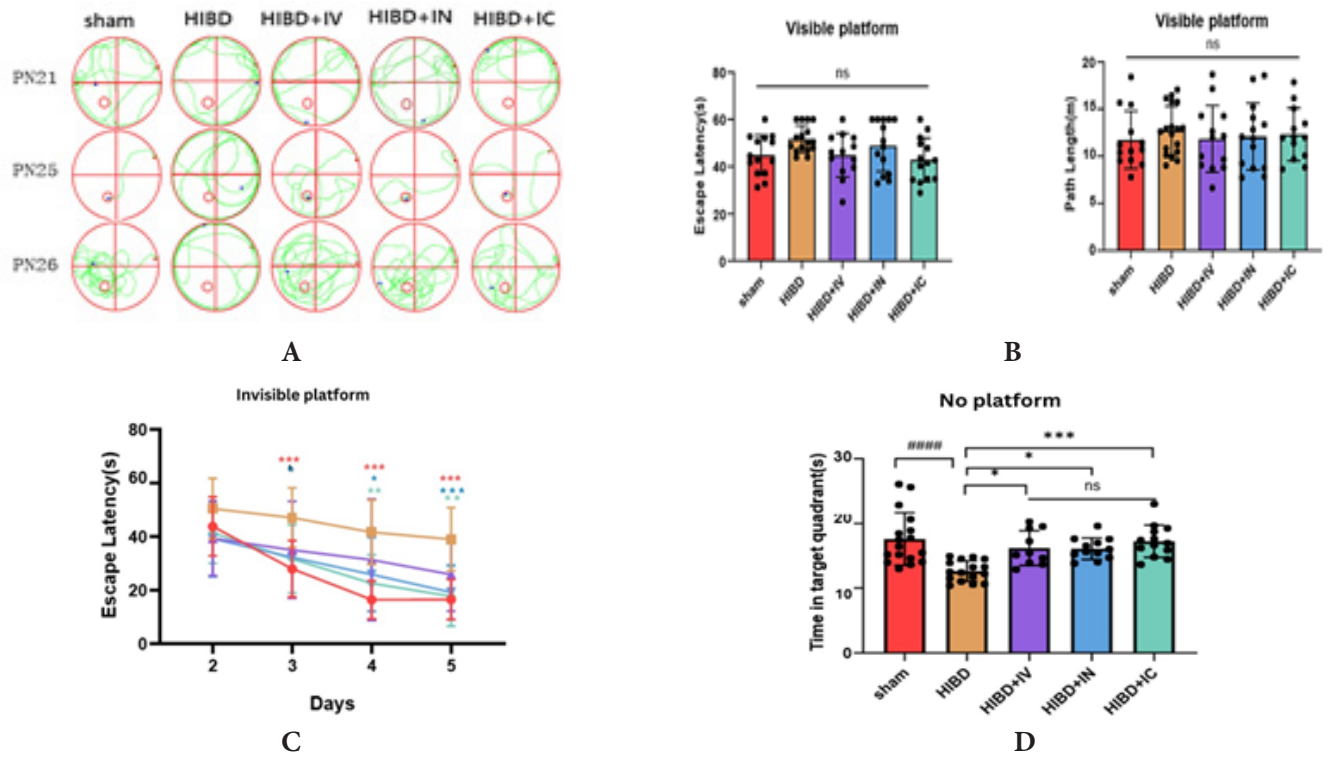


Figure 2: hUC-MSCs transplantation promote cognitive function in rats with HIBD. (A) The 1st day, the 5th day and the 6th day of the MWM were the representative swimming paths of the rats in these group; (B) No difference in escape latency and path length were observed for all rat groups during visible platform conditioning trial; (C) Rats in HIBD+IN and HIBD+IC group performed significantly better in learning than rats in HIBD group, which was nearly comparable to the sham rats during invisible platform learning trials; (D) Rats in HIBD group showed obvious memory impairment and hUC-MSCs therapy could reverse it. But no significant difference in memory performance among the HIBD+IV, HIBD+IN and HIBD+IC groups was observed during the memory test trial. **Note:** (●) Sham; (●) HIBD; (●) HIBD+IV; (●) HIBD+IN; (●) HIBD+IC. **Significance:** n=10-17 rats per group. *,**,*** represent $p<0.05$, 0.01 and 0.001 respectively compared to the HIBD group; #### represent $p<0.0001$ compared to the sham group.

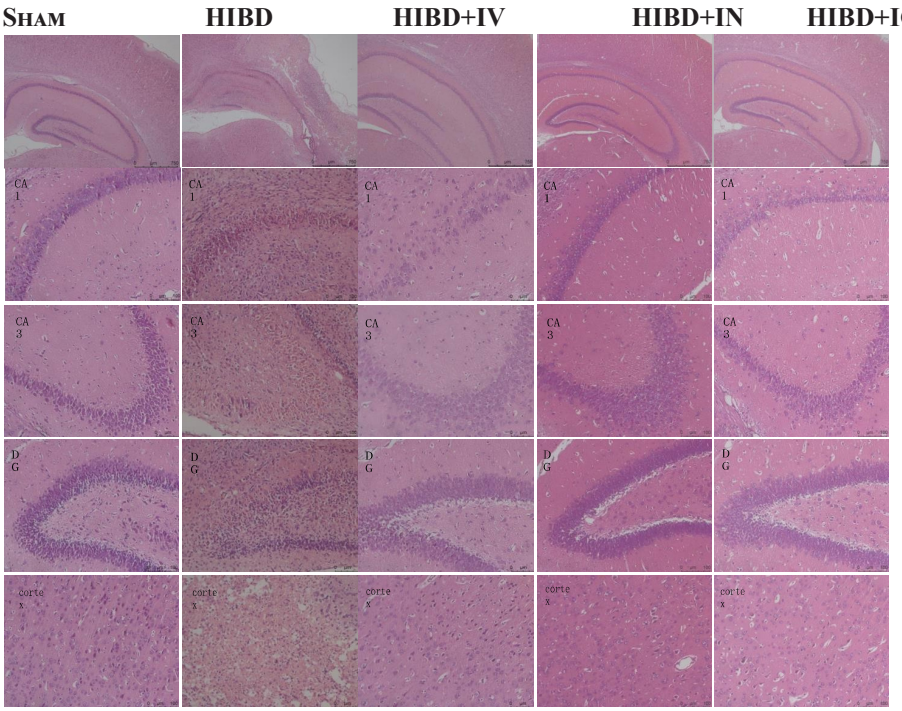
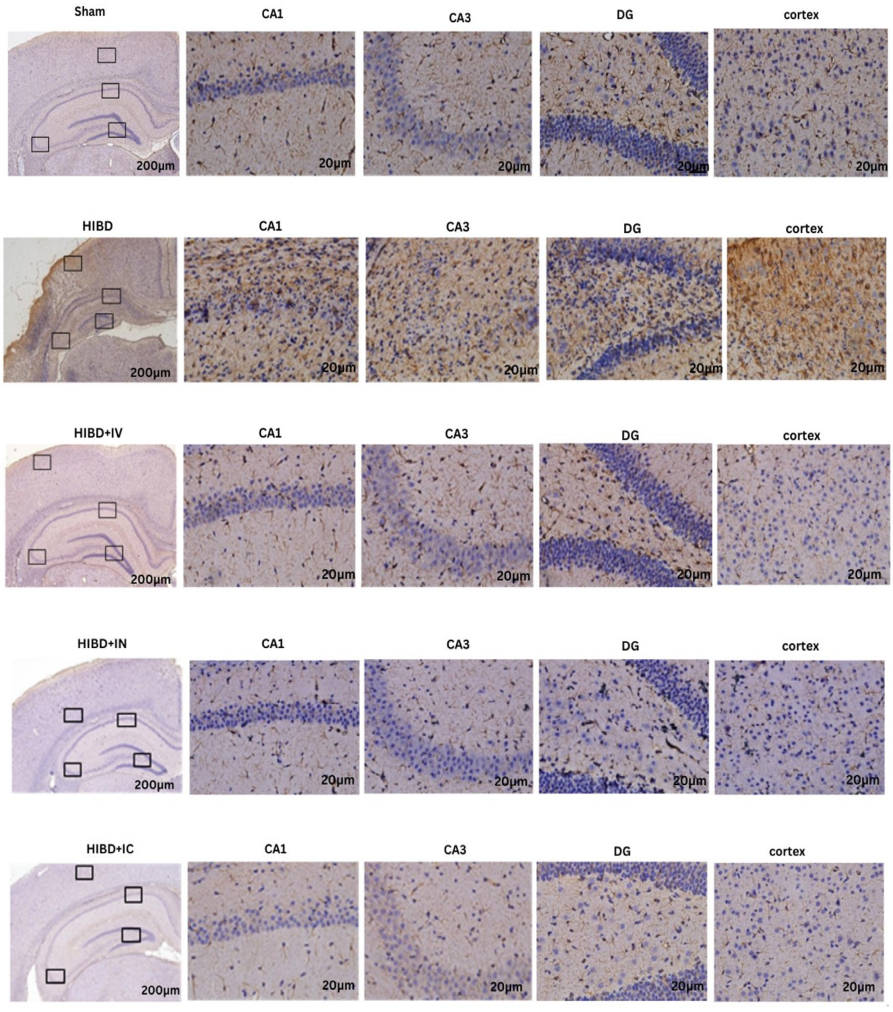


Figure 3: hUC-MSCs transplantation relieve cerebral tissue pathological changes in HIBD rats. Representative images of HE staining in the hippocampus and cortex after HI insult are shown. HE staining showed that the atrophy of damaged hippocampus and cerebral cortex became smaller, the local cell structure was disordered, the inflammatory cells infiltrated and the number of necrotic nerve cells increased. Scale bar: 750μm (40×) or 100 μm (200×).



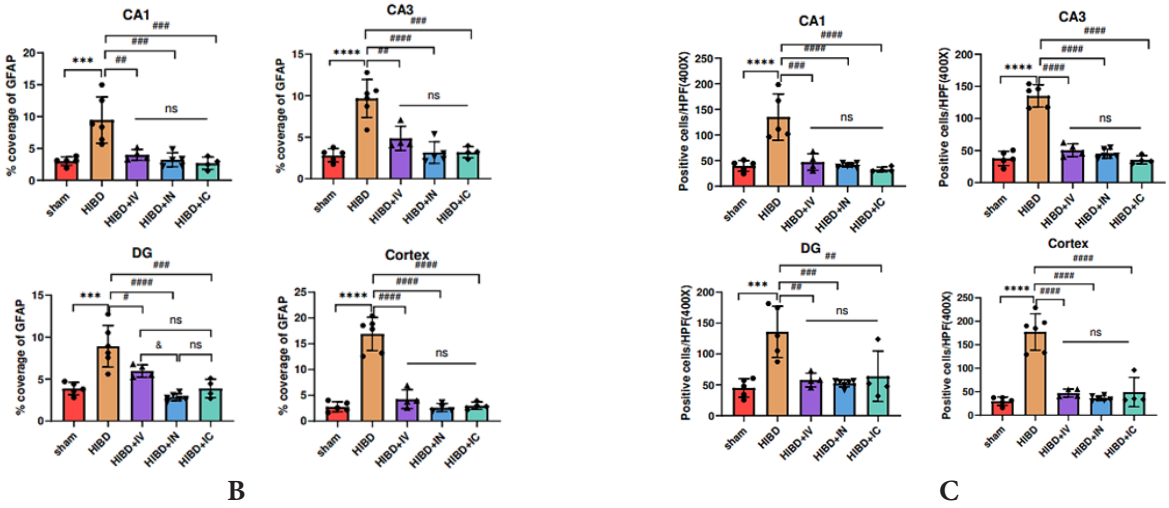
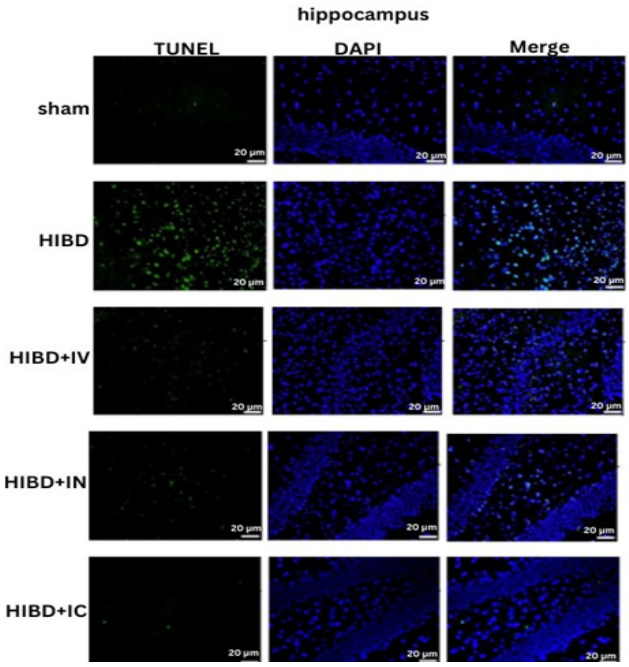


Figure 4: hUC-MSCs transplantation can reduce the proliferation of astrocytes in HIBD rats. Glial Fibrillary Acidic Protein (GFAP) immunohistochemistry was performed to assess the degree of proliferation of astrocytes in the hippocampus (CA1, CA3 and DG regions) and cortex. (A) Representative images of immunohistochemical analysis of GFAP positive cells in the hippocampus and cerebral cortex of rats in each group; (B) The % coverage of GFAP in CA1, CA3, DG and cortex; (C) The number of GFAP positive cells per high magnification (400×). Scale bar: 200 μm (40×) or 20 μm (400×). **Note:** n=4-6 rats per group. ***, **** represent p<0.001 and 0.0001 respectively compared to the sham group; $^{\#}$, $^{\#\#}$, $^{\#\#\#}$, $^{\#\#\#\#}$ represent p<0.05, 0.01, 0.001 and 0.0001 respectively compared to the HIBD group; "&" represent p<0.05 compared to the HIBD+IV group.

The apoptosis rate of nerve cells in the right cerebral cortex and hippocampus of rats in each group was detected. The results showed that the apoptosis rate of neurons in cortex and hippocampus of the HIBD group was significantly higher than that of sham group, while the apoptosis rate of three stem cell treatment groups was significantly lower than that of HIBD group. The apoptosis rate in hippocampus of HIBD+IC group and HIBD+IN group was lower than that of HIBD+IV group, but there was no significant difference between the former two groups. Besides, the apoptosis rate of HIBD+IC group on the cerebral cortex was significantly lower than that of HIBD+IV group (Figures 5A-5D).



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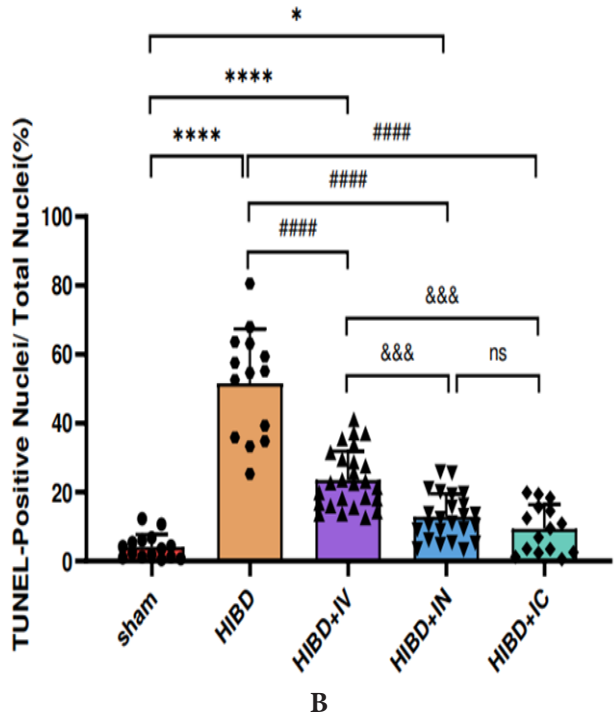

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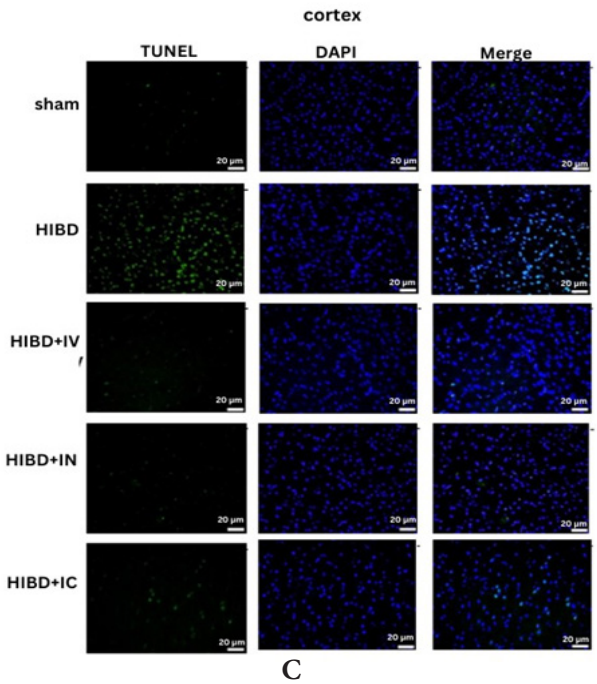

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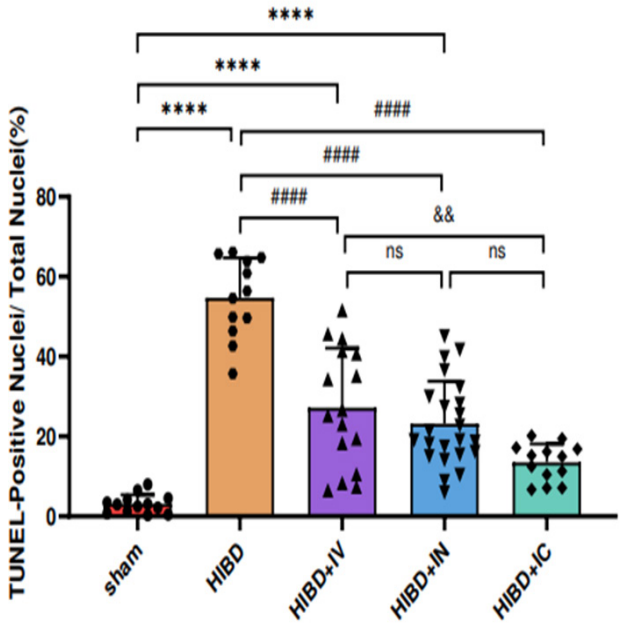

D

Figure 5: hUC-MSCs transplantation can inhibit apoptosis of neurons in HIBD rats. (A,B) Detection of apoptosis rate (TUNEL-Positive Nuclei/Total Nuclei × 100%) of neurons in the hippocampus of rats by TUNEL staining; (C,D) Detection of apoptosis rate of neurons in the cerebral cortex of rats by TUNEL staining. **Note:** n=3-5 rats per group. *,**** represent p<0.05 and 0.0001 respectively compared to the sham group; $^{\#\#\#\#}$ represent p<0.0001 compared to the HIBD group. $^{\&\&,\&\&\&}$ represent p<0.01, 0.001 respectively compared to the HIBD+IV group.

Discussion

It was known that HIBD may lead to neuropsychotic disorders such as cerebral palsy seizures and learning difficulties in children. More and more studies have shown the effectiveness of hUC-MSCs in the treatment of HIBD and related sequelae [31-33]. However, so far, most of the studies are still in the initial clinical trial stage [34], which has to be attributed to the fact that there are still many problems related to hUC-MSCs that have not been solved. For example, the most suitable dose, the most appropriate time window and the best effective way of stem cell transplantation.

There are many theories about the optimal dose and time window of stem cells transplantation. Some researchers believe that the minimum effective dose of stem cells in nasal transplantation is 0.5 × 106, the higher dose will not be more effective, but the lower dose has no obvious therapeutic effect [35]. Because the Hamilton syringe is needed in nasal or lateral ventricle transplantation and the volume of transplanting fluid is limited, the concentration of cell suspension should also be taken into account in the selection of the number of cell transplants. Higher concentration may aggregate into cell mass and affect cell activity and lower concentration may not active therapeutic effect. 5 × 10^4 cells/ul is considered to be the most appropriate cell transplantation concentration [36]. Therefore, the number of cells selected in this experiment was 0.5 × 10^6 cells. The problem of transplantation time also needs to be considered. Premature transplantation may not be conducive to the survival of stem cells in the brain microenvironment, late transplantation may result in glial scar formation and a large number of irreversible necrosis of neurons. So we decided to do the transplantation on the 7th day after the injury [37]. In order to make the results more reliable, this experiment unified the dose and time of transplantation of stem cells in three groups.

It was known that perinatal hypoxia-ischemic encephalopathy mainly causes motor and cognitive impairment in children [38]. And the cognitive function of the individual mainly includes feeling, perception, memory, thinking and so on. Through the construction of HIBD rat model, we found that the behavior of rats after hypoxia and ischemia was also manifested in the decline of motor balance and cognitive function (learning and memory). The motor and balance ability of rats in each group were compared by hanging wire and vertical pole test (Figure 1). It was found in PN10 that the rats in HIBD group showed obvious motor backwardness and lack of balance ability. On the 7th day after transplantation of stem cells (PN21), the three ways showed obvious therapeutic effect, especially the IC transplantation showed better motor function improvement effect than IV transplantation in the hanging wire test, but there was no significant difference compared with IN transplantation. It is unclear whether this is related to the IC-transplanted cells can reach the local part of brain injury more. Similar results have been shown in other experiments [39,40] and stem cells tracking techniques have been used to determine that IC transplantation is faster and more numerous than IV transplantation [41,42]. In the vertical pole test, transplantation of stem cells through three ways improved the motor balance ability of rats, but the degree of improvement among the three pathways did not show significant statistical difference. Therefore, we speculate that the functional areas of the brain affected by intervention of stem cells in different ways may be different. This was also verified by the MWM test. HIBD rats exhibit significant impairment in learning memory function and stem cells intervention reduces the extent of this functional impairment, especially through the IC and IN transplantation and even the IC transplantation has a more pronounced improvement in short-term memory retention ability. It indicates that the intervention of stem cells through IC and IN has a more significant therapeutic effect on the brain tissue area in charge of learning and memory function in HIBD rats.

The brain areas in charge of learning and memory function are mainly located in the hippocampus, especially the CA1, CA3 and DG regions, while the cortex is the advanced center for further processing memory and behavioral movement. According to the overall brain tissue specimens of our experiment, the injury area of HIBD to rat brain is also mainly manifested in hippocampus and cortex. Therefore, from the microcosmic point of view, we choose to quantify the damage of hippocampus and cortex by pathological staining to compare the therapeutic effect. Glial cells in the central nervous system mainly include astrocytes, microglia and oligodendrocytes, which play an important role in maintaining human health activities. Among them, astrocytes seem to play a more extensive role [43]. When HIBD occurred, the release of various harmful factors increased, astrocytes and microglia were activated and the expression of inflammatory factors was up-regulated, resulting in neuronal necrosis and apoptosis [44]. In the process, astrocytes proliferation is thought to last longer, play a more significant role and damage more [45]. Therefore, the protection of the nervous system from the reactive proliferation of astrocytes seems to play a greater role. The expression of GFAP in astrocytes is up-regulated after central nervous system injury, so it is widely used as a marker of the responsiveness of astrocytes [46]. We detected the repair of neuronal necrosis in cerebral cortex and hippocampus by hUC-MSCs transplantation by HE staining and detected the reactive proliferation of astrocytes by GFAP. We found that the transplantation of stem cells through IV, IN and IC could significantly repair the injured and necrotic neurons in the cerebral cortex and hippocampus of rats and decrease the proliferation of reactive astrocytes. The improvement of the area of astrocytes in DG area in HIBD+IN group was more obvious than that in HIBD+IV group. Besides, as previously studied [47], the blow to rats by HIBD also includes inducing neuronal apoptosis. In this study, the number of apoptotic cells in the cerebral cortex and hippocampus of rats decreased significantly after stem cell transplantation, indicating that stem cell transplantation can reduce the apoptosis of nerve cells induced by HIBD. Among them, the reduction of apoptotic neurons in the hippocampus was more obvious in the HIBD+IC and HIBD+IN group than in the HIBD+IV group, whereas it was more obvious in the HIBD+IC group for apoptotic neurons in the cortex. It means that the intervention of hUC-MSCs through lateral ventricle and nasal cavity has a better effect on improving the apoptosis of nerve cells in the brain tissue of HIBD rats than through intravenous intervention, which is basically consistent with our behavioral results.

To sum up, our study shows that transplantation of stem cells by three ways has therapeutic effect on HIBD. In terms of neurological recovery, damaged tissue repair, astrocytes proliferation and neuronal apoptosis, the therapeutic effect of IC and IN interventions was stronger in HIBD rats compared to the IV intervention route, while there was no significant difference between the first two intervention routes. Previous studies on the optimal pathway for stem cells intervention have also been conducted, focusing on the peripheral (venous, arterial) pathway and the local (lateral ventricular) pathway. During the same 7-week time frame, IC transplantation maybe is preferable to IV transplantation for delivery of hUC-MSCs during subacute phases of stroke [39]. This is similar to our results. IC intervention is the direct delivery of all stem cells into the brain using a brain stereotaxic instrument and is undoubtedly the fastest and the way to maximize the number of cells reaching the local area of brain injury. And clinical experiments have shown that interventional therapy through IC route

has certain curative effect on the short-term movement [48]. However, when performing lateral ventricular interventions, we also need to be aware of the side effects associated with the intervention route itself. In addition to direct puncture injuries and infectious complications, the sudden delivery of fluid and extracorporeal cells directly into the brain may also cause secondary damage by triggering an immune response in the brain. Besides, IC transplantation requires a high level of instrumentation and operator skill. IN transplantation is a non-invasive treatment method, which will hardly cause damage to the body and is easy to be repeated. It is a promising treatment method. And it has now been shown to have significant therapeutic effects in neurological disorders [49,50]. IN-transplanted cells can ignore the blood-brain barrier and rapidly pass through the olfactory nerve into the brain [51,52]. Moreover, IN-transplanted stem cells could still be detected locally in brain tissue several months later [53]. This seems to indicate that the IN pathway is more advantageous in terms of long-term therapeutic effects. Therefore, the combination of safety, efficacy and compliance may make IN intervention of stem cells more valuable in clinical applications compared to IC intervention. However, research on IN-transplanted stem cells is still mainly focused on animals, including non-human primates and has been preliminarily shown to be effective [25], although the relevant clinical evidence is still lacking.

The main purpose of our study on the effect of different stem cell intervention pathways on the repair of HIBD is to provide further reference value for the application of stem cells in clinical practice. Therefore, in addition to taking into account the therapeutic effect, we also need to include enforceability, acceptability and low adverse reactions in our thinking. Our study shows that IC as well as IN are superior to IV routes in terms of therapeutic efficacy, while there is no statistically significant difference between the first two comparisons. When applied to clinic, IN transplantation may be a more performant, more acceptable and less invasive approach. However, IN intervention with stem cells is still an emerging treatment and further research is needed to determine whether it is feasible to apply it in humans, as well as the appropriate dose, duration and preparation before the intervention. Additionally, even we have established a systematic evaluation method and system through this experiment, due to financial and time constraints, we have not done further research on the differentiation and homing of hUC-MSCs from each transplantation pathway in rats, nor the optimal transplantation dose and time window. And in the future, we can investigate more deeply in terms of therapeutic mechanisms, the optimal transplantation dose and time window, to facilitate the construction of clinical guidelines [54-57].

Conclusion

Conclusively, this report overall, hUC-MSCs transplantation can reduce brain injury and motor and cognitive impairment caused by HIBD and transplantation through the intracerebral and intranasal is more effective. In the future, if applied to clinical practice, intranasal transplantation may be an option that can be treated multiple times and is more compliant and convenient. However, it is worth noting that the stability and safety of intranasal transplantation still need to be further studied.

Ethical Approval

All the animals' experiments are approved by the ethical review of animal welfare in Hubei University of Medicine.

Authors' Contributions

Linyan Zhou conducted the experiments and wrote the initial draft, Kun Zheng conduct some experiments and revised the draft, Ruibo Zhang, Guangzhen He, Jinyun Xu, Hao Jiang, Lan Ren, Miao Zhou and Liang Zhao contributed on experiment design and draft revision. Yan Liao and Zeqin Fu contributed on human umbilical cord mesenchymal stem cells culture. Wenting Liu and Jiaowei Gu supervised the whole project.

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Competing Interests

The authors declare no conflict of interest.

Availability of Data and Materials

The data and materials are available for academic use upon request.

Ethics Statement

The experiment was approved by the Animal Ethics Committee of Hubei University of Medicine.

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