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Effects of ultrasound-combined microbubbles on hippocampal AchE fibers in rats

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ABSTRACT

Objective: To investigate the protective effect of ultrasound-combined microbubbles on hippocampal acetylcholinesterase (AchE) fibers in rats. **Methods:** According to random digits table, 60 SD rats were divided into two groups, marrow stromal cells (MSCs) intracranial transplantation group and MSCs intracranial transplantation + ultrasonic microbubbles group. Marrow stromal cells were cultivated and isolated *in vitro*; 12 weeks after transplantation, spatial learning and memorizing abilities of rats were assessed by Morris water maze; AchE staining method was used to observe changes in density and appearance of AchE staining positive fibers in hippocampal CA1 region. **Results:** There was asignificant increase in spatial learning and memorizing abilities of rats in MSCs intracranial transplantation + ultrasonic microbubbles group. Hippocampal AchE staining suggested an increase in the density of AchE staining positive fibers in MSCs intracranial transplantation group; the fibers were regular, intact and dense. Density of hippocampal AchE positive fibers was negatively correlated with the escape latent period and was positively correlated with percentage of the time needed to cross each platform quadrant. **Conclusions:** Better promotion of spatial learning and memorizing abilities of rats in MSCs intracranial transplantation + ultrasonic microbubbles group may be related with the protective effect of ultrasound-combined microbubbles on hippocampal acetylcholine fibers.

1. Introduction

Marrow stromal cells (MSCs) have potency of multi-directional differentiation and self-renewal ability in the bone marrow[1-4]. MSCs can differentiate into vascular endothelial cells, nerve cells, *etc*[5-10] across the embryonic layer. This research observed the effects of ultrasonic microbubbles opening blood brain barrier on the intracranial migration of MSCs and the protective effects of MSCs intracranial transplantation on acetylcholinesterase (AchE) fibers.

2. Materials and Methods

2.1. Isolation and cultivation of MSCs

MSCs were obtained from femurs of adult SD rats, isolated by density gradient centrifugation and cultured by adhesion method. Cells of second and third generation were used for cell transplantation.

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2.2. Establishment of forebrain ischemic rats model

2.2.1. Research subjects

60 male SD rats weighting from 245-265 g, all of which were SPF were chosen, and were obtained from the Experimental Animal Center of Third Military Medical University. Rats were divided into two groups according to random digits table: MSCs intracranial transplantation group (n=30) and MSCs intracranial transplantation + ultrasonic microbubbles group (n=30).

2.2.2. Forebrain ischemia model establishing method

Four Vessels Blockage method was used for the establishment. Model building was thought to be successful if loss of righting reflex, whiteness of eyeballs, tachypnea and coma were observed in 60 seconds after bilateral carotids occlusion.

2.3. Ultrasonic microbubbles opening blood brain barrier

According to the results in preliminary experiment, GE vivid 7 ultrasonoscope was used as the implement. Expose time was 5 minutes. Acoustic contrast agent was lipid membrane halothane microbubble contrast agent "Fat fluorocarbon" prepared by department of ultrasound. After injection of 0.2 mL microbubbles into vena caudalis of rats, cranial bone of rats was irradiated by ultrasound probe for 5 minutes.

2.4. Intracranial transplantation of marrow stromal cells

Transplantation was made one week after model building succeeded. In MSCs intracranial transplantation + ultrasonic microbubbles group, 0.5 mL MSCs (contains 5×10^6-5×10^7 cells) was injected into vena caudalis of rats using one 1ml syringe after blood brain barrier was opened by ultrasonic microbubbles. Same amount of MSCs was injected into rats in MSCs intracranial transplantation group.

2.5. Morris water maze assessing spatial learning and memorizing abilities

Mottis water maze consists of a round water pool and an auto-camera system and was used to assess spatial learning and memorizing abilities of rats. Directed sailing test: each rat was dropped into the water pool one day before the test, being left to swim freely for 120 seconds in order to familiarize the maze. It took four days to complete the test; each day was divided into two stages (morning and afternoon). Rats were trained four times in each time part, and the arithmetic mean of the four trainings made the value of the time part. Escape latent period of rats (time from dropping to finding and climbing up the platform) was recorded in detail. Space exploring test: the platform was removed on 5th day of the test, each rat was dropped into the pool facing the wall at a randomly selected point, and its swimming path was recorded by camera for 120 seconds. Number of times of rats crossed the corresponding position of the platform and the time needed to cross each quadrant in two groups were computerized and calculated.

2.6. AchE staining

After all the rats had completed the water maze test, rats in two groups were anesthetized and processed with 4 centi-degree 30% sucrose solution and 4% PFA solution of pH 7.3. Continuous coronal frozen sections were made in the area from 2.5mm to 3.5mm behind the marked spot, each of which was 40 μm thick. AchE staining was colorized according to the histochemical method recommended by Hedreen *et al*[4].

2.7. Statistical analysis

Statistics were processed with SPSS 10.0 for Windows, $P<0.05$ was recognized as significant. Distributing characteristics of AchE positive fiber layers were ascertained by the comparison between histochemical stained sections and adjacent Nissl and HE stained sections. Grid testing system was used to calculate the number of intersections of AchE positive fibers in hippocampal CA1 region and testing lines. Density of AchE positive fibers was demonstrated with number of intersections of positive fibers and testing lines within 0.01 mm^2 (N/0.01 mm^2). t test and ANOVA were used for statistical analysis.

3. Results

3.1. MSCs culture

As shown in Figure 1, MSCs adhered firmly one day after the culture. Figure 2 showed that it was fusiform MSCs that grew well in the fourth generation. The appearance of cells was like fibroblasts, with its dense, whirlpool shaped

pattern.

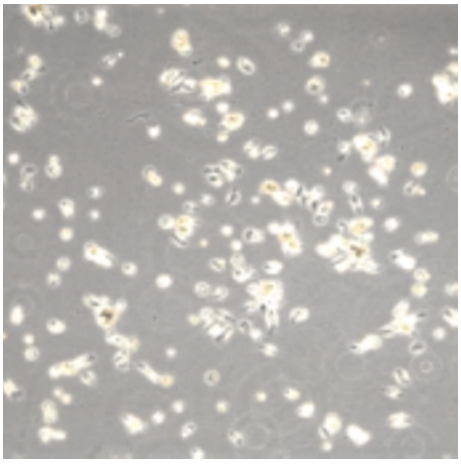

Figure 1. 1 d after cultivation of MSCs ×200.

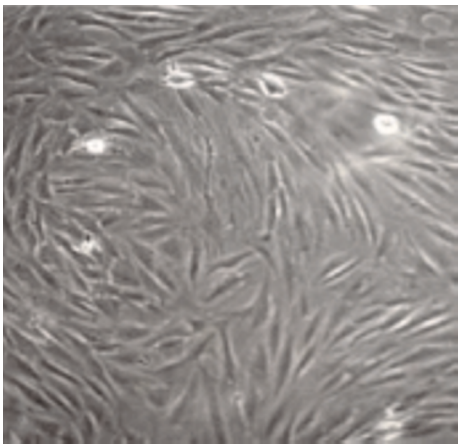

Figure 2. 4th generation of MSCs ×200.

3.2. Successful model establishment

Figure 3 shows that after the building of forebrain ischemic rats model succeeded, necrosis of pyramidal cells in hippocampal CA1 region was observed in HE staining.

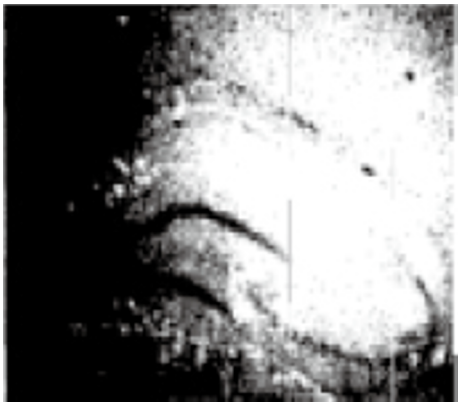

Figure 3. HE staining of rat hippocampal region fibers ×100.

3.3. Amount of GFP marked MSCs

Large amount of MSCs permeating the blood brain barrier was observed after BBB was opened by ultrasonic microbubbles (Figure 4) and small amount of GFP marked MSCs were seen in brain tissue when BBB was not opened (Figure 5).

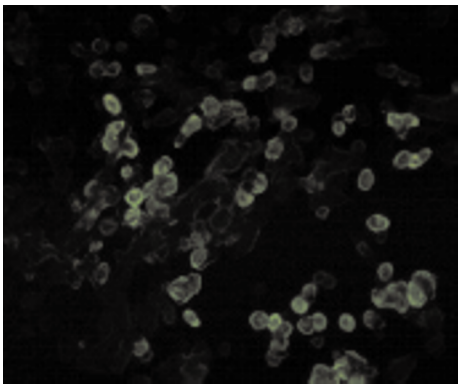

Figure 4. Large amount of GFP marked MSCs in brain tissue after BBB was opened by ultrasonic microbubbles ×400.

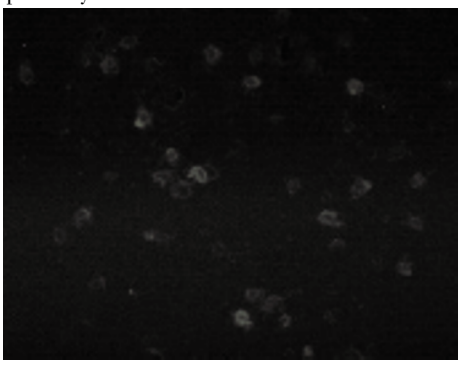

Figure 5. Small amount of GFP marked MSCs in brain tissue when BBB was not opened by ultrasonic microbubbles ×400.

3.4. Results of water maze test

ANOVA analysis of the dynamic latency showed that the difference between Group A and Group B had statistically significant difference (Figure 6).

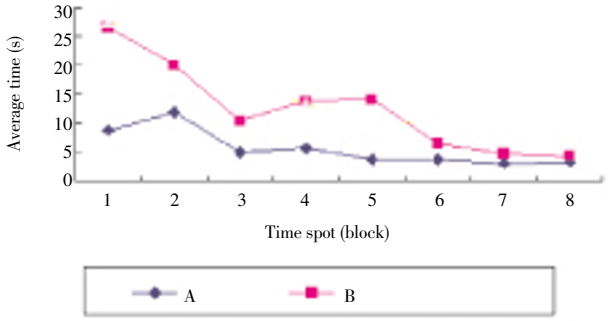

Figure 6. Changes in average escape latent period of rats in two groups in every block.

Results showed that the comparison of number of times of crossing the object between two groups had statistically significant difference (7.24±3.47 *vs.* 11.85±3.36) (P<0.05);

comparison of the percentage of the time needed to cross each platform quadrant between two groups had statistically significant difference (P<0.05) (Table 1).

Table 1

Percentage comparison of the time needed to cross each quadrant ($\overline{x}$± sd).

Groups	Percentage of the time needed to cross each quadrant (%)			
	Ⅰ	Ⅱ	Ⅲ	Ⅳ
A	22.01±2.74	25.03±7.68	32.36±4.63	24.19±4.58
B	21.53±2.63	23.96±7.52	46.29±5.27*	22.98±4.38

*P<0.05. Group A is MSCs intracranial transplantation group, Group B is MSCs intracranial transplantation + ultrasonic microbubbles group.

3.5. Changes in number and appearance of hippocampal AchE fibers

AchE positive fibers in hippocampal CA1 region could be seen clearly in MSCs intracranial transplantation group in March. And there were many fibers (Figure 7). Regular, intact, dense AchE positive fibers in hippocampal CA1 region in MSCs intracranial transplantation + ultrasonic microbubbles group in March could be observed.

Figure 7. MSCs intracranial transplantation group, AchE staining × 400.

Figure 8. MSCs intracranial transplantation + ultrasonic microbubbles group, AchE staining ×400.

On the 1st after the transplantation, comparison of the density of AchE positive fibers in hippocampal CA1 region between two groups had statistical significant difference (50.2±1.7 *vs*. 53.7±1.4) (P>0.05). However, 12 weeks after the transplantation, the comparison of the density of AchE positive fibers in hippocampal CA1 region in two groups had statistical significant difference (61.3±2.3 *vs*. 146.8±17.7) (P<0.05).

3.6. Relationship between layer density of AchE positive fibers and spatial learning &memorizing abilities of rats

Aalysis of the relationship of the variables showed that density of AchE positive fibers in the hippocampus was negatively correlated with the escape latent period (r=0.93) and were positively correlated with percentage of the time needed to cross each platform quadrant (r=0.85).

4. Discussion

Today stroke is the most important reason for disabilities and deaths in China, of which cerebral ischemia occupies a large proportion. However, up to now treatment of cerebral ischemia has still been limited[11-14]. Cell transplantation has attracted more and more attention as a method in the restoration of neural function after cerebral ischemia[15,16]. MSCs have high potency of self-renewing and multi-directed differentiation and are thought to be ideal donator cells[17-19].

Decrease in learning and memorizing abilities is common in stroke patients, while these abilities are closely related with learning and memorizing[20-25]. AchE is the hydrolytic enzyme of acetylcholine (Ach). AchE and Ach are highly conformable in basal forebrain, therefore AchE histochemistry can be used as an indirect index to reflect Ach[26-29]. In this research, spatial learning and memorizing abilities of rats 12 weeks after the transplantation in MSCs intracranial transplantation+ultrasonic microbubbles group were much better than those in MSCs intracranial transplantation group (P<0.01) in the same period. AchE staining showed that in both groups, the number of AchE positive fibers in hippocampal CA1 region molecular layer 12 weeks after the transplantation was larger than it was one day after the transplantation, while more significant increase of AchE positive fibers in MSCs intracranial transplantation + ultrasonic microbubbles group was observed, whose difference had statistical significance (P<0.01). The result suggests that transplantation of MSCs can promote the hyperplasty of AchE positive fibers, and ultrasonic microbubbles can promote MSCs to migrate intracranially.

Density of AchE positive fibers in the hippocampus was negatively correlated with the escape latent period and was positively correlated with percentage of the time needed to cross each platform quadrant. This result suggests the the relationship between spatial learning and memorizing abilities of rats and increase in the density of AchE positive fibers. Promotion of the amelioration of spatial learning and memorizing abilities of rats by MSCs transplantation may be related with the promotion of hyperplasty of AchE positive fibers.

Contemporary views about the mechanism of the amelioration of the behavior of rats are: 1. MSCs can take the place of dead neural cells; 2. MSCs can interact with the neural tissue in their residential place and lead to the production of certain cytokines, such as CSF, NTF, and SCF, which can promote the restoration of neural function. Various growth factors appear after cerebral hemorrhage[30], such as bFGF, BD-NF which are induced to be produced because of cerebral ischemia. The production of these cytokines, on the other hand, is beneficial for the survival and proliferation of MSCs and can induce the migration of MSCs to damaged areas, reducing the apoptosis of cells in ischemic penumbra; 3. MSCs are directly involved into the formation of new vessels.

The effect of MSCs transplantation on cerebral ischemia is limited in the past, mainly because of the existence blood brain barrier which blocks the migration of MSCs into intracranial ischemic areas. Therefore the problem of how to improve the migration of transplanted cells into ischemic areas is in desperate need to be resolved. Ultrasonic microbubbles can open BBB safely and efficiently as well as reversibly and can therefore promote the migration of transplanted cells through BBB into ischemic areas. The mechanism of opening BBB function of ultrasonic microbubbles may be related with the cavitation of microbubbles.

In this research, ultrasonic microbubbles opening BBB significantly promoted MSCs to migrate into intracranial areas and then promoted the amelioration of behavioral of forebrain ischemic rats. Significant improvement in spatial learning ability of rats in MSCs intracranial transplantation + ultrasonic microbubbles group was observed compared with subjects in MSCs intracranial transplantation group while there was no significant difference in spatial memorizing ability within two groups. It shows that transplanted MSCs have promotional function in restoring neural function of forebrain ischemic rats. Transplanted MSCs have promotional function on the hyperplasty of AchE positive fibers. There is certain relationship between the amelioration of spatial learning and memorizing abilities of forebrain ischemic rats and increased density of AchE positive fibers, which then suggested the relationship between the promotion of AchE positive fibers hyperplasty and the amelioration of spatial learning and memorizing abilities of forebrain ischemic rats by MSCs transplantation.

Conflict of interest statement

We declare that we have no conflict of interest.

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