RESEARCH ARTICLE

Combined acupuncture and *HuangDiSan* treatment affects behavior and synaptophysin levels in the hippocampus of senescence-accelerated mouse prone 8 after neural stem cell transplantation

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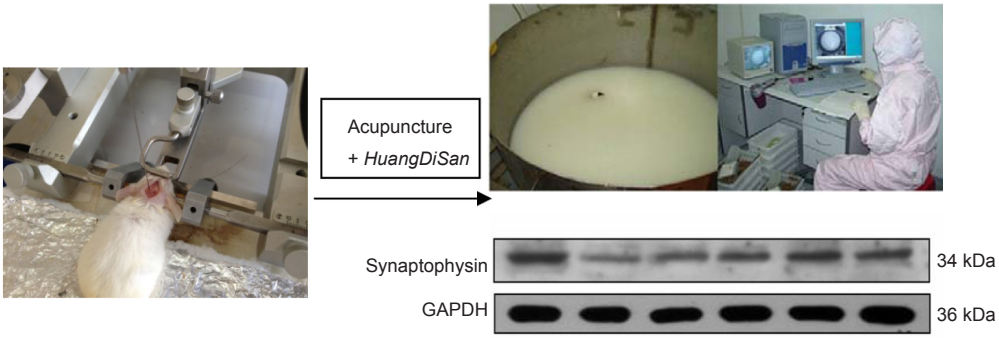
Graphical Abstract

Combination of acupuncture and *HuangDiSan* might be the most optimal method for treating cognitive impairment after neural stem cells transplantation in the hippocampus of SAMP8 mice



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Abstract

Sanjiao acupuncture and *HuangDiSan* can promote the proliferation, migration and differentiation of exogenous neural stem cells in senescence-accelerated mouse prone 8 (SAMP8) mice and can improve learning and memory impairment and behavioral function in dementia-model mice. Thus, we sought to determine whether *Sanjiao* acupuncture and *HuangDiSan* can elevate the effect of neural stem cell transplantation in Alzheimer's disease model mice. *Sanjiao* acupuncture was used to stimulate *Danzhong* (CV17), *Zhongwan* (CV12), *Qihai* (CV6), bilateral *Xuehai* (SP10) and bilateral *Zusanli* (ST36) 15 days before and after implantation of neural stem cells (5×10^5) into the hippocampal dentate gyrus of SAMP8 mice. Simultaneously, 0.2 mL *HuangDiSan*, containing Rehmannia Root and Chinese Angelica, was intragastrically administered. Our results demonstrated that compared with mice undergoing neural stem cell transplantation alone, learning ability was significantly improved and synaptophysin mRNA and protein levels were greatly increased in the hippocampus of mice undergoing both *Sanjiao* acupuncture and intragastric administration of *HuangDiSan*. We conclude that the combination of *Sanjiao* acupuncture and *HuangDiSan* can effectively improve dementia symptoms in mice, and the mechanism of this action might be related to the regulation of synaptophysin expression.

Key Words: *nerve regeneration; neurons; neurodegeneration; Alzheimer's disease; microenvironment; Chinese medicine; behavior; neural stem cell transplantation; synaptophysin; neural regeneration*

Introduction

Neural stem cells (NSCs) show great promise for the treatment of damaged nerves in inherited and injury-related diseases (Elliott Donaghue et al., 2014; Novak et al., 2016; Ma et al., 2017). Endogenous NSCs are usually in a resting state and can be activated under certain physiological or pathological stimuli (Harris et al., 2016; Yu et al., 2016). After implantation into the brain, exogenous NSCs can repair and replace damaged nerve cells, and secrete various factors that promote the proliferation and survival of NSCs (Xiong et al., 2017). However, NSC self-renewal, activation, proliferation and differentiation are mainly dependent on the surrounding microenvironment (Llorens-Bobadilla and Martin-Villalba, 2017; Wang et al., 2017). When the environment

changes, the self-renewal, proliferation, and differentiation capacity of stem cells is greatly altered (Park et al., 2015). Thus, improving the outcome of exogenous NSC transplantation is an urgent problem to be solved.

Encephalopathy can potentially be treated by NSC transplantation, aided by acupuncture to promote NSC proliferation and differentiation through the Jing-Qi-Shen conversion, marrow filling and spirit clearing (Zhao et al., 2015). Based on the theory of "Sanjiao dysfunction inducing aging", Professor Han (First Teaching Hospital of Tianjin University of Traditional Chinese Medicine) developed *Sanjiao* acupuncture and *HuangDiSan* prescription for the treatment of Alzheimer's disease and vascular dementia, with a good curative effect (Yu et al., 2006; Zhao et al., 2017).

Our previous study shows that *Sanjiao* acupuncture can promote the proliferation, migration and differentiation of endogenous NSCs in the main distribution area, the subventricular and subgranular zones, in senescence-accelerated mouse prone 8 (SAMP8) mice (Cheng et al., 2008). *Sanjiao* acupuncture improved learning and memory impairment, as well as behavior in dementia-model mice, and has a positive effect on NSC migration and expression of differentiation-related proteins and genes (Cheng et al., 2008). These findings indicate that acupuncture might affect the NSC microenvironment to have a positive impact on the transplantation of exogenous NSCs.

To further assess the significance of the *Sanjiao* theory in aging and dementia, and to improve treatment methods, we evaluated the effects of *Sanjiao* acupuncture and *HuangDiSan* on SAMP8 mice behavior and synaptophysin expression after hippocampal NSCs transplantation.

Materials and Methods

NSC isolation, culture, and transplantation

Embryos were removed from 12–16-day pregnant senescence-accelerated mouse resistance 1 (SAMR1) mice [provided by the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Animal production license No. SCXK (Jin) 2015-0003] under sterile conditions. Hippocampal samples were placed in NSC medium, containing 2% B27, 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 100 U/mL 100 g/mL penicillin and streptomycin, and DMEM/F12, in a 5% CO_2 incubator at 37°C. After subculture and expansion, Nestin-positive cells were evaluated, and the proliferation of NSCs was observed. Proliferating NSCs were adjusted to 1×10^6/mL, and seeded in 24-well plates with coverslips. The medium was replaced by DMEM/F12 with 20% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin to induce NSC differentiation. Immunocytochemical staining was used to confirm NSCs differentiation. A neuronal nuclei (NeuN) antibody suggested differentiation into neurons, while a glial fibrillary acidic protein (GFAP) antibody suggested differentiation into astrocytes. NSC density was adjusted to 5×10^5/μL and bilateral NSC transplantation into the hippocampal dentate gyrus cell sparse areas was conducted according to the following stereotaxic coordinates relative to the anterior fontanelle: anteroposterior: −2.06, mediolateral: ±1.75, dorsoventral: −1.75 (Blurton-Jones et al., 2009). The left and right hippocampal regions were injected with 1 μL NSCs over 1 minute. After the operation, mice were allowed to recover for 24 hours.

Group assignment of experimental animals

The experimental procedures were carried out according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1985). The study protocol was approved by the Ethics Committee of Tianjin Medical University of China (approval number: TMUaMEC 2016007). Senescence-accelerated mice (SAM) with different phenotypes were selected from a common background strain, AKR/J, in Kyoto University, Japan. Among the SAM mice, SAMP8 mice show deficits in learning and memory abilities (Takeda et al., 1981). Fifty 8-month-old clean healthy male SAMP8 mice [provided by the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Animal production license No. SCXK (Jin) 2015-0003] were randomized into five groups, with ten mice per group. Ten 8-month-old healthy male SAMR1 mice [provided by First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Animal production license No. SCXK (Jin) 2015-0003] were used as controls. Five mice were housed per cage, with free access to food and water under a 12-hour light/dark cycle at 24 ± 2°C and 45% humidity. The groups were:

a) SAMR1 control group (RC): catching-grasping stimulation.
b) SAMP8 control group (PC): catching-grasping stimulation.
c) SAMP8 NSC transplantation group (PT): 15 days of catching-grasping stimulation before NSC transplantation, and an additional 15 days of catching-grasping stimulation post-surgery.
d) SAMP8 NSC transplantation with acupuncture group (PTA): 15 days of acupuncture intervention before NSC transplantation, and an additional 15 days of acupuncture intervention post-surgery.
e) SAMP8 NSC transplantation with *HuangDiSan* group (PTD): 15 days of *HuangDiSan* administration before NSC transplantation, and an additional 15 days of *HuangDiSan* administration post-surgery.
f) SAMP8 NSC transplantation with acupuncture and *HuangDiSan* combination group (PTC): 15 days of combined acupuncture and *HuangDiSan* treatment before NSCs transplantation, and an additional 15 days of combined acupuncture and *HuangDiSan* treatment post-surgery.

Acupuncture method

Mice in the PTA and PTC groups received *Sanjiao* acupuncture at *Danzhong* (CV17), *Zhongwan* (CV12), *Qihai* (CV6), bilateral *Xuehai* (SP10) and bilateral *Zusanli* (ST36), with pre-determined manipulation methods for 30 sec-

onds. CV17, CV12, CV6, and bilateral ST36 were needled by twisting reinforcing methods, while bilateral SP10 was needled by the twisting reducing method. The localization of acupoints was based on Laboratory Acupuncture and Atlas of Animal Acupoints enacted by the Experimental Acupuncture-Moxibustion Research Association of the Chinese Academy of Acupuncture and Moxibustion (**Table 1**). All intervention protocols were implemented once a day for 15 days and suspended only on day 7.

***HuangDiSan* delivery**

Eight grams of *HuangDiSan* (composed with Rehmannia Root and Chinese Angelica; provided by the First Hospital Affiliated to Tianjin Medical University, license number: Z20100942) were dissolved in 8 mL 0.9% saline. Each mouse was gavaged with 0.2 mL using a 1 mL blunt syringe, once a day for 15 days, with a day off on day 7.

Morris water maze

After the 15-day treatments, behavioral changes were assessed using the Morris water maze (Tomás Pereira and Burwell, 2015). This consists of three parts: a circular pool, a platform, and a recording system. The pool was 90 cm in diameter, with a height of 50 cm. At the beginning of each experimental day, water made opaque with milk was added to the pool to a height of 30 cm, and adjusted to a temperature of 24 ± 1°C. The pool was divided into four quadrants (northeast, southeast, southwest and northwest). A cylindrical platform 9 cm in diameter and 28 cm high was placed in the center of any quadrant, such that the platform was 2 cm under the water. A camera was placed 2 m above the center of the pool. Swimming animals were automatically recorded and data were processed with an automatic image collection and analysis system. All equipment for the Morris-water maze test was provided by the Chinese Academy of Medical Sciences (Beijing, China). The mice were trained for the hidden platform trial for 5 days, for the probe trial for 1 day, for the reversal trial for 3 days, and for the visible platform trial for 1 day.

Hidden platform trial

The day before the trial, mice were allowed to swim freely for 90 seconds in the pool without the platform once in the morning and once in the afternoon to become familiar with the maze environment. During the trial, the platform had a fixed position; the midpoint of the platform from the pool wall was 22.5 cm. On the offside of the platform, two points at the same distance from the point of entry were selected as the entry points. Animals were placed into the water, facing the pool wall. The time elapsed from entering the water to finding the platform was recorded as escape latency. If a mouse could not find the platform within 90 seconds, escape latency was recorded as 90 seconds. Each mouse was tested twice daily using two different water entry points. Statistical analysis was performed by the arithmetic mean of two escape latency durations. The trial was performed for 5 consecutive days.

Probe trial

Twenty-four hours after the hidden platform trial, the platform was removed. Then, the mice were placed into the water from any entry point, and retention time in the former quadrant as well as the frequency of crossing the former platform was recorded for 60 seconds.

Reversal trial

The platform was moved to the quadrant opposite to that in the hidden platform trial, and the mice were assessed as in the hidden platform trial.

Visible platform trial

To eliminate sensory, visual or motor dysfunction effects on spatial learning and memory abilities, a 1-day visual platform trial was used. The platform was marked with yellow tape and placed 2 cm above the water. The tests were performed as described for the hidden platform trial.

Western blot assay

When the Morris water maze trial was over, mice were sacrificed by cervical dislocation after anesthesia, and the hippocampal tissue was dissected out and homogenized to a 10% homogenate in radioimmune precipitation assay lysis buffer. After centrifugation for 10 minutes at 12,000 × *g*, the total protein concentration was determined with a bicinchoninic acid protein assay kit. After electrophoresis and transfer to a membrane, proteins were incubated with a rabbit monoclonal anti-synaptophysin antibody (1:5,000; Abcam, Cambridge, UK) at 4°C overnight and then with a goat anti-rabbit IgG (1:8,000; Abcam) secondary antibody at room temperature for 1 hour. Blots were developed using the chemiluminescent method. Protein bands were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA) and the synaptophysin/GAPDH ratio calculated to show relative protein expression.

Real-time PCR

Total hippocampal RNA was extracted from 10% hippocampal homogenate using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 2 μg RNA was used for reverse transcription with a first-strand cDNA synthesis kit (Bio-Rad, USA). Real-time PCR was performed using the absolute quantitative $2^{-\Delta\Delta Ct}$ method with a SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) on a Bio-Rad iCycler system to detect synaptophysin gene expression.

Statistical analysis

All data were analyzed with SPSS 11.5 software (SPSS Inc, Chicago, IL, USA) and are expressed as the mean ± standard deviation (SD). Data of multiple groups were compared using one-way analysis of variance. Comparison between two

Table 1 Acupuncture points and manipulation (Zhao et al., 2013)

Points	Anatomical positions	Twisting angle (degree)	Frequency (per minute)
Danzhong (CV17)	On the anterior median line on the chest, at the midpoint between the two nipples, at the level of the 4th intercostal space	< 90	> 120
Zhongwan (CV12)	On the anterior median line of the upper abdomen, 10 mm below the xiphisternal synchondroses	< 90	> 120
Qihai (CV6)	On the anterior median line of the lower abdomen, 4 mm below the umbilicus	< 90	> 120
Xuehai (SP10)	When the knee is flexed, on the medial aspect of the thigh, the point is 3 mm above the mediosuperior border of the patella, on the bulge of the medial portion of M quadriceps femoris	> 180	< 60
Zusanli (ST36)	1 mm lateral to the anterior tubercle of the tibia, and 3 mm below the capitulum fibulae under knee joint	< 90	> 120

groups was conducted by the Student-Newman-Keuls test. A value of $P < 0.05$ was considered statistically significant.

Results

Spatial memory and cognitive changes in SAMP8 mice

The Morris water maze is used to evaluate the learning ability of animals to find the position of a hidden platform (Vorhees and Williams, 2014; Huang et al., 2016). In the hidden platform trial, compared with the RC group, the average escape latency in the PC group increased significantly from day 2 ($P < 0.05$). Compared with the PC group, escape latency in the PTC group was significantly shorter from day 3 ($P< 0.05$); from day 4, escape latencies in the PT, PTA, PTD, and PTC groups were significantly shorter compared with the PC group ($P < 0.05$). On day 5, compared with the PT group, escape latencies in the PTA, PTD and PTC groups were shorter ($P < 0.05$). Compared with PTA animals, escape latency of the PTC group was significantly shorter ($P < 0.05$). The PTC group showed significantly shorter escape latency compared with the PTD group ($P < 0.05$; **Figure 1A**).

The probe trial mainly reflects memory retention (Gómez et al., 2017). The results showed that retention time in the former quadrant of the hidden platform trial among all groups was significantly different. Compared with the RC group, retention time in the former platform quadrant in the PC, PT, PTA and PTD groups was significantly shorter ($P < 0.05$). Compared with the PC group, retention time in the former platform quadrant in the PT, PTA, PTD, and PTC groups was significantly prolonged ($P < 0.05$). Compared with the PT group, retention time of the PTA, PTD and PTC groups in the former platform quadrant was significantly prolonged ($P < 0.05$). The PTC group had a significantly prolonged retention time in the former platform quadrant compared with the PTA group ($P < 0.05$). Compared with the PTD group, retention time of the PTC group in the former platform quadrant was significantly prolonged ($P < 0.05$). The frequencies of crossing the former platform in the RC, PT, PTA, PTD and PTC groups were significantly higher compared with that in the PC group ($P < 0.05$; **Figure 1B**).

In the reversal trial for 3 consecutive days, escape latency significantly varied among different groups. Compared with the RC group, average escape latency in the PC, PT, PTA and PTD groups was significantly increased ($P < 0.05$). Compared with the PC group, average escape latency in the PT, PTA, PTD, and PTC groups was significantly shorter ($P < 0.05$). Compared with the PT group, average escape latency in the PTD and PTC groups was significantly shorter compared with the PT group ($P < 0.05$); from day 2, average escape latency of PTA, PTD and PTC groups was significantly shorter ($P < 0.05$). The PTC group showed significantly shorter average escape latency compared with the PTA group ($P < 0.05$). Compared with the PTD group, average escape latency of the PTC group was decreased significantly from day 2 ($P < 0.05$; **Figure 1C**).

Visible platform trials showed no significant difference in escape latency among groups ($P > 0.05$; **Figure 1D**).

Protein levels of synaptophysin in the hippocampus of SAMP8 mice

Compared with the RC group, the levels of synaptophysin in the PC, PT, PTA, PTD and PTC groups were significantly reduced ($P < 0.05$). Compared with the PC group, synaptophysin levels in the PTC group were significantly increased ($P < 0.05$). Compared with the PT group, synaptophysin levels in the PTA, PTD and PTC groups were not changed significantly ($P > 0.05$). Compared with the PTA group, synaptophysin levels in the PTD and PTC groups were not significantly different ($P > 0.05$; **Figure 2**).

Gene expression of synaptophysin in the hippocampus of SAMP8 mice

Compared with the RC group, synaptophysin mRNA levels in the PC, PT, PTA, PTD and PTC groups were significantly decreased ($P < 0.05$). Meanwhile, synaptophysin mRNA levels in the PTC group were significantly increased compared with the PC group ($P < 0.05$). Compared with the PT, PTA, and PTD groups, synaptophysin mRNA levels in the PTC group were significantly increased ($P < 0.05$; **Figure 3**).

Discussion

The multi-differentiation properties of NSCs may enable them to be used for replacement therapy in neurodegenerative tissues, which provides hope for overcoming degenerative diseases of the central nervous system (Lemmens and Steinberg, 2013; Zhong et al., 2016; Pen and Jensen, 2017). A large number of studies have shown that injured nerve cells are usually repaired and replaced; several types of cytokines are secreted to promote the proliferation and survival of NSCs, and cellular circuits and functions are partially regenerated after the implantation of exogenous NSCs into the brain (Ahmed et al., 2016; Jablonska et al., 2016; Ye et al., 2016; Hou et al., 2017). Transplantation of exogenous NSCs has the advantage of targeting a limited and defined site to replace injured or lost cells, promote self-repair, and restore brain function (Dong and Yi, 2010; Chen et al., 2014; Cai et al., 2015). We selected the hippocampus, which is strongly associated with dementia, for exogenous NSC transplantation, and the status of mice with dementia was considerably improved. Morris water maze visible platform trials indicated that differences in sensory, visual or motor functions did not significantly affect spatial learning or memory. SAMP8 mice had cognitive impairment in learning and memory acquisition, lower memory retention and re-learning ability. Hippocampal transplantation of NSCs significantly improved learning and memory dysfunction, memory retention, and re-learning ability of SAMP8 mice.

Sanjiao is the pathway for ascending, descending, exiting and entering and the source for the production of *Qi*, blood, essence and body fluid. As the commander for *Qi* activity, *Sanjiao* controls functional activities of internal organs to maintain normal human life activities. Therefore, the abnormal function of "*Qi* activity in *Sanjiao*" is the basic mechanism for aging (Han, 2007). Based on the dysfunction of *Qi* activity of *Sanjiao*, which is the fundamental pathological mechanism of senile dementia, Professor Han Jingxian proposed that "dysfunction of *Qi* activity of *Sanjiao* leads to aging", and created "*Sanjiao* acupuncture" and "*HuangDiSan*" prescription. Treatment was based on *Qi*, with the acupuncture acupoints CV17, CV12, CV6, SP10 and ST36. Regulating the *Qi* of *Sanjiao* modulates spleen and stomach functions, enriching *Qi* and blood, tonifying the kidney, promoting blood circulation for blood stasis removal, eliminating phlegm, improving dementia symptoms, and retarding brain aging. Acupuncture was used as the fundamental prescription, and could be changed according to different conditions. The main ingredients of *HuangDiSan* are Chinese Angelica and Rehmannia, with the effects of dredging *Sanjiao*, tonifying *Qi* and regulating blood, as well as strengthening the root and tonifying the kidney. The combination of acupuncture and *HuangDiSan* for the treatment of Alzheimer's disease as well as deficiency illness of *Qi* and blood had significant effects (Shi et al., 2015). In this study, three methods of acupuncture, Chinese medicine, and a combination of acupuncture/Chinese medicine, further improved cognitive impairment, memory retention and re-learning in SAMP8 mice. Furthermore, the effects of acupuncture combined with *HuangDiSan* were stronger than those of either modality administered alone. Thus, the above combination therapy might be the best way to improve cognitive function in SAMP8 mice after NSCs transplantation. Chinese medical interventions act upon nerve cells themselves and on their microenvironments, affecting multiple targets and processes, including proliferation, differentiation, migration, and synapse formation (Lu et al., 2014; Li et al., 2016; Nie et al., 2016; Zhang et al., 2016, 2017).

The synapse is the fundamental connection between neurons for information transmission (Barbash and Sakmar, 2017; Fink et al., 2017; Pietronigro et al., 2017). In AD patients, there is neuronal loss and reduced amounts of synaptophysin in the hippocampus (Tannenberg et al., 2004; Kirvell et al., 2006; Wang et al., 2007). As an important marker of synaptic reconstruction, synaptophysin is widely used for labeling axon terminals, reflecting the occurrence and density of synapses (Dan et al., 2008; Brewer et al., 2009; Hong et al., 2016; Pan et al., 2016; Stoyanova et al., 2016). Synaptophysin reduction in the hippocampus of SAMP8 mice indicates a decreased transport capacity of synaptic vesicles, resulting in deficient synaptic transmission and altered transmission, processing, and storage of information in the nervous system. Synapse formation and plasticity in hippocampal neurons are strongly associated with learning and memory abilities (Howland and Wang, 2008; Leal-Galicia et al., 2008; Rae and O'Malley, 2016). A decreased number of synapses in the hippocampus would affect the contact between neural pathways (Duyckaerts et al., 2009; Logue et al., 2016; Wu et al., 2016), which might lead to various dysfunctions, especially cognitive and memory deficits (Kleeman et al., 2016; Muhia et al., 2016). Functional synaptic connections can be formed between neurons differentiated from NSCs transplanted into the hippocampus of rats (Gao et al., 2007; Hou et al., 2008), as well as among neurons differentiated from NSCs *in vitro* (Liebau et al., 2007). As shown above, learning and memory, memory retention, and relearning abilities in SAMP8 mice were improved, and synaptophysin levels in the hippocampus were significantly increased after transplantation of NSCs. These findings indicate that the significant improvement of cognitive impairment in dementia model mice might be correlated with the formation of new synapses in the hippocampus. All three intervention approaches, including acupuncture, *HuangDiSan*, and combined acupuncture/*HuangDiSan*, significantly alleviated cognitive impairment and upregulated synaptophysin expression in dementia model mice. However, the combined acupuncture/*HuangDiSan* positively affected behavior and synaptophysin levels in dementia model mice. This indicates that the combination of acupuncture and *HuangDiSan* (based on the *Sanjiao* theory) is more effective than the use of acupuncture or Chinese medicine alone, and might be the optimal method for

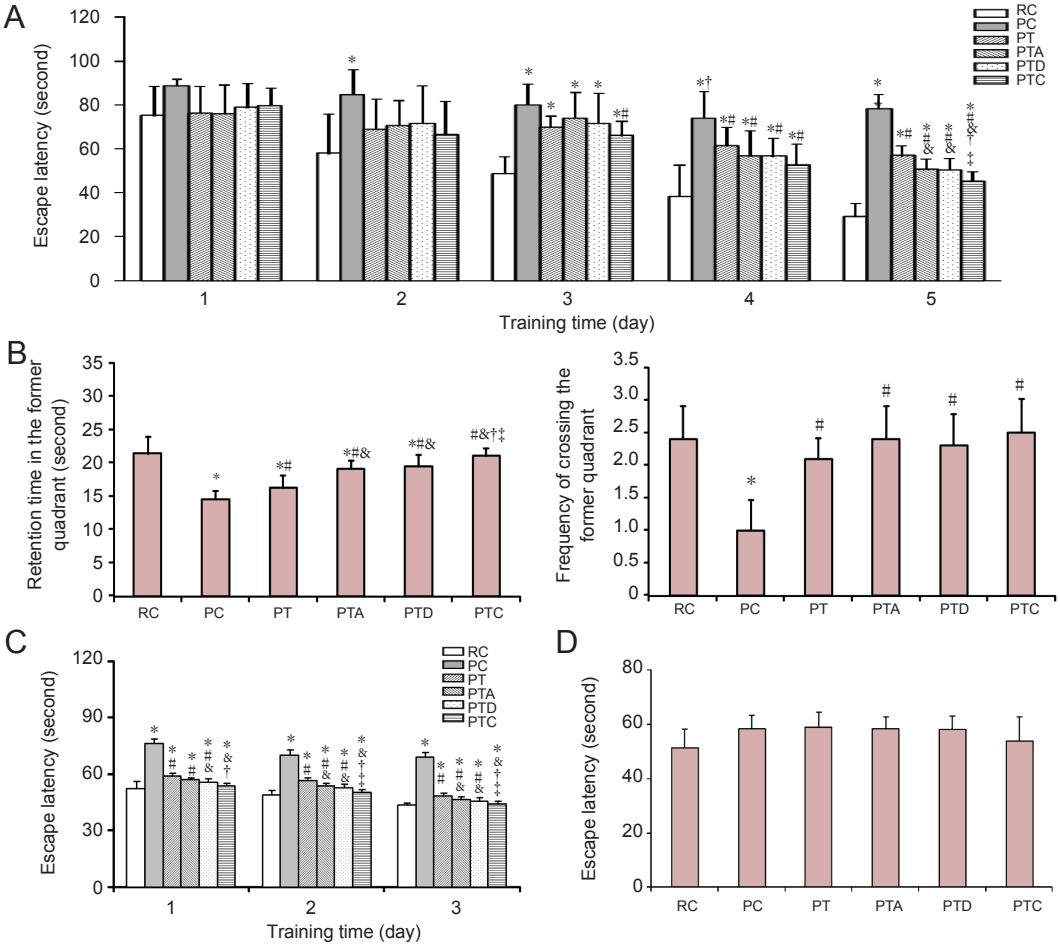

Figure 1 Morris water maze assay of SAM mice.
(A) Hidden platform trial. (B) Probe trial. Including "Retention time in the former quadrant" on the left and "Frequency of crossing the former quadrant" on the right. (C) Reversal trial. (D) Visible platform trial. All mice were trained in the hidden platform trial for 5 days. The probe trial was performed for 1 day, the reversal trial for 3 days and the visible platform trial for 1 day. Data are expressed as the mean ± SD, and were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. $*P < 0.05$, *vs.* RC; $\#P < 0.05$, *vs.* PC; $\&P < 0.05$, *vs.* PT; $\dagger P < 0.05$, *vs.* PTA; $\ddagger P < 0.05$, *vs.* PTD. RC: SAMR1 control group; PC: SAMP8 control group; PT: SAMP8 NSC transplantation group; PTA: SAMP8 NSC transplantation with acupuncture group; PTD: SAMP8 NSC transplantation with *HuangDiSan* group; PTC: SAMP8 NSC transplantation with acupuncture and *HuangDiSan* combination group. SAM: senescence-accelerated mice; NSC: neural stem cell.

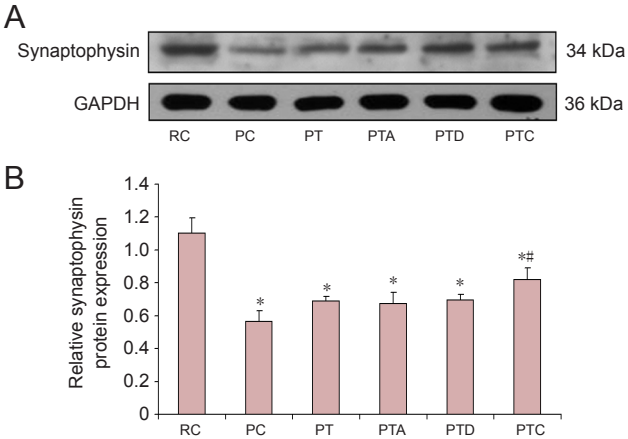

Figure 2 Western blot assay for synaptophysin protein levels.
Synaptophysin levels were assessed by western blot assays of SAM hippocampal tissues. Blots were re-probed for GAPDH to control for loading and transfer. (A) Protein bands. (B) Quantitative analysis of protein levels. Relative synaptophysin levels are expressed as the ratio of synaptophysin/GAPDH optical density values. Data are expressed as the mean ± SD, and were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. $*P < 0.05$, *vs.* RC; $\#P < 0.05$, *vs.* PC. RC: SAMR1 control group; PC: SAMP8 control group; PT: SAMP8 NSC transplantation group; PTA: SAMP8 NSC transplantation with acupuncture group; PTD: SAMP8 NSC transplantation with *HuangDiSan* group; PTC: SAMP8 NSC transplantation with acupuncture and *HuangDiSan* combination group. SAM: senescence-accelerated mice; NSC: neural stem cell; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

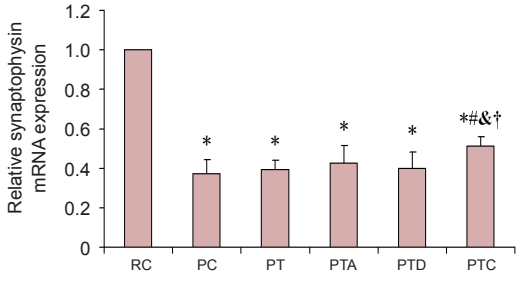

Figure 3 Real-time PCR for synaptophysin mRNA.
mRNA levels were assessed in SAM hippocampal tissues by real-time PCR. The products were quantified using β-actin as an internal reference. The relative level of mRNA was calculated as $2^{-\Delta\Delta CT}$. Data are expressed as the mean ± SD, and were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. $*P < 0.05$, *vs.* RC; $\#P < 0.05$, *vs.* PC; $\&P < 0.05$, *vs.* PT; $\dagger P < 0.05$, *vs.* PTA. RC: SAMR1 control group; PC: SAMP8 control group; PT: SAMP8 NSCs transplantation group; PTA: SAMP8 NSC transplantation with acupuncture group; PTD: SAMP8 NSC transplantation with *HuangDiSan* group; PTC: SAMP8 NSC transplantation with acupuncture and *HuangDiSan* combination group. PCR: polymerase chain reaction; SAM: senescence-accelerated mice; NSC: neural stem cell.

treating cognitive impairment after NSCs transplantation in the hippocampus of SAMP8 mice.

In conclusion, combined acupuncture and *HuangDiSan* treatment might act on components of the microenvironment that regulate transplanted exogenous NSCs, for example, promoting the secretion of nutrients necessary for injury repair, facilitating myelination of unmyelinated or new-born axons, providing substrates for axon growth, promoting synapse formation and neurotransmitter release, enhancing synaptic transmission, and improving learning ability and memory in dementia model mice.

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Research ethics: *The study protocol was approved by the Ethical Committee of Tianjin Medical University of China (approval number: TMUaMEC 2016007).*
Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
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