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Original Research Article

Effect of mesenchymal stem cell transplantation on behavior and structural changes in myelin in experimental rat autoimmune encephalomyelitis

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Abstract

Purpose: *To investigate the effect of bone marrow mesenchymal stem cell (BM-MSC) transplantation on behavior and structural changes in myelin of experimental autoimmune encephalomyelitis (EAE) in rats.*
Methods: *Wistar rats were randomly assigned to normal control, EAE, placebo injection treatment, and MSCs treatment. EAE, placebo injection treatment and MSCs treatment groups were further divided into six groups, i.e., 1-day, 3-day, 7-day, 14-day, 21-day, and 28-day post-onset. Changes in the disease status of the rats and structural changes in myelin at different time points were assessed with silver staining.*
Results: *Behavioral changes peaked between 13 to 17 days post-immunization (71.90 % incidence), while disease symptoms peaked between 3 and 5 days after onset, were sustained for about 7 days, and then eased gradually thereafter. The highest therapeutic scores and the entire course of the disease in EAE and placebo treatment groups were not significantly different ($p > 0.05$). However, in MSC treatment group, these parameters were significantly lower than in the above two groups ($p < 0.05$). In EAE and placebo treatment groups, myelin sheath lesions were obvious from day 3 to 7 but on day 14, the number of myelin sheath fragments decreased significantly. Recovery at different time points was also better than those in EAE and placebo treatment groups.*
Conclusion: *MSC transplantation shortens the course of EAE, and also reduces its severity. Thus, it has some prospects for use in the management of EAE.*

Keywords: *Bone marrow mesenchymal stem cells, Autoimmune encephalomyelitis, Transplantation, Myelin*

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INTRODUCTION

Acute disseminated encephalomyelitis (ADEM), a common central nervous system (CNS) infectious disease in children, is characterized by high incidence, rapid progression, severe symptoms and high mortality. The survivors usually have serious sequelae. Immunosuppressive agents and gamma globulin shock are commonly used for its management, but they act as mere palliatives. Cell transplantation is a recently developed treatment

strategy and it involves transplantation of exogenous cells to effect repair of CNS injury.

Bone marrow mesenchymal stem cells (BM-MSCs) are derived from various sources. They can be readily used for autologous transplantation because they are capable of self-replication and multi-directional differentiation during long-term culture *in vitro*. These cells are characterized by weak immunogenicity and ability to induce positive effect regardless of whether the transplantation is local or intravenous, thereby making them one of the most promising treatment strategies for repair of human CNS injury [1-4].

Experimental autoimmune encephalomyelitis (EAE), a classical animal model of ADEM has the same clinical, biochemical, immunological and pathological features as ADEM [5]. The aim of this study was to investigate the effect of BM-MSC transplantation on clinical behavior and structural changes in myelin of EAE rats.

EXPERIMENTAL

Materials

Complete Freund's adjuvant was a product of Sigma Aldrich Co., Ltd.; pertussis vaccine stock was purchased from Beijing Biological Products Research Institute, while silver nitrate was obtained from Sinopharm Group Shenyang, China.

Preparation of EAE animal model

Guinea pigs of both sexes (weighing 180 to 250 g), and female Wistar rats (weighing 160 to 180 g) were obtained from the Experimental Animal Center of Shengjing Hospital of China Medical University, and used for the study. This research was approved by the Animal Ethical Committee of Shengjing Hospital of China Medical University (approval no. 2018310), and was performed according to the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [6].

Preparation of complete antigen

The guinea pigs were anesthetized with intraperitoneal injection of 5 % chloral hydrate at a dose of 6 ml/kg body weight (b. wt); their occipital bone and spinal canal were excised, and the spinal cords removed. All operations were carried out on ice plate. The cauda equine was carefully removed and centrifuged. The spinal cord was homogenized with physiological saline at a volume ratio of 1:1 in an ice-bath to obtain guinea pig spinal cord homogenate (GPSCH), which was mixed with equal volume of complete Freund's adjuvant (CFA), stored at 4 ^{o}C, and used within 12 h.

Induction of EAE

The Wistar rats were anesthetized with intraperitoneal injection of 5 % chloral hydrate at a dose of 6 mL/kg b.wt, and injected with 0.1 ml stock solution of Bordetella pertussis vaccine (BPV) (containing 2×10^{10} bacteria) on their left back feet under sterile state, while 0.4 ml of the mixed solution of CFA and GPSCH was injected on their four foot pads.

Determination of neurological function score

The scores were: 0 point = no disease; 1 point = reduction of tail tension or mild clumsy gait, and double hind legs were slightly dragged; 2 points = tail had no tension or moderate clumsy gait, and there was moderate weakness of double hind legs which dragged or lacked stable posture; 3 points = severe weakness of double hind limb but still dragged, and weakness of the limb; 4 points = paralysis of double hind limbs and incontinence; 5 points = dying state.

The day of immunization with antigen was taken as 0 d, and after immunization, the rats had free access to feed and water and they were weighed daily and assessed for function scores. A score of ≥ 2 was considered as indicative of EAE.

BM-MSC culture

Male Wistar rats (100 g) were procured and used for primary cell culture. They were anesthetized and sacrificed, and their femurs removed under sterile conditions. The bone marrows were rinsed with Dulbecco's Modified Essential Medium/Ham's Nutrient Mixture F-12 (DMEM-F12) supplemented with 10 % fetal bovine serum by means of a syringe to obtain cell suspension. The culture flask was inoculated with the cell suspension and incubated in an incubator at 37 °C and 5 % CO_2. After 24 h, non-adherent cells were discarded and the culture medium was replaced with fresh medium every three days, and the suspended cells discarded. The cells were trypsinized with 0.25 % trypsin after attaining 80 % fusion, and identified using a flow cytometer.

Identification of BM-MSCs

The third generation of BM-MSCs were selected, digested with trypsin, and centrifuged (4 ^{o}C, 900 rpm for 6 min). The resultant pellet was re-

suspended in 3 ml of phosphate buffered saline (PBS) supplemented with 5 % fetal bovine serum, and divided into three equal parts: negative control, CD34, and CD90. They were centrifuged, and the supernatant discarded, while the cells were re-suspended in 200 µl of PBS, followed by the addition of 2 µl fluorescent-labeled goat anti-rat CD34 and CD90 IgG antibodies as markers, and further culturing in the dark in an incubator at 37 ^{o}C for 30 min. This was followed by the addition of 5 % serum PBS and centrifugation at 2000 rpm for 20 min. The supernatants were discarded, while 500 µl of 5 % serum PBS was added prior to reading in a flow cytometer.

Animal grouping

A total of 218 Wistar rats were randomly assigned to four groups: normal control (n = 8), EAE, placebo injection, and MSCs treatment. Except for the control group, EAE was induced in the rats by intraperitoneal injection of immune antigen. After induction, rats with functional score ≥ 2 were assigned to the EAE group (n = 144), while placebo injection and MSCs treatment groups were assigned 48 rats each. The EAE, placebo injection and MSCs treatment groups were further divided into six post-onset groups of 8 rats each: 1-day, 3-day, 7-day, 14-day, 21-day, and 28-day.

Transplantation of BM-MSCs

Third generation of BM-MSCs were washed with PBS and trypsinized. Fresh culture medium was added to stop complete shrinkage of the cells, and the cells were thereafter centrifuged (4 ^{o}C, 800 rpm for 5 min). The supernatant was discarded and the cells re-suspended in 20 µL fresh culture medium. An aliquot (1 µL) containing 1 x10^{6} cells was taken and diluted with 100 µL of fresh culture medium for counting using the Trypan blue exclusion assay. The cells were kept in an ice box. The rats were anesthetized with intraperitoneal injection of 5 % chloral hydrate at a dose of 6 mL/kg b.wt; their limbs were immobilized, with their skins and hairs sterilized.

The rats were shaven clean, and their skins cut in the middle with a sterilized knife, while bleeding was stopped with cotton swab. Their anterior fontanelles were completely exposed and the periostea treated with 3 % hydrogen peroxide. This was followed by positioning with a stereotaxic device (at the site; 1.0 mm back of anterior fontanelle and aside; 1.5 mm, and at a depth of 3.5 mm). The bilateral ventricles were injected with 10 µL of prepared cells, with the needle kept inside for 2 min, and thereafter slowly withdrawn. The rat heads were sutured, and they were returned to their cages and fed.

Collection of brain tissue

Rat brains were excised and immediately fixed in 4 % paraformaldehyde at low temperature in the dark. The tissues were dehydrated, embedded in paraffin, and sliced into sections (5 µm) using a microtome. The slices were subjected to hematoxylin and eosin **(**HE) staining, as well as myelin Bielschowsky sliver staining. Hematoxylin and eosin staining was used to reveal perivascular or sub-pial inflammatory infiltrates, while Bielschowsky silver staining was used for the screening of any demyelination.

Histopathological examination of brain tissue

Sections of the brain tissues were dewaxed with xylene and dehydrated in gradient of alcohol. They were dipped in alum hematoxylin dye for 1 min and washed within 5 min with water to remove float. Hydrochlorinated alcohol (1 %) was used to separate the red color within 15 sec and the slides were rinsed with water for 10 min for the development of blue color. Eosin solution (1 %) was used for counterstaining, while 70 % alcohol was used to separate the red color. The tissue sections were subsequently dehydrated with a gradient of alcohol concentrations, and then dewaxed with xylene, and sealed with neutral gum.

Bielschowsky silver staining procedure

Sections of the brain tissues were washed with 100 % anhydrous ethanol twice, followed by 95 % ethanol (thrice), and finally with 70 % ethanol. They were then rinsed thrice with double-distilled water, and put in 20 % silver nitrate solution preheated at 37 °C for 30 min the dark until the slices turned brown. The tissue sections were rinsed thrice with double-distilled water and subsequently immersed in 10 % formaldehyde solution until they turned yellow. They were then rinsed thrice with double- distilled water, and placed in a wet box.

The slices were dyed with silver-ammonia-ethanol solution in drops within 40 sec, and directly immersed in a freshly prepared 10 % formaldehyde solution; the dyeing was stopped when brown patches became visible under a light microscope. They were subsequently rinsed thrice with double distilled water. The sections were further fixed in 0.2 % gold chloride solution and 5 % sodium thiosulfate within 3 min, and again rinsed thrice with double-distilled water.

They were dehydrated with 95 % alcohol twice, dewaxed with xylene and sealed.

Microscopic examination of slides

This was performed using Motic Images Advanced 3.0/Olympus BX 41 photo-micrographic analysis system. Five slices on similar levels (striatum, frontal cortex, hippocampus, lateral ventricle SVZ and ependyma and other brain regions) were selected and viewed under the light microscope (x 200). Three different fields of view (x 400) were also selected, and the positive cells were counted.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) and statistical analysis was performed using SPSS (13.0). Groups were compared using Students *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Behavioral manifestations of EAE in rats

The first case of behavioral changes was observed 12 days after immunization and the peak of onset was between 13 to 17 days post-immunization. A total of 151 rats were involved (71.90 %), while the clinical symptoms peaked between 3 to 5 days after onset, were sustained for about 7 days, and gradually eased. Before the onset, sudden decline in body weights, roughened furs, and loss of appetite were observed. The initial symptoms of onset were double hind limb clumsy gait and inflexible activities, but the symptoms were gradually increased until paralysis of double hind limbs was observed. When the rats presenting with onset moved forward with their double forelimbs, the double hind limbs were dragged, accompanied by incontinence. The weights of rats in the control group were marginally increased, and no clinical manifestations of EAE were observed throughout the period of 28 days.

Outcome of HE staining

Infiltrations of inflammatory cells (mainly lymphocytes) were observed in brain tissues of rats in the EAE group. Proliferation of vascular endothelial and glial cells, swelling, and cell destruction were also observed, with inflammatory cells clustered around blood vessels forming a typical "cuff". Meningeal inflammation was also observed, but there were no structural abnormalities in the brains of rats in the control group (Figure 1).

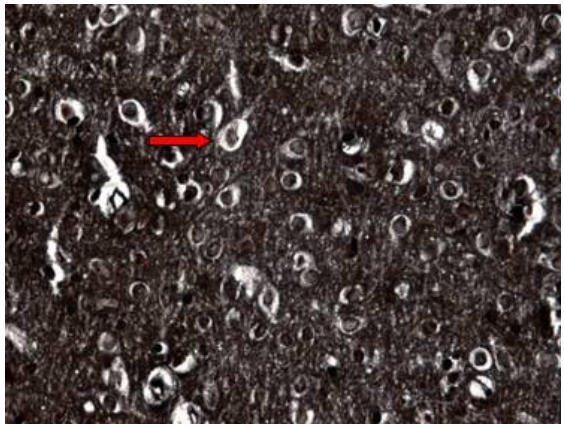

Figure 1: Normal 6 - 8 weeks old rat brain myelin staining results

Outcome of silver staining of brain tissues

There was no demyelination of brain tissues of rats in the normal group, and cross sections of their nerves were wheel-shaped, with blue central axes, and red myelin sheaths, while their shapes were round or oval. However, in the EAE group, different degrees of demyelination were observed in the white matter: some were single demyelinating lesions, while others were large or fusion demyelinating lesions. The axon showed vacuolar changes or fracture, and there was spherical swelling (axonal oval) on the fracture section (Figure 2).

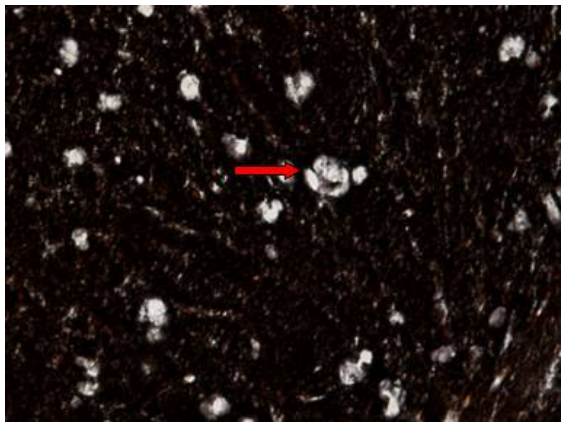

Figure 2: Myelin disintegration

Primary and subculture of MSCs

After 48 h of inoculation, adherent cells began to proliferate and their morphologies revealed homogeneous spindle shapes, and at days 4 and 5, the proliferation reached maximum. At this time, the cell processes were more obvious, the morphology of each cell generation was relatively uniform, and mostly long spindle-shaped (Figure

3). The fewer the cells were, the more scattered they were, and formed mass-like (colony-like) growth. However, when the cell population was multiplied, they were arranged in a spiral, reticular and radial form forming more consistent spindle shapes. After 2 to 3 passages, the cell purity was more than 95 %. The results of cell identification showed that CD34 was negative, while CD90 was positive (i.e., MSCs).

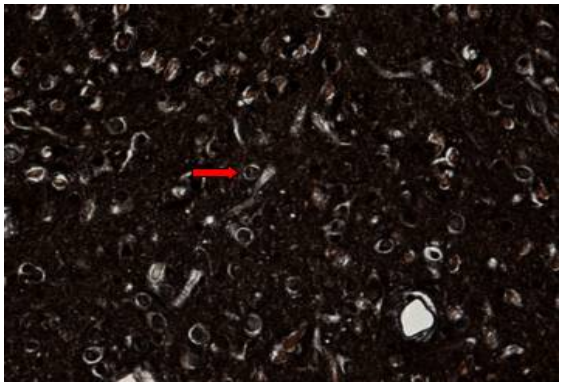

Figure 3: Myelin recovery after transplantation

Effect of transplantation of MSCs on disease symptoms in rats

The entire course of the disease in EAE and placebo treatment groups lasted for 12.13 ± 1.25 and 11.96 ± 1.32 days, respectively, and there was no significant difference between them (p > 0.05). The entire course of disease in MSCs treatment group lasted for 4.32 ± 0.63 days, and was significantly lower than the corresponding course of disease in the EAE and placebo treatment groups (p < 0.05). The rats were scored daily, and the highest therapeutic scores of rats in the EAE and placebo treatment groups were 3.87 ± 0.23 and 3.62 ± 0.28, respectively (p > 0.05). The highest therapeutic score in the MSCs treatment group was 2.35 ± 0.18, and was significantly lower than that of the EAE group (p < 0.05; Table 1). The changes in therapeutic scores in EAE, placebo and MSCs treatment groups are shown in Figure 4.

Table 1: Clinical symptoms of rats for EAE, placebo and MSCs treatment groups

Group	Duration of symptoms (days)	Highest mean therapeutic score
EAE	2.13 ± 1.25	3.87 ± 0.23
Placebo	11.96 ± 1.32$^{\triangle}$	3.62 ± 0.28$^{\triangle}$
MSCs treatment	4.32 ± 0.63$^{※★}$	2.35 ± 0.18$^{※★}$

$^{\triangle}P$ > 0.05, compared to placebo treatment and EAE groups; $^{※}p$ < 0.05, compared to the MSCs treatment and EAE groups; $^{★}p$ < 0.05, compared to the MSCs treatment and placebo treatment groups

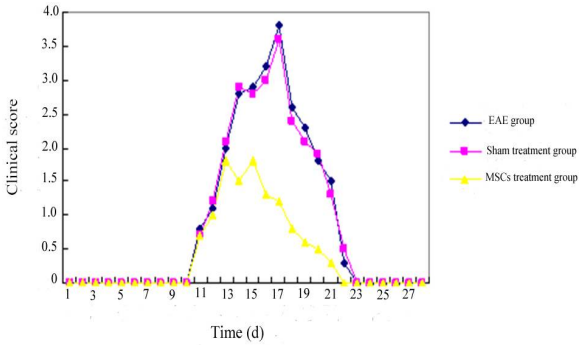

Figure 4: Changes in therapeutic scores of EAE, placebo and MSCs treatment groups. ***Note:*** Rhombus = EAE group; square = placebo treatment group; triangle yellow curve) = MSCs treatment group

Effect of transplantation of MSCs on structural changes in myelin

A total of 10 visual fields were randomly selected under 200-fold optical microscope, and edema of the myelin sheath, stratification, fragmentation, vacuolization, and myelin fragments clustered into block mass were used for myelin injury count. In the control group, myelin sheath samples were neatly arranged (without edema, stratification, fragmentation and formation of vacuolization). However, in EAE and placebo groups, myelin sheath lesions were obvious from day 3 to 7, and were presented as myelin disintegration or range expansion, diffuse distribution of myelin sheath lesions, significantly disordered tissue structure, and vacuolization of part of the myelin sheath.

After 14 days, the number of myelin sheath fragments in EAE group was significantly reduced. At day 1, the myelin sheaths of rats in the MSCs treatment group presented a tendency of widespread destruction, but recovered from day 3. The recovery at different time points was better than those in the EAE and placebo treatment groups. The quantitative analyses of the number of white matter and myelin sheath damage are shown in Table 2 and, respectively.

DISCUSSION

Acute dissimilated encephalomyelitis (ADEM) which is common in children, is an inflammatory demyelinating disease that affects the brain and spinal cord, and its pathogenesis is not known. The most common view regarding its pathogenesis is that immediately following non-specific viral infection or vaccination, molecular simulation mechanism leads to inflammatory reaction in the small venous sinuses of central nervous system (CNS).

Table 2: Number of myelin sheath damaged at different time points in each group

Group	Number of days					
	1	***3***	***7***	***14***	***21***	***28***
EAE	12.00 ± 2.00	18.00 ± 3.00	16.00 ± 4.00	10.00 ± 3.00*	8.00 ± 2.00*	4.00 ± 1.00*
Placebo	13.00 ± 4.00	19.00 ± 3.00	14.00 ± 3.00	9.00 ± 2.00*	6.00 ± 3.00*	4.00 ± 2.00*
MSCs treatment	12.00 ± 3.00	8.00 ± 2.00▲△	6.00 ± 2.00▲△	4.00 ± 1.00▲△	3.00±1.00▲△	2.00 ± 1.00▲△

*$P < 0.05$, compared to day 1 in the same group; ▲$p < 0.05$, compared to MSCs treatment and EAE groups; △$p < 0.05$, compared to the MSCs treatment and placebo groups

Some viral proteins on skin lesions such as myelin basic protein (MBP) and myelin protein lipoprotein (PLP) are structurally similar to myelin protein. The sensitized T cells adhere to CNS vascular endothelial cells through systemic circulation releasing inflammatory cytokines that change the permeability of blood-cerebrospinal fluid barrier (BBB) for ease of passage of sensitized cells. This enhances the migration of lymphocytes to the central region, thereby causing immune damage.

Mononuclear macrophages, as antigen-presenting cells (APCs), display on their surfaces peptides released from the digestion of antigens by major histocompatibility complex II molecule (MHC-II), and secrete interleukin 1 (IL-1) and other cytokines, thereby stimulating T-cells to produce other cytokines. Some cytokines can make astrocytes and endothelial cells to express MHC-II molecules and become APCs. Antigen-MHC-II complex interact with helper T-cell membrane receptors to activate helper T-cells, thus stimulating their proliferation to release cytokines. These cytokines further activate B-cells and cytotoxic T-cells resulting in pathological damage.

Adhesion factors, chemokines, matrix metalloproteinases and other cytokines play important roles in the development and progression of ADEM [7-10]. Experimental autoimmune encephalomyelitis (EAE) is a delayed-type hypersensitivity autoimmune disease that occurs in experimental animals and confined in the nervous system. It is a demyelinating disease that is induced by isomorphic, allogeneic, and heterogeneous nervous tissues and is confined to the white matter of the CNS. It has the same clinical, biochemical, immunological and pathological characteristics as ADEM, and so, it is the classical animal model of ADEM [1].

Researchers have attempted to establish EAE model with animals such as rats, mice, guinea pigs, monkeys and rabbits, but the use of rats has been most successful. Among the animals used to establish EAE models, SJL/J mice and Lewis rats are most sensitive, and they are the most commonly used. However, SJL/J mice and Lewis rats are expensive and so, most researchers use the non-sensitive Wistar rats [11].

In the present study, CFA and GPSCH were mixed in equal proportions, and used as antigen, together with simultaneous injection of BPV on the footpad to establish Wistar rat EAE model. The rats showed acute onset, and clinical symptoms began to show at day 12 post-immunization, with 71.90 % of them having onset at day 19, while the peak of onset was between 13 to 17 days post-immunization. The acute onset was manifested as reduced activity, loss of appetite, and decreased tail tension, accompanied by symptoms of paralysis associated with incontinence, or death. However, at day 23, the symptoms began to gradually ease. Results of HE staining showed there were significant infiltrations of inflammatory cells (mainly lymphocytes) around the white matter blood vessels of CNS in rats with onset, and revealed “cuff-like” changes, an indication that EAE rat model was successfully established, thus providing a good foundation for further investigation.

At present, there is no special treatment for ADEM, and the available treatment strategies include immuno-, gene and stem cell therapies. Immunotherapy involves the use of glucocorticoids and high-dose immunoglobulins. Although immunosuppressive agents have certain therapeutic effects on ADEM, their efficacies and safety of long-term use need to be further studied because of their side effects. In recent years some level of progress has been reported in animal models treated with gene and stem cell therapies [12], but not in human experiments because of their high-tech and expensive characteristics.

Stem cell therapy for the treatment of ADEM involves induction of stem cells, and differentiation and transplantation *in situ*. Induction of stem cells *in situ* by the administration of exogenous substances such as genes, proteins, and small molecules can regulate regeneration of nerve and glial cells.

Stem cell transplantation makes use of neural stem cell and MSCs because they can be derived from different sources, and are readily available. These cells are capable of autologous transplantation, self-replication and are multi-directional during long-term culture *in vitro*. Possession of weak immunogenicity and positive effects whether through local or intravenous transplantation make BM-MSCs one of the most promising treatment strategies for repair of human CNS injury.

In the present study, therapeutic symptoms were used as criteria to assess the effect of transplantation of MSCs in EAE rats. The duration of EAE was significantly shortened in MSCs treatment group, and rats in this group recovered more quickly than those in EAE and placebo treatment groups. The therapeutic score for rats in MSCs treatment group was significantly lower than those of EAE and placebo treatment groups.

CONCLUSION

The results of the present study show that MSC transplantation shortens the course of EAE, and also reduces its severity. Thus, it has prospects for use as a treatment strategy for EAE.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

This work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Hua Wang; Yu-Ying Fan, Feng-Hua Yang collected and analysed the data; Jun-Mei Zhang wrote the text and all authors have read and approved the text prior to publication.

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