# An experimental study on the treatment of diabetes-induced cognitive disorder mice model with exosomes deriving from mesenchymal stem cells (MSCs)

**Wenjing Zhao[1], Haifang Zhang[2], Jilin Yan[1]* and Xiaohong Ma[3]**
[1]Neurology Department No.1, Affiliated Hospital of Hebei University of Engineering, Handan, China
[2]Handan Emergency Rescue Command Center, Handan, China
[3]Department of Neurology, Xinxiang Central Hospital, Xinxiang, China

**Abstract**: The occurrence rate of senile dementia among diabetes patients is high, but there is still no effective therapeutic method. This study aimed to explore curative effect of exosomes deriving from MSCs treating diabetes-induced cognition impairment. After BM-MSCs culture was purified, Western Blot and electron microscope were used to identify exosomes which were injected into diabetes mouse through intracranial injection. Purified exosomes in Western blot analysis and electron microscope observation were verified. After therapy with exosomes deriving BM-MSC, mouse cognition impairment and histologic abnormity were recovered. Exosomes deriving from BM-MSCs can be a new preparation treating cognition with application prospect.

**Keywords**: Mesenchymal stem cells (MSCs), exosome, cognition impairment.

## INTRODUCTION

Cognition impairment related to diabetes is a world difficult problem, as epidemiologic studies have reported that occurrence rate of senile dementia among diabetes patients is 3 to 4 times of that of non-diabetes patients (Leibson *et al*., 1997 and Ott *et al*., 1999). Different mechanisms of causing senile dementia related to diabetes have already been put forward such as hyperglycemia and hypoglycemia, abnormities of insulin effect like insulin shortage and insulin resistance, vascular anomaly and oxidative stress of central nervous system (Wrighten *et al*., 2009 and Kawamura *et al*., 2012).

Similar to other complications of diabetes, correction of these abnormities will not surely improve cognition of patients (Sakata *et al*., 2012). There are also reports o different impairments of the central nervous system related to I-type diabetes in animals such as neuron damage of oxidative stress, reducing axonal plasticity of hippocampus, variation of glutamate neurotransmitters and swollen stellate cells which cause dysfunction of abnormal neuronal activity (Revsin *et al*., 2005, Hernández-Fonseca *et al*., 2009, Aragno *et al*., 2005 and Son *et al*., 2015). II-type diabetes model shows abnormity of mitochondrial function, reducing axon completeness and reducing insulin signal transduction in hippocamal region (Carvalho *et al*., 2015, Min *et al*., 2012 and Agrawal *et al*., 2014). Hence, the main problem causing cognition impairment in diabetes may be damaged neurons and stellate cells.

MSCs has functions of restoring damaged tissues, inhibiting inflammatory reaction and regulating immune system, which have become bases for MSXs to treat all kinds of diseases (Prockop, Oh, 2012 and Ma *et al*., 2014). Research indicates that intravenous injection of BM-MCSs or BM-MSCs conditioned media into STZ and high-fat diet induced diabetes mouse can restore diabetes-induced hepatocyte damage (Nagaishi *et al*., 2014).

Research also indicates that the quantity of MSCs reaching damaged tissues is quite little and it's deemed that MSCs restoring damaged tissues may not be direct effect, instead, it is about effect generated by some secreted cytokines or exosomes. Exosomes are small membrane vesicles secreted by cells with size being 40-140nm, consisting of functional messenger mRNA, miRNAs and protein (Thery, 2011 and Thery *et al*., 2002). Exosomes are released from all kinds of cells and execute physiological functions through cell-cell communications (Morel *et al*., 2013).

In CNS, exosomes of neuron source exert the effect of regulating stellate colloid glutamic acid transport protein 1 (GLT 1) and they act on axons (Korkut *et al*., 2013). On the other hand, exosomes of stellate cell source protect on nerves (Taylor *et al*., 2007) and improve growth of neuraxis and survival of neurons (Wang *et al*., 2011). Exosomes deriving from MSCs have been identified as factors of MSC secretom which can reduce tissue damage and enhance tissue repair of cardiovascular disease, lung injury, acute kidney injury and nerve and blood vessel injury after stroke (Bian *et al*., 2014, Zhu *et al*., 2014, Gatti *et al*., 2011 and Xin *et al*., 2013).

In this study, we separated exosomes from BM-MSC culture and injected into diabetes induced mouse through intracranial injection in order to study the influence of exosomes deriving from BM-MSCs on study and memory.

**Corresponding author:* e-mail: xiaohongmacn@163.com

## MATERIALS AND METHODS

### *Animals*

All operations in this study were conducted in accordance with operation guidebook of experimental animals in our college. C57BL/6 J was purchased from Henan Center of Experimental Animals, and age of mouse was 13 weeks. STZ (150mg/kg; Wako, Osaka, Japan) which was dissolved in citrate buffer solution (pH 4.5) was used for intraperitoneal injection to induce hyperglycemia. The control group was directly conducted with intraperitoneal injection of citrate buffer solution. Blood sugar concentration of STZ diabetes mouse was more than 300 mg/dL. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Eighth Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinxiang Central Hospital.

### *Separation of BM-MSCs eosomes*

Mouse BM-MSCs was purchased from NanoMed Biotech Co. Ltd., Shenzhen, China. In order to separate exosomes, conventional culture medium was replaced by substitution for bovine serum (EXO-FBS-250 A-1, System Biosciences, Mountain View, CA, USA) which didn't contain exosomes (Xin *et al*., 2013). When MSCs (the $4^{th}$ generation) cells converged by 60-80%, the cells were continued to be cultured for 48h. Then culture media were collected and exosomes were separated under 4°C collected cultures were firstly centrifuged at 1,000×g for 10min, and then large cell debris and dying cells were removed. Then the supernatant was centrifuged at 10,000×g for 30min to remove small cell debris. In order to obtain sediments containing exosomes, the supernatant was centrifuged at 100,000×g for 3h. Then the supernatant was discarded, the sediments were re-suspended with 0.25M sucrose dissolved in 20mM HEPES, pH 7.4, then loaded at 2.25, 2.0, 1.75, 1.5, 1.25, 1.0, 0.75 and 0.5M gradients. Then it was centrifuged at 100,000×g for 16h, 9 components of corresponding sucrose gradients were collected, diluted by 20mM HEPES, pH 7.4 and then centrifuged at 100,000×g for 1h. The sediments were collected and re-suspended with PBS and then preserved under -80°C for standby application.

### *Arterial cannula operation and intracranial injection of exosomes*

10 weeks after STZ intraperitoneal injection, stainless steel cannula (0.4mm diameter) was inserted into mouse ventricle to cause cerebral injury. 12 weeks after injection, 2μL artificial cerebrospinal fluid (142mM NaCl, 5mM KCl, 2mM $CaCl_2 \cdot 2 H_2O$, 2mM $MgCl_2 \cdot 6 H_2O$, 1.25mM $NaH_2PO_4$, 10mM D-glucose, 10mM HEPES, pH 7.4) containing 0.5μg exosomes was injected into cranium once per day for continuous five days, artificial cerebrospinal fluid was taken as the control. BCA experiment was used to test protein concentration of exosomes.

### *Morris water maze experiment*

This experiment was completed during 9:00-13:00. The equipment was an annular pool (diameter 1.2m, height 30cm), water depth 18cm (25±1°C). All kinds of viewing angles were provided in experimental area, the whole process maintained constant. Digital cameras with automatic tracking systems (Any-maze, Stoelting, Wood Dale, IL, USA) were installed around it to obtain data (Logicool, Tokyo, Japan) and these equipments were fixed 1.7m above the annular pool. An acrylic ester platform was placed around. On the visible training platform (Day 0), mice were put on the platform to evaluate their swimming ability and vision. On a hidden training platform (Day 1-4), this task was used to evaluate studying depth. During this period, the mic would did for four times per day with interval being 1h, 60s each time, and the mice had to swim until they climbed onto the platform. After reaching the platform, mice could stay for 15s. If they failed to find the platform within 60s, the experiment ended. Mice were placed by the experimenter gently for 15s. On the $5^{th}$ day, the platform was removed and memory of mice would be evaluated. Each mouse would start from offside of the previous experiment, mice would search for the platform within 60s, and the time was recorded.

## STATISTICAL ANALYSIS

Data was expressed by mean value ± standard deviation. Unmatched *t*-test or unilateral ANOVA was used to analyze statistical difference between the two groups. When evaluating 2 factors, we adopted bilateral ANOVA statistical analysis. $P<0.05$ indicates that the difference is statistically significant.

## RESULTS

### *Identification of exosomes deriving from BM-MSCs*

In order to test whether exosomes were successfully purified from BM-MSCs culture, Western Blot analysis and electron microscope analysis were made on common protein markers of exosomes, After centrifugation at 100000×g, obtained sediments CD63 and HSP70 were positive (fig. 1A), centrifugation was made again and sucrose gradient purification was implemented. Gradient which had positive reaction to HSP70 was located at 1.11-1.15g/mL, which was identical with previous reports (Zhuang *et al*., 2011) (fig. 1B). These positive components were collected as the final products. Electron microscope was used to observe the final products with diameters being 40-100nm, which was also feature of exosomes (fig. 1C).

### *Water maze experiment*

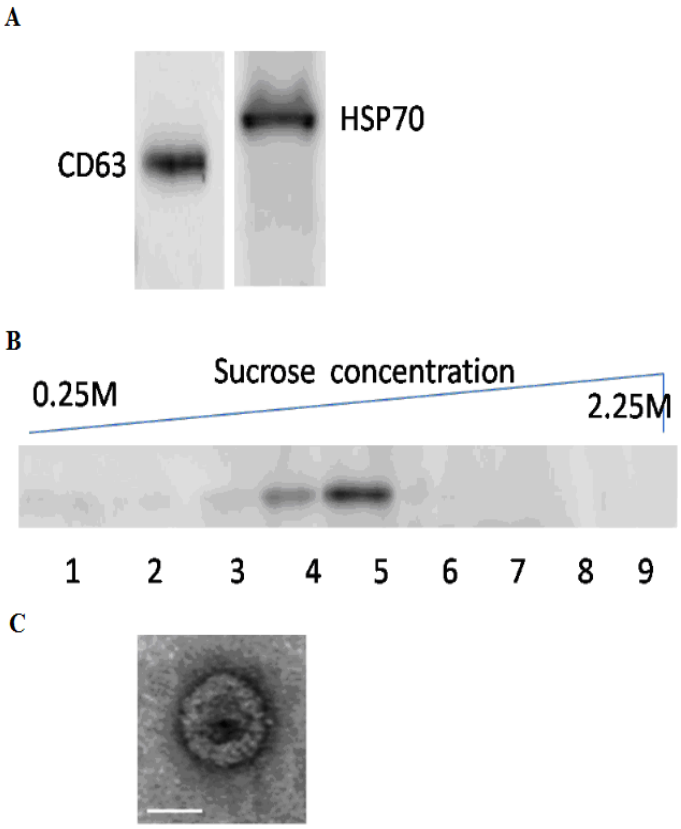


**Fig. 1**: Identification of exosomes deriving from BM-MSC culture.
A: Western Blot analysis method was used to analyze common markers CD63 and HSP70 of exosomes; B: Western Blot analysis method was used to analyze separation component HSP70 protein of sucrose density gradients; C: Electron microscope was used to observe exosomes, and length of scaleplate was 50nm.

In this study, exosomes purified from BM-MSCs culture were injected into cranium 12 weeks after STZ injection, water maze experiment was conducted for continuous five days (fig. 2A). 12 to 13 weeks after STZ injection, there was no change of mice weight or blood glucose level (not displayed in data). In hidden platform training of water maze, STZ+ mice showed long delay of escaping when compared with mice in sham operation group. Escaping delays at $2^{nd}$, $3^{rd}$ and $4^{th}$ time of STZ mice with exosomes injection were significantly shorter than those in STZ+ control group (fig. 2B). In three groups of mice, their swimming speeds were not different (fake operation group: 0.1657±0.0062 ms-1; STZ+group: 0.1588±0.0073 ms-1; STZ+exosomes group: 0.1664±0.0053 ms-1). No difference of mice in the three groups was found in training modules on the visible platform. In the whole explorative experiment, time on target platform of STZ+ group reduced. Comparatively speaking, mice with STZ+ exosomes took longer time in target quadrant.

## DISCUSSION

12 weeks after STZ injection, study and memory disorders of diabetes-induced mice were tested, at this time point, BM-MSCs or exosomes were started being used in the therapy. According to the study, intravenous injection of BM-MSCs was conducted for four times within two weeks (Nagaishi *et al*., 2014), while exosomes were injected into cranium for continuous five days. Study indicated that exosomes marked by IR Dye 800 disappeared from the cerebrum 24 hours after intranasal injection (Zhuang *et al*., 2011). Hence, exosomes were used every other 24 hours to maintain intracephalic high concentration. Purity of exosomes separated from BM-MSCs culture media was tested by Western Blot, and common exosomes markers CD63 and HSP70 were tested out (Pols, Klumperman, 2009 and Lancaster, Febbraio, 2005). Sucrose density range was 1.11-1.15g/mL and it was used for separation of exosomes, and it could be different from small vesicae (1.05-1.12g/mL) of Golqi apparatus source or those (1.18-1.25g/mL) of endoplasmic reticulum source (Thery *et al*., 2006). In section 1.11-1.15g/mL, we obtained HPS70 positive parts, which indicated that exosomes had been successfully purified from BM-MSC culture. According to other studies, this study used 0.5μg exosomes for mice intracranial injection (Thery *et al*., 2006).

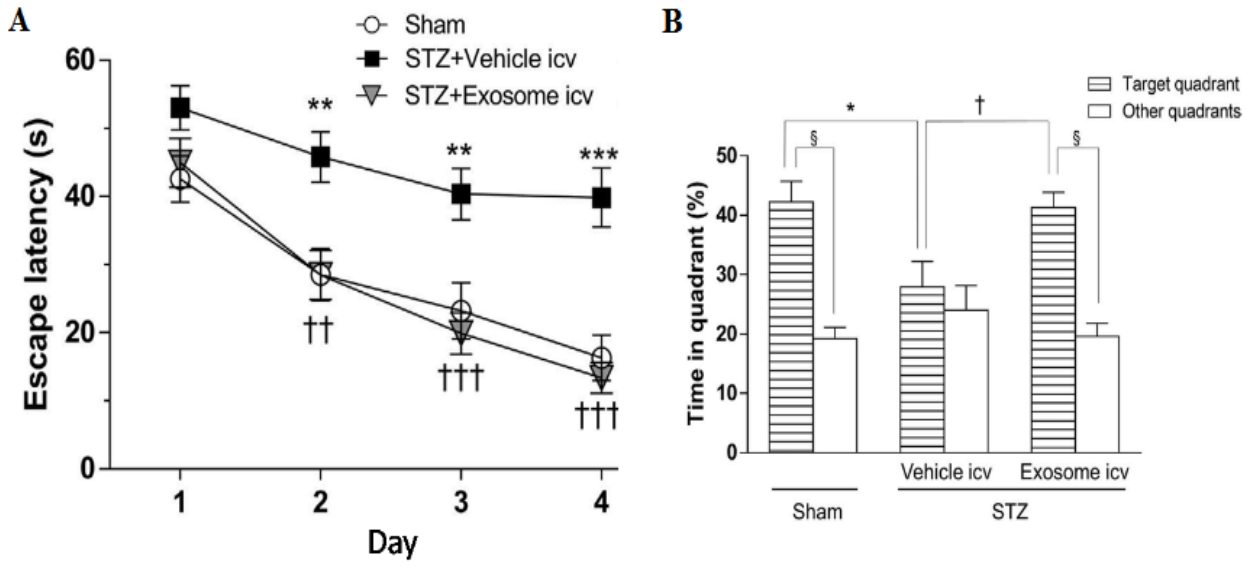


**Fig. 2**: Water maze experiment.
A: In hidden training experiment, STZ+ mice had longer escaping delay than those in the control group, while STZ+ exosome mice shortened escaping delay on the 2nd, 3rd and 4th days when compared with STZ mice. **meant p<0.05, *** meant p<0.001.
B: In the investigative experiment, STZ+ mice occupation in target quadrant obviously reduced, while occupation in quadrant of mice in the control group and STZ+ exosome group was obviously higher than that in STZ+ group. † meant p<0.05, § meant P < 0.0001.

STZ diabetes mice needed more time to seek for the platform, which indicated that their spatial learning ability was weakened. In the explorative experiment, STZ mice took less time on quadrant of the platform, which indicated that their memory was out of balance. Obviously, study and memory injury of STZ mice was caused by cognition impairment but not defect of sensorimotor, as no difference between mice in the control group and STZ mice was found in visible platform or suspending line test for up and bottom muscle strength, even if in vivo was still hyperglycemia. Normal mice injected with BM-MSCs didn't show influence on cognitive function. For STZ mice, they were injected with 0.5μg exosomes through intracranial injection per day for continuous five days, reversion of cognition impairment was found, but intravenous injection of $2.5\times10^5$ BM-MSCs had no effect. This different might be because (1) it's estimated that $2.5\times10^5$ BM-MSCs contained 0.1μg exosomes; (2) injection approaches of the two were different, one was intravenous injection, and the other was intracranial injection. Recent studies certified that exosomes deriving from BM-MSCs could restore endotoxin-induced acute lung injury (Zhu *et al*., 2014), union of skin wound (Zhang *et al*., 2015), injury caused by lower limb ischemia (Hu *et al*., 2015), etc. These studies certified that exosomes deriving from BM-MSCs could restore tissue injury as BM-MSC did, its mechanism might be that it was easier for exosomes to get through circulatory system to reach injured tissues, cellular internalization of injured tissues would then promote tissue repair. Previous studies indicated that BM-MSCs restoring tissues was probably because these cells secreted cytokines and exosomes, it was possible the reason why MSCs could restore injured tissues but it was hard to find MSCs in injured tissues. In the meantime, it also revealed that exosomes might replace MSCs to restore injured tissues with higher safety performance than living cells. This study didn't specify how exosomes of intracranial injection affected neurons and stellate cells or identify that specific miRNA or protein in exosomes had restoring effect. These problems need further study.

## CONCLUSION

Taken together, intracranial injection of exosomes separated from BM-MSCs could reverse diabetes-induced mice cognition disorder, and this study provided a new idea of using exosomes deriving from BM-MSC to treat cognition disorder.

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