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Ahmet Turan Isik[1], Turgay Celik[2], Ali Ugur Ural[3], Murat Tosun[4], Gokhan Ulusoy[5], Birsen Elibol[6]

[1]Center for Aging Brain and Dementia, Department of Geriatric Medicine, School of Medicine, Dokuz Eylul University, Izmir, Turkey, [2]Faculty of Pharmacy, Yeditepe University, Istanbul, Turkey, [3]Department of Hematology, Gulhane School of Medicine, Ankara, Turkey, [4]Faculty of Medicine, Department of Histology and Embryology, Afyon Kocatepe University, Afyonkarahisar, Turkey, [5]Department of Clinical Pharmacology, Gulhane School of Medicine, Ankara, Turkey, [6]Faculty of Medical Science, Department of Biological Science, Bezmialem Vakif University, Istanbul, Turkey

Background and Aim: Bone marrow-derived mesenchymal stem cells (BM-MSCs) are one of the sources of adult stem cells being explored for potential use in repairing neurodegenerative disorders. In this study, it was aimed to investigate the useful effects of BM-MSCs therapy on the streptozotocin-induced neurodegeneration in rats.
Materials and Methods: Adult female Wistar rats were bilaterally injected intra-cerebroventricularly with streptozotocin (3 mg/kg) for neurodegeneration. Water maze tests were used to monitor spatial learning and memory. One or two intravenous injections of BM-MSCs were administrated to rat via the tail veins. At the end of the study, all rats were sacrificed for histological evaluation and immunohistochemistry.
Results: Streptozotocin group demonstrated a significant increase in escape latency in comparison with both control groups (Sham and Saline), whereas rats treated with BM-MSCs exhibited a decrease in escape latency in comparison with streptozotocin group. The percentage of time spent in the target quadrant and the mean number of platform crossings did not change in all the groups. BM-MSCs administration improved spatial learning but not memory. However, improvement in neuronal cells in hippocampal CA1 region was only observed in the rats treated with BM-MSCs twice as opposed to the rats treated with BM-MSCs once or with saline.
Conclusions: In this study, mesenchymal stem cells therapy failed to improve the streptozotocin-induced neurodegeneration like Alzheimer's disease in rats.

Keywords: Neurodegeneration, Mesenchymal stem cell, Alzheimer's disease, Experimental model

Introduction

Current Alzheimer's disease (AD) therapies can only reduce symptoms, rather than modifying or preventing the initiation of the disease.[1] Researchers have to think of ways to deliver potentially neuroprotective molecules and to replace the cells and tissue lost due to neurodegeneration.[2,3] The discovery of multipotent neural stem cells (NSCs) in adult brain[4] revolutionized the theory of neurogenesis, and it provides promise for clinical applications of stem cell strategies in AD therapy.[3]

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are one of the sources of adult stem cells being explored for potential use in repairing neurodegenerative disorders.[5] The ability of BM-MSCs to differentiate into neural cells when transplanted into the brain was initially demonstrated by Blau's laboratory.[6] In order to demonstrate their high degree of accessibility and plasticity,[5,7] the therapeutic potential of BM-MSCs has recently been studied under various pathological conditions of the central nervous system. Furthermore, it was reported that BM-MSCs could promote the reduction in amyloid beta (Aß) through the microglia activation in the AD brain, suggesting a potential therapeutic agency against AD.[7]

Here, BM-MSCs were administrated into the tail vein of rats that first were intracerebroventricularly (ICV) injected with streptozotocin (STZ). The STZ compound is known to inhibit insulin receptor function, and to create persistent oxidative stress, mitochondrial dysfunction, impaired energy metabolism and activation of pro-apoptotic signalling pathways. All these effects of STZ are similar to AD-type neurodegeneration.[8,9]

In addition, adult central nervous system (CNS) stem cells exhibit three main features which make them attractive candidates for transplantation: firstly, they are

Correspondence to: Ahmet Turan ISIK, Dokuz Eylul Universitesi Tıp Fakültesi Geriatri Bilim Dalı 35340 Balçova Izmir/Turkey. Email: atisik@yahoo.com

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'self-renewing'; secondly, they have proliferative character (undergoing mitosis); and thirdly, they are multipotent for the different neuronal and glial subtypes.[10] The hippocampus is a target for endogenous neural stem cell migration and has an obvious role in spatial navigation and episodic memory. The main hippocampal areas, the dentate gyrus (DG) and the cornu ammonis (CA3 and CA1), have complex anatomy and functions, which are difficult to understand. Neurons from DG, CA3 and CA1 display different molecular, morphological and physiological features.[11]

The goal of this study was to investigate the effects of the BM-MSCs therapy in the STZ-induced neurodegeneration in rats. Specifically, the engraftment capability of stem cells and the effects of BM-MSCs therapy on the cognitive symptoms in this animal model AD were assessed. Additionally, we also aimed to obtain histological and immunohistochemical data to support the clinical potential of this approach for treating AD.

Material and Methods

Animals and laboratory conditions

Adult female Wistar rats (225–275 g) were placed in a quiet, temperature and humidity-controlled room (22 ± 3 °C and 62 ± 7% relative humidity) in which 12-h cycles (12 h light -12 h dark) were maintained (lights on at 07:00 am). They were fed *ad libitum* with a standard dry rat diet and tap water. The Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA) and the Declaration of Helsinki were followed during these experiments.

Intra-cerebroventricular injection of artificial cerebrospinal fluid and STZ

Artificial cerebrospinal fluid (aCSF) (3 mM KCl, 140 mM NaCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.3 mM NaH_2PO4, 1 mM Na_2HPO4, 3 mM D-glucose) was prepared, and the STZ (Sigma, St Louis, USA) was freshly dissolved in aCSF and administered as reported in our previous study.[9]

Before surgery, animals were weighed and the appropriate dose for anaesthesia was calculated. Animals were anesthetized with a mixture of xylazine and ketamine hydrochloride (3 and 75 mg/kg, respectively, IP). The head of the mouse was stabilized in the stereotaxic frame using the ear bars and stereotaxic injection of aCSF or aCSF with STZ has been used to establish STZ-induced neurodegeneration rat models. aCSF or aCSF with STZ were stereotactically injected into the right lateral ventricle using the following stereotaxic coordinates: Bregma AP -0.8 mm, ML -1.5 mm, VD + 3.5 mm.

The STZ groups were given a bilateral ICV injection of STZ (3 mg/kg) in a total volume of 20 µl. The aCSF was injected bilaterally (ICV;a 20 µL) to each rat, and this procedure was repeated on the 3rd day) in the SHAM group. Approximately, 20 µL solution was infused bilaterally into the ICV zone of the brain hemisphere, using a single syringe infusion pump (UltraMicroPump III World Precision Instruments, Inc. USA, Programmable Syringe Pump) connected to the cannula via injection tubing, and this procedure was repeated on the third day.[9] 2–5 min after the end of the infusion, we retracted the cannula slowly to avoid backflow of the injected solution to the surface and removed the animal from the stereotaxic frame. The burr holes in the scull were closed with bone wax, and the skin incisions were sutured. Next, we injected sterile saline solution (30 ml per kilogram body weight) subcutaneously to avoid dehydration of the animal after the surgery, and subsequently, we injected the same amount of glucosate solution (5% glucose) to improve the animal feeding immediately after surgical procedure. Finally, we kept the animal warm on a temperature-controlled heating pad (~37 °C) until its full recovery. Once the animal recovered, we returned it to a clean cage and put wet food pellets in the cage for easy access to food. Animals were put back into their home cages, with access to food and water *ad libitum*.

Study design

In the beginning of study, STZ-induced neurodegeneration rat model was checked that model is running by a preliminary research, as we did in our previous work.[9] The impaired cognitive performance or neurodegeneration was evaluated by the test of water maze acquisition at 21st day of STZ injection period. After checking the model by a preliminary study, we started the study. STZ-induced neurodegeneration model was produced in rat by bilateral ICV injection of STZ at first and third days of the experiment.

The animals in the STZ groups were divided into three, respectively, Group I (STZ + 1×10^6 BM-MSCs), Group II (STZ + saline) and Group III (STZ + 2×10^6 BM-MSCs). The animals of non-STZ groups were divided into two, Group IV (Sham) and Group V (Control), control groups. At 21st and 28th days of study, the BM-MSCs or saline were injected once a week by tail vein to Group I, II and III (as shown in Table 1).

To evaluate the cognitive performance or impairing of cognitive function of animals, all the rats (operated and non-operated) were tested in the Morris water maze for consecutive 4 days at 39th, 40th, 41st and 42nd days of study. A daily session of four trials was given to all groups for 4 consecutive days. Ten days after the completion of the acquisition sessions, the animals received a single 60 s probe trial for memory retention. Afterwards, all rats were sacrificed and their brains were prepared for histological examination of transfected MSCs.

Isolation and culture of bone marrow-derived mesenchymal stromal cells

For the preparation of the MSCs, the bilateral femur and tibias of a male Wistar rat were excised under general anaesthesia of ketamine hydrochloride (Alfamine® 10%, 75 mg/kg, IM; Alfasan Int. B.V., Woerden/Holland) and

Table 1 Study design, the time course for ICV STZ treatments, BM-MSC treatments and behavioural testing

		Treatment		Water maze test	
Groups	ICV injection	21st Day	28th Day	39th–42nd Days	52nd Day
Group I (STZ + 1xBM-MSC)	STZ Injection 1st and 3rd days	BM-MSC	Saline	Training	Probe
Group II (STZ + Saline)		Saline	Saline	Training	Probe
Group III (STZ + 2xBM-MSC)		BM-MSC	BM-MSC	Training	Probe
Group IV (Sham)	[a]CSF Injection 1st and 3rd days	Saline	Saline	Training	Probe
Group V (Control)	None	None	None	Training	Probe

[a]CSF: artificial cerebrospinal fluid, BM-MSC: bone marrow-derived mesenchymal stem cells, ICV: intra-cerebroventricular, STZ: streptozotocin.

xylazine hydrochloride (Alfazyne® 2%,10 mg/kg, IM; Alfasan Int. B.V., Woerden/Holland). Both ends of the bones were cut, and the bone marrow cells were obtained by flushing the cavity with modified Eagle's medium (MEM). Lipid particles and bone spicules were discarded by centrifuging the solution at 2500 rpm for 10 min. The remaining pellet including the marrow cells were incubated in flasks containing Dulbecco's MEM (DMEM; Biological Industries, Israel), 1% penicillin + streptomycin, L-glutamine and 10% foetal bovine serum (FBS) at 37 °C in 5% CO_2 for 72 h. The flasks were previously covered with FBS in order to prevent possible adhesions of mono-nuclear cells. The whole media were removed from the flasks to discard non-adherent hematopoietic cells and to enable the growth of the stromal cells (adhered to the flask). The media were renewed every three days, and the cells were passaged after reaching a confluence of 70%. The adherent cells were released from dishes with 0.25% trypsin in 1 mmol/L sodium ethylenediaminetetraacetic acid (Sigma, St Louis, MO, USA), split 1:3, and seeded onto fresh plates. Cultured MSCs were observed under inverted microscope to assess the level of expansion and to verify the morphology at each culture medium change. The stromal cells were harvested and used after the forth passage for the implantation experiments described below.

Transfection of MSC by green fluorescent protein (GFP)

Transfection of MSCs with Monster GFP Vector System (Promega) was conducted using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions.

Briefly, on the day before transfection, 5×10^6 cells were seeded per 60 mm dish in 5 ml medium containing 10% serum and incubated at 37 °C in 5% CO_2 incubator. On the day of transfection, 1 μg DNA was diluted with the DNA-condensation buffer, Buffer EC, to a total volume of 150 μl. Then, 8 μl of enhancer was added and mixed by vortexing for 1s. This mixture was incubated at room temperature for 5 min and then spun down for a few seconds to remove drops from the top of the tube. Later, 25 μl of Effectene transfection reagent was added to the DNA-enhancer mixture and mixed by vortexing for 10s. The samples were incubated for 10 min at room temperature to allow transfection-complex formation. While complex formation was in process, the growth medium was gently aspirated from the plate, and the cells were washed once with 4 ml phosphate-buffered saline. Then, 4 ml of fresh growth medium was added onto the cells, and 1 ml of growth medium was added to the tube containing the transfection complexes drop-wise onto the cells in the 60-mm dishes. The dish was gently swirled to ensure uniform distribution of the transfection complexes. The GFP-transfected MSCs were used in the experiment. The light density of the MSCs in the biopsy specimen was obtained by fluorescent microscopy.[12]

Behavioural cognition tests (Morris water maze)

The water maze (MWM) acquisition sessions and probe trials were tested as in our previous study.[9,13] Firstly, the cognitive performance of STZ-treated rats was tested with a MWM acquisition task on the 21st day of the treatment (as a preliminary study demonstrated cognitive impairment by STZ at this prime period). Then, five study groups were designed with another training protocol to examine the effects of BM-MSC treatment in the rats treated with STZ. On the 40th day, the pre-training period of two successive days was started. On the 40th and 41st day, the rats were placed for 60 s on the escape platform, which was at the centre of one of the quadrants in the pool without water. On the 42nd day, the rats were again placed on the same platform under the same conditions, except that the pool was filled with water. When the rats climbed off the platform, they were guided back onto the platform. Acquisition testing started the following day.

During the acquisition testing period, the escape platform was located in the centre of the southwest quadrant and all the rats were given a daily session of four trials for four consecutive days. The rat was placed in the water facing the pool wall at one of four randomly determined starting locations (north, west, east or south) for each trial. When the rat climbed onto the platform, it was allowed to stay on for 30 s. If the rat did not find the platform within 60 s, then it was guided onto it and allowed to remain there for 30 s. Next, it was returned to its heated cage for a 240-s inter-trial interval. After the final trial, the rats were dried with a towel and placed in a holding cage under a heating lamp before returning to their home cages. All of the rats were given a daily session of four trials for four consecutive days. Ten days after the completion of the acquisition sessions, the animals received a single 60-s probe trial for memory retention. The animals had one trial with the platform removed from the pool for testing the memory function on the retention day (day 52 of the STZ injection or day 31 following the BM-MSCs treatment). At this time, two parameters were recorded: time spent in the target area, and latency of the first crossing through the place where the platform was previously located.[14]

Histological examination and immunohistochemistry

Histology

After the rat's brains were totally removed, they were immediately put into 2% gluteraldehide + 10% buffered neutral formalin solution (1:1) for fixation. After 1-week fixation period, all the brains were histologically processed and embedded in paraffin. Then, 5 μm thickness slides were taken from all these blocks and mounted on positively charged slides for immunohistochemistry and normal slides for histochemistry.

For the evaluation of general brain tissue's histological features, some of each groups' slides were stained with 1% toluidine blue stain. For staining, the slides were firstly deparafinized, then rehydrated and stained with 1% toluidine blue solution and decolorized in absolute alcohol and at last cleared in xylene and mounted with Entellan (all the chemicals were purchased from Sigma, Fremont, CA).

Immunohistochemistry

Some of each groups' slides were stained with anti-GFP antibody (Abcam ab6556) for detecting GFP-labelled stem cells in brains' section using immunohistochemistry. For immunohistochemical staining, first antigen retrieval was made with 10 mM citrate buffer, pH 6.0 in microwave. 10% H_2O_2 was used for inactivation of endogen peroxidase in the slides. Then, the slides were reacted with primary rabbit polyclonal antibody (Abcam ab6556). After overnight incubation in +4 °C, the slides were stained with horse radish peroxidase detection system and DAB substrate system was used for chromogen (above all chemicals except primary antibody were obtained from Labvision Corporation, Fremont, CA). Mayer's hematoxylin (Sigma, St. Louis, MO) was used for counterstaining. At last, all slides were dehydrated, mounted with Entellan (Sigma, St. Louis, MO) and evaluated under light microscope (Nikon E600, Japan). Furthermore, apoptotic cell death in brain tissues was detected by terminal TDT-mediated dUTP-biotin nick-end labelling (TUNEL) technique using a commercial kit (TDT-Fragel DNA fragmentation detection kit, Calbiochem, Darmstadt, Germany). For staining, the recommended method was used by supplier. Only during staining, all the kits' chemicals were used. Positive control slides were taken from same supplier.

Image analysis

After staining with 1% toluidine blue, all the slides were evaluated under light microscope (Nikon Eclipse E600, Japan). During evaluation, under x40 objective magnification, the neurons in 5 different areas were counted for each slide. Mean values of neurons for each slide were used for statistical analysis. For counting, Nikon NIS Elements D4 Image Analysis Software was used.

Statistical Analysis

The data were presented as means ± standard error of mean. The group differences in the escape latency for days in the acquisition task of Morris water maze were analysed using two-way repeated ANOVA, and the group differences in the probe trials were analysed using one-way ANOVA. For multiple group comparison, Tukey's posthoc test was also used to analyse the group differences of the data collected during successive training days, retention trials. In the histopathological examination, statistical analysis of data was also performed using a one-way ANOVA and Tukey's posthoc test. Differences and correlations were considered significant at $p < 0.05$. All data were analysed by Statistical Packages for the Social Sciences (SPSS) for Windows 15.0.

Results

Behavioural analysis

The reliability of STZ-induced neurodegeneration was first assessed, and the cognitive impairment of treated rats was found to be similar to what was obtained in our previous study.[9] Then, to investigate the effects of BM-MSCs administration on cognition, we performed the MWM test on rats weakly treated either with one or two doses of BM-MSCs.

In the acquisition component of the test, the escape latencies showed significant differences among the groups ($F_{(4,33)} = 2.857$; $p < 0.039$). Significant decreases were also observed at the end of the four days, $F_{(3,99)} = 33.077$, $p < 0.01$ (Fig. 1A). Group II showed impaired performance in this test when compared to Sham and Control groups whereas Groups I and III showed similar performance with Sham and Control groups for each training day (Fig. 1A).

Ten days after the trainings (or on the 52nd day of study), all the groups were subjected to a probe trial in which rats swam in the pool with the platform removed as a putative measurement of memory retention. For each probe trial, the performance of the rat was analysed for 60 s. The percentage of time spent in the target quadrant was found to be not decreased in Group II (STZ + Saline) when compared to Groups I, III, IV and V ($F_{(4,34)} = 2.436$; $p < 0.069$) (Fig. 1B). The mean number of platform crossings was also similar for all groups (Fig. 1C). The swim speed did not differ significantly between groups, and the distance data were similar to the escape latency data (data not shown).

Histological analysis

The general histological evaluation of control slides (Group V) indicated that the neurons in general (Fig. 2A)

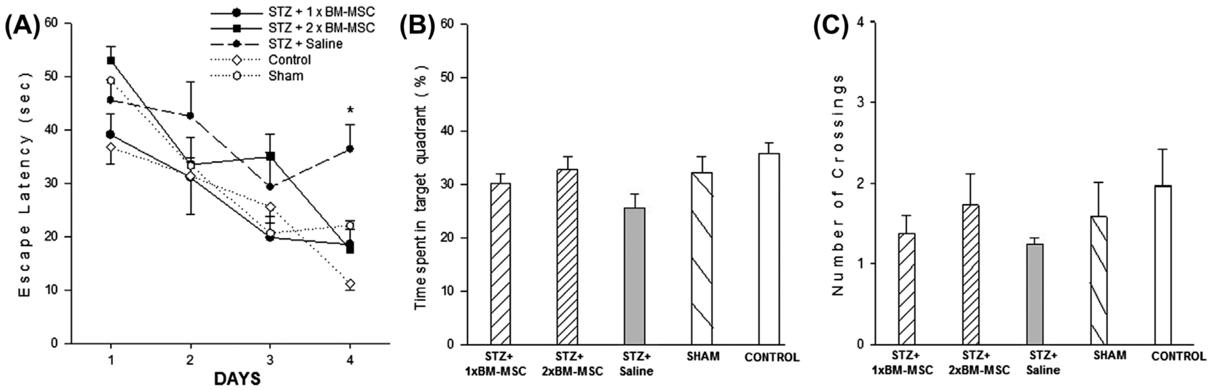

Figure 1 BM-MSCs therapy treatment reverses spatial working memory deficits in STZ-induced neurodegeneration rat model in the Morris water maze test. 1A. Escape latency in the visible-platform training. A significant difference was detected among groups during 4 days of visible-platform training. *$p < 0.05$ indicates a significant difference from the Groups I, III, IV and V [(Repeated measure two-way ANOVA; (treatments: $F(4, 33) = 2.857$, $p < 0.039$; days: $F(3, 99) = 33.077$, $p < 0.01$; trials: $F(3, 117) = 17.37$, $p < 0.001$) and the interaction between treatments × day ($F(4, 33) = 2.545$, $p < 0.058$)]. 1B. Probe trails, Group II not showed statically longer escape latencies in the invisible-platform training when compared with Groups I, III, IV and V. 1C. Number of times rats cross the platform position during probe trail
(**Group I:** STZ + 1×10^6 BM-MSCs, **Group II:** STZ-Saline, **Group III:** STZ + 2×10^6 BM-MSCs, **Group IV:** Sham, **Group V:** Control) **BM-MSCs:** Bone marrow-derived mesenchymal stem cells, **STZ:** Streptozotocin.

and particularly in the CA1 region of hippocampus (Fig. 2B) were structured regularly.

A polarity loss was observed on neurons of the CA1 region of hippocampal areas of Group IV rats. The structure of their pericarions was found to be irregular since features such as shape deformation or hyperchromasia of nuclei were detectable (Fig. 2C). Also some areas presented dead neurons.

In Group II, the histological features of neurons were deteriorated and cell polarity was almost lost in the CA1 region of hippocampus (Fig. 2D). The number of neurons was found to be clearly decreased, and dead neurons were detected in many areas of hippocampus. Furthermore, some neurons presented shrunken hyperchromatic nuclei or loss of nuclei in different areas.

An apparent improvement in the histological features of Group I brain tissues was obtained since in addition to many dead or damaged neurons with hyperchromatic nuclei and irregular pericarions, some small neurons polar in shape and with regular pericarions were also detected in different areas of the CA1 of hippocampus (Fig. 2E). Our observations also revealed a clear decrease in the number of neurons in CA1 region.

In Group III, it was observed that histological features of brain tissue and hippocampus were healed when compared with Sham, Groups I and II. The number of healthy neurons in CA1 region was clearly increased, healing (Fig. 2F). There were many dead neurons among these neurons.

All the values of the number of neurons have been summarized in Fig. 3. The statistical analysis revealed that the number of neurons in the CA1 areas of hippocampus was significantly decreased in Sham and Groups I, II and III when compared with Control group (respectively, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.03$). On the other hand, it was detected that the number of neurons were significantly increased in Group III when compared with Groups I, II and IV (respectively, $p < 0.003$, $p < 0.001$ and $p < 0.001$), even if this increase was not enough to reach the values of the Control group. Moreover, it is important to notice that less neurons were observed in Group I when compared to Groups III, IV and V ($p < 0.002$ for each comparison) and that their numbers were similar to the neuron number of Group II ($p > 0.05$). Furthermore, a better increase in the neuron number in CA3 areas was observed for Group III when compared to Groups I and II ($p < 0.001$ for both), but this increase was not enough to reach the values of Control and Sham groups ($p < 0.002$ for both).

GFP (+) and apoptotic neurons were observed in all groups (Fig. 4).

Discussion

In this study, it was demonstrated that BM-MSCs therapy failed to reduce all of the STZ-induced neurodegeneration in rats, although increased numbers of hippocampal neurons were observed in the BM-MSC-treated rats.

Currently, restorative approaches such as stem cell therapy are being considered as an important new development for the potential management of AD, because current therapies are not curative and provide only temporary benefits.[2,7] Among the therapies, intracerebral engraftment strategies using MSCs in animal models with neurodegenerative disorders, other than AD, have been shown to reduce deficits.[15–17] It was pointed out that MSCs can induce and increase the number of activated microglia, which supports the hypothesis that MSCs might be an effective therapy for reducing Aß deposits in AD patients. Thus, induced microglial phagocytosis by MSCs is associated with reductions in plaque formation, which raises the

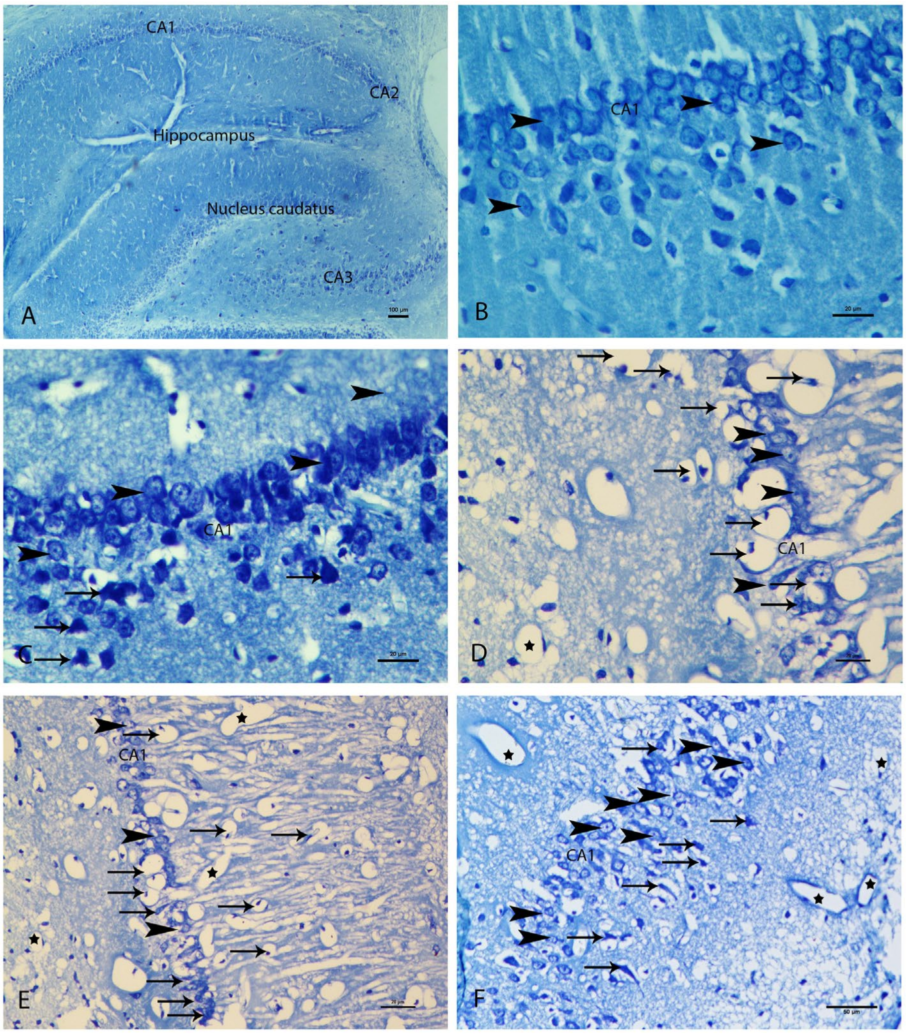

Figure 2 Histolopathological evaluation of the brains. 2A. The histopathological view of CA1, CA2 and CA3 areas of hippocampus in Group V (Control). It is seen that all the histological features of brains' tissue are regular. Hippocampus, nucleus caudatus and other neural structures are seen in normal appearance. 1% toluidine blue. x10. 2B. The histopathological view of CA1 area of hippocampus in Group V (Control). There are many healthy stellate neurons with round nuclei and prominent nucleoli (Arrowheads). 1% toluidine blue. X40. 2C. The histopathological view of CA1 areas of hippocampus in Group IV (Sham). It is seen that there are many healthy (Arrowheads) and some dead neurons in this region. In addition, a few dead neurons, which have shrunken hyperchromatic nuclei and lost its nucleoli, shrunken or elongated morphology, are observed (Arrows). 1% toluidine blue. X40. 2D. The histopathological view of CA1 areas of hippocampus in Group II (STZ-Sal). It is seen that there are many dead neurons in CA1 region of hippocampus (Arrows). The number of healthy neurons (Arrowheads) is decreased clearly. (Asters = vessels) 1% toluidine blue. X40. 2E. The histopathological view of CA1 areas of hippocampus in Group I (STZ + 1 × 10⁶ BM-MSCs). There are some new neurons in CA1 region. However, the structure of this region is not good. The number of neurons is very low. (Asters = vessels) 1% toluidine blue. X40. 2F. The histopathological view of CA1 areas of hippocampus in Group II (STZ + 2 × 10⁶ BM-MSCs). There are many healthy neurons in CA1 region. Also organization of neural tissue is very well when compared with Sham, STZ and Group I (STZ + 1 × 10⁶ BM-MSCs). (Asters = vessels) 1% toluidine blue. X40
(**Group I:** STZ + 1×10^6 BM-MSCs, **Group II:** STZ-Saline, **Group III:** STZ + 2×10^6 BM-MSCs, **Group IV:** Sham, **Group V:** Control) **BM-MSCs:** Bone marrow-derived mesenchymal stem cells, **STZ:** Streptozotocin.

possibility that certain inflammatory processes might serve to inhibit AD-type pathology.[5] However, neurogenesis in AD varies between neurogenic region and disease stage [18] and Aß oligomers may impair functional maturity of stem cells. Therefore, it should be kept in mind that the potential negative effect of the toxic host microenvironment in AD may pose a limitation for regenerative therapy.[19,20] MSCs have been derived from a variety of human tissues, including bone marrow, foetal blood, foetal liver, cord blood, amniotic fluid and adult peripheral blood. All these MSCs have retained their ability to differentiate into multiple lineages, including bone, cartilage, fat, muscle, nerve, glial and stromal cells.[21] The BM-MSCs have been able to escape allogeneic immune responses.[22] Among other studies, it has been shown that BM-MSCs can be successfully transplanted into a rat model of late onset AD, with cognitive impairments and neurodegeneration induced by ICV administration of STZ.[9] In our previous study,[9] it was reported that curcumin therapy was effective in reducing cognitive impairment and in improving the IGF-1 level in this STZ model of AD.

In this study, it was suggested that the BM-MSCs therapy improves working memory or learning function, while it does not work on memory in the rats; however, neuronal

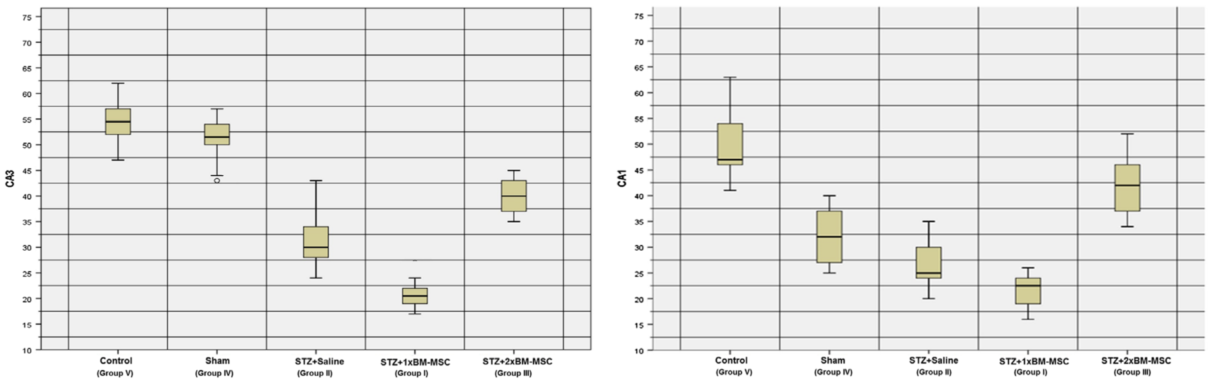

Figure 3 The mean values of the number of hippocampal neurons. The number of neurons in CA1 areas of hippocampus of Sham and Groups I, II and III were decreased when compared with Control group (respectively, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.03$). The number of neurons was increased in Group III (STZ + 2xBM-MSC), when compared with Sham, Groups II and I (respectively, $p < 0.003$, $p < 0.001$ and $p < 0.001$). The number of neurons in CA3 areas of Group III (STZ + 2xBM-MSC) was increased when compared with Groups I and II ($p < 0.001$ for both), but this increase was less than Control and Sham groups ($p < 0.002$ for both)

(**Group I:** STZ + 1×10^6 BM-MSCs, **Group II:** STZ-Saline, **Group III:** STZ + 2×10^6 BM-MSCs, **Group IV:** Sham, **Group V:** Control) **BM-MSCs:** Bone marrow-derived mesenchymal stem cells, **STZ:** Streptozotocin.

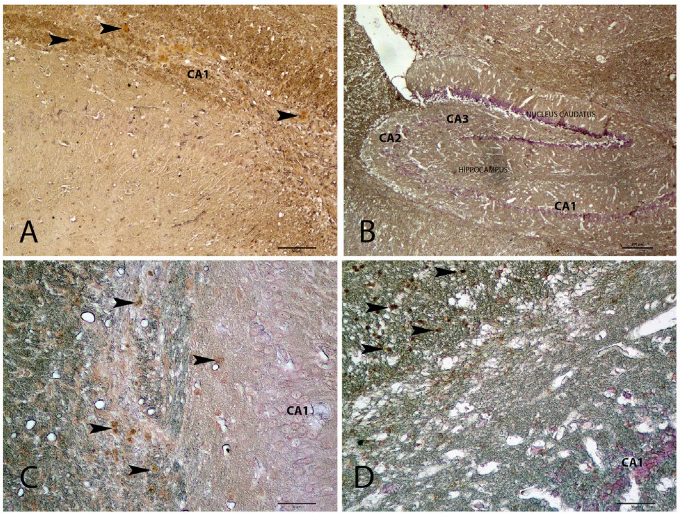

Figure 4 GFP (+) and apoptotic cells. 4A. The apoptotic cells (Arrowheads) in hippocampus of Group II. There are many dispersed apoptotic cells in CA1 region and close places Bar = 50 µ. Fragel *in situ* apoptosis detection kit (Calbiochem). 4B. The apoptotic cells (Arrowheads) in hippocampus of Group III. The number of apoptotic cells in CA1 region and close places is less than those of Group II. But there are some apoptotic cells in distant places. Bar = 200 µ. Fragel *in situ* apoptosis detection kit (Calbiochem). 4C. General view of hippocampus CA1 region in Group II. There are many GFP + cells (Arrowheads) in CA1 region. Bar = 50 µ. Anti-GFP staining (Abcam ab6556). 4D. General view of brain tissue in Group III. General microscopic structure of brain is better in view than Group II. Almost no GFP + cells are seen especially in higher magnification. Bar = 50 µ. Anti-GFP staining (Abcam ab6556)

(**Group I:** STZ + 1×10^6 BM-MSCs, **Group II:** STZ-Saline, **Group III:** STZ + 2×10^6 BM-MSCs, **Group IV:** Sham, **Group V:** Control) **BM-MSCs:** Bone marrow-derived mesenchymal stem cells, **STZ:** Streptozotocin.

replacement might be provided by intravenous BM-MSCs therapy. Similar to our results, Harting[23] reported that the intravenous infusion of MSCs neither resulted in significant acute or prolonged cerebral engraftment of cells nor modified the recovery of motor or cognitive function at two- and fourteen-day post-infusion in rats given traumatic brain injury, after the BM-MSCs therapy. In that study, it was also reported that, due to the pulmonary first-pass effect, less than 4% of the infused MSCs were likely to transverse the pulmonary microvasculature and reach the arterial circulation, which may have limited the efficacy of the MSCs therapy. However, in another study, foetal human NSCs were directly injected into the traumatic injured rat brain. It was reported that human NSCs grafts improved cognitive function of rats in this acute traumatic brain injury model and that grafted cells survived and differentiated into neurons and expressed and released glial-cell-line-derived neurotropic factor *in vivo*, which may help protect host cells from secondary damage and aid host regeneration of neurons.[24] This remarkable improvement in cognitive functions may result from the type of neurodegeneration being modelled and the type of stem cells used, as well as the fact that these cells were administered directly into the hippocampus. Although stem cell transplantation directly into the brain provides more cells, it is also a highly invasive method. In this study, it was demonstrated that GFP + BM-MSCs were observed in the brain and that the total number of neurons in both CA1 and CA3 areas of hippocampus significantly increased following transplantation. However, we were not able to demonstrate that the GFP + cells co-expressed specific neuronal or glial biomarkers to support our hypothesis of an increase in glial reaction.

Clearly, it was reported that BM-MSCs therapy via intravenous delivery was not able to improve all of the STZ-induced neuropathology; maybe a different administration route would be more effective. Although it was reported that the intravenous administration routes of BM-MSCs, a less invasive delivery method, have significant side effects, such as massive systemic distribution or entrapment of applied cells in peripheral organs,[23–26] Panchenko et al. reported that MSCs are able to reach into the brain in Alzheimer-type neurodegeneration model via intravenous administration.[27] Although it is a less invasive delivery method, it should be considered that it is not possible to predict how much of the administered number of stem cells reaches the brain via the rat tail vein. In addition to reduce the possible disadvantages of intravenous route and to avoid the complication of direct injection into brain, intranasal[25,26] or intraarterial[28] delivery of BM-MSCs may serve as a viable alternative. It is reported that this strategy provides an effective non-invasive method for cell delivery to the central nervous system.[25,26]

Moreover, it has also been demonstrated that over-expression of IGF-1 in the brain results in persistent increases in the total number of neurons and synapses in the DG, indicating that IGF-1 promotes both neurogenesis and synaptogenesis in the developing hippocampus *in vivo*.[29] Similar to these results, it was also shown that increasing IGF-1 with curcumin therapy improves memory and cognitive performance in STZ-induced neurodegeneration in rats and that improvements in IGF-1 levels could be a sign of the initiation of neurogenesis.[9] Therefore, combining the BM-MSCs transplantation and the agents, which can improve both brain structure and functionality in the AD models of neurodegeneration, appears to be a rational approach for future considerations.

The present data support the importance of considering morphological and behavioural data when evaluating pathological changes in hippocampus. Further studies are needed, including stereological estimates of total neuronal number, hippocampal volume and cell proliferation in the hippocampus following MSC transplants. Our findings of highly heterogeneous qualitative immunohistochemical properties offer some new insights into the functional reorganization and structural changes of the hippocampus in the STZ rat model of AD.

Conclusion

In this study, mesenchymal stem cells therapy failed to improve the STZ-induced AD-like neurodegeneration in rats. However, BM-MSCs may be potentiated by adjunctive therapies with other treatment strategies. The development of BM-MSCs therapy for the replacement of cells and lost tissues due to neurodegeneration in AD is still in its early stages, and further studies will be needed before it can be tested on humans.

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Notes on contributors

Ahmet Turan Isik, MD, is a professor whose research interests include Neurodegenerative disease, cognitive function, and cognitive aging in elderly. The author has pubished articles on Cognitive aging and cognitive impairment in elderly patients and new therapeutic approaches for experimental neurodegeneration models.

Turgay Celik, MD, is a professor whose research interest includes Neuropharmachology. The author has published articles on Neuropsychopharmacology/experimental neurodegeneration models.

Ali Ugur Ural, MD, is a professor whose research interest includes Stem Cell Therapy. The author has published articles on Stem cell therapy for hematologic malignancies.

Murat Tosun, MD, is a professor whose research interest includes Neurocytology. The author has published articles on CNS cytology.

Gokhan Ulusoy, MD, is an assistant professor whose research interest includes Psychopharmachology. The author has published articles on Psychopharmacologic drugs.

Birsen Elibol, PhD, is an assistant professor whose research interest includes Experimental Psychology. The author has published articles on Experimental measurement of cognitive function.

ORCID

Ahmet Turan Isik http://orcid.org/0000-0001-5867-6503

References

1 Gauthier S. Trial designs and outcomes to monitor novel therapeutics in Alzheimer's disease. In: Cuello AC, editor. Pharmaceutical and Mechanisms in Alzheimer's Therapeutics. New York, NY: Springer; 2008. p. 28–35.
2 Sugaya K. Possible use of autologous stem cell therapies for Alzheimers disease. *Curr Alzheimer Res*. 2005;2(3):367–76.
3 Isik AT. Late onset Alzheimer's disease in older people. *Clin Interventions Aging*. 2010;5:307–11.
4 Gould E, Tanapat P, Hastings NB, Shors TJ. Neurogenesis in adulthood: a possible role in learning. *Trends Cognit Sci*. 1999;3(5):186–92.
5 Lee JK, Jin HK, Bae J. Bone marrow-derived mesenchymal stem cells reduce brain amyloid-β deposition and accelerate the activation of microglia in an acutely induced Alzheimer's disease mouse model. *Neurosci Lett*. 2009;450:136–41.

6 Brazelton TR, Rossi FM, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. *Sci*. 2000;290(5497):1775–79.
7 Zhang J, Li Y, Chen J, Cui Y, Lu M, Elias SB, *et al*. Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp Neurol*. 2005;195(1):16–26.
8 Salkovic-Petrisic M, Hoyer S. Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach. *J Neural Transm Suppl*. 2007;72:217–33.
9 Isik AT, Celik T, Ulusoy G, Ongoru O, Elibol B, Doruk H, *et al*. Curcumin ameliorates impaired insulin/IGF signalling and memory deficit in a streptozotocin-treated rat model. *Age*. 2009;31(1):39–49.
10 Siebzehnrubl FA, Vedam-Mai V, Azari H, Reynolds BA, Deleyrolle LP. Isolation and characterization of adult neural stem cells. *Methods Mol Biol*. 2011;750:61–77.
11 Ropireddy D, Bachus SE, Ascoli GA. Non-homogeneous stereological properties of the rat hippocampus from high-resolution 3D serial reconstruction of thin histological sections. *Neuroscience*. 2012;205:91–111.
12 Baran Y, Ural AU, Avcu F, Sarper M, Elci P, Pekel A. Optimization of transfection of green fluorescent protein in pursuing mesenchymal stem cells *in vivo*. *Turk J Hematol*. 2008;25:172–75.
13 Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*. 1984;11:47–60.
14 Szabados T, Dul C, Majtényi K, Hargitai J, Pénzes Z, Urbanics R. A chronic Alzheimer's model evoked by mitochondrial poison sodium azide for pharmacological investigations. *Behav Brain Res*. 2004;154(1):31–40.
15 Boillee S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, *et al*. Onset and progression in inherited als determined by motor neurons and microglia. *Sci*. 2006;312(5778):1389–92.
16 Cho YH, Kim HS, Lee KH, Lee YE, Chang JW. The behavioral effect of human mesenchymal stem cell transplantation in cold brain injured rats. *Acta Neurochir Suppl*. 2006;99:125–32.
17 Bantubungi K, Blum D, Cuvelier L, Wislet-Gendebien S, Rogister B, Brouillet E, *et al*. Stem cell factor and mesenchymal and neural stem cell transplantation in a rat model of Huntington's disease. *Mol Cell Neurosci*. 2008;37(3):454–70.
18 Perry EK, Johnson M, Ekonomou A, Perry RH, Ballard C, Attems J. Neurogenic abnormalities in Alzheimer's disease differ between stages of neurogenesis and are partly related to cholinergic pathology. *Neurobiol Dis*. 2012;47(2):155–62.
19 Jin HK, Bae JS, Furuya S, Carter JE. Amyloid beta-derived neuroplasticity in bone marrow-derived mesenchymal stem cells is mediated by NPY and 5-HT2B receptors via ERK1/2 signalling pathways. *Cell Proliferation*. 2009;42:571–86.
20 Kwak YD, Marutle A. Involvement of notch signaling pathway in amyloid precursor protein induced glial differentiation. *Eur J Pharmacol*. 2011;650:18–27.
21 Mıao Z, Jın J, Chen L, Zhu J, Huang W, Zhao J, *et al*. Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. *Cell Biol Int*. 2006;30:681–7.
22 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005;105(4):1815–22.
23 Harting MT, Jimenez F, Xue H, Fischer UM, Baumgartner J, Dash PK, *et al*. Intravenous mesenchymal stem cell therapy for traumatic brain injury. *J Neurosurg*. 2009;110:1189–97.
24 Gao J, Prough DS, Mcadoo DJ, Grady JJ, Parsley MO, Ma L, *et al*. Transplantation of primed human fetal neural stem cells improves cognitive function in rats after traumatic brain injury. *Exp Neurol*. 2006;201(2):281–92.
25 Danielyan L, Schäfer R, von Ameln-Mayerhofer A, Buadze M, Geisler J, Klopfer T, *et al*. Intranasal delivery of cells to the brain. *Eur J Cell Biol*. 2009;88(6):315–24.
26 Danielyan L, Schäfer R, von Ameln-Mayerhofer A, Bernhard F, Verleysdonk S, Buadze M, *et al*. Therapeutic efficacy of intranasally delivered mesenchymal stem cells in a rat model of Parkinson disease. *Rejuvenation Res*. 2011;14(1):3–16.
27 Panchenko MM, Poltavtseva RA, Bobkova NV. Localization and differentiation pattern of transplanted human multipotent mesenchymal stromal cells in the brain of bulbectomized mice. *Bull Exp Biol Med*. 2014;158(1):118–22.
28 Chua JY, Pendharkar AV, Wang N, Choi R, Andres RH, Gaeta X, *et al*. Intra-arterial injection of neural stem cells using a microneedle technique does not cause microembolic strokes. *J Cereb Blood Flow Metab*. 2011;31:1263–71.
29 O'kusky JR, Ye P, D'Ercole AJ. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J Neurosci.* 2000;20(22):8435–42.