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Intrahippocampal Transplantation of Undifferentiated Human Chorionic-Derived Mesenchymal Stem Cells Does Not Improve Learning and Memory in the Rat Model of Sporadic Alzheimer Disease

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Abstract: ***Background and Objective:*** Alzheimer's disease (AD) is a progressive neurodegenerative disorder with consequent cognitive impairment and behavioral deficits. AD is characterized by loss of cholinergic neurons and the presence of beta-amyloid protein deposits. Stem cell transplantation seems to be a promising strategy for regeneration of defects in the brain.

Method: One of the suitable type of stem cells originated from fetal membrane is Chorion-derived Mesenchymal Stem Cells (C-MSCs). MSCs were isolated from chorion and characterized by Flowcytometric analysis. Then C-MSCs labeled with DiI were transplanted into the STZ induced Alzheimer disease model in rat.

Results: Nissl staining and behavior test were used to assess the efficacy of the transplanted cells. Phenotypic and Flowcytometric studies showed that isolated cells were positive for mesenchymal stem cell marker panel with spindle like morphology.

Conclusion: Learning and memory abilities were not improved after stem cell transplantation. C-MSCs transplantation can successfully engraft in injured site but the efficacy and function of transplanted cells were not clinically satisfied.

Keyword: Alzheimer's disease, Hippocampus, Chorion, transplantation.

1. INTRODUCTION

Alzheimer's disease (AD) is known as a progressive neurodegenerative disorder which leads to irreversible and progressive memory impairment. As a financially costly disease, Alzheimer's disease has globally developed many economic and social impacts [1, 2] Accumulation of amyloid- β plaques and neurofibrillary tangles associated with loss of neurons in hippocampus and cortex are significant features for characterization of the disease [3, 4]. Moreover, degeneration of cholinergic neurons in basal forebrain and impairment of cholinergic functions are subsequently occurring in AD patients with loss of memory [5]. Degeneration or dysfunction of basal forebrain cholinergic neuron offspring is often accompanying with learning and memory disorders. Since the pharmacological and cognitive therapies are not so effective, it seems that stem cell-based approaches for regeneration of the damaged tissue have become one of the promising alternatives [6]. Having self-renewal properties and multilineage differentiation capacity, Stem/ progenitor cells have gained the focus of interest in regenerative medicine. The ability of resident neural progenitor cells to regenerate hippocampal and cortical neurons in lower species demonstrates the possibility of endogenous progenitor cells to regenerate the lost neural circuits [7].

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However, application of these cells in repair of brain defects has some limitations due to their relatively low number in adult nervous tissues. Stem cell-based therapy for AD faces with many complications, and the best stem cell line is unresolved [8-10]. Embryonic stem cells (ESCs) or their spinoffs, functionally integrated neurons, have been suggested as capable therapies for AD [11].

Chorion, as an easy accessible extraembryonic tissue, is an ethically approved cell reservoir which contains mesenchymal stem cells with self-renewal properties and neural differentiation capacity. These cells are harvested by non-invasive earnings and maintained *in vitro* as undifferentiated cells for more than 10 passages . MSCs are able to engraft into the brain and differentiate after transplantation in situ into neurons and glial cells [12]. Neural differentiation ability of MSCs is identified for the ability to stimulate the neurogenesis of neural progenitors and survival of neural cells by expressing neurotrophic factors [13]. Furthermore, transplantation of MSCs in the central nervous systems inhibits apoptosis and supports neurogenesis (proliferation and differentiation) of 'host' neural cells and stem cells in the engrafted site [14]. MSCs were therefore suggested as candidates for treating a variety of neurodegenerative diseases, in particular, Parkinson's disease, multiple sclerosis, cerebral hemorrhage and brain cancer [15, 16]. Since there is no clinically known risk for teratoma formation after application of these cells in cell therapy, this type of stem cell may offer opportunities for cell replacement therapies in neurodegenerative medicine [17].

Here, in the current study, we focus on transplantation of C-MSCs into STZ- induced rat sporadic model of Alzheimer´s disease. We set out to determine whether C-MSCs transplantation could be useful for AD treatment or not.

2. MATERIALS AND METHODS

2.1. Animals

All protocols and procedures followed the guidelines approved by the ethical committee of Iran University of Medical Sciences (IUMS). Adult male Wistar rats were provided from the animal lab of IUMS. Animals were maintained in standard housing conditions on a 12-h light/dark cycle with free food and tap water. They were kept at a constant temperature of 22°C. A total number of forty male Wistar rats, weighing about 220-250g, with normal passive avoidance test behavior were used in the study. The animals were randomly divided into three equal groups; including model group (STZ-induced with no treatment), sham group (lesion plus Cell vehicle injection), C-MSCs–treated group (lesion treatment with C-MSCs injection).

2.2. Animal Modeling

Rats were anesthetized by intraperitoneal administration of ketamine/xylazine (50 mg/kg). For modeling Alzheimer's disease, the animal head was first positioned in a stereotactic apparatus. After a midline sagittal incision in the scalp, burr holes were drilled in the skull on both sides over the lateral ventricles according to the Paxinos and Watson atlas (0.8 mm posterior to bregma; 1.5 mm lateral to sagital suture; 3.6 mm beneath the surface of the brain). The rat was given a bilateral ICV (Intracerebroventricular) injection of Streptozotocin (STZ; Sigma, USA). To do that, STZ was dissolved in distilled water and a solution of 25mg/ml was made just before the injection. The dose of 3mg per kg was injected on day one and day3. In the control group, artificial CSF composed of 2.9mM KCl; 147mM NaCl; 1.7mM $CaCl_2$, 2.2mM dextrose and 1.6mM $MgCl_2$ was injected on the same days [18, 19].

2.3. Isolation and Culture of Human Chorion- derived MSCs (C-MSCs)

The protocol to isolate C-MSCs was approved by the ethical committee of Iran University of Medical Sciences. Full-term placenta was used after obtaining maternal informed consent. According to our lab protocol (Faghihi *et al*. 2016) human chorion tissue was washed twice in phosphate-buffered saline (PBS; Gibco, Germany) and then cut into small pieces. The small fragments were digested using 0.3 % type II collagenase solution (Gibco, Germany) at 37 °C for 30 minutes. After cell filtration using 100μm cell strainer, the enzyme was deactivated using the same volume of PBS. The cell suspension was centrifuged at 1200 rpm for 5 min. Then, the number of 5000 cell/cm^2 was plated in tissue culture flasks (TPP, Switzerland) containing the expansion medium composed of DMEM-F12 supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 1000 μg/ml streptomycin (all from Gibco, Germany). Cells were incubated at 37 °C and 5 % CO_2. The medium was changed every 3 days. After a few days, the flasks were washed to remove unattached particles. At 70- 80 % confluence, the cells were plated into new culture plates. The expanded cells at passage 3-4 were used for the following experiments

2.4. Characterization of Human C-MSCs

To confirm mesenchymal stemness properties of the isolated cells, the following experiments were carried out at passage three. A number of 10^5 of the isolated cells was incubated with 10 % goat serum at 4 °C for one hour. Then the serum was removed and the cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies against human CD44, CD45, CD34, CD73, and CD90 (all from BD bioscience, USA) at 4 °C for 40 min. Isotope-matched antibodies were used as a control. Positive expression was defined as the level of fluorescence greater than 95%. The expression of the mentioned cell surface antigens was assayed by Becton-Dickinson flow cytometer and the data were analyzed using Flomax software.

2.5. Cell Transplantation

Two weeks after making rat model of Alzheimer´s disease, the animals were anesthetized by intraperitoneal injection of ketamine/xylazine (50 mg/kg). Prior to cell transplantation, the cells including C-MSc were labeled with DiI (Invitrogen, USA) Confluent C-MSCs detached by trypsin: EDTA (1: 25), counted and plated in 6-multiwell plates at a density of 4 × 105 cells/ml. For CM-DiI dye tracking, after trypsinization, cells were washed with PBS and incubated for 3 min with a 1: 250 CM-DiI dye. After this procedure, cells were centrifuged again, the excess of fluorescent dye was

removed and immediately before transplantation, the pellet was re-suspended, then the head of each animal was mounted on a stereotaxic frame (Stoelting Co, USA). The skin was shaved and sterilized with iodine. A spindle-shaped median incision was made on the scalp and the periosteum was dissected. Hemostasis was achieved with 1% hydrogen peroxide to expose the sagittal suture and coronal suture. Bregma was used as a reference point for the following stereotaxic coordinate. After making burr hole, a number of 4×10^5 cells were injected using a 5µl Hamilton syringe (26-gauge needle) into CA_1: (AP: -3/80, ML: 2, DV: 2/6) relative to bregma. As much as 1µl of cell suspension was infused per min at each site with 5 min intervals for absorption. The holes were sealed after cell transplantation and washed with normal saline. To prevent infection, gentamicin (Iran) was spread over the incision.

2.6. Behavior Test

The apparatus used for passive avoidance (PA) test is composed of a two-structure box; including an illuminated compartment and a dark box (30×30×30 cm), equipped with a grid floor and separating with a sliding door (30×15 ×25 cm). The floor of the dark chamber could be electrified by a shock generator. All the animal groups were first habituated to the apparatus (n=6). To do that, each rat was placed in the illuminated compartment for 10 seconds, and then the sliding door was raised. Upon entering the dark compartment, the rat was taken from the dark box into the home cage. Each rat was placed in the illuminated compartment of the apparatus and left for 3 min to habituate to the apparatus. Then 50 Hz, 1.2 mA constant current shock was applied for 1.5 s through the floor grid of the dark box. After 20s, the rats were moved to their home cages. To determine long-term memory, a retention test was performed twenty-four hours after training. To do that, each rat was placed in the illuminated box for 10s, the door was opened, and the latency of entering to the dark compartment (step-through latency; maximum 300s) and the time spent in the dark were recorded as a measurement of retention performance (Time in Darkness).

2.7. Assessment of Histological Changes

Animal perfusion was done by injection of saline solution followed by injection of cold paraformaldehyde solution 8% through the ascending aorta. The volumes were calculated based on each animal´s weight. Then the brains were removed and fixed in paraformaldehyde solution 8% for 4 hrs. The brain tissues were cryoprotected in sucrose solution (18% in phosphate-buffer) for at least 24 hrs. at 4ºC. Coronal sections with 10mm thickness were cut in a cryostat at the level of hippocampus. The sections were mounted on Cillian-coated slides, and dried at room temperature.

Coronal sections with hippocampus region were deparaffinized and incubated in 0.5% cresyl violet solution for 3–5 min. The slides were soaked in distilled water and dehydrated in 95 and 100 percent alcohol. The sections were cleared in xylene and mounted by DPX mounting medium. Images were taken using light microscopy (Nikon E 800 microscope image analysis system). To quantify cell density, both healthy and pyknotic cells were counted on at least three sections with 40× magnification.

2.8. Statistical Analysis

All Results were expressed as the Mean ± SE. All analyses were performed using Graph Pad Prism software version 5 program (Graph Pad Software, Inc., CA USA). P values less than 0.05 were assumed significant. Statistical significance was performed on the data using one-way analysis of variance (ANOVA) The specific tests used are described in the Figure legends.

3. RESULT

3.1. Characterization of Human C-MSCs

Human C-MSCs were fibroblastic shape at primary culture. This morphology was kept at subsequent cultures (Fig. **1**). At passage three, the cells could express CD73, CD44, and CD90 antigens; while they were negative for hematopoietic markers; including CD45 and CD34. The isolated cells were also capable to differentiate into osteoblasts, and adipoblasts in appropriate inducing media (Fig. **2**).

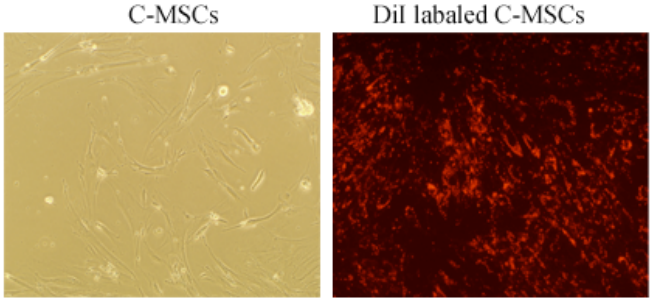

Fig. (1). Isolation and characterization of human chorion-derived mesenchymal stem cells. A) Fibroblast-like cells are shown in primary culture at passage #3 (magnification×10). B) labeled C-MSCs with DiI staining

3.2. Histological Evaluation

After labeling of human C-MSCs using CM-DiI. The cells were transplanted to the receiving rat brains (Fig. **3**). The cresyl violet staining was performed on rat hippocampal CA1 sections to show healthy and pyknotic neurons in right and left sides of this region. Healthy neurons had a bright nucleus with a large nucleolus and clear cytoplasm. pyknotic neurons were sickle-shaped with the dark fragmented nucleus. The number of healthy neurons remarkably decreased in STZ treated rat brains as compared with total cell number (Fig. **4**). Transplantation of C-MSCs into ICV-STZ models did not increase the number of healthy cells. In addition, histological analysis data showed that the grafted cells were successfully migrated and engrafted into the CA 1 Hippocampus (Fig. **5**)

3.3. Behavioral Test: Passive Avoidance Test

In all experiments, retention trial, step-through latency (STL) and the time spent in dark compartment (TDC) were recorded during passive avoidance test. Two months after transplantation of C-MSCs, the retrieval trial was performed (n=6 in each group). Passive Avoidance (PA) retrieval results indicated that the latency of the control group (healthy group) was significantly more than the model (*; $p<0.05$;

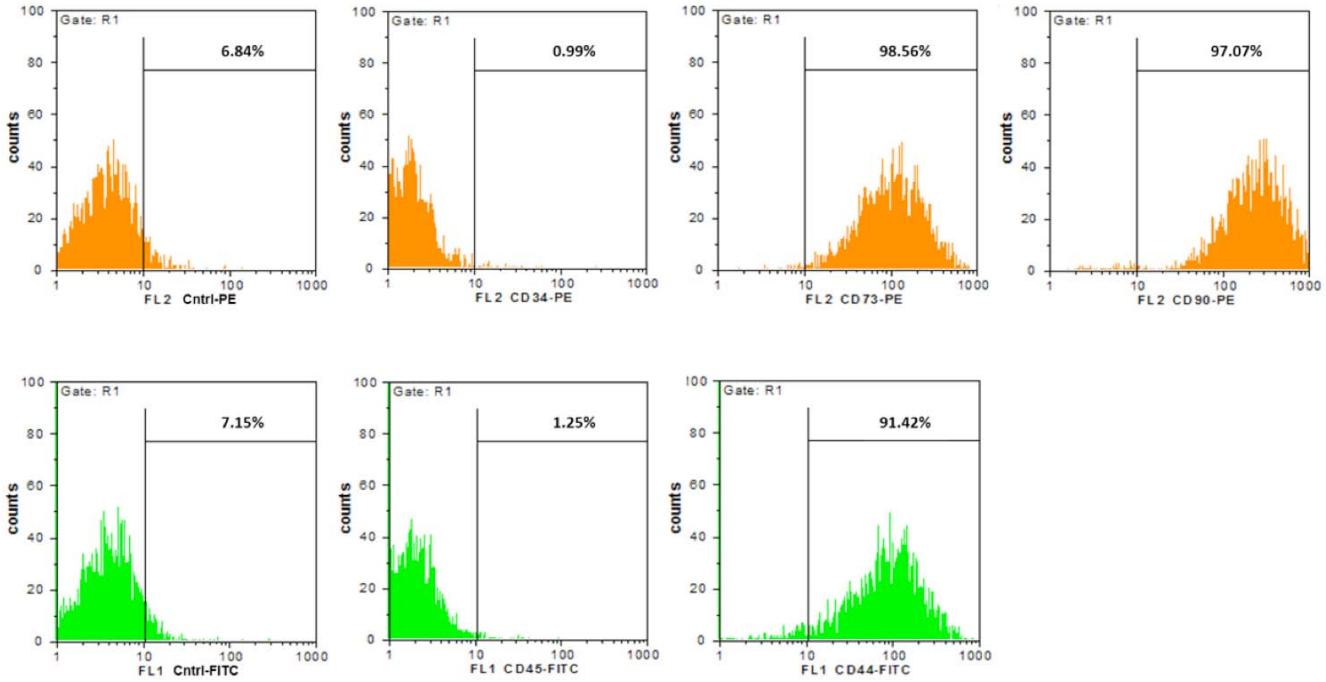

Fig. (2). Cytofluorimetric analysis of the isolated cells. Flowcytomery analysis showed that the cells positively expressed CD90, CD44, and CD73 antigens, but they were negative for hematopoietic markers; including CD45, CD34

Fig. (3). C-MSCs injection site in animal model. After a midline sagittal incision in the scalp, burr holes were drilled in the skull on both sides over the lateral ventricles according to the Paxinos and Watson atlas and the rats were given a bilateral ICV injection of Streptozotocin.

Fig. **6**). Tukey's multiple comparison test showed that there was no significant difference in STL (*; $p<0.05$) and TDC (***; $p<0.001$) between all groups. Although the time spent in STL and TDC was not changed after transplantation of C-MSCs, no significant difference was observed in STL and TDC compartments between C-MSCs treated group and the model (Fig. **6**).

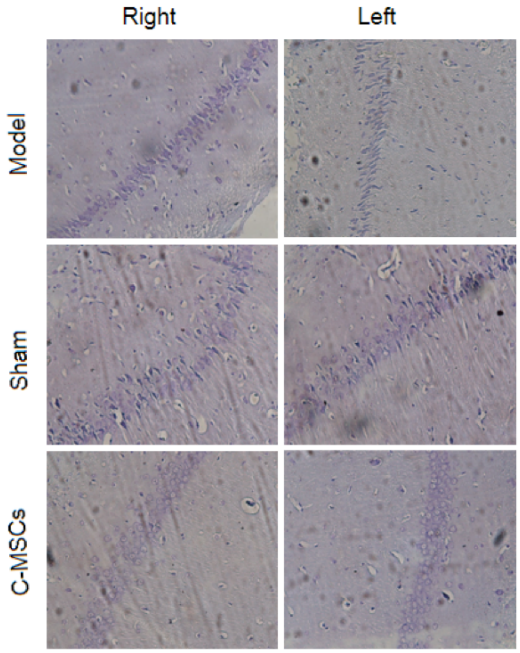

Fig. (4). Histological assessments with Nissl staining. The effect of C-MSCs on the density of neurons in hippocampal CA1 region of ICV-STZ treated rat brain were studied at both right (R) and left (L) sides of hippocampal CA1 region in sham, model and C- MSCs treated groups.

4. DISCUSSION

MSCs were initially isolated from bone marrow by Friedenstein *et al.* (1976). Recent studies have demonstrated that MSCs have the potential to differentiate into adipocytes, osteocytes and chondrocytes *in vitro* [20]. In this study, we isolated and expanded fibroblast-like cells from the chorion membrane successfully. Using immunocytochemistry, we found that these cells expressed high levels of MSC markers (CD73, CD90, and CD44) and negative for hematopoietic markers (CD34 and CD45).These results suggest that the cells from Chorion are very similar to the mesenchymal stem cells**.**

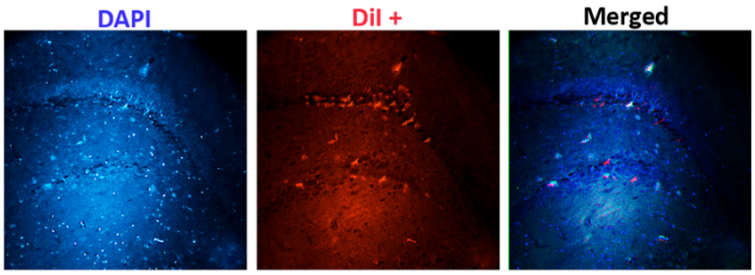

Fig. (5). Cell survival and migrating assay. DiI positive cells (red) were administrated to CA1 region of ICV-STZ treated rat brain. As represented in the figure, DiI positive cells are found in the CA1 three months after transplant. (Cell nuclei were stained with DAPI)

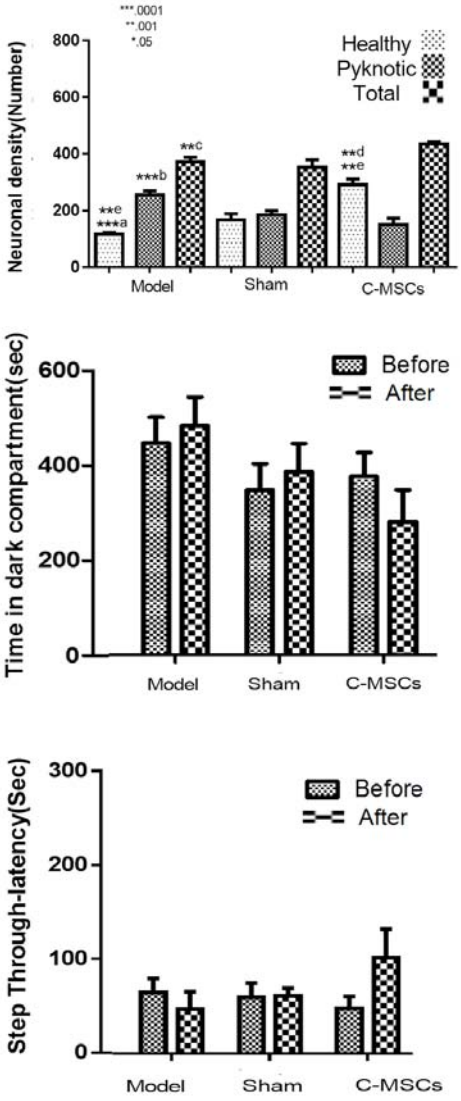

Fig. (6). Behavior evaluation after cell transplantation. Neuronal density was determined using Nissl staining. Data showed that there is a significant difference between three groups (a; difference between model and CMSCs groups in number of healthy cells, b; difference between model and CMSCs groups in number of pyknotic cells, c; difference between model and CMSCs groups in number of total cells, d; difference between sham and CMSCs groups in number of healthy cells, e; difference between model and CMSCs groups in number of healthy cells. The effect of transplantation of C-MSCs on passive avoidance retention at day 30. Step through latency (a; STL) and b time spent in dark compartment (b; TSD) were measured for each rat. Each point shows the mean ±S.E.M; n=6. #P<0.001 different from the healthy group. *P<0.05 different from model group.

As a chronic neurodegenerative disorder, Alzheimer's disease (AD) is characterized by progressive memory loss and diminished cognitive ability. Loss of cholinergic neurons in basal forebrain and hippocampus, besides disruption of the hippocampal outflow tracts, impair memory tasks in AD patients [21, 22]. Despite the existence of a few numbers of somatic neural stem/ progenitor cells in the adult human brain, this organ is less potent to repair itself upon injury [23, 24].

Basically, stem cells are dependent on their microenvironment to differentiate into particular phenotype; however reports indicated that for disorders with specific neuronal and/or glial phenotypes, immediate progenitors of the desired cell types would need to be specified to the particular type of interest for the appropriate treatment of that condition [25].

Stem/progenitor cell transplantation seems to be a promising strategy for regeneration of defects in the brain [26]. A recurrent theme in the design of clinically meaningful cell therapeutic strategies is ensuring the proper pairing of disease targets with the right donor phenotypes, i.e., those able to achieve functionally effective cell replacement and circuit repair in the disease environment. Yet a number of recent efforts have promulgated the use of donor cells that may be ill-suited for the disease targets to which they have been applied. Previous studies showed transplanted neural stem cells (NSCs) differentiated into neurons and astrocytes and improved the learning and memory deficits of AD animals [27-29]. In another study, Yu *et al*. produced functional dentate granule neurons from human iPSCs. They examine the possibility of adding new dentate neuronal progenitors to hippocampus. They Corrected cognitive dysfunctional by regionally restricted pathologies [30].

We also examined the effect of transplantation of C-MSCs on working memory deficits in STZ-ICV rat sporadic model of Alzheimer disease. The hippocampus plays a major character in memory and spatial navigation. In AD patients the hippocampus is one of the first regions of the brain to suffer damage. Therefore, for this reason, C-MSCs have been transplanted into the hippocampus. The STZ models of AD may not always carry the true pathology of the disease. Even the STZ models for AD exhibit in neuronal damage due to perturbations of energy metabolism the most common sporadic nature of the disease, rather represent rarer familial mutation. Hence we have chosen the A induced neurotoxicity model which is the most common pathological features in AD. Monisha Sharma and *et al*. have described localized neuroinflammation, oxidative stress and neuronal loss fol-

lowed by memory impairment in rat upon acute intracerebroventricular injection of STZ [31].

We first labeled the C-MSCs to track them. After transplantation into the bilateral CA1 hippocampus of rat model of AD, some of these C-MSCs survived and successfully engrafted in injured site. C-MSCs transplantation into CA1 region did not change Aβ pathology. Similarly, Gu *et al*., also reported that transplantation of mouse neural stem cell-derived CNLs into hippocampus did not alter Aβ plaques in APP/PS1 model of AD [32, 33]. According to Isakova IA *et al.* findings, similar to our study, MSCs tracing in normal brains shows a variety of transplant sites, ranging from the hippocampus to the forebrain [34]. Up till now grafted MSCs typically do not survive in the CNS, and early reports of their ability to harvest mature neural phenotypes have been largely discredited. For itself, the use of MSCs would seem logically partial to short-term immune modulation in settings for which pharmacological immune suppression has been found ineffective or otherwise ill-advised, such as in SCI and stroke [35, 36].

According to the results obtained from Nissl staining, Transplantation of C-MSCs (**; $p<0.001$) into ICV-STZ models, could not increase the number of healthy cells. Yet grafted MSCs typically do not survive in the adult human CNS, and early reports of their ability to produce mature neural phenotypes have been largely discredited. As such, the use of MSCs would seem logically limited to short-term immune modulation in settings for which pharmacological immune suppression has been found ineffective or otherwise ill-advised, such as in SCI and stroke [37]. Unclear mechanisms by which MSCs exert their immunosuppressive actions and the variability of those effects as a function of disease environment and duration, all serve to limit the utility of MSCs as clinical therapeutics. Whereas they may well prove beneficial in accelerating recovery from acute conditions exacerbated by central inflammation, such as stroke and relapsing multiple sclerosis—in each of which they are already under clinical assessment [38].

The improvement in cognitive function was correlated with enhanced STL (step through latency) and decrease in TD (time in darkness) compartments in Passive avoidance test. Our comparison test showed that there was no significant difference in STL between the groups treated with C-MSCs and the ICV- STZ model. Ridley and his colleagues reported that transplantation of rodent fetal cholinergic neurons into the cortex of monkeys with lesions at Meynert could recover the cognitive abilities [39]. According to Yun *et al*., mice Placenta-derived mesenchymal stem cells may improve memory deficient in mouse model of Alzheimer's disease. In another experimental study in which cholinergic input to the hippocampus was disrupted, transplantation of iPSC-derived cholinergic neuronal cells could significantly recover memory tasks [40]. In this esteem, it is related to Darsalia *et al*. study which showed the effect of number and time of transplanted cells which triggers their survival in stroke induced rat brains. This is facilitated by differential cell distribution, neuronal differentiation and proliferation in the implanted region [41]. We suggested that a low dose of cells possibly will not sufficiently mount a therapeutic effect. Likewise, higher cell dose possibly aggravates immunomodulatory response,as these cells are derived from human origin.

CONCLUSION

Human Chorion- derived mesenchymal stem cells (C-MSCs) are potent cell candidate with ease of procurement and no ethical limitations which can be used in cell therapy approaches but our study showed that this type of cell is not suitable for neurodegenerative disorder treatment. Actually, the satisfied results may be acquired following some criteria including long-term assessments or interval injection, transplantation of specialized cells and combination therapy with trophic factors or drugs. Furthermore, transplantation of C-MSCs may not provide an efficient alternative in cell therapies for recovery of memory deficits and restoration of cholinergic functions in AD. Future studies should further analyze the long-term effect of MSC transplantation on behavior, distribution and should discover the potential influence of MSCs modes of administration (that is, repeated and systemic injections) in various animal models of AD.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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