Mesenchymal Stem Cells Therapy Improved the Streptozotocin-Induced Behavioral and Hippocampal Impairment in Rats

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Abstract
Sporadic Alzheimer’s disease (sAD) is the most prevalent neurodegenerative pathology with no effective therapy until date. This disease promotes hippocampal degeneration, which in turn affects multiple cognitive domains and daily life activities. In this study, we hypothesized that long-lasting therapy with mesenchymal stem cells (MSC) would have a restorative effect on the behavioral alterations and cognitive decline typical of sAD, as they have shown neurogenic and immunomodulatory activities. To test this, we chronically injected intravenous human MSC in a sAD rat model induced by the intracerebroventricular injection of streptozotocin (STZ). During the last 2 weeks, we performed open field, Barnes maze, and marble burying tests. STZ-treated rats displayed a poor performance in all behavioral tests. Cell therapy increased exploratory behavior, decreased anxiety, and improved spatial memory and marble burying behavior, the latter being representative of daily life activities. On the hippocampus, we found that STZ promotes neuronal loss in the Cornus Ammonis (CA1) field and decreased neurogenesis in the dentate gyrus. Also, STZ induced a reduction in hippocampal volume and presynaptic protein levels and an exacerbated microgliosis, relevant AD features. The therapy rescued CA1 neurodegeneration but did not reverse the decrease of immature neurons, suggesting that the therapy effect varied among hippocampal neuronal populations. Importantly, cell therapy ameliorated microgliosis and restored hippocampal atrophy and some presynaptic protein levels in the sAD model. These findings, by showing that intravenous injection of human MSC restores behavioral and hippocampal alterations in experimental sAD, support the potential use of MSC therapy for the treatment of neurodegenerative diseases.

Keywords Sporadic Alzheimer’s disease · Mesenchymal stem cell · Cognitive function · Microglia · Synaptic proteins

Introduction
Alzheimer’s disease (AD) is the most common cause of dementia, accounting for an estimated 60 to 80% of cases [1]. It is characterized by a progressive loss of memory and cognitive functions. The predominant cases are sporadic Alzheimer’s disease (sAD), which is multifactorial and involves several etiopathogenic mechanisms. Neuroinflammation, head trauma, impaired brain glucose/energy metabolism, and diabetes are some of the risk factors for sAD [2]. Anatomical signs of AD include progressive brain atrophy, particularly in the hippocampus. Specifically, the Stratum Radiatum (SR) and the pyramidal layer of Cornus Ammonis 1 (CA1) are hippocampal regions highly susceptible to AD [3–5]. However, other hippocampal areas may present alterations, such as the dentate gyrus (DG), where the granular cells show morphological changes [6]. Importantly, these degenerative modifications...
correlate with cognitive decline in AD, supporting their potential as early biomarkers [3].

Novel and effective therapeutic approaches for sAD are urgently needed, which should be tested pre-clinically in animal models of the disease. A rodent model that mimics many aspects of sAD-type neurodegeneration has been generated by an intracerebroventricular (icv) injection of streptozotocin (STZ) [7–10]. Several studies have provided evidence that a single icv-STZ injection in middle-aged rats reduces the central metabolism of glucose and concomitantly impairs cognitive function and extensive neuroinflammation involving activation of both astrocytes and microglia [11–16].

There is an increasing interest in the therapeutic potential of mesenchymal stem cells (MSC) in the brain, as they are able to modulate inflammatory responses [17–19] and migrate to injury sites [20–22]. In particular, birth-associated tissues represent a very promising source and are under study as advantageous candidates for cell therapy due to their lower donor variability, faster doubling time, and ready availability, avoiding the need of invasive procedures and eliminating other ethical concerns [23–27].

Even though there have been some works on the effect of MSC therapy to treat neurodegenerative diseases [28–32], in vivo studies for sAD are still limited. The goal of the current study was to evaluate the neuroprotective potential of human umbilical cord MSC against the deleterious effects caused by the rat brain exposure to STZ as a sAD model. To this end, we assessed the effects of chronic intravenous (iv) injection of MSC on cognition and behavior in this model. In addition, since the hippocampus is particularly affected in AD, we focused our analysis on this brain region. Specifically, we studied SR volume, mature and immature neurons, glial cells, and synaptic markers to support the potential of systemic MSC therapy for the treatment of sAD.

Material and Methods

Animals

For this work, 3-month-old male Sprague-Dawley (SD) rats weighing 280–330 g (INIBIOLP, School of Medical Sciences, University of La Plata, La Plata city, Argentina) were used. Three animals were housed per cage in a temperature-controlled room (22–24 °C) on a 12-h light/12-h dark cycle (lights on 7 am–7 pm) with food and water available ad libitum.

Isolation of MSC

MSC were isolated from human umbilical cord perivascular tissue obtained from healthy donors at the Hospital Universitario Austral (Pilar, Buenos Aires, Argentina) as previously described [24]. MSC were characterized according to the International Society for Cellular Therapy guidelines [33], as previously described [20].

Experimental Design

On experimental day 0, animals were anesthetized with ketamine hydrochloride (40 mg/kg; ip) plus xylazine (8 mg/kg; im) and placed in a stereotaxic apparatus. Rats were randomly divided into three experimental groups, 7 animals each: (A) SHAM, each rat received a bilateral icv injection (5 μl/ventricle) of artificial cerebrospinal fluid (aCSF) (120 mM NaCl, 3 mM KCl, 1.15 mM CaCl2, 0.8 mM MgCl2, 27 mM NaHCO3, and 0.33 mM NaH2PO4, pH 7.4). (B) STZ and (C) STZ + MSC (Fig. 1a). Each rat of groups B and C received a bilateral icv injection of STZ (Sigma-Aldrich, CAS#18883-66-4) at a dose of 3 mg/kg. A STZ volume of 5 μl/ventricle was administered. The stereotaxic coordinates for icv infusion were measured as 0.92 mm posterior to bregma, 1.5 mm lateral to sagittal suture, and 3.9 mm beneath the brain surface [34]. Following surgery, a single dose of ampicillin was injected.

For the MSC therapy, animals were injected in a tail vein; each rat of C group received an iv-saline suspension of 1 ml containing 1 × 106 MSC on days 24, 42, 60, and 78 (every 18 days). Cell therapy started on day 24 based on our previous studies showing that at this stage, the STZ rats already display cognitive deficits.

For the open field (OF) test protocol, rats were individually placed into the center of the open arena on day 86 and allowed to explore the apparatus for 5 min. After the 5-min test, rats were returned to their home cages. Two days later, rats were submitted to the Barnes maze (BM) protocol for 4 days, which consisted on the habituation plus 3 days with 2 acquisition trials (ATs) each. On day 92, 24 h after the last AT (AT6), rats were submitted to the probe trial (PT). On the following day, the Marble burying (MB) test was performed. All rats were euthanized on day 95 by rapid decapitation.

Exploratory and Anxiety Behavior

The OF test [35] consisted of a square box (65 × 65 × 45 cm), whose floor was divided into 16 equal squares. Rats were placed in the center of the arena and allowed to explore freely for 5 min. The behavioral variables were the following:

Crossing: number of grid lines crossed by the rat with all four paws. A high frequency of this behavior indicates increased locomotion and exploration and/or a lower level of anxiety.

Center square entries: frequency of entry to the inner area by the rat with all four paws into the central square. A high frequency/duration of this behavior indicates high exploration and low anxiety [36, 37].
Grooming: time a rat spent scratching or licking itself. This behavior has proved to be useful in the behavioral investigation of anxiety and stress [38].

Spatial Memory Assessment

The modified BM protocol used here was previously documented [15, 39]. In brief, it consists of an elevated black acrylic circular platform, 122 cm in diameter, containing 20 holes around the periphery. The escape hole is numbered as hole 0 for graphical normalized representation purposes, while the remaining holes are numbered 1 to 10 clockwise, and −1 to −9 counterclockwise. A 90-dB white-noise generator and a white-light 500-W bulb provided the escape stimuli from the platform. At the beginning of the experiment, rats were habituated to the task. An AT consists of placing a rat in the starting chamber, located at the center of the platform for 30 s; the chamber is then raised, the aversive stimuli (bright light and high pitch noise) are switched on, and the rat is allowed to freely explore the maze for 120 s. The purpose of ATs is to train the rats to find the escape box (hole 0). The PT is similar to an AT except that the escape box is removed; its purpose is to assess recent spatial memory retention in 120 s. Behavioral parameters assessed were as follows:

Spatial learning parameters:

Latency. Time a rat spent since its release from the start chamber until it enters the escape box (during an AT), or until the first exploration of the escape hole (during the PT).

Errors. Number of explorations of holes different from the escape one. Each exploration of an incorrect hole is counted as an error, provided that the rat lowers its nose below the plane of the table surface.

Spatial memory parameters:

Hole exploration frequency. The explorations number for each hole of the maze, during the PT.

Exploration frequency in goal sector (GS). The sum of explorations number for the hole 0 divided by the sum of total explorations number, during the PT.

The seeking behavior for the escape hole was used to categorize rats’ search strategies per trial based as follows [40]:

Direct-to-goal. Scored when all exploration-involved holes were within two holes from the goal and fewer than 3 total errors were made.

Serial search. Scored when at least 3 errors were made and 75% of holes explored were adjacent or within 1 hole of each other and when animals searched them in order.

Marble Burying Behavior

MB test is based on the observation that rodents bury either harmful or harmless objects (e.g., glass marbles) in their bedding [41]. Marble burying is considered as a species-typical behavior and has been related to hoarding in rats [41]. This test was carried out 94 days after icv-STZ injection. Individual subjects were placed in a housing cage (30 × 30 × 17 cm) with 5 cm of fresh hardwood chip bedding. An array of 16 glass marbles (1.5 cm in diameter, arranged in a 4 × 4 grid) was evenly spaced over the surface. The number of marbles buried during a 30-min period was analyzed. In this procedure, a marble was considered buried if at least 2/3 of the marble were covered with bedding [42].

Brain Processing and Immunohistochemistry

Brains were rapidly removed and divided into right and left hemispheres. The left hemispheres were fixed in phosphate-buffered para-formaldehyde 4% (pH 7.4) overnight at 4 °C. Afterwards, brains were reserved in cryoprotectant solution (30% ethylene glycol, 1% polyvinylpyrrolidone, 30% sucrose, in phosphate buffer 0.1 M, pH 7.4) at −20 °C until use for immunohistochemistry. For this aim, brains were cut coronally in 40-μm-thick sections with a vibratome (VT1000S; Leica Microsystems). From the right brain hemispheres, the hippocampus was carefully dissected as previously described [43] and stored at −80 °C until Western blot (WB) analysis.

Immunohistochemistry

All immunohistochemistry techniques were performed on free-floating sections. For each animal, separate sets of sections were processed using the following antibodies: goat anti-Doublecortin (DCX) polyclonal antibody (immature neurons marker; 1:250; Santa Cruz Biotech c-18, Dallas, TX); mouse anti-NeuN antibody (neuronal marker; 1:850; Millipore Cat#MAB377); rabbit anti-Iba1 polyclonal antibody (microglial cells marker, 1:1000; Wako Cat#016-20001); and rabbit anti-Gfap polyclonal antibody (astrocyte marker; 1:1500; DAKO Cat#Z0334). Briefly, after overnight incubation at 4 °C with the primary antibody, sections were incubated with biotinylated horse anti-goat antibody (1:300; Vector Laboratories Cat#BA-9500), goat anti-mouse antibody (1:300; Vector Laboratories Cat#BA-9501), and biotinylated horse anti-rabbit antibody (1:300; Vector Laboratories Cat#BA-9500).
Cat#BA-9200), or a goat anti-rabbit antibody (1:300; Vector Laboratories Cat#BA-1000), as appropriate, for 120 min, rinsed and incubated with avidin-biotin-peroxidase complex (1:500; Vector Laboratories Cat#PK-6100) for 90 min and then incubated with 3,3-diamino benzidinetetrahydro-chloride (DAB) (Vector Laboratories Cat#SK-4600). DAB revealed sections were counterstained with cresyl violet (Nissl staining) as described elsewhere [39].

Finally, sections were dehydrated and mounted with Vectamount (Vector Laboratories) and used for image analysis.
Image Analysis

As mentioned earlier, we focused our stereological assessment on NeuN(+)-mature neurons of the CA1, DCX(+)-immature neurons in the DG, and Iba1(+) microglial cells and GFAP(+) astrocytes cells in the SR. The SR upper limit is the CA1 pyramidal layer, lower limit is Stratum Lacunosum Molecular, and lateral limit is the Stratum Lucidum of the dorsal hippocampus [34]. The total number of cells was estimated using a modified version of the optical dissector method [6]. Individual estimates of the total cell number (n) were calculated according to the following formula: \( n = RQR \cdot \frac{1}{\text{ssf} \cdot \text{tsf}} \), where RQR is the sum of counted cells, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction, as previously described [39, 44]. All morphological parameters were assessed unilaterally in the left hippocampus. The microscopical analysis was performed with an Olympus BX-51 microscope attached to an Olympus DP70 CCD video camera (Tokyo, Japan). In each hippocampal block, one out of six serial sections was selected in order to obtain a set of non-contiguous serial sections spanning the dorsal hippocampus.

Volume of the Stratum Radiatum

In order to assess the volume of the SR, a stereological approach on rat brain coronal sections was carried out. Four NeuN immunoreactive (NeuNir) sections were sampled, with a separation of 240 µm along the anterior-posterior axis. Our region of interest was set up as the area delimited by the CA1 pyramidal layer as the upper limit, the Stratum Lacunosum Molecular as lower limit and a width of 520 µm as lateral limit, whose midpoint was the peak point of curvature in the pyramidal layer.

Immature Neurons Analysis

Doublecortin immunoreactive (DCXir) cell number was assessed in the DG, which includes the hippocampal subgranular zone and granular cell layer [34], as previously described in the “Volume of the Stratum Radiatum” section, with asf = 1, ssf = 1/6, and tsf = 1. Four DCX-stained sections per animal were sampled. Estimates were based on counting DCXir cell bodies as they came into focus.

Mature Neurons Analysis

Mature neurons were detected as NeuNir cells. We determined NeuN immunoreactive area (IRA) and considered a decrease within as an overt sign of sAD-mediated neurodegeneration. For this purpose, 5 sections per animal and 3 fields per section corresponding to NeuNir neurons in the CA1 pyramidal layer were digitally segmented using the Image Pro Plus v5.1 software (IPP, Media Cybernetics). For each calculation, background was determined by manually adjusting the system density window as previously described [45], until only the NeuNir neurons were selected. For each animal, the sum of immunoreactive areas was multiplied by the section sampling number (NeuNir area × 6).

Microglial Cell Analysis

Microglial cells were identified as Iba1 immunoreactive (Iba1ir) cells in the hippocampal SR. The Iba1ir cell number was estimated as previously described in the “Volume of the Stratum Radiatum” section, with asf = 0.387, ssf = 1/6, and tsf = 1. Five sections per animal and three corresponding SR fields per section were analyzed. Iba1ir cells were morphologically classified as types I, II, III, IV, and V based on previously documented criteria [15, 46]. Types I, II, and III were categorized as non-reactive glia, whereas types IV and V were considered reactive. The Iba1ir reactive and non-reactive cell percentage of each animal group was calculated.

Astrogial Cell Analysis

Morphological assessment of GFAP immunoreactive (GFAPir) astrocytes was performed in the SR. To estimate the GFAP IRA occupied by cell bodies and processes, GFAPir astrocytes were segmented as described in the “Microglial Cell Analysis” section; for each animal, the sum of immunoreactive areas was multiplied by the section sampling number (GFAPir area × 6). The total number of cells was estimated as previously described in the “Volume of the Stratum Radiatum” section, with asf = 1, ssf = 1/6, and tsf = 1. Five sections per animal and three corresponding SR fields per section were analyzed. Additionally, to study branching complexity, GFAPir astrocytes were submitted to the Sholl analysis [47]. The length of the astrocyte processes and their branching complexity at every distance from the soma were averaged, and these output data were used for the statistical analysis between groups as we have previously described [15].

Western Blot Analysis

Sample Preparation

In order to obtain protein lysates, right hemi-hippocampi were homogenized with precooled RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 8, and appropriate protease inhibitors, pH 7.4). Finally, protein concentration was measured by Bradford protein assay. Bovine serum albumin (BSA, 0.1–1 mg/ml) was
used as a standard. Samples were aliquoted and stored at −80 °C.

Immunoblotting

Equal amounts of protein (50 μg) for every sample were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked by incubation in 5% non-fat milk in Tris-buffered saline/Tween-20 (TBS-T) for 1 h at room temperature and then incubated with primary antibodies against synaptotagmin 1 (SYT1) (1:200; mAb 48 (asv 48), DSHB), synaptotagmin 2 (SYT2) (1:150; Znp-1, DSHB), synaptophysin (SYP) (1:200; Santa Cruz Biotechnology, sc-17750), synaptic vesicle protein 2 (SV2) (1:200; SV2, DSHB), glutamic acid decarboxylases (GAD) (1:200; Santa Cruz Biotechnology, sc-73614), and β-actin (1:1000; Santa Cruz Biotechnology, sc-47778), overnight at 4 °C. Then, membranes were washed with TBS-T and incubated with the secondary antibody conjugated with horseradish peroxidase (1:20000, Jackson ImmunoResearch Laboratories, Inc., #115-035-003) for 3 h at room temperature. After being washed with TBS-T, membrane visualization was performed with Super Signal West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific, #34577) on a Chemidoc Image Station (Bio-Rad, Hercules, CA, USA). Relative optical density of protein bands was analyzed using a gel documentation system. Sample loading for SYP and SYT1 was normalized to relative density of the Vinculin band. Sample loading for SYT2, SV2, GAD65, and GAD67 was normalized to relative density of the β-actin band.

Statistical Analysis

For BM latency, errors and search strategies analysis data from each AT were averaged; these parameters together with Iba1ir reactive and non-reactive cells were analyzed by two-way analysis of variance (ANOVA) with repeated measures. The remaining behavioral and stereological data were analyzed by one-way ANOVA. Tukey’s multiple comparison post-hoc tests were used where appropriate. All analyses were conducted by statistical software, GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

MSC Therapy Improved Exploratory, Anxiety, and Species-Typical Burying Behavior

The OF test is a widely used procedure for examining the behavioral effects of treatments and anxiety. In the OF test, STZ injection induced an increase in grooming behavior, which was reversed by the stem cell treatment (one-way ANOVA $F_{(2,18)} = 5.379; P = 0.0147$) (Fig. 1b) and a decrease in the number of quadrant crossings, also restored by the therapy (one-way ANOVA $F_{(2,18)} = 13.05, P = 0.0003$) (Fig. 1c). The STZ treatment led to a decrease in the number of entries to the inner area; the STZ + MSC group displayed a higher number of entries than STZ group, but this difference was not significant (one-way ANOVA $F_{(2,18)} = 3.858, P = 0.0403$) (Fig. 1d). In the marble burying test, the STZ group buried fewer marbles; this species-typical burying behavior was improved by the MSC therapy (one-way ANOVA $F_{(2,18)} = 12.72, P = 0.0004$) (Fig. 1e).

MSC Therapy Improved Spatial Learning and Memory

In order to evaluate hippocampal-dependent spatial behavior, we performed the BM test. The groups displayed no statistically significant difference in learning performance, as depicted by the ATs learning curves for both errors and latencies (two-way ANOVA group factor $F_{(2,18)} = 0.3304, P = 0.7229$; $F_{(2,18)} = 0.7565, P = 0.4837$, respectively) (Fig. 2a, b). Regarding memory evaluated in the PT, the STZ group made more errors than the SHAM control; interestingly, upon MSC therapy, the subjects made significantly fewer errors (one-way ANOVA $F_{(2,18)} = 4.944, P = 0.0194$) (Fig. 2c). The same tendency was observed for the PT latency, though no significant differences were found for this parameter (one-way ANOVA $F_{(2,18)} = 2.952, P = 0.0779$) (Fig. 2d). We also analyzed the escape strategies of the animals in the second trial of each experimental day. STZ group inclined towards a random strategy to escape the maze (Fig. 2e, f); the therapy promoted a shift of some STZ subjects towards a serial strategy (two-way ANOVA group factor $F_{(2,18)} = 4.167, P = 0.0326$). In order to get a clear rendering of the number of hole explorations of each animal during the PT, we constructed sunflower plots, from where we observed that the STZ subjects explored all holes at random to a greater extent. This behavior was restored by the MSC therapy, since the exploration was shifted to the goal sector (Fig. 2g). The STZ treatment caused a decrease in the percentage of exploration frequency in the GS; the STZ + MSC group displayed a higher number of this parameter as compared with STZ group, but this difference was not significant (one-way ANOVA $F_{(2,18)} = 4.413, P = 0.0276$) (Fig. 2h).

MSC Therapy Restored Hippocampal Volume and CA1 Neuron Count but Did Not Reverse Neurogenesis Fall

The sAD pathological process involves neuronal changes in the histological structure and volume of the hippocampus. Thus, we examined the hippocampal neuronal populations and the SR volume. STZ injection induced a frank decrease
in the SR volume, which was reversed by the stem cell therapy (one-way ANOVA $F(2,18) = 6.021, P = 0.0100$) (Fig. 3a–d). The same result was observed for NeuN (+) neuron immunoreactive area in the CA1 (one-way ANOVA $F(2,18) = 6.215, P = 0.0089$) (Fig. 3e–h). The analysis of DCX (+) immature neurons revealed that the STZ injection decreased neurogenesis rate dramatically, which was not reversed by the stem cell therapy (one-way ANOVA $F(2,18) = 82.42, P < 0.0001$) (Fig. 3i–l).

MSC Therapy Ameliorated Microgliosis but Not Astrogliosis of the Hippocampus

In order to evaluate the immunomodulatory effect of MSC in the brain, we studied the microglial and astroglial cells. We confirmed that STZ injection produced an overwhelming inflammatory gliosis. GFAP immunoreactive area assessment showed a vast increase in the STZ group; this astrogliosis could not be reversed by the MSC therapy (one-way ANOVA $F(2,18) = 30.53, P < 0.0001$) (Fig. 4a–d). In order to figure out whether the cause of this increment of immunoreactive area was hyperplasia or hypertrophy, we estimated GFAP(+) cell number. We did not find any significant difference in the GFAP(+) astrocytes number in the experimental groups (Supplementary Fig. S1A. One-way ANOVA $F(2,18) = 1.772, P = 0.1983$), suggesting that the mechanism of astrogliosis was hypertrophy, rather than hyperplasia.

In addition, we recorded no differences in SR branching complexity, neither in the mean length of astrocyte processes, nor in the number of branches emerging from the cell soma in the Sholl analysis (Supplementary Fig. S1B-D. Two-way ANOVA group factor $F(2,36) = 59.39, P < 0.0001$, respectively). This result indicates that entire arbors were conserved 3 months after STZ-icv administration.

By means of Iba1 immunohistochemistry, we recorded an important rise in the total number of Iba1 cells in STZ and STZ + MSC groups (Supplementary Fig. S2. One-way ANOVA $F(2,18) = 10.86, P = 0.0008$). As we evaluated cell morphology, STZ displayed a significant increase in the percentage of reactive microglia (activated stage) together with a decrease of non-reactive microglia (resting stage); these STZ-induced modifications were ameliorated by MSC therapy (two-way ANOVA group factor $F(2,36) = 59.39, P < 0.0001$) (Fig. 4e–h).
Synaptic Protein Levels After MSC Therapy

Since synaptic dysfunction of the hippocampus is characteristic of AD, we set out to evaluate whether MSC therapy restored the levels of relevant synaptic proteins in the sAD model. STZ rats displayed a significant reduction in SYT1 (Fig. 5d), SYP (Fig. 5e), and GAD 65 (Fig. 5i) protein levels, which was reversed by the MSC therapy (one-way ANOVA $F_{(2,18)} = 1.195$, $P = 0.0067$; $F_{(2,18)} = 7.792$, $P = 0.0036$; $F_{(2,18)} = 6.019$, $P = 0.0100$, respectively). SYT2 protein level was decreased in STZ rats, while STZ + MSC group did not differ from the SHAM control (one-way ANOVA $F_{(2,18)} = 5.350$, $P = 0.0150$). Of note, we did not find any significant difference in SV2 and GAD67 protein levels (one-way ANOVA $F_{(2,18)} = 3.101$, $P = 0.0696$; $F_{(2,18)} = 2.962$, $P = 0.0772$).
Discussion

In the current study, we used icv-STZ injection in rats, a model widely accepted due to its recapitulation of several AD pathological features and behavioral phenotypes, which was previously validated by us and others [2, 7–16].

To the best of our knowledge, MSC administration in the icv-STZ rat model of sAD has been hitherto poorly investigated [48, 49]. Among the therapies, intracerebral engraftment strategies using MSC in animal models of neurodegenerative disorders, other than AD, have been shown to improve cognitive deficits [28, 30, 32, 50]. In fact, we have previously...
shown that icv-MSC therapy exerts neuroprotective effects [51] and cognitive improvement [51, 52] in the aging rat. In the present study, however, we chose a less-invasive route for delivery of MSC (intravenous), which allowed us to perform a long-term treatment employing repeated (4 in 10 weeks) MSC injections in the AD model. Importantly, in order to minimize the risk of emboli formation, we resuspended the cells in a large volume of saline and injected them very slowly and carefully at a rate of 10 μl/s [53].

Recent works have shown the safety of this injection paradigm [53], with clear clinical advantages over direct central nervous system (CNS) administration [54]. Thus, in a study in cynomolgus monkeys, no stem cell transplantation toxicity was found after umbilical cord MSC injection once every 2 weeks, for 6 weeks [55]. Most importantly, in a phase I clinical trial, a research group evaluated the safety of intrathecal and intravenous transplantation of autologous bone marrow cells in children with cerebral palsy. They were evaluated for motor and cognitive functions, and finally by MRI, which showed this procedure was safe [56].

Furthermore, other studies have indicated that iv-MSC can migrate and localize into brain regions in different models of ischemic stroke [57, 58], Huntington’s disease [59], and AD [60]. Moreover, some initial research suggested that MSC engrafted at the site of injury could differentiate into neuronal cells [61–63]. However, the importance of engraftment and frequency of transdifferentiation remains controversial. In fact, it is also known that iv administered MSC accumulate in the lungs and have a short systemic survival time [64]. For that reason, only a small percentage of implanted MSC could reach the inflamed tissue, suggesting that their therapeutic action might be due to the bioactive molecules with immunomodulatory and trophic activities that they secrete [65, 66]. More recently, it has been also demonstrated that monocytes and neutrophils contribute to the clearance of MSC from the lungs by phagocytosis and, subsequently, these cells migrate via the blood stream to other body sites. In turn, phagocytosis of MSC induced functional changes in monocytes, which then modulated the adaptive immune cell compartment [67]. In line with this, previous studies have indicated that MSC lead to neuroprotection via interaction with non-neurological organ systems such as the spleen in traumatic brain injury [68]. Taking all these into account, we suggest that the MSC therapeutic effect could be mediated by soluble factors or other cells such as microglial cells or monocyte/macrophages.

During the OF test, the natural tendency of animals in a new environment is its exploration. In line with previous findings that demonstrated the exploratory and anxiety behavior decline in this model [69, 70], rats receiving STZ presented higher grooming time and less crossing activity, indicative of their poor exploratory behavior. We also observed an increase in their anxiety behavior, as represented by the lower entries to the inner area. MSC rats improved these parameters as they were similar to those of the control group, corroborating the beneficial effect exerted by MSC therapy against exploratory and anxiety behavior impairment. It is worth noting that, before the initiation of cognitive assessment, we evaluated motor performance and no significant differences were found in icv-

![Fig. 5](image-url)
STZ animals (data not shown), in accordance with our previous studies and others [15, 71–73].

Deterioration in the ability to perform “activities of daily living” is an early sign of AD. It has been proposed that the species-typical behavior in laboratory rodents could be considered equivalent to these human activities [74, 75]. Rats bury familiar and unfamiliar objects (marbles) placed into their home cages as a species-typical behavior [41, 75]. Recently, it was observed that the integrity of the hippocampus is vital for the performance of species-typical tasks of mice [74]. In this work, icv-STZ leads to decreased burying in the MB test. In line with this observation, previously reported results showed that transgenic AD mice (Tg-APP/PS1) buried fewer marbles than non-transgenic mice [76]. Importantly, MSC therapy significantly ameliorated the marble burying behavior in STZ rats. Therefore, we suggest the treatment improves hippocampal function, which is consequently reflected in this species-typical behavior recovery.

We observed that, 3 months after icv-STZ, BM spatial learning of the rats was fairly preserved; contrarily, STZ rats showed an affected spatial memory, as evidenced by a rise in errors and an increasing trend of latency in the PT. These results are in accordance with our previous study performed within a month after icv-STZ [15] and also replicated the spatial memory deficits observed in the Morris water maze [77, 78]. The present results demonstrated that MSC treatment was able to improve the aforementioned STZ-induced memory deficits observed in the PT. Moreover, we found that MSC therapy showed a tendency to restore goal seeking activity in the strategies carried out along the ATs and PT (i.e., less use of random search strategy) and improved memory retention during the PT, two components that contribute to the magnitude of the goal hole exploration frequency.

Several studies proposed that volume and neuronal loss in CA1 of the hippocampal formation are early hallmarks of AD and are strongly correlated with cognitive status [3, 4, 15, 79, 80]. In a recent report, STZ induced neurodegeneration in the hippocampus, particularly in the CA1 area, 21 days after icv injection [81]. In our previous work, we similarly observed a decrease in NeuN immunostaining in STZ rats, which we suggested to be related to neuronal death in the dorsal hippocampus [15]. In accordance, in the present work, our results show that 3-month icv-STZ induces a SR volume reduction and CA1 pyramidal neurons loss. Interestingly, we observed that MSC therapy restored both SR volume and neuron loss. It is likely that the paracrine signaling of MSC could ameliorate structural SR and CA1 atrophy of STZ-induced hippocampal neurodegeneration and cognitive impairment.

The DG niche of adult born neurons is susceptible to deterioration under several factors, e.g., aging and AD [44, 82]. In this regard, the STZ injection produced an overt decrease in the neurogenesis rate, which had been previously observed [83, 84]. Since MSC therapy improved cognitive function of several and varied orders, our results give rise to two theses: either DG adult neurogenesis is dispensable to carry out the cognitive tasks hereby evaluated, or, if not, therapy is able to circumvent or compensate for this hippocampal feature by reactive microglia regulation and the protection of mature neurons and synapses. Further studies are needed to investigate these possibilities in detail.

It is well-established that neuroinflammation plays a significant role in AD [85–88]. In the CNS, astrocytes and microglial cells are often activated in neurodegenerative diseases. As mentioned above, the icv-STZ caused a frank increase in hippocampal gliosis, which is in line with previous studies at different time points post injection [15, 89–91] and it seems to contribute to cognitive deficits in the STZ-induced sAD model [83].

In a previous report, 25 days after STZ-icv, we observed an increased GFAPir area without change in GFAPir cell number [15], a similar effect observed in the present study, 95 days post injection. This suggests that, in this STZ model, hippocampal astrocytes are hypertrophied. In this line, AD patients were found to display GFAP hyper-reactivity with signs of cellular hypertrophy in the hippocampus [92] and in the cerebral cortex [93]. Based on our current work, MSC therapy did not exert a pronounced effect on the astrocytic population.

One relevant feature of AD and the senile brain is the progressive development of reactive microglia [94, 95]. Previously, we evaluated the effect of MSC on the senile rat brain, where we found that MSC therapy did not change the total number of microglial cells but reduced the proportion of reactive cells instead [51]. In accordance with this previous work, iv-MSC therapy decreased the reactive microglial cells, although it actually failed to reach the SHAM levels. Thus, we suggest that the neuroprotective effect of MSC could be mediated by the conversion of microglial cells to a different phenotype. It is known that microglial activation is heterogeneous and has been categorized into two opposite types: pro-inflammatory M1 phenotype (classical activation) and anti-inflammatory M2 phenotype (alternative activation) [96].

In line with this hypothesis, MSC have been shown to reprogram M1-to-M2 switching in microglia and to improve neuron survival [30, 97, 98]. Additionally, it has been reported that MSC are able to maintain the resting phenotype or to control microglial activation through their production of several factors [99]. MSC have also been shown to inhibit activated microglia proliferation in vitro by modulating the cytokine response [100].

Consequently, MSC impact on microglia, either by secreting paracrine molecules or by direct cell-to-cell contacts, can cover different aspects given the multiple MSC’s capacities. Thus, the aforementioned anti-inflammatory/immunomodulatory [17–19, 97, 100–102] and regenerative [103, 104] properties could inhibit M1 microglia proliferation, whereas activated microglia could be converted to the M2 phenotype.
Since the determination of cytokines, M1/M2 microglia population, and MSC integration were beyond our scope, further studies will be needed to elucidate the effect of MSC on microglia.

It has been established that synaptic loss, a widely characterized feature in AD, is a major correlate of cognitive impairment [105], suggesting a causal role for dwindling synaptic integrity in the etiology of the disease [106–108]. In the present study, we observed decreased levels of presynaptic proteins in the hippocampus of STZ animals. Interestingly, MSC treatment restored the levels of SYT1, SYP, and GAD65 synaptic markers, indicating that the cell therapy confers protection against synaptic proteins loss. Although the specific mechanism remains to be further studied, the synaptic proteins reduced in STZ group are known to affect the neuron transmitter release, thereby affecting the physiological functions of the neurons [108–112]. As we discussed before, the hippocampal volume is highly involved in behavioral functions. Consequently, cell therapy presumably restores the physiological functions of the hippocampal neurons by protecting the synaptic integrity.

It was previously shown that microglial processes make intimate but transient connections with neuronal synaptic elements [113, 114]. In our work, we observed a long-lasting hippocampal microglial activation in the STZ rats, which has been shown, to be related to pro-inflammatory cytokines release by other studies. This in turn contributes to cognitive function impairment [115–118]. MSC treatment prevented cognitive damage in the STZ rats, as demonstrated in the behavioral tests, whose mechanism we speculate to be related to reduced inflammation and synaptic protection.

A diabetes rodent model widely used consists of intraperitoneal (ip) injection of STZ. This induces a vast increase in blood glucose levels and similar effects as our icv-STZ model in the brain and cognition. Two recent studies on this model have revealed interesting findings, mostly in line with our current work. Thus, in one work, bone marrow-derived mesenchymal stem cells were iv injected in STZ ip injected mice [119]. This cell therapy led to a shorter latency in finding the target quadrant in the Morris water maze, as well as to a higher time spent in the target quadrant. In the hippocampal CA1, they found the therapy increased NeuN cell count, SYP levels, and decreased Iba1 total cells, thus counteracting STZ effects. Interestingly, they observed the same effects in the hippocampus by replacing the cell therapy with an iv injection of the purified exosomal fraction of the stem cells, suggesting that the therapeutic effect was mediated by secreted factors inside the exosomes. The same group published 2 years later a study which showed that an enriched environment (EE) alleviated cognitive deficit and restored CA1 NeuN immunoreactivity and SYP levels in the ip-STZ model. The authors suggested a mechanism for this effect would be an anti-inflammatory action of endogenous bone marrow MSC-derived exosomes, which, under EE, produce great amounts of miR-146a [120]. Even though this is a different model of cognitive deficit, it provides strong evidence that MSC exosomes carry relevant neuroprotective molecules, which is of great interest towards novel therapies against AD.

Overall, the present data show that the iv-MSC injection is a promising therapy to restore behavioral decline, a characteristic symptom of sAD. Our study supports the concept that the neuroprotection of the injected MSC is exerted by their restorative capacity on structural hippocampal atrophy, neurodegeneration and, partially, microgliosis. STZ-activated microglia release a variety of pro-inflammatory and cytotoxic factors whose accumulation is thought to contribute to the loss of neurons. However, a relevant finding is that hippocampal microgliosis may play a significant role in burying behavior recovery together with an improvement of synaptic damage.

The development of MSC therapy for neurodegenerative diseases is still in its early stages and future studies should be pursued to unravel specific mechanisms of this promising treatment. In this regard, based on recent and growing evidence, MSC exosome therapy would provide a fair trade-off between treatment efficacy and animal cell engraftment-related risks.

Conclusion

In summary, the results obtained from our study reveal the effectiveness of repeated intravenous administration of human umbilical cord MSC to treat behavioral impairment and neurodegeneration caused by icv-STZ in rats, at 3-month post-injury. Our data demonstrate a MSC potential in the treatment of neurodegenerative diseases such as sAD by regulating microglial activation and rescuing hippocampal volume reduction, mature neuron loss, and synaptic proteins levels.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.
Statement on the Welfare of Animals All applicable international, national, and/or institutional (INIBIOLP’s Animal Welfare Assurance #A5647-01) guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. IACUC, Protocol #P03-03-2016.

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