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Mesenchymal Stem Cell-Derived Exosomes for Treatment of Autism Spectrum Disorder

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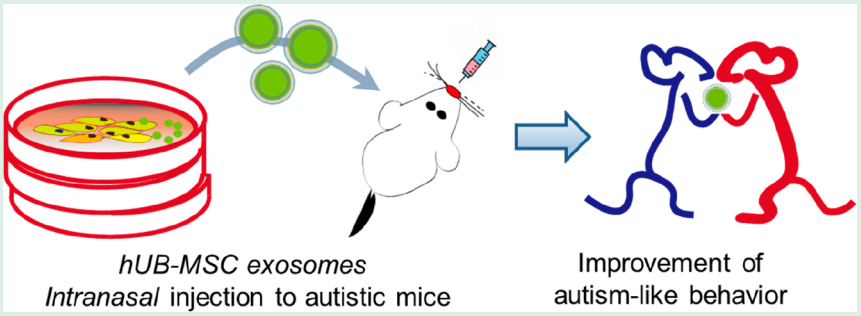

ABSTRACT: Recent breakthroughs in the field of stem cell therapy have brought hope to the treatment of mental diseases. Animal experiments and clinical studies have shown that transplantation of mesenchymal stem cells (MSCs) has a positive effect on the treatment of autism spectrum disorder (ASD). However, the therapeutic efficacy of the MSC transplants was primarily associated with the signals and molecules secreted by the MSCs. Exosomes, for example, the secreted organelles from MSCs, carry bioactive molecules of the MSCs that are essential for the therapeutic effects in ASD treatment. This then inspires us to explore the intranasal delivery of MSC exosomes to brain tissues for the treatment of ASD. Exosomes from human umbilical cord mesenchymal stem cells (hUC-MSCs) that efficiently enter the brain tissue through the intranasal route restore the social ability of the mice and correct the repeated stereotyped behaviors and other abnormal phenotypes in the offspring of valproic acid (VPA)-treated mice, which show autism-like symptoms. The therapeutic efficacy can be attributed at least partially to the anti-inflammatory effect of the MSC exosomes. This work thereby reports brain-specific delivery of hUC-MSC exosomes, as a cell-free therapy to relieve autism-related phenotypes, providing a promising direction for the treatment of mental development disorders.

KEYWORDS: *exosome, human umbilical cord mesenchymal stem cells, autism spectrum disorder, intranasal, inflammation*

INTRODUCTION

Autism, also known as the Autistic Spectrum Disorder (ASD), is a type of neurodevelopmental disorder that begins before the age of 3 and is characterized by social communication disorders; stereotyped, repetitive behaviors; and so on.[1] The incidence of autism from the past decade has increased significantly, now at a rate of nearly 200 000 cases per year. In 2018 alone, 1 in 59 children in the United States has autism, and the number of autistic patients in China currently exceeds 10 million, including more than 2 million under age of 14.[2,3] Due to the severe blockages in the neurological and mental development process, 75% of the typical autistic patients have mental retardation, and 50%−70% of the patients have lifelong developmental disabilities in adulthood, causing a huge economic and mental burden on society and families.[4−6]

Although the prevalence of autism is increasing, effective prevention and treatment options are still lacking. Research on autism has focused on finding genetic factors related to the corresponding neurodevelopmental abnormalities. However, due to the unclear pathological mechanism and the complex diversity of the disease, treatment of autism at present mainly relies on special education (including sensory integration training, game training, and structured education therapy) to improve children's skills in various aspects and promote their environmental adaptability and self-care ability.[7] The adjuvant drug therapy is limited to the control of certain symptoms and fails to fundamentally reverse the condition of autistic patients.[8] Other therapies, for example, targeting intestinal microbes using probiotics or fecal bacteria transplantation have also attracted attention.[9] However, most therapies cannot effectively improve the core symptoms of autism. Furthermore, currently no drugs are approved by the FDA at this stage.

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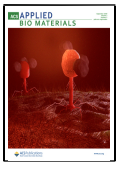

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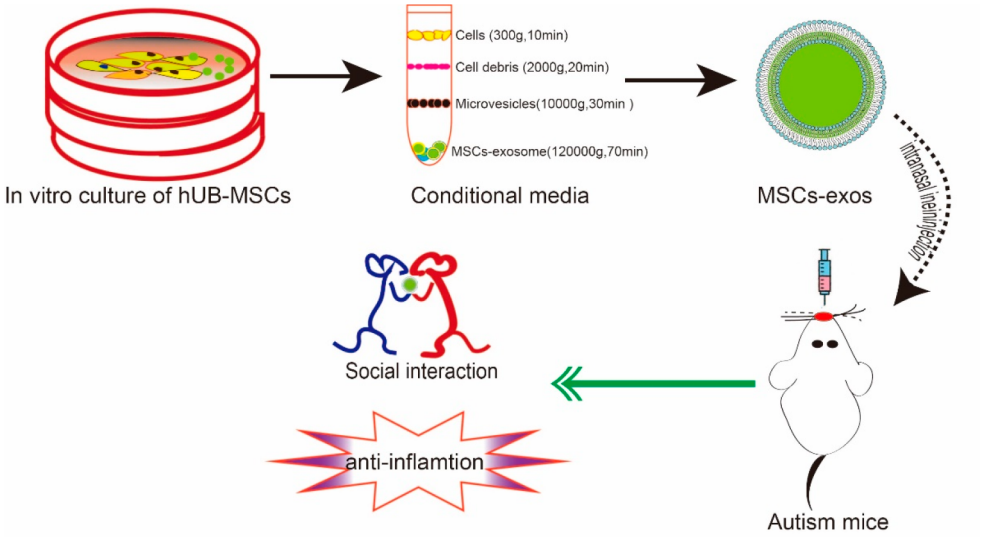
Scheme 1. Schematic Illustration Showing That MSC-Derived Exosomes through Intranasal Administration Ameliorate Autism-Like Behaviors in a Mice Model



In recent years, breakthroughs in the field of stem cell therapy have brought new hope for patients of mental diseases. Mesenchymal stem cells (MSCs) are derived from early emerging mesoderm; because of their multidirectional differentiation potential and immunomodulatory effects, MSCs have been used in regenerative medicine. Ha et al. reported that transplantation of adipose-derived MSCs into the brain of newborn pups can reduce repetitive and anxious behaviors and improve social defects in mice exposed to valproic acid (VPA) before birth.[10] Another study showed that MSC transplantation can restore hippocampal neuronal development in mice exposed to VPA.[11] Also, transplantation of human-derived MSCs outside the brain of BTBR autistic mice can improve their core ASD behaviors, including stereotypes, cognitive rigidity, and social behavior.[12,13] Fabio et al. proposed a new treatment plan by combining expanded umbilical cord blood cells and MSCs to treat autism, and this method has entered clinical trials. The latest clinical data disclosed in 2019 now show that after intravenous injection of MSCs in 8 autistic children, the CARS (Child Autism Rating Scale) score and ATEC (Autism Treatment Evaluation Checklist) score significantly decreased, and the symptoms of autism have significantly improved without noticeable side effects.[14] Dawson et al. conducted a phase I clinical trial of a single intravenous injection of autologous stem cells in 25 children aged 2 to 5 years with ASD. Clinical characterization found that most of the ASD behaviors improved significantly within the first 6 months.[15]

Notwithstanding such promising effects, a noticeable observation of MSC therapy, however, is that only a small amount of MSCs actually proliferate and differentiate into the desired cell type at the transplantation site.[16] The therapeutic efficacy of transplanted MSCs also is independent of the physical contact between MSCs and the local tissue. Therefore, it is believed that MSC functions through secreted molecules or vesicles, especially exosomes. Exosomes are membrane-enclosed vesicles secreted from cells with sizes of 30−150 nm and play important roles in cell−cell communication and the regulation of intercellular signals by delivering miRNA, mRNA, and proteins from the donor cells to the recipient cells.[17,18] Therefore, similar as MSCs, exosomes from MSCs (MSC-Exos) can suppress inflammation, restore key cellular functions, initiate tissue repair and regeneration, and contribute to the treatment of wounds, inflammations, bone and joint diseases, and so on.[19,20]

Treating diseases related to the central nervous system with exosomes derived from stem cells as a surrogate of stem cell therapy offers multifold advantages. A recent study showed injection of neural stem cell-derived exosomes into the lateral ventricle can increase the proliferation of hippocampal neurons, and stimulate synaptic growth and neural circuit development in the rat brain.[21] In addition, MSC-Exos can also reverse the neurodevelopmental defects caused by the MECP2 mutation in Rett syndrome, indicating that exosomes may have a therapeutic effect on neurodevelopmental disorders.[21] Inspired by these discoveries, here we envision that MSC-Exos can be used for the treatment of neurodevelopmental disorders, including ASD. Herein, we developed an intranasal delivery of exosomes from human umbilical cord mesenchymal stem cells (hUC-MSCs) and explore the effect on the autism-related behaviors in VPA-induced animal model (Scheme 1). Our results show that the exosome treatment rescues social deficits, reduces repetitive behaviors, and regulates neuroinflammation in VPA mice. Intranasal administration of MSC-Exos therefore holds promise as a new noninvasive, cell-free, and organelle-based therapy for ASD.

RESULTS

1. Characterization of hUB-MSC-Exos. The hUB-MSCs were extracted from umbilical cord tissue and exhibited a spindle-shaped morphology (Appendix, Figure S1). Some cell surface proteins have served as characteristic markers to define the identity of exosomes.[22,23] Expression of the cell surface antigens was probed by flow cytometry; a fluorescent signal of 10° has been set as the threshold of high expression. The results showed that the cells express very low level of CD34, CD45, and HLA-DR (<5%) and high level of CD73 and CD105 (>95%) (Figure 1A). Cells of the third passage were used for collection and purification of exosomes. Exosomes were collected by a sequential ultracentrifugation method (details are described in the Materials and Methods). The purified exosomes were characterized using transmission

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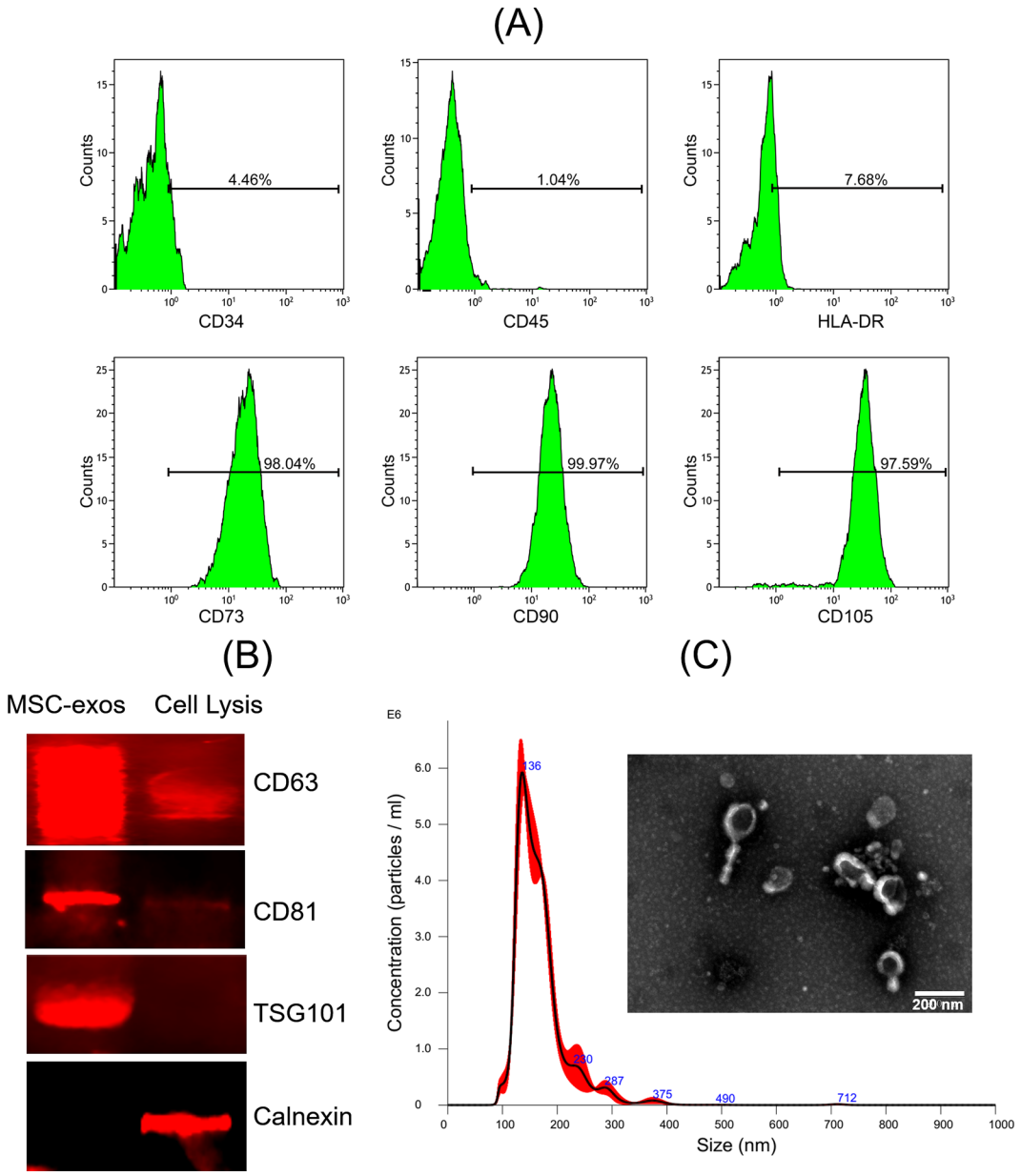

Figure 1. Purification of hUB-MSC derived exosomes (hUB-MSC-Exos). (A) Analysis of the hUB-MSC surface markers by flow cytometry. (B) Western blotting analysis of the purified exosomes showing enriched levels of exosome markers. (C) The morphology and size of hUB-MSC-Exos measured by TEM and NanoSight. Scale bar, 200 nm.

electron microscope (TEM), NanoSight, and Western blotting. Exosome markers such as CD63, CD81, and TSG101 were found (Figure 1B). TEM images showed that most particles exhibit a typical cup-shaped or round morphology. The size distribution was determined by NTA (Appendix, Figure S2). The average diameter of the exosomes was measured to be 136 nm (Figure 1C). These results indicate the successful culturing of hUB-MSCs and the purification of hUB-MSC derived exosomes (hUB-MSC-Exos).

2. Brain Uptake of MSC-Exos Following Intranasal Administration. Next, we explored the entrance of MSC-Exos into the brain through the intranasal route. Near infrared (NIR) imaging also showed that the intranasal administration delivered DiR-labeled MSC-exosomes into the brain, whereas tail vein injection primarily resulted in liver and kidney (Figure 2), consistent with the previous report.[24] Twelve hours after intranasal administration of Dil dye-labeled MSC-exosomes to mice, the brain tissue was collected. Cryosections (10 μm) were cut serially and subjected to a confocal microscope. Dil labeled exosomes were visible in the brain tissue (Figure 3A), indicating that the exogenous MSC-Exos could translocate from the nasal region to the brain. Dil-labeled MSC-Exos were found in both cortical and hippocampal cells primarily in the cytoplasm (Figure 3B,C). This work demonstrates that intranasal administration is an effective delivery route for MSC-Exos to enter the brain tissue.

3. Amelioration of Repetitive Behaviors in Mice Treated with MSC-Exos. Mice exposed to valproic acid (VPA) during pregnancy will cause sodium valproate syndrome in the offspring, which may cause nerve damage and behavioral defects, and exhibit deficit behavior resembling the core symptoms of autism. We first established an autism mouse model by exposing the pregnant mice to VPA by intraperitoneally injection of sodium valproate at 12.5 days.

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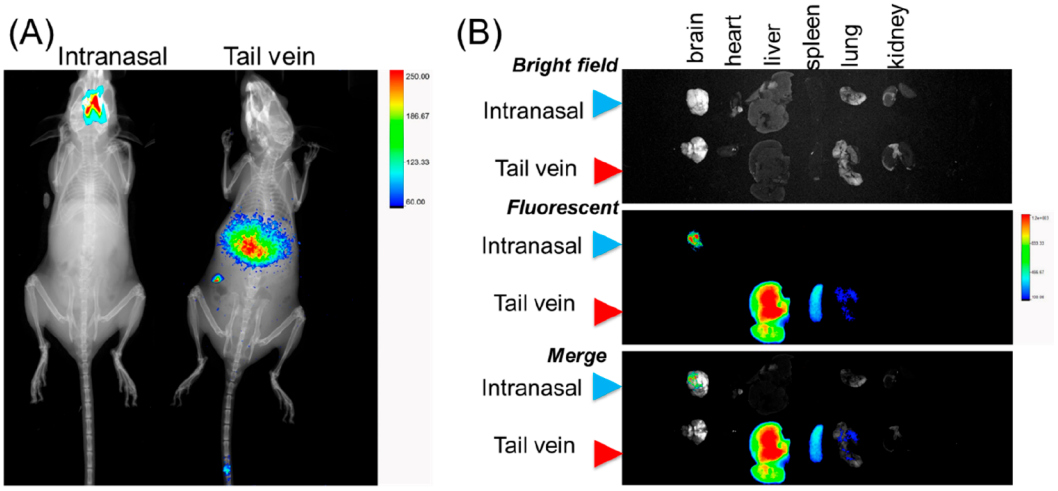

Figure 2. Intrabrain delivery of MSC-Exos through intranasal route. (A) NIR images of live mice administered with DiR-labeled exosomes through intranasal route and tail vein injection route 3 h after exosome administration, respectively. (B) Microscopic images of different organs in two administration routes shown as bright field, fluorescent images, and merged images, 12 h after exosome administration. Intranasal injection of MSC exosomes shown diffused into the brain, whereas tail vein injection to shown distribution primarily in the major organs.

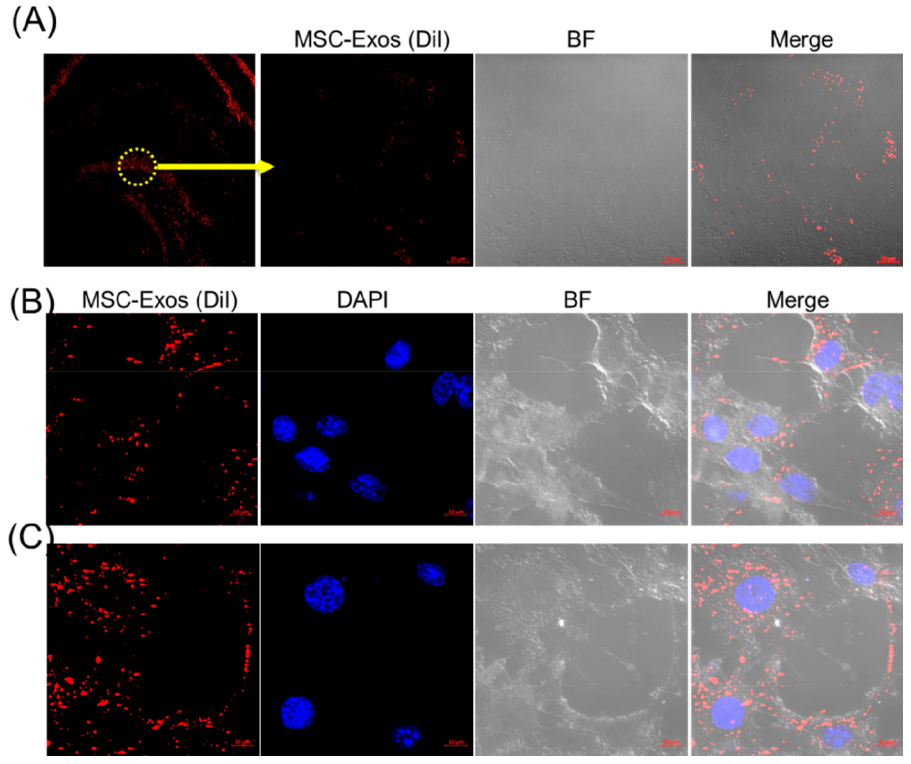

Figure 3. MSC-Exos into the brain through intranasal administration. (A) Representative cryosection images from the mouse brain. Representative images of cells from cortical (B) and hippocampal regions (C). Red channel showing Dil-stained exosome. Scale bars, 10 μm.

After screening, the male offspring were chosen as the autistic model for further study (VPA mice) (Figure 4A). VPA mice were randomly selected and divided into a model group and MSC-Exo treatment group, with 12 animals in each group. Twelve normal male mice were selected to form the control healthy group. The MSC-Exo treatment group were intranasally given 100 μg of MSC-Exos for 4 weeks each time, starting from the age of 6 weeks. The control healthy group were also treated with equal doses of MSC-Exos similarly. The model group were given equal volume of physiological saline.

VPA mice display various autism-like traits, including social deficits, repetitive behavior, and cognitive impairments, that recapitulate autistic syndromes in humans. For example, self-grooming is a typical repetitive behavior in autism mice. Control healthy mice did not display any noticeable differences in the amount of time spent on self-grooming after treatment with MSC-Exos. VPA mice exhibited significantly longer time on self-grooming. MSC-Exos treatment on VPA mice reduced the repetitive grooming behaviors (Figure 4B). Also, the repetitive rearing is analyzed as a measure of stereotyping. There was also a marked increase in the number of rearing

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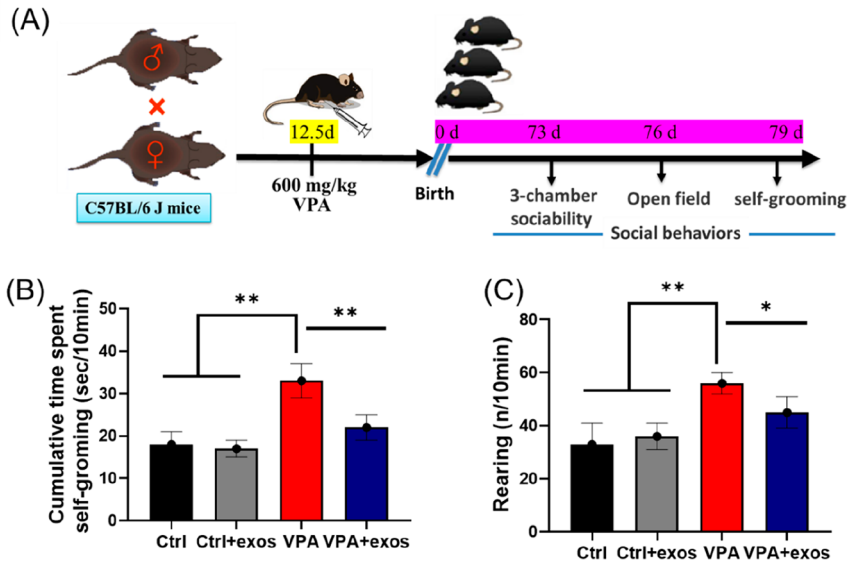

Figure 4. Amelioration of repetitive behaviors. (A) Schedule illustration of the prenatal valproic acid-induced mouse model of autism, and MSC-Exo treatment followed with behavioral studies and biochemical assessments. (B) Quantification of the cumulative time spent on repetitive self-grooming in 10 min. (C) Quantification of rearing frequency. Animals are from at least 8 litters. *, $P < 0.05$, **, $P < 0.01$, two-way ANOVA. Graphs represent mean ± s.e.m.

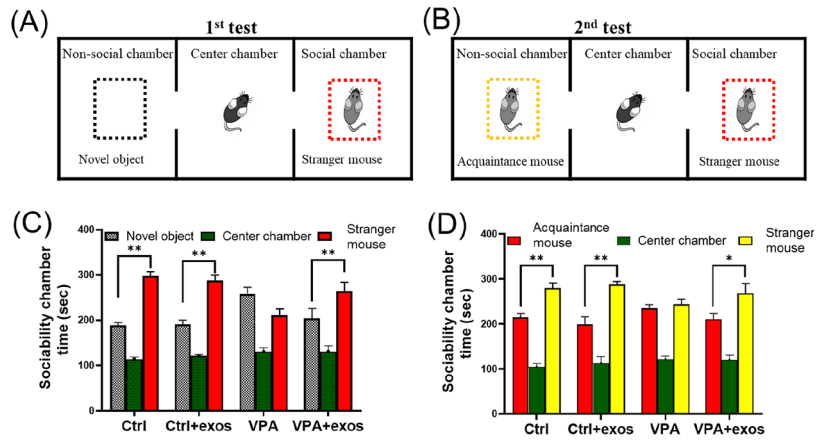

Figure 5. Behavioral studies of VPA mice in three-chambered social tests. (A) and (B) Schematic illustrations of the social interaction tests 1 and 2. (C) Quantification of the spent in the three chambers in Test 1 in 10 min. (D) Quantification of the spent in the three chambers in Test 2 in 10 min. *$p < 0.05$, **$p < 0.01$. $n \geq 8$.

episodes in the VPA mice compared to the control healthy animals. In comparison, MSC-Exos-treatment significantly reduced the number of rearing behavior (Figure 4C). Altogether, this set of experiments showed that MSC-Exo treatment alters the repetitive behaviors of autism mice.

4. Amelioration of Autism-Like Sociability Defect. A sociability defect is one of the most disabling symptoms in autism disorders. We first established a 3-Chambered Social Test to evaluate sociability. Briefly, this test is conducted in a three-chambered apparatus in which the test mouse could move freely between a right chamber which houses an unfamiliar mouse of the same age (stimulus mouse), an empty middle chamber, and a left chamber which contains either a novel object or another unfamiliar mouse. After 10 min of acclimatization, the test mice were allowed to explore all three chambers for 10 min. The time that the test mouse spent in the chamber containing the stimulus mouse versus the time in the empty chamber was measured as sociability (Figure 5A). The sociability test showed that VPA mice spent equal amount of time in the stimulus mouse chamber and the novel object chamber. The control healthy mice however spent significantly more time exploring the stimulus mouse than the novel object. In contrast, VPA mice showed no preference for the two-side chamber, which reflects the decreased sociability (Figure 5B). Interestingly, VPA mice treated with MSC-Exos spent significantly more time exploring the novel mouse 1 than the novel object ($p < 0.01$).

In a second experiment, we positioned an "acquaintance mouse"—a mouse that the test mouse is familiar with—in the left chamber and a "stranger mouse" in the right chamber. We then measured how the test mouse spends its time in the three chambers (Figure 5C). Similarly, control healthy mice spent more time on the "stranger mouse" than the "acquaintance mouse", but the VPA mice spent a similar amount of time on either chamber, showing a lack of social interest. MSC-Exo treatment reverted the trend of VPA mice and regained social interest in the "stranger mouse" (Figure 5D). Altogether, these sets of experiments show that VPA treatment induces a lack of

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social interest in the offspring mimicking autism-like social defects and MSC-Exo treatment effectively reverted this trend.

5. Spontaneous Exploratory Behavior in the Open Field (OF) Test. Next, we evaluated the spontaneous exploratory behavior of different groups of mice using the open field (OF) test. In the OF test, mice were allowed to freely explore a flat arena. The distance traveled and the time spent in the inner 14 cm-area in 10 min were was tracked and recorded (Figure 6A). No difference was found in the total

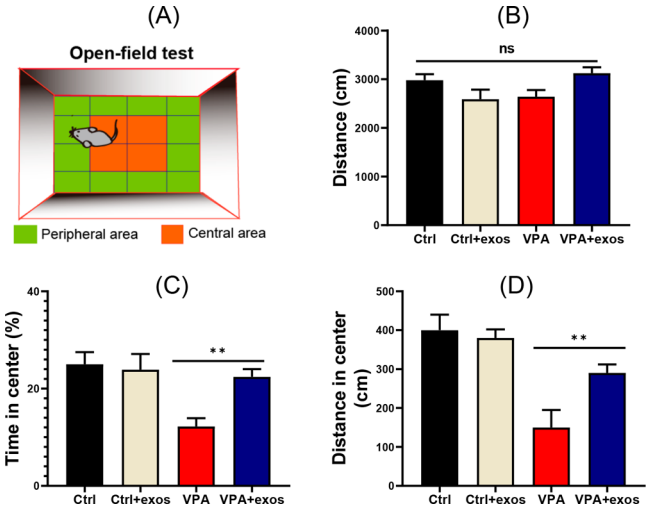

Figure 6. Anxiety measurement in the OF test. (A) Schematic illustration of the open field arena. (B) Total distances traveled in the OF test. (C) Quantification of the time spent in the center area. (D) Quantification of the distance traveled in the center area (D). Results shown are mean ± SEM ns, no significance. $^{**}p < 0.01$. $n \geq 8$.

distance traveled in the field between the four groups, suggesting that VPA mice or exosome treatment did not impair the locomotor activity (Figure 6B). However, when we measured the distance and the time spent in the center area of the open field, the VPA mice showed significantly lower preference in the center, but rather staying in the periphery area, which suggests an anxiety-like behavior. However, the MSC-Exo treatment reverted the flexibility of the VPA mice (Figure 6C,D). The results indicate that treatment with MSC-Exos could reduce the anxiety-related behaviors in VPA mice. Although not a core symptom, hyperactivity is occasionally reported in individuals with autism.

6. Inhibition of Inflammation in the Brain. Autism is known to associate with the overproduction of pro-inflammatory cytokines (i.e., IL-1β).[25] These cytokines can trigger cellular inflammatory responses. One of the possible effect of the transplanted MSCs was due to their anti-inflammation properties.[26,27] We next explore whether MSC-Exos could provide anti-inflammatory and neuroprotective activity in the brain, as one possible explanation of the antiautism effect. We assessed an array of inflammatory cytokines in the brain tissue homogenates at the end point of the experiment. The proinflammatory cytokines IL-1β, TNF-α, and IL-6 were found to significantly increase in the VPA mice as compared with the control healthy group (Figure 7A–C). Intranasal administration of MSC-Exos significantly reduced the level of these proinflammatory cytokines. In contrast, the production of the anti-inflammatory cytokines IL-10 in VPA mice was increased in the MSC-Exo treatment

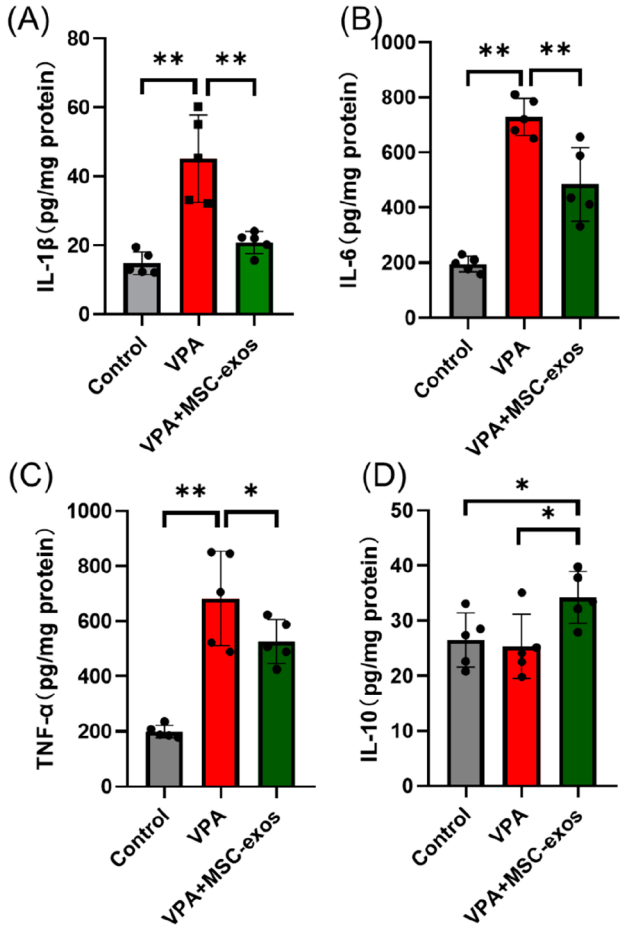

Figure 7. Pro-inflammatory and anti-inflammatory factors in different mouse groups. The expression levels of IL-β (A), IL-6 (B), TNF-α (C), and IL-10 (D) of a control group, VPA mice group, and VPA mice treated with MSC-Exo group were compared. $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$.

group. Taken together, we reason that one of the possible explanations of the therapeutic effects of MSC-Exos on the autistic phenotypes in VPA model is through the inhibition of inflammation in the brain.

CONCLUSIONS

Autism affects the social ability of children and adults to varying degrees. Although scientists have discovered hundreds of genetic variations associated with autism, it is still difficult to clearly analyze the causes of autism from the perspective of genetics, biochemistry, or other environmental factors. Each patient has one or more genetic mutations, and a common pathological pathway has not been found, which makes the development of targeted therapeutics a difficult task. In addition to small molecule drugs, stem cell-derived exosomes or macrovesicles have been reported to be new therapeutic candidates of autism.[18] Delivery of the nanosized exosomes to the brain is a critical step. Recently, intranasal administration has been shown to be an effective and noninvasive route to deliver exosomes into the brain, and achieve beneficial treatment toward Alzheimer's disease, status epilepticus, and other neuro-disorders.[28−30] Also this method allows small molecule drugs, for example, anti-inflammatory drugs encapsulated within exosomes to be delivered into the brain to treat inflammation.[24]

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In comparison with MSCs from bone marrow, fat, synovium, synovial fluid, umbilical cord, placenta, and other tissues, umbilical cord mesenchymal stem cells are easy to isolate, culture, expand, and purify. Another important feature of umbilical cord MSCs is the ability to secrete immune blocking factors such as IL-I0 and TGF-β to suppress pathological immune responses.[31−33] Therefore, the umbilical cord-derived exosomes used in this study are potential reagents as cell-free, noninvasive therapies.

Our results here suggest that MSC-Exos regulate neuroinflammatory factors in the brain. Existing studies have shown that exosomes from the serum of patients with ASD cause human microglia to release more proinflammatory cytokine IL-1β.[27,34] Triggering the neuroinflammation is therefore possibly responsible for the pathophysiology of ASD, so exerting inflammation inhibition through exosomes derived from stem cells may be a possible mechanism for exosomes to exert therapeutic effects. Our current study demonstrated that MSC-Exos could ameliorate autistic-like repetitive behavioral phenotypes and social defects. Although much more remains to be resolved, our work here presents a possible route for a noninvasive treatment of ASD. Comparing with MSCs, MSC-Exos are more stable, easier to store and to transport, and also a better clinical choice due to the advantages in immunogenicity, oncogenicity, clinical safety, and ethics.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). All mice were cultivated in a room controlled at 22 °C, light: dark period of 12 h: 12 h, with free access to drink water and standard laboratory diet.

Culture of Human Umbilical Cord MSCs (hUB-MSC). Umbilical cord tissue near the placenta was removed after cesarean section as previously set by our lab.[35,36] The umbilical cords were separated and cut into small pieces after removing the outer membrane and blood vessels, digested with collagenase II for 6 h, centrifuged, and subjected to adherent culture to obtain primitive hUB-MSC cells. Antibodies used in the FACS analysis of surface mark include antihuman CD45 (#555482, BD biosciences), mouse antihuman CD90 (#561969, BD biosciences), and mouse antihuman CD73 (#562430, BD biosciences).

Purification and Identification of Exosomes. Supernatants of hUB-MSC cell culture were collected and ultracentrifugated sequentially at 300*g* for 10 min, 2000*g* for 20 min, 10 000*g* for 30 min, then followed by ultracentrifugation at 120 000*g* for 70 min at 4 °C to isolate pellets that contained exosomes. The EVs were resuspended with PBS and ultracentrifuged again at 100 000*g* for 70 min and then suspended. The concentration of exosomes was measured by bicinchoninic acid assay (BCA, Thermo). Nanoparticle tracking analysis (NTA; NanoSight NS300, Malvern Instruments, Malvern, U.K.) were used to analyze particle size and distribution. The exosome pellet was resuspended in PBS at a concentration of ∼1 μg protein/ml. After further dilution, the exosome preparation was injected into the instrument and videos were recorded for 60 s per sample (n = 5). The ultrastructures of exosomes were staining with uranyl acetate and characterized by transmission electron microscopy (TEM, JEM-1200EX, Japan). The surface markers (CD9, CD63, and CD81) of the exosomes were analyzed by Western blotting to detect the exosomal surface markers correspondingly.

Western Blotting. Proteins were extracted from exosomes or cells in modified RIPA lysis buffer (50 mM Tris pH 6.6, 1 mmol/LEDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, %Triton X-100) containing a mixture of proteinase inhibitors on ice. Protein concentration was determined by a BCA Kit. Equal amounts of proteins were subjected to 10% SDS-PAGE, transferred to a Immobilon-FL PVDF membrane (Sigma-Aldrich, MO, U.S.A.), and incubated with the following primary antibodies: CD63 (cat no. ab216130, Sigma-Aldrich), 1:1000; CD81 antibody (cat no. ab109201, Abcam), 1:1000; anti-TSG101 antibody (cat no ab30871, Abcam), 1:1000; then IRDye 680 conjugated goat antirabbit IgG (H + L) (LI-COR Biosciences, NE, U.S.A.) was used as the secondary antibody. The fluorescent signals were visualized with the Odyssey Imaging System (LI-COR, Bad Homburg, Germany).

Tracking of MSC-Exos in the Brain. For the evaluation of exosome uptake in the brain tissue after treatment, exosomes were labeled with Dil or DiR dyes. DiR (1,10-dioctadecyl-3,3,30,30-tetramethyl indotricarbocyanine iodide) and Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine perchlorate) are lipophilic dyes that fluorescently label exosomes by incorporating into the lipid bilayer. The dyes are weakly fluorescent in water but highly bright when inside the membrane. Briefly, exosomes were incubated with 1 μM DiR or Dil in solution (Molecular Probes, Eugene, OR, U.S.A.) at 37 °C for 90 min. Free dyes were removed from labeled exosomes by centrifugation through spin columns with membranes of MWCO 3000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Labeled exosomes were injected into the nasal cavities of mice. Coronal mouse brain sections (20 μm) were cut on a Leica cryostat (Leica Biosystems, IL, U.S.A.) at −20 °C. The cryosections were thaw-mounted in OCT solution, dried for 1−3 h at RT under a continuous stream of air, and thereafter stored at −20 °C or immediately detected by Leica TCS FLIM SP8 (Wetzlar, Germany) equipped with laser at 550 nm for the Dil label. Neurons derived from primary hippocampus and cerebral cortex after 12 h postintranasal injection Dil-MSC-exos brain were cultured in the wells of 24-well plates at 37 °C for 12 h. After they were washed with PBS, cells were fixed with 4% PFA for 15 min. The nuclei were stained with DAPI, and confocal microscopy was used to detect the fluorescent signals. For *in vivo* imaging, intranasal or tail vein injection of DiR-labeled hUB-MSC-Exos was recorded using Bruker Xtreme imaging system in live mice and in dissected organs.

Behavioral Testing. Behavioral testing was completed with mice of 10 to 11 weeks of age. Tests were performed with mice of the identical ages and at the same time point in the circadian cycle for all the mice. Each mouse was individually placed into the tested cages and allow to freely habituate for 10 min.

Open-Field Test. The device is divided into 16 cells; the 4 cells in the middle are the central cells, and 12 cells are the surrounding cells. During the adaptation stage, the tested mouse was put into the white acrylic open-field box (40 × 40 × 30 cm) for 10 min as described.[37] We recorded the time spent of tested mice cross the central grid and periphery areas in the open-field by video-tracking recording for 20 min. The cross-grid criterion is all four limbs cross the lattice border. Standing and climbing standards: both front limbs are off the ground and stay at least 2 s. Time spent in the center zone and

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the frequency to enter the center zone were tracked with Noldus EthoVision system.

Three-Chamber Test. The three-chamber test, developed for social interaction of animal, was performed as described previously.[38] Briefly, the subject mice were given 10 min habituation in the center chamber, followed by in all three apparatus for 10 min. After habituation, the stranger mouse (unfamiliar C57BL/6 with the same age) was placed in a small container located in the corner of one side chamber, and the object was placed in another empty container in the opposite side chamber. Subject interaction was recorded for 10 min.

Preparation of Brain Extract and Evaluation of Cytokine Levels. Mouse brain was homogenized in ice-cold lysis buffer (50 mM Tris−HCl, pH 7.4; 1 mM EDTA; 100 mM NaCl; 20 mM NaF; 3 mM Na3VO4; with 1% (v/v) Nonidet P-40; and protease inhibitor mixture). Lysates were centrifuged at 12 000*g* for 15 min at 4 °C, the supernatant was collected, total protein concentration was calculated by BCA Protein Assay Kit, and the lysate was diluted to the required concentrations. The levels of TNF-α, IL-1β, IL-6, and IL10 were quantified using commercially available ELISA kits (MultiSciences Biotech Co. Ltd., Hangzhou, China), following the manufacturer's instructions. A standard curve was generated for each plate and used as calibration curve to determine the concentrations of the indicated cytokines (Appendix, Figure S3).

Statistical Analysis. All statistical analyses were determined using GraphPad Prism (8.0.2; GraphPad Software, La Jolla, CA). Statistical significance comparing two groups with parametric data was assessed by a two-tailed *t* test and one-way ANOVA, followed by Holm's post hoc test. Error bars on plots represent ± s.e. A p-value < 0.05 was considered significance level.

Study Approval. Collection of the umbilical cord samples was approved by the Ethic Committee of Shenzhen Second People's Hospital. Written informed consents were acquired from all the umbilical cord donors. All the animal experiments were approved by the Ethics Committee of Shenzhen Institute of Mental Health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.0c00831.

> Morphology of MSCs from human umbilical cords; size distribution of the exosomes; calibration curves for the ELISA analysis (PDF)

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Y.L., J.X., and J.L. designed research; Y.L., X.X., X.L., M.L., H.C. performed research; Y.L., L.D., and J.L. analyzed data; Y.L. and J.X. wrote the paper.

Notes

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