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Original Article

# Human umbilical cord mesenchymal stem cell-derived exosomes promote neurological function recovery in rat after traumatic brain injury by inhibiting the activation of microglia and astrocyte

Lianxu Cui [a], Wei Luo [a], Wenkang Jiang [b], Haomin Li [a], Junrong Xu [c], Xiaocui Liu [c], Bingyun Wang [b], Jinhui Wang [c, *], Guoqiang Chen [a, *]

[a] The First People's Hospital of Foshan, Foshan, Guangdong Province, 528000, China
[b] School of Life Science and Engineering, Foshan University, Foshan, Guangdong Province, 528225, China
[c] Guangdong VitaLife Biotech Co., Foshan, Guangdong Province, 528251, China

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ABSTRACT

Traumatic brain injury (TBI) is a serious neurological disorder with increasing worldwide incidence. Emerging evidence has shown a significant therapeutic role of mesenchymal stem cells (MSCs) derived exosomes on traumatic brain injury with broad application prospects as a cell-free therapy. However, a comprehensive understanding of its underlying mechanism remained elusive. In this study, umbilical cord mesenchymal stem cells (UCMSCs)-derived exosomes (UC-MSCs-Exo) were isolated by ultracentrifugation and injected intraventricularly in a rat model of TBI. Our results showed that UC-MSCs-Exo promoted functional recovery and reduced neuronal apoptosis in TBI rats. Moreover, UC-MSCs-Exo inhibited the activation of microglia and astrocytes during brain injury, thereby promoting functional recovery. However, the effect of UC-MSCs-Exo on the content of plasma inflammatory factors in rats was not significant. Collectively our study suggested that UC-MSCs-Exo promotes the recovery of neurological function in TBI rats by inhibiting the activation of microglia and astrocytes, providing a theoretical basis for new therapeutic strategies for central nervous system diseases.


## 1. Introduction

Traumatic Brain Injury (TBI) is the physiological damage to the brain structure or function caused by external force and attributed to high morbidity and mortality [1,2]. Patients with TBI often present severe motor and cognitive impairment [3]. It has been reported that during TBI external forces directly damage neurons, glial cells, blood vessels, axons, and dendrites, resulting in excitatory cytotoxicity, mitochondrial damage, inflammation, brain edema, and increased intracranial pressure, ultimately leading to the cascade of multiple pathophysiological mechanisms [4]. However, most drug treatments are limited by the blood–brain barrier. Whereas treatments such as mild hypothermia, hyperbaric oxygen therapy, and decompressive craniectomy can only relieve secondary reactions, but cannot effectively reduce neuronal apoptosis [5]. Nonetheless, in recent years regenerative medicine i.e., stem cell therapy has shown significant therapeutic efficiency in the treatment of traumatic brain injury [6–8]. Particularly, Mesenchymal stem cells (MSCs) hold the advantage over other stem cells due to their multidirectional differentiation potential, rapid proliferation, and differentiation ability, easy isolation and culture procedures, and autologous transplantation without immunogenicity [9,10]. Besides, Umbilical Cord Mesenchymal Stem Cells (UC-MSCs) (derived from neonatal umbilical cord tissue) have been known to release various nutritional factors and cell repair factors, improve neurological function, reduce apoptosis, and promote angiogenesis, which suggest their significant repair effect on the neurological deficit caused by TBI [11–13], yet, the precise mechanism remained elusive.

* Corresponding authors.
*E-mail addresses:* wangjinhui@vtlife.cn (J. Wang), 13929981788@139.com (G. Chen).
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Recently reported studies have shown that MSC- derived exosomes can effectively improve functional recovery and promote the brain remodelling [14]. Nonetheless, exosomes (that are extracellular vesicles with lipid bilayer membrane structure and an average diameter of 40–150 nm) possess biologically active substances such as proteins, lipids, and nucleic acids, and plays an important role in intercellular communication, maintenance of homeostasis and the occurrence and development of diseases [15]. Compared with MSCs, exosomes have the characteristics of good biocompatibility, long in vivo half-life, and easy extraction and storage, so they have gradually become a research hotspot [16,17]. Thus, in the present study, Umbilical Cord Mesenchymal Stem Cell-derived Exosomes (UC-MSC Exo) were isolated and injected into the ventricle of TBI rats to study the mechanism through which UC-MSCs-Exo repair the TBI.

## 2. Material and methods

### 2.1. Isolation and identification of UC-MSCs-Exo

UC-MSCs were provided by Guangdong VitaLife Biotechnology Co., Ltd (Foshan, China). Briefly, the UC-MSCs in the 3rd-5th generation were cultured in an exosome-free complete medium [MEM-a (Gibco, USA), 10% fetal bovine serum (ExCell, China) and 1% double antibody (Gibco, USA)], the cell culture supernatant was collected, and the UC-MSCs-Exo were isolated by ultracentrifugation and purified using an exosome purification reagent and concentration system (Exojuice, WeinaBio, China). The morphology of exosomes was observed by transmission electron microscopy, the particle size and concentration of exosomes were analyzed by flow nanometer, and the specific proteins (CD9, CD63, CD81, and TSG101) related to exosomes were detected by Western blot.

### 2.2. Animal experiments and behavioral assessment following TBI

All experimental procedures were approved by the Laboratory Animal Ethics Committee of Foshan University, and the experimental protocol were performed according to the ethical guidelines of animal protection and welfare. For an animal model of TBI, forty-five SPF-grade female SD rats weighing 200–220 g, were purchased from the Guangdong Provincial Medical Laboratory Animal Center. Before creating the TBI model, the rats fasted for 9 h without food or water. Furthermore, SD female rats were randomly divided into 3 groups i.e., UC-MSCs-Exo group, TBI group, and Sham (open skull without injury to the meninges) groups, with 15 rats in each group. The TBI model was established using an improved Feeney free-fall method, where a 20 g weight fell from a height of 30 cm to hit the dura mater. After 24 h, rats in the UC-MSCs-Exo group were injected with UC-MSCs-Exo (200ug) in a volume of 20 ul; rats in the TBI group were injected with an equal volume of PBS; rats in the Sham group were not treated. To evaluate the neurological deficits of the rats in each group, the behavioral function of the rats was evaluated by mNSS on 3, 7, 14, and 21 days after modeling by the field researcher (who did not know the experimental animal grouping).

### 2.3. qRT-PCR

Brain tissue was extracted by triazole method, using Takara's PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) for reverse transcription into cDNA, and Takara's TB Green PCR Master reagent for real-time fluorescence quantitative PCR. The operation steps were carried out according to the manufacturer's instructions. The expression of Bcl-2, Bax, GFAP, and IBA1 genes in each group was detected and the PCR primers are shown in Table 1.

### 2.4. ELISA

Three days after modeling, blood was collected by an orbital venous plexus puncture. After standing at room temperature for 30 min, samples were centrifuged at 3000 rpm for 5 min to separate plasma, followed by the detection of TNF-α, IL-6, and IL-10 content in plasma according to the instructions of the ELISA kit (Enzyme Immunobiology, China).

### 2.5. HE staining

For HE staining and tissue immunofluorescence, five rats in each group after modeling were taken, and the brain tissue (at the lesion site and the surrounding 3 mm area) was cut with a scalpel, fixed, dehydrated, embedded, and made into tissue sections with a thickness of 4 μm followed by HE staining and observed under the light microscope. The morphological and structural changes and the number of cells in the brain tissue were observed to determine the damage and recovery of the brain tissue of the rats in each group.

### 2.6. Immunohistochemical staining

Paraffin sections were deparaffinized, boiled in 10 mM citrate buffer for 10 min for antigen retrieval, 3% H2O2 solution was used to block the endogenous peroxidase. After serum blocking for 30 min, primary antibodies GFAP (1:200) and IBA1 (1:100) were added and incubated overnight at 4 °C. Thereafter, sections were rinsed with PBS 3 times followed by the addition of secondary antibody and incubated at room temperature for 2 h, observed and photographed after counterstaining with DAPI.

### 2.7. Western blot

10 μg of total exosome protein was resolved by 10% SDS-PAGE and then transferred to PVDF membrane (Millipore, USA). Then membranes were blocked with 5% skimmed milk for 2 h followed by incubation with primary antibody (diluted at 1:1 000) overnight at 4 °C. Thereafter, the membrane was washed 3 times with TBST, and incubated for 1 with respective horseradish peroxidase-conjugated secondary antibody (dilution ratio of 1:5 000). Then it was washed 3 times with TBST, and a chemiluminescent reagent (Tanon, China) was dropped into the membrane. Finally, protein expression was observed with a chemiluminescence gel imaging analyzer (Tanon, China) (Monoclonal antibodies to TSG101, CD9, CD63 and CD81 were from Affinity Biosciences Affinity, USA).

**Table 1**
The forward (F-) and reverse (R-) primers used in this study.

| Gene | Gene names | primer (5′–3′) | Product length (bp) |
|---|---|---|---|
| GFAP | GFAP-F | AGAGGAAGGTTGAGTCGCTGGAG | 145 |
| | GFAP-R | AGAGCCGCTGTGAGGTCTGG | |
| Iba1 | Iba1-F | AGCGAATGCTGGAGAAACTTGGG | 84 |
| | Iba1-R | CCTCGGAGCCACTGGACACC | |
| Bcl-2 | Bcl-2-F | GAACTGGGGGAGGATTGTGG | 80 |
| | Bcl-2-R | GGGGTGACATCTCCCTGTTG | |
| Bax | Bax-F | GTCCTCACTGCCTCACTCAC | 189 |
| | Bax-R | GTTTATTGGCACCTCCCCCA | |
| GAPDH | GAPDH-F | TTCCTACCCCCAATGTATCCG | 270 |
| | GAPDH-R | CCACCCTGTTGCTGTATCCATA | |

## 3. Results

### 3.1. Identification of UC-MSCs-Exo

Our results from fluoroscopic electron microscope indicated that isolated and purified UC-MSCs-Exo was cup shape (Fig. 1A); NTA particle size analysis shows that its size is 30–150 nm, concentrated at 69.75 nm, and the average particle size was 71.20 nm (Fig. 1B). While western blotting results confirmed the expression of UC-MSCs-Exo specific exosome surface proteins i.e., TSG101, CD81, CD63, and CD9 (see Figure C), which proved that the isolated vesicles were exosomes.

### 3.2. Evaluation of neurological deficits by mNSS score in rats

As shown in Fig. 2A, the mNSS scores of the rats in the UC-MSCs-Exo group were significantly lower than those in the TBI group at 3, 7, 14, and 21 d after modeling ($P < 0.01$), and were significantly higher than those in the Sham group ($P < 0.01$). 0.01).

### 3.3. Expression of Bcl-2/Bax apoptosis-related genes

RT-qPCR results (Fig. 2C) showed that the expression of Bcl-2/Bax in the UC-MSCs-Exo group was significantly higher than that in the TBI group after 21 days of modeling ($P < 0.01$).

### 3.4. Expression of glial cell markers GFAP and IBA1

Further, we aimed to determine the expression of glial cell markers ie., GFAP and IBA. Our results from RT-qPCR showed (Fig. 3A) that 21 days after modeling, mRNA expressions of the GFAP and IBA1 in the UC-MSCs-Exo group were significantly lower than those in the TBI group ($P < 0.01$). Besides, immunofluorescence results showed that the fluorescence expression intensity of GFAP protein (Fig. 3B)

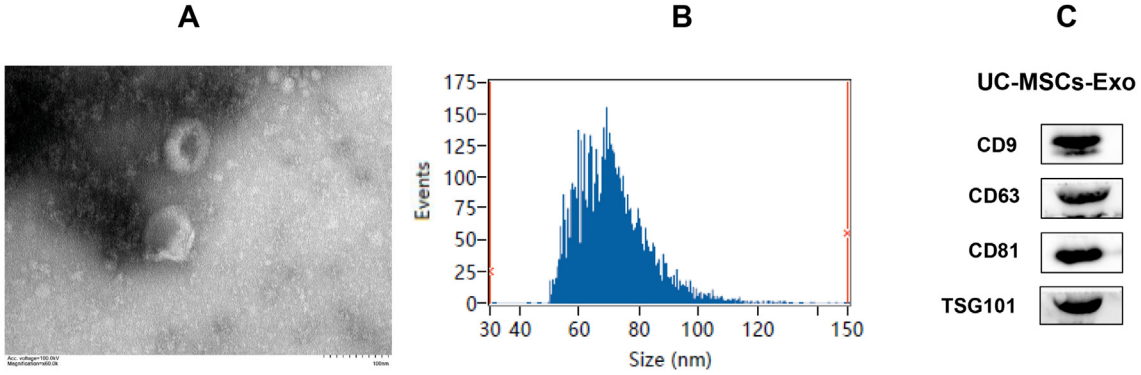


**Fig. 1.** Identification of UC-MSCs-Exo. **A:** The exosomes were cup-shaped as observed by fluoroscopy electron microscope; **B:** The size of NTA particle size was 30–150 nm, concentrated at 69.75 nm, and the average particle size was 71.20 nm; **C:** Western Blot detection showed that UC-MSCs-Exo expressed specific exosome surface proteins TSG101, CD81, CD63 and CD9.

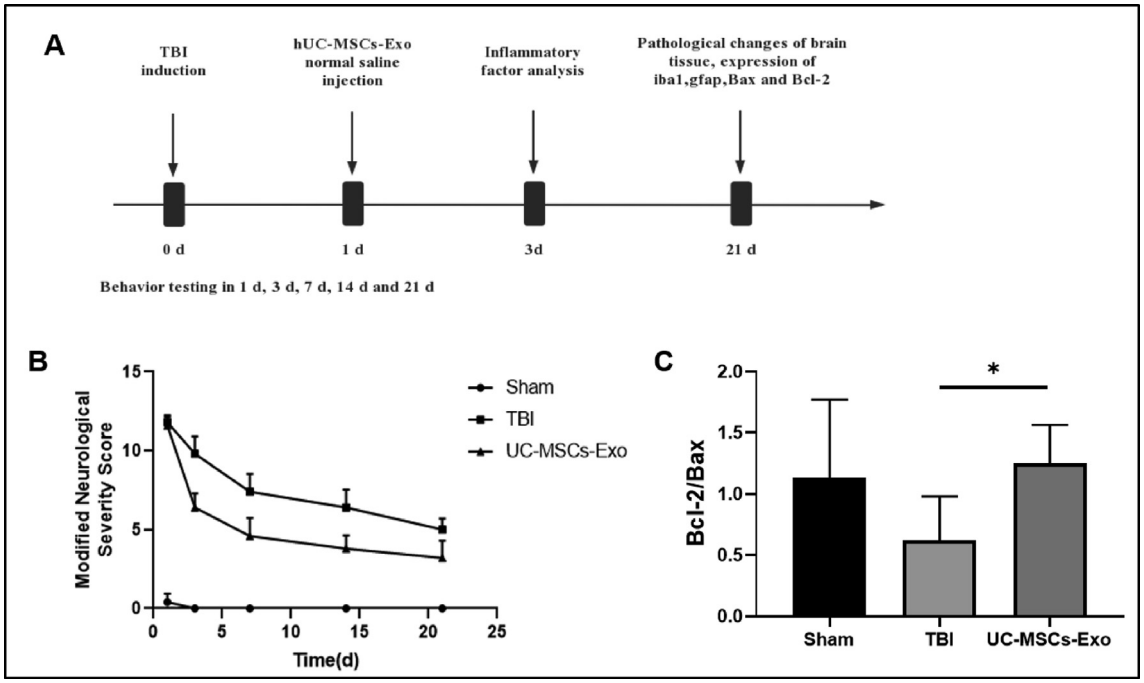


**Fig. 2.** UC-MSCs-Exo promotes neurological recovery in TBI rats. **A:** Schematic diagram of the experimental procedure. **B:** mNSS measures sensorimotor function. **C:** The expression of Bcl2/Bax apoptosis-related genes was detected by qPCR. * indicates $p < 0.05$.

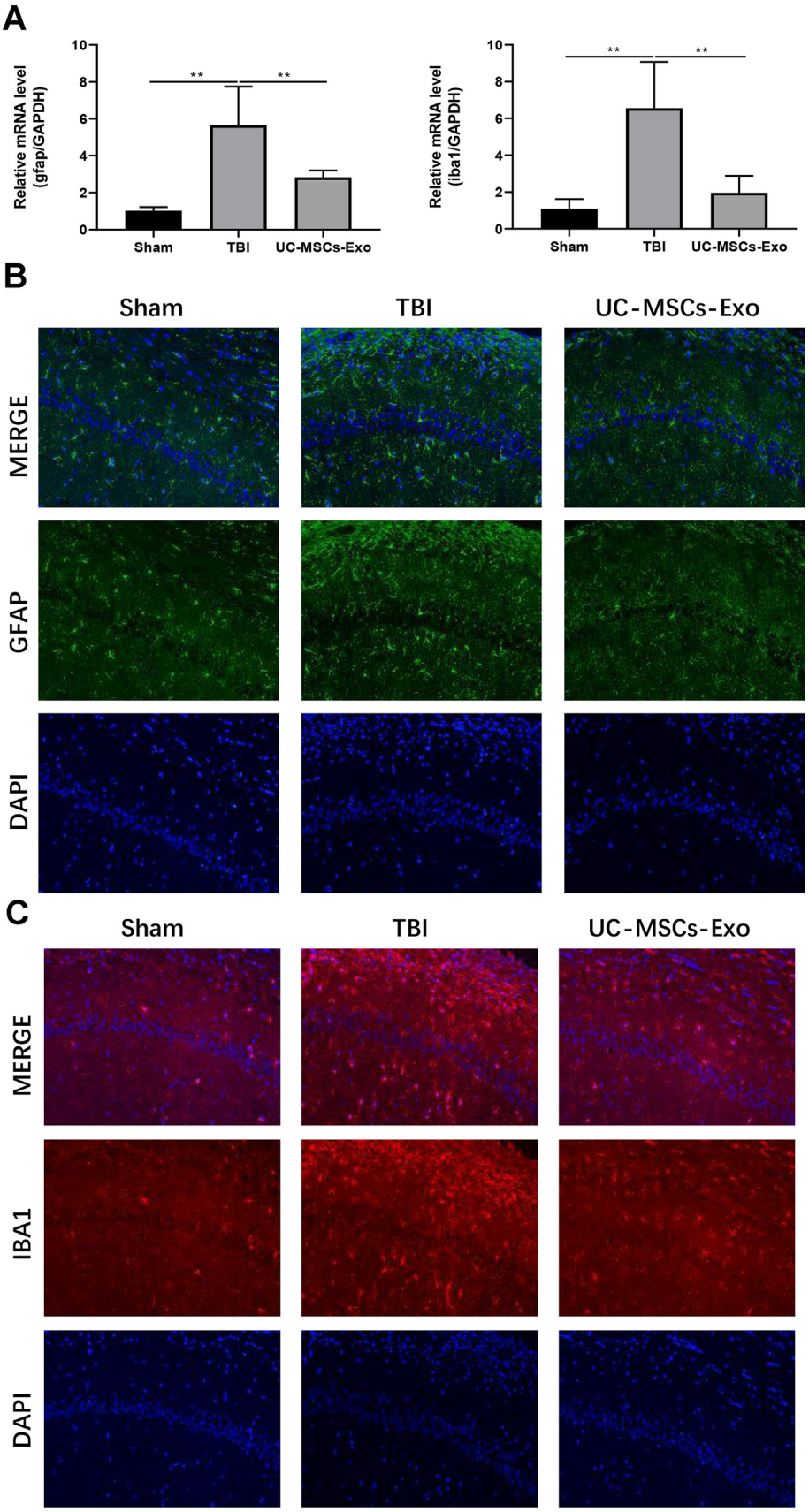


**Fig. 3.** UC-MSCs-Exo inhibits the expression of GFAP and IBA1. **A:** The mRNA expression of astrocyte marker GFAP and microglia marker IBA1 in brain tissue was detected by qPCR; **B**–**C:** The localized expression of astrocyte marker GFAP and microglia marker IBA1 protein was detected by immunofluorescence. *indicates $p < 0.05$; **indicates $p < 0.01$.

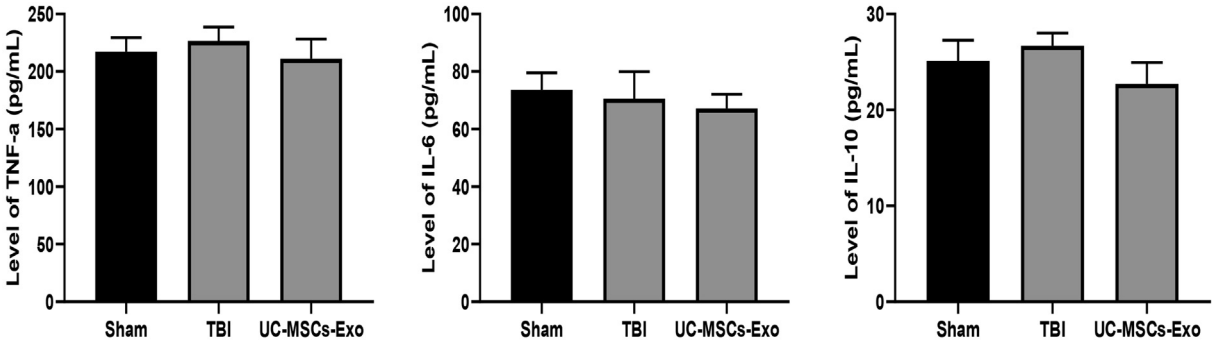


**Fig. 4.** UC-MSCs-Exo intervention did not affect the levels of inflammatory factors in rat plasma. There was no significant difference in the expression levels of inflammatory factors tumor necrosis factor α, interleukin 6 and interleukin 10 in the plasma of the three groups of rats ($P > 0.05$).

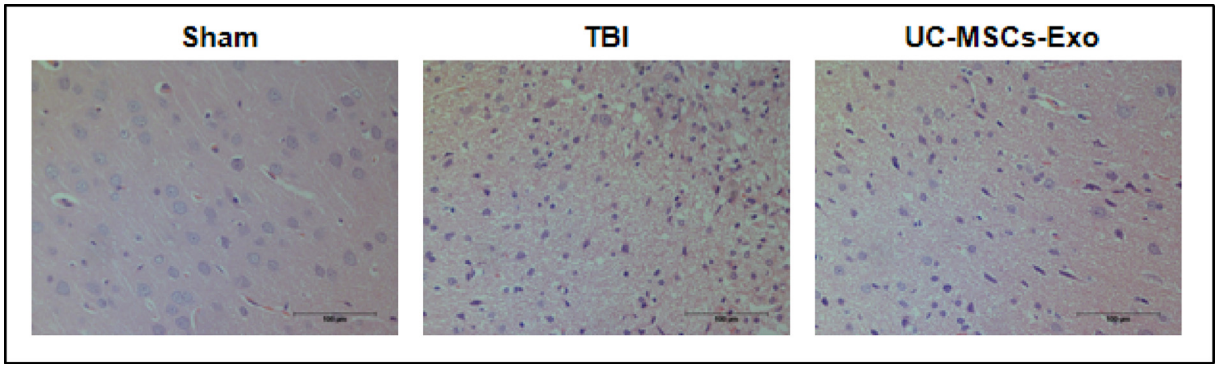


**Fig. 5.** HE staining of rat brain tissue. Rats in the TBI group had extensive brain tissue defects, edema, decreased number of nerve cells, irregular shape of nuclei, inconspicuous nucleoli, dark red staining of cytoplasm, necrosis of a small part of neurons, fragmented and condensed nuclei, nuclear staining deepened. The brain tissue defect area, edema and neuronal apoptosis of the UC-MSCs-Exo group were improved to varying degrees.

and IBA1 protein (Fig. 3C) in the UC-MSCs-Exo group was lower than those in the TBI group after 21 days of modeling. Collectively, these results showed that the activation of glial cells in the rat brain was significantly reduced after the intervention of UC-MSCs-Exo, suggesting its beneficial role in neural repair.

### 3.5. Expression of inflammatory factors TNF-α, IL-6, and IL-10

The expression of inflammatory factors TNF-α, IL-6, and IL-10 was determined by using the ELISA**.** Our results showed that there was no significant difference in the expression levels of inflammatory factors TNF-α, IL-6, and IL-10 in the plasma of the three groups of rats ($P > 0.05$) (Fig. 4).

### 3.6. HE staining

HE staining results indicated that the structure of the brain tissue of the rats in the Sham group was normal and significant pathological changes were found. Whereas rats in the TBI-model group exhibited large area defects and edema of brain tissue, a large number of glial cells were activated, the number of nerve cells was reduced, and the shape of the nucleus was irregular. A large number of activated glial cells were observed while the number of nerve cells was comparatively low, the shape of the nucleolus was not obvious, the cytoplasm showed dark red staining, a small part of neurons was necrotic, the nuclei were fragmented and condensed, and the nuclear staining was deepened. However, in the UC-MSCs-Exo group, we found that the damaged area of brain tissue, edema, and neuronal apoptosis was reduced to varying degrees as well as the number of the activated glial cell was less as compared to the TBI model without the treatment of UC-MSCs-Exo (Fig. 5).

## 4. Discussion

Traumatic Brain Injury (TBI) is a common brain injury caused by mechanical force such as sudden acceleration or deceleration [18,19]. The occurrence and development of TBI are complex and due poor self-repairing ability of the central nervous system there is no standard treatment available to treat traumatic brain injury [20,21]. Nonetheless, recent studies have demonstrated that MSCs possess the multi-directional differentiation ability to promote tissue repair and regeneration, thus suggesting an effective treatment for the repair of TBI nerve injury [22,23]. Besides, recent studies have indicated the efficiency of MSCs-Exo, a cell-free treatment method, which holds advantages over MSCs therapy in terms of production, storage, transportation, and biological safety while ensuring the therapeutic effect [24,25]. Though preclinical studies using the TBI rat model have also shown therapeutic effects of MSCs-Exo derived from other sources i.e., bone marrow [26] and adipose tissues [27] on nerve repair by significantly promoting the recovery of nerve function, however, the underlying mechanism remained unclear. The present study shows that UC-MSCs-Exo is as efficient as MSCs-Exo derived from bone marrow and adipose, in the treatment of TBI.

It has been indicated that TBI disturbs the balance of Bcl-2/Bax which increases the apoptotic rate of neurons. However, in the present study, the level of Bcl-2/Bax was significantly increased after UC-MSCs-Exo intervention, reflecting that UC-MSCs reduced neuronal apoptosis. Moreover, was craniocerebral injury has also been attributed to the neuroinflammation-induced release of cytokines and excitatory cytotoxic substances; however, our data showed that UC-MSCs-Exo exhibited no significant effect on the release of inflammatory factors. Surprisingly, the results of real-

time quantitative PCR and immunofluorescence analysis showed that the mRNA and protein levels of astrocyte and microglia markers GFAP and IBA1 were significantly decreased after UC-MSCs-Exo intervention, indicating that UC-MSCs- Exo has inhibitory effects on astrocyte and microglia hyperactivation.

Nonetheless, it has been indicated that glial cells in the central nervous system play key roles in neural development, tissue repair, and homeostasis [28,29]. Under normal physiological conditions, astrocytes and microglia function by clearing debris, recycling neurotransmitter molecules, and supporting transneuronal and cellular communication to maintain neuronal function [30,31]. However, the occurrence of traumatic brain injury can lead to excessive activation of astrocytes and microglia, which can lead to the formation of glial scarring, thereby aggravating the secondary brain injury [32]. This study found that UC-MSCs-Exo could exert a therapeutic effect on TBI by inhibiting the overactivation of astrocytes and microglia.

In conclusion, our study suggests that UC-MSCs-Exo can improve the neurological function of traumatic brain injury rats. Due to their key role in mediating intercellular signal transduction and information exchange, MSCs derived exosomes are considered as promising candidate for the diagnosis and treatment of traumatic brain injury. Recently, a growing number of studies have proved that miRNAs carried by UC-MSC-Exo play a critical role in tissue repair and remodeling as well as treatment of other diseases. Therefore, our next work will focus on sequencing and studying the miRNAs of UCMSC-Exo.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

## Author contributions

L.X. C. designed the study, analyzed, and interpreted the data and prepared the manuscript. W. L., W.K. J., H.M. L., J.R. X., and X.C. L. performed the experiments and interpreted the data. B.Y. W., J.H. W., and G.Q. C. interpreted the findings.

## Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Foshan University.

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## Declaration of competing interest

No.

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