An Acad Bras Cienc (2022) 94(3): e20210564 DOI 10.1590/0001-3765202220210564
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOMEDICAL SCIENCES

Exosomes carried miR-181c-5p alleviates neuropathic pain in CCI rat models

YU ZHANG, GUANGYAO YE, JINGSONG ZHAO, YUEBO CHEN, LINGSI KONG, CHAOXU SHENG & LIYONG YUAN

Abstract: Mesenchymal stem cells (MSCs) derived exosomes (Exos) are one of the most promising candidate for the treatment of this condition. However, the underlying molecular mechanism remains uncertain. Here we investigated the therapeutic effect of exosomal miR-181c-5p (Exo$^{miR\text{-}181c\text{-}5p}$) on a rat model of neuropathic pain induced by sciatic nerve chronic constriction injury (CCI). In this study NP model was established using the CCI method. NP levels were assessed using PWT and PWL. Microarray analysis and RT-PCR were used to determine the relative expression of miR-181c-5p. MSC-derived exosomes were extracted using the total exosome isolation reagent characterized by WB and NTA. MiR-181c-5p was loading into Exos using electroporation. The inflammation response in microglia cells and CCI rats were assessed by ELISA assay respectively. Our study demonstrates that miR-181c-5p expression was obviously decreased in a time-dependent manner in CCI rats. MiR-181c-5p was effectively electroporated and highly detected in MSC-derived Exos. Exo$^{miR\text{-}181c\text{-}5p}$ internalized by microglia cells and inhibit the secretion of inflammation factors. Exo$^{miR\text{-}181c\text{-}5p}$ intrathecal administration alleviated neuropathic pain and neuroinflammation response in CCI rats. Taken together, Exo$^{miR\text{-}181c\text{-}5p}$ alleviated CCI-induced NP by inhibiting neuropathic inflammation. Exo$^{miR\text{-}181c\text{-}5p}$ may be a valid alternative for the treatment of neuropathic pain and has vast potential for future development.

Key words: exosome, miR-181c-5p, neuropathic pain, neuroinflammation.

INTRODUCTION

NP is one of the most common types of chronic pain, which redefined as “pain resulting from a lesion or disease in the peripheral or central nervous system” by The International Association for the Study of Pain in 2008 (Scholz et al. 2019). Various etiologies including direct nerve trauma; central nervous lesions; viral infections and metabolic diseases (such as diabetes or multiple sclerosis) may lead to NP (Zhou et al. 2017, Chauhan et al. 2018). Although some studies believe that NP is related to neuroinflammation (Sommer al. 2018). Up to now, the mechanisms of NP are poorly understood. NP afflicts approximately 6% of the population in the world every year (Scholz et al. 2019). Despite the great morbidity, NP has a finite number of therapeutic options. Current treatment strategy such as physical, cognitive, pharmacological, and interventional approaches appear to be invalid in controlling this condition (Scholz et al. 2019, Finnerup et al. 2015, Baron et al. 2016). Many reports suggested that only about 30% of the patients who receive treatments, can get relief before pain inevitably reappears (Scholz et al. 2019). Therefore, to establishment a new disease-modifying treatment strategy is necessary for the treatment of NP.

MSC is a type of self-renewing and multipotent stromal cells, which can be obtained from various sources including embryonic, muscle, bone marrow, and adipose tissue (Bartolucci et al. 2017). MSCs transplantation, as a potentially effective strategy, has been applied to treat various diseases (Arzouni et al. 2018, Xiao et al. 2018). Some articles have reported that MSCs could differentiate into neuron-like cells and have shown neuroprotective effect in a variety of nerve injury models (De Simone et al. 2019). In addition, a few recent studies have shown that autologous MSCs transplantation relieve NP in the CCI model (Al-Massri et al. 2019). However, the mechanism of this therapeutic process has not been explicitly explained. One possible mechanism for the therapeutic process is that MSCs inhibited neuroinflammation and activated endogenous repair mechanisms. Furthermore, recently, accumulating evidence has demonstrated that the therapeutic benefits of cell-based therapy result from the release of Exos (Phinney & Pittenger 2017, Spees et al. 2016). Exos are small extracellular vesicles range in size from 50 to 120 nm, which released by all kinds of cells including MSCs (Phinney & Pittenger 2017). Exos convey various cargo including lipids, proteins, mRNA and miRNA molecules to recipient cells for cell-to-cell communication (Wortzel et al. 2019). A couple of researchers have confirmed that MSC-derived Exos have similar therapeutic functions as MSCs including anti-inflammatory effects (Zhuang et al. 2011, Steinbichler et al. 2019). Compared to MSCs, MSC-derived Exos are preferred for clinical application due to their ease of storage, delivery, and management (Xu et al. 2016). Thus, exosome therapy would be a promising therapeutic strategy for neuropathic pain patients, which could be further optimized for its clinical application.

MicroRNAs are a class of small non-coding RNAs responsible for gene expression by translational inhibition or mRNA degradation. MiRNAs participate in the pathological process in many diseases (Alexander et al. 2015, Men et al. 2019). Accumulating evidence has confirmed that miRNAs play an important role in the anti-inflammation process by regulating target genes in a variety of signaling pathways (Men al. 2019). Additionally, recent findings suggest that the expression of various miRNAs, including miR-30a, miR-124, miR-125 and miR-183, are induced in neuropathic pain (Xu et al. 2014, Grace et al. 2018, Peng et al. 2017). Some target pathways regulating by these miRNAs have been identified, including mTOR/VEGF, SGK3 and SLC17A6, respectively (Xu et al. 2014, Peng et al. 2017). A recent study reported that miR-181c-5p could attenuate excessive inflammation in an animal model (Wei et al. 2019). In the current study, we investigated the potential function of Exos carried miR-181c on NP in a rat CCI model. We found that Exos carried miR-181c could attenuate neuropathic pain after CCI. Moreover, we report that the therapeutic effect of miR-181c is achieved by regulating neuroinflammation.

MATERIALS AND METHODS

Animals

Sprague-Dawley (SD) rats (male, weight 200–250 g) were supplied by the Guangzhou Medical Animal Laboratory Center, China. All rats were singly housed in separate cages under approved conditions with 24 ± 3°C, natural light/dark cycles, and feed with conventional rat feed and sterilized water. Cages were changed twice weekly. All animal experiments in the current study were approved by the Institutional Laboratory Animals Committee of Ningbo No.6 Hospital and conducted following The

Guidelines for Laboratory Animal Care and Use by Ningbo No.6 Hospital.

Neuropathic pain models

To create NP models, CCI surgery was performed as described previously (Gao et al. 2017). All surgeries were completed in a sterile environment under a surgical microscope. SD rats were anesthetized using sodium pentobarbital (50 mg/kg), and the left sciatic nerve was loosely ligated with 4-0 chromic gut sutures in four regions. Sham procedure involved equal process without sciatic nerve ligation.

Intrathecal Catheter Insertion

A polyethylene catheter was inserted into the subarachnoid space of the spinal cord to L4-L6 as previously described (Jia et al. 2020). On day 0, day 3 and day 7 after CCI surgery, 10μL (1.2mg/mL) Exo ($Exo^{scramble}$), exosomal miR-181c ($Exo^{miR\text{-}181c}$) and negative controls were injected via the implanted intrathecal catheter. Following catheter implantation, rats with spinal cord injury were excluded from the experiment.

Behavioral testing

PWT and PWL was tested using a set of electronic von Frey Aesthesiometer (IITC Life Science Inc., WH, USA) on 0, 3, 7, 14 and 21 days after the CCI operation. Rats were placed on a metallic device covered with a plexiglass observation box. The clear paw withdrawal was recorded and then averaged. The Analgesia Meter Model 336 (IITC Life Science Inc., WH, USA) was used to detect thermal hyperalgesia. A heat generator was put under the plantar surface of each rat, then the latency of paw withdrawal was averaged. Both tests were repeated three times and performed in a blind fashion.

MicroRNA microarray

Total RNA of rat dorsal horn tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer. The quantity of RNA samples was examined by the Agilent 2100 Bioanalyser (Agilent, Chengdu, China), and RNA integrity was verified by gel electrophoresis. MiRNA expression analysis was performed using the RiboArray platform as described previously. This microarray platform is designed based on a locked nucleic acid (LNA) technology. Cluster analysis were performed by gplots. Differentially expressed microRNAs were identified through fold change filtering. Statistical analysis using T test, and P-value<0.05 and fold change >2 were considered significantly differentially expressed. Microarray images were generated by R software.

Cell culture

MSCs isolation and culture

Bone marrow tissue of young SD rats were collected for MSCs isolation as previously published (Mead & Tomarev 2017). In brief, both the tibiae and femora were dissected and the bone marrow was flushed with cold Dulbecco's modified Eagle's medium F12 (DMEM/F12, Invitrogen, CA, USA) supplemented with 5% fetal bovine serum (FBS, Gibco, CA, USA). After filtration and centrifugation, the MSC-pellets were resuspended and cultured in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin (Invitrogen, CA, USA). MSCs were kept in a humidified condition at 37°C, and supplied with 5% CO_2. All non-adherent cells were removed, and culture medium was replaced every 3 days. MSCs identification was performed by flow cytometry. Monoclonal antibody CD90, CD29, CD34, and CD45 (Invitrogen, CA, USA) were used for verification of cell surface antigens.

Spinal microglia cell isolation and culture

Microglia cells were obtained following a recently published protocol (Yip et al. 2009). In brief, Rats were sacrificed, the dorsal horn tissue samples with blood vessels removed were collected. The fresh tissue was minced and dissociated by incubation for 30 min in collagenase IV (150 μg/mL) at 37°C. Spinal cord tissues were centrifuged at 800g for 10 min. The isolated microglial cells were plated in a 12-well plate previously coated with poly-D-lysine, and cultured in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen, CA, USA). Cultures were maintained at 37°C in a 5% CO_2 atmosphere.

MSC-derived exosomes isolation and identification

mMSC-derived Exos were isolated and purified using a previously described method (Thomi et al. 2019). Briefly, MSCs at passages 3-6 were cultivated in DMEM/F12 added with 10% Exo-free FBS for 48 h before culture medium collection. The supernatant was centrifuged at 2000 x g for 30 min at 4°C and then passed through a 0.22 μm filter to remove cellular debris. Exos in this study were extracted using ultracentrifugation (100,000g for 120 min at 4°C). Purified Exos were preserved in phosphate buffer saline (PBS) for the following experiments. The morphological features of Exos were observed by transmission electron microscope (TEM, Thermo Fisher Scientific, CA, USA). MSC-derived Exos were identified using CD63 and TSG101 (BD Biosciences NJ, USA) by Western blot. Nanoparticle tracking analysis (NTA) was performed with a NanoSight LM10-HSB instrument (A&P Instrument, United Kingdom) according to the manufacturer's instructions.

Exosomes loading

Exos were loaded with miR-181c using electroporation performed on a Gene Pulser II system (Bio-Rad, CA, USA) as previously described. In brief, Exos at a total protein concentration of 20 μg were mixed with 800 nM of miR-181c. The Exo-miRNA mixture was electroporated using a pulse 20 ms and 500 voltage. Then, the mixture was incubated at 4°C overnight. To remove free-floating miRNAs, Exos were washed two times in PBS with 100,000g centrifugation for 2 h at 4°C. Finally, Exos pellets were preserved by PBS and stored at -80°C.

Exosomes internalization

Exos were labelled with DiI lipophilic dye (Invitrogen, CA, USA) as described previously. 250 μg of purified Exos were incubated with 1 μM DiI at 37°C for 30 min in dark. After removed excess dye, labelled Exos were collected by ultracentrifugation at 100000g for 120 min. Recipient microglia cells were incubated with DiI-labelled Exos (10 μg) for 6h. After removed the supernatants and washed with PBS, the samples were fixed with 4% paraformaldehyde (PFA, Invitrogen, CA, USA) for 10 min at room temperature (RT). Then samples were incubated with 4',6-diamidino-2-phenylindole (DAPI, 1 : 1000, Invitrogen, CA, USA) for 5 min at RT, and visualized under an inverted fluorescence microscope (Leica DMI6000B, Germany).

In vitro lipopolysaccharides (LPS) stimulation

Microglia cells were plated at 2×10^5 cells/well in a 12-well plate at 37°C supplied 5% CO_2. The following experiments were performed. First, Microglia cells were incubated in presence of 20ng/ml LPS (Sigma, CA, USA) with or without MSC-derived Exos for 6 h. Cell lysates were collected to detect the expression levels of miR-181c after MSC-derived Exos treatment. Second, to evaluate if MSC-derived Exos inhibit microglia cells inflammation in response to LPS, proinflammatory cytokines were examined. Protein levels of interleukin-6 (IL-6), IL-1β, TNF-α,

and cyclooxygenase-2 (COX-2), in the conditioned media from microglia cells were detected by ELISA kit (R&D Systems, MN, USA).

Quantitative RT-PCR analysis

Total RNA was isolated from the samples by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions. The reverse transcription reactions were performed using the PrimeScript® miRNA cDNA Synthesis kit (TaKaRa, Dalian, China) or the PrimeScript RT reagent kit (TAKARA, Dalian, China). RT-PCR was performed using SYBR Green qPCR Master Mix kit (Thermo Scientific, Shanghai, China) to detect the expression of miR-181c. The Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, CA, USA) was used. U6 was served as a control for the expression of miRNA. The primers are presented as follows: miR-181c (forward: 5'-ATGGTTCGTGCGAACATTCAACCT GTCGG-3'; reverse: 5'-GCAGGGTCCGAGGTATTC-3') and U6 (forward: 5'-GCTTCGGCAGCACATATACTAAT-3'; reverse: 5'-CGCTTCACGAAT TTGCGTGTCAT-3'). The relative expression levels of target genes was calculated using the $2^{-\Delta\Delta}$Ct method.

Western blot

Western blot was performed as described previously. The protein concentrations was quantified by BCA assay (Thermo Scientific, Shanghai, China). A total of 30 μg protein were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difuoride PVDF membranes (Millipore, MA, USA). Primary antibodies, including CD63 (1:1000, BD Biosciences NJ, USA) and TSG101 (1:1000, BD Biosciences NJ, USA) were used for incubation with the PVDF membranes at 4 °C overnight. The PVDF membranes were then incubated in the secondary antibody for 1 h at RT. The membrane was developed by ECL Plus reagents (Thermo Scientific, Shanghai, China) and exposed under the ChemiDoc XRS system (Bio-rad-CA, USA).

Statistical analyses

All parameters were represented as the mean ± SD from at least three independent experiments. One way ANOVA and Two way ANOVA were performed to compare the differences of two groups and multiple groups respectively. The difference was considered significant as P value is less than 0.05 ($P < 0.05$). GraphPad Prism 6.0 software (GraphPad Software, CA, USA) was used for statistical analyses and images generation.

RESULTS

MiR-181c-5p was downregulated in CCI rats

NP model was successfully established by CCI. As shown in Fig. 1a, a notable decline was shown both in PWT and PWL within 3 days after CCI surgery. This decline peaked on day 7 and lasted for 21 days after surgery. Microarray analysis was performed to identify the differentially expressed microRNAs in NP progression. Using a paired t-test, a total 130 microRNAs were significantly changed (change fold>2, $P<0.05$) on days 7 after surgery. The results exhibited the top 20 differentially expressed miRNA between the CCI group and the sham group (Fig. 1b). Among these miRNAs, miR-181c was one of the most prominently down-regulated miRNAs. Then, we detected miR-181c expression levels at days 0, 3, 7, 14, 21. As displayed in Fig. 1c, the expression of miR-181c was dramatically decreased in a time-dependent manner in CCI rats. These results manifested that miR-181c was involved in the pathogenesis of NP.

Characterization of MSC and MSC-Derived Exosomes

MSCs were isolated and cultured *in vitro* as described previously. The isolated MSCs were

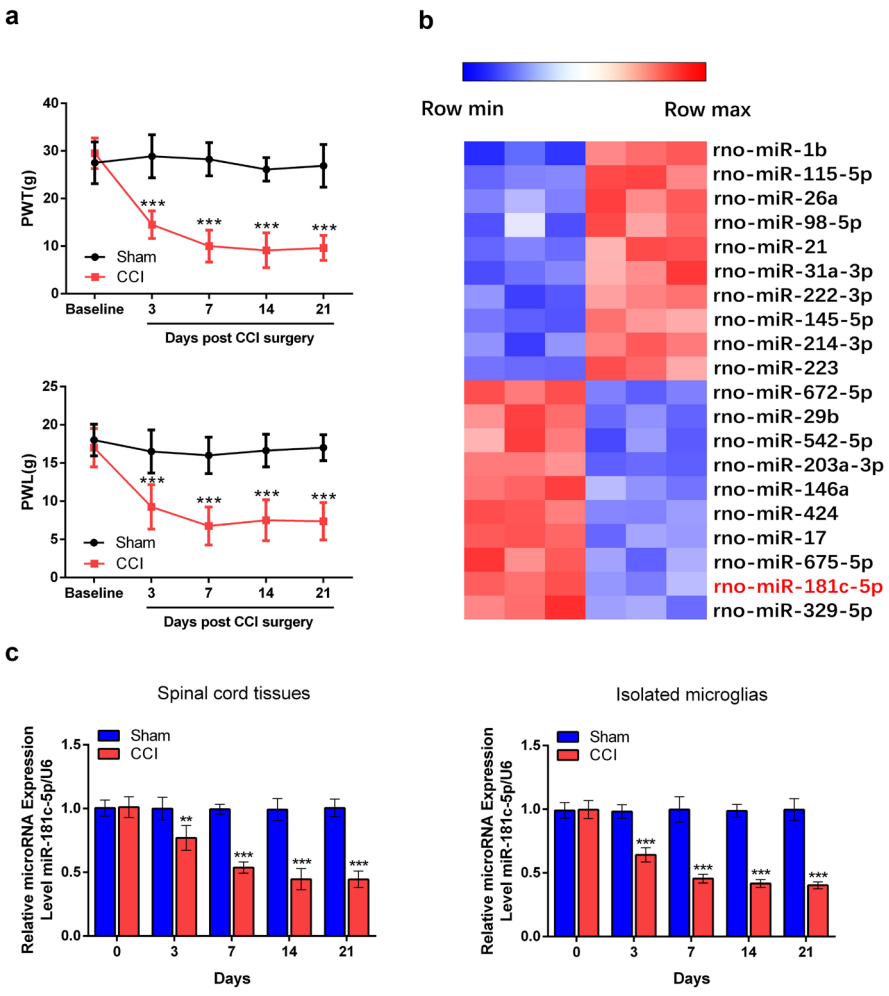

Figure 1. miR-181c was dysregulation in spinal cord under NP conditions. a. The effect of CCI surgery on mechanical allodynia and thermal hyperalgesia was tested at day 0, 3, 7, 14, 21. b. Hierarchical clustering image was generated to exhibit expression level of miRNAs after CCI surgery. c. RT-PCR analysis was performed to examine the expression of miR-181c after CCI surgery. **P < 0.01 , ***P < 0.001*vs.* Sham group.

observed under an optical microscope and exhibited a typical spindle-shaped morphology (Fig. 2a). We then examined the expression of cell surface markers, the result from flow cytometer analysis showed that the isolated cells highly expressed markers of MSCs (CD90, CD29), but not the hematopoietic or endothelial markers (CD34, CD45) (Fig. 2b). Exos were separated from medium by ultracentrifugation as described in Materials and Methods and identified using TEM, WB and NTA analysis. TEM analysis revealed that the Exos were a classical "cup-shaped" morphology (Fig. 2c). Further, we verified the expression of exosomal markers CD63 and TSG101 in isolated Exos by WB analysis (Fig. 2d). Moreover, NTA analysis indicated that the diameters of these particles mainly ranged from 40 nm to 110 nm, similar to previously described Exos (Fig. 2e). Taken together, the results demonstrate that the characteristics of isolated Exos in our study met the classical criteria for Exos.

Exosomes electroporated with miR-181c and uptake by microglia cells

MSC-derived Exos were loaded with miR-181c using a Gene Pulser II system as described previously. The data of RT-qPCR analysis suggested that miR-181c expression level in Exos was obviously upregulated after transferred with miR-181c mimics, but significantly decreased when electroporated with miR-181c inhibitor (Fig. 3a). To verify the uptake of Exos by microglia cells,

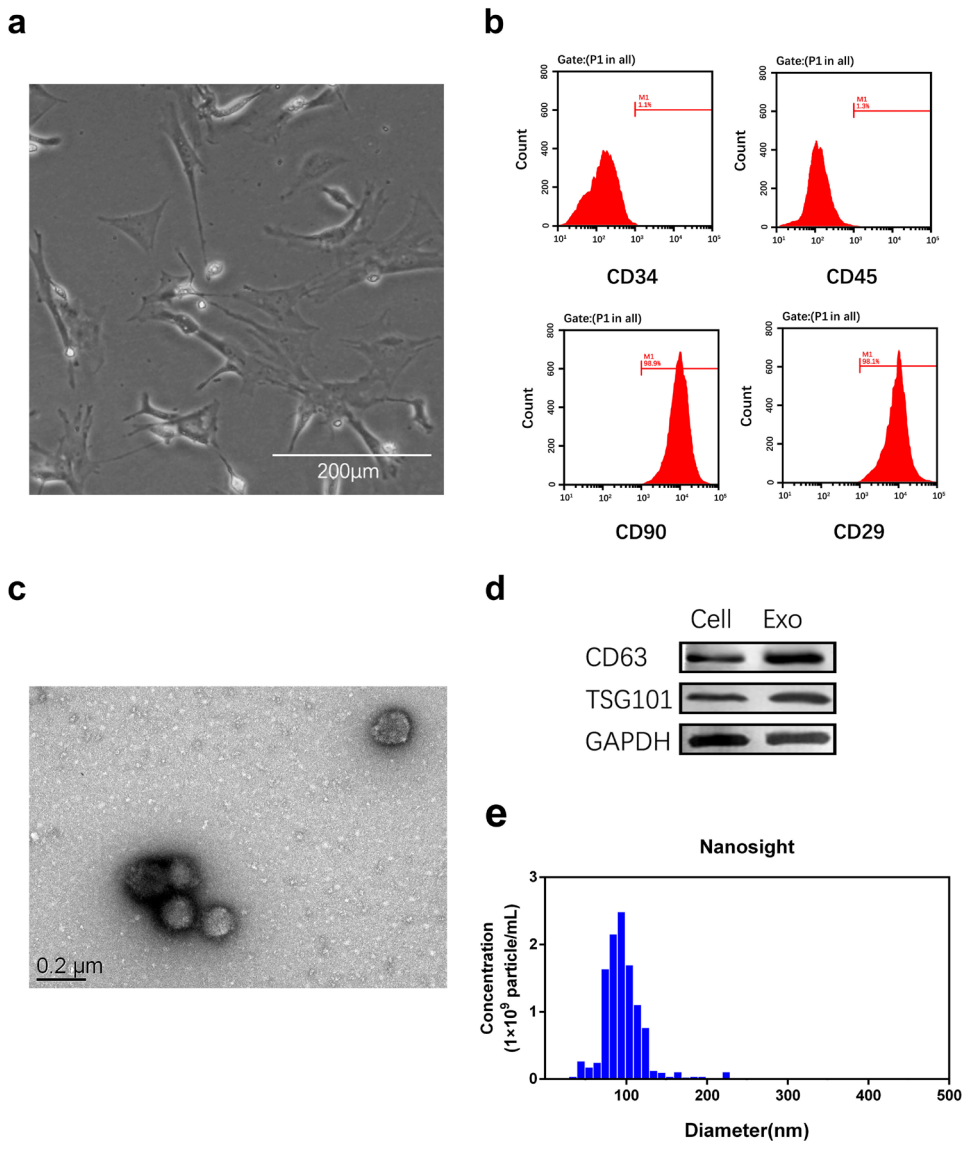

Figure 2. Identification of MSCs and MSC- derived Exos. a. Morphology of MSCs observed under an optical microscope. Scale bar=200μm. b. Flow cytometry analysis of isolated MSCs, positive expression of CD90, CD29 but a lack of expression of CD34, CD45. c. Morphology of MSC-derived Exos observed under a TEM. Scale bar=0.2μm. d. WB analysis of CD63 and TSG 101 protein expression in MSCs and Exos. e. NTA analysis measured by NanoSight.

Exos were labeled with fluorescent carbocyanine dyes CM-Dil (red) and incubated with microglia cells *in vitro*. After 6h of coincubation, nuclei of microglia cells were stained with DAPI (blue). Using a fluorescence microscope, we provided qualitative evidence that the Exos were successfully absorbed by microglia cells as displayed in Fig. 3c. RT-qPCR were used to evaluate the expression of miR-181c in microglia cells after incubated with Exos electroporated with miR-181c mimics or inhibitor, respectively. We found that a significant increase of miR-181c expression in microglia cells taking up Exos loaded with miR-181c mimics when compared with negative controls and microglia cells taking up Exos loaded with miR-181c inhibitor.

$Exo^{miR\text{-}181c}$ alleviated inflammatory response in microglia cells

Accumulating evidence showed that neuroinflammation is involved in NP development. To confirm the relationship between miR-181c and neuroinflammation, in our study, microglial cells were stimulated with

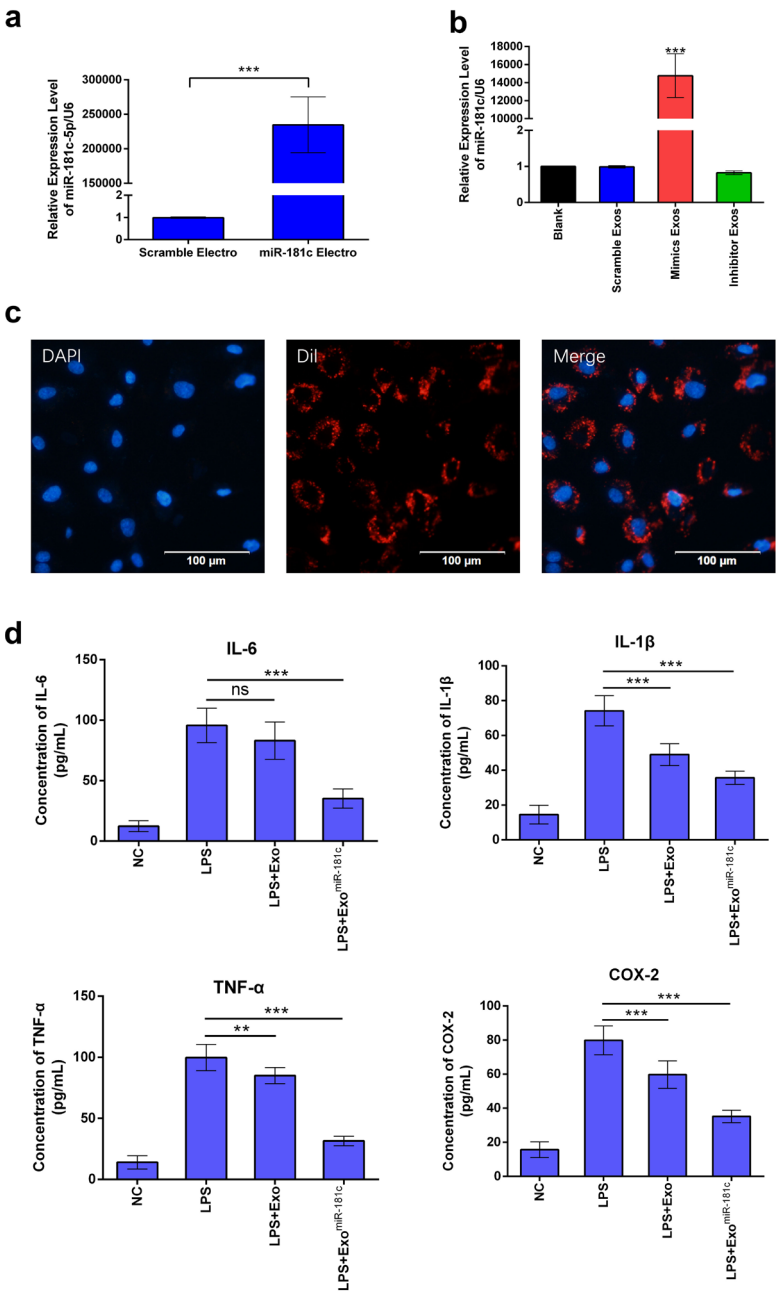

Figure 3. Uptake of Exos and alleviates LPS-induced inflammation in microglia cells. **a.** The expression level of miR-181c was detected by RT-qPCR after miR-181c mimics, inhibitor electroporation in Exos. **b.** The expression level of miR-181c was detected by RT-qPCR after Exos co-incubation for 6 h in micraglia cells. **c.** DiI-labelled Exos taken up by microglia cells observed under fluorescence microscope. Scale bar=100μm. **d.** Protein levels of IL-6, IL-1β, TNFα and COX-2 in microglial cells were tested by ELISA. **P < 0.01 , ***P < 0.001*vs.* LPS group.

LPS (0.5 ng/ml) for inflammation induction. Then, we measured inflammation factors including IL-6, IL-1β, TNF-α and COX-2 protein expression. As showed in Fig. 3d, ELISA data exhibited that Exo$^{miR-181c}$ dramatically inhibited protein expression of IL-6 IL-1β, TNF-α and COX-2 in microglia cells. These results revealed that Exo$^{miR-181c}$ significantly restrained the inflammation *in vitro*.

Exo$^{miR-181c}$ alleviated NP in CCI rats

To investigate the therapeutic effect of Exo$^{miR-181c}$ on neuropathic pain, the rats were randomly divided into four groups (sham, CCI, Exo^{scramble}, Exo$^{miR-181c}$). As exhibited in Fig. 4a, Exo$^{miR-181c}$ was intrathecal administered to the rats on day 0, day 3, and day 7 after CCI surgery, whereas CCI and Exo^{scramble} served as controls. miR-181c

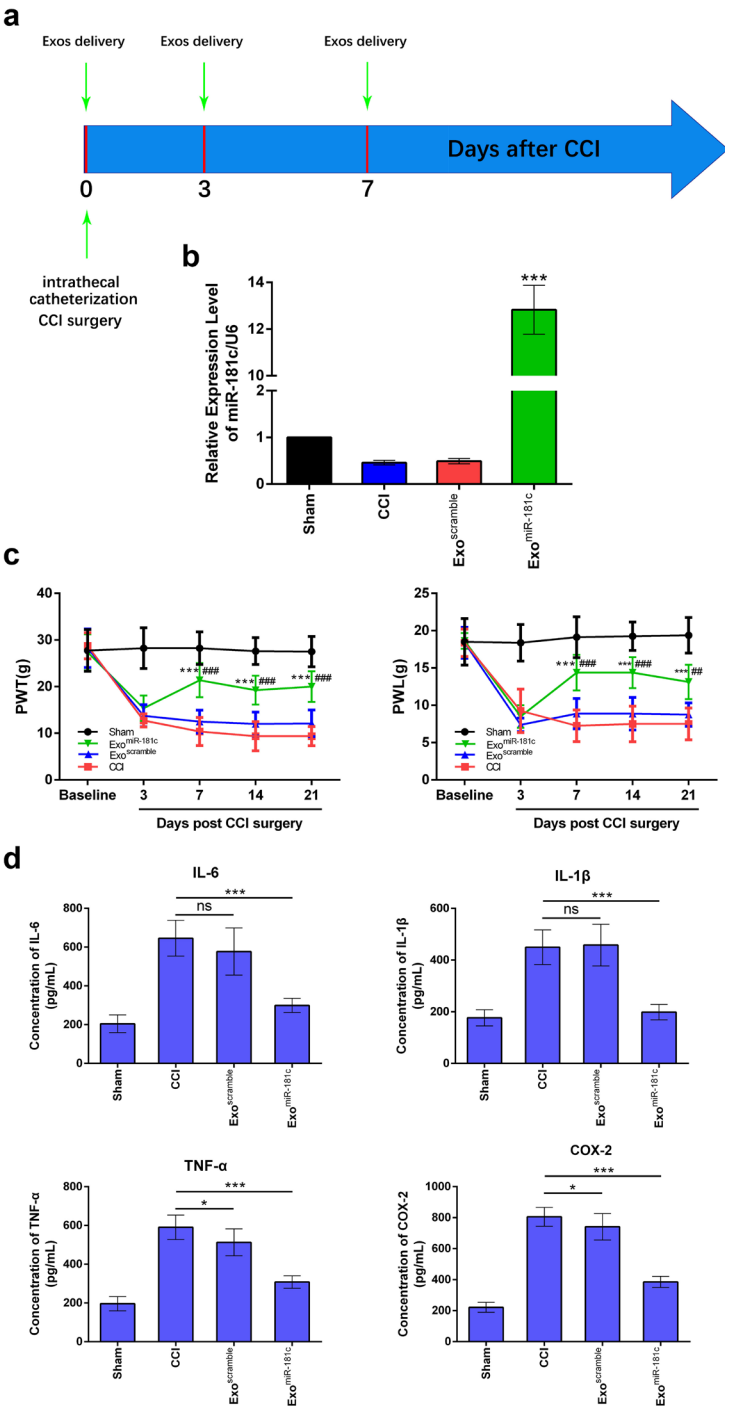

Figure 4. Exos$^{miR\text{-}181c}$ delivery alleviated NP in CCI rats. **a.** Schematic diagram of the time sequence of the experiment. **b.** The expression level of miR-181c was detected by RT-qPCR after Exos$^{miR\text{-}181c}$. **c.** The effect of Exos$^{miR\text{-}181c}$ on NP was tested by PWT and PWL (n=12). **d.** Protein levels of IL-6, IL-1β, TNFα and COX-2 in spinal cord were tested by ELISA. *P< 0.05, ***P < 0.001*vs.* CCI group. ### P < 0.001*vs.* Exos^{scramble} group.

overexpression was verified using RT-qPCR. We found that miR-181c expression was dramatically upregulated in Exo$^{miR\text{-}181c}$ group compared with Exo^{scramble} and CCI group (Fig. 4b). Additionally, we explored the changes of pain behavior after intrathecal administration. The results showed that both Exo$^{miR\text{-}181c}$ and Exo^{scramble} attenuated CCI-induced mechanical allodynia and thermal hyperalgesia. Furthermore, compared to Exo^{scramble} group, Exo$^{miR\text{-}181c}$ have better therapeutic effect on NP (Fig. 4c). These results suggested that

$Exo^{miR\text{-}181c}$ intrathecal administration inhibited NP in CCI rats.

$Exo^{miR\text{-}181c}$ inhibited neuroinflammation in CCI rats

Previous sections showed that $Exo^{miR\text{-}181c}$ relieved inflammation response in microglial cells stimulated with LPS. To further verify the therapeutic effect of $Exo^{miR\text{-}181c}$ on neuroinflammation *in vivo*, we used the ELISA assays to detect levels of IL-6, IL-1β, TNF-α, COX-2 protein expression in spinal cord of different groups on day 7 after CCI surgery. Our data revealed that the expression of inflammation factors including IL-6, IL-1β, TNF-α and COX-2 were elevated in all CCI treatment groups. In addition, $Exo^{miR\text{-}181c}$ intrathecal administration, compared with $Exo^{scramble}$ intrathecal administration, significantly decreased the inflammatory response in CCI rats (Fig. 4d). These data suggest that $Exo^{miR\text{-}181c}$ relieves neuropathic pain in CCI rats *via* modulating neuroinflammation.

DISCUSSION

In present study, we investigate the potential influence of $Exo^{miR\text{-}181c}$ on NP. We firstly observed that miR-181c expression level in CCI rats was greatly down-regulated in spinal cord tissues and microglial cells compared with rats in sham groups. We further found that $Exo^{miR\text{-}181c}$ attenuated LPS-induced inflammatory response in microglia cells. Moreover, we provided the first evidence showing that Exos loaded with miR-181c significantly reduced systemic neuroinflammation. These findings indicated that $Exo^{miR\text{-}181c}$ give an inspiring hope of therapeutic molecules for the treatment of NP.

NP is a worldwide severe chronic disease caused by disorder of nervous system, and its morbidity is increasing in the past decades (Scholz et al. 2019). Despite various pharmacological agents (nonsteroidal anti-inflammatory drugs, opioid analgesics, anticonvulsants, etc.) have been designed to attenuate NP. However, only few patients could get relief from current therapies. In addition, patients using these pharmacological agents are always accompanied by the risk of tolerance and addiction (Calvo et al. 2019). Therefore, it is necessary and urgent to explore the pathological mechanisms and develop a new effective strategy for NP therapy. MSCs transplantation is proposed as therapeutic agents for various diseases, including ischemia diseases, metabolic diseases, autoimmune diseases and tissue injury (Xu et al. 2019). Recent evidence suggests that MSCs transplantation can reduce inflammation in many disease models. MSCs have been found to prevent microglia cells proliferation, inhibit graft rejection and support post-traumatic-stroke regeneration (Doeppner et al. 2015). Nowadays, MSCs represent potential candidate in the treatment of NP both in basic experimental and clinical research. Our previous study revealed that early delivery of MSCs attenuates NP by reducing inflammation. The benefits of MSCs transplantation can be achieve *via* directly tissue regeneration or paracrine. MSCs release large amounts of extracellular vesicles (EVs), including Exos, and these Exos are currently considered as important regulators of cellular communication. Exos carry mRNA, microRNA, lipids, and protein to the recipient cells for molecular signals modify in the body. Recent evidence have demonstrated that MSC-derived Exos confer the similar benefits as MSCs through transporting bioactive factors particularly miRNAs in inflammatory response (Xu et al. 2019). Compared to MSC, Exos have shown higher promise for clinical application because following advantage: (i)Exos are easier to management and store. (ii)Exos are non-immunogenic and have lower risk of tumor formation. (iii) Exos can be easily genetically

modified (Ludwig et al. 2019). Thus, Exos have the potential to be exploited as a novel therapeutic strategy for NP.

MicroRNAs are small noncoding RNAs and that act as post-transcriptional mediators of gene expression via silencing or degrading mRNA in normal cellular function as well as in pathological processes (Jonas & Izaurralde 2015). It is widely accepted that miRNAs play a crucial role in various processes of cell biological. Recently, several studies have demonstrated the dysregulation of microRNAs in spinal cord under NP conditions, and these results are consistent with ours in the current study. Using a miRNA microarray analysis, we found a total 130 miRNAs were significant changed (change fold>2, $P<0.05$) in the spinal cord tissues after CCI surgery. Among these miRNAs, miR-181c exhibited the most significant decreased which were detected by RT-PCR analysis. Interestingly, recent findings suggest that miR-181c is abundant and widely distributed in the nervous tissues, and it is highly involved in the pathological process of neuroinflammation. For instance, X. Li et al. reported that delivery of miR-181c decreased TLR4 expression and subsequently reduced NF-κB/p65 activation, which inhibited the secretion of inflammatory factors in a burn-induced excessive inflammation model (Li et al. 2016). Our current results and those of others suggested that miR-181c might be a critical molecule to mediating pain syndromes. Considering the fact that Gene transfection, a potential and valuable method, was widely used to regulate gene expression in recent years, we hypothesized that loading miR-181c into MSC-derived Exos may help us cure NP. In current study, we investigated the effect of $Exo^{miR\text{-}181c}$ on the sensitivity for mechanical and thermal stimulus. Our results suggest that $Exo^{miR\text{-}181c}$ dramatically inhibited mechanical allodynia and thermal hyperalgesia induced by CCI surgery.

While enormous efforts have been made, there is still a lack of understanding about mechanisms underlying the pathogenesis of NP. It is generally accepted that the neuroinflammation triggered by nervous system trauma is a critical event of this pain syndrome. Microglia cells are the principal resident immune cells that maintenance of the microenvironment homeostasis in nervous tissue. Many studies have reported that microglial cell activation is the early event that mediates neuroinflammation in NP. In response to neural injury, microglial cells release inflammatory factors such as IL-6, IL-1β, TNF-α and COX-2, which have been proved important for pain behavior. Therefore, a further research on microglial cell activation is necessary for understanding the development of NP. Blocking microglia cell function may have therapeutic effects in neuroinflammatory diseases. Here, we assume $Exo^{miR\text{-}181c}$ as a potential modulator of microglia cells. To verify our hypothesis, an in vitro model of microglia cells stimulation with LPS was performed. Our data suggest that $Exo^{miR\text{-}181c}$ inhibit the activation of microglia cells upon LPS stimulation by preventing secretion of IL-6, IL-1β, TNF-α and COX-2.

In conclusion, the evidence presented here suggests that genetically modified Exos can be easily internalize by microglia cells and prevent inflammatory cytokines secretion. Moreover, intrathecal administration of exosomal miR-181c could be a valid alternative for the treatment of NP and has vast potential for future development.

Acknowledgments

This study was supported by the natural science foundation of Ningbo (No. 2019A610281).

Abbreviations

NP	neuropathic pain
MSCs	Mesenchymal stem cells

Exos exosomes
CCI chronic constriction injury
PWT paw mechanical withdrawal threshold
PWL paw withdrawal latency
TEM Transmission electron microscope
NTA Nanoparticle tracking analysis

REFERENCES

AL-MASSRI KF, AHMED LA & EL-ABHAR HS. 2019. Mesenchymal stem cells therapy enhances the efficacy of pregabalin and prevents its motor impairment in paclitaxel-induced neuropathy in rats: Role of Notch1 receptor and JAK/STAT signaling pathway. Behav Brain Res 360: 303-311.

ALEXANDER MR ET AL. 2015. Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. Nat Commun 6: 7321.

ARZOUNI AA ET AL. 2018. Using Mesenchymal Stromal Cells in Islet Transplantation. Stem Cells Transl Med 7(8): 559-563.

BARON R, BINDER A, ATTAL N, CASALE R, DICKENSON AH & TREEDE RD. 2016. Neuropathic low back pain in clinical practice. Eur J Pain 20(6): 861-873.

BARTOLUCCI J ET AL. 2017. Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial. Circ Res 121(10): 1192-1204.

CALVO M ET AL. 2019. The Genetics of Neuropathic Pain from Model Organisms to Clinical Application. Neuron 104(4): 637-653.

CHAUHAN P, SHENG WS, HU S, PRASAD S & LOKENSGARD JR. 2018. Nitrosative damage during retrovirus infection-induced neuropathic pain. J Neuroinflammation 15(1): 66.

DOEPPNER TR, HERZ J, GORGENS A, SCHLECHTER J, LUDWIG AK, RADTKE S, MIROSCHEDJI K, HORN PA, GIEBEL B & HERMANN DM. 2015. Extracellular Vesicles Improve Post-Stroke Neuroregeneration and Prevent Postischemic Immunosuppression. Stem Cells Transl Med 4(10): 1131-1143.

FINNERUP NB ET AL. 2015. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol 14(2): 162-173.

GAO SH, SHEN LL, WEN HZ, ZHAO YD & RUAN HZ. 2017. Inhibition of Metabotropic Glutamate Receptor Subtype 1 Alters the Excitability of the Commissural Pyramidal Neuron in the Rat Anterior Cingulate Cortex after Chronic Constriction Injury to the Sciatic Nerve. Anesthesiology 127(3): 515-533.

GRACE PM, STRAND KA, GALER EL, MAIER SF & WATKINS LR. 2018. MicroRNA-124 and microRNA-146a both attenuate persistent neuropathic pain induced by morphine in male rats. Brain Res 1692: 9-11.

JIA Q, DONG W, ZHANG L & YANG X. 2020. Activating Sirt1 by resveratrol suppresses Nav1.7 expression in DRG through miR-182 and alleviates neuropathic pain in rats. Channels (Austin) 14(1): 69-78.

JONAS S & IZAURRALDE E. 2015. Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet 16(7): 421-433.

LI X, LIU L, YANG J, YU Y, CHAI J, WANG L, MA L & YIN H. 2016. Exosome Derived From Human Umbilical Cord Mesenchymal Stem Cell Mediates MiR-181c Attenuating Burn-induced Excessive Inflammation. EBioMedicine 8: 72-82.

LUDWIG N, WHITESIDE TL & REICHERT TE. 2019. Challenges in Exosome Isolation and Analysis in Health and Disease. Int J Mol Sci 20(19): 4684.

MEAD B & TOMAREV S. 2017. Bone Marrow-Derived Mesenchymal Stem Cells-Derived Exosomes Promote Survival of Retinal Ganglion Cells Through miRNA-Dependent Mechanisms. Stem Cells Transl Med 6(4): 1273-1285.

MEN Y ET AL. 2019. Exosome reporter mice reveal the involvement of exosomes in mediating neuron to astroglia communication in the CNS. Nat Commun 10(1): 4136.

PENG C ET AL. 2017. miR-183 cluster scales mechanical pain sensitivity by regulating basal and neuropathic pain genes. Science 356(6343): 1168-1171.

PHINNEY DG & PITTENGER MF. 2017. Concise Review: MSC-Derived Exosomes for Cell-Free Therapy. Stem Cells 35(4): 851-858.

SCHOLZ J ET AL. 2019. The IASP classification of chronic pain for ICD-11: chronic neuropathic pain. Pain 160(1): 53-59.

SIMONE U, SPINILLO A, CALONI F, GRIBALDO L & COCCINI T. 2019. Neuron-Like Cells Generated from Human Umbilical Cord Lining-Derived Mesenchymal Stem Cells as a New In Vitro Model for Neuronal Toxicity Screening: Using Magnetite Nanoparticles as an Example. Int J Mol Sci 21(1): 271.

SOMMER C, LEINDERS M & UCEYLER N. 2018. Inflammation in the pathophysiology of neuropathic pain. Pain 159(3): 595-602.

SPEES JL, LEE RH & GREGORY CA. 2016. Mechanisms of mesenchymal stem/stromal cell function. Stem Cell Res Ther 7(1): 1-13.

STEINBICHLER TB, DUDAS J, SKVORTSOV S, GANSWINDT U, RIECHELMANN H & SKVORTSOVA. 2019. Therapy resistance mediated by exosomes. Mol Cancer 18(1): 1-11.

THOMI G, SURBEK D, HAESLER V, JOERGER-MESSERLI M & SCHOEBERLEIN A. 2019. Exosomes derived from umbilical cord mesenchymal stem cells reduce microglia-mediated neuroinflammation in perinatal brain injury. Stem Cell Res Ther 10(1): 1-16.

WEI D, JIANG M, LIN P, YANG H, DANG Y, YU Q, LIAO D, LUO D & CHEN G. 2019. Potential ceRNA networks involved in autophagy suppression of pancreatic cancer caused by chloroquine diphosphate: A study based on differentially-expressed circRNAs, lncRNAs, miRNAs and mRNAs. International journal of oncology 54(2): 600-626.

WORTZEL I, DROR S, KENIFIC CM & LYDEN D. 2019. Exosome-Mediated Metastasis: Communication from a Distance. Dev Cell 49(3): 347-360.

XIAO C ET AL. 2018. Transplanted Mesenchymal Stem Cells Reduce Autophagic Flux in Infarcted Hearts via the Exosomal Transfer of miR-125b. Circ Res 123(5): 564-578.

XU R, GREENING DW, ZHU HJ, TAKAHASHI N & SIMPSON RJ. 2016. Extracellular vesicle isolation and characterization: toward clinical application. J Clin Invest 126(4): 1152-1162.

XU S, LIU C & JI HL. 2019. Concise Review: Therapeutic Potential of the Mesenchymal Stem Cell Derived Secretome and Extracellular Vesicles for Radiation-Induced Lung Injury: Progress and Hypotheses. Stem Cells Transl Med 8(4): 344-354.

XU Y, ZHANG X, PU S, WU J, LV Y & DU D. 2014. Circulating microRNA expression profile: a novel potential predictor for chronic nervous lesions. Acta Biochim Biophys Sin (Shanghai) 46(11): 942-949.

YIP PK, KAAN TK, FENESAN D & MALCANGIO M. 2009. Rapid isolation and culture of primary microglia from adult mouse spinal cord. J Neurosci Methods 183(2): 223-237.

ZHOU CH, ZHANG MX, ZHOU SS, LI H, GAO J, DU L & YIN XX. 2017. SIRT1 attenuates neuropathic pain by epigenetic regulation of mGluR1/5 expressions in type 2 diabetic rats. Pain 158(1): 130-139.

ZHUANG X ET AL. 2011. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Mol Ther 19(10): 1769-1779.

How to cite

ZHANG Y, YE GG, ZHAO JS, CHEN YB, KONG LD, SHENG CX & YUAN LY. 2022. Exosomes carried miR-181c-5p alleviates neuropathic pain in CCI rat models. An Acad Bras Cienc 94: e20210564. DOI 10.1590/0001-3765202220210564.

Manuscript received on April 13, 2021;
accepted for publication on October 20, 2021

YU ZHANG
https://orcid.org/0000-0001-6634-6803

GUANGYAO YE
https://orcid.org/0000-0002-8466-3746

JINGSONG ZHAO
https://orcid.org/0000-0003-1270-1309

YUEBO CHEN
https://orcid.org/0000-0002-2556-5273

LINGSI KONG
https://orcid.org/0000-0002-9569-066X

CHAOXU SHENG
https://orcid.org/0000-0002-0887-8382

LIYONG YUAN
https://orcid.org/0000-0002-1054-6336

Department of Anesthesiology, Ningbo No.6 Hospital, Ningbo 315040, Zhejiang, PR China

Correspondence to: **Liyong Yuan**
E-mail: yuanl1973@yeah.net

Author contributions

GY Y and Y Zhang performed the experiments and analyzed the data; JS Zhao, YB Chen, LS Kong and CX Sheng helped perform the experiments; LY Yuan designed the overall study and drafted the manuscript.