Acta Scientific Neurology (ISSN: 2582-1121)

Volume 4 Issue 8 August 2021

Research Article

Conditioned Medium from the Stem Cells of Human Exfoliated Deciduous Teeth Rescues White Matter Injury in Neonatal Rats

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Received: June 11, 2021

Published: July 14, 2021

Abstract

Background and Aim: White matter injury (WMI) is the most common type of brain injury in premature infants. Hypoxic ischemia (HI) is the major cause of neonatal WMI and in that case oligodendrocytes (OLs) are the main involved cells. Stem cell transplantation which can differentiate into OLs or promote endogenous OLs survival or differentiation is considered to be the optimal strategy for the treatment of WMI. Specifically, the neural crest-derived origin of stem cells from human exfoliated deciduous teeth (SHEDs) predisposes the cells to be optional candidates for the treatment of nervous system diseases. Recently, the conditioned medium from SHEDs (SHED-CM) has also garnered more and more attention as it can replace most of the paracrine roles of SHEDs. In the present study, we aim to explore the effects and related mechanisms of SHED-CM on the treatment of neonatal WMI.

Methods: SHEDs were cultured *in vitro* and SHED-CM was collected and concentrated through ultrafiltration. Postnatal day 3 SD rats were subjected to HI to establish the neonatal WMI model. SHED-CM was intraventricularly injected into the rats. Then Morris water maze was used to examine the behavioral performance and MBP immunostaining was used to evaluate myelin formation of the rats. To explore related mechanisms, microglia polarization and promyelinating factors expression in rat brain were also examined through qRT-PCR.

Results: SHED-CM promoted brain myelination and enhanced the behavioral performance of WMI rats. It promoted microglia polarization from M1 to M2 and up-regulated promyelinating factors such as Fgf1, Timp3 and Bdnf after HI in rat brain.

Conclusion: SHED-CM rescues WMI in neonatal rats, partly through promoted microglial polarization from M1 to M2 and up-regulated promyelinating factors after HI in rat brain. SHED-CM might be an optional agent for the treatment of neonatal WMI.

Keywords: White Matter Injury; Human Exfoliated Deciduous Teeth; Conditioned Medium; Myelin; Microglia Polarization

Introduction

Premature birth is a global public health problem that affects the early growth and development of human beings and many health issues in adulthood [1]. White matter injury (WMI) is the most common type of brain injury in premature infants and the main cause of neurological sequelae [2]. The pathogenesis of WMI in premature infants is not completely clear, and there is a lack of

specific treatment in clinic. Therefore, it is urgent to find an effective treatment strategy for premature infants with WMI.

Oligodendrocytes (OLs) are closely related to the formation of nerve myelin [3]. In the process of brain development, OLs differentiate into oligodendrocyte precursor cells (OPCs), premyelinating oligodendrocytes (pre-OLs), immature OLs, and then mature OLs. Next, mature OLs form myelin sheath and deposit on axons to maintain the integrity of axons and ensure the rapid and effective transmission of nerve signals along the axons [4].

Human preterm infants are prone to white matter injury at 23-32 weeks of gestation, and the corresponding OLs are in the differentiation stage of OPCs to pre-OLs [5]. OPCs and pre-OLS are very sensitive to hypoxic ischemia (HI), which is an major cause of white matter injury in the brain [6]. In the case of HI, OLs are the main involved cells and cannot regenerate after death [7]. Therefore, exogenous stem cell transplantation which can differentiate into OLs or promote endogenous OLs survival or differentiation is considered to be the optimal strategy for the treatment of WMI.

Nowadays, many types of stem cells have been used in the exploration of the treatment of neonatal brain damage. Among them, stem cells from human exfoliated deciduous teeth (SHEDs) are worth expecting. SHEDs are reported to originate from the cranial neural crest during the embryonic period, simultaneously expressing early mesenchymal, neuroectodermal, and certain embryonic stem cell markers [8]. SHEDs are highly proliferative, multi-potent, and self-renewing cells which actively secrete a broad range of trophic and immunomodulatory factors, giving them significant therapeutic potential in the treatment of diseases [9]. As SHEDs originate from the neural crest, they possess a much higher innate neurogenic potential, such as neural marker expression, differentiation into neural cells, and production and secretion of neurotrophic factors, than most other adult stem cells [10]. In the last decade, SHEDs-based therapies have emerged as a novel therapeutic option in a variety of neurological disorders such as Parkinson's disease [11]. Recently, the conditioned medium from SHEDs (SHED-CM) has also garnered more and more attention, as it can replace most of the paracrine roles of SHEDs [12]. However, the roles of SHED-CM in the treatment of WMI remain unclear. In the present study, we establish a neonatal WMI rat model, and explore the effects and related mechanisms of SHED-CM on the therapy for WMI.

Materials and Methods

Ethics approval statement

This study was approved by the Research Animal Care Committee of Sichuan University, China. All procedures on animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No.8023).

Animals and brain damage modeling

Postnatal day 3 (P3) Sprague-Dawley rats (half male and half female, average weight 8 - 12g) were purchased from Sichuan Dashuo Animal Science and Technology Co., Ltd (Chengdu, China).

The WMI model was established to mimic premature WMI using the following classical procedure [2]: First, P3 rats were fixed on their backs after general anesthesia. The neck skin was then longitudinally incised for a length of about 1cm, and the right carotid artery was exposed and ligated after separation from glands and muscle tissue. After surgery, the rats were returned into an incubator for 30 min to recover. Then, they were placed in an 8%-oxygen and 92%-nitrogen cabin (8% O_2 and 92% N_2) with a gas flow rate of 3 L/min for 2h to induce WMI. Rats were kept on a heating pad during surgical procedures to maintain the body temperature at 36 - 37°C. The rats of the sham group were only subjected to neck incision for dissociating the right carotid artery, without ligation or hypoxia. Following surgery, all rat pups were returned to their cages.

Culturing and characterization of SHEDs

SHEDs were obtained from the Sichuan Neo-life Stem Cell Biotech Inc, China. They were from the exfoliated deciduous of 6 - 8 years old children and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine se-rum (Gibco) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin; Gibco) at 37°C and in 5% CO_2 atmosphere.

Flow cytometry was performed to characterize SHED. 10^6 cells from cultivated human exfoliated teeth in the 5th passage were incubated with the following conjugated antibodies against human cell surface molecules: CD90, CD105, CD166, CD73,CD29, CD44, CD34, CD79α, and HLA-DR (PharMingen-BD Biosciences, USA), conjugated with FITC (Santacruz, USA) or PE (PharMingen-BDBiosciences, USA). Data acquisition was performed using the FACSAria III flow cytometer (BD Biosciences, USA) and 10,000 events were analyzed using FACS Diva 6.1.3 software (BD Biosciences, USA).

Preparation of conditioned medium from the stem cells of human exfoliated deciduous

SHEDs at passages 3 - 5 were washed with phosphate buffer saline (Gibco) and transferred to a serum-free DMEM culture medium. After 48 hours, the CM was harvested and centrifuged at 1,500 rpm for 5 minutes. The supernatant was re-centrifuged at 3,000 rpm for 3 minutes, followed by collection of the second supernatant. The CM was concentrated using ultrafiltration with a cut-off of 3 kDa (Millipore, Bedford, MA, USA), the ultrafiltrate was measured for protein concentration using the BCA protein assay kit (Pierce) and adjusted to 125 ng/μl protein, then stored at −80°C. Serum-free DMEM processed with ultrafiltration was used as the control.

SHED-CM treatment

The rats were randomly divided into four groups: a sham-operated group (sham); a HI model group (HI); a group receiving SHED-CM treatment (CM); and a group receiving serum-free DMEM treatment (DMEM). To establish the SHED-CM group, rats were injected with 4ul of SHED-CM (500 ng) at P4, P11 and P18 using a Hamilton syringe needle (Hamilton, USA) via the lateral ventricle, located 2 mm posterior and 2 mm lateral (right) from the bregma with a 2 mm needle depth. The dosage of SHED-CM was determined according to the previous study [13]. DMEM group received 4μl of serum-free DMEM at P4, P11 and P18.

Morris water maze

Behavioral testing using the Morris water maze (MWM) was performed from P29 to P34. The maze was a 120 cm-wide and 50 cm-high round pool with a black wall. The water was kept at 25°C and 30 cm deep. A 12 cm-wide cylindrical platform was set 2 cm below the water surface and 25 cm away from the wall in the first quadrant. Four trials were carried out per day at 30 min intervals. These were performed for five days at the same time each day. The rats were placed in one of the four quadrants, each time facing the pool wall to search for the underwater platform. If a rat found the platform within 90s, it was made to sit on the platform for 20 s. If the rat did not find the platform, it was guided to the platform for 20s, and the escape latency was recorded as 90s. For the probe trials conducted on day 34 after HI, the platform was removed, and the rats were allowed to swim from the third quadrant for 90s. The times for crossing the platform quadrant were recorded. An automatic camera system was set up above the pool and recorded each test route. Data were analyzed using Smart 3.0 Software (Panlab, USA). During the trials, constant light intensity and silence were maintained, and object placement was kept constant. The test was performed by an observer blinded to the group assignments.

MBP immunostaining

At P14, rats were sequentially perfused with 0.9% normal saline and 4% paraformaldehyde (100 mL each), after which the brains were extracted and post-fixed in a 4% paraformaldehyde solution for 24 - 36h at 4°C, and then embedded in 3% agarose in ddH_2O. The brains were cut into coronal sections (thickness 10 μm) and mounted on poly-L-lysine-coated slides. The expression of MBP was examined to reflect the myelination level of the brain from rats at P14. After deparaffinization, 10 μm coronal sections were rehydrated. Antigen retrieval was performed in a pre-heated 10 mM sodium citrate buffer (pH 6.0) for 30 min. After blocking with 1% bovine serum albumin and 0.3% cold fish skin gelatin in 0.1% Tween-20 TBS (all Sigma-Aldrich, Germany), slides were incubated with primary antibodies overnight at 4°C followed by appropriate secondary antibody incubation for 1h at room temperature. The primary antibody is mouse anti-MBP monoclonal antibody (1:1000, arigo). Imaging was performed using a confocal laser scanning microscope (Olympus, Japan) and FV-ASW-3.1 software (Olympus). The mean staining intensity of MBP in CC was counted for each field with a 20 x objective lens (field size, 0.95mm2), using the Image J software. In each animal, three regions (medial, middle, and lateral) along the CC were analyzed in 3 nonadjacent sections (~100 μm apart). Six animals per group were analyzed and the data were averaged per field.

qRT-PCR

qRT-PCR was used to examine the mRNA expression profiles of GFAP and Iba1, the pro-inflammatory factors (Interleukin IL-1beta, iNOS, IL-6 and IL-12), the anti-inflammatory factors (Arginase-1, CD206, TGF-beta) at P7 and the promyelinating factors (Fgf1, Timp3, Pleiotrophin, Tmsb4x, Bdnf, Vegfc) at P14 in the brain. The epsilateral brain was extracted and the cortex was carefully removed. Total RNA was isolated from the remnants using an RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed with SuperScript III (Invitrogen, Carlsbad, CA). The levels of mRNAs were evaluated by quantitative PCR (qPCR) using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression values were determined using the DDCT method. GAPDH was used as the housekeeping gene. Results were normalized to the sham group. Three independent assays were performed. The primers used are shown in table 1.

Gene	Primer sequence
GFAP	Forward: TCCAGATCCGAGAAACCAGC Reverse: CCGCATCTCCACCGTCTTTA
Iba1	Forward: CAGAGCAAGGATTTGCAGGGA Reverse: CAAACTCCATGTACTTCGTCTTGA
IL-1β	Forward: CAGCTTTCGACAGTGAGGAGA Reverse: TTGTCGAGATGCTGCTGTGA
iNOS	Forward: AGATCCCGAAACGCTACACTT Reverse: TGCGGCTGGACTTCTCACTC
IL-6	Forward: ATTCTGTCTCGAGCCCACCA Reverse: CTGAAGGGCAGATGGAGTTGA
IL-12	Forward: AGAAGTACTCAGTGGCGTGC Reverse: GGTGGGTCCGGTTTGATGAT
ARG1	Forward: ACATCAACACTCCGCTGACAACC Reverse: GCCGATGTACACGATGTCCTTGG
CD206	Forward: CGGGGACTTGGGCTGTATTC Reverse: GCCGTGAGTCCAAGAGTTGA
TGF-β	Forward: TGCGCCTGCAGAGATTCAAG Reverse: AGGTAACGCCAGGAATTGTTGCTA
Fgf1	Forward: CCAGCACAGTTTGGAACACG Reverse: ACACATGGCTCCCCTCTTTG
Pleiotrophin	Forward: AACAAAGGCAGCCTGCTAGT Reverse: TCGACGTTGCTGCTGGTATT
Tmsb4x	Forward: CAGCTCGCTCAGCTCCTTC Reverse: TGCATATTGGCGGCGCT
Timp3	Forward: GCAACTCCGACATCGTGATCC Reverse: TCACCAGCTTCTTTCCCACC
BDNF	Forward: CTGCGCCCATGAAAGAAG Reverse: CCAGCAGCTCTTCGATCA
Vegfc	Forward: TGCCGGTGCATGTCTAAACT Reverse: TTAGCTGCCTGACACTGTGG

Table 1: The primer sequences for qRT-PCR.

Statistical analysis

Data are presented as the mean ± SD. All data were tested for normality using the Shapiro-Wilk test, and all data passed the test. A Student's t-test was used when comparing between two groups. Analysis of variance (ANOVA) with Fisher's post-hoc test was used when comparing more than two groups. A p-value of less than 0.05 was defined as significant.

Results

Characterization of isolated SHEDs

Flow cytometry showed that all SHEDs highly express MSC markers CD90 (100%), CD105 (100%), CD166 (100%), CD73 (100%), CD29 (100%), CD44 (100%), but have little expression of the hematopoietic progenitor markers of CD34 (2%), CD79α (1.9%), and HLA-DR (0.7%) (Figure 1). This result indicated that SHEDs have the characteristic of mesenchymal stem cell.

Figure 1: Characterization of SHEDs.

Flow cytometry analysis of the surface markers of CD90, CD105, CD166, CD73, CD29, CD44, CD34, CD79α, and HLA-DR with flow cytometry. SHEDs highly express mesenchymal stem cell markers CD90, CD105, CD166, CD73, CD29, CD44, but have little expression of the hematopoietic progenitor markers of CD34, CD79α, and HLA-DR.

SHED-CM enhanced the behavioral performance of WMI rats

The Morris water maze (MWM) test was conducted to examine the behavioral performance of rats, by calculating the average es-

cape latency and platform crossing times during swimming. It was shown that the average escape latency was greatly decreased from P31to P33, and the frequency of platform crossing was significantly increased at P34, in the SHED-CM group compared with the HI and DMEM group, suggesting that SHED-CM treatment contributes to behavioral improvements in terms of learning and memory in rats (Figure 2).

Figure 2: SHED-CM enhanced the behavioral performance of WMI rats.

A MWM test was conducted to evaluate the spatial learning and memory of rats. Rats in the SHED-CM group took significantly less time to find the underwater platform in the first quadrant from P31 to P33, and crossed the former platform location more frequently compared to the HI and DMEM group (*p < 0.05). Data are presented as the mean ± SD from 3 independent experiments, with n = 8 for each group in each experiment.

SHED-CM promoted brain myelination after HI in rats

We examined the myelination of brain white matter by assessing the expression of MBP through immunostaining, especially in CC. At P14, the expression of MBP was significantly enhanced in the SHED-CM group compared with the DMEM group (Figure 3).

SHED-CM promoted microglial polarization from M1 to M2 after HI in rat brain

qRT-PCR showed that GFAP and Iba were increased after HI, SHED-CM treatment decreased Iba 1 expression but did not affect GFAP expression, and DMEM did not change the expression of both Iba 1 and GFAP (Figure 4a), suggesting that SHED-CM decreased microglia activation but not affected astrocyte reaction. We next examined whether SHED-CM affected microglial polarization after HI. We assessed gene expression profiles associated with M1/M2 microglial polarization and observed a significant increase in M1 associated proinflammatory gene expression for IL-1beta, iNOS, IL-6 after HI (Figure 4b), whereas M2 associated anti-inflammatory gene expression for Arginase-1, CD206, TGF-beta was not significantly changed (Figure 4c). SHED-CM treatment significantly decreased the expression of M1 associated proinflammatory gene (Figure 4b) but increased M2 associated anti-inflammatory gene (Figure 4c), indicating that SHED-CM promoted microglial polarization from M1 to M2 after HI in neonatal rat brain.

Figure 3: SHED-CM promoted myelination after HI in rat brain.

Immunostaining showed that the expression of MBP in CC at P21 was decreased after HI, SHED-CM treatment significantly enhanced their expression, whereas DMEM had no such effect. P < 0.05, compared the SHED-CM group with HI group.

SHED-CM up-regulated promyelinating factors after HI in neonatal rat brain

Promyelinating factors play important roles in modulating myelin formation after injury [14], so we further examined whether SHED-CM affected the expression of promyelinating factors Fgf1, Timp3, Pleiotrophin, Tmsb4x, Bdnf and Vegfc after HI in neonatal rats at P14. It showed that Fgf1, Timp3, Bdnf and Vegfc decreased but Pleiotrophin and Tmsb4x were not affected in the ipsilateral brain excluding cortex after HI. After SHED-CM treatment, the expression of Fgf1, Timp3 and Bdnf was up-regulated but Vegfc remained at a low level. DMEM did not affect the expression of these promyelinating factors (Figure 5).

Discussion and Conclusion

In the present study, we established a neonatal WMI rat model, and explored the effects and related mechanisms of SHED-CM on the therapy for WMI. We found that SHED-CM promoted brain myelination and enhanced the behavioral performance of WMI rats, at

Figure 4: SHED-CM promoted microglial polarization from M1 to M2 after HI in rat brain.

(a) In the ipsilateral brain hemispheres excluding cortex, qRT-PCR showed that GFAP and Iba 1 were increased after HI, SHED-CM treatment decreased Iba1 expression but did not affect GFAP expression, and DMEM did not change the expression of both Iba 1 and GFAP. $P < 0.05$, compared the SHED-CM group with HI group. Data are presented as the mean ±SD from 3 independent experiments, with n = 8 for each group in each experiment. HI, hypoxia-ischemia; CM, conditioned medium from SHED.

(b) In the ipsilateral brain hemispheres excluding cortex, qRT-PCR showed that the M1 associated pro-inflammatory genes of iNOS, IL-1beta and IL-6 were significantly upregulated in animals with HI injury. SHED-CM treatment significantly decreased the expression of these pro-inflammatory genes, whereas DMEM did not affect their expression. $P < 0.05$, compared the SHED-CM group with HI group. Data are presented as the mean ± SD from 3 independent experiments, with n = 8 for each group in each experiment.

(c) In the ipsilateral brain hemispheres excluding cortex, qRT-PCR showed that the M2 associated anti-inflammatory genes of Arginase-1, CD206, TGF-beta remained at baselines in animals with HI injury. SHED-CM treatment significantly increased the expression of these anti-inflammatory genes, whereas DMEM did not affect their expression. $P < 0.05$, compared the SHED-CM group with HI group. Data are presented as the mean ± SD from 3 independent experiments, with n = 8 for each group in each experiment.

least partly through promoted microglial polarization from M1 to M2 and up-regulated promyelinating factors after HI in rat brain.

It has been reported that different activation states of microglia play central roles in the pathogenesis of WMI [15]. Microglia can be divided into two phenotypes, namely proinflammatory M1-type cells and anti-inflammatory M2-type cells, and can be switched between M1 and M2, depending on changes in the environment. This process is called polarization [16]. Our results suggested that SHED-CM might improve inflammatory environment of WMI through modulate microglia polarization.

Besides inflammatory reaction, promyelinating factors also participate the constitution of micro-environment for WMI and recover. Goebbels S., *et al.* have reported many promyelinating factors such as Fgf1, Timp3, Pleiotrophin, Tmsb4x, Bdnf and Vegfc which play critical roles in regulating myelin formation [14]. Therefore, we further examined whether SHED-CM affected the expression of these promyelinating factors. We found that three of them, Fgf1,

Figure 5: Promyelinating factors expression in ipsilateral brain hemispheres excluding cortex from rats.

In the ipsilateral brain hemispheres excluding cortex, qRT-PCR showed that promyelinating factors Fgf1, Timp3, Bdnf and Vegfc decreased but Pleiotrophin and Tmsb4x were not affected after HI in P14 rats. After SHED-CM treatment, the expression of Fgf1, Timp3 and Bdnf was up-regulated but Vegfc remained at a low level. DMEM did not affect the expression of these promyelinating factors. $P < 0.05$, compared the SHED-CM group with HI group. Data are presented as the mean ± SD from 3 independent experiments, with $n = 8$ for each group in each experiment.

Timp3 and Bdnf were significantly up-regulated after SHED-CM treatment, suggesting that SHED-CM might directly promote myelin formation.

Compared to SHED transplantation, the use of SHED-CM has remarkable advantages. It eliminates concerns with regard to tumorigenesis and immune reactions [17]. Besides, it resolves the ethics issues surrounding cell therapies. Moreover, as SHED-CM can be stocked, it is convenient for its immediate application and may be used in the acute stages of diseases. Taken together, SHED-CM is an optional agent for the treatment of neonatal WMI. Its effectiveness and safety are worth to be further examined in clinics in the future.

Funding

This study was funded by National Natural Science Foundation of China (81971428, 81971429).

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