Effect of hUC-MSCs on the NLRP3/Caspase-1 Pathway in APP/PS1 Mice

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Abstract [**Objectives**] To investigate the effect of human umbilical cord mesenchymal stem cells (hUC-MSCs) on the NOD-like receptor protein 3 (NLRP3)/cysteinyl aspartate specific proteinase (Caspase-1) pathway within the cerebral cortex of a mouse model of Alzheimer's disease (AD). [**Methods**] Twelve 6-month-old female APP/PS1 mice were randomly assigned to two groups: the model group (MOD, $n=6$) and the hUC-MSCs treatment group (MSC, $n=6$). Six 6-month-old C57BL/6N mice were utilized as a control group (CON, $n=6$). All mice underwent caudal vein injections of hUC-MSCs. Following a 4-week treatment, the mice from each group were euthanized. The expression levels of NLRP3, Caspase-1 protein, and mRNA in the cerebral cortex of each group were assessed using Western blotting and real-time fluorescence quantitative PCR assays. [**Results**] The results of immunoblotting showed that the expression levels of NLRP3 and Caspase-1 proteins in the MOD group were significantly higher than those observed in the CON group. Furthermore, the expression levels of NLRP3 and Caspase-1 proteins in the MSC group were found to be lower than those in the MOD group. Additionally, the findings from real-time fluorescence quantitative PCR assay demonstrated that the mRNA levels of NLRP3 and Caspase-1 in the MOD group were elevated compared to the CON group. Conversely, the mRNA levels of NLRP3 and Caspase-1 in the MSC group were reduced in comparison to the MOD group. [**Conclusions**] hUC-MSCs have the capacity to modulate the expression of the NLRP3/Caspase-1 pathway within the cerebral cortex of APP/PS1 mice. This modulation may be associated with the neuroinflammatory processes mediated by hUC-MSCs in the brains of APP/PS1 mice.

Key words Alzheimer's disease (AD), Human umbilical cord mesenchymal stem cells (hUC-MSCs), APP/PS1 mice, NLRP3 inflammasome

1 Introduction

Alzheimer's disease (AD) encompasses a category of insidious neurodegenerative disorders that are characterized by a progressive and chronic decline in cognitive function, as well as memory impairment[1]. The two primary pathological hallmarks of AD are the accumulation of β-amyloid (Aβ) plaques and the formation of neurofibrillary tangles (NFTs), which are composed of hyperphosphorylated tau protein[2-3]. The accumulation of Aβ in the brain is associated with AD, which results in significant memory impairment in affected individuals. This pathological process disrupts calcium homeostasis, leads to intracellular calcium overload, and initiates apoptosis, ultimately resulting in synaptic dysfunction and neuronal death[4]. Neuroinflammation is currently recognized as a significant factor in the pathogenesis of AD[5]. The nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasome, which senses Aβ and tau protein aggregation, initiates inflammatory signaling pathways that contribute to chronic inflammation and cellular apoptosis. This process exacerbates the physiopathological alterations associated with AD and results in cognitive decline. Consequently, the activation of NLRP3 inflammasomes is pivotal in eliciting inflammatory responses and the pathological characteristics of AD[6-7]. The transplantation of human umbilical cord mesenchymal stem cells (hUC-MSCs) has been demonstrated to be an effective therapeutic intervention for AD. This approach enhances cognitive function in AD models by decreasing Aβ deposition, safeguarding neuronal integrity, and facilitating neurogenesis. Numerous animal studies utilizing AD models have substantiated the therapeutic efficacy of various interventions. However, the underlying mechanisms of action remain inadequately understood. It is posited that these interventions may exert therapeutic effects through the secretion of neurotrophic factors, promotion of angiogenesis, immunomodulation, anti-inflammatory actions, and other mechanisms[8]. This study aimed to investigate the effect of hUC-MSCs on the NLRP3/Caspase-1 pathway in APP/PS1 mice, based on the established correlation between AD and NLRP3 inflammasomes. The objective was to elucidate the mechanisms by which hUC-MSCs may contribute to the treatment of AD, thereby providing a theoretical foundation for the clinical application of hUC-MSCs.

2 Materials and methods

2.1 Materials

2.1.1 Experimental animals and grouping. Twelve 6-month-old SPF grade female APP/PS1 mice, each weighing between 20 and 25 g, were acquired from Beijing HFK Bioscience Co., Ltd., under certificate of conformity [SCXK (Beijing) 2019-0008]. Six female C57BL/6N mice, aged 6 months and weighing between 22 and 25 g, were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd., as indicated by certificate of conformity [SCXK (Beijing) 2021-0006]. The mice were subsequently bred in the Experimental Animal Centre of Chengde Medical University, in accordance with the guidelines outlined in [SCXK (Hebei) 2022-002]. All experiments received approval from the Animal Ethics Committee of Chengde Medical University (CDMU-LAC-20220401-001). Mice were maintained in a controlled barrier environment with a temperature of (25 ± 2) ℃ and a relative humidity of (55 ± 5)%. The animals were subjected to a 12-h light/dark cycle and were provided with food and water *ad libitum*. Experiments were conducted following a 7 d acclimatization period during which the mice were fed. A total of 6 C57BL/6N mice served as the control group (CON group, $n=6$). Addition-

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ally, 12 APP/PS1 mice were allocated into two groups: 6 mice in the model group (MOD group, $n=6$) and 6 mice in the hUC-MSCs treatment group (MSC group, $n=6$), based on the random number table method.

2.1.2 Materials. hUC-MSCs were procured from Beijing Hongxin Stem Cell Biotechnology Co., Ltd.

2.2 Methods

2.2.1 Recovery and counting of hUC-MSCs. The hUC-MSCs freezing tube was removed from the −80 ℃ ultra-low temperature freezer and promptly placed in a 37 ℃ water bath. The tube was gently agitated to facilitate thawing. Subsequently, the surface of the tube was disinfected with alcohol before transferring it to the biosafety cabinet for the opening of the freezing tube. The cell suspension was aspirated into a centrifuge tube containing 1 mL of normal saline and subsequently centrifuged at 1 000 rpm for 5 min. The supernatant was then discarded, and the rinsing procedure was repeated three times. The cells were subsequently resuspended in 1 mL of normal saline and thoroughly blown to create a saline suspension of hUC-MSCs. 10 μL of the aforementioned cell suspension was diluted to 1 mL using normal saline. The diluted cell suspension was subsequently stained with an equal volume of Trypan Blue reagent for 1 min. Following this, the number of viable cells (N) was quantified using a cell counting plate. Total cellular score/mL = $N/4\times10^4$. A saline suspension of hUC-MSCs was prepared at a concentration of 2.5×10^6 cells/mL, determined by the number of viable cells intended for subsequent injections.

2.2.2 Caudal vein injection of mouse. The mice from each group were positioned in a mouse fixator. The tail of each mouse was cleansed with an alcohol-soaked cotton ball to enhance the visibility and dilation of the caudal vein. Subsequently, a needle was inserted into the thin skin of the mouse tail, specifically at the lower third of the most central vein, allowing for the puncture of the caudal vein to facilitate injection. The mice in the MSC group were administered 0.4 mL (10^6) of a saline suspension of hUC-MSCs, which was extracted using a 1 mL disposable syringe. The cell suspension was slowly injected, ensuring that there was no resistance during the administration process. A small amount of blood was observed in the syringe needle following the injection, indicating that the injection into the mouse caudal vein was successful. The mice in the CON and MOD groups received equal volumes of sterile saline for caudal vein injection. Following the injection, cotton balls were applied to the injection site to control bleeding, and all mice were carefully returned to their cages. These procedures were conducted once a week, resulting in a total of four interventions over the course of four weeks.

2.2.3 Brain tissue sampling. The mice in each group were anesthetized, and the fur was shaved along the median line at the foramen magnum of the occipital bone to expose the entire cranium. The cranium was then carefully removed, and the mouse cerebral cortex was detached and placed in sterile EP tubes. These tubes were subsequently encapsulated and stored in a refrigerator at −80 ℃ for preservation.

2.2.4 Protein immunoblotting assay. A total of 20 mg of mouse cortical tissue was utilized for grinding, followed by extraction to determine the protein concentration. Subsequently, the proteins underwent electrophoretic separation and were transferred to a PVDF membrane. The membrane was then blocked with skimmed milk for 1 h. Primary antibodies against NLRP3 and Caspase-1 (dilution 1 ∶ 3 000) were applied and incubated overnight at 4 ℃. Following this, a secondary antibody (dilution 1 ∶ 5 000) was added and incubated at 4 ℃ for 1 h. ECL chromogen was employed to develop the color, and images were captured using a chemiluminescent imaging system. The relative expression of each group of proteins was analyzed using ImageJ software.

2.2.5 Real-time fluorescence quantitative PCR assay. 20 mg of mouse cortical tissue was homogenized, and RNA was subsequently extracted utilizing the TaKaRa MiniBEST Universal RNA Extraction Kit. The concentration of the extracted RNA, along with the $OD_{260/280}$ optical density values, was measured. The qualified RNA was reverse transcribed into cDNA utilizing the TaKaRa PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time). Subsequently, a real-time fluorescence quantitative PCR assay was performed using the cDNA as a template. The specific primer sequences employed in this study are provided in Table 1.

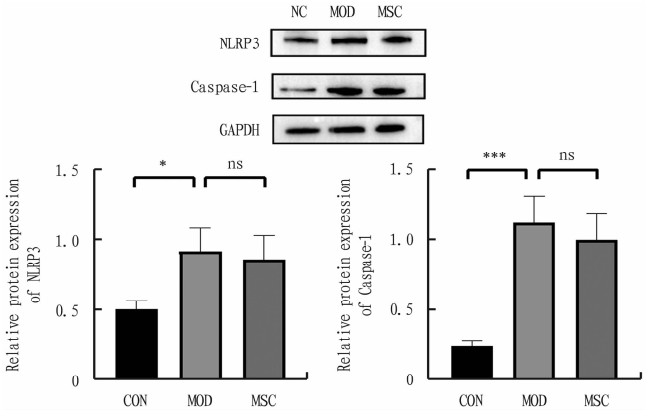
Table 1 Primer sequences

Target gene	Upstream sequence	Downstream sequence	Fragment size//bp
NLRP3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG	141
Caspase-1	ACAAGGCACGGGACCTATG	TCCCAGTCAGTCCTGGAAATG	237
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123

2.2.6 Statistical analysis. The experimental data were analyzed utilizing SPSS 27.0 statistical software, and the results were statistically summarized as mean ± standard deviation ($\bar{x}\pm s$). If the combined data from the two samples conformed to a normal distribution and exhibited equal variances, a two independent samples t test was employed to compare the groups. Conversely, if the combined data did not conform to a normal distribution or if the variances were unequal, the Wilcoxon rank-sum test was utilized for the analysis of the two independent samples. A statistically significant difference was determined at a P value less than 0.05. Graphical representations were generated using GraphPad Prism 9.5 software.

3 Results and analysis

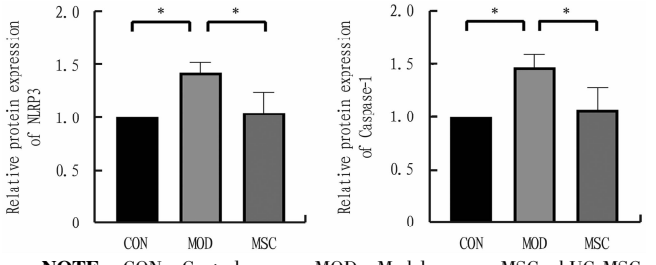
3.1 Results of protein immunoblotting assay of mice in each group The results of the protein expression of NLRP3 and Caspase-1 in mice across the various groups were analyzed, and histograms depicting the relative protein expression levels of NLRP3 and Caspase-1 was generated (Fig. 1). The data indicated that the protein expression levels of NLRP3 and Caspase-1 in the mice were significantly lower in the MSC group compared to the MOD group. The protein expression levels of NLRP3 and Caspase-1 in the mice from the MOD group were significantly elevated in comparison to those in the CON group, with a statistically significant difference ($P<0.05$).



NOTE CON. Control group; MOD. Model group; MSC. hUC-MSCs group; $^{*}P<0.05$; $^{***}P<0.001$; ns: $P>0.05$.

Fig. 1 Relative protein expression of NLRP3 and Caspase-1

3.2 Results of real-time fluorescence quantitative PCR assay of mice in each group Based on the results of the relative mRNA quantitative expression of NLRP3 and Caspase-1 in mice across the various groups, histograms depicting the relative mRNA expression levels of NLRP3 and Caspase-1 were generated (Fig. 2). The results indicated that the mRNA expression levels of NLRP3 and Caspase-1 in mice from the MOD group were significantly elevated in comparison to those in the CON group. Furthermore, the mRNA expression levels of NLRP3 and Caspase-1 in mice from the MSC group were significantly reduced when compared to the MOD group, with the difference being statistically significant ($P<0.05$).



NOTE CON. Control group; MOD. Model group; MSC. hUC-MSCs group; $^{*}P<0.05$.

Fig. 2 Relative mRNA expression of NLRP3 and Caspase-1

4 Discussion

AD is a progressive neurodegenerative disorder that profoundly impacts the quality of life of affected individuals and exacerbates the burden on their caregivers[9]. AD is clinically characterized by a progressive deterioration in memory and cognitive function, accompanied by challenges in daily living activities[10]. Currently, there are no effective preventive or curative interventions available. Consequently, clinical practice primarily focuses on symptomatic and comprehensive treatments designed to alleviate the symptoms associated with cognitive decline and to delay the progression of the disease. This underscores the necessity for the exploration of novel approaches to address this pressing issue[11]. MSCs are pluripotent stem cells characterized by their ability to self-renew and differentiate into various cell types. In animal models of AD, MSCs have been shown to enhance neurogenesis, alleviate memory deficits, mitigate synaptic dysfunction, and reduce neuroinflammation[8]. hUC-MSCs exhibit characteristics indicative of an earlier and more youthful stage of embryonic development. They demonstrate higher yields, are devoid of ethical concerns and invasive procedures, and possess the capability to secrete a diverse array of multifunctional factors[12]. It has been demonstrated that the transplantation of hUC-MSCs enhances cognitive function in mice with AD. The underlying mechanism appears to be associated with a reduction in oxidative stress and the facilitation of nerve regeneration in the hippocampus[13-14]. While limited information is available regarding the effects of hUC-MSCs on the mouse cerebral cortex, this study will initiate its investigation in the cortex to further elucidate the potential mechanisms by which hUC-MSCs may contribute to the treatment of AD mice. Neuroinflammation is a significant factor contributing to AD, and among the various components involved in the pathogenesis of AD, the NLRP3 inflammasome complex has been the most extensively studied to date[15]. The NLRP3 inflammasome is a multi-protein complex consisting of NLRP3, ASC, and the precursor of Caspase-1, with the NLRP3 protein assuming a central role in its function. Research has demonstrated that elevated expression levels of the NLRP3 and Caspase-1 genes promote the development of senescence lesions in the brains of APP/PS1 transgenic mice[16]. NLRP3 inflammasome is capable of detecting PAMPs or DAMPs. This detection initiates the formation of Caspase-1 in microglial cells, which subsequently enhances the production and secretion of inflammatory cytokines, specifically IL-1β and IL-18. This process contributes to the establishment of an inflammatory milieu within the brain. Furthermore, the activation of the NLRP3 inflammasome has been closely linked to the deposition of Aβ and the pathophysiology of tau protein[7,17]. Consequently, a more in-depth exploration of the role of the NLRP3 inflammasome in AD and its underlying mechanisms may yield novel therapeutic strategies for the intervention of AD. Western blotting and real-time quantitative PCR were employed to assess the expression levels of NLRP3 and Caspase-1 in each group of mice, at the protein and mRNA levels, respectively. The results indicated that treatment with hUC-MSCs significantly decreased the expression of NLRP3 and Caspase-1 in APP/PS1 double transgenic mice. Research has demonstrated that the activation of the NLRP3 inflammasome restricts partial microglial clearance, while deficiencies in NLRP3 and Caspase-1 enhance the phagocytosis of plaques by microglia[18]. Xu *et al.* [19] demonstrated that UC-MSCs provide protection to microglia and neurons against the neurotoxic effects of Aβ by modulating the expression of autophagy-related proteins in microglia and increasing the levels of Aβ-degrading enzymes. This regulatory mechanism facilitates Aβ phagocytosis and helps to avert apoptosis. Consequently, this study will further investigate the mechanisms by which hUC-MSCs modulate the secretion of the NLRP3 inflammasome via microglial activity, thereby influencing the progression of AD. In summary, our research demonstrates that hUC-MSCs regulate the expression levels of the NLRP3 inflammasome in the cerebral cortex of APP/PS1 transgenic mice. This provides both a theoretical framework and an experimental basis for future investigations into the potential of stem cell transplantation as a therapeutic approach for AD.

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nursing and it was statistically significant ($P<0.001$), but the increase in the intervention group was greater and close to normal. After receiving "One Disease, One Product" care for 3 weeks, the score of the intervention group was significantly higher than that of the control group, and the difference between the two groups was statistically significant ($P<0.001$); the difference between the two groups during follow-up 3 months after discharge was statistically significant ($P<0.001$) (Table 2).

Table 2 BI scores of patients in both groups ($n=120$, points, $\bar{x}\pm s$)

Group	Pre-care	Post-care	Follow-up
Intervention	23.23 ± 6.48	78.25 ± 10.27$^{\triangle\#}$	92.87 ± 15.33$^{\triangle\#}$
Control	25.79 ± 7.32	62.43 ± 9.82$^{\triangle}$	73.27 ± 12.51$^{\triangle}$

4 Discussion

With the aging trend of the population becoming more and more serious, the prevalence of cardiovascular and cerebrovascular diseases, especially stroke, is rising. *The China Stroke Prevention and Treatment Report* 2016 shows that the prevalence of stroke in China increased from 0.40% in 1993 to 1.23% in 2013[5]. Clinical reality shows that most stroke patients die not of the disease itself, but of its complications. More than 22% of stroke patients have dysphagia, and dysphagia seriously affects patients' activities of daily living and is closely related to adverse rehabilitation outcomes of stroke[6-7]. Through the "One Disease, One Product" nursing program, we conducted specialized nursing assessment when admitted to the hospital, to improve their diet as soon as possible, implemented safe eating skills, comprehensive rehabilitation measures and systematic feeding training for patients with swallowing disorders to improve the quality of life of patients. The results of this study showed that standardized screening of swallowing disorders in stroke patients and evaluation of swallowing dysfunction at admission and after 3 weeks of care indicated that the specialized nursing was superior to routine care in neurosurgery. The Barthel index (BI) was used to assess the patient's activity of daily living before the onset of stroke, after 3 weeks of "One Disease, One Product" care and during follow-up after 3 months, and it was found that the evidence-based "One Disease, One Product" specialized nursing program can continuously improve patients' activities of daily living.

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