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Umbilical cord-derived mesenchymal stem cell conditioned medium reverses neuronal oxidative injury by inhibition of TRPM2 activation and the JNK signaling pathway

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Abstract

In this study, we aimed to clarify the protective effect of umbilical cord-derived mesenchymal stem cell conditioned medium (UC-MSC-CM) on neuronal oxidative injury and its potential mechanism. Neuronal oxidative damage was mimicked by H₂O₂ treatment of the HT22 cell line. Immunofluorescence staining was used to analyze the expression of cleaved Caspase3 and TRPM2. Cytotropic factors secreted by UC-MSCs were analyzed by enzyme-linked immunosorbent assay. Whole-cell patch clamping was used to detect TRPM2-like currents. Western blotting was utilized to analyze the protein levels of SOD, p-ERK1/2, p-JNK1/2/3 and cleaved-Caspase-9. The number of cleaved-Caspase-3-positive cells and protein expression of Caspase-9 induced by H₂O₂ treatment were decreased by UC-MSC-CM treatment. Furthermore, SOD protein expression was increased in the MSC-CM group compared with that in the H₂O₂ group. The H₂O₂-induced TRPM2-like currents in HT22 cells were attenuated by MSC-CM treatment. In addition, H₂O₂ treatment downregulated the expression of p-JNK protein in HT22 cells, and this the downward trend was reversed by incubation with MSC-CM. Thus, we showed that UC-MSC-CM protects neurons against oxidative injury, possibly by inhibiting activation of TRPM2 and the JNK signaling pathway.

Key Words: mesenchymal stem cells, conditioned medium, TRPM2, neuronal apoptosis, MAPK

1. Introduction

Stroke is the second leading cause of death worldwide and a major contributor to disability. Ischemic stroke, which is the most common type of this event[1], disrupts the production and clearance of reactive oxygen species (ROS), leading to their accumulation in brain tissue. This, in turn, results in the destruction or changes in lipids, proteins and nucleic acids in a cascade reaction that ultimately causes neuronal death[2]. However, no effective therapeutic strategies have yet been developed to prevent or treat oxidative stress injury.

Mesenchymal stem cells (MSCs) have been shown to exert antioxidant functions in experimental animals[3]. In recent years, MSCs have been widely used in experimental investigations of nerve injury repair because of their strong regeneration potential and ability to improve the microenvironment by secreting a variety of cytokines[4]. MSC-conditioned medium (MSC-CM) is heterogeneous and contains various soluble factors that can reduce lipid peroxidases generation, while promoting antioxidant enzyme production[5-8]. However, the mechanism by which MSC-CM protects neuronal cells against ischemic injury remains to be elucidated.

Transient receptor potential melatonin 2 (TRPM2) belongs to the TRP family and is a non-selective cation channel that allows the entry of calcium ions[9-11]. Studies have shown that H₂O₂ and ROS activate TRPM2 to induce the accumulation of calcium ions in cells, leading to cell death[12]. Importantly, recent studies have shown that TRPM2 channels play an important role in ischemic stroke, with TRPM2 activation further aggravating the condition[13-15].

In this study, we investigated the protective effect of umbilical cord-derived MSC-CM on H₂O₂-induced neuronal injury, and the potential roles of TRPM2 inhibition and the MAPK pathway in the underlying mechanism.

2. Materials and Methods

2.1 HT22 cell culture

HT22 cells were purchased from Bena Chuang Lian Biotechnology Co., Ltd (Beijing, China).and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) containing 5% fetal bovine serum (FBS; Biological Industries, Israel) and incubated at 37°C under 5% CO₂ in a humidified atmosphere.

2.2 Preparation of human umbilical cord-MSC-CM

1 This study was approved by the Ethics Committee of the General Hospital of Ningxia
2 Medical University, China. Human umbilical cord-MSCs (UC-MSCs) were isolated from the
3 umbilical cord connective tissue collected from healthy patients after obtaining informed
4 consent. 3×10^5 UM-MSCs were seeded in 100mm culture plate containing Ultra Culture
5 Serum-free Medium (Lonza, Switzerland) supplemented with 2% Pall Ultrosor G Serum
6 Substitute (Pall, USA), and cultured in the CO₂ incubator maintained at 37°C with 5% CO₂
7 and a humidified atmosphere. Medium was changed every 3 days. At approximately 90%
8 confluence, cells were passaged and 3×10^5 UM-MSCs reseeded in a new 100mm culture plate.
9 1×10^6 UC-MSCs at passages 4 were seeded in a new 100mm culture plate. At 60%–70%
10 confluence, the medium was changed and the cells cultured for a further 24 h before harvesting
11 the conditioned medium (CM). The CM was filtered (0.22- μ m pore size) to remove cellular
12 debris and concentrated using ultrafiltration units with a 3-kDa molecular-weight cutoff
13 (Millipore, Burlington, MA) to obtain the MSC-CM.

14 15 2.3 Experimental design

16 HT22 cells were divided into the following experimental groups: Normal: culture in
17 complete medium; H₂O₂: treated with 100 μ M H₂O₂ for 1 h; MSC-CM: administration of UC-
18 MSC-CM after treatment with 100 μ M H₂O₂ for 1 h; SP600125 (a JNK inhibitor) and
19 PD98059 (an ERK inhibitor): starved with 0.5% serum for 6 h, and cultured in the presence
20 of the inhibitors for 24 h (SP600125 50 μ M; PD98059 60 μ M; MCE, USA);
21 SP600125/PD98059 + H₂O₂: cultured in the presence of the inhibitors for 24 h before the
22 addition of 100 μ M H₂O₂ for 1 h; SP600125/PD98059 + MSC-CM: cultured in the presence
23 of the inhibitors for 24 h before the addition of 100 μ M H₂O₂ for 1 h, when UC-MSCs-CM
24 were added.

25 26 2.4 Enzyme-linked immunosorbent assay (ELISA)

27 Cystatin SA, CD200 R1, Pax3, HIF-1 β , Neuroligin2, FGF-21, HGF, VEGF, BDNF, EGF,
28 GDNF and NT3 were detected in MSC-CM and Fibroblast-CM using commercial ELISA kits
29 (Cloud-Clone Corp, China) according to the manufacturer's instructions.

30 31 2.5 Whole-cell patch clamp experiment

32 HT22 cells were cultured on Matrigel coated cover slides in 35mm culture dishes containing
33 DMEM supplemented with 5% FBS. The prepared HT22 cell slides were recorded in the

whole-cell mode at room temperature. The cells were perfused continuously with DMEM supplemented with 5% FBS. The pipette solution contained (mM): 125 potassium D-gluconate, 8 NaCl, 2 Mg ATP, 0.3 Na GTP, 0.2 EGTA and 10 HEPES, with the pH adjusted to 7.2–7.4 using KOH. The recording glass electrodes were pulled from the borosilicate glass using a P97 laser electrode puller (Sutter Instruments Company, USA), and the resistance between the electrode tip and cell membrane was 8–12 MΩ. Cells were held at a potential of –60mV and current-voltage relations were obtained from voltage ramps from –80 to +80 mV (50 ms duration). Membrane currents were digitally sampled at a frequency of 10 kHz and low-pass filtered at 2–5 kHz. H₂O₂ (100 μM; Yantai, China) was used to activate the TRPM2 channels. Data acquisition was performed using EPC-10 Patch Clamp Amplifier (HEKA Elektronik, Germany). The results were analyzed and plotted using GraphPad Prism software, version 7.04.

2.6 Immunofluorescence staining

HT22 cells were fixed for 10 min with 4% paraformaldehyde (Biotopped Life Sciences, China), washed with PBS and permeabilized with 0.5% Triton-X in PBS for 1 h at room temperature. Non-specific binding was reduced by incubation with blocking buffer (0.5% Triton-X in PBS+5% normal goat serum) for 1 h at room temperature. Cells were then incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: anti-Caspase3 (1:1,000, cat. no. ab49822; Abcam, UK), anti-TRPM2 (1:500, cat. no. ab11168; Abcam). Cells were then incubated for 3 h at room temperature with the following fluorescently-labeled secondary antibody diluted in blocking buffer: Cy3-labeled goat anti-rabbit (1:1,000, cat. no 111-165-003; Jackson ImmunoResearch Laboratories, USA). Cells were incubated with 4',6' diamino-2-phenylindole dihydrochloride (DAPI) (1:4; Solarbio, China) for 3–5 min at room temperature and washed with PBS before collecting images under a fluorescence microscope (Olympus, Japan) in the dark.

2.7 Western blot analysis

Total proteins were extracted from the HT22 cells using IP Lysis buffer (cat. no 87787; Thermo, USA) with protease and phosphatase inhibitors (cat. no. 78441; Thermo, USA). The concentration was determined using the bicinchoninic acid (BCA) method (cat. no. P0012; Beyotime, China). Equal amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% and 15% gels (cat. no. PG112 and PG114; Epizyme Biotechnology, China) and transferred to polyvinylidene fluoride

membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% non-fat milk for 2 h and incubated overnight at 4°C with the following primary antibodies: anti-SOD (1:1,000, cat. no. ab183881; Abcam), anti-pJNK1/2/3 (1:1,000, cat. no. ab219584; Abcam), anti-pERK1/2 (1:1,000, cat. no. ab201015; Abcam) and anti-Caspase9 (1:1,000, cat. no. WL01551; Wanleibio, China), and anti-β-actin (1:10,000, cat. no. 66009-1-Ig; Proteintech, USA). The membranes were then incubated for 1 h at room temperature with the following secondary antibodies: IRDye680RD goat anti-rabbit (1:20,000, cat. no. 926-68071; Licor, USA), IRDye680RD goat anti-mouse (1:20,000, cat. no. 926-68180; Licor) and IRDye800CW goat anti-mouse (1:20,000, cat. no. 926-32351; Licor). The signal on the membrane were visualized by the Odyssey infrared scanner (Licor, USA) and optical densities were quantified by Image J software. β-actin was used as a loading control.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 7.04. Data are presented as mean ± standard deviation (SD) of at least three experiments. One-way analysis of variance (ANOVA) and repeated measures ANOVA were used for multivariate data analyses. $P < 0.05$ was set as the threshold for statistical significance.

3. Results

3.1 UC-MSC-CM protects HT22 cells against H₂O₂-induced neuronal apoptosis

To examine the protective ability of UC-MSC-CM, we treated H₂O₂-stimulated HT22 cells with UC-MSC-CM for 24 h. Compared with the H₂O₂-treated group, the density of HT22 cells was higher after treatment with UC-MSC-CM, although there was no morphological difference between the cells in the two groups ($P < 0.05$, Fig. 1A). Fluorescence immunostaining showed that the number of Caspase-3 positive cells was significantly lower in the UC-MSCs-CM group compared with that in H₂O₂-treated group ($P < 0.05$, Fig. 1B and C). Furthermore, Western blot analysis showed that the expression level of cleaved-Caspase-9 protein was significantly lower in the UC-MSC-CM group compared with that in H₂O₂-treated group ($P < 0.05$, Fig. 1D and E).

3.2 UC-MSC-CM is composed of cellular growth factors and mediates anti-oxidative functions

The cytotropic factors secreted into the UC-MSC-CM were analyzed by ELISA. Many growth factors, such as EGF, BDNF, GDNF and HGF, were detected in the UC-MSCs-CM (Fig. 2A). Western blot analysis showed that the expression level of SOD protein was significantly higher in UC-MSC-CM group compared with that in the H₂O₂-treated group ($P < 0.05$, Fig. 2B and C).

3.3 UC-MSC-CM suppresses TRPM2-related currents

TRPM2 can be activated by H₂O₂-induced oxidative stress[16]. Fluorescence immunostaining confirmed the expression of TRPM2 in HT22 cells, with no marked differences in the number of TRPM2-positive cells and the cellular location of TRPM2 between the groups (Fig. 3A). Whole-cell patch clamp measurement were showed that H₂O₂ stimulation of HT22 cells evoked a non-selective inward current with current–voltage properties similar to those that are characteristic of TRPM2[17]. This H₂O₂-induced TRPM2-like current in HT22 cells was attenuated by treatment with UC-MSC-CM (Fig. 3B)

3.4 The neuroprotective effects of UC-MSC-CM are mediated via the JNK signaling pathway

To explore the role of the H₂O₂-related MAPKs signaling pathway in the mechanism by which UC-MSC-CM protects HT22 cells against H₂O₂-induced injury, we analyzed the expression of p-JNK, p-P38 and p-ERK proteins. Western blot analysis showed that the expression level of p-JNK protein was significantly decreased after treatment with H₂O₂ and that this trend was reversed by subsequent treatment with UC-MSC-CM ($P < 0.05$, Fig. 4A and B) accompanied by a decreased in the expression level of cleaved-Caspase 9 protein ($P < 0.05$, Fig. 4C and D). The expression level of p-ERK protein was also decreased after treatment with H₂O₂, and the levels were unchanged by subsequent treatment with UC-MSC-CM (Fig. 4D and E). The protein expression of p-P38 has not been detected in each group (data not shown).

4. Discussion

Under conditions of cerebral ischemia, the excitotoxicity and subsequent ROS overproduction can damage neuronal structures, leading to cell death. Many studies have indicated that MSC transplantation is a promising therapy for cerebral ischemia. To date, several studies have evaluated the neuroprotective effects of MSC-CM in various diseases and many showed positive results[18]. Pre-clinically, MSC-CM has been shown to exert cellular protective and regenerative effects[19]. Recently, MSC-CM has been identified as one of the

1 key components of the neuroprotective mechanisms of MSCs and can effectively ameliorate
2 ischemia/reperfusion-induced brain injury by promoting angiogenesis, regulating immune
3 responses, and inhibiting neuronal apoptosis [20, 21]. In accordance with other reports, we
4 provided further evidence for MSC-CM treatment as a potential approach to the preservation
5 of neuronal integrity and function. Our study showed that the MSC-CM treatment protected
6 HT22 cells from H₂O₂-induced oxidative injury by decreasing apoptosis and increasing the
7 cellular anti-oxidative ability.

8 Cerebral ischemia-induced TRPM2 activation triggers abnormal intracellular Ca²⁺
9 accumulation and cell death, which in turn causes irreversible brain damage[22]. Thus,
10 TRPM2 has emerged as a new therapeutic target for ischemic stroke. TRPM2 inhibitors have
11 been in preclinical development to prevent pathological Ca²⁺ overload[23-25]; however,
12 successful development of specific inhibitors with good pharmacokinetic properties is a long
13 and challenging process. Interestingly, we observed that MSC-CM contributes to the
14 suppression of TRPM2 activation, indicating that MSCs secrete endogenous TRPM2
15 inhibitors. To identify such a component in the complex MSC-CM, we analyzed secreted
16 protein expression and detected high levels many cytokines that may modulate the function of
17 TRPM2 and warrant further investigation.

18 In eukaryotic cells, the MAPK signaling pathway regulates gene expression and processes
19 such as cell proliferation, differentiation, apoptosis and stress response, and components of the
20 pathway, including ERK, JNK, and p38, have been identified as therapeutic targets for many
21 diseases[26-28]. Accumulating evidence supports a role for the MAPK signaling pathway in
22 the pathogenesis and development of ischemic stroke[29, 30]. However, the upstream and
23 downstream kinases of the MAPK signaling pathway are complex and have many influencing
24 factors. Many studies have shown that the MAPK signaling pathway is the key modulator of
25 ROS-related apoptosis[31, 32]. Therefore, we investigated the potential of MSC-CM to
26 modulate the MAPK signaling pathway. Following treatment of HT22 cells with H₂O₂,
27 expression levels of both p-ERK and p-JNK were decreased. We showed that subsequent
28 MSC-CM treatment effectively prevented the H₂O₂-induced decrease in JNK expression, but
29 had no effect on ERK expression. Furthermore, the protective effect of MSC-CM treatment
30 against the H₂O₂-induced decrease in JNKs expression was accompanied by a decrease in
31 neuronal cell apoptosis. These findings are consistent with previous reports that JNKs play
32 roles in neuronal development and neurite growth[33, 34].

1 In this study, we evaluated the effects of UC-MSCs-CM on neuronal oxidative damage. We
2 showed that UC-MSCs-CM protects neurons against oxidative injury, possibly by inhibiting
3 activation of the TRPM2 and JNK signaling pathway.

4 5 **Data accessibility statement**

6 The data used to support the findings of this study are included in this article.

7 **Competing interest**

8 The author(s) declared no potential conflicts of interest with respect to the research,
9 authorship, and/or publication of this article.

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14 **Author contribution**

15 Xueyun Liang and Jianguo Niu contributed to the conception of the study and revised the
16 article; Jianqiang Yu helped perform the analysis with constructive discussion; Yan Wang
17 performed the main part of experiment and wrote the manuscript draft; Jiaxin Liu, Baocong
18 Yu, Yiran Jin, Jiahui Li and Xiaona Ma performed the experimental work.

19 **Ethical approval**

20 All procedures performed in this study were approved and in accordance with the ethical
21 standards of Ethics Committee of the General Hospital of Ningxia Medical University.

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Figure Captions

Figure 1. UC-MSCs-CM protected HT22 cells against H₂O₂-induced neuronal apoptosis.

A: Representative images of HT22 cell morphology, scale bar = 100 μ m; B: Representative images of immunofluorescence staining of cleaved Caspase-3, scale bar = 20 μ m; C: Percentages of the Caspase-3-positive cells; D: Representative images of Western blot analysis of cleaved-Caspase-9; E: Relative expression analysis of cleaved-Caspase-9. Data represent the mean $\pm$ SD. **P* < 0.05 compared with Normal group, #*P* < 0.05 compared with MSCs-CM group.

Figure 2. UC-MSC-CM is composed of cellular growth factors and possessed antioxidative ability. A: ELISA analysis of the concentration of various factors in the supernatant of MSCs-CM and fibroblast-CM; B: Representative images of Western blot analysis of SOD; C: Relative expression of SOD. Data represent the mean $\pm$ SD. **P* < 0.05 compared with Normal group, #*P* < 0.05 compared with MSCs-CM group.

Figure 3. UC-MSC-CM suppressed TRPM2-related currents. A: Representative images of immunofluorescence staining of TRPM2, scale bar = 20 μ m.; B: I-V curve of TRPM2 showing changes in membrane potential.

Figure 4. UC-MSCs-CM protection of HT22 cells was mediated via the JNK signaling pathway. A: Representative images of the Western blot analysis of the protein expression of p-JNK1/2/3 protein expression; B: Relative expression of p-JNK1/2/3; C: Representative images of the Western blot analysis of cleaved-Caspase-9 protein expression; D: Relative expression of cleaved-Caspase 9; E: Representative images of the Western blot analysis of p-ERK1/2 protein; F: Relative expression of p-ERK1/2. Data represent the mean $\pm$ SD. **P* < 0.05 compared with Normal group, #*P* < 0.05 compared with MSCs-CM group.

Figures

Figure 1

UC-MSCs-CM protected HT22 cells against H₂O₂-induced neuronal apoptosis. A: Representative images of HT22 cell morphology, scale bar = 100 μ m; B: Representative images of immunofluorescence staining of cleaved Caspase-3, scale bar = 20 μ m; C: Percentages of the Caspase-3-positive cells; D: Representative images of Western blot analysis of cleaved-Caspase-9; E: Relative expression analysis of cleaved-Caspase-9. Data represent the mean $\pm$ SD. *P <0.05 compared with Normal group, # P <0.05 compared with MSCs-CM group.

Figure 2

UC-MSC-CM is composed of cellular growth factors and possessed antioxidative ability. A: ELISA analysis of the concentration of various factors in the supernatant of MSCsCM and fibroblast-CM; B: Representative images of Western blot analysis of SOD; C: Relative expression of SOD. Data represent the mean $\pm$ SD. * P <0.05 compared with Normal group, # P <0.05 compared with MSCs-CM group.

Figure 3

UC-MSC-CM suppressed TRPM2-related currents. A: Representative images of immunofluorescence staining of TRPM2, scale bar = 20 μ m.; B: I-V curve of TRPM2 showing changes in membrane potential.

Figure 4

UC-MSCs-CM protection of HT22 cells was mediated via the JNK signaling pathway. A: Representative images of the Western blot analysis of the protein expression of p JNK1/2/3 protein expression; B: Relative expression of p-JNK1/2/3; C: Representative images of the Western blot analysis of cleaved-Caspase-9 protein expression; D: Relative expression of cleaved-Caspase 9; E: Representative images of the Western blot analysis of p-ERK1/2 protein; F: Relative expression of p-ERK1/2. Data represent the mean $\pm$ SD. * P <0.05 compared with Normal group, # P <0.05 compared with MSCs-CM group.