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Basic Study

Hypoxia and inflammatory factor preconditioning enhances the immunosuppressive properties of human umbilical cord mesenchymal stem cells

Li H *et al*. Hypoxia and inflammatory factor pretreated UC-MSCs

Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have great potential for the treatment of various immune diseases due to their unique immunomodulatory properties. However, MSCs exposed to the harsh inflammatory environment of damaged tissue after intravenous transplantation cannot exert their biological effects, and therefore, their therapeutic efficacy is reduced. In this challenging context, for the development of MSC-based therapies with increased immunomodulatory capacity and transplantation efficacy, an *in vitro* preconditioning method is needed.

AIM

To simulate the injury-induced environment *in vitro* using a combination of hypoxia and inflammatory factor preconditioning to determine whether this preconditioning increases the immunosuppressive properties of MSCs without affecting their biological characteristics.

METHODS

Umbilical cord MSCs (UC-MSCs) were pretreated with a combination of hypoxia (2% O_2) exposure and supplementary inflammatory factors (interleukin-1β, tumor necrosis factor-α, interferon-γ) for 24 h. Flow cytometry, polymerase chain reaction, enzyme-linked immunosorbent assay and other experimental methods were used to evaluate the biological characteristics of the pretreated UC-MSCs and to explore changes in the immunosuppressive ability of the pretreated UC-MSCs in coculture with immune cells.

RESULTS

The results showed that the pretreated UC-MSCs were elongated and that their viability, proliferation and size were not affected by pretreatment. In addition, the expression of coagulation-related tissue factors was significantly reduced, while that of other surface markers remained unchanged. Similarly, mitochondrial function and

integrity were retained. Although pretreatment promoted the apoptosis and senescence of UC-MSCs, we found that the expression of genes and proteins related to immune regulation was increased, and the proliferation rates of peripheral blood mononuclear cell and natural killer (NK) cells and the toxicity induced by NK cells were inhibited to varying degrees.

CONCLUSION

In summary, our preconditioning method led to higher immunosuppressive effects of MSCs without damaging their biological characteristics.

Key Words: Mesenchymal stem cells; Umbilical cord; Preconditioning; Hypoxia, Inflammatory factors; Immune regulation

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Core Tip: Mesenchymal stem cells (MSCs) are potential candidates for treating many immune diseases due to their unique immunomodulatory abilities, but a low survival rate and weakened function after venous transplantation reduces their treatment potential. Therefore, our study reveals a combination pretreatment method based on hypoxia exposure and inflammatory factor treatment *in vitro* that simulates the harsh *in vivo* environment to protect MSCs from injury after intravenous transfusion and promote high immunosuppressive effects of MSCs.

INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have been shown to have a variety of biological properties, such as self-renewal[1], multilineage differentiation[2,3], immunomodulation functions[4,5], tissue repair effects[6], anti-ageing and regeneration

activity[7]. Furthermore, due to the abundance and ready availability of MSCs[8], they do not lead to rejection after allografting[9]; thus, MSCs have great economic value, importance for in the field of cell therapy and broad application prospects. Although many questions remain unanswered, the immunomodulatory effects of MSCs make them candidates for cell-based tissue repair and disease treatment[10-12]. For example, MSCs have great potential in treatments for graft-*versus*-host disease[13], cardiovascular disease[14,15], liver disease[16,17], systemic lupus erythematosus[18], autoimmune encephalomyelitis[19] and spinal cord injury[20]. Studies have shown that the immunomodulatory ability of MSCs is regulated mainly by cytokines and other soluble factors. These cytokines include prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α-stimulated gene protein 6 (TSG6), inducible indoleamine 2,3-dioxygenase (IDO), interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1)[21]. They contribute to MSC-mediated immunosuppression by inhibiting natural killer (NK) cell proliferation, cytotoxicity and cytokine secretion[22], inhibiting T-cell proliferation, promoting regulatory T cell production[5,23,24], and inhibiting B-cell proliferation and immunoglobulin release[25,26].

It has been shown that when MSCs are injected into the body and migrate to damaged tissues or organs, the activation of innate immune cells leads to the increased release of chemokines and cytokines (such as TNF-α, IL-1α and IL-1β)[27] and is accompanied by the development of a harsh environment caused by hypoxic stress[28]; thus, the activity of MSCs is reduced. Therefore, *in vitro* preconditioning culture developed through hypoxia exposure and inflammatory factor supplementation can effectively mimic the *in vivo* microenvironment. Preconditioning MSCs *in vitro* activates various signaling pathways, influencing the biological activities of MSCs *in vitro* and *in vivo*, thereby preparing them to survive the harsh environment to which they are subjected *via in vivo* administration and protecting them from damage[29,30]. In addition, preconditioning can induce MSCs to secrete immunosuppressive molecules to target innate and adaptive immune cells, thereby mediating immune regulation by releasing water-soluble factors[31,32]. Therefore, pretreatment is an adaptive strategy to enhance

the efficacy of MSC therapy, which can not only prolongs the survival and function of MSCs after transplantation but also endows them with higher immunomodulatory activity levels. Studies have shown that preconditioning with inflammatory factors [such as TNF-α and interferon (IFN)-γ] can increase the immunoregulatory ability of MSCs[33,34]. Hypoxia preconditioning is often used to enhance the therapeutic effect of MSCs by inducing the expression of survival genes, chemokines, growth factors, and angiogenic factors (such as vascular endothelial growth factor), enabling the MSCs to adapt to the harsh environment of damaged tissue[35,36].

Although different preconditioning methods have been shown to successfully enhance cell properties and increase their function, safety concerns are worthy of attention. In contrast to the aforementioned positive outcomes of pretreatment, improper pretreatment can exert a negative impact on cell morphology and function, damage mitochondrial function[37], and affect the expression of MSC surface markers. For example, previous studies have shown that tissue factor (TF/CD142) binds to coagulation factor VII/VIIB to initiate the exogenous coagulation system. The percentage of TF expressed on the cell surface is strongly correlated with procoagulant activity[38]. MSCs expressing surface TF show high coagulant activity and are promote coagulation of blood or plasma[39]. Therefore, high expression of TF on MSCs increases the risk of thrombosis after intravenous injection[40-43].

We found that most studies have been focused on bone marrow- or adipose-derived MSCs (AD-MSCs), but human umbilical cord-derived MSCs (hUC-MSCs) are more suitable for clinical research and large-scale use because they are not associated with ethical problems, are abundant and highly proliferative. Therefore, we chose hUC-MSCs to study. To our knowledge, studies using a combination of hypoxia (2% O_2) exposure and inflammatory factor (IL-1β, TNF-α, IFN-γ) treatment to precondition hUC-MSCs are rare, and our study supports current theory. Our aim was to simulate the injury-induced environment *in vitro* using a combination of hypoxia and inflammatory factor preconditioning to determine whether this preconditioning

increases the immunosuppressive properties of MSCs without affecting their biological characteristics.

MATERIALS AND METHODS

Extraction and culture of UC-MSCs

The umbilical cord and cord blood were obtained with the approval of the Medical Ethics Committee of Shanxi Medical University and the consent of the donors, and our experiment was carried out in accordance with the Declaration of Helsinki.

Umbilical cord tissue was provided by one hospital, and the donors gave informed consent. After obtaining the umbilical cords, two veins and one artery were removed, and the Wharton's jelly was cut into pieces and placed on a 10 cm^2 petri dish (Nice) with 5 mL of DMEM/F12 culture medium (Thermo Fisher Scientific) containing 2.5% serum substitute (Shanxi Yinshi Cell Technology). Approximately 14 d later, the UC-MSCs were passaged, and P4 generation cells were used for experiments. UC-MSCs were cultured in a carbon dioxide incubator (Thermo Scientific) at standard oxygen tension with 5% carbon dioxide, 95% air, and 37°C (*i.e.*, normoxia). When UC-MSCs were 70%-80% confluent, a mixture of IFN-γ (R&D), TNF-α (R&D) and IL-1β (PeproTech) was added to the medium. Then, the cells were immediately placed into a three-gas incubator (Panasonic Japan) with 2% O_2, 5% CO_2, and 93% N_2 at 37 °C (*i.e.*, hypoxia). After 24 h, primed UC-MSCs (PUC-MSCs) were obtained. Therefore, our experiments were divided into two groups: A control group (UC-MSCs) with no treatment and an experimental group (PUC-MSCs) in which the cells were treated by hypoxia exposure and inflammatory factor supplementation.

Extraction and culture of peripheral blood mononuclear cells

Umbilical cord blood was obtained and centrifuged at 700 × *g* for 10 min, and the plasma was extracted and placed into a water bath at 56 °C for heat inactivation for 30 min. Then, it was removed from the water bath, centrifuged at 850 × *g* for 10 min, and placed in a refrigerator at 4 °C for later use. The blood cells were resuspended in

phosphate buffered saline (PBS) and mixed. The cell suspension was slowly added into a centrifuge tube containing Ficoll human peripheral blood lymphocyte isolation medium (Tianjin Haoyang Biological Products Technology Co., Ltd.). After centrifugation at 400 × *g* for 30 min, white cells were extracted and washed twice with PBS. The cells [(1-2) × 10^6 cells/mL] were inoculated in peripheral blood mononuclear cell (PBMC) medium containing 10% of the heat-inactivated autologous plasma (Shanxi Yin Cell Technology Co., Ltd.) in the presence or absence of UC-MSCs or PUC-MSCs. The PBMCs were treated with 100 U/mL IL-2 (PeproTech). For coculture experiments, PBMCs were inoculated with UC-MSCs or PUC-MSCs so that the cells were in direct contact at a PBMC/MSC ratio of 3:1, which is the appropriate proportion of cells to obtain MSC-mediated inhibition of PBMC proliferation.

Extraction and culture of NK cells

Umbilical cord blood was obtained and centrifuged at 700 × *g* for 10 min, and the plasma was placed into a water bath at 56 °C for heat inactivation for 30 min. Then, the plasma was removed, centrifuged at 850 × *g* for 10 min, and placed into a refrigerator at 4 °C for later use. The blood cells were resuspended in PBS, mixed, and incubated at room temperature for 20 min with RosetteSepT NK Enrichment Cocktail. The cell suspension was slowly added into a centrifuge tube containing Ficoll human peripheral blood lymphocyte isolation medium (Tianjin Haoyang Biological Products Technology Co., Ltd.). After centrifugation at 400 × *g* for 30 min, white cells were extracted and washed twice with PBS. The cells [(1-2) × 10^6 cells/mL] were inoculated into NK medium containing 10% heat-inactivated autologous plasma (Shanxi Yin Cell Technology Co., Ltd.). NK cells were cultured with or without UC-MSCs or PUC-MSCs and treated with 100 U/mL IL-2 (PeproTech).

Cell survival rate and cell size detection

Double fluorescence acridine orange/propidium iodide (AO/PI) cell viability counting was performed. The AO/PI reagent consists of the DNA-binding dye AO, which

fluoresces green, and the DNA-binding dye PI, which fluoresces red. AO can pass through a complete cell membrane and enter the nuclei of all cells (living and dead cells), emitting green fluorescence. PI can only pass through an incomplete cell membrane and enter the nuclei of dead cells, resulting in red fluorescence. After mixing 10 μL of cell suspension with 10 μL of AOPI reagent, the viability and size of the cells in suspension were measured by a Countstar Rigel S2 (Shanghai Rui Yu).

Monoclonal antibodies and cytofluorometric analysis

Flow cytometry analysis of UC-MSCs and PUC-MSCs was performed. The cells were resuspended in staining buffer after centrifugation, and the corresponding antibodies were added to the buffer, and these cells were incubated for 30 min, neutralized and washed 1-2 times. The UC-MSC or PUC-MSC antigen markers (BD) were CD105-APC, CD90-FITC(BD), CD73-PE, CD142-PC5.5, CD29-PE, CD34-PE, CD14-PE, CD45-APC, human leukocyte antigen (HLA)-ABC-APC, CD47-APC, CD166-PE, CD44-PE, and CD31-APC.

Reactive oxygen species measurement

Mitochondrial reactive oxygen species (ROS) production was measured by flow cytometry (Beckman) using an ROS detection kit (Biyuntian). 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was diluted in serum-free medium at 1:1000 to a final concentration of 10 μmol/L. After collection, the cells were suspended in diluted DCFH-DA at a cell concentration of 1 million to 20 million/mL and incubated in an infrared carbon dioxide incubator (Thermo Fisher) at 37 °C for 20 min. The cells were turned over and mixed every 3-5 min so that the probe was in full contact with the cells. The cells were washed with serum-free cell culture solution 3 times to fully remove excess DCFH-DA. Finally, the results were detected by flow cytometry.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was detected using MMP detection reagent JC-1 (Beyotime). The culture medium was removed, and the cells were washed twice with PBS. JC-1 staining solution (1 mL) was added, mixed thoroughly, and incubated with cells in an infrared carbon dioxide incubator (Thermo Fisher) at 37 °C for 20 min. During the incubation period, JC-1 staining buffer (1×) was prepared by adding approximately 4 mL of distilled water to approximately 1 mL of JC-1 staining buffer (5×), and the buffer was placed in an ice bath. After incubation at 37 °C, the supernatant was removed, and the cells were washed twice with JC-1 staining buffer (1×). Then, 2 mL of the cell culture solution was added. Images were obtained with a fluorescence inverted microscope (CKX53, Olympus).

Real time-polymerase chain reaction

TRIzol (Invitrogen) was used to lyse the cells, total RNA was extracted, and an ultratrace nucleic acid protein detector (Shanghai Jiapeng) was used to determine the RNA concentration. Then, mRNA expression levels were quantitatively analysed on a real-time polymerase chain reaction (RT-PCR) instrument (Bio-Rad) using a One Step TB Green PrimeScript PLUS RT– PCR Kit (Takara) to measure the levels of the following transcripts: Catalase, haem oxygenase (HO-1), stanniocalcin-1 (STC1), hemeoxygenase-1 (HOMX1), B-cell lymphoma 2-associated protein (Bax), B-cell lymphoma 2 (Bcl2), silencing information regulator 2-related enzyme 1 (SIRT1), P53, P16, P21, PGE2, kynurenine (KYN), IDO, IL-1 receptor antagonist (IL-1ra), cyclooxygenaese-2 (COX2), IL-10, TGF-β1, HLA-G5, TSG-6, ligands for programmed cell death 1 (PD-L1). All primer sequences for qRT-PCR are listed in Table 1.

Enzyme-linked immunosorbent assays

UC-MSCs and PUC-MSCs supernatants were collected and centrifuged at 210 × *g* for 5 min, and the suspended cells were removed and stored at -80 °C. The protein levels of PGE2, TSG6, IDO, IL-10 and TGF-β secreted by UC-MSCs and PUC-MSCs were measured by enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Eliret

Biotechnology Co, Ltd.). Finally, the optical density (OD) value of each well was measured on the basis of enzyme label (Thermo Scientific) absorption at a wavelength of 450 nm.

Annexin V-FITC apoptosis detection

UC-MSCs and PUC-MSCs suspensions were obtained and washed twice with PBS. Annexin V-FITC apoptosis reagent (Biyuntian) was added and incubated with the cells at room temperature for 10-20 min in the dark, and then, flow cytometry was performed. Annexin V-FITC emits green fluorescence, and PI emits red fluorescence.

β-galactosidase (SA-β-gal) activity detection

The cell culture medium was removed, the cells were washed once with PBS, and 1 mL/well β-galactosidase (SA-β-gal) staining fixation solution (Solarbio, United States) was added and incubated at room temperature for 15 min. The cell fixative was removed, and the cells were washed 3 times with PBS for 3 min each time. The PBS was removed, and 1 mL of staining solution was added to each well. The plate was incubated at 37 °C overnight and sealed with plastic wrap to prevent evaporation. The positive expression of blue particles was observed under an inverted microscope (CKX53, Olympus), and the number of positive cells among 100 cells was counted. Each experiment was repeated three times.

Proliferation experiment

After 3 d of coculture NK cells in the presence (or absence) of UC-MSCs or PUC-MSCs in culture, prepared on the basis of 3:1 ratio direct contact method, were prelabelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye (BioLegend). The fluorescence intensity of CFSE was measured by flow cytometry.

Cell growth and proliferation

UC-MSCs and PUC-MSCs were collected for DNA extraction, and the DNA concentration was determined by an ultramicro nucleic acid protein detector (Shanghai Jiapeng) to evaluate the cell proliferation rate. PBMCs were cultured in the presence or absence of UC-MSCs or PUC-MSCs at a ratio of 3:1. The number of cells (Countstar Rigel S2 Shanghai Rigyu) was counted by a cell imaging analyser for 5 consecutive days, and the growth curve was plotted.

Cytotoxicity of K562 target cells

NK cells were cultured alone or in direct contact with MSCs (NK: UC-MSCs or PUC-MSCs = 3:1 ratio) for 72 h. Then, the NK cells were collected and cocultured with CFSE-stained (Biolegend) K562 target cells for 4 h at the effective target ratio of 1:5. All cells were collected and washed twice with PBS. K562 cell apoptosis was analysed by PI staining (Biyuntian), and the fluorescence intensity was measured by flow cytometry.

Statistical analysis

PSS 19.0.0 and GraphPad Prism 7 (GraphPad Prism, United States) statistical software were used for data processing, and the experimental data are expressed as the mean ± SD. A t test was performed to compare the two datasets, and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of hypoxia and inflammatory factor pretreatment on the morphology, size, proliferation and viability of UC-MSCs

We evaluated the effects of hypoxia and inflammatory factor pretreatment on the morphology, vitality and size of UC-MSCs. The morphological differences between UC-MSCs and PUC-MSCs were compared under a microscope when cell confluency reached 80% to 90%. Untreated UC-MSCs appeared as either short rods or long spindles in an adherent state and were arranged in a spiral pattern. After 24 h of exposure to hypoxia and inflammatory factors, the morphology changed from cells being short and

rod-like to thin and elongated (Figure 1A), but the proliferation rate did not change (Figure 1B). We further evaluated the effects of pretreatment on the viability and size of UC-MSCs using AOPI staining and a cell imaging analyser. The viability of UC-MSCs (Figure 1C) in suspension was 93.59% ± 3.87% (n = 5), the mean diameter (Figure 1D) was 18.216 ± 0.78 μm (n = 5), the viability of the PUC-MSCs (Figure 1C) was 92.89% ± 4.13% (n = 5), and the mean diameter (Figure 1D) was 18.628 ± 0.76 μm (n = 5). These results showed that pretreated MSCs became elongated, the measured viability decreased slightly (P = 0.84), and the cells were slightly enlarged (P = 0.47), but these differences were not statistically significant.

MSC surface marker expression

We further evaluated whether preconditioning altered the cellular phenotype. We analysed the expression of MSC surface markers by flow cytometry, and UC-MSCs (Figure 2A) and PUC-MSCs (Figure 2B) from three donors showed positive surface expression of CD105, CD90, CD73, CD29, CD166, CD47 and HLA-ABC. The results were negative for the markers CD31, CD45, CD14 and CD34. The results showed that the phenotypes of PUC-MSCs and UC-MSCs were consistent and had the same characteristics. Interestingly, the expression of CD142 (59.3%) (Figure 2B) in PUC-MSCs was significantly lower than that in UC-MSCs (99.6%) (Figure 2A).

Effect of pretreatment on the mitochondrial function of UC-MSCs

ROS play key roles in the proapoptotic signaling cascade, and ROS in excess attack the mitochondrial membrane and lead to the loss of MMP, leading to the apoptosis of MSCs[44]. Therefore, in this study, flow cytometry was used to examine the effect of pretreatment on ROS production in MSCs. Intracellular ROS levels showed a three-fold increase in the fluorescence intensity of DCFH-DA in PUC-MSCs compared to the levels in untreated UC-MSCs (Figure 3A), but all the effects were within the range showed in positive controls. The expression levels of STC1, HO-1, catalase and HOMX1 were increased (Figure 3B), indicating that the antioxidant capacity of the cells was increased

and that the effectively of mitochondrial ROS removal was increased, which increased antioxidant defence effects and prevented ROS-induced DNA damage. In addition, maintaining a stable MMP (Ψm) is essential to ensure efficient ROS clearance and prevent apoptosis or other stress-related events caused by excessive ROS[45]. Therefore, we further determined whether hypoxia and inflammatory factor preconditioning induced dysfunction of MMP in UC-MSCs. We used the sensitive fluorescent probe JC-1 to evaluate the effect of pretreatment on cells. The change in the cell membrane potential of UC-MSCs was determined by a change in the red fluorescence of JC-1 to green fluorescence. Microscopically, untreated UC-MSCs showed a normal MMP, as shown by the red fluorescence staining with JC-1 (Figure 3C). However, the red and green fluorescence of PUC-MSCs showed no significant difference from that of UC-MSCs, and the MMP showed no significant change. Similarly, the red/green fluorescence ratio of JC-1 was detected by flow cytometry, and the same assay results were obtained (Figure 3D). These results showed that mitochondrial function in UC-MSCs was not damaged by hypoxia and inflammatory factor pretreatment.

Hypoxia and inflammatory factor preconditioning induces apoptosis and senescence in UC-MSCs

The expression of Bax decreased and the expression of Bcl2 and SIRT1 increased after pretreatment, suggesting that the anti-apoptotic capacity increased after pretreatment (Figure 4A). The expression of the senescence-related genes P53, P16 and P21 was upregulated by pretreatment (Figure 4B). Apoptosis was analysed by flow cytometry after Annexin V-PI staining. We compared the proportion of cells that were positive for both Annexin V-FITC and PI (necrotic cells), and the apoptotic index of PUC-MSCs (15.08% ± 4.11%) was significantly increased (Figure 4C) compared with that of UC-MSCs (6.22% ± 3.03%). Next, we investigated the effect of preconditioning on cell senescence. In terms of the gold standard for evaluating cell senescence, SA-β-gal has been the most widely accepted marker since Dimri first published its use in 1995[46], and so, we used SA-β-gal staining to evaluate the cell senescence ratio. An inverted

microscope showed that compared with that of UC-MSCs (4.83% ± 1.34%), the level of SA-β-gal in PUC-MSCs was increased (14.83% ± 1.57%) (Figure 4D). These results suggest that hypoxia and inflammatory factor preconditioning not only induces apoptosis and senescence of UC-MSCs but also increases anti-apoptotic factor levels.

Hypoxia and inflammatory factor preconditioning enhances the immunosuppressive properties of UC-MSCs

MSCs are pluripotent stem cells that have been shown to be promising cells for tissue regeneration due to their ability to self-regenerate and differentiate and broad immunomodulatory properties[1-5]. We evaluated whether the immunomodulatory activities of UC-MSCs were increased after pretreatment. The nucleic acids in MSCs were extracted after cell lysis and analysed by PCRs. The results showed that the expression of PGE2, IDO, KYN, COX2, IL-10, TGF-β1, TSG-6, HLA-G5, and PD-L1 was elevated in PUC-MSCs compared with untreated UC-MSCs. IL-1ra expression was slightly elevated, but the difference was not statistically significant (Figure 5A). Since paracrine activity is a key mechanism underlying MSC effects, we evaluated the bioactive factor secretion levels of PUC-MSCs and compared it with those of untreated UC-MSCs. We assessed MSC paracrine function by measuring the levels of immunosuppression-related soluble factors released into the culture supernatant; these factors included IDO, PGE2, TGF-β1, TSG-6, and IL-10. The protein levels of these factors were measured by ELISAs. The protein levels of IDO, PGE2, TGF-β1 and TSG-6 in the PUC-MSC supernatant were significantly increased, while the protein level of IL-10 was slightly increased, but the differences were not significant (Figure 5B). In summary, these data strongly suggest that UC-MSC preconditioning with hypoxia and inflammatory factors upregulates the expression of soluble immunomodulatory factors and enhances their immunomodulatory activity.

Hypoxia and inflammatory factor preconditioning increases the immunosuppressive properties of UC-MSCs

Considering the high expression of immunomodulatory molecules observed in PUC-MSCs, we evaluated their immunosuppressive capacity. We evaluated the immunosuppressive capacity of UC-MSCs and PUC-MSCs using PBMC and NK cell proliferation assays. We plotted the growth curves of PBMCs and observed the changes in the growth dynamics of PBMCs after PBMCs cultured alone or co-cultured with UC-MSCs or PUC-MSCs (Figure 6A). The results showed that either UC-MSCs or PUC-MSCs significantly inhibited the proliferation of PBMCs compared with PBMCs cultured alone. However, PUC-MSCs showed stronger inhibition than UC-MSCs, further indicating that pretreatment enhanced the immunosuppressive abilities of UC-MSCs. In addition, we further investigated MSC-mediated inhibition of NK cell function and evaluated the inhibition of NK cell proliferation *in vitro* in the presence of the two MSC populations. In the presence of IL-2, CFSE-stained NK cells were cultured alone or with UC-MSCs or PU-MSCs, and the CFSE fluorescence intensity was analysed by flow cytometry after 3 d. The proliferation rate of NK cells was reduced in cocultures with UC-MSCs or PUC-MSCs compared with that of NK cells cultured alone, but the PUC-MSC group exerted stronger inhibitory effects on NK cells (Figure 6B).

Finally, we evaluated the effect of UC-MSCs pretreatment with hypoxia exposure and inflammatory factor treatment on the cytotoxic activity of NK cells. NK cells were cultured alone or cocultured with UC-MSCs/PUC-MSCs at a 3:1 ratio for 72 h. Then, NK cells were collected and incubated with CFSE-stained K562 target cells at an effective target ratio (E:T = 1:5) for 4 h. The cells were collected, and K562 cell apoptosis was analysed by PI staining. The results showed that NK cells that were cocultured with UC-MSCs or PUC-MSCs showed lower cytolytic activity, and the inhibition of NK cell-mediated cytotoxic activity was higher in the PUC-MSCs than in the UC-MSCs (Figure 6C).

DISCUSSION

Intravenous injection of MSCs has been increasingly used in clinical research and has shown great potential in the treatment of various diseases. Because of their

immunomodulatory functions, MSCs have become a promising alternative treatment for inflammatory diseases. MSCs come from a wide range of sources. Currently, the most studied MSCs are derived from bone marrow and adipose tissue, followed by those from umbilical cord tissue[47]. Compared with those from other sources, MSCs derived from umbilical cord are abundant, easy to collect, genetically stable, and do not readily mutate. Therefore, MSCs from umbilical cord has broad application prospects in the field of cell therapy[48-50]. Therefore, we selected UC-MSCs to study.

In addition to the effects of their origin, MSCs exhibit decreases in their biological performance when subjected to vein transplantation and exposure to the harsh inflammatory environment of damaged tissue[29]. Studies have shown that preconditioning cells by exposing them to the external environment can enhance their therapeutic effects by preparing them for the harsh conditions they encounter when injected into the body. Antebi *et al*[51] showed that hypoxic preconditioning of bone marrow MSCs enhanced the therapeutic function of these cells. Gorgun *et al*[34] analysed the effects of hypoxia and inflammatory factor (TNF-α, IL-1α) pretreatment on the angiogenic potential of AD-MSCs. In addition, Rodriguez *et al*[52] pretreated AD-MSCs with a mixture of inflammatory factors under anoxic culture conditions, which significantly enhanced their functional characteristics and immunosuppressive and immunoregulatory functions.

In this study, we developed a combination strategy using hypoxic with a mixture of TNF-α, IL-1β, and IFN-γ preconditioning to enhance the immunomodulatory capacity of hUC-MSCs. To our knowledge, this is a previously unanalysed combination pretreatment. In this study, we demonstrated that this preconditioning approach successfully mimicked the harsh inflammatory environment and effectively enhanced the immunosuppressive function of MSCs. To simulate hypoxia, we cultured UC-MSCs in a special three-gas incubator containing 2% O_2, 5% CO_2 and 93% N_2 at 37 °C for 24 h. These are the gas conditions that we had previously evaluated and found to be most suitable for preserving the functional characteristics of MSCs. To simulate the harsh inflammatory environment, a mixture of IL-1β, IFN-γ and TNF-α that was

independently developed in our laboratory was used. After pretreatment, we found that the cells became elongated, but their proliferation, viability and size did not change significantly. Notably, Klinker *et al*[53] recently demonstrated that the morphological characteristics of MSCs were significantly correlated with their immunosuppressive ability and can be used to predict their overall immunosuppressive effects[53]. The results of the study by Klinker *et al*[53] showed that the pretreated UC-MSCs became elongated and had stronger immunosuppressive ability, which was consistent with the results of our study.

Although different forms of preconditioning have been shown to successfully enhance cell properties and increase cell function, safety concerns are still worthy of attention. Pretreatment may have negative effects on cell function, and so, we used flow cytometry to observe the phenotype of MSCs. The expression of specific molecular markers on the surface of MSCs can be used as indicators of cell differentiation potential, lineage commitment, ageing, and therapeutic function[54]. The International Society for Cell and Gene Therapy defines minimum standards for MSC characterization, and most experiments are conducted on this basis[55]. However, we examined more positive surface markers on MSCs. We found that UC-MSCs and PUC-MSCs from three donors retained high expression levels of the surface markers CD105, CD90, CD73, CD29, CD166, CD47 and HLA-ABC and that the results were negative for CD31, CD45, CD14, and CD34 expression. These findings indicated that UC-MSCs retained MSC properties after pretreatment. Interestingly, we found that the expression of CD142 was significantly decreased after pretreatment. Oeller *et al*[39] found that UC-MSCs showed higher procoagulant activity than fat-derived MSCs, and UC-MSCs showed extensive TF (CD142) expression and long-lasting clotting; a higher cell number significantly increased clot formation, which was partially dependent on coagulation factors[43]. Our study showed that the expression of CD142 in UC-MSCs without treatment exceeded 99%, and the expression of CD142 was significantly decreased after pretreatment (59.3%). Our experiment showed significantly reduced the expression of TF, which has been shown to be closely related to procoagulant activity[38]. However,

Rodriguez *et al*[52] demonstrated that bone marrow-derived MSCs (BM-MSCs) and AD-MSCs that were pretreated with a combination of hypoxia and inflammatory factors showed increased expression of CD142, which was inconsistent with previous studies[53]. We determined that the origin of MSCs may be inconsistent, and their heterogeneity and differences between culture systems may affect cell expression. However, there was significantly reduced expression of CD142 in our study. In addition, studies have suggested the use of anticoagulants or genetic methods to inhibit TF activity for the clinical application of MSCs to maximize clinical benefits for patients, which also underscores the importance of insights into the mechanisms underlying safety issues related to nonhaematopoietic cell transplantation[43].

The efficacy of MSCs depends on the full function of their mitochondria, which can be damaged after cell exposure to harmful environments[56]. Therefore, we examined the effects of hypoxia and inflammatory factor pretreatment on mitochondrial function. ROS levels were increased after pretreatment but were within the range of those in the positive controls. In addition, the increased expression of antioxidants such as STC1, HO-1, catalase and HOMX1 can eliminate ROS to maintain cell redox homeostasis[57]. Previous studies have shown that both HMOX1 and catalase play key roles in protecting cells from ROS-induced damage[58,59]. Therefore, although the levels of ROS were increased after pretreatment, the antioxidant capacity of the cells also increased correspondingly, and the removal rate of ROS was increased. In addition, the MMP ($\Delta\psi m$) plays a key role in important mitochondrial functions, and its dissipation is an indicator of mitochondrial dysfunction[60]. Maintaining a stable MMP (Ψm) is essential for ensuring efficient ROS clearance and preventing apoptosis or other stress-related events caused by excessive ROS. Therefore, we examined the MMP and found that there was no change in the MMP after pretreatment. In summary, although the levels of ROS were increased after pretreatment, the antioxidant capacity was also increased, and the MMP did not change, indicating that there was no damage to mitochondrial function after pretreatment.

Next, we examined the effect of pretreatment on apoptosis and senescence in MSCs and found that the apoptosis index of UC-MSCs increased to a certain extent after pretreatment. Galleu *et al*[61] showed that all patients can receive apoptotic MSCs for *in vivo* injection and that these cells can induce receptor-mediated immune regulation. Apoptosis of MSCs is critical to their therapeutic functions[62]. BCL-2, BAX and SIRT1 genes play important roles in the apoptosis pathway. In this study, we found increased expression of the SIRT1 gene, which protected cells from apoptosis by activating autophagy[63]. The ratio of BAX/BCL-2 is closely related to the apoptosis potential of cells[64]. In this study, an inverse proportional relationship between BAX and BCL-2 indicated that MSCs exerted stronger anti-apoptosis effects after pretreatment. This may indicate that the cells were protecting themselves from the harmful inflammatory environment. In addition, MSCs showed increased SA-β-gal activity and p53, P16 and p21 expression after hypoxia and inflammatory factor pretreatment, suggesting that MSCs underwent senescence after pretreatment. Salminen *et al*[65] found that ageing MSCs exert stronger immunosuppressive effects. The experimental results showed that even when some of the pretreated UC-MSCs underwent senescence and apoptosis, these effects did not reduce their immunoregulatory abilities, and even apoptotic and senescent MSCs showed increased immunosuppressive abilities.

The pleiotropic effect of MSCs is mostly mediated by soluble paracrine factors, and active paracrine factors produced by these cells regulate cellular immunity when they come into contact with the host[66]. Therefore, we investigated the response of the paracrine factors involved in the immune regulation of MSCs to preconditioning. Many bioactive molecules produced by MSCs, such as IDO, PGE2, IL-10, TSG-6, and TGF-β1, effectively regulate innate and adaptive immunity and play a key role in the immunosuppressive effect of MSCs[67]. The detection of immunomodulation-related genes showed that the expression levels of PGE2, KYN, IDO, COX2, IL-10, TGF-β1, TSG-6, HLA-G5 and PD-L1 were significantly increased in addition to that of IL-1ra. In addition, the protein levels of PGE2, TSG-6, TGF-β1 and IDO in the supernatant were significantly increased. The IL-10 level was slightly increased, but the difference

between the pretreatment and control groups was not significant. These results showed that preconditioning can promote the production of immunomodulatory paracrine factors in UC-MSCs and increase their immunomodulatory effect.

Finally, we studied the immunosuppressive effects of MSCs on PBMCs and NK cells. We found that UC-MSCs and PUC-MSCs inhibited the proliferation of PBMCs and NK cells, but PUC-MSCs exerted a stronger inhibitory effect. Some work has been done to characterize the interaction of BM-MSCs with NK cells[22,68,69]. However, the interaction of UC-MSCs with NK cells has been largely unexplored. Our results add to the knowledge of the immunosuppressive effects of UC-MSCs[70,71]. In addition, we found that PUC-MSCs exerted a stronger inhibitory effect on NK cell-mediated cytotoxic activity. Reportedly, IDO and PGE2 or TGF-β produced by MSCs are critical for inhibiting the cytotoxicity of NK cells[22,72]. PUC-MSCs may exert stronger inhibitory effects on NK cell-mediated toxicity due to higher expression of IDO, TGF-β and PGE2.

We acknowledge that this study has limitations. Whether the combination of hypoxia (2% O_2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) is superior to other preconditioning methods is unclear. However, our study shows a preconditioning strategy that adds to the existing experimental options to use in further research. In addition, whether these cells exhibit superior immunomodulatory functions under harsh *in vivo* inflammatory conditions remains to be demonstrated and is the focus of our future work. Moreover, although TF expression is reduced by preconditioning, the mechanism is unclear. Thromboembolism may still have significant clinical consequences for patients who receive MSCs after preconditioning. Therefore, for whole-body cell administration, it is still necessary to use anticoagulants during the clinical application of MSCs to inhibit TF activity and increase the clinical benefits to patients.

CONCLUSION

In conclusion, we successfully developed an *in vitro* preconditioning method that mimics the impaired environment through a combination of hypoxia (2% O_2) and

inflammatory factors (IL-1β, TNF-α, IFN-γ) to enhance the immunosuppressive ability of UC-MSCs without compromising their biological characteristics. Most notably, this approach greatly reduced the expression of the clotting-related TF in MSCs, which was a surprising result.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have great potential in the treatment of a variety of immune-related diseases due to their unique immunomodulatory and anti-inflammatory abilities. However, after intravenous transplantation, MSCs cannot effectively exert their biological effects when they encounter a harsh environment *in vivo*, which reduces the efficacy of cell therapy. To increase the efficacy of transplantation, appropriate pretreatment methods are particularly important.

Research motivation

Although a variety of pretreatment methods are used to increase the efficacy of MSC transplantation, suitable and effective *in vitro* pretreatment methods are still worth studying.

Research objectives

To evaluate whether umbilical cord MSCs (UC-MSCs) pretreated with hypoxia exposure and inflammatory factors show enhanced immunosuppressive effects without affecting cell biological characteristics.

Research methods

In this study, we used a combination of hypoxia (2% O_2) and inflammatory factors (interleukin-1β, tumor necrosis factor-α, interferon-γ) to pretreat UC-MSCs for 24 h to simulate the *in vivo* injury environment. Then, we comprehensively evaluated the

biological properties of pretreated UC-MSCs and investigated their immunosuppressive properties.

Research results

Our results showed that compared with UC-MSCs, pretreated UC-MSCs were morphologically elongated, but their viability, proliferation and size were not affected, the expression of coagulation-related tissue factors was significantly reduced, and mitochondria maintained their function and integrity. Although some cells underwent apoptosis or senescence, polymerase chain reactions and enzyme-linked immunosorbent assays revealed a significant increase in the levels of immunomodulation-related factors. Coculture with peripheral blood mononuclear cell and natural killer cells exerted a stronger immunosuppressive effect.

Research conclusions

The combined pretreatment of hypoxia exposure and inflammatory factors enhanced the immunosuppressive ability of MSCs but did not affect the biological characteristics of these cells.

Research perspectives

Our study provides new strategies for the preconditioning of UC-MSCs.

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1	Debanjana Chatterjee, Nicole Marquardt, Dejene Milkessa Tufa, Guillaume Beauclair et al. "Role of gamma-secretase in human umbilical-cord derived mesenchymal stem cell mediated suppression of NK cell cytotoxicity", Cell Communication and Signaling, 2014 Crossref	22 words — < 1%
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