Jujuboside A inhibits oxidative stress damage and enhances immunomodulatory capacity of human umbilical cord mesenchymal stem cells through up-regulating IDO expression

CHEN Ji-Cong1Δ, XIAO Hong-He1Δ, ZHANG Qiang1, KONG Liang1, WANG Tian-Min1, TIAN Yu1, ZHAO Yu-Meng1, LI He1, TIAN Jin-Ming1, WANG Cui2*, YANG Jing-Xian1*

1 School of Pharmacy, Liaoning University of Traditional Chinese Medicine, Dalian 116600, China; 2 Department of Neurology, Dalian Municipal Central Hospital Affiliated to Dalian Medical University, Dalian 116033, China

Available online 20 Jul., 2022

[ABSTRACT] Impaired immunomodulatory capacity and oxidative stress are the key factors limiting the effectiveness of mesenchymal stem cell transplantation therapy. The present study was aimed to investigate the effects of jujuboside A (JuA) on the protective effect and immunomodulatory capacity of human umbilical cord mesenchymal stem cells (hUC-MSCs). Hydrogen peroxide was used to establish an oxidative damage model of hUC-MSCs, while PBMCs isolated from rats were used to evaluate the effect of JuA pre-treatment on the immunomodulatory capacity of hUC-MSCs. Furthermore, Hoechst 33258 staining, lactate dehydrogenase test, measurement of malondialdehyde, Western blot, high-performance liquid chromatography; and flow cytometry were performed. Our results indicated that JuA (25 μmol·L−1) promoted the proliferation of hUC-MSCs, but did not affect the differentiating capability of these cells. JuA pre-treatment inhibited apoptosis, prevented oxidative damage, and up-regulated the protein expression of nuclear factor-erythroid factor 2-related factor 2 and heme oxygenase 1 in hUC-MSCs in which oxidative stress was induced with H2O2. In addition, JuA pre-treatment enhanced the inhibitory effect of hUC-MSCs against abnormally activated PBMCs, which was related to stimulation of the expression and activity of indoleamine 2,3-dioxygenase. In conclusion, our results demonstrate that JuA pre-treatment can enhance the survival and immunomodulatory ability through pathways related to oxidative stress, providing a new option for the improvement of hUC-MSCs in the clinical setting.

[KEY WORDS] Human umbilical cord mesenchymal stem cells; Jujuboside A; Immunomodulation; Oxidative stress; Indoleamine 2,3-dioxygenase

[CLC Number] R965

[Introduction] Human umbilical cord mesenchymal stem cells (hUC-MSCs) is a type of adult stem cells derived from umbilical cord tissue. The cells have a strong self-replication capacity and can differentiate into adipocytes, osteocytes, and chondrocytes under the appropriate conditions [1]. Current research interest in these cells is driven by their powerful immunomodulatory effect, non-invasiveness, easy availability and rich resources [2]. According to previous studies, hUC-MSCs exerted therapeutic effect on multiple autoimmune and inflammatory diseases, such as systemic lupus erythematosus [3], Crohn’s disease [4], type 1 diabetes [5] and rheumatoid arthritis [6]. Despite broad applicability, the cells showed relatively short length of effective time. Therefore, it is necessary to establish a method to promote efficacy and prolong the duration of the therapeutic effect, so as to facilitate the clinical application of hUC-MSCs.

MSCs exerted therapeutic effect on multiple autoimmune and inflammatory diseases, such as systemic lupus erythematosus [3], Crohn’s disease [4], type 1 diabetes [5] and rheumatoid arthritis [6]. Despite broad applicability, the cells showed relatively short length of effective time. Therefore, it is necessary to establish a method to promote efficacy and prolong the duration of the therapeutic effect, so as to facilitate the clinical application of hUC-MSCs.

Growing evidence demonstrates that the clinical efficacy of hUC-MSCs is largely dependent on their immunomodulatory capacity rather than differentiation capacity [7]. The immunomodulatory effects of MSCs can be affected by cytokines secreted within the microenvironment, especially under the pathological conditions. Indoleamine 2,3-dioxygenase (IDO), a cytokine secreted by hUC-MSCs, was reported to induce macrophage differentiation toward the M2 type, resulting in increased levels of interleukin-10, decreased levels of interferon-gamma (IFN-γ) and inhibited prolifera-
tion of natural killer (NK) cells. IDO also exerted regulatory effect on adaptive immunity by suppressing the proliferation of CD4+ cells [8].

Additionally, the hostile environment in the host made it difficult for hUC-MSCs to survive and exert their therapeutic effects. Although low concentrations of reactive oxygen species were demonstrated to promote the proliferation and differentiation of stem cells, MSCs tended to be exposed to strong oxidative stress conditions and high concentrations of ROS, which resulted in damage in proteins, DNA and lipids [9, 10]. Multiple studies have indicated that oxidative stress inhibited the proliferation of MSCs, induced early senescence and reduced the adaptation of the host immune system [11]. Thus, inhibiting oxidative stress damage and enhancing the immunomodulatory capacity of hUC-MSCs may be effective ways to improve its efficacy.

Jujuboside A (JuA), a triterpene saponin, is a major bioactive component of a traditional Chinese medicine Ziziphi Spinosae Semen and may be a candidate molecule for enhancing the therapeutic effects of hUC-MSCs. Previous studies have demonstrated that JuA exhibited multiple biological activity, including anti-apoptotic [12], anti-inflammatory [13], anti-oxidant [14], neuro-protective [15, 16] and anti-tumor [17] effects. In a previous study, we found that Shenzao Jiannao oral liquid (SZJN), a herbal formula consisting of Ziziphi Spinosae Semen and other herbal medicines and containing JuA, markedly inhibited oxidative stress and reduced the apoptosis of neural stem cells in a mouse model of Alzheimer’s disease [18]. Furthermore, supporting the use of JuA in this application, a growing number of studies have shown that natural small molecules were effective in promoting the function of MSCs [19]. For example, quercetin combined with hUC-MSC effectively inhibited tumor necrosis factor-α/interferon-γ-induced activation of peripheral blood mononuclear cells [20]. Given these findings, we speculate that JuA may have the potential to inhibit oxidative stress damage in hUC-MSCs and enhance their immunomodulatory capacity.

This study was designed to assess the potential of JuA in protecting hUC-MSCs against oxidative stress injury and enhancing their immunomodulatory capacity. Specifically, we investigated the effects of JuA on the biological properties, protective effects, and immunomodulatory capacity of hUC-MSCs cultured in vitro. In addition, we measured the effect of JuA on anti-oxidantion and IDO to elucidate the underlying mechanisms involved. The results demonstrated that JuA pre-treatment inhibits the oxidative damage and enhances the immunomodulatory capacity of hUC-MSCs. These effects will be beneficial for further improvement of the clinical efficacy of hUC-MSCs.

Materials and Methods

Materials

JuA (MUST-21040711) was obtained from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Cell Count Kit-8 (CCK-8, G021-1) and Hoechst 33342 staining solution (A033-1-2) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein quantification kit (07061890429) and anti-IDO antibody (090320120407) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The assay kits for oil red O staining (20201011), alizarin red staining (20200908), lactate dehydrogenase (LDH, 20201103) and malondialdehde (MDA, 20201026) were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The anti-nuclear factor-erythroid factor 2-related factor 2 (Nrf2, WL02135), anti-heme oxygenase 1 (HO-1, WL02400), anti- BCL2 (WL03847), anti-BAX (WL01637), anti-caspase 3 (WL02117) and anti-GAPDH (WL01114) antibodies were purchased from Shenyang Wanlei Bio-Technology Co., Ltd. (Shenyang, China). High performance liquid chromatography (HPLC) was performed on an Agilent 1100 system coupled to a G1316ab photodiode array detector (Agilent Technologies, Wilmington, Delaware, USA).

Animals

Three-month-old Sprague-Dawley rats were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China, license number: SCXK(Liao)2020-0001). All rats were maintained in a designated animal care room (temperature: 25 ± 2 °C; humidity: 65% ± 5%, 12-h light/dark cycle) with access to food and water ad libitum. The rats were acclimated in the room for one week prior to experimentation. All experiments involving animals were approved by the Animal Care and Welfare Committee of Liaoning University of Traditional Chinese Medicine (license number: SYXX(Liao) 2019-0004).

Culture and characterization of hUC-MSCs

The hUC-MSCs were purchased from Shenyang First Cyto Co., Ltd. (UC1209WXX001; Shenyang, China) and cultured in low glucose Dulbecco’s modified Eagle’s medium (L-DMEM) containing 10% fetal bovine serum (FBS) and 100 U·mL−1 penicillin and streptomycin. Passage 3 hUC-MSCs were collected, washed with PBS and suspended at a density of 5 × 10^6 cells/mL. To 100 μL of this cell suspension was added 5 μL of fluorescently labeled CD19, CD11b, CD34, CD45, CD73, CD90, CD105 or HLA-DR monoclonal antibodies. The tubes were mixed with slight shaking and incubated at room temperature for 30 min in the dark and washed three times with PBS. Additional PBS (500 μL) was added to resuspend the cells, and surface antigens were detected by flow cytometry.

In addition, the capacity of hUC-MSCs was detected to differentiate towards adipocytes and osteoblasts. In brief, hUC-MSCs were inoculated in 96-well plates at a density of 5 × 10^4 cells/well and placed in a cell culture incubator (37 °C, 5% CO₂). Oil red O staining was used to evaluate adipogenesis on day 14, while alizarin red staining was used to assess osteogenesis on day 21 [21].

Cell viability assay

The hUC-MSCs (passages 3, 5, and 10) were plated into 96-well plates at a density of 5 × 10^3 cells/well in L-DMEM
Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of rats using a rat peripheral blood lymphocyte isolation kit. In brief, after anesthetizing the rats, the abdominal cavity was opened, and blood samples were collected via the abdominal aorta. The collected blood was diluted with an equal volume of blood diluent solution, then transferred to a sterile tube containing lymphocyte separation solution, and centrifuged at room temperature for 30 min. PBMCs were collected and transferred to culture in RPMI-1640 complete medium supplemented with 10% FBS and 1% penicillin and streptomycin. The isolated PBMCs were activated using 10 μg·mL⁻¹ of phytohaemagglutinin (PHA) and used in subsequent experiments.

**PBMC inhibition assay**

The activated PBMCs (5 × 10⁴ cells) were co-cultured without or with hUC-MSCs at different ratios (20 : 1, 10 : 1, and 5 : 1) in 96-well plates in RPMI-1640 complete medium supplemented with 10% FBS and 1% penicillin and streptomycin. Three days later, the medium containing PBMCs was transferred to a new 96-well plate and a CCK-8 kit was used to detect the proliferation of PBMCs according to the manufacturer’s instructions.

A transwell device was used to measure whether the inhibitory effect of hUC-MSCs on PBMCs was dependent on direct cell-to-cell contact. The activated PBMCs (1 × 10⁵ cells) were directly or indirectly co-cultured with hUC-MSCs (1 × 10⁵ cells) in RPMI-1640 complete medium. Three days later, the PBMCs were collected and tested using a CCK-8 kit.

The hUC-MSCs were plated into 96-well plates at a density of 5 × 10⁴ cells/well in L-DMEM complete medium for 6 h, and then pre-treated with or without JuA (25 μmol·L⁻¹). Two days later, the medium was replaced with RPMI-1640 complete medium supplemented with 10% FBS and 1% penicillin and streptomycin. The activated PBMCs were added at 5 × 10⁴ cells/well. Three days later, the PBMCs were collected, and a CCK-8 kit was used to detect the effect of JuA on the ability of hUC-MSCs in inhibiting PBMC activation.

**T-lymphocyte subtype differentiation assay**

Passage 3 hUC-MSCs were incubated in a 24-well plate at a density of 1 × 10⁵ cells/well for 6 h and then pre-treated with or without JuA (25 μmol·L⁻¹). Two days later, the activated PBMCs were added to co-culture at 1 × 10⁵ cells/well. Three days later, the PBMCs were collected and incubated with different antibodies (anti-CD4-APC, anti-IFN-γ-PE, anti-CD4-PerCP, anti-IL-17A-PE, anti-CD4-FITC, and anti-CD25-APC) in the dark for 10 min. Subsequently, the PBMCs were centrifuged at 1500 r·min⁻¹ for 3 min, suspended in 500 μL PBS, and tested by a flow cytometer.

**IDO expression in hUC-MSCs**

Passage 3 hUC-MSCs were incubated in 6-well plates at a density of 1 × 10⁶ cells/mL for 6 h, and then pre-treated with or without 1-methyl-tryptophan (0.5 mmol·L⁻¹) for 2 h. Then, the cells were treated with or without JuA (25 μmol·L⁻¹). Two days later, the activated PBMCs were added at a ratio of 1 : 10 for co-culture. After treatment for three
days, the cells were collected and lysed with RIPA lysis buffer containing PMSF. Western blot assay was carried out using an anti-IDO antibody to detect the expression of IDO in the hUC-MSCs.

**IDO activity assay**

Passage 3 hUC-MSCs were incubated in 24-well plates at a density of $1 \times 10^5$ cells/mL for 6 h, and then pre-treated with or without 1-methyl-tryptophan ($0.5 \text{ mmol} \cdot \text{L}^{-1}$) for 2 h. Then, the cells were treated with or without JuA ($25 \text{ μmol} \cdot \text{L}^{-1}$). Two days later, the activated PBMCs were added at a ratio of 1 : 10 for co-culture. After treatment for three days, the supernatant was collected, 5% perchloric acid was added and the mixture was centrifuged at 10 000 r·min$^{-1}$ for 5 min. Subsequently, the supernatant was filtered through a 0.22 μm microporous membrane, and the filtrate was separated by HPLC. A Tdikma platisol column (250 mm × 4.6 mm, 5 μm) was used in HPLC, and the mobile phase consisted of 92% acetonitrile and 8% ultrapure water supplemented with 15 mmol·L$^{-1}$ sodium acetate. The injection volume was 20 μL. The flow rate was 1 mL·min$^{-1}$, the column temperature was 25 °C, and the detection wavelength was 360 nm from 0 to 10 min and transferred to 280 nm from 10 to 15 min.

**Statistical analysis**

All data are expressed as mean ± SEM. One-way ANOVA was performed to compare data from different groups, and statistical plots were drawn using GraphPad Prism 7 software (GraphPad software, California, USA). $P < 0.05$ was considered statistical significance.

**Results**

**Identification of hUC-MSCs**

Single cell-derived clones were arranged in a swirling pattern and gradually came into contact (Fig. 1A). Osteogenic and adipogenic differentiation assays were used to detect the multi-directional differentiation capacity of the cells. After adipogenic induction, vacuolar lipid droplets with enhanced refraction were observed and stained orange with oil red O (Fig. 1C). The cells appeared as mineralized and calcified nodules after osteogenic induction, and the calcified nodules were stained red with alizarin red (Fig. 1D). Flow cyto-

Fig. 1 Characterization of hUC-MSCs. (A–B) Morphology of hUC-MSCs. (C) Adipogenesis of hUC-MSCs measured by oil red O staining. (D) Osteogenesis of hUC-MSCs measured by alizarin red staining. (E) Phenotype of hUC-MSCs tested by flow cytometry (All the scale bar = 100 μm)
metry was used to detect the expression of surface markers on hUC-MSCs. The results showed that CD73, CD90 and CD105 were positively expressed in hUC-MSCs, while the cells were negative for CD19, CD11b, CD34, CD45 and HLA-DR expression, suggesting the cell presented a typical MSC immunophenotype (Fig. 1E). Overall, these data suggest that the cultured cells in the present study were confirmed to be hUC-MSCs.

**JuA improves the proliferative capacity of hUC-MSCs**

The effects of JuA on the basic biological properties of hUC-MSCs were evaluated. These properties included morphology, proliferative ability, and multidirectional differentiation ability. The CCK-8 assay was used to detect the effect of JuA on the proliferative ability of hUC-MSCs. The results suggested that the self-renewal ability of the cells in each group after the addition of different concentrations of JuA (6.25, 12.5, 25, 50, and 100 μmol·L\(^{-1}\)) for 48 h was not significantly different, compared with passage 3 hUC-MSCs in normal culture (Fig. 2A, \(P > 0.05\)). Furthermore, the effect of JuA on the self-renewal ability of passage 5 and passage 10 hUC-MSCs was examined. Interestingly, the proliferation ability of passage 5 hUC-MSCs cultured with JuA (25 and 50 μmol·L\(^{-1}\)) for 48 h was significantly improved, compared with normal cultured cells (Fig. 2B, \(P < 0.05\)). This pro-proliferative effect of JuA was also demonstrated in passage 10 hUC-MSCs (Fig. 2C, \(P < 0.05\)).

Therefore, 25 μmol·L\(^{-1}\) JuA was chosen as the appropriate concentration for our experiments to evaluate the effect of JuA on the morphology and multidirectional differentiation ability of hUC-MSCs. We found that JuA improved the morphology of hUC-MSCs at passages 15 and 20 (Fig. 2D). Moreover, the results also showed that JuA did not alter the multidirectional differentiation ability of hUC-MSCs (Figs. 2E−2F). The above results demonstrated that JuA does not reduce the stemness of hUC-MSCs and enhances their proliferation ability.

---

**Fig. 2** JuA improved the proliferative ability of hUC-MSCs. (A−C) Passages 3, 5, and 10 hUC-MSCs were pre-treated with or without JuA (25 μmol·L\(^{-1}\)) for two days and the cell viability were measured by CCK-8 assay. Data are expressed as mean ± SEM (\(n = 6\)). \(P < 0.05\) vs Con group. (D) Effect of JuA (25 μmol·L\(^{-1}\)) on the morphology of passages 5, 10, 15, and 20 hUC-MSCs. (E) Effect of JuA (25 μmol·L\(^{-1}\)) on the adipogenesis of passage 3 hUC-MSCs, scale bar = 100 μm. (F) Effect of JuA (25 μmol·L\(^{-1}\)) on the osteogenesis of passage 3 hUC-MSCs, scale bar = 100 μm.
JuA inhibits H$_2$O$_2$-induced apoptosis of hUC-MSCs

H$_2$O$_2$ has been reported to induce apoptosis in MSCs at different concentrations and time points [23]. To establish a stable in vitro model of oxidative stress, the CCK-8 assay was used to screen the appropriate H$_2$O$_2$ concentration and the time of action. The survival of hUC-MSCs in serum-free medium was detected after treatment with 62.5, 125, 250, 500, and 1000 μmol·L$^{-1}$ H$_2$O$_2$ for 12 h. The results showed that all concentrations of H$_2$O$_2$, except 62.5 μmol·L$^{-1}$, effectively inhibited the survival of hUC-MSCs, and 250 μmol·L$^{-1}$ H$_2$O$_2$ was chosen as the appropriate modeling concentration because of its moderate effect (Fig. 3A, P < 0.01). Accordingly, hUC-MSCs were exposed to 250 μmol·L$^{-1}$ H$_2$O$_2$ for different times, and the results showed that incubation for 8, 10 and 12 h resulted in substantial apoptosis, and 10 h was chosen as the appropriate modeling time as moderate effect was seen (Fig. 3B, P < 0.01). Therefore, we concluded that incubation with 250 μmol·L$^{-1}$ H$_2$O$_2$ for 10 h is suitable for establishing an in vitro oxidative stress model.

To investigate the effect of JuA on H$_2$O$_2$-induced apoptosis of hUC-MSCs, the survival rate of differentially treated cells was examined. Here, we found that treatment with JuA rescued H$_2$O$_2$-induced decreases in cell survival; and the survival of cells treated with H$_2$O$_2$ and two concentrations of JuA was significantly higher than that of cells treated with H$_2$O$_2$ and vehicle (Fig. 3C, P < 0.05).

Furthermore, the levels of the apoptosis-related proteins cleaved caspase-3, Bcl-2, and Bax were also examined. Western blot results showed that JuA decreased the level of cleaved caspase-3 and increased the ratio of expression of Bcl-2 to Bax. The changes were significantly different in the JuA-treated cells, compared with the control group (Figs. 3F–3H, P < 0.01). Similar results were obtained by Hoechst 33258 staining, where JuA significantly reduced the number of ap-
optic cells (Figs. 3D–3E, \(P < 0.05\) or 0.01). Therefore, we concluded that JuA can inhibit \(\text{H}_{2}\text{O}_{2}\)-induced apoptosis of hUC-MSCs and facilitate their survival. **JuA ameliorates \(\text{H}_{2}\text{O}_{2}\)-induced oxidative stress damage of hUC-MSCs**

Under the pathological conditions, oxidative stress induced damage is a key reason that results in the decreased efficacy of hUC-MSCs upon transplantation. Therefore, the impact of JuA treatment on \(\text{H}_{2}\text{O}_{2}\)-induced changes in the levels of the oxidative stress related molecules LDH and MDA was examined. The results showed that the cellular levels of LDH and MDA significantly increased after co-incubation of hUC-MSCs with \(\text{H}_{2}\text{O}_{2}\). However, we found that pre-treatment with JuA reversed this elevation, and the differences between JuA-treated cells and control cells were statistically significant (Figs. 4A–4B, \(P < 0.05\) or 0.01).

Subsequently, we examined the protein expression of Nrf2 and HO-1 in each treatment group, so as to assess whether the Nrf2/HO-1 signaling pathway was activated by JuA in hUC-MSCs. The Nrf2/HO-1 signaling pathway exerts various oxidative damage-related effects such as increasing the production of anti-oxidants and mitigating mitochondrial damage. Our results suggested that \(\text{H}_{2}\text{O}_{2}\) significantly decreased the proteins levels of Nrf2 and HO-1 in hUC-MSCs. However, treatment with JuA up-regulated the expression of both Nrf2 and HO-1 (Figs. 4C–4E, \(P < 0.05\) or 0.01). Therefore, it is reasonable to conclude that JuA can inhibit oxidative stress damage in hUC-MSCs, which is associated with activation of the Nrf2/HO-1 signaling pathway. **JuA enhances the immunomodulatory capacity of hUC-MSCs**

Immunomodulatory capacity is one of the unique biological properties of MSCs. MSCs have been reported to regulate the body’s intrinsic and adaptive immunity, including reducing the secretion of inflammatory cytokines, inhibiting the proliferation of lymphocytes, and regulating the differentiation of T-lymphatic subpopulations. To investigate the effect of JuA on the immunomodulatory capacity of hUC-MSCs, its inhibitory effect on PHA-induced activation of PBMC was examined. The results showed that hUC-MSCs inhibited the abnormal activation of PBMC, which was positi-
Fig. 5 JuA enhanced the immunomodulatory capacity of hUC-MSCs. (A) Activated PBMCs were co-cultured without or with hUC-MSCs at different ratios for three days. The viability of PBMCs was measured by CCK-8 assay. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs PBMC group. (B) Activated PBMCs were directly or indirectly co-cultured with hUC-MSCs for three days. The viability of PBMCs was measured by CCK-8 assay. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs PBMC group. (C) The hUC-MSCs were pre-treated with or without JuA (25 μmol·L⁻¹) for two days, and activated PBMCs were directly co-cultured with or without hUC-MSCs for three days. The viability of PBMCs was measured by CCK-8 assay. Data are represented as mean ± SEM (n = 3). ##P < 0.01 vs PBMC group; *P < 0.05 vs PBMC + UCMSC group. (D), (F) and (H) The hUC-MSCs were pre-treated with or without JuA (25 μmol·L⁻¹) for two days, and activated PBMCs were directly co-cultured with or without hUC-MSCs for three days. Subtype differentiation of T lymphocytes detected by flow cytometry. (E), (G) and (I) Quantitative analysis of the percentage of Th1 cells (E), Th17 cells (G), and Tregs (I). Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs PBMC group.
ively correlated with the number of hUC-MSCs (Fig. 5A, \( P < 0.05 \) or 0.01).

Additionally, a transwell device was used to evaluate whether the inhibitory effect was dependent on direct cell-to-cell contact. We observed that the proliferation of PBMCs without direct exposure to hUC-MSCs was also inhibited, which however was slightly weaker than that produced by direct exposure (Fig. 5B, \( P < 0.05 \) or 0.01). In addition, the ability of hUC-MSCs in inhibiting PBMC proliferation was enhanced by the addition of JuA, but JuA itself had no inhibitory effect on PBMCs (Fig. 5C, \( P < 0.05 \) or 0.01). Therefore, it was suggested that JuA enhances the inhibitory effect of hUC-MSCs on abnormally activated PBMCs.

Furthermore, flow cytometry was used to explore the effect of JuA on the ability of hUC-MSCs in regulating the differentiation of T lymphocyte subpopulations. We observed that hUC-MSCs inhibited the differentiation of T lymphocytes to Th1 cells and Th17 cells and promoted their differentiation into Treg cells. Meanwhile, JuA had no effect on the ability of hUC-MSCs in regulating T lymphocyte lineage differentiation (Figs. 5D–5L, \( P < 0.05 \) or 0.01). These results demonstrated that JuA enhances the immunomodulatory capacity of hUC-MSCs.

**JuA enhances the immunomodulatory capacity of hUC-MSCs by up-regulation of IDO expression**

IDO is a rate-limiting enzyme in the kynurenine pathway, which performs a protective function by catalyzing the oxidative cleavage of tryptophan and regulating the levels of tryptophan and its metabolites. In order to explore the potential mechanisms by which JuA enhances the immunomodulatory capacity of hUC-MSCs, we evaluated the protein expression of IDO in hUC-MSCs with and without JuA treatment. An inhibitor of IDO, 1-MT, was also utilized in this experiment. The results showed that the expression of IDO protein significantly increased in hUC-MSCs after co-cultured with activated PBMC. After pre-treatment with JuA, the expression of IDO remarkably increased, compared with hUC-MSCs without pre-treatment with JuA (Figs. 6A–6B, \( P < 0.05 \) or 0.01).

Tryptophan is an essential amino acid, and tryptophan starvation can lead to suppression of T lymphocyte activation, while kynurenine, a metabolite of tryptophan, can directly lead to apoptosis of T lymphocytes. Therefore, in order to measure the impact of JuA on these important compounds, HPLC was used to quantify the levels of tryptophan and kynurenine in the supernatants of cells treated with JuA and of control cells. Our results indicated that hUC-MSCs co-cultured with activated PBMC showed significant increases in IDO activity which was correlated with a substantial decrease in tryptophan content and enhanced the kynurenine content in the cell supernatant. The specificity of the promotion of increased IDO activity by JuA was demonstrated through blocking the effect by the IDO inhibitor 1-MT (Figs. 6C–6E, \( P < 0.05 \) or 0.01). Therefore, it is suggested that the enhanced immunomodulatory capacity of hUC-MSCs by JuA is associated with the up-regulation of IDO expression.

**Discussion**

Cell therapy based on MSCs is considered to be a promising strategy for the treatment of autoimmune diseases, but the pathological microenvironment of the host impairs the immunomodulatory capacity of these cells, which greatly limits their clinical efficacy. Therefore, it is important to identify tools to rescue this impaired immunomodulatory capacity, so as to improve clinical outcomes. Mounting evidence suggests that IDO is an important mediator of immune regulation in hUC-MSCs, and can function through suppressing excessive activation of T lymphocytes and decreasing the release of pro-inflammatory factors. In this study, we demonstrated that JuA enhanced the immunomodulatory capacity of hUC-MSCs through promoting the expression of IDO. Furthermore, JuA significantly inhibited oxidative stress damage and ameliorated apoptosis in hUC-MSCs.

To date, bone marrow remains the most common source of MSCs; however, the acquisition of cells in this way utilizes an invasive procedure that may aggravate the pre-existing condition or injury. In addition, it has been shown that the self-replicative capacity, immunomodulatory capacity, and differentiation potential of bone marrow mesenchymal stem cells (BM-MSCs) decline with the increasing of donor age. Therefore, finding new sources of MSCs may stimulate the development of more potent treatments. Compared with BM-MSCs, hUC-MSCs have been demonstrated to have stronger immunoregulatory and proliferative abilities in vitro experiments. These advantages suggested that hUC-MSCs are a suitable alternative to BM-MSCs. Here, we cultured and identified hUC-MSCs, and the results of characterization were consistent with the international common criteria for the identification of MSCs. It is also noteworthy that JuA effectively promoted the proliferation of hUC-MSCs without damaging or changing their multidirectional differentiation ability in vitro.

Oxidative stress negatively impacts survival and ultimately induces apoptosis in MSCs after transplantation. Recent studies have demonstrated that oxidative stress damage in MSCs also contributed to the impairment of their immunomodulatory capacity. An important consideration in the mechanisms leading to these negative outcomes is the relationship between the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax, which together can regulate apoptosis. If the ratio of Bcl-2 to Bax is excessively reduced, the repair capacity of DNA will also be reduced, leading to cell death. It has also been found that the expression and activation of caspase 3 is closely related to both oxidative dam-
age and a decreased Bcl-2/Bax ratio. Previous studies have shown, for example, that geraniin inhibited H$_2$O$_2$-induced apoptosis in BM-MSCs through reducing the activation of caspase 3 \[30\]. Similarly, artemisinin was found to ameliorate the excessive activation of apoptosis in BM-MSCs by activating the c-Raf-Erk1/2-p90-CREB pathway to increase the Bcl-2/Bax ratio \[31\]. In this study, we demonstrated that JuA significantly up-regulated Bcl-2 protein expression, decreased Bax levels and further inhibited caspase 3 activation. A related phenomenon was seen in the quantification of MDA and LDH, which suggested that JuA treatment ameliorated oxidative damage.

**Nrf2**, a key factor almost exclusively expressed during oxidative stress, can be activated by overproduced reactive oxygen species and initiate transcription of the gene encoding HO-1 \[32\]. It has been demonstrated that tetramethylpyrazine and astaxanthin inhibited oxidative stress and protected hUC-MSCs and adipose MSCs against H$_2$O$_2$-induced apoptosis by activating the Nrf2/HO-1 signaling pathway \[33, 34\]. Recent evidence has suggested that Nrf2 also plays a positive role in the immunomodulatory capacity of hUC-MSCs, in that it enhanced the inhibitory effect of hUC-MSCs on PHA-induced abnormal PBMC proliferation by promoting IDO secretion \[35\]. In this study, we found a significant increase in

---

**Fig. 6**  JuA enhanced the immunomodulatory capacity of hUC-MSCs through up-regulating IDO expression. The hUC-MSCs were pre-treated with or without 1-MT for 2 h, and then the cells were treated with or without JuA (25 μmol·L$^{-1}$). Two days later, activated PBMCs were directly co-cultured with or without hUC-MSCs for three days. (A) Representative images of IDO protein. (B) Semi-quantitative analysis of the protein expression of IDO. (C) Quantitative analysis of the percentage of kynurenine to tryptophan. (D) The chromatograms of mixed standards. (E) The chromatograms of samples. Data are represented as mean ± SEM ($n = 3$). $^{**}P < 0.01$ vs UCMSC group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs PBMC + UCMSC group; $^{{\&}\&}P < 0.01$ vs PBMC + JuA-UCMSC group.
the expression of both Nrf2 and HO-1 proteins after JuA treatment, suggesting that JuA may exert its hUC-MSC-protective effect partly through this signaling axis.

Immunomodulatory properties are the basis for the efficacy of MSCs in autoimmune diseases, so enhancing the immunomodulatory capacity of MSCs is of great importance. It has been demonstrated that rapamycin combined with transplantation of BM-MSCs attenuated inflammatory infiltration and demyelination in a mouse model of autoimmune encephalomyelitis [36]. BM-MSCs transduced with the gene encoding superoxide dismutase 3 significantly decreased the development of psoriasis in an imiquimod-induced mouse model [37].

Mounting evidence also suggests that MSCs can regulate immune responses through the secretion of IDO [38, 39]. IDO is an intracellular enzyme that catalyzes the degradation of tryptophan to kynurenine, and tryptophan deficiency and the presence of the tryptophan metabolite kynurenine have been shown to inhibit T cell proliferation [40]. Up-regulation of IDO activity has also been found to enhance the therapeutic effect of hUC-MSCs on experimental colitis [41]. In this study, we demonstrated that JuA enhanced the inhibitory effect of hUC-MSCs on abnormal activation of T lymphocytes, while decreased expression of IDO protein was seen, which coincided with decreased tryptophan levels and increased kynurenine content after JuA treatment. The presence of 1-MT reversed this change, suggesting that the phenomenon was dependent on IDO catalytic activity.

Previous studies have suggested that the modification of MSCs by viral vectors presents risks in clinical applications [42]. Thus, natural small molecules are more practical and safer for the improvement of cell therapy. For example, a small molecule ostehol has been shown to enhance the immunosuppressive ability of BM-MSCs by promoting the Fas/FasL system [43], and quercetin was demonstrated to activate toll-like receptor 3 to enhance the inhibition of abnormal activation of PBMCs by hUC-MSCs [44]. These reports and our findings jointly suggest that natural small molecules have a positive role in MSC-based cell therapy, which provide further evidence of the feasibility of using gene-free manipulation of MSCs in the clinical setting.

Taken together, our results suggest that JuA can enhance the immunomodulatory capacity of hUC-MSCs through promoting IDO expression and can inhibit damage due to oxidative stress. These finding will provide a new option for enhancing the clinical efficacy of hUC-MSCs.

References


Cite this article as: CHEN Ji-Cong, XIAO Hong-He, ZHANG Qiang, KONG Liang, WANG Tian-Min, TIAN Yu, ZHAO Yu-Meng, LI He, TIAN Jin-Ming, WANG Cui, YANG Jing-Xian. Jujuboside A inhibits oxidative stress damage and enhances immunomodulatory capacity of human umbilical cord mesenchymal stem cells through up-regulating IDO expression [J]. Chin J Nat Med, 2022, 20(7): 494-505.