

IPSC-MSC inhibition assessment in Raw 264.7 cells following oxygen and glucose deprivation reveals a distinct function for cardiopulmonary resuscitation

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Abstract. Hypoxia is a serious stress state. The nervous system is less tolerant to hypoxia, and cell death due to hypoxia is irreversible. With the incidence of cardiovascular disease gradually increasing, the sudden cardiac death rate is additionally increasing. Although cardiopulmonary resuscitation (CPR) is an important development, recovery is frequently poor. In a successful recovery population, ~40% of the population was in a vegetative state or subsequently succumbed to their condition, and ~20% had brain damage. Therefore, the recovery of the brain is of particular importance in CPR. Immune disorders are one of the major mechanisms of cerebral resuscitation following CPR. Studies have demonstrated that induced pluripotent stem cell-derived mesenchymal stem cells (IPSC-MSCs) have a strong immune regulatory effect during tissue repair and anti-inflammatory effects. IPSC-MSCs may inhibit the inflammatory response by means of the inflammatory reaction network to improve brain function following CPR, although the cellular and molecular mechanisms remain unclear. Macrophages are a bridge between innate immune and specific immune responses in the body; therefore, it was hypothesized that macrophages may be the important effector cell of the role of IPSC-MSCs in improving brain function

following recovery of spontaneous respiration and circulation subsequent to cardiopulmonary resuscitation. In the present study, IPSC-MSCs were applied to the oxygen and glucose deprivation (OGD) model. It was observed that intervention with IPSC-MSCs was able to alter the polarization direction of macrophages. The difference in the proportions of M1 and M2 macrophages was statistically significant at 6, 12, 24 and 48 h ($P=0.037$, $P<0.05$) in the OGD + IPSC-MSCs group (M1, $33.48\pm 5.6\%$; M2, $50.84\pm 6.9\%$) and in the OGD group (M1, $83.55\pm 7.3\%$; M2, $11.41\pm 3.2\%$), and over time this trend was more obvious. The polarization direction of macrophages is associated with the neurogenic locus notch homolog protein 1 (Notch-1) signaling pathway. In conclusion, it was observed that IPSC-MSCs may be associated with altered macrophage polarization, which may be accomplished by inhibiting the Notch-1 signaling pathway.

Introduction

Hypoxic encephalopathy caused by sudden cardiac death (1) is a critical clinical disease, and it is an important reason for the high rate of disability following cardiopulmonary resuscitation (CPR). Early identification, effective prevention, treatment of ischemic encephalopathy and improving the success rate of cerebral resuscitation are important issues that may not be ignored.

At present, studies have demonstrated that there are various reasons for the low success rate of treatment for hypoxic encephalopathy. During and subsequent to cardiopulmonary resuscitation, high levels of circulating cytokines, the activation of blood coagulation and platelets, the presence of endotoxins in plasma, and the alteration of soluble E-selectin and P-selectin have been described (2). A considerable number of studies have demonstrated that induced pluripotent stem cell-derived mesenchymal stem cells (IPSC-MSCs) have a marked therapeutic effect on hypoxic encephalopathy (3). IPSC-MSCs have exerted substantial protective effects and improvements on the survival rate of acute lung injury in animal

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experiments (4-6). In addition, intervention with IPSC-MSCs may reduce neutrophil infiltration in the lung tissue of mice with ventilator-associated pneumonia and improve the survival rate (7,8). All of the above studies have demonstrated that IPSC-MSCs exert an immune regulatory effect and an inflammatory response that balances multiple aspects of the immune inflammatory network of the body; however, the details of the function of IPSC-MSCs and the mechanism of action remain unclear (9,10). The role of macrophage differentiation, different phenotypes and functional status in inflammatory and neoplastic diseases has attracted much attention. Previous studies have demonstrated that the Notch-1 signaling pathway is associated with the differentiation, proliferation and function of a number of types of immune cells (11,12). However, the mechanisms underlying the way in which IPSC-MSCs exert their benefits are not well understood.

IPSC-MSCs may improve the recovery of the brain following CPR; however, the mechanism underlying the role of IPSC-MSCs in immune regulation, whether they are able to alter the direction of macrophage polarization, and whether they may improve the prognosis of cerebral resuscitation remains unknown. Further experimental studies are required to examine the mechanism underlying the way in which IPSC-MSCs exert their anti-inflammatory effect, and which signaling pathway results in the induction of M2 type macrophages. In the present study, Raw 264.7 cells were used to perform oxygen and glucose deprivation (OGD) to replicate the model of cerebral ischemia. Intervention by IPSC-MSCs was performed in the OGD model and the results demonstrated that IPSC-MSCs were able to regulate the polarization of macrophages via the neurogenic locus notch homolog protein 1 (Notch-1) signaling pathway. In addition, the results of the present study demonstrated that IPSC-MSCs were able to regulate the polarization of macrophages, which may be accomplished by inhibiting the Notch-1 signaling pathway.

Materials and methods

Materials. Cells, cell culture media, serum and cell culture supplements were purchased from the American Type Culture Collection (Manassas, VA, USA), EMD Millipore (Billerica, MA, USA), Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and PAA Laboratories (GE Healthcare, Chicago, IL, USA), respectively, unless otherwise stated. Antibodies against inducible nitric oxide synthase (iNOS; cat. no. ab178945), Hes1 (cat. no. ab108937), interleukin (IL)-10 (ab189392), arginase-1 (Arg1; cat. no. ab124917) and β -actin (cat. no. ab8226) were purchased from Abcam (Cambridge, UK). Antibodies against tumor necrosis factor (TNF)- α (cat. no. 11948P) and Notch 1 (cat. no. 4380P) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against allophycocyanin (APC)-cluster of differentiation (CD)197 (cat. no. 120107), fluorescein isothiocyanate (FITC)-F4/80 (cat. no. 123107), and phycoerythrin (PE)-CD206 (cat. no. 141705) were purchased from BioLegend, Inc. (San Diego, CA, USA). Horseradish-peroxidase conjugated secondary antibodies for western blot (WB) analysis were purchased from Abcam (cat. no. ab150157). Recombinant mouse vascular endothelial growth factor (VEGF) was purchased from R&D Systems Europe, Ltd. (Abingdon, UK).

IPSC-MSCs and Raw 264.7 cells co-culture. Naive IPSC-MSCs and Raw 264.7 cells were cultured in collagen-coated dishes with High Glucose Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l glucose; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Medium was changed every 2-3 days. By 3-4 days of incubation, cells had reached 70-80% confluence and were seeded into 6-well flat bottom microtitre plates at a cell density of 1x10⁶ cells/well. IPSC-MSCs (1x10⁶ cells/well) were inoculated onto the upper membrane (Transwell insert) and Raw 264.7 cells (1x10⁶ cells/well) were inoculated into the lower chamber of a conventional double cell co-culture system. The cells were divided into three groups, including Raw 264.7 cells (1x10⁶ cells/well; control group), Raw 264.7 cells (1x10⁶ cells/well; OGD group) and IPSC-MSCs (1x10⁶ cells/well) + Raw 264.7 cells (1x10⁶ cells/well; OGD + IPSC-MSCs group).

OGD. OGD was induced by exposing Raw 264.7 cells to a calibrated gas mixture of 2% CO₂, 5% CO₂ and 93% N₂ in a 3-gas incubator (Forma; Thermo Fisher Scientific, Inc.) in PBS for 0.5, 1, 2 and 4 h. The OGD modeling time was determined by the results of the Cell Counting Kit-8 (CCK-8) assay. Control cells were maintained in normal conditions (5% CO₂, 95% humidified air) in complete high glucose medium (DMEM 4.5 g/l).

CCK-8 assay. The integrity of cellular function was measured using a CCK-8 assay (formazan crystals were solubilized with 0.1 N HCl isopropanol). Raw 264.7 cells exposed to OGD were further incubated for 0.5, 1, 2 and 4 h with 10 μ l CCK-8 at 37°C in a normoxic chamber. At the end of the incubation period, absorption was detected at 450 nm, with background subtraction at 630 nm, using a microplate reader (Stat Fax-2100; Awareness Technology, Inc., Palm City, FL, USA) (13).

Flow cytometry (FCM). Cells were harvested, washed in PBS and resuspended in binding buffer, and a 0.5 ml aliquot was withdrawn for analysis. Following the addition of annexin V-FITC, APC and PE (BioLegend, Inc.), the sample was incubated for 30 min in the dark. Stained cells were analyzed using a BD Biosciences (Franklin Lakes, NJ, USA) flow cytometer. A total of 1x10⁶ cells were counted per sample, and the data were processed using Beckman Coulter Cell Lab Quanta™ SC MPL (AL510171; Beckman Coulter, Inc., Brea, CA, USA).

The CCK-8 and the cell sorting staining assays for FCM were performed in parallel in twin cultures that were subjected to identical conditions. This procedure was adopted to eliminate variations in the cell population, growth conditions and experimental procedures.

WB analysis. 10X RIPA lysis buffer (Abcam) was used for protein extraction (cat. no. ab156034) and a BCA protein assay kit was used for protein determination. A total of 20 μ g denatured protein diluted in 20 μ l solution samples were loaded on a 10% SDS-PAGE gel, and electrophoresis was run at 150 V for 1 h. Proteins were transferred to a polyvinylidene fluoride membrane (BioRad Laboratories, Inc., Hercules, CA, USA) using a Trans-Blot semi-dry transfer. Cell membranes were blocked with 5% skimmed milk in TBS-Tween 20 at 4°C

Table I. Reverse transcription-quantitative polymerase chain reaction primers.

Symbol	Gene ID	Amplicon size, bp	Primer sequence (5'-3')	
			Forward	Reverse
Nos2 (iNOS)	18126	127	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTTCGATGTCAC
Abl2 (Arg1)	11352	198	GAGCCACCGTTTTACATTGTGA	CTCGCCCACTAGGCAGTTC
Notch1	18128	227	ACACCGTGTAAGAATGCTGGA	GCCTGCTGACATGATTTTCCTG
Atcay (Hes1)	16467	157	TCCGACGACTTCCTCGACA	CACCAGGCATGTTTTTGGCG
β -actin	11461	154	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

iNOS, inducible nitric oxide synthase; Arg1, arginase 1; Notch1, neurogenic locus notch homolog protein 1; Hes1, transcription factor HES-1.

for 1 h and subsequently incubated with primary antibodies against TNF- α , iNOS, Notch1, Hes1, IL-10, Arg1 and β -actin at a dilution of 1:1,000 for 1 h at room temperature. Following incubation with the primary antibodies, the secondary antibody (1:1,000) was used and membranes were incubated at room temperature for 1 h. The membranes were treated with an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.) for 1-2 min and exposed at different exposure times. β -actin (1:5,000) was used as the loading control. Blots from 4-6 different experiments were scanned and band intensities from each blot were analyzed using Image J software (version 1.8.0_101; National Institutes of Health, Bethesda, MD, USA) and expressed relative to the β -actin signal.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from cells using TRIzol reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. For RT, 1 μ g total RNA from each sample was reverse transcribed using Superscript II Reverse Transcriptase (Takara Bio, Inc.). Aliquots of diluted cDNA (1:5) were amplified using TransStart Top Green qPCR SuperMix in a final volume of 20 μ l. The RT reaction was performed at 30°C for 10 min, 42°C for 60 min, and 70°C for 10 min. qPCR amplification was performed using a LabCycler Real-Time PCR system (SensoTech GmbH, Magdeburg-Barleben, Germany) using SYBR Green dye (Takara Bio, Inc.), and protein quantification was performed using the $2^{-\Delta\Delta C_t}$ method (14). The sequences of the primers are listed in Table I.

Data and statistical analysis. The values are presented as the mean \pm standard deviation. The control, OGD and OGD + IPSC-MSC groups were tested for normality within all the time points using repeated measures analysis of variance followed by the Tukey post hoc test. The null hypothesis was rejected at the significance level $\alpha < 0.05$. Data were analyzed statistically using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Establishment OGD model and CCK-8 assay. Co-cultured Raw 264.7 and IPSC-MSCs are presented in Fig. 1. Following

establishment of the OGD model, cell viability was determined by assessing the integrity of mitochondrial function using the CCK-8 assay. The CCK-8 assay demonstrated that the cell viability of the OGD group and the OGD + IPSC-MSC group decreased during 0-4 h. The OGD (0.59 \pm 0.02) and OGD + IPSC-MSC groups (0.61 \pm 0.01) exhibited no apparent alterations in cell viability at 0.5 h compared with the control group ($P > 0.05$); however, cell viability decreased following 1-2 h of intervention. In addition, OGD cells and OGD + IPSC-MSC cell viability decreased more significantly following intervention for 4 h. The cell viability of the OGD group (0.38 \pm 0.13) and the OGD + IPSC-MSC group (0.42 \pm 0.10) exhibited a statistically significant decrease compared with the control group (0.78 \pm 0.05) ($P = 0.032$, $P < 0.05$; Fig. 2).

Effect of OGD and IPSC-MSC intervention on Raw 264.7 macrophage polarization. To determine whether OGD and IPSC-MSC intervention affected macrophage polarization, FCM, WB and RT-qPCR analyses were performed. FCM analysis was used to objectively analyze the proportion of M1 and M2 macrophages. Following quantitative analysis, it was observed that the proportion of M1 and M2 macrophages following intervention for 4 h was statistically significant among the control group (M1, 27.59 \pm 1.3%; M2, 52.59 \pm 11.0%), OGD + IPSC-MSC group (M1, 50.02 \pm 2.4%; M2, 37.08 \pm 10.4%), and OGD group (M1, 56.97 \pm 12.8%; M2, 33.96 \pm 9.7%; $P < 0.05$). The proportion of M1 and M2 macrophages between the OGD group (M1, 83.55 \pm 7.3%; M2, 11.41 \pm 3.2%) and control group (M1, 29.34 \pm 4.1%; M2, 52.34 \pm 5.4%) was also statistically significant at 6, 12, 24 and 48 h ($P = 0.026$; $P < 0.05$), and over time the trend was more apparent. The proportion of M1 and M2 macrophages between the OGD + IPSC-MSC group (M1, 33.48 \pm 5.6%; M2, 50.84 \pm 6.9%) and OGD group (M1, 83.55 \pm 7.3%; M2, 11.41 \pm 3.2%) was statistically significant at 4, 6, 12, 24 and 48 h ($P = 0.037$, $P < 0.05$), and over time the trend was more apparent (Fig. 3). To confirm the FCM results, WB and RT-qPCR analyses were performed. WB and RT-qPCR analysis indicated a decrease in Arg1 and IL-10 in the OGD group compared with the control group at 6, 12, 24 and 48 h ($P = 0.041$, $P < 0.05$; Figs. 4 and 5A-C). In addition, a significant decrease in iNOS and TNF- α levels in the OGD + IPSC-MSC group was observed at 6, 12, 24 and 48 h compared with the OGD group ($P = 0.018$, $P < 0.05$), and significantly increased at 24 and 48 h compared with the control group ($P = 0.026$, $P < 0.05$; Figs. 4 and 5A, D and E).

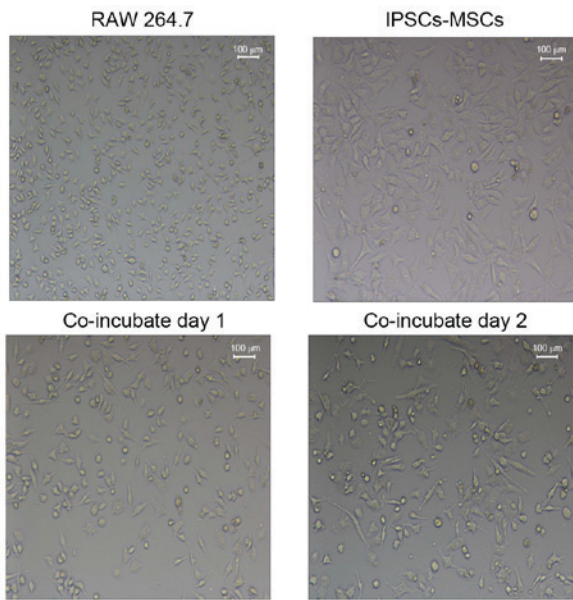


Figure 1. Cells were cultured under normal conditions (control). IPSC-MSCs and Raw 264.7 cells were co-incubated under normal conditions on days 1 and 2. Representative images from at least three independent experiments are presented (magnification, x100). IPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells.

Effects of IPSC-MSC intervention on the polarization of Raw 264.7 macrophages through the Notch-1 pathway. The Notch gene encodes a highly conserved cell surface receptor that regulates the development of a variety of biological cells, ranging from sea urchins to humans. Therefore, the present study aimed to examine the expression of Notch-1 in the OGD + IPSC-MSC group. Notch-1 protein and mRNA levels were determined in each group. WB quantitative analysis demonstrated that Notch-1 expression in the OGD + IPSC-MSC group [0.60 ± 0.05 Absorbance Unit (A.U.)] was significantly decreased at 6, 12, 24 and 48 h compared with the OGD group (1.12 ± 0.11 A.U.), and significantly increased at 24 and 48 time intervals compared with the control group (0.86 ± 0.07 A.U.) ($P=0.034$, $P<0.05$; Fig. 5A and F). Additionally, RT-qPCR analysis confirmed the effect of IPSC-MSC intervention on the polarization of Raw 264.7 macrophages through the Notch-1 pathway. A significant increase in Notch-1 in the OGD group was observed compared with the control group at 6, 12, 24 and 48 h (OGD, 3.44 ± 0.22 A.U.; $P=0.014$, $P<0.05$; Fig. 4).

Effects of IPSC-MSC intervention on Hes1 and the polarization of Raw 264.7 macrophages post-OGD. In order to further characterize the effects of Notch-1 in the OGD model, Hes1, a downstream effector of Notch-1, was examined by WB and RT-qPCR analyses. Densitometric analysis of Hes1 WB bands demonstrated a significant decrease in Hes1 expression in the OGD + IPSC-MSC group (1.69 ± 0.17 A.U.) at 6, 12, 24 and 48 h compared with the OGD group (2.01 ± 0.07 A.U.); however, Hes1 expression in the OGD + IPSC-MSC group was significantly increased compared with control groups at 6, 12, 24 and 48 h time intervals (1.58 ± 0.21 A.U.; $P=0.036$, $P<0.05$; Fig. 5A and G). Quantitative analysis of the PCR results demonstrated a significant decrease in Hes1 expression in the OGD + IPSC-MSC group (2.5 ± 0.37 A.U.) compared with the

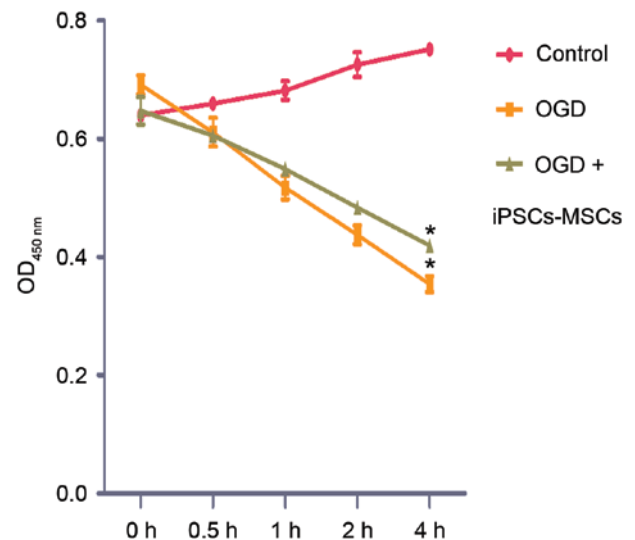


Figure 2. Control cells were incubated in a normoxic chamber. OGD cells and OGD + IPSC-MSCs were exposed to OGD (n=3). The cell viability of the OGD group and the OGD + IPSC-MSC group exhibited a statistically significant decrease compared with the control group ($P<0.05$). * $P<0.05$ vs. control group. OGD, oxygen and glucose deprivation; OD, optical density.

OGD group (3.50 ± 0.27 A.U.), and a significant increase in Hes1 expression in the OGD + IPSC-MSC group compared with the control group (1.00 A.U.; $P=0.031$, $P<0.05$; Fig. 4).

Discussion

Cerebral hypoxic ischemia injury is a primary cause of mortality and disability in emergency medicine (15). Although improvements in CPR performance and the increasing success rate in achieving recovery of spontaneous respiration and circulation in recent years, the survival and discharge rate of patients post-sudden cardiac arrest remain poor (16). However, in Denmark and the USA, greater survival and favorable neurological status were associated with hospital based post-resuscitative care guidelines; therefore, study of the associated mechanisms is required (17,18). Macrophages, a particular type of immune cell, serve a role in innate immunity, including killing pathogens. A previous study demonstrated the role of macrophage polarization direction in anti-tumor immunity, infection, immune responses, atherosclerosis, cardiovascular disease and diabetes (19). In addition, glucose tolerance abnormalities and other diseases have served an important role (19). A study demonstrated that axonal regeneration may be the primary target of and key concern with nerve injury repair (20). Previous studies aiming to promote axonal regeneration have demonstrated that the primary reason for the failure of early regeneration in the adult mammalian central nervous system (CNS) is the existence of myelin inhibitors (21,22). Studies have confirmed that macrophages/glia cells in the CNS exhibit chemotaxis, phagocytosis of myelin fragments, and that it is possible to improve the prognosis of neurological function (23,24). However, previous studies have demonstrated that macrophages mediate inflammatory responses in the process of neuronal repair, resulting in secondary neuronal damage (25,26). Therefore, in-depth examination of the regulation of macrophages in the CNS

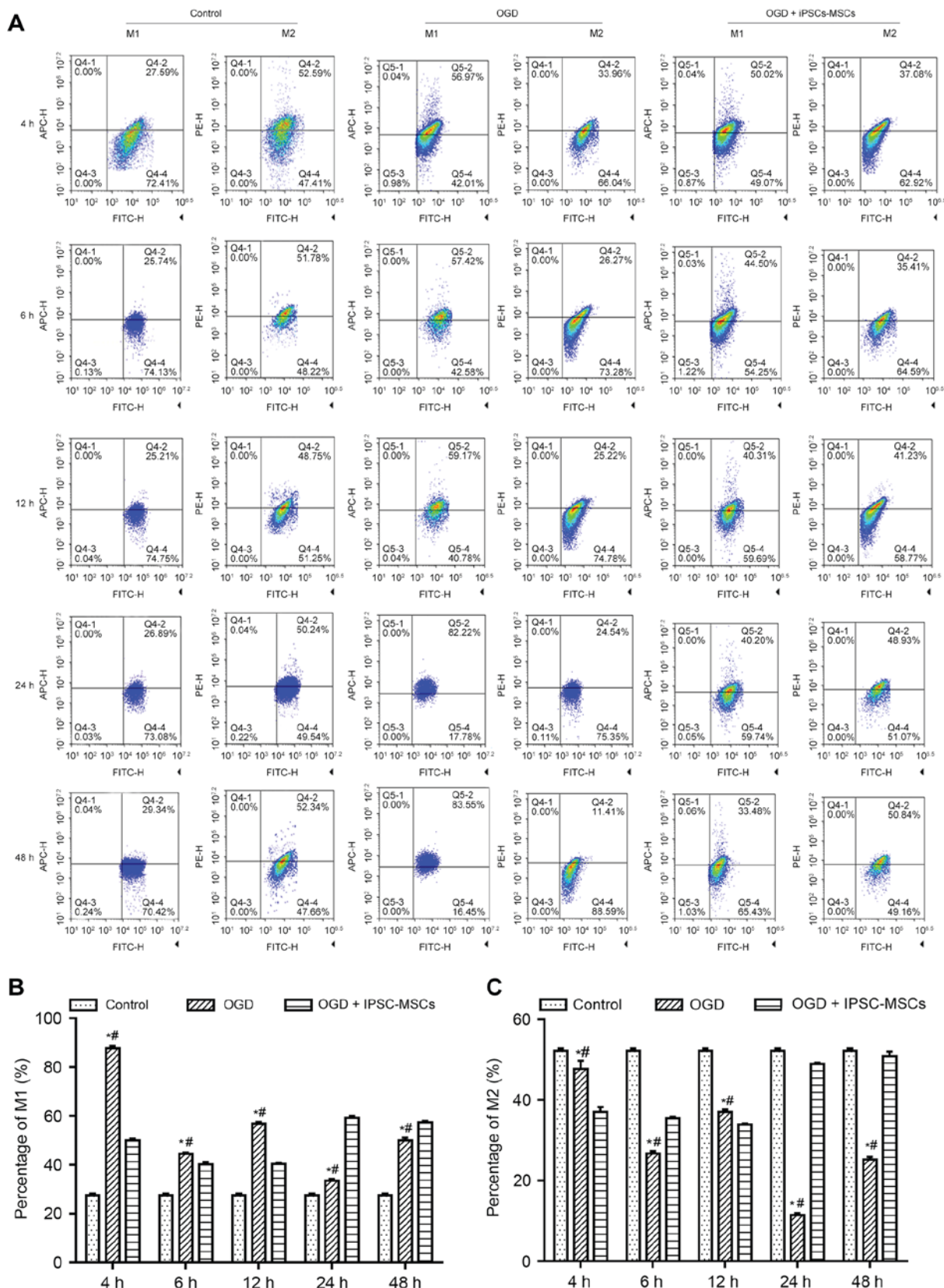


Figure 3. (A) Flow cytometry analysis of the control, OGD and OGD + IPSC-MSC groups at the 4, 6, 12, 24 and 48 h time points (n=3). The bar graphs illustrate the (B) M1 and (C) M2 macrophage proportions in the control, OGD and OGD + IPSC-MSC groups at the 4, 6, 12, 24 and 48 h time intervals (n=3). *P<0.05 vs. control group; #P<0.05 vs. OGD + IPSC-MSC group. OGD, oxygen and glucose deprivation; IPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells.

may improve the prognosis of neurological function following cardiac arrest. In the present study, the results demonstrated

that intervention with IPSC-MSCs affected the polarization of Raw 264.7 macrophages via the Notch-1/Hes1 signaling

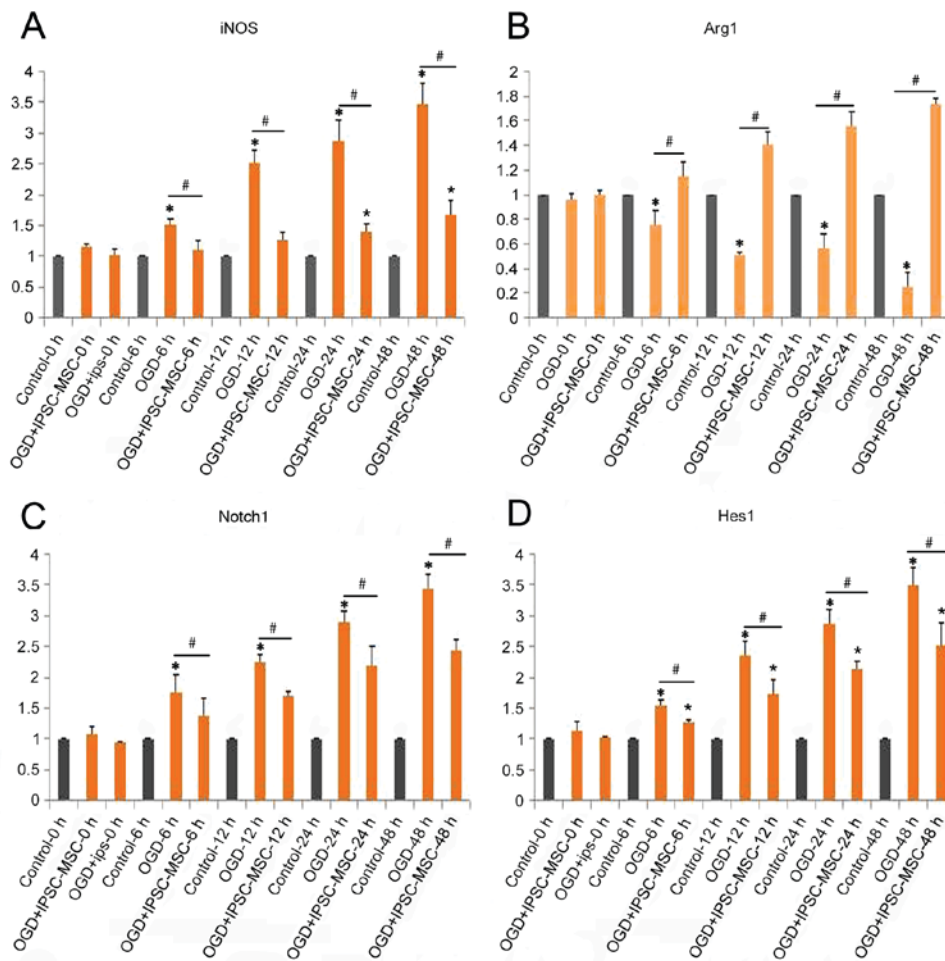


Figure 4. Reverse transcription-quantitative polymerase chain reaction analysis of the control, OGD and OGD + IPSC-MSC groups regarding the expression levels of (A) iNOS, (B) Arg1, (C) Notch1 and (D) Hes1. Each bar represents the mean \pm standard error of the mean (n=3). *P<0.05 vs. control group; #P<0.05 vs. OGD + IPSC-MSC group. OGD, oxygen and glucose deprivation; IPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; iNOS, inducible nitric oxide synthase; Arg1, arginase 1; Notch1, neurogenic locus notch homolog protein 1; Hes1, transcription factor HES-1.

pathway. However, additional experiments are required to support the present results.

In previous studies, IPSC-MSCs have been induced by an alteration in macrophage polarization direction to achieve a protective effect in inflammatory diseases (27-29). M2 macrophages serve an important role in combatting excessive inflammatory injury and tissue repair during pathogen infection (30). Following an injection of IPSC-MSCs, the expression of CD206⁺ (M2) macrophages was observed to increase in acute kidney-injured mice (31). IPSC-MSCs are able to reduce renal tubular injury, reduce interstitial fibrosis, and increase the CD206⁺/CD206⁻ proportion of macrophages in patients with unilateral fallopian tube obstruction caused by aseptic nephritis (32). IPSC-MSC intervention in a myocardial infarction model was confirmed to alter the direction of M2 macrophage polarization (33). In addition, IPSC-MSCs exert a protective effect on the phenotype and function of macrophages during severe infections. Krasnodembskaya *et al* (34) demonstrated that an injection of IPSC-MSCs may increase the proportion of CD206⁺ macrophages in the spleens of mice. A previous study demonstrated that mice lacking the Notch-recombining binding protein suppressor of hairless pathway in macrophages

produce lower levels of specific types of M1 macrophage and, therefore, exhibit a low-inflammation phenotype (35). These results indicated that IPSC-MSCs may regulate the surface molecules of macrophages to increase the levels of CD206⁺ macrophages, accompanied by the role of tissue repair and other protective effects (36-39). Therefore, it was hypothesized that IPSC-MSCs may alter the polarization of macrophages, which may be a central link in the immune regulatory network of brain resuscitation and improve outcomes following cardiac arrest.

Arg1, IL-10, iNOS and TNF- α expression levels confirmed that IPSC-MSCs may regulate the balance between inflammation and anti-inflammation, reduce the inflammatory responses of the body, inhibit inflammatory reactions, and induce phenotypic alterations and functions of macrophages (40,41). However, the mechanisms of how IPSC-MSCs alter the polarization of macrophages remain unclear.

Previous studies have determined that IPSC-MSC signaling pathways are involved in the differentiation of macrophages, including the c-Jun N-terminal kinase, phosphatidylinositol 3-kinase/RAC- α serine/threonine-protein kinase, Notch and tyrosine-protein kinase JAK/signal transducer and activator of transcription signaling pathways (42,43).

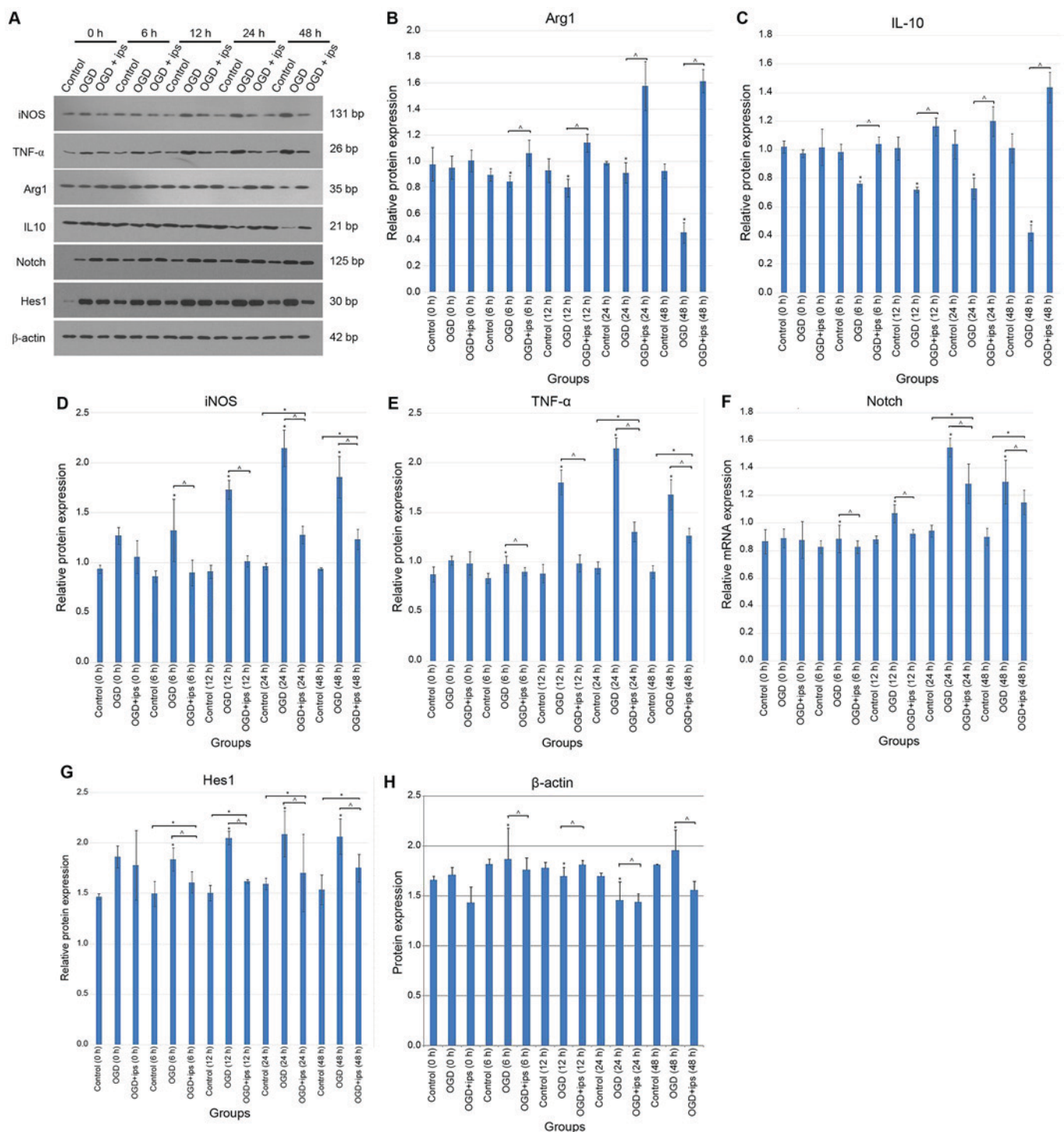


Figure 5. (A) Representative western blot analysis of the control, OGD and OGD + IPSC-MSC groups at the 0, 6, 12, 24 and 48 h time points. Quantification of the expression of (B) Arg1, (C) IL-10, (D) iNOS, (E) TNF- α (F) Notch-1 (G) Hes1 and (H) β -actin at 6, 12, 24 and 48 h time intervals for the corresponding control, OGD and OGD + IPSC-MSC groups. Each bar represents the mean \pm standard error of the mean (n=3). *P<0.05 vs. control; [#]P<0.05 vs. OGD + IPSC-MSC group. IL-10, interleukin 10; Notch-1, neurogenic locus notch homolog protein-1; Hes1, transcription factor HES-1; iNOS, inducible nitric oxide synthase; TNF- α , tumor necrosis factor- α ; Arg1, arginase 1; OGD, oxygen and glucose deprivation; IPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; OGD + ips, OGD + IPSC-MSC group.

The Notch gene encodes a highly conserved cell surface receptor that regulates the development of a variety of biological cells, ranging from sea urchins to humans. The Notch signaling pathway consists of the Notch receptor, delta-serrate-LAG-2 protein (Notch ligand), CSL protein, DNA binding proteins and other regulatory molecules. The Notch-1 signaling pathway is directly associated with

inflammation and anti-inflammatory reactions. Upregulation of the Notch-1 signaling pathway enhances the ability of macrophages to kill pathogens (44). During the activation of the Notch-1 signaling pathway, TNF- α and IL-6 expression levels increase, inhibiting IL-6, which causes Notch-1 levels to decrease and leads to a reduced inflammatory response. Therefore, Notch-1 may be an important signaling pathway

in the regulation of macrophage polarization and functional status (45). IPSC-MSC intervention may decrease the apoptosis of macrophages in the OGD model (46,47). The use of a Notch-1 receptor blocking agent or the disruption of a downstream signaling pathway may lead to a decrease in the expression of pro-inflammatory factors and an increase in the expression of anti-inflammatory factors, which cause M2 macrophages to bypass activation. The strength of the present study is that it demonstrated that intervention with IPSC-MSCs affected the polarization of Raw 264.7 macrophages via the Notch-1/Hes-1 pathway. However, there were limitations to the present study. A Notch inhibitor was not used and only macrophage polarization was observed. A grouping experiment for the dose of IPSCs-MSCs was not performed. Other channel-associated factors were not detected and, in future, *in vivo* experiments are required for functional verification.

In the present study, WB and RT-qPCR analyses confirmed that IPSC-MSC intervention affected the polarization of Raw 264.7 macrophages via the Notch-1 pathway. RT-qPCR analysis demonstrated a significant decrease in *Hes1* expression in the OGD + IPSC-MSCs group compared with the OGD group; and demonstrated a significant increase compared with the control group. Therefore, the results of the present study demonstrated that intervention with IPSC-MSCs may influence the polarization of Raw 264.7 macrophages via the Notch-1/*Hes1* signaling pathway.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XJ made substantial contributions to the conception and design of the present study. YY and DW interpreted the data. HL and YL gave final approval to the manuscript to be published and co-cultured the IPSC-MSCs and Raw 264.7 cells. ZX and JW performed RT-qPCR, western blot analysis, FCM and the CCK-8 assay.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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