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Intracerebroventricular injection of Wharton's jelly mesenchymal stem cells attenuates brain damage in rat model of hypoxia: optimization of vascular endothelial growth factor and downregulation of inflammatory factors

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Objective In this study, we investigated the effects of intracerebroventricular (ICV) Wharton's jelly mesenchymal stem cells (WJ-MSCs) injection in rat model of hypoxic brain injury by evaluating the amount of vascular endothelial growth factor (VEGF) and proinflammatory factors in hippocampus.
Methods About 24 rats were allocated to four groups of study: (1) Control and intact animals (Co), (2) sham group (Sh): animals were placed in the hypoxia chamber without inducing hypoxia and injected PBS, (3) hypoxia (H), (4) H + WJ-MSC. Hypoxia was induced by placing animals in the hypoxia chamber for 30 days (4 h a day). After 3 days of vehicle or WJ-MSCs injection, the rats were sacrificed and brain tissues were prepared for molecular and histopathological studies.
Results Despite a decrease in the gene expression of interleukin 1 beta, tumor necrosis factor-α, IL18, and the number of dark neurons in CA1 region of hippocampus in H + WJ-MSC groups compared to H ($P < 0.05$). There is an increase in all these factors in both H and H + WJ-MSC groups compared to CO and Sh groups ($P < 0.05$). The gene expression and protein concentration of VEGF increased in both H and H + WJ-MSC groups compared to Co and Sh groups ($P < 0.05$).
Conclusion Based on the findings, WJ-MSCs could reduce the number of dark neurons in hippocampus by increasing the VEGF synthesis and reducing inflammation in hypoxic condition.
Keywords hypoxia, wharton's jelly mesenchymal stem cells, proinflammatory factors, vascular endothelial growth factor, hippocampus, rat

Introduction

An environment with stable condition and high nutrient supply is required for neurons to have proper functions.[1] So, the well-established teamwork of the cells at the blood–brain barrier (BBB), first explained by Paul Ehrlich in 1885, is indispensable to form the barrier between central nervous system and peripheral environment.[2] In BBB, endothelial cells of line barrier vessels are supported by pericytes and vascular feet of astrocytes.[3]

The BBB disturbance is a mechanism defined in the pathogenesis of different neurodegenerative disorders.[4,5] Hypoxia is the reduction of oxygen that impaired the BBB *in vivo* and *in vitro*[6] as it caused the changes like vascular leakage and up-regulation of inflammatory factor and vascular endothelial growth factor (VEGF).[7] Oxidative stress has an important role in the extension of hypoxic–ischemic brain damage. Brain neurons for their qualified function require suitable environment, blood supply and homeostatic balance.[8] All of the brain injuries such as hypoxia, stroke, and ischemia are mediated by physiopathological events, which destroy BBB. Accordingly, enhanced reactive oxygen species (ROS) trigger inflammation by releasing proinflammatory factors including interleukin 1 beta (IL1β), tumor necrosis factor-α (TNFα) and IL18. During hypoxia, pericytes and astrocytes prevent disruption of BBB by keeping tight junction and preventing death of the endothelial cells.[9,10] Disruption of the tight junction, proteins lead to vasogenic edema.[11–13] VEGF is responsible for the vascular leakage and edema in the brain that occurs during hypoxia.[6]

In recent years mesenchymal stem cells (MSCs) have been used in cell therapy.[14–16] These cells can be cultured *in vitro* while retaining the potential to give rise to osteoblasts, chondrocytes, astrocytes, neurons and skeletal muscles.[17] Some evidences have indicated that stem cells do their therapeutic actions by secretion of some molecules like growth factors (GFs). Wharton's jelly MSCs (WJ-MSCs) could be effective in treating ischemic stroke[18] and hypoxia.[17] *In vitro*, MSCs release angiogenic factors like VEGF which can stimulate endothelial cells to migrate and proliferate.[19]

In this regard, we hypothesized that hypoxia induction might upregulate VEGF and inflammatory factors while single dose intracerebroventricular (ICV) injection of WJ-MSCs after 72 h as a therapeutic approach might optimize expression of VEGF and decrease inflammatory factors and dark neurons in the hypoxia rat model.

Materials and Methods

Animals

In this study all of the experiments have been approved by the Tehran University of Medical Sciences. About 24 male Wistar

albino rats, weighing 150–200 g were used in this study. Experiments were performed between 8 am and 12 am in 12 h period of light/dark, 23 ± °2C temperature and with free availability of food and water to the animals. All the steps were performed according to the instructions of Iranian Council and confirmed by the Ethical Committee of Tehran University of Medical Sciences.

Animal grouping

The animals were randomly divided into four groups as listed below:

1. Control group (Co): Intact rats without any intervention.
2. Sham group (Sh): Animals were kept in the chamber without induction of hypoxia but with ICV injection of PBS.
3. Hypoxic group (H): Animals were kept in the chamber with induction of hypoxia and ICV injection of PBS.
4. Hypoxia + injection of WJ-MSCs group (H + WJ-MSCs): Animals were kept in hypoxia chamber and 24 h after hypoxia, 10^5 WJ-MSCs were injected into both the cerebral ventricles using Hamilton syringe (AP = −0.9 mm, ML = −1.8 mm, and DV = 3.5 mm).

Induction of hypoxia

Hypoxia was induced by placing six rats in one hypoxia Plexiglass chamber (30 × 20 × 20) and revealing them to a gas mixture of 8% oxygen and 92% nitrogen for 4 h from 8 am to 12 pm for 30 days.[14] The gas composition was evaluated by an oximeter (lutron DO-5510 O_2 meter, Taipei, Taiwan). All experiments were designed to minimize animal suffering *in vivo* techniques.

Isolation and cultivation of WJ-MSCs

Umbilical cords were isolated by Explant method. Warton's jelly was chopped into small pieces (2 mm). Then, they were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) for a week and were supplemented with 15% fetal bovine serum (FBS, Gibco, USA), 1 µg/ml amphotericin B (Gibco, USA), 100 U/ml penicillin (Gibco, USA) and 100 µg/mg streptomycin (Gibco, USA). The culture medium was placed in a CO_2 (5%) incubator at 37°C. After 2 weeks, the pieces were removed, and the medium was renewed. This process was performed several times. After getting 90% confluence, WJ-MSCs were collected with 0.25% trypsin ethylenediaminetetraacetate (Gibco, USA) and the cells in the third passage were used for injection.

Flow cytometry

After isolation, WJ-MSCs were incubated for 20 min with fluorescein isothiocyanate-conjugated monoclonal antibodies against CD45 and CD90 (eBioscience, USA); then, the cells were suspended in PBS (Gibco, USA) to carry out FACS Calibur flow cytometry (BDBiosciences, USA) analysis.

RNA extraction and quantitative real-time PCR

To check the expression of VEGF, IL1β, TNFα, and IL18 genes in each group, tissues were examined using real-time PCR technique. First, the primers were designed as shown below, based on the reference sequences provided by the accession numbers of GenBank:

VEGFA: GTGACCTGAGGTTCTTTTCTGTT, IL18: ATGTCTACCCTCTCCTGT, TNFα: CACCACGCTCTTCTGTCT, IL1β: AGGCAGGGAGGGAAACACA and GAPDH: CCATGTTCTGATGGGTGTGAACCA.

Then, the entire RNA was extracted from the tissues and converted to cDNA. Then the cDNA was amplified by PCR method and examined for expression of the genes mentioned. This technique has four basic steps:

1. The total RNA from the cells was collected from each group.
2. Using the reverse transcription enzyme, the cDNA was converted.
3. The resultant cDNA was treated to remove genomic DNA with DNase I enzyme.
4. Repeated PCR in real time.

The qRT-PCR technique was used quantitatively to confirm the expression of the genes. For this purpose, the kinetics of the whole cell was first extracted using a solution of kyazol according to the synagen protocol, and exposed to DNase I (Fermentas) to ensure whether genomic DNA was contaminated. Then, the quality of the extracted RNAs was evaluated by spectrophotometry (DPI-1, Kiagen). Single-strand cDNA of Oligo dT primer (MWG-Biotech, Germany) and reverse transcription enzyme (Fermentas) were prepared based on the protocol. Each PCR reaction was performed using PCR master mix (Applied Biosystems) and SYBER Green in the ABI Step One (Applied Biosystems, Sequences Detection Systems, Foster City, CA), according to the manufacturer's protocol.

Western blotting

Lysis of hippocampus tissues was done by protein lysis buffer (Sigma, St. Louis, MO, USA). Protein concentration was determined by loading onto 8–15% sodium dodecyl sulfate polyacrylamide gels. Then these were transferred to membrane (PVDF) and incubated overnight at 4°C for binding of primary antibodies and 1 h for secondary antibodies to detect proteins (chemiluminescence reagent; Santa Cruz Biotechnology, Santa Cruz, USA).

Histopathological study

For histopathological study, the rats were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) (RaziCo, Iran), and perfused using 0.9% saline and then 4% paraformaldehyde (Sigma, USA). Hippocampus was dissected and maintained in 10% formalin, and the fixative was renewed after 12 h. The tissue samples were examined under light field microscope (Olympus, CX31, Tokyo, Japan) in ×400 magnification after Nissl staining for counting dark neurons in the CA1. Furthermore, in prepared images, the thickness of CA1 layer was measured using image processing and analysis program of ImageJ software version 1.32j (NIH, Bethesda, MD, USA).

Statistical analysis

Results were presented as mean ± SD. Data analysis was performed using one-way analysis of variance and the post-hoc Tukey's and Tamhane's T2 tests (SPSS-16 software) and $P < 0.05$ was considered significant.

Results

Flow cytometry analysis for immunophenotypic characteristics of WJ-MSCs

Based on the findings obtained from at least three independent samples, there was an up-regulation trend in the expression of CD90 (98.9%, Fig. 1a) and down-regulation trend in the expression of CD45 (1.32%, Fig. 1b) in the cultured WJ-MSCs (Fig. 1b).

Effects of ICV injection of WJ-MSCs on gene expression of proinflammatory factors of hippocampal region in hypoxic rats

A significant increase was observed in the gene expression of proinflammatory factors (including TNFα, IL1β and IL18) of hippocampus region in H and H + WJ-MSC groups in comparison to the Co and Sh groups ($P < 0.05$, Fig. 2a–c). Moreover, there was a significant decrease in the gene expression of proinflammatory factors in H + WJ-MSC group in comparison to H group ($P < 0.05$, Fig. 2a–c).

Effects of ICV injection of WJ-MSCs on gene expression and protein levels of VEGF of hippocampal region in hypoxic rats

The results of this study showed that there was a significant increase in the gene expression of VEGF in H and H + WJ-MSC groups compared to the Co and Sh groups ($P < 0.05$, Fig. 3a). Furthermore, there was a significant decrease in the gene expression of VEGF in H + WJ-MSC group compared to H group ($P < 0.05$, Fig. 3a). Based on the western blot analysis, a significant enhance was seen in protein levels of VEGF in H group compared with Co and Sh groups and also significant decrease in H + WJ-MSC group compared with H group ($P < 0.05$, Fig. 3b, c).

Effects of ICV injection of WJ-MSCs on the number of dark neurons of CA1 region of hippocampus in hypoxic rats

In H and H + WJ-MSC groups, the number of dark neurons and the thickness of CA1 layer was significantly increased in the CA1 region of hippocampus compared to Co and Sh groups ($P < 0.05$, Fig. 4a–c). In H + WJ-MSC group, the number of dark neurons and the thickness of CA1 layer in the hippocampus was significantly decreased compared to H group after one dose injection of WJ-MSCs ($P < 0.05$, Fig. 4a–c).

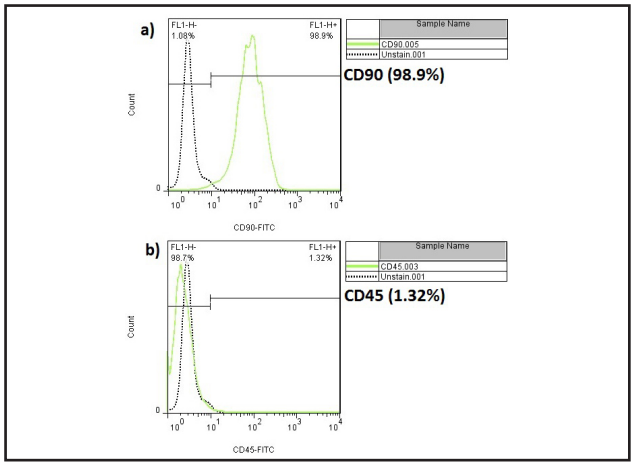

Fig. 1 **Evaluation of CD surface marker profiles of WJ-MSCs using flow cytometry. (a) Cultured WJ-MSCs were labelled with the indicated antibodies and analyzed by WJ-MSCs with down regulation trend in the expressions of CD45 and up regulation trend in the expressions of CD90 by flow cytometry assay.**

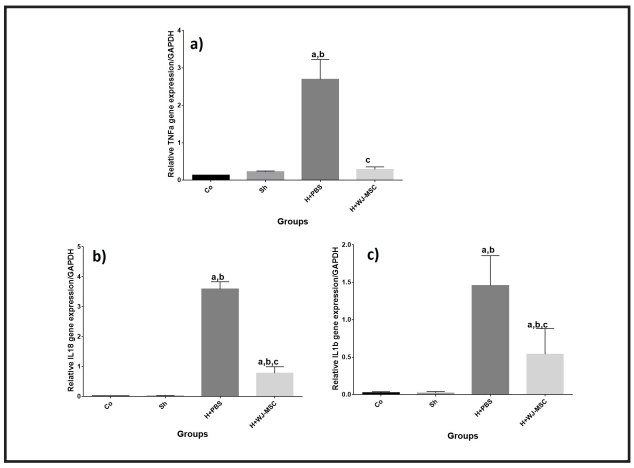

Fig. 2 **Effects of ICV injection of WJ-MSCs on gene expression of proinflammatory factors of hippocampal region in hypoxic rats. (a) TNFα, (b) IL1β and (c) IL18. a: $P < 0.05$ compared to Co group. b: $P < 0.05$ compared to Sh group, c: $P < 0.05$ compared to H group. Co: control group, Sh: Sham group, H: hypoxia group, H + WJ-MSC: hypoxia + injection of Wharton's jelly derived mesenchymal stem cells group.**

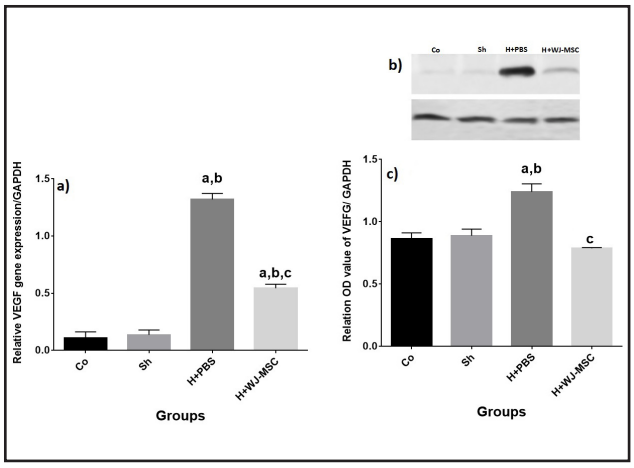

Fig. 3 **Effects of ICV injection of WJ-MSCs on gene expression of VEGF of hippocampal region in hypoxic rats. (a) Gene expression of VEGF in different group using real time PCR, b, c) protein concentration of VEGF in different group using western blot analysis. a: $P < 0.05$ compared to Co group. b: $P < 0.05$ compared to Sh group, c: $P < 0.05$ compared to H group. Co: control group, Sh: Sham group, H: hypoxia group, H + WJ-MSC: hypoxia + injection of Wharton's jelly derived mesenchymal stem cells group.**

Discussion

In the present study, we evaluated the effects of one dose ICV injection of WJ-MSCs in chronic hypoxia induced brain injury in rats. In hypoxia group, the number of dark neurons, the thickness of CA1 layer and the levels of inflammatory factor increased in the CA1 region of hippocampus following 30 day hypoxia through decreasing the oxygen supply of brain in animals using a hypoxic chamber which confirmed our model.

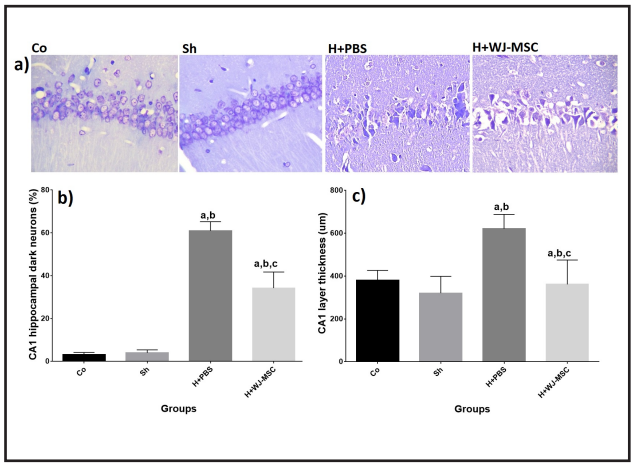

Fig. 4 **Effects of ICV injection of WJ-MSCs on the number of dark neurons of CA1 region of hippocampus in hypoxic rats. (a) Nissl staining showed the distribution of dark neurons in several groups (×400). (b) Comparing the number of dark neurons in different groups. a: $P < 0.05$ compared to Co group. b: $P < 0.05$ compared to Sh group, c: $P < 0.05$ compared to H group. Co: control group, Sh: Sham group, H: hypoxia group, H + WJ-MSC: hypoxia + injection of Wharton's jelly derived mesenchymal stem cells group.**

Dark neurons are defined as neurons with pyknotic, hyper-electron density and hyperbasophilia characteristics are recognized in the several pathological conditions such as traumatic brain injury, stroke and hypoxia.[16,20,21] Cognitive impairments are common in the brain injury due to hypoxia,[22–24] related to extending cell death in pyramidal neurons of hippocampus as a most vulnerable region to hypoxia.[25,26] It should be noticed that these neurons play an important role in learning and memory functions.[27] As well, increased the number of hypertrophic neurons induced by hypoxia increased the thickness of CA1 region. Inflammation is a critical factor in production of proinflammatory cytokines such as TNFα, IL1β and IL18 and, development of injury throughout the brain.[28,29] Lievre et al.[30] reported an increased in amount of ROS after transient hypoxia. According to the literature, it has been confirmed that leukocytes are activated and migrated into the injured area of brain and cytokines are secreted in high amounts within hours after injury in adult models of hypoxic–ischemic brain injury.[31–33] IL-1, IL-6, IL-10, TNF-α and transforming growth factor-β (TGF-β) are the most studied cytokines associated with the inflammatory responses following hypoxic–ischemic brain injury.[34] Hedtjärn et al.[28] demonstrated that hypoxic–ischemic brain injury markedly enhanced the following IL-18.[28] In ischemic stroke, T lymphocytes infiltrate into the brain and release cytotoxic molecules and proinflammatory chemokines which are responsible for early inflammation in the injured region of the brain.[35]

According to our results, VEGF increases in the hippocampus area following chronic hypoxic condition. Different studies have been performed to define the enhanced VEGF in hypoxia condition. Lennmyr et al.[36] confirmed the expression of VEGF following permanent and transient occlusion of the middle cerebral artery in the rat. Zhang et al.[37] stated that VEGF enhances angiogenesis and promotes BBB leakage in the brain of ischemic rats. Schoch et al.[6] has proved that hypoxia induced VEGF expression and this factor is responsible for vascular leakage and formed edema in the brain. Yeh et al.[38] investigated the effects of 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) against hypoxia induced BBB hyperpermeability in endothelial cell culture of adult rat brain. Their findings indicated that YC-1 may protect the BBB against hypoxia induced damage by inhibition of HIF-1alpha accumulation and VEGF production.[38] In contrast, Sun et al.[39] demonstrated that VEGF induced neurogenesis, neuroprotection and angiogenesis after focal cerebral ischemia. As well, within 24 h after hypoxic condition induced by ischemia, the endothelial cells of vessels around the injured regions begin to proliferate under the induction of several known proangiogenic mediators gene and protein expression, e.g. erythropoietin,[40] angiopoietins and Tie receptors,[40] VEGF[41] and TGF-β,[42] etc. It was suggested that angiogenesis mediated by VEGF may protect brain against reduced blood supply and restore blood stream and promote neurogenesis in the injured regions.[40]

In this study, we used one dose injection of WJ-MSCs to manage the hypoxia in animals. According to the results of this study, gene expression and protein concentration of VEGF optimized after hypoxia whereas, the gene expression of proinflammatory cytokines including TNFα, IL1β and IL18 decreased in the treated group compared to the hypoxic group.

Mesenchymal stem cells have been found to facilitate the regeneration of injured tissues by neurogenesis or releasing cytokines which reduce the inflammation[43] and enhance the protection of neurons against hypoxic condition. MSCs release bioactive agents, GFs and anti-apoptotic molecules with direct or endogenous mechanisms.[11] Munoz et al.[44] showed that implanting the bone marrow MSCs into the mouse hippocampus enhanced neurogenesis in this region. Nam et al.[45] investigated the effects of MSCs expression of matrix metalloproteinases and angiogenesis in the permanent model of rodent middle cerebral artery occlusion (MCAO). Decreased infarction volume and neurological repair were observed which relate to the increased vascular density after MSCs administration.[45] Quittet et al.[46] evaluated the effect of MSCs released VEGF in an ischemic stroke model of rat and declared that using these kind of cells to deliver GFs such as VEGF can reduce the impairment induced by stroke and could be introduced as an effective strategy to repair the brain injury following stroke. Zhou et al.[47] stated that of bone marrow MSCs which co-overexpress both BDNF and VEGF improved neuroprotection in a rat model of cardiac arrest-induced global cerebral ischemia. These differences between the results of various studies and our results can be defined by time-dependent manner of VEGF expression. The source of MSCs is important in which different studies used bone marrow MSCs in their investigations. As enhanced VEGF levels increase the permeability of BBB following hypoxia, it can be suggested that WJ-MSCs control the levels of VEGF in different pathologic conditions like hypoxia. The optimized levels of VEGF are required to increase angiogenesis, however, the mechanism of controlled and time dependent manner under the regulation of MSCs is unknown.

Immunosuppressive and anti-inflammatory effects of MSCs are proven in different studies. MSCs can release different inhibitory agents to suppress the inflammation such as indoleamine 2,3-dioxygenase, inducible nitric oxide synthase and TNF-α stimulating gene/protein 6 (TSG-6),

prostaglandin E2 (48). To show the anti-inflammatory effects of MSCs, Vendrame et al.[49] evaluated the impacts of human cord blood cells on a rat model of stroke. As well, Alizamir et al.[18] demonstrated that ICV injection of WJ-MSCs following MCAO reduced the number of dark neurons in cortical region through reducing Bax gene expression which agrees with the results of our study.

Conclusion

Based on the result of our present study, WJ-MSCs could protect the pyramidal neurons of hippocampus against hypoxic condition by optimizing the VEGF synthesis and reducing inflammation.

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Conflict of Interest

None.

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