

Associated functional motor recovery induced by Intracerebroventricular (ICV) microinjection of Wharton's jelly mesenchymal stem cells following brain ischemia/reperfusion injury in rat: Decreased dark neurons and Bax gene expression in the cerebral cortex

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Objectives Stroke is a situation caused activation of some events leading to neuronal damage and death. The proteins of Bcl-2-family are important in regulation of cell death and life. Bax, as a Bcl-2-interacting protein, is a member of this family which promotes apoptosis and cell death. Some therapeutic approaches have been introduced for the treatment of ischemic brain injury. Neuroprotection is one of the approaches for diminishing neurological deficits. We investigated the effects of intracerebroventricular (ICV) injection of Wharton's jelly mesenchymal stem cells (WJ-MSCs) on the cortex of the brain in ischemic rats.

Methods In this study, we occlude the middle cerebral artery (MCA) for induction of ischemic stroke in the brain. Rats were classified in four groups of Co, Sh, MCAo and MCAo + WJ-MSCs. Single dose of intraventricular microinjection of WJ-MSCs was injected by a Hamilton syringe. For detecting behavioral outcomes in the rats, Neurological examination was carried out. After 21 days, the animals were sacrificed and their brain tissues were removed for histopathological and molecular analysis.

Results ICV microinjection of WJ-MSCs significantly prevented apoptosis and cell death compared with MCAo group. A significant reduction in the level of Bax gene expression was observed in the MCAo + WJ-MSCs as group compared with Co, Sh and MCAo groups ($P < 0.05$). H&E staining showed considerable reduction of dark neurons in MCAo + WJ-MSCs group rather than Co, Sh and MCAo groups ($P < 0.05$).

Conclusions The results of the current study suggest that ICV microinjection of WJ-MSCs had neuroprotective effects on the brain cortex of ischemic rats by reduction of the Bax gene expression level and the number of dark neurons.

Keywords Wharton's jelly mesenchymal stem cells, Bax, dark neurons, cortex, stroke

Introduction

Ischemic stroke is a condition results from reduction of cerebral blood flow, which leads to reduction of oxygen and glucose levels in the involved regions.¹ This event causes severe disability and mortality in all over the world.² The procedure taking place in this situation, are related to some biochemical and molecular pathways.³⁻⁶ These events include excitotoxicity, inflammation, edema, and apoptosis.⁷ After stroke the neurons of cortex and hippocampus region is going to death.⁸ The occlusion of middle cerebral artery (MCA) is the most common approach to model brain ischemia/reperfusion (I/R) injury in animals mimicking human subjects.⁹

Mitochondria play a critical role in inducing apoptosis. Bcl-2 family proteins are one of the key regulators in the controlling the apoptosis via the mitochondrial pathway. These proteins effect the mitochondrial membrane permeability pore.¹⁰ Bax were reported as an accelerator of programmed cell death in 1993.¹¹ Several studies on animal models have detected the physiological roles of Bax gene and protein on nervous system.^{12,13}

Bax forms oligomers which directly effect on the release of some proteins such as cytochrome c from the mitochondrial.^{14,15} Bax function as a gateway to the intrinsic cell death, affect mitochondria by different mechanisms.^{16,17} As well, Bax

channel inhibitors prevent mitochondria activity to release cytochrome c and protect cells against apoptosis.¹⁸ It has been shown that the absence of Bax prevents more than 80% of the neuronal death after axotomy. Also, by knocking out of the Bax gene in the embryonic neurons derived from mice, an obvious resistance to cell death was seen which demonstrate a significant role for Bax in the neurons fate.^{19,20}

Stem cells are immature cells with self-renewal capacity. They can differentiate into variety of cell types of the body.²¹ Within the last years, the importance of stem cells application in cell therapy has begun to be determined.^{22,23} Also, some documents has indicated that stem cells do their therapeutic actions by secretion of some molecules, such as growth factors.²⁴ WJ-MSCs could be effective factors in targeting ischemic stroke.^{25,26}

Taking all, we focused about this hypothesis whether application a single dose of WJ-MSCs intraventricularly which injected after 24 hours of ischemia reperfusion, could decrease Bax gene expression and the dark neuron numbers in the rat model of MCAo as a therapeutic approach. Because the significance of cell regeneration in cortical neurons, we investigated this region. The examination carried out in this study include: 1) neurological examination, 2) gene expression of the pro-apoptotic protein, Bax, and 3) dark neurons in histological studies (H&E staining).

Materials and Methods

Animals

A total number of 32 male Wistar albino rats (270–300 g, 12-week-old) were purchased from the Pharmacology department of Tehran University of Medical Sciences, Tehran, Iran. Animals could have a good accessory to food and water, 12 h periods of light/darkness and $23 \pm 2^\circ\text{C}$ temperature. All steps of examination were carried out according to the instruction of Iranian Council and confirmed by the Ethical Committee of Tehran University of Medical Sciences.

Occlusion of MCA

In order to induction of cerebral ischemia in the left hemisphere, we occluded the left MCA. Briefly, animals were anesthetized with ketamine/xylazine (RaziCo, Iran), and the rat's neck was dissected for ligation of common carotid (proximal part) and external carotid arteries. In order to expose the left common carotid artery (CCA), we applied a midline neck incision. Then, CCA was isolated from its adjacent nerve, vagus. Then, according to find its bifurcation, we dissected this artery. After finding the internal carotid artery, an intraluminal 4–0 nylon monofilament (Doccol Corporation, USA) was passed from common and internal carotid arteries to go to the MCA, then this monofilament was removed after 60 min. This procedure induced brain ischemia.²⁷ One day after the induction of ischemia, WJ-MSCs (1×10^6) were injected cerebroventricularly. For analyzing gene expression and histological studies, the left cortex was dissected and removed at 21th day in both fresh and fixative form.

Animal Grouping

Rats were randomly allocated to the following groups:

Control group (Co): The normal rats without any procedure

Sham group (Sh): Sham-operated rats with ligation of left common carotid

MCAo group: Rats were subjected to occlusion for 1 h followed by 24 h reperfusion

MCAo + WJ-MSCs: Rats were subjected to occlusion for 1 h and a single dose ICV of WJ-MSCs (1×10^6) was administered 24 after reperfusion. It was injected into the left cerebral ventricle by using Hamilton syringe (bregma: AP = -0.9 mm, ML = -1.8 mm (midline), and DV = 3.5 mm deep from the dura).²⁸

Neurologic Examination

The neurologic examination was performed for each rat 24 h after procedure according to Bederson et al. study.²⁹ This neurological assessment included a grading System of 0–3. In this system, the flexion of forelimb contralateral to the injured hemisphere after suspending each rat by the tail and circling behavior of rats toward the paretic side were the criteria for evaluation. Rats with normal neurological function extend both forelimbs toward the floor. Grade 0 (Normal): there is no observable deficit; grade 1 (Moderate): we could see forelimb flexion; grade 2 (Severe): we can see decrease in resistance to lateral push and the flexion of forelimb

Table 1. Neurologic examination grading system

Normal	Grade 0	no observable deficit
Moderate	Grade 1	forelimb flexion
	Grade 2	decreased resistance to lateral push and forelimb flexion without circling
Severe	Grade 3	same behavior as grade 2, with circling

Table 2. Primer Sequence and length: reverse (R); forward (F); base pair (bp)

Primers	Sequence	Length (bp)
Bax	R: CAG CCA CAA AGA TGG TCA	18
	F: GCA AAC TGG TGC TCA AGG	18
GAPDH	R: GTGCCGTTGAATTTGCCGTG	20
	F: GGAGAGTGTTCTCTCGTCCC	20

without circling; grade 3 (Severe): all features taking place in grade 2 besides circling (Table 1).

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 (DMEM, Gibco, USA) for a week and supplemented with 15% fetal bovine serum (FBS, Gibco, USA), 1 $\mu\text{g}/\text{ml}$ amphotericin B (Gibco, USA), 100 U/ml penicillin (Gibco, USA) and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco, USA). The culture medium placed in a CO_2 (5%) incubator at 37°C . After two weeks, the pieces were removed, and medium was renewed. This process was carried out for several times. After achieving 90% confluence, WJ-MSCs were collected with 0.25% trypsin ethylenediaminetetraacetate (EDTA) (Gibco, USA) and the cells in third passage were used for injection.

Flow Cytometry

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

RNA Extraction and Quantitative Real-time PCR (qRT-PCR)

Expression of Bax gene was carried out by quantitative real-time PCR. All of the RNA was collected from cortex by Using TRIzol® reagent (Qiagen, Germany). By using the Kit for reverse transcription (First Strand cDNA Synthesis Kit, Fermentas, USA), mRNA (1 μg) converted to cDNA. Specific primer was placed on the three-color real-time PCR machine (Applied Biosystems Step One, USA) for further analysis. At first, incubation of samples was carried out at 95°C for 15 min for initial polymerase activation. In the next step, the samples tolerated the three subsequent phases: denaturation, at 94°C for 20 s; annealing, at $58\text{--}60^\circ\text{C}$ for 30 s; and elongation, at 72°C for 30 s. At last, $\Delta\Delta\text{Ct}$ technique was used for relative

quantification of data and further normalization to GAPDH and fold change for MCAo + WJ-MSCs group comparison to the control, sham and MCAo groups. The nucleotide sequences of Bax and GAPDH primer is listed in Table 2.

Histopathological Study

For the light microscopy study, the rats were anesthetized with ketamine/xylazine (RaziCo, Iran), and perfused by 0.9% saline and 4% paraformaldehyde (PFA, sigma), respectively. The brains were dissected and cut into the sections with 3–5 mm thickness. Then the sections were post-fixed in 10% formalin 72 h at 4°C. In order to light microscopy analysis, the samples were embedded in paraffin and 5 µm coronal sections were prepared by using a rotary microtome (Leica Biosystems, Milan, Italy). One section from each five section was selected and the tissue sections stained with Hematoxylin and eosin (H&E). Afterward, graded alcohol (70, 80, 90, and 100% [2 times]) was used to dehydrate the sections. Finally, they were mounted in Canada balsam and prepared for analysis. Study and survey of sections were performed by using a light field microscope (Olympus, CX31, Tokyo, Japan). In cortex field, The intact and ischemic cells considered as dark neurons, were counted in the ×400 images by using a connected camera to the microscope.³⁰

Statistical Analysis

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

Results

Morphologic Features And Flow Cytometry Analysis For Immunophenotypic Characteristics of WJ-MSCs

Based on the findings, after two weeks of expansion in medium, the cells had features of the fibroblastic spindle-like cells with up to 90% confluency (Fig. 1a). There was a down-regulation trend in the expressions of CD45 and upward trend in the expression of CD90 for WJ-MSCs. Findings are representative of at least three independent samples (Fig. 1b).

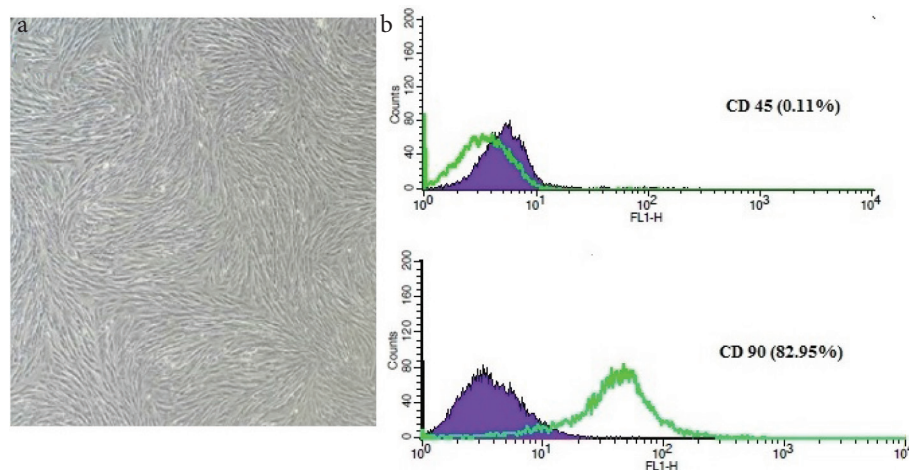


Fig. 1 Evaluation of morphology and CD surface marker profile of WJ-MSCs. (a) Fibroblastic-like morphology of WJ-MSCs. (b) Cultured WJ-MSCs were labelled with the indicated antibodies and analyzed by flow cytometry. WJ-MSCs with down regulation trend in the expressions of CD45 and up regulation trend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

Effects of WJ-MSCs ICV Injection on the Functional Motor Recovery in Rats with MCAo

According to the Figure 2, there was a significant difference in the mean grade of neurological score. Considerable decrease was recorded in the mean grade of neurological score in MCAo group compared to Co and Sh groups ($P < 0.05$, Fig. 2). Also, There was remarkably increase in the mean grade of neurological score in MCAo+ WJ-MSCs group in comparison to MCAo groups ($P < 0.05$, Fig. 2).

Effects of WJ-MSCs ICV Injection on the Bax Gene Expression in Cerebral Cortex of Rat Brains with MCAo

QRT-PCR was performed to analyze Bax gene expression. Significant differences were considered in the expression level of Bax gene. Based on turkey test, this signification was between MCAo + WJ-MSCs and MCAo ($P < 0.05$, Fig. 3).

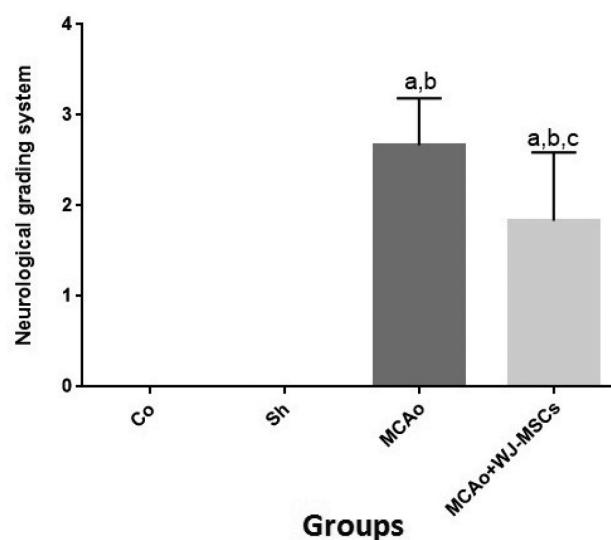


Fig. 2 Effects of WJ-MSCs ICV injection on the functional motor recovery in rats with MCAo. Bars represent means ± SE; (a) $P < 0.05$ as compared to Co group; (b) $P < 0.05$ as compared to Sh groups, (c) $P < 0.05$ as compared to MCAo group. Co: normal group with PBS ICV injection, Sh: sham-operated group with PBS ICV injection; MCAo: Ischemia induction group with PBS ICV injection, MCAo + WJ-MSCs: Ischemia induction group with WJ-MSCs ICV injection.

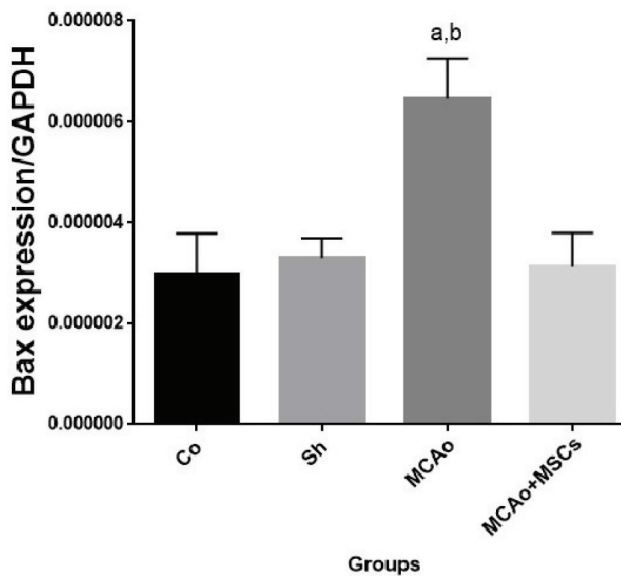


Fig. 3 Effects of WJ-MSCs ICV injection on the Bax gene expression in cerebral cortex of rat brains with MCAo. Bars represent means \pm SE; (a) $P < 0.05$ as compared to Co group; (b) $P < 0.05$ as compared to Sh groups, (c) $P < 0.05$ as compared to MCAo group. Co: normal group with PBS ICV injection, Sh: sham-operated group with PBS ICV injection; MCAo: Ischemia induction group with PBS ICV injection, MCAo + WJ-MSCs: Ischemia induction group with WJ-MSCs ICV injection.

Also, significant differences were observed between MCAo + WJ-MSCs and Co ($P < 0.05$, Fig. 3) and as well Sh groups ($P < 0.05$, Fig. 3).

Effects of WJ-MSCs ICV Injection on the Percentage of Dark Neurons in Cerebral Cortex of Rat Brains with MCAo

H&E staining showed the extensive neuronal death (dark neurons: being shrunken with an enhanced affinity for histologic dyes) in the cortex of ischemic rats (Fig. 4a). The percentage of dark neurons was determined in the prepared sections and compared in the studied groups. Based on the results, there were significant differences in the percentage of dark neurons among the groups ($P < 0.05$, Fig. 4b). According to the Tukey test, the significant differences were seen in the percentage of dark neurons of MCAo and MCAo + WJ-MSCs groups compared to Co and Sh groups ($P < 0.05$, Fig. 4b).

The correlation Between Quantitative Data Including Bax Gene Expression, the Percentage of Dark Neurons and Neurological Grading System

There was a significant correlation between quantitative data based on bivariate correlation test; Bax gene expression and percentage of dark neurons ($r = 0.827$, $P = 0.0001$, Fig. 5a), Bax gene expression and neurological grading System ($r = 0.653$, $P = 0.004$, Fig. 5b) and the percentage of dark neurons and neurological grading System ($r = 0.91$, $P = 0.0001$, Fig. 5c).

Discussion

In this study, the effects of WJ-MSCs injection on the treatment and recovery of ischemic rat brains was performed. In this way,

the occlusion of MCA was carried out due to experimental evaluations. According to the result of study, the great number of cell death (dark neurons) recorded in the cerebral cortex which confirmed our model. The occlusion of MCA, as a model of stroke, is widely used in order to analyze the mechanisms involved in ischemic stroke.^{31,32} Cerebral ischemia starts up some physiologic and biochemical events.³³ Despite the advances in clinical management, stroke is a major therapeutic challenges and considered as the second cause of death in the worldwide, and increasing investigations now suggest that cell transplantation could considerably enhance recovery.^{34,35}

The findings of present study showed that the expression of Bax increased in the MCAo group. Some investigations carried out on animal models; suggest that the apoptosis pathway which mediated by mitochondria, is involved in stroke and associated with cell death. One of this pathway is triggered by Bax activity.¹⁸ Bax activity causes cell death, but neutralized by binding of Bcl-2.³⁶ Also, some studies found that knocking out of Bax, cause decrease ischemic insults and neurological deficits.^{16,17} Moreover Krajewski et al. found that within 0.5 to 3 hours after ischemia, there is significant increase in Bax expression within neurons in different regions of the brain.³⁷

According to the results, a single dose ICV of WJ-MSCs (1×10^6) was administered 24 after reperfusion. Based on neurological assessments, we observed an improvement in the functional outcome of treated animals. Moreover, the amount of dark neurons in the cortical region decreased in this group that was strongly related to reduction of Bax gene expression and the number of dark neurons in the ischemic region in the WJ-MSCs groups in comparison with MCAo group. Thus, WJ-MSCs transplantation has an anti-apoptotic activity and can improve the neurological function.

As a therapeutic agent, stem cells or their derivatives within can transplant into the adult brain for treatment of brain diseases.²¹ By developing in regenerative medicine field, it seems that application of stem cells in the treatment of ischemic diseases, is a good effective approach.³⁸ Stem cells release growth factors, cytokines and chemokines into their surroundings and cause cell regeneration.³⁹ Different sources of stem cells have been tested for treatment of stroke. Between them, WJ-MSCs are good candidates for their immunomodulatory and neuroprotective features.^{25,40} Treatment with WJ-MSCs on ischemic stroke models can survive and release suitable neurotransmitters in addition to restore some lost functions.⁴¹⁻⁴⁷ Cali6 et al. demonstrated that transplantation of bone marrow-MSCs reduced the cell apoptosis in the hippocampus at the stroke model. Their finding was confirmed by high level of anti-apoptotic Bcl-2 gene expression in the treated group.⁴⁸

Li et al. investigated the effects of adipose-derived MSCs transplantation and found that this source decreased the cerebral ischemic/reperfusion injury through the suppressing apoptosis. They showed that there is an increased ratio of Bax/Bcl-2 at the protein level of ischemic region.⁴⁹ Additionally, the association of reduced I/R injury and regulation of some apoptotic markers levels such as Bax and Bcl-2 after MSCs therapy have been proven in a study by Chen et al. (2011).⁵⁰

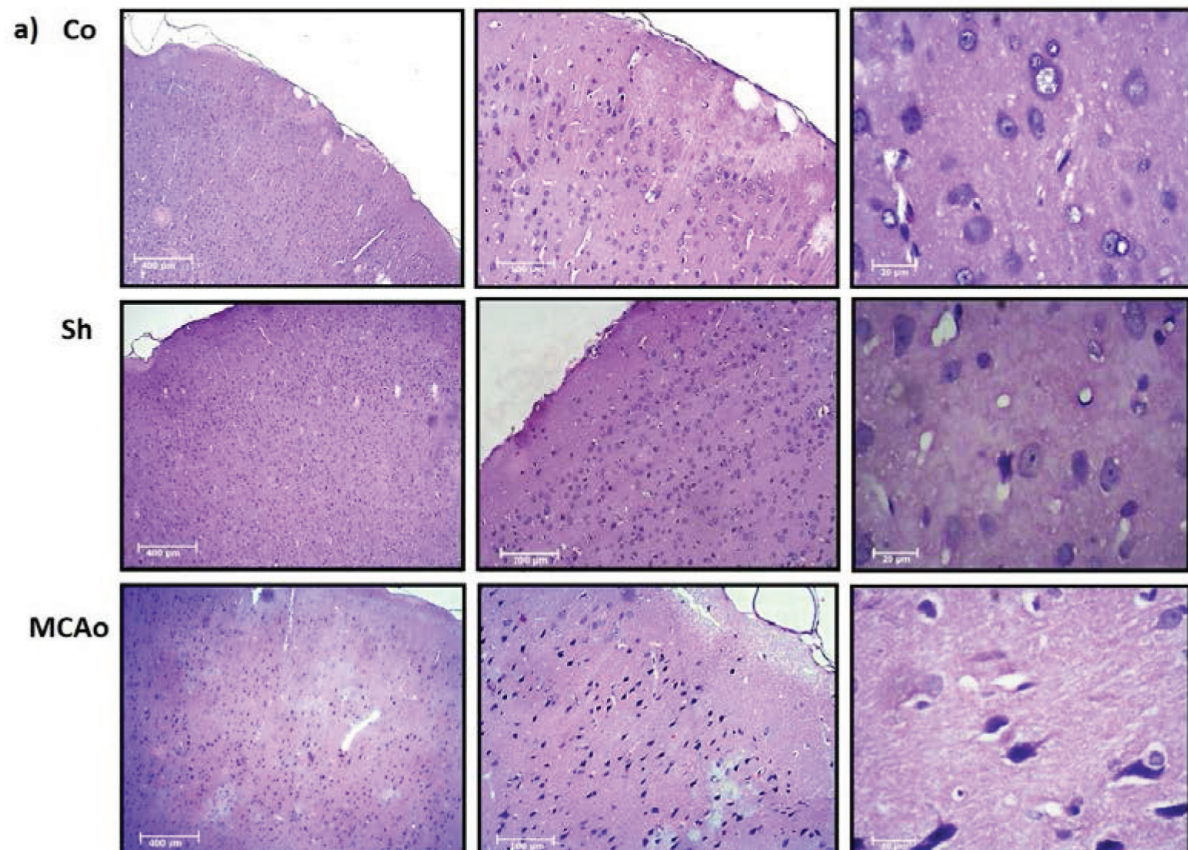


Fig. 4 Effects of ICV injection of WJ-MSCs on the percentage of dark neurons in cerebral cortex of rats with MCAo. Bars represent means \pm SE; (a) $P < 0.05$ as compared to Co group; (b) $P < 0.05$ as compared to Sh groups, (c) $P < 0.05$ as compared to MCAo group. Co: normal group with PBS ICV injection, Sh: sham-operated group with PBS ICV injection; MCAo: Ischemia induction group with PBS ICV injection, MCAo + WJ-MSCs: Ischemia induction group with WJ-MSCs ICV injection. (H&E staining, magnifications: $\times 40$, $\times 100$ & $\times 400$).

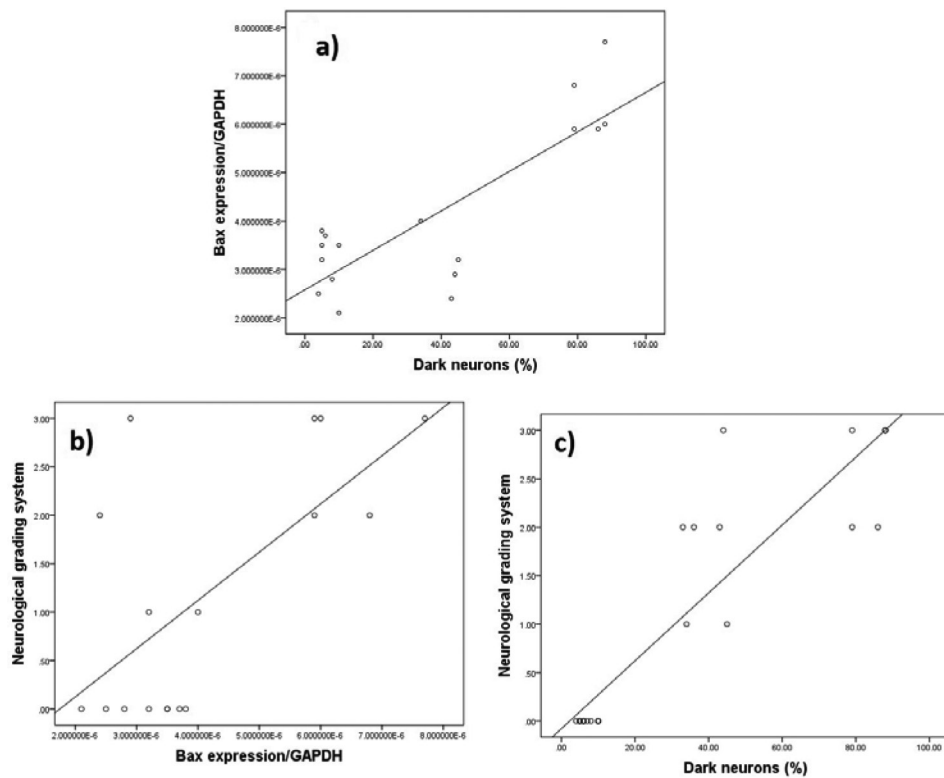


Fig. 5 The correlation between quantitative data: (a) Bax gene expression and percentage of dark neurons ($r = 0.827$, $P = 0.0001$), (b) Bax gene expression and neurological grading system ($r = 0.653$, $P = 0.004$) and (c) the percentage of dark neurons and neurological grading system ($r = 0.91$, $P = 0.0001$).

Conclusion

It seems that the injection of WJ-MSCs could be a good candidate for treatment, improvement and recovery of Brain ischemia via effecting on the pro-apoptotic gene expression level, Bax, and the number of dark neurons.

Conflict of Interest

All authors declare no conflicts of interest. ■

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