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Original article

The protective effect of bone marrow mesenchymal stem cells in a rat model of ischemic stroke via reducing the C-Jun N-terminal kinase expression

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ABSTRACT

Ischemic stroke is the main cause of disability and mortality worldwide. Apoptosis and inflammation have an important role in ischemic brain injury. Mesenchymal stem cells (MSCs) have protective effects on stroke treatment due to anti-inflammatory properties. The inhibition of the C-Jun N-terminal kinase (JNK) pathway may be one of the molecular mechanisms of the neuroprotective effect of MSCs in ischemic brain injury.

Twenty-eight male Wistar rats were divided randomly into 3 groups. Except the sham group, others subjected to transient middle cerebral artery occlusion (tMCAO). Bone marrow MSCs or saline were injected 3 h after tMCAO. Sensorimotor behavioral tests were performed 24 and 72 h after ischemia and reperfusion (I/R). The rats were sacrificed 72 h after I/R and infarct volume was measured by TTC staining. The number of apoptotic neurons and astrocytes in the peri-infarct area was assessed by TUNEL assay. The morphology of cells was checked by Nissl staining, and the expression of p-JNK was detected by immunohistochemistry and Western blot.

Behavioral scores were improved and infarct volume was reduced by MSCs 24 h and 72 h after tMCAO. TUNEL assay showed that neuronal apoptosis and astroglial activity in the penumbra region were reduced by MSCs. Also, Nissl staining showed lower neuronal apoptosis in BMSCs-treated rats compared to controls. JNK phosphorylation which was profoundly induced by ischemia was significantly decreased after MSCs treatment.

We concluded that anti-apoptotic and anti-inflammatory effects of MSCs therapy after brain ischemia may be associated with the down-regulation of p-JNK.

1. Introduction

Ischemic stroke is a major cause of long-term disability and mortality, worldwide [1]. This crippling event result from a sudden drop in brain blood flow by an embolus or a thrombus and accounts for approximately 87% of all strokes [2]. Immediately after ischemic stroke, a cascade of molecular events including excitotoxicity, increased levels of intracellular calcium, oxidative stress and inflammation is initiated that ultimately leads to apoptotic or necrotic neuronal cells death [3]. The inflammatory process is characterized by the activation of resident immune cells such as microglia and astrocytes, increasing vascular permeability, reducing the integrity of the blood-brain barrier and infiltration of peripheral immune cells including neutrophils, macrophages and T lymphocyte into the injured brain area. [4]. The inhibition of brain inflammation reduces infarct size and improves neurological function [5,6]. The blockade of apoptosis attenuates cerebral ischemic injury [7]. The primary goal of neuroprotective interventions is the protection of neurons in the surrounding area of the

Abbreviations: AP-1, Activator protein 1; Apaf-1, Apoptotic protease activating factor 1; ATF, Activating transcription factor; BDNF, Brain-derived neurotrophic factor; BMSCs, Bone marrow mesenchymal stem cells; BSA, Bovine serum albumin; CBF, Cerebral blood flow; CCA, Common carotid artery; CNS, Central nervous system; DAB, Diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; ERK, Extracellular signal-regulated kinase; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; GFAP, Glial fibrillary acidic protein; HRP, Horseradish peroxidase; ICA, Internal carotid artery; IHC, Immunohistochemistry; JNK, C-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; MCAO, Middle cerebral artery occlusion; NBF, Neutral-buffered Formalin; PBS, Phosphate-buffered saline; PE, Phycoerythrin; PI3K/Akt, Phosphatidylinositol-3-kinase/ protein kinase B, Transducer and activator of transcription 1; TNF-α, Tumor necrosis factors alpha; TPA, Tissue plasminogen activator; TTC, Triphenyltetrazolium chloride; TUNEL, Terminal deoxynucleotidyl-transferase -mediated dUTP-biotin nick-end labeling

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ischemic region [8]. There are a lot of studies about the pathophysiology, etiology, and treatment of ischemic stroke [9–11], but the range of therapeutic interventions has remained very limited [12]. Currently, tissue plasminogen activator (tPA) is the only approved pharmacological intervention with significant benefits in acute ischemic stroke. However, it is only effective in the first 4.5 h after the onset of the brain ischemia [13].

Cell therapy is known as a promising strategy in the treatment of many diseases, including. Stroke [14–16]. Mesenchymal stem cells (MSCs) are among the cells that are widely considered for their characteristics such as availability, rapidly expandable, and lack of ethical and immunological problems in allogeneic transplantation [17]. Several studies have shown the beneficial effects of MSCs transplantation in the treatment of cerebral ischemic stroke [18,19]. The mechanisms involved in MSCs–mediated therapeutic effects in cerebral ischemia are the ability to migrate to injured tissues, trans-differentiation into the neural lineage, and the production of trophic factors and cytokines by MSCs [20] suggesting the importance of paracrine signaling by MSCs [21,22]. Recent research has indicated that the transplantation of bone marrow mesenchymal stem cells (BMSCs) reduces the infarct size and improves functional outcome following a brain stroke. Despite the protective effects, the exact molecular mechanisms are unknown. Modulation of the immune system and reduction of the inflammatory response by MSCs prevents secondary damage after stroke. Also, apoptosis of neural cells after ischemic stroke can be suppressed by these cells [23–25].

The c-Jun N-terminal kinase (JNK) cascade, also known as the stress-activated signaling pathway, is a mitogen-activated protein kinase (MAPK) that is activated in response to a wide variety of stresses, including pro-inflammatory cytokines such as TNF-α [26], interleukin 1-beta [27] and many forms of environmental stress [28]. The activation of JNK leads to cell death via inflammation and apoptosis in many cell types [29]. Studies revealed that activation of JNK is a major factor in neuronal apoptosis triggered by focal and global ischemia [30]. Therefore, JNK signaling might have a role in the regulation of brain inflammation which is followed by brain I/R injury progression [30–32]. It was reported that inhibition of JNK activation may be neuroprotective by suppression of inflammation in glial cells or apoptosis in neurons or both [33].

In this study, we examined the neuroprotective effects of BMSCs transplantation against cerebral I/R injury in a rat model of focal ischemia. We also investigated the possible role of the JNK signaling pathway for MSCs effects.

2. Materials and methods

2.1. Animals and experimental groups

Adult male Wister rats weighing 230–280 g (8–10 weeks old) were maintained in a 12 h light/dark cycle at the temperature (21 ± 2) ˚C and allowed free access to food and water. All experimental protocols were approved by the Kashan University of Medical Sciences Ethical Committee by letter No. 3303, Date: 24.9.2014 and were carried out in accordance with the Directive 2010/63/EU on the protection of animals used for Scientific Purposes. A total of 28 rats were used in this study. Animals were randomly divided into 3 groups: 1. Sham-operated group (N = 8), 2. Vehicle-treated I/R group or saline group (N = 10) and 3. BMSCs-treated I/R group or MSCs group (N = 10). Rats received a single injection of MSCs 3 h after tMCAO via the tail vein and were sacrificed 72 h after I/R. The vehicle-treated group received an equal volume of saline in the same manner of MSCs treatment.

2.2. Induction of transient focal cerebral ischemia

Transient focal cerebral ischemia was conducted with the use of the intraluminal filament technique as described previously [34,35] with some minor modifications. Briefly, the rats were anesthetized by 3% isoflurane (Baxter, USA) and maintained with 2% in an oxygen/air mixture using isoflurane vaporizer (Eickemeiyer, Germany). During the experiment, body temperature was kept at 37.0 ± 0.5 ˚C using a heating pad (NARCO Bio-systems, USA). Laser Doppler flowmetry (Moor Instruments, U.K) was used to assure a successful occlusion and reperfusion of the middle cerebral artery (MCA). Laser–Doppler measuring sensors (P10d) were positioned and fixed on both hemisphere of skull surface, 1–2 mm posterior and 4–5 mm lateral to the bregma in MCA territory for detecting cerebral blood flow (CBF). A midline skin incision in the neck was performed. The left common carotid artery (CCA) was exposed and the vagus nerve was protected. Then silicon-coated monofilament (Doccol, USA) was gently inserted into the lumen of CCA and forwarded to the internal carotid artery (ICA). When about 22 mm of filament entered into the arteries, a significant drop (> 60%) occurred in CBF. At.this time, the entrance to the MCA was blocked. The filament was fixed for 60 min and CBF was monitored at intervals every 10 min. After 1 h of occlusion, the filament was pulled out and CCA was blocked, but the MCA was reperfused from Willis loop and blood flow was returned to the near of initial CBF. Then, the incision was closed and rats were killed 72 h after reperfusion for histological analysis and TTC staining. Sham-operated animals were subjected to the same anesthesia and surgical procedures but the catheter was not inserted into the CCA. The rats with more than 65% reduction in CBF were included in the study. On the other hand, the rat showed a sudden CBF reduction in both hemisphere, as a sign of hemorrhage, were excluded from the experiment.

2.3. Isolation and characterization of rat BMSCs

BMSCs were obtained from the femur and tibia bones marrow of male Wister rats by flushing method. First, the rats were deeply anesthetized with 10% chloral hydrate (3 mg/kg, intraperitoneal) and decapitated. The femur and tibia bones were removed under sterile conditions. Then, bone marrow was flushed out into a 50 ml falcon tube using a syringe containing cell culture media. The cells were expanded in a 75 cm^2 cell culture flask (SPL, Korea) by Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) that supplemented with 10% fetal bovine serum (Invitrogen, USA) and penicillin/streptomycin (Invitrogen, USA) and placed in an incubator (Memmert, Germany) with 5% CO_2, and 80% humidity at 37 ˚C temperature. After 24 h, media and non-adherent cells were discarded and replaced with fresh medium. Thus, BMSCs were isolated based on the capability of adherent to plastic cell culture flasks. When the confluence of the cells reached 80%, they were removed by trypsin-EDTA (0.25%) and expanded in two or more 75-cm^2 flasks. After three passages, cells were analyzed for surface CD markers by flowcytometry. Also, the differentiation capacity of extracted cells was examined for confirming that the cells are MSCs. The expanded cells in passage three were subjected to a specific differentiation media for differentiating to osteoblasts and adipocytes. Adipogenic differentiation medium consists of low glucose DMEM (Invitrogen, USA) supplemented with FBS and penicillin/streptomycin (same as growth media), plus 50 µg/ml ascorbic acid- 2-phosphate, 100 nM dexamethasone and 50 µg/ml indomethacin (all from Sigma, Germany) and Osteogenic differentiation medium consist of growth media addition with 10 mM glycerol-2-phosphate, 50 µg/ml ascorbic acid- 2-phosphate and 100 nM dexamethasone (all from Sigma-Aldrich). This medium was changed every 3 days. After 21 days, cells were stained with oil red and alizarin red dyes to identify adipocytes and osteoblasts, respectively. BMSCs were used for transplantation in passages 3-5. A single dose of 1×10^6 BMSCs in 1 ml saline was injected into the tail vein of rats in the cell therapy group. The rats in the control group received 1 ml saline 3 h after tMCAO.

2.4. Flow cytometry analysis

Flow cytometry analysis has been used to confirm the presence or absence of the six markers. Cells from the third passage were removed by trypsin and cell suspensions were washed twice in PBS supplemented with 0.5% (v/v) bovine serum albumin (BSA). Each one of antibodies including: PE-conjugated anti-CD34 antibody (QBEnd10; Invitrogen, Carlsbad, CA), FITC-conjugated anti-CD73 (SH-3) antibody (CiniSciences, Nanterre, France), anti-CD44 antibody, anti-CD45 antibody, anti-CD90 antibody, and PE-conjugated anti-CD105 (SH-2) antibody (Every four of them from Abcam, Cambridge, UK), were added to a suspension of 10^6 cells/ml PBS separately, and incubated for 30 min at 4 °C and protected from light. Finally, the cells were washed and analyzed by a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and results were investigated by Flowjo 7.6.1 software.

2.5. Behavioral testing

Garsia behavioral tests with minor modifications were performed 24 and 72 h after I/R as previously described [35]. Four motor tests including spontaneous activity, walking, forepaw outstretching and climbing and two sensory tests including body and head proprioception were done in all of the animals by two blinded examiner. The total scores of the tests (between 3and 18) were compared in all of animals groups.

2.6. Cerebral infarction volume

TTC staining technique was performed to determine the infarct size. Animals were deeply anesthetized with 10% chloral hydrate (400 mg/kg, intraperitoneal) and decapitated 72 h after I/R. Brains were removed rapidly and placed in a brain matrix (Zivic Instruments, USA) and cut into 2-mm thick coronal sections. Afterward, the brain sections were stained by triphenyl tetrazolium chloride (TTC) 1% in PBS (W/V) at 37° for 10 min. TTC was reduced to tri-phenyl formazan in the vital cells and its deposition causes red color but the dead cells in infarcted tissue remain pale. Then photograph was taken from tissue sections by a digital camera (Canon, Japan). Infarct area was assessed by image analysis software (Image J version 1.44p, USA). Infarcted areas of all sections were measured (mm^2), data summed and then multiplied by the distance between the sections (2 mm) in order to get the total infarction volume [4].

2.7. Western blot analysis

For western blot analysis, animals were sacrificed and their brains were removed quickly. The tissue of ischemic penumbra was dissected from 3 groups and lysed in 800 µl radioimmunoprecipitation assay buffer (RIPA buffer). An equal amount of proteins were separated in 10% SDS polyacrylamide gel. After electrophoresis, the proteins on the gel were transferred on a PVDF membrane (Roche, Germany) using a semidry transfer apparatus and blocked with 0.2% BSA in PBS for 1 h. Blots were probed with primary antibodies against beta-actin (ab8226, Abcam, 1:5.000) and pJNK (sc-6254, Santa Cruz Biotechnology, 1:200) overnight at 4 °C and subsequently incubated with secondary antibodies conjugated with horseradish peroxidase (anti-rabbit, Abcam, 1:3.000 and anti-mouse, Santa Cruz Biotechnology, 1:4.000) for 1 h. Finally, labeled proteins were observed with the ECL detection system (Biorad, USA). pJNK band density was normalized to the corresponding beta-actin band as an internal control. Protein bands were quantified using densitometry software (Image J version 1.44, USA).

2.8. Tissue preparation and immunohistochemistry

Animals were deeply anesthetized by intraperitoneal injection of 10% chloral hydrate (mg/kg,) and perfused through the left ventricle of the heart with saline and then fixed with 10% neutral-buffered formalin (NBF) (pH = 7) at 72 h after reperfusion. The fixed brains were carefully removed from the skull, sectioned into 4 mm thick slices and kept in the same fixative solution for 48 h post-fixation at room temperature. After tissue processing, brains were embedded in paraffin, 5 µm- thick coronal sections were prepared using a microtome (Diapath, Italy) and placed on silane-coated slides. Then, sections were deparaffinized, rehydrated and used for immunohistochemistry, TUNEL, and Nissl staining.

For immunohistochemistry, rehydrated sections were immersed in an antigen retrieval solution (pre-heated 0.1 M citrate buffer (pH = 6) for 20 min. Non-specific antibody binding was blocked with a superblock solution from IHC kit (UltraTek HRP-Anti Polyvalent, Scytek, USA) for 7 min at room temperature. In the next step, slides were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used for this study: mouse monoclonal anti-pJNK (sc-6254, Santa Cruz Biotechnology, USA) at 1:50 dilution, rabbit polyclonal anti-GFAP (32608, Encore, USA) at 1:1000 dilution. Then, sections were incubated in 10% H_2O_2 in methanol for 10 min to quench endogenous peroxidase activity. After several washing steps, sections were treated with biotin-conjugated secondary antibodies and subsequently with a streptavidin-peroxidase conjugate solution for 10 min. The color reaction was visualized using diaminobenzidine (DAB). For negative controls, slides underwent *identical preparation* except for the primary antibodies.

2.9. TUNEL staining

Neuronal apoptosis was delineated by using a TUNEL kit (Roche, Germany). TUNEL staining was performed according to the manufacturer's protocol. Briefly, after deparaffinization and rehydration, sections were boiled in 0.1 M citrate buffer in a microwave (pH = 6) for 10 min. After rinsing with PBS, the TUNEL reaction mixture was added on samples for 60 min in a 37 °C incubator. Samples were rinsed with PBS before they were treated with Converter-POD solution for 30 min at room temperature. DAB was used for the coloration of apoptotic cells.

2.10. Nissl staining

Initially 5 µm coronal sections were prepared for Nissl staining. The sections were placed on the slides and paraffin was removed in xylene and hydrated in ethanol. After rinsing by tap water and distilled water, they were stained with 0.1% cresyl violet (Merck, Germany) for 3-10 min, and then slides were dried and mounted. Intact neurons contained large oval nuclei while the degenerated neurons showed shrunken and pyknotic nuclei. Counts of intact-degenerating neurons were performed in two sections per animal. All IHC-, TUNEL- and Nissl-stained sections were mounted and analyzed with an inverted microscope (Nikon Eclipse Ti-U, Japan).

2.11. Statistical analysis

All data are presented as means ± SEM and analyzed with SPSS software (version 22.0, USA). For comparison, the data related to CBF repeated measurement ANOVA was used. The behavioral data were analyzed by Kruskal–Wallis test. All of the other comparisons were performed by one-way ANOVA and *post-hoc* Tukey's test. Differences were considered to be statistically significant if P value was < 0.05.

3. Results

3.1. Isolation and characterization of BMSCs

BMSCs showed a characteristic fibroblast-like cell morphology (Fig1A). The capability of bone marrow extracted cells for differentiation into osteoblasts and adipocytes were showed by culturing of the

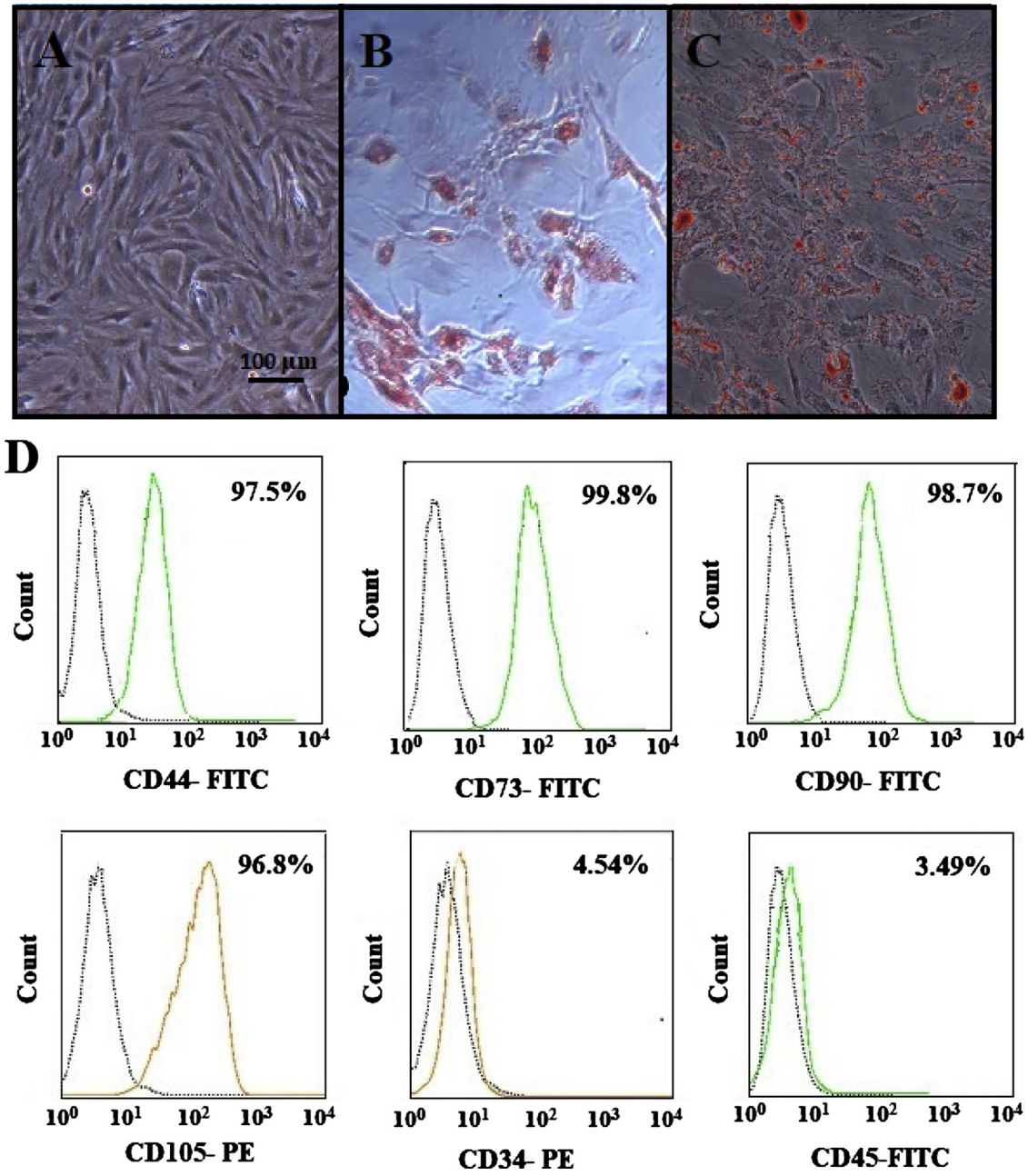

Fig. 1. MSCs identification: Morphology of bone marrow extracted cells after 3 passage (A). Potential differentiation of MSCs to bone and fat cells (B &C). Flowcytometry analysis: MSCs expressed markers CD90, CD73, CD44, and CD105 and were negative for the hematopoietic cell surface molecules CD45 and CD34 (D). Sample stained with FITC Conjugated Antibodies (green), PE conjugated Antibodies (Red), Negative Control (Black).

cells in the specific media were checked. After three weeks extra-cellular calcium depositions were showed in osteogenic media by alizarin red staining and fat vacuole were seen in adipogenic media by oil red staining (Fig1B and C). Before transplantation, the BMSCs were analyzed for cell surface markers at passage 3. Flow cytometry analysis confirmed that BMSCs were positive for the surface antigens CD44 (97.5%), CD73 (99.8), CD 90 (98.7%), and CD105 (96.8%) which are typically expressed in a variety of stem cells and had low expression for hematopoietic cell surface antigens CD45 (2.62%) and CD34 (4.52%) (Fig. 1D).

3.2. Infarct volume and behavioral scores

The infarct volume depends mainly on the blood perfusion and the time of ischemia, but secondary factors, such as oxidative stress, inflammation, and apoptosis expanded it. As expected, no infarct area was seen in the sham group but it was clearly found in the saline group. BMSCs significantly decreased the infarct volume 72 h after I/R ($P = 0.022$).

Behavioral exam indicated that ischemic stroke significantly decreased sensory and motor test scores 24 and 72 h after I/R ($P = 0.001$) but BMSCs transplantation significantly improved behavioral test 24 h ($P = 0.042$) and 72 h ($P = 0.016$) after I/R in compared to saline groups (Fig. 2).

3.3. MSCs therapy decreased cell death after ischemic stroke

To investigate the effect of BMSCs transplantation on apoptosis, TUNEL staining was done 72 h after I/R. There were almost no TUNEL positive cells in the sham group and contralateral hemisphere of saline

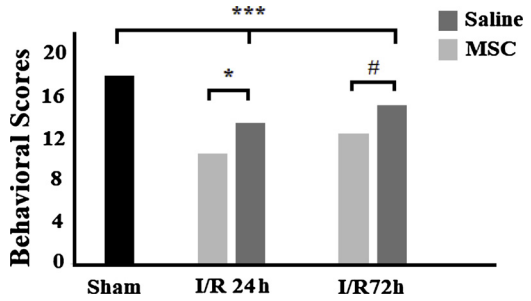

Fig. 2. Ischemic stroke significantly decreased behavioral scores (***$P < 0.001$). BMSCs improved the neurological deficits and behavioral scores significantly increased 24 h (*$P = 0.042$) and 72 h ($^{\#}P = 0.015$) after I/R in compared to the saline group.

group animals. After tMCAO, the number of TUNEL positive cells massively increased but treatment with BMSCs prevented this induction of apoptosis and reduced TUNEL positive cells ($P = 0.025$) (Figs. 3B and D).

3.4. MSCs transplantation reduced astroglial reactivity

GFAP is the principal intermediate filament protein of mature astrocytes and serves as a hallmark for reactive astrogliosis after nervous tissue injury [36]. In the sham group, most GFAP + astrocytes had thin processes 72 h after surgery (Fig. 4C), while in the control tMCAO group, GFAP + astrocytes appeared hypertrophic with thickened processes (Figs. 4A, B). The number of reactive GFAP + astrocytes was significantly reduced within the boundary zone of ischemia in the BMSCs-treated group compared with a control group 72 h after I/R reflecting a reduction in ischemia-induced astrogliosis after BMSCs treatment ($p < 0.001$) (Fig. 4D). Our data suggest a novel role of BMSCs in the modulating inflammatory responses associated with cerebral ischemia.

3.5. Morphological analysis of ischemic neuronal damage

The degree of neuronal damage was evaluated by Nissl staining. In the sham group, neurons appeared unaffected and showed round and pale-stained nuclei (Fig. 5C). In contrast, many neurons in the penumbra region of the tMCAO control group showed an aberrant morphology with shrunken cell bodies, chromosome condensation and nuclear pyknosis (Fig. 5A). Treatment with BMSCs reduced the number of degenerating neurons and significantly preserved the intact structure of neurons ($P < 0.001$) (Fig. 5B and D).

3.6. MSCs reduced JNK activation after ischemia

To confirm whether the JNK pathway is activated in the brain after focal ischemia, western blotting was performed with an antibody specific for the phosphorylated active form of JNK. Moreover, the p-JNK positive cells were stained by immunohistochemistry and quantified in the penumbra. I/R injury significantly induced activation of pJNK (Fig. 6B and D) compared to the sham group (Fig. 6F). Western blot analysis revealed approx.30% shorter protein levels of pJNK in the MSCs group compared to the tMCAO control group (Fig. 6A). Western blot results were qualitatively approved by immunohistochemistry of pJNK in control and MSCs groups (Fig. 6B–E). The administration of BMSCs significantly reduced the numbers of JNK-positive cells compared to the saline (Figs. 6C and E, $P = 0.011$).

4. Discussion

In this study, we attempted to elucidate possible mechanisms underlying the neuroprotective effects of BMSCs in an animal model of focal cerebral ischemia. The main observation of our study is that intravenously transplanted BMSCs have the capability to modulate the local inflammatory response and to decrease apoptosis in the ischemic brain most likely through inhibition of the JNK signaling cascade. tMCAO is a well-characterized stroke model inducing ischemia-related neurodegeneration in rodents [37] and thus, allows for studying potential therapeutic compounds and related molecular mechanisms. Besides the many site-specific pathological processes occurring immediately after and with a distinct time delay in the hypoxic brain area, neuroinflammation and apoptosis appear to be the major destructive events that mainly contribute to its pathogenic progression [38]. Despite the development of various therapeutic measures mainly arising from animal studies, stroke still lacks an ideal brain-suited and effective treatment [32]. MSCs have been proposed to have beneficial effects in animal models of focal cerebral ischemia [18,19]. However, the mechanisms underlying the observed reduction of the infarct volume and improvement of functional deficits are not understood.

As expected, our findings are consistent with previous reports which have shown that the administration of MSCs can reduce the infarct volume and improve the functional deficits after transient focal cerebral ischemia [25,39–41]. Apoptosis plays a decisive role in mediating neuronal death after cerebral ischemia [42] and the penumbra region is particularly vulnerable to apoptosis in the early follow-up period (hours) after the onset of reduced blood supply. Therefore, the

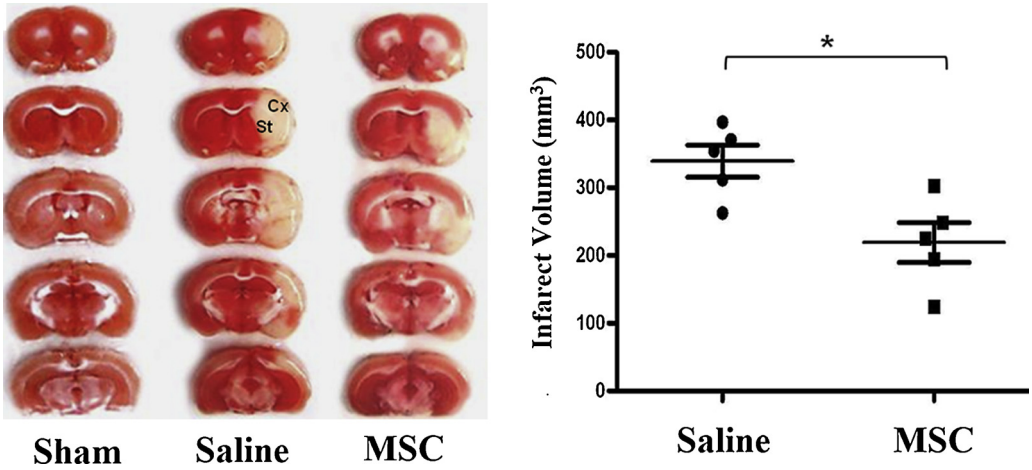

Fig. 3. Infarct Volume: TTC-stained brain slices showed a massive infarct area (white zone) 72 h after I/R. Treatment with BMSCs reduced the infarct volume. Quantitative analysis of the cerebral infarct volume indicated a significant decrease by MSCs 72 h after I/R (*$P = 0.022$). St: striatum, Cx: cortex.

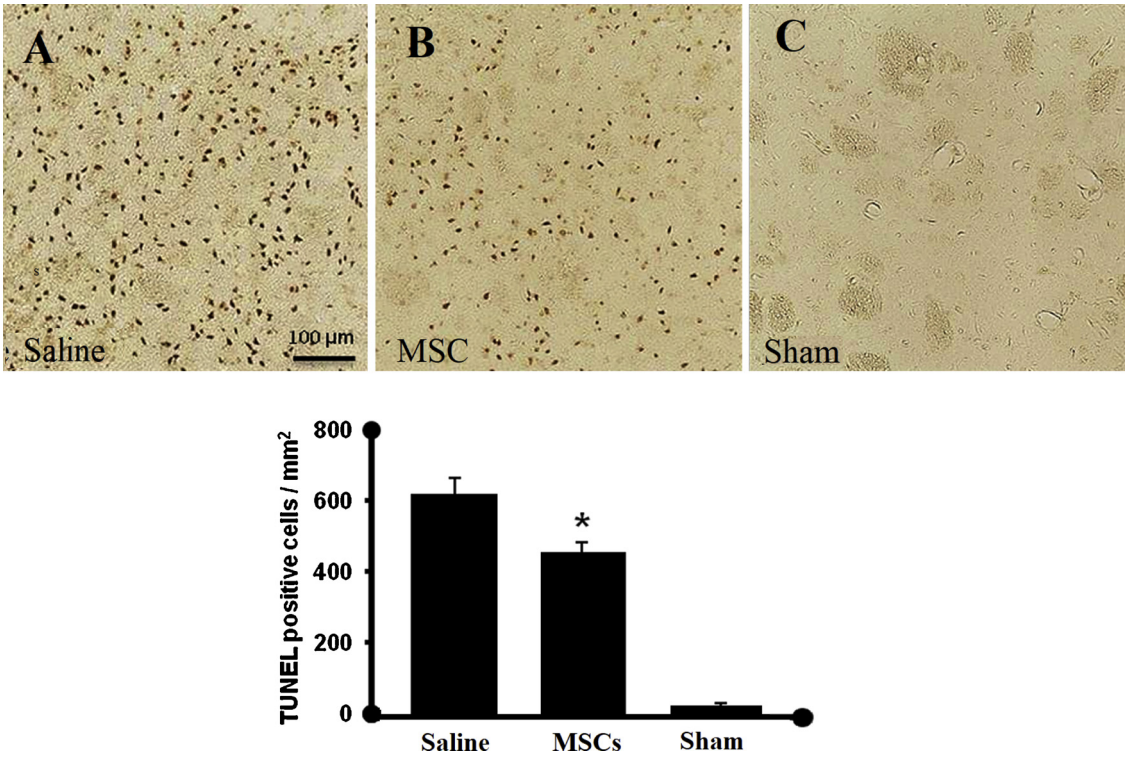

Fig. 4. Effects of MSCs on apoptosis: TUNEL staining showed an increased number of apoptotic cells in the striatum in the saline group. BMSCs significantly decreased the number of TUNEL-positive cells. (*$P = 0.025$).

attenuation of apoptosis in this brain region is one of the prime goals of therapeutic interventions [43]. Previous studies have demonstrated a protective role of MSCs transplantation against neuronal apoptosis in both permanent and transient focal ischemia models [44,45]. Here, we additionally report that BMSCs protect neurons against an ischemic insult. Evidence of this protection is that BMSCs notably increased the neuronal surviving- and decreased apoptosis rate. Isele et al. showed that BMSCs secrete a combination of multiple growth factors and cytokines that activate endogenous survival signaling pathways such as PI3K/Akt and the MAPK/ERK1/2 cascade [46]. This is supported by findings from another disease and animal model where MSCs provoke anti-apoptotic effects after myocardial infarction. This protective effect

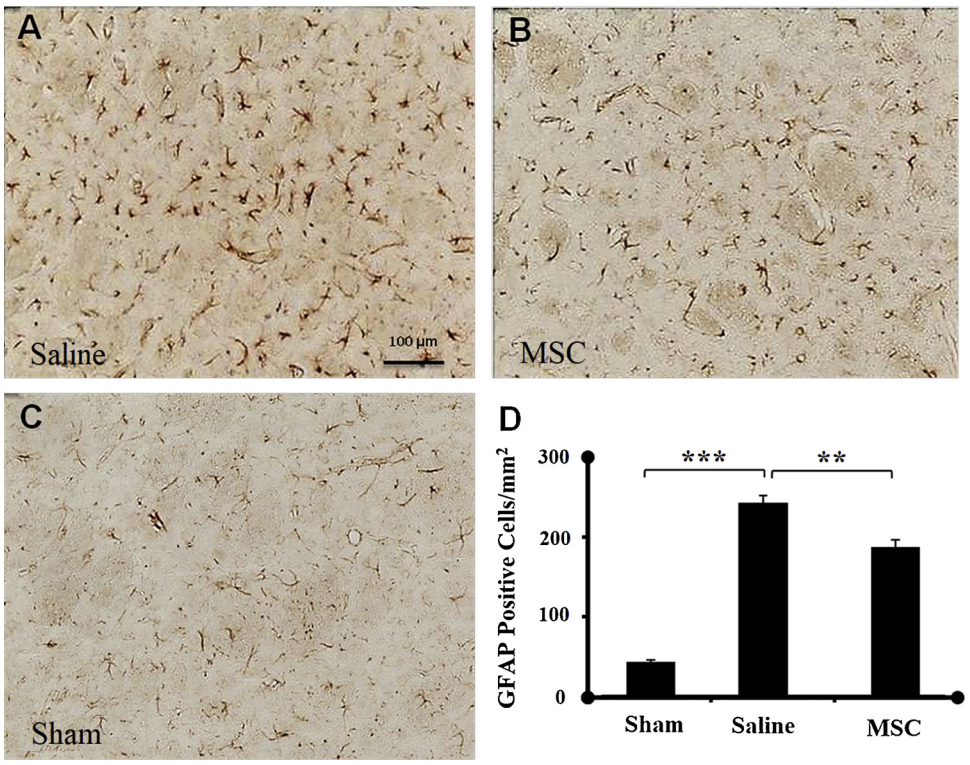

Fig. 5. MSCs therapy and astrocyte. Astrocytes are visualized by anti-GFAP staining. Ischemia significantly increased the number of reactive GFAP-positive cells in the striatum at 72 h after I/R in compared to the sham group (A, C) (***$P < 0.001$). BMSCs significantly reduced the number of GFAP-positive cells (**$P = 0.002$). The comparison of GFAP- positive cells in the ischemic penumbra was done as a number of cells/mm^2 (B, D).

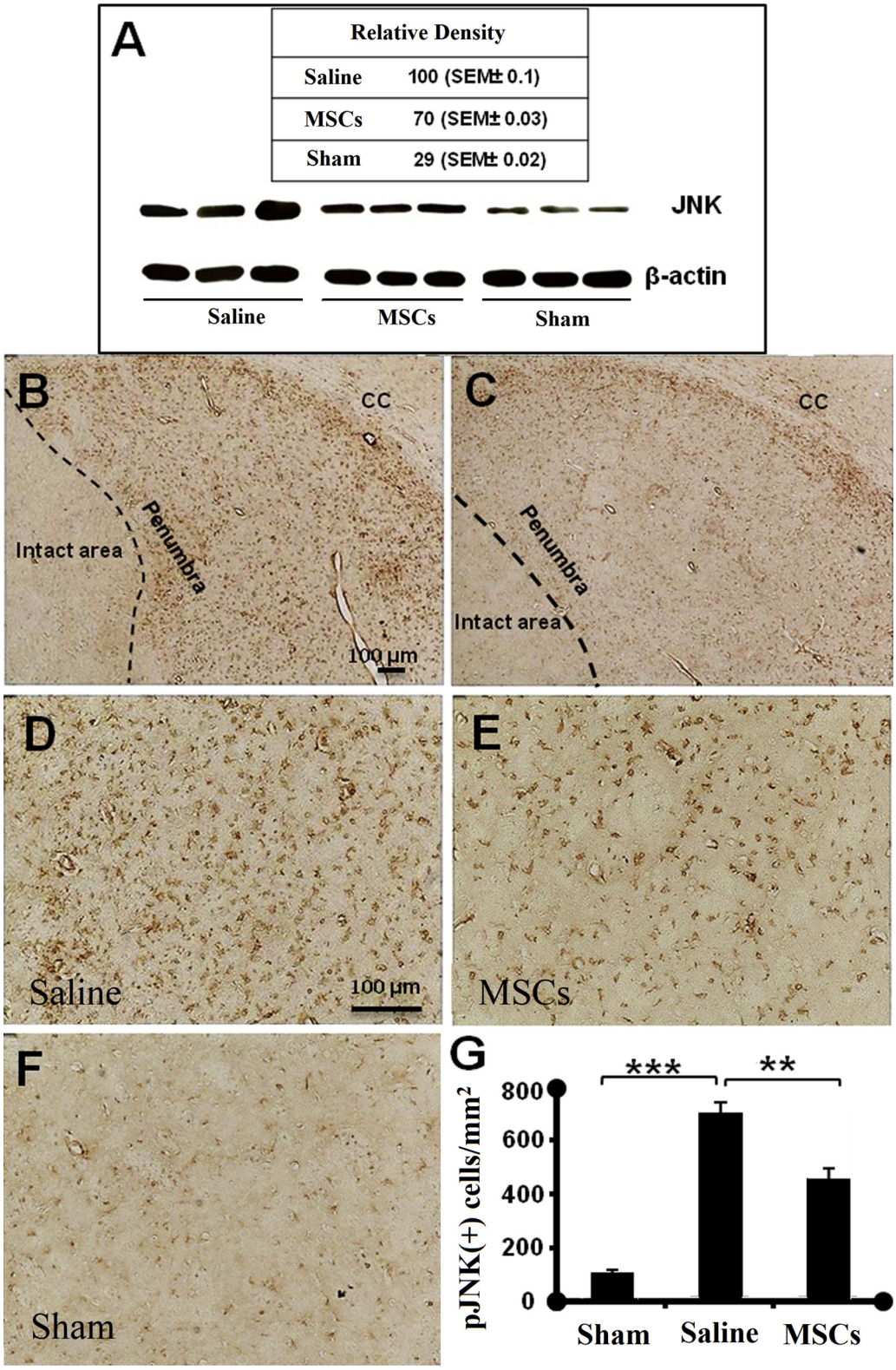

Fig. 6. Effects of BMSCs on JNK activation. (A) Western Blots result indicated a decreased expression of p-JNK in the MSCs group compared with the saline group 72 h after I/R. Immunohistochemistry results showed a lower expression of p-JNK in the penumbra region of the striatum in BMSCs treated group (C) compared to the saline group (B) 72 h after I/R (*P = 0.011) (D–E). CC: corpus callosum.

appeared to be mediated through the stimulation of mitogen-activated protein kinases pathways, including JNK, extracellular signal-regulated kinase (ERK) and p38 [47,48].

The astrocytes were activated by ischemia and converted to reactive astrocytes which have been identified to play a role in neurogenesis. [49]. Astroglial reactivity in the peri-infarct region is also an interesting and potential goal for therapeutic interventions [40]. In this study, we indicated a decreased astroglial reactivity in the peri-infarct zone of BMSCs-treated animals compared to the control group. These results are in line with a previous report by Pavlichenko et al [50] who

observed the widest glial scar in the control group and moderate glial scar in the MSCs therapy group in a rat stroke model. Another possible mechanism has been proposed by Huang et al. who showed that paracrine factors secreted by MSCs can promote astrocyte survival and down-regulate GFAP expression via the suppression of p38 MAPK and JNK [51]. Therefore, the anti-inflammatory properties of BMSCs might be a major component of its beneficial effects against I/R injury [52].

To further pinpoint putative molecular mechanisms which are involved in BMSCs-dependent neuroprotection, we investigated the JNK signaling pathway that plays a major role in ischemia-induced neuroinflammation and cell death [33]. A previous study showed the role of JNK signaling pathways in apoptosis of Jurkat T cell, which was induced by adipogenic MSCs [53]. Although the MSCs immunomodulatory effects are well known, various molecular mechanisms are mentioned that need further investigation.

In this study, the administration of MSCs clearly inhibited the increase in pJNK-expressing cells compared to tMCAO. The JNK has been demonstrated as a crucial mediator in triggering neuronal apoptosis following ischemia [30,54] and phosphorylation of the downstream molecules like c-Jun involves in its pro-apoptotic function [55]. Activation of the JNK signaling results to cell death via not only intrinsic/extrinsic apoptotic pathways, but also pro-inflammatory cytokine production [30]. The JNK phosphorylation also appears to be linked to astrocyte function/dysfunction in the penumbra after ischemia [56]. Therefore, The JNK activity seems to be important for both the immune response and apoptosis. Thus, the blockade and attenuation of JNK activation could be one pivotal mechanism against cerebral ischemia-induced brain damage [31,57] and could prevent astroglial reactivity, neuronal apoptosis [51,58].

In conclusion, the present data support the view that the administration of BMSCs in focal brain ischemia-induced animals could be neuroprotective. Taken together, our results suggested that anti-inflammatory and anti-apoptotic properties of MSCs are considerably contributed to its protective effects *via* targeting JNK pathway. These findings revealed, the JNK pathway may be introduced as an important molecular mechanism in the ischemic stroke injury.

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