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ORIGINAL ARTICLE

A Novel Cell Therapy Method for Recovering after Brain Stroke in Rats

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Background: Nowadays, stroke leads to a significant part of the adult mortality and morbidity and also it could result in some neurological deficits in the patients' lives. Cell therapy has opened a new approach to treat the brain ischemia and reduce its terrible effects on the patients' lives. There are several articles which show that the cell therapy could be beneficial for treating brain stroke. In this study, we have planned to present a new cell therapy method for stroke by administration of Mesenchymal stem cells and differentiated neural stem cells without astrocytes.
Method and Materials: The Mesenchymal stem cells were isolated from tibia and femur of a 250～300 g rat and they were cultured in DMEM/F12, 10% fetal bovine serum, 1% Pen/Strep. Neural stem cells were isolated from 14 days rat embryo ganglion eminence and were cultured in NSA media containing Neurobasal, 2% B27, bFGF 10 ng/ml and EGF 20 ng/ml after 5 days they formed some neurospheres. The isolated neural stem cells were differentiated to neural lineages by adding 5% fetal bovine serum to their culture media. After 48 hours the astrocytes were depleted by using MACS kit.
Results: The group that received Mesenchymal stem cells systemically and differentiated neural stem cells without astrocytes had the best neurological outcomes and the least infarct volume and apoptosis. It could be understood that this cell therapy method might cause almost full recovery after brain stoke.
Conclusion: Using combination cell therapy with Mesenchymal stem cells and differentiated neural stem cells with removed astrocyte could provide a novel method for curing brain stroke.

Keywords: Neural stem cells, Mesenchymal stem cells, Brain stroke, Astrocyte

Introduction

Stroke is the second most important cause of death in industrial countries and the most significant cause of adult disability around the world (1, 2). Neurological deficits contain paralysis, memory impairment, parestheisa, slurred speech; etc (3). stroke is a result of an artery occlusion that leads to lack of oxygen and glucose, this nutrient shortage could cause some irreversible damage in brain tissue (4). Inflammation caused by loss of blood perfusion plays an important role in stroke pathophysiology, secreting of some cytokines such as interleukin1 (IL-1), Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), Transforming growth factor-β (TGB-β) is resulting the post stoke inflammation (5, 6). Neuronal loss and brain damage is the secondary brain damage which the first neural loss is because of diminishing blood perfusion (7). the inflammatory cytokines could be secreted from microglia or astrocytes (8). Matrix metalloproteinases are a type of pro-

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teolytic enzyme that could make remodeling after stroke and is secreted by microglia, astrocyte, etc (9).

These days, there is only one approved drug for stroke which is rt-PA, it could be efficient according to thrombolytic effects; however, it has some side effects such as increasing the risk of bleeding and golden time limitation (10). Several hypotheses around mesenchymal stem cell therapy for stroke to account for therapeutic effects of this method have been suggested containing reducing inflammation, increasing angiogenesis, etc (11). This type of stem cells could be beneficial for reducing the inflammation and preparing the microenvironment by secreting some nutrients cytokines such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (12). Neural stem cells is another type of stem cells which has been shown a promising approach to treat stroke and they are capable of differentiating to three neural lineage including neurons, astrocytes and oligodendrocytes (13).

In this study we have planned to transplant Mesenchymal stem cells systemically in acute phase (12 hours after ischemia) for modulating the immune system and differentiated neural stem cells with astrocyte depletion transplantation 3 days after brain ischemia for promoting neural regeneration.

Materials and Methods

Animal preparation

Seventy five Sprague Dawley male rats weighting 250～300 g were selected randomly and they were divided in 7 groups (n=15) as below:

Control group: Control group which received no intervention and treatment

Sham group: Sham group which were underwent middle cerebral Artery occlusion (MCAO) operation without any treatment

Third Group: The group which received systemic Mesenchymal stem cells injection 12 hours after inducing MCAO

Forth Group: The group which received differentiated neural stem cells without astrocytes intraventicular injection 3 days after inducing MCAO

Fifth Group: The group which received differentiation neural stem cells (Neurons, Oligodendrocytes and astrocytes) intraventricular injection 3 days after inducing MCAO

Sixth Group: The group which received systemic Mesenchymal stem cells 12 hours and differentiated neural stem cells (including astrocytes) after 3 days of inducing MCAO

Seventh Group: The group which received systemic Mesenchymal stem cells 12 hours and differentiated neural stem cells without astrocytes after 3 days of inducing MCAO

The animals were kept with free access to food and water and all procedures were approved by Shiraz University of Medical Sciences ethical committee.

Mesenchymal Stem Cell Isolation, Expansion and characterization

The Mesenchymal stem cells were obtained from adult male rat femur and tibia. Briefly, the femur and tibia were isolated and the connective tissue was removed, after that the bones were flashed by Mesenchymal stem cells culture media (DMEM/F12, 10% fetal bovine serum, 1% Pen/Strep) and the culture media containing rat bone marrow was transferred to T-25 cm^2 and incubated in 5% CO_2 and 37°C. The media was changed every 2 days and after 7 days the flask was 80～90% confluent. The cultured cells were passaged by adding trypsin 0.05% and complete culture media.

Some Mesenchymal stem cells markers containing CD44 (CBL1508FMillipore), CD45 (05-1410 Millipore), CD90 (CBL1500F Millipore) and CD34 (ab192547Abcam) were assessed by immunocytochemistry method for the isolated cells.

The Mesenchymal stem cells (1000000 Cells/kg) were injected via tail vein 12 hours after inducing MCAO.

Mesenchymal stem cells Differentiation

To evaluate the multipotent features of the isolated cells before laser treatment, BMSCs were cultured inadipogenic and osteogenic differentiation medium (Miltenyi biotech) for approximately 21 days. The cells stained with Alizarin Red for osteogenic differentiation and Oil red for adipogenic differentiation.

Neural Stem Cell Isolation, expansion and characterization

The neural stem cells were isolated by dissecting rat embryo 14 ganglion eminences and adding neural stem cell culture media (Neurobasal, 2% B27, bFGF 10 ng/ml, EGF 20 ng/ml and 1% pen/strep). After 5 days some spheres had formed in the culture flask which called neurospheres.

The spheres were trypsinized and the single cells were cultured in a Poly-l-lysin coated plate and 5% fetal bovine serum was added to the neural stem cells culture media to differentiate the NSCs to three neural lineages. The neural stem cells were differentiated to neurons, oligodendrocytes and astrocytes 3 days later. The neurons and as-

trocytes were stained with β-tubulin and GFAP by immunocytochemistry method.

For preparing the differentiated neural stem cells for injecting the astrocyte were depleted with Astrocyte sorting kit (Miltenyi biotech, cat number: 130-096-052) and their depletion was assessed with flow cytometry method.

The differentiated neural stem cells minus astrocytes were transplanted stereotactically into lateral ventricle 3 days after MCAO.

Stereotactic Injection of Neural Stem Cells

The animals were anesthetized with halothane (induction 5% and maintenance 1%) and fixed to the stereotactical frame, the differentiated neural stem cells minus astrocytes were injected into right lateral ventricle at: Anterioposterior (AP)=−0.12 mm, mediolateral (ML)=1.6 mm, dorsoventricular (DV)=4.3 mm. Each rat received 200,000 cells in its right lateral ventricle.

Middle Cerebral Artery Occlusion (MCAO)

The rats were anesthetized by halothane (5% induction and 1.5% for maintenance) in the mixture of NO_2 and O_2 (50:50). MCAO induction was performed regarding to Koizumi's method. Briefly, an incision was excised in the midline of the neck and the connective tissues, muscles and salivary glands were dissected till the carotid sheath was exposed, the sheath was removed and exposed carotid artery was separated from vagus nerve, then 2 loose sutures were prepared in common carotid artery and the external carotid artery was clamped, after that a small incision was excised between the sutures on common carotid artery and a silicon coated 4.0 nylon with round tip was passed through the common carotid artery to induce the occlusion of middle cerebral artery, after 45 minutes the 4.0 Nylon was removed and the sutures were tighten up and also the clamp on external carotid artery was opened to maintain the brain blood perfusion after the operation (14, 15).

Neurological Function Assessment

Neurological function was assessed every two days for all rats during 28 days of experiment. The neurological examination was scored on six-score scale. The scores are following as below (16):

Score of 0: No neurological deficit

Score of 1: Failure to extend left forepaw completely. It shows mild focal neurological deficit

Score of 2: Circling to the left. It means a moderate focal neurological deficit

Score of 3: Falling to the left. It indicates a sever focal neurological deficit

Score of 4: Not walking spontaneously and decreasing level of consciousness.

Score of 5: Death due to brain ischemia

Histology

After 28 days, the rats were anesthetized with Halothane and were fixed with normal saline followed Paraformaldehyde 4%, cry sections (20 μm) were mounted on silicon coated slides and stained with Hematoxin & Eosin.

Apoptosis evaluation with measurement of Caspase 3 activity

Activation of ICE family proteases/caspases begins apoptosis in mammalian cells. This assay is based on spectrophtorometric evaluation of chromophore p-nitroaniline (*p*-NA) after cleavage from labeled substrate DEVD-*p*-NA. The *p*-NA light emission could be measured by using spectrophotometer at 400～405 nm. For this assay Caspase 3 assay kit from abcam company used (ab39401).

Statistical Analysis

All the data were analyzed with One-way ANOVA method with Prism 6.00 software.

Results

Mesenchymal stem cells morphology and marker assessment

The isolated rat bone marrow Mesenchymal stem cells formed few thin and long cell processes and a large nucleus in their cell body could be observed (Fig. 1). These

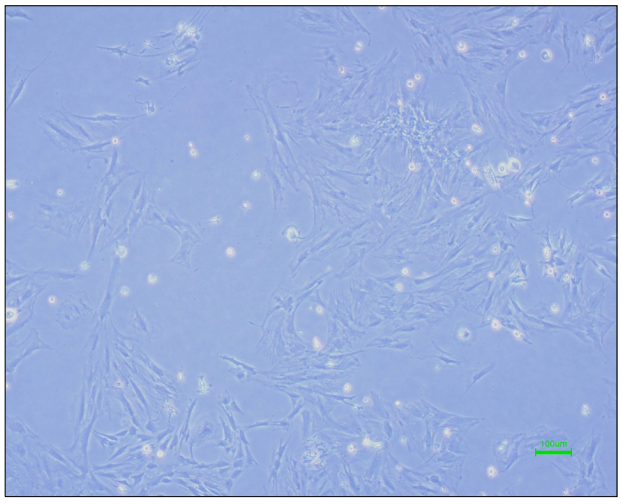

Fig. 1. Rat Mesenchymal stem cells.

cells did not express hematopoietic stem cells markers (CD34 and CD45) and also they expressed the mesenchymal stem cells markers (CD44 and CD90) (Fig. 2, Table 1).

Differentiation Assay

Differentiation to adipocyte was confirmed by morphological changes after 7 days such as vacuole production and Oil red staining after 21 days seeding in adipogenic media.

Osteogenic differentiation was started with matrix deposition after 5～7 days and was confirmed with Alizarin Red staining after 14 days (Fig. 3).

Neural Stem Cells Characterization and Differentiation

Five days after culturing neural stem cells, some spheres formed in culture flasks which were called neurospheres (Fig. 4). 48 hours after adding fetal bovine serum to the neural stem cells media they have differentiated to three neural lineages cell (Neurons, Oligodendrocytes and Astrocytes) as it could be seen in Fig. 5 the differentiated neural stem cells have different morphology due to different provided cell types (Fig. 5).

For evaluation of neural and astrocytes differentiation they were stained with anti β-tubulin III and anti-GFAP by immunocytochemistry method (Fig. 6, Table 2).

The astrocyte depletion of differentiated neural stem cells was assessed by flowcytometry method (Fig. 7).

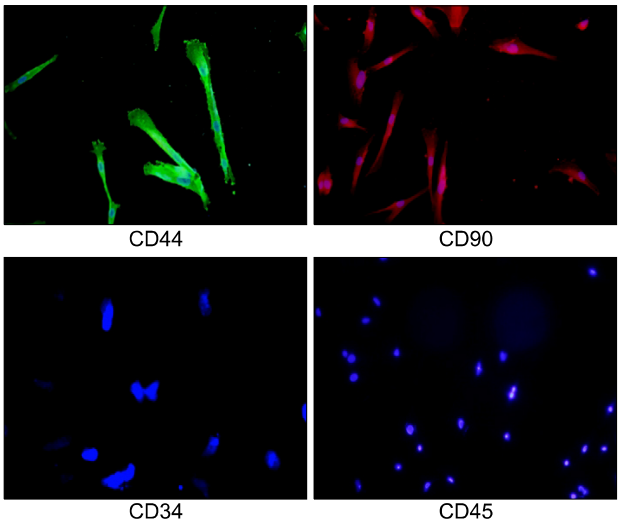

Fig. 2. Rat Mesenchymal stem cell immunocytochemistry for CD44, CD90, CD45 and CD34.

Neurological Examination

All seventy five rats were examined according to six-score scale by double blind examiner every two days during 28 days of experiment. The neurological scores were analyzed with SPSS 16.00 one-way ANOVA test and there is a significant difference between the groups which received cell therapy and the sham group. In addition, the group which received Mesenchymal stem cells and differentiated neural stem cells without astrocytes had the best neurological outcome between the groups received cell therapy with p value less than 0.05 (p value <0.05) (Fig. 8).

Histology

The coronal sections were selected with 1.2 mm interval and from the first one was 1 mm posterior to frontal pole. Damaged area was defined by some ischemic signs such as eosinophilic cytoplasm and pyknotic nuclei. The ischemic zones in the groups which were taken cell therapy

Table 1. Mesenchymal stem cells markers expression

CD44	CD90	CD 34	CD45
94.44%±2.91	84.25%±7.10	1.70%±0.84	0.94%±0.64

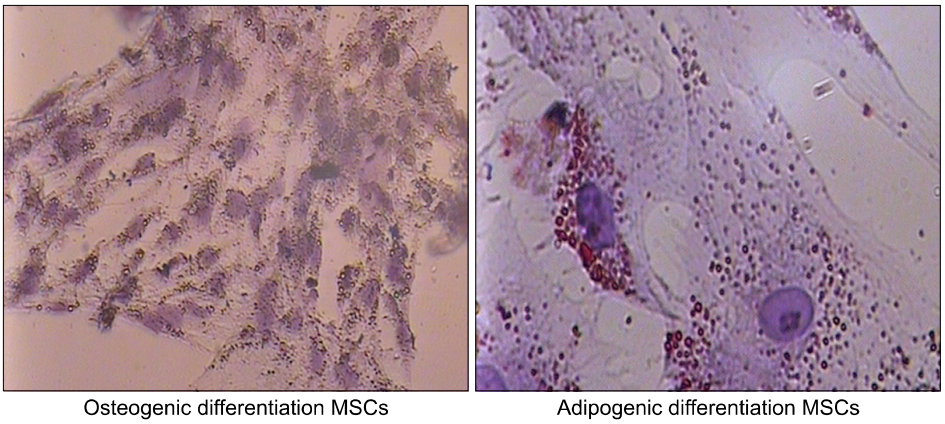

Fig. 3. Mesenchymal stem cells differentiation to adipocyte and osteocyte.

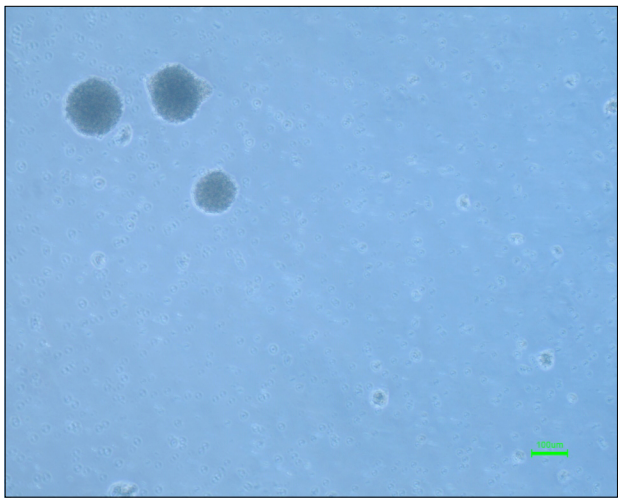

Fig. 4. Neurospheres 5 days after neural stem cell culture.

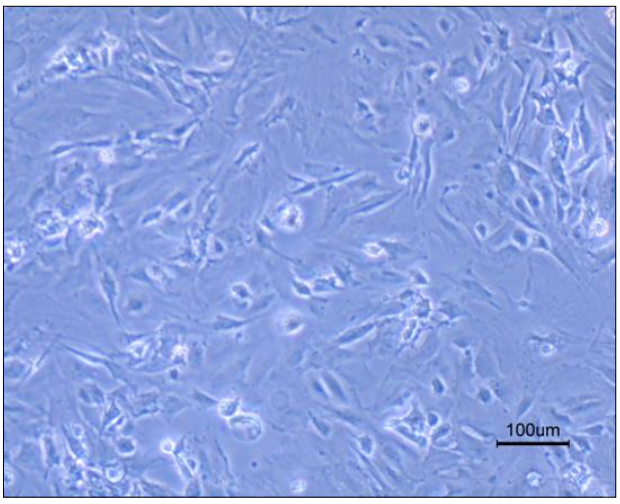

Fig. 5. Differentiated neural stem cells.

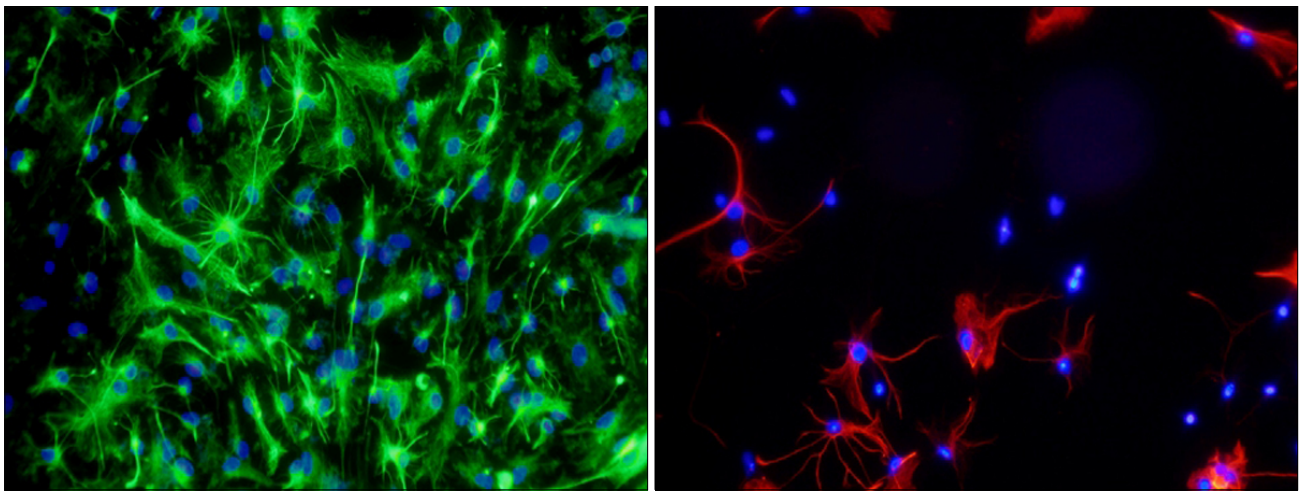

Fig. 6. Neural Stem Cell Differentiation and immunocytochemistry with GFAP and β-tubulin III antibody.

Table 2. Neural Stem Cells Differentiation to neurons and astrocytes

β-tubulin III	GFAP
11.84%±3.98	84.25%± 5.62

were significantly decreased in comparison with the sham group. Also, the group received Mesenchymal stem cells and differentiated neural stem cells without astrocytes had the least ischemic area among the cell therapy groups (40.264 mm^3±6.12) that shows it could be more effective than the other methods (Fig. 9 and 10).

Caspase 3 Activity Assay

Some stem cells are capable of reducing apoptosis by down regulating some apoptotic genes such as caspase3 (14). Caspase 3 activity was assessed by spectrophotometer at 405 nm. The higher absorbance value revealed the higher Caspase 3activity and as a result the higher incidence of apoptosis. The result indicates that the group which received Mesenchymal stem cells and differentiated neural stem cells without astrocytes had the least amount of Caspase 3 activity between the rests of the groups and as a result the lowest amount of apoptosis (0.215±0.04) (Fig. 11).

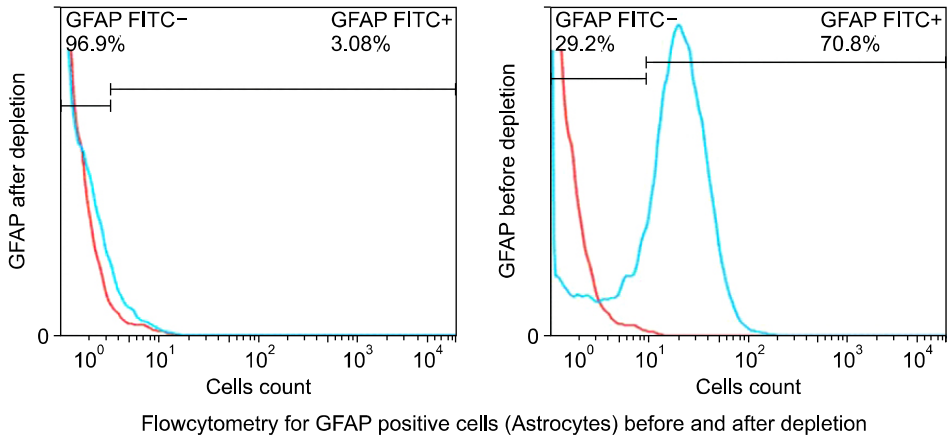

Fig. 7. Flow cytometry for assessing depletion of astrocytes. The blue line is the GFAP positive cells.

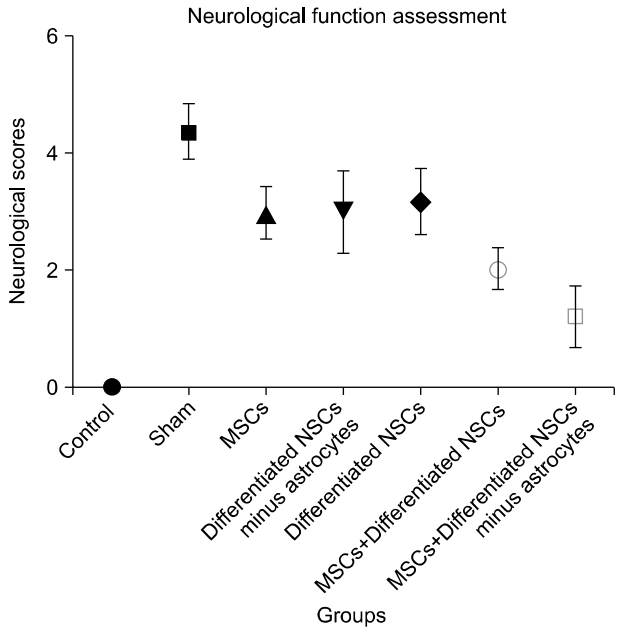

Fig. 8. Neurological scores in 28 days. The data was analyzed with One-way ANOVA and the p value is less than 0.05. The data between groups was analyzed with Tukey's multiple comparison tests.

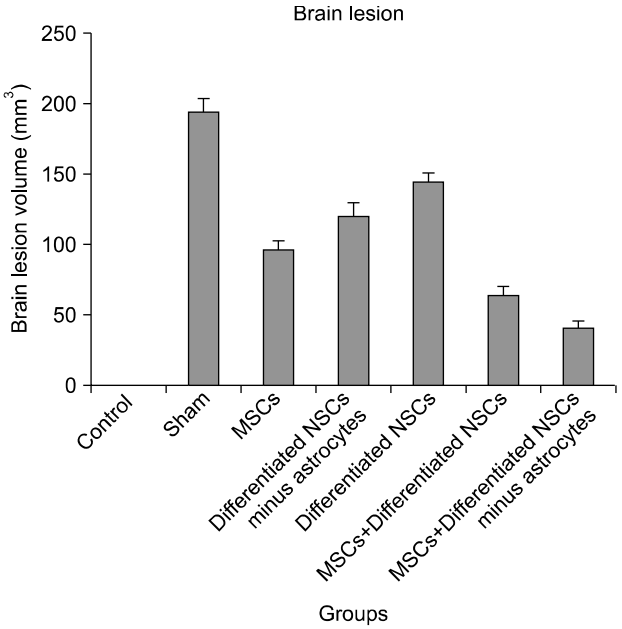

Fig. 9. H&E staining and ischemic area volume. The data was analyzed with One-way ANOVA and the p value is less than 0.05. The data between groups was analyzed with Tukey's multiple comparison tests.

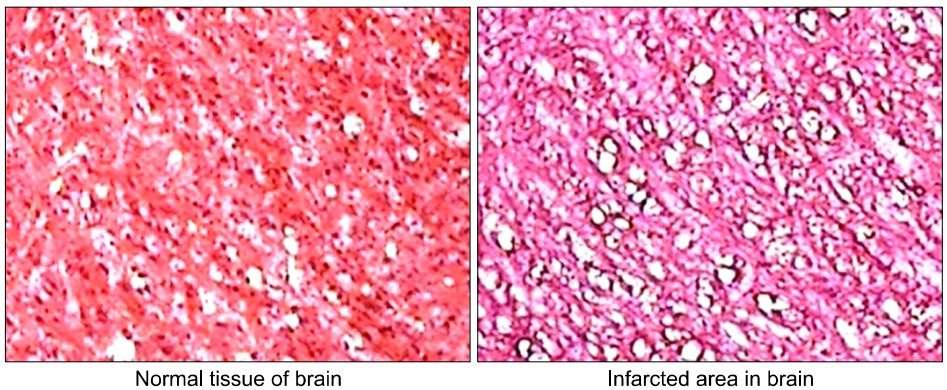

Fig. 10. H&E staining for brain tissue.

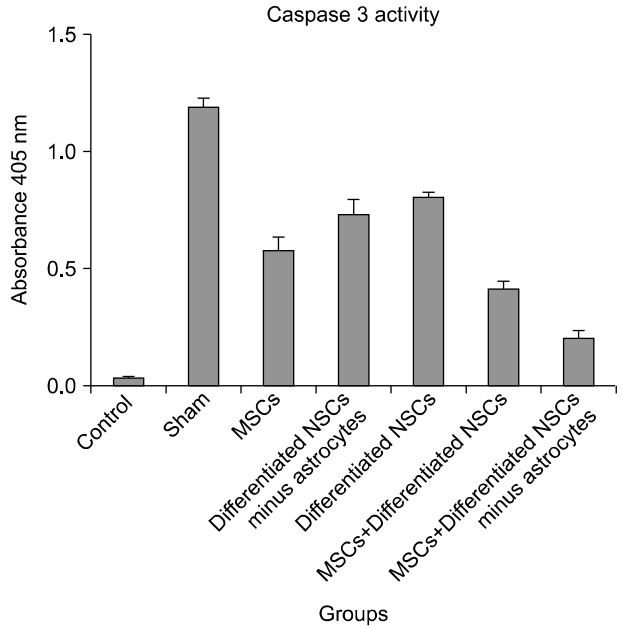

Fig. 11. Caspase 3 activity. The data was analyzed with One-way ANOVA and the p value is less than 0.05. The data between groups was analyzed with Tukey's multiple comparison tests.

Discussion

In this study we have presented a novel cell therapy method for almost treating stroke with Mesenchymal stem cells and differentiated neural stem cells with astrocytes depletion.

We have designed different cell therapy method according to different characteristics of Mesenchymal stem cells and differentiated neural stem cells and according the results the combination cell therapy with MSCs and differentiated NSCs without astrocytes could improve neurological function and also reduce the brain lesion volume and apoptosis after brain stroke.

Mesenchymal stem cells have been shown suppress T-cell activation and proliferation and also reduce T-cells response indirectly by suppression of CD 34+ progenitor cell differentiation (14, 17-19). Some studies demonstrated that Mesenchymal stem cells are capable to have anti-inflammatory effects and secret some cytokines such as interleukin 10 (IL-10) and IL-6 according to their microenvironment and some pro-inflammatory cytokines including TNF-α, IFN-λ and IL-1β are potential to induce MSC anti-inflammatory effects (20-22). Cheng et al. in 2015 have shown that Mesenchymal stem cells transplantation could improve neurological function after stroke by their ability of attenuating inflammatory factors and also modulating TGF-β (23). Calio et al. in 2014 Mesenchymal stem cells transplantation could protect the brain tissue against stress oxidative and diminish apoptosis after stroke (24). As previous studies and our results indicate, the Mesenchymal stem cells could make less volume infarct, better neurological outcomes and less apoptosis especially in acute phase of stroke. In all groups which treated with Mesenchymal stem cells alone or with other cells, neurological recovery and decreasing of apoptosis could be observed.

Neural stem cells are a type of stem cells exists mostly in subventricular zone (SVZ) and dentate gyrus (DG), they are able to migrate to degenerating area and regenerate the damaged zone by anti-inflammatory effects and differentiation to three neural lineages containing neurons, oligodendrocytes and astrocytes (25-29). Despite of mentioned positive points of neural stem cells, they promote astrocytosis (30) and it might be an obstacle for reaching an optimum effect of neural stem cells transplantation. Stroke induces a series of changes in molecular and cellular aspects in brain tissue such as increasing morphology changes, cellular proliferation and genes expressions (31-34). The expression of GFAP would be increased in stroke which leads to astrocytosis and formation of glial scares that could surround the ischemic zone and inhibit regeneration and endogenous neural stem cells migration (35-39). Our results illustrates that the groups which received differentiated neural stem cells had better outcome and less apoptosis; however by omitting the astrocyte the outcome would be promoted and apoptosis would be diminished.

According to all above, we designed an experiment to define the effects of depleting astrocyte and a new method by combining differentiated neural stem cells and Mesenchymal stem cells for treating brain stroke. As our result shows depleting the astrocytes might have positive effects on recovering after stroke and it could be developed when the Mesenchymal stem cells were injected systemically.

Potential conflict of interest

The authors have no conflicting financial interest.

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