Brief Report

Stem Cell INVESTIGATION

Effect of stem cells-based therapy on astrogliosis in stroke subjected-mice

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Abstract: This study was planned to continue our previous study to assess effect of combination therapy bone marrow stromal cells (BMSCs) with exercise (EX) and triiodothyronine (T_3) on stroke-induced astrogliosis in mice. Stroke subjected-mice were divided into five monotherapy groups including sham, control, BMSCs, EX and T_3; and three combination therapy groups including BMSCs + EX, BMSCs + T_3 and BMSCs + EX + T_3. Astrogliosis was assessed in ipsilateral hemisphere at day 7 after MCAO. Combination therapy BMSCs with EX and T_3 could significantly decrease stroke-induced astrogliosis. However, monotherapy with BMSCs or EX also improved changes of glial fibrillary acidic protein (GFAP)-positive cells following stroke. Combination therapy BMSCs with EX and T_3 didn't have any added effect on astrogliosis compared to monotherapy with BMSCs or EX. With comparing the present findings with the results of neurobehavioral functioning in our earlier study, it seems that decrease of astrogliosis could be helpful for stroke recovery.

Keywords: Stroke; astrocyte; bone marrow stromal cells (BMSCs); thyroid hormone; exercise (EX)

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Introduction

Despite plentiful researches for the handling of ischemic stroke, stroke treatment is still a challenge (1). Although, recently, stem cell therapy has offered hope for stroke recovery, it is doubted that stem cell transplantation alone can effectively improve stroke complications (2,3). For this reason, the combination of stem cell transplantation with other therapeutic approaches has been considered to improve stem cells efficacy. According to existing results, thyroid hormones or exercise (EX) could be helpful to deal with some challenges of stem cells therapy such as cell apoptosis, differentiation of stem cells into neural cells and migration of transplanted cells toward ischemic zone (2,4-9). For that reason, in this study effect of stem cell therapy in combination with triiodothyronine (T_3) and EX on astrogliosis after stroke in mice was investigated.

Pathologic situations like ischemia induce reactive gliosis in that the astrocytes become activated and undergo changes including swelling, hypertrophy of cellular processes and hyperplasia. Additionally, expression of glial fibrillary acidic protein (GFAP), known as a specific marker for astrocytes, increases (10,11).

There is debate about role of astrocytes in exacerbating or limiting stroke complications. In some studies astrogliosis was viewed as an obstacle to stroke recovery, and consequently decrease in astrogliosis was designed as an objective of therapeutic interventions (12-14). On the contrary, other studies have shown that GFAP-positive cells contribute to recovery of brain ischemia (15,16).

Because of importance of astrocytes in stroke pathophysiology, we assessed effect of post stroke treatment with bone marrow stromal cells (BMSCs) with EX and T_3 on activation of astrocytes including number of GFAP-positive cells and morphological changes in reactive astrocytes at day 7 after stroke. Since, changes of GFAP-positive cells have obviously been revealed in

strokes, knowing correlation between activation these cells with one of the vital variables in clinical situation, neurological deficit, can conduct researchers to find out whether they should stimulate or inhibit reactive astrocytes. Therefore, we assessed whether changes in astrogliosis after treatment with stem cells, T_3 and EX is in agreement with improvement of neurological score that we showed in our past study (17). Our previous findings revealed that the treatments including EX, EX + BMSCs and EX + BMSCs + T_3 could improve neurological recovery. This study aimed to evaluate effect of combination therapy BMSCs with EX and T_3 on stroke-induced astrogliosis in mice.

Method and material

Animals

In order to decrease number of experimental animals, we used brain samples of young adult male Swiss albino mice (2–3 months, 35–40 g) gathered from our previous study. All experiments were performed in accordance with relevant guidelines and regulations. The study was approved by the Research Ethics Committee of Semnan University of Medical Sciences and Health Services (ethical code number: 93.475925) and national policy for approaching animal research. We had eight experimental groups including sham (surgery without MCAO), phosphate-buffered saline (PBS) (as control group), BMSC, T_3, EX, BMSCs + T_3, BMSCs + EX, BMSCs + T_3 + EX.

Focal cerebral ischemia

Stroke was induced by occlusion of the middle cerebral artery. Briefly, after deep anesthesia with ketamine [60 mg/kg intraperitoneal (IP)] and xylazine (10 mg/kg IP), the common and external carotid arteries were ligated and a small incision was made on internal carotid artery. A silicone-coated 8-0 filament (Ethicon, England) was inserted into incision and advanced toward middle cerebral artery origin in order to decrease blood flow to 20% of baseline. Laser Doppler Flowmetry (Moor Instruments DRT4, England) was used to investigate blood obstruction. The middle cerebral artery was obstructed for 45 minutes, and then reperfusion was allowed for 7 days.

Cell preparation and administration

Cell extraction and administration was done as we described in our previous study (17). Briefly mouse stem cells isolated from the tibia and femoral marrow compartments were cultured in DMEM medium (Bio-IDEA, Iran) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and penicillin/streptomycin (100 U/mL and 100 g/mL) (Sigma, USA). The tissue culture flask was placed in a 5% CO_2 humidified incubator (Memmer, Germany) set at 37 ℃. Non-adherent cells were removed 24–72 hours later by changing the medium and plastic adherent stromal cells were remained attached to the flask surface. Cell passaging was done when the confluence was more than 80%. BMSCs at passages 3 to 5 were used for transplantation. Under aseptic conditions, 3 µL of cell suspension containing 1×10^5 BMSCs were injected into the right lateral cerebroventricle (0.9-mm right, 0.1-mm posterior, and 3.1-mm deep relative to the bregma) 24 hours after stroke (18). In PBS group, animals received 3 µL of PBS (Bio-IDEA, Iran) by intra-cerebroventricular route.

EX and T_3 injection protocols

EX groups were forced to run on a treadmill (Borj Sanat, Iran) for 30 min daily for six consecutive days, starting 24 h after induction of stroke. The running speed and duration consisted of 3 m/min for 5 min, 5 m/min for 5 min and 8 m/min for 20 min with 0° inclination. Also T_3 (Sigma, USA) (20 µg/100 g body weight) was subcutaneously administrated 24 hours after MCAO and continued for 6 consecutive days.

Tissue preparation and immunohistochemistry study

After deep anesthesia, the mice were perfused through the heart with cold saline and paraformaldehyde solution 4% in PBS. Then the brains were isolated and immersed in paraformaldehyde (Merck, Germany) solution 4% in PBS for approximately 1 week. Ten-µm sections were cut coronally from Bregma –1 mm to Bregma +1 mm. Three equal sections of each brain were washed with xylene to remove the paraffin and with graded ethanol to rehydrate. The sections were sequentially incubated in 3% H2O2 in PBS solution for 60 min, 10% goat serum (Sigma, USA), 0.5% Triton X-100, and 0.1% bovine serum albumin (Sigma, USA), in PBS solution for 30 min at room temperature, the primary antibodies (rabbit-anti-GFAP, 1:100, Biorbyt, UK) overnight at 4 ℃, the secondary antibody (biotinylated goat anti-rabbit immunoglobulin IgG, 1:100, Biorbyt, UK) for 2 h at room temperature and

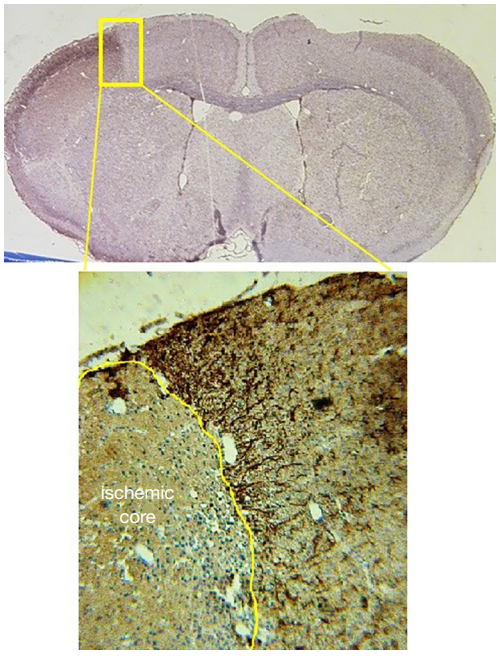

Figure 1 Histology staining to detect GFAP-positive cells (brown, DAB staining) in ipsilateral hemisphere in ischemic mice (20× magnification). Almost no GFAP-positive cells were detected in ischemic core. Conversely there was increased GFAP staining in edge of ischemic core. Yellow curve separate ischemic core and boundary zone. GFAP, glial fibrillary acidic protein.

finally avidin-peroxidase conjugate solution for 1 hour. Staining and counterstaining were respectively performed with 0.05% DAB (Sigma, USA) and hematoxylin (17).

The sections were viewed by light microscope (Reichert microscope, USA) with a ×40 magnification and then number of GFAP-positive cells was counted in a blinded manner in six visual fields per section of each sample.

Statistical analysis

The Kruskal-Wallis ANOVA on ranks was conducted to compare groups regarding number of GFAP-positive cells. Data were expressed as mean ± SEM. $P<0.05$ was statistically significant.

Results

Histological staining revealed increased GFAP staining in ischemic boundary zone in stroke-subjected mice compared to sham animals. However, there were almost no astroglial cell in ischemic core (*Figure 1*). Astrocytes in sham group had small cell bodies and thin cellular processes. Stroke led to hypertrophy of astrocytes and increase in number and diameter of cellular process in PBS group (*Figure 2*). The number of GFAP-positive cells significantly decreased in EX, BMSCs, BMSCs + EX and BMSCs + EX + T_3 groups compared to PBS group ($P<0.05$). T_3 alone or in combination with BMSCs could not decrease GFAP staining (*Figures 3*,*4*).

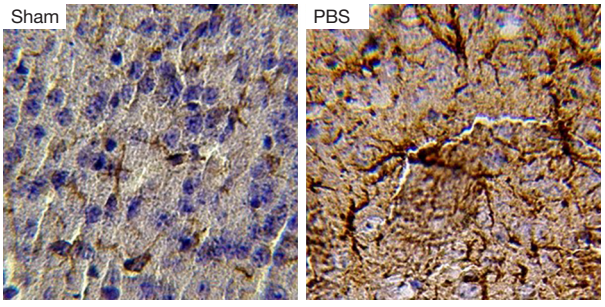

Figure 2 Morphological changes in reactive astrocytes using DAB staining (40× magnification). hypertrophy of cell body and processes and hyperplasia of astrocytes is detectable in PBS group. PBS, phosphate-buffered saline.

Monotherapies with EX and BMSCs and combination therapies with BMSCs + EX, BMSCs + EX + T_3 improve morphological changes in astrocytes. The astrocytes in these groups exhibited smaller cellular bodies and finer processes compared with the PBS group. However, astrocytes in T_3 and T_3 + BMSCs groups had a large volume, with thick branches (*Figure 3*).

Discussion

In present study, consistent with previous reports (10,11), stroke induced an increase in expression of GFAP and morphological changes of astrocytes including hypertrophy and branching. Combination therapy with BMSCs, EX and T_3 could significantly decrease reactive astrogliosis. However, monotherapy with BMSCs or EX also improved changes of GFAP- positive cells following stroke.

Present study didn't show any added effect of combination therapy than monotherapy with stem cell on reactive astrogliosis after stroke. Although existent studies have shown that thyroid hormone and EX probably potentiate BMSCs capacity by increasing survival, maturation, migration and differentiation of stem cells (2,7-9), there was no any additional impact of triplet combination therapy compared to monotherapy with stem

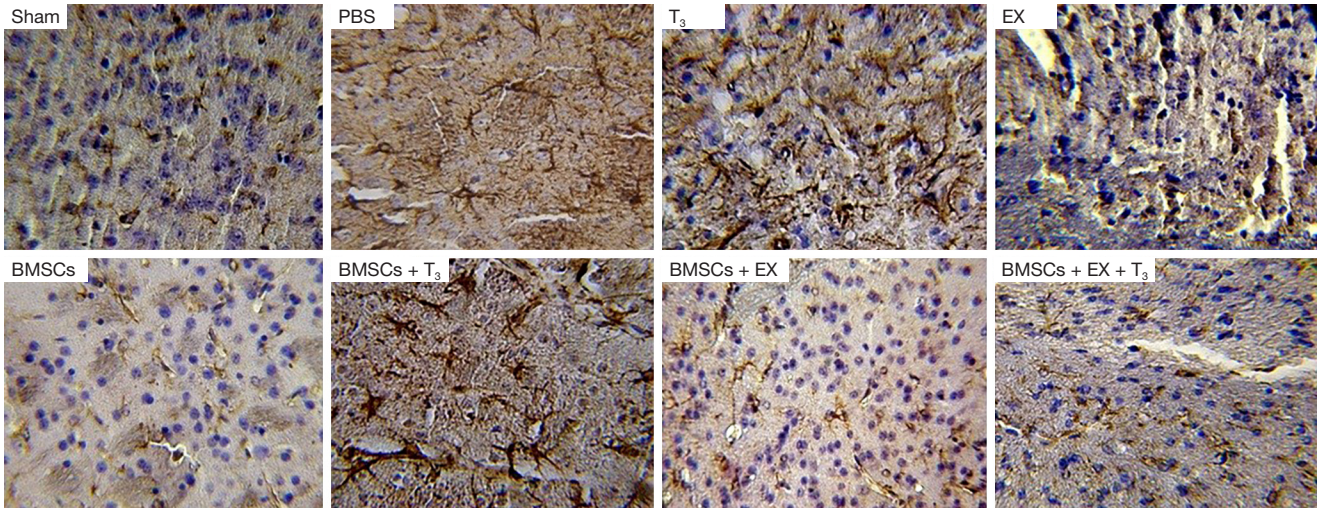

Figure 3 GFAP-positive cells (brown, DAB staining, 40× magnification) in experimental groups. Monotherapy EX or BMSCs and combination therapy BMSCs + EX or BMSCs + EX + T_3 significantly decreased GFAP-positive cells. GFAP, glial fibrillary acidic protein; BMSC, bone marrow stromal cell; EX, exercise; T_3, triiodothyronine; PBS, phosphate-buffered saline.

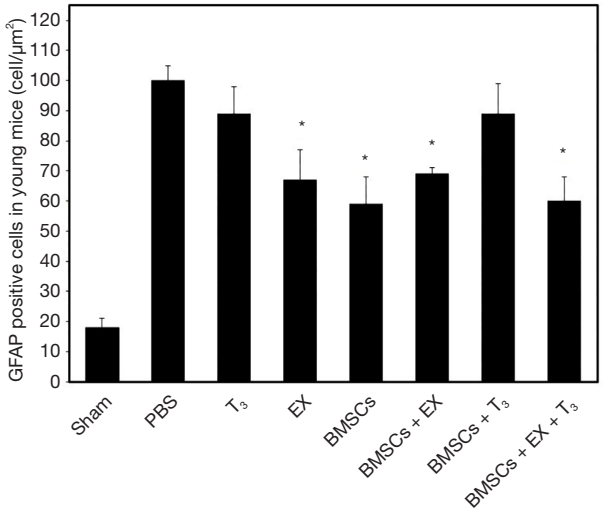

Figure 4 The number of GFAP-positive cells significantly declined in EX, BMSCs, BMSCs + EX and BMSCs + EX + T_3 groups. *, significantly different from PBS groups. GFAP, glial fibrillary acidic protein; EX, exercise; BMSC, bone marrow stromal cell; T_3, triiodothyronine; PBS, phosphate-buffered saline.

cell on GFAP- positive cells.

Our results revealed that in BMSCs group, stroke-induced astrogliosis was decreased in epsilateral hemisphere. These results could support studies that showed stem cells inhibited astroglial fate in neural progenitor cells (19) and reduced astrogliosis of ischemic astrocytes (12,20). Because of Anti-inflammatory and immunomodulatory mechanisms of mesenchymal stem cells (21), it is reasonable that ischemia-induced astrogliosis, known as an inflammatory response to stroke (22), was suppressed in BMSCs group.

Present finding showed that EX decreased GFAP expression in ischemic hemisphere at day 7 after stroke. According to the existing studies EX is able to improve stroke recovery by means of various mechanisms such as reduction in inflammation and apoptosis and improvement of angiogenesis and neurogenesis (17,23) and our results was a confirmation on anti-inflammation effects of EX on stroke brain.

Present study showed that there is no statistically significant difference between T_3 and control groups with respect to the level of GFAP expression. This finding is somewhat supported by Lee and colleagues study that had found that activations of GFAP+ astrocytes after ischemia/reperfusion were higher in the euthyroid group, compared to hypothyroid group (24). In addition, there are several studies that have showed that thyroid hormone increased GFAP-positive cells and induced morphology changes in astrocyte both *in vivo* and *in vitro* conditions (25-27). Since both stroke and thyroid hormone enhance astroglial cells, therefore it is not unexpected that stroke-induced astrogliosis do not improve in monotherapy with T_3. In contrast to T_3 monotherapy, combination therapy T_3 plus stem cells plus EX significantly diminished GFAP expression

and Astrogliosis. Although the studies showed that thyroid hormone could amplify stem cell efficacy by improvement of stem cells migration or/and differentiation (8), the effect of combination T_3 with stem cell on GFAP positive cell was not more than stem cell alone. Overall, although thyroid hormone could improve stroke recovery by the mechanisms, such as antiapoptotic mechanism (28) and the promotion of endogenous cell proliferation in brain (25), and improve stem cell efficacy by enhancing cell proliferation, migration, and maturation (7-9) our study did not show that monotherapy with T_3 impact on stroke-induced astrogliosis.

In our previous study, we have shown that EX alone or in combination with BMSCs, as well combination EX with BMSCs and T_3 decreased neurobehavioral deficit.

Conversely, monotherapy T_3 or combination T_3 + BMSCs couldn't have therapeutic effect on neurobehavioral deficit. Comparing our past and present surveys revealed that decrease of astrogliosis at day 7 after stroke in EX, BMSCs + EX and BMSCs + EX + T_3 groups was consistent with significant improvement of neurobehavioral deficit. Conversely, administration of T_3 alone or in combination with BMSCs influence neither astrogliosis nor neurological outcome. Therefore our results are to some extent in an agreement with studies that have shown that reactive astrocytes were related to poor stroke recovery and consequently the decrease in astrogliosis was correlated with recovery neurological deficit (12,29-31). Since reactive astrocytes result in exacerbation of inflammatory response, neuronal cell death (31), and forming a physical or chemical barrier to axonal regeneration (15), it seems that discovering new compound to decrease stroke-induced astrogliosis could be helpful in stroke treatment. However, treatment time to decrease astrogliosis is important because it has been shown that inflammatory responses after acute phase of stroke may help to stroke improvement (32).

In conclusion, combination therapy BMSCs with EX and T_3 didn't have an added effect on astrogliosis compared to monotherapy with BMSCs or EX. Comparing present finding with our former results to some extent showed that astrogliosis at day 7 after stroke was related to severity of neurological deficit. Therefore, it seems that decrease of astrocytes at this time could help to stroke recovery, although further researches are needed.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/sci-2020-031). Both authors report grants from Iran National Science Foundation (INSF), during the conduct of the study.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experiments were performed in accordance with relevant guidelines and regulations. The study was approved by the Research Ethics Committee of Semnan university of medical sciences and health services (ethical code number: 93.475925) and national policy for approaching animal research.

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