Bone Marrow Stromal Cells with Exercise and Thyroid Hormone Cannot Reverse Post-Stroke Injuries in Middle-Aged Mice

Running Title: Post-stroke treatment of bone marrow stromal cells with exercise and thyroid hormone

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Abstract

***Objective*:** Our previous findings showed that the treatment of stem cells alone or in combines with thyroid hormone (T3) and mild exercise could effectively reduce the risk of stroke damage in young mice. However, it is unclear whether this treatment approach can be effective in aged or middle-aged mice. Therefore, this study designed to assess whether combination bone marrow stromal cells (BMSCs) with T3 and mild treadmill exercise can decrease stroke complications in middle-aged mice.

***Materials and methods*:** Under Laser Doppler Flowmetry (LDF) monitoring, transient focal cerebral ischemia was prepared by right middle cerebral artery occlusion (MCAO) for 45 min followed by 7days of reperfusion in middle-aged mice. BMSCs (1×10^5) were injected into the right cerebral ventricle 24h after MCAO, followed by daily injection of triiodothyronine (T_3) (20 µg/100 g/day S.C) and 6 days of running on a treadmill. Infarct size, neurological function, apoptotic cells and expression levels of GFAP (Glial Fibrillary Acidic Protein) were evaluated 1-week post stroke.

Results: Post-ischemic treatment with BMSCs or with T_3 and/or mild treadmill exercise alone or in combination did not significantly change neurological function, infarct size, and apoptotic cells 7 days after ischemia in middle-aged mice ($p>0.05$). However, the expression of GFAP significantly reduced after treatment with BMSCs and/or T_3 ($p < 0.01$).

Conclusions: Our findings indicated that post-stroke treatment BMSCs with exercise and thyroid hormone cannot reverse neuronal damage seven days after ischemia in middle-aged mice. This data more confirms that age is an important variable in studies related to stroke treatment.

Keywords: Bone marrow stromal cells, Thyroid hormone, Exercise, Apoptosis, Stroke, GFAP, Middle-aged mice

Introduction:

Ischemic stroke occurs more often in aged humans, [1] and is an important reason of mortality and inability in the aged period [2]. According to clinical and animal studies, outcome and mortality after brain damage and the efficacy of neuroprotective agent are age dependent [3, 4]. It has been established that aging exacerbates brain injury and diminishes functional recovery of post ischemic damage in rodent models of cerebral stroke [5-9]. Moreover, it has also been shown that the risk of stroke increases with aging in humans and recovery after stroke lessens in older patients compared with younger ones [10, 11]. It has been indicated that increase in age was associated with inadequate collateral circulation, and consequently adverse tissue outcome and unfavorable clinical outcome after stroke [12]. Although, stroke is known as an age-related disorder, most of the experimental stroke-related studies have been done in young-adult animals. This is probably one of the reasons why stroke therapeutic interventions, which were effective in experimental animal, failed in clinical situations [4]. Therefore, use of older animal model for stroke research is clinically more applicable [13].

Thyroid hormone is necessary for maturation of the brain in the fetal period and cerebral function in adulthood through controlling the expression of many genes [14]. Recent animal and clinical studies have reported that thyroid hormone may be beneficial in management of cerebral stroke [15-17]. It has also been shown that thyroid hormone may participate in regulation proliferation, migration, and maturation of neural stem cells in the brain [18-20]. Also, it has been approved that exercise can reduce the complication of cerebral stroke in humans and animals through various mechanisms including enhancing survival of neurons, promoting production of new neurons and new blood vessels, enhancing synaptic plasticity, and inhibition of neuronal apoptosis [21-23].

Stem cell therapy is a new therapeutic strategy to improve neurogenesis and repair brain ischemic injuries that may be potentiated when combined with other neuroprotective agents [24, 25]. In this regard, some animal investigations have reported that stem cell therapy alone or in combination with other neuroprotective interventions such as exercise, can effectively attenuate ischemic injury through various mechanisms in young animal model of stroke [25-29]. Our previous findings showed that post-ischemic treatment of BMSCs combined with exercise, and thyroid hormone more efficiently reduced brain damage in young mice [30]. However, it is unclear whether this treatment approach can be effective in aged or middle-aged mice. Moreover, there is scarce and contradictory data regarding the effects of stem cells therapy alone or in combination with other neuroprotective agents on recuperation of cerebral ischemic damage in middle aged and

aged animal [4, 25, 31, 32]. Therefore, this study designed to assess whether combination BMSCs with T_3 and mild treadmill exercise can attenuate stroke induced injury seven days after ischemia in middle-aged mice.

Materials and methods

Animals and Ethics

Middle-aged male Swiss albino mice (11-12 months, 35–40g) were provided from animal center of Semnan University of Medical Sciences (SUMS), Semnan, Iran. The mice were housed in a standard condition and food and water were available freely. All tests were done in conforming to the Research Ethics Committee (ethical code number: 93.475925) and national policy for approaching animal research.

Focal cerebral ischemia

Mice were anesthetized using of ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP) and transient focal cerebral ischemia was made using of the intraluminal filament method [33, 34]. Under the surgical microscope, an incision was made on midline of neck and then right common carotid artery and its branches were isolated. Using of LDF (Moor Instruments DRT4, England), a silicone-coated 8-0 monofilament was applied to create focal cerebral ischemia. Right middle cerebral artery was blocked for 45 min and re-circulation was done for 7 days. Body temperature was kept at 37±0.5°C throughout the experiment by an electrical blanket.

Cell extraction and transplantation

Bone marrow was harvested aseptically from tibias and femurs of mice by pushing bone marrow out with Dulbecco's modified Eagle's medium (DMEM, low glucose), supplemented with fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL and 100 g/mL), to a tissue culture flask. The flask was incubated at 37 °C (5% CO2). After 72–96h, the cells were washed twice with PBS to remove the no adherent cells. After reaching 80%–90% confluence, Plastic-adherent BMSCs were isolated with 0.25% trypsin-EDTA and then replaced for 3–5 passages [35-37]. The viability of BMSCs, detected with trypan blue exclusion method, was more than 90%. Under aseptic conditions, 2µl of cell suspension (1×10^5 BMSCs) or PBS (as vehicle) was injected into the right lateral ventricle (0.9-mm right, 0.1-mm posterior, and 3.1-mm deep relative to the bregma) 24h after MCAO [38].

Exercise training and thyroid hormone injection protocols

Treadmill exercise was initiated 24 h after ischemia for 30 min and continued daily for 6 consecutive days [39]. Mild exercise included running at 3 m/min for 5 min, 5 m/min for 5 min, and then 8 m/min for 20 min at 0° inclinations (18). Thyroid hormone (T3, Sigma, USA) was administered at 20μg/100g/day [40] subcutaneously for six consecutive days, initiating 24 h following ischemia.

Neurobehavioral test

Neurological evaluation was done seven days after ischemia in all of experimental groups as shown in Tab.1 [41, 42]. Neurological function was ranked on a level of 0–14 (normal score = 0; maximum deficit score = 14). A score of 10–14 is severe; 5–9 moderate; and 1–4 mild. A subject who was unaware to the experimental groups evaluated neurological disorder.

Tab.1. Neurobehavioral test

Behavioral test	**Score**
Motor tests	
Raising the mouse by the tail:	
• Flexion of forelimb	1
• Flexion of hindlimb	1
• Head moved > 10 ° to vertical axis within 30 s	1
• Placing the mouse on the floor:	
• Inability to walk straight	1
• Circling toward the paretic side	1
• Falling down to the paretic side	1
• Abnormal movements	
• Immobility and staring	1
• Tremor (wet-dog-shakes)	1
• Myodystony, irritability, seizures, myoclonus	1
Sensory tests	
Visual and tactile placing (limb placing test to detect visual and superficial sensory)	
Moving the mouse laterally toward the table:	
• Reaching the table slowly with limbs or cannot place at all	1
Proprioceptive test (deep sensory) Pushing the paw against the table edge to stimulate limb muscles:	
• Losing the resistance	1
Reflexes	
• Absence of Pinna reflex (a head shake when touching the auditory meatus)	1
• Absence of Corneal reflex (an eye blink when lightly touching the cornea with cotton)	1

• Absence of Startle reflex (a motor response to a brief loud noise from snapping a clipboard paper)	1
Maximum points	14

Beam balance test

Beam balance test assesses sensorimotor function and balance in rodents. In this test, animals must walk across an elevated balance beam (Length: 100 cm, Width: 1.2 cm Height: 50 cm). Scoring was done in all of experimental groups as shown in Tab.2 [43-45].

Tab.2. Beam balance test

Beam balance test	Points
Balances with steady posture	0
Grasps side of beam	1
Hugs the beam and one limb falls down from the beam	2
Hugs the beam and two limbs fall down from the beam(>60 s)	3
Attempts to balance on the beam but falls off (>40 s)	4
Attempts to balance on the beam but falls off (>20 s)	5
Falls off: no attempt to balance or hang on to the beam (<20 s)	6
Maximum points	6

Experimental groups

Infarct size, neurological disorder, apoptotic cells and GFAP-positive cells were evaluated at day 7 after ischemia in eight different groups as followed (n=4-6): Group 1 (sham-operated group), surgery without MCAO; Group 2 (control group), stroke-subjected animals received PBS (2µl) Intracerebroventricularly (ICV) at 24h after MCAO; Group 3 (BMSCs group), stroke-subjected animal received BMSCs (10^5 cells, 2 µl ICV) at 24h after MCAO; Group 4 (T3 group), stroke-subjected animal received T3 (20 µg/100 g/, S.C) daily for 6 days, starting 24h after ischemia; Group 5 (EX group), stroke-subjected animal was forced to do mild treadmill exercise (running at 3 m/min for 5 min, 5 m/min for 5 min, and then 8 m/min for 20 min at 0° inclination) that was started at 24h after ischemia and continued to the 7^{th} day after MCAO; Group 6 (BMSCs + T3 group), stroke-subjected animal received both BMSCs and T3; Group 7 (BMSCs + EX group), stroke-subjected animal received both BMSCs and exercise; Group 8 (BMSCs + T3 +EX group), animal received all BMSCs, T3 and exercise.

Brain damage measurements and TUNEL assay

Under deep anesthesia, saline and then 4% paraformaldehyde was perfused transcardially at day 7 after MCAO in all of experimental groups. After decapitation, the brains were removed, immersed in 4% paraformaldehyde and then embedded in paraffin wax. Nine coronal sections of each animal brain (10 µm-thick) were provided, starting 100-µm interval from bregma -1 to +1 of the ischemic hemisphere by a microtome for brain damage measurement, TUNEL and immunohistochemistry assay.

Three coronal brain sections (10 µm thick) were used to measure the infarct area with Cresyl fast Violet staining (Nissl staining). Briefly, according to the protocol, after deparaffinized and hydration in xylene, ethanol and distilled water, brain slices were immersed in 0.5% Cresyl fast Violet (Sigma, St. Louis, MO) at 65 °C for 7 min followed by being immersed in Acetic acid 0.25% in ethanol 50% for 1 to 2 seconds and then washed. Afterward, sections were photographed using a digital camera (Cannon, Japan) and infarcted areas were calculated by an image analysis system (Motic Images Plus 2.0) and data were reported as the percentage of infracted area.

Three coronal brain sections (10 µm-thick) were used to TUNEL apoptotic cell detection using an In Situ Cell Death Detection kit, POD (Roche Diagnostic GmbH, Germany)[46]. According to the protocol, after deparaffinization, tissue was incubated with proteinase K, permeabilized with permeabilization solution, and incubated in TUNEL solution respectively. After each stage, washing was performed with PBS. The number of TUNEL-positive cells (brown) was visualized using a Reichert microscope (USA) with a ×40 magnification and counted by a blinded investigator on six non-overlapping visual fields for each section.

Immunohistochemistry assay

Three equal coronal sections of each sample were used to immunohistochemistry assay. Endogenous peroxidase activity of brain sections was extinguished with 3% H2O2 in PBS (60min) and non-specific binding was blocked with 10% goat serum, 0.5% Triton X-100, and 0.1% bovine serum albumin in PBS (30 min). The slices were incubated with primary antibody (rabbit-anti-GFAP, 1:100, Biorbyt, UK) overnight at 4°C followed by incubation with secondary antibody (biotinylated goat anti-rabbit immunoglobulin IgG,1:100, Biorbyt, UK) for 2 h at room temperature. 3, 3'-diaminobenzidine (DAB, Sigma, Germany) was used for staining GFAP-positive cells and counterstaining was done with hematoxyline eosin. The number of GFAP-stained cells (brown) was

visualized using a Reichert microscope (USA) with a ×40 magnification and counted by a blinded researcher on six non-overlapping visual fields for each section.

Statistical analysis

The statistical tests of one-way ANOVA were used for comparison among groups regarding infarct area, GFAP-positive and Tunnel positive cells. The Kruskal-Wallis ANOVA on rank and Dunn's method as post-hoc tests were used to analyze neurological scores. Results of neurological scores, beam balance are presented as median ± IQR (interquartile range) and other variable as mean ± SEM. Differences were considered statistically significant at $p<0.05$ (Sigma Stat 2.0; Jandel Scientific, Erkrath, Germany).

Results

Effect of combination BMSCs with T3 and exercise on brain lesion and neurological disorder

The percentage of infarct area in the PBS control group was 36% ± 7 at day 7 after cerebral ischemia. Treatment with BMSCs (32%± 4) or with T3 (39%±5) and or mild treadmill exercise (%29±5) alone did not significantly change percentage of infarct size ($p>0.05$, Fig. 1A-B). Additionally, combination treatment of BMSCs+T3 (37%±4), BMSCs+EX (23%±6), and BMSCs+EX+T3 (25%±7) did not change percentage of infarct size significantly compared with the PBS as control group ($p>0.05$, Fig. 1A-B).

Neurological dysfunction score was 3±1.5 in the PBS group at day 7 after cerebral ischemia. Treatment with BMSCs (2± 0.25) or with T3 (2±1.25) and/or mild treadmill exercise (2±0.5) alone did not improve neurological outcome ($H=6.233$ with 6 degrees, $p=0.398$, Fig. 2A). Furthermore, combination treatment of BMSCs+T3 (2±0.5), BMSCs+EX (2±1), and BMSCs+EX+T3 (1±1.5) did not significantly improve neurological deficits compared with the PBS as control group ($H=6.233$ with 6 degrees, $p=0.398$, Fig. 2A). In addition, scores of beam balance test were not different between experimental groups ($H=3.932$ with 6 degrees, $p=0.686$, Fig. 2B).

Effect of combination BMSCs with T3 and exercise on apoptotic cells

The number of TUNEL-positive cells (apoptotic cells) in the PBS control group was 15 ± 4 at day 7 after cerebral ischemia. Treatment with BMSCs (13± 2) or with T3 (17±2) and or mild

treadmill exercise (18±4) alone did not change content of TUNEL-positive cells ($P > 0.05$, Fig. 3A-B). Additionally, combination treatment of BMSCs+T3 (13±5), BMSCs+EX (7±3), and BMSCs+EX+T3 (9±3) did not change the number of TUNEL-positive cells significantly compared with the PBS as control group ($P > 0.05$, Fig. 3A-B).

Effect of combination BMSCs with T3 and exercise on GFAP expression

Cerebral ischemia considerably enhanced the GFAP staining in the PBS group as control (90±10) compared with the sham group (42±1) ($P < 0.001$, Fig. 4A-B). Treatment with BMSCs (43± 6) and/or T3 (54±2.9) significantly reduced GFAP stained cells ($P < 0.001$, Fig. 4A-B). Additionally, combination treatment of BMSCs+T3 (36±5) significantly declined GFAP stained cells compared with the PBS ($P < 0.001$, Fig. 4A-B).

Discussion

The main findings of this research exhibited that BMSCs transplantation alone or in combination with T3 or mild treadmill exercise did not significantly change infarct size, neurological function and apoptosis seven days after stroke in middle-aged mice. Moreover, other finding of present study indicated post-ischemic treatment only with T3, BMSCs and BMSCs +T3 significantly reduced GFAP as a marker astroglial activation and gliosis.

Extensive neuroprotective drugs and/or molecules were discovered in pre-clinical stroke studies in young animals, but most of them failed in the clinical study. One possible reason for this discrepancy may relate to the fact that most of the experimental stroke studies have been conducted in young animal, while stroke often occurs in aged human. Although use of aged animals in subject of stroke is associated with troubles such as higher mortality and morbidity, which makes researchers, have less willing to study in aged animals. However, utilize of aged animal in studies of experimental is more applicable to clinical situation of stroke. Therefore, in the present study, we used mice aged 11-12 months, which corresponds to human aged approximately 60 years.

Our data indicated that treatment with BMSCs or mild exercise failed to decrease the brain injury and recovery of neurological function 1-week after stroke in middle-aged mice. This result is in agreement with previous investigations, that showed bone marrow mononuclear cell transplantation or exercise failed to recover the brain damage in aged rats in experimental stroke [31, 47]. Moreover, our data was supported by previous researches that showed that with increasing age, vulnerability of the brain to ischemia increases and response to treatment decreases [12, 13,

48, 49]. Also, our finding is in line with a study that indicated that treatment with Apocynin (an inhibitor of NADPH oxidase), unlike with young mice, led to worsening brain damage and increase in mortality rate in aged mice [50]. However, results of present study is in contrast with our recent findings in young mice [30].

Apoptosis is an important cellular mechanism that may be involved in development of secondary ischemia damage after stroke [9]. Our study showed that the number of apoptotic cells considerably increased a week after ischemia in middle-aged mice. However, treatment with BMSC alone or combined with thyroid hormone and/or exercise could not reduce the apoptosis in ischemic area 1-week post stroke in the present study that was in line with the amount of brain damage and neurological function.

Our finding showed that treatment alone with BMSCs and/or T3 and combination therapy BMSCs+T3 significantly attenuated stroke-induced astrogliosis 7 days after stroke in middle aged mice. In line to our findings, a number of studies showed that stem cell transplantation [51, 52] and or thyroid hormone therapy could decrease GFAP expression and astrogliosis after stroke [51, 52]. Although in present study, GFAP expression decreased in BMSCs and/or T3 groups, but this decline was not associated with improved brain damage or neurological function. It is probable that other amplified pathologic mechanisms following stroke in aged brain impede therapeutic effects of decrease in astrogliosis [53].

Taken together, comparison between present findings with our previous findings in young mice [20] shows that therapeutic response to combination therapy with BMSCs, T3 and exercise is different between young and middle aged groups. Therefore, we re-emphasize that in studies of experimental models of stroke, findings obtained from aged animals are more relevant to clinical situation.

In the present study, high mortality was also a problem and about 68% of animals died before the 7thday and were excluded from present study that it was the limitation of this study. We suggest that more studies with larger sample size be done in aged and middle-aged animals.

Conclusions

Findings of present study demonstrated that post-stroke treatment BMSCs with T3 or mild exercise have no beneficial effects in recovery of ischemic injury seven days after MCAO in middle-aged mice. We suggest that in stroke research, aged animals be used for evaluation of the effect anti-ischemic potential agents against brain injury, as these results are more clinically applicable.

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Conflict of interest

We have declared no conflicts of interest.

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Legends to Figures

Fig.1. Present of brain damage (A) and microphotographs of cresyl violet staining in sham operated, PBS (control), BMSCs, EX, T_3, BMSCs+T_3, BMSCs+EX and BMSCs+EX+T_3 groups, seven days after MCAO in mice. Values are as mean ± SEM.

Fig.2. Neurological deficit score (A) and beam balance score (B) in sham operated, PBS (control), BMSCs, EX, T_3, BMSCs+T_3, BMSCs+EX and BMSCs+EX+T_3 groups, seven days after MCAO in mice. Values are as median ± IQR (interquartile range).

Fig. 3. TUNEL staining image (A) and quantitative analysis of number of TUNEL positive cells (B) in the sham-operated, PBS (control), BMSCs, EX, T_3, BMSCs+T_3, BMSCs+EX and BMSCs+EX+T_3 groups, seven days after MCAO in mice. The number of TUNEL-positive cells (brown) was visualized using a Reichert microscope with a ×40 magnification. Values are as mean ± SEM

Fig.4. Photomicrographs of GFAP-positive cells (A) and quantitative analysis of the number of GFAP-positive cells (B) in the sham-operated, PBS (control), BMSCs, EX, T_3, BMSCs+T_3, BMSCs+EX and BMSCs+EX+T_3 groups, seven days after MCAO in mice. Values are as mean ± SEM. *$p < 0.001$ compared to the PBS group. # $P < 0.001$, compared to the sham-operated group. The number of GFAP-stained cells (brown) was visualized using a Reichert microscope with a ×40 magnification.