European Review for Medical and Pharmacological Sciences 2015; 19: 1994-2000

Effect of 5-fluorouracil mobilized bone marrow regenerative cells transplantation on brain injury following focal cerebral ischemia in rats

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Abstract. – OBJECTIVE: The aim of this study was to evaluate the effect of 5-fluorouracil mobilized bone marrow regenerative cells (BMRCs) transplantation on brain injury following focal cerebral ischemia and to explore the mechanisms.

MATERIALS AND METHODS: Male Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MACO) for 120 minutes, followed by intravenous administration of DAPI-labeled 1×10^7 5-fluorouracil mobilized BMRCs at 24 h post MACO. Infarct volumes, neurological deficit score, angiogenesis and cytokine expression were evaluated at specific time after cell transplantation.

RESULTS: Comparable number of BMRCs and bone marrow cells (BMCs) were found in the infarcted area at Day 3 and Day 14 post MACO. Significant decreased infarcted volume and neurological deficit score were found in the animals receiving BMRCs. The microvessel density was significantly increased after BMRCs transplantation. Moreover, the expression of vascular endothelial growth factor (VEGF) was increased significantly after BMRCs transplantation.

CONCLUSIONS: Intravenous 5-fluorouracil mobilized BMRCs showed neuroprotective following MACO and might be considered as therapeutic choice in the treatment of transient focal cerebral ischemia.

Key Words:

Bone marrow regenerative cells, 5-fluorouracil, Cerebral ischemia, Neuroprotection.

Introduction

Ischemic brain injury resulting from diseases such as stroke is the second largest cause of death over the world[1] and a leading cause of lethality and disability in western countries and China[2]. Although great progresses have been made to know the molecular consequences of ischemic brain injury, few therapeutic treatments have proved effectively in clinical trials.

As the main hematopoietic organ, bone marrow contains various types of stem/progenitor cells, including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs)[3]. Transplantation of bone marrow cells (BMCs) has been suggested as an effective "regenerative" therapy for focal cerebral ischemia. BMCs can not only differentiate into neuronal cells, astrocytes and endothelial cells after cerebral ischemia but also activate endogenous regenerative responses in injured brain, including angiogenesis, neurogenesis and synaptogenesis[5,6]. Moreover, several combination treatment therapies have been proposed recently in order to maximize the therapeutic effect[7]. However, the heterogeneity of the bone marrow may a hindrance to achieve the optimal therapeutic effect.

Previous studies have been demonstrated that *in vivo* treatment with 5-fluorouracil (5-FU), a nucleotide analogue that is incorporated into DNA during the S-phase of the cell cycle leads to cell death of cycling cells and enhances the osteogenic potential of stromal cells *in vitro*[8,9]. By taking this advantage, Wang et al[10] proposed 5-FU could be employed for bone marrow cell purification. Therefore, appropriate treatment of BMCs will help improve the outcome. In present study, we used 5-FU mobilized bone marrow regenerative cells (BMRCs) in treatment of cerebral ischemia and examined the functional improvement in the rat middle cerebral artery occlusion (MACO) model after the transplantation of BMRCs.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (280-320 g) (certificate SCXK2006-0005; Shanghai

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Laboratory Animals Ltd Co. Shanghai, China) were used. All the rats were housed in 12-h light/12-h dark cycle conditions and subjected to MCAO. MACO was successfully achieved in 54 rats and these rats were then randomly divided into three groups: vehicle control group, BMCs group and BMRCs group. Ten male rats weighed 100-150 g were used to prepare BMCs. All the experimental procedures had been approved by the Institutional Animal Care and Use Committee of our institute.

Isolation and Labeling of BMCs and BMRCs

After tail vein injection with or without 5 FU (125 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) for 7 days, BMCs and BMRCs of male Sprague–Dawley rats were isolated according to previous description[11]. After obtaining the cells, an adequate number of BMCs and BMRCs were stained with 4',6-diamidine-2-phenylindole (DAPI) (50 mg/L; Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 60 min and the cell labeling were confirmed by microscope before transplantation.

Ischemia Model

The rats were anesthetized with 10% (chloral hydrate (3.0 ml/kg, i.p.; Sigma-Aldri St. Louis, MO, USA) and MAC l was e tablished according to the m ibed by Longa et al[12]. Briefly, the c ion ca d artery (CCA), external carotid (EC nal carotid artery (IC o carefully exposed vi midline cal incision. The ECA and the A were dou gated using a silk sutur n rubber-co round-tip nylon surg thre as inserted into the ICA (appr ately 18 m the bifurcation) via a sm puncture in the to occlude the origin he mi le cerebral artery (MCA). A silk e aro the CCA was tightened to prevent b om th ncture site. After 120 of M occlu , reperfusion was per- by w g the suture. The successful e lishme model was confirmed when the ll to the left and exhibited a circling be- h n, the rats were kept under con- t living conditions and had free access to and water. Twenty-four hours after MCAO, rat e transplanted with 1×10^7 BMCs or BMRCs by tail vein injection and in the vehicle control group the same volume of phoshate buffered saline (PBS) was injected.

Assessment the Transplanted Cells in Infarct Region

Three days or 2 weeks after transplantation, 20 μm serial coronal sections of the infr a- gions were obtained for cell survi Five sections were taken from ea at and ob served under the microscope (and DAPI positive cells were counted.

Neurologic Function eficits Assessment

On days 7, 14 a 4 afte nsplantation, neurologic funct na were sured with double bli odifi urolo Severity Score (mN method[13]. urement is a composit r, sensory a flex tests. In the seve sco injury, 1 score point is awarded for the ina to perform the test or for k of a teste ex; thus, the higher s , the more severe is the injury. Neurological ction was gr d on a scale of 0-18 (normal e 0, maxima ficit score 18)

Me f Infarct Volume

To the infarct volume, rats were killed with overdosed anesthesia 14 days after Brains were removed quickly and cryo- at ion was performed. After that, slices were immersed in 2% triphenyltetra-zoliumchloride (TCC; Sigma-Aldrich, St. Louis, MO, USA) solution, and kept at 37°C in a water bath for 30 min. The slices were then transferred to 10% buffered formalin. About 24 h later, slices were photographed and the infarct areas were measured using the Image Tool software. The infarct volume of each slice was calculated by equation as follows: $V = t \times (A1+A2+A3+A4+A5)-(A1+A2) \times t/2$ (A represents the infarct area of the slice, t represents the slice thickness).

Immunohistochemistry

Immunohistochemistry was performed using coronal frozen brain sections prepared from the rats receiving different treatment on Day 14 post MACO. Briefly, the sample sections were incubated with 10% goat serum in PBS to block nonspecific reactions, followed by incubation with rabbit polyclonal antibodies against vWF (1:50, DAKO, Carpinteria, CA, USA) as a marker of endothelial cells overnight at 4°C. Then, the sections were processed with biotinylated goat anti-rabbit IgG or anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h, followed by avidin-

biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min. The labeled secondary antibodies were visualized using diaminobenzidine. Each process was followed by several brief washes with PBS. The vWF positive cells in the cortical ischemic boundary zone were counted at five randomly chosen square fields (200 ×).

RT-PCR

Rat ischemia penumbras from these three groups were isolated as previously described[14] and total RNA were exacted by using Trizol reagent (Life Technology, Gaithersburg, MD, USA) according to manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was prepared from the RNA by PrimerScript reverse-transcriptase (Takara Biomedical, Dalian, China). Semi-quantitative PCR reactions were carried out using rat VEGF specific primers: forward primer 5'-AGA TCC ACC TCA CTG TAG CTG TGC-3', reverse primer 5'-GTG ACA TCA CCG CAG ACA AAC ATG-3'; and internal control β-actin primer: forward primer 5'-AAG TGT GAC GTT GAC ATC CGT AAA G-3' and reverse primer 5'-CAG CTC AGT AAC AGT CCT AGA-3'. Electrophoresis bands of amplification products were photographed and quantified using Image J software (Image Pro® software, Media Cybernetics, Silver Spring, USA).

Statistical Analysis

All statistics were performed using SPSS 14.0 software package (SPSS Inc., Chicago, IL, USA). All the datas were expressed as mean ± SD. Differences between individual groups were compared using analysis of variance (One-way ANOVA) and a value of $p < 0.05$ was considered statistically significant.

Results

Comparable number of BMRCs and BMCs in the Infarct area

We first evaluated the adverse effect exerted on bone marrow cells after 5-FU treatment. By comparing with the BMCs group, no significant difference was found on the rat's cortex and the infarct area (Figure 1A). Moreover, we found a comparable number of BMRCs and BMCs in the infarct area on day 3 (148.33 ± 17.56 vs. 144.00 ± 16.01, $p > 0.05$) and day 14 (49.33 ± 13.01 vs. 43.00 ± 10.08, $p > 0.05$, Figure 1B).

BMRCs Exerted Certain Neuroprotective Role in the Cerebral Ischemia

As shown in Figure 2A, the brain section in the ischemic region showing BMRCs showed less severity of injury than BMCs group and vehicle control group. The infarct size calculation showed that a significant decreasing was observed in animal received BMRCs than BMCs group ($p < 0.05$) and vehicle control ($p < 0.01$) (Figure 2B). Furthermore, the neurological deficit score was also evaluated and the results showed that a significant lower score was found in the rats of BMRCs group on day 7 and Day 14 than BMCs and vehicle control group while no significant difference was found at 24 h (Figure 2C).

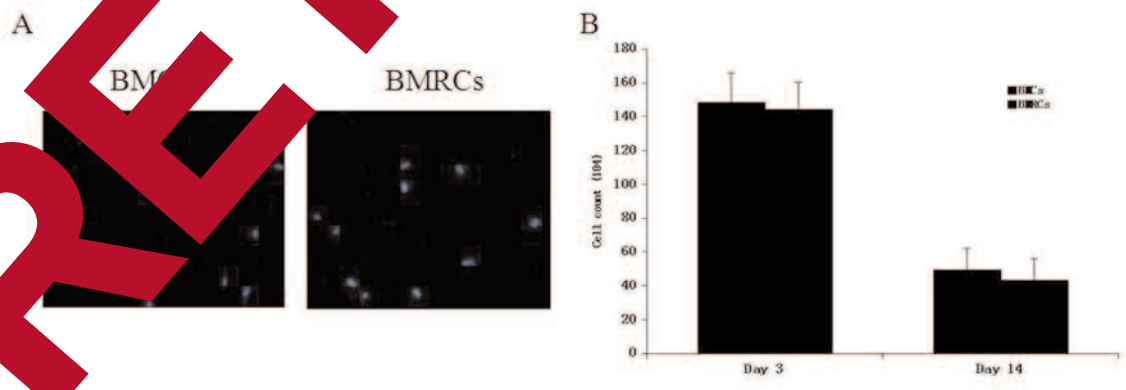

Figure 1. Comparable number of DAPI-label bone marrow cells (BMCs) and bone marrow regenerative cells (BMRCs) were found in infarcted area at day 3 and day 14 post middle cerebral artery occlusion (MACO). ***A,*** DAPI labeled BMCs and BMRCs under microscope (200×); ***B,*** Cell count of DAPI labeled BMCs and BMRCs.

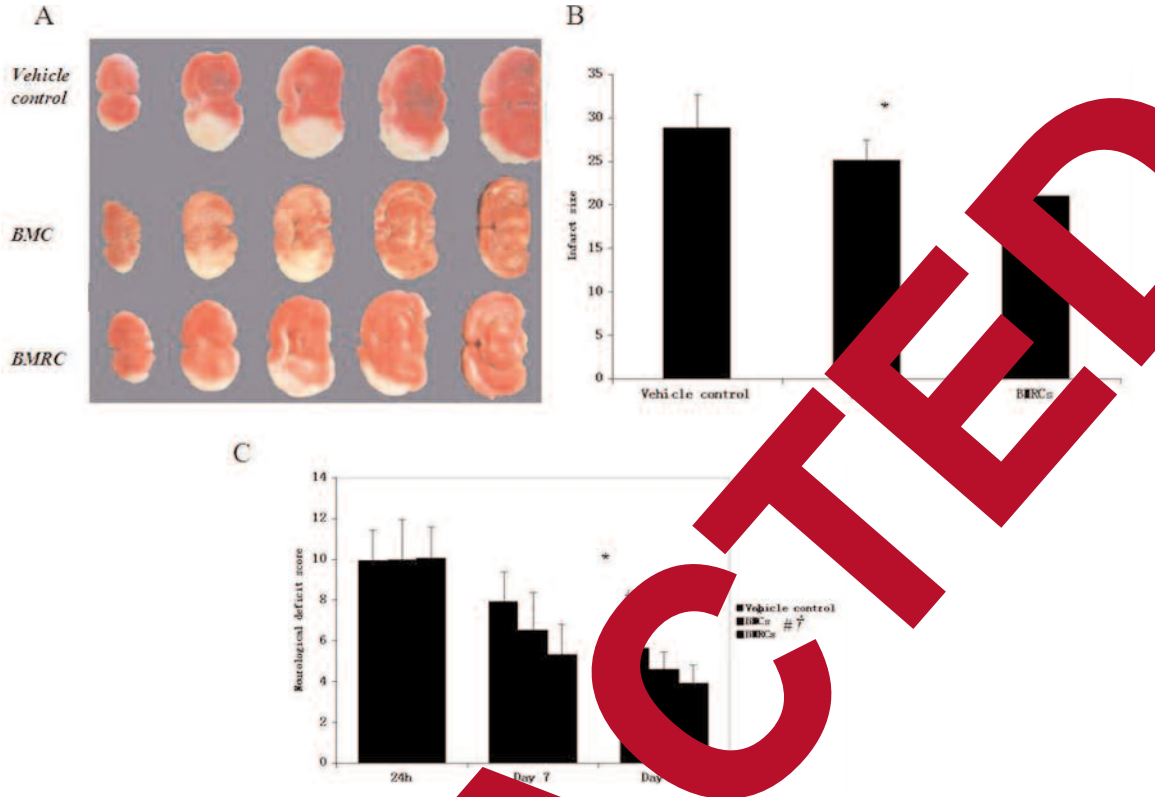

Figure 2. Neuroprotective role of transplanted [illegible] erative cells (BMRCs). ***A,*** Representative microphotographs of TTC stained brain sections in animals re[illegible]g vehi[illegible]arrow cells (BMCs) and BMRCs on day 14 post middle cerebral artery occlusion (MACO); ***B,*** Quantita[illegible]anal[illegible]of t[illegible]farct size in animals receiving vehicle, BMCs and BMRCs on day 14 post MACO model; ***C,*** Neurological [illegible]ore in animals receiving vehicle, BMCs and BMRCs at 24 h, Day 7 and Day 14 post MACO model. *represents $p < 0.0$[illegible]veen vehicle control and BMCs group; #represents $p < 0.05$ between vehicle control and BMRCs gr[illegible]nts $p < 0.$[illegible]ween BMCs and BMRCs group.

Increased Micr[illegible]sel Densit[illegible]er BMRCs Treat[illegible]

We also as[illegible]sed t[illegible]rovessel density in the experi[illegible]t rats by i[illegible]ohistochemistry analysis[illegible]e brain section a[illegible]ound that a significa[illegible]creasi[illegible]n the microvessel density in BM[illegible]group[illegible]BMCs group ($p < 0.05$) (Figure 3 [illegible]

[illegible]sed [illegible]on of VEGF was Fou[illegible] After [illegible]Cs Treatment

S[illegible]aracrine cytokines play an critical role o[illegible]utic effect of bone marrow cells, [illegible]assess the expression of VEGF in the rats re[illegible]ng BMRCs, BMC and vehicle control at 24[illegible]3 and day 7, and found that a significant increased expression of VEGF was found in BMRCs group on day 3 (p <0.05) and day 7 (p < 0.05) than BMC group (Figure 4A & B).

Discussion

Great promise was exhibited by stem cell therapy according to the experimental data which suggests it will have benefit in clinical practice[15,16]. Many different type of cells, including bone marrow stromal cells (BMSCs), BMCs, endothelial progenitor cells, umbilical cord stem cells, adipose stem cells and neural stem cells, have shown their benefic in rat models of stroke[17-19]. Transplantation of either BMMCs or BMSCs was also proved to have therapeutic effects in rat models of brain ischemia and spinal cord injury[20,21]. Preparation of BMSCs requires *in vitro* expansion to fulfill a certain amount of cells before transplantation, whereas BMCs can be obtained in a short period of time prior to administration. Thus, the use of BMCs holds some advantageous on reducing

time consuming in the treatment of acute phase transplantation. Several routes of administration to administer BMMCs in the ischemic stroke model have been reported, including intra-arterial, intravenous and intra-cerebral transplantations[22]. Intravenous administration is the most non-invasive and relative easily performed method. Therefore, we chose intravenous bone marrow transplantation in this study.

In the present study, infarct volumes assessed by TTC-stained sections were significantly smaller in the BMRCs groups (1×10^7 cells) on Day 14 compared with the BMCs and vehicle control group. In addition, the mean infarct volume was smallest in BMRCs group among the three groups. The neurological deficit score was significantly improved in the BMRC group on Day 7 and Day 14, compared with the BMCs and vehicle control group. To date, the effects of 5-FU mobilized BMRC transplantation on neuroprotection have not been elucidated yet. 5-FU, a nucleotide analogue that is incorporated into DNA during the S-phase of the cell cycle leads to cell death of cycling cells and enhances the osteogenic potential of stromal cells *in vitro*[23]. Wang et al[10] once used 5-FU to enrich and purify the mesenchymal stem cells in murine b- r-row. We employed the 5-FU here t- progenitor cells in bone marrow - nquer th possible inference exerted by ur- d cells presented in the bone marrow. Ther- we proposed here is a revised pro- l by t- MC- in the treatment of cerebr- chemia.

Angiogenesis is go- indicat- of imp- ment of cerebral is- ia[24] a- e also examined the microves- l d- he rat- h different treatmen- he mi- ssel f- ation in BMRCs grou- s significa- than BMCs group. - rted endot- progenitor cells (EP- n b- arrow can differentiate into endothelial cells a- rticipate in angiogenesis[25]. 5-FU treatm- ore EPCs could be o- ed and the angiogenesis might be enhanced - ncreased nu- er of EPCs.

urthermore, - ny researchers have shown t- e secrete- ytokines and growth factors,

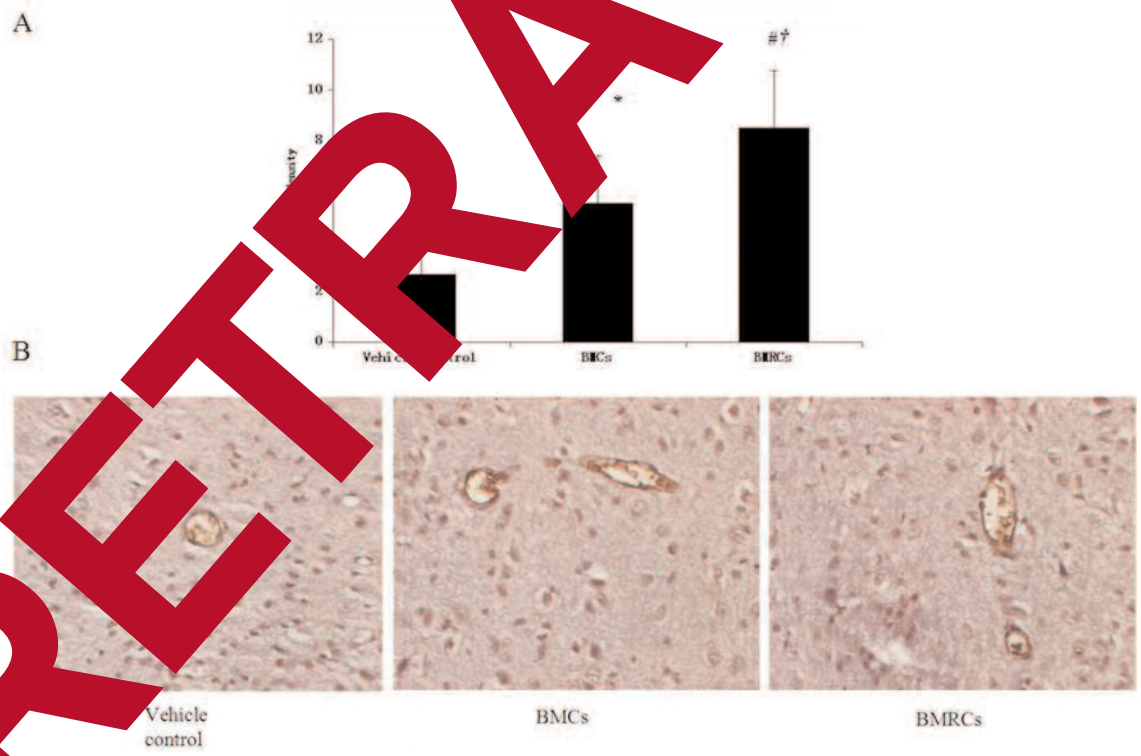

Figure 3. Pro-angiogenesis effect of transplanted bone marrow regenerative cells (BMRCs). ***A,*** The microvessel denstiy in anim- eceiving vehicle, bone marrow cells (BMCs) and BMRCs at day 14 post middle cerebral artery occlusion (MACO); ***B,*** Immunohistochemial staining of the endothelial cells in animals receiving vehicle, BMCs and BMRCs at day 14 post middle cerebral artery occlusion (MACO) (vWF staining, 200×). *represents $p < 0.05$ between vehicle control and BMCs group; #represents $p < 0.05$ between vehicle control and BMRCs group; †represents $p < 0.05$ between BMCs and BMRCs group.

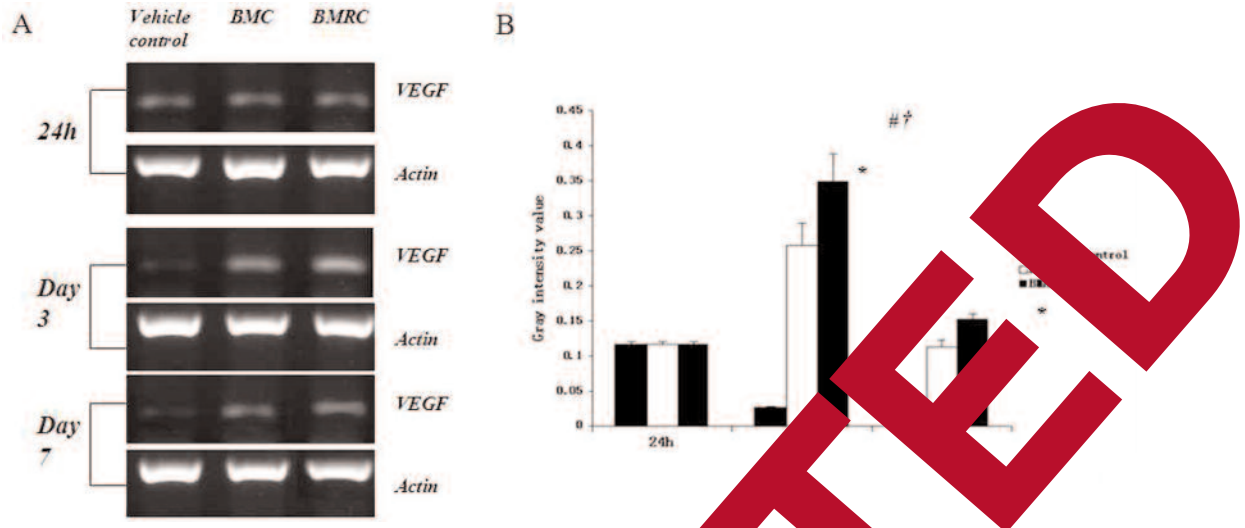

Figure 4. Increased expression of vessel endothelial growth factor (VEGF) after bone marrow regenerative cells (BMRCs) transplantation. ***A,*** The expression of VEGF in animals receiving vehicle, bone marrow cells (BMC) and BMRCs at 24 h, Day 3 and Day 7 post middle cerebral artery occlusion (MACO); ***B,*** Gray intensity value of the VEGF in animals receiving vehicle, BMCs and BMRCs at 24 h, day 3 and day 7 post MACO. *represents $p < 0.05$ between vehicle control and BMCs group; #represents $p < 0.05$ between vehicle control and BMRCs group; †represents $p < 0.05$ between BMCs and BMRCs group.

including brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), VEGF play an important role in promoting repair of the injured nerve and improve neuroprotection and vasculo-genesis[26]. We found an enhanced secretion behavior of BMRC (data not shown) in above factors. Moreover, we examined the expression of VEGF in the tissue and found a significant increased amount of VEGF in the BMRCs group.

Conclusion

We proposed here is a modified therapy by using mobilized bone marrow regenerative cells in treatment of cerebral ischemia. However, we could not identify the exact cell which contributes maximal therapeutic effect in the treatment. On the other hand, secretion profile of modified bone marrow cells or cytokine combination also be considered as therapeutic choice in cerebral ischemia treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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