

Original Article

Stroke induces the mobilization and maturation of endothelial progenitor cells

Min Cheng¹, Xuhua Liang¹, Yilin Zhang¹, Yanyan Zhao¹, Xiaobin Zhang¹, Shucun Chen², Hui Cheng³

¹College of Biopharmaceutical Engineering and Food Science, Shangluo University, Shangluo, Shaanxi, China;

²Shangluo Chinese Medicine Hospital, Shangluo, Shaanxi, China; ³Department of Emergency, Shangluo Central Hospital, Shangluo, Shaanxi, China

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Abstract: Stroke is an acute cerebrovascular ischemic incidence which causes significant mortality and morbidity worldwide. The ischemia triggers a cascade of pathiophysiological and molecular changes resulting in secondary injuries. Meanwhile, stroke also prompts endothelial progenitor cells (EPCs) to mobilize from bone marrow into circulation to facilitate the vascular repair. This study aimed at characterizing the properties of such EPCs from both stroke patients and healthy control. The level of EPCs in the mononuclear cell population was measured by flow cytometry. The morphological changes of EPCs were monitored by *in vitro* culture and confirmed by Dil-ac-LDL and FITC-UEA1-Lectin double staining. The proliferation of EPCs was analyzed by MTT assay and motility by transwell migration assay. Stroke significantly increases the levels of VEGFR2-positive EPCs in peripheral blood with higher migrating ability. The circulating EPCs from stroke patients were more differentiated towards endothelial cells compared to those in healthy individuals. At early culture stage, patients' EPCs proliferated significantly faster than those from controls while the EPCs of controls grew quicker than those of stroke patients at later stage of *in vitro* culture. In conclusion, stroke promoted the mobilization and differentiation, increased the motility, and enhanced the early stage proliferation and EPCs, which implied that later stage EPCs might be a more efficient cell therapy agent than naïve EPCs in treating stroke and other vascular ischemia.

Keywords: Stroke, endothelial progenitor cells, proliferation, migration, maturation

Introduction

Stroke is a devastating pathological event and one of the most frequent causes of death worldwide, accounting for about 11% of the total death (6.2 million in 2011) [1]. It is also the most common cause of acquired disability of adult in the world [2, 3]. Roughly 17 million people experienced a stroke in 2010 and 33 million people were alive after stroke [4]. The stroke incidence, prevalence, mortality, and disability-adjusted life-years (DALYs) lost are great and increasing [4], which imposes a great burden on the society humanly, emotionally, and economically.

Stroke is an acute onset clinical condition following a vascular insult to the brain. The most common forms of stroke are resulted from thromboembolism and *in situ* thrombosis, which constitutes 80%-85% of all strokes [5].

After vascular congestion, a chain of events occurs leading to irreversible tissue injury, including energy deficiency, loss of transmembrane ionic gradients, cell depolarization, and excitotoxicity. These processes result in rapid cell necrosis of neurons, glia, and blood vessels in the ischemic core [5]. A collateral blood supply sufficient for cell viability is transiently maintained in regions around the core (the ischemic penumbra). The penumbral tissue can be rescued by perfusion and timely recanalization following ischemic stroke is the best way to achieve good clinical prognosis [6].

Vascular endothelium is a dynamic organ that influences vascular tone, monocyte adhesion, smooth muscle cell proliferation, and platelet aggregation [7, 8]. Endothelial dysfunction is the root cause of acute vascular event such as stroke and myocardial infarction [9]. It has been shown that circulating bone marrow derived-

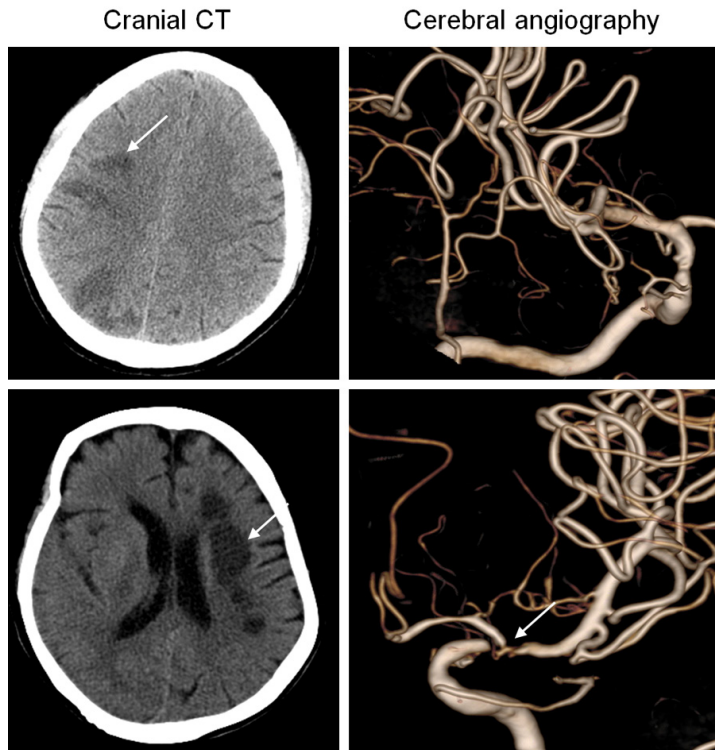


Figure 1. The images of cranial CT scan and cerebral angiography images of two patients.

Table 1. Clinical data of stroke patients and healthy subjects

	Controls	Patients
Gender		
Male	16	15
Female	14	15
Age (year)		
Average (range)	67.3 (55-70)	66.8 (55-70)
Indications		
Hyperlipidimia		11
Hypertension		17
Diabetes		5
Highhomocysteine		12
National Institutes of Health Stroke Scale (NIHSS)		
Average (Range)		23 (7-36)

endothelial progenitor cells (EPCs) are responsible for the repair of injured endothelial monolayer and its level reflects endothelial repair capacity. The status of circulating EPCs serves as a marker of endothelial dysfunction and vascular health, and the level of circulating EPCs could be used as an indicator of collective

cardiovascular risk [10, 11]. The reduction of circulating EPCs is an independent predictor of atherosclerotic disease progression and future cardiovascular events [12]. On the other hand, a quick mobilization from bone marrow and a rapid increase of circulating EPCs have been associated with Moyamoyadisease, acute myocardial infarction, and acute ischemic stroke [13-15]. However, the physiological characteristics of the EPCs after acute stroke have not been well studied. This study aimed to characterize the EPCs from stroke patients in terms of proliferation, mobility, and long-term viability in comparison to the EPCs from healthy individuals.

Materials and methods

Study subjects

This study protocol is in conformity with the Declaration of Helsinki and approved by the institution review committee of Chongqing Medical University. A signed written informed consent was obtained from each participant.

Thirty stroke patients between 55-70 years old after first stroke and confirmed as a single lesions infarction by cranial CT scan and cerebral angiography (**Figure 1**) and 30 gender and age-matched healthy individuals (**Table 1**) with the absence of previous heart and brain vascular infarction confirmed by cranial CT scan and electrocardiogram were recruited. Fifteen milliliter peripheral blood was drawn from healthy controls or from patients 7 days after stroke.

Isolation and identification of EPCs

Mononuclear cells were isolated from citrate-anticoagulated peripheral blood by Percoll density gradient centrifugation and cultured in DMEM containing 10% FBS, 10 ng/ml recombinant vascular endothelial growth factor (rhVEGF), 10 ng/ml basic fibroblast growth factor (bFGF) (both from ProSpec, East Brunswick, NJ), 100 µg/ml ampicillin, and 100 U/ml streptomycin (All media, serum, and antibiotics are from Invitrogen, Shanghai, China) at 37°C in a humidified condition with 5% CO₂.

After 10-day culture, cells were washed with PBS three times for 5 min each and incubated in complete media containing 10 µg/mL Dil-ac-LDL (Sigma, St Louis, MO) for 4 h at 37°C, 5% CO₂. Cells were then washed three times with PBS for 5 min each, fixed with 3% paraformaldehyde for 15 min, and incubated with 10 µg/ml FITC-UEA-1 (Invitrogen, Shanghai, China) at room temperature for 1 h. Cells were observed using a Nikon eclipse E600 fluorescence microscope (Nikon, Shanghai, China).

MTT assay

After specified culture time, the medium was removed and replaced with culture medium containing 0.5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, Mo.). The cells were cultured at 37°C for 4 hours before removing medium, adding 150 µl dimethyl sulfoxide (Sigma, St. Louis, Mo.), and shaking in dark for 10 min. The absorbance was measured on a microplate reader (Molecular Devices, Shanghai, China) at a wavelength of 490 nm.

The motility of EPS assessed by migration assay

The EPCs cultured 3 days *in vitro* were used in migration assay. A aliquot of 200 µl of the EPCs (5×10⁴/ml) cell suspension was placed into the upper chamber of a Corning Transwell 24 Permeable Support Culture Plate (Sigma, St. Louis, MO) and 500 µl complete medium containing 50 ng/ml rhVEGF and 10 ng/ml SDF-1α (ProSpec, East Brunswick, NJ) into the lower chamber. The cells were cultured 24 hrs before the membrane was fixed with 10% cold methanol at 4°C for 10 min. The cells on the top side of the membrane were wiped off and the membrane was stained with crystal violet. The number of migrated cells was counted in 5 random fields (×200).

Flow cytometry determination of the number of EPCs in peripheral blood

The mononuclear cells were isolated from 6 ml peripheral blood by density centrifugation to obtain and treated with 100 µl erythrocyte lysis buffer in dark for 10 min. The cells were washed with PBS and suspended in 200 µl of PBS followed by incubating with 5 µl anti-VEGFR2-Phycoerythrin (PE) in dark at room temperature for

45 min. The cells were then washed twice with PBS and resuspended in 100 µl PBS before being analyzed on a BD Accuri C6 flow cytometry (BD, Shanghai, China). The data was analyzed with CELLQuest software (BD, Shanghai, China).

Statistical analysis

The data were expressed as mean ± standard deviation. The difference between the groups was determined using a two-tailed Student's t-test. A *P* values less than 0.05 was considered statistically significant.

Results

Stroke increased the level of EPCs in peripheral blood

The average levels of VEGFR2-positive EPCs in the mononuclear cell population of stroke patients 7-day post stroke was 6.13% (ranging 4.6%-9.2%) and that of healthy controls was 3.14% (1.3%-4.9%) (*P*<0.0001) (**Figure 2**).

Stroke promoted the differentiation of EPCs into endothelial cells

The EPCs from stroke patients were able to differentiate into endothelial cells quicker than their counterpart from healthy individuals. After 12 hrs *in vitro* culture, while there were hardly any cell with morphology observed in the EPCs from controls, few EPCs from stroke patients already attached to culture plates and had round shape (**Figure 3A**). After 3 days, the patient source EPCs started to show spindle shape while the EPCs from controls had irregular shapes and evolved into spindle shape at day 7 when the majority of the EPCs from patients already possessed the morphology of cobblestone (**Figure 3A**). Double-labeling with Dil-ac-LDL and FITC-UEA-1 also showed a similar trend that EPCs from healthy controls had mostly spindle shape with those from stroke patients showed a cobblestone morphology (**Figure 3B**).

Stroke changed the growth characteristics of EPCs

The EPCs from stroke patients grew significantly faster than those from normal controls at the early stage of culture *in vitro* but their prolifera-

EPCs of stroke patients

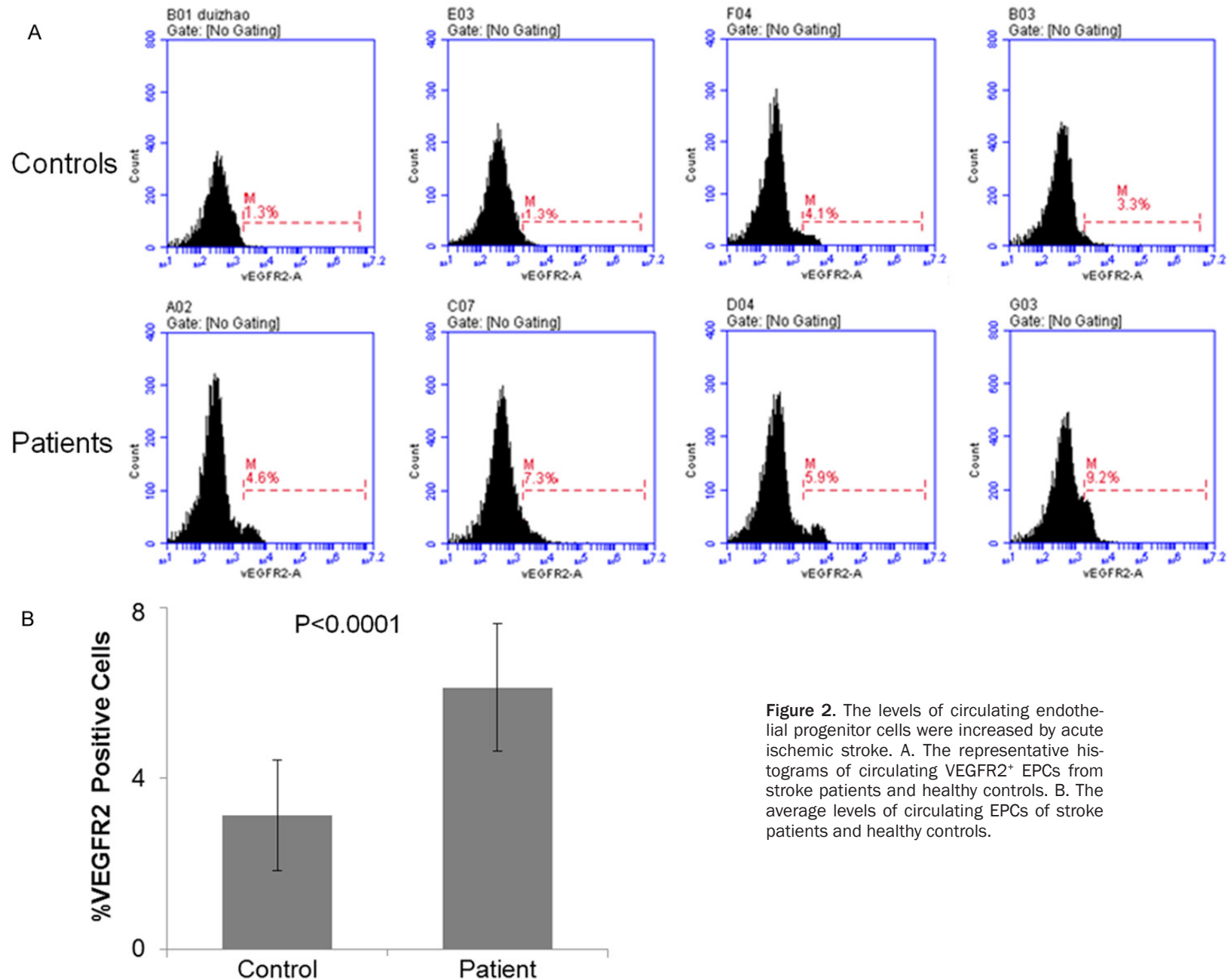


Figure 2. The levels of circulating endothelial progenitor cells were increased by acute ischemic stroke. A. The representative histograms of circulating VEGFR2⁺ EPCs from stroke patients and healthy controls. B. The average levels of circulating EPCs of stroke patients and healthy controls.

EPCs of stroke patients

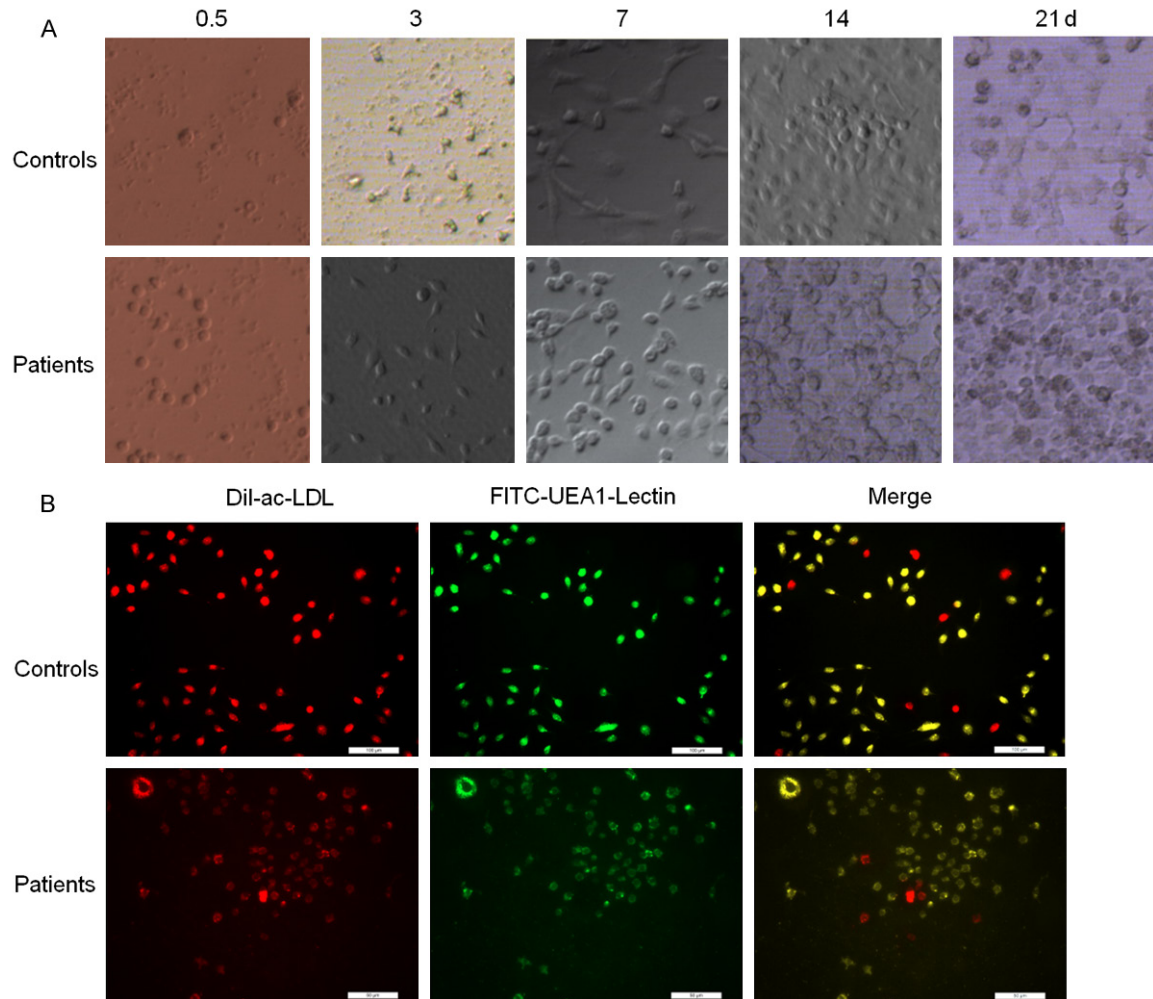


Figure 3. The growth and differentiating characteristics of EPCs from stroke patients were different from those of controls. A. The morphological changes of circulating EPCs during in vitro culture. B. The EPCs were identified by DiI-ac-LDL and FITC-UEA1-Lectin double staining after 10-day culture. The majority of EPCs from controls were showing a spindle shape while most patients' EPCs had cobblestone morphology.

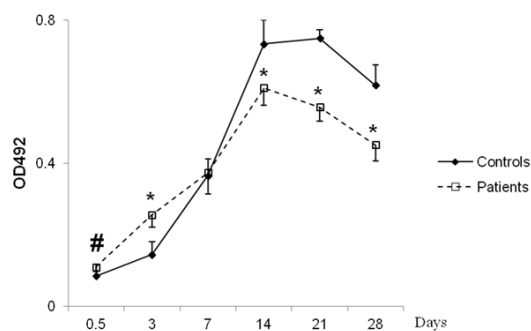


Figure 4. The proliferation rate of circulating EPCs was affected by stroke. The proliferation of EPCs was measured with MTT assay at different culture time.

tion was markedly slowed down at later culture time compared EPCs from healthy individuals

(Figure 4). The OD 490 from MTT assay for EPCs from stroke patients was 0.11 and 0.256 after 12 hrs and 3 days culture, which was significantly higher than the reading of 0.085 ($P<0.05$) and 0.144 ($P<0.01$) at those two time points for healthy controls. This growth trend was reversed after 7 days of culture (Figure 4).

Stroke enhanced the motility of the early stage EPCs

The EPCs from stroke patients showed stronger migrating ability in transwell assay, where seen an average of almost 4 times more migrated cells from patients' EPCs than that of healthy controls' EPCs (387.3 cells vs 104.5 cells, $P<0.0001$) (Figure 5).

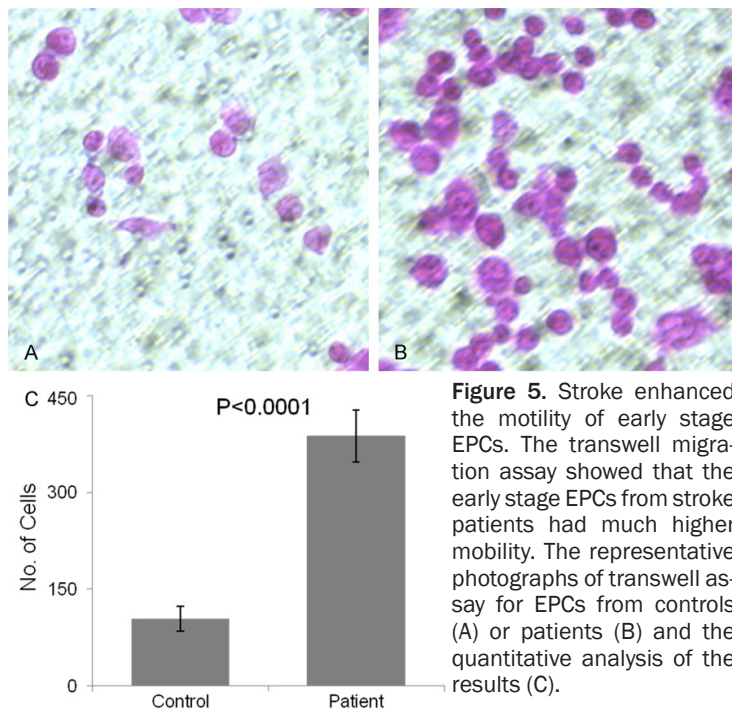


Figure 5. Stroke enhanced the motility of early stage EPCs. The transwell migration assay showed that the early stage EPCs from stroke patients had much higher mobility. The representative photographs of transwell assay for EPCs from controls (A) or patients (B) and the quantitative analysis of the results (C).

Discussion

The cerebrovascular ischemia caused by stroke causes a wide range of physiological and molecular changes. EPCs were mobilized after stroke resulting in significantly higher percentage of EPCs in the mononuclear cell population of patients than that in healthy subjects. The EPCs from stroke patients had higher proliferating power at very early stage in *in vitro* culture and exhibited higher differentiation tendency.

Acute ischemic stroke caused EPC mobilization and increased the number of circulating EPCs [15, 16] and endothelial cells [16]. The number of circulating EPCs was peaked at day 7 post-stroke and then tapered off [15, 17]. However, the levels of CD133⁺/CD34⁺ and VEGFR2⁺/CD34⁺ EPCs were found significantly decreased 2 days after acute ischemic stroke [17, 18]. The inconsistency among those studies might root from the analyzed population of CD133⁺/CD34⁺/VEGFR2⁺ EPCs [15] and those of CD133⁺/CD34⁺ [18] and VEGFR2⁺/CD34⁺ cells [17, 18]. The difference might also be due to the disease types, time points, and study population.

The level of circulating EPCs has been shown to predict the long term prognosis in ischemic stroke patients [15, 18-21] and db/db mouse

model [22], which provided the rationale for stem/progenitor cell therapy. Injecting human circulating EPCs [23], human late outgrowth EPCs [24], human umbilical cord blood-derived AC133⁺ EPCs [25], rat bone marrow derived EPCs [26], or CXCR4-primed EPCs [27] into rodents with middle cerebral artery occlusion significantly reduced infarct volume, cortex atrophy, neuronal apoptosis, and inflammation and improved neurobehavioral outcomes. Those results further confirmed the potential of using EPCs as a therapeutic agent for stroke and other acute vascular incidences.

With *in vitro* culture and Dil-ac-LDL and FITC-UEA-1-Lectin double staining [28], we demonstrated that acute stroke increased

the number of circulating EPCs, promoted their differentiation into endothelial cells, and increased their migrating ability, which would help the repair of injured vascular endothelium. The two phased *in vitro* proliferation rate of EPCs from stroke patients in comparison with EPCs from health subjects confirmed that patients' EPCs were at a later differentiation stage. It would be medically important to investigate whether transplantation of late stage EPCs could be more efficient in managing stroke than naïve EPCs.

In conclusion, the ischemic insult from stroke triggered the mobilization of endothelial progenitor cells from bone marrow into peripheral blood to increase the level of circulating EPCs. Meanwhile, stroke also changes the characteristics of the EPC population by increasing their motility, early time proliferation, and differentiation towards endothelial fate, which might help the neuronal and vascular recoveries.

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Disclosure of conflict interest

None.

Address correspondence to: Cheng Hui, Department of Emergency, Shangluo Central Hospital, 148 Beixin Street, Shangluo 726000, Shaanxi, China. Tel: +86 914 2381102; Fax: +86 914 2381102; E-mail: exitxiaobai@163.com

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