

Original Article

The association between hyperhomocysteinemia and cerebral small vessel disease

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Abstract: Objective: To evaluate whether hyperhomocysteinemia and cognitive status, vascular endothelial cell active factor levels, and endothelial progenitor cell (EPC) function are associated with cerebral small vessel disease (CSVD). Methods: A total of 97 patients with CSVD were retrospectively analyzed. According to their plasma homocysteine (Hcy) concentrations, the patients were divided into three groups: group A (Hcy <15 $\mu\text{mol/L}$, n=32), group B (15 $\mu\text{mol/L}$ \leq Hcy <20 $\mu\text{mol/L}$, n=44), or group C (Hcy \geq 20 $\mu\text{mol/L}$, n=21). The cognitive functions of the patients in the three groups were evaluated using the Montreal Cognitive Assessment (MoCA) Scale and the Minimum Mental State Examination Scale (MMSE). The vascular endothelial cell active factor levels, including serum intercellular adhesion molecule-1 (ICAM-1) and endothelin-1 (ET-1), were measured. The number, proliferation, migration, and adhesion of the EPCs in the peripheral blood of the patients in the three groups were also compared. Results: The MoCA and MMSE scores were the highest in group A and lowest in group C (all $P < 0.05$). The serum ICAM-1 and ET-1 levels were the highest in group C, and the lowest in group A (all $P < 0.05$). The number, proliferation, migration, and adhesion of the EPCs in group B and C were lower than they were in group A, and all the indicators in group C were lower than those in group B ($P < 0.05$). Conclusion: Cognitive impairment, the disturbance of vascular endothelial cell active factors, and a decrease in the number, proliferation, migration and adhesion of EPCs are found in patients with CSVD complicated with hyperhomocysteinemia.

Keywords: Hyperhomocysteinemia, cognition-endothelial, progenitor cell, cerebral small vessel disease

Introduction

Cerebral small vessel disease (CSVD) is a syndrome of clinical, pathological and neuroimaging manifestations, such as white matter hyperintensity and lacunar infarction, caused by intracranial arterioles, venules, and capillary lesions [1, 2]. CSVD is considered to be closely related to stroke and vascular dementia. It is reported that CSVD can cause 20% of ischemic strokes and 45% of dementia; thus, the early detection of this disease can effectively prevent stroke and vascular dementia [3]. The occurrence and development of CSVD are related to age, gender, hypertension, hyperglycemia, hyperlipidemia, smoking, etc. Recent studies have found that homocysteine (Hcy), as a naturally occurring amino acid, is closely related with extracellular matrix proliferation and endothelial dysfunction and seems to be able to cause vessel damage [4]. Zhang et al. have

demonstrated that Hcy can cause vessel damage by regulating the expression of cyclin A to inhibit the proliferation of endothelial progenitor cells (EPCs) [5]. Vermeer found that non-dementia elderly people with a plasma Hcy concentration of 13.8-45.0 $\mu\text{mol/L}$ had a 4.1-times higher risk of white matter hyperintensity than those with a plasma Hcy concentration of 5.0-8.5 $\mu\text{mol/L}$ [6]. Therefore, hyperhomocysteinemia is also considered to be a risk factor for CSVD. However, Seshadri et al. did not find any difference in the prevalence of white matter hyperintensity among patients with different homocysteine levels [7]. Therefore, the role of hyperhomocysteinemia in CSVD needs further confirmation. Decreased cognitive function and vascular endothelial dysfunction are typical clinical and pathological manifestations of CSVD [8]. EPCs are progenitor cells of the vascular endothelium and play an important role in the protection of vessel damage. Therefore, we se-

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lected patients with CSVD as research objects to explore the differences of cognitive function, vascular endothelial function, and the number and functions of the EPCs among patients with different plasma Hcy concentrations.

Materials and methods

Patients

A retrospective analysis was made on 97 patients with CSVD admitted to Chengyang People's Hospital Affiliated to The First Medical University of Shandong from March 2017 to October 2018. On the day of admission, these patients weren't allowed to have high protein food. In the morning of the next day, 2 ml of fasting venous blood was taken. A homocysteine detection kit (Guangzhou KOFA Biotechnology Co., Ltd., China) was used to determine the plasma Hcy concentration. Based on the study of Zhou et al. and the range of plasma Hcy concentrations in healthy people (5-15 $\mu\text{mol/L}$), the patients were divided into three groups: group A (Hcy <15 $\mu\text{mol/L}$, n=32), group B (15 $\mu\text{mol/L}$ \leq Hcy <20 $\mu\text{mol/L}$, n=44), and group C (Hcy \geq 20 $\mu\text{mol/L}$, n=21) according to the plasma Hcy concentration [10]. The general data of the patients in the three groups were collected and compared, including age, gender, medical history, etc. All the patients signed an informed consent and the Ethics Committee of Chengyang People's Hospital Affiliated to The First Medical University of Shandong approved this study.

Inclusion and exclusion criteria

Inclusion criteria: 1) Patients who, according to the consensus of experts on the diagnosis and treatment of cerebral small vessel disease published in the *Chinese Journal of Internal Medicine* in 2013, were found to have lacunar syndrome and cognitive affective disorders [11]. 2) Their cerebral white matter lesions, lacunar infarction, enlargement of perivascular space, and cerebral microbleeds were revealed using magnetic resonance imaging.

Exclusion criteria: 1) Patients who had subcortical lesions with a diameter >15 mm and subcortical infarction; 2) Patients who had marked carotid artery stenosis (>50%); 3) Patients who took medicine affecting their Hcy levels, cognitive function, and vascular activity such as folic

acid, vitamin B12, nitroglycerin, and metoprolol within 3 months; 4) Patients who had multiple sclerosis, carbon monoxide poisoning, or other central nervous system diseases; 5) Patients who had complications such as severe liver and kidney dysfunction, autoimmune diseases, or infectious diseases.

Montreal Cognitive Assessment (MoCA) scale

The MoCA test is a one-page, 30-point test that examines short-term memory recall, visuospatial abilities, multiple aspects of executive functions, language, orientation to time and place, attention, concentration, and working memory.

If the participants were educated for less than 12 years, the result could be increased by one point, but the total score could not exceed 30 points. Cognitive impairment was considered when the MoCA scores \leq 26 points. The participants were instructed to answer according to the instructions of each item in MoCA scale. The correct answers were scored, but the wrong answers were not, and the total scores of all the items were calculated.

Minimum Mental State Examination (MMSE) scale

MMSE is a 30-point questionnaire that examines orientation to time and place, attention and calculation, recall, language, registration, repetition, and complex commands. Cognitive impairment was considered when the MMSE scores \leq 26 points. The participants were instructed to answer according to the instructions in the MMSE scale. The correct answers were scored, but the wrong answers were not, and the total scores of all the items were calculated.

Detection of vascular endothelial cell active factors

A total of 2 mL fasting venous blood was collected the next morning after admission and placed for 20 minutes at room temperature. The upper serum was preserved after centrifugation at 12,000 g for 20 minutes. The contents of the serum intercellular adhesion molecule-1 (ICAM-1) (Jingmei Biotechnology Co., Ltd., Jiangsu, China) and endothelin-1 (ET-1) (Jingmei Biotechnology Co., Ltd., Jiangsu,

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China) were determined using an Enzyme-linked immunosorbent assay kit.

Detection of the number of EPCs

A total of 2 ml of heparin anticoagulation was taken the next morning after admission. The lymphocytes in the peripheral blood were separated according to the instructions of the lymphocyte separation medium (Solarbio Science & Technology Co., Ltd., Beijing, China). The APC-labeled anti-CD34 antibody (Thermo Fisher Scientific, USA, CD34-581-05) and the FITC-labeled anti-vascular endothelial growth factor receptor 2 (VEGFR2) antibody (Abcam, UK) were added to the lymphocytes, and the mixture was incubated in the dark for 10 minutes, then washed with phosphate buffered saline (PBS) and centrifuged. The supernatant was discarded, and PBS was added to prepare a single cell suspension. Flow cytometry (BD Biosciences, USA, AccuriC6) was used to determine the number of EPCs with double positivity for CD34 and VEGFR2 staining [12, 13].

Determination of the function of the EPCs

A total of 10 mL of heparin anticoagulation was taken on the morning following each patient's admission. The lymphocytes in peripheral blood were separated according to the instructions of lymphocyte separation medium (Solarbio Science & Technology Co., Ltd., Beijing, China). The cells were inoculated into 6-well plates at a concentration of 1×10^6 cells/mL using EGM-2 medium (Lonza, USA, CC-3162). The 6-well plates were coated with human fibronectin (Sigma, USA, ECM001) in advance, and the medium was replaced every 3 days. The cells were collected after digestion with 0.25% trypsin on the 7th day [14, 15]. After growing on a glass slide, the cells were fixed with polyformaldehyde for 10 min, and then incubated at 37°C for 1 h in the dark with Dil-ac-LDL (2.4 mg/mL) (Shanghai Fukang Biotechnology Co., Ltd., China, MP6013) and FITC-UEA-I (10 mg/mL) (Shanghai Fukang Biotechnology Co., Ltd., China, MP6308). Then the cells were washed with PBS and photographed using a confocal microscope.

An MTT assay was used to determine the proliferation of the EPCs. A total of 5×10^3 EPCs were inoculated into 96-well plates coated with human fibronectin. They were cultured at 37°C for 72 hours. 10 μ L of MTT solution (5 mg/mL)

(Shanghai Beyotime Biotechnology Co., Ltd., China, C0009) was added to each well, and then the cells were cultured for 4 hours. The culture medium was discarded, and 100 μ L of DMSO solution was added to the cells, which were shaken for 10 minutes. The absorbance values of each well were measured at 450 nm using a microplate reader.

A Transwell test was used to detect the cells' migration ability. A total of 2×10^4 EPCs were collected and suspended in a culture medium weighing 50 μ L, then they were added to the upper Transwell chamber, and then a 25 μ L culture medium was added to the lower chamber. The number of cells in the lower chamber was calculated under a microscope (200 \times) after incubation at 37°C for 24 hours.

Adhesion detection: The EPCs collected after digestion were suspended in 500 μ L of medium. Then the same number of cells was added to a 6-well plate coated with human fibronectin and cultured at 37°C for 30 minutes. The number of adherent cells was calculated using a microscope (200 \times).

Statistical analysis

SPSS 20.0 software was used for the statistical analysis. The measurement data are expressed as the mean \pm standard deviation. An independent *t* test was used for the comparisons of the measurement data between the two groups, and an LSD-*t* test was used for the comparisons of the measurement data among multiple groups. The enumeration data were expressed as the number of patients. The comparisons of enumeration data were performed using an χ^2 test and an χ^2 partition test. The difference was statistically significant when $P < 0.05$.

Results

General data

There were no significant differences among the three groups in terms of the general data including age, gender, hypertension, diabetes mellitus, and education duration (all $P > 0.05$). The plasma Hcy concentration in groups B and C was higher than it was in group A, and the concentration in group C was higher than it was in group B, with significant differences (all $P < 0.05$). See **Table 1**.

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Table 1. Comparison of the general data

Group	Group A	Group B	Group C	F or X ²	P
Number of cases	32	44	21		
Age (year)	65.50±10.70	64.80±11.60	67.30±13.00	0.329	0.720
Gender (n)				0.350	0.839
Male	19	29	13		
Female	13	15	8		
Height (cm)	165.44±12.07	167.29±11.35	165.91±11.70	0.255	0.776
Weight (kg)	64.58±9.330	64.16±8.55	65.40±9.62	0.134	0.875
Education duration (year)	10.29±3.61	10.86±4.07	8.95±3.72	1.751	0.179
Hypertension (n, %)	21 (65.63)	29 (65.91)	13 (61.90)	0.110	0.947
Diabetes mellitus (n, %)	7 (21.88)	12 (27.27)	5 (23.81)	0.302	0.86
Hypercholesterolemia (n, %)	16 (50.00)	24 (54.55)	12 (57.14)	0.289	0.866
Hypertriglyceridemia (n, %)	15 (46.88)	26 (59.09)	11 (52.38)	1.128	0.569
Smoking (n, %)	11 (34.38)	13 (29.55)	8 (38.10)	0.512	0.774
Drinking (n, %)	12 (37.50)	16 (36.36)	10 (47.62)	0.812	0.666
Homocysteine (μmol/L)	12.61±2.30	18.05±2.67*	22.03±2.89* [#]	88.110	<0.001

Note: Compared with group A, *P<0.05; compared with group B, [#]P<0.05.

Table 2. Comparison of the cognitive function

Group	Number of cases	MoCA scores (points)	MMSE scores (points)
Group A	32	22.17±2.09	27.23±2.45
Group B	44	21.05±1.71*	26.19±1.83*
Group C	21	19.40±1.96* [#]	24.68±2.10* [#]
F		13.530	9.266
P		<0.001	<0.001

Note: Compared with group A, *P<0.05; compared with group B, [#]P<0.05. MoCA, Montreal Cognitive Assessment; MMSE, Minimum Mental State Examination Scale.

Table 3. Comparison of the vascular endothelial function

Group	Case	ICAM-1 (ng/mL)	ET-1 (pg/mL)
Group A	32	241.47±32.39	24.55±7.68
Group B	44	267.98±31.76*	32.13±7.53*
Group C	21	295.20±38.80* [#]	48.34±9.62* [#]
F		16.520	55.760
P		<0.001	<0.001

Note: Compared with group A, *P<0.05; compared with group B, [#]P<0.05.

Comparison of the cognitive function

The MoCA and MMSE scores were the highest in group A and lowest in group C, with statistical differences (all P<0.05). **Table 2** shows that cognitive impairment is more severe with increasing plasma Hcy concentration.

Table 4. Comparison of the Number of endothelial progenitor cells

Group	n	Number of endothelial progenitor cells (cells/mL)
Group A	32	114.76±18.44
Group B	44	95.13±17.74*
Group C	21	62.67±12.88* [#]
F		59.070
P		<0.001

Note: Compared with group A, *P<0.05; compared with group B, [#]P<0.05.

Comparison of the vascular endothelial functions

The levels of serum ICAM-1 and ET-1 were the highest in group C and the lowest in group A, with statistical differences (all P<0.05). Both ICAM-1 and ET-1 are vasoconstrictive substances. **Table 3** shows that their levels increase with an increasing plasma Hcy concentration, indicating that hyperhomocysteinemia may cause endothelial dysfunction.

Comparison of the number of EPCs

The number of EPCs in groups B and C were lower than the number in group A, and the number in group C was lower than it was in group B, with statistical differences (P<0.05). **Table 4** reveals that the number of EPCs in the peripheral blood decreases with an increasing plas-

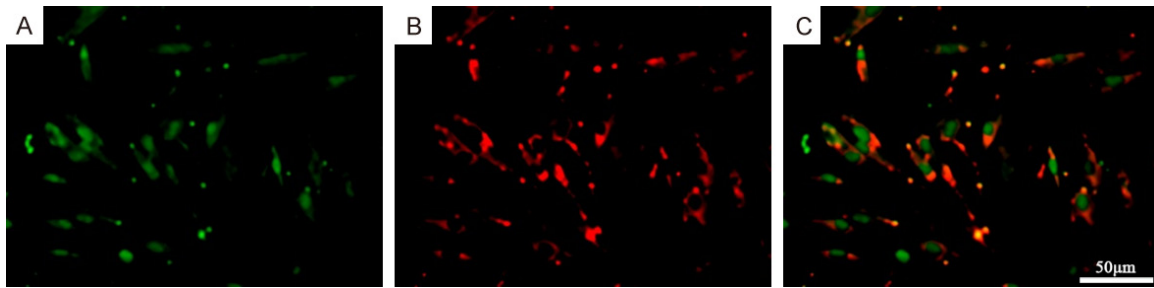


Figure 1. Identification of the endothelial progenitor cells. A. FITC-UEA-I staining (200×); B. Dil-ac-LDL staining (200×); C. Double staining (200×).

Table 5. Comparison of the function of the endothelial progenitor cells

Group	Case	Proliferation (OD value)	Number of migrated cells (200×)	Number of adherent cells (200×)
Group A	32	0.881±0.072	14.59±1.84	30.10±2.06
Group B	44	0.820±0.085*	13.13±1.52*	28.55±2.23*
Group C	21	0.744±0.069* [#]	11.82±1.95* [#]	25.48±1.67* [#]
F		19.830	16.840	31.930
P		<0.001	<0.001	<0.001

Note: Compared with group A, *P<0.05; compared with group B, [#]P<0.05. OD, optical density.

ma Hcy concentration, which may be a potential mechanism for cognitive impairment due to hyperhomocysteinemia.

Comparison of the function of EPCs

The cells with green fluorescence were positive for FITC-UEA-I, those with red were positive for Dil-ac-LDL, and those with double staining were differentiating EPCs. See **Figure 1**.

Compared with group A, the EPCs in groups B and C had lower proliferation, migration, and adhesion, and the EPCs in group C were lower than the EPCs in group B. The difference was statistically significant (all P<0.05). **Table 5** demonstrates that the function of EPCs also decreases with increasing plasma Hcy concentration.

Discussion

Recent studies have found that an elevated homocysteine level is associated with the occurrence and development of CSVD [16]. However, no unified conclusion has been reached in relevant published studies. For example, Seshadri et al. did not find a definite correlation between homocysteine and white matter hyper-

intensity presented by the typical imaging manifestation of CSVD [7]. Therefore, the role of homocysteine in CSVD needs further study.

According to their plasma Hcy concentration, the patients were divided into three groups: group A (Hcy <15 μmol/L, n=32), group B (15 μmol/L ≤ Hcy <20 μmol/L, n=44), and group C (Hcy ≥20 μmol/L, n=21). Comparing

the cognitive function in the three groups, the MoCA and MMSE scores were highest in group A, and lowest in group C, which indicated that the cognitive function of patients with CSVD decreases with an increase of plasma Hcy concentration, and hyperhomocysteinemia may aggravate the pathological process of CSVD. Yu et al. divided the patients with CSVD into a cognitive dysfunction group and a non-cognitive dysfunction group according to their MoCA scores; the results showed that the level of Hcy in the cognitive dysfunction group was significantly higher than it was in the non-cognitive dysfunction group, showing that hyperhomocysteinemia is a risk factor for cognitive dysfunction in patients with CSVD [17]. This study, based on the level of Hcy, verifies the conclusion. At present, there is no definite conclusion about the role of Hcy in CSVD [7]. The differences between relevant reports are mainly related to the number of samples, the research design, and the heterogeneity of the participants.

Vascular endothelial cell active factor disorder is a typical pathological mechanism of CSVD, which can lead to an insufficient perfusion of cerebellar vasculature, weakened local diastol-

ic function, reduced nitric oxide-mediated regulatory function, and increased blood-brain barrier permeability [18, 19]. The serum ICAM-1 and ET-1 levels in the three groups were compared in this study. The results showed that compared with group A, the serum ICAM-1 and ET-1 levels were highest in group C, followed by group B. It indicated that the serum vasoconstrictor substance levels in patients with CSVD increased with an increase of plasma Hcy concentration, which indirectly reflects the relationship between hyperhomocysteinemia and vascular endothelial dysfunction.

EPCs are precursor cells of endothelial cells, which can proliferate and differentiate into endothelial cells when the blood vessels are damaged by microorganisms and inflammation, and endothelial cells participate in the maintenance and repair of vascular function [20]. We measured the number and function of EPCs in the peripheral blood in the three groups. The results showed that the number, proliferation, migration, and adhesion of the EPCs were lowest in group C, and highest in group A. This indicated that the number and function of the EPCs in the peripheral blood of patients with CSVD were decreased with an increase in plasma Hcy concentration. These results may be related to various mechanisms of Hcy injury, including apoptosis, the induction of oxidative stress, the promotion of inflammatory factors release, the inhibition of endothelial progenitor cell paracrine, the blockade of SDF/CXCR4 signaling pathway activation, etc. [21-23].

The shortcomings of this study are as follows. 1) The relationship between Hcy and CSVD was studied by grouping patients according to the plasma Hcy concentration, but there are many interference factors in clinical research, and the relevant conclusions still need to be validated by in vitro model. 2) The long-term prognosis of the three groups was not evaluated in this study, so it was unclear whether hyperhomocysteinemia accelerates the progression of CSVD to ischemic stroke and dementia.

In conclusion, cognitive impairment, the disturbance of vascular endothelial cell active factors, a decrease in the number, proliferation, migration, and the adhesion of EPCs are found in patients with CSVD complicated with hyperhomocysteinemia.

Disclosure of conflict of interest

None.

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