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The decrease of endothelial progenitor cells caused by high altitude may lead to coronary heart disease

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Abstract. – OBJECTIVE: The purpose of this study was to explore the relationship between the number of endothelial progenitor cells (EPCs) and coronary heart disease (CHD).

PATIENTS AND METHODS: A total of 24 patients with CHD were chosen from Lanzhou City and Xianyang City, and then, 24 healthy controls who matched the CHD group in gender, age and address were chosen as control group. C-reactive protein (CRP) and c-reaction protein (hs-CRP) were detected. The levels of interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), homocysteine (Hcy), hypoxia-inducible factor-1 (HIF-1α) and stromal cell-derived factor 1 (SDF-1α) were detected.

RESULTS: The number of EPCs in control groups was both increased compared with CHD group ($p<0.05$). The number of EPCs in Xianyang control group was increased compared with Lanzhou control group ($p<0.05$). Compared with the control group, the levels of TC, LDL and CRP in the CHD group were higher ($p<0.05$). Compared with Lanzhou control group, Hcy level was decreased in Lanzhou CHD group ($p<0.05$). Compared with Xianyang control group, the levels of IL-8 and VEGF were increased, but the levels of HIF-1α and Hcy were decreased in the Xianyang CHD group ($p<0.05$). The expressions of IL-8, VEGF, Hcy and HIF-1α were increased in Lanzhou control group than the Xianyang control group ($p<0.05$). In Lanzhou CHD group, Spearman correlation analysis showed that the number of EPCs was negatively related to hs-CRP content (r=-0.631, $p<0.05$).

CONCLUSIONS: The decrease of EPCs caused by high altitude may increase the expressions of various cytokines, leading to the occurrence of CHD.

Key Words:
MiRNA-106, Pediatric osteosarcoma, PI3K/AKT signaling pathway.

Abbreviations

EPCs: endothelial progenitor cells; CHD: coronary heart disease; TC: total cholesterol; TG: triacylglycerol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; CRP: c-reactive protein; hs-CRP: c-reaction protein; IL-8: interleukin-8; VEGF: vascular endothelial growth factor; Hcy: homocysteine; HIF-1α: hypoxia-inducible factor-1; SDF-1α: stromal cell-derived factor 1; VEPCs: Vascular endothelial progenitor cells; ELISA: Enzyme-linked immunosorbent assay; SD: standard deviation.

Introduction

The pathological mechanism of coronary heart disease (CHD) is very complicated, and it is related to family genetics and environment[1,2]. Endothelial cell damage can aggravate atherosclerosis, and it is closely related to the development of CHD[3]. Vascular endothelial progenitor cells (VEPCs) are precursor cells of endothelial cells. They mobilize into the blood during tissue ischemia and vascular injury and participate in the formation of microvessels and repair of vascular endothelium[4]. It is reported that the decrease of endothelial progenitor cells (EPCs) is one of the predictors for the progression of atherosclerosis[5]. Furthermore, the number of circulating EPCs is decreased in patients with stable angina, which are increased in patients with unstable angina[6]. Additionally, the higher the number of EPCs, the lower the risk and mortality of cardiovascular disease[7]. Many growth factors, cytokines, chemokines and apoptotic agents of endothelial cells can affect the mobilization, homing, prolif-

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eration, differentiation, migration and apoptosis of EPCs[8]. However, the relationship between the number of EPCs and CHD at different altitudes is not clear. Therefore, the purpose of this study was to explore the relationship between the number of EPCs and CHD at different altitudes.

Patients and Methods

Patients

From April 2014 to December 2014, a total of 96 participants were enrolled in the Cardiology/Physical Examination Center of the Liberation Army Joint Service Support Unit 940 Hospital in Lanzhou City and the Cardiology/Physical Examination Center of Xianyang central hospital in Xianyang City. A total of 24 patients with CHD were selected as CHD group. Moreover, 24 healthy subjects who matched the CHD group in gender, age and address were selected as control group. The study was approved by the 940th Hospital of Joint Logistics Support Force of PLA [No. 2020KYLL133]. Informed consent was obtained.

Inclusion criteria: (1) long-term residents (>10 years); (2) Coronary angiography or cardiac CTA in patients with CHD was clearly diagnosed as CHD, and the standard reference from the Chinese guidelines for percutaneous coronary intervention (2012)[9]. (3) At least 2 mutually perpendicular projection images were collected for each coronary lesion with diameter ≥ 50%. Exclude criteria: patients with valvar heart disease, cardiomyopathy, diabetes, acute and chronic infection, trauma, ulcer, retinopathy, tumor, recent surgery, acute myocardial infarction in the past 3 months and acute cardiac insufficiency were excluded.

Flow Cytometry

The expressions of CD34, CD45 and KDR in peripheral blood were detected by flow cytometry. The direct immunofluorescence method was used, that is, the specific fluorescent antibody was directly added to the specimen to make it specifically bind to the antigen, and then the fluorescent antibody that did not bind to the antigen was removed. The cell ratio of double positive cells with CD34+ and KDR positive was calculated by detecting the specific fluorescent antibody combined with antigen. 5 ml peripheral venous blood were collected through forearm vein and 200 μl were added into flow tube. Then, 5 μl fluorescein isothiocyanate (FITC) labeled CD45 antibody, phycoerythrin (PE) labeled KDR antibody and allophycocyanin (APC) labeled CD34 antibody (Abcam Technology, Cambridge, UK) were added. A negative control group was set up for each sample, and only CD45 antibody was added. After mixing, the antibody was incubated at room temperature in the dark for 30 min to make the fluorescent labeled antibody fully combined with the antigen. Add another 6 ml of red blood cell lysate into each tube and react in dark for 15 min to make the red blood cells dissolve completely. After centrifugation, 500 μl phosphate buffer was added. Finally, the double positive expression rates of CD34 and KDR in endothelial progenitor cells (EPCs) were detected by flow cytometry.

Immunochromatography

Peripheral venous blood was taken when participants were fasting. The levels of total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) c-reactive protein (CRP) and c-reaction protein (hs-CRP) were determined by immunochromatography using OLYMPUS- 5421 automatic biochemical analyzer.

Enzyme-Linked Immunosorbent Assay (ELISA)

Peripheral venous blood was taken when participants were fasting. The blood was centrifuged at 3000 rpm for 5 min, and the serum was aspirated and stored at -70°C. The serum levels of IL-8, VEGF, SDF-1a, HCY, and HIF-1α were measured by ELISA using commercial kits (Sigma Chemical Co., St. Louis, MO, USA) at 450 nm as described in the instrument. The concentration of those cytokines in the serum was calculated by standard curve.

qRT-PCR

The mononuclear cells were immediately extracted by density gradient centrifugation. The total RNA was extracted by TRIzol reagent. The RNA was reverse transcribed into cDNA according to the TAKALA reverse transcription kit, and the cDNA was used as a template according to the reaction. RT-PCR amplification reaction was performed. Conditions: 95°C for 5 min, 95°C for 30 s, 55-72°C for 30 s with 30 cycles, and 72°C for 10 min. The average CT value was taken according to the formula: $^{\Delta\Delta}$CT=(CT target gene-CT internal reference) in CHD group-(CT target gene-CT internal reference) in control group; calculated multiple=2-$^{\Delta\Delta CT}$. The primer sequences

of CXCR2, CXCR4, CXCR7, and GAPDH were as follows. CXCR2: Forward primer: 5'-TCGCCGTCGTGCTCATCTTCC-3', Reverse primer: 5'-GGCGCTCACACGTCTCCTGGA-3'; CXCR4: Forward primer: 5'-GGGTTGGTAATCCTGGTC-3', Reverse primer: 5'-ATGATGTGCTGGAACTGG-3'; CXCR7: Forward primer: 5'-AACCTGGGAACTACTCGG-3', Reverse primer: 5'-CAAGACGCAGACAACACG -3'.

Western Blotting

The protein of mononuclear cells was homogenized in cold radio immunoprecipitation assay (RIPA) buffer and the protein concentration was detected by modified bicinchoninic acid (BCA) protein concentration assay kit. The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Then, the membrane was cultured with anti-HIF-1α antibody (Abcam Technology, Cambridge, UK) and second antibody. Finally, the band was exposed and Molecular Imager Chemi Doc XRS and JS-780 automatic gel imaging analysis systems were used to analyze the expression of HIF-1α.

Statistical Analysis

Data were analyzed using SPSS 24.0 (IBM, Armonk, NY, USA) statistical software. Data were expressed as means ± standard deviation (SD), and *t*-test was used for comparison between groups. $p<0.05$ was considered statistically significant.

Results

Basic Clinical Characteristics

The 96 patients with CHD were chosen from Lanzhou city and Xianyang city and then 24 patients with CHD (12 males and 12 females) were chosen from Lanzhou and Xianyang as Lanzhou CHD group and Xianyang CHD group, respectively. Furthermore, 24 healthy controls (12 males and 12 females) were chosen from Lanzhou and Xianyang as Lanzhou control group and Xianyang control group, respectively. As shown in Table I, there were no significant differences in age, gender distribution, abdominal circumference or body mass index between groups ($p>0.05$).

Comparison of the Number of EPCs in Peripheral Blood in Lanzhou and Xianyang

The number of EPCs (%) in Lanzhou CHD group was notably lower than that in Lanzhou control group ($p<0.05$). The number of EPCs (%) in Xianyang CHD group was also markedly lower than that in Xianyang control group ($p<0.05$). The number of EPCs (%) in Lanzhou control group was significantly lower than Xianyang control group ($p<0.05$) (Table II).

Flow Cytometry Results

The participants in CHD group and control group without significant difference in age, diagnosis and BMI were selected. The results of flow cytometry showed that the number of control groups in the two groups was significantly higher than that in the CHD group ($p<0.05$). In addition, there was no significant difference between the representatives of the selected groups between the two cities and between the genders (Table III).

Biochemical Indicators Test

As shown in Table IV, there was a statistically significant difference between CHD and control groups in Lanzhou city ($p<0.05$, $p<0.01$). The levels of TC, LDL and CRP in Xianyang and Lanzhou CHD groups were significantly higher than those in Xianyang and Lanzhou control groups, respectively ($p<0.05$). There were no sig-

Table I. Overall comparison of the study and control groups.

Items	Lanzhou group (n = 24)		Xianyang group (n = 24)	
	Control group (n = 12)	CHD group (n = 12)	Control group (n = 12)	CHD group (n =12)
Age [years]	55.2 ± 9	58.4 ± 10	52 ± 8.7	60.9 ± 10.3
Gender (male/female)	6/6	6/6	6/6	6/6
Abdominal circumference (cm)	75.71 ± 8.98	80 ± 5.12	77.32 ± 9.04	89.62 ± 6.4
Body mass index [kg/m^2]	21.72 ± 4.3	24.17 ± 1.35	19.29 ± 4.74	25.67 ± 3.2

Table II. Comparison of the number of peripheral blood endothelial progenitor cells in samples from Lanzhou and Xianyang (means ± SD).

Lanzhou group		Xianyang groupl	
Control group	CHD group	Control group	CHD group
0.042 ± 0.014	0.026 ± 0.012*	0.062 ± 0.028	0.042 ± 0.017*

*$p < 0.05$ *vs*. compared with control group.

Table III. Flow cytometry results of male and female in Lanzhou city and Xianyang city (%).

Items	Lanzhou group		Xianyang groupl	
	Control group	CHD group	Control group	CHD group
Male	1.002	16.002	39.006	28.006
Female	4.008	17.016	2.004	3.006

Table IV. Comparison of general and biochemical data (means ± SD).

Items	Lanzhou group		Xianyang groupl	
	Control group	CHD group	Control group	CHD group
TC (mmol·L^{-1})	1.22 ± 0.7	2.09 ± 1.36*	0.65 ± 0.1	1.48 ± 0.6*
TG (mmol·L^{-1})	2.19 ± 1.25	4.09 ± 0.98	1.78 ± 0.84	2.01 ± 1.47
HDL (mmol·L^{-1})	0.98 ± 0.26	1.47 ± 0.37*	1.68 ± 0.77	1.85 ± 0.81
LDL (mmol·L^{-1})	1.35 ± 0.1	1.96 ± 0.67	1.2 ± 0.69	3.7 ± 0.93**
CRP (μmol·L^{-1})	1.78 ± 0.8	3.2 ± 1.3**	1.34 ± 0.4	2.5 ±1.1*

*$p < 0.05$ *vs*. control group; **$p < 0.01$: *vs*. control group.

nificant differences in abdominal circumference, BMI and TG between CHD and control groups ($p>0.05$). There were no differences in age and gender between all subjects enrolled in the study ($p>0.05$).

Correlation Analysis Between the Number of EPCs and the Level of hs-CRP in Patients with CHD in Lanzhou

As shown in Figure 1, the concentration of hs-CRP in Lanzhou CHD group (n=24) was negatively correlated with the number of EPCs (%) (r=-0.631, $p<0.05$). It also showed that the more the number of EPCs in CHD group in Lanzhou, the lower the hs-CRP concentration.

Levels of Inflammatory Cytokines in Control and CHD Groups

The results showed that the expression levels of VEGF, HIF-1α and SDF-1α were higher in the control group than the CHD group ($p<0.05$). IL-8 and Hcy were highly expressed in the CHD group than the control group ($p<0.05$). However, there was no significant difference in SDF-1α expression between Lanzhou control group

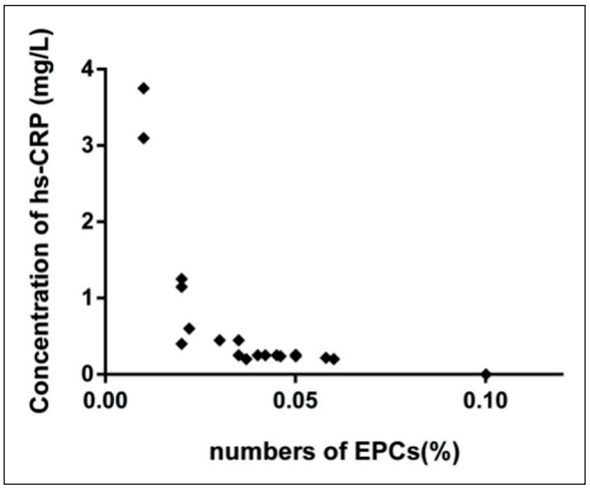

Figure 1. Relationships between numbers of EPCs and concentration of hs-CRP.

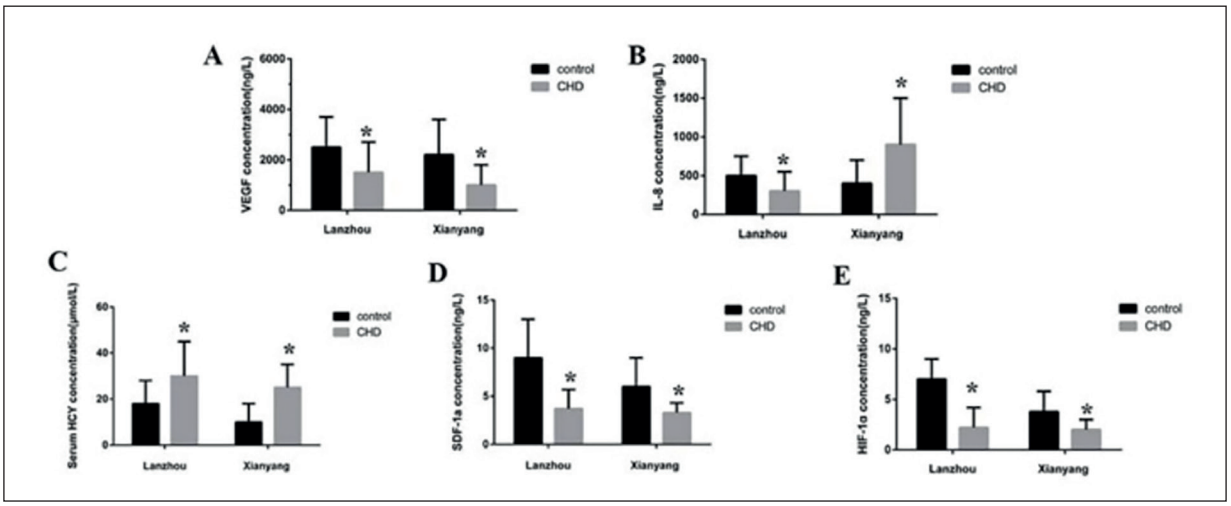

Figure 2. Relationships of VEGF (**A**), IL-8 (**B**), HCY (**C**), SDF-1a (**D**) and HIF-1α (**E**) between Lanzhou group and Xianyang group. Data are expressed as means ± SD. *p<0.05 *vs.* control group.

and Xianyang control group (p>0.05). The expressions of VEGF, HIF-1α, IL-8 and Hcy in Xianyang group were notably decreased than those in Lanzhou group (p<0.05) (Figure 2).

The Expressions of CXCR2, CXCR4, CXCR7 and HIF-1α in Monocytes

As shown in Table V, there was no difference in mRNA expression levels of CXCR4 and CXCR7 between Lanzhou group and Xianyang group (p>0.05). The mRNA expression of CXCR2 was upregulated in Lanzhou group compared with Xianyang group (p<0.05). Moreover, as shown in Figure 3, the HIF-1α protein expression in Lanzhou CHD group was higher than that in Lanzhou control group (p<0.05). The HIF-1α protein expression in Lanzhou control group was higher than that in Xianyang control group (p<0.05).

Discussion

The decrease in the number and function of EPCs is associated with the onset of CHD. It has been reported that a reduction in the number of EPCs is an independent risk factor for atherosclerosis, which plays an important role in endothelial repair[10]. The number of EPCs can predict cardiovascular adverse events[11]. CD34/KDR double positive is the main method to detect the number of endothelial progenitor cells[12]. Moreover, the number of EPCs in peripheral blood in patients with CHD was significantly lower than that in healthy controls, and the number of cell colonies and cell proliferation ability was also significantly reduced in patients with CHD[13]. Those results were consistent with our findings. In our study, we found that the number of EPCs in the two control groups was increased compared with the two CHD group. Furthermore, the number of EPCs in Xianyang control group was increased compared with that in Lanzhou control group.

There are many traditional risk factors for CHD, such as hypertension, hyperlipidemia, diabetes, hyperviscosity, lipid metabolism disorders, age, obesity, abdominal circumference. In addition to these recognized high-risk factors, there are many laboratory indicators, such as TC, LDL-C and glycated hemoglobin, which are also closely related to CHD[14]. There is often a link between these risk factors and CHD. For example, TG is a secret independent predic-

Table V. mRNA expression of CXCR2, CXCR4 and CXCR7 in monocyte.

Items	Lanzhou group	Xianyang group	*p*-values
CXCR2	0.91 ± 0.13	4.8 ± 1.87	0.03
CXCR4	0.08 ± 0.01	0.078 ± 0.03	0.14
CXCR7	0.65 ± 0.14	1.32 ± 0.39	0.32

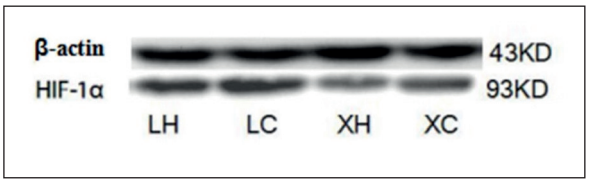

Figure 3. The protein expression of HIF-1α between the two groups. LH stands for Lanzhou control group; LC stands for Lanzhou CHD group; XH stands for Xianyang control group; XC stands for Xianyang CHD group.

tor of CHD, and is often associated with decreased HDL-C level and elevated glycosylated hemoglobin level, which are also risk factors for CHD. Some researchers found that age, hypertension, smoking, and LDL-C were related to the number of EPCs[15]. This result was consistent with our findings.

CRP is an acute phase response protein that responds to inflammatory levels, activates complement, promotes phagocytosis, and has other immune regulatory effects[16]. Scholars[17] have shown that CRP and plasma Hcy in patients with CHD are higher than healthy controls. At the same time, they found that elevated plasma Hcy level was independent risk factor for CHD. IL-8 has a chemotactic effect, which can cause a large number of T cells and macrophages to appear in the site of plaque rupture and plays an important role in the formation and rupture of atheromatous plaques[18]. This study[18] also found that serum IL-8 level in patients with CHD was higher than the control group. A study[19] found that the number of EPCs was positively correlated with the concentration of VEGF. HIF-1α is one of the key factors to regulate oxygen metabolism in cells. It is induced by changes in molecular oxygen levels in tissues and can activate the expression of many hypoxia-responsive genes, resulting in a series of hypoxic adaptive responses in the body. It showed that elevated levels of VEGF and HIF-1α may be signs of atherosclerotic plaque destabilization[20]. Chemokines play an important role in the pathogenesis of atherosclerosis, which can express in atheroma cells. Stromal cell-derived factor-1 (SDF-1α) and its specific receptor (chemokine receptor 4, CXCR4) play a key role in mediating angiogenesis, and they can promote the migration, homing and transformation of EPCs. It showed that the serum SDF-1α level in CHD group were significantly lower than those in control group[21]. The results of this study were consistent with previous studies. The study found that IL-8, Hcy, VEGF, HIF-1α and SDF-1α were significant for studying the difference in the incidence of CHD between the two places. The results were VEGF, HIF-1α and SDF-1α in the control groups of the two places were both highly expressed than CHD group, and IL-8 and Hcy in CHD groups were highly expressed than that in the control group, indicating that VEGF, HIF-1α and SDF-1α may be of interest in promoting the increase of EPCs, and IL-8 and Hcy may be meaningful to promote the reduction of EPCs. The reason may be that VEGF, HIF-1α and SDF-1α belong to anti-inflammatory factors, and IL-8 and Hcy belong to pro-inflammatory factors.

The EPCs-mediated angiogenesis process is regulated by many biochemical factors. Some studies suggest that CXCR7 can promote the proliferation, adhesion, chemotaxis and activation of downstream signaling molecules of EPCs[22]. A study[23] found that CXCR2 and its ligand CXC chemokines also participated in the mobilization, proliferation and migration of EPCs, and promoted the adhesion function of EPCs. We found that the transcription level of CXCR2 in Xianyang was higher than that in Lanzhou, which may be related to the lowering of Xianyang than Lanzhou. The transcript level of CXCR7 in Xianyang was similar to that in Lanzhou, which may be related to the smaller sample size.

This study analyzed the possible pathogenic mechanisms by measuring the expression levels of cytokines. At the same time, there were still some limitations in this study. For example, due to the degree of cooperation and financial conditions of patients, the number of samples taken from the two places was too small. In addition, there are few studies on the functional changes of EPCs in patients with CHD under altitude factors. The experimental study of selection factors is in the exploratory stage.

Conclusions

We found the decrease of EPCs caused by high altitude may increase the expressions of various cytokines and lead to the occurrence of CHD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

XQH is responsible for the guarantor of integrity of the entire study, study concepts, definition of intellectual content, manuscript preparation; JL is responsible for the guarantor of integrity of the entire study, study design, definition of intellectual content, clinical studies, experimental studies, data acquisition, statistical analysis, manuscript editing & review; CPM is responsible for the study design, literature research, clinical studies, experimental studies, data acquisition, statistical analysis, manuscript review; XLC is responsible for the experimental studies, data acquisition; CYY, CXJ and SH are responsible for the experimental studies, data analysis. All authors read and approved the final manuscript.

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