Original Article
Hypersplenism: an independent risk factor for myocardial remodeling in chronic heart failure patients

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Abstract: Background: During the progression of chronic heart failure (CHF), decreased cardiac functioning is often associated with congestion in the inferior vena vein, which in turn induces splenomegaly and subsequent hypersplenism. Hypersplenism has been shown to exacerbate endothelial dysfunction and adverse cardiac remodeling in HF mice. However, it is unknown whether this effect also occurs in CHF patients with hypersplenism. Here, we compared different patterns of myocardial remodeling between patients with and without hypersplenism. Methods: 33 CHF patients with hypersplenism were selected and carefully examined. Clinical data and baseline hemogram measurements were included in the evaluation. Another 35 CHF patients were randomly chosen as controls. All patients received formal HF treatment to ameliorate their symptoms and to preserve heart structure and functioning. Peripheral blood-derived endothelial progenitor cells (EPCs) were cultured, and the experimenters were blinded to the patients' clinical characteristics. The biological properties of the cells were then compared. The groups were also compared in terms of the free plasma hemoglobin and heme levels, endothelial adhesion molecule expression, left ventricular ejection fraction (LvEF) and cardiovascular events (re-PCI, re-myocardial infarction, stent thrombosis, stroke and death due to cardiovascular or vascular causes). Results: The free plasma hemoglobin and heme levels were significantly higher in the CHF patients with hypersplenism compared with the controls (P<0.001). Additionally, the CHF patients with hypersplenism had increased levels of VCAM-1, ICAM-1, P-selectin and E-selectin (P<0.001). Echocardiography revealed a significant reduction in the LVEF in these patients compared with the controls at the 24th month (P=0.013). During a mean follow-up period of 24±1 months, cardiovascular events were observed in 16 patients in the CHF with hypersplenism group and 9 patients in the control group. Univariate Kaplan-Meier analysis further revealed a significant difference between the groups (P=0.021). The mRNA levels of endothelial NO synthase enzyme (eNOS) in EPCs from the CHF patients with hypersplenism were significantly lower than those in the control subjects (P<0.001). We also observed decreased proliferation potential of EPCs from the CHF patients with hypersplenism (P<0.001). Further, a significant increase in TUNEL+ EPCs was observed in the CHF patients with hypersplenism after 6 h of stimulated ischemia compared with the control subjects (P<0.001). Conclusions: CHF patients with hypersplenism are susceptible to myocardial remodeling. Increased oxidative stress and endothelial dysfunction caused by excess free plasma hemoglobin and heme may partially explain this causality.

Keywords: CHF, myocardial remodeling, hypersplenism

Introduction

Chronic heart failure (CHF) not only increases the risk of cardiovascular complications and mortality but also leads to a decrease in quality of life [1]. Heart failure (HF) is a chronic clinical syndrome characterized by a reduction in left ventricular (LV) functioning and the inability to pump blood, maintain tissue perfusion, and support physiological functioning. Additionally, it is one of the most important causes of mor-

bidity and mortality worldwide [2, 3]. Optimal prevention of myocardial remodeling in patients with HF remains a challenge due to the difficulties in recognizing the presence of various clinical signs and risk factors. Several previous studies have shown that most traditional cardiovascular risk factors, including elevated blood pressure, obesity and cigarette smoking, increase the risk of death in HF patients [4, 5]. In recent studies, high brain natriuretic peptide (BNP) levels, a dilated inferior vena cava, low

sodium and high blood urea nitrogen levels were all considered independent risk factors for recurrent cardiovascular events or death in HF patients [6, 7].

Hypersplenism, which is characterized by a significant reduction in one or more cellular elements in the blood and splenomegaly, is a common manifestation in patients with portal hypertension [8]. The incidence of hypersplenism in patients with portal hypertension caused by cardiac dysfunction is high [9]. In addition, it is associated with erythrocyte rupture and chronic intravascular hemolysis, which may lead to platelet activation and thrombosis [10, 11]. However, it is still unknown whether spleen dysfunction and spleen dysfunction-induced cellular destruction play key roles in myocardial remodeling in CHF patients. In this study, we assessed whether the spleen dysfunction of CHF patients with hypersplenism serves as an independent risk factor for myocardial remodeling during the process of chronic heart failure.

Methods

Study population

All of the study participants were younger than 80 years old, with or without hypersplenism, and had been admitted to the Medical Center of Zhongda Hospital affiliated with Southeast University from 2008 to 2011 for Class III or IV CHF according to the New York Heart Association functional classification. All patients received appropriate treatment for HF according to the ESC Guidelines. Clinical data, including the baseline hemogram, concomitant diseases, history of cardiovascular or cerebrovascular diseases, smoking, blood pressure, eGFR left ventricular ejection fraction (LVEF), and drugs taken, were mostly acquired during hospitalization. This study was approved by the local ethics committee and performed in accordance with the Declaration of Helsinki. All participants provided written informed consent. For the patients with CHF, at least three of the following criteria had to be met, in addition to an increased level of BNP (cut-off point = 100 pg/ml): typical symptoms of HF (breathlessness, orthopnea, paroxysmal nocturnal dyspnea, reduced exercise tolerance, fatigue, tiredness, increased time to recover after exercise, and ankle swelling); typical signs of HF (elevated jugular venous pressure, hepatojugu-

lar reflux, a third heart sound, a laterally displaced apical impulse, and a cardiac murmur); and a reduced LVEF. Eligible CHF patients with splenomegaly, as identified by ultrasound or computed tomography imaging, and a reduction in one or more cellular elements in the blood were categorized as having hypersplenism. Subjects showing any of the following exclusion criteria were not allowed to participate in the study: ≥80 years old or <18 years old; severe liver or kidney disease; a glomerular filtration rate (GFR) of <30 ml/(min·1.73 m^2) or a Child-Turcotte-Pugh (CTP) score of ≥6; hemodynamic or electrical instability (including shock); a coagulation disorder associated with a tendency for significant bleeding; the intake of systemic immunosuppressive agents; the occurrence of other serious disorders; and a life expectancy of <6 months. Cardiovascular events were defined as re-PCI, re-myocardial infarction, stent thrombosis, stroke and death due to cardiovascular or vascular causes, all of which were in accordance with the Academic Research Consortium criteria.

Isolation and culturing of EPCs

EPCs were isolated from buffy coat specimens donated by patient volunteers from the two groups. Mononuclear cells (MNCs) were isolated by Ficoll density-gradient centrifugation. Isolated MNCs were incubated with FcR-blocking reagents and CD133 microbeads, and MACS separation (Miltenyi Biotec.) was performed. The isolated cells were then washed with 2% fetal bovine serum (FBS; Hyclone) in MACS buffer. Next, a total of 10^6 MNCs/ml were placed in 25 cm^2 culture dishes with EGM-2 medium supplemented with 10% FBS for 5 to 7 days [12]. Three weeks later, the MNCs developed spindle-shaped appearances and formed typical cell clusters. These cells were considered EPCs. The putative EPCs were further identified by the surface markers CD34, CD133, and VEGFR-2 (Becton Dickinson, USA), as shown in **Figure 1**. The cells used in this study were restricted to passages 4 through 6.

Quantitative PCR and ELISA

For quantitative reverse transcription polymerase chain reaction, total RNA was first extracted from each group of cultured EPCs with TRIzol reagent, according to the manufacturer's instructions (Invitrogen). Then, a Quan-
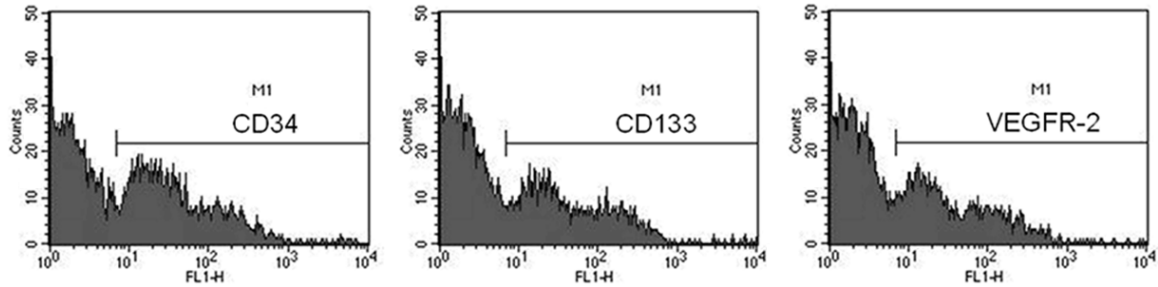
Figure 1. Three FITC-conjugated antibodies, anti-CD34, anti-CD133 and anti-VEGFR-2, were used to identify putative EPCs isolated from CHF patients with or without hypersplenism.

Table 1. Baseline Characteristics

Characteristic	CHF patients with hypersplenism (n=33)	CHF patients served as control (n=35)	P value
Age (years)	59.9±10.9	65.1±12.4	0.259
Sex (male %)	17 (51.5%)	15 (42.9%)	0.475
Smoking	3 (9.1%)	5 (14.3%)	0.710
Body mass index ($\geq$25 kg/m^2)	6 (18.2%)	10 (28.6%)	0.313
Hypertension	19 (57.6%)	15 (42.9%)	0.225
Diabetes	3 (9.1%)	4 (11.4%)	1.000
TC (mmol/L)	4.73±1.34	4.46±1.07	0.512
LDL cholesterol (mmol/L)	3.45±0.61	3.13±0.71	0.219
BNP peak (pg/ml)	827.47±89.44	771.29±72.66	0.227
eGFR (ml/min)	71.74±16.61	74.37±15.22	0.554
Hemogram			
Leukocyte (L^{-1})	7.09*10^9	6.57*10^9	0.487
Erythrocytes (L^{-1})	3.54*10^{12}	3.66*10^{12}	0.535
Platelets (L^{-1})	125.75*10^9	133.69*10^9	0.190
Hemoglobin (g/L)	85.92	119.37	<0.001
Medication, n (%)			
Furosemide/spirolactone	33 (100%)	35 (100%)	1.000
Beta-blockers	22 (66.7%)	18 (51.4%)	0.202
Statins	11 (33.3%)	15 (42.9%)	0.419
ACE inhibitors/ARB	24 (72.7%)	27 (77.1%)	0.674
Digoxin	13 (39.4%)	16 (45.7%)	0.598
Antiplatelet agents	10 (30.3%)	14 (40.0%)	0.403

Baseline Clinical Characteristics of patients. Values are mean ± SD, or n (%). TC: Total cholesterol; eGFR, estimated glomerular filtration rate; BNP, B-type Natriuretic Peptide; LDL, low-density lipoprotein; ACE inhibitors, Angiotension converting enzyme inhibitors; ARB, Angiotensin II receptor blocker.

tiTect Reverse Transcription Kit (Fermentas) was used for reverse transcription. Polymerase chain reaction was performed using Platinum SYBR Green qPCR SuperMix UDG (Applied Invitrogen) with a BIO-RAD MJ Mini Opticon Real-Time PCR System. The primer sequences were as follows: human eNOS (GenBank NM_000603.4 422 bytes), forward 5'-GTGAT-GGCGAAGCGAGTGAAG-3' and reverse 5'-CCGAGCCC-GAACACACAGAAC-3'; and human GAPDH, forward 5-CAAGGCTGAGAACGGG-AAG-3 and reverse 5-GG-TGGTGAAGACGCCAGT-3.

The concentrations of endothelial adhesion molecules (VCAM-1, ICAM-1, P-selectin and E-selectin) in the serum were measured using a human ELISA kit (R&D Systems Inc.). The free plasma hemoglobin concentration was determined using a human free hemoglobin ELISA kit (SHxinran). A Hemin Assay Kit (Sigma) was used to estimate the free heme concentration in the serum. The absorbance measurements of the test samples were compared with a standard curve. The concentrations were determined in duplicate, and the process was performed according to the manufacturer's instructions.

TUNEL assay for assessing apoptotic cells

Ischemia was then induced by incubating cultured EPCs (3×10^6/cm^2) at 37°C in an airtight, humidified glass chamber (Verretech, Lyon, France) that was ventilated with 5% N$_2$ and 95% CO$_2$ after replacing the conditioned media with nutrient-free PBS (0.15 ml/cm^2) [13]. Six hours
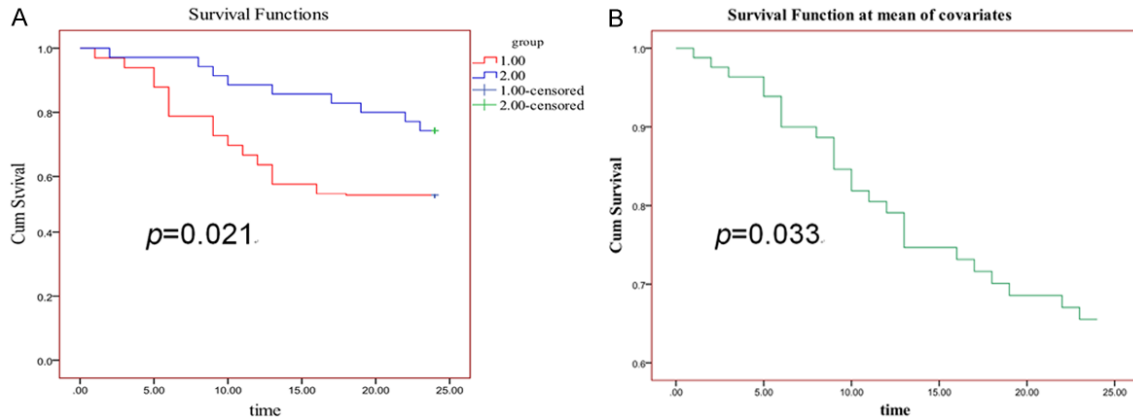
Figure 2. Univariate Kaplan-Meier and multivariate regression analyses. A. Cardiovascular events, as determined by Kaplan-Meier survival curves for CHF patients with hypersplenism (red line) and CHF patients without hypersplenism (blue line). B. Multivariate regression analysis after adjusting for confounding factors in CHF patients with hypersplenism and CHF patients without hypersplenism (control group).
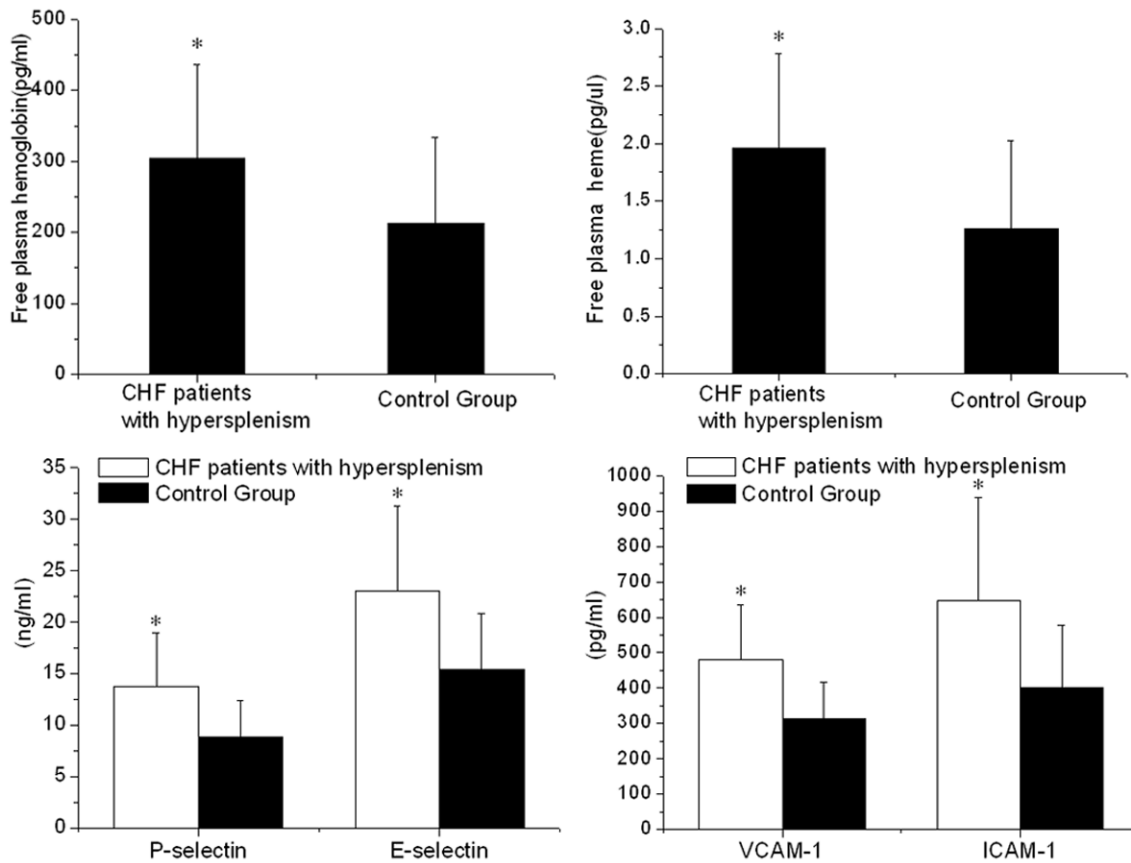

Figure 3. Comparison of the free plasma hemoglobin (pg/ml) and heme (pg/µl) levels at baseline between the two groups of patients. Endothelial adhesion molecule expression in the two groups is further shown (VCAM-1: 479.68±154.2 vs. 313.77±102.66; ICAM-1: 645.87±291.74 vs. 402.39±174.81; P-selectin: 3.71±5.28 vs. 8.82±3.64; and E-selectin 23.09±8.24 vs. 15.39±5.47). *$P<0.05$ vs. the control group.

after the induction of ischemia, a TUNEL assay was performed to detect apoptotic nuclei using the terminal deoxynucleotidyl transferase (TdT)-mediated *in situ* fluorescein-conjugated, dUTP nick end-labeling technique according to the manufacturer's protocol (Vazyme, China).
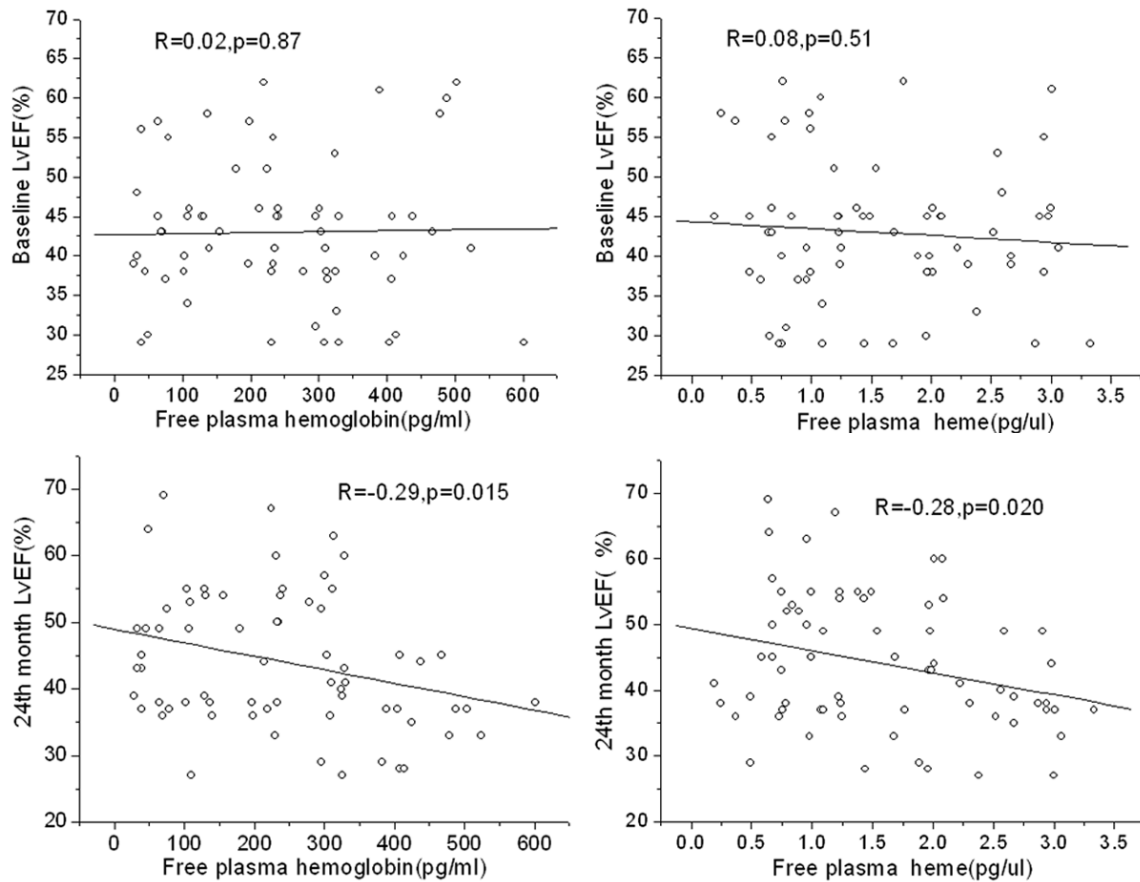
Figure 4. Measurements of the LVEF% and association of free plasma hemoglobin (pg/ml) and heme (pg/ μl) with the LVEF (%) at baseline and the 24th month in the CHF patients. LVEF: Left ventricular ejection fraction; *P<0.05 vs. LVEF% at baseline; and #P<0.05 vs. LVEF% in the control group.

Fluorescence staining was viewed by confocal fluorescence microscopy. The number of apoptotic cells was counted and expressed as a percentage of the total EPC population.

Statistical analysis

Data management and statistical analysis were performed with Statistical Package for Social Sciences software (SPSS 15.0 for Windows, SPSS Inc.). The data are expressed as the mean ± standard deviation or as the median and interquartile range when necessary. Categorical variables were compared using the χ^2 test or Fisher's exact test. For continuous data, group comparisons were performed using the unpaired t-test or Mann-Whitney U test. The log-rank Mantel-Cox test and Kaplan-Meier survival curves were used to compare event-free survival. The results were considered statistically significant if the two-sided *P*-value was ≤0.05.

Results

Baseline characteristics of the two groups

The mean age of the CHF patients with hypersplenism was 59.9±10.9 years, whereas that of the control group was 65.1±12.4 years (P=0.259). The clinical characteristics of the study population are summarized in **Table 1**. All of the patients were followed up for two years, and none were lost. No significant differences were observed between the two groups in terms of age, sex ratio, underlying diseases and previous medication usage.

CHF patients with hypersplenism experienced more cardiovascular events

During the 24-month follow-up, 16 subjects in the CHF patient group with hypersplenism and 9 in the control group experienced cardiovascular events. Therefore, a total of 25 patients
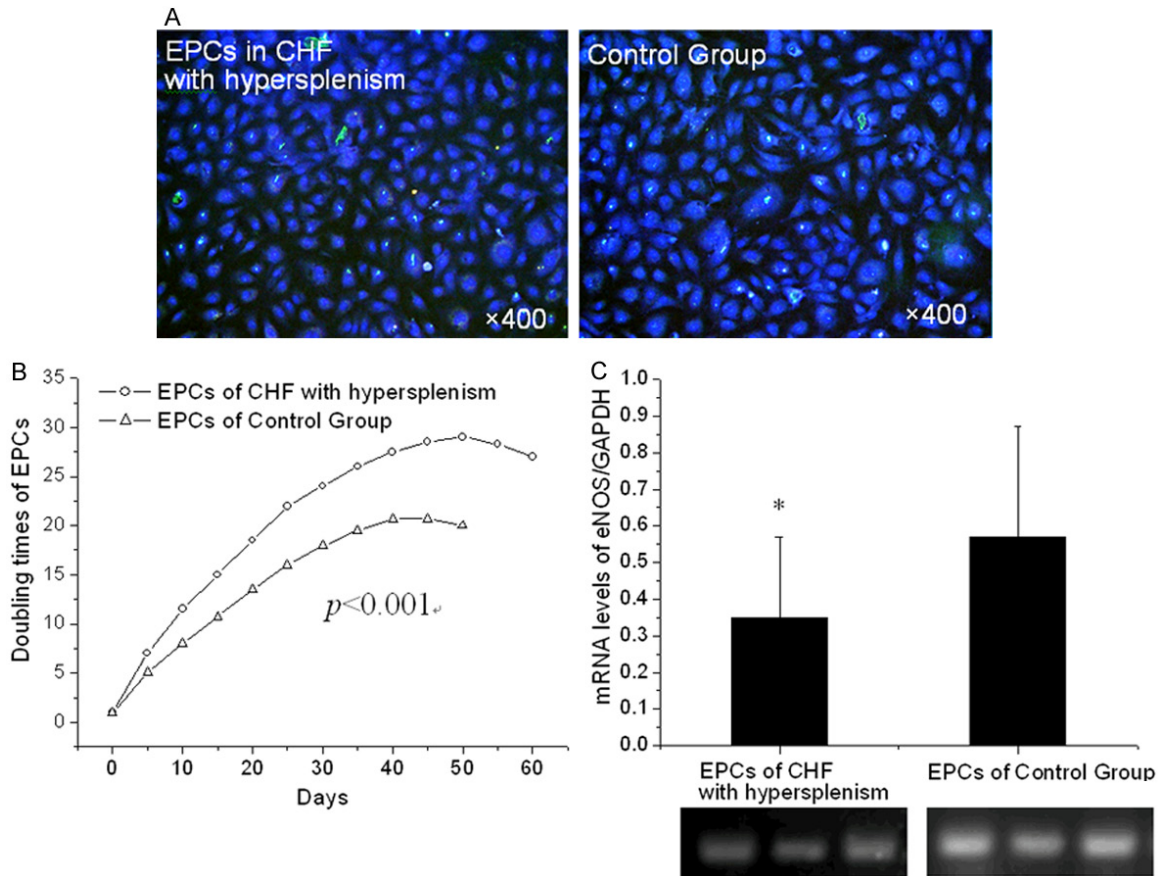
Figure 5. Assessment of EPC proliferation potential and apoptosis. A. Percentage of TUNEL+ EPCs (green) under high-powered magnification (36.5±11.8 vs. 21.2±7.4, P<0.001) in the CHF patients with hypersplenism and the controls after ischemic simulation for 6 h. B. The doubling times of EPCs and the days in culture are shown for the two groups. From the beginning of culturing to the 60th day, the proliferation capacity of EPCs from the CHF patients with hypersplenism was continuously higher than that of EPCs from the control group. C. The eNOS mRNA level in the cells derived from the CHF patients with hypersplenism is shown; *P<0.05 vs. the control group.

experienced the primary endpoint. A significant difference in the previously defined primary endpoint (P=0.021) was observed between the groups, as demonstrated by the survival curves shown in **Figure 2**. Multivariate regression analysis was further applied to evaluate the two groups after adjusting for age, sex, body mass index, hypertension, smoking, and LDL and eGFR levels, revealing the presence of a significant difference (95% CI: 0.16-0.92, P=0.033).

Assessment of free plasma hemoglobin, heme and endothelial adhesion molecule expression

Most of the CHF patients with hypersplenism showed mild to moderate anemia or reduced hemoglobin, which may have been ascribed to the destruction of red blood cells by the spleen. As expected, the free plasma hemoglobin (pg/ml) and heme (pg/μl) released by erythrocytes were greatly increased in these patients compared with the controls (304.75±130.49 vs. 212.46±121.11, P<0.001; and 1.96±0.82 vs.1.26±0.77, P<0.001, respectively). Previous studies have suggested that the destruction of red blood cells in circulation often leads to oxidative stress, endothelial activation and dysfunction [14]. Therefore, we next compared endothelial adhesion molecule expression between the two groups. As shown in **Figure 3**, the VCAM-1, ICAM-1, P-selectin and E-selectin expression levels were all much higher in the CHF patients with hypersplenism compared with the controls (P<0.001), indicating that the increased free plasma hemoglobin and heme levels caused by hypersplenism likely further exacerbated the endothelial injury and proinflammatory stress in the CHF patients.

CHF patients with hypersplenism showed excessive myocardial remodeling

The baseline and follow-up measurements of the LvEF are shown in **Figure 4**. We did not find significant differences in the LvEF% at baseline in the two groups despite the tendency toward an increased BNP level in the patients with hypersplenism (47.09%±9.15% vs. 49.57%±10.18%, P=0.296). However, it should be noted that a significant reduction in the LvEF was observed in the CHF patients with hypersplenism compared with the controls in the 24th month (41.06%±7.77% vs. 47.05%±10.62%, P=0.01). Additionally, we observed that the levels of free plasma hemoglobin and heme were significantly correlated with the LvEF (%) at the 24th month in the CHF patients with hypersplenism (r=-0.29, P=0.015; and r=-0.28, P=0.020, respectively). The differences in the LvEF between the groups and the significant correlations observed at the time point assessed may have been attributed to the presence of hypersplenism and the increased levels of oxidative stress in these patients.

Assessment of proliferation potential and apoptosis of EPCs

We further examined the potential mechanisms by which CHF patients with hypersplenism exhibit exacerbated myocardial remodeling. The EPCs from the patients were cultured and analyzed *in vitro*. The proliferation capacities of the EPCs were first observed. As shown in **Figure 5**, the cells derived from the CHF patients with hypersplenism showed a significantly decreased proliferation potential within a particular time frame compared with the controls (P<0.001). In addition, a significant increase in TUNEL+ EPCs (%) was detected in the CHF patients with hypersplenism compared with the controls after 6 h of stimulated ischemia (36.5±11.8 vs. 21.2±7.4, P<0.001). Consistent with the results above, decreased expression of eNOS mRNA (0.35±0.22 vs. 0.57±0.30, P<0.001) in the cells derived from the CHF patients with hypersplenism was further identified, as shown in **Figure 5**. Taken together, these results suggest that the excessive oxidative stress caused by hypersplenism in CHF patients may lead to increased endothelial inflammation and dysfunction.

Discussion

Heart failure is characterized by multiple relapses, including reduced cardiac diastolic and contractile function, increased apoptosis of myocyte cells and myocardial fibrosis [15, 16]. Despite the proven survival benefit of medical therapies, such as ACEI/ARBs and β-blockers, the mechanisms mediating the pathological changes that occur in heart failure have not been fully elucidated. Hypersplenism is a frequent finding in patients with CHF. It is usually asymptomatic, but it may aggravate this condition. The destruction of red blood cells is the most frequent manifestation of hypersplenism and may contribute to excessive ROS production in the progression of heart failure. The key finding of the assessment of CHF patients with hypersplenism in this study was the positive and independent relationships between cardiovascular events and hypersplenism. As shown by K-M analysis, these patients exhibited a significant trend toward experiencing cardiovascular events (defined as re-PCI, re-myocardial infarction, stent thrombosis, stroke and death due to cardiovascular or vascular causes) during the 24 months of follow-up.

To the best of our knowledge, the present report is the first to examine hypersplenism in HF patients. Considering that the duration of elevation of the free plasma hemoglobin and heme levels, which typically accompanies hypersplenism, may be associated with increased myocardial remodeling, we sought to identify the main factors contributing to aggravated heart failure in Class III or IV CHF patients according to the New York Heart Association functional classification. In the present study, we observed substantially deteriorated cardiac functioning in the CHF patients with hypersplenism; additionally, we also found that the increased levels of free plasma hemoglobin and heme induced by hypersplenism were positively correlated with myocardial remodeling in these patients at the 24th month.

It is now well accepted that CHF can lead to portal hypertension and hypersplenism and that hypersplenism increases the risks of hemolysis-driven oxidative stress and endothelial activation [17, 18]. An increasing number of studies have suggested that free plasma hemoglobin and heme play important roles in CHF

patients with hypersplenism; thus, they are interesting targets for the amelioration of myocardial remodeling [19, 20]. Consistent with other convincing data [10, 21, 22], our findings for the CHF patients with hypersplenism indicated that free plasma hemoglobin and heme are toxic to the vascular endothelium, leading to thrombus formation and vasoocclusive events. Free hemoglobin efficiently scavenges nitric oxide and reduces its bioavailability. Heme favors ROS production and promotes oxidative stress. As a result, they both aggravate the imbalance of endothelial cells, leading to endothelial dysfunction. Therefore, we further examined adhesion molecules that are expressed on vessel walls, and we observed increased levels of VCAM-1, ICAM-1, P-selectin and E-selectin. This result provides compelling evidence that hypersplenism induces excessive oxidative stress and endothelial dysfunction in the process of CHF.

Circulating EPCs are cardioprotective and may contribute to reverse remodeling by stimulating endogenous reparative pathways [23]. Endothelial dysfunction has been widely reported in patients with HF. To gain increased insight into the exact function of endothelial cells following hypersplenism in CHF patients, we conducted an *in vitro* experiment to assess EPCs derived from these patients and confirmed significantly decreased survival and differentiation capacities. We first showed that the proliferation of EPCs in the CHF patients with hypersplenism was significantly lower than that in the patients without hypersplenism. This result is in agreement with previous studies showing that the EPC count may be a prognostic factor for patients with HF [24-26]. Additionally, this result can most likely be ascribed to the high oxidative stress induced by hypersplenism in the HF patients because previous studies have shown that increased ROS levels play a key role in EPC dysfunction [27]. We further detected the decreased level of eNOS mRNA in EPCs. After 6 h of ischemic stimulation, the assessment of cell apoptosis by TUNEL assay also revealed that hypersplenism had an adverse effect on EPCs in the CHF patients, consistent with our other findings.

Although we showed that hypersplenism in CHF patients is inversely associated with myocardial remodeling and found increased levels of oxidative stress, our study still has several limita-tions. First, the sample size was relatively small. Therefore, large, multicenter studies that enroll diverse populations are needed. Second, we did not assess changes in other risk factors or determine BNP levels at the end of the follow-up period, which prevented us from exploring the effect of hypersplenism on the long-term prognosis of HF patients. Third, the results were solely observational, and future clinical studies using advanced cell labeling and imaging techniques are still needed. For these reasons, and in light of our data presented here, more studies on the mechanisms of hypersplenism in CHF patients are needed.

Conclusions

Overall, this study has revealed several novel aspects of the response of the internal environment of CHF patients with hypersplenism. Our results suggest that hypersplenism serves as an independent cardiovascular risk factor for CHF patients with a New York Heart Association functional classification of Class III or IV. Therefore, when patients with CHF are evaluated, the presence of this risk factor should be considered when possible. Furthermore, this study is the first to explore the possible mechanisms underlying the aggravation of myocardial remodeling by hypersplenism, and our findings suggest that this aggravation may be caused by excessive oxidative stress and subsequent endothelial dysfunction induced by increased levels of free plasma hemoglobin and heme in patients with hypersplenism.

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Disclosure of conflicts of interest

None.

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