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Berberine-induced decline in circulating $CD31^+/CD42^-$ microparticles is associated with improvement of endothelial function in humans

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ABSTRACT

Elevated circulating endothelial microparticles (EMPs) are associated with endothelial dysfunction. This study is to investigate whether berberine-induced fall in circulating EMPs facilitates improvement of endothelial function in healthy subjects. Fourteen healthy subjects received 1-month berberine therapy (1.2 g/d) and 11 healthy subjects served as control. Circulating EMPs were measured by flow cytometric analysis before and after therapy. Brachial artery endothelium-dependent and -independent function was assessed by flow-mediated vasodilation (FMD) and sublinqual nitroglyceride-mediated vasodilation (NMD). In vitro, human umbilical vein endothelial cells (HUVECs) were stimulated by EMPs (10^6/ml) with or without the presence of berberine (10 μM). Intracellular endothelial nitric oxide synthase (eNOS) protein expression was detected by flow cytometry. After berberine therapy, circulating $CD31^+/CD42^-$ microparticles were reduced, which was in parallel with the improvement of flow-mediated vasodilation while nitroglyceride-mediated vasodilation kept unchanged. A robust relationship was found between drop of circulating $CD31^+/CD42^-$ microparticles and increased flow-mediated vasodilation. The EMPs in vitro led to diminished eNOS protein expression in HUVECs and this EMP-mediated detrimental effect was markedly inhibited by berberine. Berberine-induced decline in circulating $CD31^+/CD42^-$ microparticles contributes to upregulation of endothelial function in healthy subjects. Deceasing EMPs may be a novel therapeutic target for the improvement of endothelial dysfunction in humans.

1. Introduction

Endothelial dysfunction plays a pivotal role in the initiation and progression of atherosclerosis which leads to high incidence of vascular disease (Behrendt and Ganz, 2002; Endemann and Schiffrin, 2004). The development of a novel method for the evaluation of endothelial dysfunction may have an important significance in the prevention of atherosclerotic vascular disease. Traditionally, measurements of endothelial function in vivo are usually dependent on assessment of nitric oxide (NO) bioavailability of endothelial origin such as flow-mediated or acetylcholine-induced endothelium-dependent vasodilation (Heitzer et al., 2005; Sinoway et al., 1989). Recently, quantitative and phenotypic change of circulating endothelial microparticles, mostly defined as $CD31^+/CD42^-$ microparticles, emerged as sensitive marker for both instant and chronic endothelial injury in response to various stimuli (Brodsky et al., 2002; Combes et al., 1999; Hamilton et al., 1990; Horstman et al., 2004; Wang et al., 2007b). Accumulating evidence indicates that endothelial microparticles may actively contribute to deterioration of endothelial homeostasis through interfering the functional and structural integrity of endothelium (Brodsky et al., 2002; Mezentsev et al., 2005). It was demonstrated that increased circulating endothelial microparticles were closely related to vascular dysfunction in patients with cardiovascular disorders (Arteaga et al., 2006; Pirro et al., 2006). We also reported that this association exists in healthy middle-aged subjects (Wang et al., 2007a), in whom diminished endothelial function has been reported to be initiated (Tao et al., 2004, 2007). However, little effort has been made to study the role of endothelial microparticles playing in endothelial dysfunction or the alteration of endothelial microparticles in facilitating the improvement of endothelial function in this population.

Berberine is a benzyl tetra isoquinoline alkaloid, which is widely used in orient world for long as an anti-microbial and an anti-diarrhoeal agent (Kuo et al., 2004). Recently, there are reports showing that berberine activates NO/cGMP pathway in smooth muscle cells and increases endothelium-dependent vasodilation in rat aortic rings (Chiou et al., 1991; Kang et al., 2002; Ko et al., 2000; Lau et al., 2001; Lee et al., 2006; Xu et al., 2008). Our laboratory has found that berberine improved endothelial integrity via increasing endothelial progenitor cells, the reservoir for endothelial repair (Xu et al., 2008a, 2008b). However, the mechanisms underlying these salutary effects remain largely unknown. For evaluation of endothelial dysfunction, there is no report on the possible effects of berberine on the alteration of endothelial microparticles or their relationship to

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endothelium-dependent vasodilation in human. Therefore, we hypothesize that one of the berberine-induced benefits is related to improvement in endothelial function via decreasing circulation of endothelial microparticles.

To address this assumption, we recruited healthy middle-aged subjects who received 1-month berberine therapy. The alteration of circulating $CD31^+/CD42^-$ microparticles was measured before and after berberine therapy. Brachial artery endothelium-dependent and -independent function was assessed by flow-mediated vasodilation and sublinqual nitroglyceride-mediated vasodilation before and after berberine therapy. To further investigate the potential mechanism involved in berberine -mediated endothelial protection, we also performed in vitro cell culture experiment to study the effect of endothelial microparticles on eNOS protein expression in HUVECs with or without the presence of berberine.

2. Materials and methods

2.1. Materials

RPMI-1640 culture medium was from Invitrogen (USA). Endothelial cell growth supplements were from Sigma (USA). Fetal bovine serum was from Hyclone (Australia). PE-conjugated monoclonal antibody against PECAM-1(PE-CD31) and FITC-conjugated monoclonal antibody against Platelet Glycoprotein Ib (FITC-CD42b) was used to identify endothelial microparticles. All the flow cytometry labeling reagents including PE- and FITC-conjugated monoclonal IgGs were purchased from Immunotech (France). Flowcount fluorospherical beads used to determine microparticles' absolute values and 1-μm calibrant beads used to define the upper size limit of the microparticles were from Beckman Coulter (USA). Berberine hemisulfate was from Alexis (Switzerland). Rabbit anti-human endothelial nitric oxide synthetase IgG(anti-eNOS IgG) was from Sigma (USA). Goat anti-rabbit FITC-conjugated IgG were from eBioscience (USA).

2.2. Procedures of clinical study

We recruited 25 healthy volunteers in the same community who were eligible for inclusion. They had no clinical cardiovascular disease history such as hypertension, ischemic heart diseases, peripheral artery disease, arrhythmia hyperlipidemia or diabetes mellitus, neither did they have impaired renal function and other major or acute pathologies. Fourteen volunteers (male:female = 6:8, age 54.07 ± 5.57 years, BMI 22.82 ± 2.95, currently smoking: 2 persons) received berberine therapy (0.4 g, 3 times per day) for 1 month, another 11 age- and gender-matched volunteers (male:female = 5:6, age 52.72 ± 4.37 years, BMI 22.56 ± 1.84, current smoking: 2 persons) were used as control group. The studied parameters were tested before and after treatment.

The study was performed in a controlled environment maintained at 23 °C after participants had rested at least for 15 min in a supine position. The subjects refrained from consuming alcohol or caffeine and did not smoke for at least 12 h before this study. The physical examination and measurement of flow-mediated vasodilation was performed. Then in the same day the venous blood sample for endothelial microparticles testing and routine laboratory tests was drawn from forearm in fasting status. Routine laboratory tests including serum total cholesterol, total triglyceride, low density lipoprotein–cholesterol, high density lipoprotein–cholesterol, and fasting plasma glucose were determined by standard laboratory methods. The protocol was approved by the Ethics Committee of Sun Yat-Sen University. All the subjects were aware of the investigative nature of the study and gave written informed consent for this study. The baseline characteristics of healthy subjects were given in Table 1.

Table 1
The characteristics of healthy subjects before and after treatments.

	Control group		Berberine group	
	Before treatment	After treatment	Before treatment	After treatment
SBP(mm Hg)	119 ± 14	120 ± 10	121 ± 12	118 ± 9[a]
DBP(mm Hg)	73 ± 10	72 ± 6	73 ± 8	71 ± 9[a]
PP(mm Hg)	47 ± 8	48 ± 5	48 ± 9	46.86 ± 7[a]
TC(mmol/L)	5.89 ± 0.89	5.32 ± 0.99	5.66 ± 0.94	4.8 ± 0.84[ab]
TG(mmol/L)	1.29 ± 0.61	1.47 ± 0.31	1.83 ± 1.04	1.65 ± 0.93
LDL-c(mmol/L)	3.48 ± 0.79	3.18 ± 0.87	3.12 ± 0.94	2.53 ± 0.66[ab]
HDL-c(mmol/L)	1.41 ± 0.22	1.26 ± 0.27	1.36 ± 0.33	1.24 ± 0.32
FPG(mmol/L)	4.79 ± 0.87	4.37 ± 1.06	4.72 ± 0.61	4.4 ± 0.53[a]
hsCRP(mg/L)	0.74 ± 0.13	0.86 ± 0.30	0.73 ± 0.15	0.63 ± 0.15
FMD(%)	8.29 ± 2.29	8.92 ± 3.22	8.14 ± 2.72	12.04 ± 3.70[ab]
NMD(%)	29.34 ± 5.07	27.76 ± 14.00	29.23 ± 12.2	30.92 ± 7.71
$CD31^+/CD42^-$ microparticles (per μL)	1150.3 ± 141.3	1112.4 ± 237.8	1305.0 ± 143.3	619.7 ± 67.1[ab]

Values are expressed mean ± S.D. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; TC, total cholesterol; TG, triglyceride; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; FPG, fasting plasma glucose; hsCRP, high-sensitive c reactive protein; FMD, flow-mediated vasodilation; NMD, nitroglycerine-mediated vasodilatation. Notes: [a] $P<0.05$ compared with berberine group before therapy; [b]$P<0.05$ compared with control group.

2.3. Measurement of flow-mediated vasodilation in the brachial artery

Flow-mediated vasodilation measurement in the brachial artery was performed with subjects in the supine position for the evaluation of endothelial function. All imaging was performed by a single, highly skilled sonographer who was unaware of the study assignment. As described previously (Raitakari and Celermajer, 2000; Tao, 2007), brachial artery diameter was imaged with a 5-12-MHz linear array transducer ultrasound system at a location 3 to 7 cm above the right elbow. The brachial artery diameters at baseline (D_0) and after reactive hyperemia (D_1) and sublingual nitroglycerine (D_2) were recorded. The flow-mediated vasodilation $[(D_1-D_0)/D_0\times 100\%]$ was used as a measure of endothelium-dependent vasodilation. The nitroglycerine-mediated vasodilatation $[(D_2-D_0)/D_0\times 100\%]$ was used as a measure of endothelium-independent vasodilatation. The repeatability coefficients of flow-mediated vasodilation and nitroglyceride-mediated vasodilation on the same person in 2-d interval were 0.93 and 0.91, respectively.

2.4. Circulating $CD31^+/CD42^-$ microparticles isolation, immunolabeling and flow cytometric analysis

Circulating $CD31^+/CD42^-$ microparticles were isolated from 4 ml of venous citrated blood drawn from forearm as described previously (Wang et al., 2007a). Briefly, the platelet-free plasma was separated from the 4 ml blood sample by centrifugation at 160× *g* (10 min) then 1000× *g* (6 min). Fifty microliters of the plasma was incubated with anti-CD31-PE (5 μl/test) and anti-CD42b-FITC (5 μl/test) antibodies or their respective isotypic immunoglobulins at room temperature for 30 min at dark with regular shaking. Samples were analyzed on an EPICS Altra(Beckman Coulter, USA) as previously described by an independent examiner who was unaware of the subject status. After using isotypic control to exclude non-specific fluorescence, events with a 0.1 to 1 μm diameter calibrated by 1 μm calibrator beads were identified in forward scatter and side scatter intensity dot representation, gated as microparticles (Fig. 1), and then plotted on one- or two-color fluorescence histograms. CD31+/CD42− microparticles were defined as elements that had a size <1 μm and >0.1 μm, positively labeled by anti-CD31-PE and negatively labeled by anti-CD42b-FITC (CD31+/CD42b- microparticles), that is B1 section in the histograms (Fig. 1B). The absolute concentration of circulating $CD31^+/CD42^-$ microparticles was calculated using the flowcount calibrator beads with known concentration provided by manufacturer,

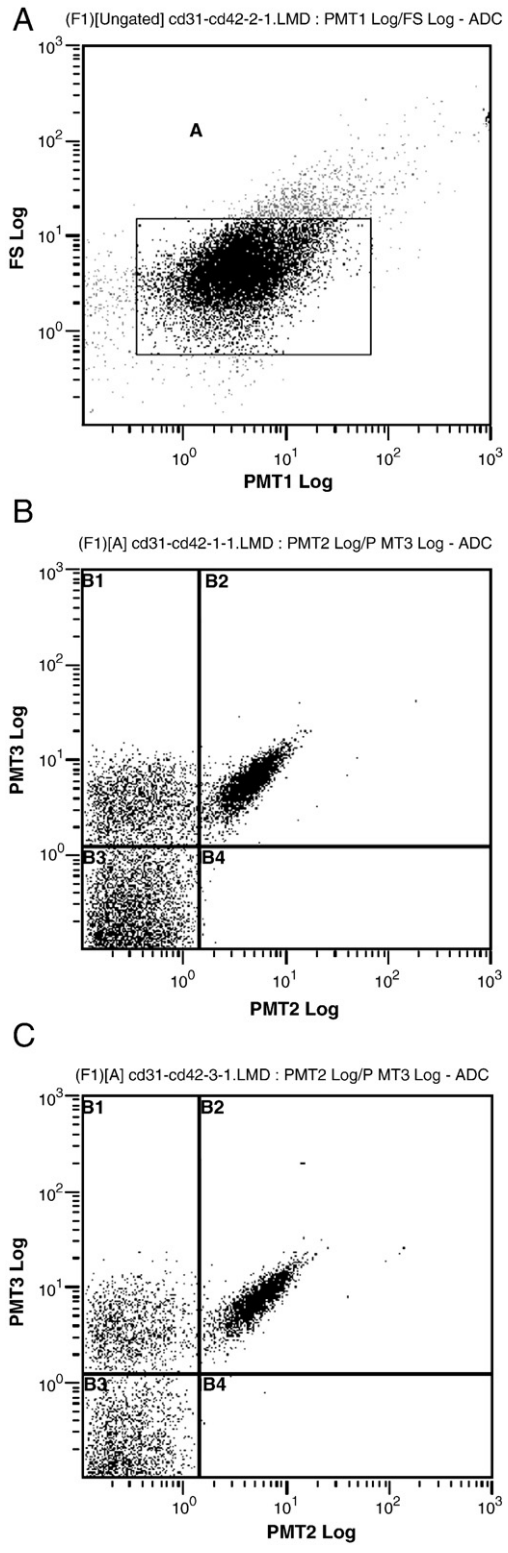

Fig. 1. The representative graphs of cytofluorometry analysis of circulating microparticles in platelet-free plasma from healthy subjects before and after berberine therapy. (A) Circulating microparticles are represented on a forward scatter/side scatter dot plot histogram. Microparticles are defined as events with a size of 0.1 to 1.0 μm gated in A window using 1 μm-diameter calibrator beads as interior criterion prior to the sample testing. (B, C) Size-selected events are plotted according to their fluorescence for specific CD42b-FITC(PMT2) and CD31–PE(PMT3) binding on a two-color scatter histograms. Events included in B1 section(CD31+/CD42b−) were considered microparticles mainly of endothelial origin, while events in B2 section (CD31+/CD42b+) were considered microparticles of platelet origin. The results of blood samples from the same volunteer indicate that the circulating $CD31^+/CD42^-$ microparticles before berberine therapy (B) were lower than that after(C) berberine therapy.

which was added (50 μl/test) into the sample right before flow cytometric analysis. The final numbers of circulating $CD31^+/CD42^-$ microparticles were expressed as microparticles per μl.

2.5. In vitro cell culture and endothelial microparticle stimulation

Human umbilical vein endothelial cells (HUVECs) were isolated from healthy umbilical vein with tripsin/EDTA as described previously (Jaffe et al., 1973; Wang et al., 2007b), and maintained in

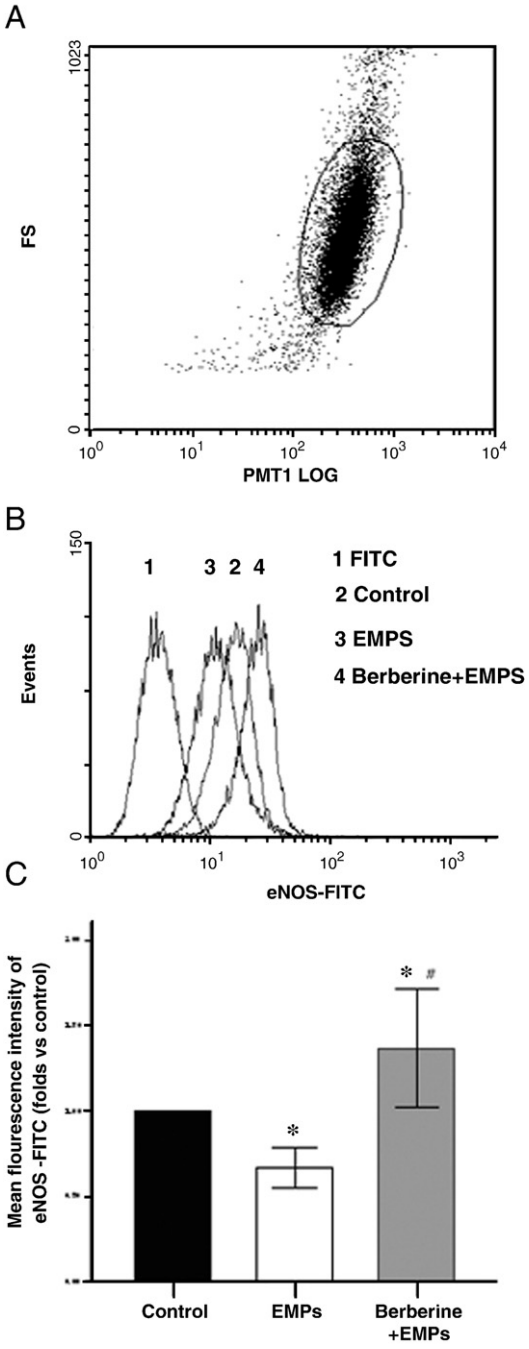

Fig. 2. Flow cytometric analysis in HUVECs detecting intracellular eNOS protein expression. (A) Flow cytometric analysis was performed on HUVECs gated on the basis of their forward (FS) and side light scatter (PMT1) with any cell debris excluded from analysis. (B) Results are expressed as histograms of relative cells counts with mean fluorescence intensity. (C) Elevated levels of EMPs(10^6/ml) significantly impaired eNOS protein expression in HUVECs. Pretreatment with berberine (10 μM) potently inhibited the effect of EMPs, largely improved eNOS protein expression. Notes: * $P<0.05$ compared with the control group; # $P<0.05$ compared with EMP group.

RPMI-1640 culture medium supplemented with 20% fetal bovine serum, 7.5% endothelial cell growth supplements, under condition of 37 °C and 5% CO_2. The HUVECs between 2nd and 5th passages grown in flasks to confluence (80%–90%) were starved (derived from FBS and growth supplements) for 4 h in cultured condition. The preparation of endothelial microparticles was proceed as previously described (Combes et al., 1999; Wang et al., 2005; 2007b). Culture supernatants from flasks containing about 10^6 cells were collected and cleared from cell fragments by centrifugation at 4300× *g* for 5 min. The supernatant was then ultracentrifuged at 200,000× *g* for 120 min at 10 °C. Pelleted endothelial microparticles were resuspended in 400 μl of RPMI-1640 medium and used immediately. Fifty μl endothelial microparticle suspension was used to undergo flow cytometry analysis using anti-CD31-PE as marker for quantification. The HUVECs were stimulated for 2 h by (1) 1640 medium ($n=6$); (2)1640 medium containing 10^6/ml of endothelial microparticles ($n=6$); (3) 1640 medium containing 10^6/ml of endothelial microparticles pretreated with berberine (10 μM) for 0.5 h ($n=6$). Then the intracellular endothelial nitric oxide synthase (eNOS) was assessed by flow cytometric analysis.

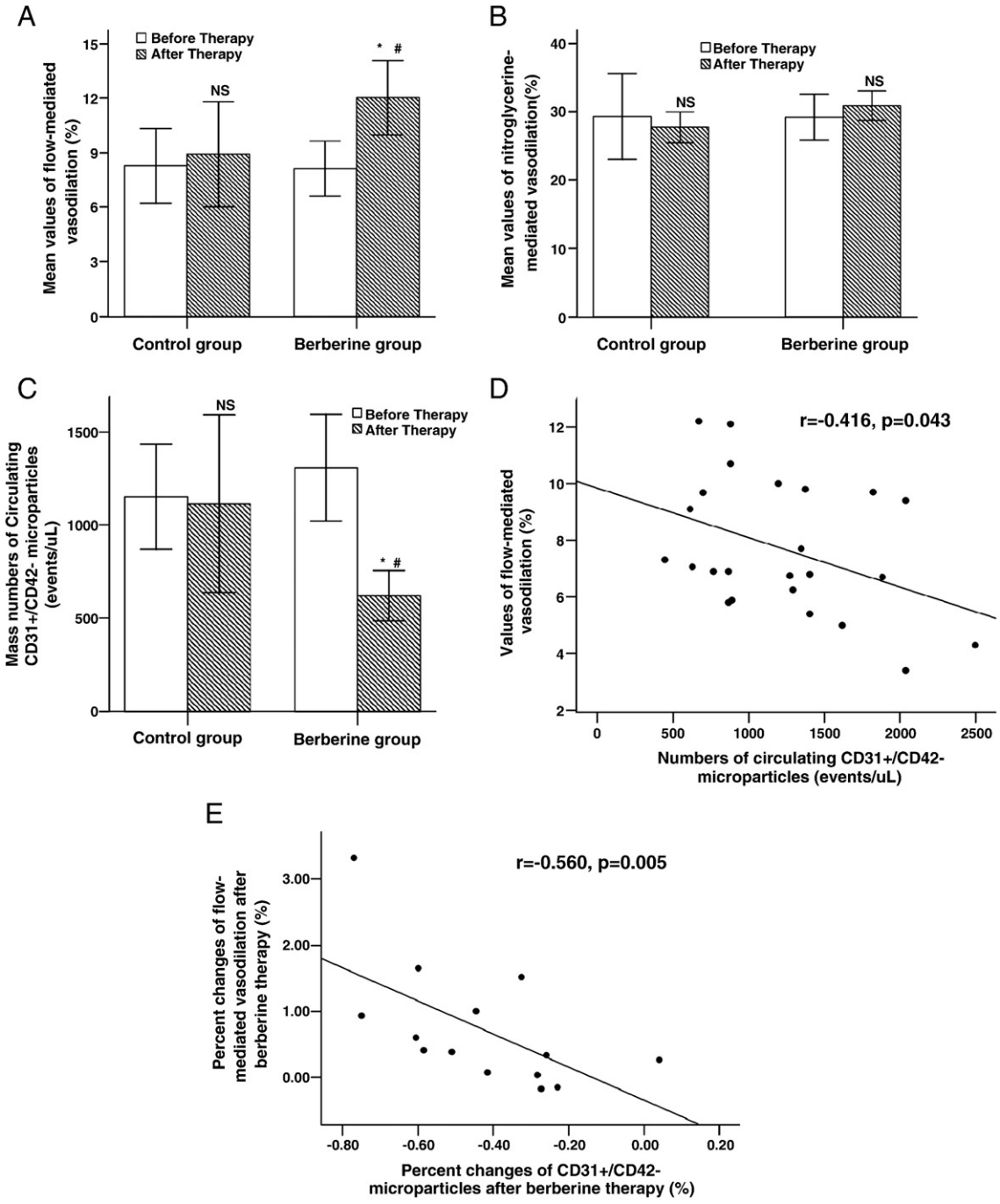

Fig. 3. Effects of berberine treatment on flow-mediated vasodilation, nitroglyceride-mediated vasodilation and circulating $CD31^+/CD42^-$ microparticles and relationships among studied parameters. (A, B, C) Bar graphs of alteration of flow-mediated vasodilation (A), nitroglyceride-mediated vasodilation (B) and numbers of circulating $CD31^+/CD42^-$ microparticles (C) in control group and berberine group. (D) Scatter plot histogram shows the correlation between values of flow-mediated vasodilation, numbers of circulating $CD31^+/CD42^-$ microparticles in 25 healthy volunteers before berberine therapy. (E) Scatter plot histogram shows the correlation between changes in flow-mediated vasodilation and circulating $CD31^+/CD42^-$ microparticles in 14 subjects after berberine therapy. Notes: $^*P<0.05$ compared with berberine group before treatment; $^{\#}P<0.05$ compared with control group.

2.6. Flow cytometric analysis for intracellular endothelial nitric oxide synthase (eNOS) protein

The detection for eNOS protein expression using flow cytometric analysis was processed as previous reported with slight modification (Havenga et al., 2001). Briefly, $1–5\times10^6$cells of one sample were harvested and transferred to polystyrene flow cytometric tubes, then washed with PBS, pelleted, resuspended in 0.5 ml of PBS, and placed on ice for 5 min. After addition of 0.5 ml of cold fixative (1% paraformaldehyde in PBS) incubation proceeded for another 5 min, followed by the addition of 2 ml PBS/0.5% BSA, cells were subsequently pelleted and washed in 1 ml PBS. Next, the cells were incubated with a 1: 50 dilution of rabbit anti-human eNOS IgG for 30 min at 4 °C in the dark, washed once, then incubated in a 1:50 dilution of goat anti-rabbit FITC-conjugated IgG (eBioscience) for 30 min at 4 °C in the dark. Isotype-identical goat anti-rabbit FITC-conjugated IgG labeling was used to exclude the nonspecific fluorescence. Then the cells were washed twice with PBS, resuspended in 0.5 ml of PBS. Expression of eNOS protein on 10,000 viable cells was then analyzed by single color flow cytometric analysis (Elite FACScan, Becton Dickinson) (Fig. 2A) equipped with an argon ion laser. Mean fluorescence intensity (MFI), which includes both the changes of eNOS on individual cell and the percentage of cells expressing eNOS protein was calculated. The repeatability coefficient of inter- and intra-group was 96% and 91%, respectively. Data were expressed as folds of MFI compared with resting control.

2.7. Statistical analysis

Data of this study are expressed as mean ± S.D. except for circulating $CD31^+/CD42^-$ microparticles as mean ± S.E.M. Statistical analysis was performed with SPSS 10.0 software for Windows (SPSS Software, Chicago, IL). In the clinical study, the characteristics between clinic visits were compared by paired Student's *t* test. Variables of $CD31^+/CD42^-$ microparticles with non-normal distribution were Log-transformed to achieve normal distribution before correlation analysis. Spearman's simple correlation coefficient between percent changes of flow-mediated vasodilation and $CD31^+/CD42^-$ microparticles as well as other studied variables was determined. Fluorescence intensity of eNOS was analysized by one-way ANOVA followed by Student's *t* test. Value of $P<0.05$ was considered statistically significant.

3. Results

The characteristics of the recruited healthy subjects before and after therapy were shown in Table 1. There were significant decreases in BP profiles, total cholesterol, total triglyceride, low density lipoprotein–cholesterol and fasting plasma glucose after berberine therapy ($P<0.05$). Most importantly, berberine significantly decreased circulating $CD31^+/CD42^-$ microparticles (Fig. 1C, 3B) and improved flow-mediated vasodilation (Fig. 3A). Meanwhile, there was no significant difference in circulating $CD31^+/CD42^-$ microparticles and endothelial function in control subjects.

In addition, values of flow-mediated vasodilation were inversely correlated with values of circulating $CD31^+/CD42^-$ microparticles before berberine therapy ($P<0.05$, Fig. 3D, Table 2). There is no other studied variable related to circulating $CD31^+/CD42^-$ microparticles and flow-mediated vasodilation before therapy (Table 2) except that the relationships between systolic blood pressure and circulating $CD31^+/CD42^-$ microparticles as well as flow-mediated vasodilation were at marginal significance. After correcting for systolic blood pressure, the partial relationship between $CD31^+/CD42^-$ microparticles and flow-mediated vasodilation was −0.422 ($P=0.064$). The changes of flow-mediated vasodilation values and that of circulating $CD31^+/CD42^-$ microparticles after berberine therapy was negatively related (Table 3, Fig. 3E), while changes of low density lipoprotein-cholesterol were positively related with changes of circulating $CD31^+/CD42^-$ microparticles ($r=0.460$, $P=0.037$). There was a negative relationship between changes of flow-mediated vasodilation and $CD31^+/CD42^-$ microparticles ($r=-0.560$, $P=0.005$, Fig. 3E).

In the in vitro experiments, compared with the resting control, endothelial microparticles (10^6/ml) stimulation significantly reduced the intracellular eNOS protein expression (0.667 ± 0.143 folds vs controls, $P<0.05$) in HUVECs (Fig. 2B and C). Pretreatment with berberine (10 μM) inhibited the effect of endothelial microparticles and enhanced the eNOS protein expression (1.368 ± 0.420 folds, $P<0.05$ vs controls; $P<0.05$ vs endothelial microparticle group) in HUVECs (Fig. 2B and C).

Table 2
Correlations between studied parameters before therapy in 25 subjects.

Spearman's rho	FMD(%)	$CD31^+/CD42^-$ microparticles(per μL)
FMD(%)	$r=1$	$r=-0.416$, P = 0.043
$CD31^+/CD42^-$ microparticles(per μL)	$r=-0.416$, $P=0.043$	$r=1$
Age(years)	$r=0.264$, P = 0.362	$r=-0.269$, $P=0.353$
SBP(mm Hg)	$r=-0.400$, $P=0.072$	$r=0.319$, $P=0.104$
TC(mmol/L)	$r=-0.262$, $P=0.310$	$r=0.325$, $P=0.162$
TG(mmol/L)	$r=-0.111$, $P=0.606$	$r=-0.005$, $P=0.979$
HDL-c(mmol/L)	$r=0.229$, P = 0.282	$r=-0.182$, $P=0.335$
LDL-c(mmol/L)	$r=-0.118$, $P=0.582$	$r=-0.041$, $P=0.831$
FPG(mmol/L)	$r=-0.158$, $P=0.786$	$r=0.246$, $P=0.191$
hsCRP(g/L)	$r=-0.230$, $P=0.315$	$r=0.211$, $P=0.668$

FMD, flow mediated vasodilation; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; FPG, fasting plasma glucose; hsCRP, high-sensitive c reactive protein.

Table 3
Correlations between changes in parameters after BR therapy in 14 subjects.

Spearman's rho	delta-FMD(%)	delta-$CD31^+/CD42^-$ microparticles(%)
Delta-FMD(%)	$r=1$	$r=-0.560$, $P=0.005$
Delta-$CD31^+/CD42^-$ microparticles(%)	$r=-0.560$, $P=0.005$	$r=1$
Delta-SBP(%)	$r=0.037$, P = 0.899	$r=0.138$, $P=0.637$
Delta-TC(%)	$r=-0.455$, $P=0.102$	$r=0.433$, $P=0.122$
Delta-TG(%)	$r=-0.152$, $P=0.605$	$r=-0.029$, $P=0.923$
Delta-HDL-c(%)	$r=-0.376$, $P=0.185$	$r=0.314$, $P=0.274$
Delta-LDL-c(m%)	$r=-0.437$, $P=0.118$	$r=0.460$, $P=0.037$
Delta-FPG(%)	$r=0.174$, $P=0.553$	$r=0.055$, $P=0.852$
Delta-hsCRP(%)	$r=0.099$, $P=0.737$	$r=-0.112$, $P=0.703$

FMD, flow mediated vasodilation; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; FPG, fasting plasma glucose; hsCRP, high-sensitive c reactive protein.

4. Discussion

The major findings of the present study were that berberine therapy was able to decrease circulating $CD31^+/CD42^-$ microparticles in healthy subjects. In parallel, flow-mediated vasodilation in the brachial artery was improved but nitroglyceride-mediated vasodilation kept unchanged. The fall in circulating $CD31^+/CD42^-$ microparticles was closely related to flow-mediated vasodilation improvement. The endothelial microparticles stimulation *in vitro* led to diminished eNOS protein expression in HUVECs and this endothelial microparticle-mediated detrimental effect was markedly inhibited by presence of berberine. The present study is the first time to demonstrate that the drop in circulating endothelial microparticles caused by berberine contributes to upregulation of endothelial function and that deceasing circulating endothelial microparticles should be a novel therapeutic target for the improvement of endothelial function in humans.

4.1. Circulating endothelial microparticles and endothelial dysfunction

It is generally accepted that well-functional endothelium maintains a non-thrombogenic surface, prohibits leukocyte attachment, and promotes vascular relaxation. Endothelial dysfunction contributes to the pathogenesis of vascular disease (Landmesser et al., 2004). Accordingly, it is essential to identify plasma markers of endothelial injury and explore novel interventions to alleviate endothelial perturbation aimed at preventing the development of vascular disease.

Recent evidence suggested that elevated plasma levels of endothelial-derived microparticles are new markers for the evaluation of endothelial function in physical condition and in various pathological status with cardiovascular disorders (Amabile et al., 2005; Bernal-Mizrachi et al., 2003; Jimenez et al., 2001; Preston et al., 2003). We previously reported that circulating $CD31^+/CD42^-$ microparticles were related with systemic vascular dysfunction in subjects without apparent cardiovascular risk factors (Wang et al., 2007a). In the present study, we also found a pronounced relationship between circulating $CD31^+/CD42^-$ microparticles and flow-mediated vasodilation in healthy subjects while other parameters were not related to flow-mediated vasodilation. Notably, systolic blood pressure showed marginally significant association with both circulating $CD31^+/CD42^-$ microparticles and flow-mediated vasodilation. To cautiously exclude the possible interfere of systolic blood pressure, we analyzed the partial relationship between circulating $CD31^+/CD42^-$ microparticles and flow-mediated vasodilation by correcting systolic blood pressure. This association was still present with marginal statistical difference ($P=0.064$), indicating that even though this association is affected by blood pressure, it may persistently exists because the endothelial microparticles are directly derived from dysfunctional endothelium.

Besides serving as a marker of endothelial injury, endothelial microparticles have been demonstrated to directly facilitate occurrence of vascular disease by initiating inflammation, coagulation, and vascular (dys)function and etc (Horstman et al., 2004; Klinkner et al., 2006). We stimulated HUVECs *in vitro* with elevated concentration of endothelial microparticles and measured the expression of the most important functional protein eNOS in HUVECs using flow cytometry. Our present data indicated that a significant down-regulation of eNOS protein expression was induced by endothelial microparticle stimulation. Taken with previous studies(Klinkner et al., 2006; Mezentsev et al., 2005), further confirm that the measurement of circulating $CD31^+/CD42^-$ microparticles is a potent indicator for the extent of endothelial injury *in vivo* and elevated endothelial microparticles per se are detrimental stimuli which may lead to poor NO bioavailability in endothelium, limiting the essential role of endothelium in maintaining integrity of vascular function. Endothelial microparticle detection and quantification as sensitive and effective marker of endothelial dysfunction is an interesting and potentially valuable tool to assess vascular risk of asymptomatic subjects. Therefore, decreasing endothelial microparticles could be a potential therapeutic target for the improvement of endothelial function.

4.2. Effect of berberine on endothelial microparticle-induced endothelial perturbation

Our experiments were the first time to investigate the influence of alteration in circulating endothelial microparticles induced by berberine on endothelial function in human. The beneficial influence of berberine on endothelial function demonstrated in animal models and in humans is drawing increasing attention (Lau et al., 2001). Despite these favorable results, the mechanism underlying berberine-mediated endothelial protection is still largely unknown. In the present study, we found that in healthy subjects berberine improved endothelium-dependent vasodilation (flow-mediated vasodilation) but not endothelium-independent vasodilation (nitroglyceride-mediated vasodilation), indicating that berberine-induced vasoprotection might largely depend on endothelial function. In parallel, circulating $CD31^+/CD42^-$ microparticles were significantly decreased after berberine therapy. A highly invert relationship was found between the alteration in circulating endothelial microparticles and that in flow-mediated vasodilation, which indicated a possibility that to decrease the release of endothelial microparticles is at least partly involved in the berberine-induced augment of endothelial function.

At the same time, we also found that berberine helped to decrease blood pressure, total cholesterol, low density lipoprotein-cholesterol, fasting plasma glucose levels in healthy subjects, which may affect both flow-mediated vasodilation and circulating $CD31^+/CD42^-$ microparticles (Table 3) (Arteaga et al., 2006; Pirro et al., 2006). To rule out the potential interfering factors we ran a partial correlation analysis and proved that the drop of circulating $CD31^+/CD42^-$ microparticles induced by berberine has substantial relationship with the improvement of endothelium-dependent vasodilation, which suggests that the benefit of berberine on endothelial function may be both in direct or indirect manners. To further prove the contribution of berberine-induced decrease in circulating endothelial microparticles to improvement of endothelial function, we performed *in vitro* cell culture experiments. HUVECs were stimulated by elevated concentration of endothelial microparticles with or with out berberine pretreatement. We found that endothelial microparticle-inducted eNOS protein down-regulation was inhibited in HUVECs pretreated with berberine. Furthermore, compared with resting controls, berberine improved eNOS protein expression despite of the stimulation of endothelial microparticles, suggesting that in addition of inhibiting the detrimental effects of endothelial microparticles, berberine might have other regulative mechanisms mediating its augment of eNOS expression so as to protect endothelium (Holy et al., 2009; Hsieh et al., 2007). However, based on the data we suggested that the berberine-mediated endothelial protection is, at least in part, related to decreasing circulating endothelial microparticles with subsequently augmenting NO bioavailability.

Taken together, we believed that berberine affects endothelial function probably through multiple pathways. On one hand, it directly suppresses circulating $CD31^+/CD42^-$ microparticles and their detrimental effects towards diminished NO bioavailability, thus helps to maintain an integrate endothelium. On the other hand, it may benefit blood pressure levels, lipid and glucose metabolism (Kong et al., 2004; Lau et al., 2001) which benefit endothelial function. These mechanisms may synergistically contribute to the maintenance of endothelial homeostasis so as to protect vascular function.

4.3. Limitations

It should be pointed out that there were some limitations in our studies. First, we recruited healthy subjects instead of patients with cardiovascular disorders and the sample size is small. Large scale of clinical studies in selected patients will be needed for further investigation. Second, our *in vitro* experiments tested the effect of berberine on EMP-induced downregulation of intracellular eNOS protein expression. But we did not investigate the down-stream pathways and other possible molecular mechanisms that may alter the NO bioavailability in endothelial cells. We previously demonstrated that EMP elevation was accompanied by impaired BH_4-dependent NO formation in HUVECs stimulated by CRP (Wang, 2007). There were also reports showing the substantial role of oxidative stress in EMP formation (Mezentsev, 2005; Brodsky et al., 2004). We therefore infer that berberine may exert its influence on EMP activities through multiple pathways, in which oxidative stress may also play an important part. Further experiments are needed to explore detailed mechanisms underlying this issue.

5. Conclusions

Our present study suggests that the elevated EMP level is not only a risk factor but also a detrimental mediator to endothelial function.

Improvement of endothelial function caused by berberine is, at least in part, via reducing circulating $CD31^+/CD42^-$ microparticles and attenuating EMP-induced endothelial dysfunction. Decreasing EMPs may be a novel therapeutic target for the improvement of endothelial dysfunction in humans.

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References

Amabile, N., Guerin, A.P., Leroyer, A., Mallat, Z., Nguyen, C., Boddaert, J., London, G.M., Tedgui, A., Boulanger, C.M., 2005. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. J. Am. Soc. Nephrol. 16, 3381–3388.

Arteaga, R.B., Chirinos, J.A., Soriano, A.O., Jy, W., Horstman, L., Jimenez, J.J., Mendez, A., Ferreira, A., de Marchena, E., Ahn, Y.S., 2006. Endothelial microparticles and platelet and leukocyte activation in patients with the metabolic syndrome. Am. J. Cardiol. 98, 70–74.

Behrendt, D., Ganz, P., 2002. Endothelial function. From vascular biology to clinical applications. Am. J. Cardiol. 90, 40L–48L.

Bernal-Mizrachi, L., Jy, W., Jimenez, J.J., Pastor, J., Mauro, L.M., Horstman, L.L., de Marchena, E., Ahn, Y.S., 2003. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. Am. Heart. J. 145, 962–970.

Brodsky, S.V., Malinowski, K., Golightly, M., Jesty, J., Goligorsky, M.S., 2002. Plasminogen activator inhibitor-1 promotes formation of endothelial microparticles with procoagulant potential. Circulation. 106, 2372–2378.

Chiou, W.F., Yen, M.H., Chen, C.F., 1991. Mechanism of vasodilatory effect of berberine in rat mesentric artery. Eur. J. Pharmacol. 204, 35–40.

Combes, V., Simon, A.C., Grau, G.E., Arnoux, D., Camoin, L., Sabatier, F., Mutin, M., Sanmarco, M., Sampol, J., Dignat-George, F., 1999. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. J. Clin. Invest. 104, 93–102.

Endemann, D.H., Schiffrin, E.L., 2004. Endothelial dysfunction. J. Am. Soc. Nephrol. 15, 1983–1992.

Hamilton, K.K., Hattori, R., Esmon, C.T., Sims, P.J., 1990. Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. J. Biol. Chem. 265, 3809–3814.

Heitzer, T., Baldus, S., von Kodolitsch, Y., Rudolph, V., Meinertz, T., 2005. Systemic endothelial dysfunction as an early predictor of adverse outcome in heart failure. Arterioscler. Thromb. Vasc. Biol. 25, 1174–1179.

Holy, E.W., Akhmedov, A., Luscher, T.F., Tanner, F.C., 2009. Berberine, a natural lipid-lowering drug, exerts prothrombotic effects on vascular cells. J. Mol. Cell. Cardiol. 46, 234–240.

Horstman, L.L., Jy, W., Jimenez, J.J., Ahn, Y.S., 2004. Endothelial microparticles as markers of endothelial dysfunction. Front. Biosci. 9, 1118–1135.

Hsieh, Y.S., Kuo, W.H., Lin, T.W., Chang, H.R., Lin, T.H., Chen, P.N., Chu, S.C., 2007. Protective effects of berberine against low-density lipoprotein (LDL) oxidation and oxidized LDL-induced cytotoxicity on endothelial cells. J. Agric. Food. Chem. 55, 10437–10445.

Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R., 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745–2756.

Jimenez, J.J., Jy, W., Mauro, L.M., Horstman, L.L., Ahn, Y.S., 2001. Elevated endothelial microparticles in thrombotic thrombocytopenic purpura: findings from brain and renal microvascular cell culture and patients with active disease. Br. J. Haematol. 112, 81–90.

Kang, D.G., Sohn, E.J., Kwon, E.K., Han, J.H., Oh, H., Lee, H.S., 2002. Effects of berberine on angiotensin-converting enzyme and NO/cGMP system in vessels. Vascul. Pharmacol. 39, 281–286.

Klinkner, D.B., Densmore, J.C., Kaul, S., Noll, L., Lim, H.J., Weihrauch, D., Pritchard Jr., K.A., Oldham, K.T., Sander, T.L., 2006. Endothelium-derived microparticles inhibit human cardiac valve endothelial cell function. Shock 25, 575–580.

Ko, W.H., Yao, X.Q., Lau, C.W., Law, W.I., Chen, Z.Y., Kwok, W., Ho, K., Huang, Y., 2000. Vasorelaxant and antiproliferative effects of berberine. Eur. J. Pharmacol. 399, 187–196.

Kong, W., Wei, J., Abidi, P., Lin, M., Inaba, S., Li, C., Wang, Y., Wang, Z., Si, S., Pan, H., Wang, S., Wu, J., Wang, Y., Li, Z., Liu, J., Jiang, J.D., 2004. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat. Med. 10, 1344–1351.

Kuo, C.L., Chi, C.W., Liu, T.Y., 2004. The anti-inflammatory potential of berberine in vitro and in vivo. Cancer. Lett. 203, 127–137.

Landmesser, U., Hornig, B., Drexler, H., 2004. Endothelial function: a critical determinant in atherosclerosis? Circulation 109, II27–33.

Lau, C.W., Yao, X.Q., Chen, Z.Y., Ko, W.H., Huang, Y., 2001. Cardiovascular actions of berberine. Cardiovasc. Drug. Rev. 19, 234–244.

Lee, S., Lim, H.J., Park, H.Y., Lee, K.S., Park, J.H., Jang, Y., 2006. Berberine inhibits rat vascular smooth muscle cell proliferation and migration in vitro and improves neointima formation after balloon injury in vivo. Berberine improves neointima formation in a rat model. Atherosclerosis 186, 29–37.

Mezentsev, A., Merks, R.M., O'Riordan, E., Chen, J., Mendelev, N., Goligorsky, M.S., Brodsky, S.V., 2005. Endothelial microparticles affect angiogenesis in vitro: role of oxidative stress. Am. J. Physiol. Heart. Circ. Physiol. 289, H1106–H1114.

Pirro, M., Schillaci, G., Paltriccia, R., Bagaglia, F., Menecali, C., Mannarino, M.R., Capanni, M., Velardi, A., Mannarino, E., 2006. Increased ratio of $CD31^+/CD42^-$ microparticles to endothelial progenitors as a novel marker of atherosclerosis in hypercholesterolemia. Arterioscler. Thromb. Vasc. Biol. 26, 2530–2535.

Preston, R.A., Jy, W., Jimenez, J.J., Mauro, L.M., Horstman, L.L., Valle, M., Aime, G., Ahn, Y.S., 2003. Effects of severe hypertension on endothelial and platelet microparticles. Hypertension 41, 211–217.

Sinoway, L.I., Hendrickson, C., Davidson, W.R., Prophet, S., Zelis, R., 1989. Characteristics of flow-mediated brachial artery vasodilation in human subjects. Circ. Res. 64, 32–42.

Tao, J., Jin, Y.F., Yang, Z., Wang, L.C., Gao, X.R., Lui, L., Ma, H., 2004. Reduced arterial elasticity is associated with endothelial dysfunction in persons of advancing age: comparative study of noninvasive pulse wave analysis and laser Doppler blood flow measurement. Am. J. Hypertens. 17, 654–659.

Tao, J., Liu, D.H., Wang, L.C., Wang, J.M., Wang, Y., Yang, Z., Lou, Z.F., Tang, A.L., 2007. Arterial elasticity identified by pulse wave analysis and its relation to endothelial function in patients with coronary artery disease. J. Hum. Hypertens. 21, 149–153.

Wang, J.M., Huang, Y.J., Wang, Y., Xu, M.G., Wang, L.C., Wang, S.M., Tao, J., 2007a. Increased circulating $CD31^+/CD42^-$ microparticles are associated with impaired systemic artery elasticity in healthy subjects. Am. J. Hypertens. 20, 957–964.

Wang, J.M., Wang, Y., Huang, J.Y., Yang, Z., Chen, L., Wang, L.C., Tang, A.L., Lou, Z.F., Tao, J., 2007b. C-Reactive protein-induced endothelial microparticle generation in HUVECs is related to BH4-dependent NO formation. J. Vasc. Res. 44, 241–248.

Wang, Y., Tao, J., Yang, Z., Tu, C., Xu, M.G., Wang, J.M., Huang, J.Y., 2005. Tumor necrosis factor-alpha induces release of endothelial microparticles from human endothelial cells. Zhonghua. Xin. Xue. Guan. Bing. Za. Zhi. 33, 1137–1140.

Xu, M.G., Wang, J.M., Chen, L., Wang, Y., Yang, Z., Tao, J., 2008a. Berberine-induced mobilization of circulating endothelial progenitor cells improves human small artery elasticity. J. Hum. Hypertens. 22, 389–393.

Xu, M.G., Wang, J.M., Chen, L., Wang, Y., Yang, Z., Tao, J., 2008b. Berberine-Induced Upregulation of Circulating Endothelial Progenitor Cells Is Related to Nitric Oxide Production in Healthy Subjects. Cardiology 112, 279–286.