EXPERIMENTAL STUDY

Serum-Derived Exosomes from Patients with Coronary Artery Disease Induce Endothelial Injury and Inflammation in Human Umbilical Vein Endothelial Cells

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Summary

Endothelial injury and inflammation have been found to be essential in the pathogenesis of coronary artery disease (CAD). Circulating exosomes are of great value as novel biomarkers for CAD. However, the role of circulating exosomes in the pathogenesis of CAD remains unclear. Thus, in this study, we aimed to examine whether circulating exosomes from CAD are involved in the endothelial injury and inflammation. The serum-derived exosomes were isolated from CAD and controls using an ExoQuick reagent, and these were then quantified by measuring the protein levels using BCA methods. The uptake of exosomes by human umbilical vein endothelial cells (HUVECs) was observed by laser scanning microscope and analyzed via flow cytometry. Then, HUVECs were treated with vehicle, exosomes from CAD (CAD-exo), and controls (ctrl-exo) in the absence and presence of vascular endothelial growth factor (VEGF). Cell viability, migration, and angiogenesis were evaluated using CCK-8 assay, scratch assay, and tube formation assay. Inflammatory factors including IL-1β, IL-6, TNF-α, ICAM-1, and VCAM-1 levels were detected via qPCR. As per our findings, no significant differences were noted in uptake of ctrl-exo and CAD-exo by HUVECs. CAD-exo suppressed cell viability in a dose-dependent manner. Compared with ctrl-exo, CAD-exo-treated HUVECs significantly suppressed migration and angiogenesis. However, CAD-exo had a stronger inhibitory effect on VEGF-induced migration and angiogenesis compared with ctrl-exo. Moreover, IL-1β, TNF-α, and ICAM-1 were determined to be significantly upregulated in HUVECs treated with CAD-exo, but IL-6 and VCAM-1 expressions were not affected. Overall, our results suggest that CAD-exo are involved in endothelial injury and inflammation, which may, in turn, cause endothelial dysfunction and potentially promote the development of CAD.

Key words: Atherosclerosis, Angiogenesis, Inflammatory factors, Pathogenesis

Coronary artery disease (CAD) has been considered an epidemic, accounting for the majority of deaths worldwide. Although remarkable achievements have been made in drug and interventional therapies in recent decades, the prevalence and mortality of CAD remain to be on the rise. It has been established that CAD is a multifactorial disease caused by the interaction of environmental and genetic factors. Traditional risk factors for CAD include age, smoking, hypertension, hyperglycemia, dyslipidemia, obesity, family history of premature CAD, and physical inactivity. Moreover, genomewide association studies together with whole-exome sequencing have identified numerous genetic risk variants associated with CAD susceptibility. However, the pathogenesis of CAD is yet to be fully elucidated.

Exosomes, which are a kind of endogenous extracellular vesicle with a diameter ranging from 40 to 100 nanometers (nm), contain a number of bioactive molecules such as proteins, nucleic acids, and lipids, which are emerging as important mediators in the intercellular communication. Recent studies identified that the bioactive molecules contained in circulating exosomes are dysregu-
lated in CAD. For example, exosomal miR-126, miR-21, and PTEN were determined to be upregulated in patients with acute coronary syndrome (ACS), whereas exosomal miR-1915-3p, miR-4507, and miR-3656 were downregulated in patients with acute myocardial infarction. Meanwhile in CAD, exosomal SOCS2-AS1 has been observed to decrease, while exosomal circ_0005540 was noted to increase. These studies suggest that circulating exosomes’ contents play an important role in the development of CAD, which are of great value as potential biomarkers and therapeutic targets for CAD. However, little was known about the role of circulating exosomes in the pathophysiology of CAD.

Atherosclerosis, the pathological basis of CAD, has been recognized as a chronic inflammatory process involving the interaction of many types of cells such as endothelial cells (ECs), macrophages, and vascular smooth muscle cells (VSMCs). ECs situated at the interface between the vessel wall and blood have been determined to play important roles in the maintenance of vascular tone and homeostasis. The exposure of ECs to most cardiovascular risk factors (e.g., hypertension and dyslipidemia) can induce endothelial injury, followed by inflammatory activation, macrophage infiltration, and VSMC activation, which, in turn, promote the development of atherosclerosis. Therefore, endothelial injury is identified as the initial process of atherosclerosis. Notably, previous studies showed that exosomes derived from macrophages and VSMCs are involved in endothelial injury and inflammation. However, whether the circulating exosomes from CAD are involved in endothelial injury and inflammation is yet to be determined. In this study, we have analyzed the protein levels of the serum-derived exosomes from CAD patients and control subjects, in order to determine whether the serum-derived exosomes could be taken up by HUVECs and whether the exosomes from CAD patients are involved in endothelial injury and inflammation. Altogether, our findings provide important insights into the regulatory role of circulating exosomes from CAD in the pathophysiology of CAD.

Methods

Study subjects: In total, 9 CAD patients and 9 age- and sex-matched control subjects were recruited at Guangdong Provincial People’s Hospital. All participants underwent coronary angiography before being recruited. CAD was defined as the narrowing of ≥50% in the left main coronary artery and ≥70% in one or several major coronary arteries. Control subjects included those with normal coronary angiograms. Inclusion criteria included men aged 40-65 years old who were diagnosed with CAD or excluded CAD by coronary angiography and agreed to join the study. Exclusion criteria included cardiomyopathy, abnormal hepatic or kidney function (transaminase and creatinine levels exceeding the upper limit of normal), malignant diseases, autoimmune diseases, acute infections, surgery, or trauma in the past 6 months. Written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee of Guangdong Provincial People’s Hospital.

Collection of blood samples: Two milliliters (mL) of peripheral blood were obtained from each participant after an overnight fast. All the blood samples were collected in serum separator tubes containing separation gel, and these were then separated into serum fractions by centrifugation at 1000 g for 15 minutes after standing for half an hour. Serum specimens were frozen at −80°C until use.

Exosome isolation and purification: The serum samples were thawed at 4°C and then used to isolate exosomes using ExoQuick. The serum specimens were thawed at 4°C and then used to isolate exosomes using ExoQuick. The serum was diluted 3000 g for 15 minutes and then further centrifuged at 12000 g for 10 minutes to remove cellular debris. All the centrifugation was performed at 4°C. Subsequently, 67 µL of ExoQuick was added to 250 µL of the centrifuged serum and incubated at 4°C for 30 minutes. Pellets of exosomes were collected by centrifuging the ExoQuick/serum mixture at 3000 g for 10 minutes. Then, the isolated exosomes were purified by elution of purification columns and collected in collecting tubes. The volume of the isolated exosomes was measured by using pipettes. To improve the accuracy of the measurement, we first estimated the volume of exosome solution according to the scale of the collecting tube and then adjusted the range of the pipettes according to the estimated volume and successfully pipetted all the exosome solution with pipette tips of 1 mL, 200 µL, and 10 µL.

Transmission electron microscope: Morphology of the isolated exosomes was observed by transmission electron microscopy (TEM). Briefly, 5 µL of exosome suspension was loaded onto a formvar-coated copper grid and fixed for at least 5 minutes. Then, the exosomes were negatively stained with 2% uranyl acetate for 5 minutes. After being air-dried, the grids were visualized via TEM (JEOL-1400 plus, JOEL, Japan) at 80 kV.

Western blot: The isolated exosomes were lysed using RIPA lysis buffer (Beyotime, China), and the protein concentrations were measured by BCA assay. The proteins (10 µg) were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred onto nitrocellulose membranes (Merck Millipore). After being blocked with 5% bovine serum albumin for 1.5 hours at room temperature, the membrane was incubated with anti-CD63 (1:1000; ab134045, Abcam, USA) and anti-Tsg101 (1:1000; ab125011, Abcam, USA) overnight at 4°C. Then, the membranes were incubated with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG (1:2000; Sigma, Germany) for 1 hour at 37°C. Finally, the protein bands were visualized by chemiluminescence using the ECL kit (Millipore).

Nanoparticle tracking analysis: The size distribution and concentration of the isolated exosomes were analyzed via ZetaView (Particle Metrix, Germany). The sample chamber was then cleaned using particle-free distilled water. Then, the exosome samples were diluted 10,000× in sterilized PBS. Subsequently, the diluted exosomes were slowly injected into the chamber and quantified using the nanoparticle tracking analysis software.

Quantification of the protein level in the serum-derived exosomes: The protein concentration of the serum-derived exosomes was determined using the BCA protein assay.
kit (Pierce, Thermo Fisher Scientific, USA) in accordance with the manufacturer’s instructions. The total protein content was calculated by multiplying the protein concentration by the volume of the isolated exosomes.

**Culture of HUVECs:** HUVECs were purchased from the Cell Bank of the Chinese Academy of Sciences. HUVEC culture was maintained in EGM-2 (CC-3162, Lonza) containing endothelial basal medium and endothelial growth media supplements in a humidified atmosphere of 5% CO2 in air at 37°C.

**Uptake of serum-derived exosomes by HUVECs:** Uptake of the serum-derived exosomes was observed via confocal laser scanning microscope and analyzed by flow cytometry. The serum-derived exosomes from control subjects and CAD patients were labeled with PKH-26 (MINI Projects and CAD patients were labeled with PKH-26 (MINI A) according to the manual’s instructions. HUVECs (1 × 10⁵) were then seeded into laser confocal dishes (Nest, China) with a diameter of 20 mm. The next day, after being washed twice with PBS, the HUVECs were incubated with the labeled exosomes for 6 hours in the endothelial basal medium. Then, the nuclei of the HUVECs were stained with DAPI (Dingguo, China). The uptake of the serum-derived exosomes by HUVECs was then visualized by confocal laser scanning microscope (Leica, Germany). The mean gray value of the images was analyzed using ImageJ software. For flow cytometry analysis, HUVECs were seeded into 24-well plate and used to analyze the percentage of cells absorbing the labeled exosomes after the incubation by flow cytometry (Beckman CytoFLEX, USA).

**Treatment of HUVECs with serum-derived exosomes:** Considering that the amount of the serum-derived exosomes isolated from each subject was insufficient to treat HUVECs, we randomly combined the exosomes of 3 subjects in the same group into one sample. As a result, 3 mixed exosome samples from the control group and 3 mixed exosome samples from the control group were prepared. HUVECs were then starved with the endothelial basal medium for 12 hours when 70%-80% confluent. After starvation, the cells were treated with the mixed exosome samples from the control group were prepared. HUVECs were then starved with the endothelial basal medium with CAD-exo, ctrl-exo, or vehicle added. Then, the cells were photographed at 0, 12, and 24 hours. The scratch areas at different time points were quantified using ImageJ software, and the cell migration rates at 12 hours and 24 hours were calculated by (scratch area at 0 hours-scratch area at 12 hours or 24 hours)/scratch area at 0 hours.

**Tube formation assay:** The angiogenesis ability of HUVECs treated with CAD-exo, ctrl-exo, or vehicle was evaluated using tube formation assay. The treated HUVECs were then seeded at a density of 5 × 10⁴ cells per well on a 48-well plate coated with Matrigel (Corning, USA). After incubation for 6 hours, the tube formation was photographed using an inverted microscope (Nikon, Japan). The photos were then analyzed by ImageJ software, and the number of nodes and the total length were recorded to assess the angiogenesis ability.

**Inflammatory factor analysis by qPCR:** Total RNA was isolated from HUVECs treated with CAD-exo, ctrl-exo, or vehicle using the TRIzol reagent (Invitrogen) per the manufacturer’s instructions. The isolated RNA was quantified using Nanadrop 2000 (Thermo Fisher Scientific, USA). Then, 1 μg of the total RNA was reverse-transcribed into cDNA by using reverse transcriptase (Toyobo, Japan) according to the manufacturer’s instructions. The expression of interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and intercellular and vascular cell adhesion molecule (ICAM-1 and VCAM-1) in HUVECs was detected via SYBR® Green Real-Time PCR Master Mix (Toyobo, Japan) with Bio-Rad CFX96 Real-Time System. Primer sequences used for qPCR amplification were shown in Table I. The mRNA expression was normalized to GAPDH and analyzed using 2^(-ΔΔCt) method. For all the qPCR experiments, samples were run in quadruplicate.

**Statistical analysis:** Statistical analysis was performed using SPSS 24.0 statistical software (Chicago, IL, USA), and graphs were generated using GraphPad Prism 7.0.

### Table I. Prime Sequences for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>ATCACGGCATCTCTCAGGACG</td>
<td>AGTCCACATTAGCGACAGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCAGACGCTGTGCAGATGAGT</td>
<td>GTGCCCCATGCTACATTGCCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CCTGCTGCACTTTGGAGTTGA</td>
<td>ACAAATGACGCTACGGCTT</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>AGACCTCAGGGGAGGTCA</td>
<td>GGGATAGGCAGGCTGTAAAAG</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>AGTAAAGGGGCGTGAAGAGAA</td>
<td>GATGACCGTCGGGAAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCTCCTCTGACTTTCAACAGGCA</td>
<td>GGTTGCCAGGGTCTTTACTCCTT</td>
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</table>

**Cell viability assay:** HUVECs were seeded into a 96-well plate at a density of 1 × 10⁵ cells per well, and the cell viability of HUVECs treated with CAD-exo, ctrl-exo, and vehicle was measured using the Cell Counting Kit-8 (CCK-8, Dojindo). The treated cells were incubated using the CCK-8 solution at 37°C for 1.5 hours. Then, the cell viability was measured using a microplate reader (Thermo, USA) by spectrophotometry at 450 nm. The cell viability assay of the HUVECs treated with each exosome sample was repeated for 6 times.

**Scratch assay:** HUVECs were grown to confluence in a 6-well plate and scratched using a 200 μL pipette tip. After the scratch, the cells were washed thrice with PBS and then cultured in the endothelial basal medium with CAD-exo, ctrl-exo, or vehicle added. Then, the cells were photographed at 0, 12, and 24 hours. The scratch areas at different time points were quantified using ImageJ software, and the cell migration rates at 12 hours and 24 hours were calculated by (scratch area at 0 hours-scratch area at 12 hours or 24 hours)/scratch area at 0 hours.
The levels of serum-derived exosomes were increased in the CAD patients. The serum-derived exosomes were absorbed into HUVECs: To determine whether the serum-derived exosomes were absorbed into HUVECs, the serum-derived exosomes labeled with PKH26 were co-cultured with HUVECs for 6 hours. The localization of PKH26-labeled exosomes (red fluorescence) in the cytosol of HUVECs (blue fluorescence) was observed under confocal laser scanning microscope (Figure 3A), which indicated that the serum-derived exosomes could be internalized by HUVECs. Flow cytometry analysis showed that nearly 100% of the cells could internalize the serum-derived exosomes (Figure 3D). Moreover, the mean gray value and the percentage of cells absorbing exosomes were not significantly different between the ctrl-exo and CAD-exo (Figures 3B, 3C).

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Results

Clinical characteristics of the study population: The clinical characteristics of the study population including 9 CAD patients (CAD group) and 9 control subjects (control group) were shown in Table II. The low-density lipoprotein cholesterol (LDLC) and the total cholesterol levels were found to be significantly higher in the CAD group than that in the control group. No significant differences were noted in terms of the other clinical characteristics between the 2 groups.

Identification of the isolated exosomes: Serum-derived exosomes from the CAD patients and controls were identified by TEM, western blot, and nanoparticle tracking analysis. TEM images showed that the isolated exosomes had a cup-shaped morphology (Figure 1A). Western blot analysis revealed that the isolated vesicles expressed exosomal marker proteins including CD63 and Tsg101 (Figure 1B). Nanoparticle tracking analysis indicated that the isolated vesicles from CAD patients and controls had median diameters of 88.4 nm and 84.8 nm, respectively (Figure 1C). These results suggested that we have successfully isolated the serum-derived exosomes.

The levels of serum-derived exosomes were increased in the CAD patients: Protein concentrations of the serum-derived exosomes from the study population were measured using BCA assay, and the total protein content was calculated by multiplying the protein concentration by the volume of the isolated exosomes. Both the exosome protein concentration and total protein content of the CAD patients (0.91 ± 0.48 mg/mL and 0.43 ± 0.23 mg) were significantly higher than that of the control subjects (0.42 ± 0.21 mg/mL and 0.2 ± 0.95 mg, P = 0.013 and 0.016, respectively, Figure 2), indicating that the levels of serum-derived exosomes increased in the CAD patients.

Table II. Clinical Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Index</th>
<th>Control (n = 9)</th>
<th>CAD (n = 9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>9/0</td>
<td>9/0</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.8 ± 7.5</td>
<td>55.2 ± 8.1</td>
<td>0.905</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.4 ± 3.6</td>
<td>24.2 ± 2.0</td>
<td>0.911</td>
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<tr>
<td>SBP (mmHg)</td>
<td>139.9 ± 7.1</td>
<td>132.7 ± 10.3</td>
<td>0.103</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>85.7 ± 9.2</td>
<td>81.2 ± 9.0</td>
<td>0.315</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>20.0 (18.5, 32.5)</td>
<td>23.0 (14.0, 37.5)</td>
<td>0.931&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>18.3 ± 4.9</td>
<td>21.8 ± 6.9</td>
<td>0.241</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>65.6 ± 11.8</td>
<td>75.4 ± 10.5</td>
<td>0.079</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.35 ± 0.84</td>
<td>5.11 ± 0.73</td>
<td>0.527</td>
</tr>
<tr>
<td>LDLC (mmol/L)</td>
<td>2.48 ± 0.63</td>
<td>3.25 ± 0.54</td>
<td>0.013</td>
</tr>
<tr>
<td>HDLC (mmol/L)</td>
<td>0.96 ± 0.16</td>
<td>0.98 ± 0.20</td>
<td>0.786</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.85 (1.21, 2.88)</td>
<td>1.54 (0.98, 2.04)</td>
<td>0.489&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.68 (3.32, 4.58)</td>
<td>4.80 (4.43, 5.44)</td>
<td>0.019&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDLC, low-density lipoprotein cholesterol; HDLC, high-density lipoprotein cholesterol; and<sup>a</sup>, non-parametric test.

The normal variables were presented as mean ± SD and were further compared using two-tailed Student t-test (for binary groups) or one-way ANOVA with Bonferroni’s correction for multiple comparisons tests (for multiple groups), whereas non-normal variables were presented as medians with interquartile range and were compared using non-parametric test. P < 0.05 was considered to be statistically significant.
Figure 1. Identification of the serum-derived exosomes isolated from the CAD patients and control subjects. A: Transmission electron microscopy showing the morphology of the isolated exosomes. B: Western blot analysis showing isolated vehicles expressed the exosomal markers including CD63 and Tsg101. C: Nanoparticle tracking analysis showing the concentration and size of the isolated vehicles. Ctrl-exo indicates serum-derived exosomes from the control subjects; CAD-exo, serum-derived exosomes from the CAD patients; and CAD, coronary artery disease.

Figure 2. Quantification of the protein levels in serum-derived exosomes from the CAD patients and control subjects. Exhibiting the protein concentration and total protein content of the serum-derived exosomes form the CAD patients ($n = 9$) and control subjects ($n = 9$), respectively. Data are presented as mean ± SD and compared with $t$-test. *$P < 0.05$. Ctrl-exo indicates serum-derived exosomes from the control subjects; CAD-exo, serum-derived exosomes from the CAD patients; and CAD, coronary artery disease.

that treated with ctrl-exo ($P = 0.015$, $P = 0.011$), but there were no significant differences in the cell migration rates between the HUVECs treated with ctrl-exo and vehicle (Figure 4B, C). Similarly, the tube formation assay showed that the number of nodes and the total length were significantly decreased in HUVECs treated with
Figure 3. Uptake of the serum-derived exosomes by HUVECs. The serum-derived exosomes were labeled with PKH-26 (red) and co-cultured with HUVECs for 4 hours. The nuclei of HUVECs were stained with DAPI (green). A: Images of confocal laser scanning microscope. Arrows: the concentration of exosomes absorbed by HUVECs. B and C: Showing comparisons of mean gray value and percentage of cells between HUVECs absorbing labeled exosomes from controls and CAD. D: The percentage of cells absorbing the labeled exosomes was detected by flow cytometry. Data are presented as mean ± SD and compared with t-test. Ctrl-exo indicates serum-derived exosomes from the control subjects; CAD-exo, serum-derived exosomes from the CAD patients; and CAD, coronary artery disease.
Figure 4. Serum-derived exosomes from the CAD patients inhibited the cell viability, migration, and angiogenesis of HUVECs. HUVECs were treated with ctrl-exo, CAD-exo, or vehicle for 24 hours. The cell viability migration and angiogenesis were evaluated using CCK-8 assay, scratch assay, and tube formation assay. For scratch assay and tube formation assay, exosome concentration of 100 μg/mL was used. A: Comparisons of relative cell viability among HUVECs treated with vehicle, ctrl-exo, and CAD-exo at different concentrations. B: Images of the treated HUVECs at 0, 12, and 24 hours after scratch. C: Comparison of the cell migration rates among HUVECs treated with CAD-exo, ctrl-exo, and vehicle. D: Images of the tube formation of the treated HUVECs co-incubated with Matrigel for 6 hours (magnification × 100). E: Number of the tube nodes. F: Total length of the tube. Data are presented as mean ± SD and compared with one-way ANOVA with Bonferroni’s correction for multiple comparisons tests. *P < 0.05, **P < 0.01 and ***P < 0.001. Ctrl-exo indicates serum-derived exosomes from the control subjects; CAD-exo, serum-derived exosomes from the CAD patients; and CAD, coronary artery disease.
CAD-exo compared to HUVECs treated with ctrl-exo ($P = 0.003$, $P = 0.004$), while no significant differences in the number of nodes and the total length were observed between the HUVECs treated with ctrl-exo and vehicle, nor between those treated with CAD-exo and vehicle (Figures 4D-F). These data suggested that the treatment of HUVECs with CAD-exo suppressed migration and angiogenesis.

**Treatment of HUVECs with CAD-exo suppressed VEGF-induced migration and angiogenesis:** To further examine whether the serum-derived exosomes have an inhibitory effect on VEGF-induced migration and angiogenesis in HUVECs, HUVECs were treated with vehicle, vehicle and VEGF, ctrl-exo and VEGF, and CAD-exo and VEGF for 24 hours in scratch assay and 6 hours in tube formation assay. In the presence of VEGF, the cell migration and angiogenesis of HUVECs treated with ctrl-exo and CAD-exo were significantly lower than that of HUVECs treated with vehicle (Figure 5). What is more, compared with the HUVECs treated with the ctrl-exo and VEGF, cell migration and angiogenesis have significantly decreased in HUVECs treated with CAD-exo and VEGF (Figure 5). These results suggested that both ctrl-exo and CAD-exo could inhibit VEGF-induced cell migration and angiogenesis, while CAD-exo had a stronger inhibitory effect on VEGF-induced migration and angiogenesis than ctrl-exo.

**Treatment of HUVECs with CAD-exo induced endothelial inflammation:** To investigate whether CAD-exo induce endothelial inflammation, HUVECs treated with CAD-exo, ctrl-exo, or vehicle for 24 hours were harvested to detect the expression levels of inflammatory factors by qPCR. As shown in Figure 6, IL-$\beta$1, TNF-$\alpha$, and ICAM-1 levels were significantly increased in HUVECs treated with CAD-exo compared to HUVECs treated with ctrl-exo ($P = 0.026$, 0.014, and 0.017, respectively), and the levels of IL-$\beta$1, TNF-$\alpha$ and ICAM-1 in HUVECs treated with ctrl-exo and vehicle were deemed comparable. Moreover, compared with the HUVECs treated with vehicle, those treated with CAD-exo had significantly higher levels of ICAM-1 ($P = 0.002$). However, IL-6 and VCAM-1 levels were not significantly different among the HUVECs treated with CAD-exo, ctrl-exo, and vehicle. These results indicated that the treatment of HUVECs with CAD-exo induced endothelial inflammation.

**Discussion**

In this present study, we aimed to determine whether circulating exosomes from CAD are involved in endothelial injury and inflammation. As per our findings, treatment of HUVECs with CAD-exo could suppress cell viability in a dose-dependent manner. Compared with ctrl-exo, CAD-exo significantly suppressed migration and angiogenesis. However, CAD-exo had a stronger inhibitory effect on VEGF-induced migration and angiogenesis than ctrl-exo. Moreover, IL-$\beta$1, TNF-$\alpha$, and ICAM-1 were found to be significantly upregulated in HUVECs treated with CAD-exo. Our findings suggest that the circulating exosomes from CAD are involved in endothelial injury and inflammation, which may, in turn, cause endothelial dysfunction and potentially promote the development of CAD.

Exosomes can be quantified by the quantification of particle number and exosomal contents such as protein, RNA, and lipid according to the MISEV2018 guidance.14 In this study, we quantified the serum-derived exosomes by measuring the exosome protein concentration and calculating the total protein content. We found that both protein concentrations and total protein content of the serum-derived exosomes increased in CAD patients as compared to control subjects. Previously, the Framingham Heart Study demonstrated that circulating endothelial microparticles levels were elevated in hypertension, hypertriglyceridemic, and metabolic syndromes,15 which are common risk factors of cardiovascular diseases including CAD. Since CAD patients had higher levels LDLc and total cholesterol than control subjects in our present study, we speculated that the increase of serum-derived exosome levels in CAD patients was probably due to the abnormal cholesterol. However, previous studies have reported that circulating exosome levels were elevated in tumor and inflammatory diseases.16,17 Therefore, circulating exosome levels may be increased not only in CAD, but also in other diseases. Additionally, the increased level of serum-derived exosomes in CAD needs to be further examined in future studies with larger sample sizes.

In our present study, we found that CAD-exo decreased cell viability of HUVECs in a dose-dependent manner, which only indicates that the more the number of exosomes, the greater the effect on cell viability. Moreover, CAD-exo of equal protein level inhibited cell migration and angiogenesis as compared to ctrl-exo, which could imply that the exosome content might also play an important role in the regulation of cell migration and angiogenesis. Recent studies have shown that circulating exosomal contents such as miRNA,18 IncRNA,19 circRNA,20 and protein21 are dysregulated in CAD, which suggest that these molecules might be essential in the pathophysiology of CAD. In addition, it has been widely reported that exosome-mediated transfer of bioactive molecules (nucleic acid and protein) plays a crucial role in EC function.14,22,23 Furthermore, previous studies have shown that exosomes regulated the migration and angiogenesis of HUVECs through transferring miRNAs that target VEGF.22,23 VEGF has been considered an important player in EC proliferation, migration, and angiogenesis. We also examined the effect of the serum-derived exosomes on VEGF-induced migration and angiogenesis in HUVECs. Surprisingly, we found that both the exosomes from controls and CAD could inhibit VEGF-induced cell migration and angiogenesis, while CAD-exo had a stronger inhibitory effect on VEGF-induced migration and angiogenesis than those from controls. Our findings suggest that the serum-derived exosomes may serve as an important player in VEGF-induced migration and angiogenesis in HUVECs. However, the mechanism by which CAD-exo inhibited migration and angiogenesis of HUVECs in the absence and presence of VEGF remains to be further examined.

Previously, Sun, et al. demonstrated that exosomes secreted by the platelets from ACS patients promoted the
Figure 5. Serum-derived exosomes inhibited VEGF-induced cell migration and angiogenesis. HUVECs were treated with vehicle, vehicle and VEGF, ctrl-exo and VEGF, and CAD-exo and VEGF for 24 hours in scratch assay and 6 hours in tube formation assay. A: Images of the treated HUVECs at 0, 12, and 24 hours after scratch. B: Comparison of the cell migration rates among the treated HUVECs (scale = 400 μm). C: Number of the tube nodes. D: Total length of the tube. E: Images of the tube formation of the treated HUVECs co-incubated with Matrigel for 6 hours (magnification × 100). Data are presented as mean ± SD and compared with one-way ANOVA with Bonferroni’s correction for multiple comparisons tests. *P < 0.05, **P < 0.01 and ***P < 0.001. Ctrl-exo indicates serum-derived exosomes from the control subjects; CAD-exo, serum-derived exosomes from the CAD patients; and CAD, coronary artery disease.
cell proliferation, migration, and expression of angiogenic factors in HUVECs. However, our study showed that the serum-derived exosomes from CAD inhibit the cell viability, migration, and angiogenesis after being internalized by HUVECs. The inconsistency in results is reasonable because the exosomes, which were used to treat HUVECs, in our study and that of Sun et al. were differently sourced (serum-derived exosomes versus platelet-derived exosomes). Serum-derived exosomes can be secreted by various cell types. Previous studies at cellular level have found that exosomes derived from many types of cells such as ECs, platelets, and macrophages could be internalized by ECs, which can have different effects on endothelial behaviors (proliferation, migration, and angiogenesis). Accordingly, it is reasonable that the serum-derived exosomes and platelet-derived exosomes had opposite effects on the proliferation, migration, and angiogenesis of HUVECs. Moreover, the abnormal proliferation, migration, and angiogenesis of ECs have been identified essential in the pathogenesis of CAD. In the early stage of atherogenesis, the decrease of EC proliferation, migration, and angiogenesis reflects endothelial injury, which induces lipid infiltration and inflammatory activation, thus, facilitating the formation of atherosclerotic plaque.

Meanwhile in the late stage of atherosclerosis, the excessive increase of EC proliferation, migration, and angiogenesis contributes to intraplaque angiogenesis, which promotes plaque growth and increases plaque vulnerability and eventually lead to adverse cardiovascular events. Therefore, circulating exosomes from CAD may cause EC dysfunction and potentially promote the development of CAD.

Besides endothelial dysfunction, inflammation also plays a crucial role in the development of CAD. There are a number of inflammatory factors involved in the pathogenesis of CAD, including adhesion factors, TNF-α, interleukin, and so forth. For instance, adhesion factors such as ICAM-1 and VCAM-1 promote monocyte infiltration, which, in turn, facilitates the activation of inflammatory responses. TNF-α, IL-1, and IL-6 have also been identified to increase the production of adhesion factors and further induce the proliferation and migration of VSMCs by upregulating the expression of platelet-derived growth factor, which, in turn, promotes the development of atherosclerotic plaque. Interestingly, several studies showed that some inflammatory factors are expressed in circulating exosomes such as ICAM-1, IL-1β, IL-6, and TNF-α.

What is more, exosome-mediated delivery of bioactive molecules such as miRNAs to ECs induces endothelial inflammation. For example, the decrease of miR-17 expression in macrophage-derived exosomes under hypotensive conditions can cause an increase of ICAM-1 expression in HUVECs. Hepatocyte-derived extracellular vesicles transfer miR-1 to ECs, which then promote E-selectin, ICAM-1, and VCAM-1 expression through activating NF-κB pathway. In this study, we found that the expression levels of IL-1β, TNF-α, and ICAM-1 were increased in HUVECs treated with CAD-exo. Based on our findings and the above-presented evidence, the expression levels of IL-1β, TNF-α, and ICAM-1 in HUVECs could be increased in 2 ways: first, transport of these inflammatory factors to HUVECs via the serum-derived exosomes; second, exosome-mediated transfer of bioactive molecules to HUVECs upregulate the expression of these inflammatory factors. Further studies are warranted to investigate how the circulating exosomes from CAD increase inflammatory factors in ECs.

This present study provides important insight into the regulatory role of circulating exosomes in the pathophysiology of CAD. However, there are several limitations of this study, which should be taken into consideration. Firstly, the serum-derived exosomes could be secreted by various cell types. Exosomes from which type of cells have an effect on endothelial injury and inflammation was not investigated in our study, which is a difficult but important question that should be addressed in future studies. Secondly, exosomes contain various bioactive molecules (protein, DNA, RNA, etc.). Although previous studies suggest that circulating exosomal contents are associated with CAD, the mechanisms underlying its pathophysiology in relation to circulating exosomal contents have not yet been well studied. Our study found that CAD-exo induced endothelial injury and inflammation. Further studies are needed to investigate the molecular mechanism of serum-derived exosomes in the regulation of endothelial injury and inflammation in CAD. Thirdly, considering age and gender may have affected the activity of the serum-derived exosomes, we electively included the study population of the same gender (male) and age group (40-65 years). Therefore, whether serum-derived exosomes from CAD patients of different gender and age group induce endothelial injury and inflammation should be further studied.

In summary, we showed for the first time that the
serum-derived exosomes could be internalized by HU-VECs, inducing endothelial injury and inflammation. Our findings provide important insight into the regulatory role of circulating exosomes from CAD in the pathophysiology of CAD. However, the origin of the cells and the key molecules of the circulating exosomes that play a regulatory role in endothelial injury and inflammation remain to be further investigated.

Disclosure
Conflicts of interest: None.

References