Procoagulant effect of extracellular vesicles in patients after transcatheter aortic valve replacement or transcatheter aortic valve replacement with percutaneous coronary intervention

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Abstract

Patients with severe aortic stenosis (AS) after replacement of the transcatheter aortic valve (TAVR) are more likely to develop thrombotic complications such as cerebral embolism and artificial valve thrombosis. However, the mechanism is not yet well defined. We aimed to explore the plasma extracellular vesicles (EVs) levels and their role in the induction of procoagulant activity (PCA) in patients receiving TAVR alone or TAVR with percutaneous coronary intervention (PCI). EVs were analyzed with flow cytometer. Markers of platelet and endothelial cell activation were quantified using selective enzyme-linked immunosorbent assay (ELISA) kits. Procoagulant activity (PCA) was assessed by clotting time, purified clotting complex assays, and fibrin production assays. Our results confirmed that EVs with positive phosphatedylserin (PS$^+$EV), platelet EVs (PEVs) and positive tissue factor EVs (TF$^+$EVs) were higher in patients following TAVR than before TAVR, particularly in TAVR with PCI. Furthermore, endothelial-derived EVs (EEVs) were also higher in patients after TAVR with PCI than pre-TAVR, however, the EEVs levels in TAVR alone patients were gradually reduce than pre-TAVR. In addition, we further proved that total EVs contributed to dramatically shortened coagulation time, increased intrinsic/extrinsic factor Xa and thrombin generation in patients after TAVR, especially in TAVR with PCI. The PCA was markedly attenuated by approximately 80% with lactucin. Our study reveals a previously unrecognized link between plasma EV levels and hypercoagulability in patients after TAVR, especially TAVR with PCI. Blockade of PS$^+$EVs may improve the hypercoagulable state and prognosis of patients.

Highlights

- Patients after TAVR, especially TAVR with PCI, suffer from increased thrombotic complications, which seriously affect the prognosis of the patients.
- Total EVs levels tend to increase after TAVR, especially TAVR with PCI, in 6 months, which positive correlation with the hypercoagulable state, and may lead to thrombosis.
- EVs play the procoagulant role in patients after TAVR, especially TAVR with PCI.
- Blockade of PS-positive EVs (PS$^+$EVs) may improve the hypercoagulable state and reduce thrombotic complications in patients after TAVR.

Introduction

Transcatheter aortic valve replacement (TAVR) is a less invasive alternative to traditional surgical aortic valve replacement (SAVR), with equivalent or superior outcomes, which is more suitable for some elderly patients and comprises 12.5% of all aortic valve replacements now [1–3]. The risk

Extended author information available on the last page of the article

of thrombosis is a major concern and a major determinant of the prognosis and quality of life post TAVR [4–6]. There is a body of supporting evidence that patients after TAVR should receive thromboprophylaxis. Previous studies have shown that anticoagulant therapy, aspirin alone and aspirin plus clopidogrel only have differences in the risk of postoperative bleeding, and there is no significant difference in thromboembolic events [7, 8]. The optimal anti-thrombotic regimen is uncertain because the specific mechanism of the formation of the hypercoagulable state after TAVR has not yet been well defined, thus further research on the mechanisms of prothrombotic state after TAVR is still urgently needed.

Extracellular vesicles (EVs) are fragments released from stimulated or apoptotic cells after plasma membrane remodeling, which have been proposed to play a role in thrombosis in patients with some cardiovascular diseases [9, 10]. The presence of phosphatidylserine (PS) and tissue factor (TF) greatly increases the procoagulant activity of EVs [10, 11]. PS^+EVs can facilitate the assembly of components of the clotting cascade by providing a membrane surface for the assembly of components of the coagulation protease cascade [12]. The presence of TF on EVs prominently increases their procoagulant activity because TF is a receptor for factor VII/ factor VIIa (FVII/VIIa) [10].

John D. Carroll et al. demonstrated a 94% increase in the number of patients receiving TAVR over the 7-year period between 2012 and 2019, which has become the dominant mode of intervention [13]. Although TAVR has been widely promoted all over the world, there are few studies on the changes in circulating EVs in patients after TAVR, and the procoagulant activity of EVs has not been extensively studied in these patients. Christian Jung et al. found that PEVs tended to increase and EEVs tended to decline after TAVR alone [14]. However, no procoagulant or inhibition assays were conducted to determine if changes in EV levels contribute to prothrombotic status in patients after TAVR. Previous studies indicated that both platelet-derived EVs (PEVs) and endothelial-derived EVs (EEVs) levels were elevated gradually after percutaneous coronary intervention (PCI) alone [15, 16]. Nevertheless, the EV levels in patients undergoing TAVR with PCI have not been extensively studied as well.

In this study, we analyzed the changes of different EVs, including total PS^+EVs, PEVs, EEVs, and TF^+EVs, as well as blood hypercoagulability indexes at 1 day, 7 days, 1 month, 3 months, and 6 months after TAVR alone or TAVR with PCI. Furthermore, we conducted coagulation function assays and inhibition assays to investigate the effect of EVs on coagulation activity in patients after TAVR alone or TAVR with PCI. Our study may help identify novel targets for thrombotic complications intervention and reduce mortality in patients after TAVR.

Methods

Subjects

In this study, patients diagnosed with severe AS by thoracic echocardiography (TTE) and undergoing TAVR were continuous selection for the study from the heart center of Qingdao Municipal Hospital from December 2019 to March 2021. A total of 68 patients met the inclusion criteria and exclusion criteria, including 53 cases receiving TAVR alone and 15 cases receiving TAVR and PCI in the same time. Thirty healthy volunteers were recruited from among the hospital staff as healthy controls in the same time. The diagnostic criteria for severe AS were: peak aortic valve velocity > 4.0 m/s or mean differential pressure > 40mmHg or continuity equation valve area < 1.0 cm^2 or valve area index < 0.6 cm^2 by chest echocardiography [17]. All the patients were implanted with the self-expandable Venus A-valve of transcatheter artificial valve produced by Hangzhou Qiming Medical Device Co., Ltd of China through transfemoral approaches, and underwent intravenous anesthesia. This study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of Qingdao Municipal Hospital, which was shown in Appendix S1. All patients signed the informed consent form. Exclusion criteria for all patients were: malignant tumors, infectious diseases, aortic dissection, and aortic aneurysm, known contraindications to radial or brachial artery puncture, unable to tolerate TAVR surgery for any reason, the lack of data due to death or other causes during follow-up.

All patients received aspirin (300 mg loading dose and 100 mg daily) and clopidogrel (300 mg loading dose and 75 mg daily) plus standard heparin to maintain an activated clotting time of > 300 s.

Materials

We used TruCount Tube, purified CD31 (clone L133.1), CD41a (clone HIP8), and CD142 (clone HTF-1) from Becton Dickinson (San Jose, CA, USA). All monoclonal antibodies were labeled with Alexa Fluro 647 or 488 in our laboratory. Polyclonal antibody against human Tissue Factor was from American Diagnostica Inc. (Stamford, CT, USA). We prepared Alexa Fluro 647 or 488 conjugated lactadherin in our laboratory. Human factors Va, VIIa, VIII, IXa, X, Xa, prothrombin, and thrombin were all from Haematologic Technologies. Tyrode's buffer, containing 1 mM

HEPES, was prepared in our laboratory and filtered through a 0.22-mm syringe filter from EMD Millipore. Chromogenic substrates S-2765 and S-2238 were from DiaPharma Group. Calibrated polystyrene latex beads (1.0 μm) were from Sigma (UK).

Protein purification and labeling

We labeled lactadherin, purified from milk, with Alexa fluor 647 or Alexa fluor 488. The proportion of fluorescein to lactadherin was 1.1–1.2/1 [18].

Collection of blood and analysis of blood hypercoagulability indexes and blood cell activation markers

Blood samples were collected from TAVR patients and TAVR with PCI patients at different time points: before TAVR, 1 day, 7 days, 1 month, 3 months, and 6 months after AS treatment. Blood hypercoagulability index and blood cell activation markers were quantified immediately using selective ELISA kits (NeoBioscience Technology, China) based on provided instructions after the collection of blood samples.

Preparation and flow cytometric analysis of EVs

Within 30 min after blood collection, samples were centrifuged for 20 min at 1500 g, and plasma was aspirated and recentrifuged for 30 min at 20,000 g. After centrifugation, we removed the EV-depleted plasma (EDP) supernatant, collected and stored the remaining EVs at the bottom of the tube at −80°C. PS$^+$EVs, PEVs, EEVs and TF$^+$EVs were characterized as lactadherin$^+$, lactadherin$^+$CD41a$^+$, lactadherin$^+$CD31$^+$41a$^-$ and lactadherin$^+$CD142$^+$. We used a trucount tube to calculate the number of MP of each type per microliter after accumulation of 10,000 gated events with the following formula: n = (C × Beadsadded) / (Beadscounted × sample volume). The 'C' in the formula represents the number of positive events after subtracting the background signal.

Coagulation time and inhibition assays of EVs

We used a KC4A-coagulometer to evaluate the PCA of EVs. To get EV-containing suspension, we mix EV-enriched suspension and Tyrode's buffer in a ratio of 1:9. We incubated one hundred microliters of EV-containing suspension with100 μl of EV-depleted plasma at 37 °C for 3 min. Then we added 100 μl of warmed CaCl2 (25 mM) to start the reaction and record the coagulation time. In the inhibition assay, 100 μl of EVs suspension was incubated with 50 μl

of lactadherin or anti-TF for 10 min at 37 °C. Record the coagulation time after the addition of 100 μl EV-free human plasma and 50 μl of warmed CaCl2 (50 mM).

Intrinsic, extrinsic FXa and thrombin formation and inhibition assays of EVs

Factor Xase and prothrombinase activity assays were performed on all samples. Inhibition assays were performed at the peak time point. For intrinsic XA formation, we incubated 10 μ L EVs suspension with fixa (1 nm), FX (130 nm), FVIII (5 nm), thrombin (0.2 nm), and CaCl2 (5 mm) in FXa buffer (10 ml 1 × Incubate in TBS containing 0.2% BSA) at 25 ° C for 5 min. EDTA (7 mm final concentration) was used to stop the reaction. The generation of FXa was measured with a Universal Microplate Spectrophotometer (Power-Wave XS; Bio-Tek, Winooski, VT, USA) set in kinetic mode at 405 nm after incubation with 10 μl of S-2765 (0.8 mM). The formation of extrinsic FXa was similar to that of intrinsic FXa except that EVs were cultured with FX (130 nM), FVIIa (1 nM), and CaCl2 (5 nM). For the prothrombinase assay, we incubated EVs with FVa (1 nM), 0.05 nM FXa (0.05 nM), prothrombin (1 μM), and CaCl2 (5 nM) in prothrombinase buffer (10 ml 1 × TBS with 0.05% BSA) for 5 min at 25 °C and then stopped the reaction using EDTA. We used the chromogenic substrate S-2238 to measure the amount of thrombin production by the standard dilution curve of thrombin as the substrate cleavage rate. For inhibition assays, EVs were pre-incubated with lactadherin (128 nM) or anti-TF at their peak time point for 10 min at 25 °C in Tyrode's buffer and then we incubated the mixture with the specified clotting factors as previously described. The formation of FXa or thrombin was appraised according to the method as above.

Fibrin formation assays

Turbidity was used to assess fibrin formation. We added EVs to recalcified (10 mM, final) EV-depleted plasma (88% EV-depleted plasma, final) in the absence or presence of lactadherin/anti-TF. We measured Fibrin production by turbidity at 405 nm using a SpectraMax 340PC plate reader.

Statistical analysis

Statistical analysis was performed using the SPSS v16.0 software or GraphPad Prism 7.0. Values were presented as mean ± standard deviation (SD) and were based on at least triplicate measurements. Student's t-test or one way ANOVA was used for statistical analysis as appropriate. Spearman's rank correlation analysis was used to determine the correlation between continuous variables. Multiple linear

regression was used to analyse the influence of confounding factors. *P < 0.05* was considered statistically significant.

Results

Subject characteristics

Clinical characteristics in 30 healthy controls and patients who underwent TAVR alone and TAVR with PCI are shown in Table 1. There were no significant differences in the means of gender, smoking, alcohol, body mass index (BMI) and the levels of HDL-C between healthy controls and patients after TAVR alone or TAVR with PCI. Concerning characteristic findings, the levels of neutrophils, leukocytes, platelets, D-dimer, fibrinogen, thrombin-antithrombine (TAT), creatinine (Cr), brain natriuretic peptide (BNP), triglyceride (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) in TAVR alone or TAVR with PCI patients were prominently higher than that in healthy controls. TAVR alone and TAVR with PCI patients did not differ with regard to age, gender, smoking, alcohol, BMI,

NYHA stage, atrial fibrillation, D-dimer, fibrinogen, TAT, Cr, BNP, blood fat, AVA and valve size. However, the proportion of diabetes mellitus, hypertension, and stroke, as well as the levels of neutrophils and platelets in TAVR with PCI patients were prominently higher than that in TAVR alone patients. On the contrary, the levels of leukocytes and BNP in TAVR alone patients were higher than that in TAVR with PCI patients.

Dynamics of PS$^+$EVs/EEVs/PEVs/TF$^+$EVs in patients undergoing TAVR alone or TAVR with PCI

Flow cytometry was used to evaluate the total number of EVs and their phenotypic characteristics. As shown in Fig. 1, compared with healthy controls, the circulating EVs levels were significantly elevated in other two groups (all $P < 0.05$; Fig. 1A-D), but there was no significant difference in circulating EVs levels between TAVR alone patients and TAVR with PCI patients before interventional therapy (all $P > 0.05$; Fig. 1A-D). When compared to pre-TAVR, the levels of total PS$^+$EVs, PEVs, and TF$^+$EVs in TAVR alone and TAVR with PCI groups were significantly increased

Table 1 Clinical features and laboratory findings of the study participants

	Control (n = 30)	TAVR alone (n = 53)	TAVR with PCI (n = 15)
Gender (M/F)	18/12	32/21	9/6
Age (years)	45.62 ± 5.23	78.25 ± 8.21$^{##}$	76.46 ± 7.95$^{##}$
Current smoking (yes/no)	11/19	21/32	6/9
Current alcohol (yes/no)	12/18	20/33	6/9
BMI(kg/m²)	22.56 ± 3.05	23.83 ± 3.56	24.21 ± 2.96
NYHA stage	—	2.25 ± 0.15	2.16 ± 0.21
Diabetes mellitus (yes/no)	—	13/40	7/8*
Hypertension (yes/no)	—	22/31	8/7*
Atrial fibrillation (yes/no)	—	8/45	3/12
Stroke (yes/no)	—	5/48	3/12*
Neutrophils (10^9/L)	3.15 ± 0.41	3.66 ± 0.48$^{#}$	3.98 ± 0.66$^{**#}$
Leukocytes (10^9/L)	5.46 ± 0.55	6.52 ± 0.62$^{#}$	6.36 ± 0.71$^{**#}$
Platelets (10^9/L)	221 ± 27.68	239.36 ± 31.35$^{##}$	257.15 ± 28.63$^{*##}$
D-dimer (mg/L)	0.27 ± 0.16	1.01 ± 0.27$^{##}$	1.04 ± 0.24$^{##}$
Fibrinogen (g/L)	2.55 ± 0.34	2.98 ± 0.41$^{#}$	3.11 ± 0.47$^{#}$
TAT (pg/mL)	355 ± 40.02	431.55 ± 52.23$^{##}$	445.36 ± 55.69$^{##}$
Creatinine(μmol/L)	68.36 ± 8.52	86.56 ± 16.25$^{#}$	89.68 ± 20.12$^{#}$
BNP(pg/mL)	51.56 ± 63.57	3662.74 ± 505.64$^{##}$	3128.05 ± 386.74$^{**##}$
TG (mmol/L)	1.55 ± 0.26	1.73 ± 0.23$^{#}$	1.76 ± 0.35$^{#}$
TC (mmol/L)	4.36 ± 0.93	5.93 ± 0.85$^{#}$	5.98 ± 0.96$^{#}$
LDL-C (mmol/L)	2.38 ± 0.79	3.53 ± 0.59$^{#}$	3.69 ± 0.69$^{#}$
HDL-C (mmol/L)	1.05 ± 0.21	1.12 ± 0.23	1.01 ± 0.24
AVA (cm^2)	—	0.45 ± 0.15	0.47 ± 0.18
Valve size (mm)	—	26.16 ± 2.86	26.36 ± 2.15

Data are presented as means ± SD or number (%). BMI: body mass index; NYHA: new york heart association; TAT: thrombin–antithrombin complex; BNP: brain natriuretic peptide; TG: triglyceride; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein; AVA: aortic valve orifice area.

P < 0.05, **P < 0.01* versus TAVR alone; $^{#}$*P < 0.05*, $^{##}$*P < 0.01* versus Control.

P < 0.05 was considered statistically significant.

A

B

C

D
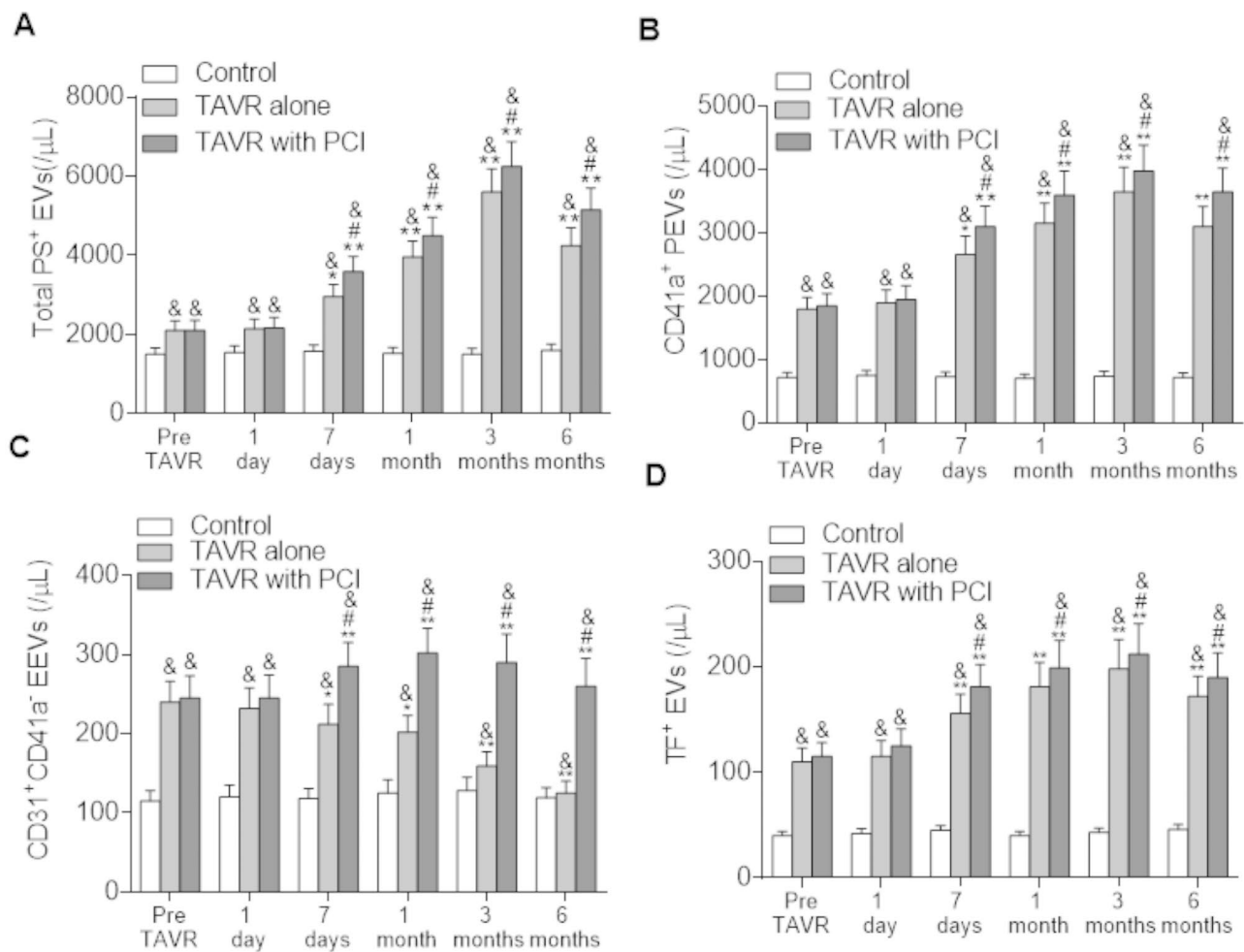
Fig. 1 Flow cytometry analyses of EVs in healthy controls (n = 30), TAVR alone patients (n = 53) and TAVR with PCI patients (n = 15) at pre-TAVR and 1 day/7 days/1 months/3 months/6 months after TAVR alone or TAVR with PCI. Events were selected for their lactadherin binding, determined by positivity for Alexa Fluor 488-lactadherin. PEVs (Alexa Fluro 647-CD41a^{+}), EEVs (Alexa Fluro 488-CD31^{+}/Alexa Fluor 647-CD41a^{−}) and TF^{+}EVs (Alexa Fluor 647-CD142^{+}) were also measured and counted. PEVs: platelet-derived extracellular vesicles; EEVs: endothelial-derived extracellular vesicles; PS^{+}EVs: phosphatidylserine-positive extracellular vesicles; TF[+]EVs: tissue factor-positive extracellular vesicles. &p < 0.05 TAVR alone or TAVR with PCI vs. healthy controls. *p < 0.01 vs. pre-TAVR. **p < 0.001 vs. pre-TAVR. #p < 0.01 TAVR with PCI vs. TAVR alone

from 7 days to 6 months after TAVR, peaking at 3 months (all P < 0.001; Fig. 1A, B, D). Furthermore, compare with TAVR alone patients, total EVs, PEVs, and TF[+]EVs in TAVR with PCI patients were greater from 7 days to 6 months after TAVR at different time points. Among them, compare with pre-TAVR, EEVs and TF[+]EVs in TAVR with PCI patients increased earlier after TAVR than in the other groups, eleevelevation started on the first postoperative day (P < 0.05; Fig. 1C, D). In addition, the levels of EEVs also started to have a remarkable increase from 7 days to 6 months, peaking at 1 month in TAVR with PCI patients (all P < 0.001; Fig. 1C). However, EEVs continuously decreased from 7 days to 6 months in TAVR alone patients (all P < 0.001; Fig. 1C).

## Multiple linear regression on total PS[+] EVs levels at 3 months after TAVR alone or TAVR with PCI

Total PS[+]EVs levels reached its peak at 3 months after interventional therapy (Fig. 1A), so we conducted multiple linear regression using total PS[+]EVs levels at 3 months after interventional therapy as the dependent variable and other factors that may affect EVs as the independent variable. As shown in Table 2, the effects of gender, age, diabetes mellitus, hypertension and atrial fibrillation on total PS[+]EVs levels were not statistically significant (P > 0.05) and receiving TAVR alone or TAVR with PCI had effects on total PS[+]EVs levels, which means that, after adjusting for gender, age, diabetes mellitus, hypertension and atrial

Table 2 Multiple linear regression on total PS+EVs levels at 3 months after TAVR alone or TAVR with PCI.

Variable	Comparison	Control	B	S.E.	Wald	Sig.
Group	TAVR alone	Control	3845.369	266.672	14.420	0.000*
	TAVR with PCI		4510.067	291.995	15.446	0.000*
Gender	female	male	-6.559	104.347	-0.063	0.950
Age			9.881	7.130	1.386	0.169
Diabetes mellitus	yes	no	43.900	136.765	0.321	0.749
Hypertension	yes	no	-174.611	126.151	-1.384	0.170
Atrial fibrillation	yes	no	-22.759	167.623	-0.136	0.892

B: represents the regression coefficient; S.E.: represents standard error; Sig.: represents statistical significance. *$P < 0.05$. $P < 0.05$ was considered statistically significant.
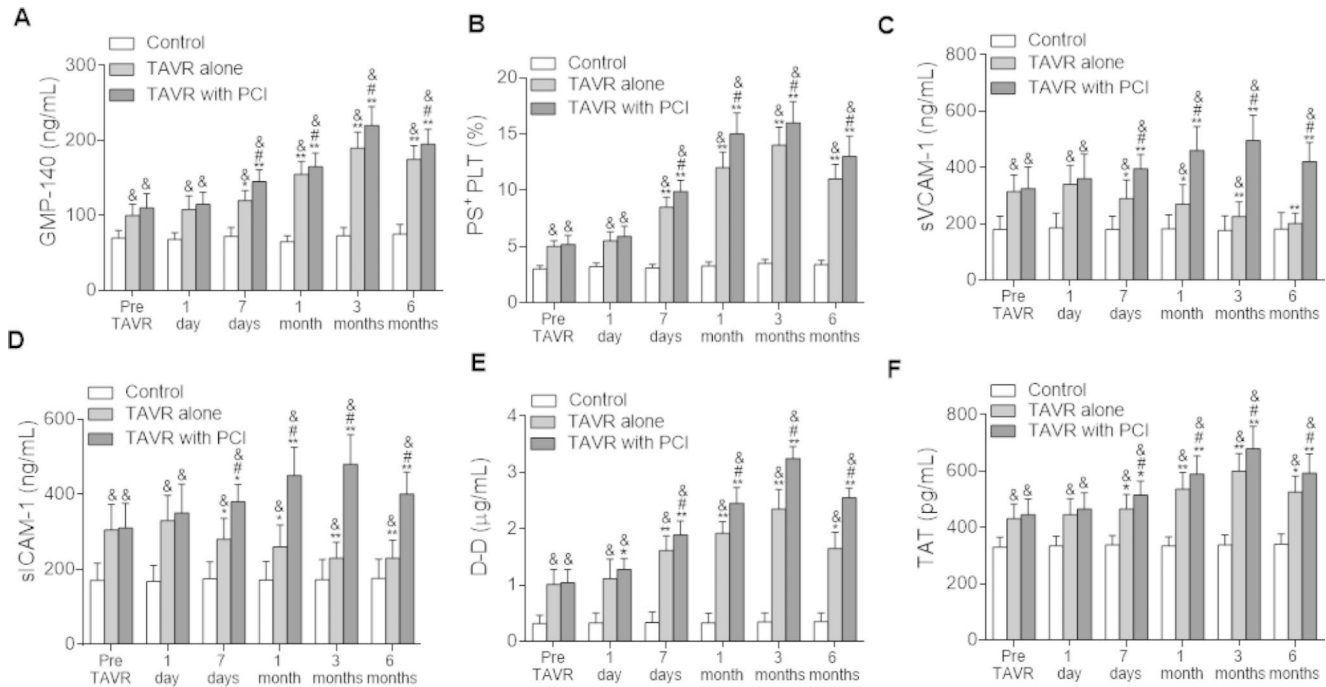
Fig. 2 GMP-140 (**A**), sVCAM-1 (**C**), sICAM-1 (**D**), D-D (**E**), TAT (**F**) and the percent of PS$^+$PLT (**B**) in blood samples from healthy controls (n = 30), TAVR alone patients (n = 53) and TAVR with PCI patients (n = 15) were measured at pre-TAVR and 1 day/7 days/1 months/3 months/6 months after TAVR alone or TAVR with PCI. GMP-140: platelet granular membrane protein 140. sVCAM-1: soluble vascular cell adhesion molecule-1. sICAM-1: soluble intercellular adhesion molecule-1. D-D: d-dimer. TAT: thrombin–antithrombin complex. $^&$p<0.05 TAVR alone or TAVR with PCI vs. healthy controls. *p <0.01 vs. pre-TAVR. **p<0.001 vs. pre-TAVR. $^#$p<0.01 TAVR alone vs. TAVR with PCI.

fibrillation by multivariate linear regression analysis, this association remained statistically significant (P < 0.05).

Changes in indicators of platelet and endothelial cell activation, and blood hypercoagulability following TAVR or TAVR with PCI

Platelet granule membrane protein-140 (GMP-140) is a specific molecular marker of platelet activation, participating in the pathophysiology of thrombosis [19]. PS is an anionic phospholipid, which is strictly located on the inner leaflet of the cell membrane and externalized to the outer surface during cell activation or apoptosis, so the ratio of PS$^+$PLT to total PLT can also be used as an indicator of platelet activation. Our data showed that GMP-140 and the ratio of PS$^+$PLT in healthy controls were lower than that in other two groups (all P < 0.05; Fig. 2A, B). Compared to pre-TAVR, GMP-140 and the ratio of PS$^+$PLT in TAVR alone and TAVR with PCI groups were observed to increase prominently from 7 days (P < 0.01) to 6 months (P < 0.001), peaking at 3 months (all P < 0.01; Fig. 2A, B). Furthermore, GMP-140 and the ratio of PS$^+$PLT in patients after TAVR with PCI were maintained at relatively higher levels than that in patients after TAVR alone (all P < 0.05; Fig. 2A, B). Soluble intracellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) are specific molecular markers of endothelial cell activation. Compared with healthy controls, sVCAM-1 and sICAM-1

Table 3 Correlation between EVs and hypercoagulability markers in patients after TAVR alone or TAVR with PCI at 3 months

	TAVR alone		TAVR with PCI	
Variable	D-dimer	TAT	D-dimer	TAT
PS+EVs	r=0.665**	r=0.585**	r=0.759**	r=0.756**
PEVs	r=0.652**	r=0.683**	r=0.756**	r=0.755**
EEVs	r=0.511*	r=0.523*	r=0.535**	r=0.565**
TF+ EVs	r=0.556*	r=0.536*	r=0.665*	r=0.635*

TAVR: transcatheter aortic valve replacement; PCI: percutaneous coronary intervention; TAT: thrombin–antithrombin complex.

*P < 0.05, **P < 0.01. P < 0.05 was considered statistically significant.

levels were significantly elevated in other groups (P < 0.05; Fig. 2C, D). In TAVR with PCI patients, sVCAM-1 and sICAM-1 had the same change as GMP-140 and PS+PLT from 7 days to 6 months after the operation. However, in TAVR alone patients, sVCAM-1 and sICAM-1 showed a steady decreasing trend from 7 days (P < 0.01) to 6 months (P < 0.001; Fig. 2C, D). Our results proved the activation of platelets and endothelial cells after interventional therapy except that patients receiving TAVR alone showed decreased endothelial cell activation, which was consistent with the PEVs and EEVs variation trend.

D-dimer (D-D) and TAT as indicators of blood hypercoagulability were also measured and tracked in our study. D-D and TAT levels in TAVR alone patients or TAVR with PCI patients were higher than healthy controls (P < 0.05; Fig. 2E, F). The levels of D-D and TAT in both groups had a significant increase from 7 days to 6 months after TAVR. Compared to TAVR alone patients, D-D and TAT levels

in TAVR with PCI patients were higher at different time points after TAVR (Fig. 2E, F). The relationships between PS+EVs/PEVs/EEVs/TF+EVs and D-D/TAT were also been analyzed. In the TAVR alone and TAVR with PCI groups, D-D and TAT had a significant positive correlation with the levels of PS+EVs/PEVs/EEVs/TF+EVs (Table 3). These data suggest that, to some extent, changes in circulating EV levels could lead to blood hypercoagulability in patients after TAVR.

PCA of EVs following TAVR or TAVR with PCI

We used clotting time (CT), intrinsic/extrinsic factor Xa, thrombin, and fibrin formation assays to test the PCA of total EVs in healthy controls and patients after TAVR or TAVR with PCI. CT in two patients groups was longer than that in healthy controls and the production of all three procoagulant enzyme complexes and the turbidity of fibrin in two patients groups were less than that in healthy controls (P < 0.05; Fig. 3A-D). As shown in Fig. 3, CT was markedly shorter, while the production of all three procoagulant enzyme complexes and the turbidity of fibrin were increased in both groups (P < 0.001; Figs. 3A-D and 4A). Patients receiving TAVR with PCI showed higher procoagulant activity than patients receiving TAVR alone. Inhibition assays were performed using 3 months following TAVR alone and TAVR with PCI, which is the highest point of coagulation activity respectively. The addition of lactadherin prolonged the coagulation times, and reduced intrinsic/extrinsic factor Xa, thrombin, and fibrin formation
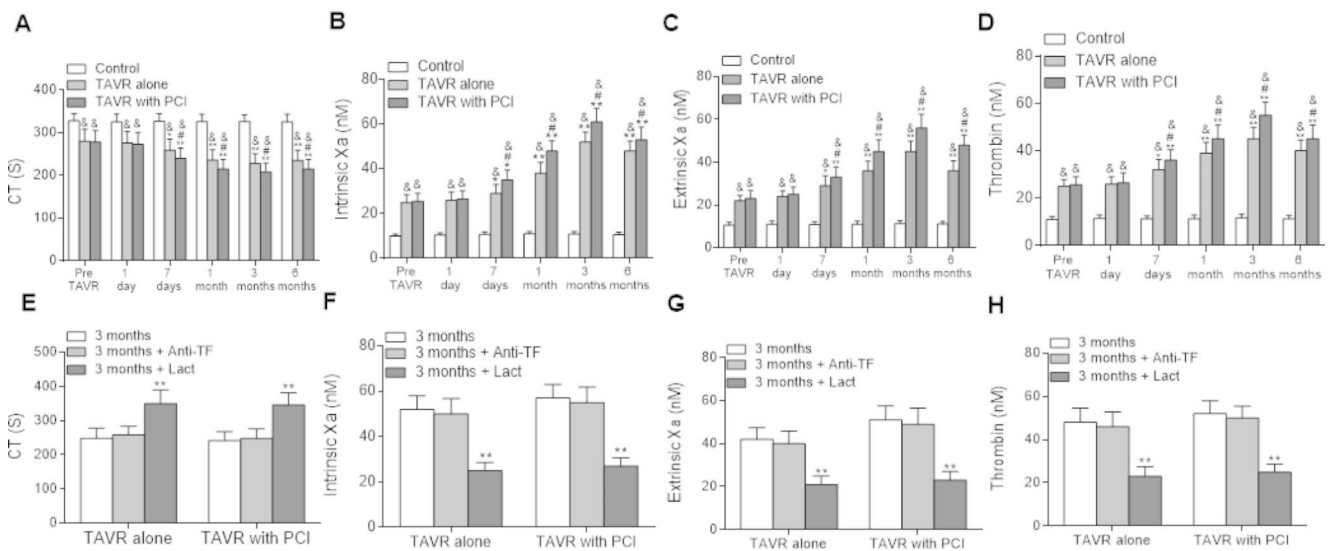
Fig. 3 The PCA of EVs isolated from healthy controls (n=30), TAVR alone patients (n=53) and TAVR with PCI patients (n=15) and inhibition assays. CT (**A**) was detected in the absence of lactadherin or anti-TF after aortic stenosis treatment. EVs was evaluated for the production of intrinsic Xa (**B**), extrinsic Xa (**C**) and thrombin (**D**). CT (**E**), intrinsic Xa (**H**), extrinsic Xa (**F**) and thrombin (**G**) were measured at

3 months with lactadherin/anti-TF disrupting the binding of procoagulant enzyme complexes to EVs. Lact: lactadherin. Anti-TF: anti-tissue factor. For A,B,C,D, &p<0.05 TAVR alone or TAVR with PCI vs. healthy controls. *p<0.01 vs. pre-TAVR. **p<0.001 vs. pre-TAVR. #p<0.01 TAVR alone vs. TAVR with PCI. For E,F,G,H, **p<0.001 3 months vs. 3 months + Lact/Anti-TF.
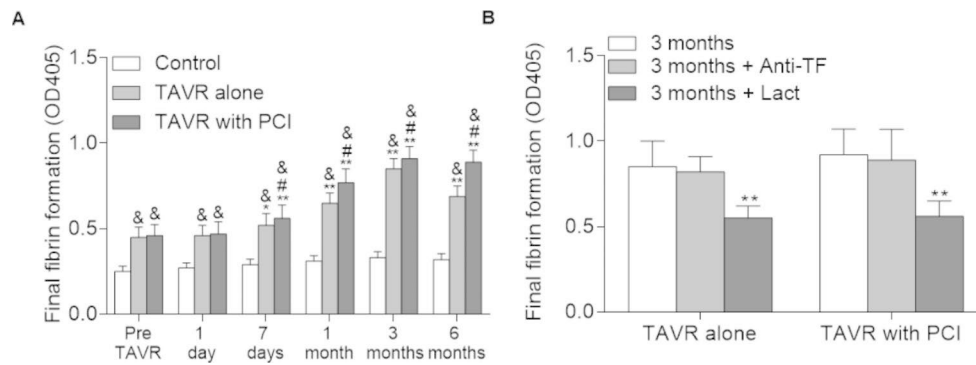
Fig. 4 A Fibrin polymerization in samples from healthy controls (n = 30), TAVR alone patients (n = 53) and TAVR with PCI patients (n = 15) was monitored by measuring turbidity at 405 nm. **B** Comparison of fibrin polymerization between 3 months and 3 months + Lact/ Anti-TF. Lact: lactadherin. Anti-TF: anti-tissue factor. For A, $^{\&}p<0.05$ TAVR alone or TAVR with PCI vs. healthy controls. $^{*}p<0.01$ vs. pre-TAVR. $^{**}p<0.001$ vs. pre-TAVR. $^{\#}p<0.01$ TAVR with PCI vs. TAVR alone. For B, $^{**}p<0.001$ 3 months + Lact/Anti-TF vs. 3 months

($P < 0.001$), whereas anti-tissue factor (anti-TF) had a negligible effect (Figs. 3E-H and 4B). Our results confirmed that the increase in total EVs levels made contributions to the high procoagulation activity after TAVR.

Discussion

In this study, we found that compared to pre-TAVR, EVs of patients remained at elevated levels from 7 days to 6 months after interventional therapy, especially TAVR with PCI, which had ruled out the influence of confounding factors. D-D and TAT levels also increased with increasing EVs, which were significantly associated with EVs levels. Furthermore, markers of platelet and endothelial cell activation showed the same trend as the EV levels within 6 months after TAVR or TAVR with PCI. Moreover, the intrinsic/extrinsic factor Xa, the formation of thrombin and fibrin experienced the same change as the EVs, while the coagulation time was the opposite. Additionally, PCA was effectively blocked after the inhibition assays with lactadherin. Taken together, we confirmed the hypercoagulable state after TAVR alone or TAVR with PCI, which can be partly attributed to the increase of EVs, and the patients undergoing TAVR with PCI had higher coagulation activity than patients undergoing TAVR alone.

The influence of EVs on PCA in patients after TAVR has started to draw people's attention. Our results showed that PS⁺EVs, PEVs, and TF⁺EVs tended to increase while EEVs tended to decline after TAVR alone, however, in TAVR with PCI patients, PS⁺EVs, PEVs, TF⁺EVs, and EEVs all tended to increase after the operation from 7 days to 6 months. However, in the previous study did not observe an increase in EVs levels in a short follow-up of TAVR [19]. Furthermore, Christian Jung al. found that although PEVs tended to increase over time after TAVR procedure, which is generally consistent with our results, circulating EVs levels do

decrease 3 months following TAVR [14]. These results are different from our findings. It may be one of the reasons for the discrepancy that we used lactadherin, a molecular probe different from previous studies, to measure EVs levels. As a probe for the detection of EVs, lactadherin is more effective and sensitive than annexin V [20]. Another reason may be that several follow-up windows in other researches are too short to detect changes in EVs levels post TAVR, because the acute injury of interventional operation, changes in hemodynamics, and shear stress after treatment may affect the production of EVs. Therefore, it takes enough time for the levels of EVs to change and reach a stable state [21, 22]. The follow-up window we choose in our research is long enough to observe a more dynamic evolution of the EV levels.

Moreover, we performed procoagulant or inhibition assays to authenticate that the increase of PS⁺EVs is responsible for high PCA after interventional therapy. Although TF can accelerate blood coagulation and TF⁺EVs were also observed to have the same changes as PS⁺EVs, in the inhibition assay, we found that the anti-TF antibody had a negative effect on the PCA of patients enrolled. In previous related studies, TF⁺EVs had not been found to play an important role in PCA, too [23, 24]. For this result, we take the possible reason into consideration that encrypted TF, with very little PCA, exposed to plasma primarily mostly decrypt through associating with PS [25, 26]. Thus, the simple addition of the anti-TF antibody does not have a significant effect on PCA in samples.

In the present study, we also found that there was a notable discrepancy in levels of EVs after treatment between the two groups. Although the preoperative EVs levels of the two groups were the same, the circulating levels of EVs in patients receiving TAVR and PCI were significantly higher than that in patients receiving TAVR alone after the operation from the 7 days. Koganti S et al. have found that the EVs levels of patients receiving PCI alone increased within

6 months, which, to some extent, was consistent with our conclusion [27, 28]. Our results corroborated the increase in EEVs levels after TAVR with PCI. Consistent with our results, Zhou BD al. also demonstrated that EEVs levels dramatically decreased immediately after PCI, and then increased gradually with time [15]. When interpreting the divergence of EEVs levels, it is maybe ascribed to the following reasons: (1) the procedure of PCI directly causes damage to vascular wall endothelial cells, resulting in increased release of EEVs; (2) EEVs have been reported to be a cause of fibrinolysis [29, 30] and after the reperfusion of the occluded coronary artery, plenty of EEVs enter the circulation promptly, so EEVs levels will rise to antagonize the increase of procoagulant PEVs.

It is well-known that, in vivo, levels of PEVs and EEVs are mainly regulated by shear stress and proinflammatory mediators [31–36]. Generally speaking, proinflammatory mediators can increase the release of PEVs and EEVs by promoting apoptosis and activation [31, 35, 36]. High shear stress can promote the release of PEVs [32–34], but there are some investigates observing the negative association between EEVs levels and arterial shear stress under pathological conditions, because high shear stress can limit EEVs release in nitric oxide (NO)-dependent regulation of ATP-binding cassette transporter A1 (ABCA1) expression and of cytoskeletal reorganization [31, 37, 38]. Thus, on the basis that TAVR can significantly reduce the shear stress on blood vessels [39], it is interesting that we observed the sustained and significant decrease in EEVs level in TAVR alone patients from 7 days to 6 months, and, consistent with our results, the previous study also provided evidence that TAVR alone procedure reduced the number of circulating EEVs [14]. This may indicate that proinflammatory mediators play a more important role in the formation of EEVs in AS patients than shear stress. Patients after TAVR received less direct stimulation to the vascular wall, which decreased circulating EMP levels. Although the relevant mechanism is not involved in this study, the effect of proinflammatory mediators on EEVs levels in AS patients receiving TAVR will be further explored in the next study.

Furthermore, we confirmed that the increase of EVs can raise the coagulation activity of patients receiving TAVR alone or TAVR with PCI, thereby increasing the risk of thrombosis. Consistent with our results, previous studies have shown that high levels of EVs can increase the risk of thrombosis in patients with coronary heart disease [40, 41]. Therefore, considering that the EVs levels of TAVR with PCI patients were higher than that of TAVR alone patients, the former has higher blood procoagulant activity and more attention should be paid to the antithrombotic therapy of them. Because the thrombotic complications of patients are mostly asymptomatic cerebral embolism

and valve thrombosis, only a few patients were willing to undergo these diagnostic tests. Therefore, we were unable to fully collect relevant data. However, clinical stroke or transient ischemic attack, one-half of which being disabling strokes, is not rare after TAVR, ranging in the randomized studies from 5 to 6% at 1 month to 8–10% at 1 year [42]. Previous studies showed that the incidence of silent cerebral embolism is much higher than that of clinical stroke, almost occurring in 75% of the cases after TAVR, which leads to a high risk for cognitive deterioration [43, 44]. In addition, Ryo Yanagisawa al. found that the accumulated incidence of hypoattenuating leaflet thickening, possibly indicating artificial valve thrombosis, was 10.0% at 6 months [6, 45]. This is consistent with the increase in EVs levels in our study in terms of time. We believe that this can also prove, to some extent, that these changes in EVs and coagulations tests are clinically relevant. In future research, we will continue to collect data on postoperative thrombotic complications in patients to help us better explore the clinical relevance between the EVs levels and thrombotic complications.

Preventing these thrombotic complications is an important measure to improve the prognosis of patients. Nevertheless, antithrombotic therapy commonly used after TAVR at present, dual antiplatelet therapy for 1 to 6 months, is totally empirical [46]. Our data demonstrated that consistent with the occurrence time of cerebral embolism and artificial valve thrombosis, EVs levels, and PCA remained at a high level from 7 days to 6 months after the operation and peaked at 3 months. In the meantime, the PCA can be blocked with lactadherin effectively. This opens up a new idea for postoperative antithrombotic therapy. Perhaps, in addition to antiplatelet therapy, anticoagulant therapy and blockade of PS$^+$EVs should also be applied in time after TAVR, especially TAVR with PCI, starting at 7 days at the latest.

Our study was a single-center and small sample size study. Therefore, from a statistical point of view, it is slightly underpowered for evaluating the clinical implication. Our follow-up was only half a year, and how EV levels change thereafter remains unclear. Simultaneously, we only investigated PS$^+$EVs, EEVs, PEVs and TF$^+$EVs, but other EVs from other sources in the blood that may increase PCA were not included in the research. Therefore, their impact on PCA after TAVR cannot be determined.

Conclusions

In conclusion, our data corroborated the existence of the hypercoagulable state in patients after TAVR and that there was an increase in EVs in TAVR alone patients and TAVR with PCI patients, which may lead to the hypercoagulable state. Moreover, patients after TAVR with PCI had higher

EV levels and coagulation activity than patients after TAVR alone. In addition to antiplatelet therapy, anticoagulant therapy and blockade of PS⁺EVs have the possibility to improve the hypercoagulable state and prognosis of patients.

Declarations

Conflict of interest Authors, HC, YS, FX, GZ, JZ, GJ, DT and JL declare to have no conflict of interest.

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