Increased blood cell phosphatidylserine exposure and circulating microparticles contribute to procoagulant activity after carotid artery stenting

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OBJECTIVE Phosphatidylserine (PS) is a major component of the inner leaflet of membrane bilayers. During cell activation or apoptosis, PS is externalized to the outer membrane, providing an important physiological signal necessary for the release of the microparticles (MPs) that are generated through the budding of cellular membranes. MPs express PS and membrane antigens that reflect their cellular origin. PS exposure on the cell surface and the release of MPs provide binding sites for factor Xa and prothrombinase complexes that promote thrombin formation. Relatively little is known about the role of PS exposure on blood cells and MPs in patients with internal carotid artery (ICA) stenosis who have undergone carotid artery stenting (CAS). The authors aimed to investigate the extent of PS exposure on blood cells and MPs and to define its role in procoagulant activity (PCA) in the 7 days following CAS.

METHODS The study included patients with ICA stenosis who had undergone CAS (n = 70), matched patients who had undergone catheter angiography only (n = 30), and healthy controls (n = 30). Blood samples were collected from all patients just before the procedure after an overnight fast and at 2, 6, 24, 48, and 72 hours and 7 days after the CAS procedure. Blood was collected from healthy controls after an overnight fast. Phosphatidylserine-positive (PS+) MPs and blood cells were analyzed by flow cytometry, while PCA was assessed with clotting time analysis, purified coagulation complex assays, and fibrin formation assays.

RESULTS The authors found that levels of PS+ blood cells and PS+ blood cell–derived MPs (platelets and platelet-derived MPs [PMPs], neutrophils and neutrophil-derived MPs [NMPs], monocytes and monocyte-derived MPs [MMPs], erythrocytes and erythrocyte-derived MPs [RMPs], and endothelial cells and endothelial cell–derived MPs [EMPs]) were increased in the 7 days following the CAS procedure. Specifically, elevation of PS exposure on platelets/PMPs, neutrophils/NMPs, and monocytes/MMPs was detected within 2 hours of CAS, whereas PS exposure was delayed on erythrocytes/RMPs and EMPs, with an increase detected 24 hours after CAS. In addition, PS+ platelets/PMPs peaked at 2 hours, while PS+ neutrophils/NMPs, monocytes/MMPs, and erythrocytes/RMPs peaked at 48 hours. After their peak, all persisted at levels above baseline for 7 days post-CAS. Moreover, the level of PS+ blood cells/MPs was correlated with shortened coagulation time and significantly increased intrinsic and extrinsic Xase, thrombin generation, and fibrin formation. Pretreatment of blood cells with lactadherin at their peak time point after CAS blocked PS, resulting in prolonged coagulation times, decreased procoagulant enzyme activation, and fibrin production.

CONCLUSIONS The results of this study suggest that increased exposure of PS on blood cells and MPs may contribute to enhanced PCA in patients with ICA stenosis who have undergone CAS, explaining the risk of perioperative thrombo-

ABBREVIATIONS aPTT = activated partial thromboplastin time; BSA = bovine serum albumin; CAS = carotid artery stenting; DSA = digital subtraction angiography; EMP = endothelial cell–derived MP; ICA = internal carotid artery; MNC = mononuclear cell; MP = microparticle; MMP = monocyte-derived MP; NMP = neutrophil-derived MP; PCA = procoagulant activity; PI = propidium iodine; PMN = polymorphonuclear leukocyte; PMP = platelet-derived MP; PS = phosphatidylserine; RBC = red blood cell; RMP = RBC-derived MP; SD = standard deviation; TBS = Tris-buffered saline.

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embolic complications in these patients. PS on blood cells and MPs may serve as an important biomarker for predicting, and as a pivotal target for monitoring and treating, acute postoperative complications after CAS.

■ **CLASSIFICATION OF EVIDENCE** Type of question: association; study design: prospective cohort trial; evidence: Class I.

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KEY WORDS carotid artery stenting; microparticles; phosphatidylserine; procoagulant activity; lactadherin; fibrin deposition; vascular disorders

CAROTID artery stenosis is known as a main cause for the development of ischemic stroke and therefore became a pivotal target for primary and secondary prevention of stroke.²⁰ Carotid artery stenting (CAS), a less invasive technique than carotid endarterectomy, has become one of the major treatment modalities for internal carotid artery (ICA) stenosis in recent years.^{4,6,15} With the use of periprocedural management, including dual antiplatelet therapy and distal protection, rates of thromboembolism following CAS are low. However, compared with carotid endarterectomy, CAS is associated with higher periprocedural complications such as stent-thrombosis, restenosis, and transient or permanent neurological deficit due to distal thrombus embolization.^{11,14,22,33,43} These complications can be debilitating and even fatal. Thus, understanding the pathophysiological mechanisms of thrombotic complications is important for early detection and intervention. Recent reports have shown that higher levels of fibrinogen and D-dimer are associated with postoperative complications after CAS.¹² However, the precise molecular and cellular mechanism involved in this hypercoagulable state has not yet been determined.

Phosphatidylserine (PS), a negatively charged phospholipid normally localized on the cytoplasmic face of cell membranes, is preferentially exposed on the cell surface during certain physiological processes, including cell apoptosis and activation. The exposure of PS serves as a scaffold for the activation of clotting factors such as thrombin and factor Xa,^{17,42} thus acting as a key catalyst in the coagulation cascade.³⁹ Nevertheless, to date, it is still unclear whether PS takes part in the pathophysiological process after CAS. Previous studies have shown that angiographic procedures and angioplasty in peripheral arteries lead to activation and inflammation of the endothelium. Following CAS, increases in P-selectin, von Willebrand factor, and endothelin-1 occur, suggesting the activation of platelets and endothelial cells.^{27,41} However, whether PS is exposed on blood cells in response to CAS and whether increases in PS exposure on these cells contribute to the coagulation cascade remain to be evaluated.

Cell activation is accompanied by the release of microparticles (MPs), which are small vesicles (diameter between 0.1 and 1 μ m) that display the specific antigens of their original cells and expose PS because of a lack of membrane asymmetry.^{26,29} MPs released by activated or apoptotic cells (such as endothelial cells, leukocytes, and platelets) have been reported to be sensitive markers of atherothrombotic disease progression and potential effectors of vascular dysfunction.^{3,21,31} Additional studies have suggested that MPs can be used to measure the severity of damage in vascular diseases.^{2,13,37} There have been only

3 studies to date that have investigated MPs in relation to carotid artery disease plaques. These reports have shown an increase in platelet-derived MPs (PMPs) in carotid atherosclerosis,²⁵ with higher levels of annexin V+ MPs associated with advanced carotid disease.⁴⁰ They have also suggested that levels of leukocyte-derived MPs and endothelial cell-derived MPs (EMPs) can be used to predict plaque instability.^{32,40} However, the serial changes in levels of circulating MPs of various origins and their role in procoagulant activity (PCA) after CAS are still unknown.

The purpose of this study was to investigate the role of PS exposure on blood cells and MPs in the pathogenesis of the hypercoagulable state in patients with ICA stenosis who have undergone CAS. We explored the changes in PS exposure on blood cells and MPs following CAS and the resulting PS-dependent PCA. We used lactadherin to detect PS externalization on cells and MPs in patients who had undergone CAS. Lactadherin binds stereoselectively to phospho-L-serine, independent of Ca^{2+} concentration and membrane phosphatidylethanolamine content.³⁵ Studies have shown that lactadherin functions as a more sensitive probe for the detection of PS-positive (PS+) cells or MPs than annexin V and acts as an anticoagulant by competing with coagulation factor V and factor VIII for membrane binding sites.^{7,34}

Methods

Patients

We enrolled 70 patients with ICA stenosis who had undergone CAS between February 2015 and February 2016. As controls, we also included 30 age-matched healthy subjects and 30 matched patients who had undergone digital subtraction angiography (DSA) but not stenting. All patients were initially evaluated at the First Hospital of Harbin Medical University. After admission, all patients received antiplatelet therapy and routine care. CAS was performed in asymptomatic patients with > 70% stenosis and in symptomatic patients with > 50% stenosis. Patients with prolonged baseline activated partial thromboplastin time (aPTT), malignant tumors, infectious diseases, connective tissue diseases, severe renal insufficiency (creatinine > 2 mg/dl), or acute inflammatory diseases or those who had undergone prior CAS or carotid endarterectomy procedures were excluded. Peripheral blood was collected in healthy controls after an overnight fast and from patients undergoing CAS just before the procedure after an overnight fast and at 2, 6, 24, 48, and 72 hours and 7 days after stent implantation; blood was collected from matched patients before and at 2, 6, and 24 hours after DSA only. The procedure protocol was approved by the

■ CLASSIFICATION OF EVIDENCE

Type of Question Association

Study Design Prospective Cohort Trial

Classification of evidence Zhao and colleagues present the results of their meticulously conducted prospective cohort study examining the relationships between carotid artery stenting (CAS), degree of carotid stenosis, and D-dimer levels on the one hand, and phosphatidylserine (PS) exposure on the outer membranes of platelets, neutrophils, monocytes, erythrocytes, and endothelial cells, the presence of PS-containing microparticles, and procoagulant activity on the other. The authors found statistically significant increases in nearly all of these outcomes in patients following CAS as compared to preprocedure values, compared to values in normal patient controls, and compared to values in patients undergoing only digital subtraction angiography (DSA). They also found baseline differences in these measures between CAS patients with symptomatic and those with asymptomatic carotid stenosis, and showed (in the laboratory) that various measures of enhanced clot formation could be abrogated by the addition of lactadherin, a specific inhibitor of PS. For its primary question, this study contains all the elements of a Class I study of association: prospective cohort design, accounting for between-group differences (pre- and post-CAS patients acted as their own controls), clearly defined primary outcomes and inclusion and exclusion criteria, and no loss to follow-up. Study quality would have been improved if the investigators measuring the PS-related outcomes had been masked to the “exposures” or risk factors of each patient (normal, DSA, or CAS and time points following CAS), but because these outcomes are relatively objective, this concern is mitigated. The authors also provide convincing evidence for a causal association between CAS and increased PS, and between increased PS and increased procoagulant activity (large effect size, biological plausibility, temporal relationship between exposure and outcome, consistency of association, outcome specific to the exposure, and coherence—6 of Hill’s 9 criteria¹⁰). The authors hypothesized that changes in PS-related measures may serve as good biomarkers for predicting undesirable vascular outcomes (stent thrombosis, stroke, restenosis) and that intervention (more aggressive anticoagulation, inhibition of PS) may improve outcomes in patients undergoing CAS. To the authors’ credit, they emphasize that these statements only represent hypotheses based on their study results. Despite their study’s Class I status, these hypotheses require, respectively, well-designed, prospective, diagnostic, and therapeutic trials in CAS patients before clinicians can act upon these results.

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local ethics committee of Harbin Medical University according to the Helsinki declaration, and written informed consent was obtained from all study participants.

Materials

Calibrated polystyrene latex beads (1.0 μm) were pur-

chased from Sigma-Aldrich. The Trucount Tube, purified CD235a (clone GA-R2), CD31 (clone L133.1), CD41a (clone HIP8), CD14, CD142 (clone HFT-1), CD66b (clone G10F5), CD3 (clone 1F4), CD19 (clone A3-B1), and mouse IgG1/IgG2a (clone $\times 40/\text{X39}$) were from Becton Dickinson Biosciences, and all monoclonal antibodies were labeled in our laboratory with Alexa Fluor 647 or Alexa Fluor 488. Polyclonal antibody against human tissue factor was from American Diagnostica Inc. Alexa Fluor 488- and Alexa Fluor 647-conjugated lactadherin were prepared 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- μm syringe filter from EMD Millipore. Chromogenic substrates S-2765 and S-2238 were from DiaPharma Group. Percoll cell separation medium was from GE Healthcare.

Protein Purification and Labeling

Lactadherin was purified from bovine milk and labeled with Alexa Fluor 647 or Alexa Fluor 488, as previously described.³⁵ The ratio of fluorescein to lactadherin was 1.2:1 or 1.1:1.

Blood Collection and Preparation of Platelets, Leukocytes, Red Blood Cells, and MPs

Blood samples were drawn with a 21-gauge needle and collected into a 5-ml tube containing 3.2% citrate. Platelet-rich plasma was prepared by centrifugation (200g, 13 minutes, room temperature) within 20 minutes of blood collection and was analyzed immediately after isolation. Leukocytes were immediately separated using a differential centrifugation gradient (with Percoll/Ficoll), as described elsewhere.³⁰ To prepare platelet-free plasma, samples were centrifuged first for 20 minutes at 1500g and then again for 2 minutes at 13,000g to remove all residual platelets.⁸ Platelet-free plasma samples were snap frozen in liquid nitrogen and then stored at -80°C until use. To isolate MPs, 250 μl of platelet-free plasma was thawed on ice for 60 minutes and then centrifuged for 45 minutes at 20,000g at 20°C .⁸ The MP-free supernatant (225 μl) was removed, and the remaining 25 μl of MP pellet was washed once and resuspended in 75 μl of Tyrode’s buffer.

Flow Cytometric Analysis of MPs

MPs were identified as previously reported.⁸ Red blood cell (RBC)- (RMPs), platelet- (PMPs), neutrophil- (NMPs), monocyte- (MMPs), T or B lymphocyte-, and endothelial cell-derived MPs (EMPs) were defined as events smaller than 1 μm and as lactadherin+ CD235a+, lactadherin+ CD41a+, lactadherin+ CD66b+, lactadherin+ CD14+, lactadherin+ CD3/19+, or lactadherin+ CD31+ CD41a-, respectively. MPs exposing tissue factor were identified as those with coexpression of lactadherin and CD142. The amount of each type of MP per microliter was calculated using a Trucount Tube (containing 48,678 beads) after an accumulation of 10,000 gated events. The number of MPs per microliter of plasma (n) was calculated using the following formula: $(C \times \text{beads}_{\text{added}})/(\text{beads}_{\text{counted}})$

$\times$ sample vol), where C represents the number of positive events with the background signal subtracted.

Flow Cytometric Analysis of PS Exposure on Blood Cells

Lactadherin was used as a probe to measure exposed PS on blood cells by flow cytometry. The concentration of platelets, leukocytes, or RBCs was adjusted to $0.5\text{--}1 \times 10^6$ cells/ml and diluted to a final volume of 200 μl in Tyrode's buffer. Alexa Fluor 488–conjugated lactadherin (5 μl) was then added to the cell suspension and incubated for 10 minutes at room temperature in the dark. Ten thousand events per sample were acquired and analyzed with BD FACSDiva software.

PCA and Inhibition Assays of Platelets, Leukocytes, RBCs, and MPs

The PCA of platelets, leukocytes, RBCs, and MPs was evaluated using a 1-stage recalcification clotting time assay in a KC4A coagulometer. A total of 100 μl of platelets (10^7), leukocytes (10^6), RBCs (10^7), or MP-containing suspension (10 μl of MP-enriched suspension diluted in 90 μl of Tyrode's buffer) was incubated with 100 μl of MP-free human plasma at 37°C . After 3 minutes, 100 μl of warmed 25-mM CaCl_2 was added to start the reaction and the clotting time was recorded. All clotting assays were performed in triplicate. Inhibition assays were performed using samples from the time point at which the number of MPs peaked (2 hours for platelets and 48 hours for the other cell types) based on a previous analysis. For those assays, 50 μl of lactadherin (final concentration 128 nM) was incubated with 100- μl cell or MP suspension for 10 minutes at 37°C . The clotting time was then recorded as above, after the addition of 100 μl of MP-free human plasma and 50 μl of warmed 50-mM CaCl_2 .⁸

Intrinsic and Extrinsic Factor Xa, Prothrombinase Formation, and Inhibition Assays

We performed factor Xase and prothrombinase activity assays on all samples and inhibition assays at the peak time point. The formation of intrinsic factor Xa in the presence of cells or MPs was performed as follows. A total of 10^4 platelets or leukocytes, 10^5 RBCs, or 10 μl of MP-enriched suspension was incubated with 1 nM factor IXa, 130 nM factor X, 5 nM factor VIII, 0.2 nM thrombin, and 5 mM Ca^{2+} in factor Xa buffer (10 ml of $1\times$ Tris-buffered saline [TBS] with 0.2% bovine serum albumin [BSA]) for 5 minutes at 25°C . The reaction was stopped by the addition of EDTA to a final concentration of 7 mM. The amount of factor Xa was determined by measuring absorbance at 405 nm using a Universal Microplate Spectrophotometer (PowerWave XS, BioTek Instruments Inc.) in kinetic mode immediately after the addition of 10 μl of the chromogenic reagent S-2765 (0.8 mM). The results were evaluated against the rate of substrate cleavage for a standard dilution of factor Xa. Extrinsic factor Xa formation was measured in the same way except that cells or MP-enriched suspension was incubated with 130 nM factor X, 1 nM factor VIIa, and 5 mM Ca^{2+} . For the prothrombinase assay, the samples were mixed with 1 nM factor Va, 0.05 nM factor Xa, 1 μM prothrombin, and 5

mM Ca^{2+} in prothrombinase buffer (10 ml of $1\times$ TBS with 0.05% BSA), and the amount of thrombin produced was measured using the chromogenic substrate S-2238. The concentration of thrombin was calculated from the absorbance per minute measurements using a standard dilution curve. To test the inhibition of coagulation complexes by lactadherin, the various cell types (platelets, leukocytes, RBCs, and MPs) were pre-incubated with lactadherin (128 nM) at their peak time point after CAS for 10 minutes at 25°C in Tyrode's buffer. The mixture was then incubated with the specified clotting factors according to the above protocols. The quantity of thrombin or factor Xa formation was assessed as previously described.⁸

Fibrin Formation Assays

Fibrin formation was quantified by turbidity, as previously described.⁵ Briefly, isolated MPs and blood cells were added to recalcified (10 mM, final) MP-depleted plasma (88% MP-depleted plasma, final) in the absence or presence of 128 nM lactadherin. Fibrin production was measured by turbidity at 405 nm in a SpectraMax 340PC plate reader.

Confocal Microscopy

Confocal microscopy was used to localize PS on the different cell types. Platelets, leukocytes, and RBCs from patients with ICA stenosis were incubated with Alexa Fluor 488–lactadherin (final concentration of 4 nM) for 10 minutes at room temperature in the dark. Cells were then washed to remove unbound dye and were imaged immediately. Observation of PS exposure on platelets and MPs was performed as previously described.⁸ To observe the location of coagulation factor binding sites, MPs were co-stained with factor Va-fluorescein-maleimide and factor Xa-EGRck-biotin (complexed to Alexa Fluor 647–streptavidin). To observe the contribution of MPs to fibrin formation at 48 hours, MP-containing suspensions (25 μl of MPs, washed twice and resuspended in 75 μl of Tyrode's buffer) were incubated with plasma (platelet-poor plasma, centrifuged at 106,000g for 1 hour at 4°C) and 3 mM calcium. Fibrin networks were imaged using Alexa Fluor 647–conjugated anti-fibrin. Background signal was calculated using a similarly labeled, isotype-matched control antibody. In addition, fibrin formation was quantified by turbidity as described.¹ Isolated MPs were added to recalcified (10 mM, final) MP-depleted plasma (88% MP-depleted plasma, final) in the absence or presence of 128 nM lactadherin. Fibrin formation was measured by turbidity at 405 nm in a SpectraMax 340PC plate reader.

Statistical Analysis

Numerical variables were tested for normal distribution with the Kolmogorov-Smirnov test. Data are expressed as the mean $\pm$ standard deviation (SD) and were statistically analyzed for significance using a Student t-test or ANOVA as required. Categorical variables were compared using the chi-square test. Line correlation analysis was used to detect any relation between PS+ blood cells/MPs and stenosis degree and D-dimer. A p value < 0.05 was considered statistically significant.

Results

Subject Characteristics

Clinical characteristics in patients just before (pre-CAS) and 48 hours after (post-CAS) the CAS procedure, in matched controls who underwent DSA only, and in healthy controls are shown in Table 1. The 3 groups were homogeneous with respect to major baseline demographics and clinical or laboratory characteristics. Levels of total cholesterol, LDL, fibrinogen, and D-dimer were significantly higher post-CAS than just before the CAS procedure or in the DSA group. Levels of C-reactive protein were also higher in post-CAS measurements than in the DSA group. Compared with controls, patients with ICA stenosis had no significant difference in clinical and laboratory characteristics.

PS Exposure on Peripheral Blood Cells

We used flow cytometry to measure the extent of PS

exposure in cells from healthy controls and patients for 7 days following CAS. At baseline (0 hours), PS exposure on platelets, neutrophils, and monocytes was significantly higher for patients who had undergone CAS than in control subjects ($p < 0.001$, respectively; Fig. 1A and B). Following CAS, PS exposure increased on platelets, neutrophils, and monocytes ($p < 0.001$, respectively) at 2 hours as compared with baseline, while PS exposure on RBCs did not significantly increase until 24 hours. PS exposure on platelets decreased after 2 hours, whereas PS on neutrophils, monocytes, and RBCs all peaked 48 hours after the CAS procedure. However, the level of PS exposure remained significantly greater than baseline at Day 7 ($p < 0.001$). Additionally, the changes in the absolute number of PS+ blood cells were similar to the percentages of PS exposure (Table 2). We also found that cellular PS levels at corresponding time points within 24 hours (all $p < 0.05$) were markedly higher in patients who had undergone CAS than in those who had undergone DSA only (Fig. 1H). Tis-

TABLE 1. Characteristics of patients with ICA stenosis undergoing CAS, matched controls undergoing DSA, and healthy subjects at study inclusion

Characteristic	Controls (n = 30)	DSA Group (n = 30)	Patients w/ ICA Stenosis (n = 70)		p Value		
			Pre-CAS	Post-CAS	p ^a	p ^b	p ^c
Age in yrs	54.25 ± 4.65	65.63 ± 4.93	63.88 ± 7.51	63.88 ± 7.51	0.17	1.0	0.74
M/F sex	15/15	15/15	35/35	35/35	1.0	1.0	1.0
Total cholesterol (mmol/L)	3.49 ± 0.35	3.2 ± 0.56	3.80 ± 0.50	5.79 ± 0.96	0.58	0.01	0.001
Triglycerides (mmol/L)	1.40 ± 0.40	2.03 ± 0.71	2.29 ± 1.07	2.69 ± 0.73	0.21	0.46	0.30
LDL (mmol/L)	1.60 ± 0.07	2.03 ± 0.05	2.19 ± 0.09	3.60 ± 0.81	0.12	0.003	0.002
HDL (mmol/L)	1.30 ± 0.20	1.21 ± 0.21	1.25 ± 0.50	0.79 ± 0.07	0.65	0.22	0.18
CRP (mg/L)	1.17 ± 0.40	2.2 ± 0.4	3.42 ± 1.10	5.90 ± 2.70	0.10	0.08	0.02
Erythrocytes (×10 ¹² /L)	4.17 ± 0.28	4.3 ± 0.16	4.56 ± 0.24	4.69 ± 0.11	0.06	0.47	0.06
Leukocytes (×10 ⁹ /L)	5.70 ± 0.47	5.54 ± 0.50	6.96 ± 1.18	7.55 ± 1.88	0.22	0.55	0.07
% Neutrophils	62.38 ± 2.50	61.5 ± 2.43	65.46 ± 4.81	64.51 ± 2.84	0.29	0.73	0.30
% Monocytes	5.07 ± 0.70	4.95 ± 0.65	5.72 ± 0.83	6.02 ± 0.66	0.30	0.62	0.10
Platelets (×10 ⁹ /L)	250.95 ± 13.96	224.50 ± 19.00	225.31 ± 19.33	227.93 ± 14.70	0.1	0.85	0.81
PT (sec)	11.3 ± 0.32	11.4 ± 0.50	11.44 ± 0.63	11.78 ± 0.30	0.72	0.39	0.34
aPTT (sec)	26.17 ± 3.19	25.90 ± 3.34	25.65 ± 2.35	28.80 ± 2.53	0.83	0.22	0.25
Fibrinogen (g/L)	2.23 ± 0.31	2.47 ± 0.43	3.07 ± 0.68	5.03 ± 0.78	0.12	0.003	0.001
D-Dimer (mg/L)	0.15 ± 0.03	0.27 ± 0.06	0.36 ± 0.13	0.68 ± 0.22	0.09	0.02	0.005
Smoker	4/30	5/30	20/70	20/70	0.13	1.0	0.31
Diabetes mellitus	—	9/30	21/70	21/70	NA	1.0	1.0
Hypercholesterolemia	—	18/30	49/70	49/70	NA	1.0	0.36
Hypertension	—	16/30	48/70	48/70	NA	1.0	0.18
Stenosis degree							
50%–70%	—	7/30	14/70	14/70	NA	1.0	0.79
>70%	—	23/30	56/70	56/70	NA	1.0	0.79
Cerebrovascular disease							
Asymptomatic disease	—	11/30	18/70	18/70	NA	1.0	0.34
Transient ischemic attack	—	15/30	42/70	42/70	NA	1.0	0.39
Minor stroke	—	4/30	10/70	10/70	NA	1.0	1.0

CRP = C-reactive protein; HDL = high-density lipoprotein; LDL = low-density lipoprotein; n = number of subjects; NA = not available; p^a = pre-CAS versus control; p^b = post-CAS versus pre-CAS; p^c = post-CAS versus DSA group; post-CAS = postprocedural (48 hours after CAS); pre-CAS = preprocedural (just before CAS); PT = prothrombin time.

Data expressed as the means ± SD, unless indicated otherwise. $p < 0.05$ was considered statistically significant.

FIG. 1. Flow cytometry and confocal microscopy of PS exposure on blood cell membranes. Comparison of PS exposure on blood cells from patients who had undergone CAS or DSA only and in healthy subjects. Cells were incubated with Alexa Fluor 488–lactadherin in the dark for 10 minutes at room temperature before flow cytometric analysis. Changes in the percent of lactadherin binding for various cell types were measured in healthy subjects ($n = 30$) and in ICA stenosis patients after CAS ($n = 70$): platelets (PLT) and RBCs (**A**) and polymorphonuclear leukocytes (PMN) and mononuclear cells (MNC) (**B**). Confocal microscopy of PS exposure on the plasma membrane of blood cells (**C–G**). Platelets, RBCs, or white blood cells (WBCs) from healthy subjects or ICA stenosis patients at 2 hours (platelets), 24 hours (RBCs), or 2 hours (WBCs) after the CAS procedure were incubated with Alexa Fluor 488–lactadherin or PI in the dark for 10 minutes at room temperature. Cells were then washed very gently to remove unbound dye. Cell membranes display green fluorescence when labeled with lactadherin, and nuclei display red fluorescence. Lactadherin staining (green) is observed on platelet membranes and MPs (white arrowheads; white arrow indicates increased degree of PS staining) (**D**) and on RBCs (yellow arrowhead) (**F**) and WBCs (**G**), but there is no staining in healthy subjects (**C** and **E**). Bar = 5 mm. Percent changes in lactadherin binding were measured in blood cells from patients in the DSA group ($n = 30$) and in those who had undergone CAS (**H**). Data displayed as mean $\pm$ SD. * $p < 0.001$ versus controls; ** $p < 0.05$ versus 0 hours; # $p < 0.05$, CAS versus DSA group. Ctr = healthy control; h = hours.

sue factor–positive (TF+) blood cells showed no significant increase as compared with baseline (data not shown). To visualize PS exposure, samples of platelets, leuko-

cytes, and RBCs were incubated with Alexa Fluor 488–lactadherin or propidium iodine (PI) (for leukocytes) and imaged using a confocal laser scanning microscope. As

TABLE 2. Absolute number of PS+ blood cells

Parameter	Erythrocyte ($\times 10^{10}/L$)	Platelet ($\times 10^9/L$)	Neutrophil ($\times 10^9/L$)	Monocyte ($\times 10^9/L$)
Control subjects	1.77 ± 0.66	1.2 ± 0.14	2.78 ± 0.13	0.10 ± 0.02
CAS patients: time after procedure				
0 hrs	3.13 ± 0.61	$4.16 \pm 0.44^*$	$5.48 \pm 0.27^*$	$0.32 \pm 0.01^*$
2 hrs	3.34 ± 1.34	$24.66 \pm 0.63^\dagger$	$6.84 \pm 2.60^\dagger$	$0.46 \pm 0.14^\dagger$
6 hrs	4.22 ± 0.10	$20.42 \pm 0.90^\dagger$	$8.22 \pm 3.11^\dagger$	$0.61 \pm 0.18^\dagger$
24 hrs	$9.73 \pm 0.49^\dagger$	$16.62 \pm 1.58^\dagger$	$11.93 \pm 2.73^\dagger$	$0.70 \pm 0.20^\dagger$
48 hrs	$19.49 \pm 5.57^\dagger$	$13.49 \pm 1.68^\dagger$	$13.31 \pm 4.86^\dagger$	$0.81 \pm 0.24^\dagger$
72 hrs	$9.3 \pm 0.14^\dagger$	$6.18 \pm 2.22^\dagger$	$13.01 \pm 2.98^\dagger$	$0.64 \pm 0.19^\dagger$
7 days	$8.17 \pm 0.45^\dagger$	$5.98 \pm 0.90^\dagger$	$8.67 \pm 1.99^\dagger$	$0.52 \pm 0.13^\dagger$

* $p < 0.05$ versus control.† $p < 0.05$ versus 0 hours.

shown in Fig. 1C–G, Alexa Fluor 488–lactadherin staining was not detected on platelet or RBC membranes from the healthy subject, while robust staining was observed on platelets, particularly on extended pseudopods and shed MPs, in the ICA stenosis patient 2 hours post-CAS. Light green fluorescence was also observed around the RBC membrane at 24 hours post-CAS. Notably, leukocytes also exhibited co-staining with lactadherin and PI 2 hours after the CAS procedure. These results confirmed that there is

increased PS exposure on blood cells in patients who have undergone the CAS procedure.

Number and Cellular Origin of MPs

Flow cytometry was used to measure the total number of MPs and their phenotypic characteristics. Our analysis showed that MPs could be derived from platelets (PMPs), endothelial cells (EMPs), monocytes (MMPs), neutrophils (NMPs), and RBCs (RMPs). Our data show the time

FIG. 2. Flow cytometric analyses of PS exposure on various cells from healthy individuals (white bars) and their dynamic changes within 7 days following CAS (gray bars). The number of total PS+ MPs (A) and MPs derived from various origins were calculated: platelets (B), neutrophils (C), monocytes (D), erythrocytes (E), and endothelial cells (F). Data are shown as the mean $\pm$ SD. * $p < 0.05$ versus controls; ** $p < 0.001$ versus 0 hours.

course of MP release during the week following CAS in patients with ICA stenosis. Total PS+ MPs increased significantly at 2 hours after CAS ($p < 0.0001$), peaked at 48 hours, and remained elevated for 1 week ($p < 0.0001$; Fig. 2A). Interestingly, we found that the changes in PS+ PMPs, NMPs, MMPs, and RMPs are consistent with the percentage of PS+ platelets, neutrophils, monocytes, and RBCs (Fig. 2B–E). Elevation of EMPs was not seen until 24 hours ($p < 0.05$, compared with 0 hours) and continued to increase over the time course (Fig. 2F). Compared with controls, patients at baseline (0 hours) had higher numbers of total PS+ MPs, PMPs, NMPs, and MMPs. The numbers of CD142+ TF+ MPs, T cell (CD3+)-derived MPs, and B cell (CD19+)-derived MPs showed no significant changes (data not shown). Additionally, the number of PS+ MPs of all types was higher after CAS than DSA ($p < 0.05$; Fig. 3).

PS+ Blood Cells and MPs in Asymptomatic and Symptomatic Patients Undergoing CAS

As shown in Table 3, we found that the levels of PS+ platelets, polymorphonuclear leukocytes (PMNs), mononuclear cells (MNCs), and MPs were higher in symptomatic patients than in asymptomatic patients at baseline ($p < 0.05$). However, levels of PS+ blood cells and MPs were not significantly different between asymptomatic

and symptomatic patients post-CAS, suggesting the CAS procedure has the same effect on PS in symptomatic and asymptomatic patients.

As anticipated, PS+ blood cells and MPs of patients with periprocedural complications (occurring within 48 hours after CAS) were all higher than those in patients without complications (Table 4). Relationships between PS+ blood cells/MPs and stenosis degree and D-dimer were analyzed. We found that stenosis degree had a significant positive correlation with levels of PS and that D-dimer had a positive correlation with PS+ platelets, RBCs, and MPs (Table 5).

PCA of Platelets, Leukocytes, RBCs, and MPs

We found levels of PS in the DSA group to be lower than those in patients who had undergone CAS and that levels are reduced to baseline levels within 24 hours. We assessed coagulation time following CAS by using a recalcification clotting time assay. At all time points, patient platelets, leukocytes, and MPs showed significantly shorter coagulation times compared with baseline ($p < 0.001$; Fig. 4A). In contrast, RBC coagulation times did not decrease until after 24 hours postprocedure ($p < 0.001$). Platelet coagulation time was lowest at 2 hours while the coagulation time of leukocytes, RBCs, and MPs was lowest at 48 hours,

FIG. 3. Flow cytometric analyses of MPs in patients who had undergone DSA only or CAS. The number of total PS+ MPs (A) and MPs derived from various origins was calculated: platelets (B), neutrophils (C), monocytes (D), erythrocytes (E), and endothelial cells (F). Data are shown as the mean $\pm$ SD. * $p < 0.05$ versus 0 hours, DSA group; # $p < 0.05$, CAS versus DSA group.

TABLE 3. Lactadherin+ blood cells and MPs in asymptomatic and symptomatic patients undergoing CAS

Parameter	0 Hrs	2 Hrs	48 Hrs	7 Days
Lact+ PLT (%)				
ACS	1.3 ± 0.09	10.5 ± 0.11	5.5 ± 0.27	2.4 ± 0.17
SCS	2.1 ± 0.16	11.0 ± 0.57	6.0 ± 0.31	2.7 ± 0.14
p Value	0.003	0.210	0.103	0.078
Lact+ RBC (%)				
ACS	0.58 ± 0.06	0.67 ± 0.03	3.90 ± 0.31	1.80 ± 0.1
SCS	0.70 ± 0.05	0.71 ± 0.04	4.44 ± 0.36	1.94 ± 0.11
p Value	0.060	0.238	0.120	0.178
Lact+ PMN (%)				
ACS	10.0 ± 0.91	14.2 ± 0.71	28.3 ± 1.40	15.6 ± 0.78
SCS	12.7 ± 0.89	15.2 ± 0.76	29.2 ± 1.46	16.1 ± 0.80
p Value	0.021	0.171	0.484	0.178
Lact+ MNC (%)				
ACS	5.3 ± 0.32	10.8 ± 0.55	19.6 ± 1.56	12.7 ± 1.25
SCS	8.9 ± 0.54	11.7 ± 0.59	20.1 ± 1.61	13.1 ± 1.30
p Value	0.001	0.125	0.719	0.720
Lact+ MPs (/μL)				
ACS	2000 ± 120	4369 ± 219	4987 ± 249	4100 ± 316
SCS	2764 ± 166	4440 ± 222	5063 ± 253	4181 ± 293
p Value	0.003	0.713	0.730	0.761

ACS = asymptomatic carotid stenosis; Lact = lactadherin; MNC = monocyte; PLT = platelet; PMN = neutrophil; SCS = symptomatic carotid stenosis.

thus inversely correlating with the percentage of PS+ cells seen by flow cytometry. Additionally, the coagulation times of CAS patients' platelets, leukocytes, and MPs at baseline were lower than those found in healthy controls ($p < 0.001$; Fig. 4B). We further investigated PCA using intrinsic factor Xa, extrinsic factor Xa, and prothrombinase assays. The activity of procoagulant enzyme complexes on platelets, leukocytes, and MPs at baseline was significantly higher than that in controls ($p < 0.001$). The changes in intrinsic/extrinsic factor Xase and prothrombinase activities of blood cells and MPs were consistent overall with the changes of PS exposure after CAS (Fig. 5A–D).

Inhibition assays were performed using the time point at which procoagulant activity peaked for each cell type (2 hours for platelets and 48 hours for leukocytes, RBCs, and MPs). Lactadherin (128 nM) prolonged the coagulation times of all cell types ($p < 0.001$, respectively; Fig. 4B). Inhibition assays of intrinsic/extrinsic factor Xase complex and thrombin formation were also performed (Fig. 5E). For blood cells and MPs, lactadherin blocked activity of the procoagulant enzyme complexes up to 80%, confirming the role of PS in supporting PCA.

Fibrin Deposition and Formation Assays

In healthy controls, platelets and leukocytes supported rapid formation of clots, whereas initial clot formation in RBCs and MPs was delayed longer than 20 minutes (Fig. 6). When analyzed by time points after CAS, the final fibrin levels of leukocytes, RBCs, and MPs increased steadily from baseline and peaked at 48 hours after CAS, while platelets peaked 2 hours after stenting, and all remained

higher than baseline after 7 days (Fig. 6A–D). The trend of fibrin production on blood cells and MPs was consistent with the number of PS+ cells and MPs. Additionally, baseline fibrin formation in platelets, leukocytes, and MPs in patients having undergone CAS was higher than in healthy controls. To determine whether PS inhibition alters fibrin formation, we treated peak samples with lactadherin prior to measuring fibrin formation. The addition of lactadherin significantly prolonged the onset of clot formation and reduced the final fibrin level by 65%.

To examine whether PS exposure relates directly to local PCA by facilitating prothrombinase assembly, confocal microscopy was used to look at the binding of fluorescence-conjugated factor Va and factor Xa to MPs from patients 48 hours post-CAS. A significant fraction of bound factor Va and Xa appeared to be co-localized on MPs, indicating that MPs are able to offer a biological surface for binding of coagulation factors, most likely through externalized PS (Fig. 6E). Because cellular PCA dictates fibrin clot formation, we sought to explore whether MPs had a similar effect. MPs were incubated with recalcified MP-depleted plasma, resulting in the formation of a dense fibrin network (Fig. 6F). We observed abundant fibrin formation spread around MPs, with the network density negatively correlated with the distance from MPs, suggesting that MPs may dictate fibrin formation through exposed PS.

Discussion

First, we first found the percentage of PS+ blood cells and the number of MPs (all types) increased after the CAS

TABLE 4. Baseline characteristics of patients with periprocedural complications within 48 hours post-CAS

Parameter	Data		
Patient No.	1	2	3
Age in yrs	70	64	75
Sex	M	M	M
Smoker	+	+	-
Diabetes mellitus	-	+	+
Hypercholesterolemia	+	+	+
Hypertension	+	+	+
Stenosis degree (%)	85	87	95
Cerebrovascular disease			
Asymptomatic disease	+	-	-
TIA	-	+	-
Minor stroke	-	+	+
Total cholesterol (mmol/L)	6.32	5.67	5.54
Triglycerides (mmol/L)	3.55	3.42	3.38
LDL (mmol/L)	4.16	3.71	3.96
HDL (mmol/L)	0.89	1.07	1.12
CRP (mg/L)	9.12	7.11	8.23
Erythrocyte ($\times 10^{12}/L$)	4.72	4.8	4.86
Leukocyte ($\times 10^9/L$)	10.88	9.4	10.08
Platelet ($\times 10^9/L$)	201	229	245
PT (sec)	11.44	11.78	12.1
aPTT (sec)	29	30.2	31.4
Fibrinogen (g/L)	5.12	5.33	6
D-Dimer (mg/L)	1.1	1.58	3.31
% PS+ PLT	11	11.6	13
% PS+ RBC	5.4	6.1	7
% PS+ PMN	38	35	32
% PS+ MNC	32	28	24
PS+ MPs (μL)	5567	5732	5897

Patient No. 1 = restenosis; Patient No. 2 = TIA; Patient No. 3 = stent-thrombosis; TIA = transient ischemic attack + = yes; - = no.

procedure, and all were higher than in the DSA group. The cell types differed somewhat in the exact time course following CAS, with platelets/PMPs peaking at 2 hours, while the other cell types peaked at 48 hours. Second, our study demonstrated that in the week following CAS, changes in PS exposure on blood cells/MPs supported dynamic changes in coagulation time, the activity of intrinsic/extrinsic Xase and prothrombinase complexes, and enhanced fibrin formation. Finally, blocking PS with lactadherin significantly inhibited the activity of procoagulant enzyme complexes and fibrin formation. The results lead us to believe that the increases in PS exposure on cells and MPs contribute to increased PCA after CAS.

Previous reports have shown no significant differences in platelet activation following CAS.¹⁸ However, we found that the levels of PS+ platelets and PMPs peaked at 2 hours after the CAS procedure was performed and remained elevated for 7 days thereafter, suggesting an early injury response. One possible explanation is that the marker used in

TABLE 5. Relationships between PS+ blood cells/MPs and clinical data at their peak time point

Parameter	PLTs	RBCs	PMNs	MNCs	MPs
Stenosis degree	$r = 0.473$ $p = 0.021$	$r = 0.361$ $p = 0.051$	$r = 0.574$ $p < 0.001$	$r = 0.543$ $p < 0.001$	$r = 0.455$ $p < 0.001$
D-Dimer	$r = 0.437$ $p = 0.034$	$r = 0.601$ $p < 0.001$	$r = 0.246$ $p = 0.507$	$r = 0.199$ $p = 0.231$	$r = 0.579$ $p = 0.001$

our study to detect PS+ platelets and PMPs is more sensitive. The immediate increase in PS+ platelets and PMPs after CAS could be attributable to the mechanical forces of stent implantation that may induce apoptotic alterations of platelet surface membranes. Our data showed that the percentage of PS+ platelets positively correlated with D-dimer. Increased PS+ platelets and PMPs may be involved in thromboembolism complications after CAS. Note that patients usually received dual antiplatelet therapy daily after CAS. In our results, we found increased platelet activation following CAS, which peaked at 2 hours. It is presumed that dual antiplatelet therapy should be considered for at least 2 hours after CAS. Thus, monitoring platelet activation could be important in preventing postoperative complications.

Similar to platelets, the levels of circulating PS+ neutrophils/NMPs and monocytes/MMPs were significantly increased and remained elevated at 7 days following the procedure, indicating that neutrophils and monocytes play an active role in the evolution of the hypercoagulant and inflammatory state and may lead to the development of in-stent restenosis after CAS. Consistent with our results, data in other studies have shown that NMPs promote coagulation by adhering to and activating endothelial cells to release inflammatory cytokines.^{9,23,24} The levels of PS+ monocytes/MMPs were increased at baseline in patients who had undergone CAS as compared with levels in the control group, suggesting neutrophils and monocytes are recruited to the intima of atherosclerotic vessel walls. Our findings indicate that PS+ neutrophils/NMPs and monocytes/MMPs may be involved in the pathophysiology of inflammation and PCA after CAS.

A previous study has shown that the erythrocyte sedimentation rate may be a predictor of long-term outcomes following CAS.²⁸ However, the role of erythrocyte activation and/or apoptosis after CAS is largely unknown. Unlike the PS+ platelets and PMPs, the PS+ RBCs and RMPs were not increased until 24 hours and peaked 48 hours post-CAS, suggesting that they may be released into circulating blood through mechanical thrombosis rupture. Indeed, our findings have shown that D-dimer was markedly increased at 48 hours post-CAS, indicating thrombosis rupture. Thus, our observations may provide new information about procoagulant mechanisms and coagulation abnormalities after CAS.

Implantation of a stent can cause endothelial disruption and abrasion. Percutaneous transluminal angioplasty leads to endothelial denuding and mechanical disruption of the atherosclerotic plaque with exposure of procoagulant plaque components to flowing blood.¹⁹ The increase in PS+ EMPs following CAS in our study confirms the

FIG. 4. Coagulation time and inhibition assay for platelets, WBCs, RBCs, and MPs. **A:** Coagulation time in platelets, WBCs, RBCs, and MPs decreased ($p < 0.05$) after the CAS procedure. Arrows indicate the time point of highest PCA and are used to illustrate panel B. **B:** Lactadherin was used to block PS and inhibit PCA in platelets, WBCs, RBCs, and MPs at the time indicated by the arrows in panel A. Coagulation time was prolonged in the lactadherin-treated samples. Data are shown as the mean $\pm$ SD. * $p < 0.001$.

presence of endothelial cell injury. The numbers of EMPs started to increase at 24 hours and remained elevated for 7 days post-CAS. Recent studies have shown that the level of MMP-9, which is exposed on EMPs, was elevated in vitro at 24 hours post-CAS,^{38,44} supporting our in vivo findings.

PS exposure on the outer membranes of blood cells and MPs serves to increase the apparent affinity of the cofactors VIIIa and Va for the enzyme factors IXa and

X, respectively. This increases the generation of factor Xa and thrombin, resulting in fibrin production and inducing increased PCA.^{16,36} Based on the role of PS in the coagulation cascade and its dynamic changes in the 7 days after CAS, we further studied its effects on PCA. Increased PS exposure on blood cells and MPs from patients who had undergone CAS increased the activity of the intrinsic and extrinsic Xase complexes and thereby shortened coagula-

FIG. 5. Formation and inhibition of procoagulant enzyme complexes post-CAS. Factor Xa and thrombin production are shown in platelets (A), WBCs (B), RBCs (C), and MPs (D). Intrinsic factor Xa formation was measured in the presence of factors IXa, VIIIa, and thrombin. Extrinsic factor Xa production was assessed in the presence of factor VIIa. Thrombin generation was assessed in the presence of factors Xa and Va. The ability of lactadherin (128 nM) to block the formation of procoagulant enzyme complexes on cells and MPs was evaluated (E). In each kind of cell and MPs, lactadherin decreased activity of the procoagulant enzyme complexes by 80%. Data are displayed as the mean $\pm$ SD. * $p < 0.05$ versus controls; ** $p < 0.001$ versus 0 hours. IIa = factor VIIa; Ex-Xa+Lac = extrinsic factor Xa+lactadherin; In-Xa+Lac = intrinsic factor Xa+lactadherin.

FIG. 6. Fibrin deposition, formation, and inhibition on blood cells/MPs and circulating MPs support factor Va/Xa binding and fibrin deposition post-CAS. Recalcified (10 mM, final) normal pooled MP-depleted plasma was added to confluent platelets (A), WBCs (B), erythrocytes (C), and MPs (D) in healthy subjects and in patients who had undergone CAS in the absence or presence of 128 nM lactadherin. Fibrin polymerization was monitored by turbidity at 405 nm. Simultaneous staining (yellow, E) of factor Va (green) and Xa (red) is observed around MPs. Converted fibrin networks were detected around MPs at 48 hours post-CAS in the presence of recalcified MP-depleted plasma (F). Bar = 2 μ m (E) and 5 μ m (D).

tion time. Blockage of PS with lactadherin inhibited procoagulant enzyme production up to 80%, decreased fibrin formation approximately 65%, and prolonged coagulation time. Our confocal microscopy data also showed that factor Va and Xa appear to be co-localized on patient MPs through externalized PS. Further, MPs mixed with recalcified MP-depleted plasma became attached to fibrin fibrils, which strongly suggests that MPs were directly incorporated into the fibrin network. Thus, we have demonstrated that increasing levels of PS+ platelets, leukocytes, erythrocytes, and MPs following CAS are associated with high PCA.

The potential limitations of this study include its limited number of subjects and the absence of patient follow-up regarding postoperative complications after 7 days. However, we made observations at 2 time points within 24 hours. Furthermore, we found that 3 of the limited number of patients had thromboembolism within 48 hours post-CAS. The clinical data of these 3 patients may be useful in the future (Table 4). Future large-scale prospective studies are needed to establish whether PS could be used as a non-invasive biomarker to guide treatment decisions in patients who have undergone CAS.

Conclusions

Our results shed new light on the potential mechanisms of coagulation abnormalities after CAS. PS+ blood cells and MPs from activated platelets, neutrophils, monocytes, RBCs, and endothelial cells changed dramatically after CAS. Stenosis degree and D-dimer showed a significant positive correlation with the levels of PS. A better understanding of these changes may provide guidance on anticoagulation therapy such that there may be a need for enhanced anticoagulant therapy for patients with excessive PCA at specific times during recovery. Further studies are also needed to explore the use of PS inhibitors to decrease PCA in the future. In our study, blockade of PS with lactadherin inhibited nearly 80% of procoagulant enzyme production and approximately 65% of fibrin formation. Thus, therapeutic strategies focusing on blockade of PS may constitute a novel therapeutic intervention after CAS when coagulation is abnormally enhanced.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Conception and design: J Shi, Zhao, Bi, Kou, H Shi, Hu, Zhou. Acquisition of data: Zhao, Wu, Si, Yao, Dong, Guo, Tong, H Shi, Tian, Hu. Analysis and interpretation of data: J Shi, Zhao, Wu, Si, Yao, Dong, Chen, Bi, Kou, H Shi, Hu, Zhou. Drafting the article: J Shi, Zhao, Si, Yao, Novakovic, Kou, Zhou. Critically revising the article: J Shi, Zhao, Wu, Si, Yao, Novakovic, Chen, Bi, Kou, Hu, Zhou. Reviewed submitted version of manuscript: Zhao. Approved the final version of the manuscript on behalf of all authors: J Shi. Statistical analysis: Zhao, Wu, Si, Yao, Guo, Tong, Tian, Hu. Administrative/technical/material support: J Shi, Zhao, Wu, Dong, Guo, Tong, Chen, Bi, Kou, Tian, Zhou. Study supervision: J Shi, Zhao, Kou, H Shi, Hu, Zhou.

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