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Novel microspheres reduce the formation of deep venous thrombosis and repair the vascular wall in a rat model

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L-Arginine (L-arg), widely known as a substrate for endogenous nitric oxide synthesis, can improve endothelial function associated with the vasculature, inhibit platelet aggregation, and alter the activity of vascular smooth muscle cells. P-selectin is a membrane component of the platelet alpha-granule and the endothelial cell-specific Wiebel–Palade body that plays a central role in mediating interactions between platelets and both leukocytes and the endothelium. The experiment was designed to evaluate the effect of novel microspheres with L-arg targeting P-selectin on the formation of deep vein thrombosis and repair of vascular wall in a rat model. Thrombosis of the inferior vena cava was induced by applying a piece of filter paper (5 mm × 10 mm) saturated with 10% $FeCl_3$ solution for 5 min. Targeted microspheres with L-arg, targeted microspheres with water, and saline were injected into the tail veins of the rats after 30 min of applying the filter paper saturated with 10% $FeCl_3$ solution. The dry weight and length of the thrombus isolated from the inferior vena cava were significantly decreased in the group with L-arg in microsphere after 24 h. No significant differences in prothrombin time, activated partial thromboplastin time, thrombin time, and fibrinogen among the groups were indicated. Images revealed that apoptosis in the vascular wall was less in the group injected with targeted microspheres with L-arg than in the other two groups at 1 and 8 d postsurgery. Meanwhile, cell proliferation was considerably excessive in the group injected with L-arg wrapped in targeted microspheres. Therefore, these novel microspheres could decrease the formation of thrombus in the early stages and in the subsequent periods of thrombosis. The microspheres can also enhance the vitality of impaired endothelial cells and reduce cell apoptosis.

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Introduction

Deep vein thrombosis (DVT) remains a major clinical problem despite decades of research. Without prophylaxis, the incidence rate of DVT ranges from 10 to 40% after medical or general surgery and even 40% to 60% after a major orthopedic surgery [1]. DVT may lead to pulmonary hypertension, recurrent thrombosis, postthrombotic syndrome, or even fatal pulmonary embolism. Surgical treatment may be an option; however, it damages the walls of the vein and vascular endothelial cells (VECs), increasing the risk of thrombotic recurrence and activation of vascular smooth muscle cells (VSMCs) [2,3].

Major prophylactic anticoagulants after hip arthroplasty include low-molecular-weight heparin and a direct Xa inhibitor (rivaroxaban), which can reduce the incidence of venous thromboembolism to 3.7 and 1.1%, respectively [4]. However, these anticoagulants can also increase the risk of bleeding and wound complications [5].

Endothelial injury, blood stasis, hypercoagulability, and simultaneous inflammatory response are identified as major precipitating factors for venous thrombosis [6]. The proliferation, transformation, and metabolism of VSMCs are regulated using nitric oxide, which is synthesized by the venous endothelial membrane. This membrane consists of VSMCs, which can maintain permeability and regulate blood platelet function. L-Arginine (L-arg), widely known as a substrate for endogenous nitric oxide synthesis, can improve endothelial function associated with the vasculature, inhibit platelet aggregation, and alter VSMC activity [7]. Endothelium-derived nitric oxide has also been proven to shorten bleeding time.

P-Selectin is a membrane component of the platelet alpha-granule and the endothelial cell-specific Wiebel–Palade body that plays a central role in mediating

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interactions between platelets and both leukocytes and the endothelium [8]. The surface expression of P-selectin can be used as a marker for platelet activation, and high levels have been described in venous thromboembolism [9]. Thus, we wrapped L-arg in microspheres to prolong the release time of nitric oxide, thereby improving the effect of the treatment; it is more stable than nitric oxide itself. Meanwhile, P-selectin-targeted microspheres can bind to sites of thrombosis in the early stages to decrease the systemic blood drug concentration, increase its permeability, and most importantly, increase the duration of the localization of the thrombus, thereby extending the duration of the therapeutic effects. The microsphere shell consists of phospholipids, a drug delivery system approved by the United States Food and Drug Administration, which can be safely used *in vivo*. Within the blood circulation, the microspheres can be delivered to the thrombus, and L-arg can be used to release nitric oxide. Redundant microspheres can be cleaned through the liver.

This study aimed to evaluate the therapeutic effects of L-arg wrapped in microspheres on venous thrombosis. We hypothesized that these microspheres can reduce the formation of DVT and repair the vascular wall in a rat model.

Materials and methods

Rats were maintained under pathogen-free conditions and were freely allowed access to food, water, and activity. All experimental protocols were approved by and conducted in accordance with the Animal Ethical Committee of Drum Tower Hospital Affiliated to Medical School of Nanjing University.

Preparation of L-arg in P-selectin-targeted microspheres

All reagents were purchased from Sigma–Aldrich (United States). L-arg in P-selectin-modified microspheres was prepared by double emulsion, following the procedure described in a previous study [10,11]. Poly(D,L-lactide) (PLA, 0.50 g) was dissolved in a methylene chloride solution (10.00 ml) at 25°C. Subsequently, an L-arg solution (2.00 ml, 0.25 mmol/l) and Tween 80 (0.05 ml) were added into the uniform PLA organic solution and sonicated continuously using an ultrasound probe at 200 W for 2 min. After the formation of the first water-in-oil (W/O) emulsion, the solution was poured into a 5% polyvinyl pyrrolidone (w/v) solution (30.00 ml) and mixed mechanically for 4 h to form a (W/O)/W stable L-arg in microsphere emulsion. To conjugate P-selectin onto the microspheres, the separated microspheres were suspended in a mesityl 2,4,6-trimethylphenyl buffer solution (50 mmol/l, pH 5.4). Activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.4 mg/ml) at room temperature, the P-selectin solution was immediately added and then incubated with a microsphere solution at −4°C. After 2 h, L-arg in P-selectin-modified microspheres were collected and then washed 3 times with phosphate buffer saline.

The mean size distribution of the microspheres was measured using Particle Sizing Systems (AccuSizer 780 A, USA) at room temperature. The morphology and structure of the microspheres were visualized by scanning electron microscopy (FESEM, FEISirion-200, Holland) under an accelerating voltage of 1.00 kV as well as by transmission electron microscopy (TEM, JEOL, JEM-2000EX, Japan). As shown in Fig. 1a and b, the microspheres with L-arg exhibit a regular spherical morphology. The mean diameter was $3.02 \pm 0.14\,\mu m$ with a polydispersity index of 0.13.

Experimental animals

A total of 45 *SD rats* (SLAC Laboratory Animal Co. Ltd., Shanghai, China) were randomly divided into three DVT groups. The rats were kept in a 12 : 12 h light–dark cycle in the animal house of Drum Tower Hospital-affiliated Medical school of Nanjing University.

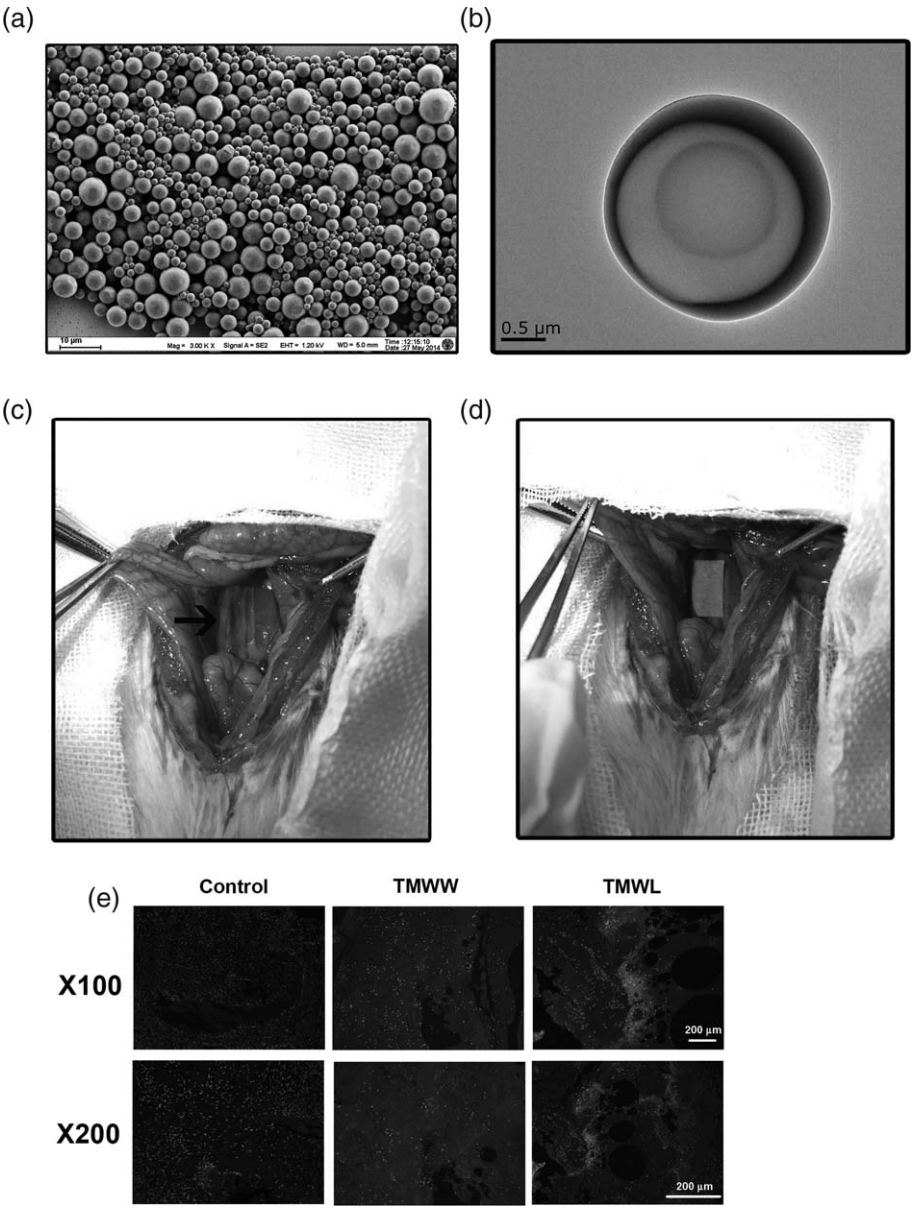
Thrombus in rat models

Thrombosis of the inferior vena cava (IVC) was induced by applying a piece of filter paper (5 mm × 10 mm) saturated with 10% $FeCl_3$ (Sigma-Aldrich, St. Louis, USA) solution for 5 min [12] (Fig. 1c and d). Rats were anesthetized with an intraperitoneal injection of ketamine (0.05 ml/kg) and diazepam (0.05 mL/kg). A midline abdominal incision was made, exposing the IVC. Vascular injuries were generated by applying a filter paper (Whatmann, 10 mm in length and 4 mm in width) saturated with 10% $FeCl_3$ on top of the IVC that was in contact with the adventitial surface of the vessel for 5 min. Targeted microspheres with L-arg (the TMWL group, 1 mL/kg, presurgery), targeted microspheres with water (the TMWW group, 1 ml/kg, presurgery), and saline (control group, 1 ml/kg, presurgery) were injected into the tail veins of the rats 30 min after filter paper saturated with 10% $FeCl_3$ solution was applied. Peripheral blood was collected in a 2.7 ml tube (Vacutainer, Becton, Dickinson and Company, USA) containing sodium citrate from retro-orbital venous plexus under ether anesthesia 0.5 h before the rats were euthanized for a coagulation test. The dry weight (mg) and length (mm) of the thrombi harvested from the thrombosed IVC were measured 24 h postsurgery.

Coagulation function test

The peripheral blood collected from retro-orbital venous plexuses after 24 h was used for coagulation function testing. In this study, four items associated with the coagulation function were detected by the clinical laboratory of Drum Tower Hospital-affiliated Medical school of Nanjing University. These items included prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), and fibrinogen (FIB).

Fig. 1



Morphology of microspheres and thrombus in rat models. (a) Scanning electron microscope; (b) transmission electron microscope; (c and d) thrombosis of the IVC was induced by applying a piece of filter paper (5 mm × 10 mm) saturated with 10% $FeCl_3$ solution for 5 min; and (e) immunofluorescence staining to detect the expression of P-selectin. Lower row represents the local amplification of upper row at 8 d postthrombosis. Scale bars: 200 μm. IVC, inferior vena cava.

Histological and histomorphometric analyses

Samples were fixed in 10% neutral buffered formalin at 4°C for 24 h and then embedded in paraffin. Serial cross-sections 5 μm thick were cut for each sample. The sections were dewaxed in xylene and rehydrated in an ethanol gradient. For histological analysis of the phenotype 1 and 8 d postsurgery, each of the five or eight sections of the specimen was sampled in a standardized manner throughout the thrombosis. The sections were then stained with hematoxylin and eosin to compare the structures of the thrombi. Terminal deoxynucleodidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling was performed to identify the apoptotic cells in the IVC wall with a commercially available kit (Promega, G3250, USA). The proliferating cells, Ki67 (Abcam, ab15580, UK) immunofluorescence-positive cells, were counted for each section. The proliferation index was calculated by the percentage of positive cells in each field. Blind scoring was performed by two independent investigators.

Ultrasonic imaging

When the thrombus model was successfully completed (after the filter paper was removed), the induced thrombus was observed by Doppler ultrasound. The ultrasonic probe detected along the IVC thrombus and the whole thrombus was included in the probe. We observed the stranded thrombus 1 and 8 d postmodeling. Subsequently, we removed the failed thrombus models, including those with venous thrombosis of varying sizes and some with total occlusion.

Statistical analysis

Statistical analysis was performed using SPSS ver. 19.0 (SPSS Inc., USA). Comparisons between groups were performed by one-way ANOVA to analyze two groups, whereas the least significant difference method was used to analyze multiple groups. Proportions were compared using Fisher exact test. $P < 0.05$ was considered significant for the differences between mean values. The numerical values for each measurement are shown as mean $\pm$ SE.

Results

L-arg in P-selectin-targeted microspheres reduces the volume of thrombi

The ferric chloride model generated venous thrombi identical in size (except for those with total occlusion) because of the similar dimensions of the filter paper (5 mm $\times$ 10 mm) saturated with 10% $FeCl_3$. The thrombosed IVC were harvested after 24 h, and the thrombi were separated to determine their dry weights and lengths. Both the dry weight and length decreased significantly in the TMWL group, as shown in Fig. 2a (weight: TMWL group 1.52 ± 0.84 mg vs. TMWW group 4.30 ± 2.07 mg vs. control group 6.10 ± 3.60 mg; length: TMWL group 2.87 ± 1.24 mm vs. TMWW group 4.20 ± 0.99 mm vs. control group 4.90 ± 1.52 mm). However, no significant differences in both dry weight and length were found between the TMWW group and the control group.

Ultrasonic imaging of venous thrombosis

Ultrasonic imaging of venous thrombosis confirmed gross morphological changes (Fig. 2b), allowing inference of conclusions similar to those previously claimed. The thrombus in the TMWL group was markedly smaller than those in the other groups, 1 and 8 d postsurgery, as shown in mid-sagittal images.

Effect on coagulation function

Coagulation function was evaluated by PT, APTT, TT, and FIB tests. No significant differences in the levels of PT, APTT, TT, and FIB were observed between the two groups (Fig. 2c).

Histological and histomorphometric analyses

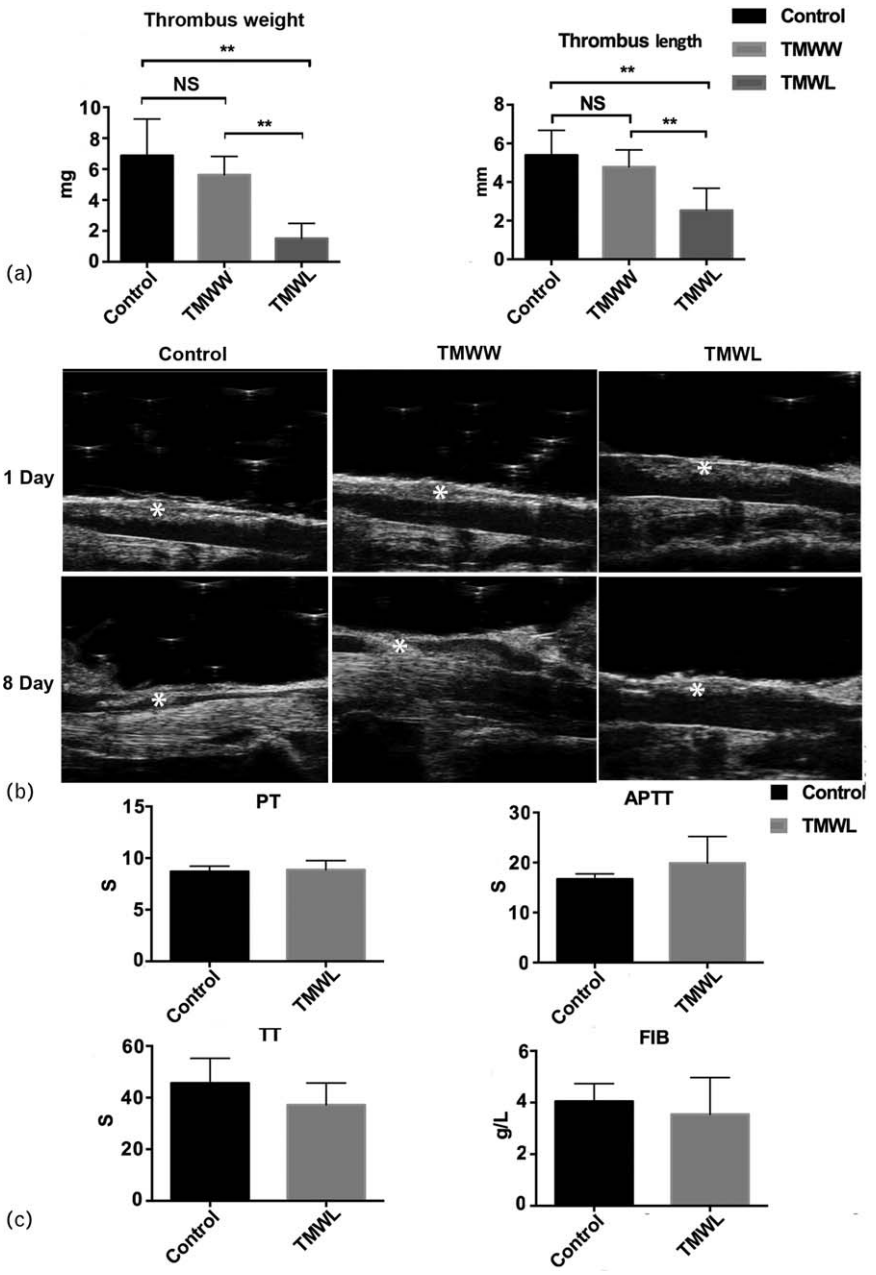
Hematoxylin and eosin staining showed that platelets, red cells, and inflammatory cells were attached to the lining of the vein walls after endothelial cell injury, 1 d postsurgery. Reduced aggregation of platelets and inflammatory cells were found in the TMWL group (Fig. 3a). In addition, the percentage of the thrombus area of the vascular cross-section was significantly decreased in the TMWL group (Fig. 3b). For matrix metalloproteinases-9 (MMP-9) protein expression, no difference was observed among the control, TMWW, and TMWL groups 1 d postsurgery; however, it increased significantly in the TMWL group compared with the other two groups 8 d postsurgery (Fig. 3c). The aforementioned results suggested that microspheres could inhibit the formation of thrombus in the early stages and in the subsequent periods of thrombosis. We performed Ki67 staining to detect the proliferation and repair of injured VECs and the vascular wall. The proliferation of cells was considerably excessive in the TMWW and control groups (Fig. 4a). A marked difference in TdT-mediated dUTP-biotin nick end labeling staining was observed, representing apoptotic cells, among the groups (Fig. 4c). The images revealed that the number of apoptotic cells was smaller in the TMWL group than in the other two groups 1 and 8 d postsurgery. This finding verified our hypothesis that TMWLs exert therapeutic effects on venous thrombosis by regulating the proliferation and metabolism of VSMCs.

Discussion

In this study, we demonstrated that L-arg in P-selectin-targeted microspheres affect the antithrombotic therapy. We wrapped L-arg in microspheres to prolong the release time of nitric oxide, thereby obtaining enhanced treatment for thrombosis; it was more stable than nitric oxide itself. P-selectin-targeted microspheres can bind to the sites of the thrombus in the early stages of the thrombotic process to decrease systemic blood drug concentration and increase its permeability (Fig. 1E). The binding time of the microspheres on the thrombus and the existing time in the blood were prominently enhanced at the same time. Meanwhile, it exhibited an efficiency in drug release and a steady degradation rate. The quantity of microspheres injected into the body was considerably, thereby avoiding air embolism and other complications.

P-selectin, a member of the selectin family belonging to the adhesion molecules [8], has a central role in mediating interactions between platelets and both leukocytes and the endothelium [13]. Studies have proven that as the largest selectin, P-selectin can stabilize initial platelet aggregates in human platelets [14,8]. After cellular activation by thrombin and oxygen-derived free radicals, P-selectin is rapidly translocated onto the cell surface from the platelet alpha-granules and the Weibel–Palade bodies specific to endothelial cells [14]. Some studies indicate that a novel small-molecule compound that specifically inhibits P-selectin can reduce both arterial and venous thrombosis in animal models [15]. Owing to

Fig. 2

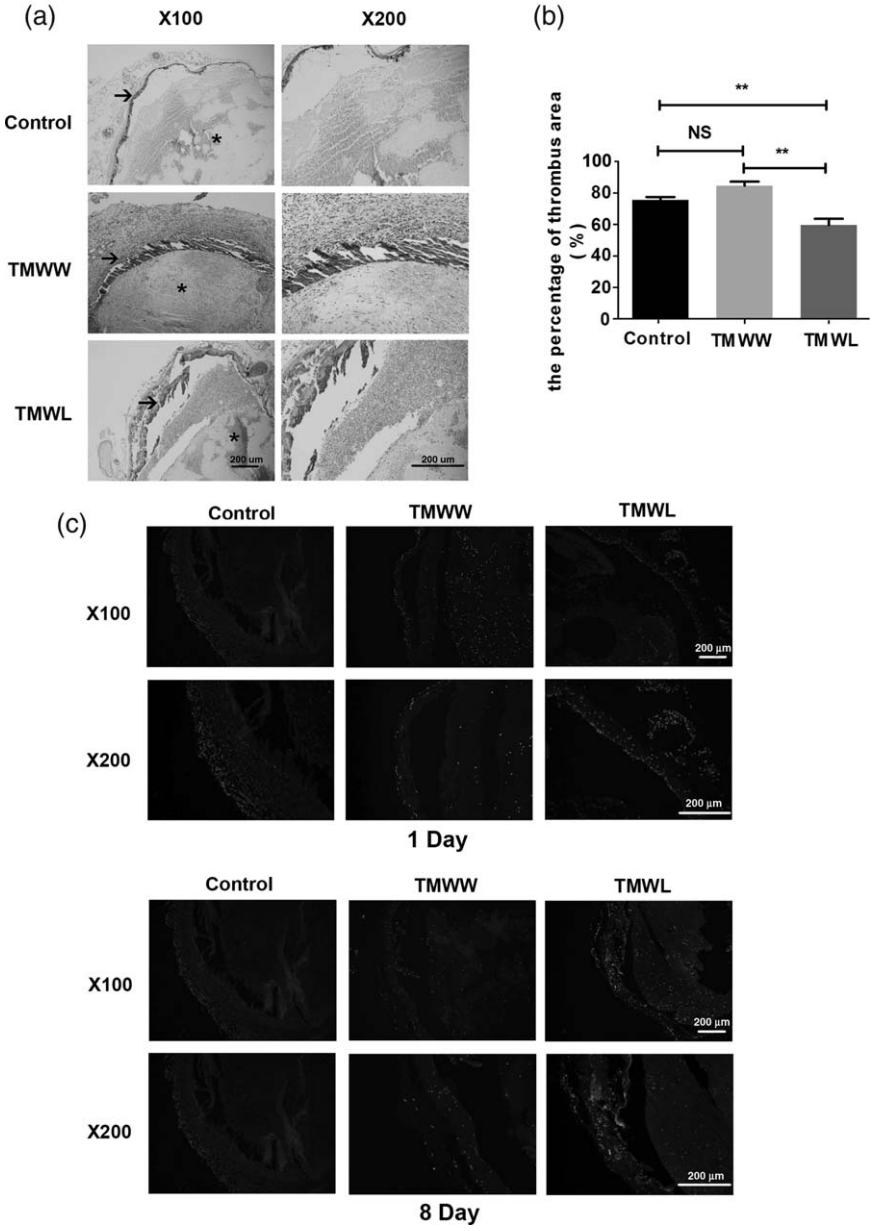


(a) Thrombus length and dry weight in the DVT group. Both thrombus length and dry weight decreased significantly in the TMWL group, compared with the TMWW group and the control group. (b) Ultrasonic diagnosis of inferior vena cava. Upper row represents three groups of blood clots (white asterisks) at 1 d postthrombosis; lower row represents three of blood clots (white asterisks) at 8 d postthrombosis. (c) Effect of coagulation function: PT, APTT, TT, and FIB of peripheral blood were tested. PT (a), APTT (b), TT (c), and FIB (d) had no statistical difference between the control and TMWL groups. Values are mean ± SD ($n = 7$), $^{*}P < 0.05$. $^{**}P < 0.01$. APTT, activated partial thromboplastin time; DVT, deep vein thrombosis; FIB, fibrinogen; PT, prothrombin time; TMWL, targeted microspheres with L-arg; TMWW, targeted microspheres with water; TT, thrombin time.

these characteristics, P-selectin significantly affects arteriovenous thrombosis and acts as a vital detection indicator [9]. In the current study, we developed L-arg in P-selectin-targeted microspheres, which provided a better therapeutic effect compared with L-arg in microspheres. The effect persisted from the early stage of thrombosis to the end.

The dry weight and length of the thrombi significantly decreased in the TMWL group by a single presurgical injection of L-arg in P-selectin-targeted microspheres. In addition, the apoptotic vascular wall cells of IVC in the TMWL group were less than those in the TMWW and control groups 1 and 8 d postsurgery. Further studies on the proliferation of vascular wall cells indicated that the

Fig. 3

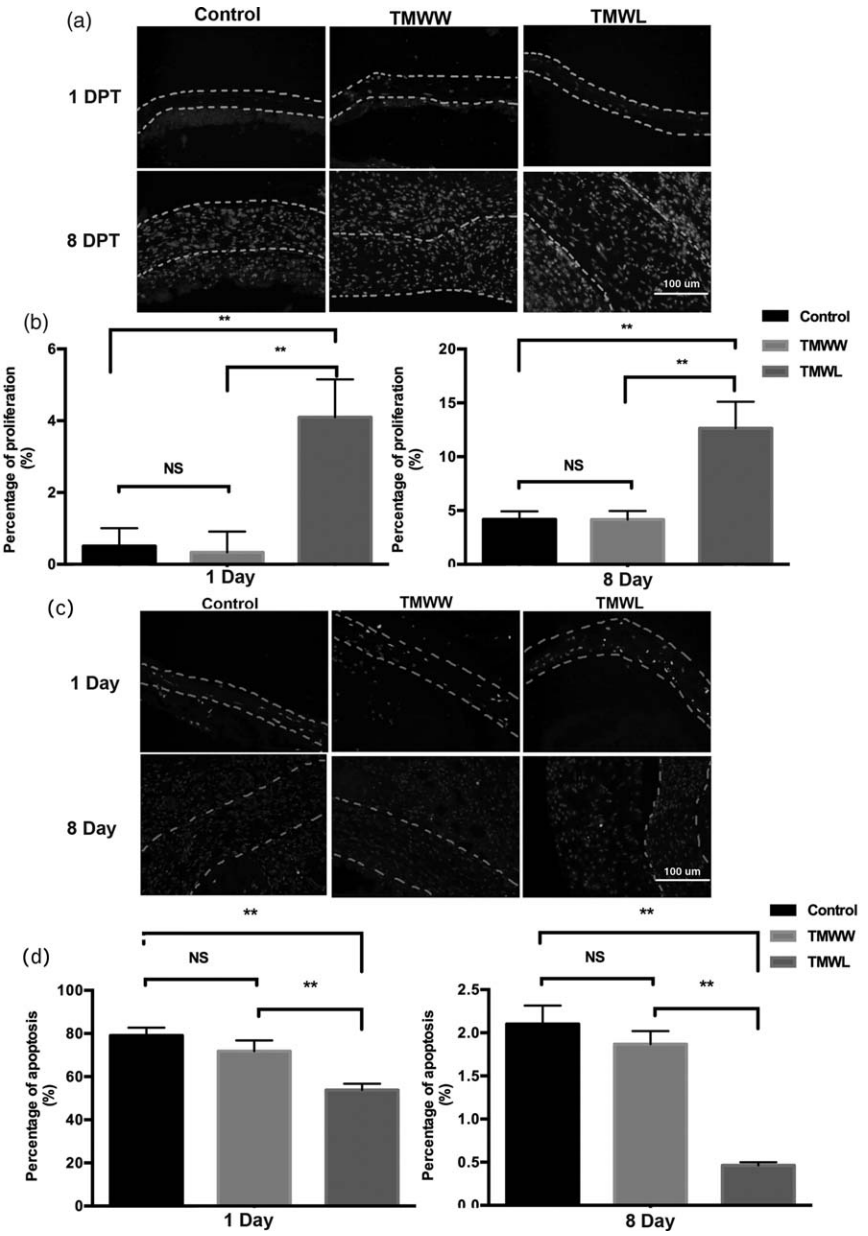


(a) H&E staining analysis: left panels show platelets, red cells, inflammatory cells, and fibrin strands attached to the lining of the vein wall at 1 d postthrombosis; right panels are the local amplifications of the left panels. (b) This figure shows the percentage of the thrombus area. The vascular cross-sectional area in the TMWL groups was significantly smaller than that in other groups. The black arrow denotes the vessel wall, and the black asterisk indicates the thrombus. Scale bars: 200 μm. Values are mean ± SD ($n = 7$), $^{*}P < 0.05$. $^{**}P < 0.01$. (c) Immunofluorescence analysis of the expression of MMP-9 at 1 and 8 d postthrombosis. Scale bars: 200 μm. H&E, hematoxylin and eosin; MMP-9, matrix metalloproteinases-9; TMWL, targeted microspheres with L-arg; TMWW, targeted microspheres with water.

percentage of cell regeneration in the TMWL group markedly increased, compared with other groups at both time points. Ultrasound imaging reflected the general condition of thrombosis and dynamically observed the changes in the thrombus. The image showing the longitudinal section of IVC provided a clearer view that L-arg in P-selectin-targeted microspheres can prevent thrombosis in the early stages and quicken thrombus degradation in the later stages.

The use of low-molecular-weight heparin for continuous anticoagulant therapy can reduce the risk of DVT and related complications with an increased risk of bleeding [5]. Rivaroxaban, an oral anticoagulant for orthopedic

Fig. 4



(a) Ki67 staining to detect cell proliferation. Upper and lower rows represent the proliferation of vascular wall cells of IVC at 1 and 8 days postthrombosis, respectively. (The area of white dotted line is the blood vessel wall; dark grey fluorescence represents the proliferation of cells) Scale bars: 100 μm. (c) TdT-mediated dUTP-biotin nick end labeling staining to detect cell apoptosis. Upper and lower rows represent the apoptosis of VECs of IVC at 1 and 8 d postthrombosis, respectively. (The area of grey dotted lines is the blood vessel wall; white fluorescence represents the apoptotic cells). Scale bars: 100 μm; b and d statistically analyzed the percentage of proliferation and apoptosis. Values are mean ± SD ($n = 7$), $^{*}P < 0.05$. $^{**}P < 0.01$. dUTP, deoxyuridine triphosphate; TdT, terminal deoxynucleodidyl transferase, TUNEL, TdT-mediated dUTP-biotin nick end labeling; VEC, vascular endothelial cell.

surgery, may induce major bleeding while reducing the incidence of venous thromboembolism and wound complications in patients undergoing total joint arthroplasty [16]. Our experiment has proven that L-arg in P-selectin-targeted microspheres only slightly influenced PT, APTT, TT, and FIB, which are important indexes of coagulation function. Microspheres of this type do not increase the risk of bleeding, which is vital in anticoagulation therapy.

In our previous study, we wrapped arginine, a nitric oxide precursor, inside the microspheres to exert a therapeutic effect; it quickened the resolution of DVT by inhibiting platelet aggregation, P-selectin expression, and fibrinogen

binding through the effects of vasodilation, vascular cell proliferation, and migration as well as by mediating inflammatory responses [17]. However, nitric oxide is an unstable molecule, which draws a significant interest in the development of materials that can provide an extended release and controlled dose of nitric oxide [18–20]. Thus, L-arg, a substrate for endogenous synthesis of nitric oxide, was wrapped in microspheres for sustained release and enhanced therapeutic effect in the current study. L-arg can reduce the increase in oxidative stress in acute pulmonary embolism [21], reverse acute hyperglycemia exhibiting intense vasoconstriction and impaired endothelial function [22], and counteract nitric oxide deficiency caused by ischemic acute renal failure [23] because of increased nitric oxide production via neurogenic nitric oxide synthase or eNOS activity [7]. A newly synthesized L-arg derivative has been shown to exert significant preventive effects on thrombosis [24]. Our experiment shows the application of L-arg in P-selectin-targeted microspheres on early anticoagulant therapy for venous thrombosis. The volume of venous thrombi significantly decreased using a single presurgical injection of L-arg in P-selectin-targeted microspheres, as expected. The microspheres also accelerated the functional recovery of blood vessel by reducing the apoptosis of vascular wall cells and the increscent percentage of cell proliferation. One important factor affecting vascular injury responses is MMPs, which play important roles in cellular proliferation and vascular repair, particularly MMP-9. It modulates collagen turnover during thrombus resolution and early wound healing [17,25].

One limitation of this research is that we did not analyze the dry weight and length of thrombus, as well as the coagulation index 8 d postsurgery owing to the difficulty in separating the thrombus from the vena cava. However, histological sectioning and ultrasound imaging 8 d postsurgery illustrated the remarkable change in the thrombus and the vascular wall. Another limitation is that the detection of proliferation and apoptosis were not specific, except for the cells in the blood vessel wall. To detect the damage and repair of VECs, we should detect its markers, such as CD31. In a further investigation, detailed studies on therapeutic parameters and the underlying mechanism will be conducted to improve the correlative studies on microspheres.

The results of our experiment suggest that L-arg in P-selectin-targeted microspheres can notably reduce the volume of DVT thrombosis in the early stages and in the subsequent period with only a slight effect on the coagulation function. In addition, data indicated that L-arg in P-selectin-targeted microspheres can significantly reduce the apoptosis of vascular wall cells and promote their proliferation, rapidly restoring the function of the blood vessels. The marked thrombolytic effect without the complications of major bleeding renders these novel microspheres ideal for the effective and safe treatment of thrombosis. However, the detailed mechanism underlying these novel microspheres needs further investigation.

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Conflicts of interest

There are no conflicts of interest.

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