

## Original Article

# Expression of P-selectin, VCAM-1, and PSGL-1 in traumatic deep venous thrombosis

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**Abstract:** The morbidity and mortality of traumatic deep vein thrombosis (DVT) show an increasing trend year by year. This research observed thrombosis by establishing rat DVT model and tested P-selectin, VCAM-1, and PSGL-1 mRNA and protein expression in blood and vena cava wall tissue. Healthy male SD rats at 7 weeks were randomly divided into control group (n = 8), sham group (n = 40) and model group (n = 40). DVT model was constructed by inferior vena cava ligation. Venous thrombosis was observed at 6 h, 24 h, 48 h, 72 h, and 168 h after modeling. RT-PCR was used to test P-selectin, VCAM-1, and PSGL-1 mRNA expression in blood. Western blot was applied to detect P-selectin, VCAM-1, and PSGL-1 protein expression in inferior vena cava wall. Immunohistochemistry was performed to determine NF- $\kappa$ B p65 expression in venous endothelial cells. Compared with sham group, obvious thrombus could be found in DVT model group at 6 h, 24 h, 48 h, 72 h, and 168 h. P-selectin, VCAM-1, PSGL-1, and NF- $\kappa$ B expression increased significantly in blood and venous wall after surgery ( $P < 0.05$ ). PSGL-1, VCAM-1, P-selectin and NF- $\kappa$ B p65 overexpressed at 6 h after DVT, of which mRNA reached peak at 24 h, while protein achieved top at 48 h and declined after 72 h. PSGL-1, VCAM 1, and P-selectin mRNA and protein expression changes were related to thrombosis process by regulating adhesion of monocytes and endothelial cells, and activating NF- $\kappa$ B signaling pathway.

**Keywords:** Traumatic deep vein thrombosis, PSGL-1, VCAM 1, P-selectin

## Introduction

The morbidity and mortality of peripheral vascular lesions deep vein thrombosis (DVT) shows an increasing trend year by year [1, 2] with complex molecular mechanism [3, 4]. It was showed that inflammation has close relationship with thrombosis [5, 6]. Cell adhesion factor is the key in the process of inflammation, thus it received most study in atherosclerosis. Intercellular adhesion molecule-1 (ICAM-1) promotes monocyte and endothelial cell adhesion. Soluble vascular cell adhesion molecule-1 (VCAM-1) overexpresses under inflammatory cytokine TNF- $\alpha$  effect [7, 8]. Selectin is a transmembrane glycoprotein, of which P-selectin, L-selectin, and E-selectin are all the member of cell adhesion molecule family. P-selectin mainly expresses in endothelial cells and activated platelets particle surface. E-selectin mainly appears on endothelial cell surface activated by cytokines. L-selectin presented on leukocyte

surface [9, 10]. Nuclear factor  $\kappa$ B (NF- $\kappa$ B) has multi-conditioning effect that can regulate a variety of inflammatory mediators, cytokines, and chemokines gene transcription level, such as IL-8, IL-6, ICAM, and VCAM, etc. It plays an important role in both inflammation and cell apoptosis [11, 12]. ICAM-1 and VCAM-1 involve in inflammation pathological process. Various inflammatory factors stimulation can increase VCAM-1 expression. At present, the role of cell adhesion factor effect and related mechanism in DVT process is still lack of investigation. This study observed PSGL-1, VCAM 1, P-selectin mRNA and protein expression in DVT model to explore their correlation.

## Materials and methods

### Experimental animals and grouping

Healthy male SD rats at 7 weeks and weighted 200~220 g were provided by the Gannan

## P-selectin, VCAM-1, and PSGL-1 in DVT

**Table 1.** Primer sequence

Gene		Sequence (5'-3')	Length (bp)
P-selectin	Forward	TTTCCTGAGTTGGGATTGTGG	100
	Reverse	CTGGCTTCTGTGGCTTGTGTTA	
PSGL-1	Forward	TGTTCCCACACTTCCTTCTGCT	160
	Reverse	TAGTCGGTATTCAAATCATCA	
VCAM-1	Forward	TGAGCGGGAAGGTGAGGAGTGAGG	509
	Reverse	CAGGATGGAGGAAGGGCTGACCAA	
GAPDH	Forward	GCTGGGGCTCACCTGAAGGG	384
	Reverse	GGATGACCTTGCCACAGCC	

Thrombus was judged based on inferior vena cava color, clot, and vein swelling degree. The blood in the inferior vena cava was collected to the PE tube (with RNALock blood stabilizer) for RT-PCR. Thrombus length and wet weight were tested. The inferior vena cava was fixed in 10% neutral formaldehyde for HE staining and Western blot assay.

### Pathomorphological analyses

Medical University (license SYXK-2013-0025). The rats were fed in SPF laboratory and the water and food both accord with standard of experimental animals. The rats were randomly divided into control group (n = 8), sham group (n = 40) and model group (n = 40).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of Gannan Medical University.

### Reagents

VCAM-1, PSGL-1, and P-selectin polyclonal antibodies were provided by Boster. NF- $\kappa$ B p65 antibody was from Santa Cruz. Secondary antibody was got from Zsbio. GAPDH protein was purchased from Shanghai Kangchen biological technology co., LTD. Primers for VCAM-1, P-selectin, and PSGL-1 were provided by Invitrogen.

### Modeling

Rat DVT model was established using inferior vena cava ligation [13]. The rats were anesthetized by 1% pentobarbital intraperitoneal injection. Abdominal peritoneum was open to isolate abdominal aorta and inferior vena cava. 4-0 surgical non-absorbent silk thread was used to ligate at 1 cm under the junction of left renal vein and inferior vena cava. Bilateral ilio-lumbar vein branches were also ligated. Sham group only isolated inferior vena cava without ligation. Control group received no treatment. The rats received normal eating and drinking after modeling.

### Sampling

The inferior vena cava was exposed at 6 h, 24 h, 48 h, 72 h, and 168 h after modeling.

Vein thrombus was observed at 6 h, 24 h, 48 h, 72 h, and 168 h after modeling. The thrombus tissue was embedded by paraffin and stained by HE for observation.

### Immunohistochemistry

Venous wall tissue was treated by paraffin section and dewaxing. NF- $\kappa$ B p65 protein expression was tested by immunohistochemical method (Ultra Vision Detection method). The slice was incubated in rabbit anti-rat NF- $\kappa$ B p65 antibody (1:1000 dilution) and developed by DAB. After sealing, the slice was analyzed by Image-pro plus System. Five random vision fields were selected to calculate the average optical density.

### Western blot

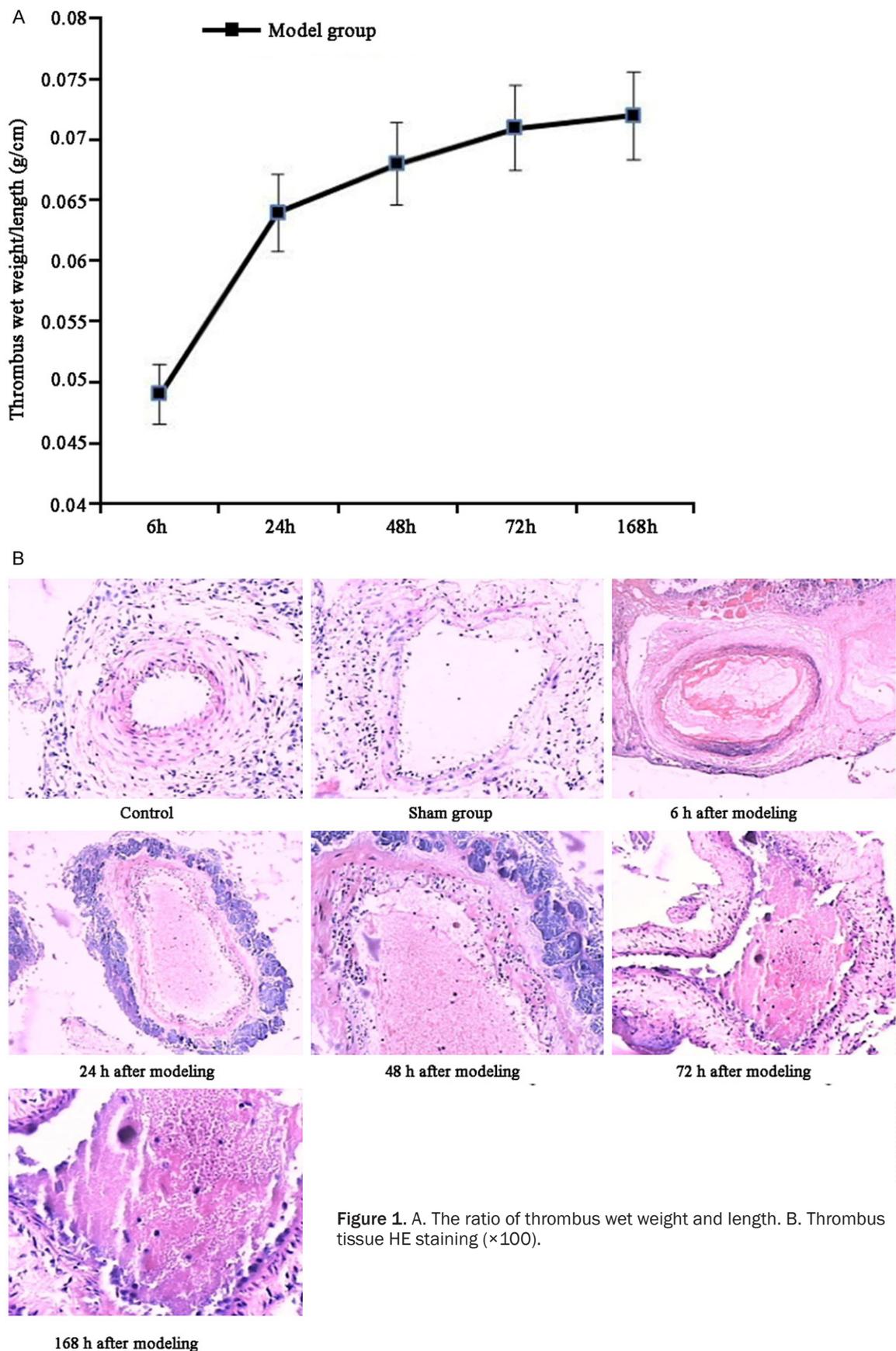
Total protein was separated by SDS-PAGE and transferred to PVDF membrane. After blocked by skim milk, the membrane was incubated in PSGL-1, VCAM-1, or P-selectin antibody (1:1000) overnight. After washed by TBST, the membrane was further incubated in secondary antibody for 1 h. At last, the membrane was developed and analyzed by Quantity One.

### RT-PCR

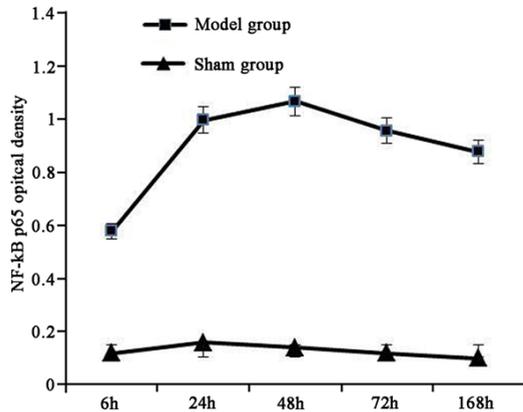
Total RNA was extracted by Trizol and quantified by ultraviolet spectrophotometer. The primers used were listed in **Table 1**. The amplification products were analyzed by agarose gel electrophoresis and expression was calculated by compared with GAPDH. All experiments were repeated for three times.

### Statistical analysis

SPSS19.0 software was applied for data analysis. The measurement data accord with normal



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**Figure 2.** NF- $\kappa$ B p65 expression in venous endothelial cells. \* $P < 0.05$ , compared with sham group.

distribution was presented as mean  $\pm$  standard deviation ( $\bar{x} \pm S$ ). One-way ANOVA and LSD test were used for data comparison.  $P < 0.05$  was considered as statistical significance.

### Results

#### *Venous thrombosis and morphologic observation*

Compared with sham group, obvious thrombus could be found in DVT model group at 6 h, 24 h, 48 h, 72 h, and 168 h. The ratio of thrombus wet weight and length was showed in **Figure 1A**. Under light microscope, red and mixed thrombus could be seen at 6 h and 24 h in model group. Vascular wall presented different degree of inflammatory cell infiltration reached peak at 24 h. Layered thrombus could be found after 72 h, while thrombus and inflammatory cells infiltration could be found at 168 h (**Figure 1B**).

#### *NF- $\kappa$ B p65 expression in venous endothelial cells*

NF- $\kappa$ B p65 mainly locates in cytoplasm and nucleus. NF- $\kappa$ B p65 expression in model group was obviously higher than that in sham group ( $P < 0.05$ ). Its level increased at 6 h after modeling, reached peak at 24 h, and declined after 72 h (**Figure 2**).

#### *P-selectin, VCAM-1, and PSGL-1 mRNA expression in blood*

P-selectin, VCAM-1, PSGL-1 mRNA expression increased significantly in blood after surgery

compared with sham group ( $P < 0.05$ ). PSGL-1, VCAM 1, and P-selectin mRNA overexpressed at 6 h after DVT, reached peak at 24 h, and declined after 72 h (**Figure 3**).

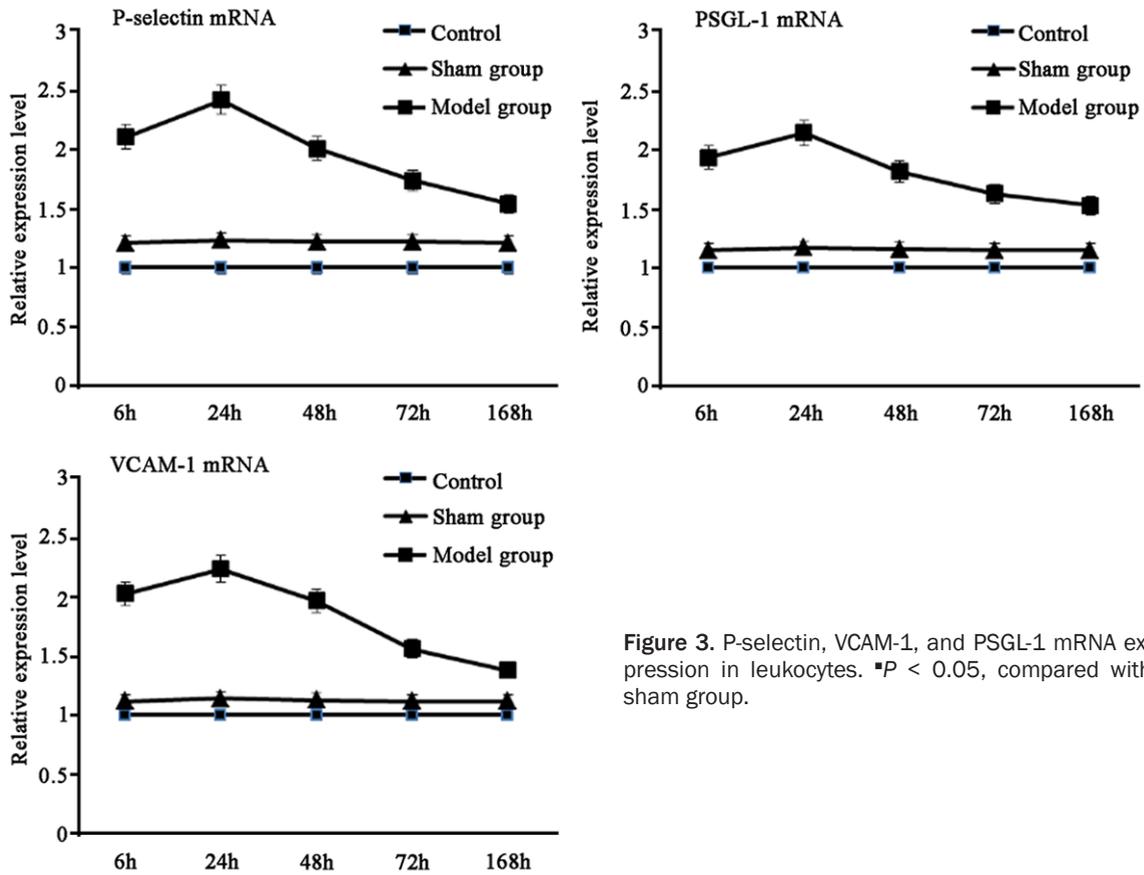
#### *P-selectin, VCAM-1, and PSGL-1 protein expression in inferior vena cava wall*

P-selectin, VCAM-1, and PSGL-1 protein expression elevated obviously in venous wall after surgery. PSGL-1, VCAM-1, and P-selectin protein overexpressed at 6 h after DVT, achieved top at 48 h, and declined after 72 h (**Figure 4**).

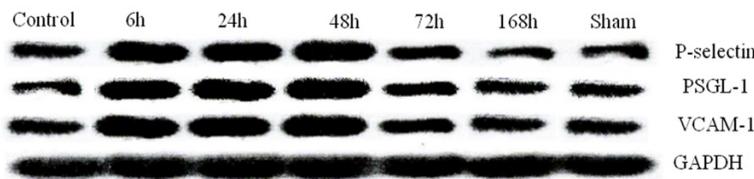
### Discussion

DVT causes vein reflux obstruction, which usually presents as lower extremity DVT. Slow blood flow, blood vessel damage, and abnormal blood components associated with DVT [14, 15]. Venous congestion leads to cell metabolism disorder, resulting in tissue hypoxia and local thrombin accumulation. Untimely correcting coagulation state may cause endothelial cells atrophy, basement membrane bareness, platelet adhesion to form thrombus core and activate coagulation material. For heparin existed on the endothelial cell surface, integral venous wall can prevent fibrin deposition. Abnormal blood composition makes blood in high coagulation state, leading to venous thrombosis [16, 17]. Leukocytes involved inflammation plays an important role in DVT process. Leukocytes adhered to endothelial cells cause endothelial injury, further triggering coagulation system activation. Leukocyte adhesion function is related to cell adhesion molecules [18, 19]. Currently, there is still lack of investigation about the role and mechanism of cell adhesion factor in DVT process. Our study observed thrombosis at different time point in DVT model, and tested P-selectin, VCAM-1, and PSGL-1 mRNA and protein expression in blood and vena cava wall tissue. The results showed that obvious thrombus could be found in DVT model group at 6 h, 24 h, 48 h, 72 h, and 168 h. Under light microscope, red and mixed thrombus could be seen at 6 h and 24 h in model group. Vascular wall presented different degree of inflammatory cell infiltration reached peak at 24 h. Layered thrombus could be found after 72 h, while thrombus and inflammatory cells infiltration could be found at 168 h, further confirming that inflammation was associated with DVT.

## P-selectin, VCAM-1, and PSGL-1 in DVT



**Figure 3.** P-selectin, VCAM-1, and PSGL-1 mRNA expression in leukocytes. \* $P < 0.05$ , compared with sham group.



**Figure 4.** P-selectin, VCAM-1, and PSGL-1 protein expression in inferior vena cava wall.

DVT begins with leukocytes adhesion and migration. P-selectin mainly mediates inflammatory cells adhering to vascular endothelial cells, neutrophils activation, and monocytes adhering to platelet. E-selectin mainly mediates the starting process of vascular endothelial cells adhering to leukocytes in inflammation [20]. Integrin family plays an important role in the beginning of the leukocytes adhering to vascular endothelial cells. L-selectin overexpresses on the surface of leukocytes and falls off under proteolytic enzyme effect after leukocytes activation. E-selectin and P-selectin bind with PSGL-1 under histamine and thrombin

effect to mediate inflammatory cells adhering to endothelial cells. VCAM-1 overexpresses in vascular endothelium under inflammatory condition by inflammatory cytokines TNF- $\alpha$  and IL-1 stimulus [21]. NF- $\kappa$ B has an important role in the inflammation by regulating multiple genes trans-

cription. Normally, NF- $\kappa$ B specific inhibitory protein I- $\kappa$ B binding with NF- $\kappa$ B dimers locates in cytoplasm. TNF- $\alpha$  or endotoxin stimulus activates I- $\kappa$ B kinase (IKK), leading to I- $\kappa$ B phosphorylation and NF- $\kappa$ B activation. NF- $\kappa$ B enters nucleus and binds with DNA to induce specific inflammatory factors gene transcription and protein synthesis, leading to inflammation [11, 12]. Generally, NF- $\kappa$ B in cytoplasm has no activity and presents as binding with I $\kappa$ B. NF- $\kappa$ B activation promotes I $\kappa$ B $\alpha$  expression, and I $\kappa$ B $\alpha$  feedback regulates NF- $\kappa$ B expression [12]. IL-1 or LPS can degrade I $\kappa$ B, releasing NF- $\kappa$ B into nucleus to bind with specific  $\kappa$ B sequence and

cause inflammatory mediators and cytokines transcription. Large amount of proinflammatory factors and inflammatory mediators result in inflammation.

Inflammation is closely related to thrombosis, and thrombosis aggravates inflammatory injury. The key in early acute inflammation injury may be related to I $\kappa$ B phosphorylation and degradation, and NF- $\kappa$ B release and translocation [11]. NF- $\kappa$ B binding site widely exists in the upstream promoter and enhancer of ICAM-1, VCAM-1, and IL-8, thus NF- $\kappa$ B activation elevates related gene expression level to promote endothelial cells and leukocyte adhesion function [19]. Our study revealed that P-selectin, VCAM-1, PSGL-1, and NF- $\kappa$ B expression increased significantly in blood and venous wall after surgery. PSGL-1, VCAM-1, P-selectin and NF- $\kappa$ B p65 overexpressed at 6 h after DVT, reached peak at 24 h, and declined after 72 h. It suggested that P-selectin, VCAM-1, and PSGL-1 play important roles in regulating endothelial cells, leukocytes and platelet adhesion. DVT is a complex process with multiple system involvement. Endothelial cells adhere to leukocytes after activation, while activated leukocytes stimulate CD40/CD40L to enhance ROS release, activate NF- $\kappa$ B signaling pathway, and promote TF secretion, leading to inflammatory factors and pro-coagulant release and thrombosis.

To sum up, P-selectin, VCAM-1, and PSGL-1 mRNA and protein expression changes in DVT model are related to thrombosis. They can regulate monocytes and endothelial cells adhesion by activating NF- $\kappa$ B signaling pathway.

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#### Disclosure of conflict of interest

None.

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