

Effect of Panax notoginseng intervention on the autophagy mechanism in a rat model of deep vein thrombosis

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Research Article

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Abstract

Objective

To establish a rat model of deep vein thrombosis (DVT) via the ligation of the inferior vena cava and investigate the intervention effect of *Panax notoginseng* (PN) on autophagy in DVT rats.

Methods

A rat model of DVT was established, and the rats were treated with PN and low-molecular-weight heparin (enoxaparin sodium injection) to observe the effect of PN on autophagy.

Results

In the model group, hematoxylin and eosin (H&E) staining revealed high levels of thrombosis and inflammatory cell infiltration. In the model group, *Beclin1*, *P62*, and *LC3* mRNA expression was significantly increased. The P-selectin content increased significantly, and the red blood cell count and hemoglobin level decreased significantly. In the model + PN (M + PN) group, H&E staining revealed reduced thrombosis formation; a small area of recanalization was observed, and one sample showed no obvious thrombosis. *Beclin1*, *P62*, and *LC3* mRNA expression was significantly downregulated. H&E staining revealed reduced thrombus formation in the model + low-molecular-weight heparin (M + LMWH) group compared with the model group; neither M + LMWH sample showed obvious thrombus formation. However, only *LC3* mRNA expression was downregulated. The content of P-selectin was decreased and the red blood cell count was increased.

Conclusion

DVT rats exhibited autophagy, and PN improved DVT by inhibiting the autophagy process.

Introduction

Venous thromboembolism (VTE) is the third most common cardiovascular disease after acute myocardial infarction and stroke¹, affecting nearly 10 million people worldwide each year². VTE is categorized as deep venous thrombosis (DVT) and pulmonary embolism (PE). Currently, effective treatment options for DVT include surgical thrombectomy, drug thrombolysis, and anticoagulation. However, these treatments may cause adverse reactions, such as bleeding, and they do not prevent the development of post-thrombotic syndrome (PTS). PTS occurs in 25 to 40 percent of patients with DVT and significantly affects their daily function and quality of life¹. Therefore, alternative therapies that may improve DVT and reduce the risk of bleeding are urgently needed. The traditional Chinese medicine *Panax*

notoginseng (PN) can promote blood circulation and stop bleeding. Recent studies have found that PN saponins (PNS), the main component of PN, have an antithrombotic effect³. In addition, our previous studies have demonstrated that large doses of PN can effectively prevent and treat VTE^{4–6} and that PN protects brain function in rats with traumatic brain injury by regulating autophagy^{7–8}. Other studies have found that autophagy plays a role in DVT⁹. Therefore, this study assessed whether PN could improve DVT by inhibiting autophagy.

1. Experimental materials and instruments

1.1 Experimental materials

1.1.1 Experimental animals Twenty male specific pathogen-free (SPF) Sprague Dawley (SD) rats were purchased from Shanghai Slack Co., Ltd. (Shanghai, China; SCXK 2022-0004; qualification certificate no. 20220004014003). Their weight ranged from 280 to 320g, and their feeding environment was maintained at a temperature range of 20–25°C with a relative humidity of 40–70%. All animal studies (including the mouse euthanasia procedures) were conducted according to the regulations and guidelines of Zhejiang Chinese Medicine University Animal Ethics as well as the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and International Animal Care and Use Committee (IACUC) guidelines. The Experimental Animal Management and Ethics Review Committee of Zhejiang Chinese Medicine University (Hangzhou, China) approved this study.

1.1.2 Experimental reagents

PN was obtained from Hangzhou Huadong Traditional Chinese Medicine Slices Co., Ltd (drug production license number: Zhe 20000070). Enoxaparin sodium (low-molecular-weight heparin, LMWH) was purchased from Sanofi Beijing Pharmaceutical Co., Ltd. (import drug registration number: H20170269, 0.4 mL, 4000 aXa IU, batch number: CS887A). Isoflurane (for pets) was purchased from Jiangsu Zhongmu Bikang Pharmaceutical Co., Ltd. (veterinary medicine number 100767015). Saline was composed of 0.9% sodium chloride and obtained from Shuanghe Pharmaceutical (lot number: 2105282A). The P-selectin enzyme-linked immunosorbent assay (ELISA) kit was obtained from Enzyme-linked Biological (item number: ml028431). Hematoxylin (item number: H9627, lot number: SLBN3249V) and erod (item number: E6003, lot no. 62R80915X) were obtained from SIGMA. A high-purity total RNA rapid extraction kit was purchased from Generay (article number: GK3016, lot no. 1703G01). The reverse transcription kit HiScript II Q RT SuperMix for qPCR (item number: R222-01, lot number: 7E092G6) and ChamQ SYBR Color qPCR Master Mix (item number: Q411-02, lot number: 7E092H6) were purchased from Vazyme. The primers were synthesized and purified by Beijing Qingke Biotechnology Co., Ltd.

1.2 Experimental instruments and consumables

The following instruments were used: a full wavelength spectrometer (SpectraMax Plus 384, American MD); a low-speed automatic balancing centrifuge (TDZ4-WS, Hunan Xiangyi); a rotary microtome (RM2235 model, LEICA Corporation); a pathological tissue drying instrument (tec 2500, Changzhou Hao

Siling Instrument Equipment Co., Ltd.); a BX43 microscope (Olympus); a thermostatic incubator (PYX-DHS500BS-II, Shanghai Yuejin Medical Equipment Co., Ltd.); a high precision spectrophotometer to measure optical density (OD) (SMA4000, Merinton); a quantitative PCR instrument (CFX Connect Real-Time PCR System); and Bio-Rad CFX Manager analysis software.

2. Experimental methods

2.1 Experimental grouping

Twenty male SPF SD rats were randomly divided into the following four groups (five rats in each group): the sham operation group (SHAM), the DVT model (M) group, the model + PN (M + PN) group, and the model + low-molecular-weight heparin (M + LMWH) group.

2.2 Animal modeling and administration

2.2.1 Establishment of animal model:

The DVT model was induced by the ligation of the inferior vena cava. The rats were first anesthetized with a gas (isoflurane) anesthesia machine. Then, under sterile conditions, the abdominal skin was cut layer by layer along the abdominal white line. After the abdominal cavity was exposed, the intestinal tube and its mesangium were moved to the right side of the abdominal cavity and fixed with cotton balls. We then continued to bluntly separate the soft tissue until the inferior vena cava was exposed and separated the inferior vena cava from the artery. A ligature (slipknot) was placed approximately 0.5 cm below the junction of the left renal vein and the inferior vena cava with a 6/0 suture; the end was retained and pulled out of the abdominal cavity. Then, a ligature (dead knot) was placed approximately 1 cm above the common iliac vein with a 6/0 suture. The abdominal cavity was closed after vein ligation. When the skin was sutured, the slipknot thread was fixed in the nodule to prevent the thread from falling into the abdominal cavity, and the slipknot was extracted outside the abdominal cavity 6 hours later. The sham group received the same operation as the DVT model group, and the other treatments were the same as the modeling operations described above except that the two vessel ligation treatments were not performed.

2.2.2 Administration:

In the M + PN group, PN was administered intragastrically twice a day at 2.5 g/kg/d (the optimal dose was selected by previous experiments) starting 4 days before operation. Intragastric administration was continued twice a day after surgery.

In the M and SHAM groups, 0.9% sodium chloride was administered instead of PN. The administration cycle and frequency were consistent with those of the M + PN group.

In the M + LMWH group, drug administration began immediately after modeling (subcutaneous injection, 4 mg/kg, twice a day).

2.3 Sampling and observation indicators

The rats were euthanized 48 hours after the model was established (calculated after slipknot removal), and then samples were taken.

2.3.1 Collection of blood for routine detection

2.3.2 H&E staining of ligation segment blood vessels: The ligation segment blood vessels were fixed in 4% formaldehyde solution for 3 to 5 days and then removed from the fixed solution and cut to a suitable shape and thickness. Next, the tissue blocks were dehydrated, waxed, encased, sliced, and baked. The tissues were observed under a microscope after staining, and the pathological changes in each sample were comprehensively described.

2.3.3 Detection of P-selectin by ELISA: P-selectin was detected according to the instructions of the P-selectin ELISA kit (Enzyme-linked organisms, item no. ml028431). The required slats were removed from the aluminum foil bag after 60 min at room temperature, and the remaining slats were sealed in a Ziplock bag and stored at 4°C. Standard, blank, and sample wells were prepared, and 50 µL of the standard was added to the standard wells at different concentrations. Subsequently, 50 µL of the sample was added to the sample well, and the blank well remained empty. In addition to the blank wells, horseradish peroxidase (HRP)-labeled detection antibody was added to the standard and sample wells, and the reaction wells were sealed with a sealing plate membrane. After incubating at 37 °C for 60 min, the liquid was discarded, and the wells were patted dry with absorbent paper. Each well was filled with 350 µL of washing liquid. After 1 min, the washing liquid was shaken off, and the wells were patted dry with absorbent paper; washing was repeated 5 times. Then, 50 µL of the substrate P-selectin was added to each well, and the solutions were incubated at 37 °C for 15 min away from light. The OD value of each well was measured at a wavelength of 450 nm with an MD SpectraMax Plus 384 spectrometer within 15 min of adding the termination solution to each well.

2.3.4 Expression of autophagy-related proteins in the iliac vein

Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression of mammalian target of rapamycin (mTOR), ubiquitin-binding protein p62 (P62), Beclin1, and microtubule-associated protein 1A/1B-light chain 3 (LC3) proteins in the left and right common iliac vein tissues.

2.3.4.1 Total RNA extraction: Total RNA was extracted with the TRIzol–centrifuge column method using a high-purity total RNA rapid extraction kit (Generay, item no. GK3016, batch: 1703G01) according to the manufacturer's instructions. Appropriate tissue samples were placed in a 1.5 mL centrifuge tube, and a steel ball was added for grinding. Then, 1 mL of TRIzol was added, and the tube was placed in a tissue grinder at 30 Hz for 120 s to fully break down the tissue. The tissue fluid was transferred to a new 1.5 mL centrifugal tube and left for 5 min at room temperature. Subsequently, 200 µL of chloroform was added,

and the tube was shaken to mix vigorously for 30 s. After letting the tube stand at room temperature for 3–5 min, it was centrifuged at 12,000 rpm for 10 min at 4 °C. The upper water phase was absorbed into another centrifugal tube, and 200 µL of anhydrous ethanol was added and mixed well. The remaining steps followed the kit instructions.

2.3.4.2 Determination of RNA purity and quantitation of RNA: With the corresponding solvent as the control (blank), 1 µL of the RNA solution was analyzed with the OD measuring instrument (high precision spectrophotometer, Merinton, SMA4000). The ratio of A260/A280, A260/A230, and continuous wavelength absorption peaks were observed; the concentration of the RNA solution was calculated; and the quality of RNA extraction was determined. The analysis revealed that A260/A280 was > 1.8 and < 2.3, which met the requirements of subsequent reverse transcription–quantitative polymerase chain reaction (RT-qPCR).

2.3.4.3 Reverse transcription test: The reverse transcription kit HiScript II Q RT SuperMix for qPCR (Vazyme, article number: R222-01, batch: 7E092G6) was used to perform the experiment; all steps were completed according to the instructions and on ice. After the experiment, the copy DNA (cDNA) was stored at – 20 °C.

2.3.4.4 Fluorescence quantitative PCR amplification experiment

(1) The primer sequence was as follows:

Primer name	Primer sequence	Base number	Product size
Rat-Beclin1-F1	GCGGCTCCTATTCCATCAA	19	100 bp
Rat-Beclin1-R1	ACCCAAGCAAGACCCCACT	19	
Rat-LC3-F1	TCGCCGACCGCTGTAAG	17	169 bp
Rat-LC3-R1	CGCCGGATGATCTTGACC	18	
Rat-P62-F1	GATAGCCTTGGAGTCGGTGG	20	228 bp
Rat-P62-R1	TCTGGTGGGAGATGTGGGTA	20	
Rat-GAPDH-F1	TGCCACTCAGAAGACTGTGG	20	129 bp
Rat-GAPDH-R1	TTCAGCTCTGGGATGACCTT	20	

(2) qPCR:

ChamQ SYBR Color qPCR Master Mix (Vazyme, item no. Q411-02, batch: 7E092H6) was used to carry out the experiment; the specific steps are detailed in the manufacturer’s instructions. After the experiment, the CFX Connect Real-Time PCR System Quantitative PCR instrument was used to read the data, and Bio-Rad CFX Manager analysis software was used to analyze the experimental data.

2.4 Statistical Analysis

SPSS 26.0 was used for statistical analysis. All experimental results are expressed as the mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was used to compare multiple samples conforming to homogeneity of variance, and the least significant difference (LSD) test was used to compare two groups. The non-parametric rank sum test was used to compare multiple samples that did not conform to the homogeneity of variance, and the Kruskal–Wallis test was used to compare two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 The DVT rat model induced by the ligation of the inferior vena cava was successfully established

In the SHAM group, H&E staining showed a clear structure of the inferior vena cava and no pathological changes.

In the M group, H&E staining showed that the intima of the inferior vena cava was seriously injured; most of the endothelial cells were shed, and the continuity of the vascular wall was interrupted. Many thrombi formed and attached to the vascular wall, and many inflammatory cells infiltrated the endovascular cortex and thrombus (Fig. 1A). Compared with the SHAM group, the M group showed a non-significant decrease in *mTOR* mRNA expression and a significant increase in *Beclin1*, *P62*, and *LC3* mRNA expression ($p < 0.05$) (Figs. 1B). Erythrocytes and hemoglobin decreased significantly in the M group ($p < 0.05$). The content of P-selectin in the M group was significantly increased ($p < 0.05$).

3.2 Comparison of the M and M + PN groups

H&E staining showed that compared with the M group, the M + PN group exhibited serious damage to the intima of the inferior vena cava; most of the endothelial cells were shed, and the continuity of the blood vessel wall was interrupted. Inflammatory cell infiltration was observed in the mixed thrombus in the M + PN group (less than that in the M group), and the center of the mixed thrombus was mainly cellulose. The phenomenon of mechanization and a small area recanalization were observed. One sample in the M + PN group showed no significant thrombosis (Fig. 2A). In addition, PN significantly downregulated the mRNA expression of *Beclin1*, *P62*, and *LC3* ($p < 0.05$). *mTOR* mRNA expression was downregulated, but no statistical difference was observed between the M and M + PN groups (Fig. 2B). In addition, PN decreased the platelet count and P-selectin level and increased the red blood cell count, hemoglobin level, and white blood cell count, but these differences were not significant.

3.3 Comparison of the M and M + LMWH groups

H&E staining revealed that compared with the M group, the M + LMWH group showed serious damage to the inner cortex of the inferior vena cava, partial shedding of endothelial cells, partial interruption of the continuity of the blood vessel wall, and inflammatory cell infiltration. In addition, fibrinoid thrombus was

identified as the main thrombus, and thrombosis was decreased compared with that in the M group. Two samples from the LMWH showed no significant thrombosis (Fig. 3A). In addition, LMWH downregulated LC3 mRNA expression ($p < 0.05$); *Beclin1* and *P62* mRNA expression was downregulated, and *mTOR* mRNA expression was upregulated; however, these differences were not significant (Fig. 3B). In addition, LMWH decreased the platelet count, white blood cell count, and P-selectin level and increased the red blood cell count and hemoglobin level, but only the red blood cell count and P-selectin level showed significant differences ($p < 0.05$).

3.4 Comparison of the M + PN and M + LMWH groups

H&E staining revealed a small area of recanalization in the thrombi in the M + PN group, and one sample showed no obvious thrombosis. Two samples in M + LMWH group showed no significant thrombosis (Fig. 4A). In addition, *Beclin1*, *P62*, *LC3*, and *mTOR* mRNA expression in the M + PN group were lower than those in the M + LMWH group, but these differences were not significant (Fig. 4B, Table 1). In addition, the platelet count, red blood cell count, and hemoglobin level in the M + PN group were lower than those in the M + LMWH group; the white blood cell count and P-selectin level were higher than those in the M + LMWH group, but these differences were not significant (Table 2).

Table 1
Expression of Beclin1, P62, LC3, and mTOR in the model + *Panax notoginseng* (M + PN) and model + low-molecular-weight heparin (M + LMWH) groups ($\bar{x} \pm s$)

Group	Number	Beclin1	P62	LC3	m-TOR
M + PN	4	0.37 ± 0.25	0.33 ± 0.22	0.22 ± 0.18	0.52 ± 0.32
M + LMWH	4	1.32 ± 1.00	0.57 ± 0.33	0.67 ± 0.41	1.08 ± 0.46
Note: No significant difference in expression was observed between the two groups ($P > 0.05$).					

Table 2
Comparison of RBC, WBC, HB, PLT, and P-selectin in M + PN and M + LMWH groups ($\bar{x} \pm s$)

Group	Number	RBC	WBC	HB	PLT	P-selectin
M + PN	5	5.65 ± 0.34	11.46 ± 1.85	124.20 ± 5.63	642.80 ± 165.18	12.54 ± 1.86
M + LMWH	5	6.37 ± 0.78	10.05 ± 2.48	130.40 ± 8.08	643.20 ± 219.88	8.06 ± 2.83
Note: No significant difference was observed in expression between the two groups ($P > 0.05$). RBC: erythrocyte; WBC: white blood cell; HB: hemoglobin; PLT: platelet.						

4. Discussion

In this study, we successfully established a rat model of DVT using the inferior vena cava ligation technique. This study assessed whether autophagy occurs in DVT rats and investigated the changes

after intervention with PN and LMWH. The results confirmed autophagy in DVT rats, and H&E staining showed a large amount of thrombosis and inflammatory cell infiltration. Furthermore, autophagy-related protein and P-selectin levels were significantly increased, and the number of red blood cells decreased. Our team's previous studies have shown that PN can improve deep vein thrombosis⁴⁻⁶. In this study, H&E staining revealed decreased thrombus formation in the M + PN and M + LMWH groups compared with that in the M group. In addition, the M + PN and M + LMWH groups exhibited reduced levels of the autophagy-related proteins LC3, Beclin1, and P62 compared with the M group; this decrease was more significant in the M + PN group, suggesting that PN can inhibit the formation of DVT by interfering with the autophagy process. No significant differences were observed between the M + PN and M + LMWH groups.

Under normal circumstances, autophagy is a metabolic process of lysosomal self-degradation that is regulated by autophagy-related genes. This process plays a key role in various physiological processes, such as cell growth and proliferation, apoptosis and senescence, the stress response, and material and energy metabolism¹⁰. Autophagy regulation requires the involvement of multiple molecules and genes¹¹; among these are LC3, Beclin 1, P62, and mTOR. LC3 is the most used marker of autophagy and can form a stable association with the autophagosome membrane¹². When autophagy was activated, LC3 protein expression increased as the autophagosome membrane content increased, that is, LC3 protein levels were positively correlated with the number of autophagosomes. Beclin1 is an important positive regulator of autophagy and significantly increases when autophagy is upregulated¹³⁻¹⁴. Ubiquitin-binding protein P62 (P62) is an important bridge between LC3 and the ubiquitination substrate that will be degraded. Theoretically, when autophagy is activated, P62 is degraded as an autophagy substrate, and its expression decreases. However, a study showed that the increase in LC3 was not consistent with the decrease in P62¹⁵. When autophagy is activated, P62 levels may increase because of a compensatory increase in the number of autophagosomes and autophagolysosomes, so autophagy activity cannot be determined by P62 expression alone¹⁶. In the present study, the level of P62 increased when autophagy was activated, which was consistent with our previous research results⁷. Finally, mTOR, a component of phosphatidyl inositol kinase-related protein kinase, suppresses autophagy; the mTOR pathway is one of the main regulators of autophagy¹⁵. In this study, the mTOR levels did not change significantly, and the differences in mTOR levels between groups were not statistically significant; therefore, we could not demonstrate that PN regulates the LC3, Beclin1, and P62 autophagy proteins through the mTOR signaling pathway. PNS, the main blood-activating component of PN, has strong anticoagulation effects, inhibiting platelet aggregation and promoting fibrinolysis³. Few previous studies have investigated the relationship between PN and DVT in terms of autophagy. A previous study found that microRNA-205 promoted DVT recalculation and resolution by regulating the protein kinase B (Akt)/autophagy pathway and matrix metalloproteinase-2 (MMP2) expression by targeting phosphatase and tensin homolog (PTEN)¹⁷.

Secondly, our results showed that the P-selectin content in the M group increased significantly in the M + LMWH group, whereas no significant change was observed in the M + PN group. P-selectin is mainly derived from the proteolytic cleavage of transmembrane P-selectin, which is shed from activated platelets

and endothelial cells. P-selectin has been studied as a potential biomarker for thrombosis, and the inhibition of P-selectin may reduce thrombosis¹⁸. In a mouse model with complete ligation of the inferior vena cava, the thrombus mass (thrombus weight /inferior vena cava length) increased by 50% in animals with elevated soluble P-selectin, whereas the thrombus mass was significantly reduced in animals with P-selectin gene deletion¹⁹. A meta-analysis showed that P-selectin inhibitors and enoxaparin had a similar ability to reduce blood clots when used to treat DVT in nonhuman primates²⁰. However, in this study, it could not be concluded that PN affected the changes in thrombi through P-selectin. Our study also found that the number of red blood cells in the M group decreased significantly. The red blood cell count increased significantly after the LMWH intervention; this increase was also observed after the PN intervention, but the difference was not significant. The decline in the red blood cell count in the M group may be explained by the predominance of entangled red blood cells in venous thrombosis^{21–22}. Studies have shown that PN has a blood-tonifying effect, and PNS can promote the proliferation of hematopoietic cells and promote the recovery of red blood cells, hemoglobin, and reticulocytes³. However, PN did not significantly increase the red blood cell count in this experiment, which may be related to the short intervention.

This study has several limitations. First, autophagy is a very complex process, and it is unclear whether the changes in autophagy were consistent at different time points after DVT. Second, we observed that PN inhibited autophagy and improved DVT; autophagy seems to cause aggravated thrombosis, but it is unclear whether autophagy could help dissolve existing thrombosis. To address the above limitations in future experiments, we will use PN autophagy flux as the research method and collect data at different time points, which will elucidate the relationship between DVT, PN, and autophagy as well as the mechanisms responsible for this relationship. This research has the potential to improve the therapeutic effect of PN for DVT and reduce its complications.

Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

Availability of data and materials The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Competing interests The authors have no conflicts of interest to declare.

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Figures

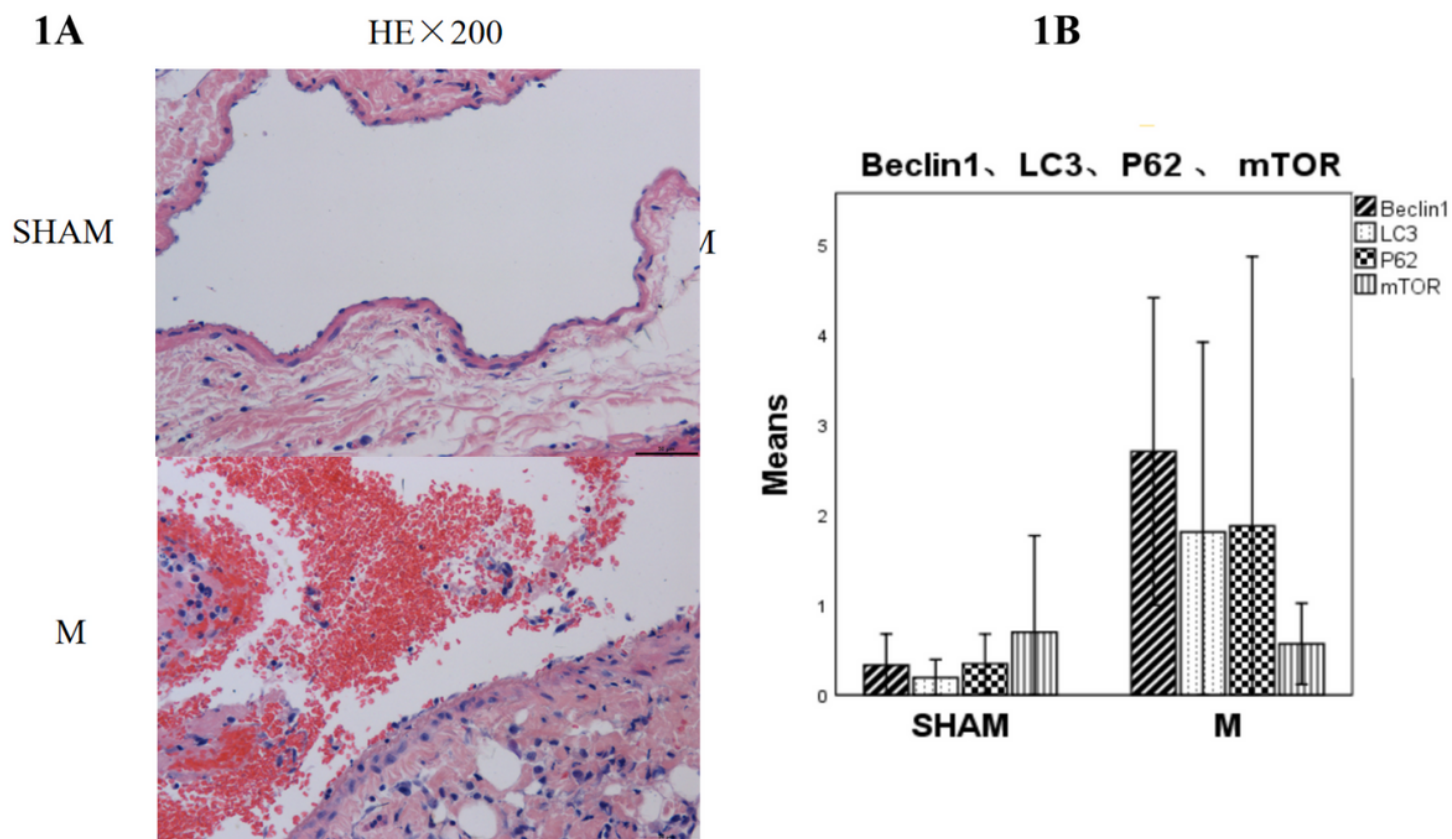


Figure 1

H&E staining revealed that compared with the sham operation group (SHAM), the model (M) group showed a large amount of thrombosis and inflammatory cell infiltration (**Figure 1A**). The expression levels of Beclin1, P62, and LC3 in the M group were significantly increased (**Figure 1B**)

2A

HE×200

2B

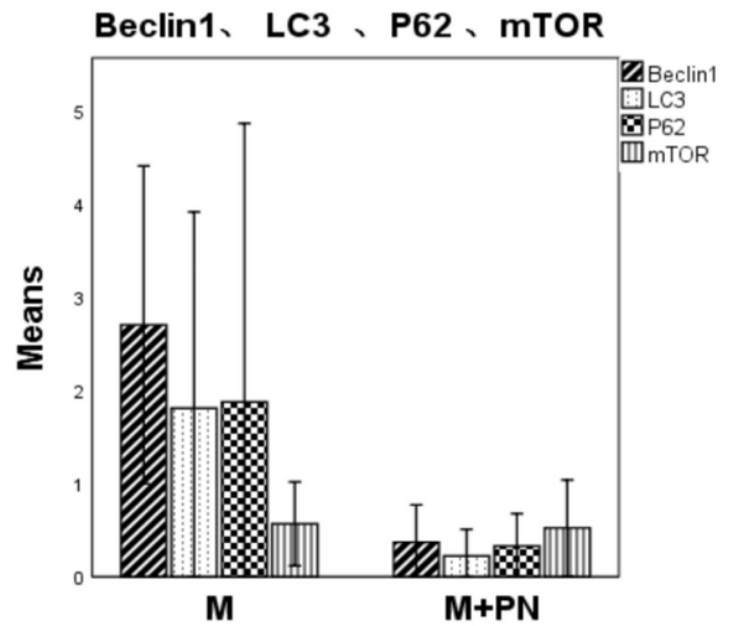
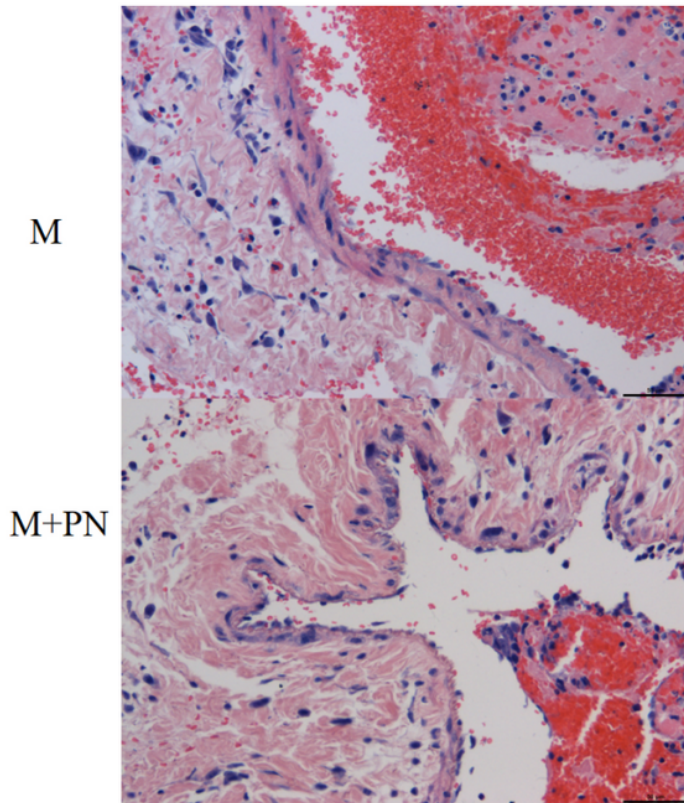


Figure 2

H&E staining revealed that compared with the M group, the model + *Panax notoginseng* (M + PN) group showed reduced thrombosis and inflammatory cell infiltration (**Figure 2A**). PN significantly downregulated the expression of Beclin1, P62, and LC3 (**Figure 2B**)

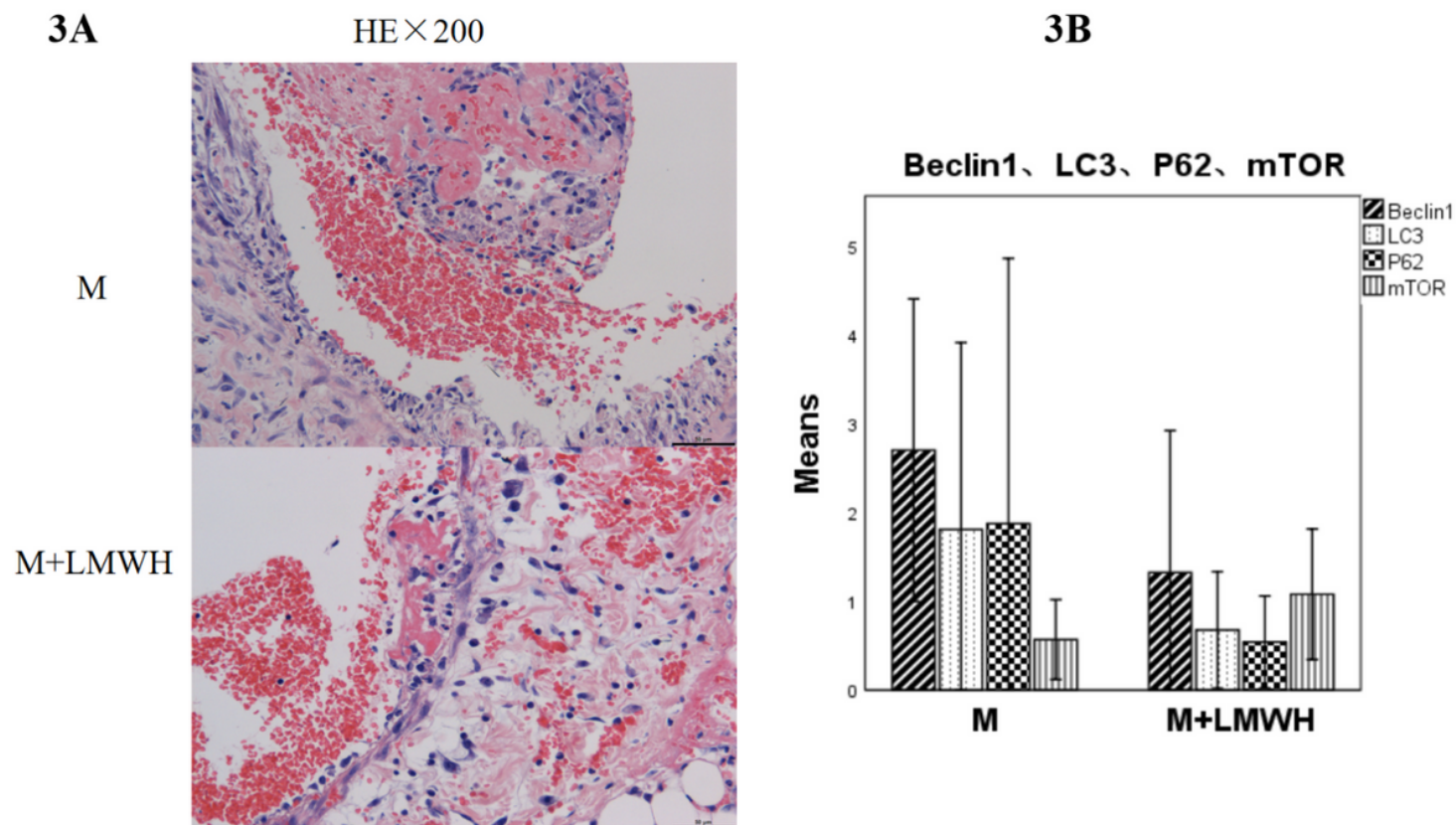


Figure 3

H&E staining revealed that compared with the M group, the model + enoxaparin/low-molecular-weight heparin (M + LMWH) group showed reduced inflammatory cell infiltration and thrombosis (**Figure 3A**). LMWH downregulated LC3 expression (**Figure 3B**).

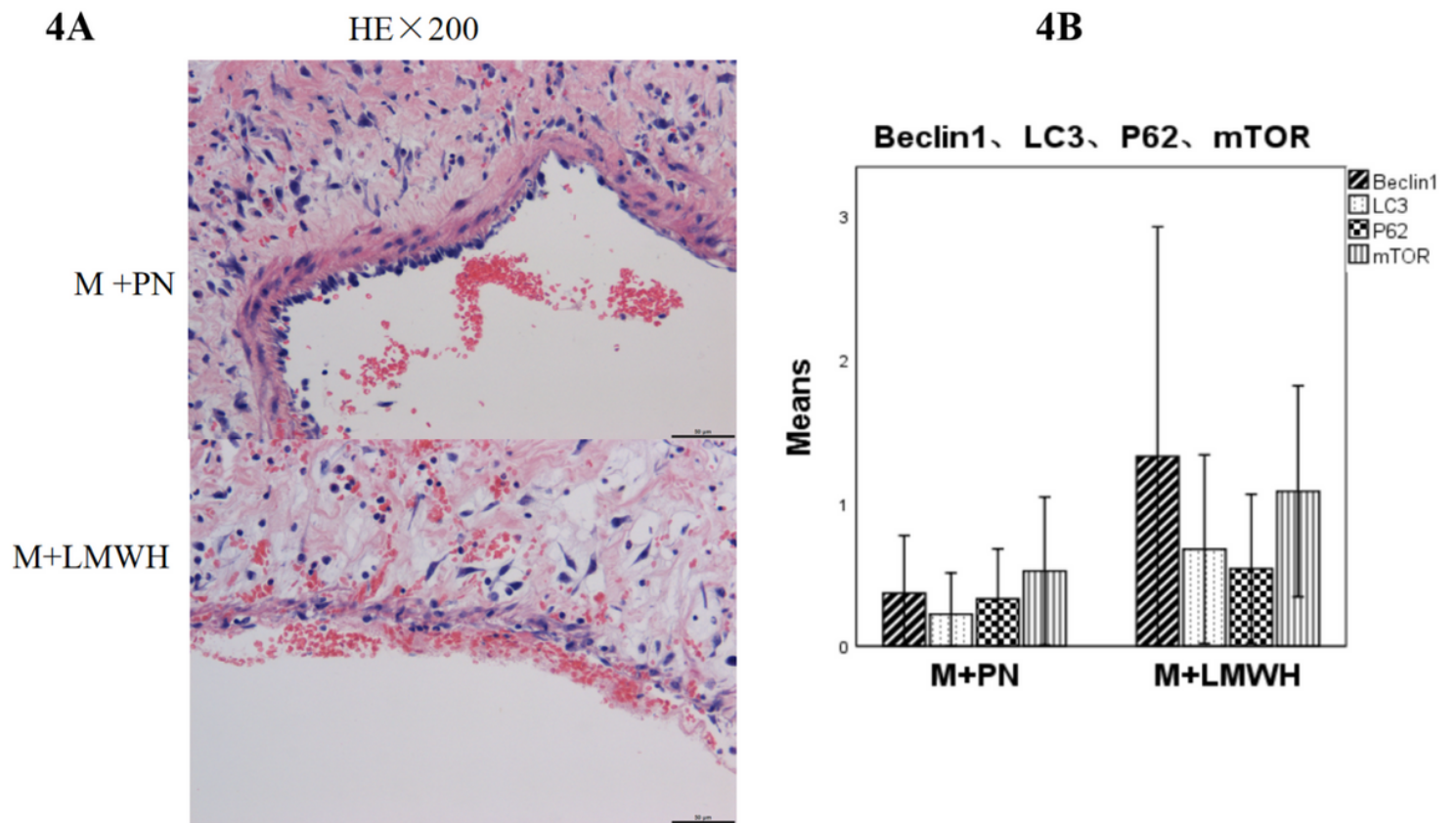


Figure 4

H&E staining of the M + PN group and the M + LMWH group (**Figure 4A**). The expression levels of Beclin1, P62, LC3, and mTOR in the M + PN group were lower than those in the M + LMWH group, but the difference was not significant ($P > 0.05$) (**Figure 4B**).