Inhibitory role of recombinant neorudin on canine coronary artery thrombosis

Running title:

[*Yu-Bin Liu 1, Xing-chen Zhou 1,Yun Liu 1, Lin Zhang 1, Ying Zhou 1 , Xiao Xu 1, Can Zheng 1, Zhuan-you Zhao 2, Chu-tse Wu 1*, Ji-De Jin 1**]

[*1 Beijing Institute of Radiation Medicine, Beijing 100850, China*

2 Center for Pharmacodynamic Research, Tianjin Institute of Pharmaceutical Reseach, Tianjin, China]

***Corresponding to:** [*Chu-tse Wu; Ji-De Jin*]

Email: *Chu-tse Wu, Email: wuct@bmi.ac.cn; Ji-De Jin, Email: jinjide505@163. com*

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What is already known

As a prodrug of hirudin, neorudin displayed antithrombin activity only after being cut by activated coagulant factor, resulting in lower bleeding, which was already proved with rat models of carotid arterial thrombosis and inferior vena cava thrombosis.

What this study adds

The antithrombosis action and lower bleeding feature of neorudin was confirmed using coronary artery thrombosis model in canine.

Clinical significance

As a new generation of anticoagulants, neorudin not only effectively inhibited various thrombosis, including coronary artery thrombosis, but also showed lower bleeding than low

molecular weight heparin, and may provide another selection for thrombus disease therapy in clinic.

ABSTRACT

The anticoagulant application is an effective treatment modality for cardiovascular diseases such as coronary heart disease, unstable angina pectoris, and myocardial infarction. In this study, the antithrombotic effect of recombinant neorudin (EPR-hirudin, EH) was evaluated using a canine model of coronary artery thrombosis. A canine model with platelet thrombosis in the left circumferent branch of the coronary artery was designed using Folt's method, and the anti-thrombus activity of EH was investigated. Femoral administration of EH intravenously had a significant dose-dependent inhibitory effect on canine coronary artery thrombosis and the effective rates were 66.7% ($P < 0.05$), 83.3% ($P < 0.05$), and 100% ($P < 0.01$) after injection of 0.3, 1.0, and 3.0 mg/kg EH, respectively. Furthermore, EH demonstrated lower bleeding, with shorter bleeding time and less bleeding loss than low molecular weight heparin (LMWH). Under the similar effect intensity of EH and LMWH (85 IU/kg), the bleeding time of the EH group at 30 min was shorter, and the blood loss at 30–120 min was less than that of LMWH ($P<0.05$ and $P<0.05$–0.001, respectively). EH had a significant dose-dependent inhibitory effect in the dose range of 0.3–3.0 mg/kg on the coronary artery thrombosis and lower bleeding side effects than LMWH with a similar antithrombosis effect.

Keywords: Animal models, Cardiovascular pharmacology, Thrombosis, Anticoagulants

1 INTRODUCTION

In recent years, the prevalence and mortality of cardiovascular diseases have risen in China, and the mortality rate of cardiovascular diseases is higher than that of cancer and other diseases, with an increase in death proportion in rural and urban residents to 46.66% and 43.81%, respectively (Hu, 2021). With the acceleration of economic development accompanying the increase of unhealthy lifestyle of residents, cardiovascular diseases have been becoming critical.

Anticoagulants are widely used in the clinic to prevent and treat various cardiovascular diseases since coronary heart disease, unstable angina pectoris, myocardial infarction, and other cardiovascular diseases are all directly related to the “hyper coagulant state” of the coronary artery (Germain et al., 2015). However, the greatest challenge of anticoagulant therapy is the high incidence of hemorrhagic events and severe bleeding, possibly threatening the life of patients (Esper et al., 2014; Sanfilippo et al., 2017). Currently, heparin and low molecular weight heparin (LMWH) are the primary anticoagulants widely used in clinics; simultaneously, their usage also often results in some adverse events such as hemolysis and anemia (Hirsh et al., 2001; Nunnelee, 1997). A direct inhibitor of thrombin, hirudin, can effectively inhibit both free and bound thrombin. It is a more beneficial antithrombotic drug than heparin in some animal models of deep vein injury (Li et al., 2020; Xu et al., 2013). However, the primary adverse effect of hirudin, the increased risk of systemic bleeding, also limited its use in the clinic (Greinacher & Warkentin, 2008; Zeymer & Neuhaus, 1995).

Therefore, to overcome the bleeding disadvantage of hirudin, a new-generation anticoagulant, recombinant neorudin (EPR-hirudin, EH) that is a targeted hirudin variant 2-

Lys47(HV2) fusion protein, was developed as a prodrug of HV2 by introducing EPR (Glu-Pro-Arg). This is recognized and cleaved using FXIa into the N-terminal of HV2 (Wu et al., 2012; Zhang et al., 2008). The antithrombotic effects of EH are exerted by releasing its active metabolite, HV2, at the thrombus site via FXIa-mediated cleavage of EPR; thus, resulting in direct inhibition of thrombin. Neorudin does not display any anticoagulant activity. When it is dissected by the corresponding coagulation factors into hirudin, neorudin demonstrates anticoagulant activity, occurring when the coagulation system is activated, and thrombus is present (Dong et al., 2020; Fareed et al., 1991). Considering the above special structure and action mechanism, EH could not only effectively inhibit thrombus formation but also reduce the risk of bleeding by increasing the specificity and efficiency of hirudin.

In this study, the anticoagulant activity of neorudin was investigated in the canine coronary artery thrombosis model, and the bleeding risks of the drug were evaluated and simultaneously compared with LMWH to provide experimental evidence for a further clinical study of the drug for the prevention and treatment of coronary artery thrombosis.

2 METHODS

2.1 Materials

EH (20 mg/bottle) was obtained from the Institute of Radiation Medicine. LMWH sodium injection (FLUXUM) manufactured by Alfa Wassermann was purchased from the Affiliated Hospital of the Academy of Military Medical Sciences. Thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) determination reagents were purchased from MDC/TECO MEDICAL. Beijing Shidi Scientific Instrument Co., Ltd

provided the fibrinogen (FG) content determination kit.

RM6300 Multichannel Physiological Recorder and MFV-3200 electromagnetic blood flowmeter were purchased from Nihon Kohden Corporation. MP100 multi-channel biological signal acquisition system was purchased from BIOPAC Systems Inc. PARBER Coagulation Factor Analyzer and 722 grating spectrophotometer was purchased from Beijing Shidi Scientific Instrument Co., Ltd and Shanghai Third Analytical Instrument Factory, China, respectively.

2.2 Animals

Fuyang Weiguang Experimental Animal Center, Anhui province of China provided the male and female beagles. Experiments conducted in the canine model were performed as per the regulations of the Animal Ethics Committee of Tianjin Institute of Pharmaceutical Research. The animal experiment in this study was approved by the Animal Ethics Committee of Tianjin Institute of Pharmaceutical Research (2018012501).

2.3 Canine model of recurrent coronary thrombosis

2.3.1 Surgical procedure

Beagles weighing approximately 4 kg were anesthetized using intravenous injection with 3% sodium pentobarbital (30 mg/kg) and were supplemented as needed to maintain anesthesia. Thereafter, endotracheal intubation and artificial respiration were provided to the beagles. Then the animal chest was open along with the fourth intercostal space on the left, exposing the heart, and the left circumflex branch of the coronary artery was separated by 2 to 3 cm. Following this, a suitable MFV-3200 electromagnetic flowmeter probe was placed in the vessel proximal end to measure the coronary flow, and a polyethylene tube filled with normal

saline was inserted into the separated femoral artery for monitoring the blood pressure using a pressure transducer. Moreover, needle electrodes were inserted into the beagle limbs to record the electrocardiogram of lead II using a bioelectrical amplifier. The femoral vein and the external jugular vein were separated for fluid infusion and medications, and blood collection, respectively. The above various analog signals were input to the MP100 system via the RM6300 multi-channel physiological recorder for real-time analog to digital conversion and data acquisition.

2.3.2 Model preparation

The model of coronary thrombosis was prepared 30 min after surgery according to the method earliest reported by Folts (Demrow et al., 1995; Folts, 1991; Toomey et al., 2002). Briefly, the coronary artery was first blocked using silk thread till the blood flow reduced to zero for 20 s and then was loosened. At this time, the blood flow was more than 2–3 times the basic flow, which was named reactive hyperemia. After the blood flow rate returned to the basic value, the coronary artery segment between the electromagnetic flowmeter probe and the silk thread used to block the coronary artery was clamped with hemostatic forceps two times, 5 s each time, to damage the vascular endothelium and expose the subintimal structure. The length of the injured coronary artery was approximately 6 mm. An adjustable 4 mm wide stenosis ring (inner diameter 2.4–3.2 mm) was attached to the middle of the injured coronary segment. The inner diameter of the ring was adjusted down so that the coronary artery reached critical stenosis and the reactive hyperemia disappeared. Under the circumstances, whatever the coronary artery was temporarily blocked and loosened, the coronary artery flow barely changed and was maintained close to the basic level. Soon after the damaged coronary arteries

reached critical stenosis, owing to endothelial damage and collagen exposure, the platelet was activated, and the platelet aggregation was formed with platelet adhesion, release, and aggregation reaction, resulting in the occurrence of acute platelet thrombosis and gradual decrease of coronary blood flow. After the decrease of blood flow reaching a certain level, the thrombus spontaneously broke off and spread out under the impact of blood flow, and then the blood flow quickly returned to the basic value. This process had been repeated, leading to cyclic flow reductions (CFRs), and an unstable angina model had been developed (Figure 1). When the flow rate dropped to zero over 1 min, and the thrombus still did not fall off, the stenosis ring should be gently shaken to make the thrombus fall off mechanically to prevent the animal from death due to severe myocardial ischemia.

2.3.3 Grouping and dosing schedule

After the model was successfully developed, the alteration of blood flow was observed and recorded continuously for 3 h, of which the value in the first hour was the baseline, and then observation was continued for another 2 h after drug administration. Thirty beagles were divided into five groups, with six dogs in each group. The three test groups were given EH at the doses of 0.3, 1.0, and 3.0 mg/kg, respectively. One-third of the drug was injected first, and the other two-third was then administered by constant speed infusion for 30 min with 30 mL of the total volume. The positive group was subcutaneously given LMWH at the dose of 85 IU/kg, and the negative control group was injected with the corresponding volume of normal saline.

2.3.4 Determination of the CFRs

A decrease in coronary flow below 30% of the base value was defined as a CFR, and the

frequency of CFRs was calculated per hour.

2.3.5 Efficiency determination of CFRs

Considering the 95% confidence limit of the CFRs number as the standard in the control group, the results in test groups were considered effective if the CFRs number was lower than the 95% confidence limit in the control group.

2.3.6 Determination of coagulation parameters

Approximately 2 mL of venous blood was collected before and after drug administration for 30, 60, and 120 min into tubes with 3.8% sodium citrate (the ratio of whole blood to anticoagulant was 9:1), and centrifuged at 4°C, 3000 rpm for 10 min. For determining TT, PT, and APTT with PARBER Coagulation Factor Analyzer and FG with 722 grating spectrophotometer, plasma was used.

2.3.7 Determination of peripheral bleeding time and wound bleeding volume

A transverse incision was placed on the inner mucosa of the beagle upper lip using a fixed deep wound cutter before and after drug administration for 30, 60, and 120 min. The blood at the incision site was absorbed gently using filter paper every 20 s. The bleeding time was recorded when the blood was no longer adherent to the filter paper. Before and after drug administration for 30, 60, and 120 min, the skin wound of 3 cm length was dissected along the muscle direction in the anterolateral abdomen of the dog. The oozed blood from the wound was absorbed using pre-weighed gauze, which was weighed 5 min later to calculate the blood loss amount.

2.4 Statistical analysis

The data were expressed as the mean± standard deviation, and significant differences between

groups were analyzed using an unpaired *t*-test ($p< 0.05$).

3 RESULTS

3.1 Efficacy of cyclic flow reductions

After intravenous administration of EH at 0.3 mg/kg, the frequency of CFRs decreased by 8.6 ±13.6%, 15 ±19.4%, 31.7 ±12.6%, 38.9 ±17.9% at 30, 60, 90, and 120 min after administration and the effective rates were 50%, 66.7%, 50%, and 66.7%, respectively. Furthermore, the number of CFRs ($P<0.05$) at 120 min was significantly different after administration between the test and negative control groups ($p<0.05$). The frequency of CFRs decreased by 11.2 ±11.7%, 24.2 ±11.4%, 44.8 ±17.3%, and 52.7 ±17.1% at 30, 60, 90, and 120 min after EH administration at 1.0 mg/kg and the effective rates were 50%, 66.7%, 50%, and 83.3%, respectively. Moreover, the number of CFRs of the treated group significantly decreased at 60, 90, 120 min after EH administration than that of the negative control group ($P < 0.05–0.01$). The frequency of CFRs decreased by 14.3 ±8.4%, 43.9 ±23.2%, 51.8 ±18.3%, and 60.4 ±12.3% at 30, 60, 90, and 120 min after EH treatment at 3.0 mg/kg administration and the effective rates were 66.7%, 100%, 83.3%, and 100%, respectively. The difference in the number of CFRs was different at 30, 60, 90, and 120 min after administration between the test and negative control groups ($P < 0.05–0.01$). Moreover, the inhibitive effect of EH on the frequency of CFRs at the dose of 3.0 mg/kg was similar to that of LMWH. The results were demonstrated in Table 1 and Figure 2.

3.2 Influence on anticoagulation parameters

The value of TT was slightly prolonged compared with control ($p<0.05–0.001$) at 30 and 60 min after administration of 3.0 mg/kg of EH and LMWH; however, there was no significant difference between the two groups (Figure 3). There was no significant alteration of PT, APTT, and FG after intravenous administration of EH.

3.3 Effects on bleeding in anesthetized dogs

The bleeding time and volume in the EH group 3.0 mg/kg were increased compared with the control group ($p<0.05–0.01$) at 60 min after intravenous administration. Moreover, the bleeding time and volume were significantly increased in the group treated with LMWH at 30–120 min after administration compared with the control group ($p<0.05–0.001$). However, EH 3.0 mg/kg showed a shorter bleeding time and a lower bleeding volume at 30 min after injection ($p<0.05$) and 30–120 min after injection ($p<0.05–0.001$), respectively compared with LMWH. The results are illustrated in Figure 4.

4 DISCUSSION

Acute coronary syndrome (ACS) owing to incomplete or complete occlusion resulting from coronary thrombosis superimposed on the atherosclerotic plaque rupture, resulting in clinical syndromes such as unstable angina (UA), non-ST elevation myocardial infarction (NSTEMI), and ST-elevation myocardial infarction (STEMI) (Farag et al., 2015; Gach et al., 2018). Currently, the treatments of ACS in the clinics are primarily implicated in percutaneous coronary intervention (PCI), antiplatelet drugs, and anticoagulants.

In general, revascularization by PCI to perform intra-coronary thrombolysis, thrombotic aspiration, and stent placement, is the primary selective therapy to a vascular complete

blockage such as refractory angina and STEMI. Moreover, anticoagulants in combination with antiplatelet drugs are applied to treat an incomplete vascular blockage such as UA and NSTEMI (Boscarelli et al., 2014). In this study, the beagle coronary artery thrombus model was developed to simulate the incomplete vascular occlusion and was utilized to determine the therapeutic efficacy of neorudin. The results demonstrated that neorudin in a dose-dependent manner inhibited the thrombosis formation and occurrence of CRFs induced by endothelium damage, which activated the intrinsic coagulation pathway (Schmaier, 2016; Wheeler & Gailani, 2016). At the beginning of the intrinsic coagulation cascade, coagulant factor XI was activated (Ponczek et al., 2020), which hydrolyzed neorudin to hirudin, a known thrombin inhibitor. However, tissue cutting wound activated the extrinsic coagulation pathway and coagulant factor XI was almost not activated in this process (He et al., 2021). Therefore, little neorudin was converted to hirudin and the increase of the bleeding time and bleeding volume of cutting wound was lower in high dose group of neorudin than LMWH group at the similar thrombus inhibiting action. The neorudin action feature, which attenuates the bleeding risk was already proved by rat models of carotid arterial thrombosis and inferior vena cava thrombosis (Wang et al., 2013) and was confirmed in the canine coronary artery thrombus model in this study. On the contrary, after administration, other anticoagulants such as LMWH will emerge in the coagulate factor inhibiting role whether the coagulation pathway is activated or not. Therefore, after treatment with LMWH, some patients often demonstrate side effects of bleeding (Hirsh et al., 2001; Schmaier, 2016; Wheeler & Gailani, 2016).

To summarize, the effective antithrombotic and low bleeding characteristics of neorudin were further confirmed in this study using a canine coronary artery thrombus model

simulating human acute myocardial ischemia. Moreover, neorudin will have a good therapeutic effect for thrombus diseases in the clinic and provide another choice for anticoagulant application in the future.

5 CONCLUSION

In the present study, neorudin pharmacodynamics was determined using a canine model of recurrent coronary thrombosis. The results demonstrated that neorudin in a dose-dependent manner inhibited thrombus formation and displayed less bleeding than LMWH.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

DECLARATION OF TRANSPARENCY AND SCIENTIFICRIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research

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Figure legends

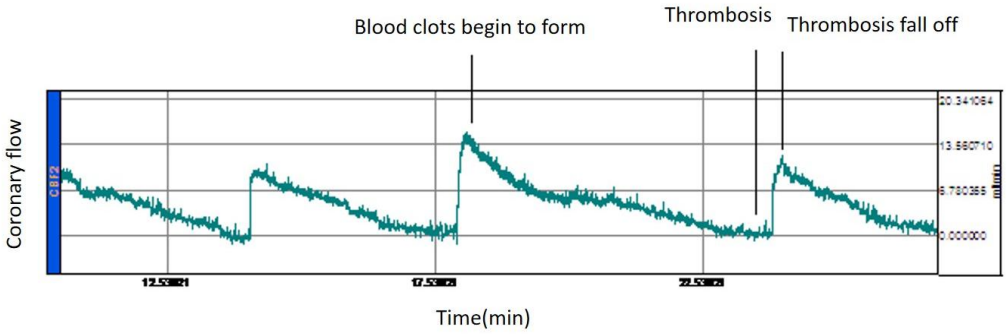

Figure 1 Legend of CFRs formation process

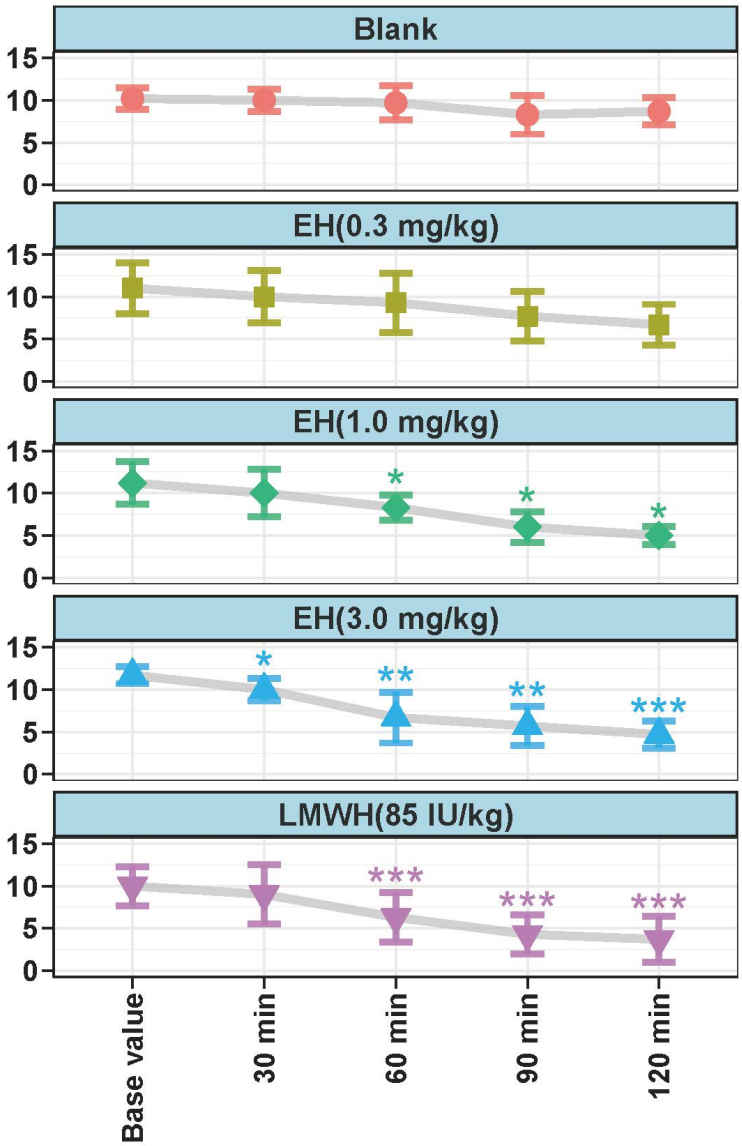

Figure 2 Effect of neorudin on the CFRs in anesthetized canine

Each data point represents the mean ± SD (n=6). *P<0.05, **P<0.01, in comparison with negative control.

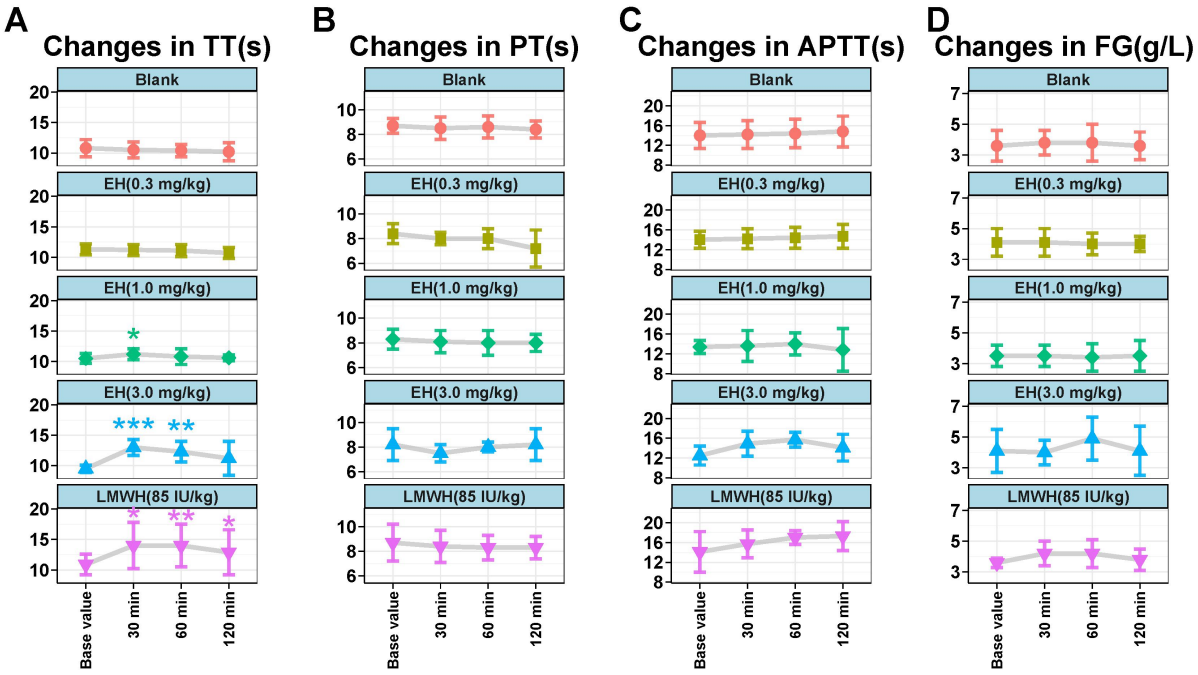

Figure 3 Effect of neorudin on TT, PT, APTT, FG in canine plasm

Each data point represents the mean ± SD (n=6). *P<0.05, **P<0.01, ***P < 0.001, in comparison with negative control; +P< 0.05, ++P<0.01, +++P<0.001, in comparison with LMWH.

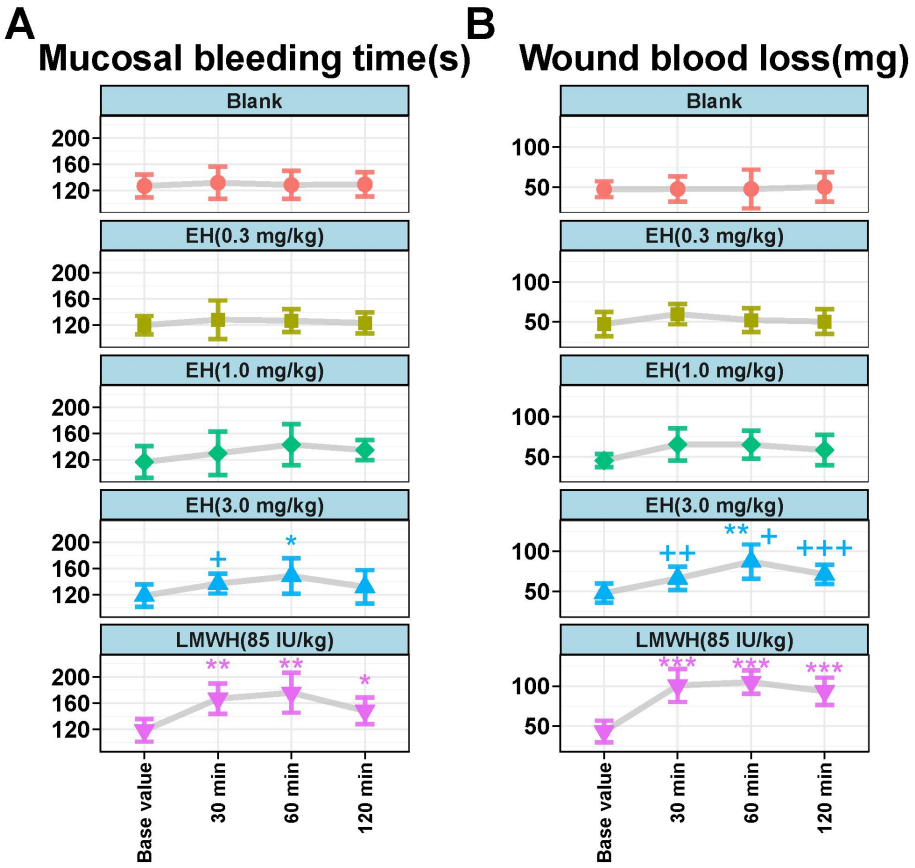

Figure 4 Effect of neorudin on bleeding time and blood loss in canine

Each data point represents the mean ± SD (n=6). *P<0.05, **P<0.01, ***P < 0.001, in comparison with negative control; +P< 0.05, ++P<0.01, +++P<0.001, in comparison with LMWH.

Table 1 The effective rate of neorudin on coronary thrombosis in anesthetized beagles

Group	Dose（mg/kg）	Number of active animals（n）				effective rate（%）			
		30	60	90	120（min）	30	60	90	120（min）
Negative control	-	0	0	0	0	0	0	0	0
EH	0.3（4.11×10^{-8} mol/kg）	3	4	3	4	50	66.7	50	66.7
EH	1.0（1.37×10^{-7} mol/kg）	3	4	3	5	50	66.7	50	83.3*
EH	3.0（4.11×10^{-7} mol/kg）	4	6	5	6	66.7	100**	83.3*	100**
LMWH	85 IU/kg	3	6	6	6	50	100**	100**	100**

*P<0.05, **P<0.01, in comparison with negative control.