

PEGylation of Lumbrokinase improves pharmacokinetic profile and enhances anti-thrombotic effect in a rat carotid artery thrombosis model

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Abstract. The present study aimed to prepare injectable Lumbrokinase (LK) with long circulation time in addition to enhanced anti-thrombotic efficacy. Following preparation, the pharmacokinetic and anti-thrombotic effect of the drug in a rat carotid artery thrombosis model was evaluated. The drug was prepared by conjugation of LK with mPEG-SC₂₀₀₀₀ as previously reported. The pharmacokinetics of the mPEG-SC₂₀₀₀₀-LK were then examined and the anti-thrombotic activity in an artery-vein bypass thrombosis rat model was evaluated. Finally, the parameters of fibrinolysis including thromboxane B₂, prostaglandin F_{1 α} , tissue plasminogen activator and plasminogen activator inhibitor-1 were compared between native LK and mPEG-SC₂₀₀₀₀-LK in a FeCl₃-induced carotid artery thrombosis rat model. mPEG-SC₂₀₀₀₀-LK was successfully prepared by PEGylation of LK with mPEG₂₀₀₀₀-SC in optimal conditions. Pharmacokinetic analysis demonstrated that the biological half-life of the mPEG₂₀₀₀₀-SC-LK increased by 2.2-fold compared with native LK. *In vivo* anti-thrombotic analysis indicated that mPEG₂₀₀₀₀-SC-LK exhibited a greater anti-thrombotic effect in artery-vein bypass thrombosis and FeCl₃-induced carotid artery thrombosis models compared with native LK. In conclusion, injectable PEGylated LK with prolonged half-life and enhanced anti-thrombotic effect is a potential anti-thrombotic agent for long-acting treatment of the thrombus diseases.

Introduction

Thrombotic disease is a common multiple disease that imperils people's life. The formation of thrombosis is a key event in the pathogenesis of myocardial infarction, stroke, and other ischemia conditions that comprise the most common and growing causes of morbidity and mortality (1). LK consists of a group of serine proteases with strong fibrinolytic activity (2,3), can dissolve fibrin either directly or indirectly by activating profibrinolysin. LK has been widely used as a novel, orally administered drug for thrombotic diseases and has caused growing interest in East Asia (4). Although some thrombolytic agents such as urokinase (uPA), streptokinase (SK) and tissue plasminogen activator (t-PA) are all typically used to dissolve clots, they are not specific to fibrin and have severe side effects such as heavy blood loss (5,6). In contrast, LK is very specific to fibrin as a substrate and does not cause excessive bleeding (7). Furthermore, some researches had shown that LK contained several active components (8,9) which could dissolve the fibrin directly or can convert plasminogen to plasmin by inducing endogenous t-PA activity to dissolve fibrin clots (10). However, The LK is an oral drug which belongs to biological products with very short half-life, it needs oral 2-3 times a day. And more importantly, given the fact that thrombotic disease is an acute illness, oral administration of the drug can not serve as first choice because of its low bioavailability and slow absorption in human body. In order to solve this problem, we have prepared it into intravenous agents by PEG modification which could improve its half-life, reduce dosing frequency. According to the results of the pharmacokinetics, the ideal result is that the drug can maintain for 12 to 24 h in the body after intravenous injection.

The attachment of proteins to polymers, especially to polyethylene glycol (PEG), is an established technique to enhance the therapeutic and biotechnological potential of numerous proteins, macromolecular carriers, oligonucleotides, and some biomolecules (11). PEGylation can increase the size and molecular weight of conjugated biomolecules and improve their pharmacokinetics. At the same time, it can protect the drug from enzymatic degradation, reduce renal clearance as well as enhance pharmacological activity *in vivo* (12-14).

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The covalent conjugation of PEG to therapeutic proteins is a clinically successful strategy for extending serum half-life to reduce administration frequency (15-17). Many biochemical agents such as tPA (18), SK (19), uPA (20), and staphylokinase (Sak) (21,22) have been modified with PEG as prodrugs for preparing injective formulation and for prolonging half-life. Currently, there are at least 10 PEGylated products with FDA marketing approval, and numerous other conjugates are entering clinical trials (23,24).

In our previous study, we investigated the optimal PEGylation condition with buffer pH of 8.0, reaction time of 5 h, reaction temperature of 0°C and molar ratio of 1:25. The primary amino group of LK and functional group of mPEG react under the buffer solution system, and finally generate PEGylated LK (Fig. 1). The *in vitro* fibrinolytic activity of both native and PEGylated LK was measured in comparison to that of uPA on fibrin plates. The results showed that mPEG₂₀₀₀₀-SC-LK was prepared successfully and had strong fibrinolytic activity *in vitro*. In this study, conjugation of LK with mPEG-SC₂₀₀₀₀ was prepared and analyzed as previously reported (25). Then, we studied the pharmacokinetics and *in vivo* anti-thrombosis efficacy of mPEG₂₀₀₀₀-SC-LK with rat carotid artery thrombosis model. This is the first systematic report to evaluate the anti-thrombotic effect of PEGylated injection form of LK in animal model.

Materials and methods

Materials and animals. LK (molecular weight of 24 KDa) was purchased from Shanghai Guoyuan Biotech Co., Ltd. mPEG-SC₂₀₀₀₀ was purchased from JenKem Technology (Beijing, China). An SDS-PAGE test kit was obtained from Beijing CellChip Biotechnology Co., Ltd., and SDS-PAGE protein standards were purchased from Bio-Rad (Hercules, CA, USA). Rat Lumbrokinase ELISA kit was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. Cyano-4-hydroxycinnamic acid (CHCA) and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, MO, USA). The standard substances used in this study were all purchased from National Institutes for Food and Drug Control. All other reagents and chemicals were of analytical grade and used as received. Male Sprague-Dawley (SD) rats were purchased from Beijing Vital River Laboratories (Beijing, China). All animal experiments were approved by the Laboratory Animal Ethics Committee at the Institute of Materia Medica at Peking Union Medical College. All procedures conformed with institutional guidelines and protocols for the care and use of laboratory animals with the Certification number of 2012/0001/SCXK for animal experiments.

Preparation of the PEGylated LK. For the synthesis of the mPEG₂₀₀₀₀-SC-LK (Fig. 1), the PEGylation was prepared as previously reported (25). Firstly, crude LK was purified using superdex 75 (1.6x25 cm, GE Healthcare) and Hi Trap DEAE FF column (GE Healthcare, Chicago, IL, USA) by Akta Purify 10 system. The purified LK and mPEG-SC₂₀₀₀₀ were then mixed at molar ratio of 1:25, added 50 mM PBS (pH 8.0) at 0°C, reacted for 5 h. Finally, the reaction was terminated by adding 11 mg/ml glycine. The reaction mixture was subjected to superdex 75 column for removal of the free PEG reagent.

Starting materials and PEGylated products were examined by HPLC to calculate the extent of PEGylation according to the following equation:

Extent of PEGylation (%) = (1 - residual peak area of LK after modification/peak area of LK before modification) x100%.

Analytical methods. Molecular weight of mPEG₂₀₀₀₀-SC-LK was measured using a MALDI-TOF-MS 4800 Plus (ABI; Thermo Fisher Scientific, Waltham, MA, USA) (14,26). Sample and matrix solution (CHCA in 70% of acetonitrile/methanol with 1% TFA) was mixed together, spotted onto a well of the sample plate and dried. The positive ion TOF detection was performed by using an accelerating voltage of 25 kV. SDS-PAGE was carried out as described by Park (26), the protein concentration was determined by Bradford assay (Bio-Rad). Native LK, purified LK and mPEG₂₀₀₀₀-SC-LK were analyzed by SDS-PAGE using 10% polyacrylamide gradient gel with standard proteins as markers. Gels were stained with coomassie blue, and then analyzed using a gel imaging system (BIO-BEST 135A; SIM International Group Co., Ltd., Los Angeles, CA, USA). The *in vitro* fibrinolytic activity of both purified LK and mPEG₂₀₀₀₀-SC-LK were measured using the method of Asturp and Mullertz as we reported previously (27).

Pharmacokinetic analysis. Male Sprague-Dawley rats (180-220 g) were divided into two groups, with each group injected intravenously with LK and mPEG₂₀₀₀₀-SC-LK (LK base dose of 25 mg/kg of body weight), respectively. Blood samples (200-300 µl) at different time were collected and centrifuged prior to analysis. For the determination of LK content, serum samples were analyzed using LK ELISA assay kit (Mlbio, Shanghai, China) following directions. The results were demonstrated as mean values ± SD. Pharmacokinetic parameters, including half-life ($t_{1/2}$), plasma peak concentration (C_{max}), peak retention time (T_{max}) and area under the curve (AUC) were calculated using a Pk solver 2.0 software.

In-vivo evaluation in rats

Anti-thrombotic effect with artery-vein bypass thrombosis model in rats. Anti-thrombotic activity of mPEG₂₀₀₀₀-SC-LK was studied in a rat model of artery-vein bypass thrombosis (28,29). Male Sprague-Dawley rats (230-280 g) were divided into three groups including negative control group, LK group and mPEG₂₀₀₀₀-SC-LK group. The rats were anesthetized with an intravenous injection of pentobarbital sodium (30 mg/kg). A skin incision was made in the neck, and a segment of the left carotid artery was separated and clamped with an artery clamp. Then a catheter with a 2.0 cm long segment of silk was inserted into the left carotid artery from the proximal end, allowed for running along the vessels for 1.0 cm, and then penetrated out from the distal end. Another end of carotid artery was tied to snip the thread, and the artery clamp was slowly loosened to restore the blood flow. Five min after the thrombus model were established, the LK and mPEG₂₀₀₀₀-SC-LK were injected intravenously via sublingual vein with the LK base dose of 25 mg/kg through 25G^{5/8} needles. Rats in negative control group were given an equal volume of normal saline. There were no side effects

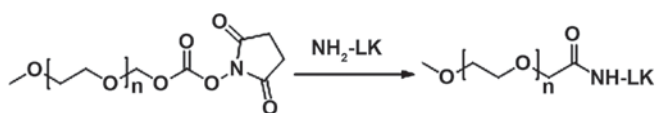


Figure 1. PEGylated modification of LK by mPEG-SC. LK, Lumbrokinase.

such as skin wound or bleeding happened after the injection. Five hours after drug administration, the thrombus formed on silk suture was isolated and weighted. The inhibition ratio of thrombosis was calculated as follow equation. Inhibition ratio = $(\text{Weight}_{\text{control}} - \text{weight}_{\text{drug}}) / \text{weight}_{\text{control}} \times 100\%$.

Anti-thrombotic effect with FeCl_3 -induced carotid artery thrombosis model in rats. FeCl_3 -induced arterial thrombus formation *in vivo* was investigated as reported (30,31), and the operating procedure was as described above. A 2x5 mm strip of filter paper saturated with 20% FeCl_3 solution was applied to the surface of the surgically exposed carotid artery for 10 min before being removed. The LK and mPEG₂₀₀₀₀-SC-LK were then injected intravenously via sublingual vein with the LK base dose of 25 mg/kg through 25G^{5/8} needles just before and 15 min after removing the filter paper, respectively. Rats in negative control group were given equal volume of normal saline. Blood samples were taken from animals 3 h after the last drug injection, and centrifuged at 3,000 g for 15 min at 4°C. The plasma TXB₂ and PGF_{1 α} concentrations were determined by ELISA, using the reagent set TXB₂ express kit (Biosamite, Shanghai, China) and PGF_{1 α} express kit (Biosamite). The plasma concentrations of t-PA were determined using a RAT tPA ELISA kit (Biosamite). The RAT PAI-1 ELISA kit (Biosamite) was used for detection of PAI-1 in plasma. All of the parameters were determined according to the manufacture's instructions.

Statistical analysis. Results were demonstrated as mean \pm SD. Statistical comparisons were performed to determine group differences through analysis of variance. Significant difference between two groups was evaluated by Student's t-test, and $p < 0.05$ is considered significant.

Results

Molecular weight determination of mPEG₂₀₀₀₀-SC-LK. The molecular weight of the purified LK and mPEG₂₀₀₀₀-SC-LK were determined to be 24045.6 Da (Fig. 2A, peak 1) and 44102.5 Da (Fig. 2B, peak 3) by MALDI-TOF mass spectrometry. The difference of molecular mass between LK and mPEG₂₀₀₀₀-SC-LK was ~ 20 kDa, indicating that one molecular of PEG was linked to one molecular of LK.

Analysis by SDS-PAGE demonstrated a band at the molecular weight of 35-50 kDa for mPEG₂₀₀₀₀-SC-LK (Fig. 3, line 1), while that of LK (Fig. 3, line 2) was at around 24 kDa. This result was in good agreement with the result of MALDI-TOF.

Pharmacokinetics. Profiles of plasma concentration vs. time for LK and mPEG₂₀₀₀₀-SC-LK were shown in Fig. 4. As shown in Fig. 4 and Table I, LK was rapidly cleared from the plasma after $t_{1/2}$ of 1.32 h. Compared to LK, mPEG₂₀₀₀₀-SC-LK showed

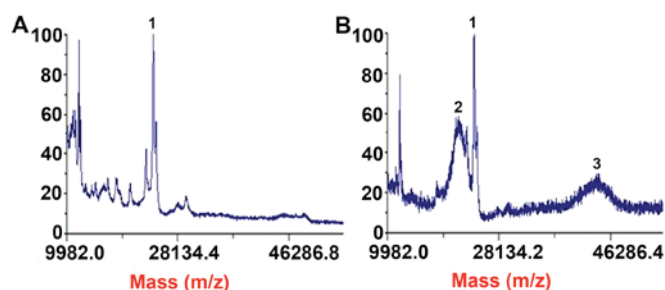


Figure 2. MALDI-TOF spectra of the purified LK (A) and mPEG₂₀₀₀₀-SC-LK. (B) In the figure, 1 was the peak of purified LK; 2 was the peak of residual mPEG₂₀₀₀₀-SC, 3 was the peak of mPEG₂₀₀₀₀-SC-LK. LK, Lumbrokinase.

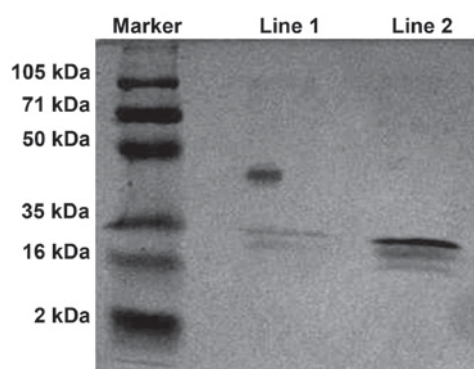


Figure 3. SDS-PAGE (10%, w/v) gel of Coomassie staining. Line 1, mPEG₂₀₀₀₀-SC-LK; line 2, purified LK. LK, Lumbrokinase.

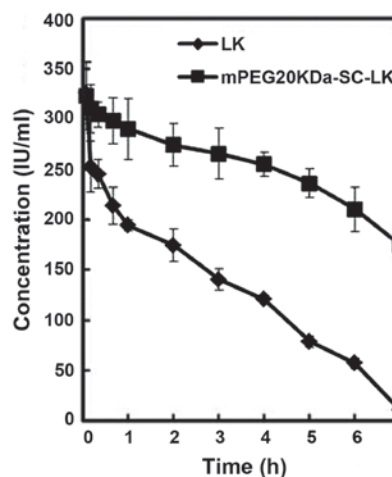


Figure 4. Mean plasma concentration-time curves of LK and mPEG₂₀₀₀₀-SC-LK. Blood clearance of the samples in rats following a single subcutaneous injection. The measurement was carried out using ELISA and each sample was measured for three times. LK, Lumbrokinase.

2.2-fold increase in $t_{1/2}$. The mPEG₂₀₀₀₀-SC-LK also displayed 3.7-fold decrease in CL. This revealed markedly prolonged serum presence of LK by PEGylation. Thus the results clearly indicated the potential of PEGylation to improve the pharmacokinetics of protein-based medicines.

In-vivo evaluation

Anti-thrombotic effect with artery-vein bypass thrombosis model in rats. In the model group, 1 h after embedding the

Table I. Pharmacokinetic parameters of LK and mPEG₂₀₀₀₀-SC-LK (n=6, mean ± SD).

Parameters	LK	mPEG ₂₀₀₀₀ -SC-LK
MRT (h)	1.90±0.75	4.19±0.87
T _{1/2} (h)	1.32±0.53	2.90±0.39
AUC (IU.h/ml)	607.86±101.23	2,246.28±586.28
CL (ml/h/kg)	1.36±0.78	0.37±0.11
V _{ss} (ml/kg)	2.58±0.69	1.54±0.86

MRT, mean residence time; T_{1/2}, half-life; AUC, area under drug concentration vs. time curve; CL, clearance over bioavailability; V_{ss}, distribution volume at steady state.

Table II. Effects of LK and mPEG₂₀₀₀₀-SC-LK on artery-vein bypass thrombosis (n=6, mean ± SD).

Group	Thrombus mass (mg)	Inhibition ratio (%)
Negative control	16.34±1.25	-
LK	5.47±1.81 ^a	13.72
mPEG ₂₀₀₀₀ -SC-LK	3.35±1.53 ^{a,b}	47.16 ^b

^aP<0.05 compared with negative control; ^bP<0.05 compared with LK group. LK, Lumbrokinase.

silk, the carotid artery became dark red, thrombosis formed, and the size of it was gradually increased. Injection of LK and mPEG₂₀₀₀₀-SC-LK inhibited the thrombus formation, with mPEG₂₀₀₀₀-SC-LK more effective than LK (Table II). In the mPEG₂₀₀₀₀-SC-LK group, 47.16% inhibition of thrombosis was observed compared to 13.72% in LK group, indicating that mPEG₂₀₀₀₀-SC-LK had stronger anti-thrombotic effect than native LK.

Anti-thrombotic effect in FeCl₃-induced carotid artery thrombosis model in rats. Completely occlusive carotid artery thrombosis was well established in rats, as presented in Fig. 5 After the injection of LK and mPEG₂₀₀₀₀-SC-LK, TXB₂ concentrations in both groups decreased compared with model group (P<0.05), and the TXB₂ content in mPEG₂₀₀₀₀-SC-LK group decreased more than that in LK group (P<0.05) (Fig. 6). The content of PGF_{1α} was higher in LK and mPEG₂₀₀₀₀-SC-LK groups when compared with the model group (P<0.05), and the PGF_{1α} content in mPEG₂₀₀₀₀-SC-LK group was higher than that in LK group (P<0.05) (Fig. 6). In the model group, ratio of t-PA and t-PA/PAI-1 activity was lower than the normal group, while the value of PAI-1 was increased in model group when compared with the normal group (Fig. 7). After administration of LK and mPEG₂₀₀₀₀-SC-LK, the activity of t-PA and t-PA/PAI-1 ratio were higher than the model group (P<0.05), while the activity of PAI-1 had no change. When compared with LK group, the activity of t-PA and t-PA/PAI-1 ratio was higher in mPEG₂₀₀₀₀-SC-LK group (P<0.05).

Discussion

Present study focused on development and evaluation of a long-acting anti-thrombosis agent through PEGylation of LK. PEGylated LK was successfully modified with mPEG₂₀₀₀₀-SC in optimal condition as we reported before (25). As the effective molecular weight of crude LK was around 24 kDa, the theoretical molecular weight of PEGylated LK was around 44 kDa. Both the SDS-PAGE and MALDI-TOF results were consistent with our expectation, suggesting that LK was successfully modified with mPEG₂₀₀₀₀-SC. The *in vitro* fibrinolytic activity of the PEGylated products was lower than that of purified LK although the values were not significantly different. Despite the trend for PEGylation to decrease *in vitro* fibrinolytic activity, this may not influence *in vivo* activity because of the longer circulation half-life of PEG modified LK.

Multiple approaches have been devised to extend the half-life and increase activity of anti-thrombotic agents in the bloodstream, such as polymer coating and the use of drug carriers. The most commonly used approach to prolong the half-life of anti-thrombotic agents involves conjugation of drugs with PEG. PEG chains form a hydrated shell that enhances drug solubility and limits renal filtration, proteolytic degradation, and immunogenicity (1). In practice, it has been reported that PEG₂₀₀₀₀-IFN and PEG₄₀₀₀₀-IFN significantly prolonged serum half-life and lowered the renal clearance rate (14,23). In our study, the serum activity of mPEG₂₀₀₀₀-SC-LK showed 2.2-fold increase in t_{1/2} than the native LK, and the mPEG₂₀₀₀₀-SC-LK displayed 3.7-fold decrease in CL. The reduction ranges of mPEG₂₀₀₀₀-SC-LK were much smaller than those of native LK. Seven h after the injection of native LK, the serum LK concentration was decreased below 10 IU/ml, while the serum mPEG₂₀₀₀₀-SC-LK concentration was remained above 170 IU/ml, indicating that mPEG₂₀₀₀₀-SC-LK had long-circulating effects and prolonged biological half-life and residence time *in vivo*. PEGylation increased the size and molecular weight of LK and improved its pharmacokinetics by protecting from enzymatic degradation, prolonging circulation time and reducing renal clearance. PEG polymer linked to LK, may creates a bulky hydrophilic shield which could efficiently mask the conjugated LK from enzymatic digestion.

LK extracted from the earthworm has been used to treat stroke and cardiovascular diseases (32,33). Recent studies (34) have shown that the fibrinolytic enzymes could dissolve blood fibrin clots and inhibit activation and aggregation of platelets. However, most studies mainly focused on the oral preparation of these enzymes. This is the first report to systematically evaluate the ant-thrombotic effect of PEGylated injection form of LK in animal model. Artery-vein bypass thrombosis animal model was widely used in evaluating properties of anti-thrombotic preparations. In our experiment, mPEG₂₀₀₀₀-SC-LK showed nearly 3.5-fold increase in the inhibition ratio of thrombosis effect compared with the native one in this rat model.

FeCl₃-induced carotid artery thrombosis model is another typical model to evaluate an anti-thrombosis agent. To investigate changes of TXB₂, PGF_{1α}, t-PA and t-PA/PAI-1 ratio, thrombi were induced by topical ferric chloride in our experiment. TXB₂ as the end product of the metabolism of

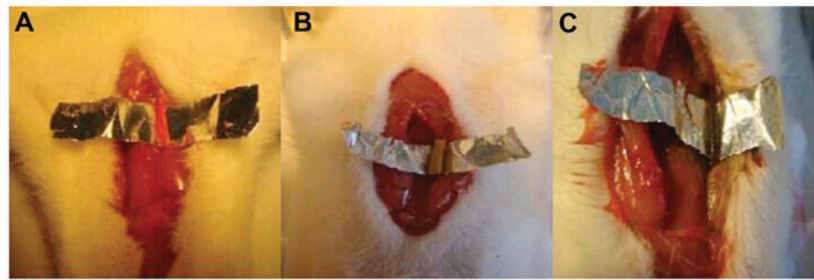


Figure 5. Induction of vascular injury in rat carotid artery. (A) Isolated the left carotid artery; (B) one piece of 2x5 mm filter paper saturated with 20% FeCl_3 was applied to surface of surgically exposed left carotid artery for 10 min. (C) Formation of thrombus.

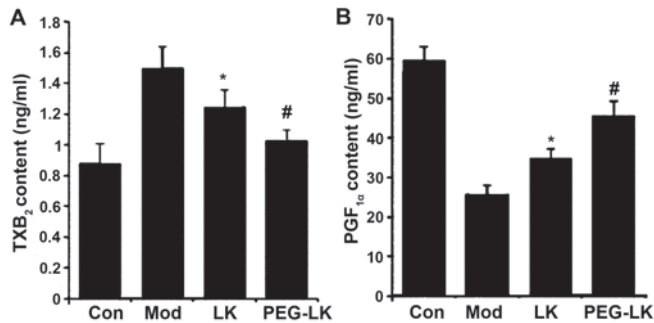


Figure 6. Comparison of content of TXB₂ (A) and PGF_{1α} (B) in rat plasma in different groups. Each value represents the mean \pm SD for three determinations. * $P < 0.05$, compared with model group; # $P < 0.05$, compared with LK group. LK, Lumbrokinase.

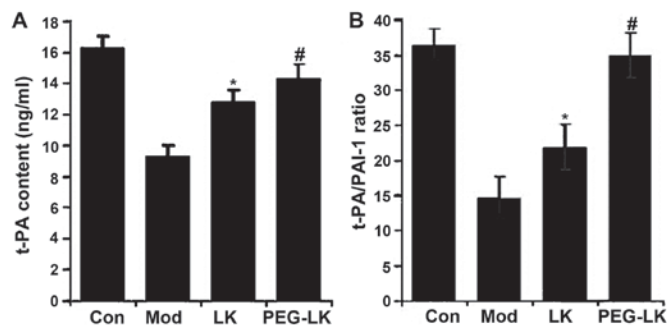


Figure 7. Comparison of content of t-PA (A) and t-PA/PAI-1 ratio (B) in rat plasma in different groups. Each value represents the mean \pm SD for three determinations. * $P < 0.05$, compared with model group; # $P < 0.05$, compared with LK group. t-PA, tissue plasminogen activator; LK, Lumbrokinase.

XA_2 (35,36), has an important role in the promotion of platelet aggregation and contraction of blood vessels. It has been reported that patients with atherosclerosis showed high plasma TXB₂ values and low PGF_{1α} content. After taking 2 weeks of LK, the plasma level of TXB₂ significantly decreased and PGF_{1α} level significantly increased in patients, suggesting that LK has a strong inhibitory effect on platelet aggregation. After the injection of LK and mPEG₂₀₀₀₀-SC-LK, we saw that the TXB₂ concentrations decreased compared to the model group, and the TXB₂ content in mPEG₂₀₀₀₀-SC-LK group decreased more than that of LK group. The content of PGF_{1α} was higher in both LK and mPEG₂₀₀₀₀-SC-LK groups when compared with the model group, and the PGF_{1α} content in mPEG₂₀₀₀₀-SC-LK group was increased greater than that in LK group.

Endogenously mediated fibrinolysis is initiated by t-PA and u-PA, the enzymes convert plasminogen to the fibrinolytic protease plasmin (37). T-PA and PAI are a pair of biological regulatory factors which are of great significance to the function of fibrinolytic system. T-PA can specifically combine with fibrin in plasma, possessing selective thrombolysis effect. PAI as a rapid inhibitor can inactivate t-PA by combining with it. It plays a decisive role in fibrinolytic system by inhibiting the role of anti-thrombosis effect. According to the literature (38), the t-PA/PAI ratio was often used as an index of anti-thrombosis. This study showed that in FeCl_3 -induced thrombosis rat model, the content of TXB₂ was increased, whereas PGF_{1α} content, t-PA content and t-PA/PAI ratio were all decreased in the model group. This is because as endothelial damage was occurred due to vascular endothelium, the platelet and coagulation system was activated and the anti-coagulant protein in the blood was consumed, leading to decreased anti-coagulant activity. Meanwhile, because of the formation of thrombus, the t-PA in the blood combined to the thrombus location decreased the activity of t-PA. As an anti-thrombosis agent, LK can directly dissolve fibrin or indirectly dissolve fibrin by activating profibrinolysin. Thus, LK can eventually reduce thrombotic diseases in patients with PAI and activate t-PA, resulting in increased fibrinolysis. After administration of the drugs, t-PA and t-PA/PAI ratio was increased, and the rising range of mPEG₂₀₀₀₀-SC-LK was greater than the native LK group. The lower *in vitro* potencies of the PEGylated LK relative to those of the native LK may be due to steric shielding of the protein by the PEG polymer with its comb-like structure (39). But when it came to *in vivo* situation, PEGylated LK presented better anti-thrombosis efficacy than the native one. Because PEGylation is able to enhance drug efficacy by reducing its clearance either through glomerular filtration or through proteolytic degradation.

Vascular thrombotic disease is a serious threat to human health which is the main cause of human disability and death. According to the results of our research, it demonstrated that mPEG₂₀₀₀₀-SC-LK exhibited greater anti-thrombosis effect in both artery-vein bypass thrombosis and FeCl_3 -induced carotid artery thrombosis models than native LK. It could effectively improve the clinical symptoms of patients, reduce morbidity and mortality, and it is an ideal drug to treat vascular thrombotic disease in clinical, such as myocardial infarction combined high blood fibrinogen, high stick sluggish blood disease, retinal vein occlusion, cerebrovascular disease,

infarction, and so on. Therefore, it is a potential anti-thrombotic agent for long-acting treatment of the thrombus diseases.

Present study showed that mPEG modified LK have prolonged half-life and strong fibrinolytic and thrombolytic activities *in vivo*. These results indicate that the PEGylated LK is a novel anti-thrombosis agent for the treatment of thrombosis diseases in clinic. These results can provide the foundation of the research of injective LK in the future study.

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