

Effect of recombinant human prourokinase on thrombolysis in a rabbit model of thromboembolic stroke

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Abstract. The aim of the present study was to investigate the efficacy of recombinant human prourokinase (rhPro-UK) on thromboembolic stroke in rabbits. A total of 210 rabbits were used in experiments. The 180 thromboembolic stroke rabbits were divided into three therapeutic time windows with six groups in each time window (n=10). The model group was administered saline, the reagent groups were administered rhPro-UK (2.5x, 5x and 10x10⁴ U/kg), and the positive control groups were administered 5x10⁴ urokinase (UK) U/kg and 4.5 mg/kg recombinant human tissue plasminogen activator via intravenous infusion at 3, 4.5 and 6 h after embolism. The remaining 30 rats (that had not undergone occlusion by autologous blood clots) served as a sham group and were administered saline. The radioactive intensity was detected using a medical gamma counter before and after the administration of the drug for 15, 30, 45, 60, 75, 90, 105 and 120 min. At 24 h after treatment, the brain samples were coronally sliced into 5 mm sections and hemorrhage was estimated used a semiquantitative method by counting the number of section faces with hemorrhaging. The plasma was collected for prothrombin time, activated partial thromboplastin time, fibrinogen and thrombin time tests using a solidification method with a blood coagulation factor analyzer. In addition, α_2 -antiplasmin (α_2 -AP) was evaluated using ELISA methods using a RT-6100 microplate reader. At the 3 h time point, the thrombolysis rate of rhPro-UK (2.5x, 5x and 10x10⁴ U/kg)

was 21.5% (P<0.05), 36.8% (P<0.001) and 55.0% (P<0.001), respectively together with patency rates of 10% (P>0.05), 40% (P<0.05) and 70% (P<0.001). Furthermore, α_2 -AP levels were reduced by 5.3% (P>0.05), 5.3% (P>0.05) and 18.1% (P<0.05). At the 4.5 h time point, the thrombolysis rate was 18.8% (P<0.05), 29.9% (P<0.01) and 49.0% (P<0.001) together with patency rates of 10% (P>0.05), 30% (P<0.05) and 50% (P<0.01), and α_2 -AP levels were reduced by 2.4% (P>0.05), 6.5% (P>0.05) and 17.8% (P<0.05). At the 6 h time point, the thrombolysis rate was 14.7% (P<0.05), 24.1% (P<0.01) and 35.7% (P<0.001) together with patency rates of 20% (P>0.05), 30% (P<0.05) and 40% (P<0.01), and α_2 -AP levels were reduced by 5.7% (P>0.05), 12.7% (P>0.05) and 22.2% (P<0.01). No significant differences (P>0.05) were identified between rhPro-UK (2.5x, 5x and 10x10⁴ U/kg) and the model group regarding hemorrhage type, size and blood coagulation factors at the different time points. Thus, rhPro-UK promoted thrombolysis and recanalization (patency rate), with reduced risk of cerebral hemorrhage, and thus exerted protective effects on cerebral ischemia rabbits.

Introduction

Urokinase (UK), also termed UK-type plasminogen activator (uPA), is a type of serine protease present in humans and other animals, used clinically as a thrombolytic agent in the treatment of severe or massive deep venous thrombosis (DVT), pulmonary embolism, myocardial infarction, and occluded intravenous (IV) or dialysis cannulas. However, UK is not particularly selective for clot-bound plasminogen (it binds almost equally to freely circulating plasminogen and clot-bound plasminogen), and causes significant fibrinogenolysis and clot fibrinolysis. To the best of our knowledge, prourokinase (Pro-UK; also termed single-chain UK-type PA, single-chain pro-UK, scu-PA, pro-UK, pro u-PA and PUK) has only been evaluated in stroke by a single study (1). Pro-UK is a zymogen with little fibrin affinity, but has an equivalent fibrin specificity to tissue PA (tPA) (2). Intra-arterial local rpro-UK infusion has previously been associated with superior recanalization in acute thrombotic/thromboembolic stroke when compared with a placebo (3). The safety and efficacy of the thrombolytic agent, pro-UK, in the treatment of DVT of the lower limbs have been investigated in an open, uncontrolled, pilot study (4). The results

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of this pilot study indicated that pro-UK was thrombolytic in DVT and that it may be administered simultaneously with a conventional heparin treatment. Recombinant human (rh) Pro-UK, is a novel type of thrombolytic, which preferentially activates plasminogen on the fibrin surface and induces fibrin-selective clot lysis. It has the advantages of more potent efficacy and less adverse reactions in comparison with other thrombolytics (5). Advantages of thrombolytic therapy using rhPro-UK for patients with acute myocardial infarction include its reliable curative effect and high safety (6). The aim of the present study was to investigate the effects of rhPro-UK in rabbit models of thromboembolic stroke at 3, 4.5 and 6 h therapeutic time windows, particularly regarding its effects on thrombolysis rate, patency rate (recanalization) and intracerebral hemorrhage.

Materials and methods

Animals. Adult male and female rabbits, weighing 2.0-3.0 kg [SCXK(JING)2014-0003] were obtained from Longan Experimental Animal Breeding Center(Beijing, China). The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Tianjin Institute of Pharmaceutical Research (Tianjin, China).

Drugs, reagents and devices. rhPro-UK (batch no. 201400401) was obtained from Shanghai Tasly Pharmaceutical Co., Ltd. (Shanghai, China). Recombinant human tissue plasminogen activator (rt-PA; batch no. 403437) was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). UK (batch no. 041603023) was obtained from Biochemical Pharmaceutical Co., Ltd. (Tianjin, China).

The rabbit α_2 -AP ELISA kit (batch no. 201606) was provided by Bio-Swamp Co., Ltd. (Wuhan, China), and assay kits for activated partial thromboplastin time (APTT; batch no. 021603A), prothrombin time (PT; batch no. 011504A), thrombin time (TT; batch no. 031601D) and fibrinogen (FIB; batch no. 041603A) were obtained from MD Pacific (Tianjin, China) Biotechnology Co., Ltd. (Tianjin, China), technetium (^{99}Tcm) sodium was obtained from Atomic High Tech Isotope Pharmaceutical Co., Ltd. (Tianjin, China).

A JC1000-PC Medical Gamma Counter was obtained from Kaipu Electromechanical Co., Ltd.(Xi'an, China), RT-6100 microplate reader was purchased from Rayto Life Science Co., Ltd., (Guangzhou, China) and a PARBER blood coagulation factor analyzer was obtained from Beijing SHIDI Scientific Instrument Company (Beijing, China).

Establishing the experimental embolism model

Embolus preparation. Embolism was established as previously described (7,8) with some modification. Briefly, labeled mixture was obtained from 0.5 ml eluted radioactive sodium (radioactive intensity, 92.5 MBq/ml) with 30 μl stannous chloride (5 mg/ml). Of this, 20 μl was added into 1 ml rabbit anticoagulant blood and incubated for 30 min at 37°C. Mixture (50 μl) with an equal volume of CaCl_2 (0.5 M) and bovine thrombin (50 IU/ml) were added into the rabbit autologous blood and a PE90 pipe (inner diameter, 0.86 mm; outer diameter, 1.27 mm) was used to collect a clot sample;

the clot was solidified at 37°C for 2 h and sliced to 10 mm. The radioactivity of the thrombus was evaluated using a JC1000-PC Medical Gamma Counter following three washes with normal saline (5 min/wash).

Establishing the embolism model. The rabbits were anesthetized using 20% urethane (1 g/kg) and fixed on the operating table. The cervical midline skin was incised and the right carotid artery, internal carotid artery (ICA) and external carotid artery (ECA) were separated. Following ligation and transection of the ECA, the modified PE90 (reducing the optical density to 0.4 mm at one end) with blood clot samples was injected with a 2 ml syringe via the ECA into the ICA. The radioactive intensity of the forelimb cortex (1.0 mm posterior and 5.5 mm lateral to the bregma) was detected using a JC1000-PC Medical Gamma Counter prior to and following embolus injection (9-11). When the radiation intensity was >2 times greater than the background signal, the model preparation for thromboembolism was considered successful (8). All rabbits were resuscitated following completion of the thrombolysis assay.

Grouping. The animals with thromboembolic stroke were randomly divided into 6 groups as follows: Model group (saline solution), the rhPro-UK (2.5x, 5x and 10×10^4 U/kg) groups and the positive groups (5×10^4 U/kg UK and 4.5 mg/kg rt-PA). In addition, the rabbits in the sham group without occlusion by autologous blood clots were administered saline solution. A total of 10 rabbits in each group were treated at 3, 4.5 and 6 h after occlusion via IV infusion.

Thrombolysis assay. The radioactive intensity was detected using a medical gamma counter before and after drug administration for 15, 30, 45, 60, 75, 90, 105 and 120 min. A thrombolysis rate >50% was considered as the patency rate (12) and calculated as follows: Thrombolysis rate (%) = $[(n_0 \times k - nt)/(n_0 \times k)] \times 100\%$ where $k = e^{-0.6931 \times (t/6.02)}$, n_0 is the radioactive intensity before administration, n_t is the radioactive intensity after different drug administration times and t is the time after administration.

Intracerebral hemorrhage assay. The animals were sacrificed 24 h after treatment under anesthesia with 20% urethane (1 g/kg). Brains were removed subsequent to perfusion and coronally sliced into 5 mm sections. The hemorrhage was estimated used a semi-quantitative method by counting the number of sections where hemorrhage was present (13-16). Each brain slice has two 'faces' and the score counting criteria were 1 for a hemorrhage on 1 'face' and 2 for a hemorrhage on 2 'faces', then the total bleeding score was calculated. Three types of hemorrhage were identified as follows: i) Hemorrhagic infarction or red speckling of an area, usually surrounded by soft infarcted tissue; ii) punctate hemorrhages or isolated small red marks within the tissue; and iii) parenchymatous intracerebral hemorrhages, a large homogeneous mass of blood within the tissue.

Blood coagulation factor determination. Blood was collected by heart puncture and anticoagulated with 3.8% sodium citrate and the plasma was obtained by centrifugation (4°C, 1,000 x g for 10 min). PT, APTT, TT and FIB were evaluated using a solidification method, according to manufacturer's

Table I. Effect of rhPro-UK on patency rate in thromboembolic rabbit models (means \pm standard error; n=10).

Therapeutic time window (h)	Group	Dose (x10 ⁴ U/kg)	Patency rate (%)
3	Sham	-	-
	Model	-	0
	rhPro-UK	2.5	10
	rhPro-UK	5	40 ^{a,d}
	rhPro-UK	10	70 ^c
	UK	5	20
	rt-PA	4.5 mg/kg	40 ^a
4.5	Sham	-	-
	Model	-	0
	rhPro-UK	2.5	10
	rhPro-UK	5	30 ^{a,d}
	rhPro-UK	10	50 ^b
	UK	5	20
	rt-PA	4.5 mg/kg	30 ^a
6	Sham	-	-
	Model	-	0
	rhPro-UK	2.5	20
	rhPro-UK	5	30 ^{a,d}
	rhPro-UK	10	40 ^b
	UK	5	0
	rt-PA	4.5 mg/kg	20

^aP<0.05; ^bP<0.01; ^cP<0.001 vs. model group. ^dP>0.05, 5x10⁴ U/kg rhPro-UK vs. UK. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator.

instructions of the PT, APTT, TT and FIB assay kits, with the PARBER Blood Coagulation Factor Analyzer. Levels of α_2 -AP were also measured via ELISA with the RT-6100 microplate reader.

Statistical analysis. Values are presented as the mean \pm standard error of the mean, normal distribution data were analyzed using one-way analysis of variance and non-normal data were evaluated using a nonparametric test, the Kruskal Wallis test. The counting data are expressed as ratios (%) and using the χ^2 test P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of rhPro-UK on thrombolysis. At the 3 h therapeutic time window, the patency rate of rhPro-UK(2.5x, 5x and 10x10⁴) was 10% (P>0.05), 40% (P<0.05) and 70% (P<0.001), and the thrombolysis rate reached 21.5% (P<0.05), 36.8% (P<0.001) and 55.0% (P<0.001), respectively. At 4.5 h post-embolism, the patency rate was increased to 10% (P>0.05), 30% (P<0.05) and 50% (P<0.01), and the thrombolysis rate reached 18.8% (P<0.05), 29.9% (P<0.01) and 49.0% (P<0.001), respectively. At 6 h post-embolism, the patency rate was increased to 20%

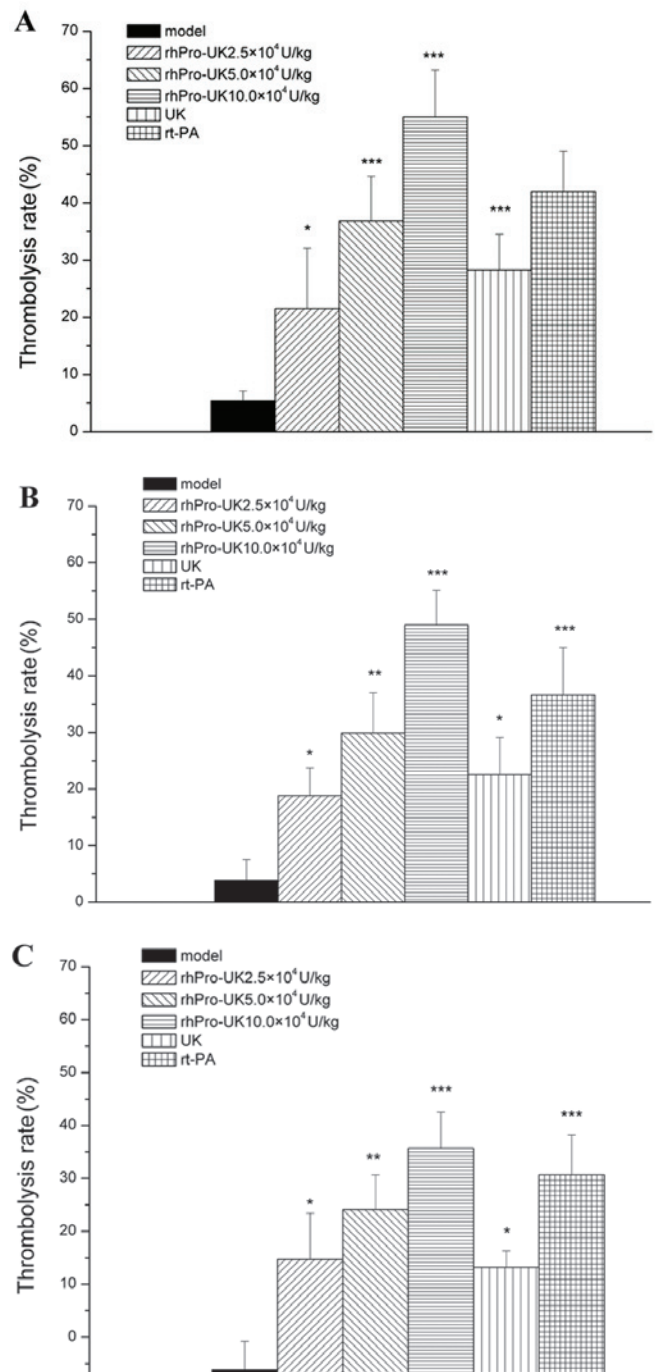


Figure 1. Effect of rhPro-UK on thrombolysis in thromboembolic rabbits. Thrombolysis rate was measured at: (A) 3, (B) 4.5 and (C) 6 h therapeutic time windows. Data are presented as means \pm standard error (n=10). *P<0.05, **P<0.01 and ***P<0.001 vs. model group. rhPro-UK 5 x 10⁴ U/kg compared with UK, P>0.05. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator.

(P>0.05), 30% (P<0.05) and 40% (P<0.01), and the thrombolysis rate reached 14.7% (P<0.05), 24.1% (P<0.01) and 35.7% (P<0.001).

At the 3, 4.5 and 6 h therapeutic time windows, the patency rate of 5x10⁴ U/kg UK was 20% (P>0.05), 20% (P>0.05) and 0% (P>0.05), respectively and the patency rate of 4.5 mg/kg rt-PA was 40% (P<0.05), 30% (P<0.05) and 20% (P>0.05). rhProUK (5x10⁴ U/kg) marginally increased the thrombolysis

Table II. Effect of rhPro-UK on thrombolysis rate (%) and thrombolysis time in thromboembolic rabbit models at the 3 h therapeutic window (means \pm standard error; n=10).

Group	Dose ($\times 10^4$ U/kg)	Pre- adminis- tration	Time after administration (min)							
			15	30	45	60	75	90	105	120
Model	-	0	3.0 \pm 1.7	3.4 \pm 2.9	3.1 \pm 2.9	5.5 \pm 4.5	2.0 \pm 2.5	4.3 \pm 3.7	5.5 \pm 4.0	5.4 \pm 1.7
hPro-UK	2.5	0	0.2 \pm 4.2	5.2 \pm 4.3	6.2 \pm 3.8	8.0 \pm 5.1	15.2 \pm 4.8 ^a	17.6 \pm 7.4	23.6 \pm 6 ^a	21.5 \pm 10.6 ^a
rhPro-UK	5	0	10.3 \pm 2.6 ^d	7.6 \pm 2.4 ^d	9.2 \pm 3.1 ^d	23.5 \pm 4.5 ^{a,d}	25.9 \pm 4.3 ^{c,d}	33.4 \pm 5.8 ^{b,d}	32.4 \pm 6.1 ^{b,d}	36.8 \pm 7.8 ^{c,d}
rhPro-UK	10	0	17.4 \pm 2.7 ^b	28.2 \pm 5.1 ^c	38.5 \pm 6.5 ^c	39.3 \pm 8.5 ^c	42.7 \pm 7.8 ^c	49.2 \pm 9.7 ^c	53.3 \pm 8.8 ^c	55.0 \pm 8.2 ^c
UK	5	0	8.8 \pm 4.8	9.7 \pm 4.6	13.6 \pm 2.8 ^a	16.8 \pm 3.8	16.8 \pm 3.4 ^a	22.1 \pm 7.1 ^a	22.2 \pm 7.2	28.3 \pm 6.2 ^c
rt-PA	4.5	0	9.5 \pm 3.4	16.1 \pm 4.4 ^a	19.1 \pm 6.9 ^a	23.8 \pm 7.6 ^a	30.8 \pm 9.2 ^c	33.4 \pm 8.5 ^b	35.6 \pm 8.1 ^b	42.0 \pm 7.0 ^c

^aP<0.05, ^bP<0.01 and ^cP<0.001 vs. model group; ^dP>0.05, 5 $\times 10^4$ U/kg rhPro-UK vs. UK. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator.

Table III. Effect of rhPro-UK on thrombolysis rate (%) and thrombolysis time in thromboembolic rabbit models at the 4.5 h therapeutic window (means \pm standard error; n=10).

Group	Dose ($\times 10^4$ U/kg)	Pre- adminis- tration	Time after administration (min)							
			15	30	45	60	75	90	105	120
Model	-	0	-3.7 \pm 3.9	-3.2 \pm 3.7	1.9 \pm 4.6	-4.3 \pm 4.9	-2.7 \pm 3.0	-0.3 \pm 3.7	1.0 \pm 5.7	3.9 \pm 3.6
rhPro-UK	2.5	0	-2.7 \pm 4.4	1.4 \pm 3.8	0.0 \pm 3.1	6.1 \pm 4.1	11.3 \pm 4.5 ^a	10.8 \pm 3.0	15.5 \pm 5.9	18.8 \pm 4.9 ^a
rhPro-UK	5	0	1.2 \pm 5.2 ^d	10.2 \pm 5.5 ^{a,d}	16.2 \pm 4.2 ^{a,d}	24.7 \pm 4.9 ^{c,d}	28.5 \pm 6.2 ^{c,d}	27.1 \pm 6.7 ^{c,d}	29.3 \pm 7.1 ^{b,d}	29.9 \pm 7.1 ^{b,d}
rhPro-UK	10	0	1.1 \pm 7.4	7.4 \pm 1.6	18.3 \pm 3.3 ^a	26.9 \pm 5.5 ^c	30.1 \pm 6.6 ^c	37.3 \pm 6.4 ^c	36.5 \pm 8.2 ^c	49.0 \pm 6.1 ^c
UK	5	0	4.8 \pm 2.5	7.6 \pm 3.4	9.4 \pm 3.8	13.4 \pm 5.5 ^a	17.0 \pm 4.3 ^b	17.1 \pm 5.2 ^a	24.4 \pm 5.9 ^a	22.6 \pm 6.5 ^a
rt-PA	4.5	0	6.3 \pm 3.9	13.3 \pm 4.5 ^b	18.4 \pm 7.9 ^a	24.8 \pm 7.8 ^c	27.0 \pm 7.9 ^c	25.7 \pm 7.5 ^b	29.8 \pm 7.6 ^b	36.6 \pm 8.4 ^c

^aP<0.05, ^bP<0.01 and ^cP<0.001 vs. model group; ^dP>0.05, 5 $\times 10^4$ U/kg rhPro-UK vs. UK. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator.

rate compared with 5 $\times 10^4$ U/kg UK (36.8 vs. 28.3%, 29.9 vs. 22.6% and 24.1 vs. 13.2%; Table I and Fig. 1A-C).

Effect of rhPro-UK on thrombolysis time. At the 3 h therapeutic time window, the thrombolysis rate of 2.5 $\times 10^4$ U/kg rhPro-UK significantly increased at 75, 105 and 120 min, at 60-120 min for 5 $\times 10^4$ U/kg rhPro-UK and at 15-120 min for 10 $\times 10^4$ U/kg rhPro-UK, respectively. In addition, UK and rt-PA increased the thrombolysis rate. The thrombolysis times of rhPro-UK(2.5x, 5x and 10 $\times 10^4$) were 117.0, 105.0 and 77.5 min, respectively and the thrombolysis times for UK and rt-PA were 109.0 and 97.5 min, respectively.

At the 4.5 h therapeutic time window, the thrombolysis rate of 2.5 $\times 10^4$ U/kg rhPro-UK significantly increased at 75 and 120 min, at 30-120 min for 5 $\times 10^4$ U/kg rhPro-UK and at 45-120 min for 10 $\times 10^4$ U/kg. Furthermore, UK and rt-PA increased the thrombolysis rate. The thrombolysis times of rhPro-UK(2.5x, 5x and 10 $\times 10^4$) were 119.5, 111.0 and 105.5 min, and were 111.5 and 101.5 min for UK and rt-PA, respectively.

At the 6 h therapeutic time window, the thrombolysis rate of 2.5 $\times 10^4$ U/kg rhPro-UK significantly increased at 75-120 min, at 30 and 75-120 min for 5 $\times 10^4$ U/kg rhPro-UK, and at 30-120 min for 10 $\times 10^4$ U/kg rhPro-UK. In addition, UK and rt-PA increased the thrombolysis rate. The thrombolysis time of rhPro-UK(2.5x, 5x and 10 $\times 10^4$) were 113.0, 111.0 and 103.5, respectively, and the thrombolysis rates of UK and rt-PA were 113.0 and 107.0 min (Tables II-IV).

Effect of rhPro-UK on bleeding. In the different therapeutic time windows, no significant difference (P>0.05) was identified between rhPro-UK (2.5x, 5x and 10 $\times 10^4$ U/kg) on hemorrhage type and number compared with the model group. At 3, 4.5 and 6 h, respectively, rhPro-UK (5 $\times 10^4$ U/kg) treatment exhibited similar hemorrhage numbers when compared with UK treatment (20 vs. 30%, 20 vs. 30% and 30 vs. 40%), and the hemorrhage size also slightly decreased (1.7 vs. 3.7, 2.2 vs. 4.4 and 2.5 vs. 4.1; Table V and Fig. 2A-C).

Table IV. Effect of rhPro-UK on thrombolysis rate (%) and thrombolysis time in thromboembolic rabbit models at the 6 h therapeutic window (means \pm standard error; n=10).

Group	Dose ($\times 10^4$ U/kg)	Pre- admini- stration	Time after administration (min)							
			15	30	45	60	75	90	105	120
Model	-	0	-4.6 \pm 3.3	0.1 \pm 1.5	0.4 \pm 3.2	2.0 \pm 4.5	-2.8 \pm 4.4	-3.8 \pm 4.2	-10.9 \pm 5.5	-6.2 \pm 5.4
rhPro-UK	2.5	0	1.7 \pm 2.7	3.4 \pm 3.7	10.9 \pm 5.2	16.8 \pm 4.0	15.9 \pm 7.2 ^a	17.7 \pm 5.5 ^b	14.7 \pm 4.2 ^b	14.7 \pm 8.7 ^a
rhPro-UK	5	0	1.9 \pm 3.2 ^d	9.0 \pm 2.7 ^{a,d}	10.1 \pm 3.4 ^d	11.5 \pm 5.8 ^d	18.7 \pm 6.0 ^{b,d}	18.0 \pm 5.8 ^{b,d}	12.7 \pm 7.9 ^{b,d}	24.1 \pm 6.6 ^{b,d}
rhPro-UK	10	0	0.2 \pm 4.3	8.9 \pm 3.7 ^a	15.2 \pm 3.5 ^b	20.9 \pm 5.7 ^a	24.9 \pm 6.0 ^c	27.3 \pm 7.0 ^c	30.1 \pm 7.2 ^c	35.7 \pm 6.8 ^c
UK	5	0	4.5 \pm 3.8	3.1 \pm 2.7	1.7 \pm 3.2	2.7 \pm 3.2	8.0 \pm 3.5	6.6 \pm 2.4	5.5 \pm 1.9	13.2 \pm 3.1 ^a
rt-PA	4.5 mg/kg	0	3.7 \pm 3.2	7.6 \pm 3.0	9.0 \pm 3.6	16.2 \pm 7.7	28.3 \pm 7.8 ^c	30.3 \pm 8.9 ^c	28.2 \pm 7.4 ^c	30.7 \pm 7.5 ^c

^aP<0.05, ^bP<0.01 and ^cP<0.001 vs. model group; ^dP>0.05 5 $\times 10^4$ U/kg rhPro-UK vs. UK. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator.

Table V. Effect of rhPro-UK on hemorrhage in thromboembolic rabbit models (n=10).

Therapeutic time window (h)	Group	Dose ($\times 10^4$ U/kg)	Hemorrhage type				Total subjects with hemorrhage (%)
			NH	PT	HI	ICH	
3	Sham	-	10	0	0	0	0 (0)
	Model	-	8	1	1	0	2 (20) ^a
	rhPro-UK	2.5	8	0	2	0	2 (20) ^b
	rhPro-UK	5	8	1	1	0	2 (20) ^{b,c}
	rhPro-UK	10	8	1	1	0	2 (20) ^b
	UK	5	8	1	0	2	3 (30) ^b
	rt-PA	4.5 mg/kg	8	1	0	1	2 (20) ^b
4.5	Sham	-	10	0	0	0	0 (0)
	Model	-	8	0	1	1	10 (10) ^a
	rhPro-UK	2.5	8	0	2	0	2 (20) ^b
	rhPro-UK	5	8	0	1	1	2 (20) ^{b,c}
	rhPro-UK	10	7	1	2	0	3 (30) ^b
	UK	5	7	0	2	1	3 (30) ^b
	rt-PA	4.5 mg/kg	7	0	2	1	3 (30) ^b
6	Sham	-	10	0	0	0	0 (0)
	Model	-	8	0	2	0	2 (20) ^a
	rhPro-UK	2.5	8	1	1	0	2 (20) ^b
	rhPro-UK	5	7	0	2	1	3 (30) ^{b,c}
	rhPro-UK	10	7	0	2	1	3 (30) ^b
	UK	5	6	1	2	1	4 (40) ^b
	rt-PA	4.5 mg/kg	7	0	3	0	3 (30) ^b

^aP>0.05 vs. sham; ^bP>0.05 vs. model group; ^cP>0.05 5 $\times 10^4$ U/kg rhPro-UK vs. UK. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator; NH, no hemorrhage; PT, punctate hemorrhage; HI, hemorrhagic infarct; ICH, intracerebral hemorrhage.

Effect of rhPro-UK on blood coagulation factor. Compared with the model group, no significant difference (P>0.05) was identified between rhPro-UK (2.5x, 5x and 10x10⁴ U/kg) on PT, TT, APTT and FIB for the different time windows. UK treat-

ment extended TT and APTT, and reduced FIB, furthermore rt-PA prolonged APTT and reduced FIB slightly. rhPro-UK (5x10⁴ U/kg) exerted lighter effects on TT, APTT and FIB when compared with UK treatment (Table VI).

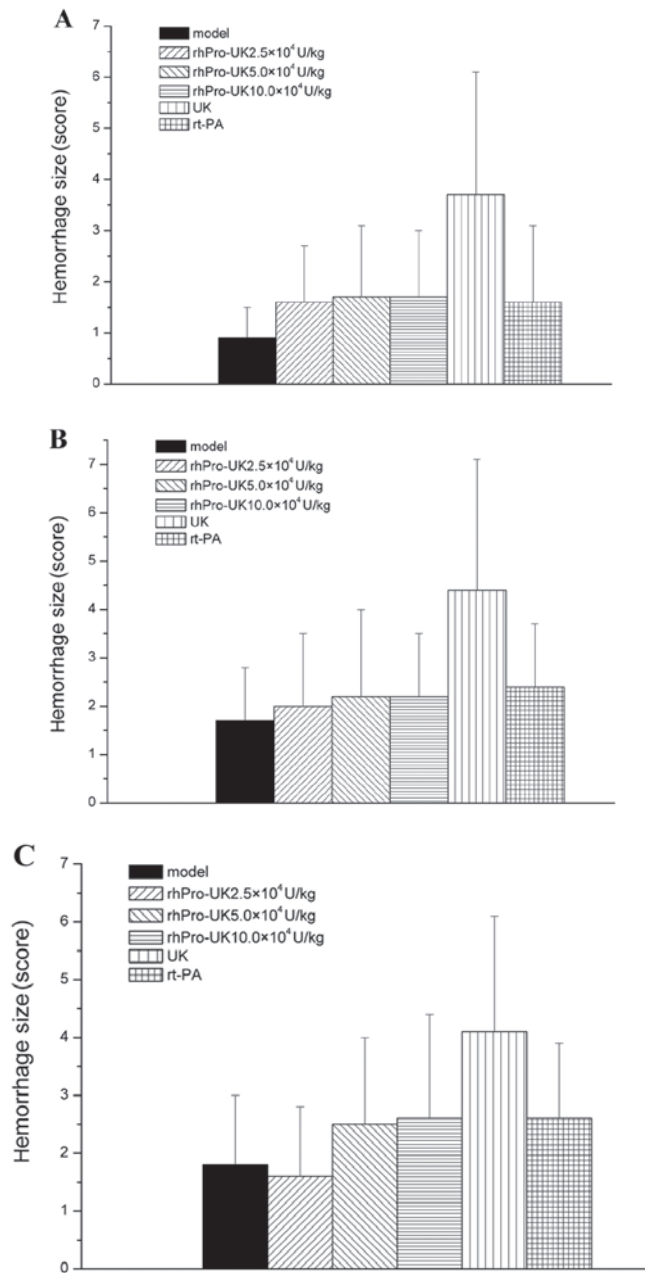


Figure 2. Effect of rhPro-UK on hemorrhage size. Thrombolysis rate was measured at (A) 3, (B) 4.5 and (C) 6 h therapeutic time windows. Data are presented as means \pm standard error (n=10). rhPro-UK groups compared with model, $P>0.05$; rhPro-UK 5×10^4 U/kg compared with UK, $P>0.05$. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator.

Effect of rhPro-UK on α_2 -AP. At the 3 h therapeutic time window, rhPro-UK (2.5×10^4 , 5×10^4 and 10×10^4 U/kg) reduced α_2 -AP by 5.3% ($P>0.05$), 5.3% ($P>0.05$) and 18.1% ($P<0.05$), respectively. In addition, UK and rt-PA reduced α_2 -AP by 29.2% ($P<0.01$) and 22.7% ($P<0.05$), respectively. Compared with UK, 5×10^4 U/kg rhPro-UK exerts a smaller influence on α_2 -AP (9.7 vs. 29.2%).

At the 4.5 h therapeutic time window, rhPro-UK (2.5×10^4 , 5×10^4 and 10×10^4 U/kg) reduced α_2 -AP by 2.4% ($P>0.05$), 6.5% ($P>0.05$) and 17.8% ($P<0.05$). Furthermore, UK and rt-PA reduced α_2 -AP by 25.3% ($P<0.01$) and 19.8% ($P<0.05$), respectively. rhPro-UK (5×10^4 U/kg) exerts a smaller influence on α_2 -AP when compared with UK (6.5 vs. 25.3%).

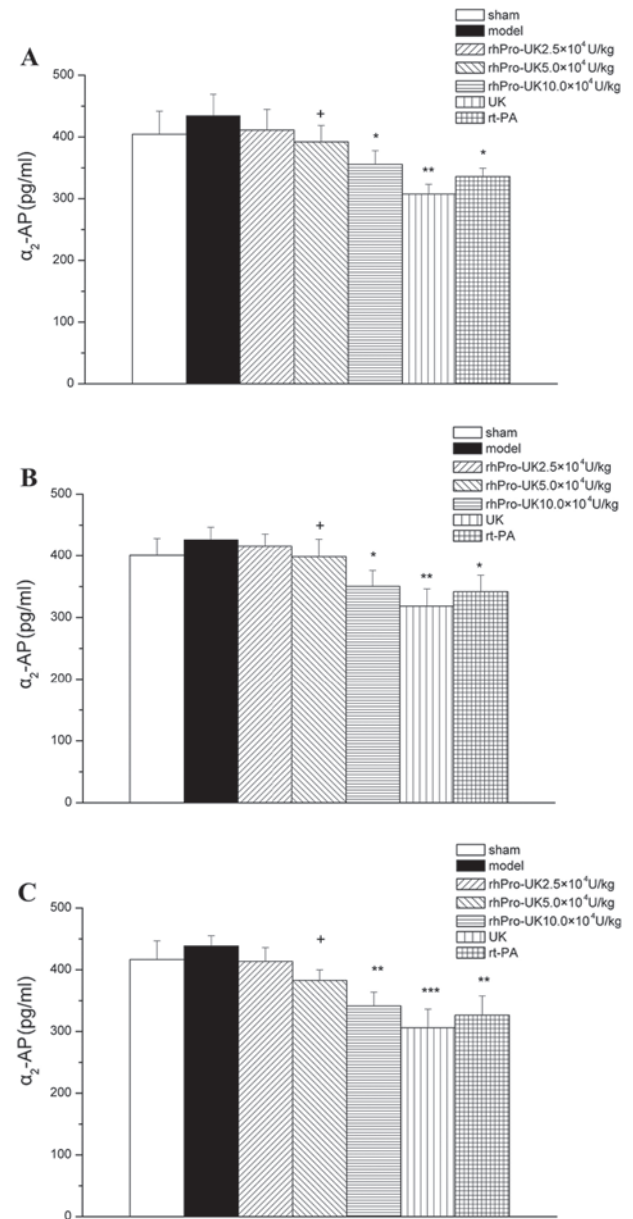


Figure 3. Effect of rhPro-UK on α_2 -AP production in thromboembolic rabbits. α_2 -AP level was measured at (A) 3, (B) 4.5 and (C) 6 h therapeutic time windows. Data are presented as means \pm standard error (n=10). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. model group; * $P<0.05$ vs. UK. Model compared with sham, $P>0.05$. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator; α_2 -AP, α_2 -antiplasmin.

At the 6 h therapeutic time window, rhPro-UK (2.5×10^4 , 5×10^4 and 10×10^4 U/kg) reduced α_2 -AP by 5.7% ($P>0.05$), 12.7% ($P>0.05$) and 22.2% ($P<0.01$). In addition, UK and rt-PA reduced α_2 -AP by 30.2% ($P<0.001$) and 25.6% ($P<0.01$). Compared with UK, 5×10^4 U/kg rhPro-UK exerts a smaller influence on α_2 -AP when compared with UK (12.7 vs. 30.2%; Fig. 3A-C).

Discussion

Stroke is the second leading cause of mortality worldwide and the number one cause of disability in the USA (17). IV tPA remains the only drug that has been approved by the United

Table VI. Effect of rhPro-UK on blood coagulation factors in thromboembolic rabbit models (means \pm standard error; n=10).

Therapeutic time window (h)	Group	Dose ($\times 10^4$ U/kg)	PT (s)	TT (s)	APTT (s)	FIB (g/l)
3	Sham	-	7.1 \pm 1.4	17.3 \pm 3.2	26.5 \pm 5.5	3.4 \pm 0.8
	Model	-	6.3 \pm 1.3 ^g	19.5 \pm 2.8 ^g	27.8 \pm 3.7 ^g	3.5 \pm 1.2 ^g
	rhPro-UK	2.5	6.7 \pm 0.8	22.1 \pm 3.0	28.2 \pm 4.0	3.6 \pm 0.6
	rhPro-UK	5	6.2 \pm 0.5	20.6 \pm 2.0 ^a	29.5 \pm 5.4 ^a	3.4 \pm 0.9 ^b
	rhPro-UK	10	6.6 \pm 0.6	21.5 \pm 4.9	31.2 \pm 4.1 ^c	3.2 \pm 0.8
	UK	5	7.2 \pm 1.2	25.3 \pm 5.9 ^d	34.0 \pm 4.3 ^e	2.4 \pm 0.6 ^c
	rt-PA	4.5 mg/kg	7.1 \pm 1.1	19.4 \pm 1.8	32.4 \pm 3.4 ^d	2.6 \pm 0.7 ^c
	Sham	-	6.2 \pm 0.5	19.7 \pm 3.7	29.6 \pm 4.6	3.3 \pm 0.8
4.5	Model	-	6.6 \pm 1.4 ^g	20.7 \pm 3.6 ^g	30.0 \pm 3.1 ^g	3.3 \pm 0.6 ^g
	rhPro-UK	2.5	6.2 \pm 0.6	19.7 \pm 1.7	28.5 \pm 4.5	3.5 \pm 0.5
	rhPro-UK	5	7.6 \pm 3.2	19.4 \pm 3.2 ^b	29.5 \pm 5.7 ^a	3.6 \pm 0.9 ^f
	rhPro-UK	10	6.9 \pm 0.9	22.4 \pm 2.8	31.6 \pm 3.0	3.4 \pm 1.0
	UK	5	6.7 \pm 1.2	24.2 \pm 4.0 ^c	34.6 \pm 5.5 ^c	2.2 \pm 0.8 ^c
	rt-PA	4.5 mg/kg	6.6 \pm 0.5	20.0 \pm 2.3	34.0 \pm 3.8 ^c	2.5 \pm 0.9
	Sham	-	6.3 \pm 0.4	18.7 \pm 2.4	26.1 \pm 3.5	3.5 \pm 0.7
	Model	-	6.4 \pm 0.5 ^g	19.2 \pm 2.1 ^g	29.0 \pm 3.4 ^g	3.5 \pm 0.6 ^g
6	rhPro-UK	2.5	6.7 \pm 0.6	19.4 \pm 3.6	29.1 \pm 5.4	3.7 \pm 0.8
	rhPro-UK	5	6.4 \pm 0.7	18.5 \pm 2.1 ^f	31.6 \pm 3.5	3.4 \pm 1.1
	rhPro-UK	10	6.6 \pm 0.8	20.9 \pm 4.0	30.7 \pm 4.3	3.3 \pm 0.9
	UK	5	6.4 \pm 0.7	23.6 \pm 3.4 ^d	34.1 \pm 5.1 ^d	2.3 \pm 0.8 ^d
	rt-PA	4.5 mg/kg	6.7 \pm 0.8	20.6 \pm 1.8	32.9 \pm 4.1 ^c	2.5 \pm 0.8 ^c

^aP<0.05 and ^bP<0.01 vs. UK; ^cP<0.05, ^dP<0.01 and ^eP<0.001 vs. model; ^fP<0.001 vs. UK; ^gP>0.05, model vs. sham. rhPro-UK, recombinant human prourokinase; UK, urokinase; rt-PA, recombinant human tissue plasminogen activator; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; FIB, fibrinogenemia.

States Food and Drug Administration for its treatment (18). However, the perception of marginal utility, high risk of intracerebral bleeding, and/or high liability associated with its administration discourage its administration (19), although the American Heart Association has deemed it an acceptable alternative therapy and many stroke centers offer it to patients within 6 h of a major acute stroke (20). These limitations reflect the requirement for more effective thrombolytic drugs. rhPro-UK has more potent efficacy and fewer adverse reactions in comparison with other thrombolytics due to the fibrin-selective clot lysis. The rhPro-UK in the present study was from Tasly Pharmaceutical Co., Ltd., generated from Chinese hamster ovary cell expression using a genetic engineering method, and is typically used to treat acute myocardial infarction (21). The present study evaluated IV thrombolysis with rhPro-UK in rabbit acute cerebral infarction at 3, 4.5 and 6 h therapeutic time windows.

The results confirmed that the thrombolysis rate and patency rate (recanalization rate) increased as the time window shortened. At 3, 4.5 and 6 h therapeutic time windows, the thrombolysis rate of 5×10^4 U/kg rhProUK was 36.8, 29.9 and 24.1%, respectively and the patency rate was 40, 30 and 30%. The thrombolysis rate of 10×10^4 U/kg rhPro-UK was 55.0, 49.0 and 35.7% and the patency rate was 70, 50, 40% at 3, 4.5 and 6 h therapeutic time windows, respectively. rhPro-UK treatment increased the thrombolysis rate slightly when compared

with UK (36.8 vs. 28.3%, 29.9 vs. 22.6% and 24.1 vs. 13.2%). Consistent with the present study, del Zoppo *et al* (3) reported a phase II randomized trial of rhPro-UK by direct arterial delivery in acute middle cerebral artery stroke, local IV rhPro-UK infusion at 5.5 h from symptom onset was associated with superior recanalization in acute thrombotic/thromboembolic stroke when compared with a placebo. In addition, Tirschwell *et al* (22) reported a PROACT II trial including 180 patients with acute ischemic stroke, despite an increased frequency of early symptomatic intracranial hemorrhage, treatment with Pro-UK within 6 h of the onset of acute ischemic stroke caused by middle cerebral artery occlusion significantly improved the clinical outcome at 90 days.

In addition, it was found that rhPro-UK (2.5×10^4 , 5×10^4 and 10×10^4 U/kg) did not increase bleeding compared with the model group ($P > 0.05$), and the hemorrhage size of the 5×10^4 U/kg rhPro-UK group was slightly decreased compared with the UK treatment group at different time points. rhPro-UK (5×10^4 U/kg) had less of an influence on PT, TT, APTT, FIB and α_2 -AP when compared with UK. This finding is comparable to a study by Zhang *et al* (23), where rhPro-UK did not effect FIB, PA or α_2 -AP, and the effect on bleeding time, clotting time and bleeding quantity per unit time was less than those of UK.

Plasmin is an enzyme that participates in fibrinolysis. α_2 -AP is a serine protease inhibitor responsible for inactivating

plasmin. Its rapid reaction with plasmin results in the formation of an inactive complex (plasmin- α_2 -AP complex; PAP), which is composed of one molecule of each component. Therefore, the method that was used for measuring α_2 -AP in the present study only indirectly reflects the actual fibrinolytic activity, and thus presents a limitation of this study. Determination of PAP may be more appropriate in future studies.

In conclusion, IV rhPro-UK exerted therapeutic effects on thromboembolic stroke rabbit models within a 6 h time frame, influencing thrombolysis and recanalization (patency rate) with reduced risk of cerebral hemorrhage.

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