

Original Article

Efficacy of a new mutated recombinant tissue-type plasminogen activator in beagles with acute coronary artery thrombi

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BACKGROUND: Development of new coronary thrombolytic agents is hot in the market. A new drug, mutated recombinant tissue-type plasminogen activator (rtPAm), is the product of mutation of tPA by changing binding loci with plasminogen activator inhibitor (PAI)-1 to reduce the degradation. *In vitro* test has demonstrated that the activity of rtPAm is much higher than rtPA in the absence of PAI. The present study is to observe the efficacy of mutated recombinant tissue-type plasminogen activator (rtPAm) in coronary thrombolytic therapy.

METHODS: A total of 30 adult beagles were equally divided into 5 groups after thrombi: vehicle group, urokinase group, rtPAm low-dose group, rtPAm medium-dose group, and rtPAm high-dose group. Thrombolytic effect and myocardial infarction were observed after thrombolytic therapy.

RESULTS: In the urokinase group, time to reperfusion was (15.8±3.8) minutes. TIMI 2 flow was demonstrated in 4 beagles, TIMI 3 flow in 2, and re-occlusion in 4 after 90 minutes respectively. In the low-dose rtPAm group, time to reperfusion was (15±4.5) minutes; TIMI 2 flow was demonstrated in 2 beagles, TIMI 3 flow in 4, and re-occlusion in 2 after 90 minutes. In the high-dose rtPAm group, time to reperfusion was (7.5±2.6) minutes. None of the beagles showed re-occlusion after 90 minutes. The infarction areas were (2.1±0.9)% in the medium-dose rtPAm group and (0.7±0.4)% in the high-dose rtPAm group, which decreased significantly than those in the low-dose rtPAm group. The aggregation rate in the medium-dose and high-dose rtPAm groups decreased significantly than that in the urokinase group.

CONCLUSION: rtPAm may serve as a thrombolytic agent with platelet-targeted fibrinolysis and antiplatelet aggregation activities.

KEY WORDS: Urokinase; RtPA; Thrombi; D-dime; Platelet aggregation

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INTRODUCTION

Human tissue-type plasminogen activator (tPA), a family member of serine protease, is a physiological activator in the blood fibrinolytic system. There are two binding sites with tPA and plasminogen respectively on the fibrin matrix. These sites in fibrinogen are hidden, and only exposed after two small peptides are resected by thrombin. The complexes formed by tPA and plasminogen can enhance the activity of plasminogen activated by tPA. The activated plasminogen dissolves fibrin for a

thrombolytic purpose.^[1] In blood circulation, tPA has been rapidly inhibited by plasminogen activator inhibitor (PAI) in order to maintain a precise balance between coagulation and fibrinolysis. When tPA is used in fibrinolytic therapy, it is promptly inhibited *in vivo* by PAI-1. Later a tPA-PAI-1 complex is possibly formed, resulting in secondary vascular embolism.^[2] Therefore it is important to develop a new tPA which blocks the binding with PAI-1.

Development of new coronary thrombolytic agents is hot in the market. A new drug, mutated recombinant

tissue-type plasminogen activator (rtPAm), is a mutational product of tPA by changing binding loci with PAI-1 to reduce the degradation. *In vitro* test has demonstrated that the activity of rtPAm is much higher than rtPA in the absence of PAI.^[3] This experiment was undertaken to observe the efficacy of rtPAm in coronary thrombolytic therapy in order to understand the effect of rtPAm *in vivo*.

METHODS

The experiment was conducted according to the European Community Guidelines for Animal Ethical Care and the Guide for Care and Use of Laboratory Animals issued by the US National Institute of Health (NIH publication No 85-23, revised 1985).

Study population and experimental design

Totally 30 adult beagles were equally divided into 5 groups by gender, with weight of (10.0±1.0) kg provided by the Hubei Provincial Center for Drug testing. Digital subtraction angiographic imaging machine, H5000 from Philips Inc., was used with 1000 mA in power. Other catheter operating equipments were the same as those used in patients. Pieces of woven copper wire net cut by the exact size of 6 mm long and 3.5 mm wide were wound and fixed around the middle of small balloons by the same technician. RtPm was provided by professor Lin-bo Ye, College of Life Science, Wuhan University, China.

The beagles were fasted for 12 hours before operation and anesthetized with thiopental sodium at an intraperitoneal dose of 40 mg/kg and a maintenance dose of 20 mg/kg per hour. The beagles were later fixed on the catheter bed in a supine position. ECG was monitored continuously in precordial leads to record arrhythmias. The right femoral artery was punctured and a 6F arterial sheath inserted in. Heparin 200 µ/kg anticoagulation was injected by the arterial sheath. A 6F coronary guiding catheter was inserted under the guidance of a guide wire. Under fluoroscopic visualization, the catheter was inserted into the opening of the left coronary artery, and then contrast medium was injected and synchronized video recorded. From the coronary guide catheter, the guide wire was inserted into the first marginal branch of the left circumflex coronary artery. A balloon wound with copper net was expanded for 10 seconds with 8 atm pressure, then the copper coil was released and fixed on the intima of the coronary artery, and the balloon was deflated and retreated from the vessel. Coronary occlusions were confirmed by coronary angiography every 5 minutes and real-time ECG monitoring after copper

coil insertion. The animals were randomly divided into 5 groups after thrombosis: vehicle group (using intravenous infusion of normal saline), urokinase group (80 000 µ/kg), rtPAm low-dose group (47 000 µ/kg), rtPAm medium-dose group (94 000 µ/kg), and rtPAm high-dose group (188 000 µ/kg). Treatments began 15 minutes after the occlusions were confirmed. All the agents were administered at an intravenous bolus dose in 30 minutes. Reperfusion was defined as TIMI (thrombolysis in myocardial infarction) grade 2 or 3 and re-occlusion defined as 0 or 1. Reperfusion assessed by angiography was confirmed every 5 minutes for up to 90 minutes after administration of the agents, and ECG was recorded. Arterial blood samples were taken through the guide catheter and venous blood samples were obtained from ear veins.

Measurement of myocardial infarction area

The animals were sacrificed by bloodletting following these experiments after 90 minutes. Their hearts were excised and cross sectioned for 1 cm thickness at 1 and 2 cm below the position of copper coil-insertion. The sections were stained with 1% TTC (2, 3, 5-triphenyl tetrazolium chloride) for 5 minutes at 37 °C.^[4] The area of myocardial infarction was identified as the area that was not stained by TTC. The area of myocardial infarction was calculated using an area measuring program.

Measurement of blood sample

Blood samples were taken for measurement of D-dimer, platelet aggregation rate, activated partial thromboplastin time (aPTT), prothrombin time (PT) and fibrinogen (Fg) concentration at different time points after thrombolytic treatment. Fibrinogen was determined with the Clauss method.^[5] Stored samples were used to measure fibrin D-dimer at the end of the study by ELISA assay and standardized for interbatch variation. APTT and PT were assayed with a Coag-A-Mate XM automated coagulation timer. Femoral artery blood was collected in 3.8% sodium citrate (9/1, v/v) from the animals before induction of thrombus, during the presence of stable thrombus, and 30, 60 and 90 minutes after the administration of the agents for measurement of platelet aggregation. Platelet-rich plasma (PRP, 2-4×10⁸ cells/ml) was obtained by centrifuging the blood at 150 g for 8 minutes at 24 °C. Platelet-poor plasma was obtained by further centrifugation at 1500 g for 15 minutes. Aggregation was determined with a dual channel light transmission aggregometer in response to ADP (20 PM) plus epinephrine (5.5 PM) or thrombin (2.5-10 units/ml).

Statistical analysis

Time to reperfusion was expressed as the value of each animal. The Steel-Dwass test and the Tukey's test were used for statistical analysis of data for intergroup comparison. Biochemical parameters and myocardial infarction area were expressed as mean±SD. A *P* value <0.05 was considered statistically significant.

RESULTS

Baseline conditions

Thrombosis of the coronary artery was formed in all beagles, resulting in occlusion of coronary blood flow. The beagles survived after the experiment. No significant differences in heart rate and blood pressure were observed before and after administration of the agents in the 5 groups of beagles (Table 1).

Thrombolytic efficacy

Thrombosis occurred in all beagles within 7 minutes, and it was stable after 15 minutes without reperfusion. In the urokinase group, thrombosis was found with reperfusion after administration of the drug for (15.8±3.8) minutes. Four beagles demonstrated TIMI 2 flow, 2 demonstrated TIMI 3 flow, and 4 demonstrated re-occlusion after 90 minutes. In the low-dose rtPAM group there was reperfusion after administration of the drug

for (15±4.5) minutes. Two beagles demonstrated TIMI 2 flow, 4 demonstrated TIMI 3 flow, and 2 demonstrated re-occlusion after 90 minutes. In the medium-dose rtPAM group, reperfusion occurred after administration of the agent for (10±4.5) minutes. All 6 beagles demonstrated TIMI 3 flow, but none of them had re-occlusion after 90 minutes. In the high-dose rtPAM group there was reperfusion after administration of the agent for (7.5±2.6) minutes. None of the beagles had re-occlusion after 90 minutes (Table 2).

D-dimer

D-dimer can be used as an indicator of thrombolysis since it is released into the blood when fibrinolysis dissolves. In consistence with time of reperfusion determined by DSA, the concentration of D-dimer in plasma increased 90 minutes after the animals were administered with urokinase or rtPAM. The concentration increased even higher in the high-dose rtPAM group (*P*<0.05) (Table 3).

Coagulation and fibrinolysis system changes

No significant changes were observed in prothrombin time (PT) and activated partial thromboplastin time (APTT) before and after administration of D-dimer in all groups, indicating that there was no change in coagulation function before and after administration of the agent. In the meantime, no significant change was observed in the concentration of fibrinogen (Fg) during the test of each group, suggesting that

Table 1. Changes in heart rate and mean blood pressure due to use of agents in beagles with induced coronary artery thrombi

Parameters	Vehicle	Urokinase	Low-dose rtPAM	Medium-dose rtPAM	High-dose rtPAM
Heart rate (bpm)					
Pre-operation	167±8	169±10	165±7	168±9	166±10
Values 15 min after coronary occlusions	166±7	166±8	165±10	169±9	163±8
Values 60 min after coronary occlusions	167±9	165±7	163±8	167±10	168±9
Values 90 min after coronary occlusions	163±8	167±9	166±8	162±7	164±9
Mean blood pressure (mmHg)					
Pre-operation	120±7	126±8	124±9	127±8	123±7
Values 15 min after coronary occlusions	123±9	125±7	128±10	126±9	124±8
Values 60 min after coronary occlusions	122±6	123±9	125±9	129±10	127±9
Values 90 min after coronary occlusions	127±7	124±8	123±7	123±8	128±7

There were no significant changes in heart rate and blood pressure before and after administration of agents in the 5 groups of beagles.

Table 2. Characteristics of thrombolysis and re-occlusion after treatment of the 5 groups of beagles

Groups	Time to occlusion (min)	Incidence of reperfusion		Time to reperfusion (min)	Incidence of re-occlusion	Time to re-occlusion (min)
		TIMI=2	TIMI=3			
Vehicle control	7.4±2.1	0	0	0	0	0
Urokinase	7.8±2.4	4/6	2/6	15.8±3.8	4/6	17.5±2.9
Low-dose rtPAM	7.1±2.7	2/6	4/6	15±4.5	2/6	17.5±3.5
Medium-dose rtPAM	7.5±2.0	0/6	6/6*	10±4.5	0/6 ^Δ	
High-dose rtPAM	7.5±2.6	0/6	6/6*	8.3±4.1	0/6 ^Δ	

Coronary angiography was performed to evaluate the incidence of coronary thrombolysis and re-occlusion after treatment with different regimens. Coronary patency was quantified using a scale of 0 to 3 described by the thrombolysis in myocardial infarction (TIMI) study group. Compared with the urokinase group, the medium-dose and high-dose rtPAM groups showed a significantly increased recanalization (TIMI=3) rate (^{*}*P*<0.05 vs the urokinase group). In the meantime, the medium-dose or high-dose rtPAM could prevent the re-occlusion significantly after 90-minute intravenous infusion (^Δ*P*<0.01 vs the urokinase group).

the concentration of rtPAM has no notable influence on the coagulation system and fibrinolysis ($P>0.05$) (Figures 1-3).

Measurement of platelet aggregation

Platelet aggregation rate reflects the ability of platelet to aggregate. Platelet aggregation rate decreased in each group after thrombosis. The rate recovered in the urokinase and vehicle groups 60 minutes and 90 minutes after their administration respectively. The rate increased more slowly in the low- and medium-dose rtPAM groups 90 minutes after administration of the agent, but significantly changed compared to the urokinase group. The aggregation rate in the high-dose rtPAM group decreased more significantly than that in the urokinase group, indicating that rtPAM could not only dissolve thrombus but also inhibit platelet aggregation (Table 4).

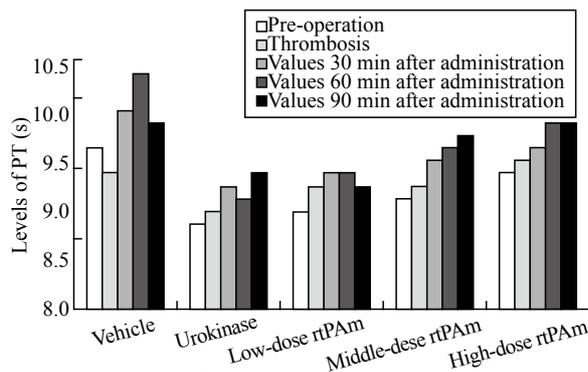


Figure 1. The changes of PT before and after administration of the agent in each group. No significant change was observed in the prothrombin time (PT) before and after the administration in all groups, indicating that there was no change in coagulation function.

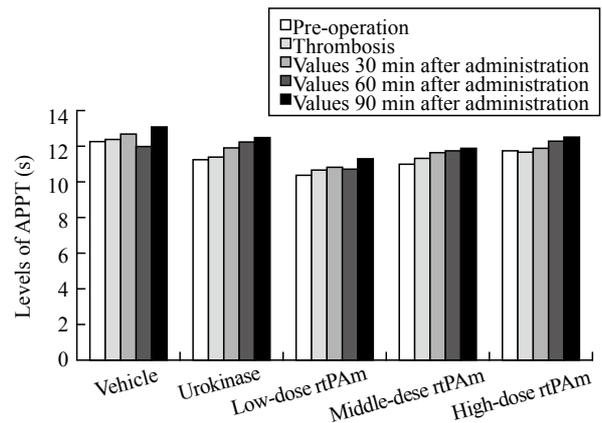


Figure 2. The changes of APTT levels before and after administration of the agent in each group. No significant changes were observed in the activated partial thromboplastin time (APTT) in all groups, indicating that there was no change in coagulation function before and after administration.

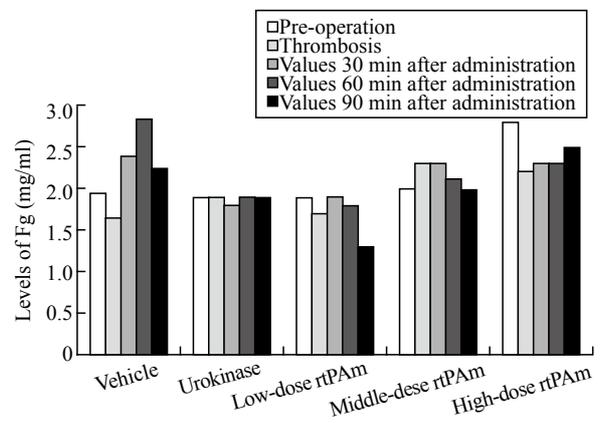


Figure 3. The changes of Fg (mg/ml) before and after administration of the agent in each group. No significant changes were observed in the concentration of fibrinogen (Fg) during the test for each group, suggesting that the concentration of rtPAM has no notable influence on the coagulation system and fibrinolysis.

Table 3. Changes of D-dimer in each group before and after administration (mg/L)

Groups	Thrombosis	Values 30 min after administration	Values 60 min after administration	Values 90 min after administration
Vehicle	0.2±0.04	0.21±0.03	0.19±0.03	0.2±0.06
Urokinase	0.21±0.03	1.4±0.35 ^Δ	1.5±0.33 ^Δ	1.8±0.37 ^Δ
Low-dose rtPAM	0.21±0.04	1.6±0.38 ^Δ	1.6±0.38 ^Δ	1.8±0.32 ^Δ
Medium-dose rtPAM	0.22±0.06	2.0±0.41	2.3±0.42	2.0±0.48
High-dose rtPAM	0.23±0.04	2.1±0.42 [*]	2.5±0.40 [*]	2.3±0.47 [*]

Compared with the vehicle group, the concentration of D-dimer in the urokinase and low-dose rtPAM groups increased significantly (^Δ $P<0.05$ vs the vehicle group). The concentration increased even higher in the high-dose rtPAM group (^{*} $P<0.05$ vs the urokinase group and low-dose rtPAM group).

Table 4. Changes of platelet aggregation before and after administration in each group

Groups	Pre-operation	Thrombosis	Values 30 min after administration	Values 60 min after administration	Values 90 min after administration
Vehicle	33±8	17±4	26±9	28±8	30±10
Urokinase	34±7	16±8	24±7	27±4	29±3
Low-dose rtPAM	32±8	15±6	19±4 [*]	18±5 [*]	28±4
Medium-dose rtPAM	40±6	14±7	17±3 [*]	18±4 [*]	25±6
High-dose rtPAM	35±7	20±7	14±3 ^Δ	10±2 ^Δ	18±3 ^Δ

Compared to the urokinase group, ^{*} $P<0.05$, ^Δ $P<0.01$.

Analysis of infarction area

The rate of myocardial infarction area in the left ventricular myocardium area was (23.5±4.7)% in the vehicle group. Only slight myocardial infarction areas were observed in the rtPAM-and urokinase groups. The rate of these infarction areas in the left ventricular myocardium was (10.3±2.4)% in the urokinase group and (9.6±2.7)% in the low-dose rtPAM group respectively. The rate of infarction areas was significantly declined in the two groups than that in the vehicle group. The rate of infarction areas was (2.1±0.9)% in the medium-dose rtPAM group and (0.7±0.4)% in the high-dose rtPAM group, both decreased significantly than those in the low-dose rtPAM group ($P<0.001$).

DISCUSSION

Thrombolytic therapy has become a conventional treatment for acute myocardial infarction (AMI), and new thrombolytic agents are the focus of drug development, but currently clinically prescribed thrombolytic drugs have such problems as delay or failure of initial reperfusion, incomplete reperfusion and re-occlusion of thrombosis. In order to solve these problems, development of new thrombolytic agents has become a hot topic. tPA is characterized by safety, thrombolytic specificity, and no immunogenicity; thus it can be widely used to treat acute myocardial infarction and cerebral disease.^[6-9] However, tPA is rapidly inhibited *in vivo* by PAI-1 when it is applied in fibrinolytic therapy. As a result, repeated high-dose intravenous infusion is needed on many occasions, thus leading to the high cost of treatment and the secondary embolism caused by tPA-PAI-1 complexes. Therefore, to develop an anti-combination of tPA with PAI is very important.^[2,10-13]

In order to prevent tPA in combination with PAI and to extend its half-time so as to enhance the thrombolytic effect of tPA, Henry^[14-16] successfully constructed the recombinant plasmid pUCT-rtPAM which could express rtPAM. In vitro test showed that the activity of rtPAM is much higher than that of tPA. These results laid a solid foundation for further development of a longer half-time thrombolytic drug and improvement of the therapeutic effects.

In this study, we implanted copper coil in the coronary artery to form thrombi. The thrombi induced by copper coil-insertion into canine coronary arteries have generally been recognized as a useful model of acute myocardial infarction to evaluate the efficacy of thrombolytic agents. This is due to that the induced

thrombi, much like many that cause acute myocardial infarction in humans, were rich in fibrin.^[17,18] In human acute myocardial infarction, the efficacy of coronary artery recanalization with thrombolytic agents diminishes with time after the onset of the events, probably due to progressive cross-linking of the fibrin networks. In our test we found that the thrombi formed in this way had the features of consistent duration, relatively similar quantity. This test demonstrated that rtPAM produced rapid recanalization of the coronary artery occluded by fresh and aged thrombi, when administered by bolus injection. The thrombolytic efficacy of low-dose rtPAM was similar to that of urokinase, both could dissolve the thrombi. Some patients demonstrated TIMI 3 flow, some had re-occlusion after 90 minutes. And the reperfusion indicators D-dimer increased significantly, but infarction area decreased in the vehicle group. The medium-dose rtPAM had a higher reperfusion rate than the same dose of urokinase. The efficacy of medium- and high-dose rtPAM was significantly better than that of urokinase, and the canines in these two groups demonstrated TIMI 3 flow, but none of them had re-occlusion after 90 minutes. The above results indicated that thrombolytic effect of an equivalent dose of rtPAM is better than that of urokinase. The concentration of D-dimer in plasma as an important indicator of thrombosis and fibrinolysis increased rapidly due to reperfusion after thrombolytic therapy. Compared to the same dose of urokinase, the medium- and high-dose rtPAM groups demonstrated a more significant increase in D-dimer concentration in plasma 90 minutes after thrombolysis, indicating better thrombolytic effect in the two groups. In the mean time, infarction area in the medium- and high-dose groups decreased more significantly than that in the low-dose and urokinase groups, and in the low-dose group and urokinase group decreased more significantly than in the vehicle group, indicating faster and better thrombolytic effect in the medium- and high-dose groups.

Thrombolytic agents incentivize platelets and further activate the coagulation system, while dissolving blood clots and leading to reperfusion. Therefore, an effective anti-platelet therapy is particularly important in the process of thrombolysis. The effect of anti-platelet aggregation is also our concern when we observe the thrombolytic efficacy of rtPAM. The results indicated that rtPAM could inhibit a dose-dependent platelet aggregation. Meanwhile rtPAM demonstrated no significant effect on clotting function in the effective dose range, and no activation of the fibrinolytic system. These facts suggest that rtPAM is a highly efficient and

relatively safe thrombolytic agent.^[15,19-21]

In conclusion, rtPA could inhibit platelet aggregation, but it could not activate the fibrinolytic system. Further clinical studies are required to determine the efficacy and safety of this mutant of rtPA.

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