

Effect of clopidogrel on tissue-plasminogen activator-induced in vitro thrombolysis of feline whole blood thrombi

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Objective—To determine if clopidogrel enhanced the thrombolytic rate of tissue-plasminogen activator (t-PA) on an in vitro feline whole blood thrombosis model.

Animals—9 purpose-bred cats.

Procedure—Blood obtained from cats before (baseline) and after treatment with clopidogrel (75 mg, PO, q 24 h for 3 days) was anticoagulated with sodium citrate (9:1 volume-to-volume ratio) to which 1 μ Ci of I^{125} -fibrinogen was added. Thrombi were formed by the addition of calcium chloride and bovine thrombin. Thrombi were placed into autologous plasma to which 0.1 mg of t-PA was added. Plasma samples were collected at different time points to determine the amount of released I^{125} -fibrin split products. Thrombolytic rates were calculated by determining the time to 25%, 50%, and 75% thrombolysis (t_{25} , t_{50} , and t_{75} , respectively). Confidence intervals for t_{25} , t_{50} , and t_{75} at baseline were compared with those after treatment.

Results—There were no significant differences in thrombolytic rates between values obtained at baseline and after clopidogrel treatment (t_{25} , 18.0 vs 18.5 minutes; t_{50} , 63.3 vs 65.6 minutes; and t_{75} , 163.0 vs 170.1 minutes, respectively).

Conclusions and Clinical Relevance—Clopidogrel did not have an effect on the rate of thrombolysis of feline whole blood thrombi induced by t-PA in this in vitro model. (*Am J Vet Res* 2004;65:715–719)

Systemic arterial thromboembolization (SATE) is common in cats and is usually associated with some form of underlying myocardial disease.^{1,4} The clinical effects are often severe and result from loss of blood flow to the affected organs. The most common site affected is the aortic trifurcation with clinical signs of posterior paralysis or paresis, signs of pain, and loss of segmental reflexes. In the natural course of disease, blood flow will often resume to the ischemic area through in vivo dissolution of the embolus, recanalization of the embolized vessel, or development of a collateral circulation network.⁵ The degree of functional return to the hind limbs depends on the amount and rate of return of blood flow.^{6,7} The return of function to ischemic organs is paralleled in human patients

with acute myocardial infarction, noncardiogenic stroke, or cardiogenic embolism (including cardiogenic thromboembolic stroke-CTES), which is similar to SATE in cats.⁸⁻¹¹ To hasten return of blood flow, thrombolytic agents such as tissue-plasminogen activator (t-PA) have been used in humans and cats.⁷⁻¹²

Platelets contribute to thrombotic and thromboembolic disease, including SATE. Early mural thrombi are initiated by platelet adhesion to altered endothelial sites, whereas the emboli are composed of platelets within a fibrin network. Altered platelet aggregation has been identified in cats with underlying cardiac disease, along with reduced collateral flow around the site of embolization in response to platelet release products such as serotonin.¹³⁻¹⁷ Platelet aggregation has been studied in cats by use of a number of agonists including arachidonic acid, collagen, adenosine diphosphate (ADP), and epinephrine.¹⁸⁻²⁰ Antiplatelet agents including aspirin and ticlopidine, a thienopyridine derivative, consistently reduce feline platelet aggregation in response to arachidonic acid and both ADP and collagen, respectively.^{19,21} There is also evidence from studies in humans and animals that thrombolytic agents can induce platelet activation, which can reduce thrombolysis rates or promote rethrombosis.^{22,23} Some antiplatelet agents, including clopidogrel, enhance in vitro and in vivo thrombolysis through alteration of the structural properties of thrombi and reduce the rate of rethrombosis.²⁴⁻³¹

The antiplatelet drug clopidogrel^a is a thienopyridine derivative that alters platelet function in multiple species.^{25,32,33} Clopidogrel is a direct antithrombotic drug that irreversibly inhibits the ADP_{2Y12} receptor along the platelet membrane inhibiting primary and secondary platelet aggregation. Clopidogrel also inhibits the ADP-induced conformational change of the glycoprotein IIb/IIIa (GPIIb/IIIa) receptor complex along with reduced secretion of platelet release products such as serotonin.³⁴ There is also evidence that clopidogrel has vasomodulatory effects and impairs myointimal proliferation in vascular smooth muscle.^{35,36} In clinical trials in humans, clopidogrel has been found to significantly reduce the risk of stroke, myocardial infarction, and vascular death.^{37,38} The parent drug has essentially no antiplatelet effect but must undergo extensive hepatic metabolism to form at least 1 active metabolite.³⁹ For this reason, in vivo and ex vivo studies are required to evaluate the drug effect on platelets. Results of a recent study^b indicate that clopidogrel can significantly impair platelet function including reduce aggregation, prolong bleeding times, and reduce serotonin release in cats. These effects are

Received August 25, 2003.

Accepted December 22, 2003.

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Presented as a poster at the 21st Annual American College of Veterinary Internal Medicine Forum, Charlotte, NC, June 2003.

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seen when clopidogrel is administered at a dosage of 18.75 to 75 mg/cat, PO, every 24 hours, with maximal effects seen within 3 days.

The purpose of the study reported here was to determine whether clopidogrel enhanced the thrombolytic rate of t-PA on an in vitro feline whole blood thrombosis model.

Materials and Methods

Cats—Nine adult neutered (5 male and 4 female) domestic shorthair cats were used for this study. Dissolution of in vitro thrombi by t-PA was performed for each cat at baseline (before clopidogrel treatment) and after 3 days of clopidogrel treatment (75 mg, PO, q 24 h). The research protocol was approved by the Purdue Animal Care and Use Committee, and all cats were adopted at the end of the study.

Formation of thrombi—Whole blood thrombi were formed by modification of a previously reported technique.⁴⁰ Five milliliters of venous blood was collected by atraumatic jugular venipuncture by use of a 22-gauge needle drawn into sodium citrate (3.8%) at 9 parts blood to 1 part anticoagulant. One milliliter of citrated blood was transferred into a test tube into which 1 μ Ci of I¹²⁵-fibrinogen^c and 12.5 μ L of 1M calcium chloride were added and then mixed. Ten microliters of bovine thrombin^d (1,000 IU/mL) was added, mixed, and then quickly aspirated into a plastic disposable pipette. Thrombus formation could be detected through the clear plastic pipettes within 10 seconds. Thrombi were permitted to mature by incubating at 37°C for 1 hour. Autologous plasma was collected by centrifuging the remaining 4 mL of blood at 3,000 \times g for 5 minutes.

Dissolution of thrombi—Dissolution of the thrombi via t-PA was performed by modification of a previously reported technique.⁴⁰ The ends of disposable pipettes were cut to permit removal of the thrombi, which were then cut into approximately 1-cm lengths. Thrombi were washed 3 times in physiologic saline (0.9% NaCl) solution to remove unincorporated I¹²⁵-fibrinogen. Thrombi were placed into 1 mL of autologous plasma in a test tube and transferred to a 37°C water bath. The test tubes were placed into a scintillation counter,^e and total counts were recorded for 2 minutes to record total radioactivity within the thrombus (total counts in 2 minutes). To approximate the plasma concentration of t-PA in a clinical situation in a typical cat, 100 μ L of 1 mg/mL t-PA^f (0.1 mg) was added to the plasma.¹² These assumptions included a cat weighing 4.5 kg with a plasma volume of 30 mL/kg⁸ and t-PA administered at a dosage of 0.5 mg/kg/h, IV, for 4 hours. Fifty microliters of plasma (duplicate 25- μ L samples) was removed from the test tube at time 0 (t₀; before addition of t-PA) and every 30 minutes for 4 hours to measure the amount of released I¹²⁵-fibrin split products (I¹²⁵-FSP). Each time sample was placed in a scintillation tube and counted in the same manner as for total thrombus radioactivity (total counts in 2 minutes). Percentage thrombolysis was determined by taking the measured I¹²⁵-FSP per microliter from each 25- μ L plasma time sam-

ple and converting that to total released I¹²⁵-FSP within the autologous plasma by multiplying by the volume of plasma. The total released I¹²⁵-FSP was then divided by the total thrombus activity. Because the timed plasma samples were not placed back in the test tube, the volume of plasma was reduced by 50 μ L and the total radioactivity of the thrombus decreased by the total amount of released I¹²⁵-FSP (both 25- μ L samples) at each time point (equations 1 and 2).

First time sample:

$$Z = ([X/25 \mu\text{L}] \cdot 1,000)/Y$$

Subsequent time samples:

$$Z = ([X_n/25 \mu\text{L}] \cdot [1,100 - 50n]) / (Y - 2X_{n-1})$$

where Z is the percentage of thrombolysis, X is the measured I¹²⁵-FSP, Y is the total thrombus radioactivity, and n is the numbered time sample from 1 through 8.

Statistical analyses—Thrombolysis and sampling data were square root and log-transformed, respectively, to achieve normality and linearity. Mean percentage thrombolysis for the 9 study cats was calculated at each

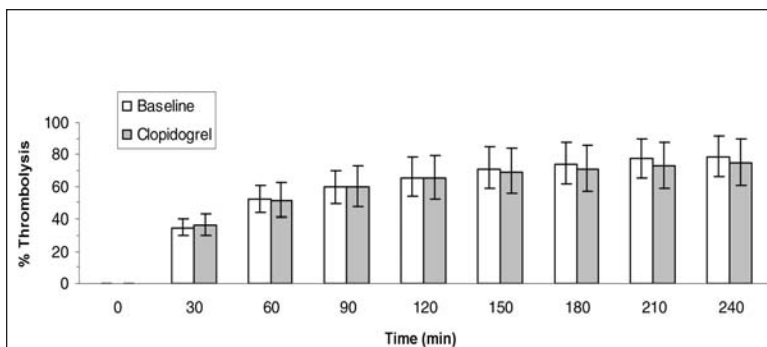


Figure 1—Mean percentage of in vitro thrombolysis before (baseline) and after cats were treated with clopidogrel (75 mg, PO, q 24 h for 3 days; n = 9). Bars represent 95% confidence intervals.

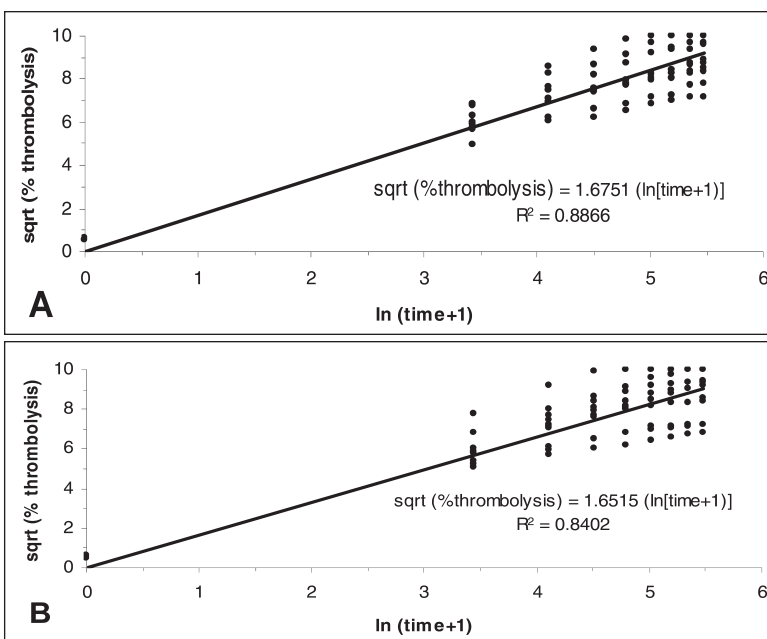


Figure 2—Association between the percentage of in vitro thrombolysis and time before (A) and after (B) cats were treated with clopidogrel (75 mg, PO, q 24 h, for 3 days; n = 9).

of the 9 sampling times, and 95% confidence intervals were estimated (± 1.96 SE) assuming normality. Composite thrombolysis data for all cats were used to calculate the time to 25%, 50%, and 75% thrombolysis (t_{25} , t_{50} , and t_{75} , respectively) for baseline and treatment data. Linear regression models were fit to the data^h by use of the following equation: $(\text{thrombolysis})^{0.5} = (a + b)(\ln[\text{time} + 1])$. The t_{25} , t_{50} , and t_{75} were predicted by solving the regression models for thrombolysis at 0.25, 0.50, and 0.75, respectively, and by use of regression coefficient (a and b) estimates. Differences in t_{25} , t_{50} , and t_{75} between values obtained at baseline and after treatment were tested for significance ($P < 0.05$) by construction of 95% prediction intervals.^h

Results

The thrombolysis pattern was consistent among the cats in which there was rapid thrombus dissolution within the first 30 minutes, followed by a slower, more linear pattern of progressive thrombolysis during the next 3.5 hours. This same pattern was seen in both the baseline and clopidogrel treatment periods (Fig 1). The t_{25} , t_{50} , and t_{75} were estimated from the lines-of-best-fit of the transformed data for each treatment period (Fig 2). There was no significant difference in the t_{25} , t_{50} , or t_{75} between baseline and clopidogrel treatment (Fig 3; Table 1).

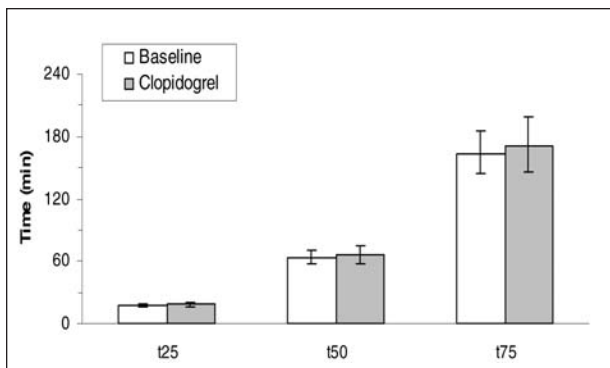


Figure 3—Estimates for the time to 25%, 50%, and 75% in vitro thrombolysis before (baseline) and after cats were treated with clopidogrel (75 mg, PO, q 24 h for 3 days; n = 9). Bars represent 95% confidence intervals.

Table 1—Mean (95% confidence interval) estimated time (minutes) to 25%, 50%, and 75% in vitro thrombolysis before (baseline) and after cats were treated with clopidogrel (75 mg, PO, q 24 h, for 3 days; n = 9)

Thrombolysis (%)	Baseline	Clopidogrel	P value
t_{25}	18.0 (16.7–19.4)	18.5 (16.8–20.3)	> 0.05
t_{50}	63.3 (57.1–70.3)	65.6 (57.8–74.4)	> 0.05
t_{75}	163.0 (143.7–186.0)	170.1 (145.9–198.2)	> 0.05

Discussion

In humans, cardiogenic embolism, including CTES, is similar to SATE in cats. The primary causes of these syndromes are blood stasis associated with cardiomyopathy, atrial fibrillation with left atrial dilatation, mitral stenosis with left atrial dilatation, and mural thrombi associated with acute myocardial infarction.^{41–47} The mural thrombi organize and superficial portions can break off which embolize to distant sites, including the brain, eyes, coronary circulation, spleen,

small or large intestines, and aortic bifurcation. Cardiogenic thromboembolic stroke is seen in 10% to 15% of patients with cardiomyopathy.⁴⁴ Platelets participate in the pathogenesis, but anticoagulants such as warfarin and heparin (both unfractionated and fractionated) are considered the treatment of choice to prevent primary and secondary CTES in humans.⁴⁸ Antiplatelet drugs can be used as adjunctive treatments; however, these agents are not recommended as the primary treatment to prevent CTES.⁴⁹ In veterinary medicine, clinical evidence appears to support this approach by the apparent lack of clinical efficacy of aspirin in the prevention of SATE in cats.^{5,7,12} A lack of clinical response is not unexpected because most studies^{49–52} demonstrating the clinical efficacy of aspirin in human medicine are targeted at preventing arterial thrombosis associated with high shear stress and platelet rich clots (PRCs), such as coronary arterial thrombosis and acute myocardial infarction, transient ischemic attacks, and noncardiogenic stroke. Platelet-poor clots are generally associated with blood stasis and are responsible for SATE and CTES, in which platelets are present within the thrombus or thromboembolus, but the primary component is fibrin.

However, it is well established that platelets do have a contributory role in SATE and CTES (as well as acute myocardial infarction, transient ischemic attacks, and noncardiogenic stroke), and this may represent a therapeutic focus. Platelets are responsible for the primary hemostasis plug and initiation of the intrinsic coagulation pathway through the formation of thrombin and other prothrombotic substances. Activated platelets also release many substances that impair thrombolysis, including the rapid inhibitor of t-PA, plasminogen activator inhibitor (PAI-1).⁵³ For these reasons, platelets shift homeostasis towards thrombosis and away from thrombolysis either at the site of thrombus formation or embolus occlusion. Antiplatelet drugs can be used as adjunctive treatments to impair the primary hemostasis aspect of thrombus formation, to shift homeostasis toward intrinsic thrombolysis, or to inhibit the platelet activation associated with extrinsic thrombolytic protocols.^{22,23} There is also evidence that some antiplatelet agents, including the thienopyridines such as clopidogrel, have a vasomodulating effect that may reduce the ischemic effect of such events.⁵⁴

Some antiplatelet agents either enhance thrombolysis or prevent reocclusion when combined with thrombolytic agents.^{24–31} However, there are some conflicting results from studies that may be explained by the model used. Collet et al²⁴ reported that the in vitro thrombolysis of PRC was enhanced by aspirin and the GPIIb/IIIa inhibitor abciximab. Results of that study indicated that platelets affect the structural properties of the PRC by increasing the permeability and viscoelasticity indexes and inducing the formation of platelet aggregates. Pretreatment of platelets with aspirin and abciximab before clot formation caused reduced indexes and enhanced thrombolytic rates. This was also seen when abciximab was added after clot formation, representing a more clinical scenario; however, it was time dependent, and the effect was lost about 10 minutes after clot formation. Aspirin did not affect thrombolysis after clot formation. These results were extremely different from

those of Bednar et al,²⁶ who found that aspirin actually impaired thrombolysis in an in vivo rabbit arterial model of thromboembolic stroke. These models are intrinsically different, and this could have influenced the results. Collet et al²⁴ used an in vitro model with a PRC and an IV preparation of aspirin. Bednar et al²⁶ used a whole blood thrombus model, which is neither platelet rich nor platelet poor. Platelets are incorporated into the clot, but RBCs and fibrin are the predominant components. The ability to recruit new platelets and the influence of the vascular endothelium are absent in in vitro models and could also result in contradictory results. Bednar et al²⁶ also evaluated ticlopidine, a thienopyridine derivative similar to clopidogrel, and found that there was no effect on thrombolysis rate but the size of the infarct was reduced when compared with t-PA alone or t-PA combined with aspirin. The reduced infarct size may have been caused by the then-unrecognized vasomodulating effect of the thienopyridines. In another study,²⁵ clopidogrel enhanced thrombolysis in an in vivo rabbit whole blood venous thrombosis model. Results of that study are different from those of Bednar et al²⁶ or the study reported here, which is difficult to explain; however, Herbert et al²⁵ used streptokinase and clopidogrel administered IV. Additionally, in the study reported here, we used a feline whole blood thrombus model, and there may be a species difference in response to this class of drug; however, this model was chosen because of the clinical importance of SATE in cats. There is also the unlikely possibility that all of the cats in this study were clopidogrel nonresponders. If this were true, an effect of clopidogrel would have not been seen because the cats acted as their own controls (baseline). However, results of a previous study^b performed in cats indicated that all cats had significant platelet inhibition at the dose of clopidogrel used here. Nonresponders have not been identified in other studies^{33,55} with different animal models. Gryglewski et al⁵⁶⁻⁵⁸ reported that thienopyridine compounds (including ticlopidine and clopidogrel) have a thrombolytic effect when administered IV, which is believed to be caused by the stimulated release of prostacyclin, t-PA, and possibly nitric oxide from endothelial cells. Obviously, these effects would be precluded by the use of our in vitro model.

Clopidogrel did not influence the rate of thrombolysis in this in vitro feline thrombosis model. The effect of using an in vivo model, in which additional platelets could be recruited and the influence of the vascular endothelium could be exerted, is unknown. Clopidogrel may also have a greater effect in a PRC model, in which there is a greater influence of platelets. This is of greater interest to human than veterinary medicine, where clopidogrel is commonly used to prevent acute myocardial infarction, transient ischemic attacks, and noncardiogenic stroke, and warrants further investigation.

^aPlavix, Bristol-Meyer-Squibb, New York, NY.

^bHogan DF, Andrews DA, Talbott K, et al. The pharmacodynamics and platelet responses to clopidogrel in the cat (abstr). *J Vet Intern Med* 2003;17:409.

^cAmersham Biosciences, Piscataway, NJ.

^dGenTrac Inc, Middleton, Wis.

^eGamma Counter 7000, Organon Teknika Corp, Durham, NC.

^fActivase, Genentech Inc, San Francisco, Calif.

^gHogan DF, Sisson DD, Solter PF. Characterization of plasma volume and neuroendocrine status in renal hypertensive cats, in *Proceedings*. 17th Annu Am Coll Vet Intern Med Forum 1999;715.

^hStatistix, version 7.0, Analytical Software, Tallahassee, Fla.

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