Temperature Affects Thrombolytic Efficacy Using rt-PA and Eptifibatide, an *In Vitro* Study

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The potential for hypothermia as a neuroprotectant during stroke has led to its increase in clinical use. At the same time, combination pharmaceutical therapies for ischemic stroke using recombinant tissue plasminogen activator (rt-PA), and GP IIb-IIIa inhibitors, such as Eptifibatide (Epf), are under study. However, there is little data on how the reactions triggered by these agents are impacted by temperature. Here, clot lysis during exposure to the combination of rt-PA and Epf is measured in an *in vitro* human clot model at hypothermic temperatures. The hypothesis is that lytic efficacy of rt-PA and Epf decreases with decreasing temperature. Whole blood clots from 31 volunteers were exposed to rt-PA (0.5 $\mu\text{g}/\text{mL}$) and Epf (0.63 $\mu\text{g}/\text{mL}$) in human fresh-frozen plasma (rt-PA+Epf), rt-PA alone in plasma (rt-PA Alone), or to plasma alone (Control), at temperatures from 30°C to 37°C, for 30 minutes. Clot lysis was measured using a microscopic imaging technique; the mean fractional clot loss (FCL) at 30 minutes was used to determine lytic efficacy. Temperature had a significant impact on FCL in clots exposed to rt-PA+Epf, with the FCL being lower at 30°C to 36°C than at 37°C. The FCL remained significantly higher for rt-PA+Epf-treated clots than Controls regardless of temperature, with the exception of measurements made at 30°C when no significant differences in the FCL were observed between groups. The use of hypothermia as a neuroprotectant may negatively impact the therapeutic benefit of thrombolytic agents.

Introduction

STROKE IS RESPONSIBLE for 134,000 deaths annually in the United States (Roger *et al.*, 2012). Therapeutic hypothermia has demonstrated neuroprotective properties in cardiac arrest and brain injury by decreasing intracranial pressure, slowing the opening of the blood-brain barrier, and reducing inflammation (Diller and Zhu, 2009; Lebedez *et al.*, 2011). Therapeutic hypothermia is therefore under investigation for its utility in ischemic stroke (Meden *et al.*, 1994; De Georgia *et al.*, 2004; Jiang *et al.*, 2006; Qiu *et al.*, 2007; Abdullah and Husin, 2011; Di Lazzaro *et al.*, 2011; Donnan *et al.*, 2011; Zhao and Steinberg, 2011). The 2004 Cooling for Acute Ischemic Brain Damage (COOL AID) study showed that hypothermia is feasible for acute ischemic stroke patients, though it did not evaluate impact on patient outcome (De Georgia *et al.*, 2004). A more recent pilot study noted that ischemic stroke patients who were treated with hypothermia for 24 hours beginning 48 hours after presentation had improved functional outcome

after 1 year than normothermic patients (Abdullah and Husin, 2011). Di Lazzaro *et al.* (2011) demonstrated that hypothermia can reduce the size of stroke-induced infarct in the brain, even when initiated 3 days after the stroke. While optimal parameters of therapeutic hypothermia, including the cooling rate and rewarming strategy, have yet to be determined (Zygun, 2007; Groysman *et al.*, 2011; Maas and Stocchetti, 2011), there is general consensus that neuroprotective benefits are maximized when hypothermic treatment is started as quickly after the initial stroke as possible and is maintained for a long duration (Maas and Stocchetti, 2011; Takeuchi *et al.*, 2011; Zgavc *et al.*, 2011).

Regardless of the use of hypothermia, recanalization of the occluded blood vessel is an essential step in the treatment of stroke. Recanalization is typically achieved by interventional mechanical clot removal or by use of a lytic therapy such as recombinant tissue plasminogen activator (rt-PA) (Connors, 2002; Brekenfeld *et al.*, 2005; Jahan and Vinuela, 2009; Zgavc *et al.*, 2011; Cohen *et al.*, 2012). Researchers are attempting to

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enhance the lytic efficacy of rt-PA in stroke by combining it with additional therapies, such as ultrasound, plasminogen, and GP IIb-IIIa inhibitors (Adams *et al.*, 1996; Alexandrov *et al.*, 2004; Meunier *et al.*, 2007, 2009, 2011). As an accepted approach to lysis in acute coronary syndromes, the combination of rt-PA and GP IIb-IIIa inhibitors, such as Eptifibatide (Epf), is being explored. The mechanism of improved efficacy of the combined approach is that while rt-PA cleaves fibrin in the fibrin mesh into fragments, the GP IIb-IIIa inhibitor blocks the GP IIb-IIIa receptor in platelets, reducing binding of platelets to each other and to fibrin (Hankey and Eikelboom, 2003; Cannon, 2006; Qureshi *et al.*, 2006; Adams *et al.*, 2008; Pancioli *et al.*, 2008; Cincinatti, 2009). Both *in vitro* and *in vivo* studies have shown preliminary success for rt-PA/GP IIb-IIIa therapies (Atar *et al.*, 2001; Collet *et al.*, 2002; Pancioli *et al.*, 2008; Meunier *et al.*, 2009; Shaw *et al.*, 2010). With increased efficacy comes the possibility that the concentration of rt-PA needed to cause clot lysis can be reduced (Shaw *et al.*, 2010; Meunier *et al.*, 2011), reducing the risk of rt-PA-induced intracerebral hemorrhage (NINDS, 1995; Patel and Mody, 1999; Tanne *et al.*, 1999). The Combined Approach to Lysis Utilizing Epf and Recombinant Tissue Plasminogen Activator (CLEAR) trial demonstrated that reduced dose of rt-PA combined with Epf can be used in place of regular-dose rt-PA with no decrease in clinical outcome (Pancioli *et al.*, 2008). The use of a combination therapy that maintains or increases efficacy while potentially decreasing the amount of rt-PA administered is very attractive for clinical use. There is also growing interest in combining these pharmaceutical options with hypothermia so that both recanalization and neuroprotection can be achieved.

A serious consequence to the use of hypothermia is that it can decrease the physiological response to certain drugs, including the only currently FDA-approved therapy for acute ischemic stroke, rt-PA (Yenari *et al.*, 1995; Adams *et al.*, 1996; Shaw *et al.*, 2007; Nielsen and George, 2011; Van Der Pals *et al.*, 2011). Despite the possibility of decreased efficacy of lytic agents, clinical research on hypothermia continues (Martin-Schild *et al.*, 2009; Hemmen *et al.*, 2010; Clifton *et al.*, 2011; Di Lazzaro *et al.*, 2011), and the effect of hypothermia on combination therapies has yet to be explored. There is a clear need to better understand how hypothermia impacts the lytic efficacy of pharmaceutical agents being used for treating stroke in routine care and in clinical trials. In this work, we report on the lytic efficacy of combined rt-PA and Epf treatment at temperatures representative of hypothermia using an *in vitro* human clot model. Our hypothesis is that hypothermia inhibits the lytic efficacy of this combined therapy.

Materials and Methods

Preparation of rt-PA, Epf, and human plasma

The rt-PA was obtained from the manufacturer (rt-PA, Activase®; Genentech, San Francisco, CA) as a lyophilized powder. Each vial was mixed with sterile water to a concentration of 1 mg/mL as per manufacturer's instructions, aliquoted into 1.0 mL centrifuge tubes (Model 05-408-13; Fisher Scientific Research, Pittsburgh, PA), and stored at -80°C . The enzymatic activity of rt-PA is stable for years when stored in this fashion (Jaffe *et al.*, 1989; Shaw *et al.*, 2009). Epf was obtained (Integrilin®; Millennium Pharmaceuticals, Inc., Cambridge, MA) as a solution at a concentration of 2 mg/mL. The

drug was stored at 4°C to 5°C to prevent degradation, as per the manufacturer's instructions. Human fresh-frozen plasma (hFFP) was procured from a blood bank in 250–300 mL units. Each unit was briefly thawed, aliquoted into 50 mL polypropylene centrifuge tubes (Model 05-538-68; Fisher Scientific Research), and stored at -80°C . Aliquots were allowed to thaw for trials; unused plasma, rt-PA, and Epf were discarded following completion of each trial.

Production of blood clots

Human whole blood was obtained from healthy volunteers by sterile venipuncture following local Institutional Review Board approval and written informed consent. The only exclusion criterion was the use of aspirin within 72 hours of the blood draw. Blood from a total of 31 subjects was used in these studies.

Samples of 1–2 mL were placed in sterile glass tubes (3 mL; VWR, Batavia, IL) and allowed to form clots in and around a small-diameter ($\sim 600\ \mu\text{m}$) micropipette (Becton, Dickinson and Company, Franklin Lakes, NJ; 20 λ) through which a segment of 7-0 silk suture (Ashaway Line and Twine Mfg. Co., Ashaway, RI) had been threaded. This is similar to clot production methods used in imaging studies by Winter *et al.* (2005) and Yu *et al.* (2000). The clots were incubated for 3 hours at 37°C , and refrigerated at 5°C for 3 days ensuring maximal clot retraction, lytic resistance, and stability (Loren *et al.*, 1989; Francis and Totterman, 1995; Shaw *et al.*, 2006). Before each trial, the micropipette was removed to produce a cylindrical clot adherent to the suture. The clot was typically 5–8 μL in volume and approximately 300 μm in width. At this size, the clots were similar in diameter to the intracerebral segments of the middle cerebral arteries (80 to 840 μm in diameter) as well as cerebral vessels, such as the recurrent artery of Heubner and its perforators ($643 \pm 237\ \mu\text{m}$ in diameter) (Marinkovic *et al.*, 1958; Tao *et al.*, 2006).

Visualization of the clot

For each trial, the clot attached to the suture was placed in a clean micropipette (Drummond Scientific Company, Broomall, PA; 200 μL), and inserted into a U-shaped sample holder composed of hollow luer lock connectors and silicone tubing (Cole Parmer, Vernon Hills, IL; outer diameter 0.125"). The sample holder was placed in an acrylic tank filled with water. Temperature was maintained during all trials using a heating element (Hagen, Mansfield, MA; 50 W). Within a trial, temperature was stable to within $\pm 0.5^{\circ}\text{C}$ of the set temperature. Set temperatures were at defined intervals from 30°C to 37°C . This range was selected to include "minimal" (35°C), "mild" (32°C – 34°C), and "moderate" (28°C – 32°C) hypothermia (Liu and Yenari, 2007; Zgavc *et al.*, 2011). Animal studies suggest that mild hypothermia tends to be tolerated best and is more effective at reducing infarct size than lower temperatures (Maier *et al.*, 1998; Huh *et al.*, 2000; Kollmar *et al.*, 2007; Zgavc *et al.*, 2011), though moderate hypothermia can also successfully reduce infarct size (Meden *et al.*, 1994); thus, the lower limit for temperatures was selected as 30°C to ensure complete coverage of the mild hypothermia range (Zgavc *et al.*, 2011).

The tank was placed over the objective of an inverting microscope (Olympus, Melville, NY) to visualize the clot. The field of view in the image was $340\ \mu\text{m} \times 260\ \mu\text{m}$ (640 pixels $\times$ 480 pixels). The entire apparatus was placed on top of a

vibration isolation table (Newport, Irvine, CA; XL-G) for mechanical isolation (Cheng *et al.*, 2005). Images were recorded at 6 frames/min using a CCD camera (Hitachi, Woodbury, NY; KP-M1A), and data were stored for later analysis on a computer (Dell, Round Rock, TX; Intel Pentium). A complete description of the imaging apparatus has been previously provided (Cheng *et al.*, 2005; Meunier *et al.*, 2007).

Determination of lytic efficacy

Light intensity transmitted through the sample clot is reduced with increased clot thickness or clot density. The CCD camera records image light intensity $I(x, z)$ at each pixel (x, z) where z is along the axis of the clot and x is along the clot diameter. By analyzing the light intensity in each pixel, the clot edges can be identified, thus enabling measurement of clot width. In brief, an edge detection routine written in Matlab 6.5 R13 (Mathworks, Inc., Natick, MA) is used to determine the positions of the two clot-plasma interfaces. The average clot width for each image was calculated by averaging the width over all z values. The clot width data were subsequently corrected for the average suture width used in these experiments ($95 \pm 15 \mu\text{m}$, based on analysis of 252 samples of the 7-0 suture), and normalized to the initial value of the clot width during the first minute (the average over the first six frames). Note that the mean of this corrected normalized clot width (CW_{NORM}) is equal to 1 during the first minute, and becomes equal to zero if the clot is totally lysed, thus leaving only the suture visible. The fractional clot loss (FCL, expressed as %) is defined as

$$\text{FCL} \equiv [1 - CW_{\text{NORM}}(30 \text{ minutes})] \times 100\% \quad (1)$$

where CW_{NORM} (30 minutes) is the average normalized clot width over the final minute of treatment. A complete description of these image analysis methods can be found in previous works (Meunier *et al.*, 2009, 2011; Shaw *et al.*, 2010).

Experimental protocol

Clots from a given donor were randomly allocated to combinations of one of five temperatures (30°C, 32°C, 34°C, 36°C, or 37°C) and one of three treatment regimes: (1) hFFP alone (Control); (2) rt-PA (0.50 $\mu\text{g}/\text{mL}$) in hFFP; or (3) combined rt-PA (0.50 $\mu\text{g}/\text{mL}$) and Epf (0.63 $\mu\text{g}/\text{mL}$) in hFFP. The dosages rt-PA and Epf used in this study were chosen based on the results of an optimization study of rt-PA and Epf concentrations: 0.50 and 0.63 $\mu\text{g}/\text{mL}$ of rt-PA and Epf, respectively, provided maximum lytic efficacy while minimizing drug volume at 37°C (Shaw *et al.*, 2010). This specific

combination of concentrations is also very similar to the average estimated plasma concentrations used in the ongoing "CLEAR in Acute Ischemic Stroke-Enhanced Regimen" (CLEAR-ER) trial: 0.6 mg/kg rt-PA (15% bolus, 85% infused over 1 hour) and 135 $\mu\text{g}/\text{kg}$ bolus of Epf coupled with an infusion of 0.75 $\mu\text{g}/\text{kg} \cdot \text{min}$ for 2 hours (Cincinnati, 2009; Shaw *et al.*, 2010). We planned to perform trials using blood from at least three subjects for a total of 10 clots for each of the 10 temperature/treatment combinations; this was found to be adequate to observe meaningful effects in our previous work (Shaw *et al.*, 2010; Meunier *et al.*, 2011). The sample size is also comparable to *in vitro* clot lysis experiments by others (Nedelmann *et al.*, 2005; Hardig *et al.*, 2008).

Individual trials began by slowly injecting 1 mL of hFFP (Control), or 1 mL of hFFP containing rt-PA alone or rt-PA and Epf, into the sample holder. At time t equal to zero, the solution was in contact with the clot. Removing the syringe exposed the ends of the sample holder to atmospheric pressure and the clot surface to a static fluid column. Clots were exposed to a specific treatment regimen for 30 minutes; previous studies have shown that the majority of thrombolysis occurs within a 30-minute window (Meunier *et al.*, 2007; Shaw *et al.*, 2008).

Statistical analysis

Data are presented as mean FCL values with 95% confidence intervals (CIs). Comparisons between groups used two-way analysis of variance with temperature and treatment effects. *Post hoc* Bonferroni-adjusted *t*-tests were used to compare between conditions. Also, analysis was performed using a mixed effects model with clot subject as a random effect, trial condition (no drugs, rt-PA alone, or rt-PA and Eptifibatide) as a fixed effect, and temperature as a covariate.

Results

A total of 416 clots were analyzed in this study. The number of clots and subjects for each treatment group at each temperature is given in Table 1. On average, 28 clots (range: 10–222) from six subjects (3–29) were used in each group. Figure 1 shows the mean and 95% CIs of the mean FCL for the three treatment groups at each of the five temperatures. For clots at 37°C, introduction of rt-PA and Epf increased the FCL from 14% (95% CI: 13–15) to 65% (57–72). As the temperature decreases, the FCLs for the clots treated with rt-PA and Epf decrease, to a minimum of 25% (95% CI: 20%–30%) at 30°C. FCLs for the control group, however, remain relatively constant, changing only slightly to a value of 24% (19%–28%) by 30°C. The analysis of variance measure demonstrated that at every temperature,

TABLE 1. MEAN FRACTIONAL CLOT LOSS (%) WITH STANDARD DEVIATIONS AND NUMBER OF SUBJECTS (N_{SUBJECT}) AND CLOTS (N_{CLOT}) FOR CLOT LYSIS MEASUREMENTS

Treatment group	Temperature (°C)				
	30	32	34	36	37
Control	24%, (17), 4/10	19%, (8), 6/13	14%, (13), 7/16	19%, (8), 4/17	14%, (12), 29/222
rt-PA	32%, (22), 3/16	31%, (21), 5/19	20%, (16), 3/10	38%, (25), 3/13	44%, (36), 6/14
rt-PA + Epf	25%, (23), 4/12	44%, (27), 6/14	37%, (23), 6/16	43%, (37), 4/12	65%, (32), 4/12

Values given are mean FCL (%), (SD), $N_{\text{subject}}/N_{\text{clot}}$.

rt-PA, recombinant tissue plasminogen activator; Epf, Eptifibatide; FCL, fractional clot loss; SD, standard deviation.

FIG. 1. Fractional clot loss (FCL) as a function of temperature with human fresh-frozen plasma (hFFP) alone (Control), 0.5 $\mu\text{g}/\text{mL}$ recombinant tissue plasminogen activator (rt-PA) in hFFP (rt-PA Alone), and 0.5 $\mu\text{g}/\text{mL}$ rt-PA and 0.63 $\mu\text{g}/\text{mL}$ Eptifibatide (rt-PA + EpF) in hFFP. Error bars denote 95% confidence intervals. *FCL (rt-PA + EpF) $\geq$ FCL (Control), $p \leq 0.05$. **FCL (rt-PA + EpF) equivalent for $T = 32^\circ\text{C}$ – 36°C , $p > 0.1$.

the FCLs for the clots treated with rt-PA and EpF were statistically significantly greater than for the control group, with the exception of 30°C ($p = 0.4$). The FCLs for the clots treated with rt-PA and EpF at 32°C – 36°C were statistically similar ($p > 0.1$), and are significantly smaller than when treated at 37°C and larger than at 30°C . The FCLs for the control group were unaffected by temperatures between 32°C and 37°C .

The mixed effects model considers temperature as a continuous variable, instead of a categorical variable as in the previous analysis. The findings of the mixed effects model are that the effect of temperature is significant, and the temperature effect is different for the different conditions.

Discussion

Our data show an effect of temperature on the lytic efficacy of combined rt-PA and EpF: treatment efficacy drops with decreasing temperature in this *in vitro* human clot model, similar to the findings of Yenari *et al.* (1995). Clot lysis with rt-PA and EpF, characterized as FCL, has maximum effect at body temperature (37°C). At each temperature, the combination of rt-PA and EpF is equivalent or superior in lytic efficacy to hFFP alone. Similar effects are seen with rt-PA alone. Clot lysis without pharmaceutical agents does not seem to have a marked dependence on temperature except at 30°C .

These results show that within the 32°C – 36°C temperature range, there is no statistical evidence for a temperature effect on the combination of rt-PA and EpF. This suggests that any temperature decrease to within this range *in vivo* will impact lytic efficacy of this treatment similarly. We do note that in this study, just below this range of statistical equivalence, at 30°C , the efficacy of combination therapy is equivalent to control. Despite its ability to reduce stroke-induced infarct size (Meden *et al.*, 1994), moderate hypothermia may well inhibit the action of rt-PA and EpF. This is in addition to the potentially fatal arrhythmias that can develop during hypothermia, increasing in likelihood as temperature drops below 30°C (Granberg, 1991), but which are not observed at higher temperatures (Lebiedz *et al.*, 2011). Hypothermia can result in additional complications as a result of reduced blood flow (Wong, 1983; Zgavc *et al.*, 2011). It remains to be seen whether the potential neuroprotective benefits of hypothermia make the reduced efficacy of rt-PA and EpF worth the trade-off.

As discussed previously, the specific concentrations of rt-PA and EpF used in this study maximize lytic efficacy *in vitro* while minimizing total drug volume, and are comparable to those used in clinical trials (Pancioli *et al.*, 2008; Cincinatti, 2009; Shaw *et al.*, 2010). Despite the strong results shown here, it remains to be seen whether this specific combination of drugs, optimized at 37°C , will also be optimal at lower temperatures, or if a different combination of concentrations would do better. However, a study by Yenari *et al.* suggests that no adjustment in drug concentration may be necessary at lower temperatures. In the study, rabbit arterial blood that had been allowed to clot for 24 hours was exposed to rt-PA at one of four concentrations (0, 0.3, 1.0, and 3.0 $\mu\text{g}/\text{mL}$) at temperatures of 25°C , 30°C , 33°C , 35°C , 37°C , or 40°C ; clot mass was measured before and after treatment, and clot mass loss was calculated (Yenari *et al.*, 1995). Results showed that clot lysis was dependent on rt-PA concentration and temperature, but the temperature effect was independent of rt-PA concentration (Yenari, 1995). The dependence of clot lysis on rt-PA concentration is confirmed by other studies (Shaw *et al.*, 2008, 2010; Meunier *et al.*, 2011). More importantly, though effects of combination with EpF were not examined, the work by Yenari *et al.* suggests that the effect of temperature on lytic efficacy of combination therapy will be similar over a range of concentrations. Thus, it is possible that a combination of drug concentrations that is “optimized” at 37°C could still give “optimal” lytic results (maximum lysis for minimum drug volume) at lower temperatures, when compared with other combinations.

Adjuvant therapy such as ultrasound can overcome temperature-induced decreases in rt-PA-driven lysis. Shaw *et al.* (2006) demonstrated that clot mass loss due to rt-PA is not significantly affected by a drop in temperature from 37°C to 33°C when ultrasound is delivered concurrently. Conversely, other studies by Meunier *et al.* (2009) and Atar *et al.* (2001) have found that adding ultrasound to the combination of rt-PA and GP IIb/IIIa inhibitors does not increase total lysis, and may even diminish the absolute amount of clot lysis. Even if the magnitude of lysis is diminished, ultrasound typically increases the rate at which the clot lyses (Meunier *et al.*, 2007, 2009). While the mechanisms by which ultrasound increases the thrombolytic rate are not fully elucidated, the proposed increased diffusion of lytics into the clot, the induction of

stable cavitation, or the induction of microstreaming vortices within the plasma (Polak, 2004; Hitchcock *et al.*, 2011) may contribute to the capacity to increase the efficacy of combination pharmaceutical therapies at low temperatures. Another possible measure for overcoming the decreased efficacy of rt-PA combined with Epf at low temperatures is the possibility of increasing drug dose; if hypothermia diminishes the therapeutic benefit, there is also the potential that it decreases the side effects, including hemorrhage. As investigators explore the use of therapeutic hypothermia in stroke, they must consider strategies to ensure adequate recanalization.

The primary limitations of this work include the fact that the *in vitro* clots are not subjected to blood flow and are therefore in a different environment than that of an *in situ* thrombus. Second, the clots are human whole blood clots and are likely different in composition to acute arterial thrombus. Third, these clots are made from the blood of healthy donors thus limiting the applicability of the results here to the more chronically ill acute stroke or myocardial infarction population. Finally, the reproducibility of the results of these *in vitro* studies in an *in vivo* setting remains to be seen.

It has been shown in this work that the lytic efficacy of combined rt-PA and Epf is dependent upon temperature in an *in vitro* human clot model. This information may prove useful in planning future clinical trials of combination lytic therapy with hypothermia in ischemic stroke, and demonstrate the importance of considering the effects of hypothermia on therapeutic activity of any medications being delivered to a patient.

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Disclosure Statement

The authors declared that no competing financial interests exist.

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