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Effect of Clot Aging and Cholesterol Content on Ultrasound-Assisted Thrombolysis

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Abstract Exposure to 2-MHz transcranial diagnostic ultrasound enhances the thrombolytic activity of intravenously administered tissue plasminogen activator (IV-tPA) in acute ischemic stroke (sonothrombolysis). However, rates of arterial recanalization vary widely, depending upon the clot burden, its location, and stroke subtype. We evaluated the influence of age and cholesterol level of the blood clots on sonothrombolysis in an in vitro model. To “age” the clots, serum was replaced by fresh blood periodically. We increased the cholesterol content of the clots by adding cholesterin to the blood. The clots were lysed by tPA and/or transcranial Doppler ultrasound sonication for 1 h. The extent of thrombolysis induced by various treatment protocols (controls, sonication, tPA, and sonothrombolysis) was evaluated with relative changes in the clot weights and in the clot structure by scanning electron microscopy (SEM) at end of the experiment. Sonothrombolysis induced significantly higher weight reduction in fresh clots (37.3 % in 2-h old clots versus 24.8 % in 10-h ones, \( p < 0.005 \)) as well as the clots with higher cholesterol levels (41.7 versus 30.6 % in normal cholesterol clots, \( p < 0.005 \)). SEM demonstrated patterns of clot dissolution among various treatment modalities. Sonothrombolysis induced better clot lysis in fresh thrombi with high cholesterol levels.

Keywords Stroke · Sonothrombolysis · Clot aging · Cholesterol

Introduction

Stroke is the second leading cause of death as well as permanent disability. The World Health Organization suggests that stroke death has increased over the years from 9.7 % in 2004 to 10.8 % in 2008 [1]. Stroke causes a rapid loss of brain functions due to the disturbance in the blood supply and can be classified into two major categories: ischemic and hemorrhagic. Ischemic strokes are caused by interruption of the blood supply, while hemorrhagic ones result from rupture of a blood vessel or an abnormal vascular structure [2]. Eighty-seven percent of strokes are ischemia caused by blood thrombus that occurs due to fibrinogen: fibrin transformation catalyzed by thrombin, calcium, and other cofactors in the blood. Fibrin monomers then polymerize to form fibrin mesh which encapsulates blood cell components to form viscoelastic blood clots [3]. Middle cerebral artery (MCA) occlusion is the most common among stroke patients since MCA is the longest cranial artery, but smaller in size [4].

Thrombolysis with intravenously administered tissue plasminogen activator (IV-tPA) or recombinant tPA (i.e., alteplase, Boehringer Ingelheim), whose dose is determined by patient’s body weight, is the only approved drug treatment for acute ischemic stroke (AIS) [5]. Prompt treatment is associated with higher chances of better functional outcome in AIS, which was demonstrated in a recently pooled analysis of various trials of IV-tPA with a 3-month outcome as 2.55 [95 % confidence intervals (CI) 1.44–4.52] for 0–90 min, 1.64 (95 % CI 1.12–2.40) for 91–180 min, and 1.34 (95 % CI 1.06–1.68) for 181–270 min in favor of the tPA group [6]. However, because of the short time window, the rate of treatment in the USA and Europe is only 2–5 % [7].
Ultrasound exposure of the occluded intracranial artery during IV-tPA infusion (sonothrombolysis) is a promising approach to facilitate recanalization in AIS. The CLOTBUST phase II trial included AIS patients treated with IV-tPA within 3-h of symptom-onset. Patients were randomized to receive either 2-h of continuous 2-MHz transcranial Doppler (TCD) ultrasound exposure or placebo [8]. Forty-nine percent of complete recanalization or dramatic clinical recovery occurred within 2 h after the administration of tPA bolus as compared to 30 % in the control group ($p=0.03$). A recent meta-analysis of six randomized ($n=224$) and three nonrandomized ($n=192$) studies demonstrated the advantage of sonothrombolysis in achieving complete recanalization (37.2 %; 95 % CI 26.5–47.9) compared with patients treated with tPA alone (17.2 %; 95 % CI 9.5–24.9) [9].

To improve vessel recanalization, a clear understanding on the clot characteristics is necessary. A thrombus usually forms around fresh atherosclerotic plaques. In contrast, an arterial embolus, an aged thrombus from the heart (especially in atrial fibrillation), travels in the arterial bloodstream. Although some in vitro experiments have already shown the effect of clot aging on the thrombolysis [10, 11], the method of leaving the sample alone in the incubator or refrigerator may not be able to mimic thrombus growth in the circulating system. Apart from the clot age, numerous factors influence the rates of clot dissolution and arterial recanalization induced by IV-tPA. Racial differences in coagulation of clot dissolution and arterial recanalization induced by IV-tPA are considered such as higher plasma concentrations of fibrinogen and plasminogen activator inhibitor among Caucasians are believed to contribute towards the higher rates of clot dissolution and arterial recanalization due to the residual fibrinogen from the replaced fresh blood clots [12]. Furthermore, higher proportion of cardioembolism among Asians is believed to contribute towards the higher rates of recanalization due to the “soft and fibrin-rich” blood clots [13]. Another important factor that influences clot dissolution by IV-tPA is cholesterol, an adherent sticky substance that increases the viscosity of blood as well as platelet aggregation [14]. Most of the morbidity and mortality consequences of thrombotic events intimately related to the development and growth within the arterial wall of lipid deposits (i.e., plaques) that are rich in cholesterol and cholesteryl esters [15]. Atheromatous embolism in the brain is one of the mechanisms leading to transient ischemic attacks and strokes. Therefore, stroke patients have a higher level of low density and very low density lipid cholesterol compared to healthy people [16].

In this study, various types of blood clots were prepared with different age and cholesterol concentration using a new method. Rheological properties of blood clot, elastic nature, and shear strength, were studied. Blood clots with 1 mg/ml cholesterol had higher shear strength (185.4±89.1 Pa) than the normal sample (108.2±34.3 Pa, $p<0.05$). Although there was no difference of thrombolysis among blood clots with ultrasound or tPA alone, combination of these modalities induced significant lysis in the clots with cholesterol levels of more than 0.5 mg/ml (41.7±2.3 %) as compared to normal clots (30.6±4.1 %, $p<0.05$). Altogether, sonothrombolysis seems to work better in fresh thrombi with higher cholesterol levels.

Materials and Methods

Blood Clot Preparation

To prepare blood clot, 0.25 ml of 100-mM calcium chloride (Sigma-Aldrich, Singapore) was mixed with 1 ml of anticoagulated horse blood (i-DNA Biotechnology, Singapore), which was used due to its commercial availability in Singapore and low cholesterol level (0.75–1.5 mg/ml). One minute later, 1.25 ml of the mixture was injected into a silicone tube (3 mm of inner diameter, Versilic, France) whose dimensions match the adult human’s MCA. Precautions were taken to avoid the introduction of air bubbles in the mixture. The silicone tube was then stored in an incubator (Lab Companion-JeioTech, Seoul, Korea) at 37ºC for a stable clot formation.

Clot Aging

To simulate the aging process of thrombus in the circulating system, serum from blood clot was carefully removed using a 3-ml Luer-lock syringe (Terumo, Singapore) with a 22G needle, and equivalent amount of fresh blood was added into the silicone tube, which was repeated bi-hourly for 10 h. So, keeping the serum at a minimum level as proposed in our method could avoid the clot retraction [17]. In addition, because of the availability of fibrinogen from the replaced fresh blood, the clot formation occurs continuously as in vivo. Subsequently, the size and weight of blood clot increase.

Clot Cholesterol Content

For assessing the relationship between tPA or tPA plus ultrasound-induced clot lysis and clot cholesterol levels, we added 0.5 and 1 mg of cholesterol, which possesses properties like low-density lipoproteins, to 1 ml of whole blood that represented an increase in the cholesterol level by 0.5 and 1 mg/ml, respectively. The borderline high risk for heart disease in the human being is about 2–2.4 mg/ml.

Measurement of Clot Elasticity

Shear strength of blood clot was measured using a rheometer (MCR 501, Anton Paar GmbH, Austria) on a 25-mm diameter parallel plate under constant vibratory [18, 19]. Blood clots in diameter of 25 mm and thickness of 1 mm were carefully taken out of the mold after 2-h incubation, placed exactly below the rheometer plate, and gently pressed to remove
excess serum. Rheological properties such as storage modulus and loss modulus were measured from samples with variable thickness (0.1, 0.2, 0.3, and 0.4 mm) using a constant strain (5 %) every 10 s for 2 min, from which mean of the stable values was used for calculations of shear strength.

Tensile modulus of the blood clot (70 mm in length and 3 mm in diameter) was measured using an electromechanical system (5566, Instron, USA) with a 50-N load cell [19]. Ends of the blood clot were attached to the center of the rubber pad, fixed and compatible to the grips. Experiments were performed at a constant strain rate of 5 and 10 mm/min in extension, respectively. Tensile modulus was then calculated from the stress–strain relationship (Bluehill, Instron).

Experimental Setup

After incubation, blood clots were removed from the silicone tube using a needle, washed thrice with isotonic phosphate buffered saline (PBS), and then dried using Whatman filter paper (VWR, Singapore), and its weight was measured by a digital balance (SBC-31, Scaltec Instruments GmbH, Germany) with a resolution of 0.1 mg. Fifty microliters of tPA (10 μg, Fitzgerald Industries, USA) was added to a 2-ml microcentrifuge tube (Greiner Bio-One, Germany) filled with 950-μl PBS to achieve the concentration of 0.5 μg/ml. Blood clot was then put into the tube, which was locked with the cap and immersed horizontally into a water bath (BS-06, Jeio Tech, Korea) at a consistent temperature of 37ºC (see Fig. 1). One hour later, the blood clot was taken out, washed with PBS thrice, and dried with Whatman filter paper to measure its weight. Thrombolysis efficiency was determined as the percentage of the loss of blood clot mass using different protocols with respect to its initial weight.

TCD ultrasound bursts (Multi-Dop, Compumedics DWL, Germany) were delivered to the blood clot placed 45 mm away from the probe, which is the average distance from the temporal bone to MCA. TCD parameters were as follows: frequency 2 MHz, Ipsita 340 mW/cm², sample volume 6 mm, and power output 100 %. A piece of temporal bone from human cadaver skull was also placed between the blood clot and TCD probe to simulate the clinical conditions [20] (see Fig. 1).

Scanning Electron Microscope

After the experiment, blood clot samples were washed with PBS thrice and fixed by 2.5 % glutaraldehyde (Sigma-Aldrich) overnight at 4ºC. Samples were washed the next day with PBS to remove glutaraldehyde, dehydrated with increasing concentration of ethanol from 25 to 100 %, and then dried using a critical point dryer (CPD 030, Bal-Tec, Germany). Samples were photographed using a SEM (JSM-5600LV, JEOL, Japan) with an appropriate magnification. In addition, the blood clot was also sectioned crossly to illustrate its structure at the center. Scanning electron microscope (SEM) was interpreted by two independent readers as poor, satisfactory, and good, blinded to the testing conditions. Thrombolysis was defined as “poor” if no significant reduction in fibrin threads was noted on the clot surface,
“satisfactory” if the fibrin threads reduced by about 50 %, and “good” if fibrin threads reduced by more than 50 % with exposed red blood cells on the surface. Inter-rater reliability for diagnosing the extent of surface thrombolysis was good (Cohen κ: 0.898, p<0.005).

Statistical Analysis

To determine the statistical difference between the test groups, Student’s t test was used in SigmaPlot 8 (Systat Software, San Jose, CA). The level of statistical significance was fixed at p<0.05. Each experiment was conducted at least six times.

Results

Effect of Clot Aging on Thrombolysis

The weights of aged blood clots at 2, 6, and 10 h after initiation of clot formation prepared with persistent serum replacement every 2 h were 37.3±2.9, 44.2±3.9, and 51.6±5.3 mg, respectively. In comparison, the weights of samples without the serum replacement decreased linearly from 33.8±3.9 mg at 6 h to 7.1±5.2 mg at 24 h (see Fig. 2). The surface and core of the aged blood clots are illustrated by SEM (Fig. 3). Red blood cells (RBCs) were surrounded and embedded in the fibrin network on the surface of blood clot 2 h after clot formation. With aging, the density and size of fibrin threads increased correspondingly. In contrast, core of the blood clot remained the same, with much less fibrin density than that on the surface. However, if there was no serum replacement in clot preparation, fibrin thread seemed to shrink. As a result, distribution of RBCs became more uniform and its concentration increased with the aging time. After 8 h, almost no fibrin threads could be found on the surface of the blood clot (Fig. 4).

With increasing age of the blood clot, thrombolysis efficiency of tPA alone decreased from 35.5±3.2 % at 2 h to 24.7±2.1 % at 10 h (p<0.05 in Table 1), which may be due to higher fibrin density on the surface that prevents the penetration of tPA into the inner part. The extension of aging up to 24 h did not change the thrombolysis efficiency considerably despite continuously increasing weight. In comparison, values of aged blood clot without serum replacement were higher (52.3±5.1 % at 10 h in Table 2), which may be due to the shrinkage of the fibrin (Fig. 4).

![Fig. 3](image1.png)

**Fig. 3** Comparison of the fibrin and red blood cells at a surface and b the cross section of blood clots 2 (left column), 6 (middle), and 12 h (right column) after coagulation initiation in the scanning electron microscope. Arrow shows the growth of fibrin threads after the clot formation. Scale is 5 μm

![Fig. 4](image2.png)

**Fig. 4** Scanning electronic microscope images of blood clot prepared without serum replacement a 4, b 8, and c 12 h after coagulation initiation. Scale is 10 μm
Effect of Cholesterol Content on Thrombolysis

Increased cholesterol concentration of 0.5 mg/ml had significantly higher shear modulus than the others at the same thickness ($p<0.05$) except blood clot with additional 1 mg/ml cholesterol and thickness of 0.1 mm (see Fig. 5). Hence, the adhesive nature of cholesterol plays a role in increasing the shear strength of the blood clot. Tensile modulus measured using different strain rates of 5 and 10 mm/min were found similar and non-significant although the mean tensile modulus of additional 0.5 mg/ml cholesterol blood clots were a little higher (35.6±6.98 Pa) than the normal ones (32.4±9.5 Pa). It shows the stress required to break the cross-linked fibrin threads is similar in different blood clots, and cholesterol did not have a great impact on it, which may be attributed to non-uniform distribution and accumulation of cholesterol.

Thrombolysis efficiencies of different treatment protocols (normal, ultrasound alone, tPA alone, and tPA + ultrasound) were compared with each other (see Fig. 6). Sonication alone did not enhance the dissolution of the blood clot, but it may enhance the permeability of the blood clot and penetration of tPA into the fibrin network for effective activation due to bubble cavitation, acoustic radiation force, and microstreaming [21]. Interestingly, increased cholesterol concentration in the blood clots led to substantially higher thrombolysis in the tPA as well as tPA + ultrasound groups. However, this influence was higher in the 0.5 mg/ml cholesterol increment group than that in 1 mg/ml one, suggesting a complicated relationship.

| Clot age (h) | Control group (%) tPA Group (%) p Value Due to tPA alone (%) |
|-------------|-----------------|-----------------|-----------------|-----------------|
| 2           | 13.0±3.9        | 35.5±3.2        | 0.001           | 22.5            |
| 6           | 14.6±1.6        | 26.7±1.7        | 0.006           | 12.1            |
| 10          | 10.1±2.7        | 24.7±2.1        | 0.009           | 14.6            |
| 12          | 15.0±5.2        | 27.6±1.6        | 0.012           | 12.6            |
| 24          | 9.9±3.9         | 27.2±3.9        | 0.003           | 17.3            |

**Table 1** Effect of the age of blood clot prepared by replacing serum periodically on thrombolysis efficiency using tPA alone

The influence of various treatment protocols was confirmed on SEM. Control samples showed no change in the amount of fibrin threads on their surface. Ultrasound alone exposed blood clots showed poor thrombolysis. However, a rearrangement of fibrin threads was observed. The evaluation of thrombolysis in tPA alone and tPA + ultrasound groups using SEM were satisfactory and good, respectively. Although sonothrombolysis patterns in blood clots with different cholesterol concentrations appeared similar, more shrinkage or breakage of fibrin threads was observed in the 0.5 mg/ml cholesterol increment group (see Fig. 7).

Since significant attenuation of ultrasound energy occurs during its travel through the skull [7], the thrombolysis experiments were repeated after placing human cadaveric temporal...
bone between the ultrasound probe and the blood clot, and a significant reduction in the clot dissolution with the presence of temporal bone was found (Table 3).

Discussion

Current determination of tPA dose is based on the patient’s weight, 0.9 mg/kg (maximum 90 mg) in predominantly Western population [5], not the characteristics of the blood clot (i.e., composition and age). Insufficient tPA will result in delayed or failure in clot dissolution and recovery of blood flow for some post-treatment symptoms, such as inability to move one or more limbs on one side of the body, to understand or formulate speech, or to see one side of the visual field. Meanwhile, overdose would increase the propensity of intracranial hemorrhage significantly, 6.4 versus 0.6 % of those given placebo [22]. However, there were some reservations about the dose for Asian patients [23], such as 0.6 mg/kg body weight and maximum 60 mg suggested on Japanese AIS patients due to the various racial differences in blood coagulation–fibrinolysis factors [12, 13]. Another reason could be the higher proportion of cardioembolic strokes in Japanese studies that respond better to the thrombolytic therapy [24]. It has been shown that “white clots” composed of platelets and fibrin are relatively resistant to thrombolysis as compared to erythrocyte-rich “red clots” in the embolic stroke and femoral artery thrombosis [25]. Our experimental study confirmed better thrombolytic activity of tPA, alone or with 2-MHz TCD ultrasound, in fresh clots that were erythrocyte-rich due to repeated blood replacement. Since fibrin threads polymerize and strengthen over time, age of the blood clot assumes a crucial consideration and influences the thrombolytic therapy in AIS. Prompt treatment of fresh blood clots with better clinical outcomes has been convincingly shown in a recent meta-analysis of all the randomized clinical trials of IV-tPA in AIS [6]. Our study suggested that higher fibrin density on the surface to prevent tPA penetration may be one of the reasons for the decreased thrombolysis in aged clot. However, various other factors might play an important role in determining the response of the clot to a thrombolytic agent.

The elastic and tensile properties of a clot are largely dependent on the fibrin threads and responsible for reversible or irreversible thrombi and emboli formation [26], and its viscoelasticity also determines the response to various treatment modalities. These characteristics in terms of mechanical properties, composition, and structure are useful in categorizing blood clot and important in developing a biochemomechanical model for AIS treatment [18, 19]. In this study, various types of blood clots due to different ages and cholesterol concentrations were prepared, and their corresponding shear strength was measured. Higher cholesterol concentrations changed the rheological properties of the clots that resulted in significantly higher rates of clot dissolutions with combined tPA and ultrasound treatment, especially for those with increased cholesterol levels of more than 0.5 mg/ml.

Elevated high-density lipoprotein cholesterol (HDL-C) and its major protein component apolipoprotein A-1 (Apo A1)

Table 3 Effect of temporal bone on thrombolytic efficiency of tPA and ultrasound

<table>
<thead>
<tr>
<th></th>
<th>Normal clots</th>
<th>Cholesterol (0.5 mg/ml)</th>
<th>Cholesterol (1 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/o temporal bone</td>
<td>28.5±4.5 %</td>
<td>36.9±4.3 %</td>
<td>34.7±3.3 %</td>
</tr>
<tr>
<td>W/ temporal bone</td>
<td>30.5±3.8 %</td>
<td>27.8±5.8 %</td>
<td>29.8±4.5 %</td>
</tr>
<tr>
<td>ρ Value</td>
<td>0.072</td>
<td>0.011</td>
<td>0.024</td>
</tr>
</tbody>
</table>
could increase plasma fibrin clot permeability and lead to the formation of plasma fibrin clots more susceptible to lysis in healthy individuals free of metabolic syndrome. No such associations were seen for other lipid variables [27]. It has been shown in the purified system that HDL and Apo A1 have anticoagulant properties and can stimulate lysis [28]. The formation of larger pores in the fibrin network by the link of Apo A1 with the fibrinogen molecule is the hypothesis. However, low HDL-C increases cardiovascular risk. Both atherosclerotic vascular disease and venous thromboembolic disease have been shown to be linked to prothrombotic fibrin clot phenotype [29]. However, the role of cholesterol on thrombolysis seems complicated (i.e., addition of 0.5 mg/ml cholesterol increases thrombolysis than normal, but there is no difference between samples with additional 0.5 and 1 mg/ml cholesterol) and needs more effort.

Although sonothrombolysis influences the thrombolytic activity of tPA, its mechanisms remain poorly understood [21]. Inertial cavitation could generate transient microjets that disintegrate thrombus mechanically. Stable cavitation may be more effective in facilitating penetration of fibrinolytic enzymes into the interior of the fibrin network of thrombus and binding to fibrin for dissolution. In contrast, US-induced thermal effect may be too mild to enhance thrombolytic effects [30]. Various in vitro experiments have shown that ultrasound enhances the thrombolytic effect of tPA by producing conformational changes such as reversible disaggregation of uncross linked fibrin fibers and microcavity formation in the shallow layers of thrombus to increase the penetration of tPA into the clot and residual flow enhancement with microstreaming and vessel dilation [31, 32]. Although SEM in our study did not show microcavities, rearrangement and thinning of fibrin threads were evident after sonication. Furthermore, SEM showed increased exposed fibrin as well as the number of openings to more interior regions. Enhancement of tPA-induced thrombolysis by ultrasound in blood clots with higher cholesterol level is interesting and suggests that the adhesive nature of cholesterol that plays a major role in increasing the shear strength of the blood clot. Although the stress required for breaking the cross-linking fibrin fibers among all types of clots was similar, fibrin threads in the clots with higher cholesterol level gradually changed to isolated fibers and fractured easily during tensile extension. Furthermore, cholesterol has higher affinity to fibrinogen and easily gets incorporated in the fibrin threads [33].

Sonothrombolysis for MCA only accounts for a part of ischemic strokes, which is due to the lack of a temporal bone window [34]. Temporal bone window failure rates occur in the rage of 8–29 % [35], among them, 39 % had bilateral window failure in a study of 624 subjects. In 182 subjects having a transient ischemic attack or minor ischemic stroke, the window failure rate is 18 % [36]. Hyperostosis, thickening of the skull’s inner table with the age, is a possible reason especially in elderly women, which has been observed in 6–12 % of adult women of all ages and in 50 % of them over 60 years old as compared with only 1 % of men [37]. Temporal bone causes high attenuation to acoustic wave; a reduction of at least 86 % of the diagnostic ultrasound energy and almost 100 % in very thin bone windows and poor bone windows, respectively. In addition, the skull also defocuses the acoustic field by a factor of approximately four through phase aberration [38]. An acoustic window exists if the ipsilateral proximal branches of the circle of Willis can be displaced can be detected using transcranial color-coded duplex sonography, and the threshold of a temporal bone thickness for obtaining acoustic window was found to be about 4 mm [39]. Although the presence of temporal bone reduces the acoustic pressure because of attenuation and distortion and the consequent sonothrombolysis efficiency, the outcome is still satisfactory for clinical application as shown in this study. Appropriate compensation of TCD power for each stroke patient by scanning the skull through CT for the measurement of thickness and properties and simulation of intracranial acoustic field may result in the reduction of variations in the MCA recanalization.

In conclusion, a new in vitro blood clot aging method was proposed to mimic the continuous thrombus growth in the circulating system. Our experimental study confirms the current understanding about the benefits of early initiation of thrombolytic therapy in AIS. Our observation regarding the facilitation of clot lysis after increasing the cholesterol level is interesting and suggests some important clues about the differential response to systematic thrombolysis due to various etiopathologies. More exhaustive evaluations and replicating these results in animal stroke models are required for testing this observation.

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Conflict of Interest The authors state that they have no conflict of interest.

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