Clot dissolution is better with ultrasound assisted thrombolysis for fresh clots with higher cholesterol content

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Abstract. Tissue plasminogen activator (tPA) remains the only drug for recanalization in acute ischemic stroke, and the dose is determined by the patient's body-weight. Properties of the blood clot as well as ultrasound exposure might affect the thrombolysis outcome. In this study, clot was prepared by mixing horse blood with CaCl₂ solution and cholesterin up to 1.0 mg/ml. To simulate the aging effect serum was replaced by fresh blood periodically. 225 IU/ml of tPA was used to initiate lysis. Clot was exposed to continuous 2 MHz transcranial Doppler ultrasound at acoustic intensity of 340 mW/cm². The weight of the blood clot increased with its age (from 37.28 ± 2.87 mg at 2 hrs to 51.56 ± 5.34 mg at 10 hrs, p < 0.05). Although no difference between clot-cholesterol levels and thrombolysis with ultrasound or tPA alone was found, combination of these modalities induced significant lysis in the clots with cholesterol levels of more than 0.5 mg/ml (clot-weight reduced by 41.68 ± 2.3%) as compared to clots with normal cholesterol (30.60 ± 4.10%; p < 0.05). Altogether, sonothrombolysis seems to work better in fresh thrombi with high-cholesterol levels.

Keywords: sonothrombolysis, tissue plasminogen activator, cholesterol, aging

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1. Introduction

Stroke causes a rapid loss of brain functions due to disturbance in the blood supply to the brain and is the fourth leading cause of death. As a result, the affected brain area leads to inability to move one or both sides of limbs, to understand or formulate speech, or to see one side of the visual field [1]. Strokes 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]. 87% of strokes are caused by ischemia, and some hemorrhages develop inside areas of ischemia [1]. With the high prevalence of stroke risk factors in our rapidly aging population, the burden of stroke will increase dramatically in the years to come, posing challenges to the healthcare system and society [3].

Current standard of care for acute ischemic stroke is the intravenous (IV) administration of tissue plasminogen activator (tPA) or recombinant tPA (i.e., Alteplase) whose dose is determined by the patient's body-weight, given within 3 hrs of symptom onset. If vessel recanalization is fast and efficient, outcome is favorable in about two-thirds of patients, and recovery is complete in half of the patients treated. Because of the short time window, the rate of treatment in the USA and Europe is only 2-5% [4].

Because the clot is the primary target of thrombolytic therapy, a clear understanding of the clot characteristics could improve vessel recanalization. In embolic stroke and femoral artery thrombosis, white clots composed of platelets and fibrin with a relative resistance against thrombolysis, whereas erythrocyte-rich clots showed higher response rates [5]. Another crucial consideration is the age of the thrombus. In thrombotic stroke, a thrombus usually forms around atherosclerotic plaques, which is quite
fresh. In contrast, an arterial embolus, an aged thrombus from the heart (especially in atrial fibrillation), travels in the arterial bloodstream and causes the blockage of an artery.

Ultrasound exposure of the intracranial vessels (sonothrombolysis) becomes a promising approach to facilitate reperfusion therapies for acute ischemic stroke. In human stroke, the CLOTBUST phase II trial showed that the combination of alteplase plus 2 hours of continuous transcranial Doppler (TCD) increased recanalization rates, producing a trend toward better functional outcomes compared with alteplase alone. Other small clinical trials also showed an improvement in clot lysis when transcranial color-coded sonography was combined with alteplase [6].

In this study, the effect of properties of the blood clot (i.e., aging and concentration of cholesterol) on thrombolysis was investigated in order to further understand and evaluate the sonothrombolysis technology. 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 (clot-weight reduced by 41.68±2.3%) as compared to clots with normal cholesterol (30.60±4.10%, p < 0.05). Altogether, sonothrombolysis seems to work better in fresh thrombi with higher cholesterol levels.

2. Methods

2.1. Blood clot preparation

To prepare blood clot, 0.25 ml of 100 mM CaCl₂ (Sigma-Aldrich, Singapore) was added to 1 ml of anti-coagulated horse blood (I-DNA Biotechnology, Singapore). Within 1 minute, 0.25 ml of the mixture was injected into a silicone tube (3 mm of inner diameter, 35 mm in length, Versilic, France), and then stored in an incubator (Lab companion-JeioTech, Seoul, Korea) at 37 ºC for 2 hours for clot formation. Furthermore, 1 mg and 0.5 mg of cholesterol (Sigma-Aldrich, Singapore) was added to 1 ml whole blood to represent 1 mg/ml and 0.5 mg/ml increase in the cholesterol level. To simulate the aging occurred in the blood clots in vivo, serum from the blood clot was carefully removed using a syringe, and then equivalent amount of fresh horse blood was added into the silicone tube, which was repeated every 2 hours after initiation of clot formation.

2.2. Measurement of Clot’s Elasticity

Shear strength of the blood clot was measured using a cone and plate rheometer (MCR501, Anton Par GmbH, Osterreich, Austria). Blood clots were prepared in a cylindrical mold with a diameter of 25 mm and thickness of 1 mm. After 2-hour incubation, the blood clots were carefully taken out from the mold and transferred to the plate of the rheometer without damage, and excess water and serum were removed. Shear modulus was measured from samples with different thickness using a constant strain (5%).

2.3. Experimental setup

After incubation, blood clots were removed from the silicon tube using a needle and washed thrice with PBS solution. The blood clots were then dried using whatman filter paper (VWR, Singapore), and its weight was measured using a digital balance (SBC-31, Scaltec Instruments GmbH, Gottingen, Germany) in a resolution of 0.1 mg. 50 µl tPA (10 µg, 225 IU, Fitzgerald Industries, USA) was added to a micro-centrifuge tube (Greiner Bio-one, Germany) filled with 950 µl of PBS solution, so the final concentration of tPA was 0.5 µg/ml. Blood clot was put into the mixture of tPA and PBS. Micro-centrifuge tube was then locked with the attached cap and immersed into a water bath (BS-06, Jeio Tech,
Seoul, Korea) with water temperature being kept at 37°C. 1 hour later, the blood clot sample was taken out from the tube, washed with PBS solution thrice, and dried by whatman filter paper to measure its weight. Thrombolysis efficiency was determined as the percentage of the loss of the blood clot mass using different protocols with respect to its initial weight. The control group means blood clot being immersed in PBS solution without tPA for 1 hour.

Transcranial ultrasound Doppler pulses (Multi-DoP, Compumedics DWL, Singen, Germany) was delivered to the blood clot (see Fig. 1) that was placed at a distance of 45 mm to the probe \( f = 2 \text{ MHz}, I_{sp}t = 340 \text{ mW/cm}^2 \). After the sonication for 1 hour, the thrombolysis of the blood clot inside the PBS solution with or without tPA was determined using the same protocol described above.

2.4. Scanning electron microscope (SEM)

After the thrombolysis experiment, blood clot samples were washed with PBS solution thrice and then fixed by 2.5% glutaraldehyde (Sigma-Aldrich, Singapore) overnight at 4°C. In the next day, samples were washed with PBS to remove glutaraldehyde, dehydrated with increasing concentration of ethanol, and then dried using a critical point dryer (CPD 030, Bal-Tec, Witten/Ruhr, Germany). Blood clot samples were examined and recorded photographically using a Scanning Electron Microscope (JSM-5600LV, JEOL, Tokyo, Japan) in an appropriate magnification.

2.5. Statistical Analysis

To determine the statistical difference between the test groups, a student’s t-test was used in SigmaPlot 8 (Systat Software, San Jose, CA). The level of statistical significance was fixed at \( p < 0.05 \).

3. Results

3.1. Effect of clot aging on thrombolysis

Figure 3. Comparison of the fibrin and red blood cells at (a) surface and (b) the cross section of the blood clots 2 hours (left column), 6 hours (middle), and 10 hours (right column) after coagulation initiation in scanning electron microscope.
Aged blood clots were prepared with constant serum replacement every 2 hours, and their weights at 2 hours, 6 hours, and 10 hours after initiation of clot formation were 37.3±2.9 mg, 44.2±3.9 mg, and 51.6±5.3 mg, respectively. Because of the availability of fibrinogen from the replaced blood, the clot formation continuously occurs in our samples. Therefore, the size and weight of the blood clot increase correspondingly. With the increase of the age of the blood clot, thrombolysis efficiency using tPA were found to decrease from 35.5±3.2% at 2 hours to 24.7±2.1% at 10 hours \( (p < 0.05) \) in Fig. 2, which may be due to higher fibrin density on the surface of the aged blood clots that prevent the penetration of tPA enzyme into the inner part of the blood clot. However, the inner part of the blood clot kept its structure at different ages of clot with much less density of fibrin than that on the surface (see Fig. 3).

### 3.2. Effect of cholesterol on thrombolysis

Shear strength of the blood clot was measured using the rheometer. Thickness of the blood clot was reduced by removing serum content. Consequently, the thin sample was densely stacked with high shear strength. Among all samples, normal blood clots were found to have lower shear strength, and the presence of cholesterol would increase the shear modulus of blood clots in the same thickness. Blood clot with 1 mg/ml cholesterol and thickness of 0.1 mm has significantly higher shear strength than those of the others \( (p < 0.05) \). Cholesterol concentration of 0.5 mg/ml had a significantly higher shear modulus with the normal clots in the same thickness.

![Figure 3. The shear modulus of the blood clot with different concentration of cholesterol and thickness.](image)

Thrombolysis efficiencies of different treatment protocols (normal, ultrasound exposure, tPA, and combination of tPA and ultrasound exposure) were compared with each other (see Fig. 5). Although low-intensity ultrasound (US) exposure alone did not enhance the dissolution of blood clot \( (p > 0.05) \), it may help the penetration of tPA into the fibrin network for effective activation \( (p < 0.05) \). In addition, it is interesting to find that the increment of the cholesterol concentration in the blood clots would lead to significantly higher thrombolysis in comparison to that of normal clots in the tPA and tPA+US exposures \( (p < 0.05) \).

Blood clots lysed using different protocols were recorded photographically using SEM and then compared with each other. Control samples were found to have more fibrin threads on their surface. Ultrasound exposed samples showed a re-arrangement of fibrin threads, but no obvious reduction on its
density. However, re-arrangement of fibrin threads may create penetration channels for the thrombolytic enzyme, such as tPA used in this study. tPA lysed samples showed red blood cells (RBCs) on their surface with significant reduction on the fibrin threads. Meanwhile, blood clot under the combined exposure to US and tPA illustrated RBCs and layers of lysed fibrin threads. Although sonothrombolysis patterns in blood clots with different cholesterol concentrations are quite similar, more shrink or breakage of fibrin for removal of RBCs are found in the cholesterified blood clot.

![Comparison of the structure](image)

Figure 6. Comparison of the structure of (a) normal, (b) 0.5 mg/ml, and (c) 1 mg/ml cholesterol blood clot after 1-hour thrombolysis by scanning electron microscope. Scale is 5 μm.

4. Discussion

Appropriate IV administration dose of tPA is of importance in clinical treatment of ischemic stroke. However, current determination of tPA dose is based on the patient’s weight, not the characteristics of the blood clot (i.e., length or weight, composition and age). Insufficient tPA will result in delayed or failure in clot dissolution and resumption 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. However, overdose of tPA would also increase the propensity of intracranial hemorrhage significantly. In our study, it has already been shown that there is apparent difference in the thrombolysis efficiencies among blood clots with different aging and concentration of cholesterol. Therefore, it is suggested to determine the appropriate tPA dose by in vivo diagnosis on the properties of the blood clot rather than only the location and type of stroke. Black-blood T1-weighted (T1W) and T2-weighted (T2W) high-resolution in vivo magnetic resonant (MR) images were obtained in a clinical 1.5T MR system to measure the thrombus signal intensity (SI) normalized to that of the adjacent muscle [7]. Thrombus appearance and relative SI revealed characteristic temporal changes in multicontrast-weighted MR images, reflecting histologic changes in the composition. Acute thrombus appeared very bright on the T2W images, facilitating the detection. The T1W images had a similar pattern with lower SI than T2W. Age definition using visual appearance was highly accurate. Therefore, magnetic resonance imaging is a promising tool to noninvasively detect arterial thrombosis, whose capability will also be investigated for detection of cranial thrombi.
Sonothrombolysis has already been investigated since 1960s. However, its mechanisms remain poorly understood [8]. Inertial cavitation (i.e., the formation and violent collapse of gas-filled bubbles in the acoustic field) could generate transient microjets that disintegrate thrombus mechanically. Stable cavitation (i.e., nonlinear periodic contraction and expansion of a gas nuclei or bubble) 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. Significant amplification of thrombolysis occurs with the addition of microbubbles (i.e., perflutren lipid micro-spheres, MRX-801) to the combination of thrombolytic drug at a dose of 1.4 ml and US as the investigation of transcranial ultrasound in the clinical sonothrombolysis (TUCSON) trial. In contrast, US-induced thermal effect may be too mild to enhance thrombolytic effects. The characteristics of bubble cavitation and penetration of fibrinolytic enzyme into the fibrin network will be studied in the next stage in order to further the understanding of sonothrombolysis, especially for different types of blood clots.

In this study, model of clot aging was established and concentration of cholesterol was increased to investigate their effects on thrombolysis. Although ultrasound exposure alone does not enhance the thrombolysis for all blood clots, tPA or combination of tPA and US induces significant lysis in the clots with cholesterol concentration of more than 0.5 mg/ml in comparison to normal ones. Altogether, it seems that fresh thrombi with higher cholesterol levels have high thrombolysis efficiencies in tPA and ultrasound exposure. Cholesterol has an affinity to fibrinogen and gets incorporated in the fibrin threads [9]. Cholesterol content in the fibrin threads may increase the pore size of fibrin threads or influence better interaction with enzyme tPA. More studies are needed to illustrate this mechanism.

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REFERENCES AND LINKS