Original Article
Zinc chelation promotes streptokinase-induced thrombolysis *in vitro*

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Abstract: Cardiovascular disorder occurs when a local blood clot obstructs an artery or a vein to its surround organs, causing related tissues to lose function and die. It is one of the leading causes of mortality and a major cause of disability. The effect of thrombolysis induced by injecting intravenous thrombolytic agents is critical for reducing tissue damages. Streptokinase (SK) is a widely used thrombolytic agent in the treatment of thromboembolism in the blood vessels. A high unit of streptokinase is used in thrombolytic therapies for thrombotic disorders and could improve tissue reperfusion. It is a potent plasminogen activator. However, safety concerns for the usage of a high unit of streptokinase have been raised for the hemorrhagic transformation. In the present study, we studied how zinc would affect streptokinase-induced thrombolysis *in vitro*, and proposed a strategy to improve streptokinase's effectiveness in promoting thrombolysis. The mice whole blood was used to form the blood clot *in vitro* by incubating with calcium at 37°C for 30 minutes. Streptokinase was used for inducing thrombolysis measured with the spectrophotometer. Zinc and its chelator, Ca-EDTA, were applied with streptokinase, respectively. Results showed that the co-application zinc inhibited the thrombolytic effect of streptokinase in a dose-dependent manner. Zinc chelator, Ca-EDTA, significantly increased the effect of streptokinase-induced thrombolysis. Our results suggest that zinc chelation improved the efficiency of streptokinase in thrombolysis. The results may have a significant clinical implication by potentially reducing the adverse effect of streptokinase application.

Keywords: Streptokinase, thrombolysis, zinc, clot, zinc chelation

Introduction

The hemostasis of circulatory system is critically controlled through the balance between coagulation and fibrinolysis system. In health condition, thrombosis is suppressed in the blood circulation, whereas it is initiated to form thrombus blocking the injured blood vessels to prevent blood loss as the first stage of wound healing [1]. However, when the hemostasis is failed to maintain, a thrombus is produced out of control resulting in the appearance of cardiovascular diseases, for example, heart attack, stroke, pulmonary embolism, deep vein thrombosis and so on [2]. Myocardial ischemia occurs when the blood clot blocks blood flow and deprives oxygen to cardiac muscles resulting in the irreversible myocardial cell damage and death which leads to myocardial infarction [3, 4]. Stroke is a neurological disorder caused by an interruption of blood flow to the brain, resulting in the deprivation of

oxygen and nutrients in brain tissues and finally dysfunction [5]. Ischemic stroke occurs when thrombosis or embolism blocks the blood supply to the brain and causes brain damage and disability, accounting for 87% of overall stroke cases [5, 6]. According to AHA/ASA data, 17.3 million deaths are caused by cardiovascular disease per year, making it the leading cause of disability and mortality worldwide [6] and this number grows every year.

Thrombolytic therapy is an approach to renovate impaired tissue function caused by thrombus-induced thrombolytic disorders through intravenous administration of thrombolytic agents [4]. Streptokinase is one of the thrombolytic agents, which was intravenously used to treat acute myocardial infarction approved by FDA in 1987.

Streptokinase (EC 3.4.99.22) is an extracellular fibrinolytic enzyme secreted by β-hemolytic

streptococci bacteria. It is a single chain protein consisting of 414 amino acids with a molecular mass of 47 kDa [7, 8]. It needs to bind to both free and fibrin bound plasminogen first to form a complex, which then converts other free plasminogen to an activate protease plasmin dissolving blood clots [9-14]. Currently, streptokinase is commonly used in the treatment of acute myocardial infarction, deep vein thrombosis and pulmonary embolism [15-18]. However, like other thrombolytic agents, the safety of streptokinase need to be concerned with preventing complications, such as hemorrhagic transformation [14, 16, 19, 20]. Therefore, a new thrombolytic strategy is in urgent demand in the aspect of not only good thrombolytic efficacy but also safety. Estimating the ideal dose of streptokinase is critical to adjust the balance of high thrombolysis efficiency and low complication rate in the treatment of cardiovascular diseases. Furthermore, some combined therapies could improve the effect of low dose streptokinase-induced thrombolysis, simultaneously preventing the appearance of combinations.

Zinc is one of the essential divalent metal ions in human body. It plays a critical role in various physiological functions, such as participating in enzyme reactions and serving as a cofactor for transcription factors [21]. Zinc has also been demonstrated a neurotoxicity role in the pathogenesis of ischemia-induced tissue injury [22-24]. Besides, zinc involves in regulating hemostasis and thrombosis. The concentration of zinc is delicately controlled in the blood. In plasma, most of the zinc is bound to proteins, and the concentration of free zinc ranges from 0.5 to 1 µM [25-27]. Platelets also involve in modulating zinc concentration. The concentration of unbound zinc ranges from 7 to 10 µM in activated platelets [28]. Once stimulated, zinc is released from α granules of platelets [29] and promotes platelet aggregation [30, 31]. Some studies also support the notion that zinc regulates coagulation through its interaction with plasma clotting factors in contact system and intrinsic pathway [26, 28, 32-38]. However, the role of zinc in thrombolysis is still under investigation. During thrombolysis, zinc is released from stimulated platelets [39-43]. In this study, we delineated the relationship between zinc and streptokinase-induced blood clot lysis *in vitro*. Zinc dampened streptokinase-induced thrombolysis. Chelation of zinc promoted the effect of streptokinase-induced thrombolysis.

Methods and materials

Specimen

Citrated blood samples collected from male CD1 mice were purchased from BioChemed services (Winchester, VA). Citrated blood was stored in the 4°C refrigerator and was used within 3 days to minimize the influence of gradually declining clotting ability. Each amount of 200 µl blood calcified with 3 µl of $CaCl_2$ (1 M) was transferred to a 0.5 ml centrifuge tube and was incubated at 37°C for 30 minutes to form a clot. After incubation, blood clots without serum were gently washed with saline for 5 times and then were randomly transferred to custom-modified cuvettes for absorbance measurement.

Streptokinase

Streptokinase (EC 3.4.99.0) from β-hemolytic *Streptococcus* (Lancefield Group C) (250000 unit) was purchased from Sigma Aldrich (St. Louis, MO). Streptokinase (250000 unit) was properly dissolved with 2.5 ml saline and was used as stock solution stored at -80°C freezer. Streptokinase was used to study thrombolytic activity *in vitro*.

Chemicals and reagents

Ethylenediaminetetraacetic acid calcium disodium salt (Ca-EDTA) was purchased from Sigma Aldrich (St. Louis, MO). Ca-EDTA was dissolved in saline to a final stock concentration at 10 mM and used to chelate the extracellular zinc. Zinc chloride was purchased from Sigma Aldrich (St. Louis, MO) and was mixed with saline to a final stock concentration at 1 mM.

Study design

Schematic diagram of the modified cuvette is shown in **Figure 1A**. The blood clot was transferred to the cuvette and was held on a nylon web, which was placed in the neck of the cuvette, during the entire experiment. Cuvettes were filled with 1.5 ml solution containing streptokinase or other chemicals. The thrombolytic activity was observed for different doses of
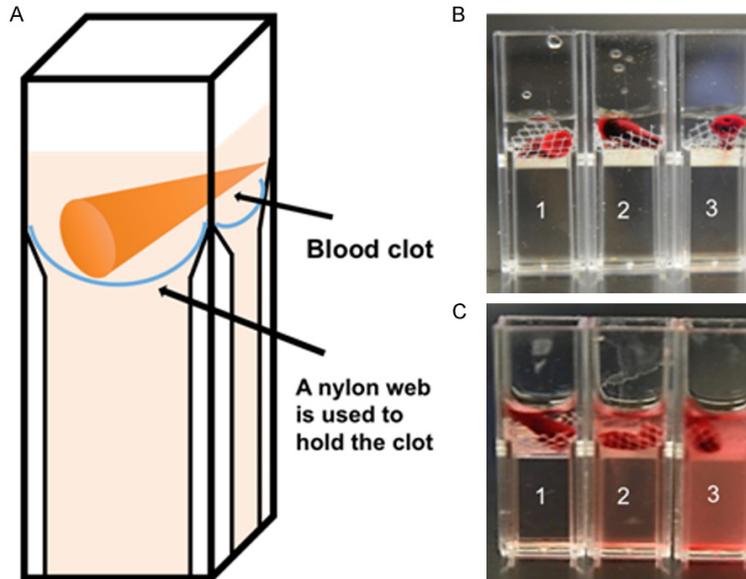
Figure 1. The measurement of thrombolysis of blood samples *in vitro* with a spectrophotometer. A. Schematic diagram of the modified cuvette with a blood clot. After incubation and rinse, the blood clots were transferred to the cuvettes and settled on the nylon net in the neck of the cuvette. B, C. Images of streptokinase-induced thrombolysis measured by the spectrophotometer. Cuvette 2 and 3 were added with two different doses of streptokinase (5000 and 7500 unit). Cuvette 1 was a control group, which clot was treated with saline. The streptokinase-induced thrombolysis was evaluated by the absorbance change. The absorbance value was measured by the spectrophotometer at 580 nm wavelength. B. At the beginning of clot lysis, the color of each cuvette was clear which meant the absorbance was low. C. After thrombolysis for 140 minutes, the color intensity of each cuvette was increased that meant the absorbance was also increased.

streptokinase, streptokinase with zinc, streptokinase with Ca-EDTA, zinc only and Ca-EDTA only groups of treatment. The color of the solution was clear at the beginning of thrombolysis, and the optical intensity was low (**Figure 1B**). With the addition of streptokinase, the clot began to lyse with the released hemoglobin precipitating down to the bottom of the cuvette and the color of solution becoming dark red with a high optical intensity (**Figure 1C**). The spectrophotometer (Biomate 3) from Thermo Spectronic (Waltham, MA, USA) was used to measure the optical intensity of solutions during thrombolysis. It detected the optical intensity of the light that passed through the cuvette below the clot. The optical intensity value was used to calculate the absorbance value by the intensity difference between total light and transmitted light. The absorbance baseline was set with the absorbance of saline without clot. The wavelength was 580 nm in the range of most sensitive wavelength of hemoglobin [44, 45]. The absorbance

value was measured every 10 minutes, and up to 140 minutes.

Statistical analysis

The absorbance changes of streptokinase-induced thrombolysis with different chemicals were analyzed by one-way ANOVA with multiple comparisons. Data are presented as mean ± standard error of the means. A *p* value ≤ 0.05 was considered to be statistically significant.

Results

Absorbance value was used to quantify the streptokinase induced blood clot lysis in vitro

After adding thrombolytic agent, the blood clot was lysed in the cuvette and released hemoglobin resulting in the increased concentration of hemoglobin in the solution. With the increased concentration of hemoglobin, the absorbance value of hemoglobin increased as well. In this study, the spectrophotometer was used to measure the absorbance of hemoglobin at 580 nm wavelength. In the beginning, the solutions were clear after the blood clots settled in the neck of cuvettes (**Figure 1B**). The hemoglobin concentration was low, which indicated the low absorbance value. With the passage of time, streptokinase induced blood clot lysis, which released the hemoglobin. The clear solution became dark resulting in an increased absorbance value (**Figure 1C**). Streptokinase induced thrombolysis in a dose-dependent manner with 5000 and 7500 unit (**Figure 1B** and **1C**, cuvette 2 and 3, and **Figure 2**). With the higher dose of streptokinase used, the more hemoglobin was released. The higher absorbance value was observed for streptokinase at 7500 unit whereas the lower one was recorded for streptokinase at 5000 unit. The absorbance values were measured every 10 minutes. The absorbance value significantly increased from the 60th minute and continued increasing to the
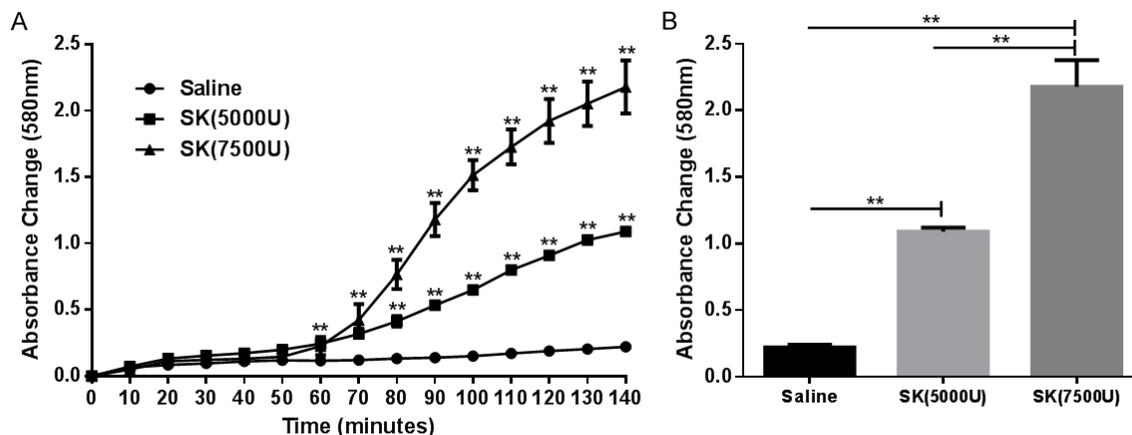
Figure 2. Streptokinase induced thrombolysis in a dose-dependent manner. A. Graph showing absorbance changes caused by different doses of streptokinase-induced thrombolysis. The clot lysis of blood samples was measured for absorbance for 140 minutes with two different doses of streptokinase (5000 and 7500 unit). Saline was used as a control group. There was a significant difference in absorbance change from the 60th minute between streptokinase treatments and saline. B. Histogram showing quantified absorbance changes after 140 minutes treatment with streptokinase and saline. The quantified absorbance change showed a significant difference between streptokinase treatments and saline and between two different doses of streptokinase. Data were presented as mean ± SEM. The numbers of samples tested: 9 in saline, 8 in 5000 unit streptokinase, and 2 in 7500 unit streptokinase. **$p \leq 0.01$.

140th minute in all streptokinase treatment groups (**Figure 2A**). Significant differences were observed in the pairwise comparisons of the final quantified absorbance values for different doses of streptokinase (**Figure 2B**). Saline was used as control (**Figure 1B** and **1C**, cuvette 1, and **Figure 2**). The steady low level of absorbance change demonstrated that saline had nearly no effect in thrombolysis.

Zinc inhibited streptokinase-induced blood clot thrombolysis

The potential role of zinc in blood clot lysis was studied through measuring spectrophotometric absorbance value change in the cuvette. Different concentrations of $ZnCl_2$ were directly added into the solution together with 5000 unit streptokinase. The decreased absorbance value indicated that zinc had an inhibitory effect in streptokinase-induced thrombolysis. The absorbance change showed that zinc decreased the efficiency of streptokinase-induced blood clot lysis (**Figure 3A**). At the low dose (10 µM), zinc inhibited blood clot lysis from the 110th minute to the end of observation. At the medium dose (50 µM), the inhibition effect occurred from the 80th minute. Zinc showed the highest inhibition from the 70th minute at the concentration of 100 µM among all three doses. At

the end of experiments, the quantified absorbance of streptokinase-induced blood clot lysis showed a 30.6%, 57.5% and 79.7% reduction with 10 µM, 50 µM, and 100 µM $ZnCl_2$, respectively (**Figure 3B**). These findings demonstrated that zinc inhibited streptokinase-induced thrombolysis in a dose-dependent manner. Thus, zinc is a critical inhibitor of streptokinase-induced thrombolysis.

Zinc chelation potentiated streptokinase-induced thrombolysis

As zinc played as an inhibitor in streptokinase-induced thrombolysis, we hypothesized that zinc chelation would promote streptokinase-induced blood clot lysis. To test it, we examined the effect of Ca-EDTA, a potent extracellular zinc chelator, in streptokinase-induced thrombolysis. The absorbance change showed an obviously separated trend in 5000 unit streptokinase with 1 mM Ca-EDTA treatment from the 60th minute compared with that in 5000 unit streptokinase only treatment. As shown in **Figure 4A**, the addition of Ca-EDTA dramatically increased the absorbance value in 5000 unit streptokinase treatment from the 70th minute and showed significantly higher amplitudes in absorbance from the 70th to 140th minute. The maximal promotion was achieved at the
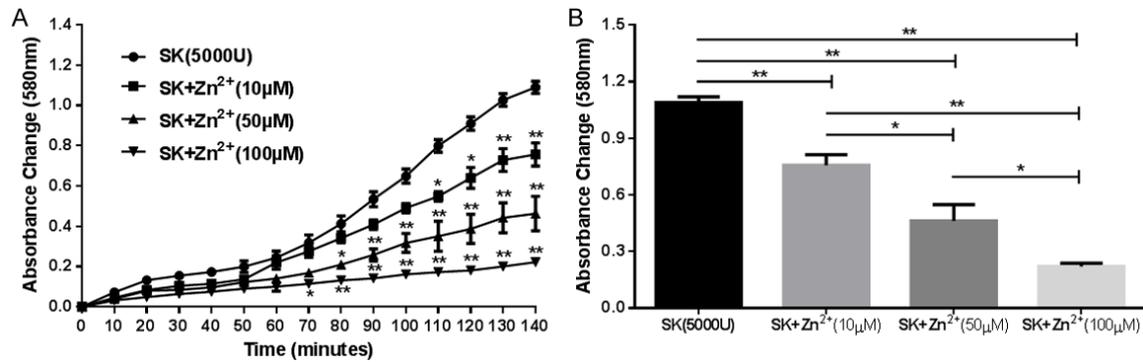
Figure 3. Zinc inhibited streptokinase-induced blood clot lysis in a dose-dependent manner. A. A graph showed streptokinase-induced absorbance change was inhibited by different doses of $ZnCl_2$. The thrombolysis of blood samples was measured for absorbance for 140 minutes in 5000 unit streptokinase with different concentrations of $ZnCl_2$ (10 µM, 50 µM, and 100 µM). The significant inhibition was observed from the 110th, 80th and 70th minute to the end between streptokinase alone and streptokinase with 10, 50 and 100 µM $ZnCl_2$ treatment, respectively. B. Histogram showing quantified streptokinase-induced absorbance changes after 140 minutes treatment with the addition of different concentrations of $ZnCl_2$. The quantified absorbance showed a significant reduction between streptokinase only and streptokinase with different concentrations of $ZnCl_2$ and between every two different doses of $ZnCl_2$. Data were presented as mean ± SEM. The numbers of samples tested: 8 in streptokinase alone, 2 in 5000 unit streptokinase with 10 µM $ZnCl_2$, 3 in 5000 unit streptokinase with 50 µM $ZnCl_2$ and 3 in 5000 U streptokinase with 100 µM $ZnCl_2$. *$p \leq 0.05$; **$p \leq 0.01$.

140th minute in streptokinase with Ca-EDTA treatment with a nearly two-fold amplitude increase in the absorbance change compared with that of streptokinase only treatment. Thus, the findings indicated that zinc chelator potentiated streptokinase-induced thrombolysis. The steady low level of absorbance change demonstrated that zinc chelator itself had nearly no effect in thrombolysis without streptokinase.

Discussion

The present study investigated the role of zinc in streptokinase-induced thrombolysis *in vitro*. The data indicated that zinc attenuated streptokinase-induced blood clot lysis through measuring the absorbance change in the cuvettes by spectrophotometry (**Figure 3**). The zinc inhibition effect showed a dose-dependent response. Treatment with zinc chelator, Ca-EDTA, potentiated streptokinase-induced thrombolysis, resulting in a two-fold increase in the absorbance change compared with that of only streptokinase (**Figure 4**). These findings unraveled zinc as a critical inhibitor in streptokinase-induced blood clot lysis.

Streptokinase-induced thrombolysis and quantitative measurement

Different strategies have been used for studying the thrombolytic activity of thrombolytic agents. *In vitro* study is one of best ways to learn it [46, 47] such as to quantify thrombolysis by spectrophotometric hemoglobin assay [48]. Streptokinase was added into the solution and bound to fibrin bound plasminogen to form a streptokinase-plasminogen activator complex, which converted substrate plasminogen to plasmin [9-14]. Plasmin cleaved fibrin into degradation products, resulting in the release of red blood cells and hemoglobin, which were measured by spectrometry at 580 nm to quantify thrombolysis in our study. The streptokinase promoted thrombolysis in a dose-dependent manner (**Figure 2**). In the experiment, different unit streptokinase exhibited significant blood clot lysis one hour later after adding into solution. The delay might be caused by the mechanism of streptokinase-induced thrombolysis. Streptokinase is a fibrin-independent plasminogen activator. It doesn't exhibit proteolytic activity by itself, but has to bind to free and fibrin bound plasminogen first to produce an activator complex to convert free plasminogen to plasmin [9-12, 49]. In our experiments, streptokinase bound to plasminogen at the surface of fibrin to form an activator complex with the low concentration of free plasminogen. The delay could be primarily caused by the specific feature of the mouse plasminogen, different from other species, such as human plasminogen that is the most efficient activated by streptokinase [10,
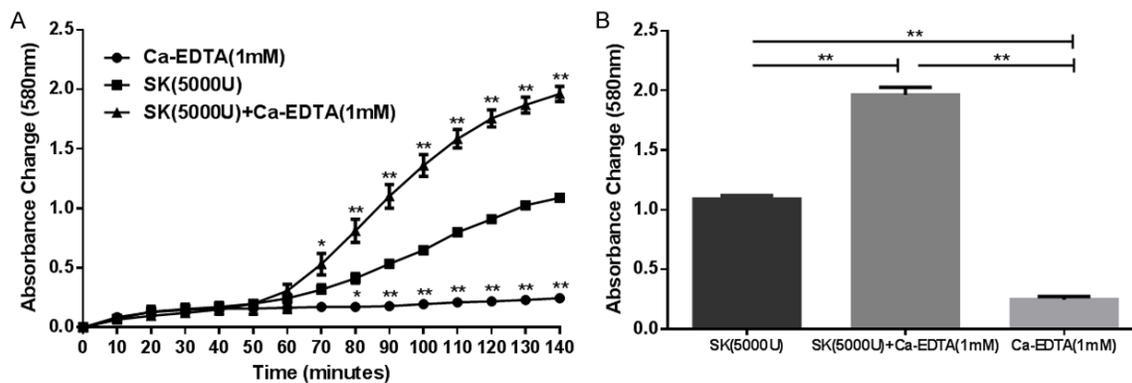
Figure 4. Zinc chelation promoted streptokinase-induced blood clot lysis. A. Graph showing increased streptokinase-induced absorbance change with the addition of zinc chelator (1 mM Ca-EDTA). The absorbance of blood samples was measured for thrombolysis induced by 5000 unit streptokinase for 140 minutes with and without Ca-EDTA. The absorbance change showed a significant difference between streptokinase only and streptokinase plus Ca-EDTA treatment from the 70th minute to the 140th minute. Ca-EDTA itself almost did not cause absorbance change. B. Histogram showing quantified absorbance changes after 140 minutes treatment with streptokinase only, streptokinase with Ca-EDTA and Ca-EDTA only. The quantified absorbance change showed a nearly two-fold promotion in streptokinase with Ca-EDTA treatment compared with that of streptokinase only treatment. Ca-EDTA itself had nearly no effect in absorbance change. Streptokinase: 5000 unit. Ca-EDTA: 1 mM. Data were presented as mean ± SEM. The numbers of samples tested: 8 in streptokinase, 7 in streptokinase with Ca-EDTA. 9 in Ca-EDTA only. *$p \leq 0.05$; **$p \leq 0.01$.

13]. Streptokinase activates mouse plasminogen slowly, thus needs more time to achieve the same effect with mouse plasminogen as that with plasminogen from other species.

Streptokinase-induced thrombolysis was inhibited by zinc

Zinc attenuated streptokinase-induced thrombolysis. Zinc-induced inhibition of thrombolysis exhibited a dose-dependent characteristic. In the present study, three doses of $ZnCl_2$ (i.e., 10, 50 and 100 µM) were applied to streptokinase-induced thrombolysis. The significant inhibition effect started from the 110th, 80th and 70th minute in 10, 50 and 100 µM $ZnCl_2$, respectively (**Figure 3A**). Furthermore, 100 µM $ZnCl_2$ caused a 79.7% reduction in thrombolysis and 50 and 10 µM $ZnCl_2$ suppressed 57.5% and 30.6% of integral streptokinase-induced clot lysis, respectively (**Figure 3B**). The mechanism of this zinc inhibition could be explained in several aspects. It is still uncertain whether zinc binds to streptokinase, but zinc might bind to this streptokinase-plasminogen activator complex to inhibit the activation of plasminogen to plasmin. Zinc, as a regulator in coagulation, prevents thrombolysis by enhancing thrombosis and hemostasis. Zinc promotes coagulation through interacting with Factor (F) XII and high-molecular-weight kininogen in contact system [32-37]. Zinc also participates in platelet aggregation. It is reported that zinc mediates fibrinogen binding to its cognate receptor on the platelet, which enhances platelet aggregation [30, 31]. Furthermore, previous studies indicated that zinc promotes thrombin-induced fibrin formation [38]. Therefore, zinc could potentiate coagulation to inhibit thrombolysis. Finally, it's interesting to note that zinc has a specific role in abrogating heparin anticoagulant property to attenuate thrombolysis. There is ample evidence that heparin interacts with histidine-rich glycoprotein in the presence of zinc, resulting in heparin neutralization and impaired anticoagulation activity [50-52].

Zinc chelation promoted streptokinase-induced thrombolysis

A high concentration of zinc is stored in plasma and platelets bound to proteins [25-28]. The plasma total zinc concentration ranges from 10 to 20 µM [53]. Local plasma zinc likely increases significantly within the vicinity of a growing thrombus [53]. Zinc is aslo present in the cytosol and alpha granules of platelets at concentrations up to 60-fold higher than in the plasma [54]. Platelets accumulate in thrombi in numbers 50- to 100-fold higher than those found in the circulating blood [55-57]. Therefore, the

concentration of zinc in clots are likely to be really high. Zinc is released from activated platelets and other damaged cells to extracellular microenvironment producing a surge in the level of free zinc [39-43]. In addition, zinc has previously been shown to be a platelet agonist, with sub-millimolar concentrations of zinc being able to induce platelets aggregation [54]. Thus, a large amount of zinc could be released during thrombolysis, resulting in a localized high concentration. A high level of zinc chelator should be used to remove this localized high concentration of zinc. If not, the thrombolytic effect will be diminished. Even more, no significant difference will be observed in the zinc chelation treatment group compared with the treatment without chelator. Ca-EDTA is a specific cell-impermeable zinc chelator that removes extracellular zinc. After the application of chelator, the efficiency of streptokinase-induced thrombolysis achieved a two-fold increase compared to streptokinase only (**Figure 4B**). The data demonstrated that extracellular zinc plays a critical role in inhibiting streptokinase-induced thrombolysis. On the one hand, zinc chelation improves streptokinase's thrombolytic effect. On the other hand, chelation also decreases the required dose of streptokinase to achieve the same effect with a high dose of streptokinase. The efficacy of 5000 unit streptokinase with zinc chelator almost reached the same thrombolytic capacity as that of 7500 unit streptokinase (**Figures 2B** and **4B**). High dose of streptokinase (1.5 million unit in clinical treatment) has a high risk of symptomatic hemorrhagic transformation in the treatment of ischemic stroke, at a rate of 8-21% in previous clinical trials [58-61]. Symptomatic hemorrhagic transformation results in an increased rate of mortality. Thrombolytic therapy combined with zinc chelator will not only reduce the required streptokinase dose to decrease the rate of hemorrhagic transformation but also achieve the same efficacy in lower dose streptokinase as that of a higher dose in the treatment of thrombotic disorders. Thus, safety concerns arising from the usage of a high unit streptokinase could be avoided by using streptokinase at a low unit along with zinc chelator.

Conclusion

According to the present study, we conclude that zinc inhibits streptokinase-induced throm-

bolysis in a dose-dependent manner *in vitro*. Treatment with zinc chelator promotes streptokinase-induced thrombolysis. Further research is needed to explore more details about the relationship between streptokinase and zinc at molecular level. Furthermore, we hope to develop a combined therapy to facilitate streptokinase-induced thrombolysis and reduce hemorrhagic transformation *in vivo*. A new thrombolytic strategy is in urgent demand in the aspect of not only good thrombolytic efficiency and safety but also financial acceptance. For example, streptokinase has a longer half-life and far better cost-effectiveness than those of tissue plasminogen activator. Thus, estimating the ideal dose of streptokinase is critical to adjust the balance of high thrombolysis efficiency and low complication rate in the treatment of cardiovascular diseases. Utilization of a proper dose of streptokinase could improve reperfusion in the treatment of thrombotic disorders.

Disclosure of conflict of interest

None.

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