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Research Article

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Tailored methods for preclinical assessment of fibrinolytic agents *in vitro*

Purnima Dey Sarkar[1], Mithun Singh Rajput[1]* and Nilesh Prakash Nirmal[2]

[1]Department of Medical Biochemistry, MGM Medical College, A. B. Road, Indore, M.P., India
[2]Center for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Health and Food Sciences Precinct, Block 10, 39 Kessels Road, Coopers Plains, Acherfield Brisbane QLD 4108, Australia

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ABSTRACT

Fibrinolytic drugs are widely used for the management of atherothrombotic diseases such as acute or prior myocardial or cerebral infarction, ischemic stroke and venous thromboembolism. Quite a lot of in vitro models have been developed to study clot lytic activity of fibrinolytic drugs, but all of these have certain limitations. There is need of a rapid method to check and quantify the clot lytic efficacy of fibrinolytic drugs precisely. In the present study, an attempt has been made to curtail two novel methods to study fibrinolysis in a simplified and easy way using standard fibrinolytic dugs, plasmin and streptokinase. Fibrin clots were allowed to form in microcentrifuge tubes using plasma separated from the whole blood from healthy mice or directly using fibrinogen and thrombin. After lysis by various doses of plasmin and streptokinase, fluid was removed and its volume was measured. Difference obtained in volume taken before and after plasma clot lysis was expressed as percentage of fibrinolysis. The validity of new-fangled methods was assessed by using various concentrations of standard drugs plasmin and streptokinase as positive control and water as negative control. The results appear to validate their claim by demonstrating a dose-dependent effect of standard test samples. The IC_{50} values for both the drugs were also found out when accessed by plasma clot lysis and fibrin clot lysis method. Thus the novel methods presented here could be reproducible, instant, quantitative, accurate, cost effective and valuable models to study fibrinolytic effect of newly developed drugs as well as known drugs. Test drugs can also be analyzed in the same way using presented methods.

Keywords: Fibrinolytic; novel method; plasmin; streptokinase

INTRODUCTION

Fibrin clot developed in the circulatory system due to failure of haemostasis renders vascular blockage and whilst recovering leads to serious consequences by thromboembolic events, including acute or prior myocardial infarction, ischemic stroke and venous thromboembolism [1]. Fibrinogen, a plasma 340 kDa glycoprotein, is converted to fibrin on limited proteolysis by thrombin further process to form fibrin clots composed of compact, highly branched networks with thin fibers, resistant to lysis [2,3]. In blood coagulation, a cascade reaction composed of many clotting factors is known to be involved and culminates in generation of thrombin which is the key enzyme responsible for conversion of soluble fibrinogen to insoluble fibrin clot [4]. As of the critical role of thrombin in thrombus formation, propagation and stabilization, an effectual inhibition of thrombin (in addition to fibrinogen and fibrin) can be a prerequisite for prevention and treatment of thrombotic disorders [5]. Low dose aspirin, statins, lowering of homocysteine, glucose lowering agents, angiotensin-converting enzyme inhibitors and suppression of inflammatory response, amplify clot permeability and susceptibility to lysis [1]. Growing evidence indicates that anomalous fibrin properties represent a novel risk factor for arterial and venous thrombotic events, particularly of unknown etiology in young and middle aged patients.

Various methods were developed to compute the fibrinolytic potential of numerous drugs such as euglobulin lysis time [6-8], fibrin plate method [9,10], colorimetric assay [11], use of fibrinolytic apparatus [12], use of a diagnostic

reagent for the detection of the activity of a fibrinolytic enzyme [13] etc. Various models have been reported which either uses complicated mathematical or computing skills, but all these methods are difficult, costly and not reasonably priced for research in small institutes, some of the methods like fibrin plate method is semi quantitative and less up to the mark [14]. The above mentioned problems demand necessitate of simple and cost effective plasma clot lytic method for measurement of fibrinolytic potential of various agents. In the present study an attempt has been made to amend *in vitro* fibrin clot lytic methods using known fibrinolytic drugs, streptokinase and plasmin.

EXPERIMENTAL SECTION

Chemicals

Plasmin from human plasma (2 units/mg protein) was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore India. A stock solution of 0.06 mg/ml of plasmin in distilled water was prepared. Stock solution undiluted, 1:2, 1:4 and 1:8 diluted by distilled water (100 µl each) were used for *in vitro* fibrinolysis studies. Lyophilized streptokinase vial of 15,00,000 IU (Bharat Biotech, Hyderabad, India) was purchased from local pharmacy, Indore, India. To the vial, 5 ml distilled water was added and mixed properly. This suspension (3,00,000 IU/ml) was used as a stock from which undiluted, 1:2, 1:4 and 1:8 diluted by distilled water (100 µl each) were used for further study. All other chemicals and reagents used were of analytical grade. (The amount of plasmin is expressed in µg/mg protein while streptokinase in IU).

Animals

Swiss albino mice (18-30 g) of either sex were used. Animals were placed randomly in polypropylene cages under standard conditions of humidity (55 ± 5%), temperature (25 ± 2 °C) and 12h/12h, light/dark cycles and free access to standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and filtered water *ad libitum*. Experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care as per the US guidelines (NIH publication #85–23, revised in 1985). Experiments were conducted after obtaining the approval from the Institutional Animal Ethics Committee constituted as the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Plasma clot lysis method

In the first method, blood was collected from the hearts of normal mice (n = 6) and mixed immediately with 3.8% tri-sodium citrate in volume ratio of 9:1. Then the mixture was centrifuged at 2500 × g for 15 min to obtain plasma [15]. Four hundred microliter of citrated plasma was added to nine different sterile microcentrifuge tubes followed by addition of 100 µl 0.2 mol/l $CaCl_2$ and allowed to stand until a firm clot was obtained. The microcentrifuge tubes, than allowed to incubate at 37 °C for 30 min to ensure the formation of fibrin clot. Each microcentrifuge tube containing plasma clot was properly labeled and 100 µl of streptokinase from stock solution along with different dilutions in sterile distilled water (1:2, 1:4 and 1:8) were added to four different tubes. One hundred microlitre of plasmin from stock solution along with dilutions in sterile distilled water (1:2, 1:4 and 1:8) were added to four another tubes. Distilled water (100 µl) was also added to one of the tubes containing clot and this serves as a negative fibrinolytic control. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, fluid obtained was removed and the volume of fluid was measured to observe the difference in volume after clot disruption. Difference obtained in volume taken before and after plasma clot lysis was expressed as percentage of fibrinolysis. The test was repeated six times with all the doses of different fibrinolytic drugs (plasmin and streptokinase) and distilled water in different blood samples.

Fibrin clot lysis method

In the second method, to prepare a fibrin clot 400 µl of 2 mg/ml fibrinogen prepared in tris buffer (pH 7.4) was added separately in nine different sterile microcentrifuge tubes. The solution was immediately mixed with 12 µl of (34 units/ml, 0.4 units) of plasminogen and 12 µl of (67 units/ml, 0.8 units) of thrombin, each prepared in tris buffer (pH 7.4). The microcentrifuge tubes, than allowed to incubate at 37 °C for 30 min to ensure the formation of fibrin clot [16]. Each microcentrifuge tube containing fibrin clot was properly labeled and 100 µl of streptokinase from stock solution along with different dilutions in sterile distilled water (1:2, 1:4 and 1:8) were added to four different tubes. One hundred microlitre of plasmin from stock solution along with different dilutions in sterile distilled water (1:2, 1:4 and 1:8) were added to four another tubes. Distilled water (100 µl) was also added to one of the tubes containing clot and this serves as a negative fibrinolytic control. All the tubes were then incubated at 37 °C for 3 h and observed for clot lysis. After incubation, fluid obtained was removed and the volume of fluid was measured to observe the difference in volume after clot disruption. Difference obtained in volume taken before and after plasma clot lysis was expressed as percentage of fibrinolysis. The test was repeated six times with all the doses of different fibrinolytic drugs (plasmin and streptokinase) and distilled water.

Statistical analysis

Data was expressed as the mean ± S.E.M. and statistical analysis was carried out employing one way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test at a $p<0.05$ significance level using GraphPad Instat (Version 3.00 for Windows 95; GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

In plasma clot lysis method (first method), plasma clots when treated by different doses of streptokinase (3750, 7500, 15000 and 30000 IU) showed 22.92 ± 0.56, 42.38 ± 0.73, 79.47 ± 0.97 and 81.25 ± 1.06% clot lysis respectively. Plasma clots when treated by different doses of plasmin (0.75, 1.50, 3.00 and 6.00 µg) showed 28.55 ± 0.23, 55.13 ± 0.59, 91.97 ± 0.65 and 94.36 ± 0.81% clot lysis respectively. Clots when treated with same concentrations of sterile distilled water (negative control) showed only negligible clot lysis (3.09 ± 0.28%). The mean difference in clot lysis percentage between each dose of fibrinolytic agents and water (control) was significant ($p<0.01$) (Table 1). The log dose-response curve represents that streptokinase exhibit an IC_{50} value of 8710 IU (antilog 3.94, as calculated from Figure 1) and plasmin exhibit an IC_{50} value of 1.22 µg (antilog 0.086, as calculated from Figure 1).

Table 1: Plasma clot lysis of blood samples of normal mice by different concentrations of fibrinolytic agents

Treatment	Dose (100 µl)	Percent fibrinolysis
Control (DW)	–	3.09 ± 0.28
Streptokinase	3,750 IU	22.92 ± 0.56*
	7,500 IU	42.38 ± 0.73*
	15,000 IU	79.47 ± 0.97*
	30,000 IU	81.25 ± 1.06*
Plasmin	0.75 µg	28.55 ± 0.23*
	1.50 µg	55.13 ± 0.59*
	3.00 µg	91.97 ± 0.65*
	6.00 µg	94.36 ± 0.81*

*Results are expressed as Mean ± S.E.M.; n = 6 in each group comparison made with control group. Data was analyzed by one way ANOVA followed by Dunnett's test. Significance: *p< 0.01 when compared with control. DW: distilled water*

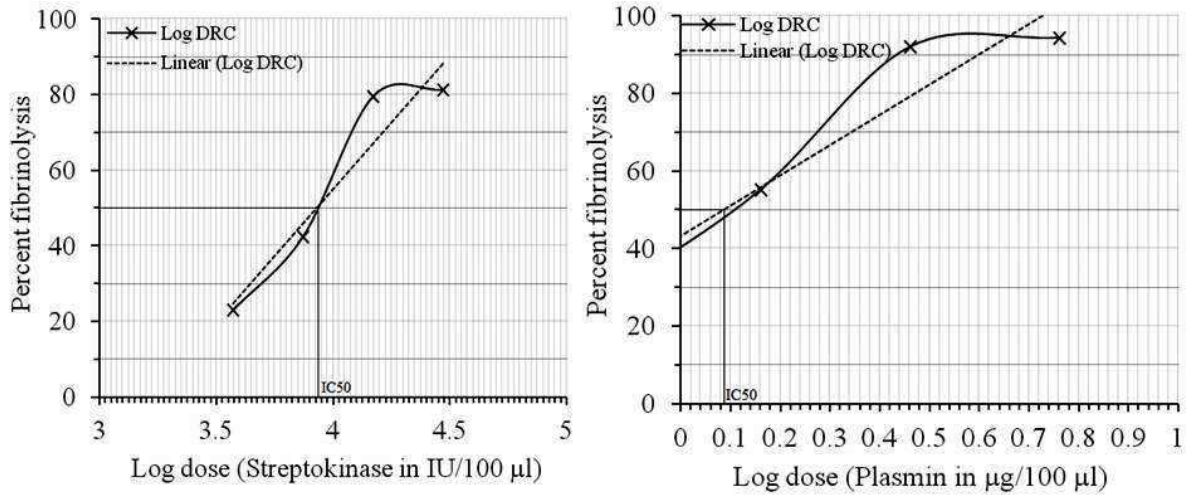

Fig. 1: Dose dependent effects and IC_{50} values of streptokinase and plasmin accessed by plasma clot lysis method

Results are expressed as Mean ± S.E.M.; n = 6 in each group comparison made with control group. Data was analyzed by one way ANOVA followed by Dunnett's test.

In fibrin clot lysis method (second method) fibrin clots when treated by different doses of streptokinase (3750, 7500, 15000 and 30000 IU) showed 19.97 ± 0.35, 43.46 ± 0.63, 82.87 ± 1.02 and 85.42 ± 0.94% clot lysis respectively. Fibrin clots when treated by different doses of plasmin (0.75, 1.50, 3.00 and 6.00 µg) showed 23.45 ± 0.19, 58.13 ± 0.36, 92.82 ± 0.54 and 95.19 ± 1.37% clot lysis respectively. Clots when treated with same concentrations of sterile distilled water (negative control) showed only negligible clot lysis (2.82 ± 0.31%). The mean difference in clot lysis percentage between each dose of fibrinolytic agents and water (control) was significant ($p<0.01$) (Figure 2). The log dose-response curve represents that streptokinase exhibit an IC_{50} value of 8318 IU (antilog 3.92, as calculated from Figure 3) and plasmin exhibit an IC_{50} value of 1.25 µg (antilog 0.1, as calculated from Figure 3).

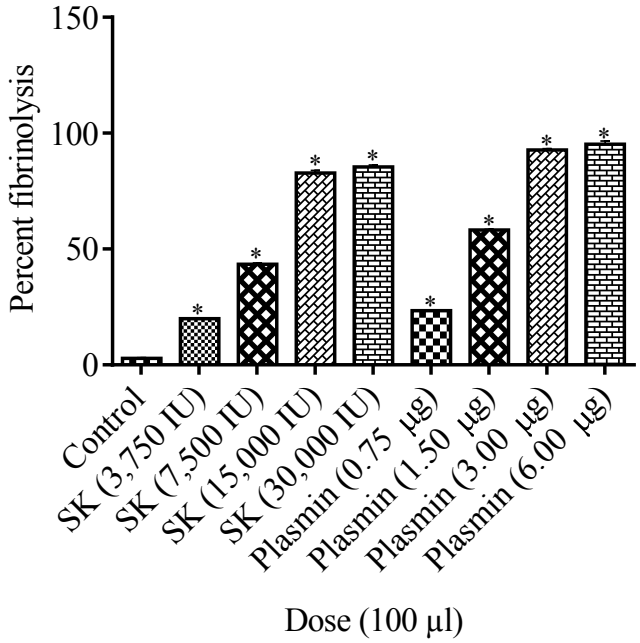

Fig. 2: Fibrin clot lysis by different concentrations of fibrinolytic agents

*Results are expressed as Mean ± S.E.M.; n = 6 in each group comparison made with control group. Data was analyzed by one way ANOVA followed by Dunnett's test. Significance: *p<0.01 when compared with control. DW: distilled water.*

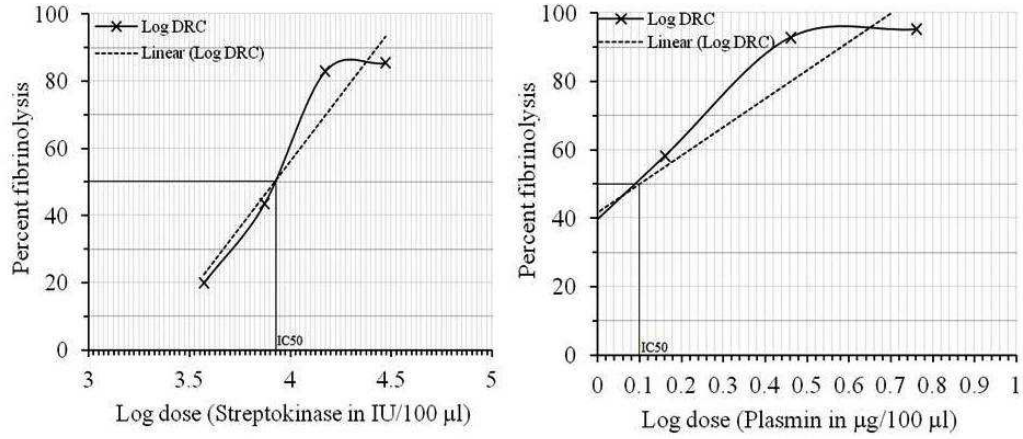

Fig. 3: Dose dependent effects and IC_{50} values of streptokinase and plasmin accessed by fibrin clot lysis method

Results are expressed as Mean ± S.E.M.; n = 6 in each group comparison made with control group. Data was analyzed by one way ANOVA followed by Dunnett's test.

This study shows *in vitro* dissolution of fibrin clots by plasmin and streptokinase, assayed by two different *in vitro* clot lysis methods amended in our laboratory. Most of the *in vitro* methods that were conventionally or currently applied to study fibrinolysis have certain limitations. Some involve complex computation and mathematical skills that too give only theoretical prediction of the outcome and most are expensive to be performed in a laboratory. In context with the current scenario we thought of developing fibrinolysis methods that would be simple and easy to perform in laboratory. Keeping this idea in the prime focus, volume of the plasma clot before lysis and after lysis was considered as appropriate determinant of calculating clot lysis percentage. In other methods, there are different parameters to analyze the extent of clot lysis. For example, turbidity determination using microtiter plate reader in euglobulin lysis test [6-8], fibrin plate method requires 18 h incubation of test drug for measurement of diameter of clear zone [9,10], quantitative measurement of plasma clot lysis by assay of Coomassie brilliant blue R-250 dye released from the clot in colorimetric assay[11], use of sophisticated fibrinolytic apparatus [12], use of a diagnostic reagent for the detection of the activity of a fibrinolytic enzyme [13], study of fibrinolytic activity by circulating fibrinolytic enzymes and monitoring the effect by calculating shear rate [17,18] etc. All these methods are sophisticated whereas, our methods are simple, cost effective and easy to perform and one can even visually observe the lysis of clots.

Streptokinase does not have a direct fibrinolytic activity and the therapeutic action is *via* the activation of blood plasminogen to the clot dissolving plasmin. Plasmin is a direct acting thrombolytic agent with a striking hemostatic safety advantage over other thrombolytic agents (plasminogen activators, streptokinase, urokinase) in animal models of thrombolysis and bleeding. In contradistinction to other thrombolytic agents, which threat bleeding at any effective thrombolytic dose, plasmin is tolerated devoid of bleeding at numerous fold higher amounts than those needed for thrombolysis. Plasmin has been safe in a current trial in patients with peripheral arterial or graft occlusion and efforts are now directed toward therapy of stroke caused by cerebral artery occlusion [19]. Owing to the above mentioned settlements plasmin has been considered in our study along with streptokinase which has been widely used as positive control. Doses for streptokinase has been adapted based on previous clot lysis studies using different models and the results of our study also found to be consisted with previous cram [20-22]. Nevertheless, the results of prior studies provide guidance for selecting appropriate initial dosages of plasmin for determination of *in vitro* fibrin clot lysis in our study. The starting dose of plasmin could be easily calculated based on relative body weight and thrombus histology [19].

Since the pathological thrombus formation in the circulatory blood vessel is known to be provoked by imbalance between the hemostatic pathway and the thrombolytic pathway, the ideal antithrombotic agents need to have not only anticoagulant activity but also fibrinolytic activity. If an agent exert only the anticoagulant activity that can interfere with thrombus formation, their efficacy would have a limitation in treatment of thrombotic diseases due to the absence of fibrinolytic activity [23]. The first method discussed above has been tailored out from the formerly accomplished study by Prasad *et al.* (2007) [20]. This curtailed method has been evolved to demonstrate the efficacy of fibrin specific agents which was the limitation of the previously published study implicating the whole blood clot. The drugs used to target whole blood clot have limited fibrin specificity [24]. The fibrin clot lacks blood cells which makes it more specific as a target for fibrinolytic agents.

The fibrin plate has been described as semi-quantitative, complicated, unreliable and difficult to use and since its introduction in 1952 by Astrup and Miillertz many modifications have been proposed in an attempt to improve the method time to time [16]. In view of these criticisms, the alternate study was carried out in order to evaluate the original method and some of the subsequent modifications. Difference obtained in volume taken before and after plasma clot lysis has been expressed as percentage of fibrinolysis in alternate studies.

The validity of new-fangled methods was assessed by using various concentrations of standard drugs plasmin and streptokinase as positive control and water as negative control. The results appear to validate their claim by demonstrating a dose-dependent effect of standard test samples.

CONCLUSION

To check the efficacy of fibrinolytic agents one can compare the data with positive and negative control. In our study we took known fibrinolytic agents plasmin and streptokinase as positive control and distilled water as a negative control. Test drug can also be analyzed in the same way. Thus the novel methods presented here could be reproducible, instant, quantitative, accurate and cost effective and effective models to study fibrinolytic effect of newly developed drugs as well as known drugs *in vitro*.

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