In-vitro and *In-vivo* Screening Methods for Antithrombotic Agents

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ABSTRACT

Thrombolytic therapy and mechanical interventions are frequently used in the treatment of thrombotic diseases. The basic problems with current thrombolytic therapy include slow and incomplete thrombolysis and frequent bleeding complications. Therefore, there is a need of continuity to develop & screen out the various natural agents that possess antithrombotic activity by using different screening methods. The experimental thrombotic methods can be classified into two groups-fibrin rich red thrombi are produced in veins by stasis or procoagulant. The platelets rich white mural thrombi are produced in artery by vessel wall injury or stenosis. Thrombosis models are usually performed in healthy animals not included atherosclerosis or throbophilias. But no one method yet to develop that completely resembles to pathophysiology of thrombosis of human beings. So it's better to evaluate any new compound in more than one model for clear clinical relevance, the pharmacological effectiveness. Presently the research in new drug discovery utilizes combinatorial chemistry, computer aided drug design, quantitative structural activity relationship, ADME-T and bioinformatics. In spite of several developments, screening and evaluation methods remain a challenge for pharmacology. This review contains numerous simplest and most widely accepted techniques of drug evaluation in precise and concise manner.

Keywords: Blood, Thrombosis, Coagulation, Clotting time, Bleeding time, Animal models.

INTRODUCTION

The process of coagulation occurs via a cascade of sequential reactions and thrombin, is the final enzyme in the coagulation cascade, that turn converts soluble fibrinogen into insoluble fibrin.[1] Thrombin also activates factor-XIII, accelerates the formation of factor-V, which increases thrombin formation, and activates platelets, thereby enhancing platelet aggregation.[2] Clotting arises from a complex interaction of various mechanisms.[3] Platelets initiate clotting by breaking and convert the

blood prothrombin into thrombin which is one of the basic substances to form the cot. Thrombosis is intravascular clotting of the blood. Slowed down circulation, atherosclerosis i.e. narrowness of lumen and roughing of the vessel walls break the thrombotic cells and finally fibrin threads are formed and a blood clot result. Heart vessels and brain vessels are often damaged and results in coronary and cerebral thrombosis respectively. Thrombosis is one of the leading causes of thromboembolic disorders and affecting a big human population worldwide. Thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are mainly responsible for morbidity and mortality in developed countries.[1] Hence, anticoagulants like heparins, vitamin-K antagonists, and their derivatives play a pivotal role in the prevention and treatment of thromboembolic disorders.[4,5] The antiplatelet agents inhibit platelet aggregation by suppressing the formation of ADP and thromboxane which are involved in platelets aggregation. Antiplatelets basically useful in arterial thrombosis, however, anticoagulants are more preferred in venous thrombosis. Although their efficacy remains undisputed, the deleterious life-threatening side effects of these drugs have also been well documented.[6,7] Plants, minerals and other dietary components may serve as the alternative sources for the development of new thrombotic agents. Various scientific reports exhibit that the consumption of dietary anticoagulants, antioxidants or phytochemicals with anticoagulant properties can ultimately reduce the risks of thrombotic disorders.[8,9] Therefore, there is a great need to evaluate newly developed antithrombotics or anticoagulants obtained from natural and synthetic resources by in-vitro as well as in-vivo or biologically. The screening techniques are used to ensure specific pharmacological activity, efficacy & safety, quantitative and qualitative availability of biologically active molecules. The current research in new drug discovery utilizes combinatorial chemistry, computer aided drug design[10,11], quantitative structural activity relationship, absorption; distribution; metabolism; excretion & toxicity (ADME-T)[12] and bioinformatics. In spite of several available techniques, development of screening and evaluation methods remains a great challenge for pharmacologists. In past, pharmacologists use whole animal and organ preparations for drug screening. By 1980s use of biochemical methods becomes popular in drug screening because these are associated with biotechnology tools. Later on computerization gave rise to novel evaluation techniques in drug research like high through put screening, cell-based assays, transgenic animals, and stem cell research.

The experimental models of thrombosis enhanced our understanding of pathophysiological changes occurs during disease development. Animal models and transgenic or gene knockout animals will allow to pinpoint the relative functional importance of single changes in specific gene products in the pathophysiological process.[13] The use of animal models of thrombosis provides a critical tool for the discovery and initial testing of novel antithrombotic drugs for thrombosis.[14] The in-vitro models also equally important in evaluation of antithrombotic agents. This review contains numerous simplest and most widely accepted techniques of drug evaluation in precise and concise manner. These techniques are often used by researchers involved in various research & development activities in the field of pharmacology, physiology, toxicology and therapeutics. Pharmaceutical industries also

use the screening techniques to evaluate the in-vivo activities of newly developed drugs.

SCREENING METHODS

(I) *In-vitro* methods
(i) Thrombin assay
(ii) Clotting time assay
(iii) Thrombin induced clotting time assay
(iv) Calcium chloride induced clotting time assay
(v) Clot lysis method

(II) *In-vitro* models

(III) *In-vivo* models
(i) Arteriovenous shunt model
(ii) Arterial thrombosis
(iii) Venous thrombosis
(iv) Coronary thrombosis
(v) Electrically induced thrombosis
(vi) Super cooling induced thrombosis
(vii) Ferric chloride (FeCl$_3$) induced thrombosis
(viii) Laser induced thrombosis
(ix) Photochemically induced thrombosis
(x) Foreign material induced thrombosis
(xi) Wire coil induced thrombosis
(xii) Eversion graft induced thrombosis
(xiii) Stasis induced thrombosis
(xiv) Homocysteine induced thrombosis
(xv) Monoclonal antibody induced thrombosis
(xvi) High fat diet induced thrombosis
(xvii) Carrageenin-induced thrombosis
(xviii) Acetic acid induced thrombosis
(xix) Adenosine diphosphate induced thrombosis
(xx) Arachidonic acid induced thrombosis
(xxi) Cuff induced thrombosis
(xxii) Mechanical trauma induced thrombosis

(IV) Bleeding models

(V) Genetic models

(I) *In-vitro* methods
There are numerous in-vitro methods like blood coagulation tests (thrombin assay; clotting time assay; thrombin induced clotting time assay; and calcium chloride induced clotting time assay), thrombelastography test, Chandler loop technique, born method, platelet aggregation method, after gel filtration, whole blood test, laser scattering test, fibrinogen receptor binding assay, euglobulin clot lysis time, flow behaviour of erythrocytes, filterability of erythrocytes, erythrocyte aggregation test, and plasma viscosity test. These are basically based on coagulation process and properties of blood components.[15]

(i) Thrombin assay[16]-the evaluating agent is added to 10 µl thrombin at 30 U/ml in distilled water. Incubate this mixture for 10 min at room temperature and then mixed 190µl 0.76 M chromogenic thrombin. The reaction is monitored at 412 nm for 4 min at 10 s intervals using a microtitre plate reader. The evaluating agent that inhibited thrombin compared to a control.

(ii) Clotting time assay[17]-clotting time assays use to determine the anticoagulant effect of drugs on human plasma. The inhibitory activity of the evaluating agent tested on thrombin, ADP, epinephrine, trypsin, bromelain and papain induced platelet aggregation. The evaluating agent (40 µl) is added to rat platelets (100 µl) and left at room temperature for 5 min. Thrombin/ADP/epinephrine (20µl) is added and the inhibition of blood clot measured at 412nm using a plate reader for 20 min at 30 s intervals. The activity of the evaluating agent expressed as % of mean inhibition.

(iii) Thrombin induced clotting time assay[18]-the evaluating agent (40µl) added to human plasma (100µl) and incubated at room temperature for 5 min. Thrombin (20µl at 5U/ml) is added and the rate of clot formation was determined by following the increase of the absorbance at 412nm microtitre plate

reader for 20 min at 30 s intervals. The evaluating agent that inhibited the rate of clot formation is determined. A negative control (2% (v/v) DMSO in saline) and a positive control like heparin may be used.

(iv) Calcium chloride induced clotting time assay[19]-this assay allows for the determination of a 50% clotting time and effect on fibrin formation. The assay is performed by adding solution of evaluating agent (40µl) to human plasma (100µl). The reaction was mixed and left to incubate for 5 min at room temperature. Clotting is induced by the addition of 20µl 0.16 M $CaCl_2$, and the reaction is followed at 412 nm with a microtitre plate reader for 2 hrs at 3 min intervals.

(v) Clot lysis method[20]-venous blood of healthy volunteers is taken and transferred in pre weighed sterile microcentrifuge tube (500µl/tube) & incubated at 37°C for 45 min. After clot formation, completely remove the serum and each tube having clot is again weighed to determine the clot weight. Each microcentrifuge tube containing clot is labelled and 100µl of streptokinase along with various dilutions in sterile distilled water (undiluted, 3:4, 1:2 and 1:3) is added to the tubes. Water is also added to one of the tubes containing clot and this serves as a negative control. All the tubes then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid obtained is removed and tubes are weighed again to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as % of clot lysis.

(II) *In-vitro* models

These are basically based on the properties and role of platelets in blood clotting. The methods are commonly used like shear flow cytometry[21], platelet adhesion and aggregation and parallel plate flow chamber test.[22]

(iii) *In-vivo* models

These models are basically used to evaluation antiplatelets, thrombolytic & antithrombotic agents in animals. The models are arteriovenous shunt model, folts model, harbauer model, electrical induced thrombosis, $FeCl_3$ induced thrombosis, thrombin induced clot formation, laser induced thrombosis, photochemical induced thrombosis, foreign surface induced thrombosis by-wire coil method, eversion graft method, arteriovenous shunt method, thread method, superfused tendon method, wessler model, disseminated intravascular coagulation model, trauma models, cardiopulmonary bypass models, extracorporeal models, experimental thrombocytopenia or leucocytopenia, collagenase induced thrombocytopenia and reversible intravital aggregation of platelets model.[15] These further discussed as-

(i) Arteriovenous shunt model[23]-the left jugular vein and right common carotid artery were cannulated with 8 cm long polyethylene cannula mounted on 25/8 needles. The shunt was assembled by connecting the two cannulae with a slightly curved 6cm long plastic tube containing a 5cm long nylon filament which be had scraped with scalped blade to render it more thrombogenic. The extracorporal circulation is maintained for 15 min during which a thrombus adheres to the nylon thread. Thrombus along with thread removed and weighted.

(ii) Arterial thrombosis-thrombus formation induced by an electric current (3 mA d.c.) for 5 min to external arterial surface by means of a d.c. stimulator. Alternatively, anodal current application of internal surface also induces thrombosis.

(iii) Venous thrombosis[24]-thrombus formation is induced by the i.v. injection of 20µg/kg rabbit brain thromboplastin, followed 10 s later by stasis of 1cm long segment of abdominal vena cava stasis was

maintained for 15 min, after which time the thrombus was removed and weighted.

(iv) Coronary thrombosis[25]-coronary thrombosis may be induced by stenosis or stenosis with the infusion of epinephrine, or stenosis with platelet activating factor or by electrical stimulation of intima with 150µA for 6 hours. The coronary thrombosis is also induced by squeezing vessel with a 2mm haemostatic clamp for 5 s. A small cylindrical plastic constrictor (2-4mm in length and internal diameter of 1.2 to 1.8mm) is placed around the artery at the site of the damage.

(v) Electrically induced thrombosis[26,27] the rabbits (2.5-3.5kg) anesthetized with ketamine (50mg/kg/h i.m.) and xylazine (10mg/kg/h i.m.). The left femoral vein and artery were isolated and catheterized. Both common carotid arteries were carefully isolated. Carotid blood flow was measured with a calibrated flow probe (3.5 mm circumference) that was linked to an electromagnetic flowmeter. A stainless steel bipolar hook electrode was placed on the carotid artery and positioned caudally in relationship to the flow probe. A piece of Parafilm (7×30mm) was placed under the electrode to protect the surrounding tissue. Thrombosis was induced by applying a direct electrical current of 4mA for 3 min to the external arterial surface, using a constant current unit and a d.c. stimulator. The carotid blood flow monitored before and after electrical stimulation. The left carotid artery served as a control artery. After the determination of the control time to occlusion, the compound or saline is given as continuous i.v. infusion via the femoral vein, starting 1 h before the electrical stimulation and continuing to the end of the test. Thrombosis was electrically induced in the right common carotid artery. When carotid blood flow was decreased to zero, the time to occlusion in minutes was noted or kept constant for 90 min (Schumacher et al., 1993). The ED_{50} value for evaluating agent may be estimated.

(vi) Super cooling induced thrombosis[28]-rat carotid artery occluded by a clamp and placed for 2 min into a metal groove which cooled to $-15^{o}C$. The vessel was compressed by weight of 200 gm. After 4 min clamp was removed and the flow re-established in the injured artery. After 4 hours remove carotid artery and weight the thrombus.

(vii) Ferric chloride ($FeCl_3$) induced thrombosis[29]-a filter paper of 2x5mm, saturated with 25% $feCl_3$ solution, applied to the carotid artery for 10 min. Measure the blood flow and weigh the thrombus after blotting.

(viii) Laser induced thrombosis[30,31]-the argon laser beam exposure of 1/30 or 1/15 s injured rabbit's arterioles of $13\pm1µm$ diameter. The number of laser shorts (with a gap 1short/2 min) required to produce a defined thrombus is determined.

(ix) Photochemically induced thrombosis[32]-intravenously injected fluorescein isothiocynate-dextran-70 (0.3ml of 10%) in rat arterioles of 15-30µm diameter and exposed to UV light of wavelength 510nm determine time of blood flow interruption.

(x) Foreign material induced thrombosis[33]-a prothrombotic foreign material activates coagulation and the platelet system. Various prothrombotic surfaces like catheters, oxygenators etc. have been used to develop experimental animal models.

(xi) Wire coil induced thrombosis[34]-a stainless steel wire coil is inserted into the lumen of the artery or vein. After 2 hrs the wire coil is removed together with the thrombus and rinsed with normal saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution in boiling water for 3 min. The protein content is determined in 100µl aliquots by the colorimetric method.

(xii) Eversion graft induced thrombosis[35]-segment of rabbit femoral or

dog left circumflex artery (4-6mm) everted and reimplanted end to end. After 15 min evaluating agent is given and after 10 min of the evaluating agent blood flow is monitored by flowmeter for 120 min.

(xiii) Stasis induced thrombosis[36]-calcium thromboplastin (0.3ml/kg) injected within 30 sec and 0.5ml saline within 15 s into 2cm jugular vein segment. Stasis is maintained for 30 min. The size of the clots is determined.

(xiv) Homocysteine induced thrombosis[37]-homocysteine is a thromboatherogenic and atherogenic agent. Homocysteine inhibits the function of thrombomodulin, an anticoagulant glycoprotein on the endothelial surface that serves as a cofactor for the activation of protein-C by thrombin.it produces both venous and arterial thrombosis. homocysteine may contribute to the development of thrombosis in patients with cystathionine beta-synthase deficiency.

(xv) Monoclonal antibody induced thrombosis[38]-protein-C is considered to be an important regulator of blood coagulation and fibrinolysis. During the production of monoclonal antibodies (MoAbs) against human protein-C in mouse ascitic fluid, one hybridoma was found to induce heavy thrombus in mice, resulting in severe haemorrhage. Intravenous infusion of the purified MoAb from this hybridoma also caused thrombosis in mice.

(xvi) High fat diet induced thrombosis[39]-Epidemiological studies suggest the high fat content of the diet to be responsible for atherosclerosis and its thrombotic complications. The high fat diet contained 20% fat (w/w) and 0.05% cholesterol (w/w) given to mice for 8 weeks. The high fat diet significantly enhanced both the thrombotic reaction and the development of atherosclerosis as compared with the low fat (7%) diet. Thrombus formation is recorded on video, analysed by computer, and the size

of thrombus was calculated by image analysis software.

(xvii) Carrageenin-induced thrombosis[40]-kappa-carrageenans is a most potent thrombogen. As the consequence of thrombosis tail infarction became visible some minutes after i.v. administration, but it was delayed for about 3 hours after the i.p. route and for about 6 hours after subplantar injection. Advantages of the carrageenin-induced thrombosis model in rats and mice are: (i) simple induction in small laboratory animals, (ii) easy observation and quantification all the time without killing the animals, and (iii) possible external testing of antithrombotic agents by applying substances on the tail.

(xviii) Acetic acid induced thrombosis[41]-the right common carotid artery (RCCA) was exposed carefully from the surrounding tissue. The RCCA was temporarily clamped using an aneurysm clip, and acetic acid was applied to generate a thrombus. After 10 min, the clip was removed.

(xix) Adenosine diphosphate induced thrombosis[42]-Adenosine diphosphate is a key agonist in haemostasis and thrombosis. ADP (0.035 mg) injected in tail vein of female mice, produce thrombosis by enhancing platelet aggregation. ADP-induced thrombi are rapidly broken down and sudden causes occlusion of pulmonary capillaries.

(xx) Arachidonic acid induced thrombosis[42]-arachidonic acid (30-100 mg/kg) in phosphate buffer solution administered in tail vein of mice induced thrombosis.

(xxi) Cuff induced thrombosis[43]-the apoE-/- mice anesthetized with ketamine/xylazine (91.0/9.1mg/kg, i.p.). The animals first underwent splenectomy: (a) the spleen was dissected through a lateral incision of the abdomen, (b) the vessels were carefully ligated using a 6.0 silk, and, (c) after the removal of the spleen, the abdomen was

closed layer by layer with a 6.0 silk. Immediately afterward, the carotid artery injury was performed. Cuffs were produced using a silastic tubing (length, 4 mm; internal diameter, 0.3mm; external diameter, 0.5 mm). The nonconstrictive cuff was placed around the left common carotid artery, and the axial edges of the cuff were approximated by the placement of 3 circumferential silk ties. The entry wound was closed, and the animals were returned to their cages for recovery from the anaesthesia. An inside diameter of 0.3mm was constrictive and resulted in stenosis of the common carotid artery.

(xxii) Mechanical trauma induced thrombosis[44,45]-a model in which forceps were used to deliver a standardized "pinch" ($1500g/mm^2$) to the surgically exposed femoral vein. Rather than measuring blood flow and occlusion time, these investigators used a miniature fiberoptic device to transilluminate the injured segment of vein while it was being visualized through a stereo microscope equipped with a closed circuit video system. Computer-assisted gray scale analysis was used to determine thrombus area and the kinetics of thrombus growth and dissolution over a 50 min period.

(IV) Bleeding models

The various bleeding time models like subaqueous tail bleeding, arterial bleeding time and template bleeding time. The animal models may be developed by transaction of rat tail 4mm from tip,[46] micropuncture of small mesenteric artery[47] and by producing an incision into the oral mucosa of dogs[48] respectively. These models are based on a simple principle as length of time for bleeding to stop from a standard incision. These are used to evaluate hemorrhagic properties of antithrombotic agents.

(V) Genetic models

The genetically altered animals that are deficient in certain proteins like FVIII, FIX, b3-integrin, GaQ, TBXA2, P-selectin, P2Y1, PAR-3, Plasminogen, tPA, uPA, involved in thrombosis and haemostasis known as knock-out models[49,50] for the evaluation & screening of antithrombotic agents.

CONCLUSION

Thrombosis is the most common single cause of death in the world. Due to this there is a necessity to develop a safe & effective antithrombotic agent. This also need a perfect and precise evaluation & screening method for newly develop molecules? The research in new drug discovery utilizes combinatorial chemistry, computer aided drug design, quantitative structural activity relationship, ADME-T and bioinformatics. In spite of several developments, screening and evaluation methods remain a challenge for pharmacology. Earlier pharmacologists use to screen drugs using whole animal models and organ preparations. By 1980s use of biochemical methods becomes popular in drug screening. This coupled with biotechnology tools and computerization gave rise to novel evaluation techniques in drug research like high through put screening, cell-based assays, transgenic animals, and stem cell research. This review provides concise information regarding the various models of screening of antithrombotic activity and would be useful for graduate and post graduate students of pharmacy, medicine and veterinary sciences.

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