EFFECT OF TIME, PH, AND DOSES TO OBTAIN IN VITRO EFFICIENT FIBRINOLYSIS WITH A NEW MICROPLASMIN. IMPORTANCE OF RESULTS FOR FUTURE IN VIVO STUDIES

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ABSTRACT

During the coagulation of the blood is important the age of the clot, because the fibrin mesh becomes more compact. Is important the doses and reperfusion time to resolve it. In this research a new microplasmina in pure fibrin clots, fibrinolytic effect was compared using different ratios times, doses and Ph, for a progressive and constant in vitro lytic activity. We used different times with constant pH and doses, constant time but variations of pH and doses, constant pH and different time and doses.
Microplasmin was provided by Agustin Joison. The biological activity is a 25 UI/ml, the final protein concentration was 0.02 mg. The activity was measured at pH 7 and 6. The microplasmin activity produces a uniform degradability of clot. There is not difference of activity either pH 7 or pH 6. The size of the fibrin being reduced from 2 cm. up to 0.2 cm.

The fibrinolytic efficacy of this new microplasmina in reducing the size of the fibrin of the clot from 2 cm to 0.2 cm is independent of the pH, but it is on the time and dose.

fibrin- plasma – thrombosis – Lysis - microplasmin

During the coagulation of the blood can form a fibrin resistant to lysis. Fibrin is a protein consisting of strands (monomers) that can branch out and form a network that encompasses elements of blood. In certain circumstances may alter the formation of fibrin and make it resistant to its lysis, increasing the risk of venous tromboembolism events or attacks such as acute myocardial infarction arterial ischemic. (1)

In the fibrin formation, fibrinogen to adsorb to certain places as resulted in a number of changes in its structure. Fibrinogen adsorbs in two orientations, depending on their concentration levels. One of them “side-on” when its level is low and when this in high concentrations. (2). In the process of fibrin polymer formation interactions N-terminal A- and B-knobs, are fundamental to the cleavage of the fibrin peptides A and B. Of the four possible interactions (A:a, B:b, A:b, and B:a), the A:a is the more actively involved in the formation of fibrin polymerization . (3).The fibrinolysis may be affected when fibrinogen levels are elevated increasing the risk of thrombosis, by its role in atherosclerotic plaque formation. Either more increases the fibrinogen levels the lysis of fibrin is more resistant. There are patients developing deep vein thrombosis (DVT) that present detectable normal qualitative fibrinogen but with defective thrombolysis.(4).

It is known that as result to the presence of free hydroxyl radical will be to produce alterations during the formation of the mesh of fibrin and thus make it resistant to the fibrinolysis. These radicals according to some studies can produce an alteration of the molecule of Fibrinogen which translates in a resistant the fibrin clot to the plasmin. Also studied in clots more than 3 hours of formation may have some type of resistance to proteolysis of fibrin. (5). the age of the clot is an important factor at the time made the thrombolysis. The older age of the clot the fibrin mesh becomes more compact and netted. In the case of diseases such as the acute myocardial infarction (AMI), after two hours of evolution are more difficult to dissolve, therefore the therapeutic window is important for the effectiveness of the thrombolytic agent. Is important the doses and reperfusion time to resolve this situation. All those components that make up the clot and fibrin, as plasminogen as well as the union of the alpha-2 antiplasmin depend on the times in which is forming. (6). Coagulation factors as XI, VIII:C, and fibrinogen have been founded elevated in epidemiologic research in thrombosis. Also high levels of Prothrombin have been correlated with arterial or venous thrombosis. Haemostatic alteration is possible when thrombin is increased too. Either elevated levels or high concentration of thrombin may be to affect a clot's structure. When clots are formed with low thrombin levels, the fibrin fibers are thick; in the other side the fibers are higher network with low thrombin concentration. (7).Another high risk of thrombosis is with high levels of fibrinogen in plasma, that to happen in diabetic patients with vascular complications in myocardial infarction, producing structural clot alteration. (8). Another environments factor where fails the fibrinolysis is the PAI-1 (Plasminogen activator inhibitor) in unstable angina. The fibrin has not bound correctly the plasminogen and the Plasminogen activator when the PAI-1 is elevated. (9, 10).

There are a lot of variables such as pH, ionic strength, levels of calcium, Fibrinogen, Thrombin, which could influence on the architecture of the fibrin mesh. When the concentration of Thrombin varies the fibrin strands change its appearance, cloudiness and thickness. For concentrations of low thrombin filaments of fibrin is thicken and cannot exercise its action of the same mix properly. On the other side, the fibrin strands are finer and less permeable to increasing its density with high levels of Thrombin. (11, 12, 13). Many investigations confirmed the relationship that exists between the fibrin structure stability and fibrinolysis on the same activity. From the physiological point of view, this relationship is very important. The increase in resistance to the fibrinolysis is associated with very dense and stable fibrin networks while the fibrinolysis increases in those clots fibrin which is unstable. Therefore many studies suggest that stability and formation of filaments of fibrin network determines the physiological responses of the fibrinolysis. (14, 15)

The physiological fibrinolysis of fibrin, is the result of an interaction between substances that produce plasmin (enzyme active), as the tissue Activator plasminogen (t-PA) and its substrate plasminogen, this last attached to the fibrin network. In regards to the fibrin its action is essential to achieve the t-PA can successfully activate plasminogen. All these mechanisms depend on the structure of the fibrin, the diameter of the fiber and the fibrin mesh density. (16). Thrombolytic therapy is a treatment used to break up dangerous clots inside your blood vessels. To perform this treatment, your physician injects clot-dissolving medications into a blood vessel. In United States, myocardial infarction (MI), has an annual incidence of 610.000 people with recurrent attacks of approximately 300,000. Average of the disease in this population is 64.5 years for men and 70 years for women. (17).

In this research a new microplasmina, fibrinolytic effect in pure fibrin clots was compared using different ratios times, doses and Ph, for a progressive and constant lytic activity. We used different times with constant pH and doses, constant time with variations of Ph and doses, or constant pH and different time and doses.

METHODS

Microplasmin was provided by Agustin Joison. Microplasmin was isolated by isoelectric precipitation and purified by ion-exchange and affinity chromatography columns. The starting material, human plasma was provided by blood centre foundation (Cordoba, Argentina). The final protein concentration was determined by folin Ciocalteu method at

750 nm (0.02 mg). The biological activity (25UI/ml), was determinate with dilution 1/5 of streptokinase solution as standard (Streptase, Germany). The Ph was measured with digital pH meter Hanna Instruments (USA), calibrated with buffer Phosphate Ph 7 and 4. The fibrinolytic activity was measured with fibrin plate method. The fibrin plate was performed adding 3 ml fibrinogen free of plasminogen. Solution was clotted in a glass dish 15 cm diameter by mixing with 0.5ml of human thrombin (Stago, USA) in 0 75 ml 0•02 M sodium borate buffer, pH 8.8 more calcium chloride 0.025 M. Pure fibrin clot was obtained by clotting fibrinogen isolated of plasmatic euglobulin fraction more calcium chloride and thrombin (Stago, USA). We used 3 fibrinolysis schemes: A: same doses (0.02 mg), same time (every 30 minutes) and different pH. (7.2 vs. 6). B: same time (every 30 minutes), same pH (7) and different doses (0.02 mg, 0.04 mg, 0.06 mg, 0.08 mg). C: same doses (0.02 mg), different time (1: every 30 min; 2: every 60 min) and same Ph (7). The microplasmin was added in different areas of the fibrin mesh at the same time. The activity was measured each 30 min until 12 hours.

RESULTS

Figure 1 shows the activity of the microplasmin (left), in relation to the addition of 150 mM Sodium Borate pH 7 (right). Figure 2 shows a normal sequence of fibrinolysis on a pure fibrin clot, the activity is progressive and produces a uniform degradability of clot. To produce it we used the isolated microplasmina every 30 min. In this assay the fibrin mesh is dissolving and breaking into small pieces releasing its internal content. When we used different pH: Left pH 7; right pH 6.0, and doses (0.02 mg) and time are constant, there is not difference in the fibrinolysis. The microplasmin was added in the middle of the clot which allowed fragment it and to continues toward at the ends of it. Not more different lysis happened in both pH (Figure 3). After to assay the fibrinolysis using the same time (each 30 min.), the same pH (7) and different doses (a: 0.02 mg, b: 0.04 mg, c: 0.06 mg, d: 0.08 mg), the fibrin clot was completely dissolved without fragmented and decreasing its size evenly (Figure 4). The microplasmin has a very specific mechanism, producing a lysis in situ; limiting its action at the same place Therefore when multiple points joined the lysis is complete, without a systemic lysis out of control (Figure 5). When performed the activity of microplasmin in relation al Sodium borate in the same conditions of pH, time and doses, the size of the fibrin on the left side being reduced from 2 cm. up to 0.2 cm. fibrin; on the right side lies within 2 cm. (Graphic 1).

DISCUSSION

This research has show how some variables may be altering or not the fibrinolytic activity of a new microplasmin. In thrombolysis treatment many studies has been demonstrated that the cost, effectivity and safe depending of type of agents, doses and time of therapeutic. Agents as Alteplase can be administered IV bolus 15 mg) followed by 0.75 mg/kg (up to 50 mg) over 30 minutes and then 0.5 mg/kg IV over 60 minutes. This activity is limited because up of 100 mg may be to produce hemorrhagic events. This scheme of Alteplase is indicated to pathology as IAM (myocardial acute infarction). Another agent to thrombosis treatment is Reteplase with two IV boluses; here the studies with an infusion of 10 units each without weight adjustment. The scheme is 10 U bolus over two minutes and after 30 minutes the second 10 U bolus over two minutes too. The treatment with Tenecteplase showed that 30 – 50 mg IV in bolus was effective after 5 seconds of infusion. Streptokinase is a protein not enzymatic used in this diseases, with infusion IV of 1.5 million U over 60 minutes or APSAC 30 U given after 5 minutes.

In this in vitro research the doses (0.02 mg for each 30 min) was similar in the test performed, we not used in bolus but the results is completely specific fibrinolysis. There is fibrinolytic activity in the euglobulin fraction, and the pH which its precipitate is fundamental to recovery optimum lytic activity. The pH variations in the euglobulin preparation encountered many problems. The assays showed that the intervals of pH either 6 to 5.3 precipitates plasminogen and plasmin with high fibrinolytic activity from euglobulin fraction. But the pH 6 was found be the better of the pH intervals. The euglobulin preparation may be provided one area to study the fibrinolytic system.

The activity of a new microplasmin have not difference either pH 7 or pH 6. Both in vitro fibrin clot were dissolved in he same time. In an in vitro model, recombinant tissue-type plasminogen activator was significantly more effective than streptokinase in dissolving 24-hour-old human blood clots. Therefore there might be a difference in the effect of time to treatment on the efficacy of these fibrinolytics with different fibrin specificity in patients with acute myocardial infarction.

In thrombosis treatment with fibrinolytic agents, is important the “window time” between symptom and initiation of therapy. The thrombolytic efficacy with different doses and drugs was analyzed in a retrospective study with angiography following. After 90 minutes of treatment initiation the reperfusion was assessed by angiography in patients with “window time” within 6 hours of symptom onset. Patency rates of patients after thrombolysis performed in two groups; one </= 3 hours and the other > 3 hours between symptom the event and start of the therapy; then was compared the two groups. There was no difference when Thrombolysis starts in Myocardial Infarction (TIMI) grade 3 perfusion after added Alteplase (72.5% vs. 76. 3%) and reteplase (63.6% vs 63.2%) between the 2 groups. In the other side, patients treated with streptokinase (36.8% vs 27.6%), anisoylated plasminogen streptokinase activator complex (59. 5% vs 34.8%), and urokinase (62.3% vs 41.7%), TIMI 3 patency decreased with the increasing interval between symptom onset and initiation of therapy. When we used different times intervals either each 30 min or 60 min, not change the efficacy of the fibrinolysis. We think that this microplasmin have the 3 D structure, allows it to self-regulation and produce the desired effect regardless of the time that we use. After in vitro test, our results in vivo research in rabbit’s model thrombosis using this information showed a security, specific lysis without hemorrhagic events. Since the treatment of thrombotic disease by antithrombotic drugs may be associated with bleeding complications, a local delivery technique for administration of the drug may be useful. The high-dose drug delivery reduced systemic coagulability. Thus, local delivery of an antithrombotic agent can reduce the thrombus size in the coronary and iliac arteries without having any significant influence on coagulability. When the microplasmin is added to the thrombus, the activity of it reduces the size of the clot until 80 % during the fibrinolysis. In vivo treatment in patients with thrombotic events, is priority the reperfusion in the less time possible. For that reduction the size of clot is important to save tissue with infarction. The microplasmin reduces the size very well compared when the fibrin is treated with sodium borate solution. The experience with thrombolytic agents with thrombosis in patients as newborns, pregnancy for example Our results of the fibrinolytic potential with this microplasmin in vitro after adding at different doses or times can be helpful to establish dosage guidelines for thrombolytic therapy in these people. Thrombolytic therapy aims to dissolve blood clots by restoring vessel patency. Blood clots are compact structures of protein fibrin meshwork with incorporated blood cells. Their susceptibility to thrombolysis is associated with their structure and viscoelastic properties. Though thrombolytic treatment is nowadays widely used in clinical practice, blood clots and their susceptibility to thrombolysis are still being extensively studied with various experimental techniques. The reduction of the size of the mesh of fibrin from 2 cm to 0.2 cm using the microplasmin in doses exposed during trials, allow an approximation of activity that would have the microplasmin in vessels with total or partial thrombosis.

There is not a Conflict of Interest Disclosure Agustin Joison designed the research and performed research, contributed vital new reagents Federico Gallo contributed with his vascular experience

CONCLUSION

We conclude from our data that the fibrinolytic efficacy of this new microplasmina in reducing the size of the fibrin of the clot from 2 cm to 0.2 cm is independent of the pH, but is dependent on the time and dose used. At respect In this regard were able to observe that the fibrinolysis carry out too, applying schemes with equal dose fractionated in intervals of time without having to administer in bolus, what would make this microplasmin an safer and effective agents. Also this model in vitro can give us an approached idea for use it in a future in therapy in patients who suffer these thrombotic diseases

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