DEVELOPMENT OF A DEVICE AND AN EXPERIMENTAL PROTOCOL TO PRODUCE THROMBI *IN VITRO.*

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Abstract. *In the present study an experimental system has been devised to produce in vitro thrombi, similar to in vivo arterial thrombi. There is an important clinical interest in thrombi destruction (thrombolysis). Structurally, clots differ markedly from arterial thrombi. Usually, a general feature of studies in this area is that clots, rather than thrombi, have been examined. The lack of a standardized method for obtaining thrombi in vitro impairs the comparison of the results obtained in different studies making difficult the applications of these results for similar situations in vivo. There are few in vitro procedures available for the study of thrombi productions. A device similar to the Chandler Loop has been developed. The device consists of a rotating cylinder, tilted to an angle of 23 degrees, where a circular tube was rotated. The tube was filled with blood taken from healthy adults, and was animated to rotational frequency of 16 rpm, with flow rate of 38.4 + 4.1 cm3/min, during thirty minutes. The resultant thrombi were weighted, measured and analyzed in its macroscopic characteristics. Seventy-two thrombi of six volunteers were studied; 48 (66.7%) presented macroscopic characteristics similar to human thrombi formed in vivo. They had a dense form, with a white head and a red tail. Factors as time, rotation and flow rate were very important factors in the production of typical arterial thrombi. The device was effective to produce in vitro thrombi with a similar structure to arterial in vivo thrombi.*

Keywords: *Bioengineering, Thrombi, In vitro, Device, Chandler.*

1. Introduction

Cardiovascular diseases are one of the main mortality causes in Brazil and in the western world. Because thrombi are involved in the pathogenesis of cardiovascular diseases there has been much interest in their formation as well as in the factors that interfere in their destruction (thrombolysis).

Thrombus is the scientific designation for a pathologic clot formed inside the blood vessel (Houaiss, 2001) and thrombosis refers to the pathological process of clot formation (Cotran, Kumar and Robbins, 1994). Thrombi are formed predominantly by clusters of erythrocytes, platelets and fibrin, which is a protein derived from the coagulation process. Thrombi differ from clots by their stiffness and for having a point of attachment and a fibrin tangle in their composition (Cotran, Kumar and Robbins, 1994).

The morphologic and biochemical structure of the thrombus is clearly influenced by different flow conditions inside the blood vessel. This can be demonstrated by the differences between an arterial thrombus formed in high flow and pressure conditions, normally present in the arteries, compared to a venous thrombus formed in milder conditions. Arterial thrombi display apparent laminations, which are produced by the contrast between dark layers containing red cells and pale areas formed mainly by platelets and fibrin. They are usually occlusive, grayish, friable and firmly attached to the blood vessel's wall. In addition, these thrombi are commonly superimposed on an atherosclerotic lesion and more difficult to be destroyed. On the other hand, venous thrombi, formed in areas of low flow rate, could be mistaken by simple clots, since, like the clots, they contain more erythrocytes and fewer platelets than the arterial thrombi, exhibiting reddish color and not adhering to the blood vessel wall. Thrombi vary in size and shape and usually have an occluding head followed by a tail. In arterial circulation the tail builds up in the opposite direction of blood flow and on the venous side it develops in the same direction of the flow (Cotran, Kumar and Robbins, 1994). The specific characteristics of arterial and venous thrombi will influence on sensitivity of a thrombus to lysis once they possess different structures.

It is difficult to evaluate accurately the shape and the macroscopic structure of thrombi, since they have irregular external surface, rubbery consistence, and cannot be appropriately visualized inside (Bergen, 2004). In the last years, some authors have suggested the use of nuclear magnetic resonance to study the thrombus three-dimensional geometry and its inner structure (Blackmore et al., 1990; Blinc et al., 1994). Thrombi microscopic and biochemical aspects are better known than their macroscopic and structural characteristics.

Studies that investigate the stability of thrombi to lysis frequently employ thrombi obtained *in vitro* by different methods. Thrombi can be produced from whole blood or plasma, using static or dynamic methods, with or without the addition of anticoagulant. The method employed can alter the morphologic structure of the thrombi and consequently its susceptibility to destruction (Robbie et al., 1997). The use of different techniques in different studies limits comparisons between these experiments. In addition, some methods frequently used represent a poor model for human thrombi formed *in vivo*. Therefore, it is important to know the technique used in order to compare the results of different studies and to recognize if it is a method capable of simulating *in vivo* thrombi characteristics. Frequently, the technique used is not detailed nor its influence in the final results is discussed. A general feature of studies in this area is that clots formed in static blood, rather than thrombi, have been examined (Robbie et al., 1997). As a matter of fact, it seems that a standard method for obtaining thrombi *in vitro* has not been established.

The most mentioned techniques used to form thrombi are summarized below:

- Blood is incubated in plastic tubes and allowed to clot and retract at room temperature for a period of two to four hours (Porter et al., 1996; Wyshelesky et al., 2001).
- Whole blood added to 10 UI of synthetic thrombin is centrifuged at 15 rpm for three hours at room temperature (Nedelmann et al., 2002).
- 7 ml of whole blood and sodium citrate are separated into 3 ml of plasma by rotating at 1500 rpm for 10 minutes at room temperature. 0,7 ml of calcium chloride at 0,25 M is added to the plasma. This method obtains a thrombus rich in fibrin and platelets (Mizushige et al., 1999).
- 10 ml of whole blood is centrifuged at 4200 rpm for 15 min and the serum is separated from the resultant clot (Behrens et al., 1999).

We can note that most of the mentioned methods are static techniques.

Chandler, in 1958, described that definitive features distinguish a thrombus formed *in vivo* from a clot obtained *in vitro* by static methods. He was the first to propose a technique to produce an *in vitro* thrombus in a moving column of blood, simulating blood flow.

In 1997, Robbie et al. performed a detailed microscopic and immunohistochemical comparison between static blood clots prepared in the test tube, thrombi formed in the Chandler Loop Circulation and human thrombi formed *in vivo*. The Chandler thrombi presented a defined and compact structure, separated in two portions: a dense head of platelets surrounded by erythrocytes, leucocytes and fibrin, and a tail composed of fibrin. They showed very similar morphology to human thrombi formed *in vivo*, in contrast to static clots that lacked defined structure and had a homogeneous distribution of erythrocytes and fibrin. In the immunohistochemical analysis the authors compared the contents of the Plasminogen Activated Inhibitor (PAI-1) between the clots and thrombi. The importance of PAI-1 is that it has a profound effect in the resistance of thrombus to lysis. Robbie et al. (1997) showed that a static clots contain up to 100 times less PAI-1 then an *in vivo* thrombus, what explains its larger susceptibility to lysis. The thrombi obtained by the Chandler's Method showed a great concentration of PAI-1 in the head, extremely similar to human thrombi. Robbie et al. (1997) concluded that the Chandler's Method provide an appropriate model for the study of thrombolysis, in contrast to static methods, and offered the best model to simulate *in vivo* thrombi both structurally and in terms of PAI-1 content. In the present study an experimental system has been devised to produce *in vitro* thrombi similar to thrombi formed *in vivo*.

2. Material and Method

Blood Collection.

Blood samples were collected from six volunteers according to the following criteria: healthy individuals; age between 20 and 40 years; male; no recent drugs use; negative history of cardiovascular, thromboembolic or hemorrhagic diseases.

Whole blood was collected by venipuncture using 28 x 5 mm needles and tube for vacuous collection (Vacutainer®). Three tubes of 4,5 ml with citrate 3,8% (anticoagulant) were collected from each volunteer and maintained homogenized.

Device.

The thrombi were obtained through a rotation device with circular loops filled out by blood. The device was developed at the UFMG Laboratory of Bioengineering (LABBIO) and shows similar characteristics to the Chandler's Method. Loops were constructed from PVC, internal diameter 3 mm and length 34 centimeters as (Fig.1). The two ends of the tube were then brought together and joined by a small piece of 4 mm of internal diameter PVC tube. A resin cylinder with 100 mm of diameter was thinned with ten equally spaced sulci to receive loops. This cylinder was positioned in a metal foil, sloping 23 degrees in relation to the horizontal plan (Fig. 1). A gear system coupled the cylinder to the engine that was connected to a source allowing variation of speed and rotation direction.

Figure 1. a) Loop b) Device

Experimental Protocol.

After the blood collection, 1 ml of citrated blood was placed in the loop and recalcified with 0,1 ml of calcium chloride 0,25 M (Biolab®) during soft homogenization. The tubes were then sealed immediately to form the loop that was positioned in the device and rotated at 16 $\pm$ 2 rpm for 30 minutes. The calculated system's flow was of 38.43 $\pm$ 4.8cm^3/min.

After 30 minutes, the loops were removed from the device and kept at 37^0 ($\pm$ 1) C for at least 2 hours to allow the occurrence of clot retraction phenomenon. After this period, the thrombi were carefully removed from the loop with the injection of 5 ml of 0,9% NaCl, washed with more 5 ml of 0,9% NaCl and laid in paper filter for drying at room temperature for five minutes. Then the thrombi were weighed in a precision scale (Mettler Toledo A.G 204) and had their length determined.

3. Results

Twelve thrombi were obtained from each volunteer resulting in a total of 72 thrombi. Among these, 48 (66.7%) presented typical characteristics and 24 (33.3%) non-typical characteristics. The typical thrombi are characterized by a cylindrical or conical shape, compact structure and a firm consistence composed of a white head and a red tail. The non-typical thrombi lacked defined structure and presented with a homogeneous red color (Fig.2).

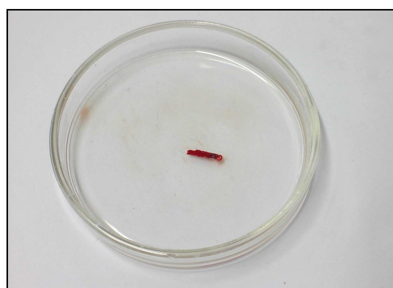

Figure 2. a) Typical thrombus b) Non-typical thrombus

The gelatinous and brittle consistence of the non-typical thrombi made the measurement of its mass and length impossible.

The average mass of the typical thrombi was 245 ± 44mg, and the average length was 8.5 ± 1.8 mm. Among 72 thrombi, 42% (30) totally occluded the PVC tube interrupting the blood flow. The remaining 58% did not interrupt the flow of the loop.

4. Discussion

There are evidences that thrombi prepared under static conditions consist in a poor model for human thrombi formed *in vivo* and are not appropriated for the study of thrombolysis (Robbie et al., 1997).

In 1958, Chandler described the differences between a blood clot obtained *in vitro* and an *in vivo* thrombi showing that clots prepared in vitro did not show microscopic similarities to the thrombi removed from *in vivo* blood vessels formed in a moving stream of blood. The Chandler's Method is an artificial circulation consisting of the following steps:

- 1 milliliter of whole venous blood was drained directly into a polyvinyl tube, 25 cm in length and 0,375 cm in internal diameter.
- Soon after the tube had been filled with blood the two ends were brought together and joined by a plastic collar to form a loop.
- The loop was then placed on a turntable tilted to an angle of 23 degrees, and rotated at 17 rpm. Chandler used an inclined record player for this purpose (Fig.3). The sloping rotation created a blood column that moved in the opposite direction to that of the tube.
- When the blood was coagulated and the thrombus was large enough to occlude the lumen, the blood column became static and moved around in the direction of rotation tube. At this time, an end point was established and the thrombus was removed from the apparatus.

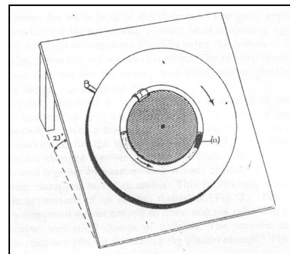

Figure 3. Chandler device

Chandler's thrombi were cylindrical and conical, measuring 10 mm long and 3 mm in diameter. The typical thrombi obtained in this experiment presents similar morphologic characteristics to those described by Chandler (1958), with a white head followed by a red tail, and they were similar to human thrombi formed *in vivo*(Fig.4). The mass (245 ±44 mg) and length (8.5 ± 1.8 mm) values were also similar to that described by Chandler.

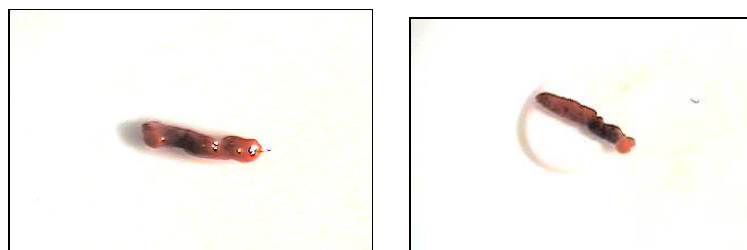

Figure 4. Typical thrombi

Chandler obtained 100% of typical thrombi, while our device obtained 66,7% of typical thrombi and 33,3% of non-typical thrombi. These results could be influenced by some factors including the presence of air in the loop, lack of coagulant's homogenization, the correct sealing of the loop and the flow rate. Chandler tested different speeds, volume of blood and angles, and concluded that speed was the most important element to obtain a typical thrombus. The device developed in this report supported ten loops that were simultaneously submitted to rotation, optimizing the capacity device, which was able to form ten thrombi at the same time. However, to introduce a new loop in the device, the rotation had to be interrupted altering the flow. This can explain the differences in efficiency between Chandler's device and the one developed in this study. In our protocol, as suggested by Robbie et al, the whole blood received

anticoagulant (citrate) and was recalcified with calcium chloride (1997). Conversely, Chandler used whole blood without anticoagulant, what can also interfere in the different efficiency between the two devices. Robbie et al. (1997) did not mention the efficiency of their device.

Of the 22 Chandler's thrombi, 15 (68%) completely occluded the loop's lumen causing a static blood column and 7 (32%) lead to partial lumen occlusion, maintaining the movement of the blood in the opposite direction of the tube. In this experimental protocol 42% of thrombi totally occluded the PVC tube interrupting the blood flow and the remaining 58% did not interrupt the flow. Probably the differences in the flow were the reason for the different results.

We noted that even typical thrombi, of similar weight and size, varied in their macroscopic morphologic structures (Fig.5). Some thrombi were more compact and firmer and the red tail and the white head exhibited different sizes and locations. It seems that these structural differences can contribute to raise the coefficient of variation of *in vitro* studies on thrombolysis. However these variations approximate them to the conditions found *in vivo*, where thrombi are not identical.

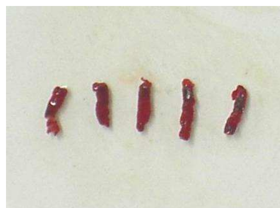

Figure 5. Differences between typical thrombi

5. Conclusion

The typical thrombi obtained in this experiment presents with similar morphologic characteristics to those described by Chandler (1958), with a white head followed by a red tail, and were similar to human thrombi formed *in vivo.*

The device and experimental protocol developed in this report is an appropriate model system to produce thrombi *in vitro* for the study of thrombolysis.

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7. Responsibility notice

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