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THE USE OF ULTRASOUND AND MICROBUBBLES FOR THROMBUS FRAGMENTATION.

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Abstract. *Thrombosis in the cardiovascular system is the leading cause of mortality and morbidity in the western world. Although other therapies are effective for thrombi fragmentation (thrombolysis), there is a need for a simpler, safer, less expensive, and noninvasive method. The use of ultrasound and microbubbles represents a completely different approach to thrombus dissolution. This new method still faces critical challenges such as technical limitations and the complex interaction between the physical parameters of ultrasound, microbubbles and living tissues. The purpose of this work was to review the current available bibliography and to evaluate, in vitro, the efficacy of ultrasound of 1 MHz and microbubbles to induce thrombus fragmentation. Thirty-six thrombi, obtained through the Chandler Loop, were submitted to one of the following treatments: 0,9% NaCl, continuous ultrasound, pulsed ultrasound, microbubbles, continuous ultrasound combined with microbubbles or pulsed ultrasound combined with microbubbles. The variable measured was the fibrinolysis index obtained through the thrombus mass. The largest indexes of thrombus fragmentation were obtained on the group subjected to continuous ultrasound combined with microbubbles, and the smaller one in the thrombus subjected solely to microbubbles. Significant statistical difference was noted between these two groups (P<0,001). The remaining groups did not present significant statistical difference.*

Keywords: *Thrombus, Thrombolysis, Ultrasound, Microbubbles, Bioengineering.*

1. Introduction

Thrombosis is involved in the pathogenesis of cardiovascular diseases, which are the main mortality cause in Brazil and in the developed countries. Thrombi formed inside of arteries lead to the interruption of the circulation causing, among other illnesses, acute myocardium infarction and cerebral vascular accidents (strokes). The current available treatments for thrombosis are thrombolytic drugs and surgical interventions. However, both of them have high rate of failure and are effective only when applied in the first hours after the thrombus formation (Francis, 2001). The limited effectiveness of currently available modalities of treatment stimulates the search for a new method for thrombi destruction.

In the last two decades, ultrasound has emerged as an alternative approach to the traditional treatments of thrombosis (Atar et al., 1999). The discovery that ultrasound enhances the effectiveness of thrombolytic drugs and the recent advances in ultrasonic technology has encouraged new studies in this area. In the nineties, microbubbles composed of protein or lipid capsules filled out with gas were produced to be used as ultrasound contrast agents. These new microbubbles were capable of circulating freely through the bloodstream (Castier, 2001). Some studies observed that microbubbles, depending on the ultrasonic parameters applied, suffered acoustic cavitation, causing erythrocytes and platelets destruction. Since this observation, the therapeutic applications of microbubbles in thrombolysis started to be studied (Unger et al., 2002). In 1995, Tachibana and Tachibana published the first study presenting the ability of microbubbles associated to ultrasound to induce thrombolysis. Ever since, it was postulated that intravenous infusion of microbubbles followed by the local emission of ultrasonic waves, could possibly result in a faster treatment for thrombosis, with low cost, low complexity and few side effects. Thrombolysis using ultrasound and microbubbles presents many advantages, as the fact of being an exclusively mechanic method, with few side effects and easy execution when used with a transcutaneous device (Birnbaum et al., 1997; Culp et al., 2001). The lysis rate reached

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with ultrasound and microbubbles has been similar to the rates obtained with ultrasound associated to thrombolytics drugs, suggesting a possibility for the use of this exclusively mechanic method for thrombi lysis (Porter and Xie, 2001).

The study of thrombolysis induced by ultrasound and microbubbles still faces challenges, as the complex interactions between physical parameters of the ultrasound, microbubbles and live tissue. Limitations in the methods used include: uncertainties about the best ultrasonic parameters (Culp et al., 2001), limited information of the exact mechanism of action (Chen, Matula and Crum, 2002) and the fact that most of the experiments used recent thrombi, what does not correspond to *in vivo* clinical situations (Culp et al., 2001).

The main studies about thrombolysis induced by ultrasound and microbubbles are cited in Tab. 1.

Table 1. Researches on thrombolysis that used ultrasound and microbubbles.

Autor	Type	Micro-bubble	Drug	Freq.	Time min	Intensity/ Power	Mode	Variable measured	Results	Side effects
Tachibana e Tachibana (1995)	in vitro	Albunex®	Uro.	170 kHz	3	0,5 W/cm^2	P	Mass	+	-
Porter *et al.* (1996)	in vitro	PESDA	Uro.	20 kHz	2	40 W/cm^2	C	Mass	+	-
Nishioka *et al.* (1997)	in vitro	DDFP	-	24,8 kHz	3	2,9 W/cm^2	C	Mass	+	Fragments of 3.3 µ
Nishioka *et al.* (1997)	in vivo	DDFP	-	20 kHz	10 to 40	1,5 W/cm^2	C	Angiogra-phy	+	Minimum vessel and skin lesion.
Wu *et al.* (1998)	in vitro	MRX-408	Uro.	1 MHz 20 kHz	...	...	...	image	+	-
Wu *et al.* (1998)	in vivo	MRX-408	Uro.	1 MHz 20 kHz	30	...	...	image	+	-
Birnbaum *et al.* (1998)	in vivo	PESDA	-	37 kHz	15 to 60	160 (W) 103(kPa)	P	Angiogra-Phy	+	Normal D-Dimer
Kondo *et al.* (1999)	in vitro	SH-U508A	tpa	10 MHz	10	1 W/cm^2 0,5 W/cm^2	C e P	Mass Image	+ CW = PW	-
Mizushige *et al.* (1999)	in vitro	DDFP SH-U508A	tpa	10 MHz	10	1 W/cm^2 0.33 (MPa)	C	Mass Image	+ DDFP>	-
Birnbaum *et al.* (1999)	in vitro	Optison	-	19,5 kHz	2	11 W	P	Mass	+	-
Culp *et al.* (2001)	in vivo	PESDA	-	1 MHz	30	0.6 W/cm^2	C	Angiogra-Phy	+ Small thrombi	No side effects
Porter *et al.* (2001)	Dogs in vitro	PESDA	-	1 MHz 40 kHz	8	1 W/cm^2	C e P	Image	+ C = P	-
Porter *et al.* (2001)	in vivo	PESDA	-	1 MHz 40 kHz	30 to 60	1 W/cm^2	C	Image	1 MHz > 40 kHz	Transitory arrhythmia
Siegell *et al.* (2001)	In vivo	PESDA DDFP	-	20 kHz 37 kHz	15 to 60	1,5 W/cm^2	C	Angiogra-phy	+	-

Legend: (P) – Pulsed-wave; (C) – Continuous- wave; (-) not applicable; (...) not quoted; (+) presence of significant thrombolysis; Uro - Uroquinase.

It is observed that the study of ultrasonic thrombolysis associated with microbubbles is still incipient, counting with a small number of studies. However, the results are encouraging with few side effects. The difficulties in the analysis of these works are the diversity of methodologies, vast variations in frequency (20 kHz to 10MHz), intensity (0,5 to 40 W/cm^2), acoustic parameters, microbubbles class and methods of thrombus obtaining. In spite of the comparisons of different studies being limited by these variations, they indicate several possible combinations of parameters increasing the potential of clinical application.

Ultrasonic and microbubbles induced thrombolysis is a noninvasive mechanical method, promising for clinical applications in peripheral, cerebrovascular and coronary thrombosis. New microbubbles able to connect specifically to thrombi should enhance the clinical applications of this thrombolytic technique (Atar et al., 1999). *In vivo* studies are still necessary to determine the effectiveness of this method in humans. Probably, this method will be an option to increase the effectiveness of other available thrombolytics agents as well as it will be a safe alternative for patients with contraindications to fibrinolytic drugs (Porter and Xie, 2001).

The aim of this study was to test *in vitro* the efficiency of 1MHz ultrasound with albumin microbubbles in thrombolysis induction. An experimental device was developed with a technique similar to Chandler Loop (Chandler, 1958) to produce *in vitro* thrombi similar to human thrombi formed *in vivo*.

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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 drug 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 in an homogenized condition.

Experimental protocol.

After blood collection, six typical thrombi were obtained from each volunteer. Six modalities of treatment were evaluated, using one thrombus obtained from each volunteer. A total of thirty-six thrombi were studied. Just before the treatment the thrombus was weighed to find the initial mass. Then, they were immersed in a polyethylene tube filled out with 0,9% NaCl and submitted, for twenty minutes, to one of the six experimental groups: 1-Continuous-wave ultrasound (3,77W/cm^2); 2- Pulsed-wave ultrasound (0,75 W/cm^2); 3- Microbubbles; 4- Microbubbles + continuous-wave ultrasound; 5- Microbubbles + Pulsed-wave ultrasound; 6-only 0,9% NaCl (control group).

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. 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 carved with ten equally spaced sulci to receive the 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 with an electric motor that was connected to a source allowing variation of speed and rotation direction.

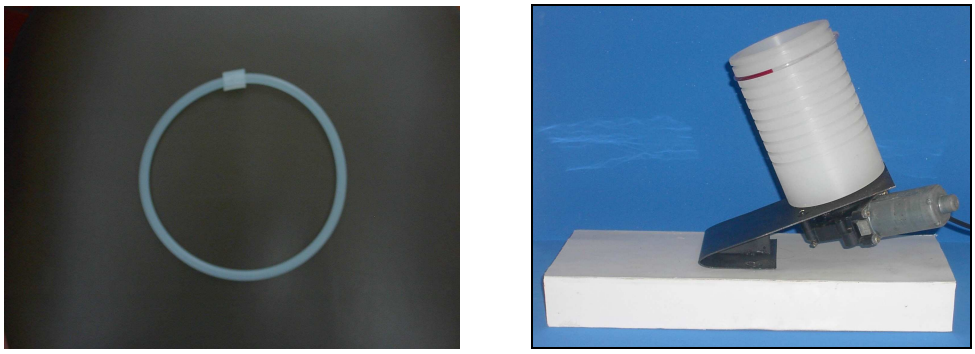

Figure 1. a) Loop b) Device

Thrombus formation.

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 min. 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 C for at least 2 hours to allow the occurrence of clot retraction phenomenon and submitted to one of the six groups of treatment.

Thrombi mass and fibrinolysis rate.

Just before the treatment, each thrombus was carefully removed from the loop with the injection of 5 ml of 0,9% NaCl, washed with 5 ml of 0,9% NaCl and laid in filter paper for drying at room temperature for five minutes. Then the thrombus had its length determined and was weighed in a precision scale (Mettler Toledo A.G 204). The mass obtained before the treatment was called Initial Mass (M_{in}).

After receiving the treatment, the thrombus was washed again with 10 ml of NaCl 0,9%, dried for five minutes at room temperature, and weighed. This residual thrombus mass was called Final Mass (M_f).

The reduction of the thrombus mass quantified the extent of thrombolysis, which was expressed as a percentage of the decrease in thrombus weight, called Fibrinolysis Rate (*FR*). The *FR* was calculated as the difference between the initial and final thrombus weights divided by the initial weight, Eq.(1).

$$FR = \frac{(M_{in} - M_f)}{M_{in}} x100 \quad (1)$$

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Ultrasound.

A commercially available physical device was used for the emission of ultrasonic waves (Sonomaster Standard®, KW Eletrônica). The frequency used was 1 MHz. The equipment was submitted to calibration. The experiment used both the continuous and pulsed-wave ultrasound. The acoustic power was checked in a radiation force balance and presented a mean value of 8.31 $\pm$ 0.94 W. The transducer had a planar circular surface with an area of 4 cm^2 (specified by the manufacturer). The transducer effective emission area was checked at the Ultrasound Laboratory of Mecatronic Engineering Department at São Paulo University using a calibrated hydrophone. The values of area of traverse section of the beam were: at transducer's face, 3.34 cm^2; at 1 cm of transducer's face, 2.44 cm^2; at 3 cm, 2.24 cm^2; at 5 cm of distance, 2.21 cm^2; and at 7 cm of transducer's face, 2.20 cm^2. The transducer focal length was 7cm, so this dictated where the thrombus was placed.

The average temporal intensity was calculated (average acoustic power 8.31W per the effective area, at seven centimeters of the transducer 2.2 cm^2). It was 3.77 W/cm^2 when the ultrasound was emitted in continuous-wave. In the pulsed-wave, considering an operation factor (Duty cycle) of 1/5 (2.0 ms "ON" and 8.0 ms "OFF") the average acoustic pressure was 1.66W and the temporal intensity calculated was 0.75 W/cm^2.

Microbubbles.

The microbubbles used in this study were kindly provided by ECOR Clinic - Rio de Janeiro, where they are experimentally used in echocardiography studies. They consist of a mixture of fluorocarbon, decafluorobutan and 20% human serum albumin (Fig. 2) and were used in a dose of 2 ml, at 8^0 C. These microbubbles are similar to PESDA (Perfluorocarbon-Exposed Sonicated Dextrose Albumin), composed of albumin, dextrose and perfluorocarbon exposed to sonication, which are a class of microbubbles approved by the Food and Drug Administration for experimental research (Culp et al., 2001; Grayburn, 2002; Porter et al., 2001). The technique employed was described by Castier, 2001.

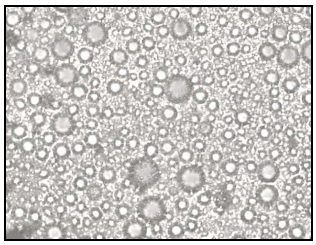

Figure 2. Microbubbles seen by optical microscopy.

Experimental bench.

An acrylic box (30 x 20 x 20 cm) was filled out with degassed water adjusted to 37^0 C. The transducer was attached in the upper lid of the box and immersed in water. The thrombus was located at 7 cm from the transducer, placed in a thin polyethylene tube filled whit 0,9% NaCl and sealed with PVC film. The opposite side of the transducer received an absorptive material (1 cm of silicon rubber, Dow Corning® 3120 added of catalyst number 1), reducing possible reflective waves (Fig.3). The thrombi that were not treated with ultrasound were positioned in parallel to the ultrasound transducer and were protected with absorptive rubber. 7 ml of 0,9% NaCl were used in the treatments that didn't receive microbubbles, and 5 ml in the treatments with microbubbles.

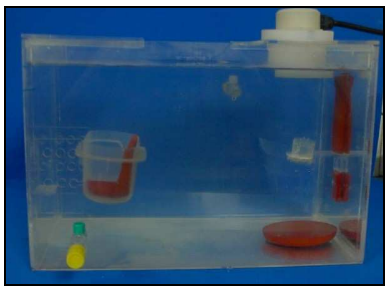

Figure 3. Experimental Bench. At left is the compartment for treatments without ultrasound. At right, ultrasound transducer, polyethylene tube, support and rubber.

Statistical analysis.

Fibrinolysis rate in each group is presented as mean and standard error of means. Since we had highly unstable variables with high standard deviations, the test of Kruskal-Wallis was used (Sampaio, 2002).

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3. Results

Mean values of fibrinolysis rates of each experimental group are shown in the Tab. 2.

The greatest fibrinolysis rates were reached in the group treated with continuous-wave ultrasound plus microbubbles and the smallest rates in the thrombi treated only with microbubbles. The difference between these two groups was statistically significant. There were not statistically significant differences between the other groups.

Table 2. Means values and standard deviation of the fibrinolysis rates

Treatment	*n*	*Fibinolysis Rate %*	*Standard Deviation %*
Continuous- wave ultrasound (3,77W/cm^2)	6	7,44	7,38
Physiologic Serum 0,9% (07 ml)	6	8,77	4,33
Pulsed-wave ultrasound (0,75 W/cm^2)	6	6,66	3,30
Microbubbles (02ml + 05ml de SF0,9%)	6	2,99	8,11
Microbubbles+Continuous-wave ultrasound	6	11,59	5,5
Microbubbles+Pulsed-wave ultrasound	6	7,6	3,7

In the groups that received microbubbles and continuous or pulsed-wave ultrasound, the thrombi were clearly submitted to pressure gradients and flows that threw them to different positions inside the tube, suggesting the occurrence of acoustic cavitation. These thrombi movements lasted for 60 to 120 sec and did not occur in the groups of ultrasound or microbubbles alone.

4. Discussion

Different methods are used to obtain thrombi *in vitro.* 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). There are evidence that thrombi prepared under static conditions are a poor model for human thrombi formed *in vivo*, not representing an appropriate model system for the study of thrombolysis (Robbie et al., 1997). 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). In view of the fact that clots are more susceptible to lyse than thrombi, this difference can impair comparisons between reports that used different methods.

Robbie et al., in 1997, demonstrated that the Chandler's method offered the best model to simulate morphologic and biochemical structure of a human thrombus. These observations lead us to develop a dynamic method to obtain thrombus, similar to Chandler's Loop. The thrombi obtained in this experiment exhibited a white head followed by a red tail, with macroscopic morphologic characteristics similar to those described by Chandler (1958), demonstrating that the method developed was effective. The thrombi were similar to thrombi formed *in vivo*, being appropriate for study of thrombolysis.

It was also observed that although typical thrombi have similar weight and size, they varied in its macroscopic structure. Some thrombi were more compact and firmer and others varied in the position and size of the red tail (Fig. 1). 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.

Wyshelesky et al. (2001) and Nedelmann et al. (2002) showed that the thrombolytic effect of ultrasound depends on the age of the tested blood clots, with the greatest effect in fresh thrombi. Nedelmann et al. (2002) also suggested a decline of the thrombolytic effect after three to six hours of thrombi formation. In our study, thrombi of different ages were submitted to diverse treatments. This difference was minimized alternating the order of treatment. At the beginning of the treatment the thrombi had mean age of 4:13h. All the thrombi were treated within the period of 2:45h to 6:44h. The mean age was similar in all treatment groups, showing that the technique of switching the treatments order was effective, not favoring any specific treatment.

The fibrinolysis rate was used to calculate the reduction of the thrombus weight after each treatment, as described by several authors (Birnbaum et al., 1999; Kondo et al., 1999; Mizuzhige et al., 1999; Nishioka et al., 1997; Porter et al., 1996; Tachibana and Tachibana, 1995). Fibrinolysis rates with high standard deviations were obtained in this work, similarly to those obtained in other reports. We believe that other methods could be employed to quantify the thrombus lysis, for instance, image studies (Wu et al., 1998; Porter et al., 2001) or flow measures, which could reduce the instability of the variable. Besides, an appropriate statistical analysis should be applied for these variables, and the results should be interpreted and compared with caution.

Intending to test a cheap and simple method, we opted to use the broadly available 1 MHz ultrasound equipment used in physiotherapeutic treatments with a transcutaneous transducer. It is known that acoustic cavitation is not relevant when the environment is submitted only to the 1 MHz ultrasound (Poliachik et al., 1999). In this experiment,

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the 1 MHz ultrasound alone did not show significant capacity of lysis because it did not induce cavitation. The fibrinolysis rate of thrombi exposed only to continuous or pulsed-wave were not statistically different from the others groups.

The ideal frequency for thrombolysis induced by ultrasound and microbubbles has not been established. Initially, low frequency ultrasound seemed more promising because it presented low attenuation, better penetration of tissues and larger capacity to induce cavitation and lysis. However, it is also associated whit more side effects (Atar et al., 1999; Francis, 2001). Nevertheless, the association of high frequency (1 MHz) ultrasound and microbubbles has been effective in thrombolysis of *in vivo* and *in vitro* thrombi, even without addiction of a fibrinolytic drug, without significant side effects (Culp et al., 2001; Porter et al., 2001). Porter et al., in 2001, confirmed this finding comparing, *in vitro*, the attenuation of 40 kHz to 1 MHz ultrasound through the cranium showing that the frequency of 1 MHz ultrasound was as effective as 40 kHz when microbubbles (PESDA) was added. They believed that the capacity of thrombolysis induced by high frequency ultrasound was maintained in spite of the great attenuation due the fact that microbubbles were able to induce acoustic cavitation to acoustic pressures much smaller than the ones initially supposed.

The use of high ultrasound intensities is associated to the greatest incidence of harmful effects *in vivo* (Francis, 2001; Thresholds, 1998). As microbubbles reduce the intensity necessary to induce acoustic cavitation, they allow the use of intensities as low as 0,6 W/cm^2, decreasing the possibility of side effects caused by high intensities (Culp et al., 2001). The appropriate intensity varies according to the ultrasound frequency and the microbubble class (Shi et al., 2000). The intensity levels necessary to induce acoustic cavitation are not defined for each class of microbubbles. Porter et al. (2001) had already shown that, in spite of the intensity decrease in the pulsed-wave, continuous and pulsed-wave ultrasound were equally effective in the ultrasonic thrombolysis by 1 MHz and microbubbles. In this experimental protocol, the two average temporal intensity levels used, 3,77W/cm^2 in the continuous-wave and 0,75 W/cm^2 in the pulsed-wave, induced acoustic cavitation.

The capacity of microbubbles to increase the effectiveness of the ultrasonic thrombolysis is demonstrated in several studies *in vitro* and *in vivo* (Tab.1). It is known that the microbubbles reduce the necessary energy for the induction of acoustic cavitation, facilitating the occurrence of this phenomenon (Birnbaum, 2001; Porter and Xie, 2001; Tachibana and Tachibana, 1995). Acoustic cavitation, both stable and transient, has been pointed out as one of the mechanisms responsible for the increase in the ultrasonic thrombolysis. Acoustic cavitation could influence thrombolysis inducing microstreaming vortices of sufficient magnitude to lyse blood cells (Tachibana and Tachibana, 1995; Porter and Xie, 2001), increasing the local pressure gradient (Nishioka et al., 1997) and by a direct erosive effect on the thrombus (Birnbaum, 2001; Culp et al., 2001).

The group submitted to continuous-wave ultrasound with microbubbles presented statistically significant superior fibrinolysis rates compared to the group submitted to microbubbles only. Nevertheless, the addition of microbubbles to continuous and pulsed ultrasound did not result in statistically significant difference when compared to the other groups (0,9% NaCl, continuous-wave ultrasound alone or pulsed-wave ultrasound alone). The similarity between the groups of ultrasound alone and ultrasound with microbubbles can be attributed to the short period of acoustic cavitation in the latter. The interruption of the thrombus oscillations indicated that all microbubbles had been destroyed and the phenomenon of acoustic cavitation was no longer present indicating that the mechanism of action responsible for the thrombi lysis had been interrupted. The influence of the interruption of acoustic cavitation in the rate of thrombolysis had already been noticed by Culp et al. (2001). The early interruption of the cavitation seems to explain the reason why there was no statistically significant between these two groups in spite of the presence of acoustic cavitation. It shows that the infusion of microbubbles in *bolus* or in continuous flow rate can alter its availability for cavitation and consequently its lysis capacity (Culp et al., 2001; Porter et al., 2001). The best form of microbubbles infusion should still be established. An experimental model that supplies a continuous flow of microbubbles seems to be more effective and will probably result in larger fibrinolysis rates.

The findings described here were compatible with the literature, showing that the association of continuous ultrasound to microbubbles increases the fibrinolysis rate when compared to the microbubbles alone. None of the previously published reports presented similar methodology, limiting the comparison of the present study with the others. The instability of the fibrinolysis rate can be considered as a limitation of this study, as well as of the previous ones reported in the literature.

5. Conclusion

The method used in this work was capable of obtaining thrombi similar to the arterial ones. The greatest fibrinolysis rates were reached in the group that was submitted to continuous-wave ultrasound plus microbubbles and the smallest rates, in the thrombi treated only with microbubbles. The microbubbles addition to the continuous ultrasound (1MHz, 3,77W/cm^2) and to the pulsed-wave ultrasound (1Mhz, 0,75 W/cm^2) was capable to induce acoustic cavitation, suggesting that this association can induce thrombolysis. However, to improve the results, we believe that microbubble infusion should be made in a continuous way and that methods, other than fibrinolysis rates, should be used to quantify fibrinolysis.

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7. Responsibility notice

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