

A MINIMALLY INVASIVE ULTRASOUND THERAPY BASED ON TIME-
REVERSAL ACOUSTICS FOR THE TREATMENT OF DEEP VEIN
THROMBOSIS

A Thesis

Presented to the Faculty of the Graduate School
of Cornell University

In Partial Fulfillment of the Requirements for the Degree of
Master of Science

by

Adithya Sagar Gurram

January 2013

© 2013 Adithya Sagar Gurram

ABSTRACT

Deep Vein Thrombosis is a clotting disorder that usually leads to the formation of clots in veins deep within the legs. Deep Vein Thrombosis generally leads to a life threatening condition called pulmonary embolism where the clot breaks away and blocks the arteries of lungs. DVT affects about 600,000 people in United States and causes approximately 300,000 deaths per year. Traditional DVT treatments have relied on using anticoagulants to prevent the recurrence of DVT but not in its treatment. Here we have developed a minimally invasive model that uses low-powered ultrasound as a therapeutic aid. We have used the principles of time-reversal acoustics to develop an ultrasound modality that thrombolyses the clot without the aid of any thrombolytic. We hypothesize that this modality would aid in the development of a treatment methodology that would reduce the dosage of thrombolytics and anti-coagulants without reducing the efficacy of treating the thrombus.

BIOGRAPHICAL SKETCH

“Dubito ergo cogito; cogito ergo sum”

The desire to understand the meaning of existence has always been the driving force underlying Adithya's choice of endeavors. Science he believes is the closest to providing something resembling an answer to the questions he seeks.

His interest in science and technology led him to pursue engineering at the prestigious Indian Institute of Technology at Guwahati where he majored in biotechnology and graduated at the top of his class in 2009. Subsequently in the summer of 2009 he worked in the Joel Sussman Lab at Weizmann Institute of Science.

He thereafter returned to India and took up a job as a technology consultant with iRunway where he worked with Fortune 500 firms analyzing cutting edge technologies while offering solutions in multi-million dollar patent litigations.

His stint with iRunway made him understand the value of research to society and thus he proceeded to Cornell University to pursue graduate studies.

Under the supervision of Prof. Olbricht he successfully completed his Master of Science from the Department of Biomedical Engineering and is now

pursuing his doctoral studies from the School of Chemical and Biomolecular Engineering at Cornell.

ACKNOWLEDGEMENTS

“It is our choices, Harry, that show what we truly are, far more than our abilities”. Believing this to be a maxim, I feel incredibly blessed to be surrounded by people who have not only given me the luxury of making difficult choices but also to see through those choices irrespective of their consequences.

Firstly I would like to acknowledge my advisor Prof. Olbricht for his tremendous support and faith in me. It simply would not have been possible to complete my research without his encouragement and guidance. His remarkable ability to offer profound insights during the briefest of our meetings greatly helped me to tackle problems inside and outside of work. I would also want to thank Prof. Doerschuk for the absolutely engrossing conversations that we have had. His deep and expansive knowledge about nearly any topic under the sun, his infectious enthusiasm were always inspiring and a source of great motivation during tough times.

This project would never have had happened without the vision of Dr. George Lewis. It was his work on low powered ultrasound for therapeutic purposes that led to my application of Time Reversal Acoustics to Deep Vein Thrombosis.

I am very thankful to my lab mates Steve and Elijah for their invaluable contributions to the DVT project. It was a pleasure to have them as company especially while focusing ultrasound in large animals. I am also quite thankful to all my lab members Sabrina, John, Martin, Eileen, Sruthi, Lilia, Justin, Rohit Khullar, Yin Ying for creating a superb environment where doing research was fun.

I am very grateful to Artann Labs for providing us with the TRA systems. I am quite thankful to AngioDynamics for the blood clots and other necessary equipment and to Cornell Vet School for replenishing our stock of clots to carry out further experiments. I am especially thankful to Ethan and Karl Roneker for their efforts in preparing the clots. I would also like to thank Dr. Bhupinder Singh and Dr. Mary Martin from the Vet School for guiding me and making the entire paperwork easy.

I have been very fortunate to have had some of the most awesome people I have known as my friends here at Cornell. They go beyond a thanks or any sort of acknowledgement.

I cannot imagine my life at Cornell without Anil, Rohith, Vidhya, Ranjith, Raghu and Tanvi. Fun and otherwise they have always been around and have been a family outside of home.

I am also extremely grateful to Manjari for her resourcefulness and for her infinite patience to put up with my requests however trivial they might be.

Best things in life are often unexpected. I will always be grateful to Akanksha for changing everything and for showing me that happiness is a choice.

Above all and everything I am incredibly fortunate to have a fantastic family that is always tremendously supportive and am more than grateful to them for teaching me that the value of education lies beyond materialistic pursuits.

TABLE OF CONTENTS

1. Deep Vein Thrombosis	1
1.1 Haemostasis and Fibrinolysis	4
1.1.1 Coagulation	4
1.1.2 Fibrinolysis	10
1.2 Disruption of hemostatic equilibrium and thrombosis.....	12
2. Treatment Strategies for Deep Vein Thrombosis	15
2.1 Ultrasound in thrombolysis.....	23
3. Materials and Methods	28
3.1 In vitro porcine clot preparation.....	28
3.2 Sensitivity calibration of 'beacon' transducer	29
3.3 TRA based ultrasound thrombolysis	32
3.4 Ultrasound focusing in large animals using Time Reversal Acoustics ..	39
4. Results	43
4.1 Focusing Experiments in cylindrical reverberator	43
4.2 Thrombolysis	44
4.3 Focusing experiments in equine head	46
5. Discussion	49
6. References	53

LIST OF FIGURES

Figure 1.1: (Left) Venous thrombus formation in the cusp of veins [Image source: Thrombosis adviser – Bayer Pharma AG ⁷] (Right) Venous thrombus [Image source: Patel et al. ⁶]	1
Figure 1.2: A pulmonary embolus [Image source: Patel et al. ⁶]	2
Figure 1.3: An overview of steps in hemostasis [Image source: Casey G et al. ³]	5
Figure 1.4: Initiation of coagulation cascade by the intrinsic and extrinsic pathways [Image source: Norris et al. ⁸]	6
Figure 1.5: Secondary structure of thrombin (PDB ID: 1MKX) represented as a cartoon. The above structure contains two molecules of thrombin. The one on left is an inactive form of thrombin and the one on right is the corresponding active form. The inactive form is cleaved into the active form exposing serine residue which can cleave fibrinogen to form fibrin ⁷⁸	8
Figure 1.6: Fibrinolysis Overview-Cleavage of single chain tissue plasminogen activator (sc-tPA) and single chain urokinase plasminogen activator (sc-uPA) by plasmin to two chain tissue plasminogen activator (tc-tPA) and two chain urokinase plasminogen activator tc-uPA. Activation of plasminogen to plasmin by tc-tPA and tc-uPA. Subsequent cleavage of fibrin by plasmin [20].PAI =Plasminogen activator Inhibitor that inhibits activation of plasminogen by binding to tc-tPA and tc-uPA; α 2-PI= α 2-plasmin inhibitor, α 2-MG = α 2 microglobulin inhibit plasmin [Image source: Cesarman-Maus Gabriela et al. ²⁰]	10
Figure 1.7: The balance between thrombus formation and degradation is carefully regulated by several factors. Coagulation and fibrinolysis are linked through thrombin, thrombomodulin and thrombin-activatable factor inhibitor [Image source: Nesheim et al. ²²]	12
Figure 2.1: Targets of anticoagulant drugs in the coagulant cascade [Image source: Brenner et al. ¹]. Direct factor Xa inhibitors (in pink) like rivaroxaban, apixaban, edoxaban, fondaparinux and YM150 act on Factor Xa. Dabigatran etexilate is a thrombin inhibitor (in blue). UFH (grey) and anti-thrombin (cyan) inhibit thrombin, factor XIIa, factor Xa, factor IXa, factor VIIa. VKA like warfarin used in long term anticoagulation inhibits factors II, VII, IX and X.	16
Figure 2.2: A and B represent generation of rapidly flowing back-directed saline jets that flow back into the outflow channels from the tip of catheter. The high velocity creates a low pressure region and thus draws the thrombus into the catheter [Image source: Lin et al. ⁶]	21

Figure 2.3: A typical electro-magnetic signal received from the 'beacon' transducer [Image Source: US Patent 7713200].....26

Figure 2.4: A typical time-reversed ultrasound signal generated in response to the signal from the 'beacon' [Image Source: US Patent 7713200].....27

Figure 3.1: A typical porcine blood clot used for thrombolysis. The clot is enclosed in a silicone tube of diameter 1.2 centimeters approximately. The tube is clamped at both ends with plastic clamps as shown in the image.28

Figure 3.2: Schematic showing the setup for the calibration of beacon transducer. The hydrophone and the beacon transducer are placed at the same distance (Rayleigh distance) from the ultrasound transducer. Both of them lie in the same plane so that the acoustic pressure experienced by both is the same. The ultrasound transducer is connected to the function generator whereas the hydrophone and beacon are connected to a digital oscilloscope.29

Figure 3.3: Experimental setup for calibrating the 'beacon' transducer. The pictures (L&R) depict the overall setup containing 1) ONDA low frequency hydrophone 2) Piezo Electric Ceramic Ring Transducer 3) Digital Oscilloscope 4) Function generator 5) Ultrasound 'beacon' transducer. These components are connected according to the schematic shown in Figure 3.2.....30

Figure 3.4: Schematic showing the arrangement of various components for ultrasound assisted thrombolysis using Time Reversal Acoustics. The beacon is placed inside a reverberator. The reverberator is surrounded by external transducers which are connected to a power amplifier circuit which is powered both by an AC and DC source. The TRA electronics (powered by the AC source) is connected to the beacon transducer and the amplifier circuit. A computing interface controls the acoustic parameters based on signals received from TRA electronics.32

Figure 3.5: A ballistics gel based cylindrical reverberator (Left) containing a UNI*FUSE catheter (cyan colored). Right (top) shows the initial position of the blood clot. Right (bottom) shows the blood clot in its final position in the reverberator with the catheter containing the ultrasound beacon transducer placed in it.....33

Figure 3.6: (Left) The reverberator with clot placed inside the narrow cylindrical channel within the reverberator. (Middle) The reverberator surrounded by ultrasound transducers. (Right) A close up of transducers placed around the reverberator containing the clot. The blue ultrasound gel is at the interface of reverberator and the transducers.....35

Figure 3.7: A low-impedance multi-channel power amplifier circuit.	36
Figure 3.8: Using 1 MHz reverberator and ultrasound “beacon” transducer to focus ultrasound energy within a horse brain.....	40
Figure 3.9: (Left to Right) Focusing ultrasound energy in a horse brain using (Left) Hydrophone infusion catheter smart needle and 1 MHz reverberator (Middle) 190 KHz transducers and ultrasound “beacon” transducer (Right) 190 KHz transducers and multiple ultrasound “beacons”.....	41
Figure 4.1: A highly enhanced time-reversed ultrasound signal at a frequency of 342 KHz (Left); Continuous burst of these ultrasound signals at 342 KHz for thrombolysing the clot in the cylindrical reverberator (Right); X-axis represents time in microseconds with the origin at the centre of X-axis. Y-axis represents peak-peak to pressure amplitude in kilo pascal (kPa) with origin at the centre of the Y-axis.....	43
Figure 4.2: Loss in clot mass when exposed to TRA enhanced ultrasound and loss in clot mass when not exposed to ultrasound in a) Clots where thrombin was added b) thrombin was not added.	44
Figure 4.3: Showing change in size of a blood clot pre and post thrombolytic treatment focusing ultrasound using Time Reversal Acoustics. (Left) Blood clot post treatment which is approximately 0.6-0.7 cm in diameter and 2 cm in length (Right) Blood clot pre-treatment which is of approximately 1-1.2 cm in diameter and 4.8 cm in length.	45
Figure 4.4: A highly enhanced time reversed signal obtained at 191 KHz and 342 KHz using 190 KHz external transducers and ultrasound ‘beacon’ transducer.	46
Figure 4.5: A time-reversed signal focused at a location in the equine head using hydrophone infusion catheter smart needle and 1 MHz reverberator.	46
Figure 4.6: Time reversed signal obtained using hydrophone infusion catheter smart needle and 1 MHz reverberator.....	47
Figure 4.7: Time reversed signal obtained using two beacon transducers and 190 KHz transducers.....	48

Chapter 1

1. Deep Vein Thrombosis

Deep Vein Thrombosis (DVT) refers to abnormally coagulated blood in one of the deep veins of the leg (Figure 1.1) or the arm. It commonly results in valvular dysfunction and chronic swelling. Untreated thrombus often gets fragmented leading to fatal pulmonary embolism (PE) [6] (Figure 1.2).



Figure 1.1: (Left) Venous thrombus formation in the cusp of veins [Image source: Thrombosis adviser – Bayer Pharma AG ⁷] (Right) Venous thrombus [Image source: Patel et al. ⁶]



Figure 1.2: A pulmonary embolus [Image source: Patel et al.⁶]

Rudolf Virchow described 3 factors popularly known as the Virchow's triad that contribute to venous thrombosis. These factors are 1) Venous stasis 2) Activation of coagulation and 3) Vein Damage

The obstruction or slowing of venous blood also known as venous stasis results in an increase in viscosity and leads to the formation of microthrombi. The microthrombi grow larger and obstruct blood flow. Activation of coagulation or development of a hypercoagulable state occurs due to a biochemical imbalance resulting from an increase in tissue activating factor or decrease in plasma antithrombin. Vein damage which is endothelial may be intrinsic or can occur from external trauma. All these factors

generally result in the development of an early thrombus that interacts with endothelium. This causes local cytokine production, leukocyte adhesion leading to abnormal clotting or thrombus production. These factors cause an imbalance between the coagulation cascade and fibrinolysis leading to the propagation of thrombus [6].

1.1 Haemostasis and Fibrinolysis

1.1.1 Coagulation

In a normal healthy individual blood flows as a liquid. However in the event of an injury to any vessel a complex pathway is initiated that leads to the recruitment of blood platelets and fibrin to form a blood clot thereby stopping the blood flow [8, 9]. This phenomenon of stopping the blood flow or hemorrhage is called hemostasis. This process generally involves vasoconstriction, platelet response and coagulation [10, 12]. On subsequent repair of the damaged blood vessel, the clot is dissolved by a process called fibrinolysis [8]. The process of vasoconstriction and platelet response, followed by coagulation and fibrinolysis are sometimes referred to as primary, secondary and tertiary hemostasis respectively [12].

In the event of an injury to the blood vessel there is a reflex contraction of circular layers of smooth muscle surrounding the endothelium thus causing vasoconstriction. Vasoconstriction reduces the extravascular blood flow as well as the local blood flow [12, 13]. Immediately thereafter there is a platelet response which leads to the recruitment of platelets at the site of injury. The platelets adhere to the exposed sub endothelial collagen at the site of injury. In areas of high flows, the glycoprotein 1b-IX receptors on the platelets bind to the sub endothelial collagen through the von Willebrand factor (vWF) [11-13]. In areas of low flow the platelets will adhere to collagen and fibronectin [13]. The binding causes the platelets to change shape activating a collagen receptor called glycoprotein IIb/IIIa [12]. Subsequently they release a number

of chemical factors prominent of which are thromboxane A₂, serotonin and adenosine-di-phosphate (ADP) [10-13]. The release of these factors further augments the vasoconstriction, makes the platelets sticky and clumps them together. This step subsequently leads to the initiation of the clotting cascade [10]. The platelet aggregation is further enhanced through the production of thrombin by the coagulation cascade. The aggregation is mediated and stabilized by fibrinogen which binds to glycoprotein IIb/IIIa leading to the formation of primary platelet plug [12].

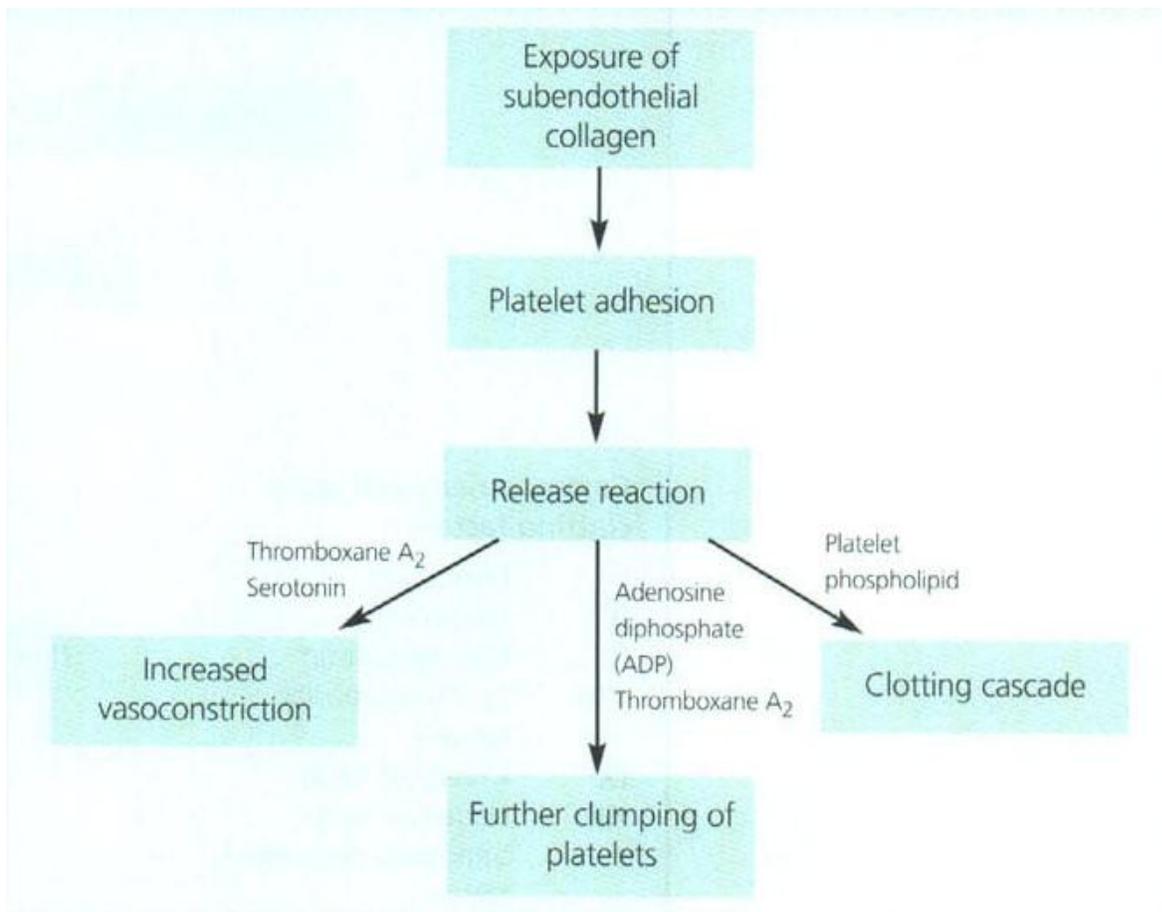


Figure 1.3: An overview of steps in hemostasis [Image source: Casey G et al.³]

The next stage of hemostasis is the initiation of coagulation of blood through a) the extrinsic or tissue factor pathway and b) the intrinsic pathway. Though it was earlier believed that both the pathways are equally involved in the initiation and propagation of coagulation cascade it has now been realized that the extrinsic pathway plays a more significant role [8].

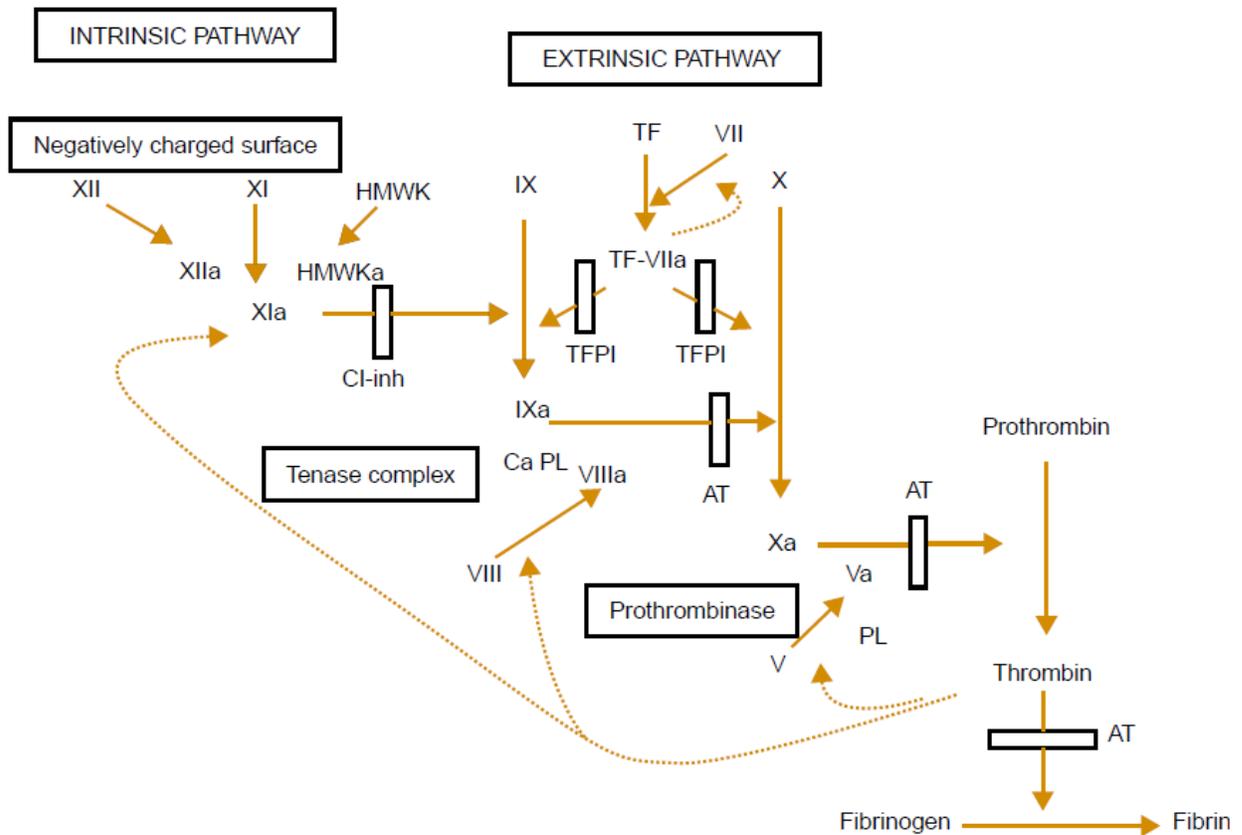


Figure 1.4: Initiation of coagulation cascade by the intrinsic and extrinsic pathways [Image source: Norris et al.].

Extrinsic pathway

Extrinsic pathway or the tissue factor pathway is the predominant blood coagulation pathway that is triggered when a membrane bound protein called tissue factor (TF) comes into contact with a factor called VII (VIIa is the activated form) present in the

plasma [1,4]. Cells (sub endothelial pericytes and fibroblasts) [4] express TF in the event of a vascular injury and come into contact with factor VII, a serine protease. The contact mediates the conversion of factor VII to its activated form VIIa. Cells like the monocytes and smooth muscle cells are also triggered to produce TF by cytokines produced in the event of an injury. This event leads to the formation of a TF-VIIa complex which initiates a signaling cascade as shown in Figure 1.4. The TF-VIIa activates factor IX to IXa and X to Xa. The TF mediated extrinsic pathway is regulated by two inhibitors- the Tissue Factor Pathway Inhibitor (TFPI) that blocks the TF-VIIa activation of factor X and anti-thrombin which inhibits thrombin and factor Xa. Thus only when TF is exposed at a high concentration to overcome the inhibition by TFPI and anti-thrombin does the coagulant cascade proceed forward through the extrinsic pathway [8].

Intrinsic pathway

In the intrinsic pathway auto-activation of a factor XII to XIIa takes places through binding to a negatively charged surface [8]. Factor XIIa subsequently causes the conversion of prekallikrein to kallikrein and of factor XI to XIa [8,14]. Simultaneously cleavage of high-molecular weight kininogen (HMWK) also occurs to produce HMWKa. These changes subsequently cause the activation of factor IX to IXa. Defects in factor XI are associated with improper fibrinolysis and thrombosis [8].

Factor IX activated through the intrinsic or extrinsic pathway leads to the formation of a complex called tenase complex [8]. Factor IXa forms the tenase complex with factor

VIIIa, calcium and phospholipids and thereby activates factor X to Xa. von Willebrand factor(vWF) binds to the platelets and activates factor VIII to VIIIa [15]. Thrombin can also activate factor VIII [9]. The activated factor Xa participates in the formation of a prothrombinase complex which consists of factor Xa, factor Va (activated through anionic phospholipids or thrombin) and a phospholipid membrane surface. This leads to the conversion of prothrombin to α -thrombin, a serine protease. The conversion of prothrombin to thrombin occurs through an intermediate called meizothrombin. Meizothrombin formed by the cleavage at Arg 320 [8] which if further cleaved at Arg 271 produces α -thrombin and prothrombin fragment 1.2 [8 ,17].

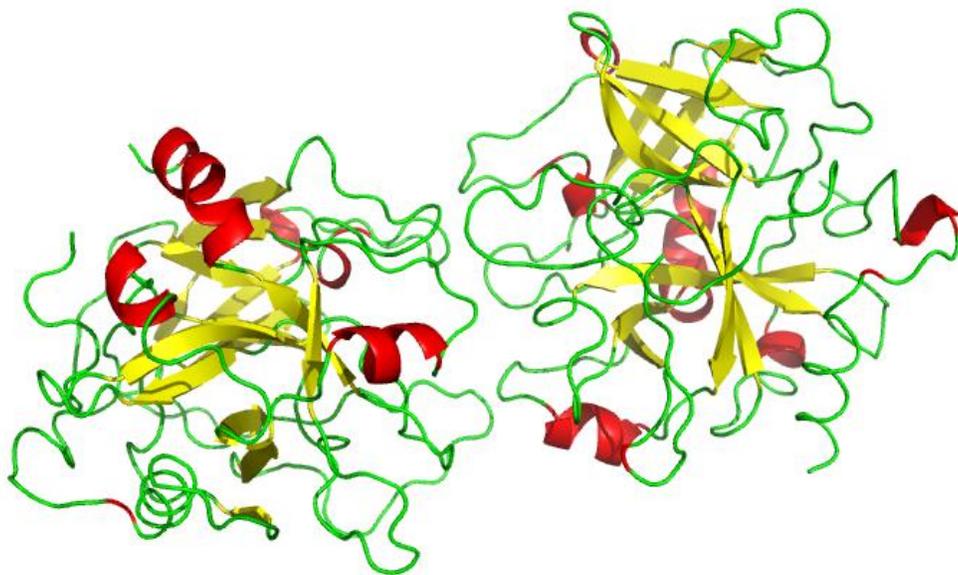


Figure 1.5: Secondary structure of thrombin (PDB ID: 1MKX) represented as a cartoon. The above structure contains two molecules of thrombin. The one on left is an inactive form of thrombin and the one on right is the corresponding active form. The inactive form is cleaved into the active form exposing serine residue which can cleave fibrinogen to form fibrin⁷⁸.

The activated thrombin plays many roles in hemostasis. It is responsible for the formation of fibrin from fibrinogen, activation of platelets, coagulation factors and activation of inhibitors like protein C and thrombin activatable fibrinolysis inhibitor (TAFI) [8 ,18]. The activated thrombin converts soluble fibrinogen to an insoluble fibrin polymer. This forms a clot that plugs the site of the injury and leads to wound healing. A number of regulatory mechanisms are in place to ensure that the clotting takes place only at the site of injury and that no inadvertent activation of clotting takes place. The heparin-antithrombin III (ATIII), protein C and thrombomodulin protein S and tissue factor inhibition pathways are the natural anti-coagulant mechanisms.

1.1.2 Fibrinolysis

The fibrinolytic system is an enzymatic system that is responsible for dissolution of fibrin clots in the blood vessels [8, 19, 20]. The system comprises of a proenzyme called plasminogen, which is converted to an active form called plasmin by different plasminogen activators (PA's). The two prominent plasminogen activators are tissue-type plasminogen activator (t-PA found in tissues) and urokinase-type plasminogen activator (u-PA).

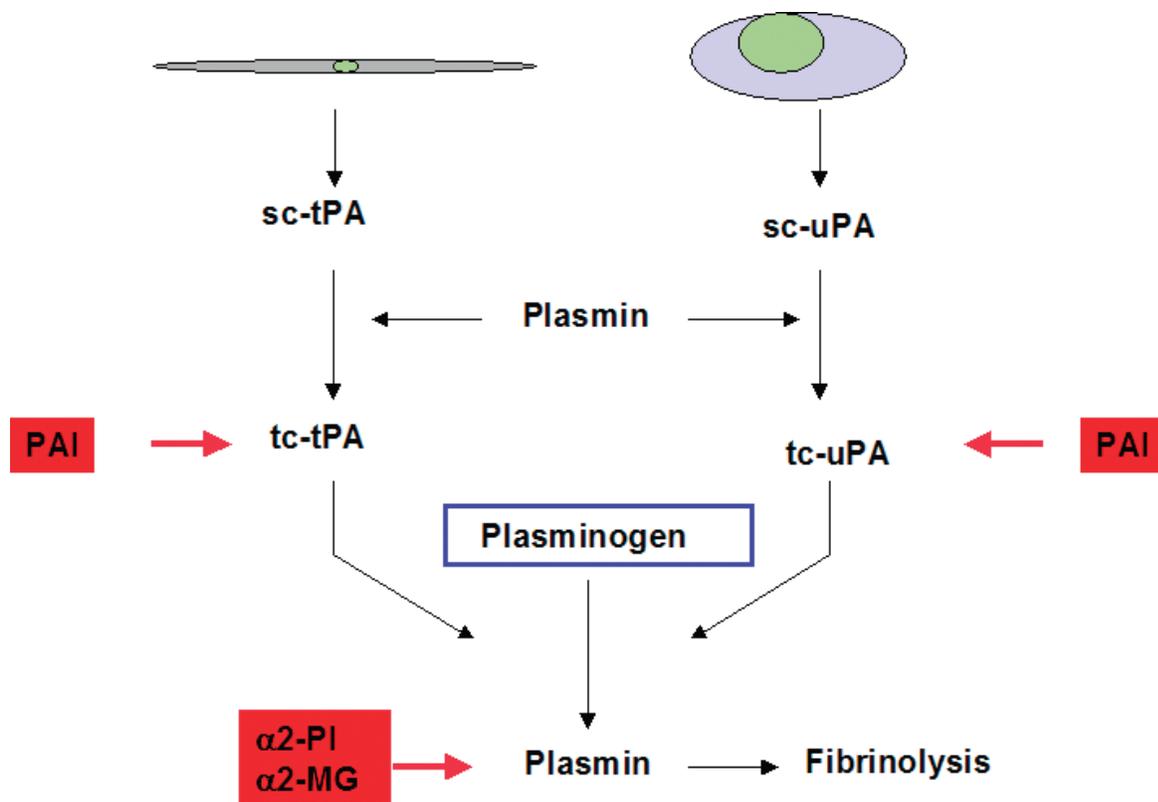


Figure 1.6: Fibrinolysis Overview-Cleavage of single chain tissue plasminogen activator (sc-tPA) and single chain urokinase plasminogen activator (sc-uPA) by plasmin to two chain tissue plasminogen activator (tc-tPA) and two chain urokinase plasminogen activator tc-uPA. Activation of plasminogen to plasmin by tc-tPA and tc-uPA. Subsequent cleavage of fibrin by plasmin [20].PAI =Plasminogen activator Inhibitor that inhibits activation of plasminogen by binding to tc-tPA and tc-uPA; α 2-PI= α 2-plasmin inhibitor, α 2-MG = α 2 microglobulin inhibit plasmin [Image source: Cesarman-Maus Gabriela et al.²⁰]

The physiologic PA's activate plasminogen at the surface of fibrin. Activation of plasminogen in plasma occurs by the cleavage of an Arg-Val bond. The cleaved plasminogen contains lysine binding sites that interact with lysine and 6-amino hexanoic acid. These sites mediate specific binding of plasminogen to fibrin and the interaction of fibrin with α 2-antiplasmin and play an important role in fibrinolysis. Fibrin bound plasmin (protected from α 2-antiplasmin) degrades the fibrin of the thrombus. The physiologic PA's - t-PA and u-PA activate the plasminogen in different ways. t-PA is a serine protease with a single polypeptide chain containing 135 amino acids. t-PA is activated in presence of fibrin where plasminogen and fibrin are sequentially adsorbed onto fibrin creating a ternary complex. The high affinity of t-PA for plasminogen in presence of fibrin converts plasminogen to its active form called plasmin which degrades fibrin. Urokinase initially exists as a single chain pro-urokinase (scu-PA). Upon hydrolysis by plasmin it is converted to a two chain urokinase (tcu-PA). The activated tcu-PA thereby activates fibrin bound plasminogen which subsequently degrades fibrin. Fibrinolytic system is inhibited primarily by α 2-antiplasmin and plasminogen activator inhibitor respectively. α 2-antiplasmin is the primary physiologic inhibitor of plasmin in blood plasma whereas plasminogen activator inhibitor-1 (PAI-1) inhibits the physiologic plasminogen activators t-PA and u-PA. Thrombin bound to thrombomodulin activates thrombin-activatable fibrinolysis inhibitor (TAFI) which reduces plasmin activity by making fibrin more resistant to t-PA mediated fibrinolysis [20, 22].

1.2 Disruption of hemostatic equilibrium and thrombosis

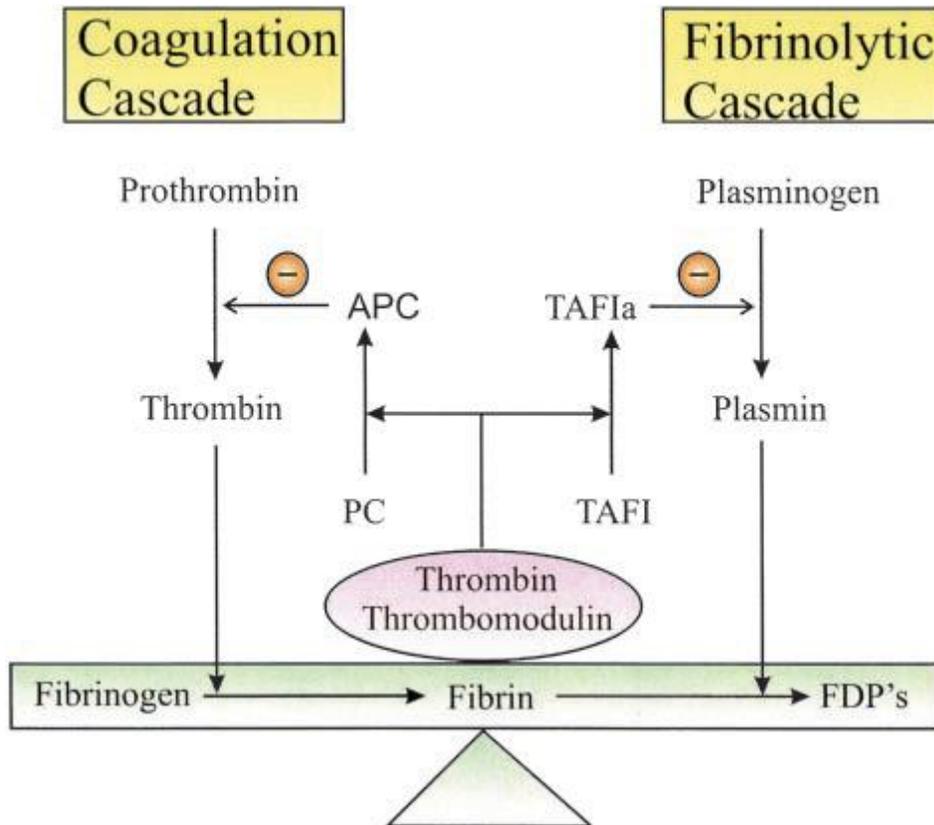


Figure 1.7: The balance between thrombus formation and degradation is carefully regulated by several factors. Coagulation and fibrinolysis are linked through thrombin, thrombomodulin and thrombin-activatable factor inhibitor [Image source: Nesheim et al.²²]

The thrombus formation and fibrinolysis are in equilibrium and occur continuously at a microscopic level. Regulation of coagulation is important to prevent formation of massive thrombi. In order to do so a number of regulatory mechanisms are in place. Further coagulation takes place only on the surface of activated cells and platelets. Tissue factor pathway inhibitor (TFPI) and anti-thrombin are important proteins regulating coagulation. However it is the protein C anticoagulant pathway which is believed to play an important role in preventing coagulation at microscopic level. Thrombin bound to thrombomodulin activates protein C (APC) and forms a complex with protein S. The protein S-APC complex inactivates both factor Va and factor VIIIa

thereby inhibiting prothrombinase and tenase activity [8, 75]. In cases of APC resistance a mutation called Leiden mutation occurs on factor V where in Arg 506 is replaced by glutamine. Inactivation of factor V that generally occurs through cleavage at Arg 506 and Arg 306 is now inefficient due to this mutation thereby conferring an inherited risk towards thrombosis. APC resistance is believed to be the most common inherited risk of thrombosis [8]. Anti-thrombin III deficiency is another major inherited disorder that leads to recurrent VTE [76]. Apart from these, mutations in protein C, protein S, endothelial protein C receptor (EPCR) seem to impart risk towards thrombosis [8].

Apart from genetic factors injury, stasis and other procoagulant factors disturb thrombosis-fibrinolysis balance and lead to a macroscopic thrombus that is obstructive in nature. In cases of trauma or surgery the level of anti-thrombin III (AT III) is decreased (especially in total hip replacement when the levels to a very low extent) increasing the chances of coagulation. The thrombus formation usually takes place behind valve cusps or at venous branch points [7]. According to certain studies low flow sites carry the greatest risk for development of venous thrombi [25, 26]. The thrombus incorporates inflammatory cells into the clot resulting in fibroelastic intimal thickening at the site of thrombus [27] leading to valvular dysfunction and overall vein thrombosis. Most of the patients who have experienced acute DVT experience venous stasis, valvular reflux and chronic edema [28]. Chronic venous insufficiency develops in 29-79% of patients with an acute DVT [7, 30, 31]. Natural healing process is another mechanism that contributes to venous insufficiency. Inflammatory reaction and fibrinolysis break down the thrombus in the process altering the valves and venous walls. This process predisposes the damaged, incompetent, underlying valves to

venous reflux. The inflammatory reaction breaks down the collagen and elastin making the venous wall noncompliant [8, 33-38]. Incomplete removal of an obstructive thrombus with venous damage perpetuates venous insufficiency eventually leading to post thrombotic syndrome.

Venous thrombi also break off from their location and travel to through the right heart to pulmonary artery causing ventilation perfusion defect and cardiac strain leading to pulmonary embolism (PE). PE occurs in 10% of patients with acute DVT and results upto 10% of hospital deaths [7, 27, 28].

Chapter 2

2. Treatment Strategies for Deep Vein Thrombosis

Deep Vein Thrombosis (DVT) and the subsequent Post Thrombotic Syndrome (PTS) pose a major health hazard in most parts of the world. Venous thromboembolism (VTE) which encompasses DVT and pulmonary embolism (PE) affects more than 600,000 people per year and causes 300,000 deaths per year in the United States. In the European Union the incidence is higher at over one million resulting in 500,000 deaths per year [1-3]. Approximately 250,000-300,000 cases of VTE in the United States have a first incidence of Deep Vein Thrombosis [3, 4]. DVT affects about 25% of patients undergoing all major surgical procedures with a mortality rate of about 12% [4]. The recurrence of DVT after a non-surgical first incidence is about 18% in first 2 years and 30% in 8 years. Nearly 20-50% of patients develop post thrombotic syndrome after the incidence of DVT in the first two years [2, 5].

The need to prevent PE and PTS has brought forth a DVT management strategy rooted in the view of the disease as a condition that leads to pulmonary embolism and recurrent venous thromboembolism [6, 40]. This notion has primarily led to a treatment methodology that focused on the use of anticoagulant drugs to prevent recurrence of DVT.

Also at a physiological level it has been well established that hypercoagulability, stasis of blood flow and venous endothelial injury (Virchow's triad) are the major factors

responsible for incidence of DVT. However at the molecular level it is influenced by interaction between various genes, inflammation, lipid biology and other incompletely characterized factors. Owing to this incomplete characterization and the view that the disease is more a condition that leads to PE and recurrent VTE, conventional DVT treatment has primarily relied on the use of anticoagulants to prevent thrombus formation [40].

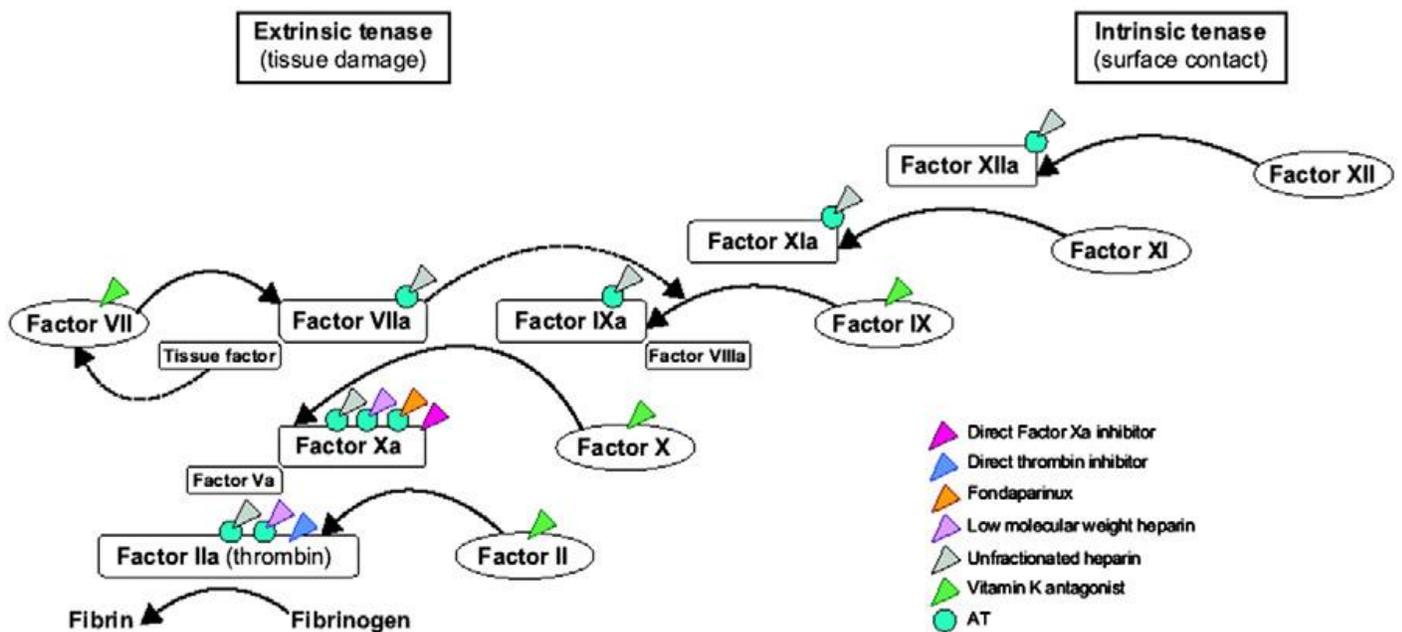


Figure 2.1: Targets of anticoagulant drugs in the coagulant cascade [Image source: Brenner et al.¹]. Direct factor Xa inhibitors (in pink) like rivaroxaban, apixaban, edoxaban, fondaparinux and YM150 act on Factor Xa. Dabigatran etexilate is a thrombin inhibitor (in blue). UFH (grey) and anti-thrombin (cyan) inhibit thrombin, factor XIIa, factor Xa, factor IXa, factor VIIa. VKA like warfarin used in long term anticoagulation inhibits factors II, VII, IX and X.

The initial treatment involves the use of anticoagulant drugs like unfractionated heparin (UFH), low molecular weight heparin (LMWH) or pentasaccharide fondaparinux followed by the long term use of Vitamin K antagonist (VKA) such as warfarin. Generally UFH, LMWH or fondaparinux are given for 5 days subcutaneously and then discontinued if

the International Normalized Ratio (INR) is 2.0 or above for more than 24 hours [1, 51]. Subsequently VKA is administered for 3 months or more based on risk of bleeding and INR values. Randomized trials have shown that anticoagulant treatment with UFH and LMWH has a risk of recurrent DVT 4%, bleeding 3% and PE 2% [52, 53].

An anti-coagulant based treatment though effective has several issues associated with it, apart from the risk of excessive bleeding (approximately 2% have major bleeding in first three months and 1-3% thereafter with a fatality rate of 13% [54]). UFH also binds to plasma proteins and cells and is known to cause heparin-induced thrombocytopenia and osteoporosis. Heparin-immunoglobulin G complexes are formed that bind to Fc receptors on platelets, activating them. These are removed during circulation causing thrombocytopenia. This process also generates platelet-derived microparticles which have procoagulant activity [1].

Several studies have shown that anticoagulant treatment is not efficient in preventing the patients from developing post thrombotic syndrome (PTS) [39, 40, 43, 44]. 75% of patients with symptomatic DVT develop PTS in 5-10 years when using anticoagulation treatment alone [64, 65]. Anticoagulants usually do not completely resolve a thrombus. At a molecular level the factors behind the development of PTS are still not clearly understood. Inflammatory and growth factor mediators, extracellular matrix-derived factors, blood-borne elements are some of the factors that increase the thickness of the cell wall and reduce the compliance, thus promoting the development of PTS [40]. Though PTS at the microscopic level is not completely understood, at the macroscopic

level it has been proved that incomplete resolution of thrombus after the incidence of DVT leads to PTS [4, 39, 40, 44]. The presence of thrombus after DVT leads to PTS through obstruction and inflammation. The thrombus physically blocks the venous blood. Subsequently there is an inflammatory response to this thrombosis that directly damages the venous valves, alters the adjacent vein wall and leads to valvular reflux. Distal deep veins also dilate and become incompetent. Obstruction and the resulting valvular reflux cause ambulatory venous hypertension which in turn leads to edema, tissue hypoxia, injury, progressive calf dysfunction, subcutaneous fibrosis and skin ulceration [40]. Thus it is extremely essential for the early and efficient removal of the thrombus to prevent PTS and ensure long term healthy venous function [39]. Systemic embolism, chronic venous insufficiency, soft tissue ischemia and non-oral administration are other issues associated with anti-coagulant treatment [6].

With all these drugs (especially long term use of VKA) there is a substantial risk of bleeding which varies from patient to patient. Several new generation anticoagulants are being developed that can be administered orally like rivaroxaban, apixaban, edoxaban and YM150 which are direct factor Xa inhibitors, dabigatran etexilate a direct thrombin inhibitor. Though the new-generation anti-coagulants show promise of reducing the risk of recurrent VTE they still are in clinical phase and show some new disadvantages. A major limitation in the use of these drugs is the lack of any specific compounds that reverse the anti-coagulant effect. Also at times the oral route of administration is a problem in patients who have undergone major surgery. The effects of these medications have not been studied in patients with heart disease, cancer, and

in patients who have undergone valvular replacement etc. These anticoagulants being small molecules also cross the placental barrier and hence cannot be used during pregnancy [1].

Systemic/pharmacologic thrombolysis is a treatment strategy where the thrombus is dissolved using a fibrinolytic drug given via an intravenous line distant from the affected limb [40]. Streptokinase has been a popular first generation fibrinolytic drug that was proved to be more effective in addressing VTE than anticoagulants like heparin [40]. However the bleeding complications are much more frequent and more intensive when compared to anti-coagulant treatment. Hence the drug is not used in the current practice. Recombinant tissue plasminogen activator (rt-PA) is a drug with greater fibrinolytic activity than other fibrinolytic agents and is currently the preferred drug systemic thrombolysis [40]. However studies have shown that the systemic administration route is not the best possible administration route to attain therapeutic drug concentration to lyse the clots. It was also observed that this administration route leads to extracranial bleeding [40]. Intracranial bleeding has also been noted with the use of fibrinolytic drugs with death and impairment as infrequent effects [6].

The advent of non-invasive vascular imaging using Duplex ultrasound and helical CT scanning, ultrasound facilitated catheter access into venous system, drug delivery through catheter systems facilitated the development of endovascular techniques to treat DVT [39,40]. Modern endovascular techniques to treat DVT consist of catheters to deliver drugs at the site of thrombosis, mechanical thrombectomy angioplasty and

stenting of venous obstructions [6]. Endovascular techniques aim to prevent PE, recurrent VTE and PTS and reduce drug dosage by enhancing concentration of the drug locally [6, 40].

Catheter direct thrombolysis (CDT) is a popular endovascular technique which uses a multiside hole catheter to dispense fibrinolytic drug at the site of thrombosis [40, 46, 47]. After the clot lysis underlying veins are evaluated by venography and any venous obstructive lesion identified is treated with balloon angioplasty or stent placement [40]. Some studies have shown that when compared to thrombectomy CDT treatment resulted in less residual thrombus, greater valvular competence, improved endothelial function [55] and also have been more effective than systemic thrombolysis [56,57]. A major limitation with CDT is the longer infusion times (1 to 3 days) to treat DVT. Studies have also reported extensive bleeding in certain cases (2%-3% of cases) using low doses of rt-PA [40, 48, 49, 50].

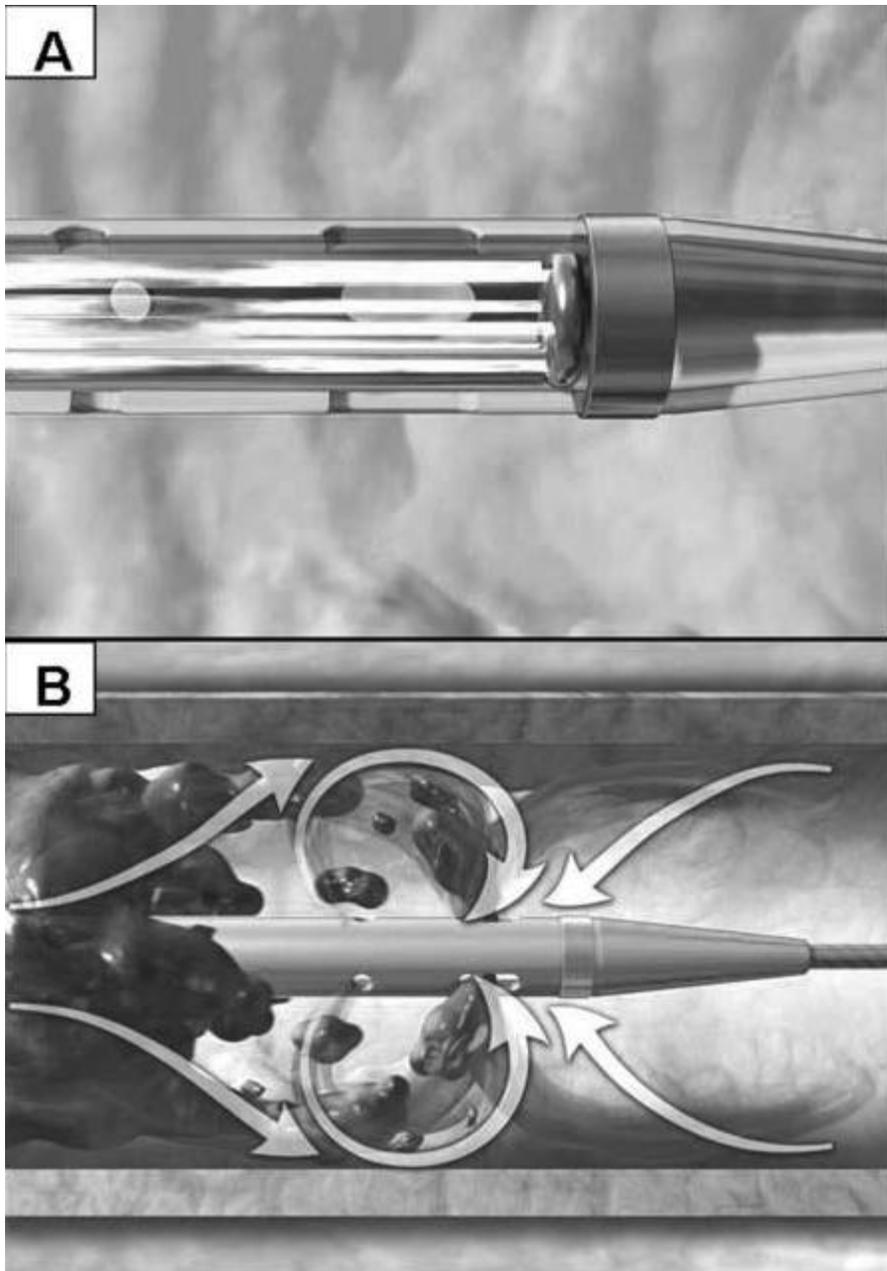


Figure 2.2: A and B represent generation of rapidly flowing back-directed saline jets that flow back into the outflow channels from the tip of catheter. The high velocity creates a low pressure region and thus draws the thrombus into the catheter [Image source: Lin et al.⁶]

Percutaneous mechanical thrombectomy is another method for thrombus removal that uses mechanical means to remove the thrombus. These methods are primarily used as an adjuvant to CDT or pharmacologic thrombolysis [6, 40, 45]. The combined treatment

methods are referred to as pharmacomechanical catheter directed thrombolysis or the PCDT and pharmacomechanical thrombectomy or the PMT. These methods promise reduced treatment times and removal of large thrombus volumes within a short period of time [40]. AngioJet system (Possis Medical, Minneapolis, MN) and the Trellis device (Bacchus Vascular, Santa Clara, CA) are two of the popular thrombectomy systems. The AngioJet system is based on Bernoulli's principle where thrombus is drawn into catheter using backward flowing saline jets [4] (Figure 2.2).

There are two general methods of PCDT application. One utilizes thrombectomy devices along with traditional CDT reducing the fibrinolytic drug dose and time of infusion [40, 58]. The other are the single session PCDT methods that rapidly disperse thrombolytic drug and remove the thrombus in a single 1-3 hour procedure [26,40,59,]. Major bleeds have been reported in 2-4% of the patients and symptomatic PE has been observed in cases where aggressive mechanical thrombectomy has been used [6]. However no conclusive studies have been performed that ascertain the efficacy and the safety of PCDT methods [40]. All patients undergo anticoagulant treatment irrespective of whether an endovascular procedure is performed [40].

2.1 Ultrasound in thrombolysis

Conte and de Lorenzi first showed the effect of ultrasound on biological tissue in 1940 and in 1965 Anschuetz and colleagues used ultrasound for atherosclerotic plaque ablation [41, 62]. Rosenschein and colleagues first demonstrated the application of ultrasound to disrupt intravascular thrombi in 1990. They reported that the fibrin matrix was disrupted within the thrombi using ultrasound energy [4, 60, 61]. Subsequently over the last couple of decades ultrasound based thrombolysis has emerged as a promising endovascular alternative to treat DVT and as an adjunctive to pharmacological thrombolysis and anticoagulation treatment. US accelerated thrombolysis has low bleeding rates, enhanced clearance of thrombus with low treatment times. The drug dosage is low thereby this treatment reduces the bleeding complications [77].

Ultrasound is believed to trigger fibrinolysis due to 1) acoustic cavitation 2) microstreaming 3) mechanical effects 4) intracellular microcurrents 5) thermal warming 6) increased clot permeability. The mechanism of thrombolysis varies with the mode of delivery of ultrasound whereas the extent of lysis depends on the mode of delivery, acoustic parameters like frequencies used, energy delivered, pulsed wave or a continuous wave, the depth of penetration, magnitude of microstreaming, stability of cavitation, the thrombolytic drug used, microbubble agents used [4,41,62].

Currently ultrasound based thrombolysis technologies may be categorized into three categories – (1) intravascular catheter delivered external transducer ultrasound (2)

intravascular catheter-delivered transducer-tipped ultrasound (3) transcutaneous ultrasound [4, 41]. Intravascular catheter-delivered external transducer ultrasound uses an electrical generator that powers the ultrasound transducer which then transmits this ultrasound energy to a metal wire probe (of different lengths) causing a rapid motion in the surrounding fluid [41]. The rapid motion of the probe is said to cause acoustic cavitation, micro streaming and a mechanical perturbation thus initiating the fibrinolytic cascade. The range of frequencies are however limited to 19 KHz-50 KHz. The transducer-tipped ultrasound method utilizes a piezo electric element at the tip of the catheter which allows for frequencies to be delivered in the range of 0.1 MHz-2.0 MHz. The ultrasound intensity ranges from 0.6-2.0 W/cm². At this intensity micro streaming is believed to play an important role in fibrinolysis with acoustic cavitation taking a minor role. The ultrasound delivered in this manner also aids in the increase of clot permeability and promotes clot lysis by enhancing the diffusion of fibrinolytic drug (into the clot (Eg. EKOS Endowave system) [4, 42, 77]. Both these methods are however invasive and limited by the size of the ultrasound transducer in the latter or probe wire in the former. The size of the probe wire and transducer restrict the frequencies that can be applied and place a limitation on the amount of ultrasound energy delivered to the site of lysis.

Transcutaneous ultrasound thrombolysis is a non-invasive procedure that is readily used in patients with acute myocardial infarction or stroke [41]. Unlike transcatheter thrombolysis wherein the invasiveness causes time delay, logistic and technical issues during clinical implementation, this approach is faster and can be easily deployed. It is

believed that transcutaneous ultrasound enhances drug transport into the clot through micro streaming, acoustic cavitation and precavitation via the oscillation/vibration of micro bubbles [41, 63]. The major limitations of this system are the inability to focus ultrasound at a specific location, dissipation of energy through skin and other tissue, possible injury to the skin and tissue resulting from exposure to non-focused ultrasound and longer treatment time. The inability to focus also limits the delivery of therapeutic ultrasound at the desired location.

Apart from thrombolysis, several applications of ultrasound in medicine require focused signal that is localized to a specific tissue. However various heterogeneities across different tissues, the interfaces and boundaries that affect the speed of the wave and absorption of energy, reflection, refraction and scattering across biological tissues greatly limit the focusing of external ultrasound energy. Delivery of ultrasound energy using an internal transducer is invasive and carries the potential risk of causing internal damage. The biggest disadvantage however is the transducer size which limits the amount of energy delivered, the frequencies applied and the delivery location. One of the goals of the current research is to overcome these limitations by using an ultrasound focusing methodology that is based on the principle of time-reversal acoustics.

In the late 90's Mathias Fink pioneered the concept of Time Reversal Acoustics (TRA) that allowed for focusing of ultrasound energy in highly inhomogeneous media. The method allowed for an acoustic signal to be focused spatially and temporally in a highly inhomogeneous media. TRA is based on the principle that in a non-dissipative

heterogeneous medium the wave equation is invariant. Thus for a given set of divergent waves that are reflected, refracted and scattered there exists a time-reversed version which retraces these exact paths and converges at the original source [68, 76]. The ability to focus in highly heterogeneous media helps localize ultrasound energy to a specific location and limits the exposure to other tissues. TRA focusing also allows for a

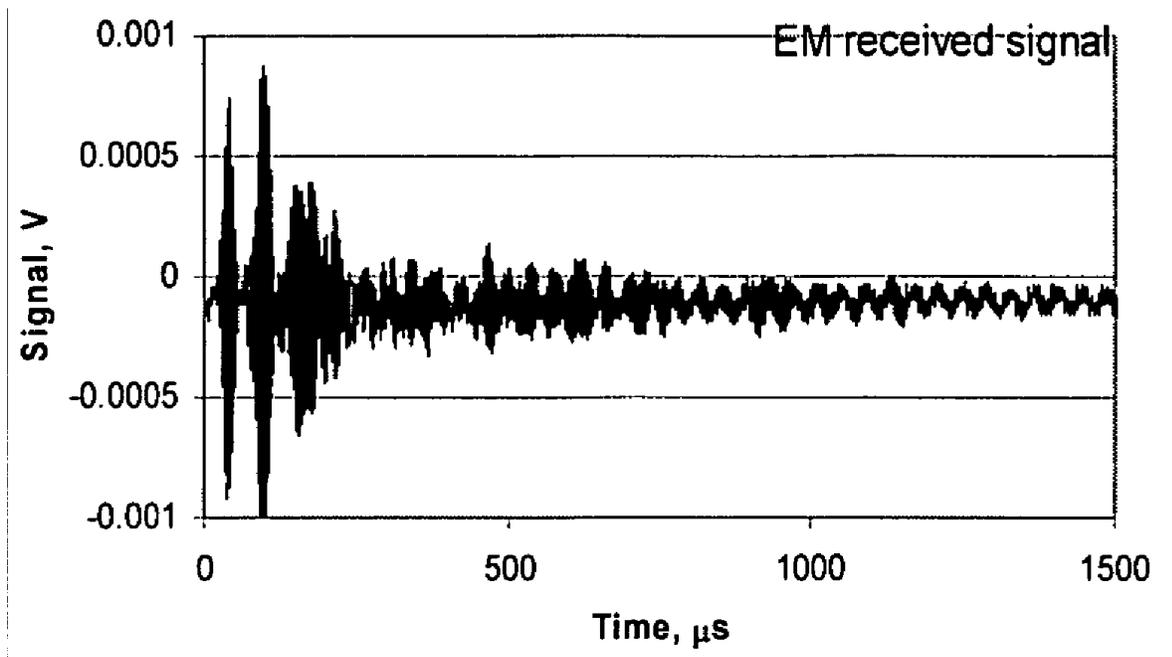


Figure 2.3: A typical electro-magnetic signal received from the 'beacon' transducer [Image Source: US Patent 7713200].

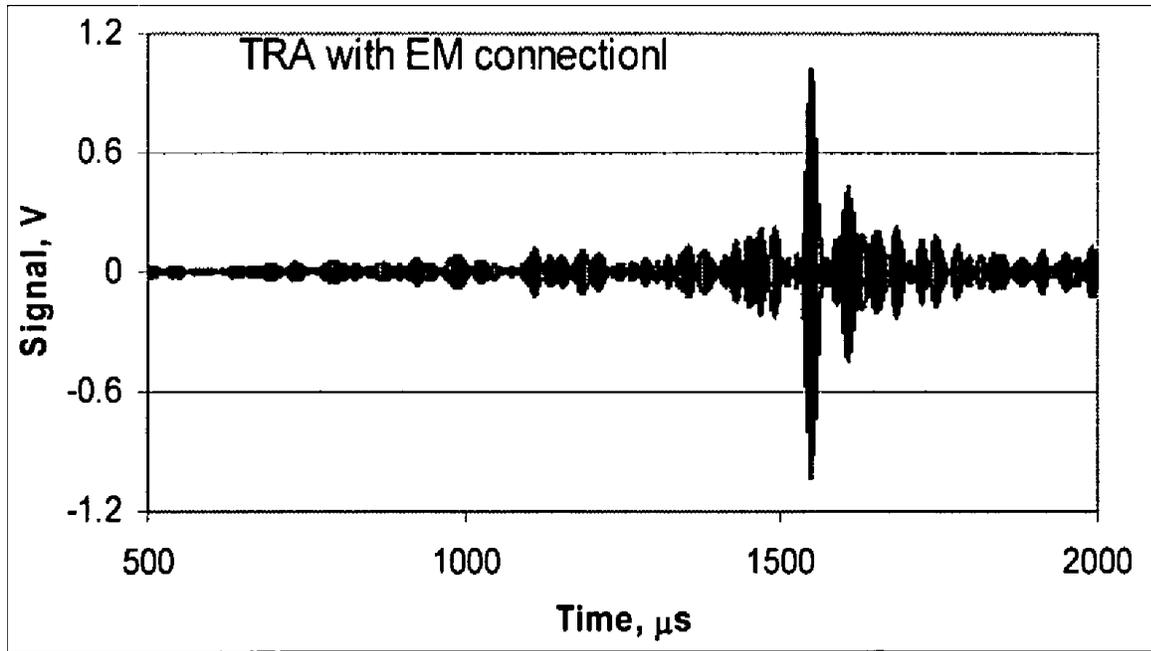


Figure 2.4: A typical time-reversed ultrasound signal generated in response to the signal from the 'beacon' [Image Source: US Patent 7713200].

better spatial focusing than other focusing systems. The focal volume approaches the spherical diffraction limit. TRA focusing can also dictate the type of waveform (spherical or elliptical etc.) This helps in controlling the mechanism by which ultrasound can affect thrombolysis [70]. For any focusing of ultrasound using TRA an important requirement is the presence of an entity at the required location that provides necessary feedback. Generally a hydrophone or a highly reflective target is used as a beacon. The presence of such a beacon at the site often limits the TRA based focusing [70]. To overcome the above limitation the system contains a small piezo electric transducer at the tip of the catheter (which can be adjusted according to the vessel size) that is inserted into the clot. The piezo electric transducer acts as a beacon which pin points the location of clot. Thereafter external transducers using the principle of time-reversal acoustics focus the ultrasound energy at the beacon thereby greatly enhancing clot lysis.

Chapter 3

3. Materials and Methods

3.1 In vitro porcine clot preparation



Figure 3.1: A typical porcine blood clot used for thrombolysis. The clot is enclosed in a silicone tube of diameter 1.2 centimeters approximately. The tube is clamped at both ends with plastic clamps as shown in the image.

Accessing the carotid artery of the donating pig, approximately 15-20 ml of blood is collected in a silicone tube of inner diameter 1.2 centimeters with a clamp on one end. After filling the tube (with approximately 20ml of blood) the other end is clamped. The thrombus is allowed to form at room temperature for about 1hour. Subsequently excess plasma is discarded and the clots are stored at approximately 5 °C. The donating pigs were 15 weeks old and weighed 120lbs. The blood was collected as per the regulations of Institutional Animal Care and Use Committee (IACUC) protocol. 12 tubes of clots were prepared according to this protocol.

To an additional set of 12 tubes, reconstituted thrombin (Sigma Aldrich) having a concentration of 100U/ml was added. Each tube containing 15-20ml of blood was mixed with approximately 0.75ml of reconstituted thrombin.

3.2 Sensitivity calibration of 'beacon' transducer

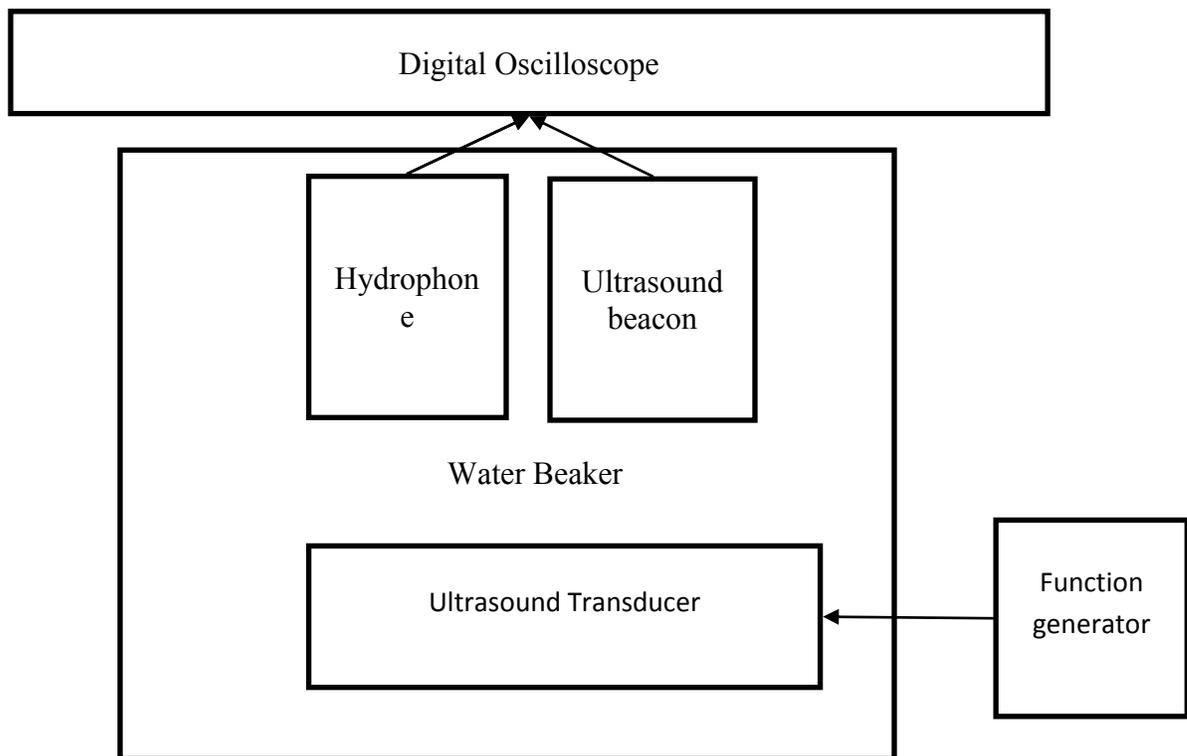


Figure 3.2: Schematic showing the setup for the calibration of beacon transducer. The hydrophone and the beacon transducer are placed at the same distance (Rayleigh distance) from the ultrasound transducer. Both of them lie in the same plane so that the acoustic pressure experienced by both is the same. The ultrasound transducer is connected to the function generator whereas the hydrophone and beacon are connected to a digital oscilloscope.

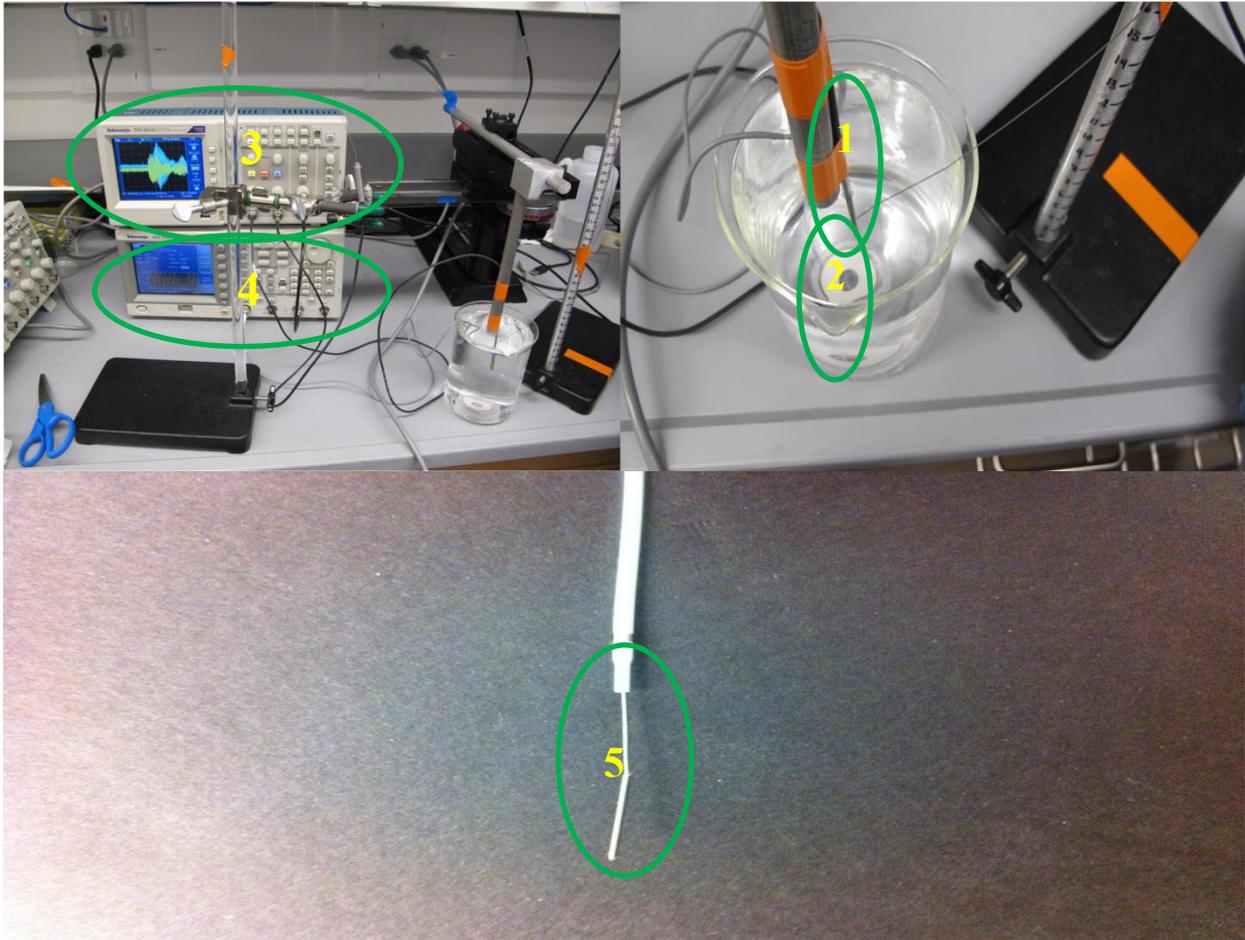


Figure 3.3: Experimental setup for calibrating the 'beacon' transducer. The pictures (L&R) depict the overall setup containing 1) ONDA low frequency hydrophone 2) Piezo Electric Ceramic Ring Transducer 3) Digital Oscilloscope 4) Function generator 5) Ultrasound 'beacon' transducer. These components are connected according to the schematic shown in Figure 3.2.

A piezo electric ceramic ring transducer with resonant frequency of 190 ± 5 KHz (SMR 2809t60118, STEMiNC- Steiner and Martins, Inc.) is placed within a glass beaker filled with water and is connected to a function generator. The ultrasound 'beacon' transducer and the low frequency hydrophone (ONDA HNR-1000 S/N: 1557) are placed at the Rayleigh distance ($\frac{OD^2}{4\lambda_w}$) where OD is outer diameter of the transducer and λ_w is wavelength of sound in water) of the ultrasound transducer (OD 28mm x ID 9mm x Th 6mm). The setup ensures that the beacon and the transducer are in the same plane.

Thus the pressure experienced by the hydrophone and the beacon is the same. The low frequency hydrophone and the ultrasound beacon transducer are then connected to two different channels of the oscilloscope. A sine wave of frequency 342 KHz is applied and the corresponding peak-peak voltages are measured in both the channels. Since $\text{Sensitivity}(S) * \text{Pressure}(P) = \text{Voltage}(V)$, we obtained the sensitivity of the beacon as $1.0126e-6 \text{ V/Pa}$ (given the sensitivity of hydrophone at 330 KHz is $0.56845e-6 \text{ V/Pa}$).

3.3 TRA based ultrasound thrombolysis

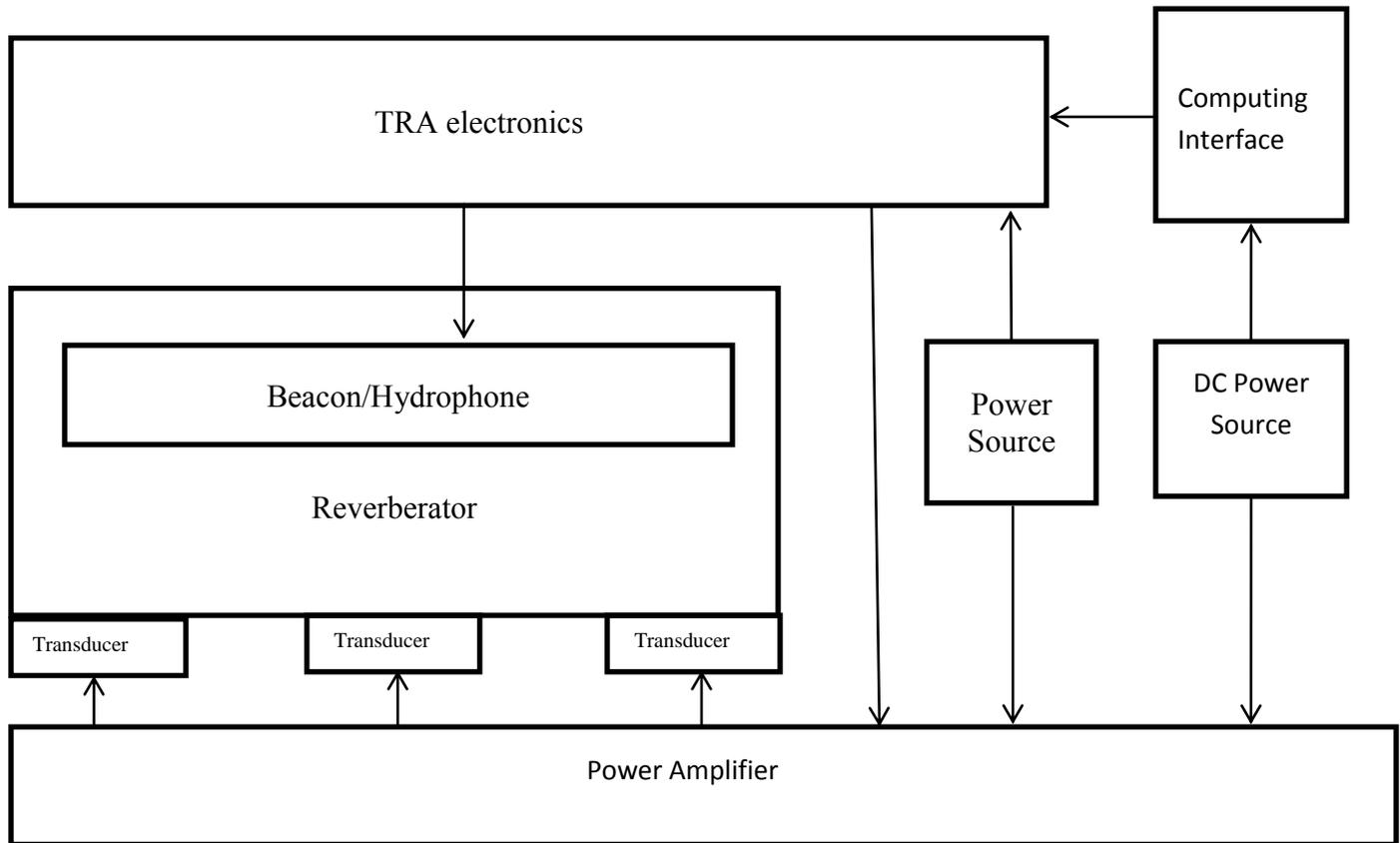


Figure 3.4: Schematic showing the arrangement of various components for ultrasound assisted thrombolysis using Time Reversal Acoustics. The beacon is placed inside a reverberator. The reverberator is surrounded by external transducers which are connected to a power amplifier circuit which is powered both by an AC and DC source. The TRA electronics (powered by the AC source) is connected to the beacon transducer and the amplifier circuit. A computing interface controls the acoustic parameters based on signals received from TRA electronics.

The beacon transducer (which also acts as a hydrophone) is placed within the cylindrical reverberator (made of ballistics gel) and connected to the TRA electronics through a female BNC connector. The TRA electronics is connected to an AC power source and a computing interface. The computing interface contains the software that controls the various parameters associated with TRA focusing.

The cylindrical reverberator (Figures 3.5 and 3.6) is surrounded with transducers (temperature regulated at 25°C) that are in turn connected to a low impedance power amplifier circuit. The amplifier circuit is also connected to the TRA electronics. A DC power source powers the computing interface housing the TRA software and the coolant fans of the amplifier circuit. The alternating power source provides the voltage across various circuit elements.

The reverberator used in the given experimental setup is shown in the Figure 3.5. It is a ballistics gel based cylinder with an outer diameter of about 14 cm containing a narrow cylindrical channel of approximately 1.2 cm within which the clot to be thrombolysed is placed.

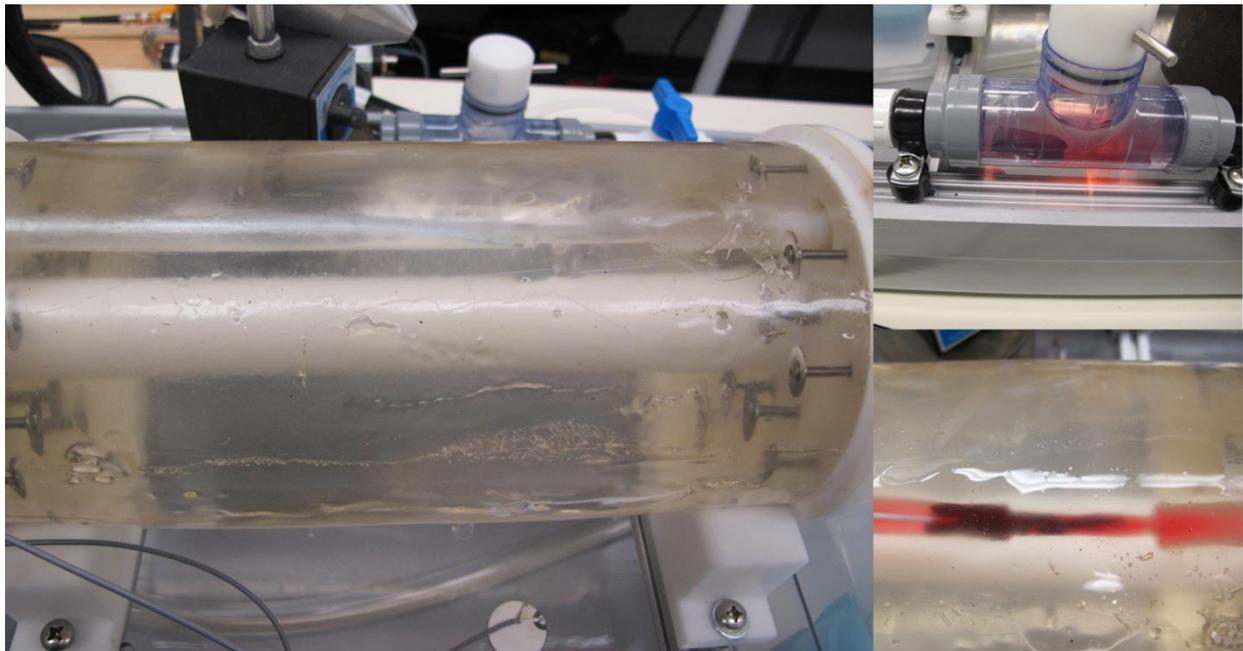


Figure 3.5: A ballistics gel based cylindrical reverberator (Left) containing a UNI*FUSE catheter (cyan colored). Right (top) shows the initial position of the blood clot. Right (bottom) shows the blood clot in its final position in the reverberator with the catheter containing the ultrasound beacon transducer placed in it.

The clots are removed from the silicone tubes and placed in a petri dish for measurement. The weight of the clot is measured as per equation (1)

Weight of clot [WC] = (Weight of petridish+ Weight of clot) [WP]-Weight of petridish containing residual blood [WR] - (1)

The blood clot (approximately 1cm in diameter) is then placed in a broad cylindrical tube as shown above in Figure 3.5 (Top right). The tube is connected to the reverberator through a silicone tube (approximately 1.4 cm in diameter) that leads into a cylindrical channel within the reverberator (Bottom right). Using a pump (115V, 1.1A, 1phase) that drives water through this system, the blood clot is slowly driven into the final position in the cylindrical channel as shown in Figure 3.5 (Bottom left). Prior to placing the clot, the system of tubes and cylindrical channel is flushed with water to remove any air bubbles. A UNI*FUSE catheter (Angiodynamics Inc., Catheter size 5F) is inserted through the cylindrical channel so that it reaches the beginning of the clot. Subsequently the beacon transducer connected to the TRA electronics is inserted into the catheter such that the piezo electric element of the beacon transducer sits in the middle of the clot.

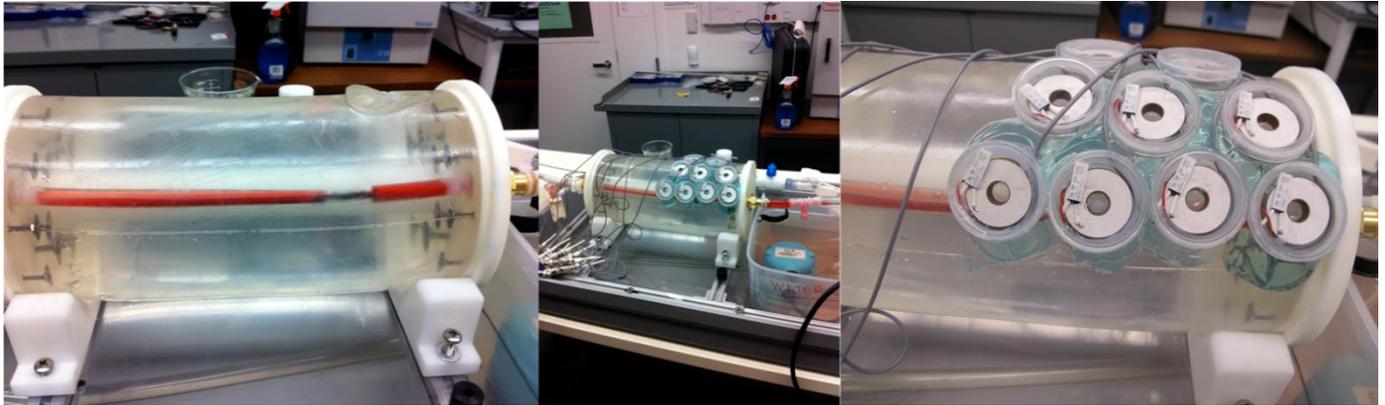


Figure 3.6: (Left) The reverberator with clot placed inside the narrow cylindrical channel within the reverberator. (Middle) The reverberator surrounded by ultrasound transducers. (Right) A close up of transducers placed around the reverberator containing the clot. The blue ultrasound gel is at the interface of reverberator and the transducers.

After the clot is placed within the reverberator, 9 piezo electric ceramic ring transducers with resonant frequency of 190 ± 5 KHz (SMR 2809t60118, STEMiNC- Steiner and Martins, Inc.) are placed around the reverberator (shown in Figure 3.6). The transducers are housed in a plastic material for protection and isolation. In order to minimize the loss of ultrasound energy through the reverberator, ultrasound gel is applied at the transducer-reverberator interface. This enhances ultrasound coupling and minimizes the energy losses.



Figure 3.7: A low-impedance multi-channel power amplifier circuit.

As shown in the schematic above in Figure 3.4, the transducers and the TRA electronics are then connected to a multi-channel, low-impedance power amplifier circuit (shown in Figure 3.7). There are 16 power MOSFETs per channel. Each channel is controlled by the TRA electronics to deliver ultrasound either continuously or in a pulsed fashion, at the required frequency and duty cycle for a given period of time. The amplifier is powered by two +/- 30V 10 Amp power supplies that control the power to the external transducers and one 8V, 2 Amp power supply that enables the fans, integrated circuits and the general amplifier. The BNC input of the amplifier accepts 2 to 5 volt transistor-transistor logic signals supplied by the TRA electronic unit and amplifies the signal up to a maximum of +/- 30 volt push-pull square wave drive signal. The output of each amplifier channel is connected to an ultra-flexible coax cable terminated with a female BNC connector [73].

After supplying all components with appropriate power sources and ensuring that all the connections are made properly the system is turned on. The DC power source is set to at a constant voltage of 8V and the alternate power source is set to operate around 24V.

The parameter file located on the computing interface controls various acoustic parameters associated with the TRA electronics. The initial sweep i.e. a library of initial ultrasound pulses, the sensitivity value, duty cycle, time of operation are initialized as 20 KHz-1MHz, $1.0126e-6$ V/Pa 92%, 180000s respectively with a pulsed mode of operation.

An initial wave signal (a transistor-transistor logic excitation signal from the range of ultrasound pulses) is applied to a transducer connected to the TRA channel through the TRA electronics. The signal then passes through the reverberating medium and is detected by the beacon. The piezo electric transducer of the beacon which is located at the center of the blood clot receives the initial signal and provides an electro-magnetic feedback to the TRA electronics. The TRA electronics receives this signal; time reverses the signal and stores the time-reversed signal. This step is subsequently repeated for each of the transducers connected to the different channels of the amplifier circuit. After having received signals from each of the transducers, time-reversing them and storing the time-reversed signals, simultaneous time-reversed signals (synchronized with the initial signal) from all the transducers are emitted at once. This generates an enhanced time-reversed signal that is directed towards the piezo electric transducer element of the beacon transducer located at the center of the clot [71, 72,

73]. From the set of signals that generate enhanced time reversed signals the one generating the best signal to noise ratio is selected for thrombolysis by the TRA electronics.

The TRA system uses a graphical user interface that is based on a Matlab® program working in conjunction with the TRA electronics. The TRA electronics communicates with the PC through a 2 channel USB2 interface and is capable of operation with up to 95% duty cycle [73]. The user initiates the MatLab® command window using the function call to the main program TRA_UI ('expert'). This function initializes the entire TRA system. Once initialized, the GUI enables the selection of frequency, pulse duration, pulse repetition, duty factor for the duration of the experiment in real time [73]. In our study we have already fixed these parameters within the parameter file instead of using the GUI.

After thrombolysis the clot is removed and its mass is measured according to equation (1). The loss in clot mass is measured for three groups 1. Controls clots where no ultrasound was applied 2. Clots with 100U/ml thrombin added 3. Clots with no thrombin added.

3.4 Ultrasound focusing in large animals using Time Reversal Acoustics

To investigate the ability to localize/focus ultrasound using time reversal acoustics in large animals, TRA based ultrasound focusing was carried in a 45lb cadaveric equine head. A 2cm by 2cm hole was drilled into the cadaveric skull between the forelock and the poll region exposing the dura mater.

Four different experimental modalities were considered to demonstrate the focusing of ultrasound

1. *Beacon transducer and 1MHz reverberator*

Using a schematic similar to Figure 3.4 the beacon transducer is placed inside the equine brain as shown in Figure 3.8. A metal reverberator housing 1MHz PZT-4 piezoelectric crystals is placed in contact with the equine brain as shown in the Figure 3.8. To ensure ease of insertion for the catheters a metal bore was used to create a cylinder column within the equine brain. The column was then filled with saline to ensure a proper coupling between the reverberator and the beacon transducer. The beacon was inserted with a 5 French (Fr) introducer sheath at an insertion distance of 5 cm. The reverberator was placed at the medial part of the equine forehead and coupled with ultrasound gel. Ultrasound was delivered using this modality and subsequent focusing results were observed.



Figure 3.8: Using 1 MHz reverberator and ultrasound “beacon” transducer to focus ultrasound energy within a horse brain.



Figure 3.9: (Left to Right) Focusing ultrasound energy in a horse brain using (Left) Hydrophone infusion catheter smart needle and 1 MHz reverberator (Middle) 190 KHz transducers and ultrasound “beacon” transducer (Right) 190 KHz transducers and multiple ultrasound “beacons”.

2. Hydrophone infusion catheter smart needle and 1 MHz reverberator

Using the experimental setup described in case 1, the beacon transducer is replaced with a hydrophone infusion catheter smart needle. To ensure proper coupling, the insertion site of the needle was supersaturated with saline. Ultrasound was delivered using this modality and subsequent focusing results were observed. The corresponding setup can be seen in Figure 3.9 (Left)

3. Beacon transducer and 190 KHz ultrasound transducers

Using the same experimental setup as described previously in case 1, the 1MHz reverberator is replaced with a set of 9 piezo electric ceramic ring transducers with resonant frequency of 190+/- 5 KHz (SMR 2809t60118, STEMiNC- Steiner and Martins, Inc.). Subsequently ultrasound energy was delivered to observe the corresponding focusing results. The corresponding setup can be seen in Figure 3.9 (Middle).

4. Multiple beacon transducers and 190 KHz ultrasound transducers

This experimental modality used the same setup described in case 1. However two beacon transducers were used instead of one to focus ultrasound. The corresponding setup can be seen in Figure 3.9 (Right).

Chapter 4

4. Results

4.1 Focusing Experiments in cylindrical reverberator

To investigate if ultrasound at low frequencies can be localized to a specific location, the initial sweep was limited to a range between 20 KHz-1.5 MHz. Enhanced time-reversed acoustic signals were obtained at 180 KHz, 342 KHz, 720 KHz and 1 MHz. Amongst these the frequencies focusing ultrasound at 342 KHz resulted in a signal with the highest signal to noise ratio (Figure 4.1). Choosing 342 KHz as the operating frequency TRA enhanced thrombolysis was performed.

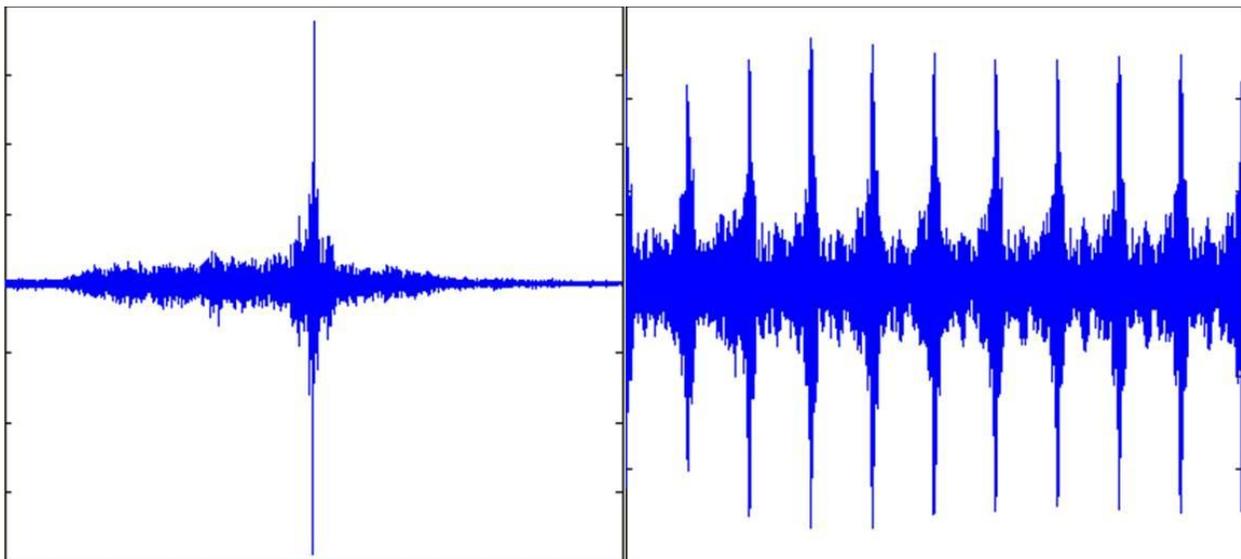


Figure 4.1: A highly enhanced time-reversed ultrasound signal at a frequency of 342 KHz (Left); Continuous burst of these ultrasound signals at 342 KHz for thrombolysing the clot in the cylindrical reverberator (Right); X-axis represents time in microseconds with the origin at the centre of X-axis. Y-axis represents peak-peak to pressure amplitude in kilo pascal (kPa) with origin at the centre of the Y-axis.

4.2 Thrombolysis

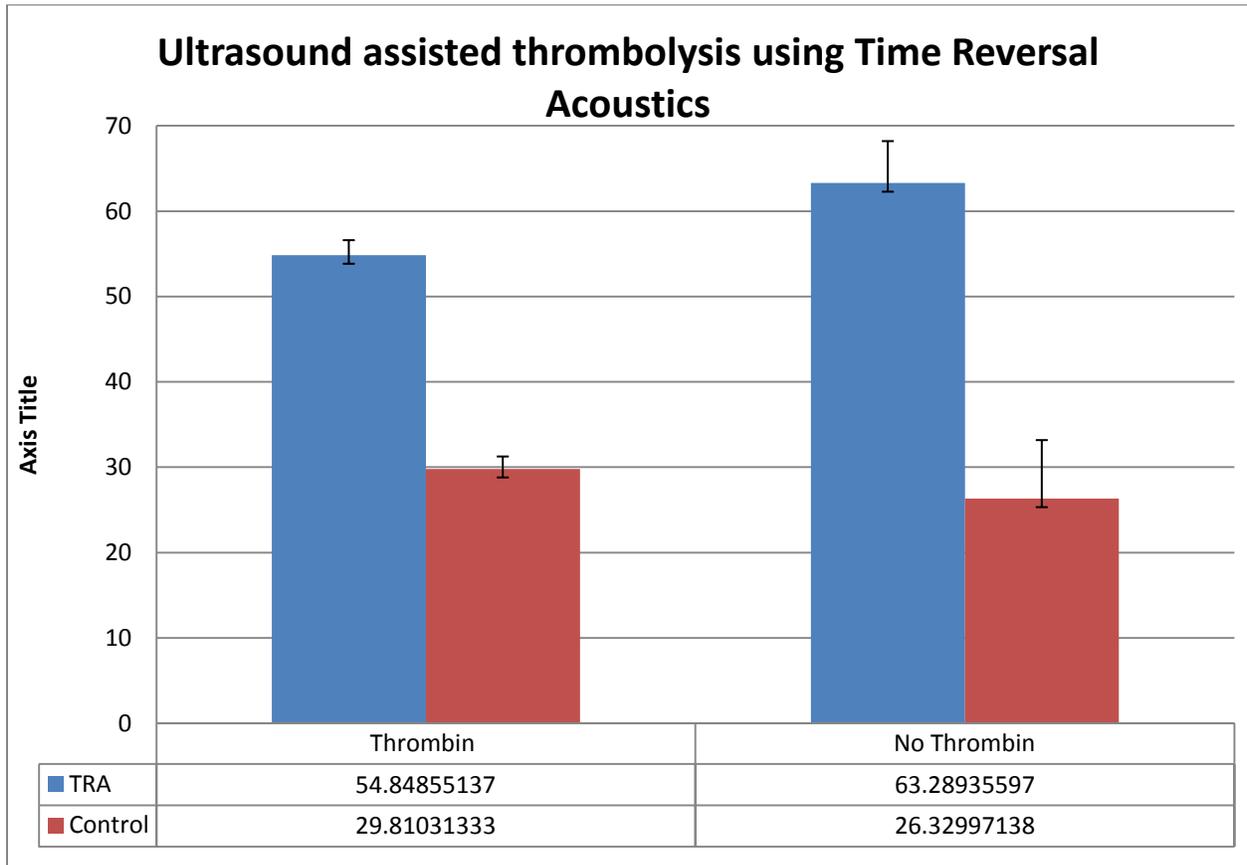


Figure 4.2: Loss in clot mass when exposed to TRA enhanced ultrasound and loss in clot mass when not exposed to ultrasound in a) Clots where thrombin was added b) thrombin was not added.

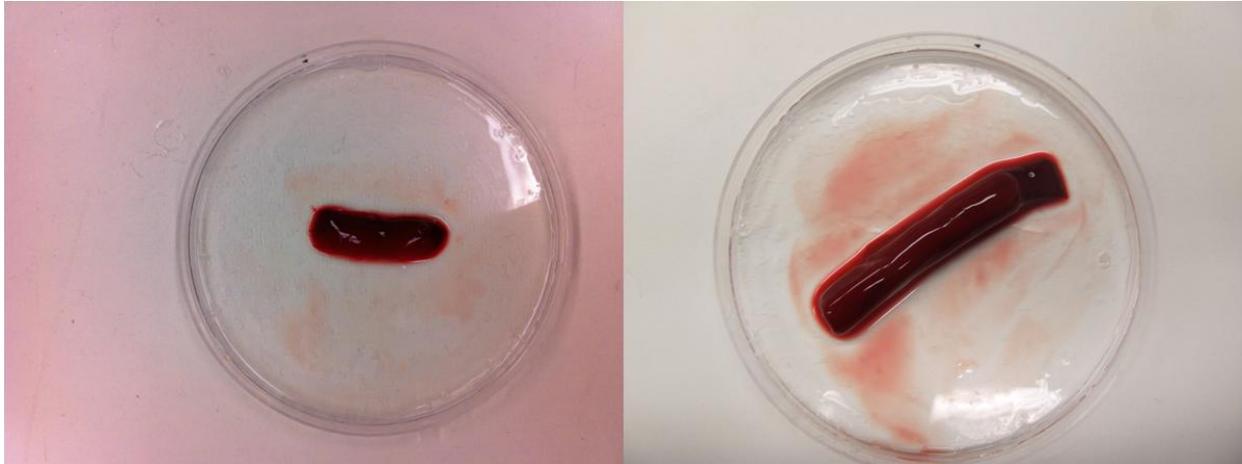


Figure 4.3: Showing change in size of a blood clot pre and post thrombolytic treatment focusing ultrasound using Time Reversal Acoustics. (Left) Blood clot post treatment which is approximately 0.6-0.7 cm in diameter and 2 cm in length (Right) Blood clot pre-treatment which is of approximately 1-1.2 cm in diameter and 4.8 cm in length.

The clots in the cylindrical reverberator were exposed to time-reversed ultrasound signals at 342 KHz (spherical focal volume of $4.59e-8 \text{ m}^3$) for a period of 1800 seconds. The duty cycle of treatment was set at 92%. The peak-peak pressure varied from 28KPa-45KPa per transducer. The intensity varied from $5.5\text{mW}/\text{cm}^2$ to $8\text{mW}/\text{cm}^2$. The clots (N=7) that were exposed to this ultrasound exhibited a mass loss of approximately 63.23% ($p < 0.00005$ with respect to controls). The control clots which were placed under similar conditions but not exposed to any ultrasound had a mass loss of 26.33%. Using the same acoustic parameters clots (N=5) with additional thrombin (100 U/ml) were exposed to ultrasound. These set of clots had a loss of mass of 54.85% ($p < 0.0014$ with respect to controls). The corresponding mass loss for control clots was 29.81% (Figure 4.2). Figure 4.3 shows a change in the size of a blood clot pre treatment and post treatment.

4.3 Focusing experiments in equine head

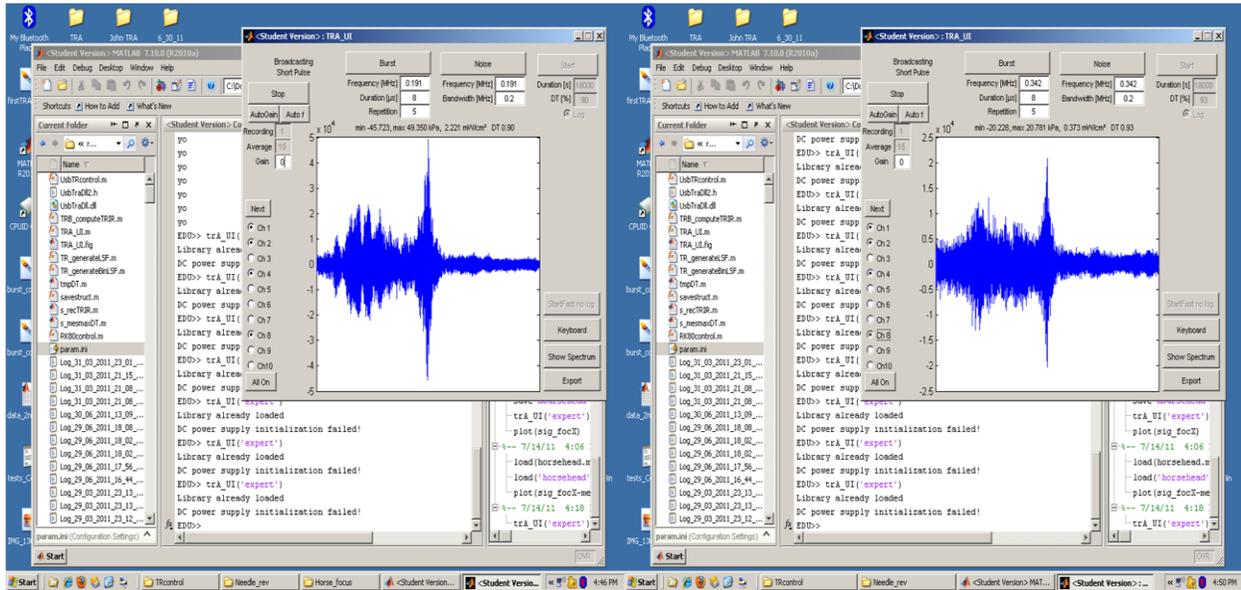


Figure 4.4: A highly enhanced time reversed signal obtained at 191 KHz and 342 KHz using 190 KHz external transducers and ultrasound ‘beacon’ transducer.

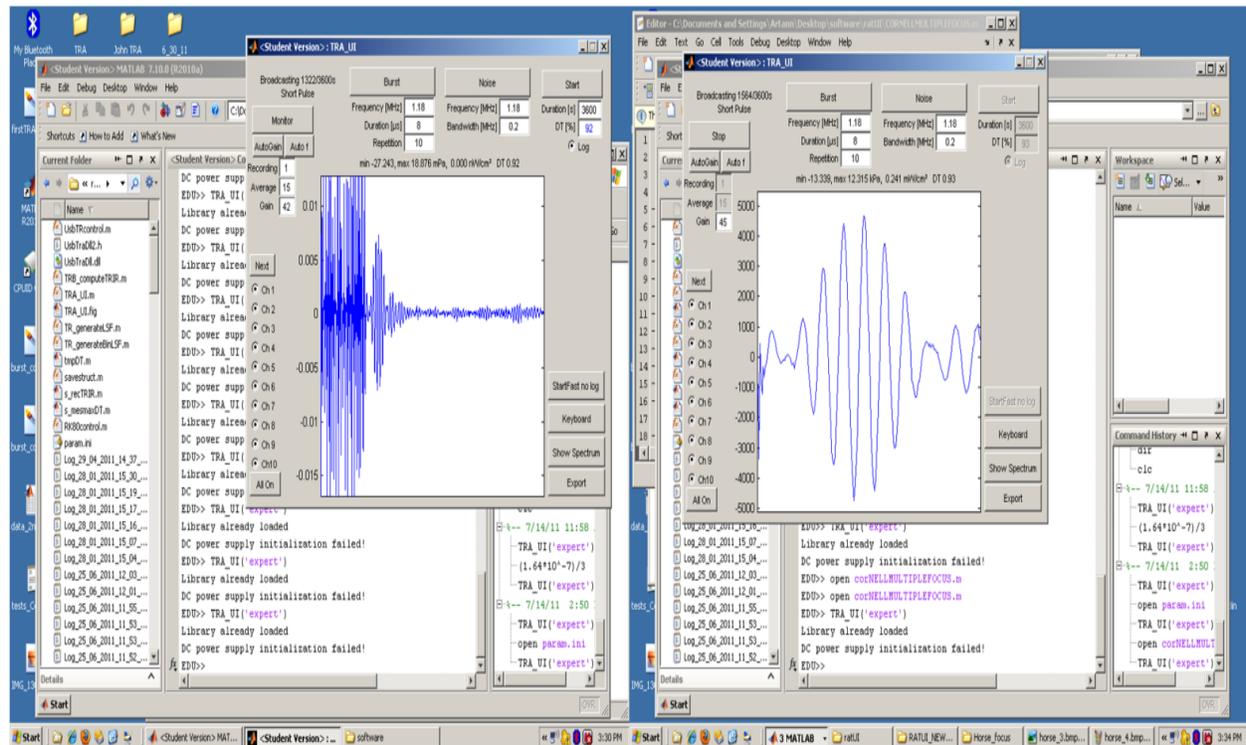


Figure 4.5: A time-reversed signal focused at a location in the equine head using hydrophone infusion catheter smart needle and 1 MHz reverberator.

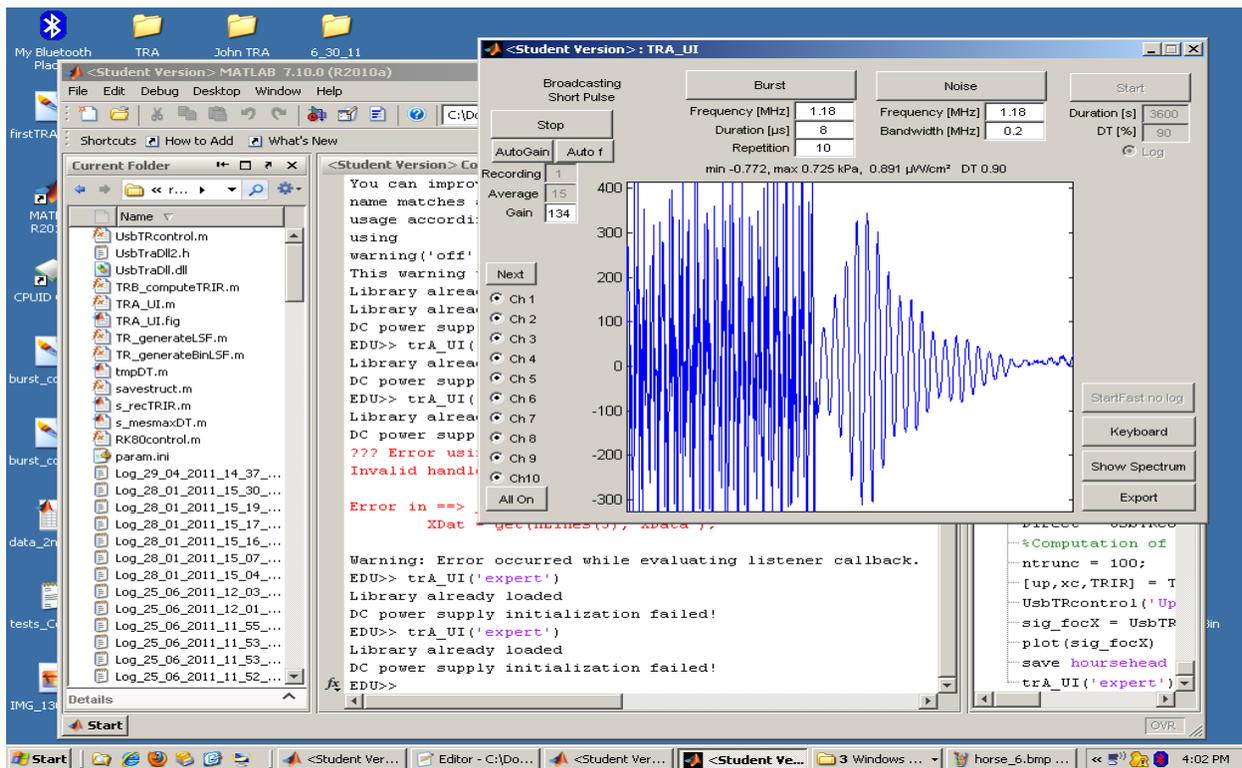


Figure 4.6: Time reversed signal obtained using hydrophone infusion catheter smart needle and 1 MHz reverberator.

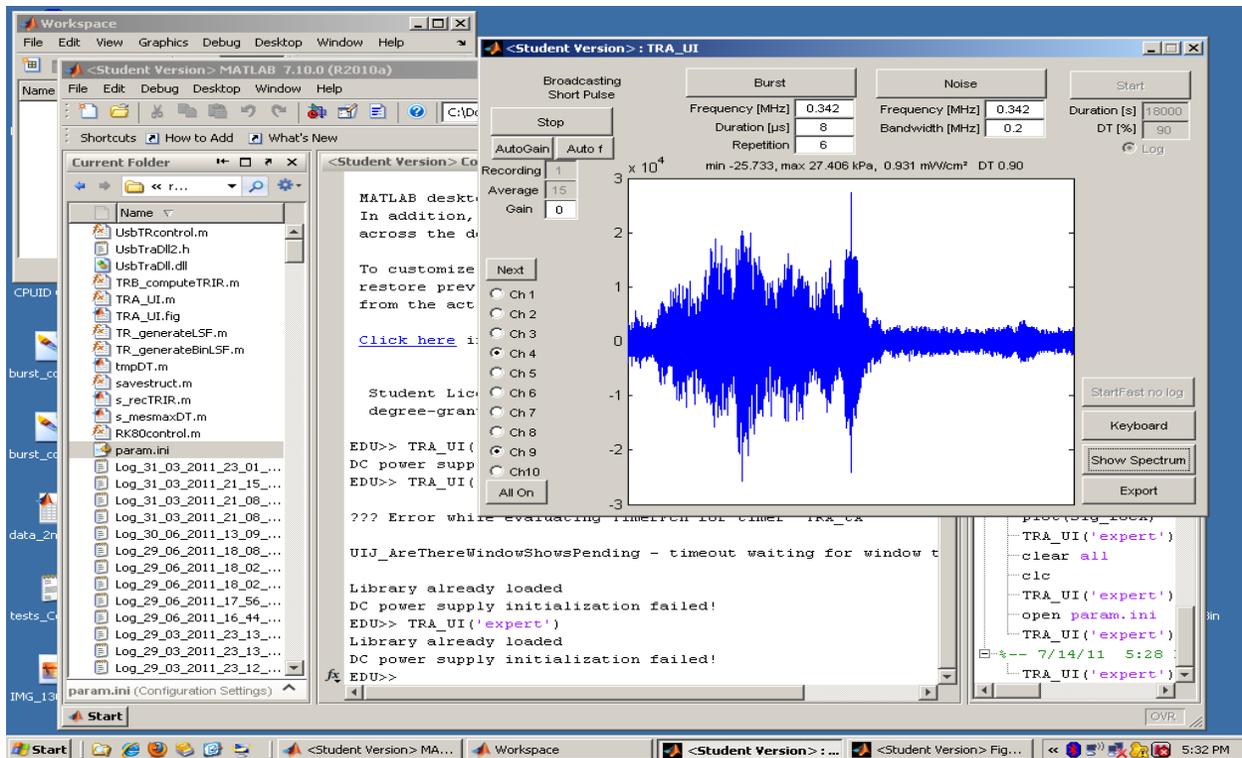


Figure 4.7: Time reversed signal obtained using two beacon transducers and 190 KHz transducers.

Using the four different experimental setups described in the methods we investigated if TRA based ultrasound could be localized within large animals and if an enhanced time-reversed signal can be obtained which is comparable to the one used in an *in vitro* setting. The experimental setup which used the ultrasound 'beacon' transducer with 190 KHz transducers gave (Figure 4.4) highly focused time reversed signals at 191 KHz and 342 KHz respectively. The experimental setting (Figure 4.7) wherein two ultrasound beacons are inserted in the equine brain surrounded by 190 KHz external transducers also resulted in a focused ultrasound signal. However the peak-peak amplitude was noticeably smaller when compared to the case where only one beacon transducer was used. The other two cases which involved the hydrophone infusion catheter smart needle (Figures 4.5 and 4.6) resulted in focused signals with high signal to noise ratios.

Chapter 5

5. Discussion

Ultrasound based thrombolysis over the years has evolved to play a more prominent role in Deep Vein Thrombosis. However there are still several challenges associated with this treatment methodology. Invasive ultrasound treatments are limited by the size of transducer which is dependent on the size of vessel. This in turn places a limitation on the frequencies and energy delivered thereby reducing the effectiveness of treatment. Transcutaneous ultrasound lacks the ability to deliver focused energy at a specific location [41]. This greatly reduces its effectiveness to deliver any therapeutic ultrasound or to enhance drug delivery. Therapeutic ultrasound (0.7 MHz to 5 MHz) generally has relied on delivering high intensity ultrasound energy at the treatment location. High intensity focused ultrasound (HIFU) achieves the desired effect through thermal heating with temperatures upto 60°C [77]. This is not desirable with respect to DVT as there is a chance of damaging the tissues that lie between the transducers and the clots due to thermal heating [79]. Also the small size of vessels limits placed a limitation on delivering focused high intensity ultrasound energy without any thermal dissipation to neighboring tissue. The system we have presented has tried to overcome the above limitations by using a minimally invasive system that delivers highly focused low intensity external ultrasound energy. The low intense ultrasound avoids the effect of hyperthermia that is seen in HIFU.

We investigated if ultrasound energy at a low frequency (<1.5 MHz) and low intensity (<0.5 W/cm²) could be focused at the desired location using time reversal acoustics. We successfully demonstrated that it is possible to do so *in vitro* and in large animals. We also showed that it is possible to focus ultrasound at low frequencies (191 KHz and 342 KHz) in both the cases. The study also showed that though a focused signal was obtained in both cases the intensity was lower in animal studies. This result can be explained by the attenuation of already low-intensity ultrasound by equine skull and other tissues. By increasing the number of ultrasound transducers and also by removing the design limitation on the low-impedance amplifier circuit (current design has 10 channels and allows an operational voltage of only 30 V) it may be possible to generate an enhanced signal similar to those *in vitro* conditions. The focusing experiments within the equine head also demonstrated the remarkable ability of focusing ultrasound at a specific location using Time Reversal Acoustics.

Thrombolysis using the above acoustic parameters (Frequency-342 KHz, Duty Cycle-92%, Repetition rate -10) showed a significant loss in clot mass in both sets of clots, one where thrombin was added and the one without additional thrombin. Previous studies reported [42] no loss in clot mass when using ultrasound alone at low frequencies of 120 KHz and 1 MHz. We report for the first time a significant loss in clot mass when using TRA based ultrasound at 342 KHz without a thrombolytic drug. We hypothesize that cavitation and microstreaming could be the dominating mechanisms that cause this thrombolysis. We also hypothesize that at these frequencies there should be increased clot permeability leading to better penetration of thrombolytics into

the clot. Thus we could see a reduction in the dosage of anticoagulants and thrombolytics resulting from ultrasound aided thrombolysis and ultrasound aided penetration. For the given set of porcine clots we have also seen that the thrombolysis seemed to be a constant (about 55% for thrombin added clots and around 63% for clots with no thrombin). Given a near constant clot size and thrombin concentration within the clots it is possible that the extent of thrombolysis is also a constant.

We also observed that clots with additional thrombin had a reduced loss in clot mass as compared to the ones where no thrombin was added (54.85% vs 63.29%). Previous studies have shown that ultrasonic energy causes thrombolysis by disrupting the fibrin matrix [4, 53, 54]. It has also been reported that the amount of thrombin affects the extent of cross linking in the fibrin matrix. Increase in thrombin concentration reportedly increased the fiber diameter in the fibrin network [24]. Thus it is expected that the thrombin group exhibit lesser loss in clot mass as compared to the no thrombin group when exposed to the same ultrasound energy. It is also expected that at these frequencies the time-reversed ultrasound plays the dual role of acting as a therapeutic agent and as an enhancer of fibrinolysis by promoting drug dissipation. Studies have already shown that [42, 73] thrombolysis is enhanced at certain frequencies in presence of a thrombolytic drug. Thus the additional therapeutic nature imparted to the ultrasound due to time-reversal acoustics would further enhance the thrombolysis. In addition the low frequencies reduce the harmful bioeffects of ultrasound like heating, have deeper penetrating power, lower attenuation as compared to higher frequencies [40].

The study is limited due to the *in vitro* nature of the experiment and the use of porcine blood clots. However it is expected that this treatment model would have an enhanced

effect in humans due to higher plasminogen (0.8-1.2 U/ml in humans) concentration as compared to pigs (0.08-0.5 U/ml in pigs) and also due to an actively recruiting thrombus [42]. Ultrasound could also increase clot permeability to plasminogen and other thrombolytic drugs. Thus loss of clot mass is expected to be higher in human beings.

6. References

1. Brenner B, Hoffman R. Emerging options in the treatment of deep vein thrombosis and pulmonary embolism. *Blood Reviews*. 2011; 25: 215–221.
2. Cohen AT, Agnelli G, Anderson FA, Arcelus JI, Bergqvist D, Brecht JG, et al. Venous thromboembolism (VTE) in Europe. The number of VTE events and associated morbidity and mortality. *Thromb Haemost* 2007; 98: 756–64.
3. Heit JA, Cohen AT, Anderson FA. on Behalf of the VTE Impact Assessment Group. Estimated annual number of incident and recurrent, non-fatal and fatal Venous Thromboembolism (VTE) Events in the US. *Blood* 2005; 106:910a.
4. Lin PH, Ochoa LN, Duffy P. Catheter-Directed Thrombectomy and Thrombolysis for Symptomatic Lower-Extremity Deep Vein Thrombosis: Review of Current Interventional Treatment Strategies. *Perspect Vasc Surg Endovasc Ther* 2010 22: 152.
5. Kahn SR, Ginsberg JS. Relationship between deep venous thrombosis and the postthrombotic syndrome. *Arch Intern Med* 2004; 164:17–26.
6. Patel K. Deep Vein Thrombosis and Management. *Medscape Reference-Drugs, Diseases and Procedures*. 2011; Retrieved July 2, 2012, from <http://emedicine.medscape.com/article/1911303>
7. Venous thrombus formation. 2012. Retrieved August 6, 2012 from <http://www.thrombosisadviser.com/en/image.php?image=venous-thrombus-formation&category=vte>
8. Norris, Lucy A. Blood Coagulation. *Best Practice & Research Clinical Obstetrics and Gynaecology* 2003; Vol. 17, No. 3, pp. 369-383

9. Horne, McDonald. Overview of hemostasis and thrombosis. Current status of antithrombotic therapies. *Thrombosis Research* 2005; 117, 15-17
10. Casey G. Hemostasis, Anticoagulants and fibrinolysis. *Nursing Standard* 2003; 18, 7, 45-51
11. Tanaka Kenichi A., Key Nigel S., Levy Jerrold H. Blood Coagulation: Hemostasis and Thrombin Regulation. *Anesthesia and Analgesia* 2009; Vol. 108, No. 5, pp. 1433-1446
12. Introduction to hemostasis. Retrieved July 2, 2012, from <http://ahdc.vet.cornell.edu/clinpath/modules/coags/introf.htm>
13. Boon GD. An Overview of hemostasis . *Toxicologic Pathology* 1993; Vol. 21 No. 2, pp. 171-179
14. In Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN et al (eds) *Haemostasis and Thrombosis, Basic Principles and Clinical Practice*, 4th edn, pp 3–16, New York: Lippincott/Williams & Wilkins, 2001.
15. Neisheim M, Pittman DD, Giles AR et al. The effect of plasma von Willebrand factor on the binding of human factor VIII to thrombin activated platelets. *Journal of Biological Chemistry* 1991; 266: 17 815–17 820.
16. Hamer RJ, Koedam JA, Beeser-Visser NH et al. The effect of thrombin on the complex between factor VIII and von Willebrand factor. *European Journal of Biochemistry* 1987; 167: 253–259.
17. Doyle MF & Haley PE. Meizothrombin: active intermediate formed during prothrombinase-catalysed activation of prothrombin. *Methods in Enzymology* 1993; 222: 299.

18. Brummel KE, Paradis SG, Butenas S & Mann KG. Thrombin functions during tissue factor induced blood coagulation. *Blood* 2002; 100: 148–152.
19. Collen D., Lijnen HR. Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* 1991; 78:3114-3124
20. Cesarman-Maus Gabriela, Hajjar A. Katherine. Molecular Mechanisms of fibrinolysis. *British Journal of Hematology* 2005; 129, 307-321
21. Takada Akikazu, Takada Yumiko, Urano Tetsumei. The physiological aspects of fibrinolysis 1994; Vol. 76, No. 1, pp. 1-31
22. Nesheim M. Thrombin and fibrinolysis. *Chest* 2003; 124:33S-39S
23. Booth A. Nuala. Fibrinolysis and Thrombosis. *Bailliere's Clinical Haematology* 1999. Vol. 12, No.3 , pp. 423-433
24. Kolev Krasimir, Machovich Raymund. Molecular and cellular modulation of fibrinolysis *Thromb Haemost* 2003; 89: 610–21
25. GIBBS NM. Venous thrombosis of the lower limbs with particular reference to bed-rest. *Br J Surg*. Nov 1957;45(191):209-36.
26. Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. *J Clin Pathol*. Jul 1974;27(7):517-28.
27. Beyth RJ, Cohen AM, Landefeld CS. Long-term outcomes of deep-vein thrombosis. *Arch Intern Med*. May 22 1995;155(10):1031-7.
28. Kistner RL, Ball JJ, Nordyke RA, Freeman GC. Incidence of pulmonary embolism in the course of thrombophlebitis of the lower extremities. *Am J Surg*. Aug 1972;124(2):169-76.

29. Aronson DL, Thomas DP. Experimental studies on venous thrombosis: effect of coagulants, procoagulants and vessel contusion. *Thromb Haemost.* Dec 17 1985;54(4):866-70.
30. Monreal M, Martorell A, Callejas JM, Valls R, Llamazares JF, Lafoz E, et al. Venographic assessment of deep vein thrombosis and risk of developing post-thrombotic syndrome: a prospective study. *J Intern Med.* Mar 1993;233(3):233-8.
31. Strandness DE Jr, Langlois Y, Cramer M, Randlett A, Thiele BL. Long-term sequelae of acute venous thrombosis. *JAMA.* Sep 9 1983;250(10):1289-92.
32. Haenen JH, Wollersheim H, Janssen MC, Van 't Hof MA, Steijlen PM, van Langen H, et al. Evolution of deep venous thrombosis: a 2-year follow-up using duplex ultrasound scan and strain-gauge plethysmography. *J Vasc Surg.* Oct 2001;34(4):649-55.
33. Meissner MH, Caps MT, Zierler BK, Bergelin RO, Manzo RA, Strandness DE Jr. Deep venous thrombosis and superficial venous reflux. *J Vasc Surg.* Jul 2000;32(1):48-56.
34. Meissner MH, Caps MT, Zierler BK, Polissar N, Bergelin RO, Manzo RA, et al. Determinants of chronic venous disease after acute deep venous thrombosis. *J Vasc Surg.* Nov 1998;28(5):826-33.
35. Meissner MH, Manzo RA, Bergelin RO, Markel A, Strandness DE Jr. Deep venous insufficiency: the relationship between lysis and subsequent reflux. *J Vasc Surg.* Oct 1993;18(4):596-605; discussion 606-8.

36. Caps MT, Manzo RA, Bergelin RO, Meissner MH, Strandness DE Jr. Venous valvular reflux in veins not involved at the time of acute deep vein thrombosis. *J Vasc Surg.* Nov 1995;22(5):524-31.
37. Johnson BF, Manzo RA, Bergelin RO, Strandness DE Jr. Relationship between changes in the deep venous system and the development of the postthrombotic syndrome after an acute episode of lower limb deep vein thrombosis: a one- to six-year follow-up. *J Vasc Surg.* Feb 1995;21(2):307-12; discussion 313.
38. Johnson BF, Manzo RA, Bergelin RO, Strandness DE Jr. The site of residual abnormalities in the leg veins in long-term follow-up after deep vein thrombosis and their relationship to the development of the post-thrombotic syndrome. *Int Angiol.* Mar 1996;15(1):14-9.
39. Vedantham S. Interventional Approaches to Deep Vein Thrombosis. *American Journal of Hematology.* 2012; 87:S113–S118.
40. Popuri RK, Vedantham S. The Role of Thrombolysis in the Clinical Management of Deep Vein Thrombosis. *Arterioscler Thromb Vasc Biol.* 2011;31:479-484.
41. Atar S, Rosenschein U. Perspectives on the Role of Ultrasonic Devices in Thrombolysis. *Journal of Thrombosis and Thrombolysis.* 2004; 17(2), 107–114.
42. Holland CK, Vaidya SS, Datta S, Coussios C, Shaw GJ. Ultrasound-enhanced tissue plasminogen activator thrombolysis in an *in vitro* porcine clot model. *Thromb Res.* 2008; 121(5): 663–673.
43. Grewal NK, Martinez JT, Andrews L, Comerota AJ. Quantity of clot lysed after catheter-directed thrombolysis for iliofemoral deep venous thrombosis correlates with postthrombotic morbidity. *J Vasc Surg* 2010; 51:1209–1214.

44. Comerota AJ. Thrombolysis for deep venous thrombosis. *J Vasc Surg* 2012; Vol. 55, Number 2:607–611
45. Comerota AJ, Thom RC, Mathias SD, et al. Catheter-directed thrombolysis for iliofemoral deep venous thrombosis improves health-related quality of life. *J Vasc Surg* 2000; 32:130–137
46. Vedantham S, Grassi CJ, Ferral H, Patel NH, Thorpe PE, Antonacci VP, Janne d’Othee BM, Hofmann LV, Cardella JF, Kundu S, Lewis CA, Schwartzberg MS, Min RJ, Sacks D; for the Technology Assessment Committee of the Society of Interventional Radiology. Reporting standards for endovascular treatment of lower extremity deep vein thrombosis. *J Vasc Interv Radiol*. 2006;17:417– 434.
47. Semba CP, Dake MD. Iliofemoral deep venous thrombosis: aggressive therapy with catheter-directed thrombolysis. *Radiology*. 1994;191:487–494.
48. Shortell CK, Queiroz R, Johansson M, Waldman D, Illig KA, Ouriel K, Green RM. Safety and efficacy of limited-dose tissue plasminogen activator in acute vascular occlusion. *J Vasc Surg*. 2001;34:854–859.
49. Grunwald MR, Hofmann LV. Comparison of urokinase, alteplase, and reteplase for catheter-directed thrombolysis of deep venous thrombosis. *J Vasc Interv Radiol*. 2004;15:347–352.
50. Mewissen MW, Seabrook GR, Meissner MH, Cynamon J, Labropoulos N, Haughton SH. Catheter-directed thrombolysis for lower extremity deep venous thrombosis: report of a national multicenter registry. *Radiology*. 1999;211:39–49.

51. Patel K. Deep Vein Thrombosis and Management. Medscape Reference-Drugs, Diseases and Procedures. 2011; Retrieved July 2, 2012, from <http://emedicine.medscape.com/article/1911303>
52. Kearon C, Kahn SR, Agnelli G, Goldhaber S, Raskob GE, Comerota AJ. Antithrombotic therapy for venous thromboembolic disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest. Jun 2008;133(6 Suppl):454S-545S
53. Burke DT. Prevention of deep venous thrombosis: overview of available therapy options for rehabilitation patients. Am J Phys Med Rehabil. Sep-Oct 2000;79(5 Suppl):S3-8.
54. Merli GJ. Prophylaxis for deep venous thrombosis and pulmonary embolism in the surgical patient. Clin Cornerstone. 2000;2(4):15-28.
55. Linkins LA, Choi PT, Douketis JD. Clinical impact of bleeding in patients taking oral anticoagulant therapy for venous thromboembolism: a meta-analysis. Ann Intern Med. Dec 2 2003;139(11):893-900.
56. Rhodes JM, Cho JS, Gloviczki P, Mozes G, Rolle R, Miller VM. Thrombolysis for experimental deep venous thrombosis maintains valvular competence and vasoreactivity. J Vasc Surg. 2000;31:1193-1205.
57. Laiho MK, Oinonen A, Sugano N, et al. Preservation of venous valve function after catheter-directed and systemic thrombolysis for deep venous thrombosis. Eur J Vasc Endovasc Surg. 2004;28:391-396.
58. Comerota AJ, Aldridge SC. Thrombolytic therapy for deep venous thrombosis: a clinical review. Can J Surg. 1993;36: 359-364.

59. Kim HS, Patra A, Paxton BE, Khan J, Streiff MB. Adjunctive percutaneous mechanical thrombectomy for lower-extremity deep vein thrombosis: clinical and economic outcomes. *J Vasc Interv Radiol.* 2006;17: 1099–1104.
60. Lin PH, Zhou W, Dardik A, Mussa F, Kougias P, Hedayati N, Naoum JJ, El Sayed H, Peden EK, Huynh TT. Catheter-direct thrombolysis versus pharmacomechanical thrombectomy for treatment of symptomatic lower extremity deep vein thrombosis. *Am J Surg.* 2006;192:782–788.
61. Rosenschein U, Frimerman A, Laniado S, Miller HI. Study of the mechanism of ultrasound angioplasty from human thrombi and bovine aorta. *Am J Cardiol.* 1994;74: 1263-1266.
62. Rosenschein U, Rozenszajn LA, Kraus L, et al. Ultrasonic angioplasty in totally occluded peripheral arteries: initial clinical, histological, and angiographic results. *Circulation.* 1991;83:1976-1986.
63. Atar S, Luo H, Nagai T, Siegel RJ. Ultrasonic thrombolysis: Catheter-delivered and transcutaneous applications. *Eur J Ultrasound* 1999;9:39–54.
64. Francis CW, Onundarson PT, Carstensen EL, et al. Enhancement of fibrinolysis in vitro by ultrasound. *J ClinInvest* 1992;90:2063–2068.
65. Saarinen J, Kallio T, Lehto M, Hiltunen S, Sisto T. The occurrence of the post-thrombotic changes after an acute deep venous thrombosis. A prospective two-year follow-up study. *J Cardiovasc Surg (Torino).* Jun 2000;41(3):441-6.
66. Elliott G. Thrombolytic therapy for venous thromboembolism. *Curr Opin Hematol.* Sep 1999;6(5):304-8.

67. Sarvazyan AP. Wireless beacon for time-reversal acoustics, method of use and instrument containing thereof. US Patent 7713200. 2005
68. Sarvazyan AP. Ultrasound-assisted drug delivery method and system based on time-reversal acoustics. US Patent 7985184. 2010
69. Fink M, Montaldo G, Tanter M. Time reversal acoustics in biomedical engineering. *Ann. Rev. Biomed. Eng.* 2003;5:465–497. [PubMed]
70. Sutin A, Sarvazyan A. Spatial and temporal concentrating of ultrasound energy in complex systems by single transmitter using time reversal principles. *Proc. World Congress on Ultrasonics; Paris, France.* 2003. pp. 863–866.
71. Sarvazyan AP., Emelianov S. Wireless beacon for Time-Reversal Acoustics, method of use and instrument containing thereof, US Patent 7713200. 2005
72. Sarvazyan AP. Ultrasound-assisted drug delivery method and system based on time-reversal acoustics. US Patent 7985184. 2010
73. Lewis GK Jr., Guarino S, Gandhi G, Filinger L, Lewis GK Sr., Olbricht WL, Sarvazyan A. Time-reversal Techniques in Ultrasound-assisted Convection-enhanced Drug Delivery to the Brain: Technology Development and In Vivo Evaluation. *Proc Meet Acoust.* 2011 July 10; 11: 020005–020031
74. Walker FJ & Fay PJ. Regulation of blood coagulation by protein C system. *FASEB Journal* 1992; 6: 2561–2567.
75. Thaler E, Lechner K. Antithrombin III deficiency and thromboembolism. *Clin Haematol* 1981; 10: 369-90

76. Parikh S, Motarjeme A, McNamara T, et al. Ultrasound-accelerated thrombolysis for the treatment of deep vein thrombosis: initial clinical experience. *J Vasc Interv Radiol* 2008;19 : 521–528
77. Ter Haar G. Ultrasound Focal Beam Therapy. *Ultrasound in Med. & Biol.* 1995, Vol. 21. No. 9, pp. 1089-1100
78. The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.
79. Dubinsky TJ. , Cuevas C., Dighe MK., Kolokythas O., Hwang HJ. High-Intensity Focused Ultrasound: Current Potential and Oncologic Applications *AJR* 2008; 190:191–199