In vitro and in vivo investigation of enzymatic clot lysis through non-thermal mechanisms using focused ultrasound waves as an adjunct to thrombolytic drug tenecteplase and in synergy with microbubbles

A thesis submitted to the graduate faculty in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Biomedical Engineering

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Abbreviations and terms used

Abbreviation	Definition
Abib	Abciximab immunobubbles
ABS	Acrylonitrile Butadiene Styrene
AC	Alternating Current
AIS	Acute Ischemic Stroke
AP	Acoustic Power
ARF	Acoustic Radiation Force
BBB	Blood Brain Barrier
CAD	Computer-Aided Design
CLOTBUST	Combined Lysis of Thrombus in Brain Ischemia Using Transcranial Ultrasound and Systemic TPA
CW	Continuous Wave
DAQ	Data Acquisition
DDC	Drug Delivery Catheter
DF	Duty Factor
DFOV	Displayed Field Of View
DSPA	Desmoteplase Salivary Plasminogen Activator
ECASS	European Cooperative Acute Stroke Study
ELIP	Echogenic Liposomes
Epf	Eptibatide
ETL	Echo Train Length
FDA	Food and Drug Administration
FDP	Fibrin Degradation Product
FOV	Field Of View
FRFSE	Fast Recovery Fast Spin Echo
FUS	Focused Ultrasound
GRE	Gradient Echo
HIFU	High Intensity Focused Ultrasound
ia	Intra-arterial
ICH	Intracranial Haemorrhage

IMS	International Management of Stroke
I_{PA}	Pulse Average Intensity
I_{SA}	Spatial Average Intensity
I_{TA}	Temporal Average Intensity
I_{TP}	Temporal Peak Intensity
iv	Intra-venous
MBs	Micro-Bubbles
MCA	Middle Cerebral Artery
MI	Mechanical Index
MR	Magnetic Resonance
MR-ARF	Magnetic Resonance Acoustic Radiation Force
MRgFUS	Magnetic Resonance guided Focused Ultrasound
MRI	Magnetic Resonance Imaging
mt-PA	monteplase tissue Plasminogen Activator
NEX	Number of Excitations
NIH	National Institute of Health
NINDS	National Institute of Neurological Disorders and Stroke
n/s	Not specified
Nsib	non-specific immunobubbles
PD	Proton Density
PRF	Pulse Repetition Frequency
PRP	Pulse Repetition Period
PRFS	Proton Resonance Frequency Shift
PW	Pulsed Wave
PZT	Lead Zirconate Titanate
rt-PA	recombinant tissue Plasminogen Activator
SAH	Subarachnoid haemorrhage
SD	Standard Deviation
sICH	symptomatic Intracranial Haemorrhage
SK	Streptokinase
SPGR	Spoiled Gradient
TCD	Transcranial Doppler
TCCD	Transcranial Color-Coded Duplex
TE	Echo Time

TIMI	Thrombolysis In Myocardial Infarction
TMM	Tissue Mimicking Material
TNK-tPA	Tenecteplase tissue Plasminogen Activator
TOF	Time Of Flight
tPA	tissue Plasminogen Activator
TR	Repetition Time
TRUMBI	TRanscranial low frequency Ultrasound-Mediated thrombolysis in Brain Ischemia
TUCSON	Transcranial Ultrasound in Clinical Sonothrombolysis
USC	Ultrasound Core
UCA	Ultrasound Contrast Agent
UK	Urokinase
USgFUS	Ultrasound guided Focused Ultrasound
WHO	World Health Organization
WFUMD	World Federation of Ultrasound in Medicine

Dedication

This thesis is dedicated to the memory of my father Socrates, who passed away prematurely almost three decades ago and to my mother Maria, without her enormous personal sacrifice and prays of day and night, none of my success would be possible.

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Abstract

In this thesis, the beneficial effect of ultrasound (US) energy is utilized for accelerating thrombolysis efficacy through non-thermal mechanisms. The outcome of the research, can be applied in the future for clinical use, providing alternative techniques (either noninvasive or invasive), for treating ischaemic stroke under MR guidance.

To study the effect of US on thrombolysis efficacy, three different in vitro circulating flow clot models were developed. Fully retracted porcine blood clots were treated with US waves as an adjunct to thrombolytic drug tenecteplase (TNK-tPA), in the presence or absence of microbubbles (MBs). Each one of the flow clot models used, was designed to reproduce a different physiologic situation of middle cerebral artery (MCA) occlusion, since it is the most common cause of stroke. In all the proposed treatment protocols, temperature elevation at beam focus never exceeded 1 ^{0}C, providing that the contribution of thermal mechanisms to clot lysis was negligible.

The first model, was reproducing a deep-seated MCA occlusion in a brain tissue. To provide a more realistic clinical environment, the study was conducted into a brain tissue mimicking phantom. Flow rate was set to 20 % of the maximum value occur in an open MCA and TNK-tPA dose was not exceeding the 30 % of the average maximum concentration in blood. Using 1.18 MHz focused US (FUS) waves, various experimental parameters that influence thrombolysis efficacy, were optimized. Maximum thrombolysis efficacy was observed when FUS pulses were used as an adjunct to thrombolytic drug in the presence of MBs. With this technique, 370 mg of clot mass was removed in 30 min, which may not be enough to achieve significant clinical benefits. However, further improvement was done (increase of flow rate and TNK-tPA concentration), to enhance thrombolysis efficacy.

The second model, was reproducing a MCA occlusion occurred superficially. Flow rate was increased four times and TNK-tPA concentration was doubled. The optimum operating mode parameters obtained before, were employed in this study in order to investigate the impact of frequency and acoustic intensity on thrombolysis efficacy. Study findings established that higher FUS frequencies (1.18 MHz), are associated with enhanced thrombolysis compared to lower frequencies (0.6 MHz). Also, a linear increasing dependence between acoustic intensity and thrombolysis efficacy was observed. After 30 min of treatment with 1.18 MHz FUS exposures in synergy with TNK-tPA and MBs, 1050 mg of clot mass was removed, which should be sufficient for timely recanalization of an occluded cerebral artery.

The third model, was reproducing a MCA occlusion, treated invasively with a catheter-directed US device. The efficacy of two small planar ultrasonic transducers (operating at frequencies 3.7 and 5.2 MHz respectively), on clot lysis was evaluated. The values of flow rate and TNK-tPA concentration were kept the same with those used in the previous study. Using 3.7 MHz US waves in association with TNK-tPA and MBs for 30 min, 700 mg of clot mass was removed, showing that potentially, intravascular sonothrombolysis with such a transducer can be an effective method for treating stroke.

Finally, the knowledge gained in vitro for enhancing TNK-tPA induced thrombolysis, was translated in vivo using an animal model. A blood clot was artificially formed into the right carotid artery of a rabbit, mimicking a MCA occlusion in humans. The complete flow blockage as well as the recanalization procedure were monitored using a 3D time of flight MR angiography. This novel technique, clearly demonstrated that the combination of 1.18 MHz FUS pulses with MBs, strongly accelerated the action of TNK-tPA, leading through non-thermal mechanisms to full artery recanalization within 19 min.

Keywords: ultrasound, stroke, clot, TNK-tPA, MBs, agar, intravascular, MRgFUS.

1. Introduction

1.1. Stroke

Stroke is an important public health concern, since is one of the most frequent cause of mortality, morbidity and disability of population in developed countries [1-2]. According to World Health Organization (WHO), stroke is the number two cause of death worldwide after heart disease [3]. It is estimated, that 15 million people suffer from stroke worldwide each year [4] and of these, nearly six million die and another 5 million are left permanently disabled [5]. In 2012, stroke killed 6.7 million people out of an estimated of 56 million deaths worldwide, making it roughly responsible for one-ninth of all global deaths [6]. Stroke is the leading cause of long-term disability in adults causing a tremendous burden on the healthcare system of each country, as it is associated with expensive long-term rehabilitation care [7]. Each year, millions of stroke survivors have to adapt on a new way of life with severe limitations on their daily living activities.

Stroke, sometimes called "brain attack", is caused when there is a blood flow disturbance to a part of the brain due to an occluded blood vessel. As a result, the blood supply is interrupted or severely reduced, known as ischemia. Therefore, the resupply of blood flow to the affected area is crucial, since the lack of oxygen to the affected part of the brain can lead within minutes to brain cells death (tissue necrosis) and brain damage. The effects of a stroke depend on which part of the brain occurs and how severely affects it. The longer the time that area remains without blood supply, the less chance to survive or function properly, as tissue necrosis increases with the duration of occlusion and ischemia. If this part of the brain is severely injured, sudden death can be caused. In 2014, Meretoja et al. [8], estimated 1.8 days of added healthy life benefit for each minute reduction in time to treatment.

In a patient affected by an ischemic stroke it is possible to distinguish the three types of tissue with abnormal blood flow, as presented in figure 1.1:

1. The core, which is a zone of death cells due to under-perfusion, where dense ischemia early infarction occurs.
2. The penumbra, which is a zone with hypo-perfused tissue surrounding core, where moderate ischemia and delay infarction occurs. Penumbra remains salvageable if revascularisation occurs in a timely manner [9].

3. The benign oligemia, which is a zone between the unaffected area and the ischemic penumbra, where blood flows below normal range but not at risk of infarction. Even without prompt reperfusion, tissue in this zone usually survives the ischemic insult [10].

Consequently, the most vital factor for the recovery of a stroke patient, is to resupply blood flow to the penumbra region of stroke before brain cells begin to die.

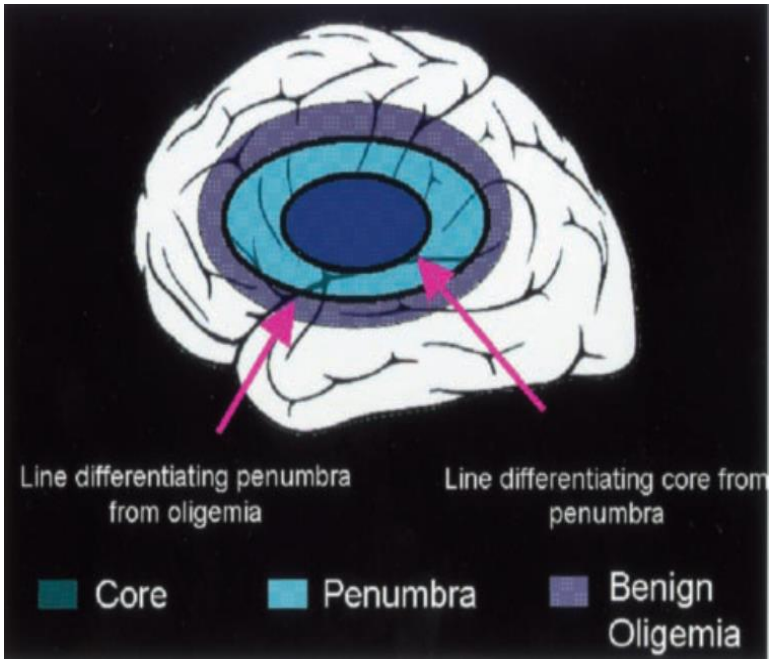

Figure 1.1: The concentric, four-compartment, brain ischemia model. The core represents a tissue compartment that is irreversibly damaged, surrounded by the penumbra that is at risk of infarction. Between the unaffected area and the penumbra, there is an area of benign oligemia that usually survives the ischemic insult, even without prompt reperfusion [10].

The pathological background for stroke may be either due to ischemic or haemorrhagic disturbances of the cerebral blood circulation (figure 1.2). Ischemic stroke or cerebral infarction occurs when the blood supply to part of the brain is interrupted or severely reduced due to full or partial occlusion of a blood vessel, typically caused from a blood clot (thrombus). Haemorrhagic stroke occurs when a weakened blood vessel in the brain ruptures, causing blood leakage either within the brain, or into the space surrounding it. Bleeding into the space surrounding the brain is called a subarachnoid haemorrhage (SAH) and can be caused by a ruptured aneurysm. Bleeding within the brain tissue itself is called an intracerebral haemorrhage and is primarily caused by hypertension. About 87% of strokes are caused by ischemia and the remaining roughly 13 % by haemorrhage [11].

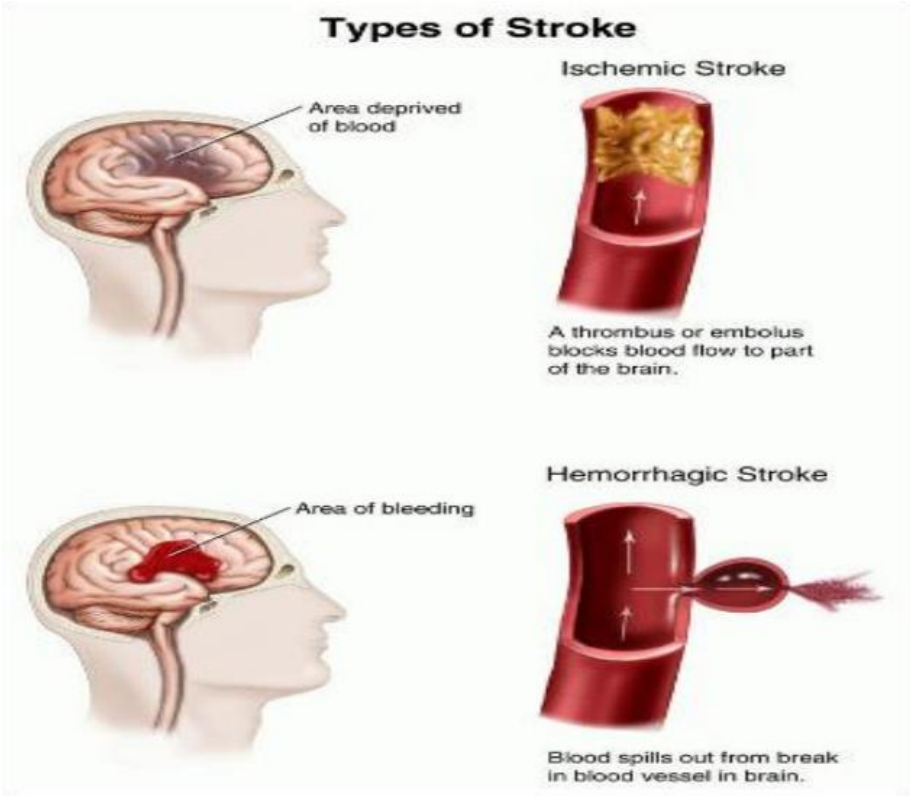

Figure 1.2: The two types of stroke, the ischemic and hemorrhagic stroke [12].

There are two types of ischemic stroke due to a blood clot (thrombus) occlusion, thrombotic stroke and embolic stroke:

1. A thrombotic stroke occurs when a stationary thrombus or blood clot formed in one of the arteries supplying blood to the brain (cerebral arteries), blocks the blood flow, causing thrombotic stroke.
2. An embolic stroke occurs when a blood clot formed in a part of the body other than the brain (usually the heart), breaks off and a part of it travels through the bloodstream to the brain until it reaches a brain vessel small enough to deny its passage and thus becomes lodged. This trapped clot, also known as embolus, blocks the blood flow causing embolic stroke.

Thus, the critical need of an occluded cerebral vessel due to a blood clot, either local (thrombotic) or after migration (embolic), is to relieve ischemia as quickly as possible. Each type of stroke demands different treatment course. For haemorrhagic stroke, the treatment is focused to control the bleeding, to reduce the pressure on the brain and to stabilize blood pressure. For ischemic stroke, the treatment is focused to restore the blood flow to the brain by intravenous (iv) therapy, i.e. the use thrombolytic drugs (clot dissolving), to break up the thrombus [13].

1.2. Thrombolytic therapy

Healthy endothelial cells secrete tissue plasminogen activator, an enzyme that converts proenzyme plasminogen (circulates in the blood) into thrombolytic enzyme plasmin. Plasmin is a serine protease that is capable of breaking down (hydrolyses) the fibrin molecules of blood clots (thrombi) to fibrin degradation products (FDP), leading to clot dissolution [14].

Consequently, thrombolytic drugs are plasminogen activators (PAs), of either human origin, which are identical to the naturally occurring endogenous fibrinolytic enzyme tissue-type PA (tPA), found in vascular endothelial cells in humans [15], or urokinase-type PA (UK) [15], or bacterial products such as streptokinase (SK) [16]. Since a blood clot consists of a structural matrix of fibrin fibres with entrapped platelets and red blood cells, thrombolytic drugs activate fibrin-bound plasminogen on the surface of the clot, improve plasmin formation and hence enhance thrombolysis**.** Of course, it is important to note that PAs sometimes referred as "fibrinolytic agents", also activate circulating plasminogen in plasma and the releasing plasmin can cause an unwanted systemic fibrinolytic state that can lead to undesirable bleeding problems. Although all PAs enhance the endogenous formation of fibrinolytic enzyme plasmin, they differ in fibrin specificity (selectivity) and for that reason are classified into fibrin selective such as tPA and non-fibrin selective such as SK and UK [14]. For instance, the affinity of tPA for plasminogen is significantly increased in the presence of fibrin, so plasmin formation on the clot surface is higher compared to that in the plasma. Despite its affinity for fibrin, tPA still activates circulating plasminogen, leading to undesirable bleeding (fibrin selectivity of tPA is relative not absolute). However, the low affinity for circulating plasminogen, makes the family of tPA a more desirable thrombolytic drug than SK and UK. So, are currently used clinically for breaking down the fibrin contained in a clot, for acute myocardial infarction, cerebrovascular thrombotic stroke and pulmonary embolism.

Thrombolytic therapy has evolved from the first generation thrombolytics such as UK [17] and SK [18], to newer thrombolytics also known by specific chemical names such as the second generation alteplase [19], or the third generation reteplase [20] and tenecteplase [21]:

1. Alteplase (Activase®; rt-PA) is a recombinant form of human tPA, commonly known as rt-PA, reflecting its method of manufacture. It has a short half-life (~5 min) and therefore is usually administered as an iv bolus followed by an infusion (second generation) [19].

2. Reteplase (Retavase®) is a genetically engineered, smaller derivative of recombinant tPA that has increased potency and is faster acting than rt-PA. It is usually administered as iv bolus injections (third generation) [20].
3. Tenecteplase (TNK-tPA) has a longer half-life and greater binding affinity for fibrin than rt-PA. Because of its longer half-life, it can be administered by iv bolus (third generation) [21].

Although third generation thrombolytic agents offer pharmacokinetic and hemodynamic advantages over rt-PA, have not been approved yet by the United States Food and Drug Administration (FDA), for the treatment of acute ischaemic stroke (AIS). At present, rt-PA is the only thrombolytic treatment for AIS approved by the FDA [19] and is administered iv at a dose of 0.9mg/kg, with a maximum dose of 90 mg. Ten percent of the medication is given as a bolus and the remainder is infused over 60 min [22]. Nevertheless, there are severe restrictions in rt-PA administration. The most important is that according to the NINDS (National Institute of Neurological Disorders and Stroke) study, the drug should be administered within the first three hours of stroke's onset in order to be beneficial [19]. However, in 2009, the European Cooperative Acute Stroke Study 3 (ECASS 3), demonstrated that patients treated with iv rt-PA in the 3–4.5 hour window, also showed improved outcome compared to placebo, with no increase in mortality. Although professional organisations in the United States and many regulatory agencies worldwide have recommended that the time requirement should be extended from 3 hours to 4.5 hours [23], the FDA has declined to approve this expanded time window.

The most important to underline, is that not all the patients with ischemic stroke can be treated with thrombolytic drug rt-PA, due to protocol exclusion. Except form the time window restriction, patients that have taken heparin in the previous 48 hours, or had another stroke in the previous 3 months, or surgery within the past 14 days, or suffer from hypertension, should be excluded from thrombolytic therapy. It is estimated that less than 5 % of patients with ischemic stroke receive iv rt-PA and of these, only 30 to 40 % achieve early recanalization. Furthermore, due to the low efficacy of thrombolytic therapy, the recanalization is complete and sustained in less than 20 % of the treated patients [24-25]. Moreover, many studies have shown that rt-PA administration leads to intracerebral haemorrhage [26-27]. As a result, even without any side effect, often cerebral vessels are still obstructed after the treatment [28]. Numerous studies have shown that in a significant number of patients, iv thrombolysis with rt-PA remains ineffective, with poor

recanalization rates, especially for large proximal vessels, as compared with distal smaller vessels [26]. More specifically, the recanalization rates achieved with iv administration of rt-PA for proximal, large-vessel arterial occlusion, range from only 10 % for internal carotid artery (ICA) occlusion to 30 % for MCA occlusion [27]. All these reasons have created the need for the development of new alternative methods for the treatment of stroke that would be less restrictive, more safe, easy to use and more efficient.

1.3. Sonothrombolysis

Mathematical models of fibrinolysis predict that clot lysis occurs along a front where the concentrations of plasminogen activator generate a sufficient plasmin concentration [29]. As theoretically predicted by Zidansek and Blinc 1991 [30], Zidansek et al. 1993 [31], Diamond and Anand 1993 [32] and experimentally shown by Blinc et al. [33], clot lysis based solely on diffusion of fibrinolytic enzymes into the clot is a very slow process that could last for days instead of a few hours as required for successful thrombolytic therapy. Furthermore, Diamond and Anand 1993 [32] have shown in their theoretical model, that thrombolytic therapy is physically limited by diffusion as no "driving force" exists to promote drug permeation since blood flow is stagnant or absent near the clot surface.

This "driving force" was found in the mid-70s to be the application of ultrasound (US) energy. Trübestein et al. 1976 [34], first reported that mechanical pressure waves produced by US energy enhance the efficacy of thrombolytic agents, a process known as US enhanced thrombolysis. Since then, the ability of US energy to increase thrombolysis has been investigated extensively both in vitro and in animal models by several investigators [35–39].

The use of US energy to enhance thrombolytic therapy, also referred as sonothrombolysis, represents a completely different approach with unique features. This new approach appears to be very attractive, since it could offer unique potential benefits for patients, such as faster and more complete clot dissolution as well as lower dose of thrombolytic agent with resulting reduction in associated bleeding risks [40]. Whether delivered through a catheter (intravascularly), or transcutaneously (noninvasively), the effects of US are limited to the insonified area. Catheter-based devices have the advantage of localizing treatment to the clot, without any interference with the tissue. By applying this therapeutic technique, more energy can be delivered to the clot and unnecessary side effects such as thermal heating can be avoided. On the other hand, intravascular

sonothrombolysis, is time delayed and carries an increased risk of bleeding due to damage of the vessel wall and infection [41].

In 2004, Atar and Rosencschein [42], examined all available by that time, techniques and methods of US thrombolysis. According to their review, the potential advantages of noninvasive sonothrombolysis over catheter alternatives are significant because bleeding risks from catheters are eliminated and faster treatments may be achieved as the time delay to intervention could be dramatically decreased.

1.4. Effects of US on clot lysis

The effects of US on thrombus dissolution vary with the energy level delivered, as described by Polak 2004 [43]. As it is shown in figure 1.3, at very low energies or intensities (< 0.5 MPa), US promotes the motion of fluid, an effect called microstreaming. It is possible that this effect agitates the blood close to the occluding clot and promotes the mixing of rt-PA. As a result, the concentration of the agent that is in contact with the thrombus is increasing effectively. At moderate energies or intensities (0.5-4 MPa), US increases permeation of rt-PA into clot and binding to fibrin mesh that forms the occlusive lesion. At high energies or intensities (> 4 MPa), the onset for cavitation activity is exceeded creating gaps in the fibrin mesh, that increases the permeation of rt-PA into clot.

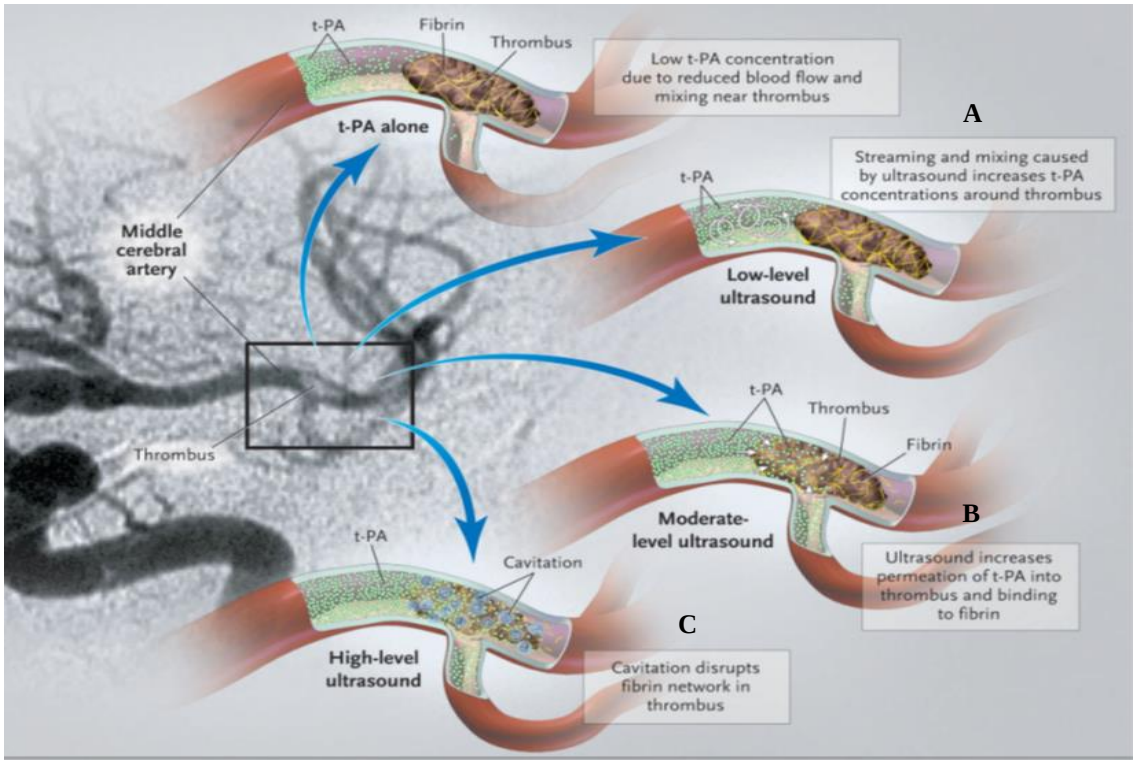

Figure 1.3: A) Low level US increases the concentration of the agent around clot due to microstreaming. B) Moderate level US increases drug's permeation into clot and binding to fibrin. C) High level US disrupts the fibrin network in clot due to cavitation activity [43].

Similarly, Sakharov et al. 2000 [44], also showed in their study that acoustic streaming led to thrombolysis enhancement, which is equivalent to that induced by mild stirring of thrombolytic solution around the thrombus. If the streaming is significant, both in absolute terms and relative to the existing blood flow, it can act as a 'mild stirrer' enhancing the supply of a PA and plasminogen from outside the clot exterior, directly to the clot surface.

Although the mechanism of action of sonothrombolysis is not fully understood, numerous studies demonstrated that exposure to US increases both the uptake and depth of penetration of the activator to fibrin, through mechanisms that improve drug transportation [43–45], causes reversible alteration of the fibrin structure [46–49] and increase the binding of thrombolytic agent to fibrin [50–52]. The mechanisms for US enhancement of clot lysis and the contribution of each one of these mechanisms to thrombolysis efficacy are well reviewed by Bader et al. 2016 [53].

Exposure to US can be used with any thrombolytic agent and at any site where the appropriate US field can be delivered. US enhances thrombolysis during pulsed (instead of continuous), exposure and over a wide range of frequencies and intensities. Choices of frequencies for the majority of therapeutic US reside in the range of 40 kHz-2 MHz due to lower skull attenuation and are typically lower than those for diagnostic applications, which are normally in the range of 2-10 MHz.

The influence of operating parameters on US induced thrombolysis was investigated extensively by Schäfer et al. 2005 [54]. Study results have shown that the thrombolytic efficiency of US is directly depended on intensity and inversely depended on frequency. Higher US frequencies lead to greater energy attenuation, while higher intensities are associated with increased thermal effects. For this reason, the majority of sonothrombolysis models so far (both in vitro and in vivo), were developed using low frequencies and low intensities.

Due to the increased interest in sonothrombolysis, there are a lot of experimental studies focusing on the significant enhancement of enzymatic thrombolysis by the simultaneous application of US. For instance, Devcic-Kuhar et al. 2002 [55], examined the effects of standing and travelling US wave fields on thrombolysis enhancement and showed that the enhancement of clot dissolution is much more pronounced in travelling than in standing acoustic waves. Another experimental study published by Pfaffenberger et al. 2003 [56], also suggested that an intermittent (pulsed instead of continuous)

application of a 2 MHz high frequency US using a travelling wave field would be the most potent application for lysing blood clots.

In chapter three, several published reports in the field of sonothrombolysis are summarised in three tables (in vitro, in vivo and clinical), since 1992. This comprehensive review, provides an overview of the different protocols applied so far by researchers on US thrombolysis studies.

1.5. In vitro and in vivo studies

In the past two decades, increased evidence of US ability to accelerate thrombolysis rate with adjunctive PAs in vitro, have been reported [57–59]. Kimura et al. 1994 [57], Spengos et al. 2000 [58] and Holland et al. 2008 [59], showed that external application of US increased thrombolysis in vitro. Kimura et al. 1994 clarified that the mechanism of clot lysis when subjected to a combination of thrombolytic drug and US leads to degradation of fibrin, allowing a quantitative measurement of the enhancement of clot lysis.

Also, Spengos et al. 2000 suggested that transcranial application of 1 MHz pulsed-wave US may accelerate reperfusion and recanalization rate of occluded intracerebral vessels, by enhancing rt-PA mediated thrombolysis. Holland et al. 2008 explored various US settings to determine the thrombolytic efficacy of US therapy with and without rt-PA administration. Using an in vitro model, they demonstrated that treatment with US as an adjuvant to rt-PA can significantly increase clot dissolution by as much as 104 % compared to rt-PA alone.

Moreover, many in vivo studies like Lauer et al. 1992 [60], Kashyap et al.1994 [61] and Suchkova et al. 2000 [62], have shown that the combination of thrombolytic drugs and US accelerates fibrin degradation and causes a significant enhancement of thrombolysis. The study by Lauer et al. 1992, demonstrated that 1 MHz intermittent US enhances rt-PA induced clot lysis in a rabbit jugular vein thrombosis model. Similarly, Kashyap and colleagues showed in their study that noninvasive percutaneous application of 1 MHz pulse wave US significantly accelerated SK induced fibrinolysis, in a rabbit ear model of small vessel injury. Furthermore, Suchkova et al. 2000, showed that the combination of SK with low intensity 40-kHz US in a rabbit model, markedly accelerates fibrinolysis, improves tissue perfusion and reverses acidosis, effects that would be beneficial in the treatment of acute thrombosis. In the former studies, the range of

intensities varied from 0.2–2.0 W/cm^2 (spatial peak, temporal average intensity-I_{SPTA}), while the range of frequencies varied from 20 kHz to 2 MHz, using unfocused US.

Another promising method for the treatment of vascular thrombosis is the targeted delivery of fibrinolytic agents to the site of occlusion such as the entrapment of rt-PA into liposomes. This method, provides a selective targeting to improve the efficacy of fibrinolytic agents [63-64]. Shaw et al. 2009 [63] have shown that the combination of 120 kHz US with rt-PA-loaded echogenic liposomes, significantly enhanced lytic treatment efficacy. In another study, Laing et al. 2011 [64], using a rabbit aorta model, demonstrated that liposomes loaded with rt-PA in combination with Doppler US treatment, can significantly enhanced thrombolysis.

1.6. MBs enhanced sonothrombolysis

In addition to thrombolytic drugs, US contrast agents (UCA) have been studied as a way to further enhance the effect of US, on the lysis of blood clots. UCA are micron-sized gas bubbles widely used to improve the signal-to-noise ratio in US image acquisition. Beyond their routine diagnostic use as UCA, there is growing interest in the therapeutic potential of these MBs, since many studies have shown that may also improve the lytic efficacy of sonothrombolysis. The use of MBs to enhance thrombolysis, was first introduced by Tachibana and Tachibana 1995 [65]. The authors, demonstrated that the presence of albumin MBs can further accelerate fibrinolysis induced by 170 kHz US plus UK, in an in vitro human blood clot model.

The administration of MBs in combination with US and rt-PA, may further improve thrombolysis as a result of cavitation mechanisms. Acoustic cavitation, is the formation of gas bubbles within US field that undergo oscillation and disruption. When oscillating under US exposure, MBs can increase rt-PA permeation inside the clot due to stable cavitation [66] or can cause directly a mechanical breakdown of the clot surface due to inertial cavitation [67].

The last 20 years, several in vitro and in vivo studies have presented that the effectiveness of thrombolytic drugs can be increased when combined with US and MBs [68-69]. Using either high [68] or low excitation frequencies [69], treatments combining US, MBs and rt-PA achieved greater mass loss than rt-PA alone or rt-PA with US. For instance, Cintas et al. 2004 [68], using an in vitro model found that the application of MBs, strongly accelerates rt-PA induced fibrinolysis of clots exposed to high excitation frequency (2 MHz US). In contrast, Datta et al. 2008 [69], exposed in vitro human blood

clots using low excitation frequency (120 kHz US). Study results showed that the greatest clot mass loss was also observed for clots treated with US plus rt-PA and MBs.

Concerning the in vivo studies, Brown et al. 2011 [70], using a rabbit embolic stroke model, demonstrated that the combination of low-dose or no-dose rt-PA and US plus MBs, improved thrombolytic treatment with a strong trend to decrease infarct volumes and significantly decreased intracranial haemorrhage (ICH), outside the area of infarct.

Another area of investigation was focused on clot lysis using only US in synergy with MBs, for patients that are ineligible for rt-PA administration. An in vitro study by Nishioka et al. 1997 [71], showed that US in combination with MBs can induced clot lysis without the use of thrombolytic drugs. Besides, Culp et al. 2011 [72], using a rabbit embolic stroke model, demonstrated that MBs sonothrombolysis with no exogenous rt-PA, produces significant improvement in strokes without apparent side effect. Study results along with the results of a long series of animal studies conducted by the author in the past, have shown that human trials without rt-PA (for patients where rt-PA is contraindicated), were justified.

As cavitation is an important mechanism of thrombolysis with US and MBs, specifically targeting bubbles may enhance this effect, through close proximity of the cavitating bubbles to the thrombus. A challenging option may be the formation of MBs, which can be attached to vascular thrombi by using ligands and receptors that are incorporated in bubble's shell (figure 1.4). Studies have demonstrated that MBs can be concentrated at the surface of clot by attaching a glycoprotein receptor antagonist, which increases MBs proximity to blood clot, leading to enhanced thrombolysis [73-74].

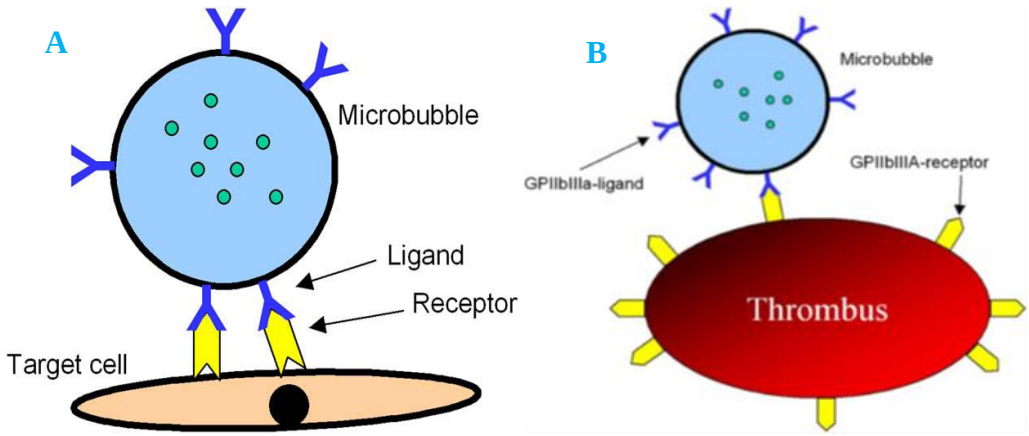

Figure 1.4: A) MB can be attached to a target tissue by using ligands and receptors, which are incorporated in its shell. B) MB attached to a target blood clot [67].

1.7. Role of FUS in clot lysis

A relatively new approach to sonothrombolysis is the use of FUS from an external source. This novel, emerging, therapeutic modality has received attention lately as it can deposit energy into millimetre sized focal volumes within the body, without harming adjacent normal tissue. Many studies, either bench-top [75–77] or animal [78–80], have shown that the use of FUS for clot distraction is enhancing thrombolysis. In 2006, Frenkel et al. [75] evaluated the effect of FUS on clot destruction with and without rt-PA, using an in vitro human blood clot model. No significant increase in thrombolysis from FUS treatment alone was seen but they demonstrated that treatment with FUS in combination with rt-PA increases thrombolysis by 50 % compared to treatment with rt-PA alone. In 2007, the same group conducted in vivo studies using a rabbit marginal ear vein thrombus model [78]. Once again, FUS treatment alone showed no significant increase in thrombolysis, but the group observed improved rates of clot reduction using pulsed FUS in synergy with rt-PA.

Recently, the use of FUS has been proposed as a stand-alone method for clot lysis (in the absence of thrombolytic drugs). In the study by Hölscher et al. 2011 [81], human whole blood clots in vitro were ablated though the skull using pulsed FUS. The conclusion of the study was that even in the absence of thrombolytic drug, FUS alone can be utilized to destroy clots. In another study, Wright et al. 2012 [82], showed that FUS either in vitro or in vivo can cause clot lysis within minutes when used as a stand-alone method. Even though this would eliminate side effects from thrombolytic drugs and hence reducing the risk of haemorrhage, the potential for bleeding with this approach requires further investigation.

So far, none of the experimental studies on sonothrombolysis has claimed enzymatic clot lysis within minutes, based on non-thermal effects. To our knowledge, it is speculated that clot lysis within minutes should be probably due to thermal mechanisms.

1.8. MRgFUS

In the last few years, an innovative new technology is emerging, which offers FUS treatments under magnetic resonance (MR), guidance and monitoring. This new quickly developing technology is commonly used by the term MR guided FUS (MRgFUS). The idea of using MRI to guide and monitor a real time non-invasive FUS treatment was originally introduced by Hynynen et al. in 1993 [83], who demonstrated that lesions

produced in dog's thigh muscle in vivo were clearly visible inside the gantry of an MRI. Studies followed this concept shown that the contrast between necrotic tissue and normal tissue is superior when compared with US technology, verifying that MRI is an excellent tool for FUS systems guidance [84-85]. Since then, MRI became the preferred monitoring tool because not only provides the possibility to monitor a therapeutic effect close to real time, but also to monitor temperature elevation as well as focus navigation based on MRI derived thermometry. This rapidly expanding technology has many applications across the spectrum of non-invasive FUS treatments, especially in the domain of oncology.

Lately, the possibility of using MRgFUS for treating stroke has become an important area of investigation, since stroke is the primary focus of sonothrombolysis research due to the high mortality rate associated with it. In 2012, Burgess and colleagues, used MRgFUS for clot dissolution in a rabbit embolic stroke model [86]. Study results have shown that this technique could dramatically reduce restoration flow time, leading to a significant increase in the number of patients, which benefit from MRgFUS thrombolysis treatment.

As MRgFUS sonothrombolysis relies on non-thermal mechanisms to achieve clot lysis, MR thermometry is not useful for treatment monitoring in real time. In 2013, Durst et al. [87], evaluated different imaging sequences in order to extract the optimal imaging sequence, for real time monitoring of in vitro clot sonothrombolysis by MRgFUS. Study findings have identified T2-weighted imaging as the optimum imaging sequence to verify that a clot had been lysed.

A major limitation of all transcranial US techniques is the beam distortion due to the inhomogeneous thickness and density of the skull bone and the heating of the skull and near-field soft tissues caused by the acoustic signal absorption. As a result, the acoustic energy that reaches the deeply located lesions, decreases proportional. To overcome these limiting factors, an innovative MRgFUS head system, has been developed. This prototype helmet-type brain system (figure 1.5A), is equipped with a hemispheric multi-element array transducer that produces a sharp focus at the target lesion, while it simultaneously distributes heating across a larger surface area (Exablate 4000, Insightec, Haifa, Israel). In addition, an active cooling system that circulates cold degassed water (about 15 ^{0}C), is placed between the patient's head and transducer. The clinical set up is shown in figure 1.5B.

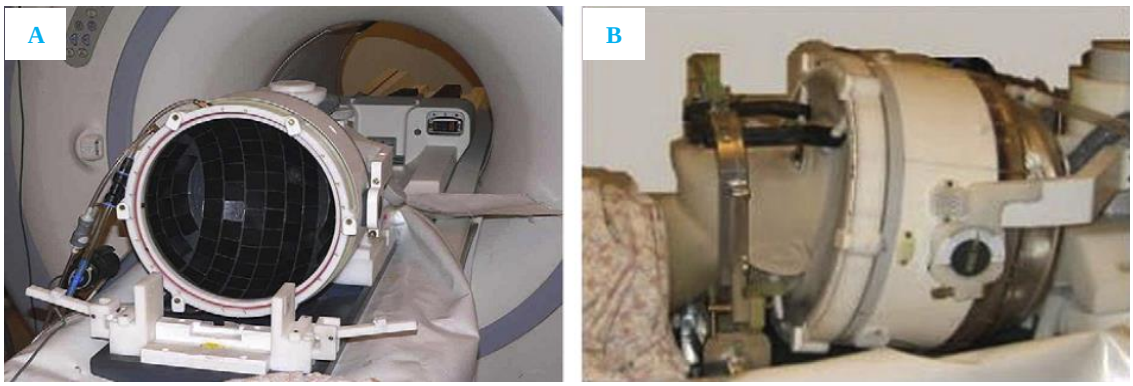

Figure 1.5: MRgFUS head system. A) The helmet-type hemispheric transducer with 1,000 single piezo elements. B) Patient set up prior to positioning inside the MRI scanner [88].

1.9. Clinical trials using sonothrombolysis

The enhancing effect of US on rt-PA mediated lytic efficacy, which have been demonstrated in many in vitro and in vivo studies, have led to clinical trials of Transcranial Sonothrombolysis in acute ischemic stroke (AIS) patients. In the last 10-15 years, there have been some significant clinical trials concerning the application of therapeutic US in the treatment of stroke. Although preclinical studies have been very encouraging, the results from clinical trials have shown that further research in the field of sonothrombolysis has to be done.

Two different approaches regarding sonothrombolysis for recanalization have been evaluated (figure 1.6): (1) a noninvasive transcranial approach using a Doppler in combination with rt-PA [89–93] and (2) an invasive endovascular approach using an US emitting catheter for simultaneous intra-arterial (ia) delivery of rt-PA and US [94–98].

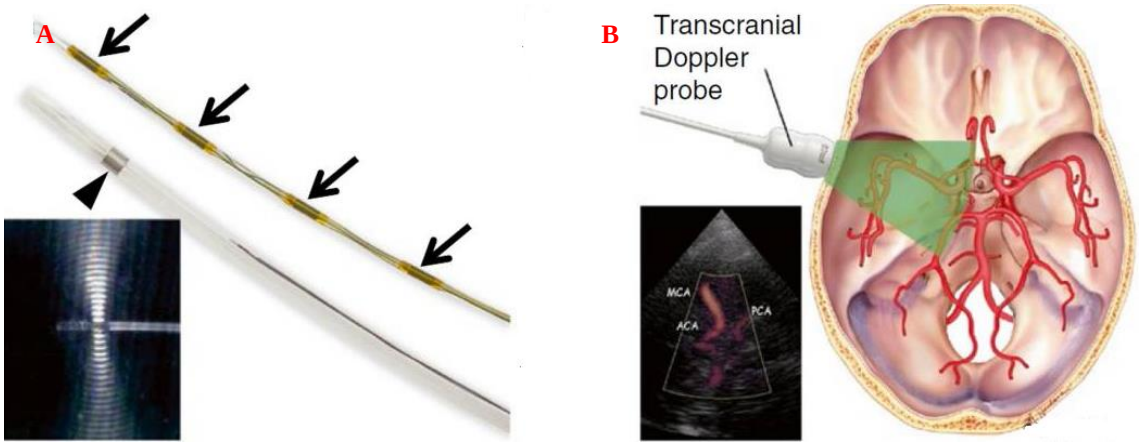

Figure 1.6: A) Invasive approach using US assisted thrombolysis catheter. B) Noninvasive approach using transcranial Doppler [53].

Although low-frequency US (< 1 MHz) is a good choice for skull penetration [99], a clinical trial using transcranial 300 kHz US plus rt-PA, the TRanscranial low-frequency

Ultra-sound-Mediated thrombolysis in Brain Ischemia (TRUMBI) trial, was ended prematurely due to a significant increase (36 %), in symptomatic intracranial hemorrhage (sICH) within 3 days of treatment [89]. Since then, low frequency US has not been available for therapeutic purposes in clinical trials.

One of the first clinical studies, using high frequency (2 MHz) low intensity Transcranial Doppler US (TCD), was the Combined Lysis of Thrombus in Brain Ischemia Using Transcranial US and Systemic rt-PA (CLOTBUST) trial, by Alexandrov et al. [90]. The purpose of this phase II randomized multi-center international clinical trial, was to evaluate the effects of TCD ultrasonography in conjunction with systemic administration of rt-PA in patients who had AIS due to occlusion of the MCA. After 2 hours of continuous TCD monitoring, increased recanalization rates were observed in 49 % of patients treated with TCD + rt-PA compared to 30 % of patients treated with rt-PA alone.

Similarly, in another smaller clinical trial by Eggers et al [91], patients with acute proximal MCA occlusion were treated with 1.8 MHz transcranial color-coded duplex (TCCD) technology in association with rt-PA. TCCD transducers generate multiple small beams at dual emitting frequencies, one for Doppler (1.8 MHz) and one for gray scale imaging (4 MHz). Study results, also demonstrated improved recanalization rates when TCCD was combined with rt-PA in patients with AIS, compared to patients treated with rt-PA alone.

The last few years, sonothrombolysis clinical research has been focused on the boosting effect of MBs in clot dissolution. The safety and effectiveness of various types of MBs in combination with transcranial US and rt-PA have been clinically evaluated since 2006.

Molina et al. [92], combined 2-MHz TCD with rt-PA and MBs in patients with AIS. The study showed promising results since the patients treated with the triple combination of 2 MHz TCD, rt-PA and MBs, exhibited higher recanalization rate compared with patients treated either with rt-PA plus TCD or rt-PA alone.

Based on the encouraging results from the first study, Molina et al. [93], performed a second clinical trial, the Transcranial Ultrasound in Clinical Sonothrombolysis (TUCSON). In this study, stroke patients were treated with 2 MHz TCD in combination with rt-PA and continuous infusion of newly developed MBs, such as the C_3F_8 perfultren-lipid microspheres-"MRX-801" (ImaRx Therapeutics, Inc., Tucson, Arisona). Theoretically, the smaller size of these newer and more stable microspheres (1-2 μm), allows permeation through the thrombus, which may further

enhance recanalization process, when combined with iv administration of rt-PA plus TCD monitoring. Although at low dose of MBs, 50 % reperfusion enhancement was observed, when the dose was double, reperfusion enhancement was ceased and hemorrhaging occurred. As a result, the clinical trial terminated with the recommendation that the use of low doses MBs in sonothrombolysis may be safe, but higher doses when administered with rt-PA may increase sICH rate.

Another series of clinical trials, investigated the safety and feasibility of endovascular sonothrombolysis for treating patients with AIS. The first experience with endovascular sonothrombolysis for patients with AIS, was published by Mahon et al. [94]. They used the EKOS Micro-Infusion Catheter, a novel technology for ia delivery of rt-PA along with low intensity US from the tip of the catheter, in order to accelerate thrombolysis. EKOS Infusion Catheter [100], consisted of a Drug Delivery Catheter (DDC) and an US Core (USC) (figure 1.6). The DDC had a pattern of holes to deliver uniform drug flow along and around the treatment zone and a central lumen to accept the USC and to deliver coolant fluid to the USC during operation. Six US transducer elements, each with dimensions 2.0 x 0.40 x 25 mm, were mounted at evenly spaced 1 cm intervals along the USC to form the treatment zone. The combination of ia rt- PA administration with endovascular US, was applied continuously for 60 min. Partial or complete recanalization was detected in 57 % patients and no adverse effects were observed during the therapy.

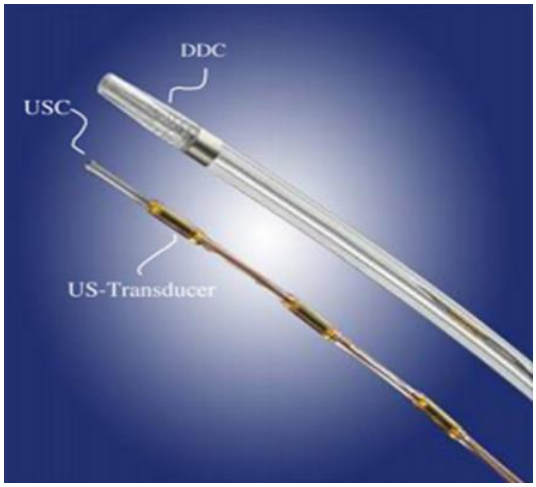

Figure 1.7: EKOS Infusion Catheter [100].

To bridge the gap between early initiation of iv therapy and faster recanalization of ia therapy, a combined iv/ia thrombolysis approach was designed to achieve higher rates of early and successful reperfusion. This approach was evaluated in the International Management of Stroke (IMS) I and II clinical trials, sponsored by the National Institute

of Health (NIH). The first phase of the study (IMS I) was completed in 2002 [95], followed by the second phase (IMS II), which was completed in 2005 [96]. Both studies have shown that this combined approach may be more effective in recanalization than standard iv rt-PA alone.

Additionally, in the IMS II trial, the feasibility and safety of intravascular sonothrombolysis for treating patients with AIS was investigated [96]. More than half of the patients in the IMS II study, were treated with the EKOS system along with ia infusion of rt-PA, in order to accelerate thrombolysis. The study showed that this combined approach may be more effective in recanalization than standard iv rt-PA alone.

Based on the positive findings of IMS I and IMS II clinical trials, the NIH has sponsored in 2006 the largest interventional ischemic stroke clinical trial so far, called IMS III [97]. Nevertheless, IMS III trial was terminated prematurely in 2012 when the results shown no substantive difference in benefit between stroke patients receiving endovascular therapy after iv rt-PA, as compared with iv rt-PA alone.

Another clinical study by Kuliha et al. [98], was also designed to confirm the safety and efficacy of intravascular sonothrombolysis for treating stroke patients. Study results demonstrated that complete or partial recanalization of brain artery was achieved in 77 % of the patients treated with the EKOS system along with ia infusion of rt-PA.

Finally, a different focus for sonothrombolysis research concerns clinical trials for those stroke patients who are ineligible for rt-PA [101]. Based on both in vitro and in vivo studies, which have shown that US energy (with or without MBs) can induce clot lysis, the authors of this study evaluated the effects of TCD on patients with acute MCA occlusion and contraindications for rt-PA administration. Even though the results of the study were promising, need to be confirmed in larger clinical trials.

1.10. Aims and objectives

The aims and objectives of the thesis are listed below:

i. To review protocols used in sonothrombolysis studies aiming to give an overview of the different protocols used so far in the field of sonothrombolysis and to investigate the impact of several aspects involved, on thrombolysis outcome.

ii. To use fully retracted blood clots, obtained by natural coagulation of fresh porcine blood, in order to study the effect of US energy on thrombolysis efficacy.

iii. To design circulating flow clot models, reproducing in vitro the physiological situation of a MCA occlusion occurred either deeply into a brain tissue mimicking phantom or superficially.

iv. To measure thrombolysis efficacy the blood clots will be treated with US waves as an adjunct to thrombolytic drug TNK-tPA, in the presence or absence of MBs.

v. To determine and evaluate various experimental parameters (acoustic and physical), that influence the thrombolytic efficacy of the 3rd generation thrombolytic drug TNK-tPA.

vi. To establish an optimised treatment protocol that through non-thermal mechanisms maximises the thrombolytic activity of TNK-tPA leading to enhanced thrombolysis.

vii. To evaluate the thrombolytic efficacy of two small planar ultrasonic transducers (operating at frequencies 3.7 and 5.2 MHz respectively).

viii. To evaluate the thermal capabilities of a small flat rectangular ultrasonic transducer, operating at 4 MHz frequency, with the use of two different gel phantoms fabricated to mimic brain tissue.

ix. To optimise treatment protocol obtained in vitro must be translated in vivo using a carotid artery model under MR monitoring. The aim of the study is the fast and complete recanalization of a fully occluded carotid artery of a rabbit, without excess heating.

1.11. Chapters outline

This section gives an overview of each chapter.

Chapter 2: Physics of Ultrasound and Biological Tissue Effects

This chapter covers the physics of US as well as the biological effects generated from the interaction of US waves with targeted tissue. It refers to the basic ultrasonic quantities, to the generation and the types of US waves and to image guidance. Also, it describes the FUS induced biological effects and their underlying thermal and non-thermal mechanisms, which are the foundation of FUS therapies such as hyperthermia, thermal ablation, sonoporation and sonothrombolysis.

Chapter 3: Review of Protocols used in Ultrasound Thrombolysis

This chapter focuses on the review of different protocols used so far in the field of sonothrombolysis. These protocols are summarised in three tables

(in vitro, in vivo and clinical), providing detailed information concerning clot model, thrombolytic drug, treatment mode, sonication parameters, evaluation method, thrombolysis outcome, side effects and conclusions.

Chapter 4: Determination of the Experimental Parameters that Influence Ultrasound Induced Thrombolysis in Vitro

In this chapter, various physical and acoustic properties that influence the thrombolytic activity of the 3rd generation thrombolytic drug TNK-tPA, are determined. In addition, the different circulating flow clot models used to evaluate in vitro the effect of US energy (either focused or unfocused), on clot lysis are also described.

Chapter 5: Focused Ultrasound Enhanced TNK-tPA Mediated Thrombolysis into a Brain Tissue Mimicking Phantom

In this chapter, an in vitro flow clot model is developed, reproducing the physiological situation of MCA occlusion, occurred 4 cm deep into a brain tissue. For this purpose, fully retracted porcine blood clots are embedded into a brain tissue mimicking phantom and treated with FUS pulses either alone or as an adjunct to TNK-tPA, in the presence or absence of MBs. The objective is to establish an optimized treatment protocol that maximizes the permeation and binding of thrombolytic drug into the clot, leading to increase degree of thrombolysis, without excess heating.

Chapter 6: The Enhancing Effect of Focused Ultrasound on TNK-Tissue Plasminogen Activator Induced Thrombolysis of a Superficial Target Using an in Vitro Circulating Flow Model

In this chapter, an in vitro flow clot model is developed, reproducing a MCA occlusion occurred superficially. The optimized experimental parameters obtained in the previous chapter are employed in this one in order to investigate the impact of frequency and acoustic intensity on thrombolysis efficacy.

Chapter 7: Microbubble Based Sonothrombolysis using a Planar Rectangular Ultrasound Transducer

In this chapter, an in vitro flow clot model is developed, reproducing a MCA occlusion, treated invasively with a catheter-directed US device. The aim is to evaluate the efficacy of two small planar ultrasonic

transducers, operating at frequencies 3.7 and 5.2 MHz respectively, on clot lysis.

Chapter 8: Evaluation of a Small Flat Rectangular Therapeutic Ultrasonic Transducer Indented for Intravascular Treatment of Atherosclerosis

This chapter evaluates the thermal capabilities of a small flat rectangular, MRI compatible ultrasonic transducer, operating at a frequency of 4 MHz. Transducer's efficacy is tested in two different gel phantoms fabricated to mimic tissue. A polyacrylamide hydrogel phantom is used to visualize the size and shape of the thermal lesion and an agarose based gel phantom is used to measure temperature elevation.

Chapter 9: MRI Guided Sonothrombolysis in Vivo Using a Rabbit Carotid Artery Model

In this chapter, the enhancing effect of 1.18 MHz FUS waves on TNK-tPA induced thrombolysis in the presence of MBs, is evaluated in vivo under MR monitoring, using a carotid artery model. The aim of the study is the fast and complete opening of the occluded artery of the rabbit, without excess heating.

Chapter 10: Conclusions and Future Directions

Conclusions and future direction are presented in this chapter.

2. Physics of Ultrasound and Biological Tissue Effects

2.1. Introduction

US biophysics studies the interactions between US waves and biological tissues. The type of each interaction such as transmission or reflection, specifies the way that biological tissues affect US waves and provides the basis for US applications (diagnostic or therapeutic). On the other hand, US can induce biological effects (bioffects), to tissues through mechanisms that are separated into thermal and non-thermal. If US waves can alter biological tissues in a controllable way, which means without any cause of undesirable side-effects, then can be used for many therapeutic applications.

2.2. Basic ultrasonic quantities

US, are pressure waves that transport energy from one location to another, in a medium. As US waves propagate through a medium, apply pressure to its particles, causing them to oscillate back and forth around their rest position. Due to the bonding between the particles, the motion is transmitted to the neighbouring particles. If the particles are widely separated (gas), US propagation will be strongly reduced. When the particles get closer together, create wave fronts of compression (high or positive pressure) and when they separate, create wave fronts of rarefaction (low or negative pressure). As a result, US energy is propagated in a medium through these series of repeating compression and rarefaction regions. These mechanically pressure waves can be described through a sinusoidal function, which is characterized by the following properties (figure 2.1):

Wavelength (λ): is the distance travelled by one oscillation (cycle), or is the distance between two consecutive maximal compressions or rarefactions. It is measured in meters (m).

Frequency (f): is the number of times a particle oscillates per second. It is measured as cycles per second and the unit is hertz (Hz).

Period (T): is the time occupied by one oscillation. It is expressed in seconds (s) and is the reciprocal of frequency:

$$\mathbf{T} = \frac{\mathbf{1}}{\boldsymbol{f}} \qquad (2.1)$$

Propagation Velocity (c): is the speed that sound waves propagate through a medium and depends on tissue density and compressibility (slower in gasses, faster in liquids, and

fastest in solids). Speed is the constant that relates wavelength (λ) to period (T) and is expressed by the Wave Equation:

$$c = \lambda f \qquad (2.2)$$

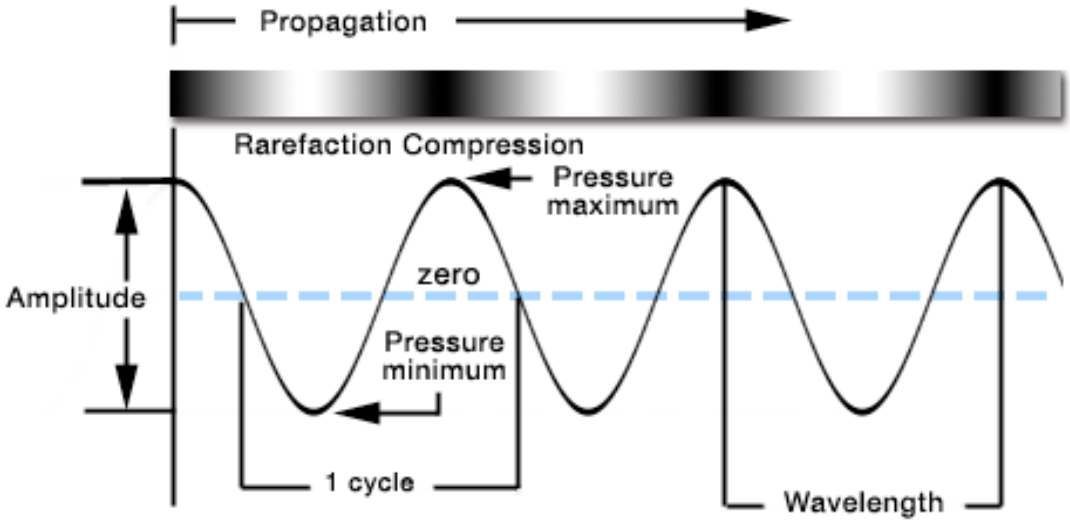

Figure 2.1: Physical parameters of US wave and their relations [102].

For medical applications, the propagation velocity (c) in all soft tissue is assumed to be constant and equal to: $c = 1540\ m/s$.

Amplitude: is the maximum distance a particle moves back or forth (displacement), i.e. the maximum height above or below the baseline value. It is expressed in decibels (dB), which is a logarithmic scale.

Pressure amplitude (P): amplitude is also measured in units of pressure, which is described by the peak positive (P_p) and peak negative pressures (P_n).

Acoustic Power (AP): is the amount of acoustic energy generated per unit time. Energy is measured in joules (J).

Acoustic intensity (I): is the power flowing through a unit area. It is expressed in Watts/m^2 or mW/cm^2. For a sinusoidal wave, the instantaneous acoustic intensity $I(t)$ is given by:

$$I(t) = \frac{P(t)^2}{2\rho c} \qquad (2.3)$$

where $P(t)$ is the instantaneous pressure amplitude and ρ is the physical density of the medium.

2.3. Generation of US waves

US waves are generated from small devices called transducers or probes, which are constructed of a piezoelectric crystal. Piezoelectric crystals have the property to convert electrical energy to mechanical energy and vice versa, a phenomenon known as the piezoelectric ("pressure electric") effect. When an alternating current (AC) voltage is applied across the opposite surface of a piezoelectric crystal, its shape changes with polarity, causes the crystal to expand and contract (vibrate), at the same frequency (f) as that of the applied field. This leads to the generation of compressions and rarefactions of US pressure waves. The most commonly used piezoelectric crystal by all medical US transducers is lead zirconate titanate (PZT), because it exhibits strong piezoelectric effect and can be manufactured in almost any shape. The resonance frequency of a transducer, also referred to as the centre frequency **(f)**, is determined by the thickness **(d)** of the crystal:

$$\mathbf{f = \frac{c}{2d}} \qquad \mathbf{(2.4)}$$

Thus, thinner crystals generate higher frequencies, while thicker generate lower frequencies.

To improve transducer's efficiency, a matching layer is added to the external face of the crystal, providing acoustic coupling between the crystal and the patient. The thickness of this matching layer should be equal to a quarter of the US wavelength, to maximise energy transmission through the layer in both directions.

With only air behind the crystal, the internal reverberations within the crystal due to US reflection would be very strong and would continue long after the end of the voltage pulse. This unwanted ringing can be much reduced by having a backing (damping) layer behind the PZT, made of a material with the ability to absorb US such as tungsten powder embedded in epoxy resign.

2.3.1. Focused transducer

US beams can be either focused or unfocused depending on the curvature of the surface of the piezoelectric crystal. If the surface is flat, the beam will be unfocused. Using a curved surface piezoelectric crystal with a radius r, beam focusing is achieved, i.e. the beam becomes convergence and narrow in the point which has the distance r to all points on the transducer surface [103]. The location where the beam reaches its minimum diameter is termed focus or focal point. The beam can be focused at a

predetermined distance from the transducer face, which is determined by both the frequency of US wave and the diameter (D) of the transducer. This predetermined distance, is termed focal depth or focal length. At focus, the beam has its maximum intensity (figure 2.2). With focused transducers, a great amount of energy is delivered to a precise location within the body without damaging the surrounding healthy tissues.

For therapeutic applications such as brain treatments, a specially designed transducer has been developed, known as phased array. It comprised of a spherical transducer with multiple small piezoelectric crystals arranged with separate connections, which can be pulsed individually. This type of transducer offers versatility in beam steering and focusing, by applying variable delay time.

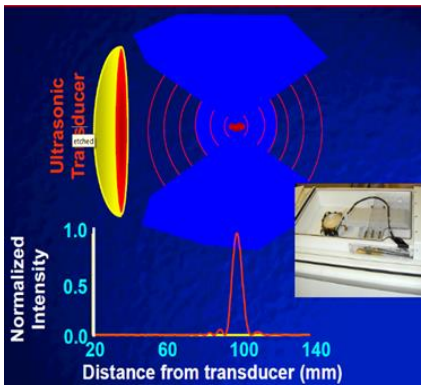

Figure 2.2: Focused transducer [103].

2.3.2. Phased-array transducer

With the use of an array of small piezoelectric crystals, multiple pressure waves are generated each of which can be pulsed separately. The timing of firing of each crystal is electronically controlled in order to precisely steer and focus the beam (electronic beam steering), as it is shown in figure 2.3. The development of phased array transducers, make possible for transcranial FUS treatments, to overcome focal distortion of the target volume due to heterogeneity in skull thickness and density.

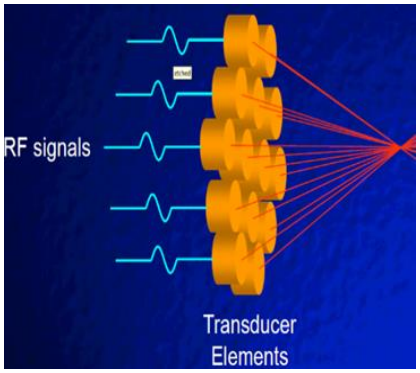

Figure 2.3: Focusing and steering capabilities of phased arrays by controlling the phase and amplitude of the RF signal driving each crystal [103].

2.4. US wave types

The classification of US waves is based on the direction the particles in the medium are oscillating, relative to the direction of energy transfer (propagation) and the material properties in which they travel. In other words, US waves are classified upon the way they travel and transfer their energy. Longitudinal waves occur when the particles oscillate along (back and forth) the direction of propagation (bungee cord).

Shear waves occur when the particles oscillate transverse (vertically up and down) to the direction of propagation (guitar string) [104]. In general, longitudinal waves travel through all kinds of materials such as gases, liquids and soft tissue, while shear waves travel only through solid material such as bone. Shear waves are quickly attenuated in soft tissues because their attenuation is higher compare to that of longitudinal waves. At interfaces between different types of tissue, longitudinal waves may be converted into shear waves when the wave incidence is not normal to the interface, which is important at the interfaces between soft tissues and bones.

Two types of modes are used to generate US waves for medical US:

1. Continuous wave mode (CW)
2. Pulse wave mode (PW)

A continuous wave is produced when the US transducer is continuously excited at constant amplitude over time, containing an infinite number of maximum and minimum amplitude peaks with the same value, also called the P_p and P_n. Since in CW mode, US energy is produced all the time, US intensity is constant over time. A PW is produced when the US transducer is excited only for a short period of time (pulse "ON") followed by a pulse "OFF" period and both the "ON" and "OFF" pulses are repeated in succession throughout the sonication period.

2.5. Pulsed US intensities

While a continuous US wave is sufficiently described by frequency (f), period (T), wavelength (λ) and propagation, for pulsed wave US several more terms are needed such as duty factor (DF), pulse duration (τ), and pulse repetition frequency (PRF). Pulse duration (τ), is the time length of a single pulse and is given by the number of cycles per pulse (N) times the period (T):

$$\tau = NT = \frac{N}{f} \tag{2.5}$$

DF, is the fractional amount of time that the pulse is activated and is given by the ratio of the pulse duration to the pulse repetition period **(PRP)**:

$$\mathbf{DF} = \frac{\boldsymbol{\tau}}{\mathbf{PRP}} = \boldsymbol{\tau}\mathbf{PRF} \qquad (2.6)$$

where, pulse duration is the time from the beginning to the end of a pulse, **PRP** is the interval between pulses. The reciprocal of **PRP** is **PRF**, which is the number of pulses per unit of time.

In pulsed mode operation, intensity may be calculated in several different ways, taking into account different power measurements and the spatial distribution of energy. Since pulsed wave intensity varies with time and distance across the beam, it can be expressed as peak or average intensity, either spatially (within a specified cross-sectional area) or temporally (within a specified exposure time). The temporal peak intensity (I_{TP}) is the maximum value of intensity measured in a pulse and the temporal average intensity (I_{TA}) is the measured average over the entire PRP. The spatial peak intensity (I_{SP}) is the maximum value of intensity at a specific location in the US beam. The spatial average intensity (I_{SA}), is the average value of intensity across the beam's cross-sectional area. The pulse average intensity (I_{PA}), is the measured average over pulse duration. Combining the above temporal and spatial considerations, the six intensities of importance are the following:

1. Spatial average, temporal average ($I_{SATA} = I_{TA}$/beam area)
2. Spatial peak, temporal average ($I_{SPTA} = I_{SATA} \times I_{SP}/I_{SA}$)
3. Spatial average, pulse average ($I_{SAPA} = I_{SATA/DF}$)
4. Spatial peak, pulse average ($I_{SPPA} = I_{SATA} \times I_{SP}/I_{SA}$)
5. Spatial average, temporal peak ($I_{SATP} = I_{SP}$/beam area)
6. Spatial peak, temporal peak (I_{SPTP})

The lowest value of intensity is the spatial average, temporal average (I_{SATA}), which is obtained by dividing the temporal average power by the area of the transducer face, i.e. it averages in space and time. The spatial peak, temporal peak intensity (I_{SPTP}) is the highest value of the temporal peak intensity in the beam area (does not average either in space or time). The measurements which are most relevant to known bioeffects mechanisms are I_{SPTA} and the $I_{SPPA.}$ The spatial peak, temporal average intensity (I_{SPTA}), is the time-averaged intensity at the position of the spatial peak, is used to estimate the thermal bioeffects of US and it is important in safety considerations. The spatial-peak, pulse-averaged intensity (I_{SPPA}), is the intensity that exists during a pulse at the position

of the spatial peak and is related with the likelihood of mechanical bioeffects, such as cavitation.

2.6. Interaction of US with tissue

As US beam propagates through different media of a body, encounters tissue types with varying physical and acoustic properties. Reflection occurs at a boundary between two different tissue types. At each boundary, an amount of US energy is reflected back and the remaining is transmitted, which means it continues deeper in the body. As an US wave travels deeper in the body, it becomes weaker, i.e. the amplitude of the wave decreases.

For diagnostic applications, it is the detection of the reflections that forms an US image. For therapeutic applications, the goal is to minimize the reflected amount of energy, so the required amount of energy to cause the desired biological effect, to be transmitted to the target.

Every medium has its own characteristic property, which is called the acoustic impedance (Z) and is the product of the speed of sound in the medium (c) and the physical density of the medium (ρ):

$$\mathbf{Z} = \boldsymbol{\rho c} \tag{2.7}$$

The acoustic impedance (Z) is also defined as the ratio of acoustic pressure (P) at a point in the medium to the particle velocity (v) at the same point. Therefore, the particle velocity:

$$v = \frac{P}{Z} \tag{2.8}$$

The maximum displacement of the particles of a medium from their zero position with amplitude (ξ_{max}) is defined as the ratio of the particle velocity (v) to the angular frequency of the wave $(\omega = 2\pi f)$:

$$\xi_{max} = \frac{v}{\omega} \tag{2.9}$$

By combining equations 2.3, 2.7 and 2.8 with 2.9 the maximum particle displacement in a medium is given by:

$$\xi_{max} = \frac{1}{\pi f}\sqrt{\frac{I}{2\rho c}} \tag{2.10}$$

The amount of reflection depends on differences in acoustic impedance (z) between media. Maximum transmission occurs when the acoustic impedance on either side of a

boundary is equal. The greater the difference in impedance at a boundary, the greater the amount of US energy reflected and the less transmitted. This is the reason why US energy does not pass from air into water or vice versa. Due to that, is very critical for all FUS systems to have an appropriate acoustic interface between the transducer and the target in order to minimize the difference in impedance. The coupling media used for this purpose include various oils, gels or degassed water. Any bubbles filled with air or gas in the path of the US beam can cause reflection, which means that the transmission will not be effective. Therefore, bubbles should be removed during setup procedures. Acoustic impedance of some common types of human tissue in relation to density, sound propagation and absorption coefficient are shown in table 1 below [102]:

Table 1: Values of acoustic impedance, density, sound propagation and absorption coefficient of some biological tissues

Material	Density (ρ) ($kg.m^{-3}$)	Propagation velocity (c) ($m.s^{-1}$)	Acoustic Impedance (Z) ($kg.m^{-2}.s^{-1}$)	Absorption coefficient (α) ($dB.cm^{-1}.MHz^{-1}$)
Air	1.2	330	400	1.64
Blood	1060	1570	1.61×10^6	0.18
Skull Bone	1810	3360	6.10×10^6	20
Brain	1030	1560	1.55×10^6	0.85
Liver	1060	1570	1.66×10^6	0.9
Lung	400	650	0.26×10^6	40
Muscle	1070	1580	1.70×10^6	1.2

2.7. Frequency as a function of depth and sharpness

The amplitude of an US wave decreases with distance as it propagates into an attenuating medium such as tissue. This attenuation in intensity is either due to absorption or scattering. Absorption represents the part of US energy that is converted into heat, while scattering represents the part that changed direction. The intensity of an US wave is attenuating exponentially and is described by the following relationship:

$$I = I_0 e^{-\mu x} \qquad (2.11)$$

with I_0 being the initial intensity and I the intensity at any point x along the propagation direction. The attenuation coefficient μ describes the loss in intensity per unit length x. The attenuation coefficient of tissue increases as a function of frequency (**f**) and is described by the following relationship:

$$\mu = a f^{b} \qquad (2.12)$$

with **a** and **b**, being constants dependent on tissue type

From equation 2.12 is derived that as the US frequency increases, the absorption per unit length (heating) increases and hence the depth of penetration decreases. At a frequency of 1 MHz, the attenuation coefficient in soft tissue is approximately 0.7 dB/cm, whereas at 2 MHz, is double (1.4 dB/cm), indicating that is linearly related with frequency. The attenuation coefficient for soft tissue can be expressed mathematically by the value of 0.5 dB/cmMHz.

The optimal frequency for therapeutic US systems varies by treatment and is a balance between depth of penetration and sharpness of the focus. As US frequency decreases, the penetration of the beam increases but the creation of a sharply defined thermal focus becomes difficult. For this purpose, most of sonothrombolysis models were developed using low frequencies in order to penetrate the skull.

2.8. Thermal energy transfer

The thermal energy (heat) produced by the absorption of US energy, is transferred to biological tissue through the following two mechanisms:

1. Relaxation absorption, which is the most important
2. Classical absorption, which is less important

Relaxation absorption is the mechanism of thermal energy transfer that is dominant in US clinical frequencies and is described by the following relationship:

$$\beta r, \mathbf{tissue} \propto \sum_{n} \frac{f^2}{1 + (\frac{f}{fr, n})^2} \qquad (2.13)$$

where β_r is the relaxation absorption coefficient, f is the operating frequency and $f_{r,n}$ are the various relaxation frequencies present in the tissue. The relaxation frequency (f_r) of each type of tissue corresponds to a relaxation time $(\tau = \frac{1}{f_r})$, which is the time required for elastic forces within the tissue to return the particles to their equilibrium position after having been displaced (either compressed or rarefacted), by a pressure wave. If the elastic forces are moving the particles to their equilibrium position (left) at the same time the next compression wave arrives, which tries to move the particles to opposite direction (right), then the two forces applied to particles are destructive and the amount of energy extracted from the pressure wave is maximum. In contrast, if the elastic forces are moving the particles to their equilibrium position (left) at the same time the next rarefaction wave

arrives (left), then the two forces applied to particles are constructive and the amount of energy extracted from the pressure wave, is minimum.

Classical absorption is the second mechanism of thermal energy transfer and is less important. Classical absorption occurs due to the friction between particles in the tissue. It is proportional to the square of the operating frequency and is described by the following relationship:

$$\beta_{classical} \propto f^2 \qquad (2.14)$$

The friction between particles in a biological tissue as they are displaced by the passage of a pressure wave converts the US energy into heat and thus is proportional to the viscosity of the medium. For thermal applications of therapeutic US such as tumour ablation where the key point is temperature elevation at the focus of the beam, classical absorption is the dominant mechanism for heating [105].

2.9. Image guidance

Image guidance is very crucial in FUS surgery in order to verify that the patient is correctly positioned and to ensure that the therapeutic target and surrounding structures have been identified and localized. Also, real-time image guidance can provide targeting feedback during treatment. A technique known as thermometry can be used in thermal therapies to ensure accurate targeting and to monitor temperature changes especially in sensitive structures such as the brain.

Currently, FUS systems are using either US or MR technique for image guidance. US guided FUS, sometimes referred as USgFUS systems [106], rely on diagnostic US imaging for target localization and post procedure verification. MR guided FUS, sometimes referred as MRgFUS systems [106], use MR for target localization and post procedure imaging for verification as well as real time monitoring of temperature changes through a technique called MR-thermometry [107]. This is due to the fact that MR parameters such as longitudinal relaxation time T_1, transverse relaxation time T_2, proton density (**PD**), diffusion coefficient (**D**), and proton resonance frequency (**PRF**) are temperature dependent.

In general, temperature has a larger effect on T_1 than T_2, while the temperature dependency of proton density is much weaker. Among all these MR parameters, **PRF** is the most reliable indicator of temperature change. The excellent linearity with field strength and good temperature dependence of **PRF** as well as its tissue type independence for most soft tissues has made this thermometry method the most commonly used. In **PRF**

thermometry technique, the temperature change is determined using phase changes in gradient echo (GRE) pulse sequences. Temperature change is relative to a baseline condition, so images acquired during treatment are subtracted from baseline images acquired prior to temperature elevation. The change in temperature is described with the following formula:

$$\Delta T = \frac{\Delta\varphi\ (T - T_0)}{\gamma\alpha\ B_0 TE} \qquad (2.15)$$

where, $\Delta\phi$ is the absolute phase change of the MR signal at a starting and final temperature T and T_0 respectively, γ is the gyromagnetic ratio, B_0 is the main magnetic field, TE is the echo time and α is the PRF change coefficient.

MR-thermometry based on PRF phase mapping method, can provide quantitative temperature measurements over the temperature range of interest used in FUS treatments, which are independent of tissue type and can be acquired at almost real-time using customized gradient echo (GRE) pulse sequences. PRF thermometry works best when the baseline and treatment images are perfectly aligned [107]. Any misalignment, caused by patient movement or internal organ motion, edema, structural changes and deformation of the treated tissue due to thermal coagulation, might cause artifacts in the resulting thermal map. Additionally, PRF phase mapping cannot be used to measure temperature within fatty tissue (e.g. breast), as the phase dependence of fat is almost independent of temperature [107].

2.10. Biological effects of FUS

The interaction of US waves with the targeted tissue can produce a variety of biological effects (bioeffects) that can be beneficial in a wide range of therapeutic applications. Bioeffects are classified into:

1. Thermal effects (continuous wave exposure), cause changes related with temperature elevation as a direct result of US energy absorption in tissue. Thermal effects occur when tissue is damaged due to heating such as thermal ablation treatment.
2. Non-thermal or mechanical effects (pulsed wave exposure), cause changes related with the mechanical interactions between US energy and tissue. Non thermal effects occur when tissue is damaged due to mechanical forces and can be categorized as primary mechanical effects such as acoustic radiation force (ARF) or secondary mechanical effects such as acoustic cavitation. ARF refers to the transfer of momentum from the propagating US wave in an attenuating medium. ARF can induce displacement in tissue

and motion in fluids. Cavitation activity refers to both bubble formation and oscillation due to an acoustic pressure and is divided into stable and inertial cavitation. Stable cavitation due to sustained bubbles motion generates microstreaming of the surrounding fluid, while inertial cavitation due to violent collapse of bubbles is associated with effects such as shock waves and liquid microjets. The non-thermal mechanisms underlying the biological effects induced when MBs are excited by FUS energy, are well presented in figure 2.4.

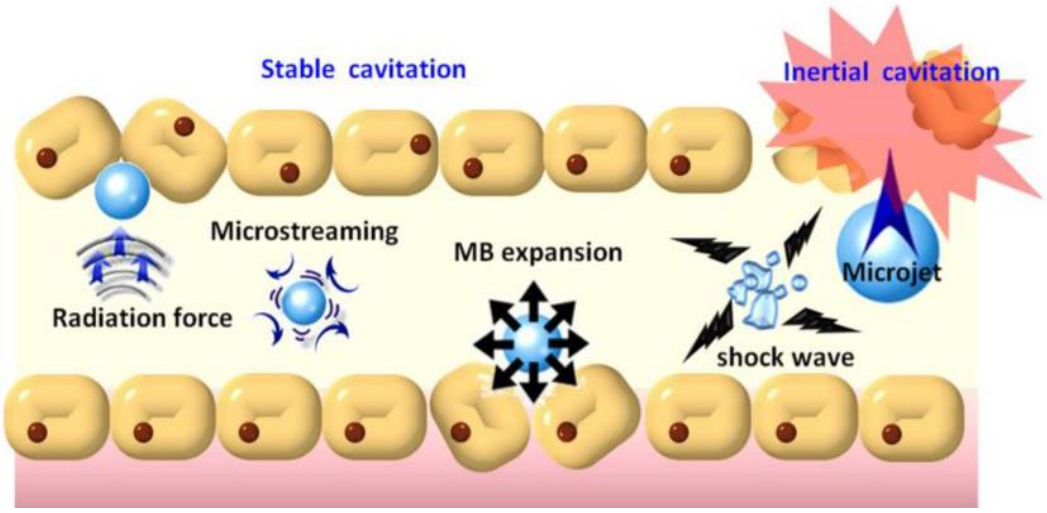

Figure 2.4: In stable cavitation, radiation forces, microstreaming and shear stresses resulting from bubble oscillation can cause localized micron sized stretching of cells membrane, leading to increased cell permeability. In inertial cavitation, shock waves from violent bubble implosion, cause cell membrane disruption [88], [108].

2.11. Thermal effects

US absorption in tissue causes microscopic-scale frictional heating, generated by shearing due to longitudinal compression and rarefraction pressure variations of the US. The friction between the molecules causes temperature elevation. The amount of temperature increase, which can be measured with techniques such as MR thermometry, depends on DF, intensity, frequency, type of tissue etc. It should be noted that when used for tissue ablation, FUS thermal effects are significantly different from traditional hyperthermia therapies as used in oncologic settings. Ablative FUS achieves much higher temperatures in a much shorter amount of time, and in a more localized area of tissue. Thermal effects causing ablation of tissue are currently the primary effects used in FUS.

2.12. Non thermal effects

2.12.1. Acoustic radiation force

Is a physical phenomenon associated with the interaction of a propagating US wave with a biological tissue. As a result, the medium is subjected to a mechanical force, which has the ability to deform it (tissue), or to set it in motion in the direction of propagation (fluid) [53]. Even though this tissue displacement induced by radiation force is small, is detectable and can be used in Magnetic Resonance Acoustic Radiation Force Imaging (MR-ARFI). This imaging technique uses highly motion sensitized gradients to measure tissue displacement within a resolution of micrometre (μm) and thus the position of the focal point of an US beam can be detected in MRI without significant tissue heating. The acoustic radiation force **(ARF)** generated in tissue due to the transfer of momentum from the propagating FUS wave, can be written in terms of the absorption coefficient of the medium (**α**), transmitted frequency **(f),** intensity at beam focus (**I**) and speed of sound in medium (**c**):

$$ARF = \frac{2\alpha f I}{c} \qquad (2.16)$$

2.12.2.Acoustic streaming

As US waves propagate through a fluid, can transfer momentum to the fluid upon interaction, thus creating a force, which depends on viscosity, absorption coefficient and US energy. This force is set the fluid in motion, creating a circular flow along the propagation of US wave. Acoustic streaming can induce high velocity gradients at the boundary between fluid and more rigid structures such as thrombi, which in turn causes shearing stresses, promoting the exposure of the fibrin network to vigorous drugs [55]. The shear stress associated with a velocity gradient **(grad V)** is given by:

$$\mathbf{S = n\,grad(V)} \qquad (2.17)$$

where **n** is the viscosity of water at 37 °C

2.12.3.Acoustic cavitation

Is the interaction of US waves with microscopic gas bubbles, commonly known as MBs. In order for cavitation to occur in a medium, the presence of pockets containing gas at locations termed cavitation sites is required. When the negative pressure of the rarefaction cycle is high enough to exceed the tensile strength of medium, the particles are pulled apart and gas is drawn out from the cavitation sites, forming bubbles. Once a

bubble is formed, oscillates in phase with the applied US field and grows with time until it reaches unstable size and collapses [109]. A graphical summary of the of bubble formation, growth and collapse over several cycles is shown in figure 2.5.

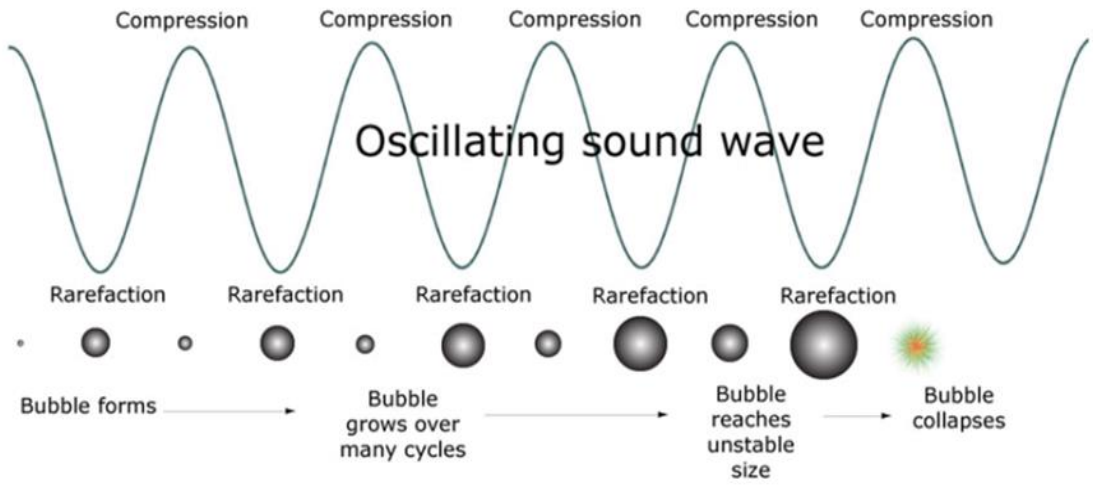

Figure 2.5: Bubble forms, oscillates in phase with the applied US field, grows with time and collapses when it reaches unstable size [109].

Two possible cavitation phenomena may occur, which are triggered above a certain threshold value, depending on the frequency of the US wave (figure 2.6):

1. Stable cavitation: At low acoustic pressures, bubbles oscillate at, or near their resonance size for a long period of time.
2. Unstable or inertial cavitation: At higher acoustic pressures, bubbles rapidly expand, become unstable and collapse violently during the compression cycle of US wave over a very short period of time.

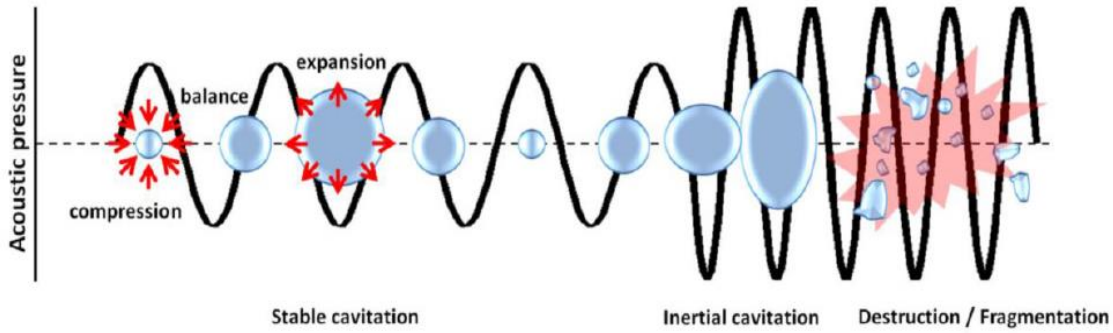

Figure 2.6: Effect of acoustic pressure on MBs oscillation and explosion [108], [110].

A simple relationship that can relate the resonance size of the bubble with the frequency is given by equation:

$$F \times R \approx 3 \qquad (2.18)$$

where, **F** is the frequency in Hz and **R** is the bubble radius in m. Note that this equation gives only a very approximate theoretical resonance size [111].

The pressure amplitude required to initiate stable oscillations is always lower than that required for inertial cavitation. Therefore, the threshold for the onset of inertial cavitation is reached with a small increase in the pressure amplitude of stable cavitation.

The ability of an US wave to induce cavitation is given by the Mechanical Index **(MI)**, which is an estimation of the biological effects induced by an US wave. **MI** is defined as the $\mathbf{P_n}$ (MPa), divided by the square root of the US transmitted frequency **f** (MHz):

$$\mathbf{MI} = \frac{\mathbf{P_n}}{\sqrt{\mathbf{f}}} \qquad (2.19)$$

2.12.4.Microstreaming

Is a phenomenon that occurs in fluids when a gas bubble undergoes direct oscillation due to US exposure. During oscillation, the gas bubbles displace small amount of fluid surrounding them, causing rapidly moving streams around their surfaces (eddying circular currents), termed microstreams. The fluid velocity is greatest near the bubble surface and decreases with distance from the bubble. Due to this velocity changes, a gradient exists in the vicinity of fluid around the oscillating bubble. Strong velocity gradients, form shear forces on the side of the cell near the bubble, which can damage cell membrane [112]. Therefore, shearing forces resulting from oscillating gas bubbles close to a rigid structure (thrombus or brain barrier), may disrupt or tear biological tissues [113].

2.12.5. Shock waves and liquid microjets

At high enough acoustical pressures, bubbles oscillations become highly nonlinear, the bubbles expand to a certain size (unstable) and then, their radius decrease rapidly and collapse violently [88]. The stored energy is released in the form of shock waves within the bubbles and surrounding fluid. This violent collapse also produces high velocity liquid microjets. Biological tissues exposed to shock waves and high velocity microjets, experienced stresses large enough to damage them (cell membrane disruption) [67], [88].

2.13. Therapeutic applications of FUS

The interaction of FUS with the targeted tissue can induce different biological effects as a result of either thermal or non-thermal mechanisms. These localised biological effects are determined by the type of tissue and the acoustic parameters such as power, exposure time, and mode (continuous or pulsed). By using either thermal energy to destruct tissue (thermal ablation), or mechanical energy to increase the precise delivery of drugs to targets in the body (sonoporation) or to dissolve blood clots (sonothrombolysis), FUS can be applied to treat noninvasively a wide range of different diseases [114].

2.13.1. Sonoporation

Cell membranes often prevent large molecules such as drugs or genes to pass into the cells and take effect. Low intensity US, alters temporarily cell membrane permeability and enhance the absorption of these molecules. When non thermal mechanisms such as stable cavitation occur at the boundary of cell membrane, have sufficient strength to modify the permeability of cell membranes by creating pores, allowing the therapeutic molecules to enter into the cell (figure 2.6). Additionally, the microstreaming produced, increases the flow of fluid in cell's environment enhancing cellular uptake. This effect, known as sonoporation can increase the efficacy of drugs and genes in precise areas in the body. Potential applications taking advantage of this effect include drug delivery, gene therapy and selective opening of the blood-brain-barrier (BBB).

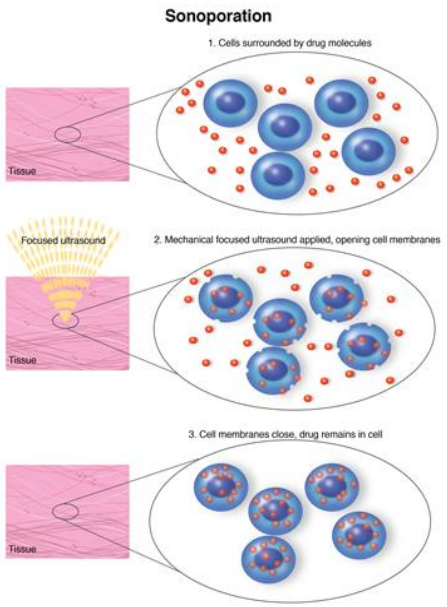

Figure 2.7: FUS alters temporarily cell membrane permeability allowing large therapeutic molecules such as drugs to pass into the cells [114].

2.13.2. Sonothrombolysis

FUS, either alone, or as an adjunct to thrombolytic agents and/or in combination with microbubbles (MBs), has been shown to enhance thrombolysis and dissolve blood clots (figure 2.7). US energy is capable of accelerating clot breakdown via mechanisms that include thermal heating acoustic radiation force and cavitation. This could play a significant role in thrombolytic therapy such as the treatment of stroke, since there is a low risk of side effects outside the focal zone, although a high amount of energy is deposited at beam focus.

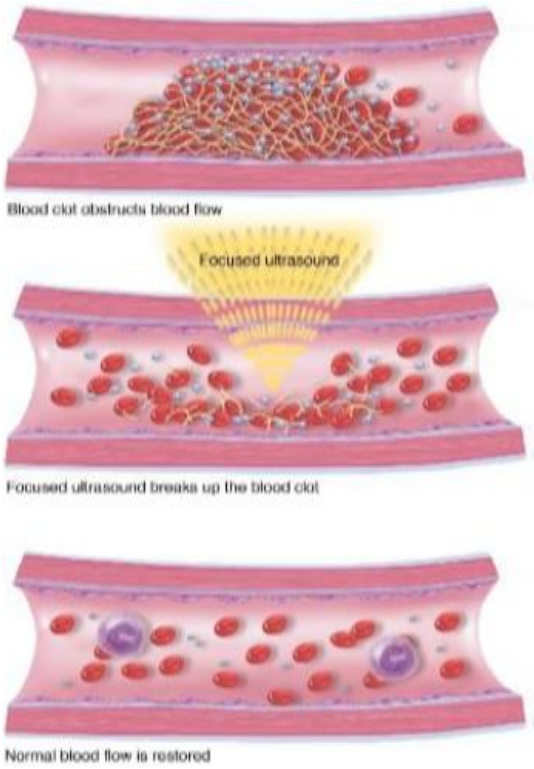

Figure 2.8: FUS is capable of breaking down the fibrin matrix of blood clots [114]

3. Review of protocols used in ultrasound thrombolysis

3.1. Introduction

One of the early investigators who used US energy to accelerate the fibrinolytic activity of thrombolytic drugs in vitro, was Lauer et al. 1992 [60]. Later, more in vitro studies [59], [115], [116], have shown that US applications, improved thrombolysis induced by thrombolytic agents (sonothrombolysis). The knowledge on US thrombolysis was enhanced by performing experiments in animals [78], [117], [118]. When sufficient data were collected, this research was translated into clinical trials [91], [119], [120].

This chapter focuses on the review of protocols used in thrombolysis studies with US. The protocols of several published reports are summarised in three tables (in vitro, in vivo and clinical), providing detailed information concerning clot model, thrombolytic drug, treatment mode, sonication parameters, evaluation method, thrombolysis outcome, side effects and conclusions.

3.2. Aim of the review

This review aims to give an overview of the different protocols used so far in the field of sonothrombolysis and to investigate the impact of several aspects involved, on thrombolysis outcome.The review provides information regarding: (1) the clot model used (human or animal for the in vitro studies and type of occlusion for the animal and clinical studies), (2) the US technique applied such as external or internal (catheter based) and focused or unfocused, (3) the use of flow system (only in vitro), (4) the temperature (only in vitro), (5) the type and concentration of thrombolytic drug used, (6) the treatment mode (US alone, drug alone, US + drug, US + MBs, and US + drug + MBs), (7) the sonication parameters applied, such as frequency, intensity or acoustic power or negative pressure, pulse repetition frequency (PRF), duty factor (DF) and treatment time, (8) the evaluation method used to estimate study's outcome, (9) the effect of treatment on clot lysis and (10) the main conclusions derived.

3.3. Methodology

This review is divided into three categories (in vitro, in vivo and clinical) and provides a comprehensive compilation of protocols used during US thrombolysis studies with or without thrombolytic drugs and/or MBs, since 1992.

Published reports on thrombolysis with US that are available in PubMed (www.ncbi.nlm.nih.gov/pubmed), were collected. Information in several aspects of the protocols used in the studies examined, were also extracted. In animal studies as well as in clinical trials, the following info was needed: treatment time, evaluation method, treatment's outcome, side effects and main conclusions. In the in vitro compilation, the additional info needed was temperature.

There is an absence in standardization about the necessary info collected from each study due to different methods/measuring units used by the investigators. For example, the output of US transducer is specified in different units (intensity, acoustic power, negative pressure etc.) and the treatment's outcome is quantified by different evaluation methods such volume reduction, fibrin degradation products (FDP), lytic rate, recanalization rate, etc. Furthermore, in some cases experimental parameters like temperature, PRF and DF are not specified.

3.4. Analysis of results

Table 1 lists the in vitro studies, which include info that describe the clot model used (human or animal clot, flow system, focused or unfocused US waves, external or internal US device, temperature, type and concentration of thrombolytic drug). Also, info is provided regarding the treatment mode (US, drug, MBs), along with the sonication parameters applied (frequency, output, PRF, DF and treatment time). Moreover, the evaluation method, the treatment's outcome and the main conclusions are presented as well.

Table 2 lists the in vivo studies, which include info that describe the clot model used (type of occlusion, focused or unfocused US waves, external or internal US device, type and concentration of thrombolytic drug). Also, info is provided regarding the treatment mode (US, drug, MBs) and the sonication parameters applied (frequency, output, PRF, DF and treatment time). Moreover, the evaluation method, the treatment's outcome, the side effects and the main conclusions are also presented.

Table 3 lists the clinical studies, which include info that describe the clot model used (type of occlusion, focused or unfocused US waves, external or internal US device, type and concentration of thrombolytic drug). Also, info is provided regarding the treatment mode (US, drug, MBs) and the sonication parameters applied (frequency, output, PRF, DF and treatment time). Moreover, the evaluation method, the treatment's outcome, the side effects and the main conclusions are presented as well.

3.5. Review of protocols used in ultrasound thrombolysis

Table 2: In vitro protocols used in US thrombolysis

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Human	rt-PA (3000 IU/mL)	37		Yes							50	Measured volume reduction	33 %	[60]
					Yes		1 MHz	1.75 W/cm^2 I_{SATA}	Intermittent	50	50		50 %	
				Yes							200		62 %	
					Yes		1 MHz	1.75 W/cm^2 I_{SATA}	Intermittent	50	200		91 % US combined with rt-PA caused a significant enhancement of thrombolysis compared with rt-PA alone.	
Human	rt-PA (1 µg/mL)	37		Yes							60	Measured volume reduction	8 %	[115]
					Yes		27 kHz	1 W/cm^2	70	10	60		17 %	
					Yes		40 kHz	1 W/cm2	70	10	60		20 %	
					Yes		100 kHz	1 W/cm2	70	10	60		15 % US in the range of 27 to 100 kHz is effective in accelerating fibrinolysis at intensities and pulsing conditions that minimize the probability of heating and cavitation.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human	rt-PA (3000 IU/mL)	36		Yes							60	Measured volume reduction	22.7 %	[56]
					Yes		2 MHz	1.2 W/cm^2	cw	100	60		49 % (travelling)	
					Yes		2 MHz	1.2 W/cm^2	cw	100	60		34.8 % (standing)	
					Yes		2 MHz	1.2 W/cm^2	1, 10, 100, 1000	50	60		46.4, 39.8, 44, 34 % Intermittent application of a 2 MHz high frequency US using a travelling wave field would be the most potent application for lysing blood clots. No effect of PRF on clot lysis.	
Human	rt-PA (3000 IU/mL)	37	Yes				1.95 MHz	1.2 W/cm^2 I_{SATA}	1	50	60	Measured volume reduction	25.3 %	[116]
				Yes							60		19.9 %	
					Yes		1.95 MHz	1.75 W/cm^2 I_{SATA}	1	50	60		35.2 % US enhances thrombolysis by affecting the distribution of rt-PA within the clot.	
Human	rt-PA (3.15 µg/mL)	37		Yes							30	Measured lytic rate	0.5 µm/min	[121]
					Yes		120 kHz	0.35 MPa	1667	80	30		3.4 µm/min US treatment + rt-PA significantly enhanced the mean lytic rate (580% change), compared with rt-PA treatment alone.	
Human	rt-PA (3.15 µg/mL)	37		Yes							30	Measured lytic rate	7 µm/min	[122]
					Yes		120 kHz	0.35 MPa	1667	10, 20, 50, 80	30		15, 25, 50, 65 µm/min The lytic efficacy of clots exposed to rt-PA and US increases with increasing DF.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Porcine	rt-PA (107 µg/mL)	37		Yes							30	Measured volume reduction	12 %	[59]
					Yes		120 kHz	0.35 MPa	1.7 k	80	30		19.1 %	
					Yes		1 MHz	0.35 MPa	1.7 k	100	30		25 %	
					Yes		1 MHz	0.35 MPa	1.7 k	10	30		22 %	
													Both 120-kHz and 1-MHz pulsed and cw US enhanced rt-PA thrombolysis in a porcine whole blood clot model.	
Human	rt-PA (10 µg/mL)	37		Yes							30	Measured volume reduction	16 %	[123]
					Yes		120 kHz	0.35 MPa	1667	50	30		31 %	
													120-kHz US substantially increases the lytic efficacy of rt-PA for almost all rt-PA concentrations.	
Human	rt-PA (3.15 µg/mL)	37		Yes							30	Measured volume reduction	31 %	[63]
					Yes		120 kHz	0.35 MPa	1667	50	30		71 %	
				Yes		Yes (t-ELIP)					30		48 %	
					Yes	Yes (t-ELIP)	120 kHz	0.35 MPa	1667	50	30		89 %	
													The addition of 120 kHz US with t-ELIP substantially increases the rate of lysis compared with either tPA or t-ELIP alone.	
Human	rt-PA (0.5-3.15 µg/mL)	37		Yes							30	Measured volume reduction	38-44 %	[124]
					Yes		120 kHz	0.18 MPa	1667	50	30		50-70 %	
					Yes		2 MHz	0.47 MPa	10.5 k	13	30		52-58 %	
													Combination treatment with rt-PA is more effective than rt-PA alone in human whole blood clots.	

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Fibrin gel	rt-PA (0.1 µg/mL)	25			Yes		1 MHz	2 W/cm^2	100	50		Measured binding ratios	US exposure accelerates rt-PA binding, alters binding affinity, and increases maximum binding to polymerized fibrin	[125]
Fibrin gel			Yes				1 MHz	4 W/cm^2	c.w.	100	15	Measured fiber density Measured fiber diameter	> 65 % < 27 % US exposure causes reversible disaggregation of uncrosslinked fibrin fibers into smaller fibers, an effect that may alter flow resistance and create additional binding sites for fibrinolytic components, improving fibrinolytic efficacy.	[51]
Human	rt-PA (0.1 µg/mL)	37		Yes							240	Measured uptake rate	8.2 %	[46]
					Yes		1 MHz	4 W/cm^2	c.w.	100	240		15.5 % Exposure to US increases uptake of rt-PA into clots and also results in deeper penetration.	
Human	rt-PA (2 µg/mL)			Yes							60	Measured fibrin degradation product, D-dimer (FDP-DD)	957 ng/mL	[57]
					Yes		300 kHz	0.07 W/cm2	c.w.	100	60		1669 ng/mL	
					Yes		1 MHz	0.4 W/cm^2	c.w.	100	60		1727 ng/mL The combination of drug and US leads to degradation of fibrin, allowing a quantitative measurement of the enhancement of clot lysis. A high correlation was observed between the FDP-DD produced with the rate of decrease in clot weight.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human Flow system	UK (400 U/mL)	37		Yes							60	Measured volume reduction	18.7 %	[126]
					Yes		1 MHz	2.5 W/cm^2	c.w.	100	60		52.5 % C.W. US at 1 MHz and an intensity of 2.5 W/cm2 accelerates urokinase-induced thrombolysis and reperfusion.	
Human	SK (5000 U/mL)	37		Yes							65	Measured reperfusion rate	100 %	[127]
					Yes		170 kHz	0.5 W/cm^2 (I_{SATA})	c.w.	100	34		100 %	
				Yes							39		100 %	
					Yes		1 MHz	1 W/cm^2 (I_{SATA})	c.w.	100	20		100 % US exposure of a type and intensity that may be transmitted transthoracically accelerates the thrombolytic process (for both frequencies used).	
Human HIFU	rt-PA (10 µg/mL)	37	Yes				1 MHz	60 W	1	10	30	Measured volume reduction	33 %	[75]
				Yes							30		53.1 %	
					Yes		1 MHz	60 W	1	10	30		63.2 % The rate of tPA-mediated thrombolysis can be enhanced by using pulsed HIFU exposure.	
Human	rt-PA (1.0 µg/mL)	37		Yes							60	Measured volume reduction	12.8 %	[35]
					Yes		1 MHz	1, 2, 4, 8 W/cm^2	c.w.	100	60		18, 19.3, 22.8, 58.7 % US at 1 MHz potentiates enzymatic fibrinolysis by a non-thermal mechanism. Thrombolysis efficiency increases with intensity.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human	UK (200, 2000, 5000 µg/mL)	room	Yes				1 MHz	2.2 W/cm^2	n/s	n/s	30	Measured volume reduction	18 %	[128]
				Yes							30		19, 44, 50 %	
					Yes		1 MHz	2.2 W/cm^2	n/s	n/s	30		41, 55, 61 %	
	SK (50, 250, 2000 µg/mL)		Yes				1 MHz	2.2 W/cm^2	n/s	n/s	30		26 %	
				Yes							30		37, 50. 62 %	
					Yes		1 MHz	2.2 W/cm^2	n/s	n/s	30		45, 61, 73 %	
													The use of external US has the potential to increase both efficacy and rate of thrombolysis. Thrombolysis efficiency increases with drug dose. Thrombolytic activity of SK more effective than UK.	
Human	rt-PA (1.0 µg/mL)	37		Yes							60	Measured volume reduction	23 %	[129]
					Yes		2.2 MHz	0.5, 1, 2, 4, 8 W/cm^2	n/s	n/s	60		31, 40, 48, 69, 88 %	
													US accelerates enzymatic fibrinolysis by increasing transport of reactants through a cavitation-related mechanism. Thrombolysis efficiency increases with intensity.	
Human	rt-PA (66.7 µg/mL)	37		Yes							25	Measured volume reduction	7.24 %	[126]
					Yes		0.5 MHz	8 W/cm^2	c.w.	100	25		26.7 %	
													US waves accelerate rt-PA-induced thrombolysis and reperfusion.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human	rt-PA (1 µg/mL)	37		Yes							60,120	Measured volume reduction	13, 37 %	[130]
					Yes		40 kHz	0.25 W/cm^2	c.w.	100	60,120		39, 93 %	
					Yes		40 kHz	0.75, 1, 1.5 W/cm^2	c.w.	100	60		58. 75, 77 %	
													40-kHz US significantly accelerates enzymatic fibrinolysis with excellent tissue penetration and minimal heating. Thrombolysis efficiency increases with intensity.	
Human	rt-PA (3000 U/mL)	37		Yes							60	Measured volume reduction	23.8 %	[55]
					Yes		1 MHz	1.2 W/cm^2	c.w.	100	60		34.8 (standing)	
				Yes							60		11.3 %	
					Yes		2 MHz	1.2 W/cm^2	c.w.	100	60		24.5 % (travelling)	
													Travelling US waves enhanced thrombolysis (116.8%), which is significantly more than standing US waves did (46 %).	
Human	rt-PA (3 µg/mL)	37	Yes				20 kHz	0.35 W/cm^2	c.w.	100	10	Measured volume reduction	41.8 %	[131]
				Yes							20		49.1 %	
					Yes		20 kHz	0.35 W/cm^2	c.w.	100	10		65.8 %	
													The use of low-frequency US alone, has the potential to induce thrombolysis. Combination of US with rt-PA is superior to either treatment alone.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human	rt-PA (3.15 µg/mL)	37		Yes							30	Measured volume reduction	15.6 %	[132]
				Yes + Epf.							30		28 %	
					Yes		120 kHz	0.18 MPa	1667	80	30		44.4 %	
					Yes + Epf.		120 kHz	0.18 MPa	1667	80	30		30.3 %	
													Although the addition of eptifibatide enhances the lytic efficacy of rt-PA alone, the efficacy of US and rt-PA is greater than that of combined US, rt-PA and eptifibatide exposure.	
Human	UK (2 mg/mL)	37		Yes							720	Measured volume reduction	43 %	[133]
					Yes		211.5 kHz	0.25 W/cm^2	c.w.	100	720		61 %	
													Low-frequency US transmits well through human temporal bone and enhances thrombolysis.	
Human Through skull	rt-PA (100 µg/mL)	37	Yes				33.3 kHz	0.5 W/cm^2	n/s	n/s	60, 180	Measured volume reduction	39.96, 44.09 %	[134]
			Yes				71.4 kHz	3.4 W/cm^2	n/s	n/s	60, 180		32.24, 35.17 %	
				Yes							60, 180		46.55, 56.27 %	
					Yes		33.3 kHz	0.5 W/cm^2	n/s	n/s	60, 180		51.04, 67.89 %	
					Yes		71.4 kHz	3.4 W/cm^2	n/s	n/s	60, 180		46.23, 60.47 %	
													Transcranial application of US can shorten the recanalization time of intracerebral vessel occlusion by increasing rt-PA-mediated thrombolysis. Thrombolysis efficiency increases with time.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human	rt-PA (3.15 µg/mL)	33, 37	Yes				120 kHz	3.2 W/cm^2	1667	80	30	Measured volume reduction	7.5, 7.2 %	[135]
				Yes							30		8.6, 12.4 %	
					Yes		120 kHz	3.2 W/cm^2	1667	80	30		21.2, 22.7 %	
													The efficacy of US enhanced thrombolysis decreases at temperatures below the body baseline temperature of 37 ^{0}C.	
Human Through temporal bone	rt-PA (10 µg/mL)	37	Yes				1.8 MHz	1.6 MI	n/s	n/s	60	Measured volume reduction	41 %	[136]
				Yes							60		70.8 %	
					Yes		1.8 MHz	1.6 MI	n/s	n/s	60		78.7 %	
													Diagnostic transcranial US with rt-PA, enhances thrombolysis.	
Human Through skull HIFU Flow system		n/s	Yes				220 kHz	111 W/cm^2	2.5	50	0.5	Measured volume reduction	76.1 % (flow)	[81]
		n/s	Yes				220 kHz	111 W/cm^2	2.5	50	0.5		29.9 % (no flow)	
													Transskull HIFU for immediate clot lysis without the need of further drugs and disregarding individual skull bone characteristics is feasible.	
Human	UK (1200 IU)	37		Yes							60	Measured volume reduction after incubation	40.6 %	[35]
					Yes		48 kHz	5-6 kPa	n/s	n/s	60		59.2 %	
				Yes							60, 120		8.9, 46.7 %	
					Yes		225 kHz	30 mW/cm^2	n/s	n/s	60, 120		37.3, 61.1 %	
													US energy enhanced fibrinolysis with UK, especially in the early phase of lysis. Thrombolysis efficiency increases with time and decreases with frequency.	

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Bovine Through skull Flow system	rt-PA (100 µg/mL)	37 37		Yes	 Yes		 1 MHz	 0.35 W/cm^2 (I_{SPTP})	 16 k	 41.6	29.3 17.1	Measured recanalization rate	100 % 100 %	[137]
					Yes		185 kHz	1.27 W/cm2 (I_{SPTP})	c.w.	100	14.1		100 % Transcranial application of low frequency, c.w. US may accelerate reperfusion and shorten the recanalization time.	
Bovine Through skull Flow system	rt-PA (100 µg/mL)	37		Yes	 Yes		 1 MHz	 0.35 W/cm^2 (I_{SPTP})	 16000	 41.6	30 30	Measured recanalization rate	30 % 90-100 % Transcranial application of 1 MHz US may accelerate reperfusion and recanalization rate of occluded intracerebral vessels.	[58]
Human Through skull HIFU Flow system		24	Yes				220 kHz	13.7, 27.4, 54.8, 136.8 W/cm^2 (I_{SPTA})	0.5, 5, 50, 500	5, 10, 20, 50	0.5	Measured volume reduction	10.3-27.2 % 17.1-42.9 % 30-59.6 % 48.7-59.2 % Using transcranial HIFU, significant thrombolysis can be achieved within sec and without the use of lytic drugs. Longer DF in combination with longer pw seem to have the highest potential to optimize clot lysis efficacy.	[138]
Human FUS		n/s	Yes Yes				550 kHz 535 and 565 kHz	200 W 110 W	3.7 3.7	10 10	5 5	Measured volume reduction	80 % 80 % The power needed to achieve 80 % of thrombolysis with a monofrequency excitation is reduced by the half with a bifrequency excitation.	[139]

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Human	rt-PA (60 kU/mL)	37		Yes							60	Measured volume reduction	36.7 %	[140]
					Yes		2 MHz TCCD	0.179 W/cm^2	n/s	n/s	60		40.8 %	
					Yes		2 MHz TCD	0.457 W/cm^2	n/s	n/s	60		40.4 % Although clot lysis rate after I h treatment with rt-PA alone was significant, a slight increase of weight loss was detected under the application of US + drug.	
Human HIFU		37	Yes				230 kHz	1000 W	1000	10	0.5	Measured volume reduction	82 % After sonication, the clot was nearly completely lysed.	[87]
Human Through skull HIFU Flow system		37	Yes				220 kHz	29.71-193.24 W/cm^2 (I_{SPTA})	2,5	50	0.5	Measured volume reduction	4.55-74.83 % Transcranial sonothrombolysis could be achieved within sec in the absence of rt-PA and without producing relevant clot fragmentation, using acoustic output powers of <400 W.	[141]
Bovine		n/s	Yes				500 kHz	I_{SPTA} > 35 W/cm^2	200	4	4	Measured volume reduction	91 % External HIFU thrombolysis for periods of ≤ 5 min appears to be a safe and effective method to induce thrombolysis.	[142]

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Rabbit Flow model		room	Yes				1.51 MHz	185 W	1	0.1	0.33	Measured volume reduction	99.2 % HIFU thrombolysis is feasible as a means of restoring partial blood flow in thrombus occluded arteries in the absence of thrombolytic agents.	[82]
Human	rt-PA (10 µg/mL)	37	Yes				1 MHz	4 W/cm^2 (I_{SATA})	c.w.	100	60	Measured volume reduction	6.8 % @ 0 atm	[143]
				Yes							60		21.8 % @ 0 atm	
					Yes		1 MHz	4 W/cm^2 (I_{SATA})	c.w.	100	60		39.3 % @ 0 atm US is ineffective in increasing fibrinolysis without a fibrinolytic agent present. An 80% increase in clot lysis occurs when US and agent are both present (no overpressure).	
Porcine	rt-PA (107 µg/mL)	37		Yes							30	Measured volume reduction	13 %	[144]
					Yes		120 kHz	0.15 MPa	1667	80	30		13.7 % (< SC)	
					Yes		120 kHz	0.24 MPa	1667	80	30		26 % (SC)	
					Yes		120 kHz	0.36 MPa	1667	80	30		20.7 % (SC+ IC) Significant enhancement of thrombolysis correlates with presence of cavitation. Stable cavitation appears to play a more important role in the enhancement of thrombolysis.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human Catheter type Flow system	rt-PA (300 µg/mL)	37		Yes							10	Measured volume reduction	13.6 %	[145]
					Yes		1.7 MHz	62.46 W/cm^2 (I_{SPPA})	30	8.5	10		21.4 %	
					Yes		1.7 MHz	180.02 W/cm^2 (I_{SPPA})	40	4	10		46.1 % Thrombolysis efficiency increases with intensity.	
Human	rt-PA (96 µg/mL)	37	Yes			Yes	120 kHz	0.32 MPa	1667	80	30	Measured volume reduction	6.3 %	[69]
				Yes							30		13.0 %	
					Yes		120 kHz	0.32 MPa	1667	80	30		16.0 %	
					Yes	Yes	120 kHz	0.32 MPa	1667	80	30		26.2 % MBs administration further increases the effect of US on rt-PA induced thrombolysis.	
Bovine	rt-PA (1000 IU/mL)	37	Yes			Yes	500 kHz	0.7 W/cm^2	c.w.	100	1	Measured volume reduction	25.8 %	[146]
				Yes							1		24.2 %	
					Yes		500 kHz	0.7 W/cm^2	c.w.	100	1		24.9 %	
					Yes	Yes	500 kHz	0.7 W/cm^2	c.w.	100	1		29.2 % MBs have slightly accelerated the thrombolytic effect of rt-PA.	
Human Catheter type	rt-PA (5000 IU/mL)	37	Yes			Yes	1.7 MHz	4.9 W/cm^2 I_{SATA}	n/s	n/s	30	Measured volume reduction	0.95 %	[66]
				Yes							30		7.68 %	
					Yes		1.7 MHz	4.9 W/cm^2 I_{SATA}	n/s	n/s	30		11.10 %	
					Yes	Yes	1.7 MHz	4.9 W/cm^2 I_{SATA}	n/s	n/s	30		14.41 % SC plays an important role in MB-enhanced US accelerated rt-PA mediated thrombolysis.	

Clot model	Drug	Temp. (^{0}C)	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Ref.
			US alone	Drug alone	US + Drug									
Human Flow system	rt-PA (20 µg/mL)	37	Yes				2 MHz	455 mW/cm^2 I_{SPTA}	5000	n/s	30	Measured volume reduction	6.1 %	[68]
			Yes			Yes		455 mW/cm^2 I_{SPTA}	5000	n/s	30		10.9 %	
					Yes		2 MHz	455 mW/cm^2 I_{SPTA}	5000	n/s	30		13.1 %	
					Yes	Yes	2 MHz	455 mW/cm^2 I_{SPTA}	5000	n/s	30		30.7 % The application of MBs strongly accelerates lysis of clots exposed to low-intensity US with rt-PA.	
Rabbit		25	Yes			Yes (3 µm)	1 MHz	0.1 W/cm^2	100	20	30	Measured volume reduction	18 %	[147]
			Yes			Yes (1 µm)	3 MHz	2 W/cm^2	100	20	30		18 % Sonothrombolysis efficacy was achieved at twenty-fold lower intensity with 3 µm MBs (0.1 W/cm2) than with 1 µm MBs (2.0 W/cm2).	
Porcine Flow system	rt-PA (7.1 µg/mL)	37		Yes							30	Measured volume reduction	29 %	[148]
				Yes		Yes	120 kHz	0.44 MPa	c.w.	100	30		34 %	
					Yes	Yes	120 kHz	0.44 MPa	c.w.	100	30		83 % (SC) SC nucleated by an infusion of MBs enhances rt-PA thrombolysis without apparent treatment-related damage.	
Human Flow system	rt-PA (3 µg/mL)	37		Yes							60	Measured clot diameter loss	6.6 µm/min	[149]
	rt-PA (0.3 µg/mL)				Yes	Yes	1.6 MHz	600 kPa	0.33	33	60		5.9 µm/min The combination of US, MB and a low dose of rt-PA (0.3 µg/mL) is as effective for thrombolysis as is a high dose of rt-PA (3 µg/mL) alone.	

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Porcine Flow system	rt-PA (3 µg/mL)	37		Yes							30	Measured volume reduction	25.6%	[150]
					Yes	Yes	1 MHz	1 MPa	0.2	0.002	30		55.7 % The US + MB + rt-PA treatment showed dramatically higher lytic efficacy than rt-PA treatment alone.	
Porcine Flow system	rt-PA (1 µg/mL)	37	Yes			Yes	1 MHz	1.5 MPa	0.34	0.17	20	Measured lytic efficacy In terms of pressure change (thrombotic occlusion = 40 mm Hg)	6 mm Hg	[151]
					Yes	Yes	1 MHz	1 MPa	0.34	0.17	20		2 mm Hg Similar lytic efficacy was achieved at 1.5 MPa without rt-PA as was at 1.0 MPa with rt-PA.	
Porcine Flow system			Yes			Yes	1.6 MHz	0.2 (MI)	n/s	n/s	10	Measured volume reduction	54 % (20 µs PD)	[152]
			Yes			Yes	1.6 MHz	0.2 (MI)	n/s	n/s	10		33 % (5 µs PD) Slightly prolonging the pulse duration (PD) on a diagnostic transducer improves the degree of sonothrombolysis that can be achieved without fibrinolytic agents at a lower MI.	
Human Flow system	rt-PA (0.32-3.15 µg/mL)	37.3		Yes							10	Measured lytic rate	0.8-2 %/min	[153]
					Yes		120 kHz	0.44 MPa	Inter.	62.5	30		0.8-2 %/min	
					Yes	Yes	120 kHz	0.44 MPa	Inter.	62.5	30		2-5.5 %/min MBs administration significantly enhanced lytic rate. Both SC and radiation force are mechanistically responsible for the process of clot lysis.	

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Human Flow system	rt-PA (3 µg/mL)	37		Yes							60	Measured fibrin degradation product (FDP)	51.7 %	[154]
					Yes	Yes	1 MHz	528 mW/cm^2 I_{SPTA}	0.8	40	60		53.2 % (SC)	
					Yes	Yes	1 MHz	323 mW/cm^2 I_{SPTA}	0.8	8	60		57.2 % (SC+IC)	
					Yes	Yes	1 MHz	3 mW/cm^2 I_{SPTA}	0.8	0.08	60		50.9 % (SC+IC)	
					Yes	Yes	1 MHz	45 mW/cm^2 I_{SPTA}	0.8	0.08	60		66.3 % (IC) Both SC and IC, resulting from the US–MB interaction, increased the efficacy of rt-PA with respect to fibrin degradation.	
Porcine FUS Flow system	rt-PA (3.5 µg/mL)	37	Yes			Yes	1 MHz	20 W	100	10	30	Measured volume reduction	31 %	[76]
				Yes							30		45%	
					Yes		1 MHz	20 W	100	10	30		56.2%	
					Yes	Yes	1 MHz	20 W	100	10	30		69.5 % MBs administration further enhanced the beneficial effect of FUS on TNK-tPA mediated thrombolysis.	

Clot model	*Drug*	*Temp. (^{0}C)*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main Conclusion*	*Ref.*
			US alone	*Drug alone*	*US + Drug*									
Porcine Flow system	rt-PA (7 µg/mL)	37		Yes							30	Measured volume reduction	29.2 %	[77]
					Yes		0.6 MHz	60 W	100	10	30		45.8 %	
					Yes		1 MHz	20-60 W	100	10	30		39-62,5 %	
					Yes	Yes	1 MHz	60 W	100	10	30		87.5 %	
													1 MHz FUS frequency is associated with enhanced thrombolysis compared to that of 0.6 MHz. An increase linear relationship between acoustic power and thrombolysis efficacy was exhibited. The combination of MBs + FUS strongly enhanced the thrombolytic efficacy of TNK-tPA.	

Table 3: In vivo protocols used in US thrombolysis

Clot model	*Drug*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main conclusion*	*Side effects*	*Ref.*
		US alone	*Drug alone*	*US + Drug*										
Rat Jugular vein occlusion	rt-PA (1 mg)		Yes							50, 200	Measured volume reduction	20, 30 **%**		[60]
				Yes		1 MHz	1.75 W/cm^2	Intermittent	50	50, 200		41, 55 %		
	rt-PA (2 mg)		Yes							50, 200		28, 40 %		
				Yes		1 MHz	1.75 W/cm^2	Intermittent	50	50, 200		45, 50 % Intermittent US administered during a 200-min period showed a trend toward enhancement of rt-PA-induced fibrinolysis without causing thermal changes or inducing tissue damage. Thrombolysis efficiency increased with drug dose.		
Rat MCA stroke	rt-PA (10 mg/kg)		Yes							60	Measured relative infarct volume reduction	34 %	18 % ICH	[155]
	rt-PA (5 mg/kg)			Yes		25.6 kHz	0.6 W/cm^2	n/s	20	60		51 %		
	rt-PA (10 mg/kg)			Yes		25.6 kHz	0.6 W/cm^2	n/s	20	60		68 % US treatment in addition to rt-PA is more effective than single rt-PA treatment in reducing infarct volume and safe with regard to bleeding. Thrombolysis efficiency increased with drug dose.	20 % ICH	
Rabbit Embolic stroke HIFU		Yes				1.5 MHz	255 W	1	0.1	0.33	Measured reperfusion rate	0 % (0/3)		[86]
		Yes				1.5 MHz	415 W	1	0.1	0.33		50 % (2/4)		
		Yes				1.5 MHz	550 W	1	0.1	0.33		70 % (5/7) HIFU, as a stand-alone method, can cause effective thrombolysis and does not damage the targeted vessels.	20 % ICH	

Clot model	*Drug*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main conclusion*	*Side effects*	*Ref.*
		US alone	*Drug alone*	*US + Drug*										
Rabbit		Yes			Yes	1.5 MHz	88-137 W	1	0.1	0.33	Measured	78 % (7/9)	22 % ICH	[156]
MCA		Yes			Yes	1.5 MHz	88 W	10	0.1	0.33	recanalization	50 % (1/2)		
Stroke											rate	Droplets reduce the IC threshold and enable IC-mediated clot lysis to occur at lower power levels.		
HIFU														
Rabbit		Yes				1.51 MHz	185 W	1	0.1	0.33	Measured flow	0 % (0/5)		[82]
Femoral		Yes				1.51 MHz	215 W	1	0.1	0.33	restoration	50 % (1/2)		
artery											rate			
occlusion		Yes				1.51 MHz	300 W	1	0.1	0.33		63 % (5/8)	13 % ICH	
HIFU												HIFU thrombolysis is feasible as a means of restoring partial blood flow in thrombus-occluded arteries in the absence of thrombolytic agents. Increased power resulted in increased flow restoration rate.		
Rabbit	SK			Yes		37 kHz	160 W	91	n/s	15	Measured	40 % (6/15)		[157]
Iliofemoral	(25000 U/kg)			Yes		37 kHz	160 W	91	n/s	30	recanalization	67 % (10/15)		
artery				Yes		37 kHz	160 W	91	n/s	45	rate	87 % (13/15)		
occlusion				Yes		37 kHz	160 W	91	n/s	60		100 % (15/15)		
												Transcutaneous concentrated US which significantly enhances streptokinase induced thrombolysis in vivo can be delivered without concomitant tissue damage. Recanalization rate increases with treatment time.		
Rabbit		Yes				37 kHz	160 W	91	n/s	60	Measured	0 % (0/5)		[158]
Iliofemoral					Yes	37 kHz	160 W	91	n/s	60	recanalization	0 % (0/10)		
artery		Yes			Yes	37 kHz	160 W	91	n/s	15	rate	30 % (3/10)		
occlusion		Yes			Yes	37 kHz	160 W	91	n/s	30		50 % (5/10)		
		Yes			Yes	37 kHz	160 W	91	n/s	45		70 % (7/10)		
		Yes			Yes	37 kHz	160 W	91	n/s	60		100 % (10/10)		
												In vivo arterial clot dissolution can be achieved with i.v. MBs and transcutaneous US. Recanalization rate increased with treatment time.		

Clot model	*Drug*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main conclusion*	*Side effects*	*Ref.*
		US alone	*Drug alone*	*US + Drug*										
Rabbit Marginal ear vein occlusion HIFU	rt-PA (1 mg/kg)	Yes				1 MHz	40 W	1	5	15	Measured relative clot size at 5 h post treatment	90 %		[78]
			Yes							15		78 %		
				Yes		1 MHz	40 W	1	5	15		4 % rt-PA mediated thrombolysis can be significantly enhanced when combined with non-invasive pulsed-HIFU exposures.		
Rabbit Femoral artery occlusion	SK (15000 U/kg) bolus followed by an infusion of 15,000 U/kg per h.		Yes							120	Measured volume reduction	13 % (2/15)		[117]
				Yes		1 MHz	2 W/cm^2	c.w.		120		53 % (9/17) Externally applied, low-intensity US can significantly enhance thrombolysis in a rabbit arterial model.		
Rabbit Femoral artery occlusion	rt-PA (200 µg/5 mg of lipid)	Yes			Empty ELIP	5.7 MHz	1.25 MPa	5 k	n/s	2	Measured recanalization rate at 15 min post treatment	27 %		[64]
			Yes		rt-PA ELIP					2		60 %		
				Yes	rt-PA ELIP	5.7 MHz	1.25 MPa	5 k	n/s	2		100 % Doppler US treatment enhances the thrombolytic effect of rt-PA loaded ELIP, resulting in earlier and more complete recanalization rates.		
Rat MCA stroke	rt-PA (1.2 mg/animal)		Yes							32	Measured volume reduction	45 % (9/20)		[118]
				Yes		490 kHz	0.8 W/cm^2	c.w.	100	32		76.2 % (16/21) Low frequency transcranial US under appropriate conditions could be an effective and safe method of treatment for ischemic stroke.		

Clot model	*Drug*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main conclusion*	*Side effects*	*Ref.*
		US alone	*Drug alone*	*US + Drug*										
Rabbit Femoral artery occlusion	mt-PA (1.2 mg/animal)		Yes							32	Measured recanalization rate	16.7 % (2/12)		[159]
				Yes		490 kHz	0.13 W/cm²	c.w.	100	32		66.7 % (6/9) Low frequency and low intensity transcranial US enhanced thrombolysis by mt-PA.		
Rabbit Femoral artery occlusion	SK (15000 U/kg) as bolus followed by an infusion of 15000 U/kg/h	Yes				40 kHz	0.75 W/cm²	c.w.	100	120	Measured reperfusion rate	< 7 %		[62]
			Yes							120		7 %		
				Yes		40 kHz	0.75 W/cm²	c.w.	100	120		83 % 40-kHz US at low intensity markedly accelerates fibrinolysis and also improves tissue perfusion and reverses acidosis, effects that would be beneficial in treatment of acute thrombosis.		
Rabbit MCA stroke HIFU	rt-PA (1.mg/ml/kg)		Yes							120	Measured recanalization rate	100 %		[79]
				Yes	Yes	1 MHz	20 W/cm² (I_{SATA})	10	10	70		100 % HIFU in combination with rt-PA dissolved clots.		
Rabbit MCA stroke HIFU	rt-PA (1.mg/ml/kg)			Yes	Yes	1 MHz	20 W/cm² (I_{SATA})	10	10	70	Measured recanalization rate	100 % Therapeutic US in synergy with rt-PA dissolve clots.		[80]
Rabbit Hindlimb occlusion		Yes			Yes	1 MHz	0.031 W/cm² (I_{SATA})	0.33	0.17	10	Measured recanalization rate	67 %		[160]
		Yes			Yes	1 MHz	0.031 W/cm² (I_{SATA})	0.33	0.17	20		100 % Long-pulse-length US with MBs has a		

Clot model	*Drug*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main conclusion*	*Side effects*	*Ref.*
		US alone	*Drug alone*	*US + Drug*										
												therapeutic effect on microvascular perfusion.		
Rabbit	rt-PA		Yes							74	Measured	15-50 %		[161]
Femoral artery occlusion	(30 µg/kg/min)			Yes		1 MHz	6.3 W/cm^2 I_{SPTA}	c.w.	100	33	initial reflow	15-50 % Although time to initial reflow was shortened by US, it was associated with less reperfusion and more reocclusion in this model.		
Rat	rt-PA	Yes				1-3 MHz	1.7 (M.I.)	n/s.	n/s	60	Measured	40 %		[162]
MCA stroke	(10 mg/kg)	Yes			Yes	1-3 MHz	1.7 (M.I.)	n/s.	n/s	60	recanalization	59 %		
			Yes							60	rate	77 %		
				Yes		1-3 MHz	1.7 (M.I.)	n/s.	n/s	60		88 %		
				Yes	Yes	1-3 MHz	1.7 (M.I.)	n/s.	n/s	60		96 % Recanalization rate with rt-PA alone is better than US alone. Recanalization rate significantly increased with the combination of US + drug. Recanalization rate increased even more when US was combined with rt-PA + MBs.		
Rat		Yes				2 MHz	1.56 MPa	150	5	30	Measured	1.70 µg/mL		[163]
Carotid artery occlusion		Yes			Yes (Nsib)	2 MHz	1.56 MPa	150	5	30	plasma D-dimer concentrations	2.31 µg/mL		
		Yes			Yes (Abib)	2 MHz	1.56 MPa	150	5	30		3.91 µg/mL US in combination with abciximab immunobubbles (Abib) induces thrombolysis without lytic agents that is superior to insonation of non-specific immunobubbles (Nsib).		

Clot model	*Drug*	*Mode*			*MBs*	*Freq.*	*Output*	*PRF (Hz)*	*DF (%)*	*Time (min)*	*Evaluation method*	*Main conclusion*	*Side effects*	*Ref.*
		US alone	*Drug alone*	*US + Drug*										
Rabbit Embolic stroke	rt-PA (0.8-0.9 mg/kg)	Yes				1 MHz	0.8 W/cm² (I_{SATA})	100	20	60	Measured infarct volume	1 %	56 % ICH	[70]
				Yes		1 MHz	0.8 W/cm² (I_{SATA})	100	20	60		0.13 %	61 % ICH	
		Yes			Yes	1 MHz	0.8 W/cm² (I_{SATA})	100	20	60		0.2 %	19 % ICH	
				Yes	Yes	1 MHz	0.8 W/cm² (I_{SATA})	100	20	60		0.09 %	26 % ICH	
												The ability of MBs to reduce rt-PA requirements may lead to lower rates of hemorrhage in human stroke treatment.		
Rabbit Embolic stroke	rt-PA (0.9 mg/kg)		Yes							60	Measured infarct volume	2.2 %	45 % ICH	[164]
				Yes		1 MHz	0.8 W/cm²	n/s	20	60		1.7 %	50 % ICH	
		Yes			Yes	1 MHz	0.8 W/cm²	n/s	20	60		0.8 %	36 % ICH	
												Sonothrombolysis without rt-PA using MBs is effective in decreasing infarct volumes.		
Rabbit Embolic stroke	rt-PA (0.9 mg/kg)	Yes				1 MHz	0.8 W/cm²	n/s	20	60	Measured infarct volume	0.97 %	56 % ICH	[165]
			Yes							60		0.14 %	48 % ICH	
				Yes		1 MHz	0.8 W/cm²	n/s	20	60		0.15 %	73 % ICH	
		Yes			Yes	1 MHz	0.8 W/cm²	n/s	20	60		0.20 %	19 % ICH	
				Yes	Yes	1 MHz	0.8 W/cm²	n/s	20	60		0.10 %	36 % ICH	
												Treatment with MB+US following embolization decreased the incidence of ICH and efficacy was similar to tPA in reducing infarct volume.		
Rabbit Iliofemoral artery occlusion	SK (25000 U/kg))	Yes				20 kHz	1.5 W/cm²	n/s	n/s	60	Measured patency rate	0 % (0/6)		[166]
			Yes							60		6 % (1/17)		
				Yes		37 kHz	160 W	n/s	n/s	60		100 % (15/15)		
				Yes	Yes	20 kHz	1.5 W/cm²	n/s	n/s	60		87 % (13/15)		
		Yes			Yes	20 kHz	1.5 W/cm²	n/s	n/s	60		76 % (13/17)		
		Yes			Yes	37 kHz	160 W	n/s	n/s	60		Non-invasive transcutaneous US can greatly enhance the effect of clot dissolution with thrombolytic drugs and/or MBs.		

Table 4: Clinical protocols used in US thrombolysis

Clot Model	No of subjects	Drug	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Side effects	Ref.
			US alone	Drug alone	US + Drug										
Stroke patients MCA occlusion	12	rt-PA (0.9 mg/kg)			Yes (TCD)	Yes	2 MHz	n/s	n/s	n/s	120	Measured complete and partial recanalization rate	50 % (6/12) 33 % (4/12) MBs reached and permeated beyond occlusions with no increase in sICH suggesting the feasibility of further studies	0 % sICH 0 % sICH	[167]
Stroke patients MCA occlusion	12		Yes (TCD)				2 MHz	n/s	n/s	n/s	60	Measured complete recanalization rate	30 % (4/12) Sonothrombolysis using 2 probes and bilateral monitoring is safe but not more effective than standard sonothrombolysis.	0 % sICH	[168]
Stroke patients MCA occlusion	6		Yes (TCCD)				2 MHz	415 mW/cm^2 (I_{SPTA})	n/s	n/s	30	Measured partial recanalization rate	83 % (5/6) High rate of early partial recanalization during continuous exposure to 2-MHz US without rt-PA.	0 % sICH	[169]
Stroke patients MCA occlusion	111	rt-PA (0.9 mg/kg)		Yes							120	Measured complete recanalization rate	24 % (9/36)	5.5 % sICH	[92]
					Yes (TCD)		2 MHz	n/s	n/s	n/s	120		41 % (15/37)	2.7 % sICH	
					Yes (TCD)	Yes	2 MHz	n/s	n/s	n/s	120		55 % (21/38) MBs administration induces further acceleration of US-enhanced thrombolysis.	2.6 % sICH	
Stroke patients MCA occlusion	26	rt-PA (0.9 mg/kg)			Yes (TCCD)		2 MHz	189 mW/cm^2	n/s	n/s	60	Measured complete recanalization rate	53 % (8/15)	7 % sICH	[170]
					Yes (TCCD)	Yes	2 MHz	189 mW/cm^2	n/s	n/s	60		64 % (7/11) MBs enhanced TCCD monitored rt-PA thrombolysis lead to a greater immediate clinical improvement.	9 % sICH	

Clot Model	No of subjects	Drug	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Side effects	Ref.
			US alone	Drug alone	US + Drug										
Stroke patients MCA occlusion	25	rt-PA (0.9 mg/kg)	Yes								60 60	Measured complete and partial recanalization rate	21.4 % (3/14-complete) 0 % (0/14- partial) 27.3 % (3/11-complete) 18.2 % (2/11-partial) Transcranial TCCD with rt-PA showed a higher grade of recanalization compared with rt-PA alone.	7 % sICH 36 % sICH	[91]
				Yes (TCCD)			2-4 MHz	179 mW/cm^2	n/s	n/s					
Stroke patients MCA occlusion	126	rt-PA (0.9 mg/kg)	Yes								120 120	Measured complete recanalization rate	30 % (19/63) 49 % (31/63) Continuous TCD augments rt-PA induced arterial recanalization.	5 % sICH 5 % sICH	[171]
				Yes (TCD)			2 MHz	750 mW/cm^2	n/s	n/s					
Stroke patients MCA occlusion	55	rt-PA (0.9 mg/kg)		Yes (TCD)			2 MHz	n/s	n/s	n/s	120	Measured complete recanalization rate	36 % (20/55) Complete recanalization within 2 hours after rt-PA bolus is a feasible goal for thrombolysis given with TCD monitoring.	6 % sICH	[119]
Stroke patients MCA occlusion	37	rt-PA (0.9 mg/kg)	Yes								60 60	Measured complete and partial recanalization rate	11.1 % (2/18-complete) 11.1 % (2/18-partial) 15.8 % (3/19-complete) 42.1 % (8/19-partial) Transcranial US in combination with rt-PA accelerates recanalization in MCA occlusion, compared to rt-PA alone	5.6 % sICH 15.8%sICH	[172]
				Yes (TCCD)			1.8 MHz	179 mW/cm^2	n/s	n/s					
Stroke patients MCA occlusion	26	rt-PA (0.9 mg/kg)	Yes								90 90	Measured recanalization rate (both complete and partial)	50 % (6/12) 29 % (4/14) Low frequency US combined with rt-PA showed an increased rate of sICH.	0 % sICH 36 % sICH	[89]
				Yes			300 kHz	700 mW/cm^2 (I_{SPTA})	100	5					

Clot Model	No of subjects	Drug	Mode			MBs	Freq.	Output	PRF (Hz)	DF (%)	Time (min)	Evaluation method	Main Conclusion	Side effects	Ref.
			US alone	Drug alone	US + Drug										
Stroke patients Proximal intracranial occlusion	35	rt-PA (0.9 mg/kg)		Yes							90	Measured complete and partial recanalization rate	33 % (4/12-complete) 25 % (3/12-partial)	0 % sICH	[93]
					Yes (TCD)	Yes (1.4 mL)	2 MHz	n/s	n/s	100	90		67 % (8/12-complete) 17 % (2/12-partial)	0 % sICH	
					Yes (TCD)	Yes (2.8 mL)	2 MHz	n/s	n/s	100	90		45 % (5/11-complete) 0 % (0/11-partial) MBs can be safely combined with systemic rt-PA and US at a dose of 1.4mL.	27 % sICH	
Stroke patients MCA occlusion	52	rt-PA (0.9 mg/kg)	Yes (TCCD)				2-4 MHz	208 W/cm^2 (I_{SPPA})	5 k	0.7	45	Measured complete and partial recanalization rate	97 % (36/37-complete) 0 % (0/37-partial)	2.7 % sICH	[173]
				Yes							45		67 % (10/15-complete) 7 % (1/15-partial)	6.6 % sICH	
					Yes (TCCD)		2-4 MHz	208 W/cm^2 (I_{SPPA})	5 k	0.7	45		80 % (12/15-complete) 7 % (1/15-partial) Recanalization rate of continuous TCCD monitoring of MCA occlusion in combination with rt-PA was lower compared to that of TCCD monitoring alone.	6.6 % sICH	
Stroke patients MCA occlusion Catheter type	81	rt-PA (60 mg IV + 22 mg IA infusion)		Yes							120	Measured recanalization rate (both complete and partial)	56 % (33/59)	6.6 % sICH	[174]
					Y es		1.7 MHz	n/s	n/s	n/s	120		73 % (24/33) EKOS micro-infusion catheter is a reasonable and easy-to-use tool for re-opening occluded intracranial arteries. Additionally, the delivery of intra-arterial rt-PA or other thrombolytic drugs via a standard micro-catheter remains an excellent option.	9.9 % sICH	

3.6. Discussion

This compilation is very useful since numerous aspects associated with the US protocols examined (in vitro, in vivo and clinical), that are used in thrombolysis, were investigated. The review protocols are summarised in tables providing all necessary data in terms of clot model, treatment mode, sonication parameters, evaluation method, thrombolysis efficacy and side effects. In addition, the main conclusions derived from each study are presented as well.

Although the mechanisms behind US enhanced thrombolysis are not very clear, it is evidenced that exposure to US increases the uptake and depth of penetration of thrombolytics into clots [46], causes additional binding sites due to reversible disaggregation of fibrin fibres [51] and increases the binding of thrombolytic agents to fibrin [125].

The influence of temperature on clot lysis was only investigated in vitro. In most of the experimental studies, the temperature during sonication was kept constant at 37 ^{0}C, which sufficiently explains that clot lysis occurred through non-thermal mechanisms. In a few studies, the temperature at the target was not specified. It is well established that temperature plays an important role on clot lysis, since thrombolysis efficacy decreases at temperatures below the body baseline temperature of 37 ^{0}C [135]. Therefore, it will be useful in the future, for all the in vitro studies to be conducted at 37 ^{0}C.

Different thrombolytics such as UK, SK, rt-PA, TNK-tPA, etc. in various concentrations have been investigated. Studies have shown that US energy can accelerate the thrombolytic activity of any thrombolytic used and that the extent of thrombolysis is dependent on drug's concentration [128], [153]. The most common thrombolytic drug used by the researchers was rt-PA, since it is the only thrombolytic treatment approved for AIS. Although rt-PA was administered in various concentrations between 0.1-300 μg/mL, the majority of the above mentioned studies were conducted at a concentration of 3.15 μg /mL, which is the average concentration of the drug in human blood.

The current review, shows that the thrombolytic efficacy of drug alone is better than that of US alone [75], [136], [175]. Therefore, we assume that US energy as a stand-alone method for clot lysis is not effective and should be applied in synergy with thrombolytic drugs to enhance thrombolysis. However, a few studies [82], [87], [141], demonstrated that using US alone (in the absence of thrombolytic drug), can achieve almost complete clot lysis within seconds. Taking into consideration the very high level

of acoustic powers used in their studies, in association with the fact that the temperature elevation at the target was not specified in their results, we suspect that most likely the protocols applied have entered into thermal mechanisms of thrombolysis. This suspicion, is supported by the results of many other researchers [75], [136], [175], who exhibited reduced thrombolytic efficacy using US alone, although prolonged exposure times were used in their studies.

Some other studies, focused their research on the effect of travelling vs. standing acoustic waves on clot lysis, demonstrating that travelling acoustic waves enhanced thrombolysis significantly more than standing waves did [55-56].

It is well known that US frequency in conjunction with acoustic intensity, exert a major effect on clot lysis. Different US frequencies ranged from 20 kHz to 5.0 MHz and intensities (either low or high), have been employed for sonothrombolysis studies. A number of studies indicated that lower US frequencies (in the kilohertz range), are more efficient in sonothrombolysis over higher frequencies (in the MHz range), since they exhibited improved tissue penetration and greater acceleration in fibrinolysis [35], [124], [137]. Considering that some other studies showed that thrombolysis efficacy increases as the level of acoustic intensity increases [129], [130], [176], it is reasonable to come to the conclusion that the thrombolytic efficacy of US waves is directly dependent on acoustic intensity and inversely dependent on frequency.

There is enough evidence from in vitro [69], animal [162], and clinical [92] studies, indicating that the administration of MBs further enhance the effect of US on enzymatic thrombolysis induced by thrombolytic agents. The findings of this review demonstrated that the boosting effect of MBs in clot dissolution, correlates with the presence of cavitation mechanisms and most specifically with stable cavitation, which appears to play a more important role in MB mediated sonothrombolysis [66], [144], [148]. However, a study performed by Molina et al. [93], showed that there is a safe limit on the administered dose of MBs, which should not be exceeded since it is associated with an increased risk of sICH rate. Therefore, it is recommended that MBs should be administered in combination with rt-PA at low doses (1.4 mL), in order to enhance thrombolysis and avoid unnecessary adverse health effects, such as sICH.

Animal studies, as well as clinical trials, have shown that the most common side effect of sonothrombolysis was sICH. Apart from the use of high dose of MBs, the application of low frequency US might increase the rate of sICH. The TRanscranial low-frequency Ultrasound-Mediated thrombolysis in Brain Ischemia (TRUMBI) clinical trial

[89], which was designed to treat stroke patients with transcranial 300 kHz US plus rt-PA, was ended prematurely due to a significant increase in sICH rate. Since then, low frequency US has not been available for therapeutic purposes in clinical trials. Additionally, our investigation showed that the risk of sICH rate increases with the concentration of thrombolytic drug [155] and the level of acoustic intensity [86], [156].

The effect of time is very critical on thrombolysis efficacy, since early recanalization is the key of therapeutic success in the treatment of vascular thrombosis. In this review, the exposure times used in the in vitro studies varied from 0.5 to 240 min. In the animal studies, the treatment times ranged from 0.5 to 120 min, while in the clinical trials the continuous monitoring of the patients was between 30 and 120 min. Several studies [60], [134], showed that most of the clot mass was removed within the first 60 min of treatment and beyond that time the efficacy of thrombolytic treatment was decreased significantly. This phenomenon, might be caused due to the fact that the concentration of the thrombolytic drug in the blood after 60 min of treatment, decreases dramatically leading to a significant reduction on enzymatic fibrinolysis rate. Therefore, at least for the experimental studies, 60 min of treatment must be a sufficient exposure time and should not be exceeded.

The most common protocol used in clinical trials so far for the treatment of patients with AIS due to MCA occlusion, was the continuous monitoring of the patients with high frequency (2 MHz) low intensity (< 750 mW/cm^2) diagnostic transcranial US in combination with rt-PA [169-170]. Using this protocol, higher recanalization rates exhibited compared to those with rt-PA alone.

3.7. Conclusions

Despite the absence in standardization, the heterogeneity and the limitations of the reviewed studies, useful information concerning the ability of US energy to enhance thrombolysis efficacy either alone or as an adjunct to thrombolytic drugs and/or in combination with US contrast agents, was extracted. Although preclinical studies have been very promising, the results from clinical trials have shown that further research in the field of sonothrombolysis has to be done, in order to improve recanalization rates and clinical outcomes.

More specifically, this comprehensive review has provided strong evidence that sonothrombolysis research for the treatment of stroke should be focused in the field of therapeutic MRgFUS. Additionally, the prospect of co-administration of thrombolytic

drugs with MBs is especially encouraging and therefore for better outcomes adjuvant therapies should be developed for future clinical studies. Based on these findings, a fully-characterised sonothrombolysis model must be produced dedicated to use:

1. High intensity FUS pulses under MR guidance and monitoring for fast and accurate beam targeting, temperature monitoring, post therapy evaluation.
2. Phased array technology in order to overcome problems such as beam defocusing due to the inhomogeneous thickness and density of the skull bone and skull/near-field soft tissues heating, caused by the acoustic signal absorption.
3. Non thermal mechanisms to avoid the contribution of thermal mechanisms in clot lysis
5. A 3rd generation thrombolytic drug with longer half-life and higher thrombolytic potency than rt-PA
6. Systemically administered MBs to further increase FUS enhanced sonothrombolysis

3.8. Summary

A summary table providing the most important studies in sonothrombolysis related to my thesis (in vitro, in vivo and clinical), is shown below. The table presents the main experimental parameters applied in each study and identifies the aspects that influence thrombolysis efficacy and were not explored by the investigators. These aspects will be evaluated in this research.

Table 5: Most important studies in sonothrombolysis and gaps in knowledge

Most important studies		Freq. (MHz)	Mode	Output (Intensity)	Drug (rt-PA)	MBs	Identification of aspects that influence thrombolysis efficacy and were not explored
In vitro	Holland et al. 2007	1	US	Low	Yes	No	The contribution of thermal effects, high intensity, FUS pulses, flow rate, MBs administration and parametric studies on clot lysis.
Flow model	Cintas et al 2004.	2	US	Low	Yes	Yes	The contribution of thermal effects, high intensity, FUS pulses and parametric studies on clot lysis
	Frenkel et al. 2006	1	FUS	High	Yes	No	The contribution of thermal effects, flow rate, MBs administration and parametric studies on clot lysis.
Flow model	Wright et al. 2011	1.5	FUS	High	No	No	The contribution of thermal effects, thrombolytic drug, MBs administration and parametric studies on clot lysis.
In vivo	Stone et al. 2007	1	FUS	High	Yes	No	The contribution of thermal effects, MBs administration and parametric studies on clot lysis.
MRgFUS	Burgess et al. 2012	1.5	FUS	High	No	No	The contribution of thermal effects, thrombolytic drug, MBs administration and parametric studies on clot lysis.
Clinical	Alexandrov et al. 2004	2	TCD	Low	Yes	No	The contribution of thermal effects, FUS waves, high intensity, MBs administration and parameters optimisation on clot lysis.
	Molina et al. 2009	2	TCD	Low	Yes	Yes	The contribution of thermal effects, FUS waves, high intensity, MBs administration and parametric studies on clot lysis.

4. Determination of the experimental parameters that influence ultrasound induced thrombolysis in vitro

4.1. Introduction

In this chapter, the experimental parameters that influence US induced thrombolysis were determined. Using an in vitro flow model, fully retracted porcine blood clots were exposed to a realistic unidirectional circulating pulsatile flow and were treated with either focused or unfocused US waves, either alone or as an adjunct to thrombolytic agent TNK-tPA and in the presence or absence of MBs. The objective of the study was to establish the optimum treatment protocol that maximizes the uptake, the depth of penetration and the binding of the thrombolytic agent to the fibrin of the clot, leading to enhance thrombolysis, without excess heating.

In order to succeed this, the impact of operating acoustic parameters such as frequency, acoustic power (AP), pulse length, PRF, DF and treatment time, on thrombolysis efficacy was investigated. Additionally, the effect of various physical parameters on thrombolysis efficacy, was also explored. These are, the US energy, the temperature, the TNK-tPA concentration, the flow-rate, the formation of standing or travelling acoustic waves, the acoustic properties of the medium and the administration of MBs

4.2. In vitro clot preparation

Blood clots were obtained by natural coagulation of fresh porcine blood, collected from healthy pigs. Blood clots were prepared by aliquoting blood samples of the same volume into pre-weighed tubes, tubing or custom made containers (depending on the study). The blood aliquots were incubated in a 37 °C water bath for 3 hours before refrigerated at 5 °C for 72 h, to ensure maximal clot retraction [59].

The decision to use or reject the formed clots was based on their appearance. Normally should be dark red in color, since venous thrombi are mainly composed of fibrin and red blood cells, known as “red thrombi”. In 2007, Holland et al. [59], demonstrated that aged clots (2–16 day old), exhibited significantly lower mass loss when exposed to rt-PA alone, compared to clots that were one day old. Thus, in all subsequent experimental studies, only clots that were one day old were used.

After clot formation, the produced serum was completely removed from each sample with great caution in order not to disturbed the formed clot. Then, each container comprising clot was weighed again to determine the net mass clot weight (C_{NW}):

$$C_{NW} = W_C - W_T \quad (4.1)$$

where, W_C is the weight of the container plus the clot and W_T is the weight of the container alone. When the treatment was terminated, clot's residual was carefully removed from the container and was left to dry on a filter paper for 60 min before weighted again to obtain the mass clot removed due to thrombolysis.

The efficacy of thrombolytic treatment (T), was measured either as the relative reduction in the mass of the clot before and after treatment:

$$T = \frac{W_{in} - W_{fi}}{W_{in}} X100 \quad (4.2)$$

where, W_{in} is the initial clot weight and W_{fi} is the final clot weight, or was measured in mg of mass clot removed:

$$T = W_{in} - W_{fi} \quad (4.3)$$

In all experimental studies, the mass of the clot before and after treatment was measured on a precision digital balance (Scaletec, SM001, Heiligenstadt, Germany).

4.3. Preparation of TNK-tPA

TNK-tPA (Boehringer, Ingelheim, Germany), is a 3rd generation modified tissue plasminogen activator that is more fibrin-specific and more resistant to plasminogen activator inhibitor. Due to higher thrombolytic potency and longer half-life than rt-PA, TNK-tPA can be administered as an iv bolus [21], [177]. TNK-tPA was obtained as powder, mixed with sterile water as per manufacturer's instructions. Although this thrombolytic agent is an approved safe drug for acute myocardial infarction, due to its safe administration and ease of use, it can be a new choice for AIS treatment [178]. Figure 4.1 shows the time-dependence of plasma concentrations of alteplase (rt-PA) and tenecteplase (TNK-tPA), at 30, 40 and 50 mg in a subset of patients from the Thrombolysis in Myocardial Infarction (TIMI) 10B trial. This trial included 886 patients and was designed to compare prospectively the efficacy and safety of rt-PA and TNK-tPA. Systemic exposure to TNK-tPA at all of these doses, as measured by the peak plasma concentration and area under the curve, was higher than for a 90 min infusion of 100 mg rt-PA. Due to the improved fibrin specificity of TNK-tPA, this increased systemic

exposure did not compromise clinical safety and resulted in less serious bleeding events compared to rt-PA [179].

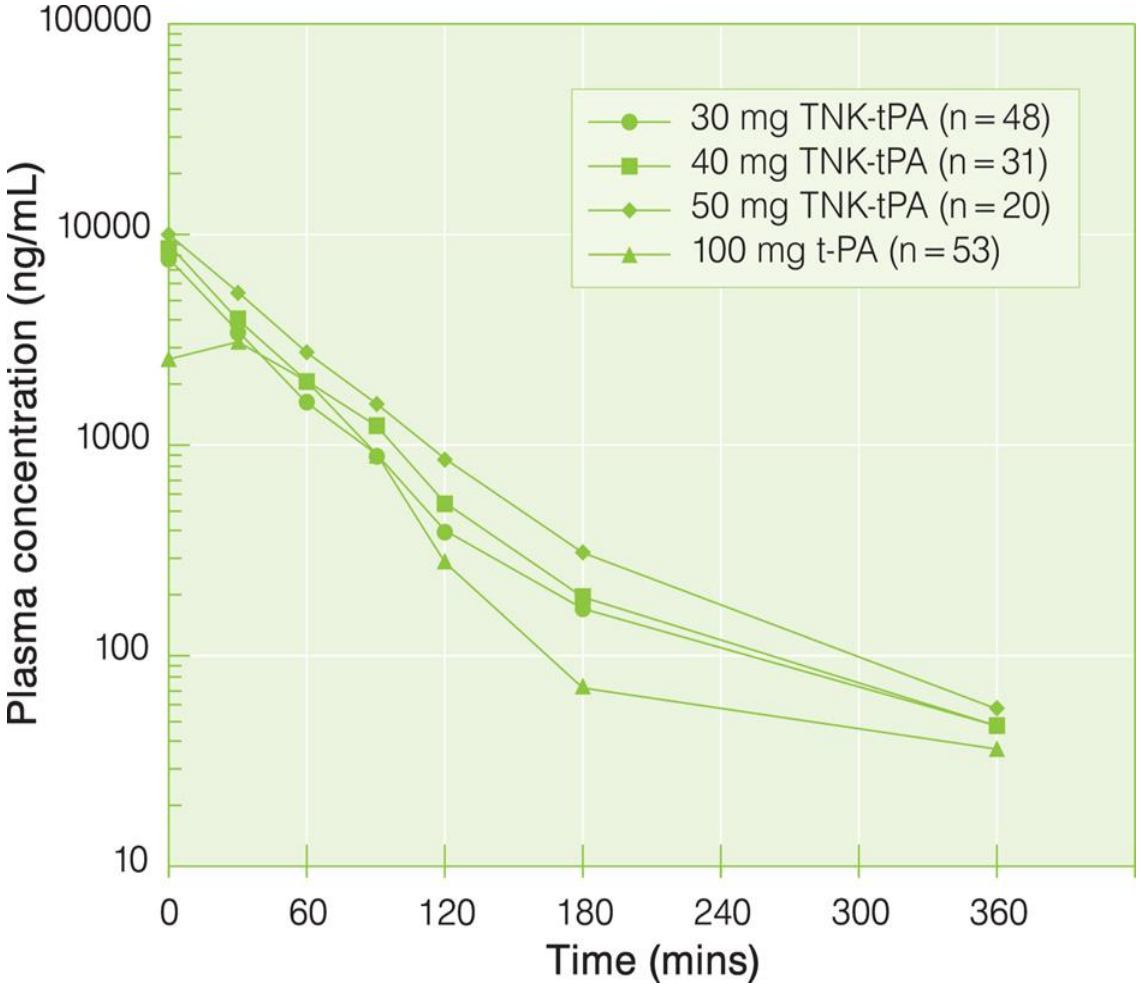

Figure 4.1: Blood concentrations of TNK-tPA vs. rt-PA over time in the TIMI 10B trial. Mean plasma concentration vs. time for the 3 doses of TNK-tPA (30, 40 and 50 mg), given as bolus and for 1 dose of rt-PA (100 mg), given as bolus and 90 minute infusion [179].

4.4. Preparation of SonoVue MBs

To study the effect of cavitation nuclei on sonothrombolysis efficacy, UCA were used. UCA are micron-sized MBs, with inert, high molecular weight gas core, surrounded by a lipid, surfactant, or biocompatible polymer shell. SonoVue (Bracco, Milan, Italy) MBs contain a sulphur hexafluoride (SF_6) gas core, which is a very stable molecule. The shell consists of a highly flexible phospholipid membrane, allowing the MBs to change the size and shape. The composition of the shell determines the stiffness of the bubbles and their resistance to rupture in the US field. As a result, MBs decreased solubility and low diffusion coefficient, prolong their lifespan within the circulation. The mean size of these MBs is 2.5 μm, and the resonance frequency ranges between 1 and 4 MHz [180]. SonoVue was obtained as kit with a vial containing a lyophilized powder (25 mg) and a

syringe prefilled with sterile saline solution (5 mL). The powder and the saline solution were mixed as per manufacturer's instructions, a manual process that takes less than one min. The resultant suspension with a concentration of 1-5x10^8 MBs/mL, was injected in the reservoir of the circulating flow system on a constant rate, in order to replace the bubbles that were destroyed in process and sustain cavitation.

4.5. Experimental apparatus

The experiments were carried out in an Acrylic tank (42 X 24 X 23 cm^3) filled with degassed water to prevent reflections, that may affect the propagation of the beam. Since this research covered both focused and unfocused US experimental studies, different US systems were involved.

For FUS studies, the US system used was consisted of two single element spherically shaped focused transducers, made from piezoelectric ceramic (Piezotechnologies, Etalon, Lebanon, IN, USA). The transducers were driven by a radio frequency (RF) generator/amplifier (750 W, JJ&A Instruments, Duvall, WA, USA). The one transducer used operates with 0.6 MHz centre frequency, while the other one operates with 1.18 MHz centre frequency. The focal length and the diameter of both transducers were 100 mm and 40 mm respectively. The acoustic output of the transducers was measured with an US power meter (UPM-DT100N, Ohmic Instruments Co. Easton MD 21601).

For unfocused US studies, the US system consisted of three flat rectangular ultrasonic transducers, operating at 3.7, 4.0 and 5.2 MHz respectively. The active size of each transducer was 2 X 10 mm^2 and the transducer material was made out of Pz26-type piezoceramic (Ferroperm, Kvistgaard, Denmark) with epoxy backing. The electrical signal was produced by a function generator (Agilent technologies, 33220A 20 MHz Function/Arbitrary Waveform Generator, Englewood, CO, USA) and was amplified by a radio frequency amplifier (AR Research, 75A250, Souderton, PA, USA).

The container comprising clot was fixed on a custom made plastic holder and immersed into the water tank. The rear surface of the container was lined with an absorbing rubber to minimize reflections and to prevent the generation of standing waves, since the enhancement of clot dissolution is much more pronounced in travelling than in standing acoustic waves [55].

The physiological situation of flow in a MCA occlusion, was reproduced by exposing the clots to a circulating pulsatile flow using a Masterflex peristaltic pump (Cole

Parmer, 7518-40, Vernon Hills, IL, USA). The flow system was consisted of a reservoir connected to a peristaltic pump and the container with the clot. The outlet tubing from the reservoir after passing the peristaltic pump, where the pulsatile flow was generated, was connected to the upper left side of the container. The inlet tubing was connected to the opposite right side, in order to sustain circulation. The reservoir was filled with degassed water (fluid for the flow system) along with TNK-tPA.

The transducer was mounted on the arm of an automated robotic system **(**VXM, Velmex Inc, Bloomfield, NY, USA**)**, immersed in the water tank and arranged opposite the blood clot. The robotic system has 3 user-controlled degrees of freedom, capable to guide the transducer in X, Y and Z direction with sub-millimetre accuracy.

All custom made 3D plastic models used in this research such as containers, holders, needles, arm of the robotic system, etc. (figure 4.2), were manufactured using a Stratasys 3D printer (FDM400, Eden Prairie, Minnesota, USA). The machine uses a common thermoplastic material known as Acrylonitrile Butadiene Styrene (ABS). This raw material satisfied the prerequisite of building MR compatible 3D models, since ABS is a non-magnetic material.

4.6. Beam positioning

For FUS studies, a custom made plastic needle with length equal to the focal length of the transducer (figure 4.2A), was attached at the transducer's face (figure 4.2B).

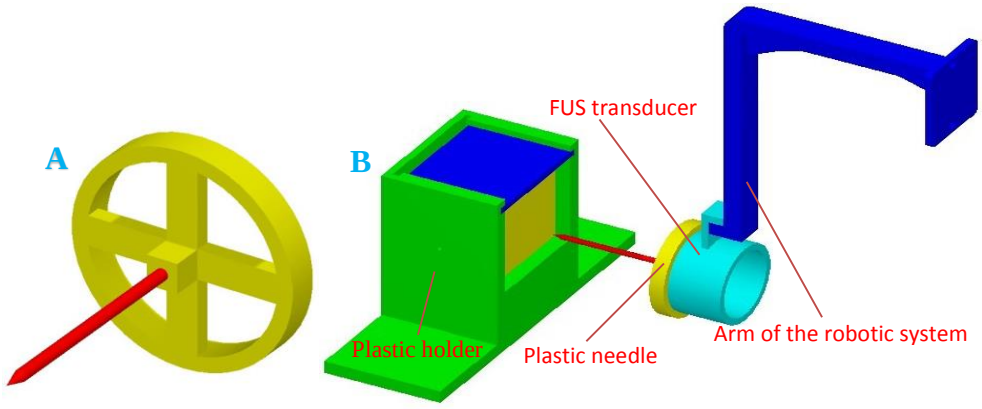

Figure 4.2: The attachment of the plastic needle (A) on the transducer's face (B)

Then, the needle was moved by the robotic system until its tip touched the target. Beam focus was always positioned at clot's edge in order to increase the uptake, penetration and binding of the thrombolytic drug into the clot. When it was confirmed that the focus of the US beam was positioned precisely on target, the plastic needle was carefully removed.

Two different flow models were designed to study the effect of FUS on thrombolytic efficacy. In the case where the blood clot was formed into a plastic tubing mimicking MCA occlusion occurred 4 cm deep into a brain tissue, precise beam positioning was achieved when the tip of the needle touched anteriorly the mid-height of the tubing at clot's edge (figure 4.3).

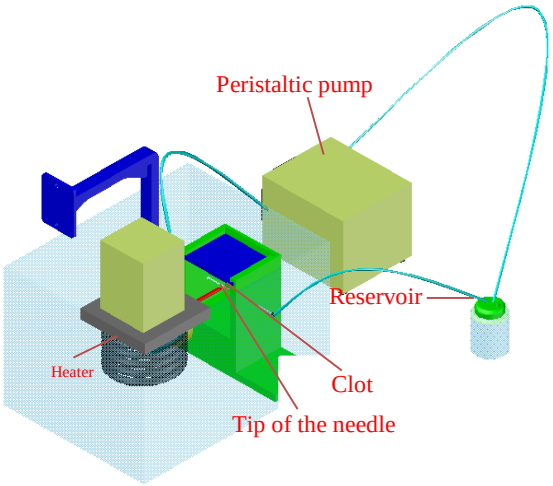

Figure 4.3: Beam positioning for FUS studies.

In the case where the blood clot was formed into a container mimicking MCA occlusion, occurred superficially, precise beam positioning was achieved when the tip of the needle touched anteriorly the clot's edge (figure 4.4).

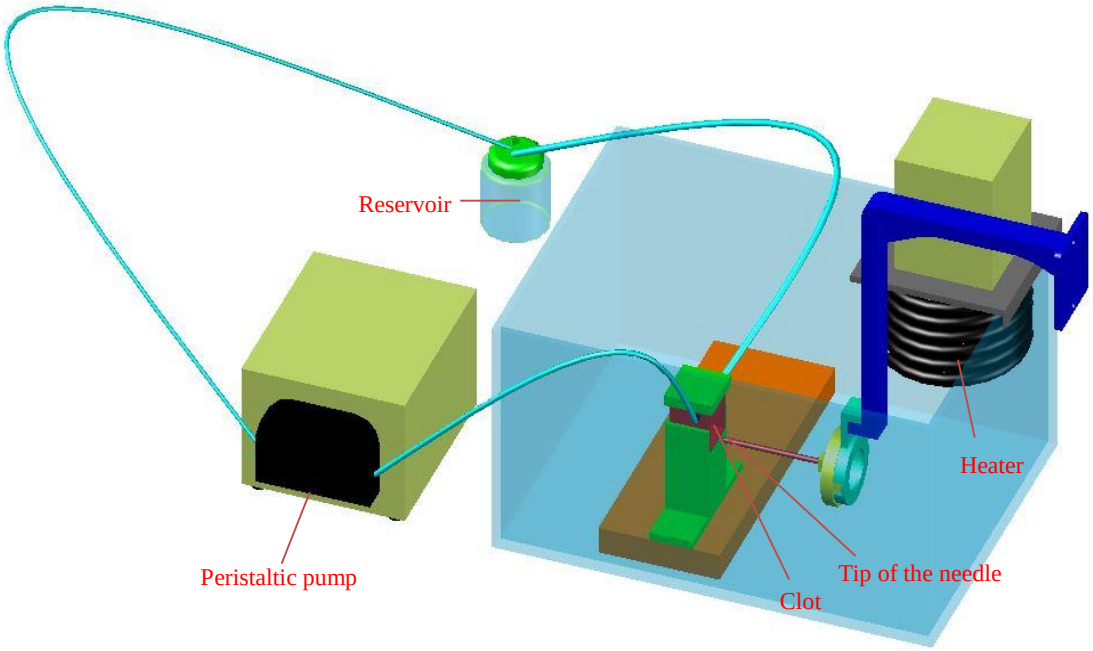

Figure 4.4: Beam positioning for FUS studies.

For unfocused US studies, the flow clot model was designed to study a MCA occlusion treated invasively with a catheter-directed US device. Precise beam positioning was achieved when the transducer was placed as close as physically possible to the proximal portion of the clot, since the acoustic pressure decreases rapidly with distance due to the high frequencies used (figure 4.5).

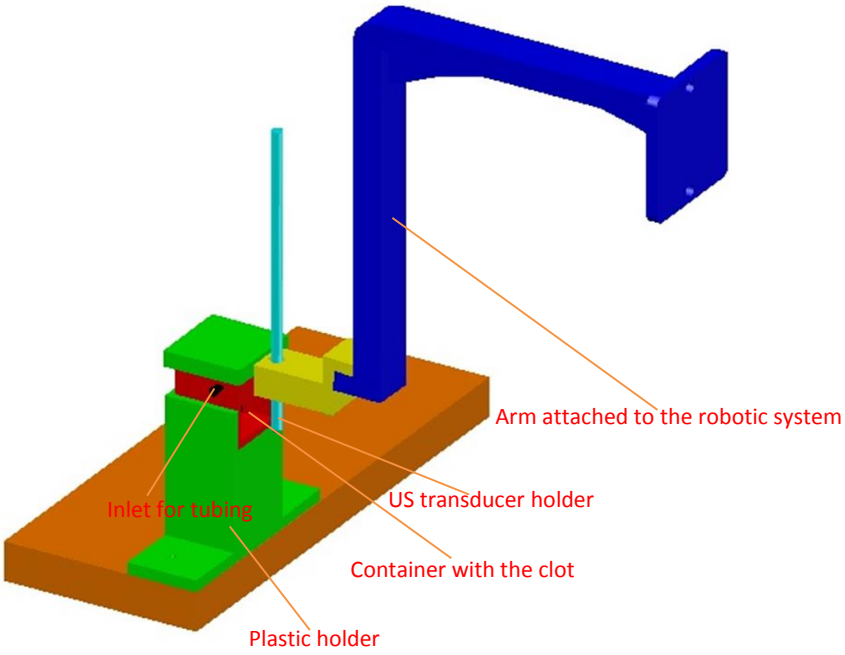

Figure 4.5: Beam positioning for unfocused US studies.

4.7. Experimental protocol

The main aspect for establishing a treatment protocol was to ensure the negligible contribution of thermal effects to clot lysis, i.e. providing that thrombolysis occurred mainly through non-thermal mechanisms.

Regarding thermal mechanisms for biological effects of US, Miller and Ziskin 1989 [181], suggested that a temperature rise of 1-2 ^{0}C in an afebrile patient would not be likely to have a damaging effect. However, for exposures resulting in temperature rises of greater than 2 ^{0}C, the duration of exposure becomes an important consideration in risk/benefit assessment. Moreover, a comprehensive review on thermal bioeffects by the World Federation of Ultrasound in Medicine (WFUMD), concluded that "Based solely on a thermal criterion, an US exposure that produces a maximum temperature rise of 1.5 ^{0}C above normal physiological levels (37 ^{0}C), may be used without reservation in clinical examinations" [182].

Based on the above mentioned thermal criterion, in all experimental studies, the temperature increase within the clot was maintained even lower, i.e. not to exceed 1˚C (called safe temperature). This means that during sonication, the temperature of the medium surrounding the clot was held constant with a localized temperature increase of 1˚C, at the target. For the assessment of heat produced during sonication, a thermocouple (OMEGA Engineering, INC. Stamford, Connecticut, USA), was fixed at the position of target. The thermocouple was connected to a data acquisition-DAQ interface (National Instruments Corporation, Austin, Texas, USA), that fed real time temperature measurements to a computer.

Pulsed US protocols to ensure that clot lysis occurred mainly through non-thermal mechanisms, were applied. Each sonication set, included a pulse "ON" period followed by a pulse "OFF" period and both the "ON" and "OFF" pulses were repeated in succession for the duration of the treatment. In each experiment, only one parameter was varied, while all others parameters were kept constant provided that no excessive heating (> 1 ^{0}C) was produced (figure 4.6).

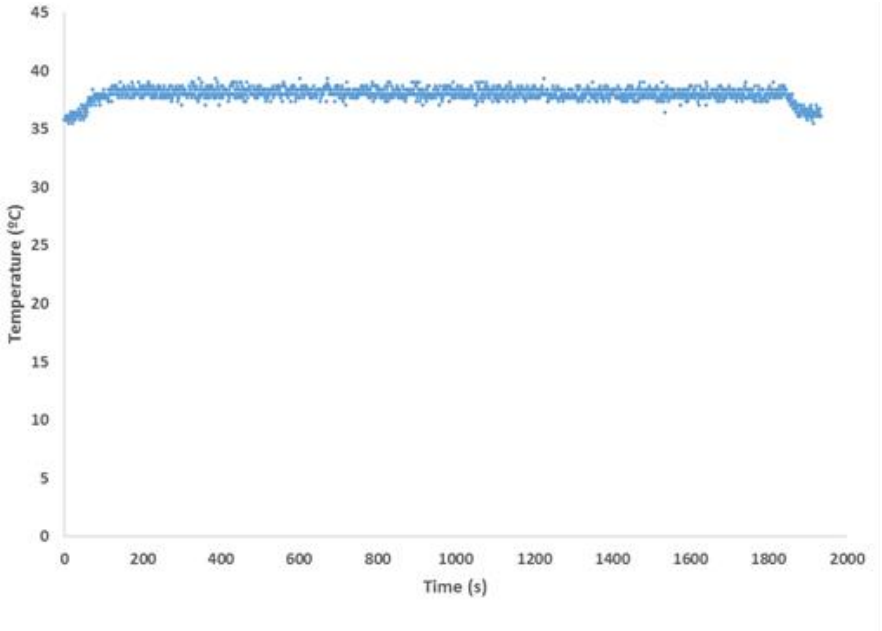

Figure 4.6: Time temperature profile at 37 ^{0}C baseline value, using 1.18 MHz FUS with 10 % DF, 60 W AP and 30 min sonication time.

The optimal frequency for US applications varies by treatment and is always a trade-off between the depth of penetration and the sharpness of the focus. For transcutaneous sonothrombolysis, frequencies up to 2 MHz are employed as a best trade-off between focus and penetration, while for intravascular sonothrombolysis, where

smaller penetration depths are required, frequencies greater than 2 MHz have been employed.

The acoustic parameters that mainly control heating are the AP, which is the amount of power sent to the transducer´s surface and the DF, which is the fraction of time that the US system is transmitting. Although longer DFs achieve better thrombolysis efficacy, the use of shorter DFs (≤ 10 %) is very attractive, since thrombolysis efficacy can be enhanced by increasing AP levels and the desired reduction in heating can be achieved. Since the temperature at the target was continuously monitored, the value of AP was set to the appropriate level, where no excess heating was produced ($\Delta T \leq 1\ ^0C$).

All experiments were conducted in a circulating unidirectional flow system, simulating blood flow in MCA occlusion. The peristaltic pump was set to maintain the appropriate flow rate for each study and degassed water was used as a fluid for the closed-loop flow system. The values for flow rate and TNK-tPA concentration were selected to be well below the maximum levels occurred in a physiological situation of MCA occlusion, following iv thrombolysis with thrombolytic drug.

During treatments, the temperature in the water tank was kept constant at 37 ^0C, using a heating element (Thermo Scientific, SC001, Haake, Germany). For each experimental study, a group of 5 clots was used. The group of clots treated with neither US exposure nor TNK-tPA, was served as the control, or untreated group.

4.8. Determination of the experimental parameters that influence thrombolysis efficacy

To maximize the effect of US on TNK-tPA mediated thrombolysis it was necessary to determine and evaluate all experimental parameters (both operating and physical), involved in this technique. Therefore, for the establishment of an optimised treatment protocol for therapeutic purposes, it was critically important to elucidate the role that each one of the following experimental parameters plays on thrombolysis efficacy.

Effect of frequency

The effect of frequency on thrombolysis efficacy was investigated in both focused and unfocused US studies. In all studies, two groups of clots were treated with US + TNK-tPA. For focused US studies, mass loss data were collected from clots exposed at 0.6 MHz compared with clots exposed at 1.18 MHz. For unfocused US studies, mass loss

data were collected from clots exposed at 3.7 MHz compared with clots exposed at 5.2 MHz respectively.

Effect of acoustic power

To examine the effect of AP on clot lysis, three groups of clots were treated with FUS + TNK-tPA. The degree of thrombolysis was determined for clots treated at AP of 20, 30 and 60 W respectively.

Effect of pulse length and PRF

To assess the effect of pulse length and associated PRF on clot lysis, two groups of clots were treated with FUS+TNK-tPA. Thrombolysis efficacy was measured as the relative reduction in the mass of the clots treated with 1000 Hz, and 100 Hz.

Effect of DF

To explore the effect of DF on thrombolysis efficacy, two groups of clots were treated with FUS+TNK-tPA. Data for clot lysis were collected from clots treated with a DF of 5 % compared to clots treated with a DF of 10 %.

Effect of sonication time

To evaluate the effect of sonication time on thrombolysis efficacy, three groups of clots were treated with FUS+TNK-tPA. Clot mass loss was assessed for exposure times of 15, 30 and 60 min respectively.

Effect of each modality alone and with synergy

To determine the enhancing effect of either focused on unfocused US waves on clot lysis, the decrease in clot weight of the group treated with US + TNK-tPA was compared with that of the group receiving the same TNK-tPA concentration but no US.

Effect of temperature

To study the effect of temperature on the lytic efficacy of TNK-tPA, three groups of clots were treated with TNK-tPA alone. Percentage mass loss was measured for clots treated at 37, 39 and 41 ^{0}C respectively.

Effect of TNK-tPA

To study the effect of TNK-tPA concentration on thrombolysis efficacy, two groups of clots were treated with TNK-tPA alone. Mass loss data were collected from clots treated at a concentration of 2.0 μg/mL compared to clots treated at a concentration

of 3.5 μg/mL. When the drug dose was established, the percentage decrease in clot weight of the group treated with TNK-tPA alone was compared with the control (untreated) group.

Effect of flow rate

To investigate the effect of flow rate on thrombolysis efficacy, two groups of clots were treated with FUS+TNK-tPA. Percentage mass loss was measured for clots exposed at a flow rate of 10 mL/min compared with clots exposed at a flow rate of 30 mL/min.

Effect of standing waves

To estimate the effect of standing acoustic waves on thrombolysis efficacy, two groups of clots were treated with FUS+TNK-tPA. Data on clot mass loss were taken for clots treated with and without the use of absorber, which prevents the formation of standing waves.

Effect of medium

To examine the effect of the acoustic properties of the medium on thrombolysis efficacy, two groups of clots were treated with FUS+TNK-tPA. Data on clot mass loss were gathered for clots treated 4 cm deep into a brain tissue mimicking phantom, compared with clots treated into degassed water.

Effect of bubbles

The administration of UCA in the form of gaseous MBs, as a way to further enhance the thrombolytic activity of TNK-tPA, was studied for both focused and unfocused US waves. Data on mass loss were collected from two groups of clots. The one group was treated with US+TNK-tPA, while the other one was treated with US+TNK-tPA and MBs. For FUS studies, a 0.5 mL bolus of MBs was injected in the reservoir of the flow system at the beginning of insonation. Since the half-life of the bubbles is between 3-6 minutes [108], [110], the procedure was repeated every 5 min, for the duration of a 30 min treatment (i.e. a total amount of 3 mL was used). For unfocused US studies, a 1.0 mL bolus of MBs was injected in the reservoir of the flow system at the beginning of insonation and the procedure was repeated every 6 min, for the duration of a 30 min treatment (i.e. a total amount of 5 mL was used). Moreover, the effect of MBs dose on the degree of clot lysis was investigated by comparing the percentage of

thrombolysis enhancement occurred at a dose of 3 mL with that occurred at a dose of 5 mL.

4.9. Statistical analysis

A paired difference test (Wilcoxon signed-rank test) was performed to determine if differences between pairs of experimental groups examined, follow a symmetric distribution around zero.

For comparisons between pairs of groups, the following procedure was followed:

1. The sample size, i.e., the number of pairs n = 5.
2. For pairs i = 1-5 the measurements were denoted as $\mathbf{X_{1,i}}$ and $\mathbf{X_{2,i}}$.
3. The difference between pairs $|X_{2,I}-X_{1,i}|$, and sgn $(X_{2,I}-X_{1,i})$ was calculated, where sgn is the sign function.
4. Pairs with $\mathbf{|X_{2,i} - X_{1,i,}| = 0}$ were excluded. The remaining pairs were ordered from smallest absolute difference to largest absolute difference and were ranked, starting with the smallest as 1. Ties receive a rank equal to the average of the ranks they span. $\mathbf{R_i}$ denotes the rank.
5. The test statistic **W** was calculated as it is shown below:

$$\mathbf{W = \sum_{i=1}^{n}[sgn(x_{2,i} - x_{1,i})R_i]} \qquad \mathbf{(4.4)}$$

Differences that yielded a test statistic |W| value > $W_{0.05,n}$ were considered to be significant.

4.10. Summary

A summary table with the experimental parameters (operating and physical), investigated in this research in order to optimise through non-thermal mechanisms their thrombolytic efficacy for potential therapeutic purposes, is provided below:

Table 6: List of experimental parameters that influence thrombolysis efficacy

Operating Parameters	*Physical Parameters*
Frequency	*US energy alone* *US energy + TNK-tPA* *US energy + TNK-tPA + MBs*
Acoustic Power	*Temperature*
Pulse length	*TNK-tPA concentration*
Pulse Repetition Period	*Flow rate*
Pulse Repetition Frequency	*Standing waves*
Duty Factor	*Attenuating medium*
Sonication time	*MBs administration and dose*

5. Focused ultrasound enhanced TNK-tPA mediated thrombolysis into a brain tissue mimicking phantom using an in vitro flow clot model

5.1. Introduction

The low and incomplete recanalization performance of thrombolytic therapy in patients with AIS has created the need to use US energy in synergy with thrombolytic drugs (sonothrombolysis), as a way to increase the degree of clot lysis. Recently, the use of FUS to enhance the activity of thrombolytic drugs has become an important area of investigation in sonothrombolysis, due to accurate clot targeting and low risk of side effects outside the beam focus.

To evaluate the thrombolytic efficacy of FUS on thrombolysis induced by thrombolytic agent TNK-tPA, an in vitro flow clot model was designed reproducing the physiological situation of MCA thrombosis, occurred deep into brain tissue. In order to succeed this, fully retracted blood clot samples were embedded 4 cm deep into brain tissue mimicking phantoms. As a result, the FUS waves at beam focus were significantly attenuated, due to absorption and scattering. The objective was to establish an optimized treatment protocol for 1.18 MHz pulsed FUS exposures that maximizes the permeation of thrombolytic agent into the clot, leading to increase degree of thrombolysis, without excess heating.

For this purpose, the role of various experimental parameters involved in this technique, was evaluated. These are: the TNK-tPA concentration, the FUS energy, the flow rate, the exposure time, the pulse length, the PRF, the DF, the formation of standing waves, the acoustic properties of the medium and the administration of MBs.

5.2. In vitro experimental set up

In this study, a set up as reliable as possible to the real situation of MCA thrombosis occurred 4 cm deep into a brain tissue was designed. Blood clots were formed into low density plastic tubing (4 mm inner diameter, 0.85 mm wall thickness), mimicking occluded cerebral arteries. Each tubing containing clot was positioned through a custom made ABS plastic holder (figure 5.1A) and immersed into a water tank filled with degassed water. A brain tissue mimicking phantom was constructed into a custom made

ABS plastic mould (figure 5.1B). The phantom was specially constructed to accommodate a clot tubing through it, reproducing a MCA occlusion occurred 4 cm deep into a brain tissue.

Figure 5.1: A) Custom made plastic holder, B) Custom made plastic mould for phantom's construction.

5.2.1. Brain tissue mimicking phantom

The ideal material for constructing a tissue mimicking phantom should have the same range of speed of sound as well as attenuation and scattering coefficient as soft tissues. Therefore, in our case a brain tissue mimicking phantom was constructed to simulate the biological properties of brain tissue [183]. Agar gel was selected as the base of the phantom, as it ensures mechanical characteristic similar to that of soft tissue. The agar powder used (Himedia Laboratories, Mumbai, India), produced a gel flexible enough to withstand high intensity US compression forces without cracking. To control the gel's scattering coefficient, crystalline silica dioxide powder (Merck Millipore, Darmstadt, Germany), was added as scattering material to reproduce the acoustic properties of brain tissue. Furthermore, evaporated milk was also added to the mixture in order to raise its attenuation coefficient to the average value of the brain tissue, which is approximately 0.6 dB/cmMHz [184]. The mixture was stirred and heated until agar melted (90 °C) and then poured in a custom made ABS plastic mould, specially designed to create a cavity (4 mm in diameter and 30 mm in height), at phantom's bottom. When the mould was completely filled with the mixture, was stored overnight at 4 ^{0}C to gellify. After the precise positioning of the US beam on target, the agar-evaporated milk-silica gel phantom, which was designed to fit exactly into the plastic holder, was placed with great caution over the tubing (figure 5.2). It should be noted that the distance between the clot and the front surface of the phantom was 4 cm.

5.2.2. Unidirectional circulating flow system

To provide a more realistic clinical environment, the physiological situation of flow was reproduced by exposing blood clots to a unidirectional circulating pulsatile flow.

The outlet tubing from the reservoir of the flow system after passing the peristaltic pump was connected to a Y-shaped connector. The one end was connected back to the reservoir (inlet tubing), in order to sustain circulation and the other end was connected to the clot tubing, embedded into the brain tissue mimicking phantom. The distal end of the tubing was terminated with a 3-way connector to ensure that the clot was not moving during treatment.

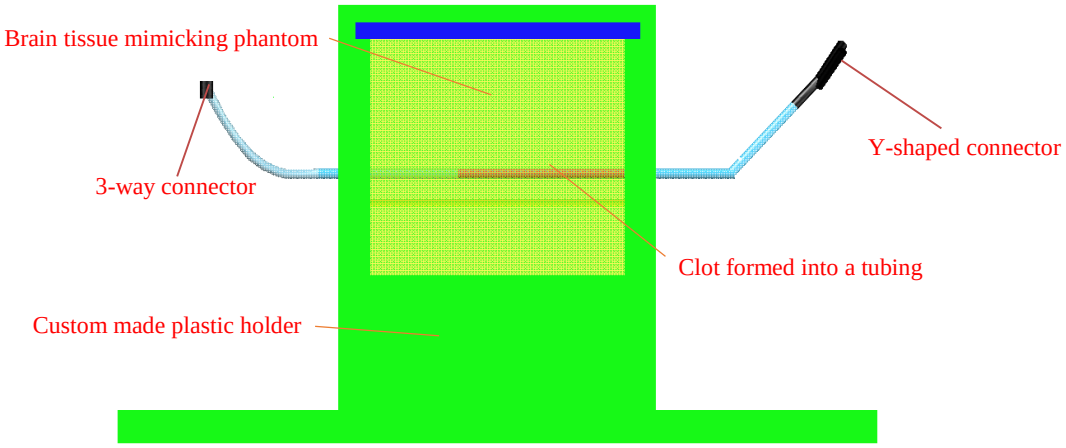

Figure 5.2: Placement of brain tissue mimicking phantom over the clot tubing.

The overall experimental set-up for in vitro FUS exposures into a brain tissue mimicking phantom, using a flow clot model is shown in figure 5.3.

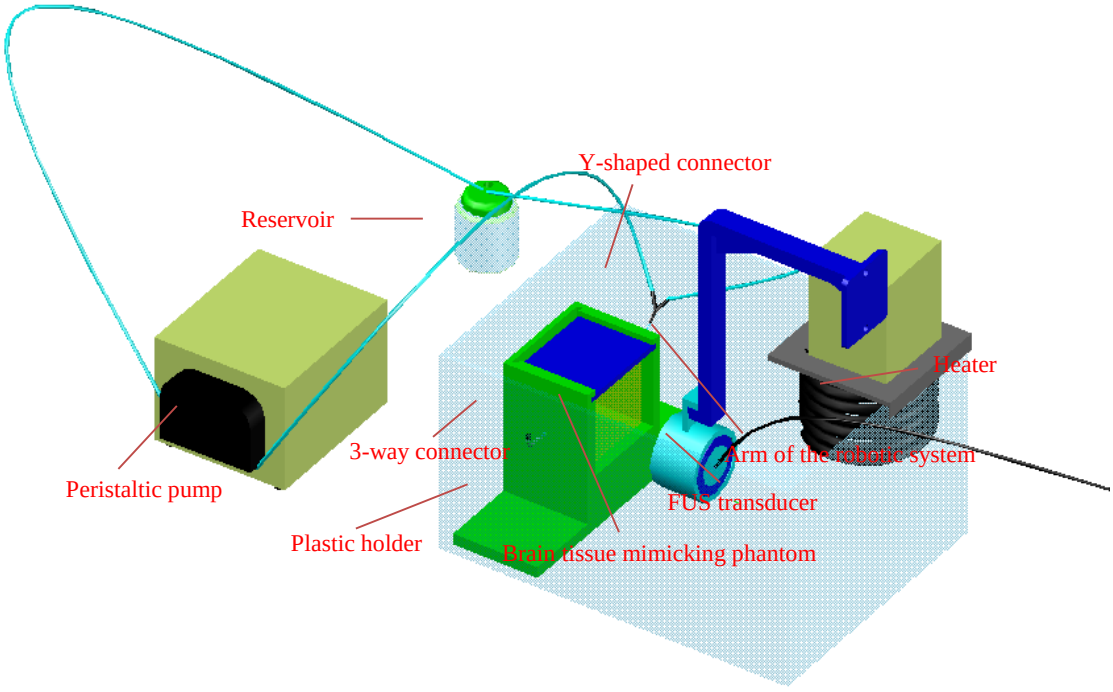

Figure 5.3: Experimental set-up for FUS exposures into a brain tissue mimicking phantom, using an in vitro flow clot model.

5.3. Optimum frequency

Although low US frequencies (< 1 MHz), penetrate better with minimal heating, an experimental kHz delivery system in combination with rt-PA resulted in a significant increase of intracerebral haemorrhage in patients with AIS, due to the formation of standing waves at and away from the therapeutic target locations [89], [133]. The increased potential of low frequency transcranial thrombolysis to induce standing waves, can be associated with the occurrence of longer wavelengths and reduced absorption in the brain tissue [185].

Motivated by these observations, a higher US frequency was examined as the best approach for eliminating the induction of standing waves in the skull without increasing the risk for producing adverse biological effects, such as thermal heating. Taking into consideration the above mentioned studies in association with the study by Spengos et al. 2000 [58], where they suggested that transcranial application of 1 MHz pulse wave US, may accelerate reperfusion and recanalization rate of occluded intracerebral vessels, by enhancing rt-PA mediated thrombolysis, it seems that an US frequency in the range between 1-1.5 MHz is the best choice for transcranial sonothrombolysis, as it penetrates the temporal bone with less attenuation than higher frequencies and hence, reduced heating [186-187]. Based on the above findings, all experimental studies in this chapter were conducted using 1.18 MHz FUS frequency.

5.4. Optimisation of treatment protocol

The effect of each one of the following experimental parameters on thrombolysis efficacy (see equation 4.2 in chapter 4), was evaluated. Values of thrombolysis (T) as a function of the experimental parameters examined are presented as mean ± SD. To make sure that the contribution of thermal mechanisms to clot lysis was negligible, the value of AP was set to the maximum possible level that maintained a temperature elevation at beam focus of 1 ˚C. The 1.18 MHz FUS transducer was driven in a pulsed mode with a DF of 10 % and a PRF of 100 Hz. This resulted in a pulse “ON” period (1 ms) followed by a pulse “OFF” period (9 ms). The sonication time was fixed at 30 min and the peristaltic pump was set to the lowest flow rate achievable (10 mL/min). Temperature measurements at the focus were taken every 1 second.

5.5. Results

Effect of TNK-tPA concentration

To explore the effect of TNK-tPA concentration alone, mass loss data were collected from clots treated at a concentration of 2.0 μg/mL compared to clots treated at a concentration of 3.5 μg/mL. When the drug dose was established, the percentage decrease in clot weight of the group treated with TNK-tPA alone was compared with that of the control (untreated) group. Figure 5.4 shows the thrombolysis vs. TNK-tPA concentration for treatment time = 30 min and flow rate = 10 mL/min.

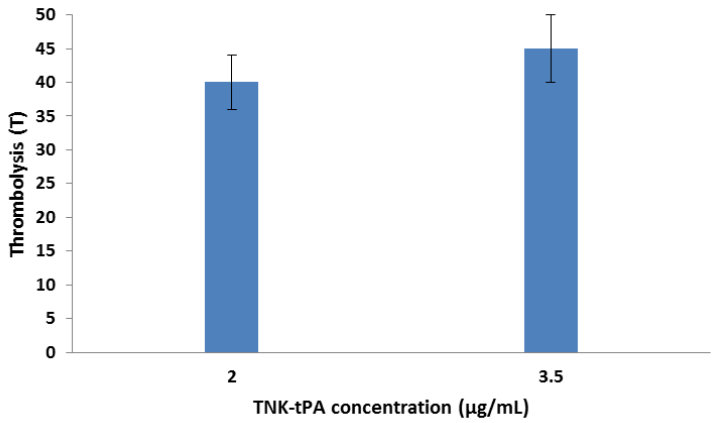

Figure 5.4: The effect of TNK-tPA concentration on thrombolysis efficacy. Experimental parameters: treatment time = 30 min, flow rate = 10 mL/min, water bath temperature = 37 ^{0}C, no FUS and no MBs.

Effect of each modality alone and in synergy

Next, the effect of each modality alone (FUS, TNK-tPA) and in in synergy was evaluated. Figure 5.5 shows the effect of no FUS, no TNK-tPA (control), TNK-tPA alone, FUS alone and FUS+TNK-tPA for f = 1.18 MHz, DF = 10 %, AP = 20 W, Pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL and no MBs. This result shows that even with no treatment there is a 30 % reduction in mass due to the placement of the clot in the experimental setup. Treatment of FUS alone or TNK-tPA alone has some effect, but clearly the combination of the two has enhanced effect.

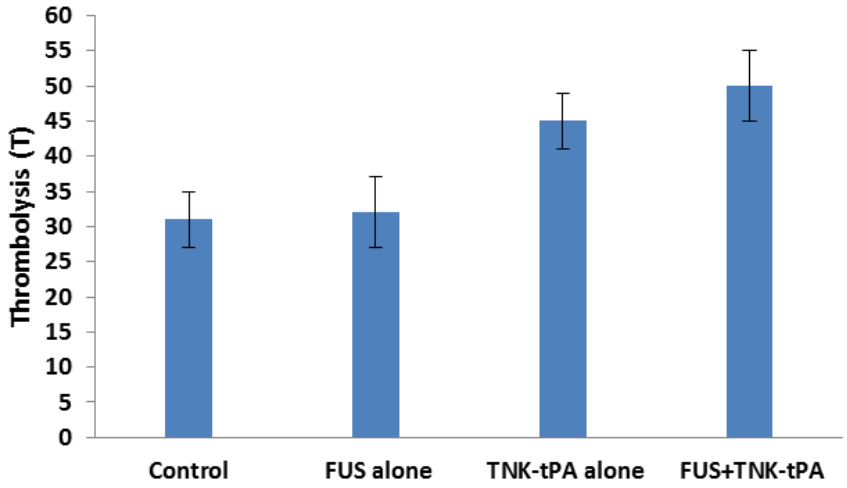

Figure 5.5: The degree of thrombolysis in untreated clots, in clots treated with FUS alone, in clots treated with TNK-tPA alone and in clots treated with FUS+TNK-tPA. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, water bath temperature = 37 ^{0}C and no MBs.

Similarly, by subtracting thrombolysis achieved in the control group, the enhancing effect of FUS on TNK-tPA mediated thrombolysis was assessed. Figure 5.6 shows the thrombolysis vs. FUS for f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL and no MBs. The reduction on clot mass is almost double with TNK-tPA and FUS compared to TNK-tPA alone. Therefore, in all subsequent experiments the synergy of TNK-tPA and FUS was implemented.

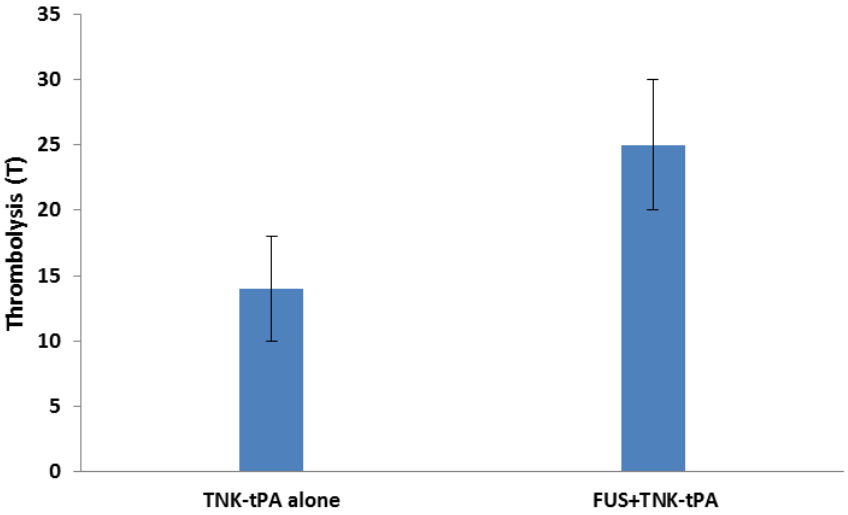

Figure 5.6: The enhancing effect of FUS on TNK-tPA mediated thrombolysis. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL, water bath temperature = 37 ^{0}C, and no MBs.

Effect of flow rate

To investigate the effect of flow, 2 different rates were used (10 mL/min and 30 mL/min). Figure 5.7 shows the thrombolysis vs. flow rate for f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, TNK-tPA concentration = 3.5 µg/mL and no MBs.

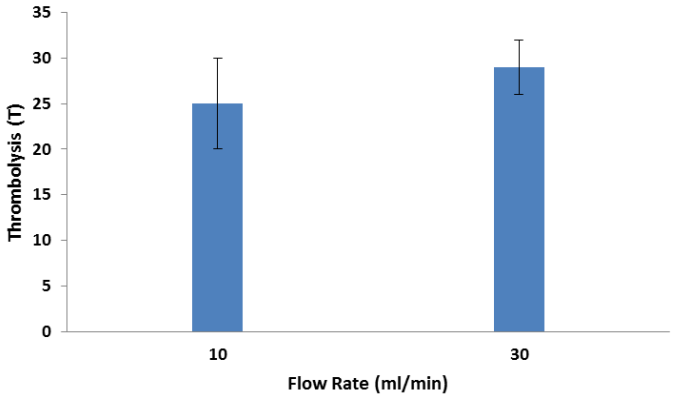

Figure 5.7: The beneficial role of flow rate on thrombolysis efficacy. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, TNK-tPA concentration = 3.5 µg/mL water bath temperature = 37 ^{0}C, and no MBs.

Effect of sonication time

The effect of sonication time was evaluated at 15, 30 and 60 min respectively. Figure 5.8 shows the thrombolysis vs. time for f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL and no MBs.

This result shows that saturation was achieved in 30 min, therefore in all subsequent experiments the 30 min duration was used.

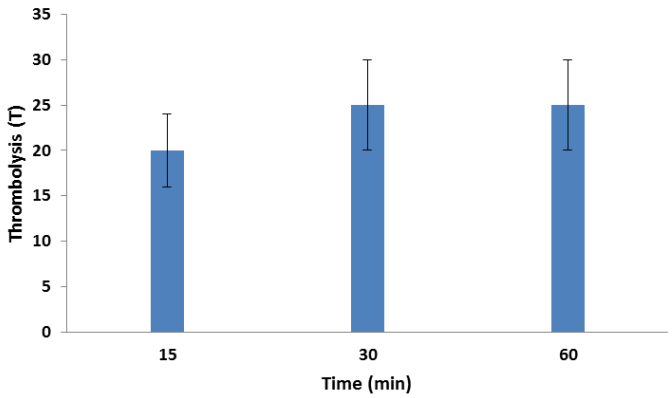

Figure 5.8: The effect of time on thrombolysis efficacy. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of pulse length-PRF

The effect of pulse length was estimated, using 2 different pulse durations (1 ms and 100 ms). Figure 5.9 shows the thrombolysis vs. pulse length for f = 1.18 MHz, DF = 10 %, PRF = 100 and 1 Hz, AP = 20 W, treatment time = 30 min, Flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL and no MBs.

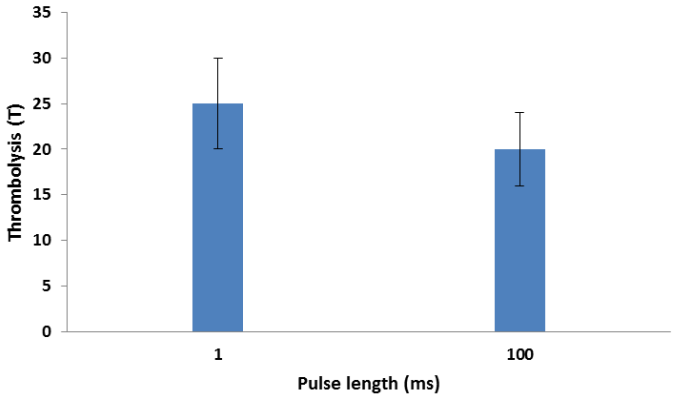

Figure 5.9: The effect of pulse length on FUS enhanced TNK-tPA induced thrombolysis. Experimental parameters: f = 1.18 MHz, DF = 10 %, PRF = 100 and 1 Hz, AP = 20 W, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of DF

The effect of DF was assessed at values of 5 % and 10 % with AP 40 W and 20 W respectively, thus establishing the same temporal average intensity. Figure 5.10 shows the thrombolysis vs. DF for f = 1.18 MHz, pulse length = 0.5 ms and 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL and no MBs. Obviously, DFs < 10 % did not play an important role on thrombolysis efficacy.

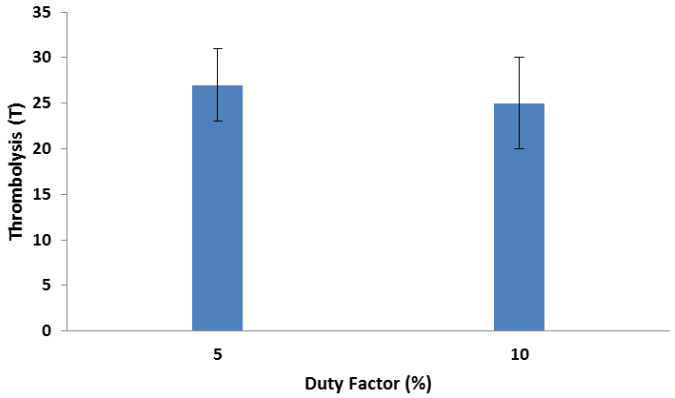

Figure 5.10: Relative thrombolysis enhancement as a function of DF. Experimental parameters: f = 1.18 MHz, AP = 20 W, pulse length = 0.5 and 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of standing waves

The effect of standing as well as travelling acoustic waves was estimated by using an absorbing medium. Figure 5.11 shows the thrombolysis vs. presence or absence of absorbing material for f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL and no MBs.

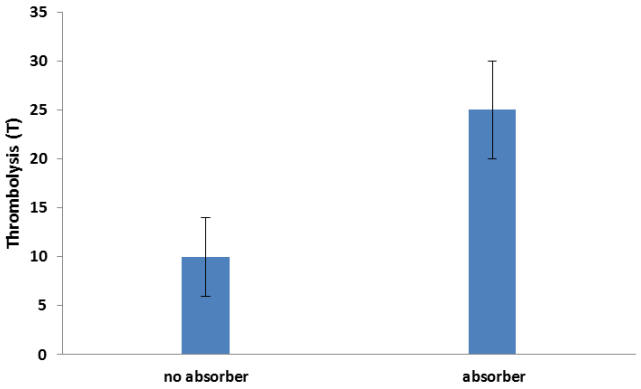

Figure 5.11: The impact of standing acoustic waves on thrombolysis efficacy. Experimental parameters: f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of medium

The effect of the medium was studied using water and agar. Figure 5.12 shows the thrombolysis vs. medium for f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL and no MBs.

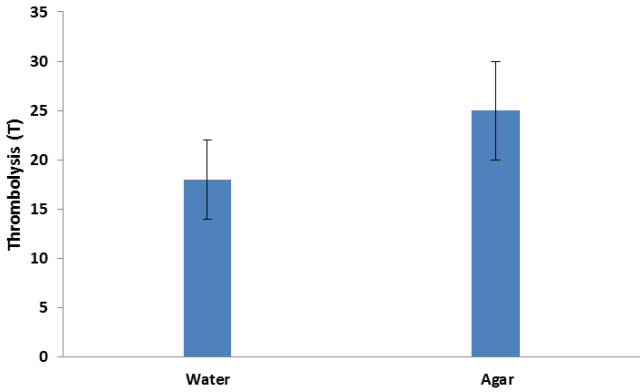

Figure 5.12: The influence of the medium on thrombolysis efficacy. Experimental parameters: f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 µg/mL water bath temperature = 37 ^{0}C, and no MBs.

Effect of bubbles

The effect of cavitation nuclei was investigated by injecting 2.5 mL of MBs (0.5 mL/5 min). Figure 5.13 shows the thrombolysis vs. MBs for f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL and MBs rate = 0.5 mL every 5 min.

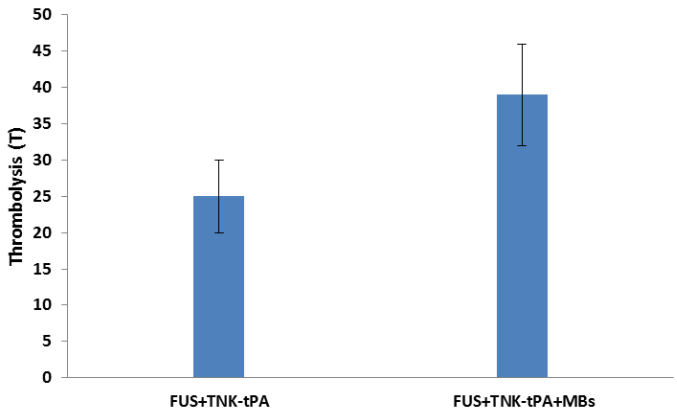

Figure 5.13: Thrombolysis enhancement due to MBs administration. Experimental parameters: f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, Treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL, water bath temperature = 37 ^{0}C and MBs rate = 0.5 mL every 5 min.

5.6. Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test. This paired difference test was used to test for significant differences on thrombolysis efficacy between the following pair groups:

1. TNK-tPA (2 μg/mL) group with TNK-tPA (3.5 μg/mL) group
2. Control group with TNK-tPA alone group
3. TNK-tPA alone group with FUS (1.18 MHz) + TNK-tPA group
4. Flow rate (10 mL/min) group with Floe rate (30 mL/min) group
5. Sonication time (15 min) group with Sonication time (30 min) group
6. Pulse length (100 ms) group with Pulse length (1 ms) group
7. Duty Factor (10 %) group with Duty Factor (5 %) group
8. Standing waves (no absorber) group with travelling waves (absorber) group
9. Medium (water) group with Medium (agar) group
10. FUS (1.18 MHz) + TNK-tPA group with FUS + TNK-tPA + MBs group

Applying the Wilcoxon signed-rank test for the difference (of the mean) between all pairs of experimental groups examined in this chapter, it turns out that $|W| >> W_{0.05,5}$,

leading to a strong rejection of the null hypothesis. Hence, it can safely be said that in all cases, the second sample (group) has a higher population mean.

5.7. Discussion

Thrombolysis efficacy is dependent on TNK-tPA concentration and increases with increasing thrombolytic concentration as shown in figure 5.4. Treatment with TNK-tPA alone produced more thrombolysis than that achieved either in the control or in the FUS alone (figure 5.5). Clots treated with FUS plus TNK-tPA exhibited a 25 % (56.2/45), greater degree of thrombolysis compared with clots treated with TNK-tPA alone (figure 5.5). The degree of thrombolysis achieved in the control was subtracted from that achieved in the other methods. Clots treated with FUS plus TNK-tPA exhibited enhanced thrombolysis over clots treated with TNK-tPA alone, as much as 80 % (25.2/14), as it is presented in figure 5.6. Study results are in good agreement with those from another in vitro study by Frenkel et al. [75]. In 2006, Frenkel and colleagues demonstrated that clots treated for 30 min with 1 MHz FUS + rt-PA (10 μg/mL), utilizing 100 ms pulses, exhibited 19 % greater degree of thrombolysis compared to clots treated with TNK-tPA alone. Thrombolysis enhancement was increased to 50 % when the degree of thrombolysis achieved in the control group was subtracted. Although the level of the AP used in their study was significantly higher than ours, we demonstrated a 33 % (25.2/19) greater degree of thrombolysis probably due to the following effects:

1. The presence of perfusion gradient through occlusive clots due to flow. The penetration of the thrombolytic agent into clots by perfusion is much more effective than by diffusion [188].
2. The type of the thrombolytic agent. TNK-tPA has a longer half-life and greater binding affinity for fibrin than rt-PA [21].
3. The acoustic properties of the medium. We demonstrated that the enhancement of clot dissolution is much more pronounced in a brain tissue mimicking phantom than in water, possibly due to preservation of the acoustic field. Study results showed that thrombolysis efficiency is 45 % (25.2/17.4) more effective, when is conducted in the agar medium, compared to the water (figure 5.12).

The effect of flow on the speed of fibrinolysis is always considered beneficial, since it is directly responsible for increased transport and penetration of PAs into blood clots, leading to enhanced thrombolysis. In the case of full occlusion, a situation occurred in our study, lysis proceeded from the inside, due to the non-uniform permeation of PAs into

the blood clot, through the least permeation-resistant. In a highly ischemic MCA, flow rates can vary between 0 and 15 cm/s [189]. In contrary, flow rates in an open MCA (without occlusion), can be as high as 50 cm/s [190]. In our experimental study, flow rate was set 6-times lower (8.3 cm/s). A 3 fold increase on the flow rate (25 cm/sec) resulted in 17 % (29/25.2) enhancement on thrombolysis efficacy, indicating that flow rate is directly proportional to pressure gradient (figure 5.7). In addition, it should be taken into account that in our in vitro case the clot was located at a dead end and thus flow was minimal. Normally, in an in vivo case, there is also flow from the veins resulting in an increased concentration of the thrombolytic agent that is in direct contact with the clot. So, our results are underestimated with this in vitro model.

Regarding the effect of time on clot lysis, study findings showed a steep increase in thrombolysis efficiency during the first 15 min of FUS exposure, where 90 % of clot mass loss was observed. After 30 min of treatment, the peak thrombolytic activity of TNK-tPA was measured, followed by a plateau for longer exposure times, where no further thrombolysis occurred (figure 5.8). The phenomenon that no thrombolytic activity took place between 30-60 min of treatment, can be explained from the time dependence curve of TNK-tPA concentration in blood, as presented in TIMI 10B trial [179], which was designed to compare prospectively the efficacy and safety of rt-PA and TNK-tPA. The blood concentration of TNK-tPA over time in the TIMI 10B trial, shows that after 30 min of treatment the initial concentration of the agent reduces below 50 % (17-24 min half-life) and drops down to 25 % at 60 min. Taking into consideration that FUS alone did not enhance thrombolysis compared to control clots, it is reasonable to assume that that the thrombolytic activity of TNK-tPA between 30-60 min of treatment was probably very low to cause further fibrinolysis.

Pulse length and associated PRF are directly related with the degree of clot displacement and thus, the depth of penetration of the thrombolytic agent into the clot. Study findings have shown that the transmission of 1 ms pulse with 100 Hz PRF, promoted deeper TNK-tPA penetration compared to the transmission of 100 ms pulse with 1 Hz PRF, resulting in a 26 % (25.2/19.4) thrombolysis enhancement (figure 5.9). Consequently, it is more effective to transmit shorter pulses more frequently, which keep the clot in constant motion, than to transmit longer pulses less frequently, which saturate clot's displacement for an extended period of time.

Since one of the main aspects of our treatment protocol was to avoid excessive heating, short DFs values were applied (≤ 10 %). The outcome of this experiment,

demonstrated a 7 % (27.0/25.2) enhancement in thrombolysis efficiency using a DF of 5 % (AP = 40 W and pulse length = 0.5 ms), compared to that of 10 % (AP = 20 W and pulse length = 1 ms), as it is presented in figure 5.10. Thrombolysis enhancement using 5 % DF, might be due to the increased level of ARF (AP was doubled), since it is well established that there is linear increasing dependence between AP levels and thrombolysis efficacy [75]. On the other hand, since the difference in thrombolysis enhancement between DFs of 5 and 10 % is within the statistical error, further investigation is required.

Concerning the effects of standing and travelling US waves on thrombolysis enhancement, experimental results have shown a significant difference in clot mass loss in relation with the wave type applied. Study findings demonstrated that the impact of travelling acoustic waves on enzymatic fibrinolysis is much more pronounced than in standing waves, resulting in a 163 % (25.2/9.6) enhancement in thrombolysis efficacy (figure 5.11).

The largest thrombolysis enhancement was estimated for clots treated with FUS plus TNK-tPA, in the presence of MBs. A clear synergistic effect was observed on enzymatic fibrinolysis induced by TNK-tPA when FUS waves interacted with injected MBs. Study outcome demonstrated that the injection of MBs (SonoVue) on a constant rate (to replace the destroyed bubbles), enhanced thrombolysis efficacy by 53 % compared to thrombolysis induced using only FUS and TNK-tPA (figure 5.13). In 2007, Prokop et al. reported that the enhancement of clot lysis in the presence of a thrombolytic drug is related to the cavitation process caused by the combination of US and MBs [66]. It is assumed that the administration of MBs (acting as cavitation nuclei) on a constant rate to sustain cavitation, significantly reduced acoustic cavitation threshold, giving rise to cavitation activity. In 1991, Apfel and Holland [191], quantified the threshold for inertial cavitation to occur (MI > 0.7), assuming that a full range of bubble sizes were present to provide nucleation centres and linear propagation occurs. It was estimated that during this experimental study, the physical conditions to support the onset of inertial cavitation probably did not existed and hence it is presumed that MBs enhanced sonothrombolysis was based mainly on stable cavitation activity.

In the light of our findings, although cavitation activity was not measured, it is reasonable to hypothesize, that the mechanism by which the administration of MBs improved FUS enhanced TNK-tPA thrombolysis is related with cavitation based phenomena and most likely with stable cavitation.

5.8. Conclusions

In the present study, an in vitro flow clot model was developed to evaluate the influence of experimental parameters involved on thrombolysis efficiency. We have demonstrated that the application of 1.18 MHz FUS waves, accelerated enzymatic fibrinolysis of porcine blood clots induced by the thrombolytic agent TNK-tPA. In addition, the injection of MBs, was also demonstrated as a way to further enhance the effect of FUS on TNK-tPA mediated thrombolysis. In all parametric studies, the enhancement of enzymatic fibrinolysis was occurred through non-thermal mechanisms, since the temperature at beam focus never exceeded 1 ^{0}C. Although the lytic effect of FUS was clearly demonstrated, the mechanisms behind clots dissolution were not elucidated. It is speculated that ARF and cavitation activity with accompanying microstreaming, are probably the most important mechanisms of FUS that dominantly appeared in our study contributing to thrombolysis enhancement.

Study findings, which are all associated with enhanced thrombolysis, helped us to optimize the treatment protocol for 1.18 MHz pulsed FUS waves that maximizes the thrombolytic efficiency of TNK-tPA. However, since the potential objective of thrombolytic treatment is the fast and complete clot dissolution, it is possible that even with this optimization, the proposed treatment might not unblock on time large occluded volumes. Therefore, the following needs should be done in order to improve thrombolysis efficacy:

1. Study results even with the use of low flow rates and TNK-tPA concentrations, has shown a significant increase in thrombolysis efficacy. Thus, it is likely that our findings would have been even more significant, under higher levels of flow rates and TNK-tPA concentrations.
2. Beam focus was stationary during the treatment period. We hypothesize, that if the beam focus was moving with the robotic system across the long axis of the clot following clot's edge (due to clot lysis, the position of clot's edge will change with time), presumably the degree of thrombolysis rate would have been even higher.
3. The use of a transducer that produces a larger beam area on clot's surface, in order to increase the uptake, penetration and binding of the thrombolytic agent into the clot.

Therefore, further optimization parametric studies are needed, before the proposed treatment protocol translated in vivo.

5.9. Summary

A summary table with the recommended therapeutic protocol for the treatment of a MCA occlusion occurred 4 cm deep into a brain tissue is provided below:

Table 7: Recommended therapeutic protocol for the treatment of a deep-seated MCA occlusion

Experimental Parameters	*Values*
Mode	*MR compatible FUS transducer*
Frequency	*1.18 MHz*
Acoustic Power	*20 W*
Duty Factor	*10 %*
Pulse length	*1 ms*
Pulse Repetition Period	*10 ms*
Pulse Repetition Frequency	*100 Hz*
Treatment time	*30 min*
Temperature	*37 ^{0}C*
Flow rate	*10 mL/min*
TNK-tPA concentration	*3.5 µg/mL*
MBs dose	*3 mL (0.5 mL/5 min)*
Treatment's outcome	*370 mg of mass clot removed*

6. The enhancing effect of focused ultrasound on TNK- tPA induced thrombolysis of a superficial target using an in vitro circulating flow model

6.1 Introduction

Since the most common cause of stroke is due to occlusion of MCA or one of its branches, an in vitro flow clot model was developed to reproduce the physiological situation of MCA branch occlusion, occurred superficially. For this purpose, fully retracted porcine blood clots were exposed to a circulating pulsatile flow and were treated with pulsed FUS exposures as an adjunct to thrombolytic TNK-tPA and in the presence or absence of MBs. FUS waves were delivered to the target through a cellophane window to minimize absorption.

In this study, the influence of various parameters such as temperature, frequency, AP, FUS energy and MBs administration on thrombolysis efficacy, was evaluated. The objective was to obtain the optimum value for each one of the parameter examined, in order to maximize the uptake, the penetration and the binding of the drug into the clot, leading to increase enzymatic thrombolysis. Thrombolysis efficacy was measured in mg of blood clot removed, except from that concerning the effect of temperature, where it was measured as a percentage of clot lysis.

The optimum operating parameters obtained in the previous chapter such as DF, pulse length, PRP, PRF and treatment time [76], were employed in this study in order to establish a treatment protocol that through non-thermal mechanisms, maximizes the amount of mass clot removed from a superficial target.

6.2 In vitro experimental set up

Porcine blood clots were formed into custom made ABS plastic containers. The lower part of each container (where the blood was naturally coagulated), included a 20 x 30 mm^2 window, closed with a thin cellophane membrane. The container comprising clot was fixed on a custom made ABS plastic holder and immersed into the water tank filled with degassed water. The physiological situation of flow in a MCA branch occlusion, was reproduced by exposing the clots to a unidirectional circulating pulsatile flow. The outlet tubing from the reservoir after passing the peristaltic pump was connected to the upper left side of the container. The inlet tubing was connected to the opposite right side, in

order to sustain circulation. The FUS transducer was mounted on the arm of the automated robotic system, immersed in the water tank and arranged opposite to the blood clot at such a distance in order to focus the US beam precisely on the target. The overall experimental set-up to evaluate the enhancing effect of FUS on TNK-tPA mediated thrombolysis, using an in vitro flow clot model is presented in figure 6.1 below.

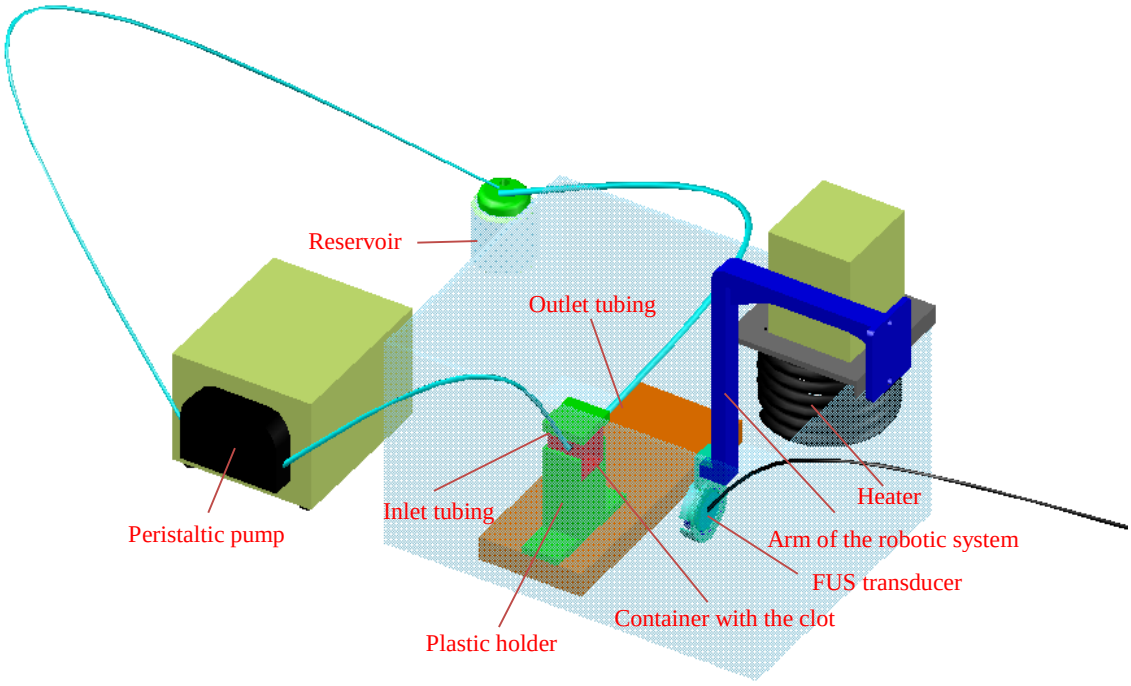

Figure 6.1: Experimental set up for direct FUS exposures, using an in vitro flow clot model.

6.3 Optimisation of treatment protocol

Once again, the main aspect for establishing the experimental protocol, was to maintain a localized temperature increase at beam focus that does not exceed 1˚C and keep the surrounding temperature constant. Values of thrombolysis (T) as a function of the experimental parameters examined such as, temperature, frequency, AP, FUS energy and MBs administration, are presented as mean ± SD.

Since the values for DF, PRF, PRP and exposure time, were kept the same as those obtained in the previous experimental study [76], the FUS transducer was driven in a pulsed mode with a DF of 10 % and a PRF of 100 Hz. As a result, each sonication set included a pulse “ON” period (1 ms) followed by a pulse “OFF” period (9 ms) and both the “ON” and “OFF” pulses were repeated in succession for the duration of a 30 min treatment (i.e., 3000 pulses).

In order to maximise thrombolysis efficacy, the levels of flow rate and TNK-tPA concentration were increased, compared with those used previously. As a result, the peristaltic pump was set to maintain a flow rate of 40 cm/s (5 fold increase) and the concentration TNK-tPA used was 7 μg/mL (2 fold increase). It is important to note that the values for flow rate and drug concentration were selected to be well below the maximum levels occurred in a physiological situation of MCA occlusion, following iv thrombolytic therapy. In such a case, the flow rate in an open MCA can be as high as 50 cm/s [190] and the average maximum TNK-tPA concentration in blood following iv bolus administration of 50 mg can be as high as 11.6 μg/mL [192].

6.4 Results

Effect of temperature

Firstly, data on the temperature dependence of TNK-tPA lytic efficacy would be useful in establishing an optimal treatment protocol. To study the effect of temperature on the lytic efficacy of TNK-tPA, 3 groups of clots were treated with TNK-tPA alone. Percentage mass loss was measured for clots treated at 37, 39 and 41 ^{0}C respectively. Figure 6.2 shows the thrombolysis vs. temperature for treatment time = 120 min, TNK-tPA concentration = 10.0 μg/mL and no flow rate. Study results demonstrated that the peak fibrinolytic activity of TNK-tPA was achieved at 37 ^{0}C.

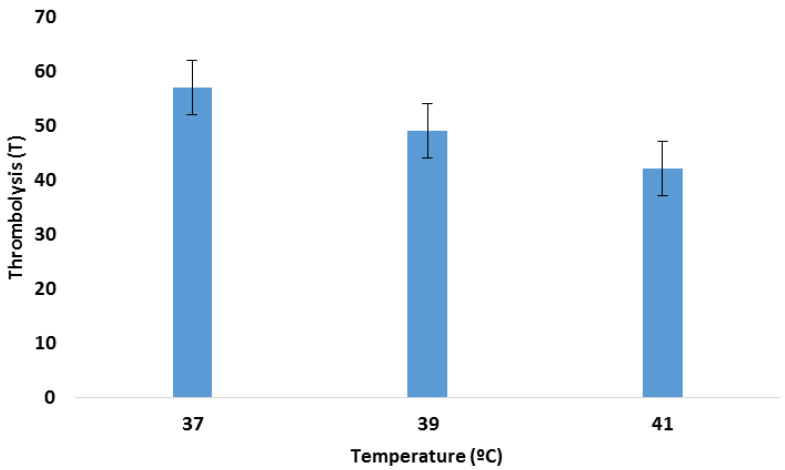

Figure 6.2: The effect of temperature on the lytic efficacy of TNK-tPA. Experimental parameters: no FUS, no MBs, no flow rate, treatment time = 120 min and TNK-tPA concentration = 10 μg/mL.

Effect of frequency

Generally, investigators use two commonly frequency ranges to enhance thrombolysis:

1. US frequencies in the kilohertz range, which are classified as low and have the advantage of better tissue penetration with reduced heating due to lower absorption rate,
2. US frequencies in the megahertz range, which are classified as high and have the advantage of sharp focus.

In this work, thrombolysis efficacy was evaluated using FUS pulses at 0.6 and 1.18 MHz respectively. To investigate the effect of frequency on thrombolysis efficacy, two groups of clots were treated with FUS + TNK-tPA. Mass loss data were collected from clots exposed at 0.6 MHz compared with clots exposed at 1.18 MHz. Figure 6.3 shows the thrombolysis vs. frequency for DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and no MBs. Since study results showed that the frequency in the kilohertz range produced less thrombolysis compared to that in the megahertz range, all subsequent experiments were conducted using a 1.18 MHz transducer.

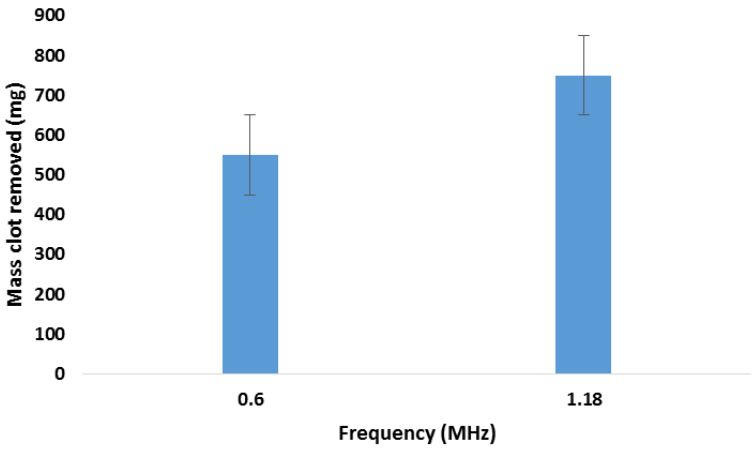

Figure 6.3: The effect of frequency on thrombolysis efficacy. Experimental parameters: DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms and PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of acoustic power

The degree of thrombolysis was examined over a range of APs up to the level where the temperature at beam focus did not exceed 1 ^{0}C. To examine the effect of AP on clot lysis, three groups of clots were treated with FUS + TNK-tPA. The degree of thrombolysis was determined for clots treated at AP of 20, 30 and 60 W respectively. Figure 6.4 shows the thrombolysis vs. AP for f = 1.18 MHz, pulse duration = 1.0 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and no MBs.

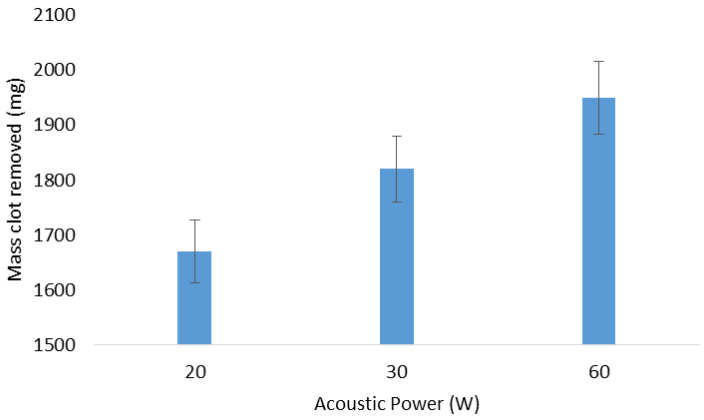

Figure 6.4: The effect of AP on clot lysis. Experimental parameters: f = 1.18 MHz, DF = 10 %, pulse duration = 1 ms, PRP = 10 ms and PRF = 100 Hz. treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of FUS energy

To determine the enhancing effect of FUS on clot lysis, the decrease in clot weight of the group treated with FUS + TNK-tPA was compared with that of the group receiving the same TNK-tPA concentration but no FUS. Moreover, the effect of each modality alone (FUS, TNK-tPA) and in in synergy was determined. Figure 6.5 shows the effect of no FUS, no TNK-tPA (control), FUS alone, TNK-tPA alone and FUS + TNK-tPA for f = 1.18 MHz, DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and no MBs. Even with no FUS, no TNK-tPA (control), the placement of the clots in the circulating flow system, resulted in 1200 mg of mass clot removed.

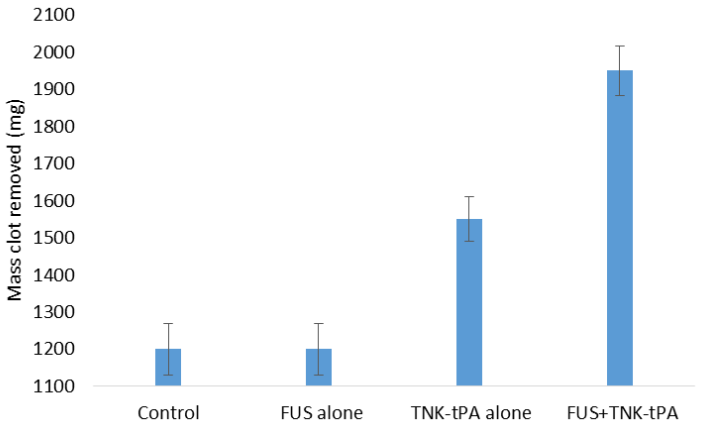

Figure 6.5: The degree of thrombolysis in untreated clots, in clots treated with FUS alone, in clots treated with TNK-tPA alone and in clots treated with FUS + TNK-tPA. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms and PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, water bath temperature = 37 ^{0}C and no MBs.

Similarly, by subtracting thrombolysis achieved in the control group, the enhancing effect of FUS on TNK-tPA induced thrombolysis was assessed. Figure 6.6 shows the thrombolysis vs. FUS for f = 1.18 MHz, DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and no MBs.

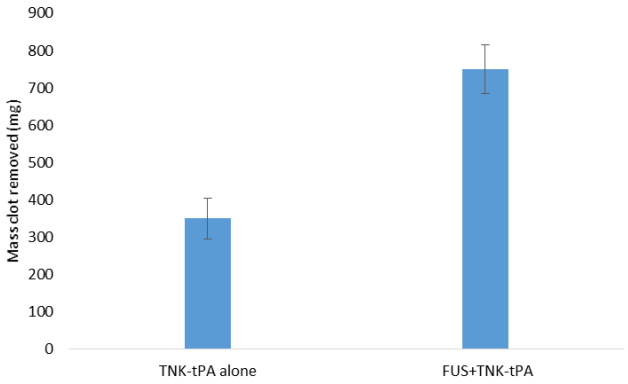

Figure 6.6: The enhancing effect of FUS on TNK-tPA mediated thrombolysis. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms and PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, water bath temperature = 37 ^{0}C and no MBs.

Effect of bubbles

The effect of cavitation nuclei on thrombolysis efficacy was explored by injecting 0.5 mL of MBs every 5 min. Figure 6.7 shows the thrombolysis vs. MBs for f = 1.18 MHz, AP = 60 W, pulse duration = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and MBs rate = 0.5 mL every 5 min.

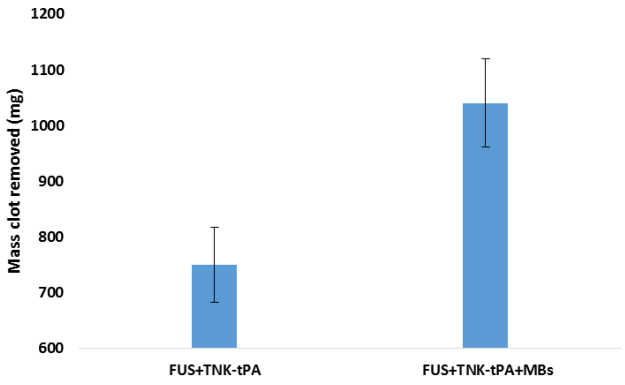

Figure 6.7: The effect of MBs administration on thrombolysis efficacy. Experimental parameters: f = 1.18 MHz, DF = 10 %, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms and PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, Water bath temperature = 37 ^{0}C and MBs rate = 0.5 mL/5 min.

6.5 Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test. This paired difference test was used to test for significant differences on thrombolysis efficacy between the following pair groups:

1. Temperature (41 ^{0}C) group with Temperature (39 ^{0}C) group
2. Temperature (39 ^{0}C) group with Temperature (37 ^{0}C) group
3. Frequency 0.6 MHz group with Frequency 1.18 MHz group
4. Acoustic Power (20 W) group with Acoustic Power (30 W) group
5. Acoustic Power (30 W) group with Acoustic Power (60 W) group
6. Control group with TNK-tPA alone group
7. TNK-tPA alone group with FUS (1.18 MHz) + TNK-tPA group
8. FUS (1.18 MHz) + TNK-tPA group with FUS (1.18 MHz) + TNK-tPA + MBs group

Applying the Wilcoxon signed-rank test for the difference (of the mean) between all pairs of experimental groups examined in this chapter, it turns out that $|W| >> W_{0.05,5}$, leading to a strong rejection of the null hypothesis. Hence, it can safely be said that in all cases, the second sample (group) has a higher population mean.

6.6 Discussion

An in vitro flow clot model was used to investigate the effect of temperature on the thrombolytic efficacy of TNK-tPA. In this model, blood clots were exposed to a certain concentration of TNK-tPA, at fixed temperatures (37, 39 and 41 ^{0}C). Temperatures below the body baseline temperature of 37 ^{0}C were not investigated because:

1. Some other in vitro studies, showed that the thrombolytic efficacy of rt-PA is reduced at lower temperatures ($T \leq 35$ °C), which anticipated for clinical hypothermic therapy [193-194].
2. Hypothermic studies showed an increased risk of hemorrhage [193].

Study results showed that temperature has a significant impact on thrombolysis in clots exposed to TNK-tPA. The study also demonstrated that the peak fibrinolytic activity of TNK-tPA occurred at 37 ^{0}C and decreases approximately 4 %/^{0}C increase in temperature (figure 6.2). These findings are in good agreement with those from other in vitro studies [194], which have shown that the fibrinolytic activity of rt-PA, decreases 5 %/^{0}C increase in temperature.

The optimal frequency for US applications is a trade-off between the sharpness of the focus and the depth of penetration. Although low US frequencies are more penetrating (due to less attenuation), does not allow energy focusing so sharply as high frequencies does. Due to their narrower focal spots, higher frequencies generate higher pressures and intensities, leading to higher ARF output [195].

In the case of fluids, ARF creates acoustic streaming [196], while in tissues it causes motion [197]. The ARF generated in tissue as a result of FUS exposures, has been reported to cause repetitive micron sized tissue displacements, creating structural changes that enable greater amounts of TNK-tPA to penetrate the clots and expose additional binding sites, leading to increase thrombolysis efficacy. In our study, clots treated with the operating frequency of 1.18 MHz exhibited 11.4 % (1950/1750), increased degree of thrombolysis compared to clots treated with the operating frequency of 0.6 MHz (figure 6.3). Therefore, according to literature, the differences in measured clot lysis may be attributed to the 175 % difference between the two operating frequencies used (1.18 vs. 0.6 MHz), since it is reported that the higher the operating frequency the higher the associate ARF [75], [198].

Regarding the effect of acoustic power on thrombolysis efficacy, a linear relationship was observed, in the range between 20-60 W (figure 6.4). Thrombolysis efficacy at AP levels above 60 W was not attempted since the temperature at beam focus would exceeded our prerequisite of 1 ^{0}C. The application of 20 W AP resulted in 1670 mg of mass clot removed. When AP was increased from 20 to 40 W, a significant increased rate in thrombolysis efficacy was achieved, leading to 1820 mg of mass clot removed. A second increase in AP from 40 to 60 W caused a further significant increase in thrombolysis efficacy with 1950 mg of mass clot removed. Our observation is supported by another in vitro study showing that a linear increase of AP is directly related with enhanced thrombolysis, due to a proportional increase in the ARF [75]. In 2006, Frenkel et al. using 1 MHz FUS pulses, demonstrated a linear increasing dependence between transmitted AP and thrombolysis efficacy, in the range of 20-80 W [75]. Similarly, Hancock et al. [49] and Wright et al. [199], using FUS pulses at 1.0 and 1.5 MHz respectively, also observed a linear correlation between transmit AP and the magnitude of clot displacement, as predicted for the generation of radiation forces [200].

Using a theoretical model described by Zanelli et al. [201], the intensity gain of our 1.18 MHz FUS transducer at beam focus was estimated and then, by applying equation 2.10, a peak clot displacement (ξ_{max}), of 256 nm was calculated at a transmit AP

of 60 W (see appendix 3). In another in vitro study published by Jones et al. 2010 [198], a peak clot displacement of 243 μm was measured with a 1 MHz, around 5 % decrease from the value calculated in this study (with 1.18 MHz), at equivalent AP. Similarly, Wright et al. [199], measured a peak clot displacement of 263 μm with 1.5 MHz at 60 W AP. Consequently, the peak clot displacement calculated in this study is in a good agreement with those measured experimentally at equivalent AP and in the frequency range between 1-1.5 MHz, showing that the generation of clot displacements through ARF, is responsible for increasing thrombolytic drug's efficacy and hence is associated with enhanced thrombolysis.

In view of the findings in the above mentioned studies, in association with the results of the present study, it is speculated that ARF is the mechanism of action that dominantly appears in the propagation of pulsed FUS waves through a blood clot and is accompanying by enhanced thrombolysis.

Treatment with TNK-tPA alone produced more thrombolysis than that achieved either in the control or in the FUS alone (figure 6.5). The effect of FUS alone on thrombolysis efficacy is practically negligible (figure 6.5). Clots treated with FUS plus TNK-tPA exhibited almost 26 % (1950/1550), greater degree of thrombolysis compared with clots treated with TNK-tPA alone (figure 6.5). When the degree of thrombolysis achieved in the control was subtracted from that achieved in the other methods, clots treated with FUS plus TNK-tPA exhibited enhanced thrombolysis over clots treated with TNK-tPA alone, as much as 114 % (750/350), as it is presented in figure 6.6.

Frenkel and colleagues[75], demonstrated in their study, that clots treated for 30 min with 1 MHz FUS + rt-PA (10 μg/mL), exhibited 19 % greater degree of thrombolysis compared to clots treated with TNK-tPA alone. Thrombolysis enhancement was increased to 50 % when the degree of thrombolysis achieved in the control group was subtracted. Although the levels of acoustic parameters applied in both studies were the same (DF = 10 %, AP = 60 W), a significant greater degree of thrombolysis was observed in our study, which might be probably due to the following effects:

1. The presence of perfusion gradient through occlusive clots due to flow. The penetration of the thrombolytic agent into clots by perfusion is much more effective than by diffusion [188].

2. The type of the thrombolytic agent. TNK-tPA has a longer half-life and greater binding affinity for fibrin than rt-PA [21].

3. The 18 % difference between the two operating frequencies used (1.18 vs. 1.0 MHz), since it is reported that the higher the operating frequency, the higher the ARF [75], [198].

The largest thrombolysis enhancement was assessed when clots were treated with FUS plus TNK-tPA in the presence of MBs. Study outcome demonstrated that the interaction of FUS waves with MBs, provided more efficient use of thrombolytic enzymes. The synergistic effect of FUS in combination with MBs on the enzymatic fibrinolysis induced by TNK-tPA, enhanced thrombolysis efficacy by 40 % (1050/750), compared to thrombolysis induced using only FUS + TNK-tPA (figure 6.7). This phenomenon can be explained by the ability of MBs to act as cavitation nuclei. As a result, the acoustic cavitation threshold is reduced, giving rise to cavitation activity. It is well established, that cavitation activity induces changes in the fibrin matrix, accelerates the transport and penetration of fibrinolytic agents into the clot and hence, increases the degree of clot dissolution [202]. In addition, it is important to bear in mind that the cavitation properties of MBs depend on their size, which influences their resonant frequency.

According to equation 2.18 in chapter 2, the theoretical resonance radius of MBs in 1.18 MHz US field, is approximately 2.5 μm. Based on this approximation and taking into consideration that MBs mean size is between 2-8 μm [108-110], it is not unreasonable to assume that the bubbles in this study were driven near their resonance frequency, which is correlated with enhanced thrombolysis [147].

Although cavitation activity was not detected in our experiments, the negligible contribution of thermal mechanisms on clot lysis, has lead us to make the hypothesis that MBs enhanced sonothrombolysis is directly related with cavitation based phenomena (non-thermal mechanisms). Since in this study, the theoretical threshold for the onset of inertial cavitation (MI > 0.7), probably never exceeded, we can postulate, that MBs enhanced sonothrombolysis is related most likely with stable cavitation.

6.7 Conclusions

In conclusion, this experimental work clearly demonstrated that thrombolysis efficacy can be significantly enhanced in vitro when pulsed FUS exposures are combined with the thrombolytic agent TNK-TPA.

Furthermore, the administration of MBs on a constant rate to sustain cavitation activity, was established as a way to further accelerate FUS enhanced TNK-tPA mediated

thrombolysis. For that reason, MBs mediated sonothrombolysis may offer a new approach to improve outcomes in patients with AIS.

Also, study results showed that the fibrinolytic activity of TNK-tPA varies with temperature and it is reasonable to say that temperature significantly affects thrombolysis in terms of both thrombolysis efficacy and haemorrhage risk.

Taking into consideration that for intracranial occlusion, low-frequency US has the potential to cause intracerebral haemorrhage in association with the results of the present study, it is confirmed that for transcranial sonication, a frequency of 1.18 MHz, is employed as a best trade-off between focus and penetration. We can assume that transcranial application with 1.18 MHz pulsed FUS waves is a good choice for treating stroke patients, since:

1. It penetrates the temporal bone with less attenuation than higher frequencies, i.e. reduces the possibility to overheat the skull [187],
2. It is not associated with any excessive risk of intracerebral haemorrhage due to formation of standing waves in the cerebral cavity [203], and
3. It improves recanalization rates and clinical outcomes [58].

Moreover, emphasis should be given to the biological effects produced when FUS energy interacts with tissue, since bioeffects are the basis for new FUS therapies. The low DF used in our experiments, lead to low energy deposition rates in tissue and hence minimal thermal rise at beam focus ($\leq 1\ ^{0}C$), making sure that no bioeffects were generated from thermal mechanisms.

As a final point, this study has shown promising results concerning the enhancing effect of FUS pulses in synergy with MBs, on TNK-tPA induced thrombolysis, since with 30 min of treatment, 1050 mg of clot was removed with no excess heating. Taking into consideration that stroke is time dependent, this thrombolytic rate should be sufficient for timely recanalization of the occluded cerebral artery. Based on the encouraging results of the study, the proposed therapeutic protocol can be applied in vivo for the treatment of a superficial target such as the fast and complete recanalization of an occluded carotid artery of a rabbit.

6.8 Summary

A summary table with the recommended therapeutic protocol for the treatment of a MCA occlusion occurred superficially is provided below:

Table 8: Recommended therapeutic protocol for the treatment of a superficial MCA occlusion

Experimental Parameters	*Values*
Mode	*MR compatible FUS transducer*
Frequency	*1.18 MHz*
Acoustic Power	*60 W*
Duty Factor	*10 %*
Pulse length	*1 ms*
Pulse Repetition Period	*10 ms*
Pulse Repetition Frequency	*100 Hz*
Treatment time	*30 min*
Temperature	*37 ^{o}C*
Flow rate	*50 mL/min*
TNK-tPA concentration	*7 µg/mL*
MBs dose	3 mL (0.5 mL/5 min)
Treatment's outcome	1050 of mass clot removed

7. Microbubble-based sonothrombolysis using a planar rectangular ultrasonic transducer

7.1. Introduction

The objective of this experimental study, was to evaluate the ability of small planar rectangular (2x10 mm^2) ultrasonic transducers, to enhance thrombolysis induced by the thrombolytic agent TNK-tPA. The intended clinical application of this study is the minimally invasive treatment of stroke patients, using intravascular ultrasonic transducers in combination with thrombolytic drugs and MBs. In future clinical trials, the transducer will be incorporated in a catheter for intravascular insertion (1-3 mm wide) and thus, the element of the transducer must be as compact as possible.

Using an in vitro circulating flow clot model, the physiological situation of flow in a superficial MCA branch occlusion, was reproduced. To provide a more realistic clinical environment of an intravascular treatment of stroke, the transducer was positioned as close as physically possible to clot's proximal surface, simulating an invasive approach of sonothrombolysis. For this purpose, fully retracted porcine blood clots were exposed to unidirectional circulating pulsatile flow and were treated with high frequency planar pulsed US waves, along with thrombolytic TNK-tPA, in the presence or absence of MBs. Degassed water was used as the fluid for the closed-loop flow system.

Two ultrasonic flat rectangular transducers were used, operating at 3.7 and 5.2 MHz respectively. A pulsed US protocol that maintained temperature elevation at the target of 1 ^{0}C was applied, providing that thrombolytic enhancement occurred mainly through non-thermal mechanisms. The impact of different experimental parameters such as US energy, frequency and MBs administration on thrombolysis efficacy, was evaluated. Thrombolysis efficacy was measured in mg of blood clot removed.

7.2. In vitro experimental set up

Porcine blood clots were formed into custom made ABS plastic containers. The lower part of each container (where the blood was naturally coagulated), included a 20 x 30 mm^2 window, closed with a thin cellophane membrane. The container comprising clot was fixed on a custom made ABS plastic holder and immersed into the water tank filled with degassed water. The planar rectangular US transducer was mounted on the arm of the automated robotic system, immersed in the water tank and positioned as close as physically possible to the proximal portion of the blood clot The overall experimental set-

up to evaluate the effect of high frequency US waves on TNK-tPA mediated thrombolysis with the use of in vitro flow clot model, is presented in figure 7.1 below.

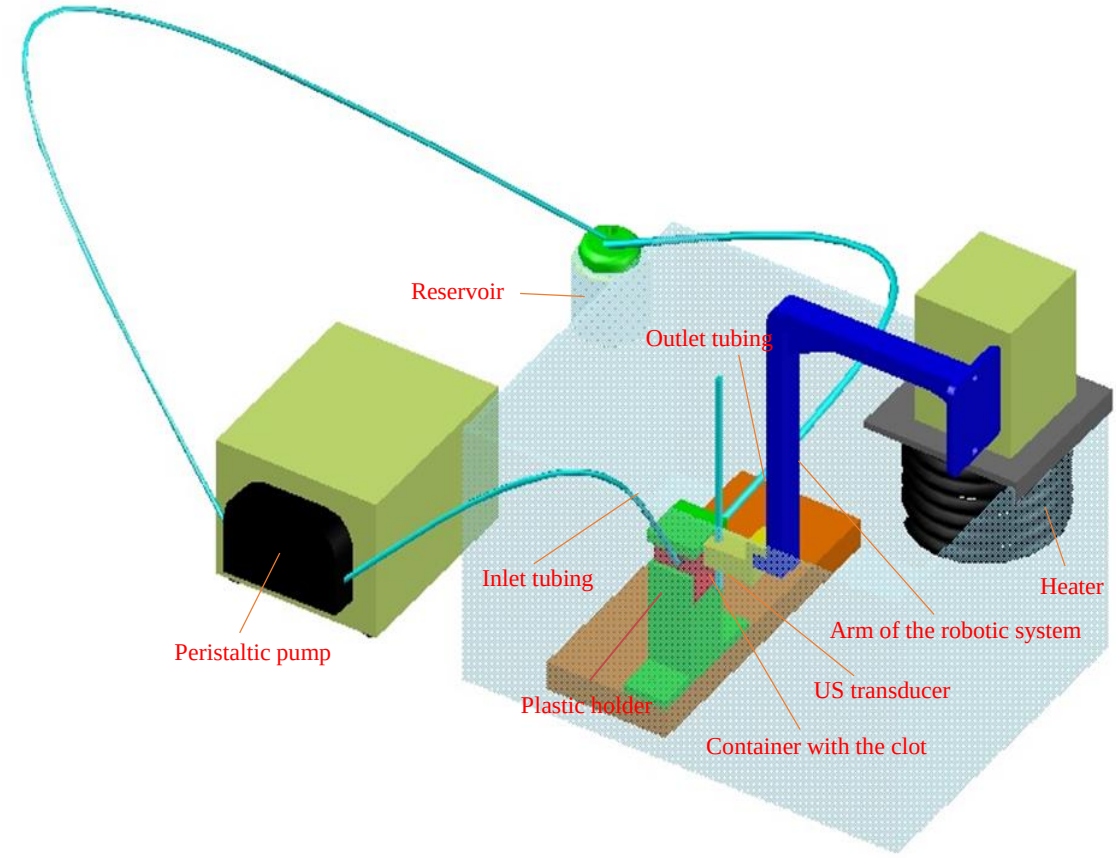

Figure 7.1: The experimental set up for in vitro sonothrombolysis, using a planar rectangular transducer.

7.3. Optimisation of treatment protocol

In all experiments, the values of thrombolysis are presented as mean ± SD. The main aspect for establishing the experimental protocol, was to maintain a temperature increase at the target that does not exceed 1 ^{0}C. The planar US transducers were driven in a pulsed mode with a DF of 10 % and a PRF of 1 Hz. As a result, each sonication set included a pulse "ON" period (100 ms) followed by a pulse "OFF" period (900 ms) and both the "ON" and "OFF" pulses were repeated in succession for the duration of a 30 min treatment. Since the temperature at the target was continuously monitored, AP was set to the appropriate level, where no excess heating was produced ($\leq$ 1 ^{0}C).

Flow rate and TNK-tPA concentration were set at the same levels as those used in the previous experimental study. The flow rate was selected to be 20 % lower than the maximum value occur in an open MCA [190], while the drug concentration was selected to be 40 % lower of the average maximum concentration in blood, following iv thrombolysis with 50 mg of TNK-tPA [192]. Hence, the peristaltic pump was set to

maintain a flow rate of 40 cm/s and the concentration of the thrombolytic drug in the flow system was 7 µg/mL.

7.4. Results

Effect of US energy

To determine the enhancing effect of US energy on clot lysis, the effect of TNK-tPA in synergy with US waves at frequencies 3.7 and 5.2 MHz respectively, was compared to that of TNK-tPA alone. Figure 7.2 shows the effect of no US and no TNK-tPA (control), TNK-tPA alone and US + TNK-tPA for f = 5.2 MHz and 3.7 MHz, DF = 10 %, AP = 20 W, pulse duration = 100 ms, PRP= 1000 ms, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration =7.0 µg/mL, temperature of the water bath = 37 ^{0}C and no MBs. Even with no US, no TNK-tPA (control), the placement of the clots in the circulating flow system, resulted in 1000 mg of mass clot removed.

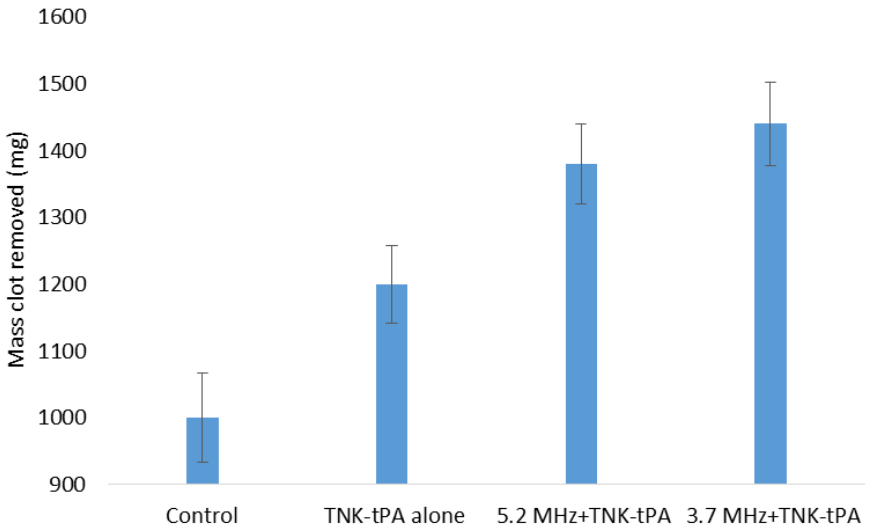

Figure 7.2: The degree of thrombolysis in untreated clots, in clots treated with TNK-tPA alone, in clots treated with 5.2 MHz US + TNK-tPA and in clots treated with 3.7 MHz US + TNK-tPA. Experimental parameters: DF = 10 %, AP = 20 W, pulse duration = 100 ms, PRP = 1000 ms, PRF = 1 Hz, treatment time = 30 min, flow rate = 50 mL/min, water bath temperature = 37 ^{0}C and no MBs.

By subtracting thrombolysis achieved in the control group, the enhancing effect of US on TNK-tPA induced thrombolysis was assessed. Figure 7.3 shows the thrombolysis with TNK-tPA alone and with US + TNK-tPA (f = 3.7 and 5.2 MHz), for DF = 10 %, AP = 20 W, pulse duration = 100 ms, PRP= 1000 ms, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and no MBs.

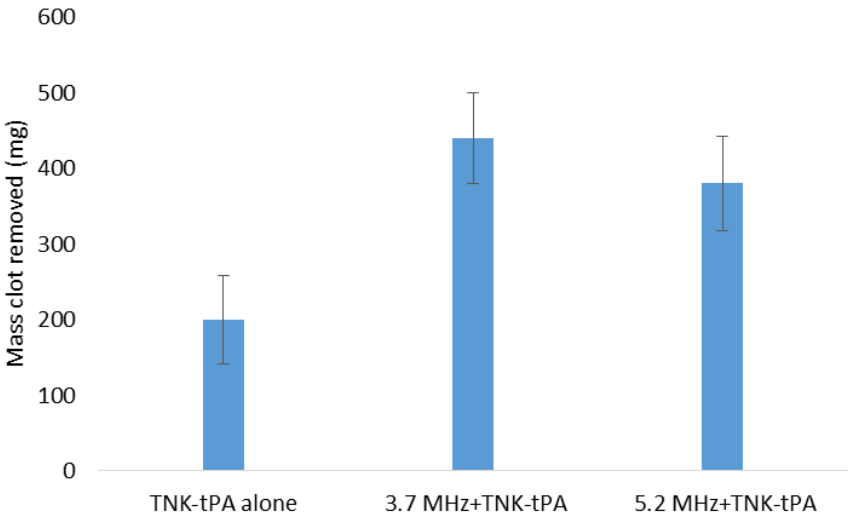

Figure 7.3: The enhancing effect of US waves on TNK-tPA mediated thrombolysis. Experimental parameters: f = 3.7 and 5.2 MHz, DF = 10 %, AP = 20 W, Pulse duration = 100 ms, PRP =1000 ms, PRF = 1 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL.

Effect of bubbles

The effect of cavitation nuclei on thrombolysis efficacy was explored by injecting 1 mL of MBs every 6 min. Figure 7.4 shows the thrombolysis vs. MBs for f = 5.2 MHz, DF = 10 %, AP = 20 W, pulse duration = 100 ms, PRP= 1000 ms, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL, temperature of the water bath = 37 ^{0}C and MBs rate = 1 mL every 6 min and figure 7.5 shows the corresponding results with f = 3.7 MHz.

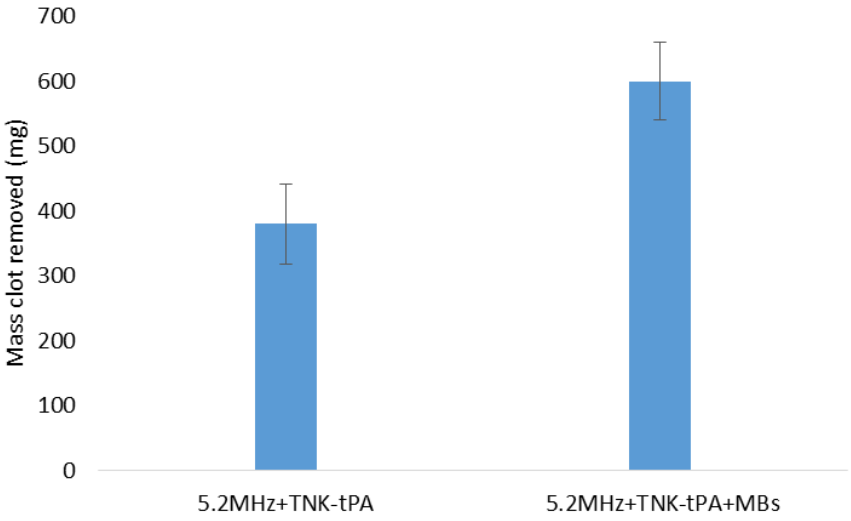

Figure 7.4: The effect of MBs administration on thrombolysis efficacy. Experimental parameters: f = 5.2 MHz, DF = 10 %, AP = 20 W, pulse duration = 100 ms, PRP = 1000 ms, PRF = 1 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 µg/mL.

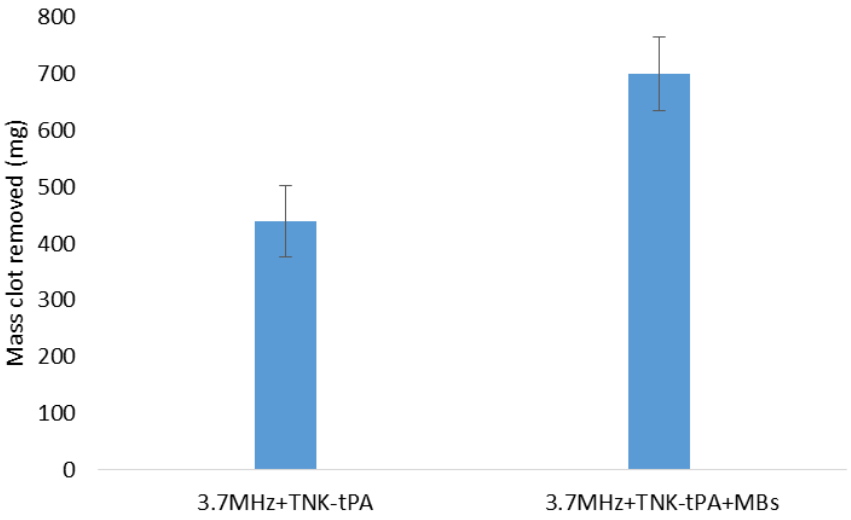

Figure 7.5: The effect of MBs administration on thrombolysis efficacy. Experimental parameters: f = 3.7 MHz, DF = 10 %, AP = 20 W, pulse duration = 100 ms, PRP = 1000 ms, PRF = 1 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 μg/mL.

7.5. Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test. This paired difference test was used to test for significant differences on thrombolysis efficacy between the following pair groups:

1. Control group with TNK-tPA alone group
2. TNK-tPA alone group with US (5.2 MHz) + TNK-tPA group
3. TNK-tPA alone group with US (3.7 MHz) + TNK-tPA group
3. US (5.2 MHz) + TNK-tPA group with US (5.2 MHz) + TNK-tPA + MBs group
4. US (3.7 MHz) + TNK-tPA group with US (3.7 MHz) + TNK-tPA + MBs group

Applying the Wilcoxon signed-rank test for the difference (of the mean) between all pairs of experimental groups examined in this chapter, it turns out that $|W| >> W_{0.05, 5}$, leading to a strong rejection of the null hypothesis. Hence, it can safely be said that in all cases, the second sample (group) has a higher population mean.

7.6. Discussion

The goal of the study was to assess whether small rectangular ultrasonic transducers in combination with thrombolytic TNK-tPA and in the presence or absence of MBs, can remove effectively blood clots. Fully retracted porcine blood clots were exposed to a unidirectional flow rate and to a certain concentration of TNK-tPA, at fixed temperature (37 °C). Small planar rectangular ultrasonic transducers operating at high

frequencies, were positioned as close as physically possible to the clot for optimal thrombolytic treatment.

To isolate the influence of frequency on thrombolysis efficacy, the experiments were performed with identical sonication parameters at frequencies 3.7 and 5.2 MHz respectively. Clots treated with 5.2 MHz US waves plus TNK-tPA exhibited 15 % (1380/1200), greater degree of thrombolysis compared to clots treated with TNK-tPA alone (figure 7.2). Clots treated with 3.7 MHz US waves plus TNK-tPA exhibited 20 % (1440/1200), greater degree of thrombolysis compared to clots treated with TNK-tPA alone (figure 7.2). In order to have a quantitative measurement of thrombolysis efficacy, the degree of thrombolysis achieved in the control was subtracted from that achieved in the TNK-tPA alone and in the US + TNK-tPA (figure 7.3). Clots treated with 5.2 MHz US plus TNK-tPA exhibited enhanced thrombolysis over clots treated with TNK-tPA alone, as much as 90 % (380/200), while thrombolysis enhancement reached 120 % (440/200), in clots treated with 3.7 MHz US plus TNK-tPA over clots treated with TNK-tPA alone.

Study results exhibited that thrombolysis efficacy decreases with higher US frequencies (figure 7.3). This is in accordance with previous work of Suchkova et al. 1998 [130] and Schäfer et al. 2005 [54], who showed that the thrombolytic efficacy of US is inversely depended on frequency. Similarly, another in vitro study by Blinc et al. [129], demonstrated that the acceleration of fibrinolysis by US was maximum at frequencies between 1 and 2.2 MHz, but decreased at 3.4 MHz, also indicating that frequency exerts a significant impact on thrombolysis efficacy.

It is well documented that the interaction of acoustic US waves with target tissue can produce either thermal or non-thermal effects, which contribute to thrombolysis enhancement. The non-thermal effects can generally be categorized into primary mechanical effects such as radiation force, or secondary mechanical effects such as acoustic cavitation.

In our experiments, the temperature increase within the clot never exceeded 1°C. As observed in the studies by Blinc et al. 1993 [129], Francis et al. 1992 [176] and Olsson et al. 1994 [127], such a minor degree of heating is insufficient to influence thrombolytic efficacy, establishing that thrombolysis enhancement occurred through non-thermal mechanisms.

Taking into consideration that in our study the contribution of thermal effects in clot lysis was negligible, it is reasonable to assume that the enhancement of enzymatic

fibrinolysis due to the application of high US frequencies, occurred primarily due to non-thermal mechanisms (e.g. radiation force). Radiation force is generated through a transfer of momentum from an acoustic US wave to the propagation medium and can initiate motion in fluids (acoustic streaming) [196] and displacement in tissues [197]. Hence, it is speculated that clot's displacements due to ARF, mediated changes in the fibrin structure that may increase the permeation and penetration of the thrombolytic drug into the clot leading to enhance thrombolysis.

When clots were treated with US plus TNK-tPA in the presence of MBs, further thrombolysis enhancement was observed. Study outcome demonstrated that the interaction of US waves with systemic administration of MBs, strongly accelerated the enzymatic fibrinolysis induced by TNK-tPA. The combination of 5.2 MHz US waves with MBs enhanced TNK-tPA mediated thrombolysis by almost 58 % (600/380) compared to thrombolysis induced using only 5.2 MHz US + TNK-tPA (figure 7.4). In addition, the combination of 3.7 MHz US waves with MBs enhanced TNK-tPA mediated thrombolysis by more than 59 % (700/440), compared to thrombolysis induced using only 3.7 MHz US + TNK-tPA (figure 7.5).

The degree of thrombolysis enhancement due to MBs administration (almost 60 %), was significantly higher compared to that obtained in a previous in vitro study conducted by the same group [77], where MBs enhanced sonothrombolysis by 40 %. Considering that in the present work, the administered dose of MBs was almost double (5 mL) compared to that administered previously (3 mL), it is reasonable to assume that thrombolysis efficacy increases with higher doses of MBs, contributing to thrombolysis enhancement.

The phenomenon of sonothrombolysis enhancement due to MBs administration, can be explained by the ability of bubbles to act as cavitation nuclei, reduce the acoustic cavitation threshold and give rise to cavitation activity. Cavitation activity can be classified as stable or inertial. Stable cavitation is associated by sustained small amplitude oscillations of the bubbles about their equilibrium, generating flow around them termed microstreaming. Inertial cavitation is associated by bubbles collapse, capable of producing shock waves free radicals, fluid jetting and erosion of materials. The pressure amplitude required to initiate stable oscillations is always lower than that required for inertial cavitation [204]. Although, cavitation activity was not detected in our experiments, we can suspect that MBs enhanced sonothrombolysis is based on cavitation related phenomena.

Since the MI at both frequencies used in this study was estimated to be well below the theoretical threshold for the onset of inertial cavitation (MI > 0.7), it is suspected that the enhancement in thrombolysis efficacy was due to stable cavitation. The microstreaming produced by stable cavitation, can promotes both the transport [69] and penetration [143] of the thrombolytic drug into the clot, leading to faster clot dissolution. Furthermore, the presence of MBs, could modify both the attenuation and sound speed of the clot [205] and thus increasing the acoustic radiation force. This phenomenon may also contribute to thrombolysis enhancement.

7.7. Conclusions

This study, presents two flat rectangular ultrasonic transducers, operating at frequencies 3.7 and 5.2 MHz respectively, which can be incorporated in a catheter and used in the future for invasive applications of therapeutic US, such as intravascular sonothrombolysis.

Study results demonstrated that intravascular sonothrombolysis using a high frequency (in the megahertz range) small planar ultrasonic transducer, seems to be a promising method for effective clot lysis and potentially, may offer a new approach for the treatment of stroke patients.

The maximum thrombolytic enhancement occurred when 3.7 MHz pulsed US waves combined with the thrombolytic agent TNK-tPA in the presence of MBs. Using this method, 700 mg of clot mass was removed with 30 min of treatment, which may be sufficient for timely recanalization of occluded cerebral arteries.

Study findings showed that thrombolysis efficacy decreases with higher frequencies and hence lower US frequencies should be employed to improve outcomes. Since the optimal frequency for endovascular treatment is a trade-off between depth of penetration and thrombolysis efficacy, US frequencies in the range between 2-2.5 MHz [129], might be a good choice for intravascular sonothrombolysis and should be further investigated.

On the other hand, a major issue is resulting from the possibility of this type of transducer to be utilized in combination with diagnostic imaging. In that case, the frequency has to be increased (probably between 20-40 MHz) [206]. Therefore, a compromised frequency needs to be derived that serves both the therapeutic and diagnostic purposes.

The acceleration of enzymatic fibrinolysis by US waves has been shown to occur mainly through non-thermal mechanisms. Consequently, it is speculated, that ARF and acoustic cavitation are the mechanical effects that dominantly appear in the propagation of high frequency pulsed US waves through a blood clot and contribute to thrombolysis enhancement.

In conclusion, although intravascular sonothrombolysis seems to be an effective treatment method for stroke, further in vitro and in vivo studies are needed to determine the optimal frequency and to confirm the safety and the efficacy of this technique, before translated into clinical trials.

7.8. Summary

A summary table with the recommended therapeutic protocol for the treatment of a MCA occlusion with a small flat rectangular ultrasonic transducer is provided below:

Table 9: Recommended therapeutic protocol for intravascular treatment of MCA occlusion

Experimental Parameters	*Values*
Mode	*Small planar rectangular ultrasonic transducer*
Frequency	*3.7 MHz*
Acoustic Power	*20 W*
Duty Factor	*10 %*
Pulse length	*1 ms*
Pulse Repetition Period	*10 ms*
Pulse Repetition Frequency	*100 Hz*
Treatment time	*30 min*
Temperature	*37 ^{o}C*
Flow rate	*50 mL/min*
TNK-tPA concentration	*7 μg/mL*
MBs dose	*3 mL (0.5 mL/5 min)*
Treatment's outcome	*700 mg of mass clot removed*

8. Evaluation of a small flat rectangular therapeutic ultrasonic transducer intended for intravascular treatment of atherosclerosis

8.1. Introduction

Atherosclerosis is a condition in which fatty material collects along the walls of arteries. This fatty material thickens and may eventually block the arteries [207]. Atherosclerosis treatment may require special surgical procedures such as balloon angioplasty and stenting [208], cutting balloon [209], atherectomy [210-211] and surgical bypass [212], to open an artery and improve blood flow.

Another technological modality with therapeutic capabilities in treating atherosclerosis, is the application of US. This technology can be used in the future for clinical trials primarily to treat plaques in the vascular system. Unstable plaques in a blood vessel are a major source of risk to cause an embolic stroke, since the plaque can breaks off and a part of it through the bloodstream can reach the brain, becomes lodged to a brain vessel and blocks the blood flow. The US device can be attached to a catheter of appropriate size and guided intravenously to the vessel for ultrasonic treatment. Care will be taken to avoid the escape of debris which may cause further vessel blockage.

In this experimental study, a small flat rectangular (2 X 10 mm^2), MRI compatible ultrasonic transducer, operating at a frequency of 4 MHz was used. The aim of the study was to evaluate the thermal capabilities of such a transducer. Transducer's efficacy was tested in two different gel phantoms fabricated to mimic tissue. A polyacrylamide hydrogel phantom was used to visualize the size and shape of the thermal lesion and an agarose based gel phantom was used to measure temperature elevation.

The thermal effects of US energy were monitored under MRI guidance, since it is considered as the optimal imaging modality due to its unsurpassed soft tissue contrast and its temperature sensitivity [83], [213], [214]. Therefore, thermal lesions were visualised with MR imaging and temperature changes were demonstrated with MR thermometry. The intended application of this type of transducer is the intravascular treatment of atherosclerosis.

8.2. Experimental set up to measure temperature elevation with a thermocouple

The active size of the transducer under evaluation is 2 X 10 mm². It operates at 4 MHz and it is made of Pz26-type PZT piezoceramic material with epoxy backing. The transducer was fixed on a custom made ABS plastic holder, which includes a cavity to accommodate the 1 mm thick coaxial cable (figure 8.1).

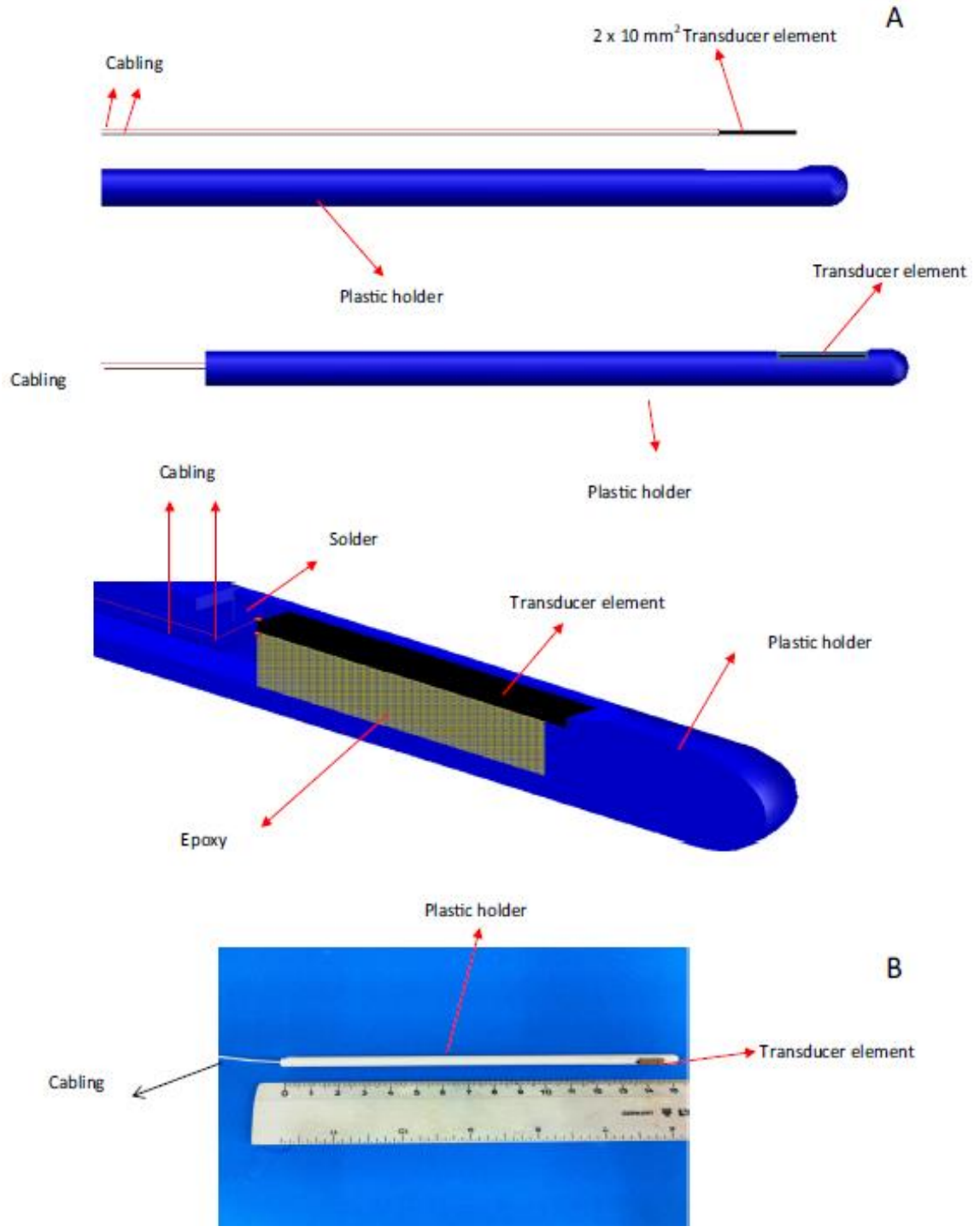

Figure 8.1: A) CAD drawing of transducer. B) Photo of the2x10 mm² prototype transducer.

The plastic holder was attached on the arm of the robotic system and immersed in an acrylic tank containing degassed water. The robotic system positioned the transducer on the surface of the tissue mimicking gel phantom. Tissue mimicking phantoms simulate the biological properties of tissue in order to provide a more realistic clinical environment. The phantom is made of agar powder, silica dioxide (SiO_2) and evaporated milk (agar: 2% w/v, silica: 1.2 % w/v, evaporated milk: 25% v/v). The details of the recipe and the steps for the preparation of the phantom are described in Menikou et al. [183]. To measure temperature elevation, a thermocouple was placed between the transducer's face and the gel phantom. The thermocouple was connected to a data acquisition (DAQ) station that sampled temperature measurements for every 1 s. The size of the thermocouple was chosen to be 50 μm, so that the interaction with the US beam was minimized. At the bottom of the phantom, an acoustic absorber was placed to reduce the reflections of the US waves. The experimental set up for the placement of the transducer on the agar/silica/evaporated milk gel phantom is shown in figure 8.2.

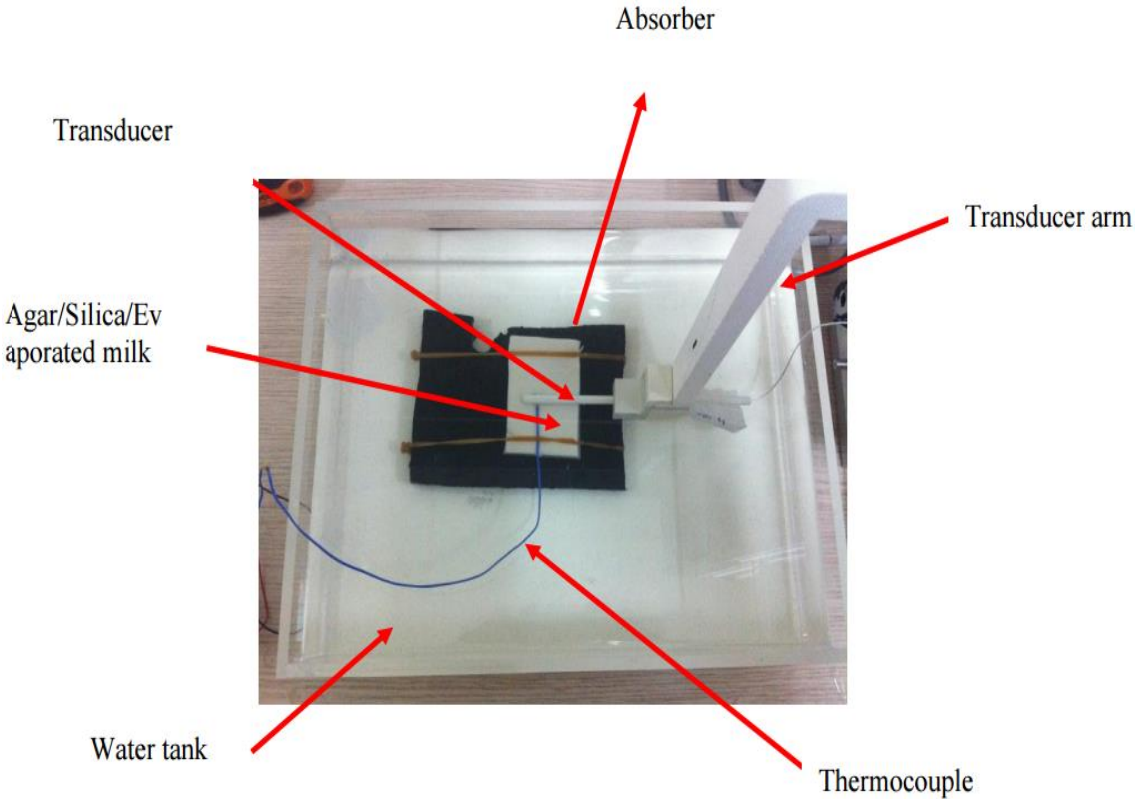

Figure 8.2: Placement of the transducer on the agar/silica/evaporated milk phantom.

Figure 8.3 shows the temperature elevation in the agar/silica/evaporated milk phantom, from a baseline value of 28 °C to temperatures as high as 93 ^{0}C within 60 s. Temperature changes were measured with a thermocouple, placed between the transducer's face and the gel phantom. The AP used was 6 W.

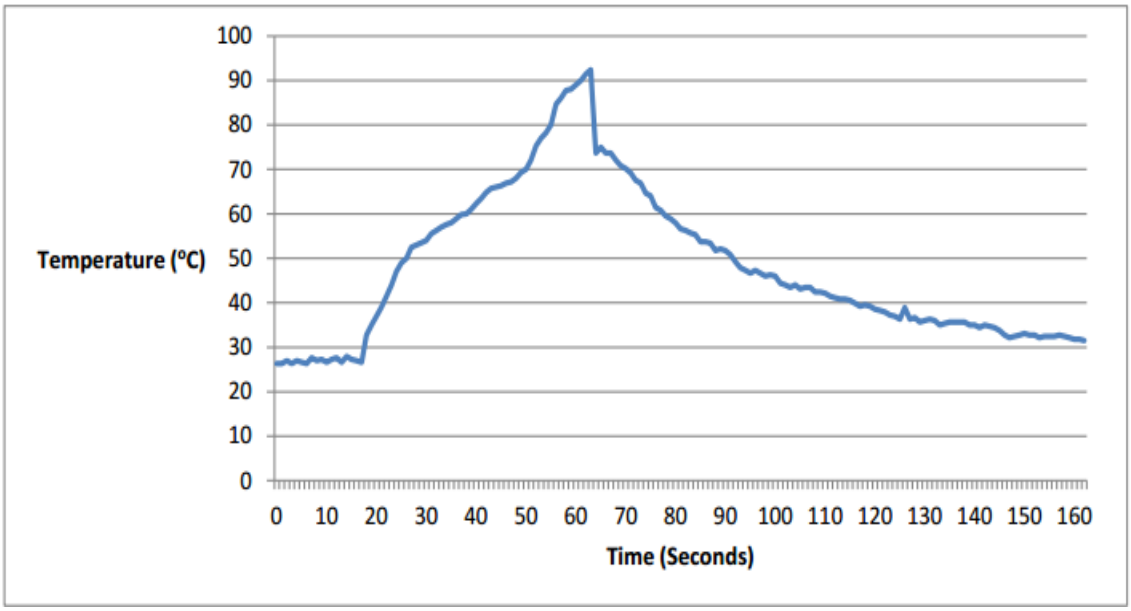

Figure 8.3: Temperature elevation in the agar/silica/evaporated milk phantom using the thermocouple placed in the face of the transducer. The acoustic power used was 6 W for 60 s.

8.3. MR thermometry

The safety and efficacy of thermal therapy requires accurate temperature monitoring throughout the treatment, which is feasible with MR based thermometry method. This method is based on temperature sensitive MR parameters such as the proton density, the diffusion coefficient (D), the spin-lattice relaxation time (T1), the spin-spin relaxation time (T2), the magnetization transfer and the proton resonance frequency shift (PRFS). Among these different temperature dependent parameters, the excellent linearity of signal with temperature over a large temperature range and the low susceptibility to tissue type, has made the PRFS method the preferred choice for MR thermometry.

Therefore, in this experimental study the temperature elevation during US exposures was estimated using the PRFS method [107]. This method relates the associated phase shift derived from the frequency shift of the MR signal due to the local temperature elevation (see equation 2.15 in chapter 2).

In a 1.5 T MR system (Signa, General Electric, Fairfield, CT, USA), the pulse sequence used to extract the thermometry maps was the T1-weighted spoiled gradient recalled echo (SPGR), with the following parameters: Repetition time (TR) = 40 ms, Echo time (TE) = 20 ms, slice thickness=2.1 mm Field of view (FOV) = 21 cm, matrix = 128x128, flip angle = 20^0, Number of excitations (NEX) = 1. Phase maps were reconstructed by calculating the phase on a pixel-by-pixel basis after combining pixel data from real and imaginary channels. Although the scanner is capable of producing

directly phase image reconstructions, the applied intra-scan gradient nonlinearity corrections induced phase interpolation problems. Transient phase shifts developed during the acquisition of non-treated (mask) and treated images were compensated by subtracting internal references in regions away from the treated site. Temperature elevation was returned by the software as the maximum value in a prescribed region of interest (ROI) that was manually positioned. Temperature-colour coded maps were produced by adjusting the colour map (blue to red) for a range of minimum to maximum ROI temperature.

The MR temperature map for the planar transducer is shown in figure 8.4. MR thermometry was performed using the 2D SPGR sequence. The temperature indicated in this graph is temperature increase. US was activated during the first 4 images. The AP used during temperature acquisition was 6 W for 60 s. The phantom used was agar/silica/evaporated milk (agar: 2% w/v, silica: 1.2 % w/v, evaporated milk: 25% v/v).

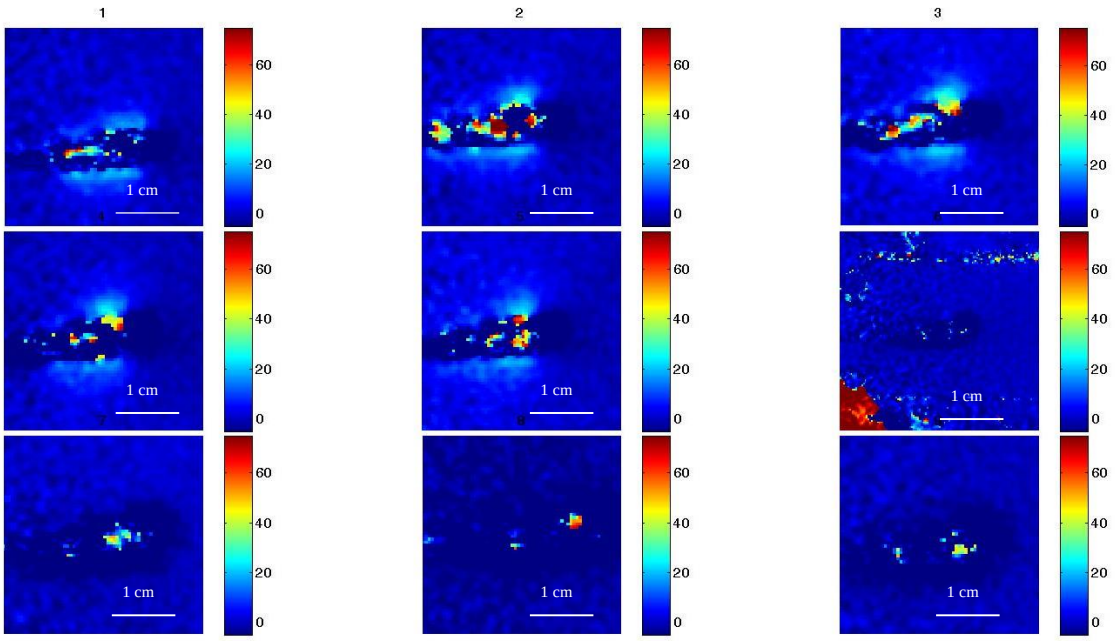

Figure 8.4: MR temperature map for the planar transducer using the 2D SPGR sequence. The acoustic power used was 6 W for 60 sec. The phantom used was agar/silica/evaporated milk. Thermal maps 1-9 represent temperature evolution with time at a temporal resolution of 12 s per frame and US was activated during the first 4 images.

8.4. In vitro experimental set up to visualize the transducer's lesion

To visualize the lesions created by this type of transducer, the experiments were carried out in a commercial polyacrylamide gel phantom (ONDA Corporation, Sunnyvale, CA, USA). This type of phantom is a crystal clear synthetic gel that produce

lesions of the same position, size and shape as those produced in real tissue due to the application of US power. The lesions appear as white, three-dimensional solid profiles inside the clear gel and can detect many defects in US systems such as beam shape variations and alignment problems.

8.5. MR Imaging

A safe and effective use of thermal therapy requires that the thermal dose to the targeted tissue be spatially localized and monitored in real time. In this work, the thermal effects of US energy were monitored under MRI guidance. The therapeutic protocol was tested using a GPFLEX coil (USA instruments, Cleveland, OH, USA). In order to evaluate the thermal capabilities of the transducer, the signal in the polyacrylamide gel phantom was acquired. High-resolution MR imaging was performed in order to evaluate the lesions of the transducer using a Fast Recovery Fast Spin Echo (FRFSE) T2-weighted fast spin echo sequence with the following parameters: TR = 2200 ms, TE= 61.2 ms, slice thickness=1.5 mm, matrix=192 x 192, FOV=17 cm, NEX=1 and echo train length (ETL) = 16.

The thermal lesion in the ONDA phantom is shown in figure 8.5. The size and shape of the lesion matches the geometry of the transducer element. An AP of 6 W was used for a duration of 60 s.

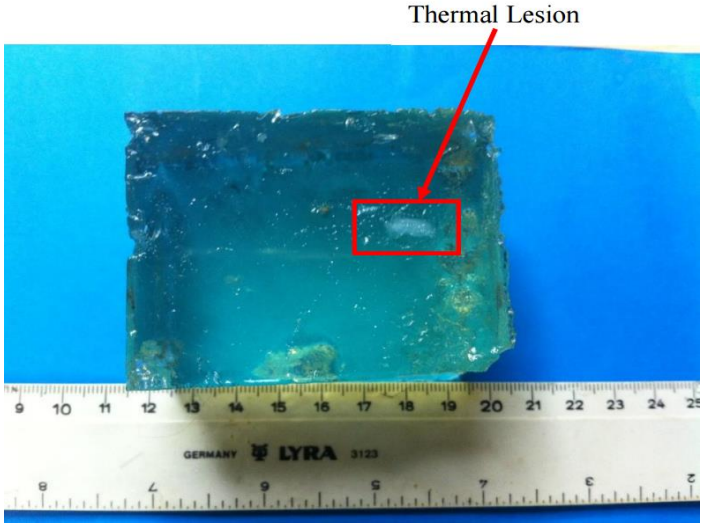

Figure 8.5: Thermal lesion in the ONDA phantom, using 4 MHz frequency, 6 W acoustic power and 60 s exposure time.

Figure 8.6 A shows lesions (deep into the gel) developed in the ONDA gel using 10 W AP for 5 s, using the 2 X 10 mm^2 transducer operating at 4 MHz. The maximum temperature as measured by the thermocouple was 92 ^{0}C. The lesion depth ranged from 1 to 2 mm (average 1.8 mm). Figure 8.6 B shows the corresponding lesions in a plane

parallel to the transducer face. The average length of these lesions was 10.5 mm, and the average width was 2.2 mm. Therefore, the lesion size slightly extends beyond the 2 X 10 mm^2 area due to the conduction effect. Figure 8.6 C shows the MRI image of the lesions shown in fig. 8.6 B using T2 weighted FRFSE. Note that because the lesion depth is small (1-2 mm) and because of the slice thickness and orientation, some lesions were not clearly shown in the MR images.

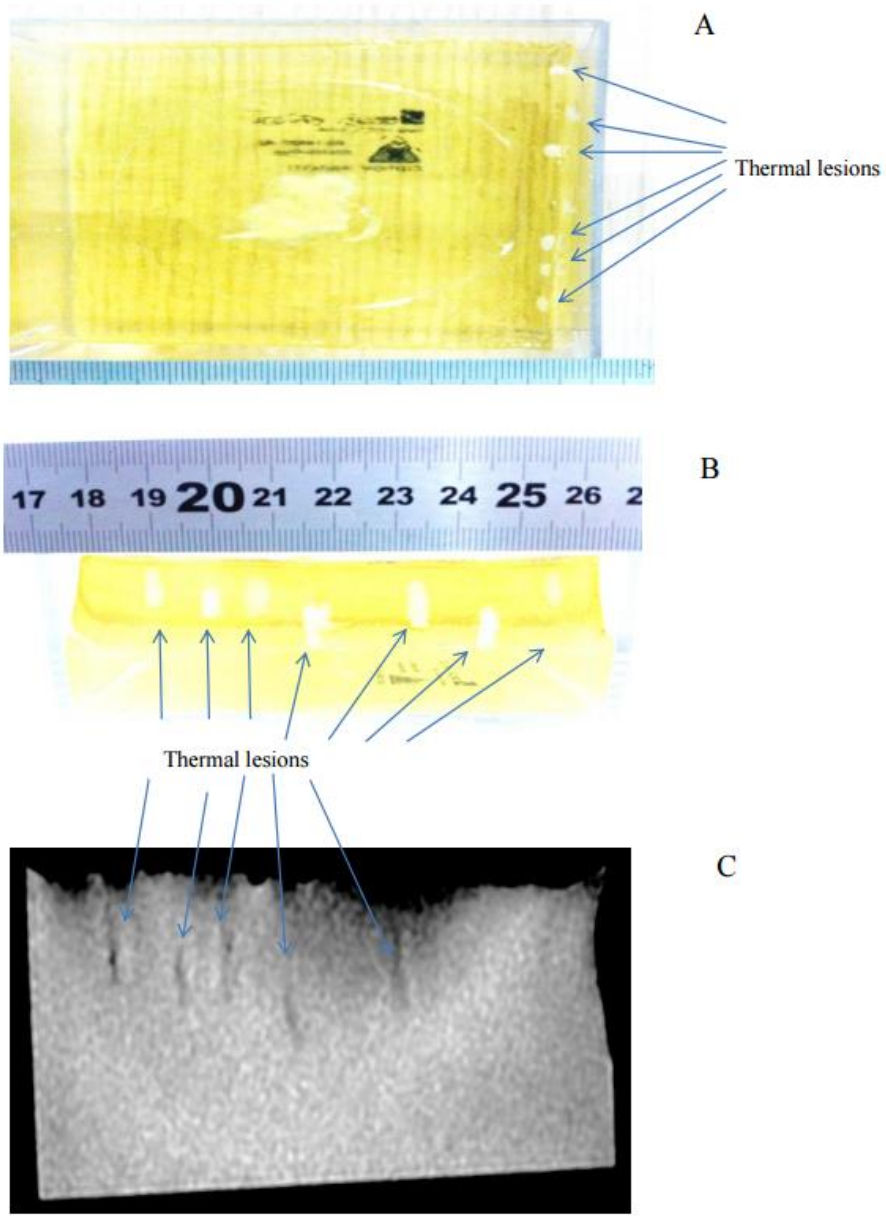

Figure 8.6: A) The use of 2 X 10 mm^2 transducer (operating at 4 MHz), developed deep lesions into the ONDA gel, by applying 10 W acoustic power for 5 s. B) the corresponding lesions in a plane parallel to the transducer face. C) MRI image of the lesions shown in figure 6B using T_1 weighted SPGR.

8.6. Discussion

The continuous transmission of acoustic energy raises temperature at the targeted tissue in the body [215]. The magnitude and duration of this temperature elevation is quantified as the "thermal dose" delivered to the targeted tissue. Different levels of thermal dose are associated with different biological outcome such as:

1. Low temperature hyperthermia, where temperatures are in the range of 43–45 °C,
2. High temperature thermal ablation, where temperatures are in the range of 50–80 °C (or higher).

In hyperthermia, US energy is used to create a low level thermal rise over several minutes or hours that kills cancer cells directly [216]. Conversely, in thermal ablation, a situation that was tested in our study, US energy is applied for a short period of time (seconds), to create a high temperature rise that coagulates the tissue and induce necrosis through protein denaturation [217].

The ability of MR imaging to define the tissue to be treated, to measure induced temperature changes [218–220], to ensure proper targeting [221] and to image the tissue after thermal treatment [222–224], makes MRI a superior modality for guiding and monitoring thermal therapies.

In this study, the thermal capabilities of a small flat rectangular ultrasonic transducer were evaluated. This type of transducer can be used in the future for intravascular applications, such as the treatment of atherosclerotic plaques with thermal ablation. The transducer was evaluated in two different gel phantoms. A commercial polyacrylamide gel phantom was used to visualize the size and shape of the thermal lesion. A custom made agar/silica/evaporated milk gel phantom was utilized to measure temperature elevation above a baseline value, either with a thermocouple or with MR thermometry.

One of the main targets in this study was to create thermal lesions of sufficient depth (1-2 mm). This lesion size is sufficient enough to potentially shrink a vascular plaque and improve blood flow, without causing harmful thermal damage to the surface tissue layer of the artery that is in close proximity to the plaque.

Due to the phenomenon of conduction, the size of necrosis in a plane parallel to the transducer's face was always slightly higher than the area of the transducer (2 X 10 mm^2). At a deeper level, the size of the lesion was smaller. This is attributed to the fact that deeper in the tissue the intensity of US beam drops due to attenuation and so does the temperature. If deeper lesions are required, then either the power of the transducer or the

exposure time has to be increased. In any case, care should be taken not to exceed temperatures above 100 ^{0}C, which is the threshold for tissue boiling regardless of the exposure time. The thermal ablation time for this application is not a major issue since in future clinical trials, the time needed to place a catheter in a vessel (10-30 min) is much longer.

With an AP of 6 W, temperatures as high as 93 ^{0}C were reached within 60 s. When the AP was increased to 10 W, thermal lesions were created in just 5 s (figure 8.6 A), indicating that AP is the main operating acoustic parameter for controlling thermal heating.

A transducer operating at the frequency of 4 MHz was chosen, although a transducer operating at higher frequencies could be more beneficial due to lower depth of penetration. However, taking into consideration that in future clinical trials, the transducer (attached to a catheter), that will be inserted through a vessel is consumable, going to higher frequencies means a more expensive and more fragile transducer.

8.7. Conclusions

In this chapter the capability of a small flat rectangular ultrasonic transducer to create thermal lesions of the appropriate shape and size within seconds, was demonstrated. The transducer can be attached to a catheter of appropriate size and guided intravenously to the vessel for ultrasonic treatment. In the future, this technology can be used for clinical trials primarily to treat atherosclerotic plaques in the vascular system. Care will be taken to avoid the escape of debris which will cause further vessel blockage. A suction mechanism incorporated to US system would be a good idea for debris collection. Such a mechanism was used in atherectomy studies to collect the removed particles [225-226].

Taking into account that the transducer used seems to be able to remove a sufficient amount of plaque within seconds without harming adjacent normal tissue (artery), in association with the fact that it is not expensive, we can assume that 4 MHz US frequency, potentially might be a good choice for the treatment of atherosclerosis.

Of course, the lesion penetration in plaque tissue might not be the same as it was shown in this gel phantom, because the ultrasonic absorption of plaque is unknown and might be quite different from the value of absorption in gel phantoms. Another, property that is not known is the thermal dose threshold of necrosis for plaque. Although this property was reported for many tissues [227], for plaque it is not known at the moment,

possibly because researchers in the past did not believe that US would have a role in plaque interventions.

8.8. Summary

A summary table with the recommended treatment protocol for visualizing the size and shape of the thermal lesion using MR imaging and for measuring temperature elevation using MR thermometry, is provided below:

Table 10: Recommended therapeutic protocol for creating thermal lesions with sufficient depth within seconds

Experimental Parameters	*Values*
Mode	*Small planar rectangular ultrasonic transducer*
Frequency	*4 MHz*
Acoustic Power	*6 W*
Exposure time	*60 s*
MR imaging T_2 *weighted Fast Recovery Fast Spin Echo (T2wFRFSE)*	*TE= 61.2 ms*
	TR = 2200 ms
	slice thickness =1.5 mm
	matrix=192 x 192
	FOV=17 cm
	flip angle = 20^0
	NEX=1
	ETL = 16
MR thermometry T_1 *weighted Spoiled Gradient Echo (T1wSPGR)*	*TE= 20 ms*
	TR = 40 ms
	slice thickness = 2.1 mm
	matrix=128 x 128
	FOV=21 cm
	flip angle = 20^0
	NEX=1
	ETL = 16
Treatment's outcome	*700 mg of mass clot removed*

9. MRI-guided sonothrombolysis in vivo using a rabbit carotid artery model

9.1. Introduction

In the last few years, an innovative new technology is emerging, which offers FUS treatments under MR guidance and monitoring, commonly used by the term MRgFUS. Lately, the possibility of using MRgFUS for treating stroke has become an important area of investigation, since stroke is the primary focus of sonothrombolysis research due to the high mortality rate associated with it. This relatively new approach to sonothrombolysis, permits a highly concentrated US energy from an external source, to be deposited into millimetre sized focal volumes within the brain, without harming adjacent healthy tissue.

In this experimental study, the enhancing effect of FUS on TNK-tPA induced thrombolysis in the presence of MBs, was evaluated in vivo using an animal model. In vivo animal models allow the full biological response of a living organism to US, thrombolytics and MBs to be assessed, including the efficacy of the treatment, the ability to guide and monitor treatment progress, physiologic alterations and the potential to evaluate healthy tissue damage.

For our in vivo study, a New Zealand adult rabbit was used weighting approximately 4 kg. An artificial method was applied to form a blood clot in the right carotid artery of the rabbit, which completely blocked the blood flow. Therapeutic US waves were delivered to the target with a MR compatible spherically focused transducer, 4 cm in diameter, focusing at 10 cm and operating at 1.18 MHz. A pulsed US protocol that maintains a temperature elevation of 1 ^{0}C in the clot (called safe temperature) was applied, in order to avoid any adverse health effects, such as thermal heating. The animal experiment protocol was approved by the national body in Cyprus responsible for animal studies (Ministry of Agriculture, Animal Services).

The blood clot was exposed to a highly FUS beam and was treated with TNK-tPA in association with MBs. The proposed treatment protocol was monitored using MR angiography. The goal of this animal study, was to achieve fast and complete flow restoration in the carotid artery of the rabbit, with no excess heating. This would be advantageous, since the speed of clot lysis for fast and complete artery recanalization is the key of therapeutic success in the treatment of stroke patients. Additionally, it would be beneficial, if successful clot lysis achieved with the use of low doses of thrombolytic

drug and MBs, as this would minimize the risk of undesirable bleeding complications to occur, during or after thrombolytic treatment.

9.2. Rabbit carotid artery model

Small animal models, such as rats or rabbits are used in sonothrombolysis due to their low cost, ease of handling, and data accumulation from previous studies. The carotid artery of a rabbit is the preferred in vivo sonothrombolysis model because it fulfils the following requirements:

1. A rabbit carotid artery has arterial dimensions that are comparable to either a smaller-sized proximal (M1) or distal (M2), segment of a human MCA or a normal-sized MCA branch.
2. Blood clot size/volume comparable to what can be expected in proximal M1 or M2 occlusions.
3. A rabbit carotid artery provides a bifurcation (common-internal/external carotid artery), to mimic MCA branching, a preferred location for embolic occlusions.
4. A rabbit carotid artery is in direct contact with FUS because no other tissues are involved and thus potential problems of US penetration are avoided.

9.3. Experimental set up

The rabbit was anesthetized using a coctail of 500 mg of ketamine (100 mg/mL, Aveco, Ford Dodge, IA), 160 mg of xylazine (20 mg/mL, Loyd Laboratories, Shenandoah, IA) and 20 mg of acepromazine (10 mg/mL) at a dose of 1 mL/kg. The animal experiment protocol was approved by the national body in Cyprus responsible for animal studies (Ministry of Agriculture, Animal Services). The neck was shaved and through an incision of the overlying tissue layers by an experience veterinarian, the right common carotid artery was exposed. The anaesthetized rabbit was set in a decubitus position into a customized MR-compatible treatment platform, with its right carotid artery on the opening of platform.

The 1.18 MHz MR compatible FUS transducer was attached on the arm of a custom made MR compatible robotic system and immersed into a tank of degassed water inside a 1.5T MR scanner (Signa 1.5 T; General Electric, Fairfield, CT). For beam targeting, the three axes robotic system positioned the transducer under the blocked artery and arranged it at such a distance in order to focus the US beam precisely on the clot.

A soft tissue mimicking material (TMM) was positioned between the rabbit's neck and the opening of the treatment platform to improve acoustic coupling and thus, ensure US wave propagation. The set up for beam positioning is shown in figure 9.1.

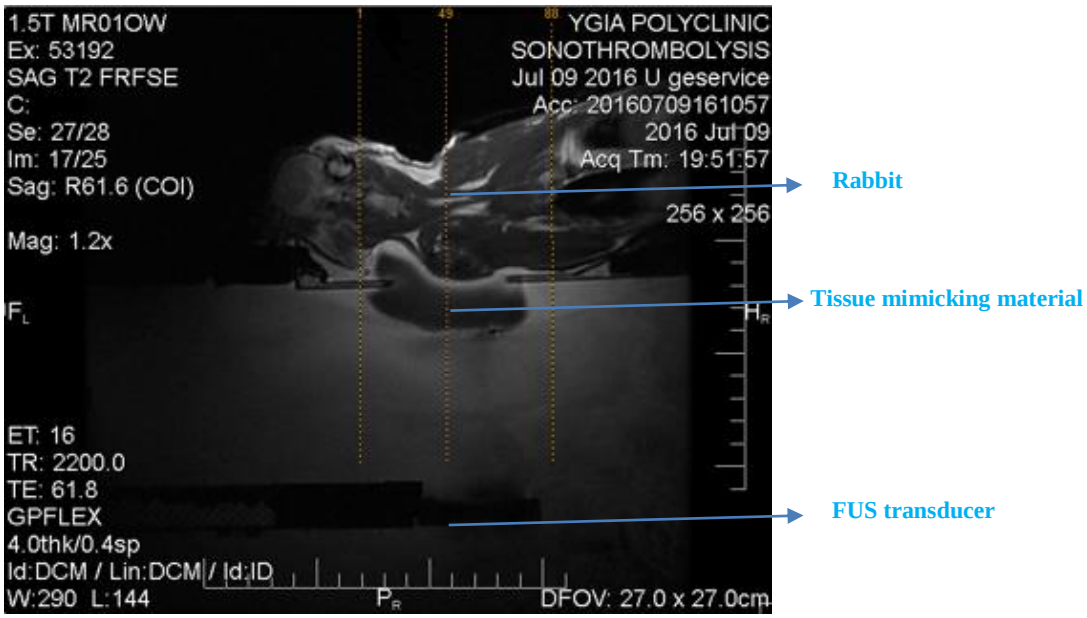

Figure 9.1: Beam positioning for MRgFUS treatment.

A schematic diagram presenting the experimental set up for in vivo sonothrombolysis using a rabbit carotid artery model under MRI guidance, is shown in figure 9.2 below.

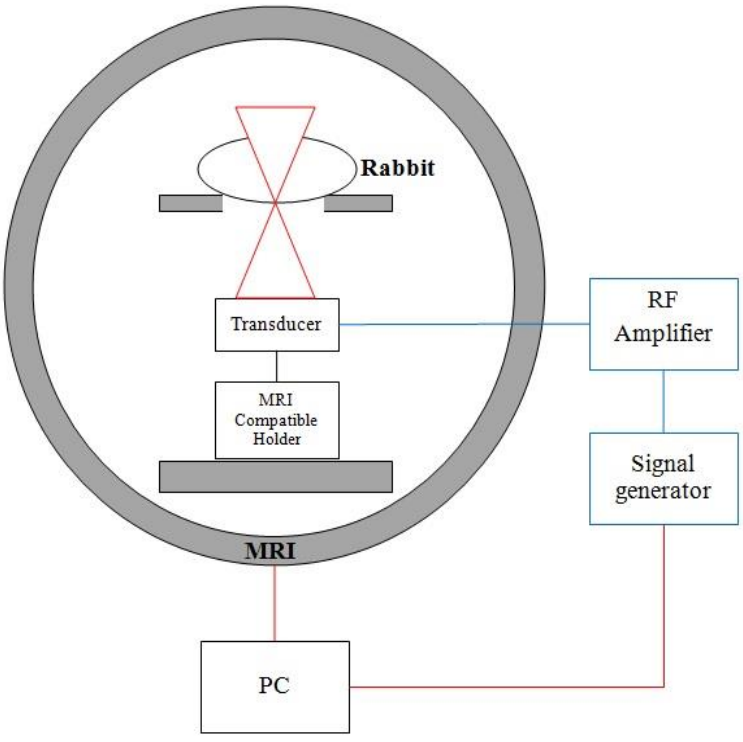

Figure 9.2: Schematic diagram of experimental set up for MRgFUS sonothrombolysis in vivo using a rabbit carotid artery model.

9.4. MR angiography

To visualize flow within rabbit's carotid artery, a 3D time of flight (TOF) MR angiography technique was applied, since it is the dominant non-contrast bright-blood method for imaging flow within vessel without the need for contrast administration. In MR, the signal from spins decreases as it expose to an increasing number of excitation pulses, until eventually a saturation value is reached. Consequently, the goal in TOF imaging, is to subject the spins of the flowing blood to only a very few excitation pulses and the spins of the surrounding stationary tissues to a large number of excitation pulses and thus, by manipulating the magnitude of the magnetization, a signal difference between blood flow and stationary tissues is achieved. This signal difference is large, since the magnitude of magnetization from the moving spins is very large as compared to the magnetization from the stationary spins. For maximum enhancement of flow, the imaging plane or slab is positioned perpendicular to the blood flow in order to minimize the TOF duration of blood through it. As a result, the moving spins enter the slab fully magnetized and experience only a few excitation pulses before flow out of this. This ensures that the signal from the blood will be relatively large, as it is continuously refreshed during image acquisition and hence, it will never experience enough excitation pulses to become saturated. In contrast, the stationary tissues remain in the slab during image acquisition, give rise to a diminished signal as the magnetization from them is saturated due to constant exposure to excitation pulses.

9.5. Mechanism of thrombus formation

Although the pathogenesis of thrombus formation in the natural condition can be either due to an acute or due to a chronic process, in animals models an artificial method is required for in vivo experimental studies. These methods are related to the physiologic mechanisms of thrombus formation as described by Virchow [228]. In response to Virchow's triad, thrombosis is the result of alterations in blood flow (stasis or turbulence), vascular endothelial injury, or alterations in the constitution of blood (hypercoagulability). In animal studies, thrombus formation can be resulted from endothelial injury (due to artificial vascular wall trauma), or from stagnant flow (due to vessel ligation).

According to literature, clot preparation methods vary widely, since there is no common agreement of how experimental thrombi used for in vivo sonothrombolysis studies, should be formed. In this study, the clot preparation technique applied to the

rabbit carotid artery model, was accomplished by causing a direct mechanical trauma to the vessel's wall. A 10 mm segment of the right common carotid artery of the rabbit was subjected to external compression with a forceps, in order to induce the release of tissue factor III, which is necessary for the initiation of thrombin formation. The blockage in blood flow due to clot formation at the segment of injury was monitored using a 3D TOF images. The signal from inflowing blood appears bright compared to background tissue (figure 9.3). On the other hand, the complete absence of signal indicated that there is no through plane flow, i.e. the carotid artery was fully occluded.

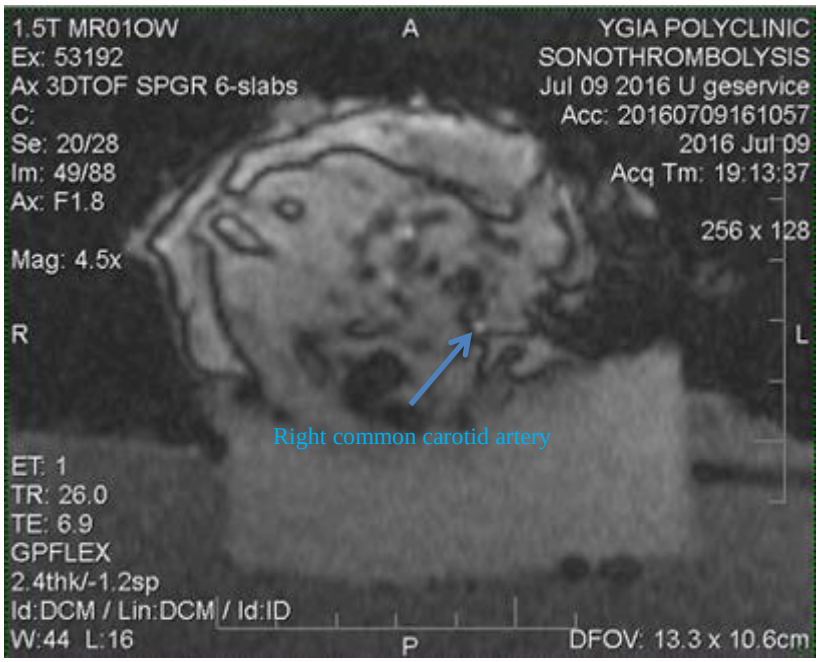

Figure 9.3: Blood flow produces bright signal in TOF images.

9.6. Treatment protocol

The optimized therapeutic protocol established in vitro for the treatment of a MCA occlusion occurred superficially, was translated in vivo using a rabbit carotid artery model. The low magnetic susceptibility FUS transducer with 1.18 MHz centre frequency, was driven in a pulsed mode with a DF of 10 % and a PRF of 100 Hz. Each sonication set included a pulse "ON" period (1 ms) followed by a pulse "OFF" period (9 ms) and both the "ON" and "OFF" pulses were repeated in succession for the duration of a 30 min treatment (i.e., 3000 pulses).

The main prerequisite of the treatment protocol was to avoid the production of excess thermal heating at the target during the sonication period. Since AP is the operating parameter that mainly controls thermal heating, the AP was set at same level with that used in the corresponding in vitro experimental study, in order to maintain a temperature

change in the clot that does not exceed 1 ^{0}C (called safe temperature). Taking into consideration that MRgFUS sonothrombolysis relies on non-thermal mechanisms to achieve clot lysis, thermometry is not useful for temperature monitoring during the procedure.

Since it is well established that thrombolytic drugs are associated with an increased risk of bleeding complications which limit their use, among the goals of this animal study was the dose of the thrombolytic TNK-tPA injected to the rabbit to be as safe as possible. A Pilot Dose-Escalation Safety Study, was designed to test the hypothesis that TNK-PA could be administered safely to patients with AIS within 3 hours of onset at doses that may be associated with improvement in clinical neurological outcome [177]. The study included dose tiers starting at 0.1 mg/kg to a maximum of 0.5 mg/kg. The results showed that doses of 0.1 to 0.4 mg/kg are safe, since no sICH occurred, while at a dose of 0.5 mg/kg, sICH was observed in 15 % of the patients and the study was halted. Based on the results of the above mentioned study, the amount of TNK-tPA administered to the rabbit was selected to be as low as 50 % of the final tier and hence, a single bolus of 0.25 mg/kg was injected in the jugular vein prior sonication.

Regarding the safety of MBs dose, a clinical trial performed by Molina et al. [93], demonstrated that although the use of low doses of MBs (1.4 mL) in sonothrombolysis may be safe, higher doses (2.8 mL) when administered with rt-PA may increase sICH rate. Based on the recommended clinical dose in humans for US imaging, which is 0.03 mL/kg [229] and by comparing blood volume of humans with rabbits, a bolus of 0.02 mL/kg was calculated to be injected iv through the ear vein prior to sonication. Every 5 min a bolus of the same dose of MBs was administered to the rabbit, in order to replace the bubbles that were destroyed in the process and sustain cavitation.

9.7. MRI parameters

The flow of right common artery of the rabbit was monitored using a 3D TOF SPGR sequence acquired by the GPFLEX flexible surface imaging coil. The 3D volume was covered by a single slab consisting of 88 locations (slices). To minimize the TOF duration of blood through the slab and hence to minimize as little as possible the saturation of the flowing spins signal, the 3D TOF slab was positioned perpendicularly to the expected flow direction.

The acquisition parameters used were the following: TR = 26 ms, TE = 6.9 ms, Echo Train Length (ETL) = 1, Matrix size = 256 x 128, DFOV = 13.3 x 10.6 cm, Slice Thickness = 2.4 mm and Interslice Spacing = 1.2 mm.

Figure 9.4 shows sonothrombolysis procedure in the carotid artery due to the application of FUS waves in synergy with MBs and TNK-tPA. Initially, it is clearly seen the complete absence of TOF flow signal, verifying the complete blockage of the artery (figure 9.4 A, B and C). During recanalization procedure the TOF flow signal was increasing gradually (figure 9.4 D and E), until it was fully recovered, indicating complete recanalization (figure 9.4 F).

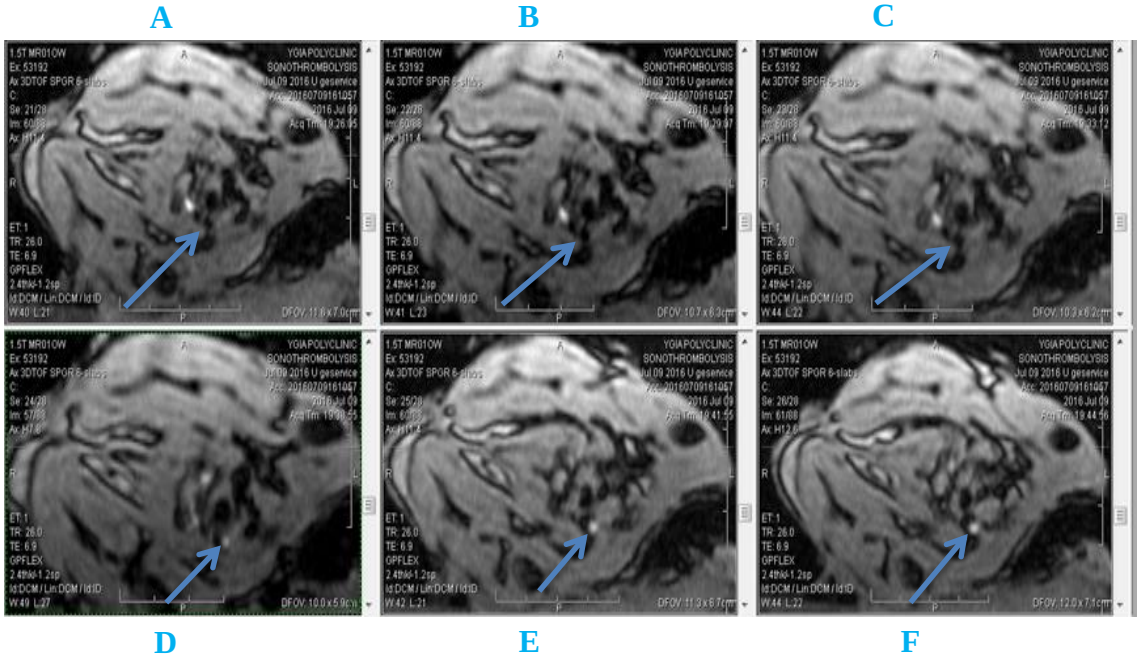

Figure 9.4: A-C) The complete absence of TOF flow signal confirmed the blockage in blood flow. D-E) The TOF flow signal is gradually increasing during recanalization procedure. F) The TOF signal is fully recovered indicating that the artery was completely opened.

9.8. Discussion

This experimental study demonstrated the ability of MRgFUS as an adjunct to thrombolytic TNK-tPA and in combination with MBs to dissolve a blood clot in vivo, using a carotid artery model. Since this proposed novel technique can be applied potentially for the treatment of stroke patients due to MCA occlusion, the carotid artery of a rabbit was the preferred in vivo sonothrombolysis model because it has arterial dimensions that are comparable to human MCA.

Using an artificial method, a blood clot was formed into the right carotid artery of the rabbit. The artery was in direct contact with FUS beam (no other tissues were involved) and hence, penetration problems due to US attenuation, were avoided. The clot

was artificially formed due to a mechanical trauma on artery's wall, which completely blocked the blood flow. Using a 3D TOF MR angiography, which is based on the phenomenon of flow-related enhancement, both the capability of the clot model to block the carotid artery as well as the ability of therapeutic FUS in synergy with MBs to enhance TNK-tPA mediated thrombolysis and hence to accelerate the recanalization rate, were monitored. MR angiography provided the opportunity to assess vessel occlusion and recanalization, since it is considered as the gold standard in MR imaging.

The optimized therapeutic protocol obtained in vitro for the treatment of a MCA occlusion occurred superficially, was employed in vivo to unblock the carotid artery of the rabbit. The goal was to achieve the maximum thrombolytic efficacy of TNK-tPA for fast and complete artery recanalization, without the production of unwanted thermal heating.

The proposed treatment protocol, provided a localized temperature increase at beam focus that never exceeded 1˚C and kept the surrounding temperature constant. As a result, no excessive heating was produced in the clot and the surrounding tissue. This temperature increase at beam focus, was insufficient to account for the increase in clot lysis rate, indicating that the accelerated rate of recanalization observed in the study, occurred primarily through non-thermal effects such as ARF and cavitation mechanisms [53].

The enhancement of clot dissolution by non-thermal mechanisms, was mainly achieved due to accelerated enzymatic action, which is consistent with prior observations from other studies, demonstrating that US at 1 MHz can potentially be applied and tolerated in vivo to accelerate therapeutic fibrinolysis [129], [176].

Study showed that for the complete opening of the artery, lower doses of thrombolytic and MBs were used, compared to the recommended doses. Since one of the major problems of thrombolytic therapy is that it is associated with an increased risk of intracranial hemorrhage, a potential benefit from the application of the proposed technique, is that without affecting the functionality of the treatment, lower doses of thrombolytic drug and MBs were used, minimizing the risk to cause hemorrhage.

The main limitations of this model was the lack of artery and surrounding tissue histology, in order to evaluate with care whether biological side effects might be caused due to this pulsed FUS regimen. Therefore, more in-depth investigation is required to confirm that this technique does not cause irreversible tissue damage, before applied to clinical trials.

Another limitation of the model was that the proposed therapeutic protocol was applied to a superficial target. In future clinical trials such as the treatment of stroke patients, tissue penetration may be a crucial factor, since occluded cerebral arteries intended as targets for this technology, occurred at depths of about 3 to 10 cm from skull's surface [175]. In such a case, because the US beam will be highly attenuated due to tissue absorption, the AP should be significantly increased in order the output at the target to be the same with that used in this in vivo model (superficial target). Taking into consideration that the acoustic parameters that mainly control heating are AP and DF, a significant increase of the AP might cause unwanted local tissue heating. Therefore, to avoid excess tissue heating, the DF should be decreased. Since studies by Suchkova et al [115] and Holland et al [59] have shown that statistically significant enhancement of the fibrinolysis rate was achieved even with a DF of 1 %, extensive parametric studies (including AP, DF, exposure time etc.) are needed before this technology apply in humans.

9.9. Conclusions

In conclusion, the use of a rabbit carotid artery model for sonothrombolysis research is feasible and mimics an arterial thrombosis, which is close to MCA occlusion in humans.

This model, clearly demonstrated that 1.18 MHz FUS waves in combination with MBs, has the potential to strongly accelerate the action of thrombolytic drug TNK-tPA and hence reducing the treatment time needed for opening a vascular occlusion, which is a critical factor for future clinical studies.

In addition, it is important to note that enzymatic clot dissolution occurred through non-thermal mechanisms that maximized the penetration and binding of the drug into the clot, leading to complete clot dissolution.

Although the proposed treatment protocol lead to fast and complete recanalization of the carotid artery, further research in to the mechanisms, safety and reproducibility of this technique are required before applied for future clinical trials, including treatment of arterial thrombosis and ischemic stroke.

9.10. Summary

A summary table with the treatment protocol applied for the complete opening of a fully occluded common right carotid artery of a rabbit is provided below:

Table 11: Recommended therapeutic protocol for the treatment of a fully occluded carotid artery of a rabbit under MR guidance and monitoring

Experimental Parameters	Values
Mode	MR compatible FUS transducer
Frequency	1.18 MHz
Acoustic Power	60 W
Duty Factor	10 %
Pulse length	1 ms
Pulse Repetition Period	10 ms
Pulse Repetition Frequency	100 Hz
Treatment time	19 min
MR angiography technique	3 D time of flight (TOF)
TNK-tPA concentration	0.25 mg/kg
MBs dose	0.02 mL/kg prior sonication and every 5 min
Treatment's outcome	Complete artery recanalization

10. Conclusions and future directions

10.1. Conclusions

In this research, the 3rd generation thrombolytic agent TNK-tPA was used. Using an in vitro clot model, its lytic efficacy was determined. Fully retracted porcine clots were treated with different concentrations of TNK-tPA in the range between 0.1-1 mg/mL for two hours. Even though study results showed that the thrombolytic efficacy of TNK-tPA increases with dose, none of the TNK-tPA concentrations used, has led to full clot lysis. Therefore, it is confirmed that due to the low efficacy of thrombolytic therapy, the use of thrombolytic drug alone is not an effective lytic treatment and hence, should be enhanced in order to increase the degree of thrombolysis. The experimental study to measure the clot lysis activity of TNK-tPA is presented in appendix 2.

This research demonstrated that temperature has a significant impact on thrombolytic efficacy in clots exposed to TNK-tPA, with the relative reduction in mass of the clot being higher at 37 °C compared to 39 °C and 41 °C respectively. According to the experimental results, the fibrinolytic activity of TNK-tPA decreases 4 % / ^{0}C increase in temperature above the body baseline temperature of 37 °C, where the peak fibrinolytic activity of TNK-tPA was observed. Based on this observation, it is recommended that all sonothrombolysis studies with TNK-tPA, should be performed at a baseline temperature of around 37 °C, in order to take advantage of the peak thrombolytic activity of the drug and also to avoid unwanted thermal heating.

The low and incomplete clot lysis achieved by thrombolytic therapy, has created the need to use FUS energy in order to improve efficacy. The use of FUS pulses for clots destruction has received attention lately, since it can deposit high amount of energy into millimeter sized focal volumes with a low risk of damaging the surrounding healthy tissue. Our experimental studies exhibited that FUS as a stand-alone method for clot lysis is not effective and should be always applied as an adjunct to thrombolytic agent TNK-tPA, in order to increase the degree of thrombolysis. Experimental results clearly demonstrated that the use of pulsed FUS waves, strongly enhanced enzymatic fibrinolysis induced by thrombolytic TNK-tPA.

Moreover, using in vitro flow clot models, the effect of flow on clot lysis was also investigated. Taking into consideration that experimental results confirmed the beneficial effect of flow on the speed of fibrinolysis, we can easily come to the conclusion that the combination of FUS waves and flow strongly enhanced fibrinolysis acceleration, since

both of them are directly responsible for increased transport and penetration of PAs into blood clots, leading to enhanced thrombolysis.

It is of great importance to note that in all experimental studies, clot lysis occurred through non-thermal mechanisms, since the main aspect of all proposed treatment protocols was to maintain temperature elevation at the target that never exceeded 1 ^{0}C. Taking into consideration that the contribution of thermal effects on clot lysis was negligible, it is reasonable to assume that ARF is the primary non-thermal mechanism that dominantly appears in the propagation of pulsed FUS waves through a blood clot and contribute to thrombolysis enhancement.

In addition, study's outcome exhibited that a frequency of 1.18 MHz produced more thrombolysis compared to 0.6 MHz. Bearing in mind that for intracranial occlusion, low-frequency US has the potential to cause intracerebral hemorrhage, in association with our experimental results, a frequency of 1.18 MHz seems to be the best trade-off between the sharpness of focus and the depth of penetration and hence, should be employed as the optimum choice for transcranial applications such as the treatment of stroke patients.

Using 1.18 MHz FUS pulses, a linear increasing dependence between transmitted AP and thrombolysis efficacy in the range of 20-60 W was observed, verifying that ARF is the mechanism of action that is directly responsible for increasing the thrombolysis rate. In view of our findings, it is not unreasonable to suggest that ARF created structural changes in clots that increased the uptake, penetration, and binding of thrombolytic agent into the clot, leading to enhanced thrombolysis.

The largest thrombolysis enhancement was assessed when clots were treated with FUS in synergy with TNK-tPA and MBs, establishing that the administration of MBs on a constant rate, is as a way to further accelerate FUS enhanced TNK-tPA mediated thrombolysis. Consequently, MBs mediated sonothrombolysis may offer a new approach to improve outcomes. Although cavitation activity was not detected in our experiments, the negligible contribution of thermal mechanisms on clot lysis, has lead us to make the hypothesis that MBs enhanced sonothrombolysis is directly related with cavitation based phenomena and most probably with stable cavitation since the physical conditions to support the onset of inertial cavitation (MI > 0.7), did not exist in our studies.

In this research, the effect of operating parameters such as DF, PRF and pulse length on thrombolysis efficacy were investigated as well. Study findings have shown that pulse length and PRF are directly related with the degree of clot displacement and thus, the depth of penetration of the thrombolytic agent into the clot, leading to thrombolysis

enhancement. More specifically, our results demonstrated that it is more effective to transmit shorter pulses more frequently, which keep the clot in constant motion, than to transmit longer pulses less frequently, which saturate clot's displacement for an extended period of time. In addition, in order to avoid excessive heating, short DF values were applied (5 and 10 %). Study results exhibited a 7 % enhancement in thrombolysis efficiency using a DF of 5 % (AP = 40 W and pulse length = 0.5 ms) compared to that of 10 % (AP = 20 W and pulse length = 1 ms). Although thrombolysis enhancement using a DF of 5 % might be due to the increased level of ARF (AP was doubled), further investigation is required to be done, since the difference in thrombolytic efficacy between the DFs examined, is within the statistical error.

The main limitation of the proposed treatment protocol considers the effect of time on clot lysis, since our in vitro experimental studies showed that sonothrombolysis treatment is a time consuming procedure. Taking into account that after the incident of stroke only 3-6 hours are available to achieve a significant clinical benefit, thrombolysis enhancement due to FUS exposures even in the presence of MBs, may not be enough for timely recanalization of an occluded cerebral artery.

Besides FUS studies, experimental studies with unfocused US were also conducted, using high frequency small planar rectangular transducers. The degree of clot lysis was explored at frequencies 3.7 and 5.2 MHz respectively and study findings showed that thrombolysis efficacy decreases with higher frequencies. Although experimental results demonstrated that intravascular sonothrombolysis using 3.7 MHz small planar ultrasonic transducers, seems to be a promising method for effective clot lysis, the employment of lower US frequencies might be more beneficial. Since the optimal frequency for endovascular treatment is a trade-off between depth of penetration and thrombolysis efficacy, US frequencies in the range between 2-2.5 MHz, potentially may offer a new approach for intravascular sonothrombolysis.

Moreover, the thermal effects of such a transducer were monitored under MRI guidance. Using 4 MHz US frequency, a small planar rectangular transducer created within seconds thermal lesions with size sufficient enough to potentially shrink a vascular plaque and improve blood flow, without harming adjacent normal tissue (artery). Taking also into account that such a transducer is not expensive, we can easily come to the conclusion that in the future, a frequency of 4 MHz, may be a good choice for the treatment of atherosclerosis.

Finally, our in vivo study showed that the use of a rabbit carotid artery model for sonothrombolysis research is feasible and mimics an arterial thrombosis, which is close to MCA occlusion in humans. This novel model, clearly demonstrated that 1.18 MHz pulsed FUS waves in combination with MBs, has the potential through non-thermal mechanisms to strongly accelerate the enzymatic fibrinolysis induced by thrombolytic TNK-tPA, leading to fast and complete opening of the artery. This significant reduction in the treatment time needed for opening a vascular occlusion, is a critical factor for future clinical studies.

To our knowledge, none of the animal sonothrombolysis studies so far has claimed complete artery recanalization in such a short period of time, through non-thermal mechanisms and based on enzymatic clot lysis using a low concentration of thrombolytic drug and a low dose of bubbles. In a similar in vivo study by Stone et al. [78], a rabbit marginal ear vein clot model was developed. This superficial target was treated with 1 MHz pulsed FUS in synergy with rt-PA (no bubbles) for 15 min and complete vein recanalization was observed at 5 hours post treatment (relative clot size was measured at 4 %). In some other animal studies, complete artery recanalization was claimed within seconds, using 1.5 MHz pulsed FUS alone [82], [86]. Taking into consideration that temperature elevation at beam focus is not stated in their studies, in association with the very high values of AP used, it is speculated that such a fast clot lysis occurred probably due to thermal mechanisms.

10.2. Future directions

Concerning the in vitro applications of FUS enhanced TNK-tPA mediated thrombolysis in the presence of MBs the following steps should be done:

1. Detailed investigation into the non-thermal mechanisms behind sonothrombolysis, such as ARF and cavitation phenomena. The understanding and identification of these mechanisms will allow us to further optimize our treatment protocol.
2. Conduction of bench-top studies using a FUS transducer that produces a wider beam area at the target, in order to treat larger volumes of blood clot. More parametric studies are needed to establish a treatment protocol that through non-thermal mechanisms will maximize the thrombolytic activity of TNK-tPA. Thrombolysis efficacy in terms of mg of blood clot removed with 30 min of treatment should be evaluated.
3. The use of a different 3rd generation thrombolytic drug with longer half-life and higher specificity than TNK-tPA, such as Desmoteplase Salivary PA (DSPA). Desmoteplase has

an extended half-life of 190 min and is more fibrin dependent and fibrin specific than TNK-tPA and thus it is of interest to be applied to stroke patients [230].

4. Further in vitro experimental studies using small rectangular planar transducers as an adjunct to thrombolytic TNK-tPA and in association with MBs are required. Thrombolysis efficacy at a wider frequency range should be explored in order to determine the optimal frequency for maximum thrombolytic enhancement.

Concerning the in vivo applications of FUS enhanced TNK-tPA mediated thrombolysis in the presence of MBs, the following steps should be done:

1. More animal models are needed to confirm the safety and reproducibility of this technique.

2. Precise beam positioning using MR thermometry. By applying real-time MR thermometry, the location of the beam compared with the blocked artery will be identified quickly, ensuring accurate targeting.

3. Careful evaluation of the biological side effects is required to confirm that this technique does not cause tissue damage, especially if cavitation (inertial) exists, which may be highly destructive. Therefore, after treatment, histological analysis of the vessel walls in the treated arteries and the surrounding tissues is needed, for any indications of damage in the endothelial cells or extravascular tissue.

4. Experimental studies for deeper targets should be conducted, since occluded cerebral arteries intended as targets for this technology, occur at depths of about 3 to 10 cm from skull's surface. In such a case, our treatment protocol established in vitro, reproducing the physiological situation of a MCA occlusion occurred 4 cm deep into a brain tissue mimicking phantom, should be translated in vivo.

5. The therapeutic protocol should be applied directly to arteries in the brain such as the MCA. The main limitation of using a single element spherical transducer for transcranial application is the defocusing of the beam and the heating of the skull near the tissue/skull interface. To overcome these limitations, a phased arrays technology should be used.

6. Intravascular sonothrombolysis studies, using an animal model. The small flat rectangular transducers will be attached on a catheter and inserted through the artery to the site of occlusion. The optimized treatment protocol obtained in vitro that maximized the mass clot removed with no excessive heating, will be applied in vivo. The time needed for complete recanalization of the occluded artery, in association with the safety of the technique will be evaluated.

Appendix 1

Journals and conference publications

Journal papers

N. Papadopoulos and C. Damianou, "In Vitro Evaluation of Focused Ultrasound-Enhanced TNK-Tissue Plasminogen Activator-Mediated Thrombolysis," *Journal of Stroke and Cerebrovascular Diseases*, vol. 25, no. 8, pp. 1864–1877, 2016.

N. Papadopoulos, C. Yiallouras, and C. Damianou, "The Enhancing Effect of Focused Ultrasound on TNK-Tissue Plasminogen Activator-Induced Thrombolysis Using an In Vitro Circulating Flow Model," *Journal of Stroke and Cerebrovascular Diseases*, vol. 25, no. 12, pp. 2891–2899, 2016.

N. Papadopoulos, G. Menikou, M. Yiannakou, C. Yiallouras, K. Ioannides, and C. Damianou, "Evaluation of a small flat rectangular therapeutic ultrasonic transducer intended for intravascular use," *Ultrasonics*, vol. 74, pp. 196–203, 2016.

N. Papadopoulos, and C. Damianou, "Microbubble-based sonothrombolysis using a planar rectangular ultrasonic transducer," *Journal of Stroke and Cerebrovascular Diseases*, 2017.

Accepted for publication

N. Papadopoulos, and C. Damianou, "Review of protocols used in ultrasound thrombolysis," *Journal of Stroke and Cerebrovascular Diseases*.

N. Papadopoulos and C. Damianou, "In vitro enzymatic clot lysis using focused ultrasound waves as an adjunct to thrombolytic drug tenecteplase and in combination with microbubbles," Springer volume, *"Translational Stroke Research."*

Conference papers

N. Papadopoulos and C. Damianou, "In Vitro Study using MR-Guided Focused Pulsed Ultrasound for Destroying clots using thrombolytic Drugs," ISTU International Symposium on Therapeutic Ultrasound, March 14-16, Tel Aviv, Israel, 2016.

Appendix 2

Development of an in vitro human clot model to study clot lysis activity of different doses of thrombolytic drug TNK-tPA

To study the efficacy of TNK-tPA, an in-vitro clot lysis model was used. This model provides an easy to perform and effective method to measure in vitro the clot lysis activity of either newly developed or already known thrombolytic drug such as TNK-tPA.

For this purpose fully retracted porcine blood clots were formed into various pre-weighted sterile tubes, carefully numbered (figure 1A). Different concentrations of TNK-tPA were added to each one of them. To measure the thrombolytic activity of each one of the concentrations used, a group of 5 clots was used. Also, a group of clots was served as the control, or untreated group. Clots no. 1-4, were treated with TNK-tPA concentrations of 0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1.0 mg/mL respectively and clot no. 5, which was served as the control was treated only with saline (figure 1B).

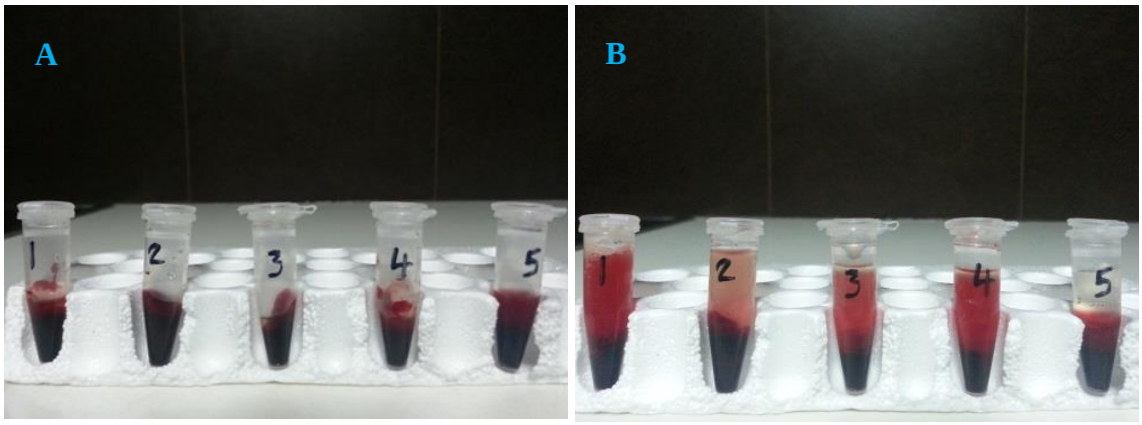

Figure 1: A) Tubes 1-5 containing blood clot samples, B) Tubes 1-4 treated with different concentrations of TNK-tPA. Tube 5 is the control clot.

The lowest concentration used was equal to the average maximum TNK-tPA concentration in blood (Cmax = 11.6 μg/mL), following iv bolus administration of 50 mg (figure 4.1). The clot samples were immersed in a temperature controlled water bath and treated at a constant temperature of 37 ^{0}C (body's baseline temperature) for 2 hours, since the treatment time for stroke is limited to around 2 hours. After treatment's termination, all tubes were taken out of the water and inverted for clot lysis observation. Clot dissolution and fluid along with the remnants of each one of the clots is clearly seen in figure 2A. Then, the clot dissolution and fluid obtained from each sample was carefully removed and the tubes were left to dry for 60 min (figure 2B). Percentage clot lysis was

measured as the relative reduction in the mass of the clot before and after thrombolytic treatment.

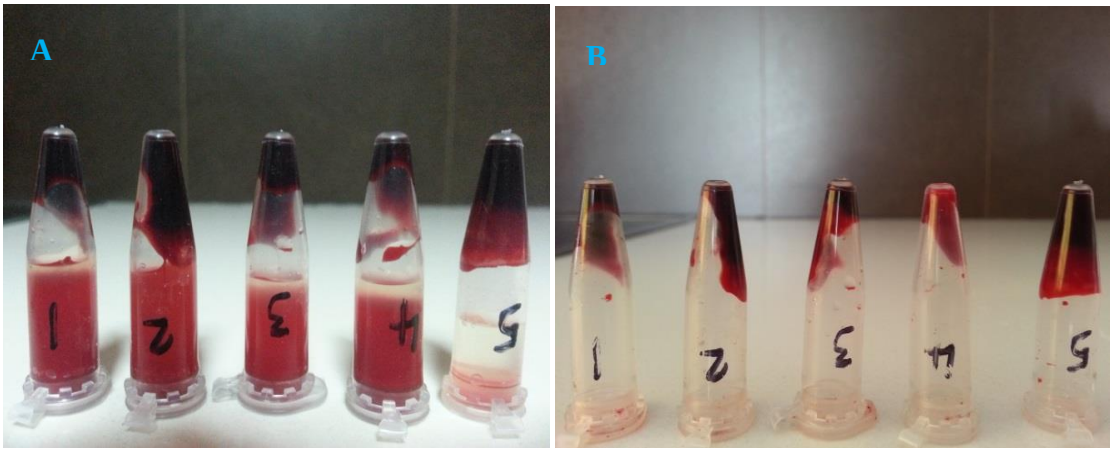

Figure 2: A) After clots dissolution, fluids along with the remnants of clots. B) Clot dissolution in tubes 1-4 with increasing order is clearly seen, whereas the control clot is intact.

Figure 3 shows the percentage clot lysis obtained from each one of the TNK-tPA concentrations used. Maximum clot lysis (87%) was obtained when the clots were treated with 1.0 mg/mL of TNK-tPA and minimum clot lysis (45 %) was obtained when the clots were treated with 0.01 mg /mL of TNK-tPA.

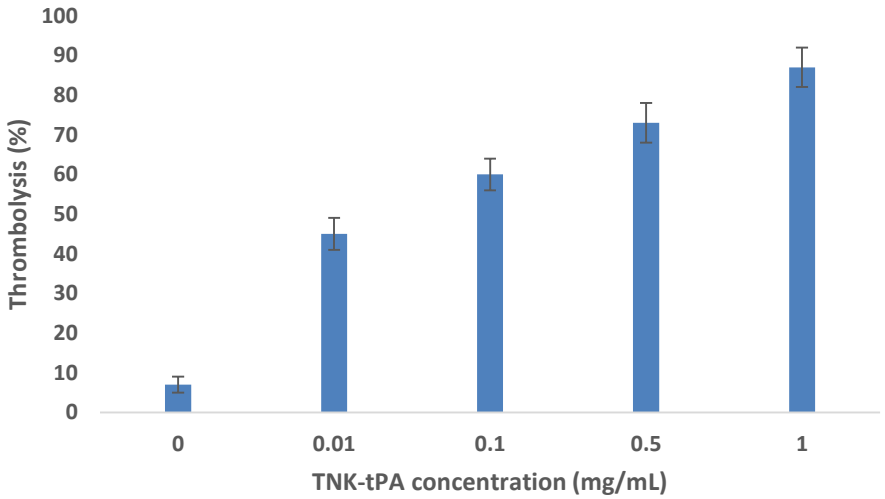

Figure 3: In vitro clot dissolution using various concentrations of TNK-tPA.

Although study results have shown that thrombolysis efficacy increases with the dose of the thrombolytic drug, none of the TNK-tPA concentrations used, led to full clot lysis. Since the average concentration of TNK-tPA in blood during 2 hour thrombolytic treatment is between 1-11 μg/ml (figure 4.1), we come to the conclusion that the use of thrombolytic drug alone is not an effective lytic treatment for thrombotic diseases, due to slow and incomplete clot lysis.

Appendix 3

Calculation of clot displacement in vitro initiated by FUS pulses

The area of the focal spot size a, is related to the transducer area A (12.57 cm^2) by:

$$\mathrm{a} = \frac{\lambda^2 f^2}{A} = 0.13\ cm^2 \qquad (1)$$

where, λ is the wavelength in water (0.13 cm), c the speed of sound in water at 37 °C (1527 $\frac{m}{s}$), f the focal distance (10 cm) and F the frequency of US waves (1.18 MHz). The intensity gain G in a lossless medium for longitudinal waves:

$$G\ (lossles) = \frac{A}{\mathrm{a}} = (\frac{AF}{cf})^2 = 97 \qquad (2)$$

The attenuation loss after traversing a distance x (10 cm) in degassed water is:

$$\text{Attenuation (loss)} = 10^{-\frac{xaF}{10}} = 0.63 \qquad (3)$$

where, the attenuation coefficient of water $\alpha =$ -0.2 dB/MHz.cm [55].

So, the net acoustic gain is the product of *(2)* and *(3)*:

$$G = (\frac{AF}{cf})^2\ 10^{-\frac{x\alpha F}{10}} = 61 \qquad (4)$$

For transmit AP = 60 W, the intensity on transducer's surface: $I = 4.77 \frac{W}{cm^2}$. So, the intensity at beam focus: $Io = 286\ \frac{W}{cm^2}$.

To calculate the maximum clot displacement (ξ_{max}), the following formula was used:

$$\xi\ \mathrm{max} = \frac{1}{\pi F}\sqrt{\frac{I_0}{2\rho_b c_b}} = 256\ nm \qquad (5)$$

Since, the values for mass density (ρ) and speed of sound (c) in clot at 37 °C are not available, the corresponding values in blood at the same temperature were used ($\rho_b = 1060\,\frac{kg}{m^3}$ and $c_b = 1530\,\frac{m}{s}$) [55].

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