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Of Ultrasound Exposure

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This dissertation is dedicated to my parents

Erik and Anita Madsen

Papers

This dissertation is based on the following papers, referred to in the text by their roman numerals:

I Low energy ultrasound exposure of the streptokinase molecule may enhance but also attenuate its fibrinolytic properties.
Madsen Härdig B, Persson HW, Olsson SB.
Accepted for publication in: Thromb Res. 2005 Jun 30; [Epub ahead of print].

II Direct action on the molecule is one of several mechanisms by which ultrasound enhances the fibrinolytic effects of reteplase.
Madsen Härdig B, Persson HW, Olsson SB.
Submitted.

III Can pulsed ultrasound increase tissue damage during ischemia? A study of the effects of ultrasound on infarcted and non-infarcted myocardium.
Olivecrona KG, Madsen Härdig B, Roijer A, Block M, Grins E, Persson HW, Johansson L, Olsson SB.
BMC Cardiovascular Disorder 2005, 5: 8.

IV Does low energy ultrasound known to enhance thrombolysis, affect the size of ischemic brain damage?
Madsen Härdig B, Persson HW, Gidö G, Olsson SB.
J Ultrasound Med 22:1301-1308, 2003; (Reproduced in this dissertation by the kind permission from the publisher: American Institute of Ultrasound in Medicine).

Abstract

The aim of this study was to further clarify the pro-fibrinolytic effects, and to explore the possible non-beneficial effects in ischemic organs, during exposure to pulsed ultrasound. This was accomplished by studies of the effects of different intensities of pulsed ultrasound exposure on the fibrinolytic properties of streptokinase and reteplase. Measurements were performed both following pre-exposure of the drugs and clots to pulsed ultrasound, and following concomitant exposure of clots and drugs.

In the exploration of pro-fibrinolytic effects during ultrasound exposure it was shown that exposure of the streptokinase molecule to low-intensity, pulsed high frequency ultrasound modulates its fibrinolytic properties. The effects were present following ultrasound exposure of streptokinase solution and during ultrasound exposure of clots and streptokinase solution concomitantly. Depending on its intensity, modulation was observed as both increased and decreased fibrinolytic effects. Pre-exposure of reteplase solution to low-intensity ultrasound induced changes in the function of the reteplase molecule associated with enhanced fibrinolytic effects. Enhancement effects were also seen when the clots were pre-exposed to high intensities of ultrasound before or concomitantly with exposure to reteplase solution, suggesting that two different intensity-dependent mechanisms are involved in ultrasound-enhanced reteplase fibrinolysis. No decreased fibrinolytic effect of reteplase depending on ultrasound exposure could be seen.

In an initial study of the fibrinolytic effects induced by a combination of Sonazoid® microbubbles and ultrasound, the effects of various ultrasound parameters on the microbubble destruction-rate was studied. It was shown that, at the same intensity level, the destruction-rate was faster outside the resonance frequency range. Five pulses were needed to achieve a fast destruction-rate. An ultrasound intensity of 0.5 W/cm^2 was shown to be the lowest intensity yielding a fast destruction-rate of Sonazoid® microbubbles.

The possible non-beneficial effects in ischemic organs of ultrasound with characteristics used to enhance fibrinolysis in vivo were studied in two different models, one using non-perfused myocardia in pigs, and one using non-perfused brain tissue in rats. It was shown that prolonged exposure of low intensity pulsed ultrasound might increase instantaneous myocardial damage. However, the same frequency, intensity and duration of pulsed ultrasound did not seem to enhance ischemic damage in non-perfused rat brain or induce any damage in the perfused rat brain.

In conclusion, ultrasound-enhanced fibrinolysis is induced by multiple mechanisms and drug specific reactions, and this study shows the importance of evaluating the effects of exposure on ischemic tissue, aiming at ultrasound-enhanced fibrinolysis.

Contents

1 Introduction

1.1 Ultrasound in medicine, a short history

In medicine, ultrasound (denoted as US) was initially applied in therapy. In the 1920s, Paul Langevin (1872 - 1946) showed that US could generate heat and destroy tissues [1]. In the 1930s, US was applied as a neurosurgical tool to destroy different parts of the brain in the treatment of Parkinson's disease (William J Fry and Russell Meyers), and to ease pain in moribund patients suffering from carcinomatosis (Peter Lindstrom). Other early applications of US were in physical and rehabilitation medicine for treating rheumatic arthritis (Jerome Gersten) and as a treatment for Meniers disease (Douglas Gordon, Mischele Arslan and Peter Wells) [2]. In the 1940s, therapeutic applications of US, considered by some to be a cure-all treatment, were used in the treatment of arthritic pain, gastric ulcers, eczema, asthma, thyrotoxicos, haemorrhoids, urinary incontinence, elephantiasis and angina pectoris. However, many of these treatments lacked scientific support [2].

In the early 1940s, the first experiments using US as a diagnostic tool were performed by the group of Karl Theodore Dussik (a neurologist/psychiatrist at the University of Vienna, Austria). They introduced the hypephonography, which was a technique in which US traversed the medium. An excited transducer was placed on one side of the head while on the other side a receiver registered the loss of intensity. This technique was designed to be used for detection of brain pathologies [3]. During the progressive development of US as a diagnostic tool in the 1950s, the introduction of newer piezoceramic materials (such as lead zirconate-titanate) had great impact, making it possible to produce transducers with better performance regarding overall sensitivity, frequency handling, coupling efficiency and output [2]. This development took place all around the world in areas renowned for brain diagnostics, ophthalmologic investigations and clinical applications in the abdomen and pelvis.

In Lund, pioneer work on echocardiography was initiated by Inge Edler and Helmut Hertz [4]. During its first 20 years, further use of echocardiography was rather slow. However, in the 1970s, the development of fibre-optic strip-charts, two-dimensional real-time scanners and the phased-area transducer started the widespread use of cardiac US as a real-time diagnostic tool [5, 6]. Development since then has been ongoing and today it includes three-dimensional animated pictures [7].

1.2 Thrombolysis, a short history

Streptokinase

In the bacteriological sphere, thrombolytic drugs were first discovered in studies of the reactions of the human body to various infections. In the early 1930s, studies showed that Lancefield group A β-haemolytic streptococci excreted a potent fibrinolytic substance, called streptococcal fibrinolysin [8]. The discovery of a plasma-lysing factor (later called plasminogen), an inactive precursor of a plasma proteolytic enzyme capable of dissolving fibrin (later called plasmin) that could be

activated by streptococcal fibrinolysin (later named streptokinase, further on denoted as SK) [9-12], led to clinical investigations of fibrinolysis. Since in many animals plasminogen resists activation by SK animal studies were not made. The first (1947) reported clinical treatment of a patient [13] was of a young man with cystic bronchiectasis who had, after surgery removal of one of his lungs, developed a high fever ascribed to blood effusion in the chest. Due to coagulation, this could not be aspirated, so SK was injected. During the next two days, 1300 ml lysed coagulum was removed and the patient cured. However, at this stage, the SK available was too impure to administer intravenously. Because of purification problems, clinical applications developed only slowly. However, in the late 1950s, Johnson and McCarty [14] showed that it was possible to lyse experimentally induced vein thrombi in human volunteers by SK infusion.

During the 1950s, better understanding of the molecular mechanism involved in SK-induced plasmin activation was achieved. It was found that SK was not a direct activator of human plasminogen [15, 16]. Instead, in the activation of plasminogen by SK, a two-step reaction was involved. However, the understanding of the function of SK is still a matter of investigation [17-21]. Pilot studies in the field of myocardial infarction showed that patients receiving SK soon after the onset of infarction did better than those receiving SK later in infarct development [22, 23]. This was followed by observations in animals of reductions in infarct size following SK infusion [24]. During the 1960s and 1970s, many clinical trials in acute myocardial infarction were performed. However, cardiologists were not easily convinced of the advantage of SK treatment. Therefore, the widespread use of SK-induced thrombolysis in clinical settings was delayed until the 1980s [25].

Tissue plasminogen activators

In 1947 a new substance produced within the body (tissue plasminogen activators denoted as t-PA) capable of degrading fibrin was discovered. Tissue plasminogen activators were originally called fibrinokinase and were discovered in animal tissue [26]. In the 1970s, it was established that t-PA, vascular plasminogen activators and blood plasminogen activators are immunologically the same [27] and also that the plasminogen activators found in blood originate mainly from the endothelial cells. In the early 1980s, the first purified human form of t-PA was obtained in small amounts from uterine tissue [28]. It was possible to purify t-PA in sufficient amounts from a stable human melanoma cell-line (Bowes, RPMI-7272) to be able to study its biochemical and biological properties [29]. (This cell-line was obtained from a patient named Bowes in 1974 who suffered from pulmonary metastasis melanoma.) This cell-line was cultured and exchanged among investigators. It was at this time established that t-PA had a specific affinity to fibrin utilizing its effects of plasmin conversion [29]. In 1983, the first studies of the possibility of cloning the t-PA gene led to the production of recombinant tissue plasminogen activators (denoted as rt-PA) [30].

During the 1980s, the first in vivo surveys of t-PA were made in studies of experimental pulmonary embolism in rabbits [31] and in myocardial infarction [32-34]. The clinical use started in patients with vein thrombosis

following kidney transplantation [35] and later in patients with acute myocardial infarction [36]. Since then, numerous clinical trials have evaluated the effects of t-PA and rt-PA mediated thrombolysis [37, 38]. Reteplase (denoted as r-PA), an rt-PA developed in the beginning of the 1990s, is produced in Escherichia coli cells by multiplication of its genetic sequence [39, 40]. Reteplase has a long half-life in vivo and has been shown to decrease the risk of intracranial bleeding compared to t-PA [41, 42].

1.3 Ultrasound-enhanced fibrinolysis

In the mid seventies, Trubestein et al started to explore the possibility for inducing fibrinolysis by exposure to US. In their initial invasive work, US was used to ablate thrombus in the absence of any thrombolytic drugs [43]. Development of US induced fibrinolysis was slow during the 1980s, but was followed by extensive work on invasive catheter-based US both in vitro and in vivo [44-47]. However the scientific investigations of enhancement of thrombolytic drugs during US exposure took over. More recently, the interest has again grown for the possibility of inducing fibrinolysis without thrombolytic drugs. During the 1980s this was done invasively [43, 44, 46, 47], but today US is applied non-invasively. Ultrasound induced fibrinolytic effects without any drugs present have been shown to occur both in vivo and in vitro [48-50].

Since thrombolytic treatment is accompanied by the risk of bleeding [51], the use of US-enhanced fibrinolysis is a way to increase the efficacy of fibrinolytic therapy. The development of non-invasive US enhancement of thrombolytic treatment started in the early 1990s. These studies showed that it was possible to increase the fibrinolytic action of different thrombolytic agents [52-54]. They were followed by work from different groups during the 1990s, with the Department of Cardiology in Lund as one of the early participants [55, 56]. During the 1990s, many in vitro and in vivo investigations showed that US-enhanced treatment of fibrinolysis worked. Further development was concentrated in two frequency-ranges one low (kHz frequencies) [52, 57-62] and the other high (MHz frequencies) [53-56, 63-69]. Clinical applications of US-enhanced fibrinolysis have been started both for treatment of cardiac vessel occlusion [70, 71] as well as cerebral vessel occlusion [72, 73].

A combination of US-enhanced thrombolytic treatment and microbubbles has been shown to further enhance fibrinolytic effects [74-78]. Studies have therefore explored the possibility of inducing fibrinolysis without thrombolytic agents, only using a combination of microbubbles and US. This has been shown to be a possible way of inducing fibrinolysis [79-85]. Other studies have documented the fibrinolytic effects of a combination of non gas-filled micro-particles and US exposure [86].

The mechanisms involved in ultrasound-enhanced fibrinolysis

That enhancement is observed in various regimes of US exposure suggests that several mechanisms are involved in US-enhanced fibrinolysis. The physiological properties of the US fields that have been suggested to account for the pro-

fibrinolytic effects observed are thermal effects, the cavitation effect and micro-streaming [57, 63, 65, 87, 88]. Documentation of physiological properties is, however, limited. One study showed enhanced effects due to increasing temperature and acoustic streaming [89], however, the influence of increasing temperature has been questioned [54]. Another study showed that only about half of the observed acceleration of thrombolysis could be attributed to inertial cavitation, thereby suggesting the existence of other mechanisms [68]. Further, US induced flow, detached fibrin from a tube wall and fragmented the fibrinogen in a gel structure. These effects were significantly reduced when the fluid was degassed. The flow effects were concluded to be related to cavitation-induced changes in fibrin gel structure [65]. One study explored differences in enhanced fibrinolytic effects between travelling and standing acoustic waves. Travelling waves were shown to enhance thrombolysis significantly more than standing waves [90].

Based on the available information, the mechanisms involved in the fibrinolytic process in high-frequency, low-intensity US-enhanced fibrinolysis have been suggested [91] to be non drug-specific [87], not to alter the sizes of plasmatic derivates or degradation products [54] and not to be caused by clot disruption [87]. Instead, the additive effect of US is supposed to cause increased transport of the lytic agent into the clot due to increased flow through the thrombus [65], caused by fibrin fibres that disaggregate of into smaller fibres [56, 92], explained by changes of drug binding affinity and to increased drug binding [67]. Recently, one study reported differences in the enhancement effects of US in blood clots made from normal subjects, compared to those made from patients with coronary artery disease medication (acetylsalicylic medication) [93].

Earlier in vitro studies of US-enhanced, SK-induced fibrinolysis have shown that these changes depend strictly on intensity [56, 94]. Several studies suggest a direct effect of US on the function of the SK molecule [95, 96]. The specific effects on SK from 1 MHz US exposure have been explored and, although a pro-fibrinolytic effect was evident at low US intensity (1 W/cm^2), no enhanced fibrinolytic effects of SK were seen at a slightly higher intensity. Further increase in intensity decreased the fibrinolytic action [56]. Another study using 3 MHz US of intensity 1.4 W/cm^2 failed to verify any enhanced fibrinolytic action of SK [94]. Furthermore, at low frequencies, US clots are disrupted mainly due to sonomechanical effects and the additive effect of SK was shown to prevail first after the US was switched off [95]. Another study suggested that low frequency, high-intensity US caused functional changes in the structure of SK [96]. Finally, one study showed that exposure to low frequency US produced an additive rather than a synergistic effect between the US and the lytic drug [97].

In studies of the effects of t-PA enhanced by US, increased fibrinolytic effects seem to be present at various intensities and frequencies of US [62, 67, 69, 89, 93, 94, 98-101]. The specific effects of the combination of exposure of clots to US during treatment with t-PA have been suggested to be increased flow in and around the clots [89, 100], induced changes in the fibrin structure [67, 93] and increased accumulation of t-PA and plasminogen on clot-surfaces [99].

The US mechanisms inducing fibrinolysis are similar to those discussed as non-beneficial. One study of the effects of combined treatment with high frequency US and abciximab on occluded cardiac vein grafts showed an increased incidence of

acute ischemic complications [71]. An effect observed already in the mid 1990s in a pilot study at the Department of Cardiology in Lund on five patients with signs of exclusive acute anterior myocardial infarctions (pronounced anterior ST-elevations) receiving SK and US treatment. Unexpectedly, one of the patients died in hospital with signs of cardiac tamponade 30 hours post US and SK treatment, while another died from circulatory arrest due to asystole 62 hours post US and SK treatment. After unsuccessful cardiopulmonary resuscitation with mechanical chest compression, diagnostic echocardiography revealed the accumulation of pericardial fluid, possibly indicating cardiac tamponade. Repeated attempts have been made to publish these data, however, these observations have remained unpublished until now. Also in neurological applications it has recently been shown that low-frequency US exposure produced increased bleeding during US mediated thrombolysis treatment [73].

These observations show the necessity of evaluating whether mechanisms of US-enhanced fibrinolysis might induce non-beneficial effects in the tissue exposed.

1.4 Mechanisms inducible by ultrasound and non-beneficial effects of ultrasound

US can produce biological effects through both thermal and mechanical mechanisms (also called non-thermal mechanisms). Mechanical effects are defined as all mechanisms that produce increases of less than 1 °C above normal physiological temperature. Thermal effects are accounted for when temperature rises more than 1 °C over normal physiological values.

Thermal mechanisms

Absorption of US energy in a medium results in heat. US-induced temperature increases depend on a number of factors such as absorption efficiency of the tissue and tissue perfusion rate in the exposed tissue. The resulting temperature increase is affected by parameters of the US exposure such as frequency, pressure amplitude, pulse duration, pulse repetition frequency and beam and scanning configurations [102]. During diagnostic imaging, US output levels are quantified as the Thermal Index (< 0.9 °C), which is a calculated index based on reasonable estimates of temperature increase [103].

The Thermal Index is subdivided into three different categories depending on the properties of the tissue exposed: the soft tissue thermal index is applied in tissue without bone, the bone thermal index applies to tissue where US impinges on bone, and the cranial bone thermal index applies when the bone is located close to the transducer (transcranial applications) [102, 103]. The high temperatures arising from US exposure are used in therapeutic setting of US, for beneficial effects of both non-destructive [89, 104] and destructive nature [105-108]. However, due to the risk for tissue damage, temperature increases are not wanted in diagnostic US [109, 110].

Mechanical mechanisms

Mechanical mechanisms that can be induced during US exposure are:

Cavitation

Microstreaming

Shock waves

Micro jets

Free radical production

Radiation force

Radiation torque

Acoustic streaming

Most often cavitational mechanisms require the presence of gas bodies in the tissue. However, cavitational mechanisms are also possible to induce in liquids free of gas bodies, but this demands very high intensities of US. The gas bodies in the tissue can either be naturally present in the body (e.g., in the lungs and intestines) or be introduced into the body by intravenous injection (e.g., in the form of US contrast agents). Cavitational mechanisms are classed in two groups. The first, non-inertial cavitation requires the repetitive oscillation of a bubble, whose equilibrium radius does not exceed twice its size, in phase with the US acoustic cycles. Non-inertial cavitation can result in heat production, in microstreaming of fluids near the bubble, in shear stress and in interactions between the bubble and surrounding tissue. The second mechanism, inertial cavitation, occurs when the US applied results in bubble oscillations greater than twice the equilibrium radius and when bubbles collapse rapidly and violently into many small bubbles. Inertial cavitation can result in extremely high local temperature increases (high temperatures leading to formations of free radicals), micro-jets and shock waves. The degree of violence of the collapse of a bubble is related to parameters such as frequency, pressure amplitude and bubble radius [102, 111]. Cavitational mechanisms have been shown to be beneficial in US-enhanced fibrinolysis [68]. However, cavitation effects are more commonly associated with non-beneficial effects of US exposure [112-117].

The radiation force is defined as the US energy absorbed by the object. Hence, if the tissue (e.g., lung tissue) absorbs only small amounts of US energy, reflecting the greater part, the force in the tissue is small, while it is large in tissues absorbing large amounts of US energy (e.g., red blood cells) [118]. In the literature, radiation force has been shown to induce changes in aortic pressure [119]. There are two phenomena related to radiation force, radiation torque and acoustic streaming. Radiation torque arises when US induces freely suspended, spherically symmetrical body rotation. It has been shown that radiation torque can cause disc-like particles to align with their axes parallel to the oscillatory velocity of the US field [120]. Acoustic streaming relates to the transfer of momentum to a liquid and it depends

on viscosity, absorption coefficient and the US energy applied. This effect can be important in the US exposure of a volume of liquid such as the urine bladder, large blood vessels or the heart [102, 111]. Acoustic streaming has been discussed as beneficial in US-enhanced fibrinolysis [89].

During diagnostic imaging, US output levels are quantified as the Mechanical Index (maximum of 1.9), which is a calculated index based of reasonable estimation of inducing unwanted US mechanical mechanism [122].

2 Aim of the study

The aim of this study was to further clarify the fibrinolytic effects following exposure to pulsed ultrasound and to explore the possibility that non-beneficial effects are induced in ischemic organs with US characteristics used for enhancement of fibrinolysis.

This was accomplished by investigating:

- changes in fibrinolytic effects following different intensities of pulsed 1 MHz frequency ultrasound pre-exposure of streptokinase solution and during concomitant exposure of clots and streptokinase solution.
- changes in the fibrinolytic effects of reteplase exposed to different intensities of 1 MHz frequency pulsed ultrasound following pre-exposure of reteplase solution and blood clots and during concomitant exposure of clots and reteplase solution.
- changes in the destruction-rate of Sonazoid® microbubbles during exposure of ultrasound of different frequencies, intensities and number of pulses.
- the possibility of increasing tissue damage in non-perfused pig myocardium following 1 hour exposure to pulsed 1 MHz frequency ultrasound.
- the possible increase of tissue damage in non-perfused rat brain following 1 hour exposure to pulsed ultrasound of 1 MHz frequency.

3 Material and Methods

3.1 Ultrasound equipment and measurements

Characterization of the US field is important for the understanding of the mechanisms involved in its interaction with biological and chemical structures. Therefore, several different US measurements were performed, with the aims of facilitating detailed knowledge of the intensity parameters and the field distribution of the applied US and of the overall performance of the various transducers used.

In all experiments, unfocused piezoelectric transducers (CERAM AB, Lund, Sweden) with resonance frequencies of 0.5, 1, 2, 4 and 6.5 MHz were used. The 4 MHz transducer was excited at three different frequencies (3.5, 4 and 4.5 MHz).

The 1 MHz transducer was used for the experiments described in I – IV, while the other transducers were used in the Sonazoid® microbubbles (denoted as MB) experiments.

In evaluating the fibrinolytic effects of exposing both SK and r-PA to US, a US configuration with a burst profile of 100 cycles repeated every millisecond (10 % duty cycle) was used (I – II). The same configuration was used for evaluating possible tissue damage (III – IV) (Figure 3.1).

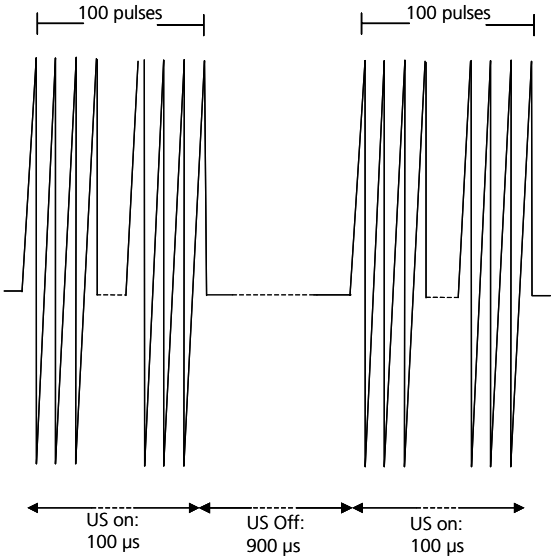

Figure 3.1. The burst profile of 100 cycles repeated every millisecond (10 % duty cycle) used in the measurements described in I – IV.

In all experiments, the transducers were excited by an electronic system consisting of a function generator (HP 3314A, Hewlett-Packard, Washington, USA) and an RF power amplifier (ENI 240L, ENI, Rochester, New York, USA) (Figure 3.2).

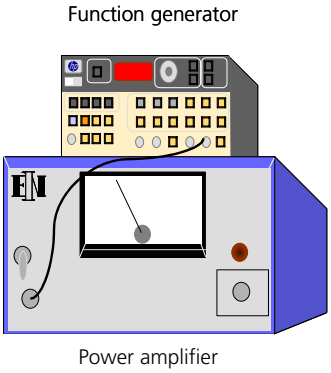

Figure 3.2. The function generator and the RF power amplifier used in all of the measurements.

The characteristics for the transducers are shown in Table 3.1.

Frequency MHz	Diameter mm	Area cm^2	Near field mm
0.5	20.0	3.10	33.0
1	16.0	2.00	42.0
2	12.7	1.30	54.0
3.5†	9.0	0.60	46.0
4†	9.0	0.60	53.0
4.5†	9.0	0.60	56.0
6.5	10.0	0.78	108.0

Table 3.1. Characteristics of the transducers used in the different studies described in this dissertation. †: the same transducer driven at different frequencies.

Ultrasound calibration and measurements

The transducers were calibrated by determining spatial-average temporal-average intensity (denoted as SATA) in W/cm^2 by measuring the total pressure of US radiation on an electrical balance (Model UPM-DT-1, Ohmic Instrumental co). The force (F) could be calculated by the equation:

$$F = \frac{h\,W}{c} \qquad (3.1)$$

where c is the speed of US in the propagating medium, (degassed water), h depends on the geometry of the target (in this case conical), and W is emitted acoustic power. A total acoustic power of 1 mW will produce a 0.69 µN force. If this is related to the electronic balance measurements made in the present studies, 1 W produces 69 mg weight [121].

After calibration, it was possible to calculate voltage settings for other intensity values using:

$$P = k \cdot U^2 \tag{3.2}$$

where P is the acoustic power (W), in this case output intensity in W/cm^2, k is a constant containing the transducer impedance, a voltage transformation constant from HP function generator to transducer via the ENI power amplifier, and a constant to convert from transducer electrical power to acoustic power output and U is the output voltage of the HP function generator. Since k is assumed constant, voltage values may be calculated by:

$$\frac{P_{calib}}{U_{calib}^2} = \frac{P_{desired}}{U_{desired}^2} \tag{3.3}$$

$$U_{desired} = \sqrt{\frac{P_{desired}}{P_{calib}}} \cdot U_{calib} \tag{3.4}$$

where $U_{desired}$ is the function generator output voltage value to be calculated to reach an acoustic force corresponding to a weight equivalent to the desired intensity, $P_{desired}$ is the intensity desired, P_{calib} is the intensity at which calibration was performed, in this case the intensity 1 W/cm^2 SATA, and U_{calib} is the function generator output voltage required to excite the transducer to an acoustic force with resulting weight corresponding to 1 W/cm^2.

Example 3.1: To calculate the output voltage to be set on the function generator for the 1 MHz transducer and an intensity of 2 W/cm^2 SATA ($P_{desired}$), the measured values P_{calib}: 1 (Wcm^2) and U_{calib}: 130 (mV output voltage) were used:

$$U_{desired} = \sqrt{\frac{2}{1}} \cdot 130 = 184$$

Thus, the voltage setting on the HP function generator should be 184 mV for the intensity 2 W/cm^2 SATA.

To determine the change in the US power after passing through the front wall of a test tube (used for MB destruction-rate measurements), thorough measurements were performed of US field distribution and peak negative pressures. In these measurements, a cylindrical plastic tube was halved and one half placed in front of the transducer at the same distance as the whole cylindrical tubes were placed during the experiments. Identical measurements were also performed without the half cylindrical plastic tube in front of the transducer.

Two sets of measurements were made. In the first set, measurements of US field distribution were made using a 0.5 mm diameter needle hydrophone. Following amplification (Precision Acoustic Ltd. United Kingdom) the signal was registered on an oscilloscope (Tektronix TDS 360, Tektronix UK, Ltd. Berkshire, United Kingdom). The digitised signals were processed using LabVIEW-based (National Instruments, Austin, Texas, USA) software developed at the Department of Electrical Measurements, Lund Institute of Technology, Lund, Sweden. The computer-controlled scanning-system enabled the hydrophone to be moved along three orthogonal axes (X, Y and Z) (Figure 3.3).

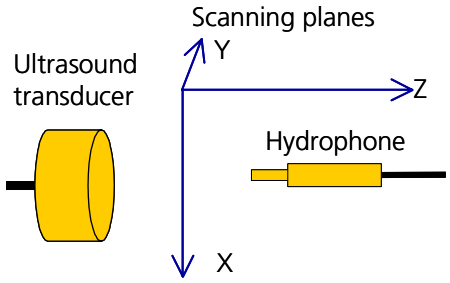

Figure 3.3. The measurement system used in the needle hydrophone measurements, consisting of a transducer and a hydrophone able to scan along 3 orthogonal axes (X, Y and Z). This setup was used in the measurements described in I-II as well as in measurements of ultrasound field distribution and peak negative pressures.

For the various transducers used (Table 3.1), the areas at the end of the near fields in the X- and Y-axes were scanned.

In the second set, measurements were made by scanning with a polyvinylidene fluoride membrane hydrophone (GEC-Marconi Hydrophone Type Y-34-3598) calibrated at the National Physical Laboratory, Teddington, England. The signals were registered on an oscilloscope (Tektronix TDS 360, Tektronix UK, Ltd. Berkshire, United Kingdom). These measurements were made to calibrate pressure values from the needle hydrophone measurements and to get correct values of acoustic pressure (in kPa) for calculating values of Mechanical Index. Mechanical Index calculations were made for measurements both with and without the half cylindrical plastic tube in front of the transducer. Values of Mechanical Index were calculated as described earlier [122].

Measurements at intensity 1 W/cm^2 SATA were performed at various frequencies and using different number of pulses as follows:

- **0.5 MHz** frequency and 2, 5 and 10 pulses/2 ms (0.2, 0.5 and 1 % duty cycle).
- **1 MHz** frequency and 2, 5 and 10 pulses/1 ms (0.2, 0.5 and 1 % duty cycle).
- **2 MHz** frequency and 10 pulses/0.5 ms (1 % duty cycle).
- **3.5 MHz** frequency and 10 pulses/0.286 ms (1 % duty cycle).
- **4 MHz** frequency and 5 and 10 pulses/0.250 ms (0.5 and 1 % duty cycle).
- **4.5 MHz** frequency and 10 pulses/0.222 ms (1 % duty cycle).
- **6.5 MHz** frequency and 10 pulses/0.153 ms (1 % duty cycle).

Ultrasound measurements related to ischemic pig heart experiments

To determine the Mechanical Index, the peak compressional pressure was measured. Measurements were also performed of the rarefactional pressure and the maximum value of spatial-peak temporal-average intensity (denoted as SPTA). Measurements were performed by scanning with a polyvinylidene fluoride membrane hydrophone as described earlier (*Ultrasound measurements related to Sonazoid® microbubble experiments page 14)*.

Measurements of the distribution of the US field yielded by a transducer similar to that used in the study were also carried out. Scanning was performed using a 0.5 mm diameter needle hydrophone and amplifier as described in the previous section (Figure 3.3) over an area of 80×30 mm^2 in the Y and Z-directions starting close to the transducer surface.

Control measurements of the rise in temperature in non-circulated pig myocardium exposed to pulsed US were performed. A 0.5 mm temperature probe was placed 1.5 cm inside an extracted pig myocardial muscle (3.0 cm thick) with no circulation [123]. The myocardial muscle was then placed in a degassed water bath heated to 37 ºC. The US transducer was placed perpendicular to and 1.5 cm from the surface of myocardial muscle and centered on the temperature probe. Exposure to pulsed US started when the water bath and muscle reached equivalent temperatures. Pulsed US (one burst of 100 pulses/ms) of 1 MHz frequency and an intensity of 1 W/cm^2 SATA was applied for one hour. Temperature measurements were performed simultaneously inside the myocardial muscle and in the surrounding water bath twice a second during the hour of pulsed US exposure.

Thorough measurements were carried out to determine how skull bone affected US field distribution and intensity loss. Measurements were performed both with and without skull bone present. In all measurements the skull bone was placed 30 mm from the transducer.

Firstly, measurements were made by scanning with a 0.5 mm diameter needle hydrophone and amplifier as described earlier (Figure 3.3). Scanning was performed over an area of 25×25 mm^2 in the Y and Z-directions starting at a distance of Z: 34 mm from the transducer surface. Over areas of 25×25 mm^2 along the X and Y-axes, two measurements were made, one at 34 mm and the other at 44 mm from the transducer.

Secondly, measurements of the loss of total radiation pressure were made on an electronic balance (Model UPM-DT-1, Ohmic Instrumental Co, St Michaels, Maryland, USA) in degassed water with and without the skull bone. Measurements were performed in degassed water. The 1 MHz transducer was excited at an intensity of 1 W/cm^2 SATA using both continuous wave and pulsed wave (100/ms).

Thirdly, measurements of the loss of intensity through the intact rat skull were performed by placing an excited transducer over the skull and, as receiver, an identical transducer was placed under the mandible. The signal was registered on an oscilloscope (Hewlett Packard, 54502A Digitising oscilloscope, Hewlett-Packard Colorado Springs Division, USA) and the reduction in peak-to-peak voltage of the received signal registered. The rat was exposed to US of frequency 1 MHz and an intensity of 0.15 W/cm^2 SATA, with continuous wave excitation. A reference measurement with the same US configuration was made in degassed water. Continuous US exposure was used to avoid problems of oscilloscope trigging during the measurements.

3.2 Fibrinolysis model and methods

In earlier studies of US-enhanced fibrinolysis, the fibrinolytic effect was explored using three different evaluation methods: Clot weight reduction [56], radio labelling of clots [90] and fibrin degradation products [124]. We have developed a new, low-cost, detailed method eliminating the need for mechanical handling of clots. To estimate the thrombolytic effects, this method measures the release of red blood cells from the fibrin network, on which the thrombolytic drugs exerts their effects (Figure 3.4).

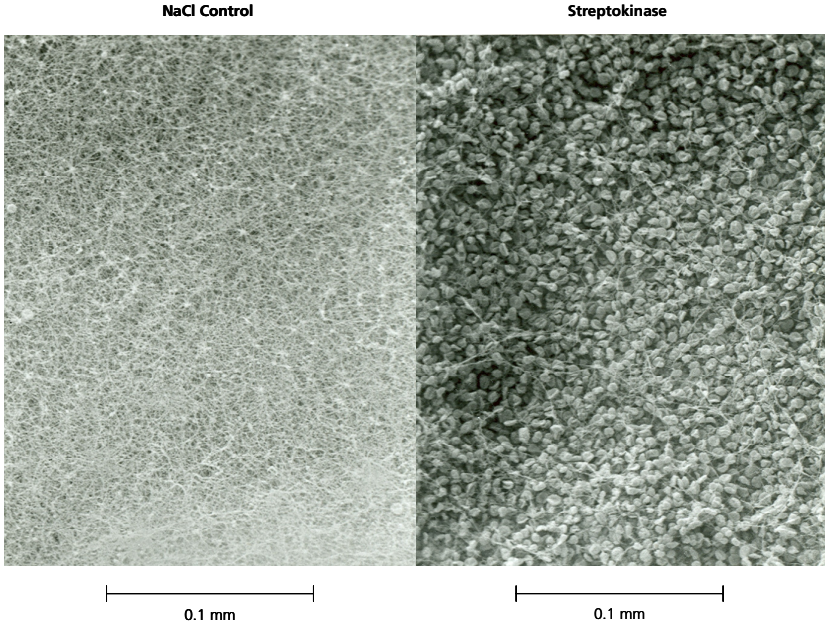

Figure 3.4. Two electron microscopy pictures of whole blood clots. Left-hand picture shows control clots exposed to 0.9 % NaCl solution and right-hand figure shows an interventional clot exposed to streptokinase solution. Note the high number of red blood cells exposed.

Clot formation

Blood clots were made using venous blood from healthy volunteers with no history of coagulation disturbances or ongoing anticoagulation medication. Blood was collected by venous puncture into 10 ml vacutainer collection tubes (BD Diagnostics - Preanalytical Sytems, Stockholm, Sweden) Solutions, without additives. The collected blood was immediately transferred to Teflon coated bottles to which 1.4 ml citrate-phosphate-dextrose adenine per 10 ml of blood had been added, resulting in immediate anticoagulation [56, 125]. The anticoagulated blood was either used within 8 hours or stored in a refrigerator at 4 °C for later use within one week.

Clots were formed by placing loosely spun yarns (length five cm and weight 55g/ 100 m) of 100 % natural wool (Peer Gynt, Sandnes, Uldvarefabrik A/S, 4300 Sandnes, Norway) diagonally within a cylindrical plastic tube (10 mm diameter and 35 mm length). Two clots were always made simultaneously and in the same manner, the first used in the intervention measurements and the second in the control measurements. After positioning the yarns, the cylindrical plastic tubes were filled with 1.4 ml of anticoagulated blood and coagulation restored by adding 56 µl of 0.45 mmol/ml calcium chloride. The tubes were then plugged, and after careful mixing, the blood was left to coagulate at room temperature for one hour (Figure 3.5).

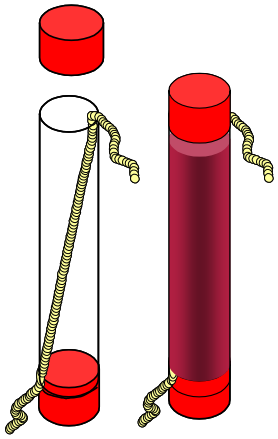

Figure 3.5. The left hand illustration shows the cylindrical plastic tubes with a wool yarn placed diagonally. The right hand illustration shows a tube filled with anticoagulated blood mixed with calcium chloride to induce coagulation.

After one hour of coagulation, the wool yarns with their associated clots (30 mm long and 10 mm in diameter) were carefully extracted. Visual inspection was made to ensure that both intervention and control clots were homogenous, faulty clots being discarded. Clots were next mounted in plastic frames (Figure 3.6) which were submerged and fixed in position in containers filled with 160 ml 0.9 % NaCl solution circulated at a rate of 999 ml/hour by a circulation pump (Sabratek, 3030 PLUS, Ardus Medical Inc. USA). To expose clots completely to US, the containers had windows of styrene sheet (0.13 mm Styrene sheet, Evergreen Scale Model, Inc. Woodinville, WA 98072, USA).

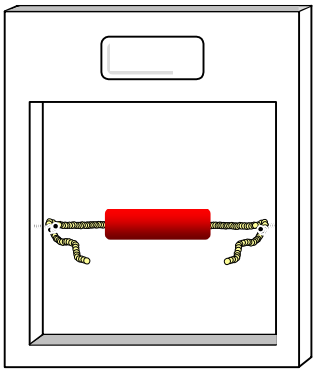

Figure 3.6. Illustration of the wool yarn with its associated clot mounted in one of the plastic frames.

The containers were placed in a water bath heated to a temperature of 37 °C by an immersion heater (MT/2, LAUDA, Königshofen, Germany). The water bath contained a fixed US transducer positioned 30 mm from the central part of one of

the containers. On the other side of the water bath, a control clot container was placed without exposure to US. To avoid reflections and US exposure of the control clots, the insides of the water bath were covered with US absorbing material reflecting less than 1 % of the incident US intensity (CERAM AB, Lund, Sweden) (Figure 3.7).

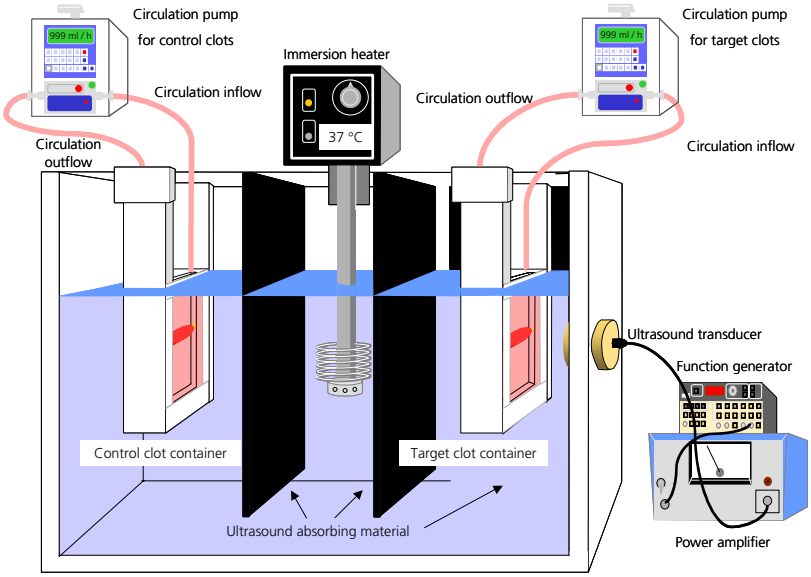

Figure 3.7. Schematic depiction of the experimental setup (I - II).

Evaluation of clot lysis

For evaluation of clot lysis, 1 ml fluid samples were taken repeatedly from each container and used to estimate the leakage of red blood cells from the clots. Samples were taken in two ways, either once every ten minutes or once every 20 minutes. To quantify the content of red blood cells in each fluid sample, Haemoglobin (denoted as Hb) was measured by dissolving the fluid samples in Drabkin's solution:

Potassium hydrogen phosphate	KH_2PO_4	2.89 g
Potassium cyanide	KCN	1 g
Potassium ferricyanid	$K_3Fe(CN)_6$	4 g
Tween 20		10 ml
Distilled water		2000 ml

inducing an instant haemolysis of the red blood cells.
Fifty ml of Drabkin's solution was further diluted with 450 ml of distilled water (1:10) before being used for evaluating clot lysis. After haemolysis, free Hb binds to cyanide to make a stable complex, cyanmethaemoglobin. The conversion of Hb is completed after 3 min in Drabkin's solution and the cyanmethaemoglobin complex formed stable for several hours [126].

The cyanmethaemoglobin concentration was then be measured by a spectrophotometer (Camspec M107, Camspec Limited, Cambidge, UK) at wavelength λ = 540 nm and Hb values calculated using the formula [127]:

$$C_{\mathrm{Hb}} = \frac{A^{540} f \mathrm{M}}{\varepsilon_{CMHb}^{540} l} \tag{3.5}$$

where C_{Hb} is the total Hb concentration (mg/l) of the sample, A^{540} is the absorbance of the cyanmethaemoglobin solution at wavelength λ = 540 nm, f is the dilution factor, M is one-quarter of the relative molecular mass of the Hb tetramer (11614.5 Da) [127], ε_{CMHb}^{540} is the millimolar absorptivity of the cyanmethaemoglobin solution at wavelength λ = 540 nm (11 liters $\mathrm{mmol^{-1}cm^{-1}}$) [127], and l is the length of the light path inside the solution (cm).

The absorbance value A^{540} was set to zero by measuring the absorbance value of 1 ml 0.9 % NaCl solution mixed with 4 ml Drabkin's solution (blank solution).

Due to the high concentrations of red blood cells in whole blood, high dilution factors were needed when measuring Hb with a spectrophotometer. Therefore, only 20 µl of blood was mixed with 5 ml of Drabkin's solution.

Example 3.2. Measurements of Hb in whole blood.

20 µl whole blood was mixed with 5 ml of Drabkin's solution (dilution factor 251) .The absorbance value, A^{540}, measured by spectrophotometer was 0.400, with a light path length of 1.1 cm. Calculation of Hb concentration was done using Equation (3.5):

$$C_{\mathrm{Hb}} = \frac{A^{540} \cdot 251 \cdot 16114.5}{11.0 \cdot 1.1} = 0.400 \cdot 367.7 \cdot 10^3 \text{ mg/l} = 147 \text{ g/l}$$

When evaluating clot lysis, the concentration of red blood cells in the clot container was much lower than in whole blood. To measure these low levels, the method to estimate Hb in urine, ascites or pleura fluids was used [128]. Thus, according to this method 1 ml of fluid was mixed with 4 ml of Drabkin's solution.

Example 3.3. Hb in clot lysis experiments:

A 1 ml sample of blood mixed with 0.9 % NaCl solution from the clot container was mixed with 4 ml of Drabkin's solution (dilution factor 5). The absorbance value, A^{540}, measured by spectrophotometer was 0.113, with a light path length of 1.1 cm. Calculation of Hb concentration in the clot container was done using Equation (3.5):

$$C_{\mathrm{Hb}} = \frac{A^{540} \cdot 5 \cdot 16114.5}{11.0 \cdot 1.1} = 0.400 \cdot 6658.88 = 752 \text{ mg/l}$$

To calculate the content of Hb (red blood cells) released from the experiment clot, the Hb concentration in the clot containers was related to the volume of the containers (initially 160 ml).

Example 3.4. Hb content in the clot container
A 1 ml sample was taken from the 160 ml container. The Hb concentration was measured to 752 mg/l (Example 3.3). The Hb content released from the experiment clot in the clot container (Hb_{rel}) was calculated using:

$$Hb_{rel} = 752 \cdot 0.160 = 120 \text{ mg}$$

Calculation of Hb content in the clot contaier with compensatory calculations of the loss of fluid volume and red blood cells from the containers caused by the repetitive sampling could be done by:

$$Hb_{rel} = 6658.88 \cdot A^{540}(n) \cdot \left(\frac{V - V_s(n-1)}{1000} \right) + \frac{V_s}{1000} \cdot 6658.88 \cdot \sum_{i=0}^{n-1} \cdot A^{540}(\text{i}); \quad (3.6)$$

where n = 1,2… is the sample number, $A^{540}(n)$ is the absorbance value of sample number (n), (where sample $A^{540}(0)$ is defined as 0),

$$6658.88 \cdot A^{540}(n)$$

is the equation for calculation of total Hb content in a fluid sample at a specified time,

$$\left(\frac{V - V_s(n-1)}{1000} \right)$$

is the equation for correction of volume loss due to repetitive samples taken, where V is the initial volume in the clot container, and Vs is the sample volume, and

$$\frac{V_s}{1000} \cdot 6658.88 \cdot \sum_{i=0}^{n-1} \cdot A^{540}(\text{i});$$

is the equation used for calculation of loss of red blood cells removed from the container in the previous fluid samples.

Example 3.5. In clot lysis experiments, Hb content in the clot container was analysed every ten minutes during one hour, in 1 ml fluid samples. The absorbance values measured were: 0.019, 0.027, 0.041, 0.077, 0.105 and 0.113. Using Equation (3.6), the Hb contents at each time instant were calculated:

$$Hb_{rel}(1) = 6658.88 \cdot 0.019 \cdot \frac{160 - 1 \cdot (1 - 1)}{1000} = 20.2 \text{ mg}$$

$$Hb_{rel}(2) = 6658.88 \cdot 0.027 \cdot \frac{160 - 1 \cdot (2 - 1)}{1000} + \frac{1}{1000} \cdot 6658.88 \cdot 0.019 = 28.5 \text{ mg}$$

$$Hb_{rel}(3) = 6658.88 \cdot 0.041 \cdot \frac{160 - 1 \cdot (3 - 1)}{1000} +$$

$$+ \frac{1}{1000} \cdot 6658.88 \cdot (0.019 + 0.027) = 43.2 \text{ mg}$$

$$Hb_{rel}(4) = 6658.88 \cdot 0.077 \cdot \frac{160 - 1 \cdot (4 - 1)}{1000} +$$

$$+ \frac{1}{1000} \cdot 6658.88 \cdot (0.019 + 0.027 + 0.041) = 80.6 \text{ mg}$$

$$Hb_{rel}(5) = 6658.88 \cdot 0.105 \cdot \frac{160 - 1 \cdot (5 - 1)}{1000} +$$

$$+ \frac{1}{1000} \cdot 6658.88 \cdot (0.019 + 0.027 + 0.041 + 0.077) = 109.5 \text{ mg}$$

$$Hb_{rel}(6) = 6658.88 \cdot 0.113 \cdot \frac{160 - 1 \cdot (6 - 1)}{1000} +$$

$$+ \frac{1}{1000} \cdot 6658.88 \cdot (0.019 + 0.027 + 0.041 + 0.077 + 0.105) = 117.7 \text{ mg}$$

Relative clot lysis

To determine relative clot lysis, the total Hb content of 1.4 ml of non-coagulated blood mixed in 160 ml 0.9 % NaCl solution was analysed for each volunteer's blood pool. The Hb contents of individual container samples were then related to the total Hb content, allowing calculations of percentage clot lysis for each sample. The percentage of cumulated relative release of Hb (denoted as CRHb) was used as measure of fibrinolytic effects.

Example 3.6: Calculation of relative clot lysis for an individual sample:

The Hb content of the last individual container sample ($Hb_{rel}(6)$ of Example 3.5) was related to the Hb contents of 1.4 ml of mixed non-coagulated blood ($Hb_{rel}(1.4)$ and relative clot lysis calculated as:

$$\text{CRHb} = \frac{Hb_{rel}(6)}{Hb_{rel}(1.4)} = \frac{117.7}{229.1} = 0.51$$

The percentage relative clot lysis after the one-hour experiment was 51 %.

The relative change of clot lysis was calculated as the difference in clot lysis between each intervention clot and its control, expressed as a percentage of the control clot lysis. The mean value was calculated for each series of measurements (I and II). In the evaluation of the effects following blood storage the absolute change in clot lysis was calculated as the difference between each interventional clot and its control expressed as the percentage difference.

Thrombolytic drugs

In evaluating US induced effects two thrombolytic drugs were used. SK was used in the experiments described in I, while both SK and r-PA were used in those described in II.

Streptokinase is a single-chain polypeptide produced by various strains of β-haemolytic streptococci. It has a molar mass of 47 kDa and is made up of 414 amino acid residues. The fibrinolytic action of SK is induced by the indirect activation of plasminogen [17].

The particular SK used in the present experiments was Streptase® (Hoechst Marion Roussel AB, Stockholm, Sweden) with a concentration of 1.5 million IU. This was diluted in 1000 ml 0.9 % NaCl solution resulting in a concentration of 1500 IU/ml. Immediately after dilution and thorough mixing, the SK solution was frozen in 10 ml dose [129, 130]. For each experiment, 3600 IU (2.4 ml) of this solution were added to the 160 ml clot container resulting in a concentration of 22.5 IU/ml. The concentration used in the experiments was taken from earlier studies of US-enhanced fibrinolysis [55, 56].

Reteplase is an rt-PA, a single-chain molecule with a molar mass of 39 kDa and comprises 355 amino acids. r-PA is produced by multiplying its genetic sequence in Escherichia coli bacteria after which it is purified. r-PA acts by a direct activation of plasminogen [131].

Rapilysin 10 U® (Roche registration limited, Welwyn Garden City, Great Britain) was used in the experiments. First, twenty units of r-PA were dissolved in 20 ml of water for injection, according to the manufacturer's specifications. This was followed by further dilution in 20 ml 0.9 % NaCl solution, resulting in a concentration of 0.5 U/ml. The diluted r-PA solution was immediately frozen in separate 2 ml doses, and for each measurement one 2 ml dose was thawed.

To determine the concentration to use, a series of dose response measurements were performed. The fibrinolytic action (using blood from five volunteers) was measured, at concentrations, 0.01562, 0.03125, 0.0625, 0.125, 0.25 and 0.5 U/160 ml 0.9 % NaCl. Fluid samples were taken every 20th minute during one hour (n = 6 pairs of clots for each concentration).

Control measurements of evaluation of fibrinolysis

Five series of control measurements were performed. In the first control series, the intrinsic fibrinolytic effect of SK in the fibrinolysis model was explored and compared with the effect of 0.9 % NaCl solution only. Clot lysis was evaluated during one hour and fluid samples were taken every ten minutes (n = 10 pairs of clots).

In the second control series, the intrinsic fibrinolytic effect of r-Pa in the fibrinolysis model was explored and compared with that of 0.9 % NaCl solution only. Clot lysis was evaluated in fluid samples taken every 10 minutes during one hour (n = 6 pairs of clots).

In the third series, the inter- and intra-individual variabilities in fibrinolytic response to r-PA were explored in fresh blood clots. For this purpose 3 to 31 clots from different blood samples from each individual were used and the fibrinolytic effect at the same r-PA concentration (0.001562 U/ml) evaluated.

In the fourth series, the inter-individual variations in response to SK (22.5 IU/ml) were evaluated in 15 individuals. Intra-individual variation was evaluated by blood sampling on different days (2 to 11 samples) in seven of the individuals.

The final series, explored the effects of storage time and cold storage (4 °C) on fibrinolytic effects of SK. Blood was stored for up to four weeks and SK induced fibrinolysis was evaluated on days 1, 2, 3, 4, 8, 11, 15, 22 and day 29.

Control measurements on blood components following ultrasound exposure

Two series of measurements were performed. In the first series the possible haemolytic effect of exposure to pulsed US on red blood cells was explored as follows: intervention clots were exposed to SK solution concomitantly with one hour of pulsed US of intensity 0.5 W/cm^2 SATA. At the same time, control clots were exposed to SK solution but without US Clot lysis was evaluated in parallel with evaluation of haemolysis, in one sample taken for each analysis (clot lysis and haemolysis) every ten minute during one hour. Following centrifugation of one of the 1 ml samples, the Hb contents of the supernatants in both SK induced and concomitant US and SK induced clot lysis were analysed. From six pairs of clots, both results from clot lysis and haemolysis measurements were compared.

In the second series, uncoagulated citrated whole blood in cylindrical plastic tubes was exposed to pulsed US of intensity 1 W/cm^2 SATA for one hour, while blood in control tubes was left unexposed during the same period. The platelet activity was measured by PFA-100® (Dade Behring Inc., Glasgow Business Community, Newark, USA) to determine if US induced platelet aggregation, which enabled a quantitative measure of platelet function during shear stress conditions [132]. Measurements of platelet activity were made before and after one hour of US exposure. Measurements were made using venous blood (4.5 ml) from ten healthy volunteers.

Fibrinolytic effects of ultrasound exposure alone

First, the fibrinolytic effect of exposure to pulsed US was explored at four intensity levels (0.125, 0.5, 1 and 4 W/cm^2 SATA). In these experiments, clots were submerged in 0.9 % NaCl solution. In most of them, six interventional clots were exposed to pulsed US while six control clots were exposed to 0.9 % NaCl solution but not to US. The exception was the series at intensity 4 W/cm^2 SATA in which 12 pairs of clots were compared.

To evaluate if plasmin in the clots was activated internally by exposure to US a second control series was performed. Six intervention clots were submerged in 0.9 % NaCl solution and exposed to pulsed US of intensity 4 W/cm^2 SATA while six control clots were exposed to 0.9 % NaCl solution but not irradiated with US. After one hour of exposure to US, the amount of Hb remaining in individual clots were recalculated enabling the effects in the second hour to be compared without being influenced by any changes induced during the first hour. In the second hour, clot lysis in 0.9 % NaCl solution in both containers was evaluated without the application of US. For evaluation of clot lysis, fluid samples were taken every 20 minutes during US exposure and every 10 minutes during the following hour.

The effect of exposure to ultrasound on the fibrinolytic effects of streptokinase

The effects of exposing SK to US at different exposure settings were explored in a series of measurements. In the first, intervention clots were exposed to SK solution concomitantly with one hour of pulsed US. At the same time, control clots were exposed to SK solution but without US. In all, eight levels of intensity were used: 0.0625, 0.125, 0.25, 0.5, 0.75, 1, 2 and 4 W/cm^2 SATA (Intensities 0.5, 1 and 4 W/cm^2 SATA, in I and intensities 0.0625, 0.125, 0.25 0.75 and 2 W/cm^2 SATA, in II). At each level six pairs of clots were analysed.

In the second series, performed to evaluate if enhanced fibrinolytic levels varied with clot age, clots were left to coagulate for 2-hours in room temperature. Clot lysis was measured during concomitant exposure of SK and US (intensity 0.5 W/cm^2 SATA). To evaluate clot lysis, fluid samples were taken every ten minutes during one hour of US exposure (n = 6 pairs of clots).

In the third measurements series, clot lysis induced by SK alone or by concomitant exposure of SK and US was followed over a 24-hour period. This was done to evaluate the effect of the prolonged period of exposure. The US intensity used was 0.5 W/cm^2 SATA. Clot lysis was studied in fluid samples taken once every hour for six hours and then at 12 and 24-hours after exposure started (n = 4 clots). In these measurements compensatory calculations were also done for the evaporation of fluid from the clot containers that occurred during the 24 hour of evaluation.

In the fourth series, pulsed US was applied to the SK solution circulating in one of the containers (without any clot). US was applied for one hour at one of three intensity levels (0.5, 1 and 4 W/cm^2 SATA). At the same time, unexposed SK solution was circulated in the other container. One clot of each pair was then submerged in the container with pre-exposed SK solution and the other in that with unexposed SK solution. Clot lysis was evaluated during the following hour (n = 12 clots at each level of intensity).

Effects of reteplase in different ultrasound exposure settings.

The effect of exposing r-PA to US in different settings was explored in various ways.

In the first series of measurements, interventional clots were exposed simultaneously to circulating r-Pa solution and pulsed US and control clots were exposed to circulating r-PA solution without US exposure. Eight intensity levels: 0.0625, 0.125, 0.25, 0.5, 0.75, 1, 2 and 4 W/cm^2 SATA were used. Clot lysis was evaluated in six pairs of clots in samples taken every ten minutes during the hour of US exposure.

In the second series of measurements, circulating r-PA solution was exposed to pulsed US for one hour before mounting the clots in the containers. Three different intensity levels were selected for evaluation. Two of them (0.125 and 4 W/cm^2 SATA) were levels at which enhanced effects was observed in the first series, and a third level (intensity 0.5 W/cm^2 SATA) at which no enhanced effects were observed. After exposing the circulating r-PA solution to US for one hour, clots were placed in either pre-exposed r-PA solution or in unexposed r-PA solution. Fluid samples were taken every 10th minutes during one hour to permit comparison of the fibrinolytic effects in both solutions. At each intensity level, results for 12 pairs of clots were compared.

In the third measurement-series, also comprising 12 pairs of clots, interventional clots were submerged in 0.9 % NaCl solution and exposed to pulsed US of intensity 4 W/cm^2 SATA, while control clots were exposed to 0.9 % NaCl solution but not US. Fluid samples were taken every 20 minutes. Following the first hour of US exposure, the amount of Hb remaining in individual clots was recalculated as in the control series (*Fibrinolytic effects of ultrasound exposure alone,* page 24). r-Pa solution was then added to both clot containers, but the solutions were not exposed to US. Fluid samples were then taken every ten minutes for another hour.

In a fourth series of measurements, target clots were submerged in 0.9% NaCl solution, exposed to pulsed US of intensity 4 W/cm^2 SATA while control clots were exposed to 0.9 % NaCl solution but without exposure to US. Clot lysis was evaluated in fluid samples taken every twenty minutes. After one hour of exposure to US, the remaining amounts of Hb were recalculated as described earlier. The clot containers were then moved so as to place the clot not exposed to US during the first hour in front of the US transducer. r-PA solution was then added to both clot containers, and US was applied to the previous control clot concomitantly with r-PA exposure while the previous target clot was unexposed. Clot lysis was evaluated using fluid samples taken every ten minutes. This switching of clots made it possible to evaluate differences in r-PA enhancement effects between pre-exposed clots and clots concomitantly exposed to r-PA and US. In the second hour, fluid samples were taken every ten minutes during exposures (n = 12 pairs of clots).

3.3 Bubble destruction model and methods

The expectation underlying our exploration of the dependency of various combinations of US exposure effects on the MB destruction-rate was that a fast destruction-rate could be connected with inertial cavitation and a slow destruction-rate could be connected to non-inertial cavitation. The results from this MB destruction survey could then be used as a guide when deciding on which frequency, duty cycle and intensity to use in future studies of MB and US induced fibrinolysis. To evaluate the effects on MB destruction-rate from modulation at different US configurations, a Coulter counter (Sysmex K-1000, Diamond Diagnostics, Holliston, USA) was used. This is a method used earlier in quantitative measurements of MB concentration [133].

Microbubbles

The MB used in this study were generously supplied by Nycomed Amersham AS (Oslo, Norway). MB have a monolayer lipid membrane and are filled with perfluorocarbone gas, a clear, colourless, chemically stable and inert gas with low solubility in water. MB have a relatively narrow size distribution, with a median diameter between 2.4 and 3.5 μm and an interquartile width-distribution of approximately 1 – 2 μm. MB resonance frequencies range from 3 to 5 MHz. They are supplied in glass vials containing 500-2000 millions MB/ml. MB are produced as an ultrasonic contrast agent. Each glass vial contains a dry substance that was dissolved in 7-10 ml sterile water. Diluted MB solution were divided between two cylindrical plastic tubes

Ultrasound exposures

Five unfocused piezoelectric transducers (CERAM AB, Lund, Sweden) with resonance frequencies of 0.5, 1, 2, 4 and 6.5 MHz were used. The 4 MHz transducer was also excited at frequencies 3.5 and 4.5 MHz.

Analyses of the effects of US, of the above described frequencies, at the same US intensity level (SATA) on MB destruction-rate were performed. Further, the effects of different US intensities on MB destruction-rate were analysed. Finally, we analysed the effects of different number of sent pulses on MB destruction-rate.

MB within cylindrical plastic tubes with were exposed to US as follows:

- **0.5 MHz** frequency:

  10 pulses/2 ms (1 % duty cycle) of intensity 0.25, 0.5, 1, 2 W/cm^2 SATA.

  5 pulses/2 ms (0.5 % duty cycle) of intensity 1 and 2 W/cm^2 SATA.

  2 pulses/2 ms (0.2 % duty cycle) of intensity 1 and 2 W/cm^2 SATA.

- **1 MHz frequency**:
  10 pulses/ms (1 % duty cycle) of intensity 1 and 2 W/cm^2 SATA.
  5 pulses/ms (0.5 % duty cycle) of intensity 1 and 2 W/cm^2 SATA.
  2 pulses/ms (0.2 % duty cycle) of intensity 1 and 2 W/cm^2 SATA.
- **2 MHz frequency**:
  10 pulses/0.5 ms (1 % duty cycle) of intensity 1 W/cm^2 SATA.
- **3.5 MHz frequency**:
  10 pulses/0.286 ms (1 % duty cycle) of intensity 1 W/cm^2 SATA.
- **4 MHz frequency**:
  10 pulses/0.250 ms (1 % duty cycle) of intensity 0.5, 1, 1,5, 2 W/cm^2 SATA.
  5 pulses/0.250 ms (0.5 % duty cycle) of intensity 1 W/cm^2 SATA.
- **4.5 MHz frequency**:
  10 pulses/0.222 ms (1 % duty cycle) of intensity 1 W/cm^2 SATA.
- **6.5 MHz frequency**:
  10 pulses/0.153 ms (1 % duty cycle) of intensity 1 W/cm^2 SATA.

The cylindrical plastic tubes were placed at the ends of the calculated near field areas of the different transducers (Table 3.1.) and exposed to US for 60 minutes, or until the MB concentrations were too low to measure.

Analytic procedure

Both cylindrical plastic tubes were placed in a tube holder and immersed in a water bath at room temperature. One cylindrical plastic tube was exposed to US while the other tube served as control and was not exposed to US (Figure 3.8).

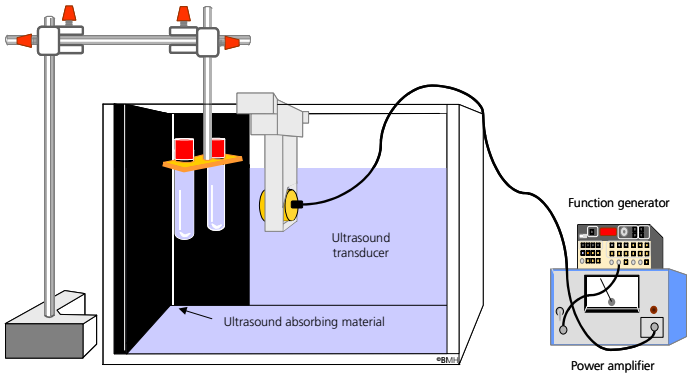

Figure 3.8. Illustration of the cylindrical plastic tubes placed in a tube holder immersed in a water bath at room temperature. One tube is exposed to ultrasound while the other serves as control and is not ultrasound exposed.

The Sysmex K-1000, Coulter counter was used to determine the number of MB left [133] at 0, 2, 4, 6, 8, 10, 15, 30 and 60 minutes of exposure to US. While determining the number of MB, the US exposure was stopped for 2 min. Each series comprises 2-8 sets of measurements.

Calculations correcting for spontaneous destruction of MB in the control plastic tubes were made by adding the reductions in the control measurements (spontaneous MB destruction) to the measured reduction in MB exposed to US:

$$R_{tot}\% = \left(\frac{b_{contr}(t)}{b_{contr}(0)} + \left[1 - \frac{b_{us}(t)}{b_{us}(0)} \right] \right) \cdot 100\ \% \qquad (3.7)$$

where $R_{tot}\%$ is the corrected value of MB at a specific time, $b_{contr}(0)$ the median number of MB at start in control measurements, $b_{contr}(t)$ the median number of unexposed MB at a specific time, $b_{us}(0)$ the median number of MB at start of US exposure and $b_{us}(t)$ the median number of MB following US exposure at a specific time Thus, the $R_{tot}\%$ was calculated at different time intervals which enabled estimation of the number of MB actually destroyed by US exposure. Concentrations were obtained as the number of MB, recalculated to show the median percentage reduction of MB over time.

Example 3.7: Calculation of median percentage reduction of MB over time for 0.5°MHz, 1 W/cm^2,2 pulses/ms:

The median number of MB in 6 sets of measurements in ultrasound exposed MB was measured to be: $b_{us}(0)$ = 188 (•10^9/L) and $b_{us}(t)$ (2,4,6,8,10 and 15 minutes) = 146, 86, 48.5, 24.5, 8.5 and 6.5 (•10^9/L). The median number of MB in 6 sets of measurements in control measurements was measured to be: $b_{contr}(0)$ = 198 (•10^9/L) and $b_{contr}(t)$ (2,4,6,8,10 and 15 minutes) = 193, 189, 185, 181, 181 and 183 (•10^9/L). The median percentage reduction over time (Rtot%(t)) corrected for spontaneous destruction of MB was calculated using Equation (3.7)):

$$R_{tot}\%(2) = \left(\frac{193}{198} + \left[1 - \frac{146}{188} \right] \right) \cdot 100\ \% = 80\ \%$$

$$R_{tot}\%(4) = \left(\frac{189}{198} + \left[1 - \frac{86}{188} \right] \right) \cdot 100\ \% = 50\ \%$$

$$R_{tot}\%(6) = \left(\frac{185}{198} + \left[1 - \frac{48.5}{188} \right] \right) \cdot 100\ \% = 32\ \%$$

$$R_{tot}\%(8) = \left(\frac{181}{198} + \left[1 - \frac{24.5}{188} \right] \right) \cdot 100\ \% = 22\ \%$$

$$R_{tot}\%(10) = \left(\frac{181}{198} + \left[1 - \frac{8.5}{188} \right] \right) \cdot 100\ \% = 13\ \%$$

$$R_{tot}\%(15) = \left(\frac{183}{198} + \left[1 - \frac{6.5}{188} \right] \right) \cdot 100\ \% = 11\ \%$$

The half-life of the MB was then defined as the time when 50 % of them had been destroyed as shown in Figure 3.9.

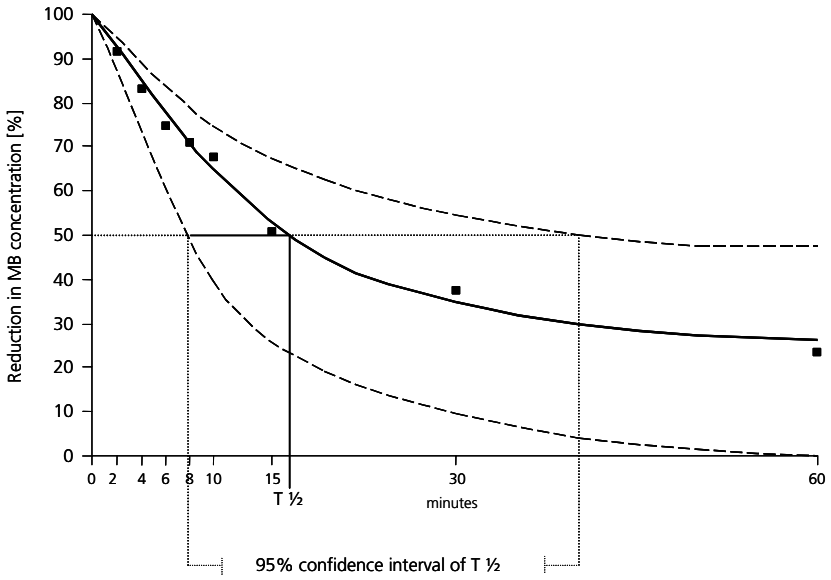

Figure 3.9. A schematic description of the calculation of half life measurements in the MB destruction-rate exploration: ■: median of measured values corrected for spontaneous destruction of MB, calculated using Equation (3.7), —: measured curves fitted to regression curve, ----: 95 % confidence interval of the regression curve. T½ is shown and 95 % confidence interval.

3.4 The Ischemic heart model and methods

To test if lengthy exposure of ischemic myocardial tissues to US could potentially be harmful, we chose an open chest porcine model with an experimentally induced myocardial infarction. This model includes a detailed histopathological evaluation of early signs of myocardial damage.

Animal preparation

Seventeen Swedish landscape pigs with weights ranging from 25 to 30 kg were used in the experiments. Before tracheotomy, anaesthesia was induced with 5-10 ml (25 mg/ml) sodium pentothal (Pentothal Sodium, Abbot Scandinavia AB, Sweden) given intravenously. Anaesthesia was maintained with a mixture of ketamine (5 mg/Kg/min) (Ketalar, Parke-Davis, Division of Warener Lambert Nordic AB, Sweden) and pancurone (Pavulone, Organon Teknika AB, Sweden) (0.3 mg/Kg/min) given intravenously. During maintenances anaesthesia the animals were ventilated mechanically (Serviventilator 900 B, Siemens Elema, Sweden). Following anaesthesia a medial sternotomy was performed and the pericardium incised. The borders of the pericardium were sutured to the skin

overlying the sternal edge. To induce ischemia, a large proximal diagonal branch of the left anterior descending artery was ligated. Ischemia was allowed to progress for 1 hour before positioning the transducers.

Transducer and ultrasound exposure

Two transducers (CERAM AB, Lund, Sweden) were placed over the myocardium, one at the non-infarcted myocardium of the anterior/apical free wall of the left ventricle and the other was in the mid/basal region of the anterior portion of the left ventricle, corresponding to the non-circulated myocardial region perfused by the ligated large diagonal branch of the left anterior descending artery. The transducers which were fixed to universal joints attached to small steel pipes on stands, were placed in fixed positions approximately 1.5 cm from the epicardium (Figure 3.10). To ensure adequate acoustic coupling, US gel (Clinical, Diagramm Halbach AG, Germany) was applied to the entire anterior portion of the heart. To compensate for loss of gel during the procedure, gel was added between the transducers and the myocardial tissue throughout the period of US exposure. The myocardium was either only exposed to a transducer (without US) or exposed with transducer and US for one hour. Pulsed US of frequency 1 MHz and intensity 1 W/cm^2 SATA was applied. In each millisecond, a burst of one hundred pulses was sent, equivalent to a 10 % duty cycle.

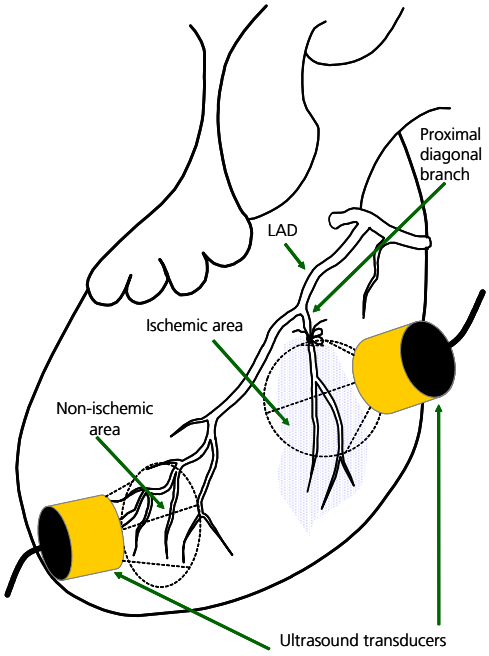

Figure 3.10. Schematic depiction of the open chest porcine model with the ligated diagonal branch of the left anterior descending artery (LAD). The shading indicates the estimated size of ischemic tissue. Ultrasound transducers were applied over both ischemic and non-ischemic areas.

The experiment was designed to investigate possible injurious effects due to pulsed US exposure, mechanical handling of the hearts and the application of transducers alone.

Altogether, 68 tissue samples from 17 pig hearts were used. These were divided up as follows:

- **17 samples**, of non-infarcted myocardium without any exposure.
- **4 samples**, of non-infarcted myocardium exposed only to the transducer.
- **13 samples**, of non-infarcted myocardium exposed to pulsed US.
- **17 samples**, of infarcted myocardium without any exposure.
- **8 samples**, of infarcted myocardium exposed only to the transducer.
- **9 samples**, of infarcted myocardium exposed to pulsed US.

Tissue preparation and histopathological evaluation

At the end of the exposure period, epicardial sutures were placed at two locations under the transducers, to indicate the diameters of the areas exposed. The pigs were then given 10 ml of potassium (2 mmol/ml, Addex-Kalium™, Pharmacia & Upjohn AB, Sweden) intravenously to induce ventricular fibrillation, which occurred momentarily following administration. The large vessels were clamped and cut using a scalpel, while the veins were ligated and cut by scissors. After extracting the entire heart, the regions of tissue indicated by the sutures were removed. Transmural samples, 5 - 10 mm in diameter, were carefully cut out with a scalpel. During this procedure, care was taken to minimize traumatic handling of the heart.

Following excision, the tissue samples were prepared for histopathological evaluation. From each sample, three slides were cut from the formalin fixed and the paraffin embedded blocks of myocardial tissue. These were then stained with Hematoxylin-Eosin (Van Gieson, and Phosphor Tungistic Acid Hematoxylin, PTAH). The samples were examined using routine light microscopy in 10 X, 20 X and 40 X enlargement by an experienced pathologist unaware of whether the tissue samples were from the infarcted or non-infarcted tissue or exposed or not exposed to US. In microscope evaluations, only common signs of tissue damage, seen during the early phase of myocardial infarction were looked for [134].

The parameters used to evaluate possible damage were:

- Eosinophilic changes in the myocyte (**Ischemia**).
- Reduction or loss of cross striation (**Loss of striation**).
- Coagulation necrosis (**Necrosis**).
- Infiltration of polymorphonuclear cells (**PMN**).

All parameters were assigned scores on a scale from 0 to 3, where 0 indicated no damage, 1 minimal change, 2 intermediate changes and 3 extensive changes in the myocardial tissue. Analyses of the damage score for each of the above parameters (Ischemia, Loss of striation, Necrosis and PMN) as well as the total sums of damage scores of all parameters were made. Thus, the total sums of damage scores had minimum and maximum possible values of 0 and 12 respectively.

3.5 The Ischemic brain model and methods

The model used was chosen on the basis of potential in evaluating whether pulsed US exposure induced increases in infarct volume during cerebral vessel occlusion. The method is a well-documented method of quantifying brain injury due to cerebral vessel occlusion [135-140].

Experimental protocol

The experiments were performed on male Wistar rats (Møllegaard Breeding Center, Copenhagen, Denmark) ranging in weight from 310 to 405 g. The rats had fasted overnight but had free access to water. A mixture of 3.5 % halothane and oxygen-nitric oxide in the ratio 30:70 induced anaesthesia. Following anaesthesia, the rats were intubated and artificially ventilated and anaesthesia maintained using 1.5 % halothane and oxygen-nitric oxide (30:70). A catheter placed in the artery of the tail permitted measurements of arterial pressure, blood sampling (PCO_2, PO_2, pH, B-glucose) and administration of anticoagulation (heparin 0.1 ml, 150 IU/ml). During the experiments, body-temperature was maintained at 37 °C using a thermistor-controlled heating blanket. Rats were randomised to one of three groups:

- In the **US group**, occlusion was monitored by laser Doppler and an excited ultrasound transducer placed over the uncovered hemispheres.
- In the **US control group**, occlusion was monitored by laser Doppler and an unexcited transducer placed over the uncovered hemispheres.
- In the **Method control group**, occlusion was verified by neurologic deficiency. Transducers were not used nor were the hemispheres uncovered.

Surgical procedure

Following anaesthesia, rats in the US and US control groups were placed in a head holder and a laser Doppler probe (Periflux System 5000, PF 5010, Perimed AB, Järfälla; Sweden) was placed near the coronal suture of the right parietal bone. This probe was used for monitoring the cerebral circulation and for verification of occlusion of the right middle cerebral artery. Next, the rats were placed in a prone position and an incision was made in the middle of the neck. The right common carotid artery was exposed by dissection and a suture was placed close to the

bifurcation of the right common carotid artery and the internal carotid artery. After ligating the external carotid artery, anticoagulant was injected and blood sampled. The distal suture around the right common carotid artery was ligated. A small incision was made close to the bifurcation of the right common carotid artery and internal carotid artery. A filament was then inserted 19 – 20 mm (in Method control group) or until flow on the laser Doppler dropped notably (in the US and US control groups) (Figure 3.11). During filament introduction, a small vascular clip was placed on the internal carotid artery to prevent bleeding. After insertion, the filament was ligated close to the bifurcation of the right common carotid artery to prevent its dislodgement and bleeding. This procedure is a modification of the intra-luminal filament model of right middle cerebral artery occlusion described by Koizumi and co-worker [136]. The filaments used in this study were made from fishing line (Stren Fishing Lines, Wilmington, DE). The tips were rounded by heating and the rounded tip-diameters ranged from 32.50 to 36.25 μm.

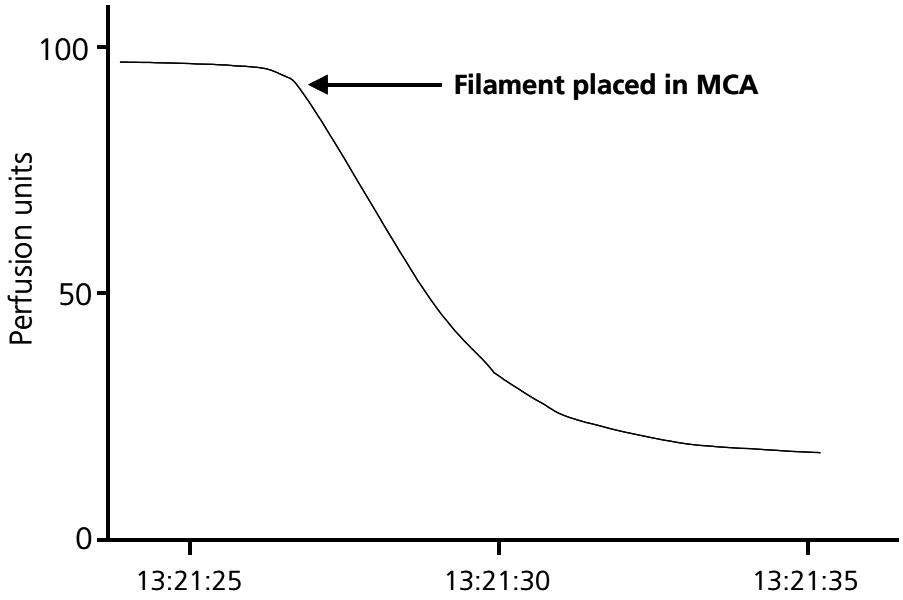

Figure 3.11. A laser Doppler measurement from one of the rats in the US group, illustrating the flow reduction induced by the filament.

After vascular occlusion, the neck wounds were sutured. Rats in the US and US control groups were then placed in the head holder, and two holes were drilled in the parietal bone between the coronal suture and the lambdoid sutures, uncovering both hemispheres. The holes had diameters of 5 mm and were separated by 7 mm. At this point, the US transducer was mounted 30 mm from the skull bone.

Rats in the Method control group were instead awakened after filament placement and placed in a recovery box. During the occlusion period, they were examined for signs of neurologic deficiency. The animals were assigned scores using a three-level scale modified from a previous study [141].

Following the 1.5 - hour of occlusion they were re-anaesthetised and the filament removed. They were then allowed to recover in the recovery box.
Of the 18 animals that underwent surgery using laser Doppler measurements, 12 were included in the study (6 in the US group and 6 in the US control group). Of the 12 animals in the Method control group that underwent surgery, six were included in the study. Causes of exclusion included bleeding complications arising while drilling the skull, in dissecting the right common carotid artery or if the vessel-rich glands on the neck were damaged. In the Method control group, only rats showing signs of neurologic deficiency were included. In the US and US control groups only rats with a substantial drop in the laser Doppler measurements were included (Figure 3.11). Rats in the US and US control groups were omitted from the behaviour test during occlusion because they were anaesthetised during this period.

Ultrasound exposure

During the 1.5-hour of right middle cerebral artery occlusion, rats in the US group were exposed to 1 hour of US of frequency 1 MHz and intensity 1 W/cm^2 SATA, with a pulse profile of 100 cycles repeated every millisecond. This is equivalent to a duty cycle of 10 %. In rats in the US control group, the transducers were placed in position but not excited. US transmission gel (Aquasonic 100, Parker laboratories Inc, Fairfield, NJ) covered the intervening gap between the skull bone and the transducer. The gel was kept in place by a piece of cardboard shaped into a cylinder. To avoid reflections from the operations table, rats were placed on US absorbing material (CERAM AB, Lund Sweden) (Figure 3.12).

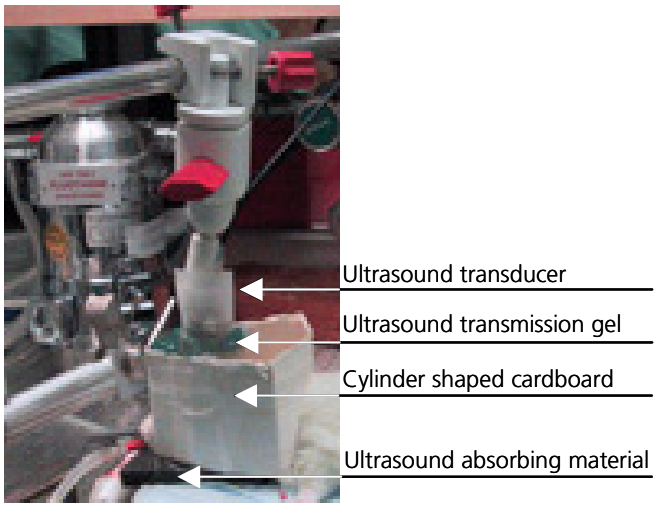

Figure 3.12. A photograph of the experimental set-up with the transducer placed in position. US transmission gel covers the intervening gap between the transducer and the skull bone. The transmission gel is kept in place by a piece of cardboard shaped into a cylinder. The rat is placed on ultrasound absorbing material.

Postoperative care

Rat body temperature was measured once every hour for at least 3 hours after recirculation. If, after right middle cerebral artery occlusion, the temperature rose above 38.5 °C, animals were placed in a cooling box at a temperature between 5 to 10 °C. Cooling was maintained for at least 2 hours after recirculation. All rats were examined for sign of neurologic deficit [141] after two hour of recirculation.

Evaluation of Brain damage by microscopy

Twenty-four hours after recirculation, rats were decapitated. The brains were quickly removed and chilled in ice-cooled 0.9 % NaCl solution for 10 minutes. The brains were then placed in a tissue slicer and sliced, starting from the frontal part of the brain. Twelve 1 mm thick slices were cut and stained with 2,3,5-Triphenyltetrazolium chloride. After staining, the slices were fixed in 4 % phosphate-buffered formalin solution as described previously [140]. This staining procedure a distinct red coloured stain in undamaged brain tissues, while the totally necrotic regions remain unstained and regions of neuronal necrosis show a mixed staining (referred to as light red). The fixed slices were digitised with a charge-coupled device video camera on monochrome film and the sizes of the unstained regions and of those with mixed staining were evaluated and measured using a video-analysing programme (NIH Image 1.61, National Institute of Health, Bethesda, MD, based on Adobe Photoshop, Adobe Systems Inc, San Jose, Ca, USA). The sizes of the infarcted regions were estimated as the total number of pixels in the infarcted regions. The numbers of pixels in a square graph paper of length 10 mm, cross-ruled in steps of 1 mm were measured in the same manner. In this way, the area of the infarcted regions for each slice could be calculated. The total volumes (V_{tot}) of the infarcted regions, the areas calculated as:

$$V_{tot} = \text{Area} \cdot 1 \cdot 12 \tag{3.8}$$

where Area is the number of pixels in a 1 mm cross-ruled graph paper, 1 is the thickness of the brain slices (mm) and 12 is the number of slices for each brain (Figure 3.13)

Measurements of sizes of infarcted regions were performed by the author (BMH) being unaware of whether the tissue had been exposed to US or not. This was achieved through renumbering of the initial randomisation. Since all brains contained a similar pattern of damage there were no ways of identifying results from particular groups.

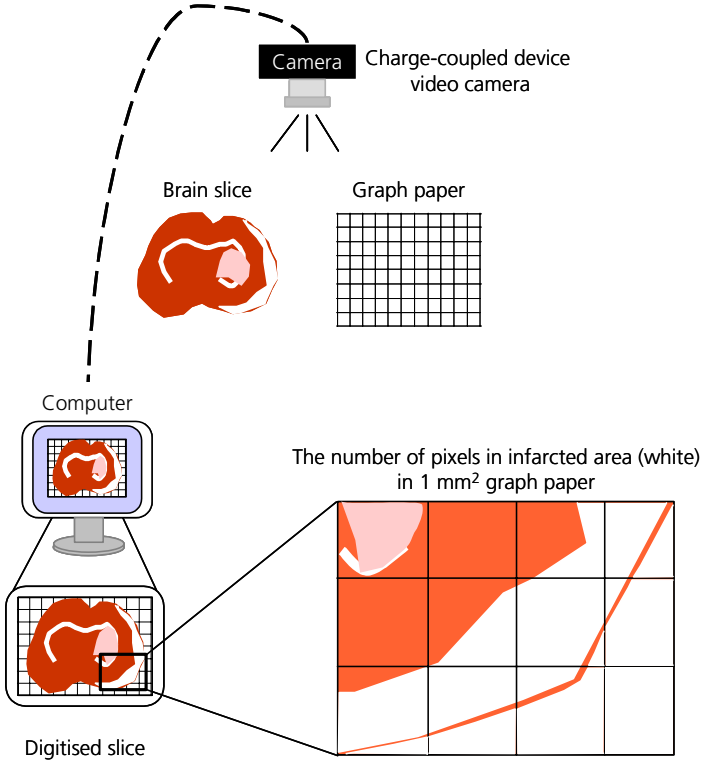

Figure 3.13. A schematic description of the method used for evaluation of brain damage (IV).

3.6 Statistical methods

In all statistical comparisons, P-values below 0.05 were considered to be significant.

- In the clot lysis experiments (I and II), the Wilcoxon paired signed rank test was used for making comparisons.
- In experiments exploring the effects of US exposure on the ischemic heart (III), the sums of the total damage scores of all groups were analysed using the Chi-squared test for goodness of fit to estimate the normal distribution.
- In the statistical analyses (III), the sums of the total damage scores were compared in two ways: effects in individual animals were compared using the paired Student's t-test while the Student's t-test was used in comparisons of groups.
- In the experiments on the effects of US exposure on the ischemic brain (IV), the Mann-Whitney U test was used to analyse statistical differences between pairs of groups.

3.7 Ethical considerations

All studies in this dissertation (I – IV) were scrutinised by an independent ethical board and approval granted as follows:

- The exploration of the mechanisms involved in US-enhanced fibrinolysis (I and II) was approved by the Regional Ethical Review Board in Lund (Approval 879/2004).
- The Ethical committee of Lund University (Approval M246/90) approved the experiments performed in studying the effects of US on the ischemic myocardium (III).
- The Ethical Committee of Lund University (Approval 1992-90) approved the measurements performed in exploring the effects of US on the ischemic brain (IV).

4 Results

4.1 Ultrasound calibrations and measurements

Transducer calibration done by electronic balance measurements

All transducers were calibrated at an intensity of 1 W/cm^2 SATA. The characteristics of the different transducers are presented in Table 4.1.

Frequency MHz	mg/1 W/cm^2	HP output voltage
0.5	214	247
1	138	130
2	89	100
3.5†	41	86
4†	41	56
4.5†	41	49
6.5	54	70

Table 4.1. Calibrated values of the transducers used in the measurements described in the text. The function generator output voltages shown are those required to excite the transducers to an acoustic force with a resulting weight (mg) corresponding to an intensity of 1 W/cm^2 SATA (†: The same transducer used but excited at different frequencies).

The validity of the calculation (Equation 3.4) was verified for all transducers. In Table 4.2 the measured values are compared with calculated ones, for the 1 MHz transducer.

Intensity W/cm^{2}SATA	Calculated value/mV	Measured value/mV
2	184	181
1	-	130
0.5	92	89
0.25	65	64

Table 4.2. Comparison between the calculated and measured calibration values using the 1 MHz transducer at different intensities. Differences were shown to be small for all transducers, therefore calculated values was allowed to be used.

Ultrasound measurements in the Sonazoid® microbubble experiments

The calibrated membrane hydrophone measurments showed that the needle hydrophone yielded values that sometimes was too high and sometimes too low. This resulted in corrections values for different frequencies, these are listed in Table 4.3.

Frequency MHz	Correction-value
0.5	1.14
1	0.82
2	1.35
3.5	1.82
4	2.24
4.5	2.07
6.5	0.69

Table 4.3. Correction values taken with the membrane hydrophone, for use in adjusting faulty values in the needle hydrophone measurements.

The changes induced in the effects of US by passage of the front-wall of the cylindrical plastic tube varied with frequency and intensity as well as with the numbers of pulses transmitted. The values of all measured variables after correction using results from the calibration measurements (Table 4.3) are presented in Table 4.4.

Frequency MHz	Number of pulses	P- with plastic tube /kPa	P- without plastic tube /kPa	MI With plastic tube	MI Without plastic tube
0.5	10	262	257	0.37	0.36
0.5	5	256	245	0.36	0.35
0.5	2	213	225	0.30	0.32
1	10	343	407	0.34	0.41
1	5	405	377	0.41	0.38
1	2	248	248	0.25	0.25
2	10	561	361	0.40	0.26
3.5	10	362	327	0.19	0.17
4	10	404	340	0.20	0.17
4	5	321	342	0.16	0.17
4.5	10	361	307	0.17	0.14
6.5	10	111	146	0.04	0.06

Table 4.4. Needle hydrophone measurements performed at various frequencies at an intensity of 1 W/cm^2 SATA. Corrected values of peak negative pressures and the resulting attenuations or increases of intensity depending on the cylindrical plastic tubes used are shown. The table also shows the calculated Mechanical Index (MI) with and without the plastic tubes present.

Ultrasound measurements in the ischemic heart experiments

In measurements with the membrane hydrophone, the SPTA was measured to be 460 mW/cm^2 and the Mechanical Index calculated [122] to be 0.41 at distances of 3.0 - 3.5 cm from the transducer surface. At the same distance the peak

compressional pressure was measured to be 0.41 MPa and the peak rarefactional pressure 0.41 MPa.

Needle hydrophone explorations of the field distribution for a transducer similar to that used in III were performed in degassed water, but exact values of intensity were not measured. The field distribution is shown in Figure 4.1.

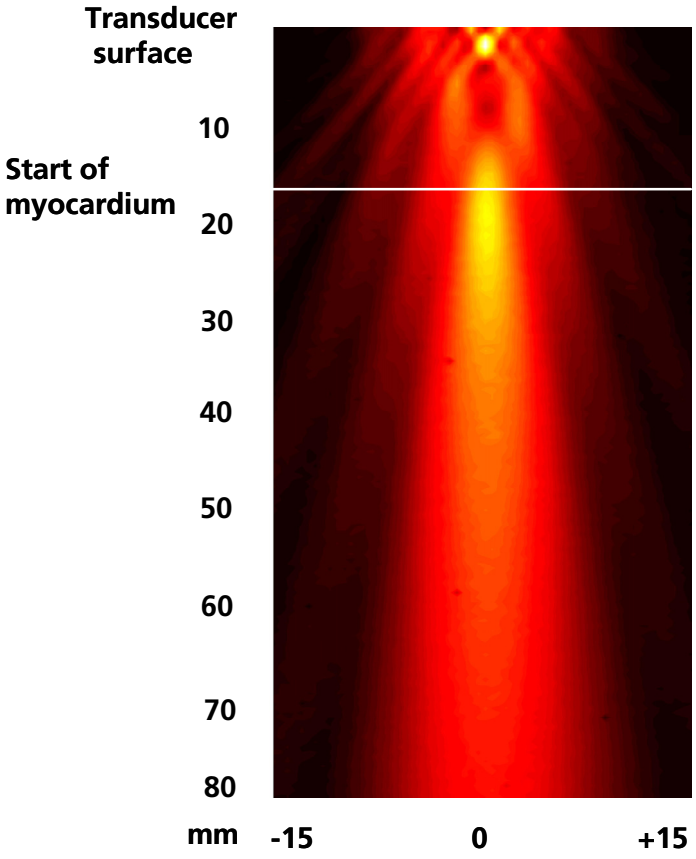

Figure 4.1. The field distribution for a transducer similar to that used in III is shown, but no exact values of intensity were measured. Scanning was performed over an area of 80×30 mm^2 in the y and z-directions starting close to the transducer surface. The transducer surface and the start of myocardium are shown.

During the first 20 minutes of exposing extracted myocardial muscle to pulsed US an increase in the temperature difference between the (non-circulated) pig myocardium and the surrounding water bath was observed. After 20 min of exposure the temperature reached a steady state difference of 0.5 °C (Table 4.5).

Time intervals	Water bath mean ± SD	Heart muscle mean ± SD	Difference in temperature
5 min steady state	36.8 ± 0.12	36.8 ± 0.03	0.0
During first 2 min of US exp.	36.7 ± 0.12	36.9 ± 0.05	0.2
During first 5 min of US exp	36.8 ± 0.12	37.0 ± 0.07	0.2
During first 10 min of US exp	36.8 ± 0.12	37.1 ± 0.08	0.3
10-20 min of US exp	36.7 ± 0.11	37.2 ± 0.03	0.4
20-40 min of US exp	36.7 ± 0.10	37.2 ± 0.03	0.5
40-60 min of US exp	36.7 ± 0.10	37.1 ± 0.03	0.5
20 min after US termination (2 min)	36.6 ± 0.12	36.7 ± 0.03	0.1

Table 4.5. Temperature increases in non-circulating myocardial muscle during ultrasound exposure as a function of time. The temperature was measured in intervals of 0.5 seconds during 1 hour and 27 min. The temperature in different time intervals is presented as mean ± SD °C. The calculated difference in temperature between the exposed myocardium and the surrounding water bath is also shown.

Ultrasound measurements in the ischemic brain model

In measurements with hydrophones, the US field distributions in water and the changes in field distribution after traversing the skull bone were determined. Exact values of intensity were not measured. The measurements showed that the transversal of skull bone caused a significant decrease in US intensity, spots of higher intensity in the US field being observed in areas a short distance from the skull bone. The distributions along different axes are shown in Figure 4.2.

Balance measurements of the intensity loss, yielded values of 2 W in the absence of skull bone and 0.99 W with it present. With pulsed-wave US, the reductions were in the same proportions: 0.19 W with bone and 0.09 W without.

In the measurements of the intensity lost traversing the intact skull, a peak-to-peak voltage reduction of approximately 90 % of the original value was noted (from 13.4 to 1.15 V).

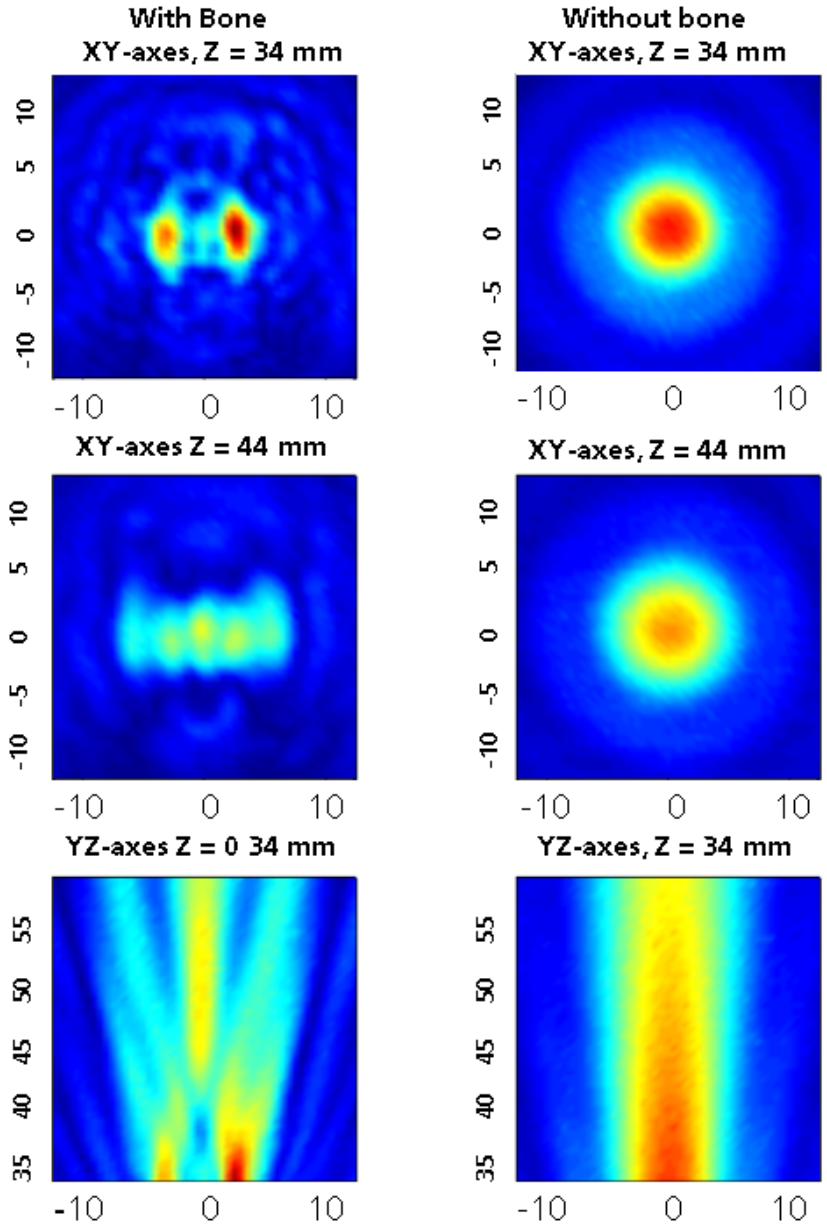

Figure 4.2. Three pairs of measurements illustrating the ultrasound field distribution where the right column shows distribution with skull bone and the left column shows distribution without skull bone. Exact values of intensity were not measured.

4.2 Ultrasound-enhanced fibrinolysis

Control measurements of spontaneous streptokinase and reteplase effects

The dose response measurements showed an increasing activity of r-PA using the concentrations, 0.01562, 0.03125, 0.0625 and 0.125 U/160 ml in 0.9 % NaCl solution. However, at the higher concentrations (>0.125 U/160 ml) the fibrinolytic activity was more stable. Therefore the concentration 0.250 U/160 ml (0.001562/ml) was used in all measurements of the fibrinolytic action of r-Pa (II) (Table 4.6).

Reteplase concentration U/160 ml	**$CRHb_{1h}$**
0.01562	16 ±3.5
0.03125	22 ±3.2
0.0625	31 ±6.2
0.125	42 ±6.3
0.25	46 ±12.1
0.5	52 ±23.5

Table 4.6. Dose response measurements for reteplase at different concentrations (U/160 ml), during one hour exposure. Data is presented as mean ±SD of $CRHb_{1h}$.

In all experiments, clot lysis evaluation showed an initial rapid release of Hb during the first 20 min (irrespectively if clots were exposed to thrombolytic drugs, thrombolytic drugs and US or 0.9 % NaCl solution) this was followed by a slower more stable release (Figure 4.3) Therefore, $CRHb_{1h}$, was used as a measure of fibrinolytic activity.

The evaluation of intrinsic SK effects in our model showed that after exposing clots for 1 hour to 0.9 % NaCl solution, the $CRHb_{1h}$ level reached 8 ±4 % (mean ± SD). In SK solution exposed simultaneously, the $CRHb_{1h}$ level reached 28 ±8%, corresponding to a mean relative change in clot lysis by about 250 % (P = 0.005) (Figure 4.3).

When intrinsic r-PA effects were measured, measurements showed that after exposing control clots for 1 hour to 0.9 % NaCl solution the $CRHb_{1h}$ level reached 19 ±2.8 %. In the r-PA solution exposed simultaneously, the $CRHb_{1h}$ level reached 36 ±7.6 %, corresponding to a relative change of about 90 % in clot lysis (P = 0.028) (Figure 4.3).

Inter- and intra-individual response to reteplase and streptokinase

Following 1 hour of r-PA exposure, inter-individual $CRHb_{1h}$ levels ranged from 18 % to 47 % (median: 31 %) while intra-individual $CRHb_{1h}$ levels ranged from 2 % to 48 % (median: 9 %). (Figure 4.4)

In evaluations of the inter-individual response to SK, results for the 15 individuals showed variations in $CRHb_{1h}$ levels from 14 % to 77 % (median: 28 %), while intra-individual $CRHb_{1h}$ levels ranged from 6 % to 29 % (Median: 16 %) (Figure 4.5 and Figure 4.6).

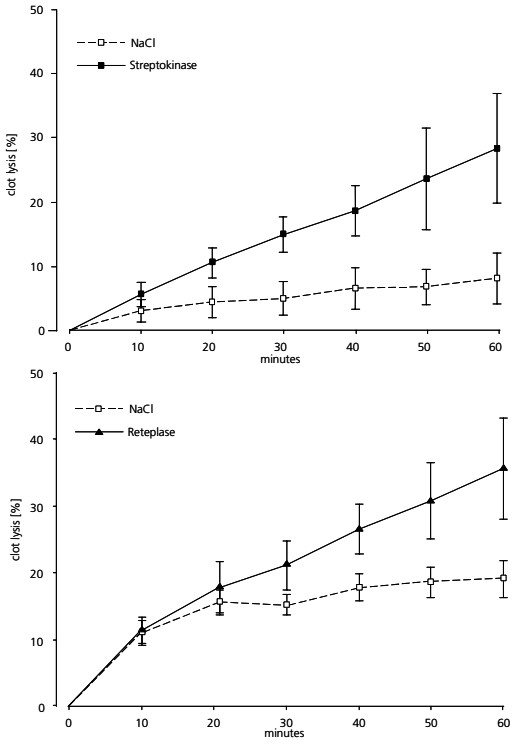

Figure 4.3. The upper figure shows the development of spontaneous clot lysis in, □: clots exposed to 0.9 % NaCl solution, and ■: clots exposed to streptokinase solution, during 1 hour. The lower figure shows the development of spontaneous clot lysis during 1 hour and the effect of exposure to reteplase solution during the same period. □: clots exposed to 0.9 % NaCl solution, ▲: clots exposed to reteplase solution. Data is presented as mean ±SD.

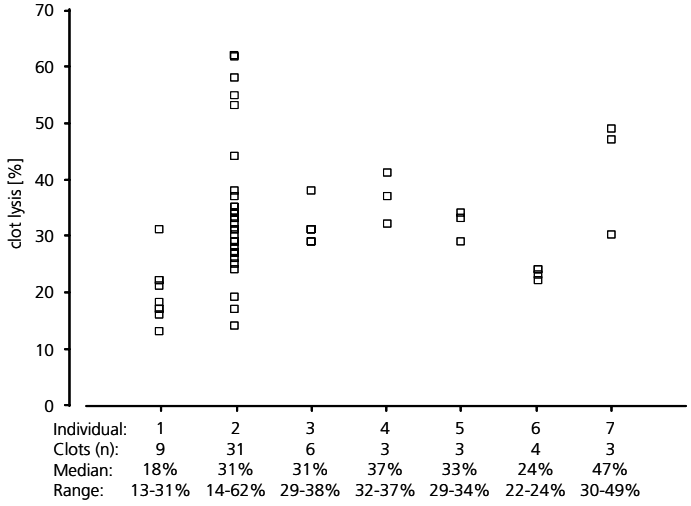

Figure 4.4. Intra- and inter-individual values of clot lysis following one hour of exposure of reteplase solution at a concentration of 0.001562 U/ml.

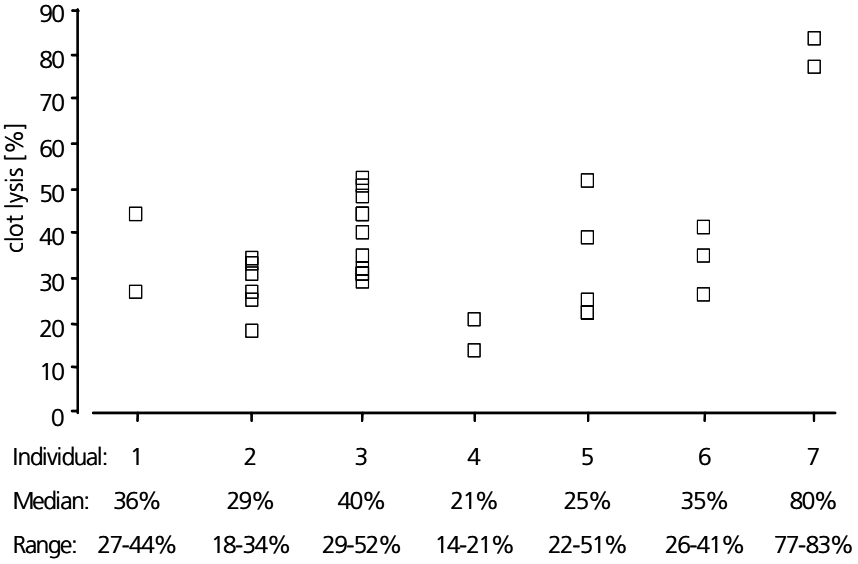

Figure 4.5. Intra-individual clot lysis following one hour exposure of streptokinase solution at a concentration of 22.5 IU/ml.

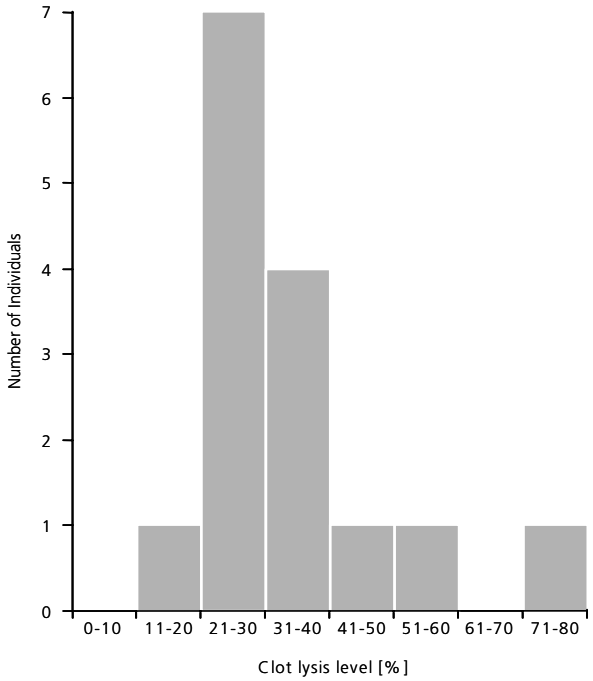

Figure 4.6. Inter-individual variability in response for 15 healthy volunteers to streptokinase treatment (22.5 IU/ml).

Following cold storage, the fibrinolytic effects of SK exposure resulted in significantly decreasing $CRHb_{1h}$ levels, from 31 ± 10 % to 27 ± 6 % between Days 1 and 2 (P = 0.041). From Day 2 to Day 8 no significant changes in fibrinolytic levels could be seen. However, $CRHb_{1h}$ levels increased during Day 8°-°15. During the last two weeks of follow-up no significant changes in the fibrinolytic effects could be seen (Figure 4.7) [142].

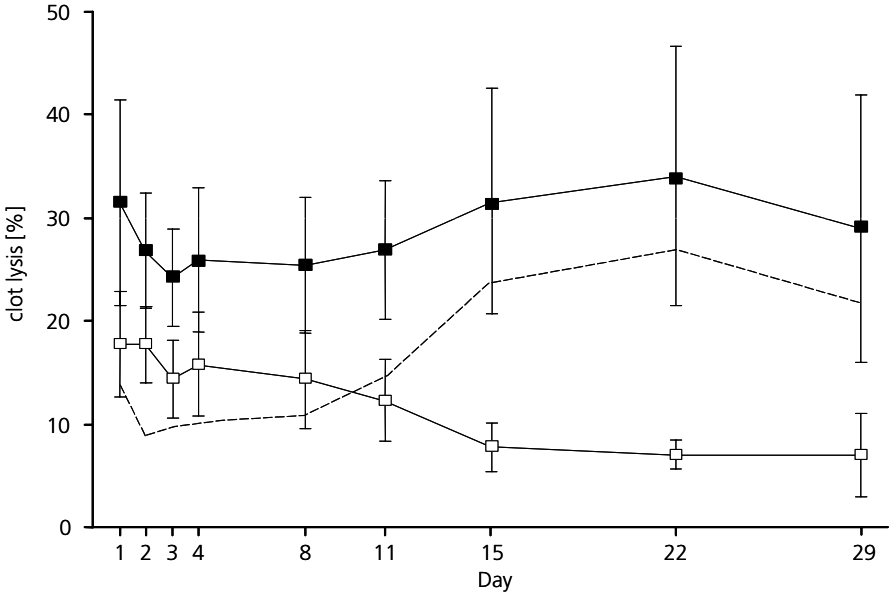

Figure 4.7. The changes in fibrinolytic effects of streptokinase induced by 4 weeks of cold storage. Note the spontaneous clot lysis in 0.9 % NaCl solution that decreased during follow up From Day 1 to 15, clots were made of blood from 12 volunteers. On day 22, clots could only be made of blood from 8 of the volunteers and on day 29 from 3 of them. ■: streptokinase treated clots, □: Clots exposed to 0.9 % NaCl solution and ---: absolute difference in clot lysis.

Effects of ultrasound on other blood components

The fibrinolytic effects from concomitant exposure of SK and US at an intensity of 0.5 W/cm^2 SATA was increased by 25 % compared to SK exposure alone. Following exposure of clots to SK the degree of haemolysis in the supernatant fluid was minimal ($CRHb_{1h}$ = 0.9 ±0.7 %). Exposure of SK treated clots to US (intensity 0.5 W/cm^2 SATA) did not increase haemolysis ($CRHb_{1h}$ 1.1 ± 0.7 %, P = 0.334) (Figure 4.8).

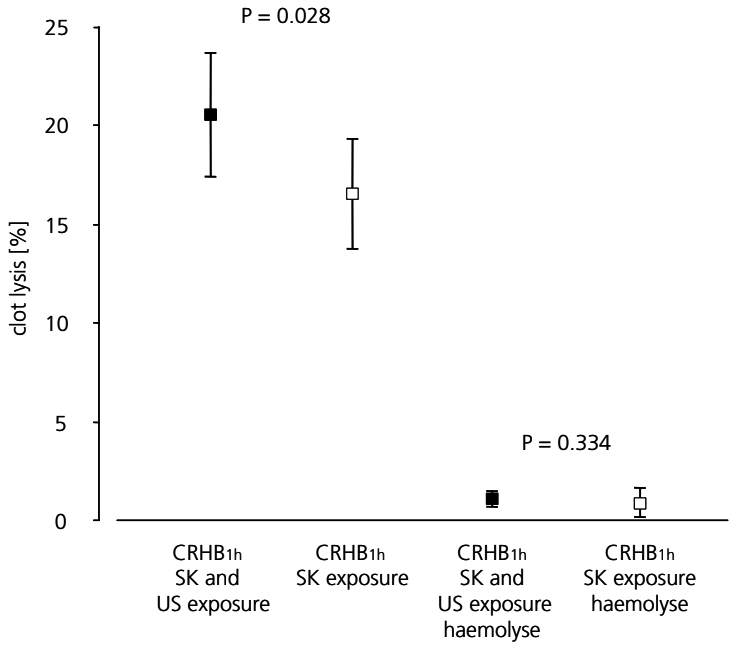

Figure 4.8. Analyses of haemolysis effects from pulsed ultrasound during streptokinase treatment. The applied ultrasound was at frequency 1 MHz and an intensity of 0.5 W/cm^2 SATA (10 % duty cycle). Data is presented as mean ±SD.

During the measurements of the effects on platelet aggregation the intensity used was 1 W/cm^2 SATA. After corrections for loss of intensity due to attenuation induced by the cylindrical plastic tubes, the intensity was corrected to 0.7 W/cm^2 SATA (Section 4.1).

No increased platelet aggregation in blood exposed to pulsed US of frequency 1 MHz at the above-described intensity could be noted (Figure 4.9).

Fibrinolytic effects of exposure to ultrasound alone

At the intensities of 0.125, 0.5 or 1 W/cm^2 SATA, no fibrinolytic effects due to exposure to pulsed US alone could be detected. However, a small but significant enhancement of fibrinolytic levels was observed following exposure to intensity 4 W/cm^2 SATA, where CRHb1h increased from 12 ±5 % in unexposed clots to 15 ±6 % in US exposed ones (P = 0.008).

In the evaluation of possible plasmin activation following US exposure, it was noted that during the first hour of evaluation, the CRHb1h increased from 13 ±5 % in unexposed clots to 18 ±5 % in exposed ones (P = 0.028). However, in the second hour, no increase in fibrinolytic levels could be detected (Figure 4.10).

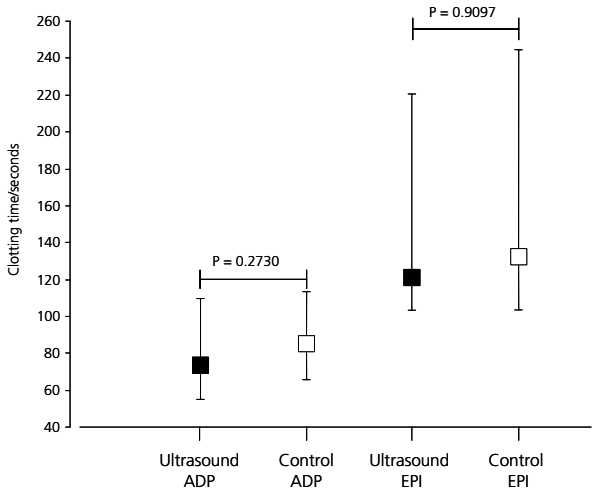

Figure 4.9. Comparison of mean clotting-times for ultrasound treated blood and control blood. Platelets were stimulated using either adenosine 5'-diphosphate (ADP) or epinephrine bitartrate (EPI). Data is presented as mean ±SD.

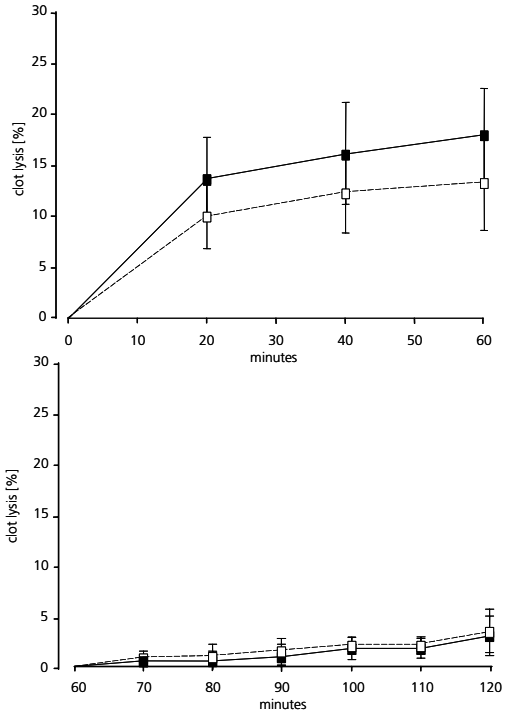

Figure 4.10. Development of clot lysis over a two-hour period. The upper figure shows haemoglobin release during the first hour while the lower figure shows the development of lysis during the second hour without ultrasound exposure. □: clots not exposed to ultrasound during the initial hour, ■: clots exposed to ultrasound (intensity 4 W/cm^2 SATA) during the first hour. Data is presented as mean ±SD.

Effects of different ultrasound exposure settings on streptokinase-induced fibrinolysis.

At intensity levels of 0.25 and 0.5 W/cm^2 SATA, increased fibrinolytic activity was seen in clots exposed to SK and concomitant pulsed US. At intensities 0.0625, 0.125, 0.75 and 1 W/cm^2 SATA, no increase in $CRHb_{1h}$ levels indicative of enhanced fibrinolytic activity could be seen. Following pulsed exposure to US of intensities 2 and 4 W/cm^2 SATA, decreased $CRHb_{1h}$ levels were seen (Table 4.7, Figure 4.11).

Effects of streptokinase and concomitant ultrasound exposure on two-hour-old clots

In the measurements in which clots were left to coagulate for two hours, a mean relative increase of 39 % (P = 0.028) in fibrinolytic activity was seen in clots exposed concomitantly to SK solution and US of intensity 0.5 W/cm^2 SATA. $CRHb_{1h}$ was 24 ±7 % in exposed clots and an $CRHb_{1h}$ level of 18 ±5 % in unexposed ones (Figure 4.12).

Intensity level W/cm^2 SATA	**$CRHb_{1h}$ in US treated clots**	**$CRHb_{1h}$ in Control clots**	**Δ lysis %**	**P-values**
0.0625	31 ±9	30 ±10	5	0.916
0.125	41 ±9	43 ±12	1	0.600
0.25	43 ±9	37 ± 6	16	0.028
0.5	48 ±9	38 ± 9	31	0.028
0.75	52 ±8	50 ± 6	5	0.600
1	46 ±7	49 ±19	1	0.916
2	17 ±1	21 ± 2	-17	0.028
4	32 ±8	44 ±12	-25	0.028

Table 4.7. Relative clot lysis expressed as cumulated release of haemoglobin during 1 h ($CRHb_{1h}$) during streptokinase exposure with different intensities of concomitant 1 MHz pulsed ultrasound. The effect on clot lysis shown as the percentual difference in mean for each group of clots. Δ lysis: the relative change in clot lysis. Wilcoxon paired sign rank test has been used in tests of significance.

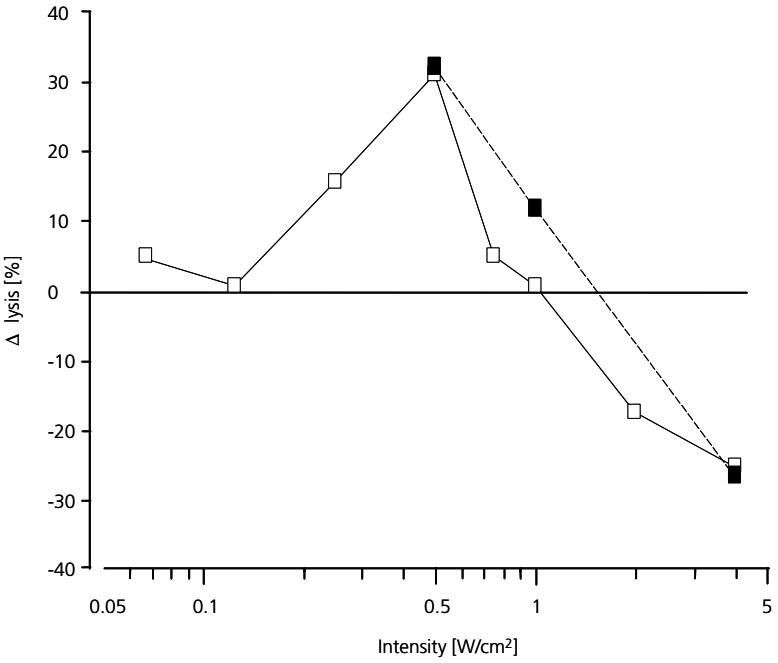

Figure 4.11. Results of comparison of the additive effects of different intensities of ultrasound on the fibrinolytic properties of streptokinase, estimated as the cumulated release of haemoglobin during one hour shown as the mean differences between target clots and control clots. □: clots exposed to streptokinase solution and concomitant ultrasound ■: clots exposed to streptokinase solution earlier exposed to ultrasound.

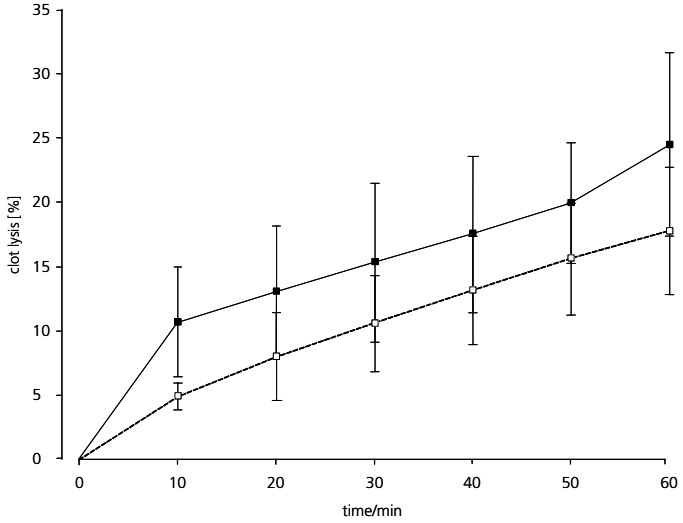

Figure 4.12. Haemoglobin release in two-hour-old clots. □: clots exposed to streptokinase solution, ■: clots exposed concomitantly to streptokinase solution and ultrasound at intensity 0.5 W/cm^2 SATA. Data is presented as mean ±SD.

Effects of streptokinase and prolonged concomitant ultrasound exposure

In evaluating the effects of prolonged exposure period, the small number of measurements made precluded statistical comparisons. The amount of fluid that evaporated during the 24 hours of exposure was 40 ±6 ml (mean ±SD). This loss was compensated for in calculations of clot lysis using the Equation 3.6. During the first 12 hours of exposure to US, the increase in fibrinolytic activity was not greater than that noted in the first hour. In the time-range 12 - 24 hours, an increasing fibrinolytic activity was seen when comparing the fibrinolytic levels in clots exposed to US with levels in unexposed clots (Table 4.8).

Effect of pre-exposing streptokinase to ultrasound

After pre-exposing SK for one hour with pulsed US of intensity 0.5 W/cm^2 SATA a relative increase of 33 % in fibrinolytic effects was noted. The $CRHb_{1h}$ level reached 27 ± 12 % in clots exposed to pre-exposing SK solution compared to $CRHb_{1h}$ levels of 21 ± 8 % in clots exposed to unexposed SK solution. When the effects of SK solution pre-exposed to pulsed US of intensity 1 W/cm^2 SATA were compared to those of unexposed SK, no enhancement of fibrinolytic activity was seen. The mean $CRHb_{1h}$ level following pre-exposure to pulsed US of intensity 4 W/cm^2 SATA was 27 ±4 % compared to 36 ±7 % in unexposed SK solution, a relative decrease of 25 % (P = 0.002) (Figure 4.11).

Exposure period	Clot lysis in US treated clots	Clot lysis in Control clots	Δ lysis %
$CRHb_{1h}$	31 ±12	23 ± 8	31
$CRHb_{2h}$	37 ±11	28 ± 8	32
$CRHb_{3h}$	46 ±13	33 ±13	41
$CRHb_{4h}$	48 ±16	36 ±12	34
$CRHb_{5h}$	50 ±16	38 ±16	34
$CRHb_{6h}$	54 ±13	41 ±13	36
$CRHb_{12h}$	51 ± 9	41 ± 6	23
$CRHb_{24h}$	61 ± 5	40 ± 8	54

Table 4.8. Relative clot lysis expressed as increased cumulative release of haemoglobin during 24 hours exposure to streptokinase solution and pulsed ultrasound of frequency 1 MHz and intensity 0.5 W/cm^2 SATA. The effect on clot lysis is shown as the percentual change in the mean for each group of clots. Δ lysis: relative change of clot lysis.

Effects of reteplase at different ultrasound exposure settings.

In the intensity-ranges 0.125 - 0.25 and 2 – 4 W/cm^2 SATA, significant enhancements of clot lysis in clots treated with r-PA and concomitant exposures to pulsed US were observed, but such enhancement was absent below intensity 0.125 W/cm^2 SATA as well as in the intensity interval 0.5 - 1 W/cm^2 SATA (Table 4.9 and Figure 4.13).

Intensity level W/cm² SATA	CRHb1h in US treated clots	CRHb1h In Control clots	Δ lysis %	P-values
0.0625	23.0 ±4.3	22.4 ±6.3	5	0.600
0.125	36.9 ±4.4	32.7 ±1.9	13	0.028
0.25	34.0 ±6.3	32.3 ±5.7	5	0.028
0.5	29.9 ±5.1	29.3 ±7.2	5	0.753
0.75	27.5 ±6.0	25.9 ±4.4	8	0.600
1	33.2 ±5.3	31.9 ±4.2	4	0.600
2	25.8 ±2.7	20.3 ±3.5	28	0.028
4	32.0 ±11.4	24.0 ±8.5	33	0.028

Table 4.9. Relative clot lysis expressed as increased cumulative release of haemoglobin during 1 h ($CRHb_{1h}$) of concomitant exposure to reteplase solution and different intensities of pulsed ultrasound of frequency 1 MHz. The effect on clot lysis is given as percentual change in the mean for each group of clots. Δ lysis: relative change of clot lysis.

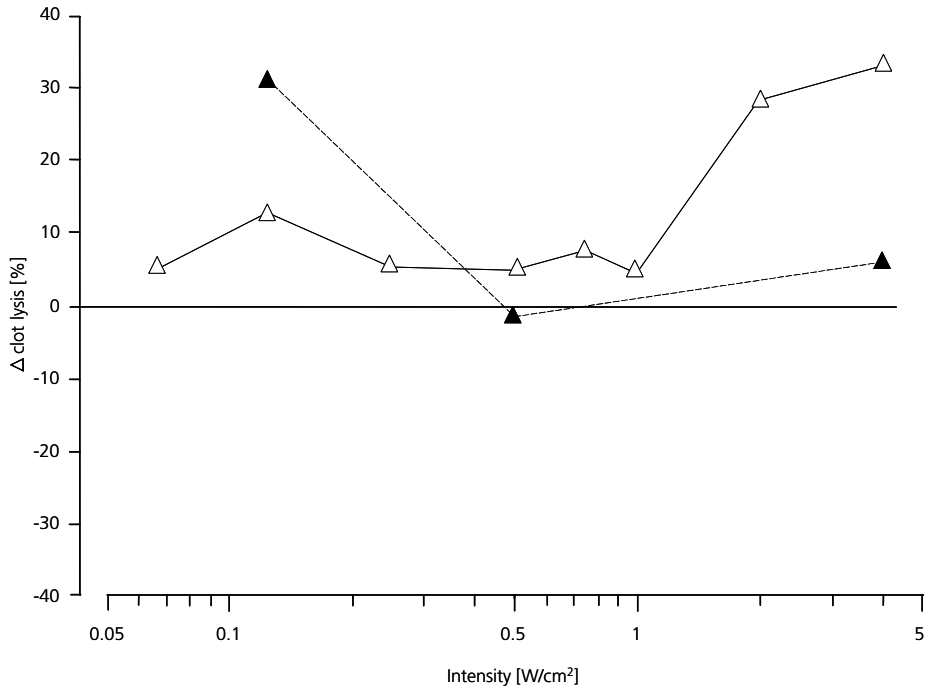

Figure 4.13. Relative changes in clot lysis levels estimated as the cumulated release of Hb during one hour shown as the mean difference between target clots and control clots caused by exposure to 1 MHz pulsed ultrasound of different intensities. Δ: clots exposed to reteplase solution with concomitant ultrasound, ▲: clots exposed to reteplase solution pre- exposed to ultrasound.

Effect of pre-exposing reteplase to ultrasound

After one hour of exposure, clots in r-PA solution which earlier had been exposed to US showed a significantly increased $CRHb_{1h}$ level at an US intensity level of 0.125 W/cm^2 SATA but not at intensities 0.5 or 4 W/cm^2 SATA (Table 4.10 and Figure 4.13).

Investigated condition	$CRHb_{1h}$ US exposure	$CRHb_{1h}$ Control	Δ lysis %	P-values
US pre-exposure of r-PAs				
0.125 W/cm^2 pre-exp. of r-PAs	47 ±10	36 ± 6	31	0.002
0.5 W/cm^2 pre-exp. of r-PAs	57 ±14	58 ±13	-1	0.346
4 W/cm^2 pre-exp. of r-PAs	48 ±11	46 ±12	6	0.388
US pre-exposure of clot				
4 W/cm^2 pre-exp. of clots in 0.9% NaCl	22 ± 4	18 ± 4	25	0.004
r-PAs effect following US pre-exp. of clot	32 ±10	21 ± 5	58	0.002
US pre-exp. of clot vs. concomitant US exp.				
4 W/cm^2 pre-exp. of clots in 0.9% NaCl	16 ± 2	14 ± 2	14	0.002
Effects of r-PAs in pre-exp vs. conc. exposed clots	20 ± 9	18 ± 7	10	0.002

Table 4.10. Relative clot lysis expressed as increased cumulated release of haemoglobin during 1 h ($CRHb_{1h}$) at different intensities of 1 MHz pulsed ultrasound in various settings of pre-exposure. In all comparisons, reteplase solution was used as thrombolytic drug. The effect on clot lysis is given as the percentual change in the mean for each group of clots. Δ lysis: relative change of clot lysis. r-PAs: reteplase solution, exp: exposure, conc: concomitant.

Effect of reteplase on clots pre-exposed to ultrasound

Clots in 0.9 % NaCl solution exposed for one hour to pulsed US of intensity 4 W/cm^2 SATA, showed a slight but significant increase in lysis. $CRHb_{1h}$ increased from 18 ±4 % in unexposed clots to 22 ±4 % in exposed ones (P = 0.004), confirming the enhanced fibrinolytic effects following exposure to US of intensity 4 W/cm^2 SATA observed in Figure 4.10. After terminating US exposure and following the addition of r-PA solution to the clot containers, a further marked increase in $CRHb_{1h}$ was noted in clots pre-exposed to US (Table 4.10 and Figure 4.14).

Comparison of the effects of reteplase on ultrasound pre-exposed clots and concomitant exposed clots

During the first hour of exposure of clots in 0.9 % NaCl solution to pulsed US of intensity 4 W/cm^2 SATA, increased clot lysis from, $CRHb_{1h}$, 14 ±2 % in unexposed clots to 16 ±2 % in ones exposed to US was seen (P = 0.002). The position of the clot containers were then switched so that clots earlier not exposed to US were now in front of the transducer being exposed to US. r-PA solution was added to both clot containers. Evaluation was made between pre-exposed clots and concomitantly exposed ones during the second hour. The latter reached a mean fibrinolytic level, $CRHb_{1h}$, of 18 ±7 % compared to $CRHb_{1h}$ levels of 20 ±9 % in pre-exposed by

US, corresponding to a small but significant relative increase of 10 % in pre-exposed clots (Table 4.10 and Figure 4.15).

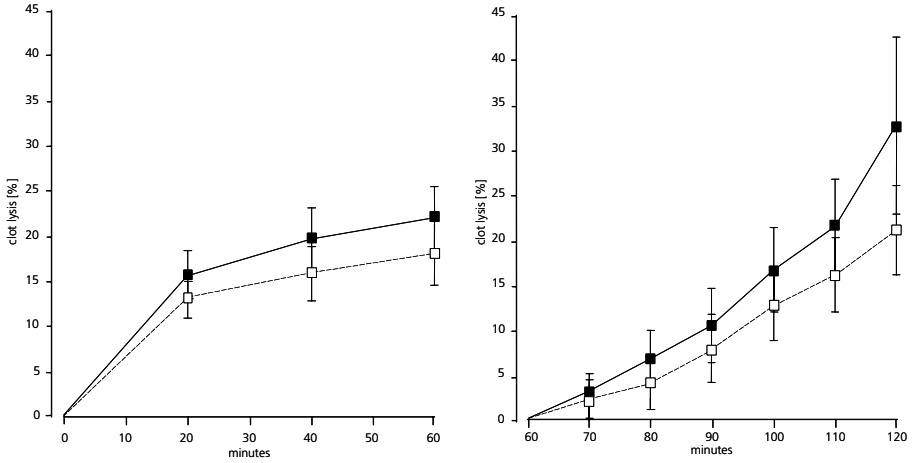

Figure 4.14. Development of clot lysis during two hours. The left-hand figure shows haemoglobin release during the first hour: ■: clots not exposed to ultrasound, □: clots exposed to ultrasound. The right-hand figure shows how the lysis develops during the following 60 minutes without ultrasound exposure, using the 60 minute lysis levels as baseline: □: clots not earlier exposed to ultrasound, ■: clots exposed to ultrasound during the first hour. Data is presented as mean ± SD.

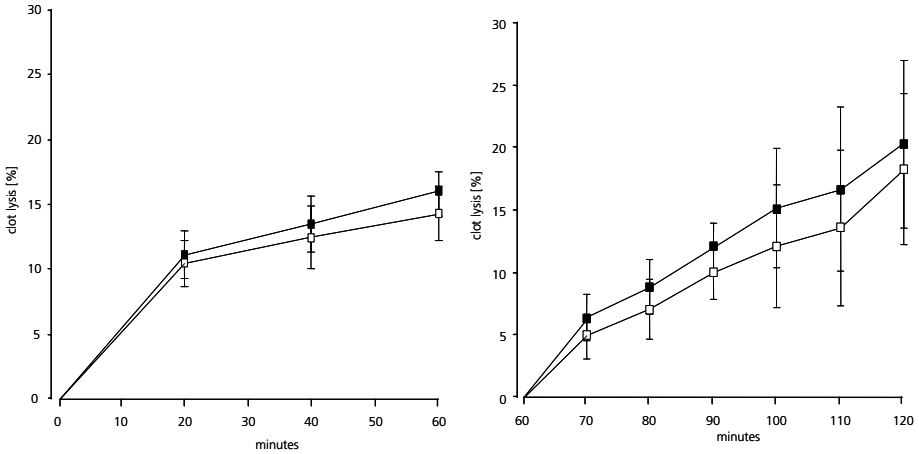

Figure 4.15. illustration of development of clot lysis during two hours. Left-hand figure shows haemoglobin release during the first hour: □: clots not exposed to ultrasound during the initial hour, ■: clots exposed to ultrasound during the first hour. The right-hand figure shows development of during the second hour, using the 60 minutes lysis levels as baseline: ■: reteplase effects in clots earlier exposed to ultrasound, □: clots concomitantly exposed to ultrasound and reteplase solution. Data is presented as mean ± SD.

4.3 Bubble destruction results

Effects on Sonazoid® microbubbles destruction-rate of different ultrasound frequencies at intensity 1 W/cm² SATA

The slowest rate of MB destruction (1 h 43 min) was observed at frequency 4 MHz. In the lower frequency range (0.5 – 2 MHz), a much faster rate of destruction was seen, at 0.5 MHz (1 min 41 s) and 1 MHz (1 min 42 s) while at 2 MHz the time for reaching 50 % destruction was 14 min 11 s. At frequencies 3.5, 4.5 and 6.5 MHz, destruction-rates were increased compared to frequency 4 MHz (3.5 MHz = 1 h 34 min, 4.5 MHz = 59 min and 6.5 MHz = 1 h 25 min)(Figure 4.16).

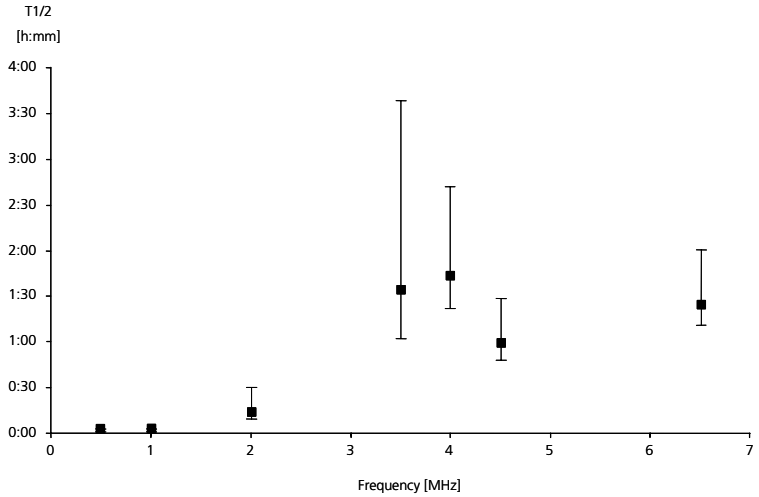

Figure 4.16. The effects of different frequencies of ultrasound (10 % duty cycle) at intensity 1 W/cm² SATA on Sonazoid® microbubbles destruction-rates. Data is presented as the time when 50 % of them had been destroyed. The error-bars show the 95 % confidence interval.

Sonazoid® microbubbles destruction-rate as function of ultrasound intensity

The effect of US on MB destruction-rates was analysed as a function of intensity at frequencies of 0.5, 1 and 4 MHz. At 0.5 MHz, the effects were measured at four intensity levels. At intensities of 0.5 W/cm² SATA and higher, high rates of MB destruction ranging from 56 s to 3 min 48 s were noted. At intensity 0.25 W/cm² SATA the time at which 50 % of the MB were destroyed was reached after 53 min. At 1 MHz, measurements made at two intensity levels (1 and 2 W/cm² SATA) yielded values of 1 min 42 s and 1 min 17 s respectively. At frequency 4 MHz the 50 % destruction-level being reached at 36 min, 1 h 59 min, 1 h 43 min and 9 h 24 min at intensities 2, 1.5, 1 and 0.5 W/cm² SATA respectively (Figure 4.17).

Sonazoid® microbubbles destruction-rate as a function of the number of pulses

MB destruction-rate as a function of the number of sent pulses was analysed at frequencies 0.5 and 1 MHz and intensity 1 W/cm^2 SATA. At 1 MHz 50 % of the MB were destroyed at 13 min 31 s using 2 pulses/ms, at 5 min 3 s using 5 pulses/ms and at 1 min 42 s using 10 pulses/ms. At frequency 0.5 MHz, the corresponding times were 4 min 34 s using 2 pulses/2 ms, 1 min 9 s using 5 pulses/2 ms and at 1 min 17 s using 10 pulses/2 ms (Figure 4.18).

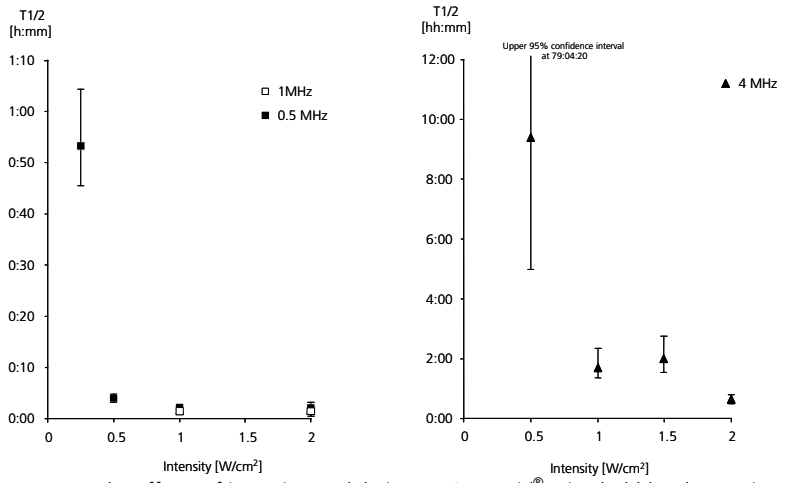

Figure 4.17. The effects of intensity modulation on Sonazoid® microbubbles destruction-rate at frequencies 0.5, 1 and 4 MHz. Data is presented as the time when 50 % of them had been destroyed. The error-bars show the 95 % confidence interval.

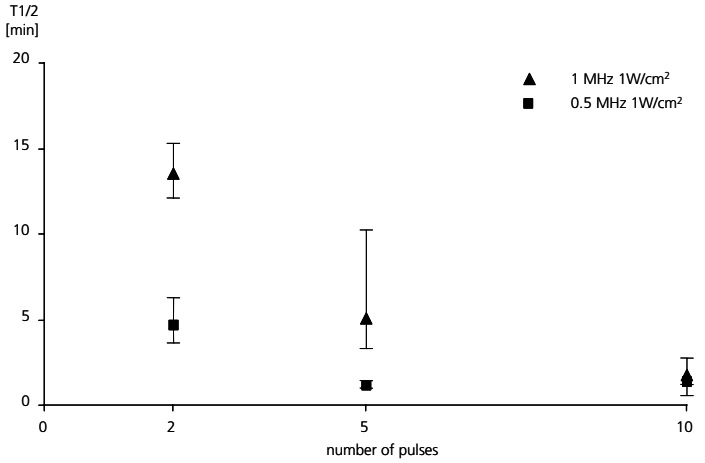

Figure 4.18. The effects on Sonazoid® microbubbles destruction-rate of modulating the number of pulse cycles at intensity 1 W/cm^2 SATA and frequencies 0.5 and 1 MHz. Data is presented as the time when 50 % of them had been destroyed. The error-bars show the 95 % confidence interval.

4.4 Evaluation of possible non-beneficial effects of ultrasound exposure

Ultrasound and its effects on myocardial tissue

During the experiments, all animals were stable as regards circulation and were under the supervision of both anaesthesia and cardiology expertise throughout the measurement-periods. Estimates of the size of infarcted areas are shown in Figure 3.10. No exact measurements of the sizes of infarcted areas were, however, performed.

Histopathological evaluation

Examples of the grade scores used in assessing tissue damage are shown in Figure 4.19.

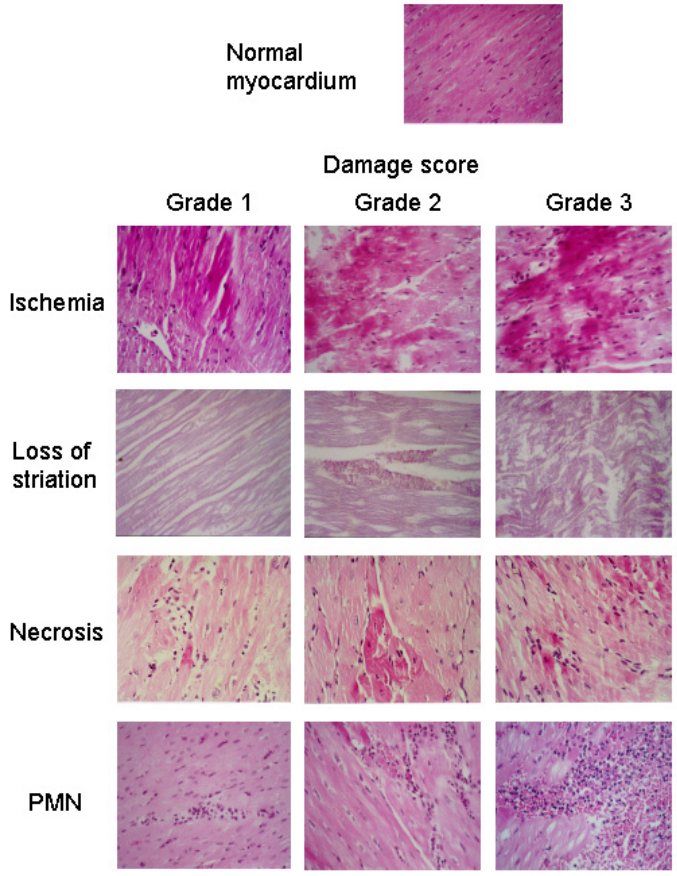

Figure 4.19. Examples of the scores of the various histopathological indicators used in assessing tissue damage: Ischemia: Eosinophilic changes in the myocyte, Loss of striation: Reduction and loss of cross striation, Necrosis: Coagulation necrosis, PMN: Infiltration of poly-morphonuclear cells.

Myocardial damage was noted already in tissue not exposed to US or transducers in all 17 perfused tissue specimens, the total sum of damage scores being 4.3 ±1.5 (mean ±SD). In comparison, the total damage scores in unexposed infarcted tissue were significantly higher (6.2 ±2.0 vs. 4.3 ±1.5), irrespective of whether the comparison used was the paired difference test (P < 0.001) or Student's t-test for group comparison (P = 0.004).

Following US exposure of non-infarcted myocardium, there was a significant increase in total damage scores, from 4.3 ±1.5 in non-exposed myocardium to 5.8 ±1.7 in US exposed myocardium (P = 0.015, Student t-test for group comparison).

Exposure to the transducer alone did not significantly affect the damage caused by myocardial infarction, the total sum of damage scores being 6.2 ±2.0 in unexposed infarcted myocardium and 6.6 ±2.1 in transducer (alone) exposed infarcted myocardium (P = 0.662, Student t-test for group comparison and P = 0.244 using the paired Student t-test).

Further, there was significant augmentation of tissue injury in infarcted myocardium exposed to US, as shown by the paired difference test (P = 0.026) and Student's t-test for group comparison (P = 0.027), total sum of damage scores being 8.1 ±1.7 in US exposed infarcted myocardium and 6.2 ±2.0 in infarcted myocardium without any exposure. The individual damage scores for the 68 tissue samples and the results of statistical analyses for all groups are shown in Figure 4.20.

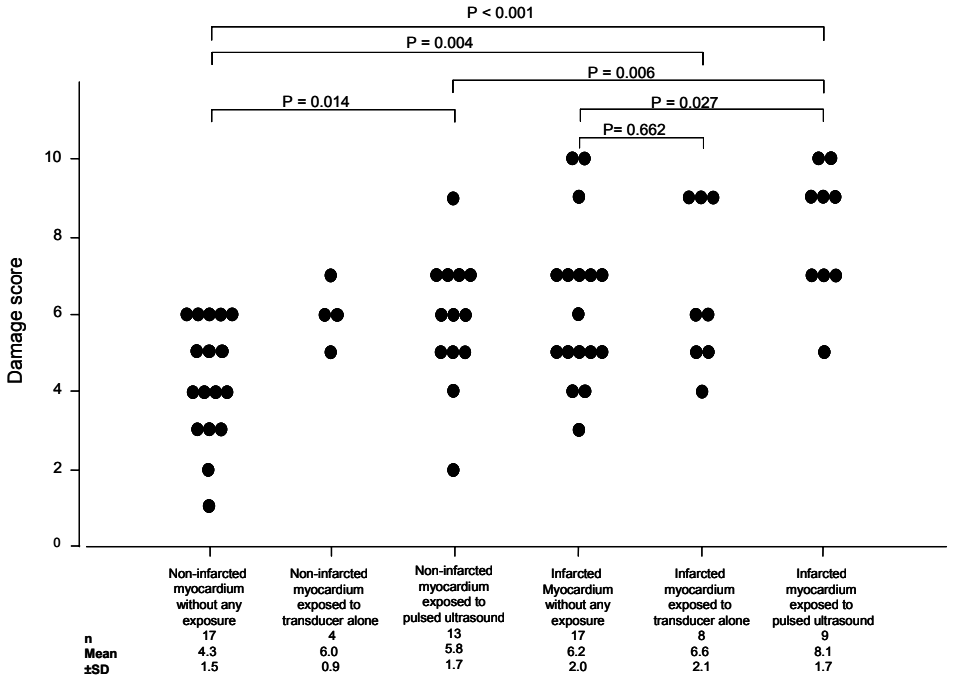

Figure 4.20. Total damage scores obtained in 68 individual tissue samples from the six different myocardial areas (see text).

The effects of ultrasound on brain tissue

The measured physiological variables are presented in Table 4.11. No significant differences in between the US and US control groups were found during the procedure. However, operation-times in the Method control group were significantly shorter than in both the US and US control groups (P = 0.004 and 0.005 respectively). Temperatures 1 hour after occlusion in the Method control group were also significantly higher than in the other groups (P = 0.006 and 0.006 respectively) while temperatures 2 and 3 hours after occlusion were significantly higher in the Method control group than in the US group (P = 0.045 in both comparisons) (Table 4.11).

	Groups					
Physiological variables	**Median US**	**Range**	**Median UCG**	**Range**	**Median MCG**	**Range**
Body weight (g)	335.0	325.0-340.0	327.5	310.0-355.0	345.0	310.0-405.0
Operation time (min)	175.0	150.0-190.0	172.5	145.0-200.0	110.0	62.0-145.0
Blood pressure (mm Hg)						
Before occlusion	87.5	80.0-108.0	92.5	90.0-100.0	105.0	90.0-118.0
After occlusion	90.0	80.0-130.0	92.5	90.0-101.0	97.5	85.0-110.0
0.5 h after occlusion	95.0	85.0-110.0	96.0	90.0-110.0	N.A.	N.A.
1 h after occlusion	99.0	90.0-130.0	95.0	90.0-107.0	N.A.	N.A.
Temperature (°C)						
Before occlusion	36.7	36.0-37.6	37.2	36.4-37.4	36.7	36.4-37.7
1 h after occlusion	37.1	36.7-37.7	37.2	37.1-37.3	38.5	38.1-39.2
2 h after occlusion	37.8	36.8-38.1	38.5	36.8-39.0	38.6	37.8-38.8
3 h after occlusion	38.0	36.8-38.7	38.3	37.7-39.2	38.1	37.5-39.1
4 h after occlusion	N.A.	N.A.	N.A.	N.A.	38.8	38.4-39.2
Filament size (µm)	35.0	33.75-35.0	33.75	32.50-36.25	33.75	32.25-36.25
pCO_2 (mm Hg)	38.4	32.3-43.3	38.4	32.3-43.7	33.9	30.5-42.4
pO_2 (mm Hg)	114.6	100.2-128.3	113.5	110.7-122.7	116.2	89.7-127.9
pH	7.419	7.352-7.468	7.410	7.367-7.456	7.443	7.412-7.477
B-Glucose (mmol/L)	4.8	2.8-5.0	4.5	3.7-5.1	4.8	3.8-5.8

Table 4.11. Physiological variables measured during the experiment US: Ultrasound group, USG: Ultrasound control group, MCG: Method control group (N.A. indicates Not Applicable).

Brain damage

Using the behavioural test no significant differences were observed in indicators of neurological deficiency between any pairs of the three groups, after two hours of recirculation.

In the microscopic evaluation, two regions of infarction were distinguished, one totally white and the other light red. The white regions were considered as regions

of total necrosis and the light red ones as indicative of selective neuronal necrosis [143] (Figure 4.21).

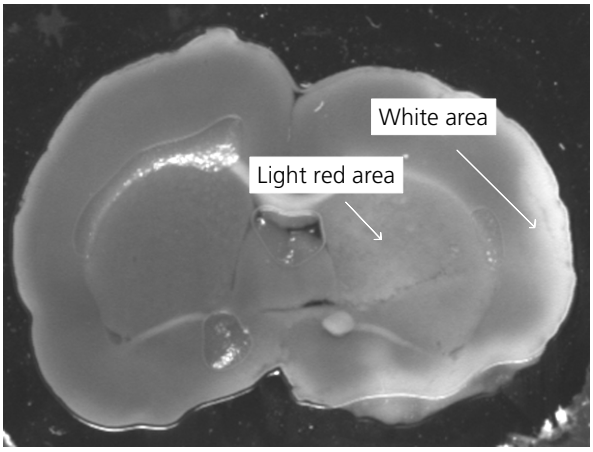

Figure 4.21. CCD video camera digitised monochromatic photo of a brain slice showing an example of the two regions that were considered infarcted. Light red indicates selective neuronal necrosis and white indicates total necrosis.

In each group, three measurements of infarct volumes were made in regions of total necrosis, regions of selective neuronal necrosis and the combined regions of both total and selective neural necrosis (Table 4.12). Statistical analyses of the differences between various pairs of groups as regards volumes of regions of total necrosis, of regions of selective neuronal necrosis and the combined volumes of both types of regions were made (Table 4.13). The left hemispheres (that were perfused during US exposure) showed no regions of necrosis in any of the three groups.

Groups/regions	Median	Range
US W	30.4	0.0-449.1
UCG W	57.0	0.0-404.6
MCG W	53.6	0.0-334.5
US L	163.7	102.0-210.0
UCG L	122.3	66.5-178.9
MCG L	151.4	90.0-258.2
US L+W	176.0	105.5-610.4
UCG L + W	203.3	66.5-485.9
MCG L +W	267.5	133.1-467.2

Table 4.12. Infarct volumes. US: Ultrasound Group, UCG: Ultrasound control group, MCG: Method control group. Three measurements were made in each group in regions. W: regions regarded as having total necrosis, L: regions regarded as having selective neuronal necrosis, and, W+L: combined regions of total and selective neuronal necrosis.

Groups/regions		Group/regions	P-values
US W	vs.	UCG W	0.873
US W	vs.	MCG W	0.689
UCG W	vs.	MCG W	0.749
US L	vs.	UCG L	0.522
US L	vs.	MCG L	0.337
UCG L	vs.	MCG L	0.749
US L + W	vs.	UCG L + W	0.631
US L + W	vs.	MCG L + W	0.749
UCG L + W	vs.	MCG L + W	0.423

Table 4.13. Results of statistical comparisons of the Ultrasound Group (US), the Ultrasound control group (UCG) and the Method control group (MCG). W: total necrosis, L: selective neuronal necrosis,.and, W+L: combined regions of total and selective neuronal necrosis.

5 Discussion

5.1 Mechanisms of ultrasound-enhanced fibrinolysis

Enhancement of drug action by US is not unique for thrombolytic drugs. Similar concepts of using US as a promoter of medical treatment have been explored in other areas of medicine. One study explored US enhancement of the cytotoxicity of chemotherapeutic agents [154]. The enhancement effects were drug specific, a modest intracellular uptake (20 %) resulting in a threefold increase in toxicity. Another report showed an increase in cytotoxicity despite a decrease in drug uptake [155]. There have also been studies of enhancement effects of US on antibiotics [156-159]. Exposure to US has been shown to reverse bacterial resistance to antibiotics [160]. The effects were concluded to be strongly dependent on frequency and intensity [159].

Thus, while US affects structures in various regions of biology and pharmacology, the explanations as to why such effects occur vary [54, 56, 65, 67, 87, 91, 92, 95-97, 144, 148, 154-161].

Effects of ultrasound on blood components

It has earlier been indicated that erythrocytes may disrupt in the clots following exposure to low frequency US exposure in the setting of US-enhanced fibrinolysis [97], suggesting the presence of an mechanism for clot disruption in this context. Erythrocyte disruption during high frequency US exposure has also been shown in non-coagulated blood [144]. We have excluded the possibility that the Hb release is caused by erythrocyte disruption at intensity 0.5 W/cm^2 SATA where enhanced fibrinolytic effects were noted in the evaluation using SK solution. We have not fully explored this phenomenon at different intensities properly and can therefore not exclude the possibility that erythrocytes disrupt during exposures to high-intensity US, as previously shown [97, 144].

Platelet activation would affect fibrinolysis, making the clots more compact [145, 146]. However, no activation of platelet could be noted during US exposure at a low intensity (0.7 W/cm^2 SATA). This indicates that the enhanced effects seen during concomitant SK and US exposure are not affected by platelet activation, a finding also verified *in-vivo* [147]. However, in other previuos studies it has been shown that it is possible to activate platelets by exposure to US [144, 148]. These studies showed that the activation of platelets depends on US frequency and intensity [144, 148]. However, since we have not explored this phenomenon properly at other intensities, we cannot exclude the possibility that platelets are activated during US exposure at the other intensities used. However, if platelet activation occurs, the enhanced effects are present despite more compact clots.

Fibrinolytic effects from exposure to ultrasound alone

No fibrinolytic effects due to exposure to pulsed US alone could be detected at intensities 0.125, 0.5 or 1 W/cm^2 SATA. However, a small but significant enhancement of the fibrinolytic levels was observed following exposure to intensity 4 W/cm^2 SATA. This effect was then reproduced twice, firstly, when the effects of r-PA in pre-exposed clots were analysed and secondly, when the effects of concomitant r-PA and US and r-PA exposure of clots earlier exposed to US were compared. That these effects were present only during US exposure and not in the following hour shows that plasmin activation was not induced by the exposure to US (Figure 4.10). This confirms earlier *in-vitro* as well as *in-vivo* studies of the possibility for fibrinolytic effects from US exposure alone [48, 50]. Earlier studies of US-enhanced t-PA mediated fibrinolysis have shown that this works through a mechanism of changes in the structure of the fibrinous network [67, 92] following US exposures.

Enhanced fibrinolytic streptokinase effects from ultrasound exposure

We have confirmed that the enhancement of fibrinolysis by US is much more complex than has hitherto been assumed [54, 56, 65, 67, 87, 91, 92]. Earlier, US-enhanced fibrinolysis by thrombolytic drugs has been interpreted as being caused by changes in fibrin structure [56, 65, 69, 90, 92, 93], temperature increase [55, 89], cavitational effects [54, 68], and to increased flow in and around clots inducing higher drug uptake [64, 65, 89, 90]. The effects have also been shown to be dependent on drug concentration [54, 63, 89] and the frequency, intensity and duty cycle of US applied [55, 56, 63, 64, 69, 87]. Finally, a recent study reported blood donor medication dependent differences in the enhancement effects seen during US exposure [93]. Thus, multiple mechanisms are presumably involved, in the US-enhancement of fibrinolysis.

In the work described in I it was shown that following exposure to pulsed US of frequency 1 MHz and intensity modulation, the SK solution shows varying sonochemical reactions. The enhanced fibrinolytic effects were noted in a narrow intensity range (0.25 – 0.5 W/cm^2 SATA). In an earlier model, we have shown inhibition of the enhanced SK effects at various intensity levels of pulsed high frequency US (1 MHz), and also that further increases in intensity actually decrease the fibrinolytic action of SK [56]. These results were confirmed (I) at 1 W/cm^2 SATA (inhibition) and 4 W/cm^2 SATA (decrease) both when clots and SK were exposed concomitantly as well as when SK solution was pre-exposed. Others [95] have also reported that US exposure may inhibit the effects of SK. In fact, exposing SK to high intensity US at kHz frequencies caused structural and functional changes in the SK molecule, resulting in lower plasmin auto activity [96]. In contrast, the present study shows an alteration of SK function following US exposure evinced by decreased or increased fibrinolytic action, both at considerably lower US intensities than used by others [95, 96].

While SK has been investigated since the 1940s [149] the mechanism of its interaction with plasminogen is still the subject of study [17-21]. Streptokinase consists of three independent folding structural domains, an α domain, a β domain

and a γ domain [19]. While all are involved in the interaction with plasminogen, the β domain is possibly the first to bind to plasminogen [18]. A possible interpretation of the results would be that the interaction between the SK structural domains and plasminogen is altered by exposure to US.
The effect of prolonged US exposure was analysed in the present work and the enhanced effects observed seem to be induced during the first hour of exposure (Table 4.8) and then followed by constant increases in both exposed and unexposed clots. However, it has been shown that when SK is diluted in 0.9 % NaCl solution it will degrade spontaneously relatively fast [130]. Thus, in the present analysis the SK effect might just be present during the first hours of evaluation. However, the fact that during the last 12 hours of US exposure the clot lysis increased in exposed clots compared to unexposed clots might indicate fibrinolytic effects from exposure to US alone. Due to the low number of clots used and the lack of knowledge of SK-concentrations, these results remain speculative, and these findings must be explored more fully in the future.

A limited analysis of the dependency of clot age (two hours) was also made. In the enhanced effects seen during concomitant SK and US exposure at intensity of 0.5 W/cm^2 SATA, no influence on clotting time could be found. This suggests that beneficial effects may well be attainable, even after delay between onset of symptoms and arrival at hospital.

In conclusion, it seems possible to modulate the stereochemistry of SK by exposing it to US of different intensity levels, and, the attenuation of the SK effect seen seems to be due to a direct effect on the SK molecule, rather than to changes in the fibrin network or circulation within or around the clot. These effects occur at US intensities below the prescribed upper limit of exposure of the human body to US energy (Mechanical Index < 1.9) [41]. The intensity levels used in the present study correspond to the Mechanical Index range 0.29 - 0.82. Therefore, during SK treatment of any thrombotic disorders, possible undesired effects of exposure to US should be considered. Thus, the local intensity of US in the human body following transcutaneous application of US is affected by several factors, such as acoustic attenuation, scattering and reflection, which are difficult to control. Using US to enhance the effects of SK in clinical situations should be recommended to be restricted until reliable calculations or measurements of local US intensities have been performed. Fortunately, although US enhancement of thrombolysis has been applied in clinical situations [42, 43], fibrinolytic agents other than SK were used.

Enhanced fibrinolytic reteplase effects from low intensity ultrasound

For the first time, it has been shown that clot lysis is significantly enhanced when using r-PA solution pre-exposed to US of intensity 0.125 W/cm^2 SATA. This enhancement could be accounted for by changes in the fibrinolytic properties of the r-PA molecule, the induced changes being stable for one hour after US termination.

Tissue plasminogen activators induce fibrinolysis after converting plasminogen to the active enzyme, plasmin, which is able to degrade fibrin. Earlier studies have documented poor plasmin activation in low fibrin content environments for native

t-PA, showing the need for t-PA molecules to bind to fibrin before a high rate of plasminogen conversion is possible [28, 150]. However, r-PA does not have this high need for bind to facilitate plasminogen conversion [131]. Thus, the most likely explanation for any changes in the r-PA structure induced by US is an increased affinity to plasminogen. However, the possibility that better r-PA affinity to fibrin structures increases the plasminogen conversion rate cannot be excluded. Clearly, the exact mechanism remains needs to be verified for r-PA.

Previous studies have shown enhanced fibrinolytic effects of t-PA in the low intensity US range (< 0.5 W/cm^2 SATA) [58, 59, 151]. However, these studies show effects increasing with intensity, a finding not seen in the present study where enhanced levels of fibrinolysis were absent in the intensity interval 0.5-1 W/cm^2 SATA. However, differences in the US frequencies and fibrinolytic drugs used make comparison difficult [58, 59, 151].

Several earlier studies have shown that the fibrinolytic effects of t-PA are enhanced by exposure of the target clots to US. The present study confirms this possibility for r-PA when exposed to 1 MHz pulsed US of high intensity. However there is probably more than one mechanism involved. Thus, pre-exposing clots to US of high intensity significantly increased the fibrinolytic action of r-PA during the following hour of evaluation of fibrinolytic effects. Thus, the current study confirms one of the mechanisms earlier recognized in connection with improved t-PA effects during US exposure [67, 93]. Thus, one possible explanation could be that US induces changes in the clot-structure that are associated with improved fibrinolytic action of r-PA. These changes are *not* induced in the r-PA molecule at the intensity levels used here, nor is US induced streaming, a mechanism earlier shown to be involved in US-enhanced t-PA induced fibrinolysis [89, 100] needed.

The effect of pre-exposure to US was also shown to induce higher enhanced effects than those induced during concomitant exposures, an observation also indicating that the possible US induced streaming produced by exposure to pulsed US of intensity 4 W/cm^2 SATA does not influence the enhanced effects to a greater degree than changes in clot-structure.

Comparison of data for reteplase and streptokinase

The present study show that, as a result of modulation of the effects of SK and r-PA by US of different intensities, fibrinolytic responses vary. The two molecules differ in weight and size, SK having a molar mass of 47 kDa and comprising 414 amino acid residues [152] while r-PA has a molar mass of 39 kDa and is comprised of 355 amino acids [131]. To enhance fibrinolysis, the structures of the two molecules require different levels of intensity (0.25 - 0.5 W/cm^2 SATA for SK and 0.125 - 0.25 W/cm^2 SATA for r-PA). A decrease in the fibrinolytic action of SK was seen when it was pre-exposed to US of high intensity (4 and 2 W/cm^2 SATA). At this intensity, there are no changes in the fibrinolytic properties of r-PA, suggesting that the structure of the SK molecule is more vulnerable than that of r-PA when exposed to US. The stability of another rt-PA molecule following US exposure has recently been studied by others, they conclude that the alteplase molecule is fully active and stable following US exposures of similar intensity ranges [153].

5.2 Ultrasound and microbubble fibrinolysis

Ultrasound in combination with microbubbles has been shown to induce fibrinolysis both in vitro [74, 83, 85, 162] as well as in vivo [77, 80-84, 163].
The measurements of MB destruction-rates made in this study showed that, at the same intensity level were faster outside the resonance frequency (3 - 5 MHz). The evaluation of the effects of the number of pulses sent showed that a burst of 5 pulses was needed to achieve a fast destruction-rate. An US intensity of 0.5 W/cm^2 SATA was shown to be the lowest intensity giving fast MB destruction. All these observations together suggests that the likeliest way of attaining inertial cavitation effects that could be beneficial in the setting of MB and US induced fibrinolysis would be a US configuration of frequency between 0.5 – 2 MHz, sent by pulsed wave of more than 0.5 % duty cycle and intensities not less than intensity 0.5 W/cm^2 SATA. A similar US setting has been shown to be efficient in US and microbubble-induced fibrinolysis in vivo. However, these studies used another US contrast agent than that used here [48, 80, 81, 163].

Since inertial cavitation involves the growth and collapse of one or several microbubbles, it is connected with extremely high local temperature increases (high temperatures leading to formations of free radicals), micro-jets and shock waves. The noninertial cavitation, in which collapse of the microbubbles does not occur, would be associated, with bubble oscillation, radiation force and also microstreaming appearing in the area around the oscillating microbubble [102, 111]. In MB-enhanced fibrinolysis both inertial cavitation and noninertial cavitation could be effective, but, since inertial cavitation is a more violent phenomena maybe the noninertial cavitation would be preferable, since it would be more controllable in a biological settings. Two studies [164, 165] have been published trying to explore these phenomena for MB. The first of these concluded that the likelihood for MB to provide inertial cavitation effects is small, while the authors of the second stated that they detected cavitation pulses on a scale sufficient to explain that MB produces inertial cavitation during US exposure at low duty cycle and intensity.

The MB are produced for contrast enhancement during US diagnostics, where the reflection of the MB is registered. This reflection will lower the incidence of US on a target such as a thrombus, making it difficult to know the exact US intensity reaching the MB in the clot. If MB were supplied to the clot containers used in the fibrinolysis model described earlier, it would be difficult to control where the MB are and if they are at the clot surface. This problem has been addressed by others, who have modified the microbubbles, making them targeted for platelets [166] or fibrin present in a clot [167]. Thus, an optimization of many parameters is needed before this concept could be introduced in the clinical setting.

5.3 Non-beneficial effects of ultrasound

Heart

We chose an open chest porcine model with an induced myocardial infarction and applied US to test our hypothesis that prolonged exposure on ischemic myocardial tissues to US *could potentially* be harmful, even when used within limits hitherto considered safe in cardiac exposures [168, 169]. However, histopathological diagnosis of tissue damage during the first hour of coronary artery occlusion was based on subtle findings and the assessment of cardiac damage used was therefore less specific than in a fully developed infarction. The total effect of the two-hour period of ischemia showed, however, a significant increase in the total damage score by 45 %. US exposure of the infarcted myocardium resulted in a significant increase of the total damage score by a further 30 %.

As earlier stated, the US-induced damage in biological tissues may involve the same mechanisms as those enhancing fibrinolysis. Heat produced in perfused tissues exposed to US within safe levels would under ordinary circumstances mainly be lost to the circulation. The reduced circulation in ischemic tissues may therefore decrease their ability to lose heat during US exposure, resulting in undesirable heating and thereby creating a thermal injury [170]. However, temperature measurements in non-circulated myocardial tissue showed only small increases (0.5 ^{o}C), well within in the limits of now used safety-rules [103].

Exposing circulated tissue to high frequency US has been shown to induce mild increase in interstitial oedema, in accumulation of polymorphonuclear cells [171], in oxidative stress in endothelial cells [172] and increased cell lysis and apoptosis in human myelomonocytic leukaemia cells [173]. It has been hypothesized that these effects may be caused by radiation force [173] and other non-thermal effects [172]. US at similar exposure settings as used in the present study have been shown to produce necrotic and cellular damage in epidermis layers in goldfish [123]. Increasing the length of the exposure period caused the damage to propagate gradually inwards the cell layers increasing the damage-score. It was concluded that the damage produced was caused by cavitational injury [123]. It has earlier been shown that anatomical structural differences and animal age can affect the sensitivity for US exposure [111], although the role of the age-dependence remains unclear [174].

Multiple studies of the beneficial effects of US used to enhance thrombolysis have been conducted in different animal models, both in the venous and arterial systems [66, 79, 88, 124, 171, 175-182]. The progress in the area has also reached humans in the clinical setting. Thus, a prospective controlled study of US augmentation of thrombolysis in myocardial ischemia failed to verify a beneficial effect [71]. In fact, the number of ischemic complications increased following low frequency US exposure. In contrast, no undesirable effects were noticed in 25 patients with myocardial infarction, who received traditional thrombolytic treatment with adjunctive exposure of low frequency US [70].

Brain

The present study was prompted by our earlier observations of enhanced myocardial damage caused by low-energy US in the ischemic myocardium (III, and unpublished data from the clinical pilot study performed in the mid 1990s).

In the myocardial study, we used a detailed histopathological evaluation that was not used in the study of the effects of US on the ischemic brain tissue. The model used is, however, a well-documented method to quantify brain injury due to right middle cerebral artery occlusion [140]. The method of staining the tissue with 2,3,5 - Triphenyltetrazolium chloride gives distinct colourings of both damaged and undamaged regions. The regions between necrotic areas and undamaged areas did not show distinct colouring, but were instead light red in colour, and were judged to be regions of selective neuronal necrosis. Complete damage probably stopped after removing the filament. We have assumed that, should the right middle cerebral artery be permanently occluded by a thrombus, the light red regions would turn into regions of total necrosis. Memezawa and co-worker [143] have shown how infarction develops over time and have used histopathological evaluation to confirm selective neuronal necrosis, their results shows that selective neuronal necrosis could be present after as much as 180 minutes of occlusion. We are well aware that the 2,3,5 - Triphenyltetrazolium chloride staining method used in this study does not revile subtle changes in the brain, but rather giving the quantity of the volume and size of the brain damage.

As stated above, as a consequence of anatomical structural differences and their ability to reflect and absorb US, it is probable that various types of tissue may have different thresholds for US-produced damage. This may explain the difference in the findings in III and in IV. Different animal models may be another explanation.

A recent clinical study of low-frequency US mediated thrombolysis showed that the incidence of bleeding was higher following US exposure during t-Pa infusion in patients with cerebrovascular occlusion [73]. Since we did not use thrombolytic drugs, increased bleeding effects from US exposures was not analysed in the present study. However, earlier clinical studies of US-enhanced thrombolysis in the setting of high frequency US have not revealed such effects following US exposure [48, 72, 183, 184].

5.4 The experimental models

Fibrinolysis model and methods

Most previous studies of US-enhanced fibrinolysis have evaluated the degree of fibrinolysis using techniques requiring measurements of clot weight before and after exposure to US [56, 57, 59, 98, 185]. Considerable mechanical handling is then required to determine the properties of individual clots at one particular time. In contrast, the method described here allows continuous evaluation of lysis without mechanical handling, diminishing markedly the number of clots to be made. Similar techniques have been used in other studies, using fibrin degradation products to measure fibrinolytic effects [90, 151]. The red blood cells trapped within the fibrin network of a blood clot are released by its degradation. Although

it cannot be used with pure fibrin clots, Hb measurement is an appealing method for estimating the progress of fibrinolysis during thrombolytic treatment in vitro.

In comparisons between different levels of fibrinolysis in control clots between experimental setups, a certain variation of $CRHb_{1h}$ is seen. In addition to the inherent variability of the present method (The Hb method has a variation of ±2.5 % [128], and the spectrophotometer has a variation of ±2 nm corresponding to a clot lysis variation of ±3 % [186]), further variability is caused by differences in the response of individual blood donors to the thrombolytic drugs used. Similar variability has been reported elsewhere [145, 146, 187, 188]. Among its possible causes, exercise levels [188], blood parameters such as endogenous levels of plasminogen activator inhibitor 1 and plasminogen [187] have been discussed. The last study [188], showed that results also depend on age. Platelets may also influence the variability by inducing a clot retraction, thereby decreasing the action of t-PA. Another factor is the number of platelets [145, 146].

Storing of blood affected the fibrinolytic action of SK both increasing and decreasing its fibrinolytic action [142]. The importance of the expected method variability induced by inter- and intra-individual as well as that induced by the cold storage is minimized by always using paired clots in any intervention, where the same levels of blood parameters influence the variability in the same manner. The effect of storing blood on the fibrinolytic action of r-PA has not been analysed. However, since r-PA acts upon the same blood parameters as were analysed for SK, the same results could be expected for both substances [17, 131].

The addition of thrombolytic solutions to pure 0.9 % NaCl solution might not be the optimal mixture of components for exploring when fibrinolytic effects are optimised. Previous studies have explored the stability of fibrinolytic effects at low concentrations of SK and other thrombolytic drugs in pure 0.9 % NaCl solution [130, 189, 190]. These studies showed that the fibrinolytic effects were stable or partly reduced, but none of the studies showed a total inactivation of the fibrinolytic effects. We have not identified any verification of the stability of r-PA in 0.9 % NaCl solution in the literature. However, our measurements evidence fibrinolytic increase by 87 % in clots exposed to r-PA solution compared to clots exposed to 0.9 % NaCl solution (Figure 4.3). Furthermore, results from earlier *in-vitro* studies in which fibrinolytic drugs were added to pure 0.9 % NaCl solution [56, 97] have later been reproduced and verified *in-vivo* [66, 175] as well as in clinical studies [70]. One earlier study showed that urokinase was stable in plastic containers if diluted in 0.9 % NaCl but not in glucose solution [191], suggestive of urokinase adhesion to the walls of the plastic materials. We have not performed any similar analysis in our model, however, the use of identical experiment setups for both target and control clots make this problem less important. In conclusion, the present study was designed to elucidate genuine changes in the fibrinolytic action of the thrombolytic compounds following exposure to US, thus not aiming at the optimised effects of each drug.

Exposure to US of frequency 1 MHz and intensity 1 W/cm^2 SATA has earlier been identified as the condition producing the highest enhancement effects of SK induced fibrinolysis [56]. That, in the model used here, the highest enhancements effect was observed at intensity 0.5 W/cm^2 SATA is due to differences in acoustic properties of the models [56, 94]. One of these earlier studies [56] used the same

cylindrical plastic tubes as those used here in US measurements done for MB experiments (Table 4.4), where a considerable loss of intensity was seen when US (1 MHz and an intensity of 1 W/cm^2 SATA) was passing one of the walls of the cylindrical plastic tubes. No loss of intensity could be seen in control measurements when US passed 1 styrene sheet of the clot containers used in the fibrinolysis studies (1 MHz frequency and intensities: 0.625, 0.125, 0.25, 0.5, 1, 1,5, 2 and 4 W/cm^2 SATA).

The US configuration used in I and II was limited to one pulse mode, a limited intensity range and a single frequency area. Also the effects of shorter periods of exposure to US have not been determined. Identification of the optimal enhancement of fibrinolysis by US was not, however, the goal of these studies.

Bubble destruction model and method

The use of cylindrical plastic tubes was shown to influence the incidence of US energy in various ways as evidenced by the measurements of the loss of intensity carried out in the hydrophone survey (Table 4.4). Thus, it is difficult to compare US parameters. However, there are even greater difficulties quantifying the MB destruction-rate in relation to US intensity parameters because of the reflection of each microbubble scattering and attenuating the incidence of US intensity.

The most important US parameter in determining the biological effects produced by MB destruction is the peak-negative pressure [192]. Thus the Mechanical Index, which considers the peak-negative pressure in relation to frequency, might be a more proper way of comparing the effects of US on bubble destruction than the W/cm^2 used in the present work. However, in studies exploring the fibrinolytic effects of US and microbubbles, the intensity parameter most often used is W/cm^2 [77, 80-84, 163].

Ischemic heart model

We found a notable amount of damage already in the samples from the non-infarcted myocardium that were not exposed to transducer or US, a finding which could be explained as the result of stress-induced damage [193-195] or mechanical handling of the heart during removal. This makes the interpretation of the results more complex, maybe there were three different types of damages present when the histopathological examination was performed (stress-induced damage, damage induced by mechanical handling and ischemic damage).

The present data are unable to disclose the true mechanism of the myocardial injury induced by pulsed US. The development of ischemic myocardial damage over time is evidenced by different and unspecific indicators of damage, some of which being reversible [196]. It was therefore not possible to estimate how the myocardial tissue in the present study would be affected after a fully developed infarction, where US might just contribute to a speed-up of the developing infarction.

Two control experiments were carried out. First, we verified that fixation of the transducer and application of US gel close to the infarcted myocardium did not significantly affect the total damage scores. Second, we explored the effect of US on

perfused myocardium. Interestingly, following exposure to US, there were significant signs of myocardial damage also in this presumed healthy tissue. However, due to the notable amount of damage already in samples from the non-infarcted myocardium, it should not be stressed that healthy tissue is damage by US exposure, it might just indicate increased stress-induced damaged. Another factor influencing the interpretation is the low number of samples (n = 4) in the group of non-infarcted myocardium exposed to transducer alone. This, being the result of study protocol misinterpretation, unfortunately necessitated the exclusion of these data from the statistical comparison.

No threshold measurements of US induced myocardial damage were performed, however the total US energy we used is in the order of 10 times higher than earlier been shown to be required to enhance the thrombolysis *in-vitro* [56]. Neither was the temperature monitored in the areas during exposure to US but instead afterwards and outside the experimental setup. The experimental setup could include reflection sites not present in the temperature measurements, which could possibly increase temperature.

Ischemic Brain model

Our measurements of US characteristics indicated that there was an important decrease in US energy after traversing the skull, both as regards total radiation pressure and in the hydrophone measurements. However, probably due to slight focusing by the skull roof, spots of higher intensity were observed in the hydrophone measurements, but these could not be connected with any distinct location of damage in the US group. There was also a considerable reduction in US intensity after traversing the whole skull. Figure 4.2 also shows a scattering of the field after traversing the skull roof. Obviously, the complexity of the US field in an intact rat brain, in which the bottom of the skull could reflect and absorb the US and possibly cause focusing phenomena, cannot be properly explored. We are thus unaware of the detailed US distribution and intensity of US within the exposed intact skull.

We used the Method control group as an extra control group to exclude the possibility that our modification of the model would enhance damage to brain tissue. We observed higher body temperatures in the Method control group than in both the US and US control groups. The US and US control groups had longer intervals of anaesthesia than the Method control group. This has been identified as a temperature-lowering component [197]. In addition, the cylindrical tube full of room-tempered ultrasonic gel over the skull could have a cooling effect on the brain. These two factors might account for the higher body temperatures in the Method control group. The temperature differences in the present study should, however, be too small to affect infarct size [198].

We used two different methods to evaluate the ischemic effect on the brain. Thus, since the US and US control groups required anaesthesia, the laser Doppler method was used while in the Method control group signs of neurological deficiency were used. We cannot exclude the possibility that these two methods identify different degrees of brain damage. However, there were no significant differences among the three animal groups using the neurological deficiency scale after two hours of recirculation.

The recirculation period for the animals was chosen on the basis of experience from other studies of right middle cerebral artery occlusion [143]. We cannot exclude the possibility that a longer recirculation period might have revealed late US-produced damage.

We did not monitor the local temperature in the brain during US-exposure and cannot therefore exclude the possibility of local temperature rise during US-exposure. There have been reports of local temperature rises in foetal brains during exposure to US [109, 110]. We could not, however, find any evidence of local injuries compatible with such a mechanism.

The number of rats in the groups analysed in IV were based on the hypothesis that US would increase damage in the non circulated hemispheres, however, since no difference between groups were found, a correct statistical comparison of a two groups showing now difference would render a large number of rats to be included in each group. The approximate number per group has been estimated (using power calculations) to be in excess of 500, beyond our economic resources and difficult to justify ethically. Therefore, this study should be considered only as a pilot one.

6 Conclusions

General conclusion

The results from this study clearly emphasise the importance of evaluating the potentially non-beneficial effects that may be induced by ultrasound when enhancing thrombolytic effects. In the setting of ultrasound exposure during cerebral ischemia, at US intensity levels verified to be beneficial in experimental studies of thrombolysis, no detectable additive damage was verified (IV). However, US of the same characteristics induced increased damage in the infarcted myocardium (III). These two findings along with the observed complexity of the mechanisms involved in ultrasound-enhanced fibrinolysis with multiple mechanisms and drug specific reactions (I and II) show that further clarification is needed before an optimal combination of parameters in the setting of high frequency ultrasound-enhanced fibrinolysis can be advised in clinical studies.

Specific conclusions

The specific conclusions reached in this study are:

- Exposure of the streptokinase molecule to ultrasound modulates its fibrinolytic properties, resulting in both increased and decreased fibrinolytic effects. That these effects are present during simultaneous exposure of clots and streptokinase solution as well as after pre-exposure of the streptokinase solution suggests a direct effect of ultrasound on the streptokinase molecule.
- Pre-exposure of the reteplase solution to low intensity ultrasound induce changes in the function of the molecule associated with enhanced fibrinolytic effects. Enhancement effects also occurred when clots were exposed to high intensity of ultrasound before or concomitantly to exposure to reteplase, suggesting that two different intensity-dependent mechanisms are involved during ultrasound-enhanced reteplase fibrinolysis.
- The destruction-rate of Sonazoid® microbubbles following exposure to ultrasound was at one and the same intensity-level faster outside the resonance frequency range. Five pulses were needed to achieve a fast destruction-rate. An ultrasound intensity of 0.5 W/cm^2 SATA was shown to be the lowest intensity yielding fast microbubble destruction.
- Lengthy exposure to low-intensity pulsed ultrasound exerting beneficial pro-fibrinolytic effects in thrombolysis may increase instantaneous myocardial damage.
- Ultrasound at parameter-values used to enhance fibrinolysis in vivo seems not to increase ischemic damage in non-perfused rat brain or to induce damage in the perfused rat brain in our model.

7 Summary in Swedish (Populärvetenskaplig sammanfattning)

7.1 Bakgrund

Ultraljud används inte bara som ett diagnostiskt hjälpmedel utan också som en behandlingsmetod. De terapeutiska områden som utforskas idag är destruktion av cancertumörer, behandling av diskbråck, ökning av antibiotikaeffekter, ökning av cellgiftseffekter och som i detta arbete, ökning av effekterna av propplösande läkemedel. Det görs även försök där ultraljud används för att lösa blodproppar utan propplösande läkemedel, då används enbart ultraljud eller en kombination av mikrobubblor och ultraljud. De effekter som åstadkoms vid ultraljudsexponering har tidigare förklarats bero på faktorer som, temperaturökning, ultraljudsinducerad strömning och kavitationseffekter. Ultraljud har visats kunna påverka fibrinnätverket så detta blir mer känsligt för propplösande läkemedel, kunna öka transporten av läkemedel in i blodproppen samt öka bindningen av läkemedel i blodproppen. De faktorer som är involverade i samband med ultraljudsförstärkt blodproppsbehandling är dock inte helt klarlagda.

Syftet med denna vetenskapliga studie var att undersöka olika ultraljudsparametrars effekter på två olika blodproppslösande läkemedel, samt att utvärdera om de ultraljudsparametrar som verkar positivt på blodproppslösande behandling kan ge upphov till skadliga effekter på hjärt- och hjärnvävnad som ultraljudsexponeras vid en behandling.

Detta gjordes genom att utvärdera:

- förändringar i de propplösande effekterna hos streptokinas vid samtidig ultraljudsexponering av läkemedel och blodpropp samt förändringar i läkemedelseffekten när detta pre-exponeras med ultraljud.
- förändringar i de propplösande effekterna hos reteplase vid samtidig ultraljudsexponering av läkemedel och blodpropp samt förändringar i läkemedlets effekter efter pre-exponering av såväl läkemedel som blodpropp.
- beroendet av ultraljudsfrekvens, intensitet och pulstågslängd på sönderfallshastigheten hos Sonazoid® mikrobubblor.
- om långtidsexponering med ultraljud ger ökade skador på icke genomblödd hjärtmuskel.
- om långtidsexponering med ultraljud ger ökade skador på icke genomblödd hjärnvävnad.

7.2 Metoder

Ultraljudsförstärkt propplösande behandling

För att utvärdera effekten av ultraljudsförstärkt blodproppslösande läkemedelsbehandling utvecklades en ny metod (I-II), där utvärdering gjordes genom att analysera utsöndringen av röda blodkroppar ur blodproppen.

När ett blodkärl skadas ansamlas blodkroppar, bland annat röda blodkroppar, och det bildas en blodpropp. Blodkropparna binds samman av fibrintrådar på vilka de propplösande läkemedlen verkar.

Blodproppar tillverkades genom att ta venöst blod från friska frivilliga försökspersoner. Efter detta tillsattes en vätska som tar bort kalcium ur blodet, för att blodet inte skulle koagulera. Blodet kunde sedan sparas i kylskåp upp till 8 dagar. Små blodproppar formades runt en garnstump genom att koagulationen återställdes efter återtillsättning av kalcium. De garninbäddade blodpropparna sänktes därefter ner i försöksbägare fyllda med koksaltlösning. Två blodproppar tillverkades samtidigt, den ena utsattes för behandling (blodproppslösande läkemedel, ultraljud eller en kombination av de båda) och den andra användes som kontroll (ingen behandling). När blodproppens fibrinnätverk löstes upp ökade antalet röda blodkropparna i koksaltlösningen. Under behandlingen togs därför en liten mängd av koksaltlösningen vid upprepade tillfälle, och koncentrationen av röda blodkroppar mättes i vätskan. Därefter jämfördes skillnaderna i utsöndring av röda blodkroppar mellan de behandlade blodpropparna och dess kontroller.

Ultraljud med en frekvens av 1 MHz (1 miljon svängningar per sekund) pulsades genom att upprepande sända 100 pulser varje millisekund. Olika intensiteters effekt av denna ultraljudskombination på propplösningen utvärderades i kombination med läkemedel. Även försök där läkemedelslösningen pre-exponerades med ultraljud (utan någon blodpropp närvarande) utvärderades genom att först ultraljudsexponera läkemedlet under en timme, därefter tillsattes blodproppen i läkemedelslösningen. På detta sätt kunde mätningar göras för att utvärdera om ultraljudet förändrat läkemedlets egenskaper. Även blodproppar pre-exponerades med ultraljud innan de kom i kontakt med läkemedlet (reteplase). Detta för att utvärdera om ultraljudsexponeringen åstadkom förändringar i blodproppen som gav upphov till ökade effekter av det propplösande läkemedlet.

Inledande försök med mikrobubblor har även utförts. Dessa försök var tänkta att ligga till grund för val av ultraljudsparametrar vid framtida propplösningsförsök med hjälp av mikrobubblor och ultraljud. Kartläggningen utfördes genom att utvärdera hur olika ultraljudskonfigurationer påverkade destruktionshastigheten hos mikrobubblorna. Mikrobubblorna som användes var Sonazoid® som är gjorda för att användas som kontrastmedel vid diagnostiskt ultraljud. Mikrobubblorna blandades i provrör där det ena provröret ultraljudsexponerades och det andra lämnades oexponerat. Mikrobubblorna har en diameter mellan 2,4 – 3,5 µm, som något mindre än blodkroppar. Likheten i storlek med blodkroppar gjorde det möjligt att räkna antalet mikrobubblor i en maskin som normalt räknar blodkroppar. Beräkningar av destruktionshastigheten utfördes därefter vid olika tidsintervall av ultraljudsexponering.

Två olika metoder användes för att utvärdera om de ultraljudsparametrar som verkar positivt vid blodproppslösning kunde medföra ökade skador på vävnad som inte var cirkulerad. I det första försöket användes en grismodell (III) där man under öppen hjärtkirurgi stängde av blodflödet i ett av hjärtats blodkärl under en timme. När skadan uppstått, applicerades ultraljudet med intensitetsnivåer som visats fungera vid blodproppslösning. Ultraljudsexponeringen pågick under ytterligare en timme. Därefter togs hjärtat ut och små områden av hjärtmuskel togs där ultraljudet varit applicerat. Som kontrollmaterial togs hjärtmuskel från områden där inget ultraljud varit applicerat. Utvärdering av skadegraden utfördes i ultraljudsexponerad hjärtmuskel och jämfördes därefter med skadegraden hos oexponerad hjärtmuskel. Skadegraden i hjärtmuskelbitarna undersöktes med hjälp av mikroskop.

I det andra försöket utvärderades effekterna av ultraljudsexponering på icke genomblödd hjärnvävnad hos råtta (IV). Cirkulationsstopp inducerades genom att placera en tunn nylontråd i ett av de kärl som försörjer hjärnan med blod. Därefter exponerades hjärnvävnaden för antingen ultraljud av tidigare nämnd effekt eller bara för ultraljudsgivaren. Detta medförde att hälften av råttorna exponerades för cirkulationsstopp och ultraljudsexponering och den andra hälften av råttorna utsattes endast för cirkulationsstopp. Efter 1,5 timmas cirkulationsstopp med eller utan ultraljudsexponering avlivades råttorna och hjärnorna togs ut. Därefter snittades hjärnorna i tolv 1 mm snitt och färgades in. Frisk hjärnvävnad färgades då röd och den skadade vävnaden lämnas ofärgad (vit). På detta sätt kunde jämförelse av storleken på de områden som skadats av cirkulationsstoppet uföras och jämföras med de områden som utsatts för både cirkulationsstopp och ultraljudsexponering.

7.3 Resultat

Ultraljudsexposition av streptokinas vid låga intensiteter (< 0,5 W/cm^2) ökade effekten av läkemedlet när detta ultraljudsexponerades före eller tillsammans med blodproppen (31 respektive 33 %). Vid höga intensiteter (> 2 W/cm^2) minskade effekten av läkemedlet (25 %) både då läkemedlet pre-exponerades med ultraljud och när blodproppen läkemedels- och ultraljudsexponerades samtidigt. I intensitetsområdet mellan höga och låga intensiteter kunde ingen förändring i läkemedelseffekten ses.

Pre-exponering med ultraljud och exponering av blodproppen för reteplase och ultraljud samtidigt ökade effekterna av läkemedlet i det låga intensitetsområdet (0,125 W/cm^2). De ökade effekterna av förbehandlingen försvann dock vid höga intensiteter, men kunde ses då blodproppen exponerades för reteplase och ultraljud samtidigt. Ökade effekter av reteplase såg även då blodproppen pre-exponerades med höga intensiteter (4 W/cm^2) av ultraljud. Ultraljudsexponering åstadkom ingen minskning av reteplaseeffekten vid någon intensitet, dock fanns det intensitetsområden där ingen ökad effekt kunde sågs.

Kartläggningen av Sonazoid® mikrobubblor visade att destruktionshastigheten var hög vid en ultraljudskonfiguration av låg frekvens och måttligt höga intensiteter, och att det fanns ett pulstågsberoende för att destruktionen skulle vara

fortsatt hög. Vid högre frekvenser var destruktionshastigheten betydligt långsammare även vid höga intensiteter av ultraljud.

Utvärderingen av eventuella oönskade effekter vid långtidsexponering med ultraljud på hjärtmuskel visade att ultraljudsexponeringen markant ökade skadorna i de områden som inte varit cirkulerade. Det fanns också skador efter ultraljudsexponering i områden som haft god cirkulation, dock är dessa resultat svårtolkade då det fanns en grundskada även i denna vävnad.

Den ultraljudskonfiguration som visat vara skadlig vid exponering av hjärtmuskel gav inga ökade skador i försöken på icke-genomblödd hjärnvävnad hos råtta. Inga skador kunde heller ses på den hjärnvävnad som haft god cirkulation under ultraljudsexponeringen. Dock är detta en liten begränsad studie som bör anses som en pilotstudie.

7.4 Slutsatser

Resultaten från studierna i denna avhandling visar på vikten av att utvärdera både de goda effekterna och de oönskade effekterna av ultraljud då detta används för att förstärka propplösande behandling.

Slutsatserna av de enskilda delarbetena är:

- att låga ultraljudsintensiteter ökar effekten av streptokinas. Dessa effekter sågs inom ett smalt intensitetsområde. Ökade effekter av läkemedlet kan uppnås genom pre-exponering av läkemedlet. Minskade effekter av streptokinase såg vid höga ultraljudsintensiteter.
- att ultraljudsexponering i kombination med reteplasebehandling ger upphov till två olika mekanismer, en där ultraljudet påverkar läkemedelsmolekylen, vilket medför ökade effekter, samt en där ultraljudet påverkar blodproppen så att strukturen förändras vilket också medför ökad effekt av läkemedlet.
- att ultraljudsexponering vid låga frekvenser ger snabb bubbeldestruktion, men vid högre frekvenser ses en långsammare destruktion. Minst 5 cyklers pulstågslängd krävs för snabb bubbeldestruktion. För fortsatt snabb bubbeldestruktion krävs en lägsta intensitet av 0.5 W/cm^2.
- att ultraljudsnivåer som visats åstadkomma positiva effekter vid propplösande behandling ger ökade skador på icke genomblödd hjärtmuskel.
- att ultraljudsnivåer som visats åstadkomma positiva effekter vid propplösande behandling inte verkar ge ökad skadeutbredning på hjärnvävnad.

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Appendix:

Original Papers

I. Low energy ultrasound exposure of the streptokinase molecule may enhance but also attenuate its fibrinolytic properties.
Madsen Härdig B, Persson HW, Olsson SB.
Accepted for publication in: Thromb Res. 2005 Jun 30; [Epub ahead of print].

II. Direct action on the molecule is one of several mechanisms by which ultrasound enhances the fibrinolytic effects of reteplase.
Madsen Härdig B, Persson HW, Olsson SB.
Submitted.

III. Can pulsed ultrasound increase tissue damage during ischemia? A study of the effects of ultrasound on infarcted and non-infarcted myocardium.
Olivecrona KG, Madsen Härdig B, Roijer A, Block M, Grins E, Persson HW, Johansson L, Olsson SB.
BMC Cardiovascular Disorder 2005, 5: 8.

IV. Does low energy ultrasound known to enhance thrombolysis, affect the size of ischemic brain damage?
Madsen Härdig B, Persson HW, Gidö G, Olsson SB.
J Ultrasound Med 22:1301-1308, 2003; (Reproduced in this dissertation by the kind permission from the publisher: American Institute of Ultrasound in Medicine).