LECITHIN COATED MICROBUBBLES ENHANCED SONOTHROMBOLYSIS OF DEEP VEIN THROMBOSIS.

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I

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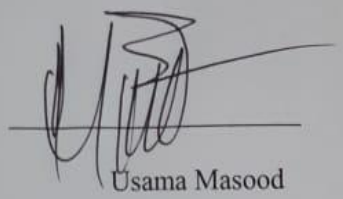

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Dedication

I want to dedicate my thesis to my late parents, Zahra Shoaib and M. Faiq Rizwan.

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Abstract

Microbubble-mediated sonothrombolysis is a promising approach for thrombus treatment. The objective of this in-vitro study was to examine the effectiveness of in vitro sonothrombolysis utilizing several combinations, including parameters of Diagnostic Ultrasounds, microbubbles, and thrombolytic drugs (Streptokinase). The thin-film hydration approach was used for the synthesis of lecithin micro-bubbles consisting of Perflurohexane core. The microbubbles synthesis was confirmed by the FTIR and SEM examination. Human whole-blood clots were synthesized exposed to different combinations of ultrasound parameters, microbubbles, and thrombolytic treatments. Sonothrombolysis was performed at diagnostic frequencies 3.5 MHz and 7 MHz at mechanical index (0.1, 0.3, 0.6, 0.9, and 1.2) at the various combination of study groups. Thrombolysis efficacy was assessed by measuring clot weight changes during 30-min US exposure, by recording the mean gray intensity from the US images of the clot by computer software IMAGE J, and spectrophotometric quantification of the hemoglobin in the effluents that were collected from a blood clot after 30minutes exposure of US. The most efficient clot lysis was obtained at US frequency 7 MHz and mechanical index of 1.2 in combination with drug-loaded microbubbles (US+Drug loaded MB). This in vitro study showed the potential use of ultrasound in combination with drug and microbubble and can further evaluate the sonothrombolysis at diagnostic Ultrasound parameters in the in vivo studies as well.

ABBREVIATIONS

SEM	Scanning Electron Microscopy
FTIR	Fourier Transmission Infrared Spectroscopy
PCL	Polycaprolactone
MHz	Mega Hertz
PFC	Perfluorocarbon
MB	Micro bubble
MI	Mechanical Index
SL	Soy Lecithin
MRI	Magnetic resonance imaging
US	Ultrasound
PFH	Perflurohexane
CVD	Cardiovascular Disorder
DVT	Deep vein Thrombosis
PET	Positron emission tomography
MRI	Magnetic resonance imaging
CT scan	Computed tomography scan.
USCA	Ultrasound contrast Agents
RBCs	Red Blood Cells

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INTRODUCTION

1.0 INTRODUCTION

Thrombosis and hemorrhage lead to sudden and possibly permanent neurological disorders, such as the ability to move the limbs, the formulation, and understanding of language and vision problems (The Lancet 2009). Due to the rising prevalence of stroke risk factors in the increasingly aging and modern populations, the mortality rates of stroke worldwide increased dramatically from 9.7 to 10.8 percent in 2004. The blockage due to blood clots in ischemic strokes, often in the middle cerebral artery, which is the longest blood artery supplying the brain with blood (Pai, Varma, and Kulkarni 2005), accounts for 87% of all strokes (Wadey et al. 2009). Thrombosis also represents the leading cause of myocardial infarction, lung embolism, and deep vein thrombosis (DVT). DVT can start without symptoms but typically occurs in the legs, leading to widespread pulmonary emboli, with complicated pathophysiology and considerable financial strains. It affects over 300 thousand patients in the United States and causes 60 thousand to 100 thousand deaths per year(Motykie et al. 2000)(Beckman et al. 2010).

1.1 Mechanism of thrombus formation and its roll in Cardiovascular disorders and deep vein thrombosis.

Thrombus formation is the situation in with blood is clotted in the blood vessels and it stops the blood flow towards the vital organ of the body. And it would lead to different diseases like cardiovascular disorders (CVD) including myocardial infarction commonly due to heart attack, angina coronary artery diseases, Brain stroke, deep vein thrombosis, and pulmonary embolism (Saric and Kronzon 2012). Myocardial infraction majority happens due to the death of the cardiac tissue. And this condition occurs due to the embolic atherosclerotic thrombi in the coronary arteries. (Cosselman, Navas-Acien, and Kaufman 2015) There are approximately more than 6 lacks cases of myocardial infarction each year with a mortality rate of 17% each year. Thrombus formation in the condition of atherosclerotic disorder having the composition of the RBCs rich fibrin mesh (Bansal 2020). Thrombus

formed that is not very stable at an early stage if it is not stabilized by the fibrin so it may delocalize and move to other locations of the body in the blood vessels transmural myocardial infarction occurs due to the complete blockage of the coronary artery that leads to the death of three layers of the tissues of heart (endocardium myocardium and epicardium (Van de Voorde et al. 2013). On the other hand, non-transmural myocardial infraction leads to the death of the myocardium layers of the tissue of the heart. cardiovascular disorders can also be characterized by the hindrance of the blood flow to the different parts of the body due to the formation of a vascular thrombus (Blood clots) (Beck et al. 1996). Bleeding from the injured blood vessels can be prevented by the formation of the thrombus fibrin and platelets are involved in the formation of the mesh at the injury site and create the clot to prevent the bleeding. During normal conditions, the blood-clotting protein fibrin is cut by the help of plasmin to initiate the normal repairing mechanism of injured vessels (Nichols et al. 2014). But some time during hypercoagulability (pre thrombus formation condition) and the hemodynamic situation lead to abnormal blood clotting in the vessels but according to the Virchow triad increase of the lipid and the cholesterol profile in the tissues of the blood, arteries are also vulnerable and lead to the formation of the thrombus because rupturing of the cholesterol and lipid-rich plaque in the tissues of arteries leading to the formation of platelets and fibrin mesh of the thrombus, thrombus formation create the hypoxic situation for the part of the body where obstruction of the blood occurs and lead to the death of the tissues (Foley, Parfrey, and Sarnak 1998). Histological analysis of the thrombus that was retrieved from the patients regardless of the location, maximum of them have same composition profile. Thromboembolism is the condition in with a thrombus is broken down into different parts and travel along the with the bloodstream and struck in the vessels and cause the blockage of the blood vessels. Several causes of the thrombus formation led to the various class of disorders major of them are cardiovascular (CVD) disorders (Rose 1981). Major causes of it unhealthy diet with a high content of fat and lipids

and maximum amount of alcohol and cigarette consumption and some of us jeopardize of CVD situation due to the side effects of certain medications like aspirin that lead to the situation that cause the changes in the hemodynamics and initiate the formation of thrombus (Verhaar, Stroes, and Rabelink 2002)

1.2 Thrombolytic Drug as front-line therapy

There are two major classes of blood clotting drugs worldwide that contain heparin and warfarin. Heparin is usually prescribed to prevent blood clots in a patient who already has thrombosis. Warfarin is recommended for patients who are at risk of developing blood clots. One of the side effects of warfarin is that patients who have been on the drug for so long should be monitored because if it exceeds the limit, the blood clotting stops at the site of the injury. The amount of medicine should be monitored. (Rosamond et al. 2007)

Recanalization of the obstructed blood vessels can be related to the functional clinical results for deep vein thrombosis brain stroke and myocardial infarction (Francis et al. 1992). Despite the location of the thrombus, thrombus breaking can speed up by thrombolytic drug or mechanical therapeutic procedure. The main mechanism of the action of many thrombolytic drugs is to break the bonds of the plasminogen to create the plasmin, and plasmin can also be used as the thrombolytic drug that induces the lyses of the fibrin and initiate the process of the thrombolysis (Hitchcock et al. 2011). Two drugs recombinant urokinase and streptokinase are used as a thrombolytic therapeutic drug, but these drugs don't have specificity for the protein fibrin and their microbial origin brings a lot of side effects. With these side effects, the medical industry moves toward a drug that should be specific for the fibrin. Recombinant Tissue plasminogen activator and its derivatives are currently in use as a primary thrombolytic drug (Shi et al. 2000).

But the administration of the recombinant tissue plasminogen activator has some strict exclusion criteria so only 1.5% of the patients of total ischemic stroke can have the rtPA. (Ricci et al. 2012)

1.4 Sonothrombolysis

Sonothrombolysis is the use of ultrasound waves to improve coagulation therapy. Since the 1980s it has been observed to use ultrasound-enhanced thrombolysis (UET). Sonothrombolysis is commonly used to improve the results of thrombolytic therapy. This treatment strategy benefits from the biomechanical effects of ultrasounds to help thrombolytic medicines spread to the blood and mechanically break down blood coagulation. The usual appropriate improvement mechanism for coagulation is that ultrasound induces stable cavitation and inertial cavitation.

Fine flow and acoustic radiation power to 'weak' fibrin coagulations temporarily and increase the diffusion of thrombolytic drugs into the blood clot, thus increasing the pace. Deep vein thrombosis is most successful in treating this condition. Several research and clinical studies have shown that ultrasound contrasting agents (MBs) can be used to improve colt lysis with tPA and thus to reduce the blood coagulation while reducing target debris, by increasing the desired amount of cavitation.

The introduction of microbubbles will increase Acoustic cavitation and thus improve the distribution and penetration of the thrombolytic drug into the blood clot and improve the recanalization with decreased complications, such as intracranial hemorrhage symptoms (Bor-Seng-Shu et al. 2012)(Ricci et al. 2012). in a clinical trial of transcranial sonothrombolysis the used ultrasound waves and tPA, it showed there has been a trend towards early recanalization and increased clinical cure rates relative to normal intravenous therapy. Furthermore, it was found that a range of commercial ultrasound devices approved US Food and Drug Administration (USFDA) can achieve satisfactory and comparable efficiency in in-vitro coagulation when the acoustic performance is high enough despite significant differences (Yufeng Zhou and Ramaswami 2014).

1.3 The Acoustic Principles of Ultrasound Contrast agents.

The phenomena of backscattering happen when the waves of the sounds hit the microbubbles and they get reflected. both of them reflected wave and a backscattered wave would have the same amplitude of the wavelength (Choudhury et al. 2017). An ultrasound contrast agent is known as the best backscattering agent that increases the gray intensity up to 30dB and backscattering phenomena are also known as the linear behavior of the microbubble as well. (Yang Zhou et al. 2013)

On other hand with the increase of the mechanical index (sound pressure) would be the result of an increase in the non-linear behavior of the USCAs (microbubble). (Samuel et al. 2012) First, e appearance of microbubble oscillations triggers harmonic oscillations gradual increase of the sound pressure brings instability in the USCAs microbubble. This is the result of the rupturing of the USCAs microbubble. (Wang et al. 2012). Due to this short high energy signal sent out due to the stimulated acoustic emission.

USCA microbubble fabricated of lipids shell is flexible that gives oscillations at the low sound frequencies and appeared with prominent effects of harmonics (Tsivgoulis et al. 2010). On other hand USCAs microbubble fabricated hard polymer shell give enhanced SAE signal when they get rupture.

If the value of the high rare fractional pressure is divided by the square root of the ultrasound frequency, the resulting value is known as the Mechanical Index (MI).(Schutt et al. 2003). Commonly in ultrasonography machines, these MI values are displayed on the screen and MI ranges from (0.1 – 2.0). if the value of mechanical index MI is low, it means that the microbubbles can stay stable while the intonation power of the microbubbles depends upon the value of the mechanical index of the ultrasounds machine if the value of the mechanical index is in between 0.2- 0.5 there will be the linear oscillation in the microbubble and if the value of mechanical index increases from 1.2- high it brings non-linear oscillations in the

microbubble and microbubble become unstable due to expand out of its limits and ultimately will get rupture. (Matsunaga et al. 2012)

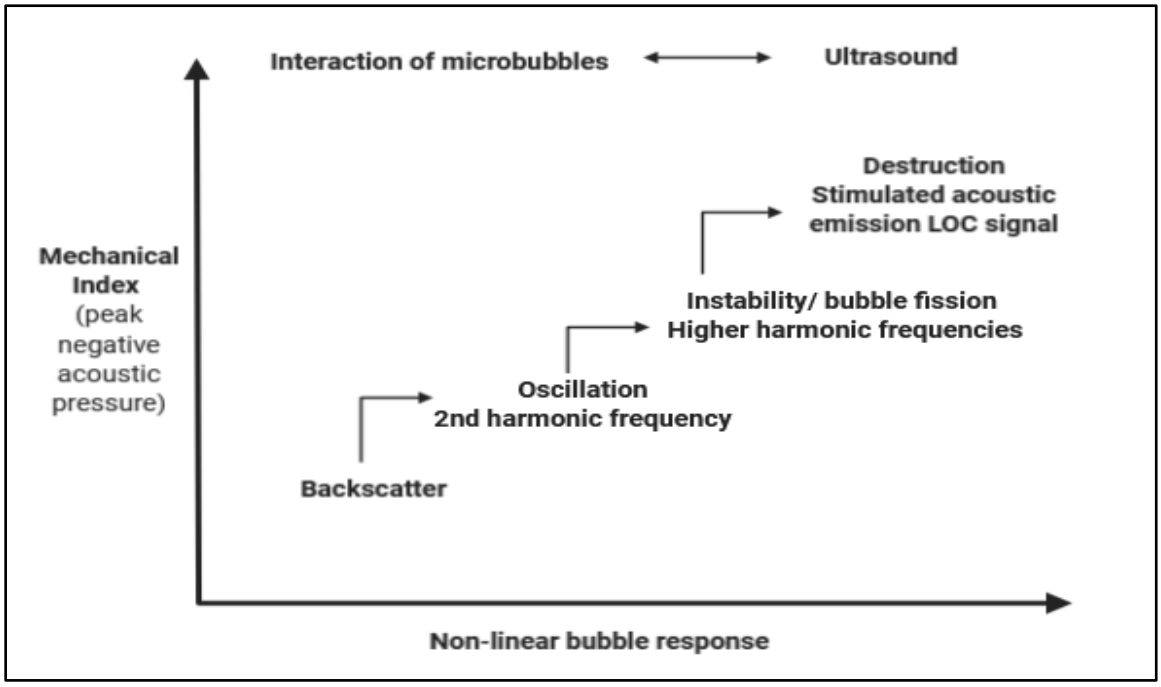

Figure 1: Behavior of microbubbles in the ultrasound field.

1.5 USCAs mediated Sonothrombolysis:

The Main Purpose of the use of eco-contrast microbubbles is to improve ultrasonic images by increasing the sound wave intensity (Vignon et al. 2013). Microbubbles undergo stable cavitation or second when a low-intensity diagnostic ultrasound beam is targeted. This increases the diagnostic quality of the ultrasound images obtained in patients as the ultrasound probe returns more sound with increased intensity and thus more information is available to the clinician (Rapoport, Gao, and Kennedy 2007). However, over the past two decades, the effects of additional treatment of microbubbles have been discovered Unlike diagnostic ultrasound, therapeutic ultrasound consists of high-intensity ultrasound (Mullin et al. 2011). The

microbubble not only oscillates but also bursts and explodes under intense ultrasound pressure. This violent reaction releases energy into the local environment Because of this energy release, the application of therapeutic ultrasound as a method of therapy destruction in the setting of acute myocardial infarction and severe ischemic stroke has been the subject of intense research in human studies If the diagnostic and therapeutic ultrasound parameters are combined in the same probe, diagnostic ultrasound with microbubble infusion can detect obstructed vessels and the treatment can see the fullness of microbubbles in vascularity after application of ultrasound impulse These intense are almost always combined with a constant dose of load or fibrinolytic agents, and this method of treatment is often called sonothrombolysis.(Nakamura et al. 2016) Unfortunately, there is a relatively high incidence of bleeding as a side effect in fibrinolytic agents. Bleeding has a negative effect, for example, causing additional hemorrhagic stroke during treatment. The main goal of sonothrombolysis research is to minimize the risk of bleeding and maximize the effectiveness of treatment, while potentially reducing treatment delays and achieving early recovery (Gao et al. 2008). When sonothrombolysis is combined with a low dose of antithrombotic equal efficacy can be achieved by reducing the risk of bleeding. It is therefore important to increase the efficacy of ultrasound treatment while reducing the dose of fibrinolytic agents. This can be achieved using microbubbles possibly even without the use of a fibrinolytic agent. (Rosamond et al. 2007).

REVIEW OF THE LITERATURE

2.1 Commercially available USCA and its generations: Zero Generation microbubble

First time in (Kakkar et al. 1969) a physician named Geamaik and shah utilized the ultrasounds contrast agents in the echocardiography. They successfully visualize the aortic root by injecting of agitated saline water that increased the backscatter and reflected sound intensity coming back to the diagnostic probe (Damianou et al. 2014). After that a series of the testing for other stable ultrasound contrast agents was started since then various contrast agents were tasted like, dextrose iodinated contrast media, indocyanine dye and hydrogen per oxide. But they all have size limitation because they all USCA are larger than size of 8um and they cannot pass through the pulmonary capillaries bed. And for the opacification of the left ventricle, it needs to be administered intra coronary or intra cardiac injection. Therefore, they can't be used in the cardiovascular disorders (Hossmann 1998).

2.1.1 First generation microbubble:

In (Hirsh and Hoak 1996) a germen pharmaceutical industry Bayer and schering was the first company who fabricated the first-generation microbubble named livovist. Composition of this USCA was 99.9 % microbubbles and 0.1 % palmatic acid and core was of air enclosed in the shell. It was found that once intravenously delivered, it spread around in the blood within a few minutes. In addition, two minutes after injection, the spleen/liver specific step could be reached. The method on how Levovist is absorbed into the liver or spleen cells, though, is a little incomprehensible, however this behavior is said to have some parallels with the absorption scenarios (Hirsh and Hoak 1996). This behavior is said to have many parallels with the scenarios of uptake; first, when the Tc-colloid is absorbed during scintigraphy, and second, when the super paramagnetic iron oxide is absorbed by the reticuloendothelial system of the liver during MRI. The microbubbles of Levovist have an average size of 2-5 μm, but about 97 percent of

these microbubbles are below 7 μm. (Datta et al. 2008a) This scale is ideal for use since the lung microcapillaries are typically 7 μm deep and this enables the 2-5 μm microparticles to travel effectively for effective CEUS picture production to the liver or left ventricle of the heart. The air in these microbubbles has small molecules that cause the blood stream to disperse from the surface of the shell. One unfortunate outcome was that the air from bubbles will dissolve into blood faster than what is best desired, because air is readily soluble in blood. A polymer-based material was used for the formation to make the microbubbles more stable, but the image consistency did not go anywhere.

2.1.2 Second generation of USCAs

Another attempt to stabilize microbubbles has been made by changing the air for inert gas for example perfluoro butane or hexafluoride sulfide (SF6), that diffused relatively slower from microbubbles, leading to the formation of ultrasounds contrast agents of the second generation. Examples included the Definity (haves octa fluoropropane gas in a lipid shell), Sonazoid (SF6 in a phospholipid shell), and Optison shell (SF6 in a phospholipid shell), (HEPS) shell of perfluoro butane (albumin shell containing octofluoropropane). Optison and Definity were only approved in cardiac therapies (Sontum, 2008). In comparison to Vue, Kupffer and vascular images with Sonazoid are available and usually take about 10-15 minutes after the administration of US contrast agencies.

2.1.3 Third generation of the microbubble

The principle of microbubbles third generation consists of the development of contrast agents and the synthesis of contrast PFC microbubbles and emulsions. The objective was to bind ligand to a microbubble shell so that it could aggregate in tissue or pathology of interest. Microbubble may be engineered to target various diseases such as thrombosis, inflammation, ischemia, and tumors.

2.2 Mechanisms of Thrombolytic Enhancement

Previously Therapeutic interaction of the ultrasounds acoustic waves with tissue is well documented. (Perler 2005) Thermal effects of acoustic interaction with tissues is categorized as the primary mechanical effect for example radiation energy and acoustic cavitation effect categorized as the secondary mechanical effect. (Pasceri, Andreotti, and Maseri 1996)

2.2.1 Primary mechanical effect:

Absorption and scattering effect of acoustic waves increases the momentum energy of tissues and as result this force is known as acoustic radiation force (Viola et al. 2004). This force brings the acoustic streaming in the fluid that is helpful in the mixing of the thrombolytic drug and drug penetration on the thrombus. Moving ultrasound intonation produces the more acoustic streaming in the fluid as compared to the standing ultrasound intonation waves. In (Westermark et al. 1999) study the promising results of an in-vitro study that traveling intonation waves of the ultrasounds has more enhance phenomena of the thrombolysis at frequency of 2 MHz rather than the standing intonation waves of the ultrasound. In addition, 1-Hz pulse repeater frequency of the passing wave was used to test thrombolytic efficacy. These acoustic streaming effects are similar to the moderate stimulation of the clot around the medium and acoustic force of radiation has also showed the displacement of the thrombus in one the sin-vitro study this study has shown the results by the in-vitro thrombus model studies and the computational studies the displacement of the thrombus in implementation of the acoustic force of the radiation at frequency of the 1 MHz (Tang and Clement 2010). and it found the relation between the mean square velocity of the displaced clot and thrombolytic rate of the human blood clot at different changing frequency of the ultrasounds. Acoustic force of the radiation also act on the microbubble in several study of sonothrombolysis has shown the introduction of microbubble in the in-vitro clot apparatus and has some promising

results of enhancement of the thrombolysis at different frequency (Suchkova et al. 1998). The main principle of acoustic waves it brings the changes in radiation frequency and movement of the microbubbles at the area of clot that increases the thrombolysis effect.

2.2.2 Thermal effect of the Ultrasound

Incident waves of the ultrasound wave on the tissue is result into the heating of the biological tissue due to absorption of the ultrasonic waves. In the invitro thrombolytic studies Arhenius temperature describes the clot lysis by the recombinant tissue plasminogen activator. Enzyme activity is dependent on the increase of the temperature and it enhances the thrombolysis (Nahirnyak, Mast, and Holland 2007). But the several clinical and Insilco computational studies shows there is negligible increase of the temperature during thrombolysis in between frequency of 0.12 – 3.5 MHz in study (Mohr et al. 1983) shows the enhancement of the in-vitro thrombolysis at ultrasound frequency of 1 MHz that was combined with the increase of the heat. But this in-vitro study fails to explain the flow of the heat around the area of the clot (Shaw et al. 2006).

2.3 Secondary mechanical effect (Acoustic Cavitation)

2.3.1 Classification of the cavitation:

For twentieth century acoustic cavitation is been the topic of the study and it's been reviewed everywhere (Molina et al. 2006). Formation and oscillation that appears in the microbubble due to the ultrasounds pressure is called acoustic cavitation. Cavitation can be of two types, stable cavitation, and inertial cavitation. (Miller, Dou, and Wiggins 2008).

2.3.2 Inertial cavitation

Inertial cavitation effect brings the high expansion in the microbubble due to the high acoustic pressure that creates the high tension in the surrounding fluid (Vignon et al. 2013). As a result of this in microbubble burst in its last stages. High density energy is created by the covering liquid and heat due to acoustic pressure. If the rupturing of the microbubble is at the surface of the liquid it would produce a jet of the liquid, speed of 1Km/sec that is associated with the mechanical effect that break the blood thrombus in the in-vitro studies. In (E. P. Stride and Coussios 2010) an in-vitro study was performed to observe the liquid jet effect at area of the microbubble and visualize the thrombolytic effect in clot after incident of the acoustic waves. Acoustically the inertial cavitation can be observed by emission of the broadband. In (Weiss et al. 2013) in-vitro study has presented the result about the relation between the broad band emission and the enhancement in the thrombolysis and in another study in (E. Stride and Saffari 2003) it showed in the results of improvement in the fibrinolysis in an in-vitro studies only when the ultrasounds acoustic pressure brings the inertial cavitation effect in the microbubble. In (Tachibana and Tachibana 1995) in the in-vivo study, broad band emission also has shown the thrombolytic effect in the rabbit and porcine. In (Barreto et al. 2013) in-vivo study of canines has showed the positive result about the enhancement of thrombolysis only when the microbubble attains the state of cavitation effect.

2.3.3 Stable cavitation

Microbubble oscillation and motion remain steady level in stable cavitation and restoring force of the microbubble offset the liquid inertia (Roy et al. 1985). To initiate the stable oscillation in the microbubble it needs acoustic pressure lower than that required to start the inertial cavitation. And due to the nonlinear oscillation

results in the production of the microstreaming in the environment of the microbubble that is not only useful of mixing and penetration of the of the thrombolytic drugs (recombinant tissue plasminogen activator and plasminogen) in the blood thrombus but also enhance the process of the fibrinolysis (Salgaonkar et al. 2009).

Broadband emission was useful for the analysis of the inertial cavitation but the stable cavitation gives harmonics (multiple of integer of fundamental), sub harmonic (fractions of rational no less than one) and ultra-harmonics (fractions of rational number of fundamentals that is greater than one) emission spectra lines in the acoustic spectra. Is useful to detect the stable cavitation.

In (Bader et al. 2012) an in-vitro study presents its results that sowed a correlation between the high thrombolytic rate and ultra-harmonics in the emission spectra. In another (Aaslid, Markwalder, and Nornes 1982) in-vitro study showed that occasionally exposure of the ultrasounds waves to optimize the stable cavitation results in the high thrombolysis rate.

In (Commander and Prosperetti 1989) in-vitro study used a model to observe the continuously reduction the size of the blood thrombus due to the stable cavitation in the Definity microbubble and that stable cavitation was confirmed due to the ultra-harmonic emission spectra. And that study successfully correlates the ultra-harmonic emission spectra and decreasing size of the blood clot.

In (Commander and Prosperetti 1989) suggest the relation between the sub-harmonic's emission spectra due to the stable cavitation and enhancement of the thrombolysis and the relation between the ultra-harmonics and the sub harmonics was explaining in (Holland et al. 2008) in this same in-vitro study and in (W. S. Smith et al. 2008) explains the importance of the stable cavitation for the enhancement of the fibrinolysis by enzymes. In another study in (Apfel 1981) was recommended some rules to understand these two types of the cavitation frost was

one must know the acoustic field second; one must know about liquid and last one must know about instantaneously changes to optimize the process of the sonothrombolysis.

2.4 Different modes of ultrasound exposure for sonothrombolysis

2.4.1 Catheter's ultrasounds mediated sonothrombolysis.

Catheters of ultrasounds are widely used for a wide range of diseases. (Pajek et al. 2014) The catheter is placed into the blood clot so that the ultrasound and local infusions of medications of thrombolysis can be mixed. Using a collection of thin unfocused transducer positioned in the lumen of the catheter, a reasonably large area of the clot the exposing with the ultrasound's radiations can be achieved. With distance from the catheter, acoustic pressure decreases quickly as the transducers are small and unfocused. Additionally, in broad thrombi caused by intracerebral bleeding, successful catheter ultrasound sonothrombolysing has been done (Newell et al. 2011)

2.4.2 Transcranial Doppler mediated sonothrombolysis

Extracorporeal sonothrombolysis is an attractive, minimally invasive approach to the treatment of ischemic stroke paired with a thrombolytic injection intravenously and/or microbubbles. The cranial bone attenuates, mirrors and distorts the wave of ultrasound (de Saint Victor et al. 2014). Bone is also a significant impediment to Trans-Cranial Sonothrombolysis. The acoustic temporal bone window is routinely used to analyze cerebral blood supply by transcranial Doppler (TCD) (Aaslid, Markwalder, and Nornes 1982). Exposure to temporal bones accelerates the rt-PA thrombolysis, or transcranial color-coded sonography (TCCS)(Cintas et al. 2004b) . Related frequencies (around 2MHz) and similar ultrasound pulse waves were used for TCD and TCCS sonothrombolysis and equivalent outcomes were achieved in

clinical trial (Saqqur et al. 2014) TCD is based on operators, and the absence of qualified operators is an obstacle to broad adoption (Alexandrov and Barlinn 2012). A sonothrombolism instrument has been designed to be easier to incorporate and less operator dependent than TCD, to address this constraint. A frame with basic landmarks is placed on the head of the patient. Eighteen transducers are attached on the head frame to direct the ultrasound waves the two most common thrombin sites through acoustic windows during an ischemic stroke. This device's acoustic parameter are based on those of the FDA-authorized TCD devices In Phase I and II clinical trials the safety and relevance of this apparatuses is demonstrated (Barlinn et al. 2013). frequency of 2 MHz Ultrasound have poor results through the bone. An estimation of the in-situ acoustic pressure is made in 2012, based on CT results, for 20 subjects treated with TCD in the TUCSON experiment. For patients clinically dependent at 3 months, the acoustic pressures measured in situ were greater. Any patients could also be exposed to inadequate sound pressure with TCD in sonification to increase thrombolysis (Adeoye et al. 2011)

2.4.3 Sub-megahertz Ultrasound mediated sonothrombolysis.

The sub-megahertz frequency spectrum enables effective acoustic propagation through the skull. In five human crane specimens (Datta et al. 2008a) evaluated a decrease in acoustic pressure in bones at 2 MHz from 77.1% to 96.6%. A decrease of the acoustic pressure at 120 kHz was observed between 22.5 and 45.5%. There is a greater degree of insonation than 2-MHz TCD for Sub-megahertz Sonothrombolysis.at 300 KHz of frequency. (Baron et al. 2009) simulated TRUMBI insonate numerically and observed that standing waves may have triggered the highest unpredictable local pressure due to acoustic reflections in the cranial cavity. Reflection from the contralateral bone and related constructive interference for 2-MHz TCD is less possible because the wave is greatly reduced as the brain tissue is propagated. The TRUMBI trial and following studies have demonstrated the need for a healthy and effective sonothrombolysis with a well-tested transcranial

ultrasound area Taking these results into account, revised transcranial sono-megahertz devices were created. Taken into account For transcranial insonation a two-frequency array was built that can conduct 2.5-MHz TCCS and emit a 500-kHz sonothrombolysis beam (Shimizu et al. 2012).This system has evaluated in-vitro the transcribable ultrasound area, and the protection of this method has been demonstrated in a stable primate model(Shimizu et al. 2012). et al. (Bouchoux et al. 2012) simulated transcranial ultrasound 120 and 500 kHz fields of head CT scans of 20 ischemic stroke patients by means of a computational model of validated acoustic diffusion. The MCA section was insonated reliably and homogeneously at both 120 kHz and 500 kHz. (Bouchoux et al. 2012) suggested a positioning method focused on external head points that did not require knowledge of the location of the thrombus and were close to an optimized technique of transducer positioning based on CT data analyzes. The local maximal acoustic pressure was well controlled and comparable to amplitude in the target M1 section of the MCA by representing the contralateral bone. Sub-megahertz trans-cranial exposure in ischemic patients without the exclusion of subjects with an inadequate time-bone window for TCD or TCCS will allow for easy and reliable insonation of the thrombus. Carefully designed and tested instruments for sub megahertz sonothrombolysis A simulation or in-vitro measures may be used to test the Tran scripted ultrasound region in many cranes. In addition, sub-megahertz sonothrombolysis systems should be tested for the positive interference arising from standing waves in a big animal model. The wave can also be used to eliminate the wave standing frequency random modulation. (Tang and Clement 2010); (Azuma et al. 2010)

2.4.4 Focused Ultrasound mediated sonothrombolysis.

Focused ultrasound thrombolysis is also a promising method. An ultrasound beam that is very oriented facilitates precise spatial targeting of the blood clot. Locally,

high-pression amplitudes with low chance of side effects may be extended beyond the target field. Additionally, clot thrombolysis based on inertial cavitation erosion can be done without a thrombolytic treatment by means of centered ultrasound Responsive thrombosis. (Maxwell et al. 2011) used the pig model to treat the thrombus using ultrasound frequency of 1 MHz similarly another in vivo study has used the rabbit model in which they used the 1.5 MHz ultrasound frequency to treat the thrombus in the femoral arteries The target region, though, is typically greater than the specified vessels. Careful experiments on the tissue underlying the blood clot are therefore necessary. It was also proposed to treat ischemic stroke using an MR-guided transcranial ultrasound-focused system ((Yamashita et al. 2009). This is a 1000-element 220-kHz hemispheric array which can generate a 4 μm transcranial focus point electronically controlled in 6 mm. The study by (Alexandrov et al. 2000) is therefore quite early, and further tests are required to determine this approach's feasibility and protection.

2.5 In-vitro and ex-vivo studies mediated sonothrombolysis.

In-vitro studies analyze the interaction of the ultrasound waves with the clot by minimizing the problem related to the biological model animals in in-vivo studies in a controlled setup. In-vitro clot models fabricated according to the prescribed protocol that were related to the biological thrombus (Mousa 2010). clot has been formulated by calcification of the blood and then provide incubation under the temperature of the 37 ^{0}C and followed by the refrigeration of 4 days for proper clot retraction (Monteith et al. 2013). In another invitro study blood clot model was prepared by addition of the thrombin in the blood plasma (Molina et al. 2009). Retraction of the human blood clot depends upon the surfaces in which clot use to incubated and changes of temperature of refrigeration, moreover clots were retracted in the clod temperature for several days after post incubation for proper

retraction. (Watson, Shantsila, and Lip 2009). The process of the thrombolysis can be detected by the mass loss of the clots that were analyzed in the ultrasound's images (Francis et al. 1992) and presence of degraded product of fibrin in the effluent. In (Chen et al. 2009) study about the sonothrombolysis on the animal model it is very important to know the effect the ultrasound waves on the animal model tissues. For this purpose, animal tissues were taken from animal model and then exposed to the waves of ultrasound and thrombolytic drugs. And as specifically study of the thrombus, excised arteries were exposed to the ultrasound waves to observe the effects of ultrasounds on the endothelium (most delicate tissue) tissues. In (Wu et al. 2014) study the sonothrombolysis on the animal model one should most assure about the response of the animal model during the treatment of sonothrombolysis and progress of the treatment moreover effects of the thrombolytic treatment and ultrasound waves and its damaging effects on the tissues of the animal model. All these parameters should be approved from the food and drug administration (Harris 2009). there are a lot of studies on thrombolysis has been made and for this a lot of the clot model has been made. All the studies were done so far on the small animal models like rodents and rabbits. To progress in the studies of the sonothrombolysis we need to study this on large animal model like primates to progress the study of sonothrombolysis of brain i.e., ischemic stroke clot fabrication initiated by the autologous incubation of the blood in the arteries of stenosis moreover laser beam of the high intensity can also be used to induce the clot formation in the animal model (Hossmann 1998). And fibrin degradation product and velocity flow of the blood in the blood taken as tools to analyze the efficacy of sonothrombolysis. (Ricci et al. 2012)

2.5.1 Ultrasound therapy without thrombolytic drug

In the therapy of the ultrasound in the absence of the thrombolytic drug was initiated by the mechanical effects of the ultrasound waves. And in this case the reduction of the clot size was observed in 10 out of the 12 models. 1 MHz of the ultrasound frequency was used in the form histotripsy pulses. (Kondo et al. 1999) evaluated the pulse form of the ultrasound waves was more effective in the thrombolysis as compared to the continuous application of thrombolysis. Moreover, in (Saric and Kronzon 2012) it was observed in the study of effectiveness of the pulsed ultrasound waves application in both in-vitro and in-vivo thrombolysis. But a mild tissue damage was also observed in the form of the hemorrhage and the denudation of the tissue of the endothelium. And this damage can be discus by the focused zone of the ultrasound's waves and the small diameter of the blood vessels. (Turgut and Bates 2000)

2.5.2 Microbubble mediated sonothrombolysis without thrombolytic drug

there are many in-vitro and in-vivo studies that evaluated the result of the sonothrombolysis. And in in-vitro study that showed the promising results of the clot lysis on upon treating the clot with ultrasound waves with microbubble (Hitchcock et al. 2011). These studies are important to show the importance of the cavitation effects of microbubble during interaction with clot at different frequency and mechanical index of ultrasound waves different size and types of the microbubble was formulated that are target specific and location specific as well, microbubble use as theragnostic agents that not only visualize the clot lyses as well as therapy agent as well. In (Birnbaum et al. 1998) showed the result of the microbubble mediated sonothrombolysis in animal model of the porcine and in (Faez et al. 2013) showed the result of microbubble mediated sonothrombolysis in

rabbit model in which carotid arteries were treated with acoustic pulse with frequency of 1- MHz and with thrombolytic drug called recombinant tissue plasminogen activator. But these studies did not show any significant volume of thrombolysis if clot model was treated with targeted and non-targeted microbubble. But in (White and Chew 2008) has shown the promising results of high-rate thrombolysis in which clot was treated with the ultrasound and microbubble but it should pe in the notice, the parameter set for the microbubble mediated sonothrombolysis would not be applicable for ultrasound thrombolysis and thrombolysis with thrombolytic drug alone.

2.5.3 Sonothrombolysis with thrombolytic drug.

Sonothrombolysis alone is the best was to overcome the contraindication of drugs used for the sonothrombolysis. But use of the acoustic waves with the thrombolytic drug is the best way to use this combination in the clinical practice (Verbeuren 2006). In this mechanism of treatment ultrasound use as the adjuvant with the low dose of the thrombolytic drug rather than using high dose of it to reduce side effects. In one of the study in 2007 research group found increase of the thrombolytic effect of recombinant tissue plasminogen effect when its use with the ultrasound of frequency of 120 KHz(Westermark et al. 1999). In another study, in (Hua et al. 2014) it was demonstrated the increase in the efficacy of the thrombolytic drug will be observed when cavitation effect was detected. So its mean cavitation effects is the prime line parameter to use the acoustic wave for thrombolysis.

2.5.4 Microbubble and thrombolytic mediated sonothrombolysis

Stabilizing the microbubble for the diagnostic purposes reducing the cavitation effect for the therapeutic efficiency for thrombolysis (Sarkar, Katiyar, and Jain 2009). (Tachibana and Tachibana 1995) first who utilized the microbubble in the sonothrombolysis. They used the USCAs named Albunes along with the acoustic

wave of 170 KHz that increased the efficacy of the thrombolysis. And thrombolytic rate was high as compared to urokinase used alone. However, these studies showed the promising result of increase of the thrombolytic effect when drug used along with the sub-megahertz acoustic waves as well as in frequency range of diagnostic range. In study of (Dwedar et al. 2014) showed the results of increase in the thrombolytic effect in the brain with color coded Doppler. In this study comparison of the treatment rt-PA+ sono- Vue microbubble with the treatment in which rt-PA used alone, so combination of the rt-PA and microbuuble showed the less edema and lesion in result as compared to the results in wich rt-PA used alone. In (Birnbaum et al. 1998) showed the comparison of microbubble + thrombolytic druge with the thrombolytic drug that used alone with ultrasound waves. Results showed significantly less infract volume of the carotid artery of rabbit when ultrasound used with drug and microbubble as compared to the treatment in which ultrasound used with ultrasounds waves. The new ex-vivo porcine carotid model was created by (Hitchcock et al. 2011), and the effectiveness of Definity and Rt-PA was significantly measuring when sub-megahertz ultrasound was subjected to rt-PA alone. This model was generalized by(Sutton et al. 2013) and suggested an improvement in thrombolysis in un retracted clots element. (Kutty et al. 2012) found that in the pig atherosclerotic model epicardial rechanneling was the highest in which Doppler pulses caused Definity cavitation in the presence of rt-PA. The drug-powered microbubbles also can improve thrombolytic effectiveness as a "theragnostic" strategy. As a vector for the treatment of pharmaceutical medications, echogenic liposomes (ELIP), which contain air-filled microbubbles, may also be used. The use of rt-PA in ELIP or t-ELIP facilitates acoustic stimulation of localized drug delivery (D. A. B. Smith et al. 2010). The increase in thrombolytic effectiveness of t-ELIP and ultrasound over the rt-PA, or combined over rt-PA, ultrasound, and Optison TM has been shown in in vitro tests (D. A. B. Smith et al. 2010). Targeted microbubbles loading rtPA and 2-MHz ultrasound, (Daffertshofer et al. 2004) thrombi-treated in the rabbit femoral artery. The recanalization of

microbubbles obtained with rt-PA was nearly close to those achieved with untargeted microbubbles and free rtPA.

MATERIALS & METHODOLOGY

3.1 Preparation of Perflurohexane emulsion

For the encapsulation of the Perflurohexane in the liposomal shell, Perfluorocarbon micellar emulsion was prepared. 10 ml of 2.5 % solution of Tween 80 was prepared and 0.5 ml of PFH was added drop by drop to this solution on a magnetic stirrer until the PFC is dissolved.

3.2 Preparations of Lecithin liposomes by thin-film hydration methods

Liposomes were prepared by the method of thin-film hydration. For this 50 mg of the soy lecithin and 10 mg of the cholesterol (Sigma) were taken in the round bottom flask and dissolved in the organic phase of chloroform. After this chloroform was evaporated under the vacuum at a temperature of 50 ^{O}C at the apparatus of the rotary evaporator until the thin film was deposited at the round bottom flask. And this film was dried for 1 hour under the vacuum. And the thin film was rehydrated with the prepared PFH emulsion. 10 ml of the Perflurohexane emulsion was added to the flask and heated above the transition temperature of the soy lecithin (Tm = 50 ^{0}C). for encapsulation of PFH with soya lecithin. The liposomal suspension was extruded three times through the vacuum filter consisting of filter paper, size of 3 micrometers to reduce the size of the liposomes. Then emulsion has purified the centrifugation at 6,000rpm for the time of 1 hour. The resultant pellet was collected and resuspended in the distilled water and the supernatant was discarded.

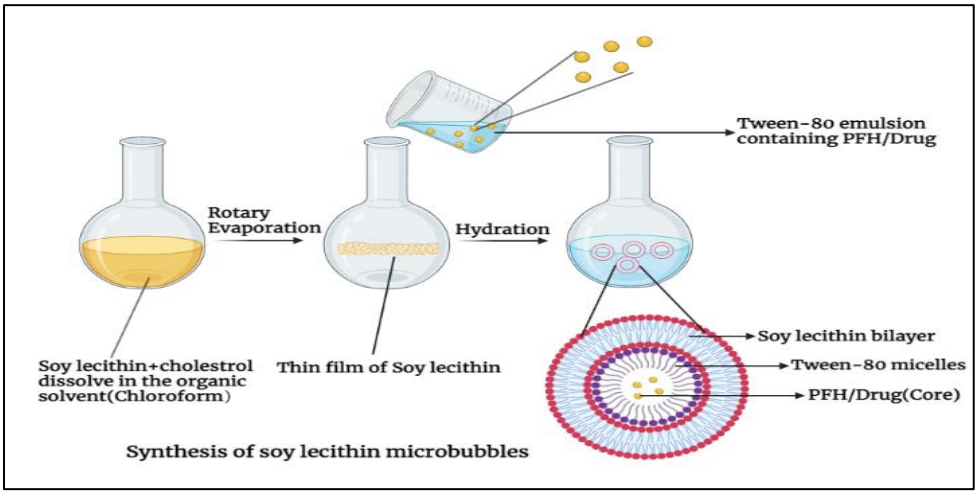

Figure 2: Schematic diagram of synthesis of lecithin microbubbles.

3.3 Preparation of the streptokinase-loaded lecithin liposomes.

The thin-film hydration method was used to prepare Streptokinase-loaded liposomes.25 mg of the soy lecithin and 5 mg of cholesterol were dissolved in the 10 ml of the organic solvent (chloroform) in the round bottom flask. A rotary evaporator was used to evaporate the solvent under vacuum conditions at the temperature of 50 °C for one hour and a thin film was deposited in the round bottom flask. And after this thin film was hydrated with the Streptokinase emulsion, this 0.5g of Streptokinase has dissolved in 10 ml of 2.5 % tween 80 solutions on the magnetic stirrer. After that rehydrated film was again set at rotary evaporator in vacuum condition for 10 minutes at a temperature of 50 °C. The streptokinase-loaded liposomal suspension was extruded three times through the vacuum filter consisting of filter paper, size of 3 micrometers to reduce the size of the liposomes. Then emulsion has purified the centrifugation at 6,000rpm for the time of 1 hour. The resultant pellet was collected and resuspended in the distilled water and the supernatant was discarded.

3.4 Free drug separation

Centrifugation was performed at 20,000g for 60 minutes at 4 °C of the temperature to separate the free or unencapsulated streptokinase from streptokinase encapsulated lecithin microbubble. Pellets were collected and washed twice with the washing buffer (pH 6 distilled water) and centrifuges again for half-hour (Panwar et al. 2010).

3.5 Reference standard preparation

By adding 1 g Streptokinase drugs to 1000 ml Dextrose water in a beaker, the standard stock solution for streptokinase was prepared. For 10 minutes the suspension was well stirred to achieve a clear solution. Four reference streptokinase standard solutions have been used for standard calibration peaks. The concentration of 0.1 0.2 0.3 0.4 and 0.5g/ml were used. The required reference solutions concentration was obtained with the formula V1C1=V2C2, as follow,

$$V1 = \frac{V2 \times C2}{C1}$$

Here,

- V1= Volume of the stock (**unknown**)
- C2= Concentration of the stock (0.5g/ml)
- V2 = total volume required for making standard sample
- C2 = the required concentration

3.6 Standard calibration curve for drug encapsulation

To find out the drug encapsulation concentration in lecithin liposomes microbubble, a standard curve ranging from 0.1-0.5 g/ml was generated for streptokinase. For this, a stock solution of 1g/ml streptokinase was prepared in dextrose water and the serial dilutions were done to find out the required range of the sample concentration (0.1-0.5 g/ml). All measurements were taken using independent samples in triplicates at the λ max = 278 nm. Then that standard curve was calibrated with the concentration of the encapsulated drug in the lecithin microbubble All the calculations was for standard curve calibration were performed at Microsoft Excel.

3.7 Drug encapsulation efficiency

UV Visible spectrophotometer was used for measuring the Streptokinase content in Lecithin liposomes at a specific wavelength of 278 nm. Blank utilized in the procedure was 95% ethanol to ensure the quality of results. For the quantification of the streptokinase, the suspension should be free first from the free drug that was present in the medium. For disruption of the lecithin liposomes, 2 ml ethanol should be added into the medium which ultimately led to drug release. Then the sample was subjected to vigorous vertexing and centrifugation at 4000 rpm for 10 min to separate the lipid precipitates. Lipid was settled as pallet while the drug was collected from supernatant which was further subjected to spectrophotometric studies. (Panwar et al. 2010).

To find out the streptokinase quantity in the lecithin liposome, a calibration curve was drawn for streptokinase. For this streptokinase was dissolved in the dextrose water and

make dilutions i.e. (range 0.1-0.5g/ml with linear equation (y=2.57x + 0.265) and used to find out the concentration of the streptokinase in the lecithin liposomes. and to find out the encapsulation efficiency of the streptokinase in the lecithin liposomes, to quantify the amount of the drug in the liposomes UV absorption of the sample was recorded and compared with the calibrated curve of the streptokinase and that experiment was performed three times and the average result values were put in the formula given below to calculate the encapsulation efficiency of streptokinase in the liposomes.

$$\text{ENCAPSULATION EFFICIENCY } (\%) \frac{\text{CONCENTRATION OF STRPTOKINASE DETECTRD IN MEDIUM}}{\text{CONCENTRATION OF STREPTOKINASE ADDED INITIALLY}} \times 100$$

3.8 Physiochemical Characterization of Microbubble Constructs

3.8.1 Scanning electron microscopy

The structure and the morphology of the microbubbles that were prepared were analyzed using the scanning electron microscope, model: (JEOL model no JSM-6490LA). And for analysis microbubble samples were sonicated and diluted, and one drop of each emulsion sample was taken and placed on the slides. After this slide was placed in the desiccator for the evaporation of the solvent and make it dry. Then each sample slide was coated with gold salts to make it more conductive and placed for SEM analysis. The voltage of the gun was 10kV and placed at 9.7 mm of distance from the sample.

3.8.2 Zeta potential measurements

Zeta sizer (Malvern) was used to measure the size of the microbubble and zeta potential at the temperature of 25^{o}C and pH = 7.4. to analyze the sample, 20 microliters of it were diluted in the distilled water of 2 ml. size, and the zeta potential of the microbubble was measured in the triplicate manner.

3.8.3 Fourier Transform Infra-Red spectroscopy (FTIR)

Spectrum – 100 was used to determine the FTIR spectra of the samples. The main purpose of the FTIR analysis is to characterize and to know the comparison of the constituent functional group and final microbubble construct. An infrared range of 4000-350 cm-1 was used to record the FTIR spectrum of all the samples at room temperature. To prepare the sample for the FTIR sample were ground it properly and blend with the powdered KBr in an approximate ratio of 1:5 (sample: KBr) and 6 tons of the pressure was applied to compress the sample for one minute in the hydraulic press to form a disc. And for samples in the liquid and semi-liquid phase, the sample was added to the KBr pellet disc dropwise to analyze it.

3.9 Blood sample

Samples of the whole blood of humans were collected from the volunteer by the process of sterile venipuncture. The cubital vein of the volunteers was used for blood samples and then stored in the sodium citrate buffered tubes and provide refrigeration till the time of their use. All volunteers were healthy, without hematological, coagulative, or bleeding disorders, and did not take hormone or contraceptive therapy or treatment.

3.9.1 Preparation of invitro blood clot model

A blood clot was prepared according to the method that was reported in the literature (Petit et al. 2012). For this volume of 2.5 ml of 100 mM molarity of calcium, chloride solution was taken, and it was mixed in the 1 ml of anticoagulated human blood that was collected from the volunteer after their consent. After 5 minutes that mixture was injected into the silicon tubes that were chosen to prepare the invitro clot model. For proper retraction of the clot, avoid the injection of air bubbles in the silicone tube. After that blood containing silicone tubes were incubated at 37 ^{0}C for a time of 3 hours and then refrigerate for 3 days for proper retraction of the clot.

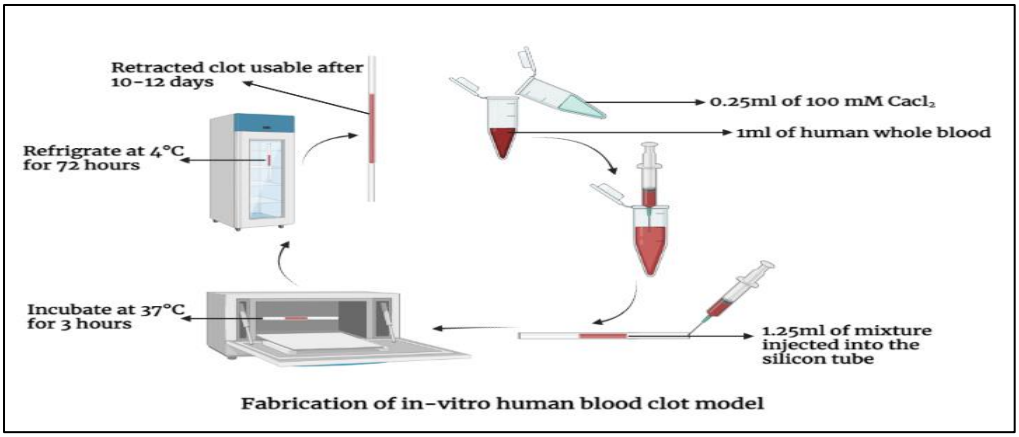

Figure 3: Schematic diagram of synthesis of clot model

3.10 In – vitro Sonothrombolysis

The prepared clot model was subjected to the process of the sonothrombolysis in diagnostic ultrasound apparatus, for this following the Ultrasounds parameters were used that are given below.

- Transducer probes= 11L4 and 6C1
- Frequency of transducers = 3.5 MHz and 7 MHz
- Mechanical index (MI) =0.1 0.3 0.6, 0.9 and 1.2
- Depth = 5 cm

In this study Blood clot model was exposed to ultrasound waves for 30 minutes in the following study groups i.e., ultrasound only, ultrasound+ lecithin microbubbles, ultrasound + streptokinase, and Ultrasound + streptokinase loaded lecithin microbubbles. And the potential effect of the sonothrombolysis of these study groups was analyzed by mean gray intensity analysis of ultrasound images of the blood clot, pre and post weight analysis of the blood clot, and hemoglobin analysis in the effluents that we collected after 30 minutes of the process of the sonothrombolysis by absorption through UV visible spectrophotometer analysis.

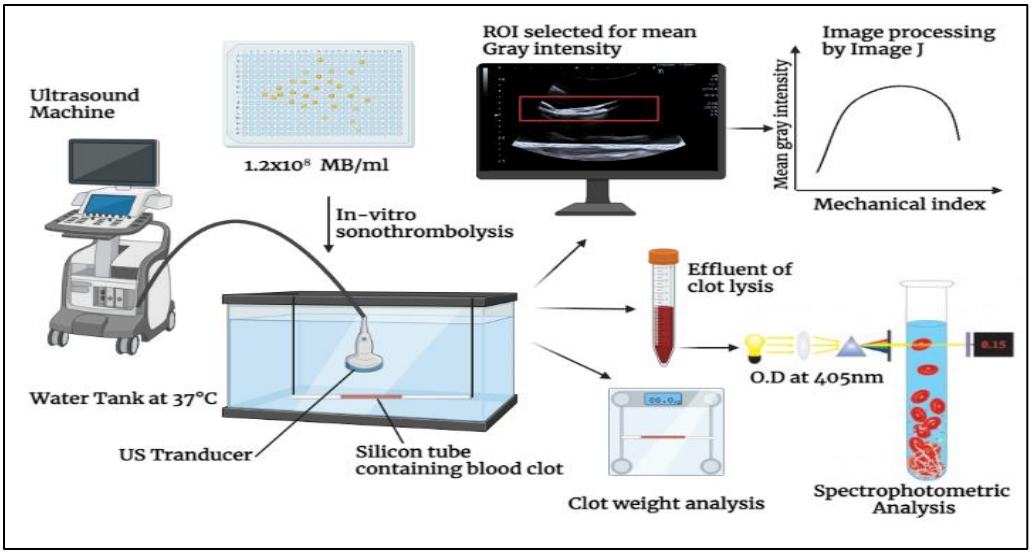

Figure 4: Schematic diagram of in vitro sonothrombolysis

3.10.1 Ultrasound Image Analysis of in vitro clot model

Ultrasound images of the blood clot that were stored during the process of the sonothrombolysis. Every image was captured after every 3 minutes of sonothrombolysis for all the groups of the study. Images of the ultrasounds analyzed with the software i.e., **IMAGE J.** mean gray intensity of the clot of interest in ultrasound images were recorded. Parameters to analyze the images i.e., Region of interest and X-Y coordinates were kept constants for all images.

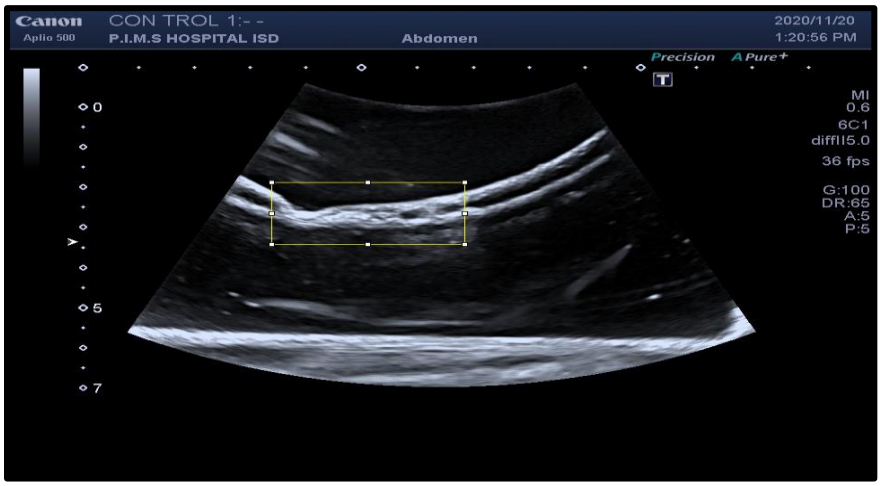

Figure 5: Mean gray intensity of ultrasound images of clot model analyzed by image J. and XY coordinates were kept specific for analysis. X1= 291, Y1= 237, X2= 542, andY2= 395

3.10.2 Weight Analysis of in vitro clot model

weight analysis of the blood clot was performed after 30 minutes of exposure to ultrasound waves. All the blood clots model was synthesized at the same weight of 4.56 g. after 30 minutes of sonothrombolysis weight of the blood clot model was recorded at scientific weighing balance.

3.10.3 Spectrophotometric analysis of Hemoglobin of red blood cells

UV visible spectrophotometer was used to record the absorbance of hemoglobin of the red blood cells that were released during blood clot lysis after the exposure of 30 minutes of ultrasound waves in various study groups. Effluents of clot lysis were collected in separated falcon tubes and absorption of hemoglobin was recorded at 405 nm wavelength at spectrophotometer

RESULTS

4.0 Results.

4.1 Scanning electron microscopy

Images of scanning electron microscopy of Perflurohexane loaded lecithin microbubbles are shown in Figure: 6 (A) A homogenous population of the lecithin microbubble with respect to size can be seen. The shape of the lecithin microbubble was spherical. in another Figure:6(B). Perflurohexane loaded lecithin microbubble forms aggregates and fused to each other that is mainly due to the not complete drying of the sample slides and not proper sonication of the sample before slid preparations.

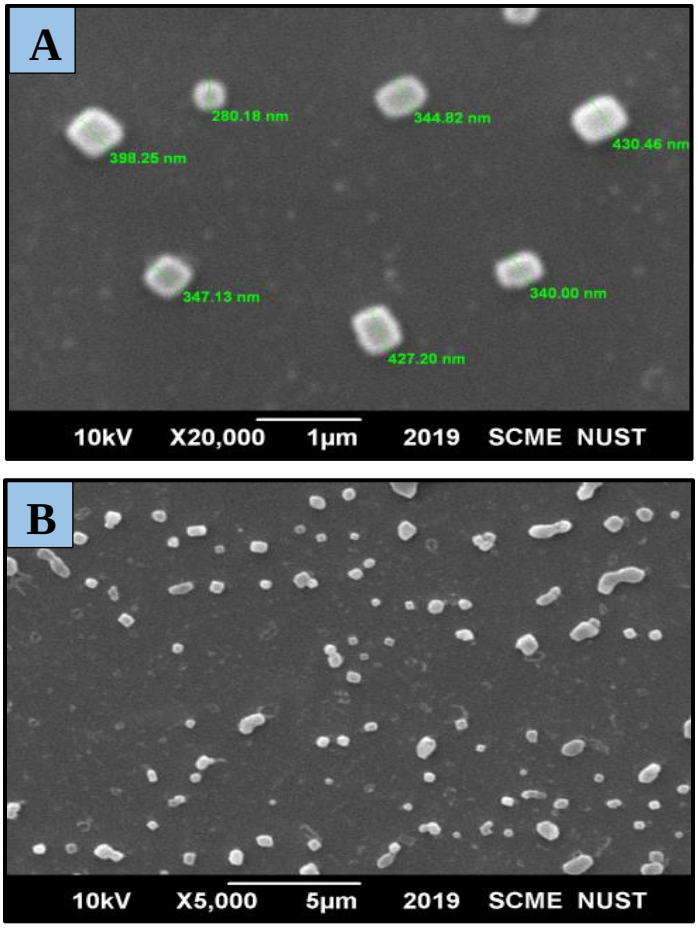

Figure 6: SEM images of Lecithin microbubbles at A) 1µm & B) 5µm

4.1.2 Lecithin MB zeta potential and zeta size

For uncoated lecithin microbubbles, the zeta was found to be -28.3 as shown in Figure: 7 (A). The average size of the lecithin microbubbles was 357.8nm ±124 as shown in Figure:7 (B)

Table 1 Zeta potential and Zeta size of Lecithin MB

Sample	Zeta size(nm)	Zeta potential	PDI
Lecithin Microbubbles	357.8±124	-28.5	0.149

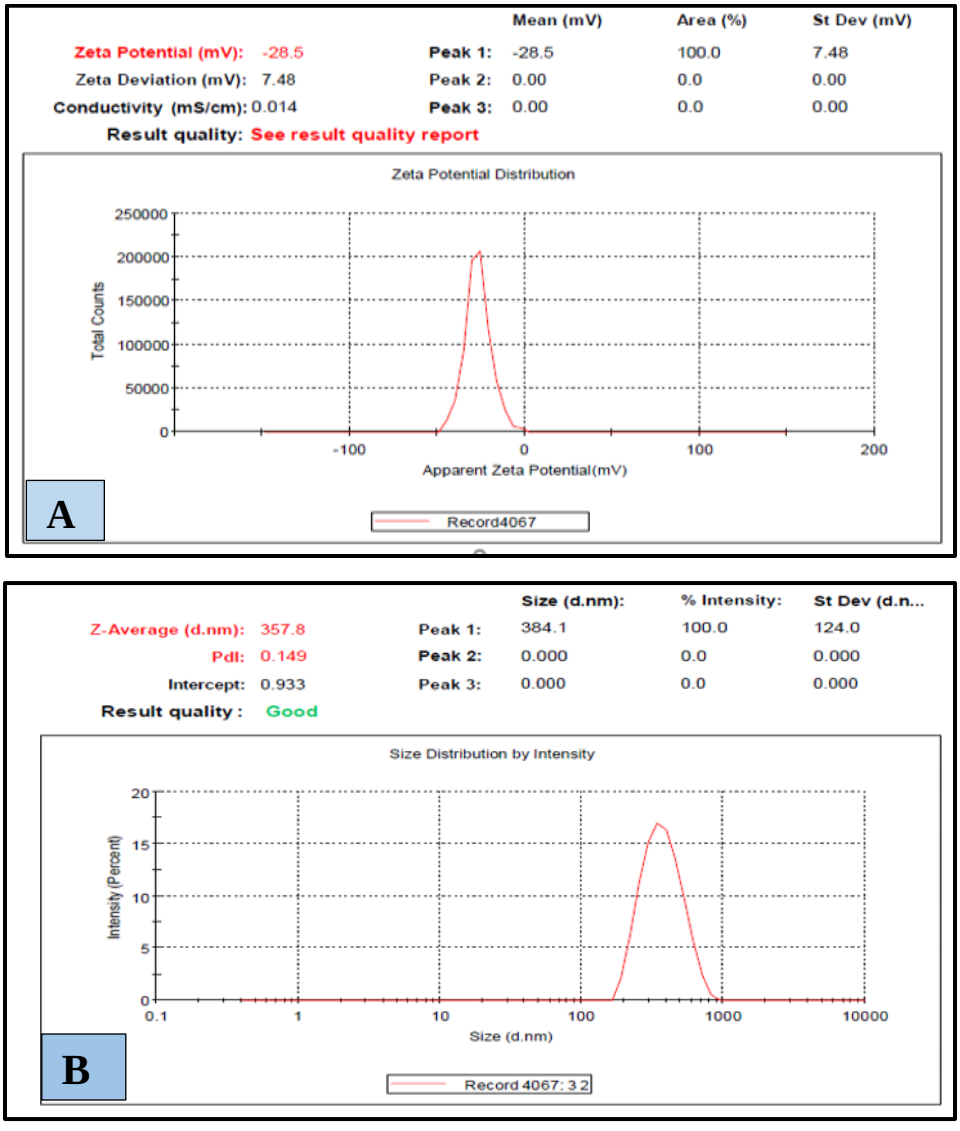

Figure 7: Zeta potential of Lecithin MB B) Zeta size of Lecithin MB

4.1.3 Fourier transform infra-red (FTIR) spectroscopy:

Fourier transform infra-red (FTIR) spectroscopy was performed to analyze the important chemical functional groups and the surface chemistry of the Perflurohexane loaded lecithin microbubble. Figure 8 (A) shows the FTIR of Perflurohexane which shows the absorption peaks band that shows the properties of the Carbon-Hydrogen stretching 2800cm^{-1} and 3000$cm^{-1.}$ The absorption band at 1270cm^{-1} and 1100cm^{-1} shows the strong stretching of carbon and fluorine (C-F). The representative spectrum of FTIR showed in Figure 8 all types of pure soy lecithin absorption peaks. Soy lecithin had typical peaks between 3436 cm^{-1} and 755 cm^{-1}. In the area, 1770-1500 cm^{-1} were apparent vibrations of bending of hydroxyl (OH) and carbonyl (C-OH) stretches. The standard ranges observed for P=O between 1200-1146 cm^{-1} and for P-O-C, 1446 cm^{-1} to 888 cm^{-1}. The soy lecithin is a mixture of different phospholipids and therefore heavy atoms exist in the region of less than 900 cm^{-1}. The dominant bands were located at 3412 cm^{-1} to 593 cm^{-1} for the pure cholesterol spectrum IR in Figure 8 (C). The properties of 2800 to 3000 cm^{-1} vibration ranges are attributed to asymmetric and Symmetric CH_2 and CH_3 vibration ranges. The presence of OH stretches is due to the strong vibration band observed at 3412 cm^{-1}. The symmetric stretching vibration of the CH_2 bond is another intense peak at 2934 cm^{-1}. In the second cholesterol ring, the double bond (C=C) is clearly seen at 1670cm^{-1}, substitution and determined significantly by the adjacent hydrogen atoms on the ring. The characteristic band observed from 900-765 cm^{-1} is the orderly bending of C-H that has the presence of aromatic substitution and the adjacent hydrogen atoms located on the ring are significantly determined. Figure 8 (D) shows a strong band at 3440cm-1 of Perflurohexane loaded lecithin MB due to its stretching of OH (strong hydrogen bonding). CH degree of absorption is due to the absorption peaks 2946-1 and 2865-1. The C=C band can be measured at 1637cm-1 suggesting binding of lecithin and cholesterol. Between main bands, 1392cm^{-1} – 1159 cm^{-1}, lecithin major groups of P=O can be seen. At 1095-975 cm^{-1}, absorption peaks correspond to the P-O-C stretch. Pure PFC showed absorption bands at 3431cm-1 (due to OH stretching), 2835 cm^{-1} (CH stretching), and 1270 to 1100 cm^{-1} peaks show strong C-F bond vibrations.

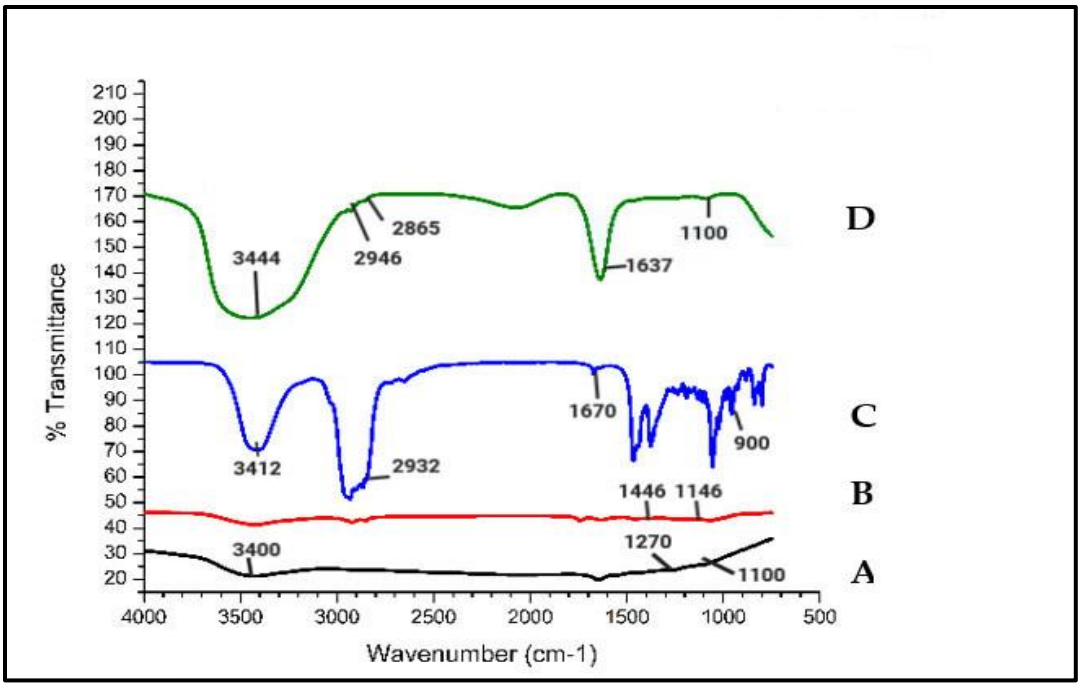

Figure 8: FTIR spectrum of A) Perflurohexane B) Soy Lecithin C) Cholesterol D) Perflurohexane loaded Soy Lecithin MB

Table 2 Interpretation of wavenumbers

Samples	**Absorption peaks(cm^{-1})**	**Inference**
A) Perflurohexane	1100, 1270 2800-3000 3400	C-F (strong) C-H (medium) OH
B) Soy lecithin	1146,1200 1446 1500 1770	P-O-C P=O C-OH OH bending
C) Cholesterol	900 1670 2934 3412	CH C=C CH_2 OH (strong)
D) Perflurohexane loaded Soy Lecithin MB	1100 1392 1637 2865,2946 3444	C-F P=O C=C C-H OH

4.2 Drug encapsulation Efficiency:

The absorbance was measured with a UV-vis spectrophotometer at 278 nm in various concentrations of streptokinase. For the standard curve for streptokinase, a graph was

generated between the absorbance of the UV spectrophotometer and the concentrations Figure:9. The concentration of the free drug in the supernatant was determined through this curve using the following equation.

$$y=mx+b$$

Therefore.

$$\frac{y-b}{m}=x$$

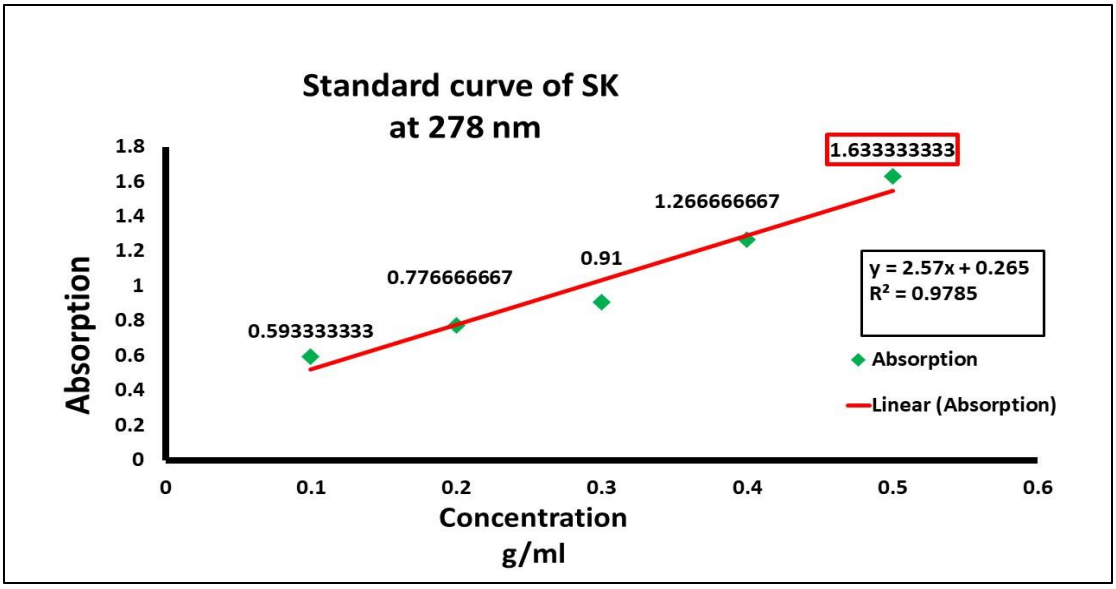

Figure 9: Standard curve for streptokinase in concentration 0.1-0.5 g/ml, for drug encapsulation efficiency analysis. Each sample was run in triplicate (n=3, STD ±0.23) and the graph was plotted against mean values.

therefore, streptokinase encapsulation efficiency in the lecithin liposomes was easily calculated. At first, the total drug for liposome preparation was 0.5 g in ml. This has resulted in 64.71% percent of lecithin liposomes being encapsulated using the EE% formula. Table 3 shows detailed results

Table 3 Drug (SK) encapsulation efficiency percentage of lecithin microbubbles, Absorbance at 278 nm of streptokinase concentration (0.1, 0.2, 0.3, 0.4 and 0.5 g/ml)

SK(g/ml)	Replicate 1	Replicate 2	Replicate 3	MEAN	SD
0.1	0.55	0.61	0.62	0.593333	0.037859
0.2	0.78	0.76	0.79	0.776667	0.015275
0.3	0.9	0.91	0.92	0.91	0.01
0.4	1.2	1.4	1.2	1.266667	0.11547
0.5	1.6	1.7	1.6	1.633333	0.057735

Table 4 Drug (SK) encapsulation efficiency percentage of lecithin microbubbles, Absorbance at 278 nm for released streptokinase in the medium

OD1	OD2	OD3	Average	SD	SK in Medium
1.1	1.2	1.1	1.133333	0.057735	0.337873

y= mx + b

y = 2.57x + 0.26

y = line intercept (Absorbance)

X = any point on the line (Drug concentration)

$$\mathbf{X} = \frac{y-0.267}{2.57}$$

$$\mathbf{X = 0.3378\,g/ml}$$

$$\boldsymbol{EFFICIENCY}(\%) = \frac{\boldsymbol{DRUG\ RLEASED\ IN\ MEDIUM}}{\boldsymbol{DRUG\ CONC.USED\ INITIALLY}} \times 100$$

$$= \frac{0.3378}{0.522} \times 100$$

$$= 64.71\,\%$$

4.3 Mean gray intensity analysis of ultrasound images of the clot of interest.

The approach of ultrasound is used for diagnosing and studying DVT's natural history. The assessment of the echogenicity of the thrombus and of the width of the thrombosis vein is now carried through the interpretation of DVT with changes of time. in vivo and in vitro

investigations demonstrated that ultrasounds picture is becoming more echogenic as thrombus organization takes place. Analysis of the ultrasound images is the subjective and operative dependent method in the investigation of chronic venous blockage.

4.3.1 In vitro Sonothrombolysis at frequency 3.5 MHz; (US) only and (US+MB)

Figure10: shows the Recoded mean grays intensity value during the process of the sonothrombolysis at in vitro clot model with ultrasound probe of frequency of 3.5 MHz at mechanical index **(MI)** (0.1, 0.3, 0.6, 0.9, and 1.2) for the time of 30 minutes at ultrasound diagnostic machine. Ultrasound Images of the clot of interest were stored after every 3 minutes during the 30 minutes' process of the sonothrombolysis. Images were processed to record the mean gray intensity of the clot of interest and plot a graph between mean gray intensity (y-axis) and time (x-axis). Figure 10: shows the plotted graph of the first study group that was ultrasound **(US) only** and Ultrasound with lecithin liposomes Microbubble **(US + MB)** at the selected mechanical index **(MI).** Figure 10: is showing the significant difference in reduction of the mean gray intensity of clot in this study group ultrasound **(US)** and Ultrasound with lecithin liposomes Microbubble **(US+MB)** at different mechanical index (MI). approximately there is the uniform reduction in the mean gray intensity at all mechanical index (MI) was seen. But there was seen a greater reduction in mean gray intensity in **(US+MB)** group than the group of **(US)** alone, Table: 5 because microbubble oscillations aided the cavitation of the thrombus and bring more lysis there is seen a sharp reduction in the mean gray intensity in Ultrasound with lecithin liposomes Microbubble **(US + MB)** at mechanical index (MI) = 0.6 at the time point of 12 minutes and reduce further till 30 minutes. In this study group of **(US) only and (US+MB)** at 3.5 MHz maximum reductions of mean gray intensity was recorded at the mechanical index of 1.2 of **(US+MB)** study group

At mechanical index 0.9 and 1.2 of Ultrasound with lecithin liposomes Microbubble **(US + MB)** repeat this trend but from the time point of the 9 minutes and reduce further the end of 30 minutes.

Table 5 is showing the reduction of the mean gray intensity of the clot during the process of sonothrombolysis at the frequency of 3.5 MHz, after 30 minutes. At mechanical index 0.1, 0.3, 0.6 0.9 and 1.2 of group of (US) only there is 2.5 %, 5.1 %, 7.5 % 9.5 %11 % reduction of the mean gray intensity and group of (US+MB) 13 %, 17%, 28 %, 39 % and 43 % respectively.

Mechanical index (MI)	**Mean gray intensity at the Start of sonothrombolysis. (US) only**	**Standard deviation (SD)**	**Mean gray intensity after 30 minutes of sonothrombolysis. (US) only**	**Standard deviation (SD)**	**Mean gray intensity at the Start of sonothrombolysis. (US+MB)**	**Standard deviation (SD)**	**Mean gray intensity after 30 minutes of sonothrombolysis. (US+MB)**	**Standard deviation (SD)**
0.1	37.5	± 3	36.5	±3.2	45.2	± 2.4	39.18	± 2
0.3	36.2	± 2.5	34.4	± 3.5	37.73	± 3	31.11	± 2.5
0.6	38	± 2.5	35.1	± 2.4	38.56	± 3.6	27.63	± 4
0.9	35.5	± 2	32.12	± 2.5	35.89	± 4	21.65	± 5.5
1.2	37.1	± 2.5	31.2	± 3.5	36.7	± 2.5	20.26	± 3.2

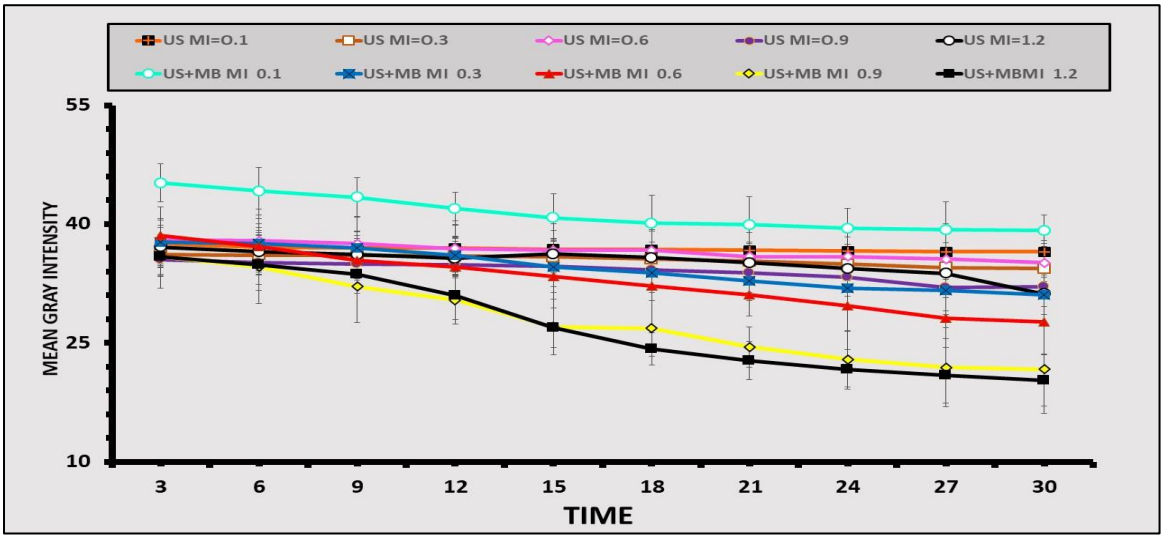

Figure 10: Mean gray intensity analysis after Sonothrombolysis at ultrasound frequency 3.5 MHz at mechanical index 0.1, 0.3, 0.6, 0.9, and 1.2 for the time of 30 minutes, Study group: US only and (US+MB).

4.3.2 In vitro sonothrombolysis at frequency 7 MHz; (US) only and (US+MB)

Figure 11: shows the Recoded grayscale mean value during the process of the sonothrombolysis at in vitro clot model with ultrasound probe of frequency of 7 MHz at mechanical index **(MI)** (0.1, 0.3, 0.6, 0.9, and 1.2) for the time of 30 minutes at ultrasound

diagnostic machine. Ultrasound Images of the clot of interest were stored after every 3 minutes during the 30 minutes' process of the sonothrombolysis. Images were processed to record the grayscale mean intensity of the clot of interest and plot a graph between mean gray intensity (y-axis) and time (x-axis). in that study group reduction of mean gray intensity were recorded on the higher frequency of 7 MHz in figure 11: in the study group of **(US)** only their reduction of mean gray intensity at all Mechanical index 0.1, 0.3 0.6 0.9, and 1.2 but at the mechanical index of 1.2 this study group shows steep reduction of the mean gray intensity at 7 MHz from the time point of 12 minutes and keep reducing till the end of the process of sonothrombolysis at the time point of 30 minutes. And the study group of **(US+MB)** showing more reduction of mean gray intensity at all mechanical index 0.1, 0.3, 0.6, 0.9 and 1.2 than study group of **(US) only** Table:6**.** In the study group of **(US+MB),** there is a steep reduction of mean gray intensity has been shown at mechanical index 0.6 that starts from the time point of 12 minutes and continue till the end of 30 minutes and at a mechanical index of 0.9 and 1.2 same trend of steep reduction of mean gray intensity that starts from the time point of the 3 minutes and continue till the end of the process of sonothrombolysis at 30 minutes. In this study group of **(US) only and (US+MB)** at 7 MHz maximum reductions of mean gray intensity were recorded at the mechanical index of 1.2 of **(US+MB)** study group.

Table 6 is showing the reduction of the mean gray intensity of the clot during the process of sonothrombolysis at the frequency of 3.5 MHz, after 30 minutes. At mechanical index 0.1, 0.3, 0.6 0.9 and 1.2 of group of (US) only there is 4 %, 6.2 %, 15%, 16 % and 21 % reduction of mean gray intensity and group of (US+MB) there is 20 %, 25% 36.5% 45% and 50.9% reduction of mean gray intensity, respectively.

Mechanical index (MI)	**Mean gray intensity at the Start of sonothrombolysis. (US) only**	**Standard deviation (SD)**	**Mean gray intensity after 30 minutes of sonothrombolysis. (US) only**	**Standard deviation (SD)**	**Mean gray intensity at the Start of sonothrombolysis. (US+MB)**	**Standard deviation (SD)**	**Mean gray intensity after 30 minutes of sonothrombolysis. (US+MB)**	**Standard deviation (SD)**
0.1	39.1	±3.55	37.53	± 3.2	44.1	± 3.3	35.15	± 3.6
0.3	38.9	± 3.5	36.48	± 1.5	43.11	± 3.5	32.3	± 2.4
0.6	40	± 2.5	34.55	± 2.4	40.56	± 3.2	25.7	± 2.5
0.9	39.19	± 2.4	33.16	± 4	36.9	± 2.5	20.11	± 3
1.2	38.22	± 3.5	30.95	± 3.5	36.9	± 3	18.1	± 1.5

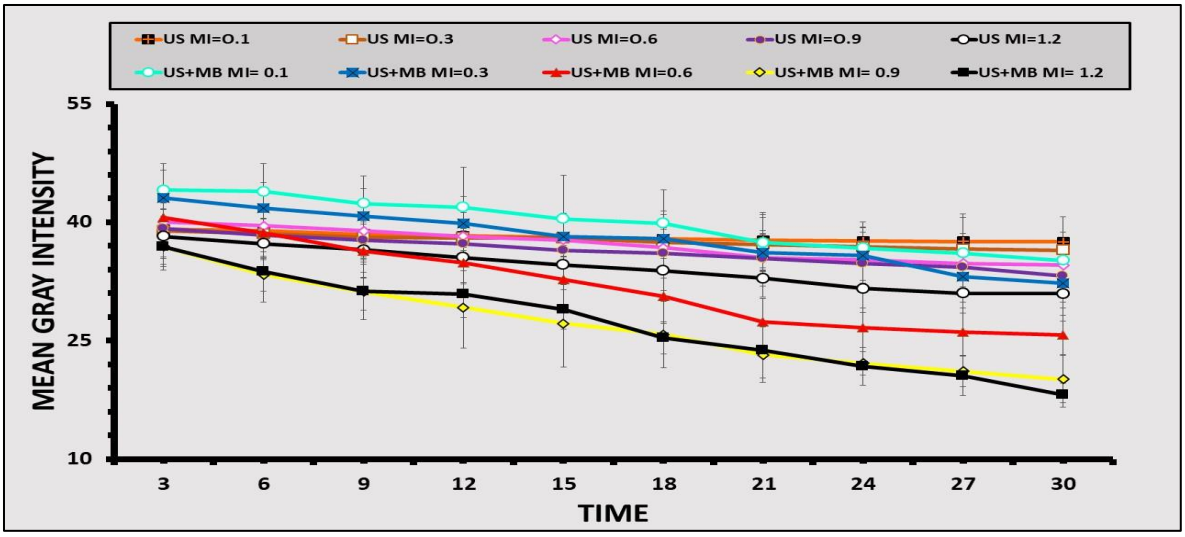

Figure 11: Mean gray intensity analysis after Sonothrombolysis at ultrasound frequency 7 MHz at mechanical index 0.1, 0.3, 0.6, 0.9, and 1.2 for the time of 30 minutes, Study group: US only and (US+MB)

4.3.3 In vitro sonothrombolysis at frequency 7 MHz; (US+ DRUG) only and (US+DRUG LOADED MB).

After the process of sonothrombolysis with the frequency of 3.5 MHZ and 7 MHz at study group of **(US) only and (US+MB)** at selected mechanical index 0.1, 0.3, 0.6, 0.9, and 1.2 we shortlisted our best ultrasound parameter at which we get our best result; maximum reduction of mean gray intensity was obtained at 7 MHz at the mechanical index of 1.2. Now next experiments of the study group **(US+ drug) and (US+Drug Loaded MB)** were performed on 7 MHz at the mechanical index of 0.1, 0.3, 0.6, 0.9, and 1.2. in Figure 12, Showing the significant reduction of the mean gray intensity during the process of sonothrombolysis. There is a significant reduction in the mean gray intensity in **(US + DRUG)** group but **(US+DRUG LOADED MB)** showing greater reduction of the mean gray intensity specifically at mechanical index 0.6 0.9 and 1.2 shows a steep reduction of mean gray intensity from time points of 18 minutes, and 12 minutes and 6 minutes and continue till the time point of 30 minutes, respectively. Table: 7 **(US+DRUG LOADED MB)** group shows a greater reduction in mean gray intensity with 7 MHz, specifically at the mechanical index of 1.2.

Table 7 :is showing the reduction of the mean gray intensity of the clot during the process of sonothrombolysis at the frequency of 7 MHz, after 30 minutes. At mechanical index 0.1, 0.3, 0.6, 0.9 and 1.2 of group of (US+ DRUG) only there is 30 %, 40 %, 45%, 50 % and 58 % reduction of mean gray intensity and group of (US+ DRUG LOADED MB) there is 59 %, 66% 81% 89% and 94% reduction of mean gray intensity, respectively.

Mechanical index (MI)	Mean gray intensity at the Start of sonothrombolysis. (US + DRUG)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis. (US + DRUG)	Standard deviation (SD)	Mean gray intensity at the Start of sonothrombolysis. (US+ DRUG LOADED MB)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis. (US+ DRUG LOADED MB)	Standard deviation (SD)
0.1	38.92	± 4.2	27.24	± 2.5	46.78	± 4.2	18.97	± 4
0.3	37.27	± 4.2	22.37	± 2.5	41.27	± 2.4	13.89	± 3
0.6	39.87	± 3.5	21.93	± 3	40.12	± 4.2	5.12	±5.12
0.9	38.29	± 3.3	19.095	± 4	37.44	± 4.2	3.55	±3.55
1.2	36.81	± 4.2	15.47	± 2.5	37.11	± 4.2	1.64	±1.64

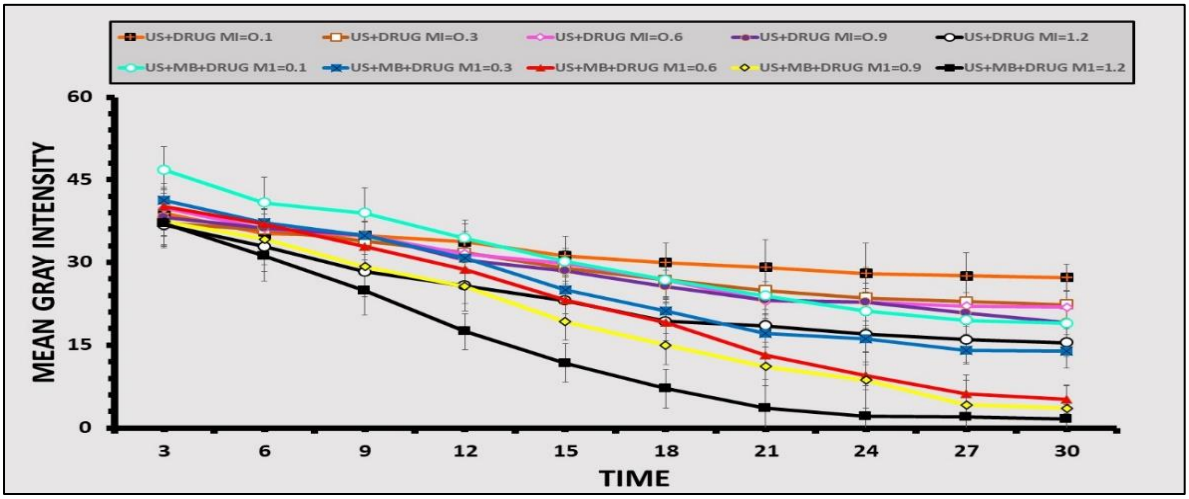

Figure 12: Mean gray intensity analysis after Sonothrombolysis at ultrasound frequency 7 MHz at mechanical index 0.1, 0.3, 0.6, 0.9, and 1.2 for the time of 30 minutes, Study group: (US+ DRUG) and (US+Drug Loaded MB)

All these study groups (US) only, (US+MB), (US+DRUG), and (US+DRUG LOADED MB) show a maximum reduction in mean gray intensity at ultrasound parameters; 7 MHz at the mechanical index of 1.2. and among these study groups, maximum reduction of mean gray intensity was recorded in the study group **(US+DRUG LOADED MB)** group at the above-mentioned ultrasound parameters **Figure:13.**

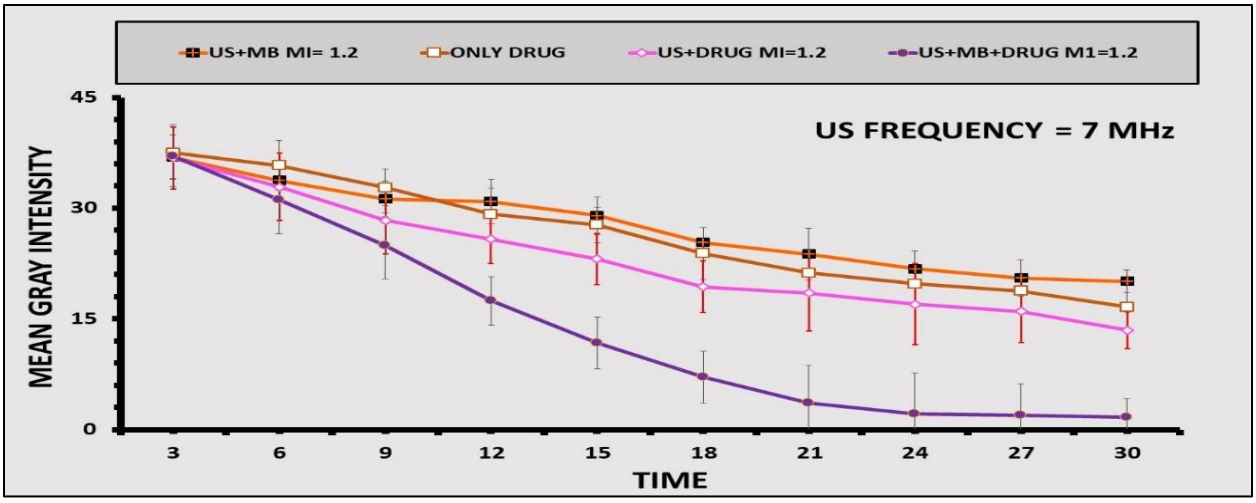

Figure 13: Mean gray intensity analysis after of Sonothrombolysis at ultrasound frequency 7 MHz at mechanical index 1.2 for time of 30 minutes, Study group: Drug only, US only (US+MB) (US+ DRUG) and (US+ drug loaded MB)

4.4 WEIGHT ANALYSIS.

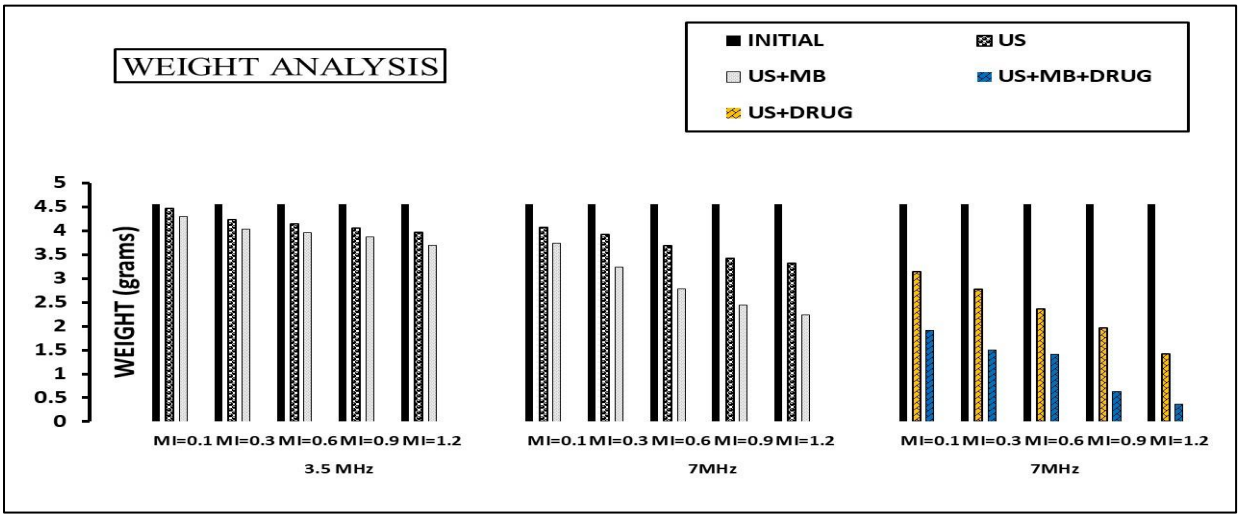

Figure 14: Weight analysis after sonothrombolysis at US frequency 3.5 MHz and 7MHz at mechanical index 0.1, 0.3, 0.6, 0.9, and 1.2. study group: (US) only, (US +MB), (US + DRUG) and (US + DRUG LOADED MB)

Weight analysis was performed of the in vitro clot model, that was used in the study group **(US) only, (US +MB), (US + DRUG) and (US + DRUG LOADED MB)** with ultrasound frequency of 3.5 MHz and 7 MHz at mechanical index 0.1, 0.3, 0.6, 0.9 and 1.2. initially, sonothrombolysis was performed at study group **(US) only, (US +MB)** with ultrasound

frequency of 3.5 MHz and 7 MHz at mechanical index 0.1, 0.3 0.6 0.9, and 1.2. maximum weight reduction was shown at the mechanical index of 1.2 with the ultrasound frequency of 7 MHz as shown in Figure. 14 in the study group of **(US) only, (US +MB), Then,** ultrasound frequency 7 MHz was selected to continue to perform sonothrombolysis at mechanical index 0.1, 0.3, 0.6, 0.9 and 1.2 at study group of **(US + DRUG) and (US + DRUG LOADED MB).** Here the maximum weight reduction was shown at mechanical index 1.2 in the study group of the **(US + DRUG LOADED MB).**

Results of weight analysis are supporting the results we get from the analysis of mean gray intensity; reduction of the mean gray intensity is corresponding to the mass loss of the in vitro blood clot after the sonothrombolysis. And the maximum reduction of the mean gray intensity was seen at mechanical index 1.2 with ultrasound frequency 7 MHz in the study group of **(US + DRUG LOADED MB)** as shown in Figures 12 and 13. Moreover, maximum weight reduction was shown at the same mechanical index 1.2 with the same ultrasound frequency of 7 MHz in the same study group **(US + DRUG LOADED MB). As** shown in Figure 14.

4.5 Spectrophotometric analysis of effluent of sonothrombolysis.

Absorption of the effluents from clot lysis that were collected after the process of sonothrombolysis at study group of **(US) only, (US +MB), (US + DRUG), and (US + DRUG LOADED MB)** With ultrasound frequency of 3 MHz and 7 MHz at mechanical indexes 0.1, 0.3, 0.6, 0.9 and 1.2 were analyzed at spectrophotometer to record the absorbance of hemoglobin of red blood cells at the wavelength of 405 nm. Detailed results of absorption of hemoglobin showed in table 8

Table 8 Absorption of hemoglobin at the wavelength of 405 nm, showing the maximum absorption (2.9 ± 0.22) of hemoglobin was recorded for a study group of **(US+ DRUG LOADED MB)** at Ultrasound frequency of & MHz and mechanical index 1.2.

MECHANICAL INDEX	US (3.5 MHz)	Standard deviation	US+MB (3.5MHZ)	Standard deviation	US (7MHz)	Standard deviation	US+MB (7MHz)	Standard deviation	US+DRUG 7MHz	Standard deviation	US+DRUG+MB 7MHz	Standard deviation
MI=0.1	2.19	±012	2.31	±0.31	2.46	±0.22	2.48	±0.33	2.48	±0.24	2.72	±0.11
MI=0.3	2.21	±0.2	2.36	±0.29	2.47	±0.15	2.51	±0.14	2.56	±0.27	2.84	±0.17
MI=0.6	2.25	±0.35	2.38	±0.27	2.47	±0.12	2.52	±0.18	2.62	±0.22	2.87	±0.15
MI=0.9	2.28	±0.19	2.44	±0.21	2.48	±0.26	2.56	±0.13	2.71	±0.34	2.9	±0.28
MI=1.2	2.35	±0.21	2.52	±0.18	2.58	±0.23	2.69	±0.19	2.73	±0.21	2.93	±0.22

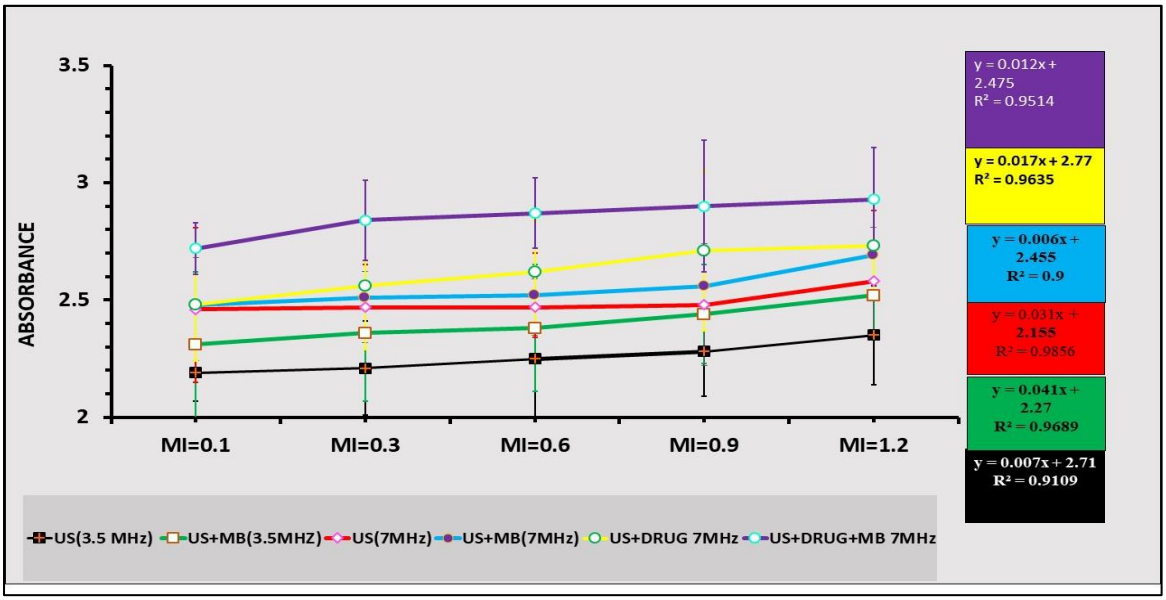

Figure 15: Absorbance of hemoglobin at 405 nm of effluent, collected after sonothrombolysis with US frequency 3.5 and 7 MHz at MI 0.1, 0.3, 0.6, 0.9, and 1.2. Study group: (US) only, (US +MB), (US + DRUG) and (US + DRUG LOADED MB

In Figure 15: It is shown that with the increase of the mechanical index, absorbance values of hemoglobin of red blood cells at wavelength 405 nm is also increasing in all study group **(US) only, (US +MB), (US + DRUG) and (US + DRUG LOADED MB)** at which sonothrombolysis was performed with ultrasound frequency of 3.5 MHz and 7 MHZ at mechanical index 0.1, 0.2, 0.3, 0.6 and 1.2. firstly, sonothrombolysis was performed with

ultrasound frequency 3.5 MHz and &7 MHz on a study group of **(US) only, (US +MB)** with a mechanical index 0.1, 0.2, 0.3, 0.6, and 1.2. the effluent of blood clot collected after the process of sonothrombolysis from these study groups was analyzed in spectrophotometer analysis to record absorbance at 405 nm. Maximum absorbance was recorded for (**US +MB) study gro**up of sonothrombolysis with ultrasound frequency of & 7 MHz at the mechanical index of 1.2 as shown in figure 15. Then shortlisted ultrasound frequency of 7 MHz continues to perform sonothrombolysis for the study group **(US + DRUG) and (US + DRUG LOADED MB).** In this study group absorbance value increasing with the increase of the mechanical index and maximum absorbance was recorded at the mechanical index of 1.2 at ultrasound frequency 7 MHz for the study group of **(US + DRUG LOADED MB).** detail results are shown in Table: 7.

DISCUSSION

5.0 Discussion

Because of the stability of Microbubbles, it takes the focus in ultrasound imaging and therapy. Several studies have produced several approaches of synthesizing microbubbles with diverse shell materials and gas core with optimal contrast improving ability (E. Stride and Saffari 2003), (Chung and Kim 2015). There are several problems regarding the microbubble like polydisperse in nature and their micron size that can only be optimized by the procedure of their synthesis. And the stability and cost of microbubble is the real topic of interest nowadays (Versluis et al. 2020). The material that we choose for fabrication has a strong impact on its stability. In this study, we fabricated the lecithin microbubbles according to the reported method in literature (Xu et al. 2011) by the thin-film hydration method. And the confirmation of the lecithin microbubble synthesis was done by the following analysis including zeta sizing, zeta potential, SEM analysis, and FTIR analysis. The Average Size of the lecithin microbubble was 357±124 nm along with the zeta potential. -28.3 mV. The physical stability of the liposomes may be enhanced with a high Zeta potential because it indicates a greater repulsive force between particles, thus reducing the possibility of aggregation. Zeta potential determination is an important physicochemical analysis of nanoparticles to estimate surface charge to understand the suspension physical stability of the nanoparticle (Joseph and Singhvi 2019). A strong positive or negative value of the zeta potential of nanoparticles indicates good physical stability of nano-suspensions since individual particles are electrostatically repulsed. In general, a zeta potential value that differentiates from −30mV to +30mV is considered to have enough repulsive force for better colloidal physical stability. On the other hand, because of van der Waal's attractive forces, the small zeta potential value may result in the aggregation of particles and the coagulation and results in Physical instability. In addition to zeta potential values also influence the physical stability of the obtained nano-suspensions, such as material properties, surfactant presence, and solution chemistry. Lecithin microbubbles appeared to be spherical with a smooth outer layer under scanning electron microscopy analysis. Some of the microbubbles appeared in an aggregated form that is mainly due to the insufficient drying of the sample slides of the microbubbles and less sonication of the sample before slide preparations. Sonothrombolysis was performed

at in vitro fabricated human blood clot model. Human whole blood from the volunteered individual was taken and utilized to create a clot model in the silicon tubes. In vitro blood clot model was then placed in ultrasound experimental setup as shown in figure 4 and performed sonothrombolysis with ultrasound parameters, i.e., frequency 3.5 MHz and 7MHz at the mechanical index of the 0.1,0.3,0.6,0.9 and 1.2 over four study group. **(US) only, (US+MB), (US+DRUG), and (US+DRUG LOADED MB)** and compared the results of these study groups to examine the efficacy of the sonothrombolysis. The probes of ultrasounds used for superficial imaging used are 10-14Mhz (Nadrljanski et al. 2013). Thyroid, breast, vascular (carotid), deep venous studies and pediatric imaging are performed at 3-10Mhz whereas muscular skeletal imaging is performed at 14Mhz probe (Markowitz 2011).

The Streptokinase drug that we use in this study is the common thrombolytic enzyme that is used in case of the myocardial infractions deep vein thrombosis and pulmonary embolism (Chuang et al. 2010). We utilized streptokinase as a drug in our study along with the Ultrasound (US) and in the loaded form inside of microbubbles to perform the sonothrombolysis. in vitro sonothrombolysis was analyzed by the measurement of the mean gray intensity of the ultrasound images of the clot of interest, pre and post weight analysis of the clot model and absorption of the hemoglobin of the red blood cells that is collected as the effluent of the clot lysis after sonothrombolysis.

Mean Gray intensity uses as a tool to distinguish DVT as being acute subacute or chronic. Acute deep vein thrombosis has a low mean gray value than subacute deep vein thrombosis which has a high mean gray intensity value. So, mean gray intensity can be used to assess the process of sonothrombolysis as grayscale mean reduces due to clot lysis. (Cassou-Birckholz et al. 2011).

According to the purpose mechanism of the behavior of the microbubble oscillation the acoustic response is considered linear with a low mechanical index (MI 0.1-0.5) since the microbubbles oscillate with an equivalent amplitude of compression and rarefaction but at MI $\leq$ 0.5 growth of the microbubble is less. And their oscillations and growth increase with the increase of the mechanical index (MI).(Greis 2014), At mechanical index $\geq$ 0.5 behavior of microbubble shifts from linear oscillation to then on a linear oscillation in

which microbubble undergo rarefaction that is greater than compression and growth of the microbubble, this behavior of microbubble aid the more clot lysis oscillatory interaction with thrombus. And at high MI destruction rate of microbubble is found to be high (Şen, Tüfekçioğlu, and Koza 2015). So, that is why in more steep reduction of the mean gray intensity was seen in **(US +MB), (US + DRUG) and (US + DRUG LOADED MB)** due to the oscillation of the microbubble in figure 10 reduction of mean gray intensity in the study group **(US +MB)** at mechanical index 0.6 starts at time point 15 minutes and at mechanical index 0.9 and 1.2 steep reduction of mean gray intensity starts from time points at 9 minutes.

In figure 11 With the increase of the frequency, 3.5 MHz to 7 MHz in the same study group **(US +MB)** time points shifted for steep reduction trends of mean gray intensity, at mechanical index 0.6 steep reduction of mean gray intensity starts from time point 12 minutes that was at 15 minutes for ultrasound Frequency of 3.5 MHz figure 10, and at mechanical index 0.9 and 1.2-time point for steep reduction of mean gray intensity started from 3 minutes and continue till the end of 30 minutes that was 9 minutes for ultrasound frequency of 3.5 MHz. So, an increase in the frequency also has an impact on reducing the time of sonothrombolysis.

In figure 12, Reduction of steep reduction of mean gray intensity was also seen in the study group **(US+ drug) and (US+Drug Loaded MB)** were performed on ultrasound frequency 7 MHz **(US+DRUG LOADED MB)** showing a greater reduction of the mean gray intensity specifically at mechanical index 0.6 0.9 and 1.2 shows steep reduction of mean gray intensity from time points of 18 minutes, and 12 minutes and 6 minutes, respectively. the main reason is microbubble oscillations in the ultrasound field and the effect of the clot lysing drug aided the cavitation of the thrombus and bring more lysis there is seen a sharp reduction in the mean gray intensity in Ultrasound.

Amount clot lysis during the process of sonothrombolysis would bring the reduction in mean gray intensity and weight of the clot model, corresponding to the value of high absorption value of hemoglobin of red blood cells in the effluent clot model that were used in the study group of the sonothrombolysis.

In previous studies, the first time the microbubbles Albunex with the thrombolytic drug Urokinase was used in the process of the sonothrombolysis along with the ultrasound frequency of 170 kHz to increase the rate of sonothrombolysis by (Tachibana and Tachibana 1995). And the combination of ultrasound with microbubble shows promising results over urokinase alone or in combination with ultrasounds. Subsequent studies confirmed the thrombolytic enhancement in-vitro, with sub-megahertz ultrasound exposure (Datta et al. 2008b), (Hitchcock et al. 2011), (Bader, Gruber, and Holland 2015) as well as with diagnostic imaging frequencies (Kondo et al. 1999),(Cintas et al. 2004a) (Xie et al. 2011). in another, in vivo studies, transcranial color-coded duplex ultrasound was used along with the microbubbles sonovue microbubbles with thrombolytic drug rt-PA showed high thrombolytic efficacy over rt-PA used alone. (Nedelmann et al. 2010). Invitro studies showed the high efficacy of thrombolysis with Ultrasound and rt-PA loaded microbubbles over rt-PA alone and rt-PA with ultrasounds (Shaw et al. 2009).

Our results correspond to the previous proved results, the combination of US, MB, and drug in sonothrombolysis is more effective as compared to the results in which US used alone along with drug or MB. In this study, the study group **(US + DRUG LOADED MB)** among other groups shows the maximum reduction of mean gray intensity at mechanical index 1.2 with ultrasound frequency 7 MHz as shown in figure 13. And the same group shows the maximum weight loss of the blood clot at the same ultrasound parameters as shown in figure 14. Spectrophotometer analysis of the effluent of blood clot shows the maximum absorption for the hemoglobin of red blood cell was recorded in **(US + DRUG LOADED MB)** at same ultrasound parameters, i.e., mechanical index 1.2 and ultrasound frequency 7 MHz as shown in figure 15. So, the combination of the drug-loaded microbubble along with the ultrasound parameters 7 MHz frequency and mechanical index 1.2 showed the high efficacy of sonothrombolysis.

CONCLUSION

6.0 Conclusion

Microbubble has great potential for targeting and drug delivery, after successful synthesis of lecithin microbubbles, confirmed by FTIR SEM and zeta analyses were used in in vitro sonothrombolysis at human blood clot model, Various combinations of the Ultrasound parameters i.e., frequency and mechanical index were used along with microbubbles and thrombolytic drugs demonstrated an increase in clot lysis. Evidence of thrombolysis was analyzed by weight loss of the blood clot, reduction of the mean gray intensity of the US images of the blood clot and increase the absorption values of the hemoglobin that were collected after the clot lysis after the 30 minutes of the Us exposure. To conduct in vitro sonothrombolysis, we have successfully built a new, easy, and effective approach. This testing method is considered highly valuable for fundamental analyses of the thrombolysis effects of ultrasounds.

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