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Rapid and Short Communication

Therapeutic effect of diagnostic ultrasound on enzymatic thrombolysis

An *in vitro* study on blood of normal subjects and patients with coronary artery disease

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Summary

If delivered at elevated intensity, ultrasound potentiates enzymatic clot dissolution; however, an elevated acoustic intensity damages vascular wall and favors reocclusion. This study's aim was to investigate whether exposure to high-frequency, low-intensity ultrasound - generated by a diagnostic scanner - enhances enzymatic thrombolysis, and if this effect differs in clots from blood of normal subjects and of patients with coronary artery disease (CAD). Venous blood samples were drawn from 10 healthy volunteers and from 10 CAD patients on chronic medical treatment, which also included aspirin. Each sample generated 2 radiolabelled clots, which were positioned in 2 *in vitro* models filled with human plasma recirculating at 37°. One clot was exposed to acetyl salicylic acid (60 μg/ml), tissue plasminogen activator (3 μg/ml) and heparin (1 IU/ml), while the other was exposed to the same medications plus ultrasound (2.5 MHz, mechanical index = 1.0) for 3 hours. Enzymatic thrombolysis was measured as solubilization of radiolabel. Normal subjects and patients did not significantly differ as to coagulation parameters, weight, volume and density of the clots, and fibrinolytic activity ($p = 0.794$). Ultrasound exposure did not influence thrombolysis in clots of normal subjects ($p = 0.367$), while it enhanced the dissolution of clots of CAD patients ($p = 0.013$). The enhancement was equal to 51% at 5 minutes, 32% at 15 minutes, 27% at 30 minutes, 20% at 1 hour and 19% at 3 hours ($p < 0.05$). Diagnostic ultrasound enhances enzymatic dissolution of clots generated from the blood of CAD patients, likely due to chronic treatment and in particular to aspirin.

Keywords

Thrombosis, ultrasound, thrombolysis, microbubbles

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Introduction

The therapeutic applications of ultrasound are currently limited to dental plaque removal, lithotripsy and physiotherapy (1, 2). However, several *in vitro* and experimental studies have demonstrated the ability of ultrasound to accelerate clot dissolution (3, 4). This effect is mainly influenced by the energy and the frequency of the beam. If high frequency (1 MHz) ultrasound is delivered at intensities (from 1 to 8 W/cm^2) well exceeding the safety limit allowed by the regulatory authorities for diagnostic scanners (0.72 W/cm^2) (5), acoustic waves potentiate enzymatic clot dissolution *in vitro* (6-9) and in experimental models of acute arterial occlusion (10, 11). However, the elevated acoustic intensity can damage vascular wall - mainly through thermal injury - thus favoring vessel reocclusion (10, 11). To limit the thermal effect of acoustic waves and to favor their penetration into the tissues, other investigators have utilized ultrasound with a frequency lower (from 27 to 500 kHz) than that of diagnostic scanners (from 1.3 to 10 MHz) (9, 12, 15). Again, ultrasound enhanced enzymatic thrombolysis but caused arterial damage

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when ultrasound intensity of 45 W/cm^2 was used for more than 5 minutes (12), while moderately high intensities (0.75-2 W/cm^2) (9, 13, 14) appeared to be safe and effective.

The aims of this study were to investigate whether ultrasound delivered by a commercially available diagnostic scanner, which operates at high frequency (2.5 MHz) and low signal intensity (0.165 W/cm^2), enhances enzymatic clot dissolution and to evaluate whether this effect differs in clots generated from the blood of normal subjects and of patients with coronary artery disease.

Materials and methods

Characteristics of patients

Venous blood samples were drawn from 10 healthy volunteers (mean age 46±13 years, 4 male), not taking any medication, and from 10 patients with coronary artery disease (mean age 71±12 years, 8 males). The diagnosis of coronary artery disease was based on the history of a previous myocardial infarction in 5 patients, of stable angina pectoris in 5 patients, and on the angiographic evidence of stenoses involving one coronary vessel in 3 patients, two vessels in 2 and three coronary vessels in 3 patients. In addition, three of these patients previously underwent coronary artery bypass surgery and one underwent percutaneous coronary angioplasty. Patients with acute coronary syndrome, those receiving intravenous medication, and those submitted to coronary angioplasty within the last month were excluded from the study. All patients had been receiving aspirin (100 mg once a day) for at least one year; additionally, 7 patients were on ACE inhibitors, 7 on statins, 7 on nitrates, 7 on diuretics, 4 on beta blockers, 2 on angiotensin II antagonists, 2 on calcium channel blockers and 9 on other medications.

Clot preparation

From each blood sample two clots were generated. Briefly, citrated whole blood (0.5 ml) was mixed with a trace amount (≈200.000 c.p.m.) of ^{125}I-radiolabeled fibrinogen (Amersham Biosciences, Germany); clotting was induced by adding calcium chloride (50 mmol/l) and 1 IU/ml of bovine thrombin (Sigma, USA). After one-hour incubation at 37°C, the clots were washed, weighted on analytic balance, counted by a gamma-counter and measured for volume. The two clots were positioned in two separate *in vitro* models.

In vitro model

Each clot was positioned into 50 ml polypropylene test-tubes (BD Biosciences, USA) filled with 5 ml of plasma, pooled from different healthy donors. As schematically illustrated in Figure 1, the tube contained an inlet and an outlet cannula that were connected by silicon pipes to a multi-channel peristaltic pump, allowing the plasma to flow at a rate of 1.0 ml/min. The test-tube was fixed in a vertical position inside a perspex box filled with water. To allow ultrasound penetration, an acoustic window was obtained from one of the box's walls by substituting the perspex with a thin silicon membrane. The transducer was leaned on this membrane, perpendicularly to the tube, with the interposition of ultrasound gel. To mimic the acoustic properties of the tissues, a multi-layered gel was interposed between the transducer and the test-tube. Each layer was made of a synthetic gel built with a polymeric matrix (Sigma Aldrich, Germany) permeated by water. Two *in vitro* models (with and without ultrasound) were located in a thermostatic bath that kept the temperature inside the test tubes at a constant value of 37°C. The temperature changes throughout the experiments were within 0.5 °C at the clot site and within 1.5° C close to the transducer.

Ultrasound exposure

Ultrasound was generated by a mechanical sector scanner (AU3, Esaote, Italy) operating at 2.5 MHz. Using a 45° sector angle and a 6 cm depth, the mechanical index was 1.0. The acoustic attenuation of the ultrasound beam was measured by a

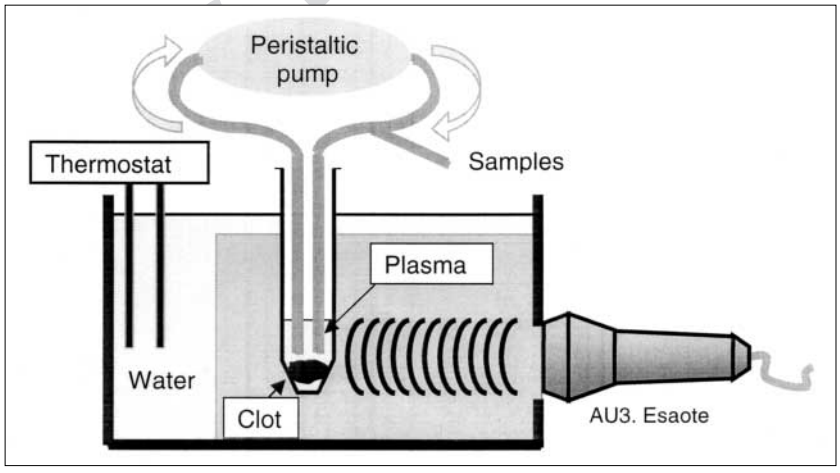

Figure 1: Schematic representation of the *in vitro* model.

Table: Physical characteristics of clots, blood cell count and coagulation parameters in normal subjects and in patients with coronary artery disease.

	Normal subjects	Patients	p value
Clot weight (mg)	256±48	255±36	0.926
Clot volume (μl)	184±49	184±21	0.947
Clot density (mg/μl)	1.42±0.14	1.38±0.11	0.319
White blood cells (nx10^3/μl)	7.041±1.649	6.736±1.572	0.687
Red blood cells (nx10^6/μl)	4.510±0.511	4.355±0.447	0.521
Platelets (nx10^3/μl)	259.40±68.50	203.77±77.43	0.115
aPTT (sec.)	33.0±2.7	33.4±2.9	0.756
Fibrinogen (mg/dl)	316.60±39.78	353.50±74.94	0.191
INR	0.98±0.05	1.00±0.05	0.292

aPTT = activated protrombin time, INR = international normalized ratio.

hydrophone in an acoustic bath. The ultrasound power in the focal zone was 0.165 W/cm^2 in water, while it became 0.099 W/cm^2 after crossing the membrane, the multi-layered gel and the test-tube. This value substantially agreed with that measured for a 2.5 cm thick layer of subcutaneous and muscular tissues (0.093 W/cm^2). Ultrasound exposure lasted 3 hours.

Pharmacological and ultrasound treatment

All the clots were treated with acetyl salicylic acid (60 μg/ml), heparin (1 IU/ml) and tPA (3 μg/ml). These medications were chosen to mimic the treatment of the patients with acute myocardial infarction and ST segment elevation (which is based on the administration of aspirin, heparin and tPA) (16, 17). In paired experiments, each clot received the same pharmacological treatment, but one was also continuously exposed to diagnostic ultrasound.

Determination of clot dissolution

To measure the release of ^{125}I from each clot and the reproducibility of measurements, two aliquots of 50 μl of plasma were removed from the circuit at time 0 and after 5, 15, 30, 60, 90, 120 and 180 minutes. At each time the clot lysis was calculated as the percent ratio (count at time t – count at time 0)/ (total clot count - count at time 0). The inter-assay coefficient of variation of this method was <4% (n=10).

Statistical analysis

The difference between normal subjects and patients with coronary artery disease regarding blood cell count, coagulation parameters and clot characteristics was tested by the analysis of variance. The difference in % clot lysis between normal subjects and patients was tested by the analysis of variance for repeated measurements, while the difference in % clot lysis activity at various time intervals was tested by the student's *t* test. Data are expressed as mean ± standard deviation. A p value ≤ 0.05 was considered to be statistically significant.

Results

Normal subjects did not differ from patients with coronary artery disease as to red and white blood cell count, platelet count, activated protrombin time, fibrinogen and international normalized ratio (Table). Similarly, the clots generated from blood of normal subjects did not differ from those of patients as

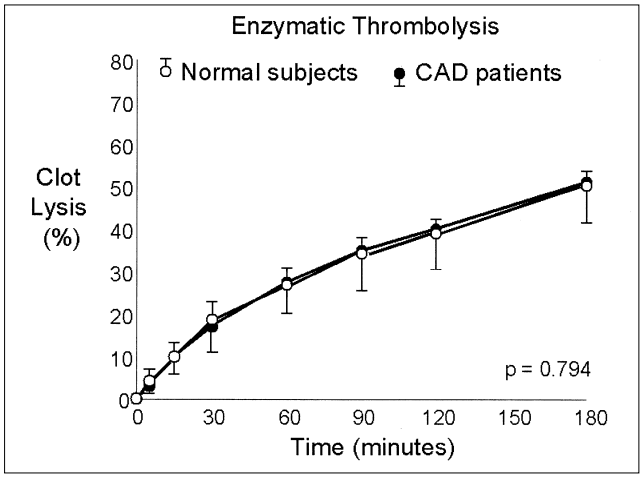

Figure 2: Enzymatic lysis in normal subjects and in patients. Comparison between clot dissolution in normal subjects and in patients with coronary artery disease. All the clots were exposed to acetyl salicylic acid (60 μg/ml), heparin (1 IU/ml) and tPA (3 μg/ml) for 3 hours in the absence of ultrasound. No difference in clot lysis of normal subjects and of patients was observed at any time.

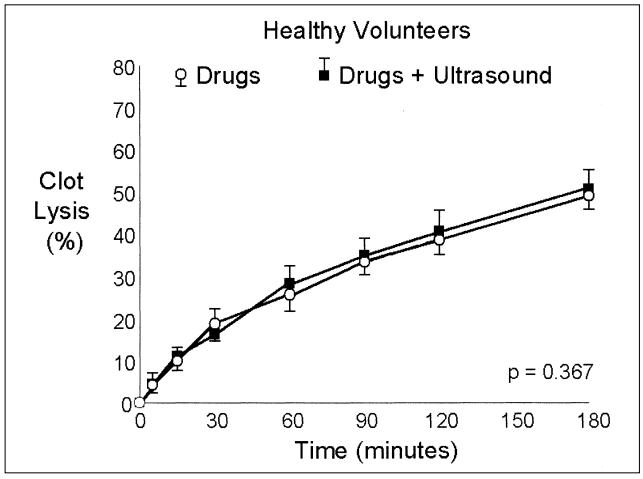

Figure 3: Ultrasound effect in normal subjects. Effects of ultrasound on enzymatic dissolution of the clots prepared with blood of normal subjects. All the clots were treated by acetyl salicylic acid (60 μg/ml), heparin (1 IU/ml) and tPA (3 μg/ml) in the presence or absence of ultrasound. No difference in clot lysis was observed at any time nor with or without ultrasound exposure.

to weight (256±48 vs 255±36 mg), volume (184±49 vs 184±21 μl) and density (1.42±0.14 vs 1.38±0.11 mg/μl) before treatment. Finally, the combined effect of aspirin, heparin and tPA on clot dissolution did not differ between normal subjects (49±4% at 3 hours) and coronary artery disease patients (51±9% at 3 hours) at any time (Fig. 2).

Continuous exposure to diagnostic ultrasound did not modify the combined effect of aspirin, heparin and tPA on the dissolution of the clots prepared with blood of normal subjects ($p = 0.367$) (Fig. 3). Conversely, ultrasound did significantly accelerate enzymatic thrombolysis of clots prepared from the blood of patients with coronary artery disease ($p = 0.013$) (Fig. 4).

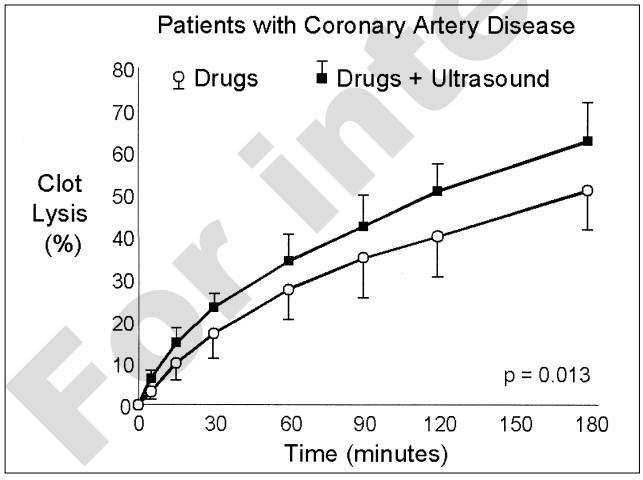

Figure 4: Ultrasound effect in patients. Effects of ultrasound on enzymatic dissolution of the clots prepared with blood of patients with coronary artery disease. The clots were treated by acetyl salicylic acid (60 μg/ml), heparin (1 IU/ml) and tPA (3 μg/ml) in the presence or absence of ultrasound. Ultrasound exposure enhanced at all times the lysis of clots from patients with coronary artery disease.

The enhancement of thrombolysis obtained with ultrasound was greater at the beginning of acoustic exposure, being 51% at 5 minutes, 32% at 15 minutes, 27% at 30 minutes, 20% at 1 hour and 19% at 3 hours ($p < 0.05$).

Discussion

This study shows that the exposure to ultrasound generated by a diagnostic scanner operating at high frequency (2.5 MHz) and low intensity (0.165 W/cm^2) enhances enzymatic lysis of clots generated from the blood of patients with coronary artery disease; diagnostic ultrasound exposure had no effect on enzymatic lysis of clots from normal subjects. In previous studies thrombolysis was enhanced by ultrasound with elevated acoustic energy (from 1 to 8 W/cm^2) which, however, can cause relevant side effects up to vessel reocclusion (4, 18). A major difference between these studies and ours is that we evaluated the effects on thrombolysis of high frequency, low intensity ultrasound, commonly utilized in diagnostic imaging. The only study in which clot lysis was accelerated by a high frequency transducer is that by Behrens et al (9), who placed pure fibrin clots in a tubing system at a constant pressure, and in which the clots were obstructing the flow. Exposure to 1 MHz ultrasound slightly accelerated clot lysis. Thus, Behrens' study differs from ours in several aspects, including the transducer frequency (1.0 vs. 2.5 MHz), the experimental model (obstructing vs. non obstructive clot) and the thrombus composition (pure fibrin vs. whole blood clots).

An intriguing finding of this study is the reason why diagnostic ultrasound enhances enzymatic lysis in patients with coronary artery disease, while it has no effect in normal subjects. Patients with coronary artery disease and normal subjects differ in so many variables, including age, that the reason for such a divergence can only be speculated upon in this study. Normal subjects and patients did not differ as to physical characteristics of thrombus and coagulation parameters. Thus, we hypothesized that ultrasound effect could be influenced by the chronic medical treatment taken by the patients. Medical therapy could make the clots of patients less "resistant" to ultrasound than those on normal subjects. Because no concomitant treatments were listed among the study exclusion criteria, the enrolled patients were taking a variety of medications. Although it is difficult to speculate on the different drugs, their dosages and the possible interactions, we are inclined to believe that aspirin could have played a role in enhancing the ultrasound effect. As a matter of fact, aspirin was the only medication taken by all patient and indeed it has been demonstrated that the treatment with aspirin alters the fibrin network structure (19). Specifically, previous studies showed that tighter fibrin gels were obtained

with plasma samples derived from patients with cardiovascular disease and with insulin dependent diabetes mellitus than from those of healthy controls (20, 21). However, the treatment with aspirin reversibly increased the permeability of the fibrin network in patients with coronary artery disease (22) and in normal subjects (23). The aspirin-induced increase in gel porosity might be beneficial to clot dissolution, making the transport of components promoting fibrinolysis more efficient (19). In addition, ultrasound reversibly increases flow through fibrin gels, a property primarily determined by the structure of the fibrin matrix clot (24). Thus, in a more porous fibrin gel due to aspirin treatment, ultrasound exposure could cause disaggregation of uncrosslinked fibrin fibers and could create additional binding sites for fibrinolytic components, with the net effect of improving the fibrinolytic efficacy. However, the effect of other medications cannot be excluded.

An interesting finding of this study is that the enhancement of thrombolysis was maximal (51%) at the beginning of ultrasound exposure - and it is well known that early vessel recanalization and tissue reperfusion can limit the effects of ischemia and preserve tissue viability. If the results of this study are confirmed *in vivo*, ultrasound-enhancement of thrombolysis could open new therapeutic perspectives in acute vascular occlusion, also considering the wide availability and the optimal safety profile of diagnostic scanners.

The observations made in this study are susceptible to further improvement on physical, pharmacological and biological grounds. In particular, the optimal transducer frequency, the intensity with the best efficacy/safety profile, the ideal acoustic pressure and the additive effects of ultrasound contrast agents (25) have yet to be defined. Furthermore, the interaction between ultrasound and aspirin or other medications should be investigated in future studies to elucidate the mechanism of action. Finally, the difference in coagulation parameters of normal subjects and patients with coronary artery disease was tested in this study only for routine tests, without exploring other specific biochemical parameters of endogenous fibrinolytic activity, clot stability and systemic inflammation.

Conclusions

This study shows that even ultrasound generated by a diagnostic scanner can produce a therapeutic effect, enhancing *in vitro* the enzymatic lysis of the clots of patients with coronary artery disease. Since diagnostic ultrasound does not produce such an effect in the clots of normal subjects, who are not taking any medications, we hypothesized that the ultrasound effect could be influenced by the chronic medical treatment. In particular, aspirin treatment could make the clots of patients less "resistant", so that even a low acoustic energy could facilitate the transport and uptake of the fibrinolytic agents into the clot; at variance, an intense acoustic energy is necessary to enhance the lysis of the more "resistant" clots of normal subjects. Despite these considerations, the exact mechanism of action was not covered by this study, and the interaction between ultrasound and different medical treatments should be investigated in further studies. Finally, if this observation made *in vitro* is confirmed *in vivo*, it could have relevant implications for the treatment of acute vascular occlusions, considering the wide availability and the excellent safety profile of the scanner utilized for diagnostic purposes.

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