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THERMALLY-INDUCED HAEMOLYSIS *IN VIVO*

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INTRODUCTION

Wong and Watmough [1] depilated the skin covering the abdomen of adult rats and directed the beam of ultrasound emitted by a one inch diameter therapy transducer under the rib cage, through the liver and towards the heart. The anaesthetised animals were subjected to continuous wave 0.75 MHz ultrasound for 5 min at SATA intensities of up to 2 W/cm^2. Blood samples were removed by cardiac puncture immediately after the ultrasound exposure and were diluted with isotonic saline before centrifuging and the spectrophotometric estimation of the amount of free haemoglobin in the plasma. Despite the large inherent variability in their measurements they observed a "threshold" spatially averaged intensity of about 0.5 W/cm^2 for the release of free haemoglobin into the plasma. The amount of haemoglobin in the plasma increased with increasing intensity above this "threshold", rising to about 5% of the value which would be obtained by complete rupture of all of the erythrocytes in the blood at a spatially averaged intensity of 2W/cm^2. Their spatial peak to spatial average intensity ratio was estimated to be about five.

Wong and Watmough [1] also directed a beam of ultrasound from a Doppler diagnostic apparatus at an artery in the rat's thorax and detected "spikes" which they interpreted as evidence of intravascular gas bubbles. They therefore concluded that the mechanism responsible for the liberation of free haemoglobin could have been some form of cavitational activity occurring within the rat's heart.

Chater and Williams [2] observed the release of free haemoglobin into the plasma of anaesthetised rabbits (presumably caused by a cavitational mechanism) after a 25 kHz tissue disruptor (Rapidis 50), driven slightly off resonance, had been pressed against the outside of the exposed but intact inferior vena cava. However, this same *in vivo* model system was not able to detect erythrocyte lysis after the vena cava had been irradiated with c.w. 0.75 MHz ultrasound for 5 min at spatially averaged intensities of up to 17.4 W/cm^2[2].

Similarly, an extensive series of *in vivo* investigations involving the exposure of the blood within the hearts of anaesthetised dogs has failed to detect any evidence of ultrasound-induced erythrocyte lysis or intravascular cavitational activity even at spatial peak intensities as high as 16 W/cm^2 at either 1.0 or 1.6 MHz [3]. Resonant bubble detectors [4] were attached to the aorta within the thorax or abdomen so as to detect the micron sized bubbles which are washed downstream when a flowing liquid is driven to cavitate at MHz frequencies [5]. These negative *in vivo* observations indicate that it is surprisingly difficult to get blood to cavitate at low MHz frequencies within the intact vascular system, even when it is provided with pre-formed micron-sized gas bubbles to act as "initiating sites"[3].

The negative *in vivo* results described above clearly contrast with the conclusions of Wong and Watmough [1] regarding the mechanism of release of haemoglobin

from erythrocytes by ultrasound. The present article therefore attempts to confirm the haemolytic effect of MHz ultrasound *in vivo* and to identify the mechanism responsible for rupture of the erythrocyte membrane.

METHODS AND MATERIALS

Inbred young adult male Sprague Dawley rats of average weight 382 ± 24 gm were anaesthetised by the intraperitoneal injection of Innovar Vet (Droperidol and Fentanyl, 0.3 ml of a 10% solution/100 gm + 0.1 ml/100 gm as needed). The entire abdominal area (dorsal and ventral) was shaved and depilated and the animal's forelimbs were bound by adhesive tape to a 1.5 by 12 cm flat bar. A rectal thermometer (Bailey, RET-2) was inserted, held in place by adhesive tape around the tail, and connected to a Bailey BAT-8 display. The flat bar was held in a clamp stand so that the animals were immersed up to their necks in a circulating water bath maintained at 38 ± 0.5°C.

Ultrasound generation

An air-backed flat piezoelectric disc 2.54 cm in diameter was mounted in a waterproof brass flange fitted with a 4 cm long and 3.2 cm internal diameter clear plastic tube. The open end of this tube was covered with an acoustically transparent window of clingfilm and the tube was filled with degassed water to provide a water path. This water was continuously circulated through a 0.5μm pore size filter to remove any debris or bubbles. This arrangement was designed so as to (1) facilitate orientation of the ultrasound beam, (2) to cool the transducer, and (3) to minimise cavitational activity (bubble generation) between the transducer and the animal. The transducer was driven by a Tektronix SG503 signal generator, amplified by an ENI A150 power amplifier, and monitored by a Fluke 8920A RMS voltmeter.

Ultrasound dosimetry

The ultrasonic field emitted by the transducer was measured under free field conditions by means of a beam scan and integration technique. A Dapco NP10A90 hydrophone was used to plot the field and measure the spatial peak (SP) intensity and a 5 cm diameter absorbing target radiation force balance was used to measure the total power output at a distance of 10.8 cm from the transducer face. The spatial average (SA) intensity was obtained by dividing the total power from the radiation force measurements by the radiating area of the transducer obtained from the field plots.

The transducer was driven at its fundamental thickness resonance (1.04 MHz) and its nominal third harmonic (3.4 MHz). The 3dB and 6 dB beamwidths were 8.6 and 14.1 mm at 1.04 MHz and 10.9 and 18.0 mm at 3.4 MHz and the SP/SA intensity ratios were 3.9 at 1.04 MHz and 2.6 at 3.4 MHz. The exposure intensities were calculated from the square of the monitored voltage. The plane wave shock parameter [6] was less than unity for all exposure conditions(based on an 8 cm transducer-to-skin distance and on the spatial average intensities used).

Experimental procedure

Each animal's rectal temperature was found to have been lowered by the anaesthesia and depilatory procedures, and so they were not exposed to ultrasound in the water bath until their core temperature had returned to 37 ± 0.5°C. Each animal received two 10 min ultrasound exposures directed towards its liver and diaphragm, one on each side of its xiphisternum. Any ultrasound that passed straight through the animal was absorbed in a slab of SOAB rubber. Blood

samples (typically 5 ml) were removed by cardiac puncture, using syringes containing a small volume of 1,000 IU/ml heparin, immediately after sonication. The animals were then sacrificed with an overdose of barbiturate before necropsy and the removal of portions of the liver for histopathological investigation.

Haematological investigations

One half ml aliquots of the blood were added to 4.5 ml each of isotonic (295 mOsm/l) and hypotonic (180 mOsm/l) saline solutions and thoroughly mixed. The saline solutions and the remainder of the original blood sample were centrifuged for 5 min at 3,000 rpm and the supernatants removed for the estimation of free haemoglobin by its absorbance at 540 nm using a Bausch and Lomb Spectronic 70 spectrophotometer. The plasma samples were routinely filtered through 0.45 μm pore size Micropore filters to remove chylomicra and erythrocyte fragments before the estimation of plasma haemoglobin. One hundred microlitres of the original blood samples were also added to 9.9 ml of distilled water to provide a complete osmotic haemolysis value.

Selected blood samples were also diluted with filtered isotonic saline solution and their size distribution measured using a Coulter Counter ^{R} (model Z_B with a 70 μm aperture) connected to a Coulter model P_{128} size distribution analyser. The output of the analyser was smoothed using a condenser and displayed on a chart recorder.

RESULTS AND DISCUSSION

Preliminary investigations were performed using rats having only the ventral surface of their abdomen shaved and depilated, and the animals were exposed to ultrasound while they were lying supine on a wooden bench (i.e. this duplicates the exposure situation used in [1]). These studies confirmed that therapeutic intensities of MHz ultrasound could result in the release of free haemoglobin into the plasma even though the effect was highly variable and critically dependent on the orientation of the transducer. The stomach and intestines became distended with gas if they were in the path of the ultrasound beam, and by reflecting the ultrasound could result in the production of a full-thickness burn through both layers of the body wall. This circumstance (and the analogous one where the ultrasound was reflected by the cartilagenous xiphisternum) was associated with low values of plasma haemoglobin. When the ultrasound beam was redirected so that it could enter the liver, then higher values of plasma haemoglobin were obtained which were similar whether the ultrasound was directed towards or away from the heart. Greater reproducibility of results was obtained by irradiating the animals in the water bath and subjecting them to two 10 min exposures of ultrasound, one on either side of the xiphoid cartilage.

In an earlier article [7] we showed that the posterior and post-caval lobes of the right lung extend below the heart of the rat and are interspersed between it and the diaphragm. Thus, the ultrasound beam which traverses the liver is largely reflected at the diaphragm and can not enter the animal's heart.

Nevertheless, ultrasound exposure does result in an intensity-dependent increase in the amount of free haemoglobin in the plasma. Figure 1 presents the plasma haemoglobin levels (expressed as a percentage of the value obtained by complete osmotic haemolysis) as a function of the spatially averaged intensity at 1.04 and 3.4 MHz for a total of 60 animals, including 8 sham controls. The magnitude of the effect can be seen to be greater at the higher frequency, which implies a

thermal mechanism.

Photomicrographs of blood smears from animals having detectable levels of plasma haemoglobin showed the presence of substantial numbers of haemoglobin-filled spherical bodies having a wide size distribution [7]. The smallest of these spherical bodies have such a high ratio of fat-containing membrane to their haemoglobin-filled contents that they are neutrally buoyant and are not sedimented during centrifugation. Since these small bodies would scatter a light beam and give falsely high absorbance values, the plasma samples had to be filtered through a 0.45 μm Micropore filter before being measured. Some samples contained so many of these bodies that the filters became blocked and had to be replaced.

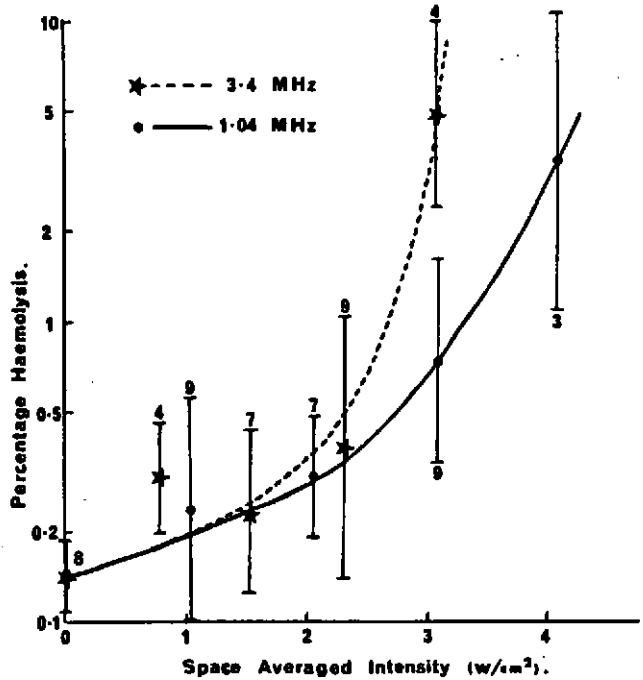

Figure 1. The free haemoglobin content of the plasma (expressed as a percentage on a logarithmic scale) as a function of the space averaged ultrasonic intensity. The error bars represent ± one standard deviation (logarithmically averaged) and the adjacent numbers refer to the number of animals in each sample.

Figure 2 presents normalised Coulter Counter^{R} size distribution analyses of aliquots of the same rat's blood both before(1) and after (2) it had been exposed *in vivo* to two 10 min exposures of 3.3 MHz ultrasound at a space averaged intensity of

2 W/cm². Both traces were obtained at the same instrument settings with 1/amplification = 1, and 1/ aperture current = 0.354 with a 70 μm diameter orifice. It can be seen that ultrasound exposure decreased the number of erythrocytes having a large cell volume and increased the proportion of erythrocytes having a smaller cell volume. Ultrasound exposure also caused a very large increase in the number of particles having volumes corresponding to cell fragments (microspheres) having dimensions less than 1 μm in diameter. The lower threshold settings of the instrument had to be set to eliminate these small particles because they were so numerous that they saturated the counters. Similar results were obtained when control blood samples were heated *in vitro* for 5 min at a temperature of 50 ± 0.5°C.

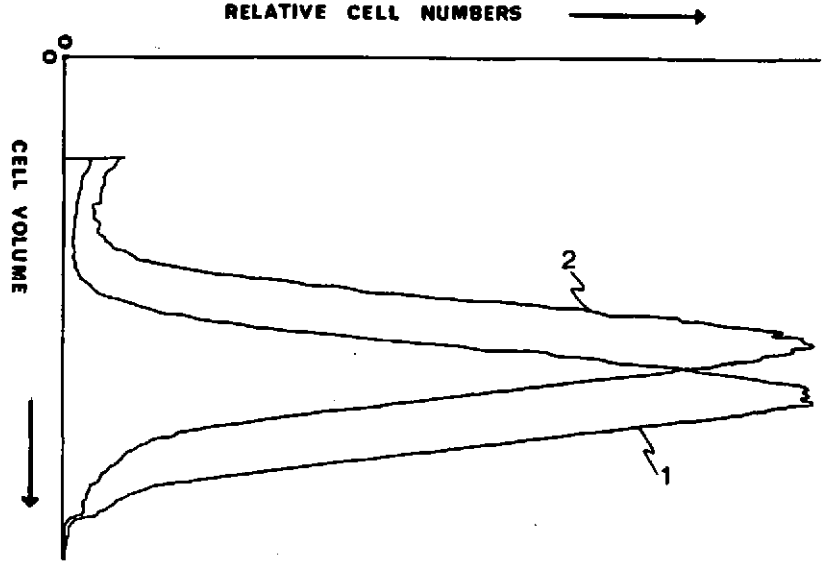

Figure 2. Normalised coulter counter displays of the relative size distribution of a rat's erythrocytes before (1) and after (2) exposure to 2 W/cm² of 3.3 MHz ultrasound for a total of 20 min.

Mammalian erythrocytes are known to fragment forming haemoglobin-filled spherical fragments (microspheres) having a wide size distribution when they are heated *in vitro* to more than 49°C [8]. These thermally damaged cell fragments would most probably be eliminated by the reticuloendothelial system if they were present *in vivo* and their haemoglobin contents would be released into the blood prior to being removed and disposed of by the liver. This possibility was investigated by heating a sample of control blood *in vitro* at 54 ± 0.2°C for 5 min and then injecting it via a tail vein into another unexposed animal. The plasma haemoglobin value of the heated blood sample was 12%, and after 0.9 ml

of this blood had been injected into a 415 gm rat (whose total blood volume was estimated to be about 38 ml), the donor animal's plasma haemoglobin should have been raised to about 0.3% just by simple dilution. If all of the microspheres and cell fragments within the heated blood were lysed *in vivo*, then the plasma haemoglobin value should be raised to about 2.4%. A blood sample removed 20 min after the injection of the heated blood sample had a plasma haemoglobin of 1.95% showing that most of the microspheres and larger cell fragments had indeed been lysed and their haemoglobin contents were present in the plasma [7]. Microscopic investigation of the blood sample taken 20 min after injection of the heated blood confirmed that some microspheres were still present and had not yet been removed from the circulation.

Since the blood samples withdrawn from the sonicated animals still contained a large number of microspheres and larger cell remnants, the plasma haemoglobin measurements presented in Figure 1 must be an underestimate of the total amount of blood cell damage caused by the ultrasound. In an endeavour to obtain a more meaningful estimate, 0.5 ml aliquots of each original blood sample were added to 4.5 ml of 180 mOsm/l saline, mixed and centrifuged before measuring the percentage lysis in the supernatant. This osmolarity was chosen so that control erythrocytes would be swollen but not ruptured whereas microspheres and cell fragments having a smaller surface area to volume ratio would be lysed. The percentage lysis observed in 180 mOsm/l saline was about 2 to 3 times greater than than observed in isotonic saline if the samples contained only a small number of microspheres (i.e. when the animals had been exposed to less than 2 W/cm^2 of ultrasound) but rose to be 5 to 8 times greater than the values presented in Figure 2 when the samples contained larger numbers of microspheres (i.e. following exposures to intensities greater than 2 W/cm^2) [7].

All of the results presented above indicate that the mechanism of haemoglobin release is thermal damage to erythrocytes as they perfuse anatomical structures (particularly the liver) being heated to more than 49°C by the ultrasound beam. This hypothesis was confirmed by histological examination of the livers of sonicated animals which showed the build up of a layer of platelets around the walls of the larger blood vessels [7] which is what would be expected if the endothelium had been damaged by heat diffusing from the surrounding tissues. Cooling the animals by reducing the temperature of the water bath to 25.5°C virtually eliminated the haemolytic effects [7].

Additional evidence supporting a thermal mechanism was obtained from experiments where the same time averaged ultrasonic intensity was delivered either as a continuous wave or as a pulsed beam. There were no significant differences between the plasma haemolyis levels obtained at 1.04 MHz when the same amount of ultrasonic energy was given as a continuous wave of spatial average intensity 1.03 W/cm^2 or as pulses 1 ms on and 2 ms off (spatial average intensity 3.08 W/cm^2) or 1 ms and 3 ms off (spatial average intensity 4.1 W/cm^2) [7].

As a final positive control, two 2.3 cm diameter nickel electrodes were coated with electrically conducting gel and bound to the depilated skin both above and below the liver. An electrical current at a frequency of 10 MHz and rms voltage of 30 v was applied for 20 min until the animal's rectal temperature was raised to a value similar to those obtained during ultrasound exposures (41°C). Both the visual appearance of the heated liver and the pattern of blood cell damage were qualitatively and quantitatively similar to those seen in sonicated animals [7].

CONCLUSIONS

Therapeutic intensities of MHz ultrasound directed into the livers of anaesthetised rats for prolonged periods *in vivo* results in the liberation of free haemoglobin into the plasma.

Microscopic examination and size distribution analyses indicated that erythrocytes from sonicated animals had fragmented in a manner analagous to that seen when blood is heated to more than 49°C *in vitro*.

The haematological findings in sonicated animals could be duplicated by injecting blood heated *in vitro* into non-sonicated animals. They could also be duplicated by heating the animal's liver by non-acoustic means.

Thus, the dominant mechanism responsible for the observed cell lysis appears to be thermal damage to the erythrocytes as they perfuse the microvasculature of anatomical structures being heated by the ultrasound beam.

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ANALYSIS OF FINE STRUCTURE IN ULTRASOUND IMAGES

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SYNOPSIS

An experiment has been built to investigate the way fine echo structure, (also called parenchymal echoes or speckle), is formed in medical ultrasound images. At present this part of the image formation not well understood. It depends in a complicated way, on the interaction between the acoustic structure of the tissue and the imaging system parameters. These include the transducer pulse and beam shapes, (which define the "Resolution Cell" of the transducer), the scanning geometry, and the signal processing used. The simplified model, used here, allows the effect of the above features to be controlled and studied seperately, where this is physically possible. This paper describes a method to measure structure in the image of a random model.

The salient features of the experiment are as follows. The tissue is modeled as a non-regular distribution of small scatterers in a gelatine substrate. Its "structure" therefore is described by the mean separation between the scatterers and their distribution. A range of transducers of the sort used clinically in static 'B' scanners is used. They have been characterised separately using a ball target. The scanning method was a simple rectilinear 'B' scan taking a series of horizontal "slices" which build up to a particular volume within the model. The geometry was repeatable for each transducer. The r.f. signal for each 'A' scan line was digitised. All subsequent signal processing (such as envelope detection), was done by a computer. The resulting ultrasound images were analysed both visually and by using well known image measures based on the Two Dimensional Fourier Transform and Autocorrelation Function.

The results of this analysis will be presented. Unfortunately it was not practical to include the results in this paper.The models had a mean scatterer separation which ranged from greater than a resolution cell to less than a wavelength of the transducer pulse. The volume scanned had dimensions of 20 x 20 x 10 mm. The transducers had pulse frequencies between 3 and 5 MHz. An image was built up from the lines of r.f. data by a process of detection, smoothing and resampling. In doing this, a number of artifacts (system effects) were identified which are believed to be common to other ultrasound imaging systems. A number of characterising features were tried on both the r.f. and detected images. These features will be compared and the ability of this particular system to resolve model structure will be shown.