Ultrasound in Med. & Biol., Vol. 8, No. 1, pp. 45–55, 1982
Printed in Great Britain.

0301-5629/82/010045-10$03.00/0
Pergamon Press Ltd.

THE EFFECT OF ULTRASOUND ON MICROVASCULAR HEMODYNAMICS IN SKELETAL MUSCLE: EFFECT ON ARTERIOLES

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(*First received* 23 *September* 1980; *and in final form* 12 *March* 1981)

Abstract—A skeletal muscle model (rat cremaster muscle) has been described for the evaluation of ultrasonic bioeffects on the microscopic vasculature. One MHz pulsed ultrasound (mark-space 100 μsec:100 μsec) was administered to the tissue at 1.25, 2.5, 5.0 and 10.0 Wcm^{-2} (temporal average) while continuously measuring lumen diameter, volume flow, and rate of vasomotion (intrinsic cyclic constriction and relaxation) in three different sizes of arterioles (100, 50, 30 μm inner diameter). No change in these parameters was noted at intensities below 5 Wcm^{-2}. Above 5 Wcm^{-2} there was a 25% decrease in diameter and a 44% decrease in volume flow in the smallest arterioles observed, as well as a 29% decrease in flow in the largest arteriole feeding the tissue. The rate of vasomotion in the smallest arterioles increased 20× in the same intensity range. None of these effects or their magnitude could be attributed to simple tissue heating in the same temperature range (34–36.5°C) as obtained with ultrasound exposures.

Key words: Ultrasound, Ultrasonic Bioeffects, Rat cremaster muscle, Microvascular, Hemodynamics, Arterioles.

INTRODUCTION

Several clinical trials have demonstrated the efficacy of ultrasound application in the treatment of skin ulcerations associated with venous stasis (Paul *et al.*, 1960; Galitsky and Leving, 1964; Dyson *et al.*, 1976). An experimental study of tissue regeneration in otherwise normal tissue (rabbit pinna) has shown that the application of ultrasound (f = 3.5 MHz, PRF = 100 Hz, 20% duty cycle) at intensities less than 1.0 Wcm^{-2} produces significantly faster regeneration when compared to sham-exposed controls (Dyson *et al.*, 1970). The enhanced regeneration could not be linked to simple changes in temperature produced by insonation. The same study also showed that insonation at 8.0 Wcm^{-2} reduced the rate of regeneration. Recent studies in our laboratory have indicated that application of continuous wave ultrasound (f = 1 MHz, 1.5 Wcm^{-2}) to infarcted canine myocardium speeds healing as demonstrated by increased vascularization and decreased dense collagen formation at 6 weeks (Franklin *et al.*, 1976). Furthermore, the severity and volume of infarcted tissue was less in the group treated with ultrasound (Franklin *et al.*, 1978).

These studies suggest that ultrasound is therapeutically beneficial in the complex process of wound healing and tissue regeneration when there is a large tissue defect. In such cases necrotic tissue must first be removed by cells involved in the inflammatory response, and then highly vascularized, collagenous tissue (granulation tissue) will fill the defect. Both processes are critically dependent upon an adequate blood supply (Robbins and Angell, 1976). It is possible, therefore, that the therapeutic usefulness of ultrasound is related to some action on local tissue perfusion. Such a hypothesis is teleologically appealing in cases of ulcerations of vascular origin and would explain the decreased infarct size found in insonated myocardium.

This ongoing study is an investigation of the effects of therapeutic levels of ultrasound on hemodynamics in skeletal muscle vaculature at the microscopic level (vessels $\leq$ 100 μm diameter). In this first of two parts on effects in normal tissue, the methodology is introduced and the interaction of ultrasound with the arteriolar vessels is described.

MATERIALS AND METHODS

Animal model

Male Wistar rats (70–90 g) were anesthetized with 7% urethane and 2% chloralose in saline (0.6 ml/100 g body wt.) delivered intraperitoneally. The animals were placed in a supine position on a water heated stage to maintain the body core temperature between

36 and 38°C. A tracheotomy was performed to insure a patent airway, and the left femoral artery was cannulated with a polyethylene cathether (PE 10) for systemic arterial pressure measurements (Statham P50 transducer).

The cremaster muscle was prepared for observation using a modification of the technique introduced by Baez (1973). A small incision was made at the tip of the scrotum on the right side, and the testicle was gently mobilized from the scrotal skin and fascia. A longitudinal cut along the antero-lateral aspect of the exposed cremaster muscle allowed the testicle to be removed from the enveloping muscle and delivered back into the abdominal cavity. The thin layer of muscle ($\sim 200\,\mu$m thick) was spread over a water-filled pedestal, 1 cm in diameter, and the tissue anchored with five sutures along the excised edges (see Fig. 1a). A bare copper-constantan thermocouple was placed under, and in contact with, the muscle for measurement of tissue temperature with a digital thermometer (Bailey Instruments, BAT-8). The exposed cremaster was continuously irrigated with a rat plasma analog (150 meq/l. Na^+, 5.8 meq/l. K^+, 6.7 meq/l Ca^{++}, bicarbonate buffer) that was bubbled with 5% CO_2, 95% N_2 to maintain pH at 7.4 and to create a low O_2 tension. The suffasant was warmed to maintain the cremaster muscle at its normal *in situ* temperature of 34.5°C.

The vascular distribution of the cremaster muscle is illustrated in Fig. 1b. A single arteriole $\sim 100\,\mu$m in diameter enters the midline of the muscle and is termed a first order arteriole (1A). Arteriolar branches from the 1A arteriole form a tree structure with 3–4 successive branching levels before giving rise to the capillaries. Branching at nearly 90° from the parent vessel, the subsequent arteriolar branches are referred to as second order (2A, $\sim 50\,\mu$m dia.), third order (3A, $\sim 30\,\mu$m dia.) and fourth order (4A, $\sim 15\,\mu$m dia.), respectively (Wiedman, 1968).

Equipment

Direct observation and insonation of 1A, 2A and 3A arterioles was accomplished by mounting the rat and stage on an Olympus microscope modified to accommodate the rat, ultrasound coupler, transducer and light source. The muscle was transilluminated with a fiber optic cable connected to a d.c. powered light source and the tissue was visualized on a closed circuit video system coupled to the microscope (see Fig. 2). The magnification observed on the video monitor was calibrated with a stage micrometer to allow direct measurements of vessel diameter from the video screen. A counter/timer displayed elapsed time in seconds on the video monitor and videotape recorder allowing off-line synchronization of the video data with arterial pressure, muscle temperature, and red blood cell (RBC) velocity which were recorded on a Gould 2400 oscillograph.

The center-line RBC velocity was measured with a velocity correlator system originally developed by Wayland and Johnson (1967). The system utilizes a photodiode pair mounted between the microscope and CCTV camera as the transducing element (Instrumentation for Physiology and Medicine). Calibration of the correlator was achieved by measuring the velocities of erythrocytes smeared on a glass plate which were rotated under the microscope at several known velocities with a d.c. motor. By using the fact that center-line velocity (v) has a constant relationship to average velocity ($\bar{v}$), for vessels down to 25 μm diameter ($\bar{v} = v/1.6$; Baker and Wayland, 1974), arteriolar volume flow (F) could be computed using the equation:

$$F = \pi r^2 \bar{v}$$

where r is the lumen radius.

Ultrasound application

An unfocused transducer (1 in. dia. PZT element) mounted in a water-cooled jacket was used to insonate the tissue. The transducer was driven at the resonant frequency (1.051 MHz) by a gated frequency synthesizer with the signal gated on for 100 μsec and off for an equal length of time. This pulsed format was chosen to suppress standing wave formation in the irradiation system. The double path length between the ultrasound source and the irradiated specimen was 22 cm. All equipment was fabricated at the Ultrasound Research Division.

The sound was coupled to the tissue on the microscope stage through a substage assembly diagrammed in Fig. 3. The sound was reflected through 90° by an optically flat glass plate which also served as an illumination port. The sub-stage assembly was attached to the microscope such that the sound field was

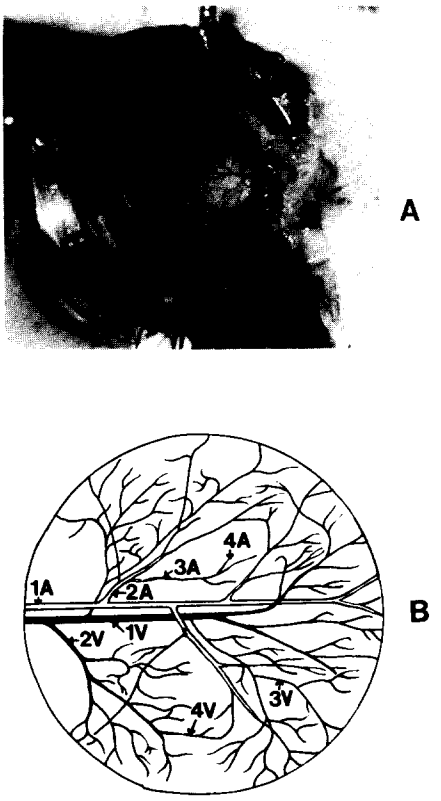

Fig. 1. (a) Photograph of the cremaster muscle prepared for observation and insonation. The muscle lies on a polymer film membrane covering a chamber filled with degassed water. The vascular distribution is a tree structure with the largest arteriole entering the muscle termed the 1A arteriole. (b) A schematic of the vascular distribution of the cremaster muscle. There are three levels of branching beyond the 1A arteriole before the terminal arterioles and capillaries. Vessel diameters decrease at each branching level (1A ~ 100 μm, 2A ~ 50 μm, 3A ~ 30 μm; 4A ~ 15 μm). Adapted from Hutchins *et al.* (1972) by permission of Academic Press, Inc. and the author.

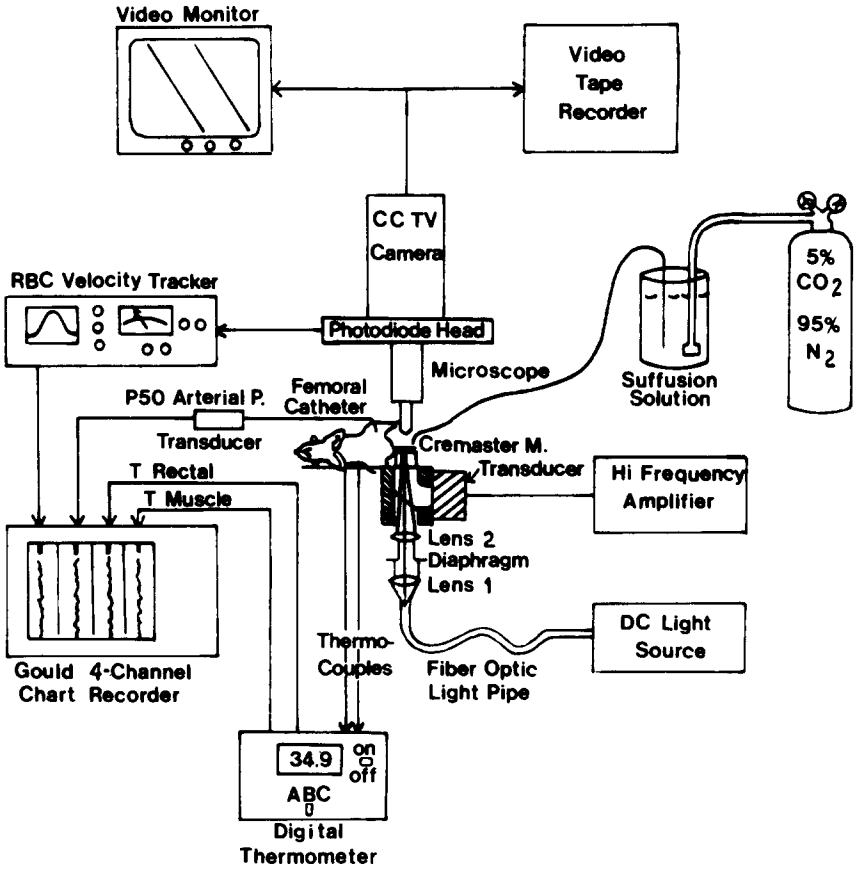

Fig. 2. Diagram of the equipment used in the microscopic evaluation of hemodynamics and the effects of ultrasound.

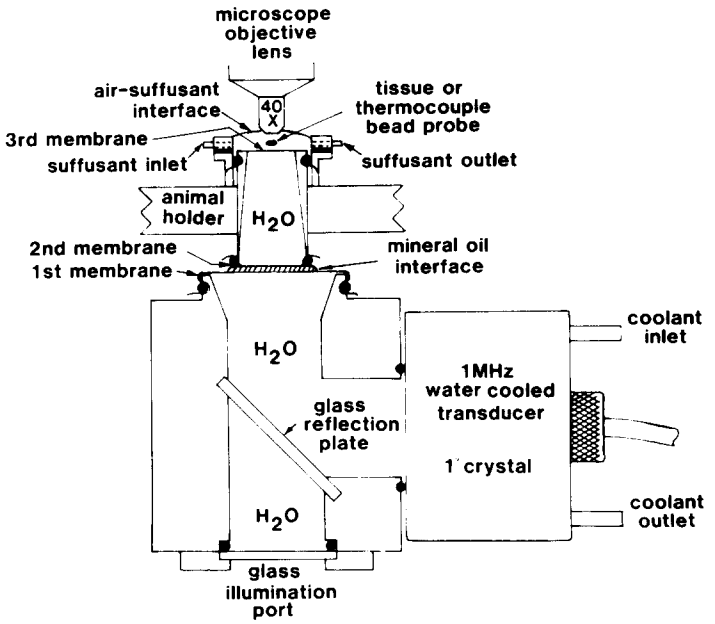

Fig. 3. Detailed diagram of the sub-stage assembly and animal holder used to provide sound coupling and illumination of the tissue. The transducer and reflection plate are fixed relative to the optical axis. The tissue may be moved in the field with coupling maintained through the mineral oil interface between the first two membranes.

fixed in position relative to the optical axis. The tissue could be moved in this field by movement of the animal holder on the stage surface. Sound coupling was maintained between the fixed sub-stage coupler and the movable pedestal by a mineral oil interface between two polymer film membranes. The entire coupler system was filled with degassed water.

The sound intensity at the level of the tissue was calibrated by means of a thermocouple junction covered with an epoxy bead 0.5 mm in diameter. Primary calibration of the thermocouple was carried out by a radiation-force method using displacement of a stainless steel ball in the sound field as the standard (Fry and Dunn, 1962). The change in voltage across the thermocouple for a 1-sec sound pulse was recorded in both the known and unknown intensity sound fields (Fry and Fry, 1954a, b). All intensity values presented are temporal averages.

In actual practice, a single blood vessel was observed on the optical axis during insonation. Calibration of the intensity at this locus was carried out with the thermocouple at the focal point of the objective. Intensity (calculated from the change in voltage across the thermocouple) vs V_{pp}^2 was plotted for four different values of voltage applied to the transducer. This was performed before each experiment in the earlier stages of the study, and the results are plotted in Fig. 4. Later studies used peak-to-peak voltage values specified by the regression line.

Figure 5 is a beam plot of the sound field at the tissue level which was made by moving the animal holder and thermocouple in the field. The plot was made with and without the microscope objective lens immersed in the suffusant solution. In all experiments it was necessary to immerse the lens to improve optical resolution although this produced distortion of the sound field. The result, however, was a flatter field between the 3 dB points.

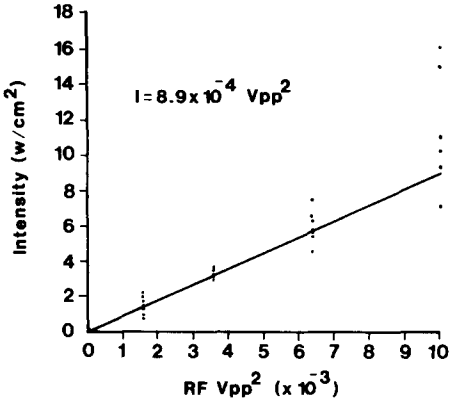

Fig. 4. Plot of intensity (Wcm^{-2}) vs RF V_{pp}^2. Intensity was calculated from changes in voltage across the thermocouple bead probe with the probe at the focal point of the microscope objective. Voltage is the peak–peak voltage applied to the transducer.

Protocols

Effect of ultrasound. Evaluation of ultrasonic bioeffects on arterioles was carried out by insonating the tissue while continuously observing a single 1A, 2A or 3A arteriole. Sound intensities of 1.25, 2.5, 5.0 and 10.0 Wcm^{-2} (temporal average) were applied in succession to the tissue. Each exposure period consisted of a 1-min control period, immediately followed by a 5-min insonation and a variable length post-exposure period to allow the tissue to normalize before increasing the intensity. The post-exposure period was increased in length from 10 min after the first exposure to 20 min following the last. Lumen diameter, RBC velocity, volume flow, arterial pressure and muscle temperature were measured every 10 sec during the control and insonation periods and every 2 min during the post-exposure period.

Thermal control. To evaluate vessel reaction to simple changes in temperature that might be created by the ultrasound exposure, a thermal control study was conducted. 1A, 2A and 3A vessels in each animal were individually observed using the same protocol as above. In place of the sound application, the temperature of the suffusant flowing over the tissue was increased to produce a linear change in tissue temperature from 34 to 38°C over the 5-min experimental period. A 10-min period was allowed between evaluations of different size vessels to allow the tissue to re-equilibrate at 34.5°C.

RESULTS

Ultrasound effects

Figure 6 graphically depicts the effect of 1 MHz pulsed ultrasound, in the range of 1.25–10 Wcm^{-2}, on arteriolar diameter and volume flow. The data are presented in terms of percent of control flow and diameter, where the control values are averages over the minute preceding the first sound exposure. The experimental values for each animal are averages over the last minute of a

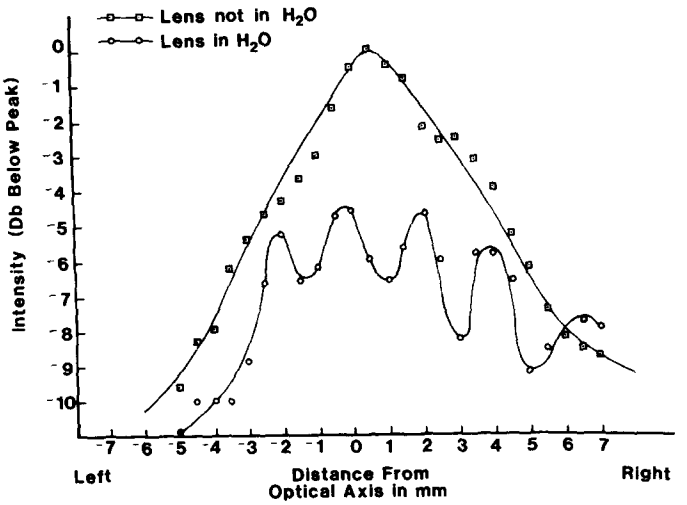

Fig. 5. Beam plot made by moving the animal holder from left to right in 0.5 mm increments with the thermocouple probe fixed on the pedestal. Placing the microscope objective lens in the suffusant overlying the thermocouple distorts the sound field.

5-min exposure at successive intensities of 1.25, 2.5, 5.0 and 10.0 Wcm^{-2}. Only one vessel was investigated in each animal so that the "n" refers to both animals and vessels (1A and 2A, $n = 5$; 3A, $n = 8$).

The largest arterioles (1A) showed little change in diameter or flow below 10.0 Wcm^{-2}. At 10.0 Wcm^{-2} there was a 29% decrease in volume flow without a significant change in 1A diameter. No significant change was seen in the second-order arterioles (2A). The greatest changes were seen in the smallest arterioles investigated (3A). Both diameter and flow were dramatically decreased at 5.0 and 10.0 Wcm^{-2}. There was an average decrease in lumen diameter of 25% and a decrease in volume flow through the individual vessels of 44%. In both the 2A and 3A there was increased average flow at 2.5 Wcm^{-2} though neither result was significantly greater than control.

In most tissues the arteriolar smooth muscle demonstrates slow cyclic contraction and relaxation, a process known as vasomotion

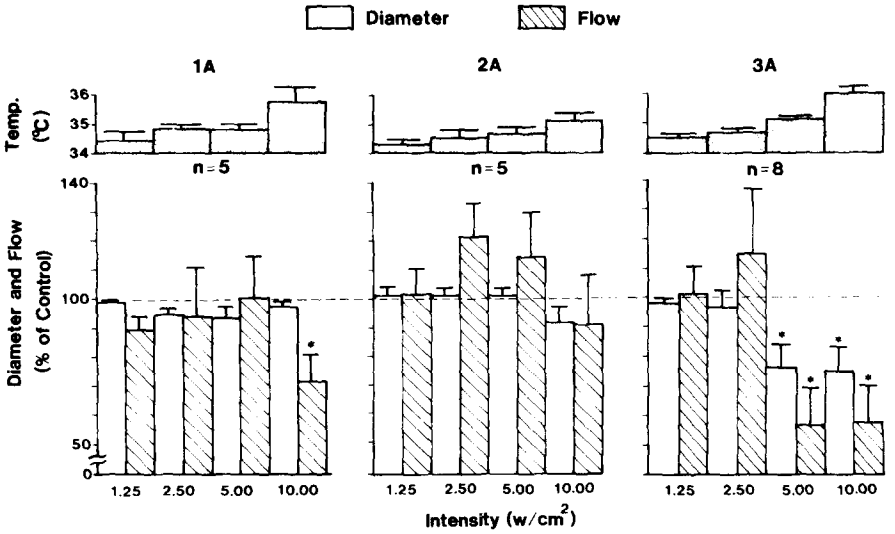

Fig. 6. Effect of ultrasound exposure on arteriolar diameter and flow. The white bars indicate changes in diameter as percentage of control. Cross-hatched bars are flow values as percent of control. Error bars are S.E.M. Diameter and flow were measured at 10 sec intervals during a 1-min control period, and during the last minute of a 5-min exposure to ultrasound at one of four intensities (1.25, 2.5, 5.0 and 10.0 Wcm^{-2}). A significant decrease in diameter and flow was seen in the 3A arterioles at 5.0 and 10.0 Wcm^{-2} ($p < 0.05$). Flow was also decreased in the 1A arteriole at 10.0 Wcm^{-2}. The number of vessels (n) studied is indicated (one vessel in each animal).

(Wiedeman, 1968). Late in the study it was noted that in most of the 3A vessels exposed to ultrasound the rate and apparent vigor of these contractions was greatly increased. Figure 7 shows the increase in the rate of contraction in 3A arterioles produced by exposure to 1 MHz pulsed ultrasound at 10.0 Wcm^{-2}. The measurements were made immediately following a 5-min sound exposure in a tissue which has previously been exposed at 1.25, 2.5 and 5.0 Wcm^{-2} according to the protocol described earlier. By way of comparison, the change in vasomotion is shown for a 3A arteriole which was heated over a 5-min period from 34 to 38°C. In both the sound and thermal study the control levels of vasomotion were the same (3–4/min). Heating the tissue to 38°C only increased the rate of contraction to 6/min whereas insonation of the tissue increased the rate to 31/min with a rise in tissue temperature to only 36°C. There was no clear dose-response relationship between the intensity of sound applied and the rate of contraction. In some cases there was a graded response; in others the maximum rate was achieved at intensities as low as 2.5 Wcm^{-2}.

Thermal effects

In order to separate the thermal and other effects of ultrasound on the arterioles, the intrinsic response of the arterioles to heat stress was investigated. Table 1 shows the results of directly increasing the muscle temperature over the same range as produced by exposure to sound at 10.0 Wcm^{-2} for 5 min. The data are reported as percent of control flow and diameter where the control period consisted of a 1-min period before heating where the temperature was held at 34.5°C. Subsequently the temperature was lowered to 34°C and raised at a constant rate of 0.8°C/min. The experimental values are averages for all values in the indicated temperature ranges. In only one case was there a significant change in flow or diameter in any of the vessels evaluated. Volume flow in the 3A arteriole was decreased by 15% in the range 35.5–36.5°C.

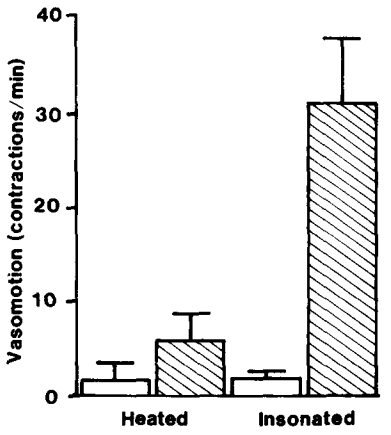

Fig. 7. Effect of direct heating (34–38°C) and insonation at 10.0 Wcm^{-2} (34–36°C) on the rate of vasomotion in 3A arterioles ($n = 6$).

The values in Table 1 were compared to the data in Fig. 6 in those cases where a significant decrease in arteriolar diameter or flow was shown due to ultrasound exposure. In all cases the decrease in flow and diameter produced by sound exposure was significantly greater than that which could be attributed to thermal effects ($p < 0.05$, t-prime test).

DISCUSSION

The data presented demonstrate that 1 MHz pulsed ultrasound produces constriction of the small arterioles (of the order of 30 μm diameter) in skeletal muscle at intensities of 5.0 Wcm^{-2} and above, with a consequent decrease in flow through those vessels. This occurs within a 5-min exposure period, and with heating of no more than 2°C above normal tissue temperature. The constrictor effect cannot be attributed to simple heating of the tissue by the sound. It is further shown that application of sound in the same intensity range produces an increased rate of arteriolar vasomotion in the smallest arterioles. No change was seen in either vasomotion or arteriolar lumen diameter in vessels larger that 30–40 μm. In spite of this there was a decreased flow at 10.0 Wcm^{-2} in the largest arteriole in the tissue.

Several caveats are in order for the interpretation of these data. First, it will be noted that the tissue temperature rise produced in the insonation experiments for the 2A arterioles was less than for the 1A and 3A experiments (Fig. 6). In retrospect this appears to be a procedural error caused by improper placement of the thermocouple on the tissue. It is possible, however, that sound delivery to the tissue was impaired in some experiments which might hide real changes in 2A diameter and flow. Secondly, it should be recognized that the cremaster muscle was not uniformly insonated. The tissue was between 1 and 1.5 cm in diameter whereas the sound

Table 1. Effect of direct heating on arteriolar diameter and flow in cremaster muscle*

Temperature	1A		2A		3A	
Range (°C)	Diameter	Flow	Diameter	Flow	Diameter	Flow
34.0-34.5	100.1±0.4	99.5±1.3	98.8±0.9	95.0±4.2	99.9±1.2	96.9±3.7
34.5-35.0	99.4±0.4	99.7±2.7	96.6±1.9	94.2±4.8	101.0±2.9	98.9±4.8
35.0-35.5	99.1±0.5	97.5±4.3	97.5±1.6	93.7±5.6	96.3±3.2	89.0±7.2
35.5-36.0	99.4±0.7	97.9±5.1	96.5±3.1	93.5±8.9	93.6±3.6	85.3±7.2**

* Diameter and flow expressed as percentage of control. Control in temperature range 34.5±0.3°C.

**Significantly less than control ($p \leq 0.05$)

field was only 0.7 cm wide at the 3 dB points. Also, in many cases the center of the field would be near the edge of the tissue so that only half the muscle would be insonated. This would probably affect the values for flow in the larger vessels if their area of distribution were outside of the sound field. A change in flow in the 3A vessels should be reflected in the larger arterioles unless the 3A branches of the larger vessels were, in fact, not insonated. Lastly, it should be recognized that it is not possible to determine from these results whether or not the constrictor effects are cumulative. In spite of these potential problems a real vasoconstrictor action of ultrasound has been shown which engenders speculation as to its mechanism and implications.

No ultrasonic probe method was used to monitor the system for possible cavitation generation. Constant visual observation and rerun of recordings showed no evidence of any bubble formation of 2 μm size or above. The optical system resolves blood platelets (2–3 μm size). In the course of the several hours involved for study of each muscle preparation, there was a generalized increase in optical opacity observed for both the ultrasound and thermal experiments (no irradiation). This opacity change was judged to be due to a generalized edema occurring in the preparation over a number of hours. No modification of optical opacity was observed for any level of ultrasound irradiation starting at the stage of maximum optical clarity. Such a change might occur if a mass of bubbles of less than 2 μm size was generated. Other studies using CW ultrasound in the range of intensities used here having an *in vitro* or partially *in vitro* character have shown evidence of cavitation (ter Haar *et al.*, 1979; Fu *et al.*, 1980). It is not certain that cavitation did not play a role in this study except that there is no optical evidence for the process, coupled with the fact that the irradiation system was operated in the pulse mode.

Two actions of ultrasound are possible suspects as the mediators of sound-induced vasoconstriction: acoustic microstreaming and direct mechanical effects (pulsed ultrasound producing a cyclic varying radiation force). Several effects might occur as a result of microstreaming. First, it is known that application of sound at intensities as low as 2 Wcm^{-2} can cause changes in cellular ion content *in vitro*, presumably due to membrane permeability changes induced by microstreaming (Chapman *et al.*, 1979). A similar effect on vascular smooth muscle might alter smooth muscle membrane potential and, consequently, smooth muscle tone. The same result might occur due to streaming without any membrane change, however, if the unstirred bundary layer at the membrane

surface were disturbed, producing an altered ion concentration profile at that locus. Another possible effect of streaming, suggested by A. C. Guyton (personal communication), would be the disruption of the plasma layer at the interface between the arteriolar epithelium and the red cell columns. This layer is thought to provide a barrier to O_2 diffusion because of the absence of hemoglobin. Any disturbance which might bring the red blood cells in contact with the wall, or simply stir this layer, would enhance O_2 transport to the vascular smooth muscle. It is easily shown that increasing oxygen delivery to the cremaster muscle results in arteriolar constriction (Guyton, 1976).

It is also possible that direct mechanical effects of the sound could produce vasoconstriction. Agitation conceivably could increase the release of norepinephrine from sympathetic nerve endings. A direct effect on the muscle is also a remote possibility since touching an arteriole with a micropipette, for instance, will induce a substantial constriction.

Clinically the vasoconstriction produced by insonation at 5.0–10.0 Wcm^{-2} is probably of little consequence because the therapeutic range of sound is roughly 0.5–3.5 Wcm^{-2} in the absence of any focusing effects. It is somewhat difficult, however, to compare the results of this study with clinical findings. Applying therapeutic sound to a muscle at 2 Wcm^{-2} could easily produce a 2°C rise in temperature. Such a temperature change required 10.0 Wcm^{-2} of sound intensity in the present study, most likely due to convective heat loss to the suffusant solution.

These results confirm experimental studies of ultrasound effects on whole limb blood flow which in general show no consistent increase or decrease in flow in the therapeutic range of insonation (Bickford and Duff, 1953; Paul and Imig, 1955; Hansen and Kristensen, 1973). One can conclude that there is no change in muscular perfusion in normal muscle associated with the application of sound below 5.0 Wcm^{-2} for muscle temperatures below 36°C. Vasoconstriction occurs above that level of intensity in the same temperature range. It is not certain, however, that the same reaction will occur in impaired tissue as the mechanism for the demonstrated therapeutic capability of sound is as yet unknown.

The significance of the tremendous increase in arteriolar vasomotion even at the lower sound intensities is unclear. Arterioles of the size investigated here are important in the regulation of local tissue perfusion to meet the metabolic demands of the tissue. The cyclic contraction and relaxation of these vessels allows a smaller volume of blood to supply a tissue's needs than if all the vessels were constantly dilated. Conceivably the rapid vasomotion could produce more rapid shifts in blood distribution to the tissue which could improve oxygenation and nutrient delivery without increasing total blood flow. Evaluation of this possibility will require direct measurement of tissue oxygen tension with the oxygen microelectrode technique.

Acknowledgements—The authors would like to thank Dr. Stephen A. Goss for his assistance in thermocouple manufacture and in the design of the substage sound coupler. Thanks also to Howard W. White for construction of the coupler and animal holder; to Luisa Manfredi for technical assistance; to JoAnne Drake for preparation of the manuscript, and to Timothy B. Yates for preparation of the illustrations. This project was supported in part by NIH Grant HL20869-03.

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