Influence of Injection Site, Microvascular Pressure and Ultrasound Variables on Microbubble-Mediated Delivery of Microspheres to Muscle

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OBJECTIVES Our objective was to test the hypothesis that the ultrasound pulsing interval (PI), microbubble injection site and microvascular pressure significantly influence the transport of 100-nm microspheres to muscle through extravasation sites created by the destruction of microbubbles with ultrasound.

BACKGROUND Microbubbles show promise as targeted drug and gene delivery agents; however, designing optimal microbubble-based therapies will require an understanding of the factors that influence the transport of microbubble-delivered, gene-bearing vehicles to tissue.

METHODS Ultrasound at 1 MHz, with a peak negative pressure amplitude of 0.75 MPa, was applied to microbubbles and 100-nm microspheres in exteriorized rat spinotrapezius muscle. Ultrasound PIs of 1, 3, 5 and 10 s, arterial microsphere injection times of 10 or 40 s and arterial versus venous injection sites were tested.

RESULTS Extravasation point creation and microsphere delivery were greatest when the ultrasound PI was 5 or 10 s. No significant differences in extravasation point creation or microsphere delivery were observed with arterial versus venous microbubble injection, but a trend toward increased microsphere delivery with arterial injection may exist. Decreasing the arterial injection time from 40 to 10 s increased microvascular pressure, which, in turn, substantially enhanced microsphere transport to tissue, without a concomitant increase in the number of extravasation points.

CONCLUSIONS The ultrasound PI and microvascular pressure significantly influence the creation of extravasation points and the transport of microspheres to tissue. These factors may be important in designing and optimizing contrast ultrasound-based therapies. (J Am Coll Cardiol 2002;39:726–31) © 2002 by the American College of Cardiology

The effective delivery of intravascular gene-bearing vehicles is dependent on site-specific targeting and subsequent transport of these vehicles across the endothelium. Recent studies have shown that the destruction of microbubble contrast agents by ultrasound may represent an effective means of targeting and transporting genes and growth factors to tissue (1–6). It has been reported that the application of ultrasound to intravascular microbubble contrast agents creates extravasation points in skeletal muscle capillaries, and that this effect is dependent on applied ultrasound power (5,7). These extravasation points permit the passage of 200- and 500-nm polymer microspheres (PMs) from the bloodstream into the tissue, and in many cases, PMs are delivered as far as 200 μm from the site of microbubble destruction (6). These results are consistent with the hypothesis that applying ultrasound to a region of interest after the simultaneous injection of microbubbles and drug- or gene-bearing vehicles that are <500 nm in diameter will deliver these vehicles deep into the tissue.

The future effectiveness of contrast ultrasound-based gene and growth factor delivery techniques may, however, be dependent on a number of factors. The purpose of this study was to quantify the influence of three of these factors—the pulsing interval (PI), microbubble injection site and microvascular pressure—on the creation of extravasation points, and the subsequent intravascular delivery of 100 nm of PMs to the muscle. These studies were performed in vivo using a rat spinotrapezius muscle preparation that allows for simultaneous microscopic visualization and ultrasound application to the muscle.

METHODS All studies were approved by the Animal Research Committee of the University of Virginia, and conformed to the “Position of the American Heart Association on Research Animal Use,” adopted by the Association in November 1984.

Ultrasound experiments. Thirty-six female Sprague-Dawley rats weighing 254 ± 11 g were anesthetized by an intramuscular injection of ketamine (80 mg/kg body weight) and xylazine (8 mg/kg). The left femoral vein and right carotid artery were cannulated. The right spinotrapezius muscle was exteriorized and arranged in a rectangular chamber containing a 1 MHz cylindrically focused, single-element ultrasound transducer aligned with the muscle thickness, as previously described (5,6,8). The transducer (Panametrics, Inc., Waltham, Massachusetts) diameter was...
with settings adjusted so that no pixels were saturated. The same settings were used for observation of each field of view in every specimen. Gray-scale histograms were obtained and used to calculate the mean gray-scale level for each specimen.

**Microvascular pressure measurements.** Rats \( (n = 5) \) were anesthetized as previously described; the spinotrapezius muscle was exteriorized; and a servo-nulling system \( (9) \) was used to take pressure measurements. A glass micropipette with a 2-μm tip was inserted into a feed arteriole with micromanipulators \( (Narshige, Tokyo, Japan) \). Pressures were continuously measured before and throughout 10-s \( (n = 5) \) and 40-s \( (n = 4) \) arterial co-injections of microbubbles and PMs. No more than two injections were performed per animal, and the injection order was alternated. Pressure measurements were analyzed with a Butterworth filter, and the mean pressure before and during injection was calculated.

**Statistics.** The PI data were analyzed using one-way analysis of variance, followed by pairwise comparisons with the Tukey \( t \) test. The injection site and duration, as well as microvascular pressure data, were compared using the Student \( t \) test. The paired Student \( t \) test was used to compare injection pressures with baseline values. Significance was set at \( p < 0.05 \).

## RESULTS

Figure 1 illustrates the appearance of discrete extravasation points, as denoted by the presence of red blood cells in the interstitial space, in treatment group C. Figure 2 depicts confocal images of PM delivery for the treatment groups \( \text{(Fig. 2A–F)} \) and a control group \( \text{(Fig. 2G)} \) in which ultrasound was applied during administration of 100 nm of PMs in a 5% albumin solution.

**Ultrasound PI.** The effects of PI on 100-nm fluorescent PM delivery to tissue are illustrated in Figure 2A through 2D, where the results for PIs of 1, 3, 5 and 10 s are shown. The fluorescence area appears greater with the 3- and 5-s PIs \( \text{(Fig. 2B and 2C)} \), as compared with the 1- and 10-s intervals \( \text{(Fig. 2A and 2D)} \). Figure 3A illustrates that PM delivery and extravasation point creation increase when the PI goes from 1 to 5 s, but they subsequently decrease when the PI is increased from 5 to 10 s. In Figure 3B, the data in Figure 3A are normalized to the number of applied ultrasound pulses. The purpose is to illustrate, over the course of an entire experiment, the contribution of each individual ultrasound pulse. The 5- and 10-s PIs exhibit significant increases in extravasation point creation, as compared with...
The 1-s PI. The 5-s interval is also greater than the 3-s interval. A significant increase in PM delivery per pulse was observed for the 5- and 10-s PIs, as compared with the 1-s interval.

**Arterial versus venous microbubble injection.** Fluorescent PM delivery after arterial and venous microbubble injection is illustrated in Figure 2B and 2F, respectively. In each case, ultrasound was applied over a 40-s duration, and the PI was set to 3 s. Total fluorescence per unit area appears increased in Figure 2B, as compared with Figure 2F. In Figure 4, it is evident that the microbubble injection site had no effect on extravasation points per unit area. Although the fluorescence intensity was approximately doubled for arterial microbubble injection, this result is significant only at p < 0.15.

**Arterial injection time and microvascular pressure.** Figure 2, A and E, illustrates the significant effect that the injection duration has on fluorescent PM delivery to tissue, with the image from the 10-s injection duration (Fig. 2E) showing a marked enhancement of total fluorescence, as compared with the 40-s duration (Fig. 2A). The key characteristic of the 10-s duration image is the wide dispersion of PMs from individual extravasation points. Figure 5A demonstrates that, with respect to the number of created extravasation points, there is no significant difference between a 10- and 40-s injection duration. The shorter injection time does, however, yield a significant increase in the delivery of fluorescent PMs. In Figure 5B, the extravasation point and fluorescence data in Figure 5A are normalized to the number of pulses. Here, a significant increase in...
extravasation points per pulse is evident. The PM delivery per pulse for the 10-s injection duration is almost an order of magnitude greater than that for the 40-s duration.

Microvascular pressures in the feed arterioles were determined to gain insight into the arterial injection data presented in Figure 5. In Figure 6, A and B, the pressure tracings for 10- and 40-s injections, respectively, are shown. Arterial injections (especially the 10-s duration) create a pressure spike that is subsequently damped by myogenic constriction. After the injections, pressures remain below baseline due to a sustained myogenic response. Figure 6C depicts the mean pressure increases for the 10- and 40-s injection durations. The pressure increase above baseline was significantly greater for the 10-s group, as compared with the 40-s group.

Control groups. For each control group, no extravasation points were created, and fluorescence was only observed inside the vessels. The intravascular fluorescence intensity was identical for each control group. Gray-scale level data for treatment groups A through F were found by subtracting the mean gray-scale level from that of the control specimens.

DISCUSSION

Gene delivery to tissue through microbubble destruction with targeted ultrasound represents a promising new treatment strategy that may be applicable to a number of diseases. Although a number of recent studies have demonstrated "proof of principle" for growth factor and gene delivery (1–4), optimization of these strategies will require a better understanding of the unique transport of therapeutic agents that occurs with these techniques. In this study, we present evidence that the PI, microvascular pressure and, possibly, microbubble injection site play an important role in governing the transport of PMs to tissue by microbubble destruction with ultrasound.

Factors influencing extravasation point creation and microsphere transport to tissue. A PI $\leq 5$ s generates the greatest PM delivery to rat skeletal muscle. This is because a PI $< 5$ s does not allow for microbubble concentration restoration, and a PI $> 5$ s, while delivering the same number of PMs per pulse, requires a greater time to generate the same total effect. In practice, a technique similar to that used for measuring blood flow with contrast ultrasound (10), wherein video intensity is quantified as a function of PI, may be used to find the shortest PI at which the microbubble concentration is restored. In a recent study, ultrasound with a 3-s interval was used for gene delivery to the myocardium through microbubbles (1). LacZ expression appeared to be quite uniform with this PI. When vascular endothelial growth factor (VEGF) was delivered to the myocardium with constant ultrasound application (3), it was noted that VEGF deposition occurred primarily in the arterioles. Although there are many potential reasons for this, it is possible that with constant ultrasound application, VEGF was delivered before reaching the downstream capillaries and venules.

Extravasation point creation by microbubble insonation, although not well understood, is certainly influenced by a
number of factors. Because extravasation points are created only in vessels in which microbubbles are physically con-
strained, the size of the microbubble relative to the mi-
crovessel lumen is clearly important. Our study addressed
this issue by comparing PM delivery with arterially injected
microbubbles with venously injected microbubbles. With
venous injection, large microbubbles are filtered in the lungs
before reaching the muscle. Arterial injections were made
upstream of the muscle; thus, they contained these larger
microbubbles. Arterial and venous injections created the
same total number of extravasation points (Fig. 4); however,
PM delivery with arterial injection was approximately two-
fold greater. Although this result is significant only at p < 0.15,
it may indicate that arterial microbubble injections
create extravasation points that are larger and permit greater
delivery vehicle transport. Venous injections are preferable
because of low invasiveness; however, further study of the
potential benefits of arterial microbubble injection is war-
ranted.

The effects of microvascular pressure on PM delivery are
addressed in Figures 5 and 6. In Figure 5A, 10- and 40-s
injections create an equivalent number of extravasation
points, because the same number of microbubbles are
injected for each case. The 10-s duration yields a four times
higher microbubble concentration; however, this is exactly
balanced by the fourfold increase in ultrasound pulses for
the 40-s injection. Given that the same number of extrav-
asation points are created, the doubling of PM delivery with
the 10-s injection (Fig. 5A). This apparent discrepancy
may have two explanations. First, initial pressure spikes
created by the 10-s injection were considerably higher than
the mean pressure, and most PM delivery may have oc-
curred at this time. Second, once exposed to positive
pressures, skeletal muscle hydraulic conductivity increases
exponentially with increasing pressure (11). Thus, after
microbubble destruction, small differences in driving pres-
sure create marked increases in delivery vehicle transport.

**Strategies for gene delivery with contrast ultrasound.**

Two promising strategies for delivering genes and growth
factors with microbubbles are emerging (1–4). The first
tells ultrasound-targeted destruction of microbubbles that
are directly bound to the molecule. Using this strategy, a
10-fold increase in beta-galactosidase activity in the rat
myocardium has been achieved using albumin microbubbles
bound to a recombinant adenovirus containing the comple-
mentary deoxyribonucleic acid (cDNA) encoding beta-
galactosidase. Skeletal muscle transfection has also been
achieved in rats through the ultrasonic destruction of
cationic lipid microbubbles housing plasmid DNA that
contains the luciferase reporter gene (2), and the deposition
of VEGF in intramyocardial arterioles and antisense oligo-
nucleotides in the kidney has been shown (3,4). A second
strategy, in which the genetic material (1), growth factor
(3) or microsphere (6) is in the blood stream but not bound
to a microbubble, attains delivery after ultrasound/
microbubble microvessel permeabilization. Although our
study reproduces this last strategy, we believe that the
transport characteristics for both strategies may actually be
quite similar.

We make this assertion based on the following argument.
First, we have observed that, after microbubble destruction,
microbubble shell fragments remain on the vessel wall (5).
Thus, it is unlikely that the previously reported delivery of
genes to tissue (1,2) was due to microbubble fragment

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**Figure 6.** Representative pressure tracings for transverse arterioles before, during and after 10-s (A) and 40-s (B) arterial injections of microbubbles and
PMs. Microvascular pressure increased during injection for both groups, but then fell below baseline immediately after injection. (C) Bar graph showing
the mean pressure increase above baseline during injection in feed arterioles. *p < 0.05 versus 40-s injection group. **p < 0.05 versus baseline.
transport. Instead, when microbubbles with bound genetic material are destroyed, the genetic material is released from the microbubbles into the bloodstream (4). Subsequently, this genetic material would be transported through vessels permeabilized by the effect of ultrasound on other microbubbles. If this is correct, the basic transport is similar, and our results are applicable to both strategies.

**Ultrasound power, frequency and capillary rupturing.**

The potential for clinical contrast ultrasound to create capillary ruptures in intact organs is controversial. We previously observed capillary rupturing in exteriorized rat skeletal muscle with 2.3-MHz ultrasound and intravenously injected microbubbles (5,6). However, at this frequency, we cannot reproduce capillary rupturing in intact rat muscle (R. J. Price, unpublished observations, 2000). Thus, in our laboratory, muscle exteriorization facilitates capillary rupturing at clinical frequencies, perhaps because tissue attenuation of ultrasound power is virtually eliminated. In preliminary studies, capillary rupturing was observed in intact muscle with 1-MHz ultrasound at an MI of 0.75 and with venous microbubble injection (R. J. Price, unpublished observations, 2000). Therefore, these values were maintained for the current study. Moreover, the ability of subclinical frequencies (~1 MHz) to disrupt the capillary endothelium and underlying basement membrane in the presence of microbubbles has been documented by other investigators (3,12,13). Independently, capillary rupturing with ultrasound at a clinical frequency (2.5 MHz) has been shown in the intact diaphragm muscle (7). However, this study was done in the mouse, whose capillary diameters may be smaller than those of rats and humans. Precise measurements of the capillary diameter are difficult, and to the best of our knowledge, they have not been made for mouse skeletal muscle. However, it is known that the mean corpuscular volume in mice is significantly less than that in rats and humans (14), perhaps indicating that capillary diameters are also smaller. This may be important because, as previously noted, the relative size of the microbubbles to the capillary lumen largely determines whether a rupture will occur. This factor should be considered when extrapolating the results of small animal studies to humans.

Given the current uncertainty surrounding contrast ultrasound-generated bioeffects, further studies quantifying microvessel permeability, as a function of ultrasound frequency and power, will be needed to optimize targeted delivery to intact organs. Based on our observations, ultrasound at clinical frequencies does not create the capillary rupturing bioeffect in intact tissues. Therefore, it seems likely that specially designed ultrasound transducers will be required for this purpose. Moreover, the effects of tissue attenuation and target depth on capillary rupturing must be studied before precise targeting can be achieved. This information can be derived from experiments that correlate the depth of capillary rupturing within a target organ to selected ultrasound variables.

**Conclusions.** Our results indicate that extravasation point creation and PM delivery are greatest when the PI is ~5 s. No significant differences in extravasation point creation or PM delivery were observed with arterial versus venous microbubble injection. However, a trend in the data indicates that extravasation points created by arterially injected microbubbles may allow for greater transport of 100-nm PMS to tissue. Increasing the convective driving pressure by decreasing the injection time substantially enhances 100-nm PM transport to tissue, without a concomitant increase in extravasation points. We conclude that the PI and microvascular pressure may be important factors in designing and optimizing future contrast ultrasound-based therapies; however, further studies on the effects of arterial versus venous microbubble injection are warranted.

**References**


