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Lung ultrasound and microbubbles enhance aminoglycoside efficacy and delivery to the lung in *E. coli*-induced pneumonia and ARDS

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Conception and design: WLL, RK, HLP, WMK; Acquisition, analysis, or interpretation of data: MGS, VM, HR, NG, JB, LB, WLL; Drafting the manuscript for important intellectual content: WLL, WMK, LB

ARDS is a devastating disorder characterized by lung microvascular leakage leading to pulmonary edema and hypoxemia (1). Its hallmark is heterogeneous involvement of the lung parenchyma, with thoracic CT scans revealing densely-consolidated areas interspersed with relatively normal appearing lung. Such heterogeneity greatly complicates management. Mechanical ventilation, although life-saving, preferentially inflates the normal regions of the lung, causing over-distension and lung damage (1). Pharmaceutical agents like antibiotics administered by inhalation distribute to the healthy rather than the injured regions of the lung, potentially causing off-target effects. By contrast, drugs administered systemically cannot target the injured lungs and are delivered to organs throughout the body– thereby predisposing to adverse effects (2). The inability to deliver therapy solely to the injured areas of the ARDS lung remains an intractable problem in critical care.

We hypothesized that thoracic ultrasound and microbubbles could be used to specifically enhance drug delivery to, and uptake by, the injured lung. Microbubbles (1-3 μm) are made up of a gas-filled core and an outer lipid shell and are used routinely as a contrast agent for echocardiographic studies, including in critically ill patients (3). When exposed to an ultrasound pulse, microbubbles undergo cavitation that induces shear stress on biological membranes within their vicinity leading to the formation of transient pores and enhanced endocytosis (4) at the plasma membranes of cells. This induces enhanced uptake of local therapeutics such as drugs or genes (5); importantly, the process is effective at increasing uptake whether the drug is encapsulated by the microbubble, bound to its surface, or even only in its vicinity. This phenomenon has been harnessed to deliver genes to ischemic limbs (5). Ultrasound of the lung has traditionally been considered of limited utility (6) since air causes reflection and scattering of ultrasound energy. We hypothesized that this very limitation can be harnessed in ARDS because ultrasound waves will preferentially penetrate damaged areas of lung (since they are filled with fluid or atelectatic), leaving normal (air-filled) areas of lung mostly unaffected (Figure 1A). In addition, by targeting the ultrasound transducer to the chest, the ultrasound energy is focused on the thorax, limiting the effect

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on other organs. Furthermore, circulating microbubbles may accumulate in injured regions due to increased endothelial permeability.

To test this hypothesis, we focused our initial efforts on aminoglycoside antibiotics using a murine model of severe gram-negative bacterial pneumonia, one of the commonest causes of ARDS. Antibiotic resistance is growing worldwide (7); many strains of enteric gram-negative bacilli are now resistant to cephalosporins and beta-lactam/beta lactamase-inhibitor combinations. While almost 100% of gram-negative isolates remain sensitive to aminoglycosides, this class of antibiotics has traditionally been avoided for treating pneumonia because of poor lung penetration and the association of systemic therapeutic aminoglycoside levels with significant nephrotoxicity and ototoxicity (8). Thus, developing an easy and safe method to increase the therapeutic index of aminoglycosides specifically in the lungs would be of great practical value.

C57BL/6 (male, 9-12 weeks) were infected intratracheally with *E. coli* (ATCC 25922) and developed significant hypoxemia and lung edema within 3 hours; histological examination confirmed extensive neutrophilic lung injury that was bilateral (Figure 1B). All work was performed with the approval of the local animal care committee (ACC 742).

To confirm the safety of microbubbles and ultrasound-mediated cavitation in this setting, we measured oxygen saturation by pulse oximetry before, during and up to 48 hours after intravenous injection of 1 X 10^9 Definity™ microbubbles (Lantheus; dose based on previous work (5)) and thoracic ultrasound administration; no antibiotics were administered at this point. Definity™ are FDA-approved lipid-coated microbubbles that are widely used in diagnostic cardiovascular ultrasound. We used a Sonos 5500 (Phillips Healthcare) ultrasound device and a S3 phased array transducer to induce microbubble cavitation, positioning the probe over the murine thorax (transmit frequency 1.3 MHz, 67V, 0.2 W, mechanical index 0.9, peak negative acoustic pressure -900-1200 kPa) with a pulsing interval of every 5

seconds for 5 minutes. Microbubbles and ultrasound were well tolerated, with the treatment resulting in no adverse effect on arterial oxygenation (Figure 1C).

To establish whether ultrasound-induced microbubble-cavitation (USMB) could enhance antibiotic delivery to the injured lung, in separate experiments we administered gentamicin (1.5 mg/kg) by intraperitoneal injection 6 hours after infection with *E. coli*. Microbubbles (1×10^9) were injected intravenously immediately afterwards and thoracic ultrasound was then applied as described earlier. Two hours later, animals were euthanized by cervical dislocation and lung homogenates were plated on agar. Each experiment was repeated three times with similar results; statistical analysis was performed using Kruskall-Wallis one-way ANOVA and Dunn's Multiple Comparison Tests (GraphPad).

Compared to gentamicin alone (which was ineffective at this low-dose), USMB caused an almost 1-log reduction in bacterial growth (Figure 2A). In contrast, administration of microbubbles and antibiotics (but without ultrasound) or ultrasound and microbubbles (but without antibiotics) were both ineffective; this rules out a direct antibacterial effect of microbubbles and/or ultrasound alone. Bronchoalveolar lavage (BAL) fluid and lung homogenates from infected mice was analyzed for gentamicin levels by ELISA (Europroxima). Thirty minutes after USMB, gentamicin levels in both BAL and lung tissue were significantly higher (≥ 2-fold) in USMB-treated animals *vs.* gentamicin-alone controls (Figure 2BC). Thus, USMB enhanced antibiotic delivery and bacterial killing. Interestingly, USMB was able to enhance gentamicin delivery despite the theoretical limitation of hypoxic vasoconstriction in injured lung regions, perhaps due to the extent of infected and injured lung in our model. With that caveat, while our model mimics pneumonia-induced ARDS (lung edema, bilateral injury, hypoxemia), it is possible that USMB-mediated antibiotic delivery would be beneficial in cases of localized (e.g. lobar) pneumonia as well.

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Microbubble contrast agents are safe and have been extensively used in critically ill (3) and ARDS (9) patients. Similarly, thoracic ultrasound is now widely used for diagnosis of barotrauma, pulmonary edema and lung consolidation (10)(11, 12). There is growing literature in other fields showing that ultrasound-induced cavitation of microbubbles can dramatically enhance gene transfection and drug delivery (5, 13); however, its use for the treatment of ARDS has remained unexplored due to preconceived negative notions of lung ultrasound feasibility.

Given the limited mobility of ARDS patients, the potential utility of bedside ultrasound-mediated microbubble therapy is high. In principle, USMB-induced drug delivery is targeted specifically to the injured areas of the lung, in essence providing precision medicine to the most critically ill patients. In this report we have focused on a very pragmatic goal - the enhanced delivery of aminoglycoside antibiotics in pneumonia to improve their therapeutic index. Further research will determine if USMB can enhance the delivery of other therapeutic cargoes to the injured lung.

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Competing Interests
HLP, RK and WLL are listed as co-inventors on a patent application related to this work.

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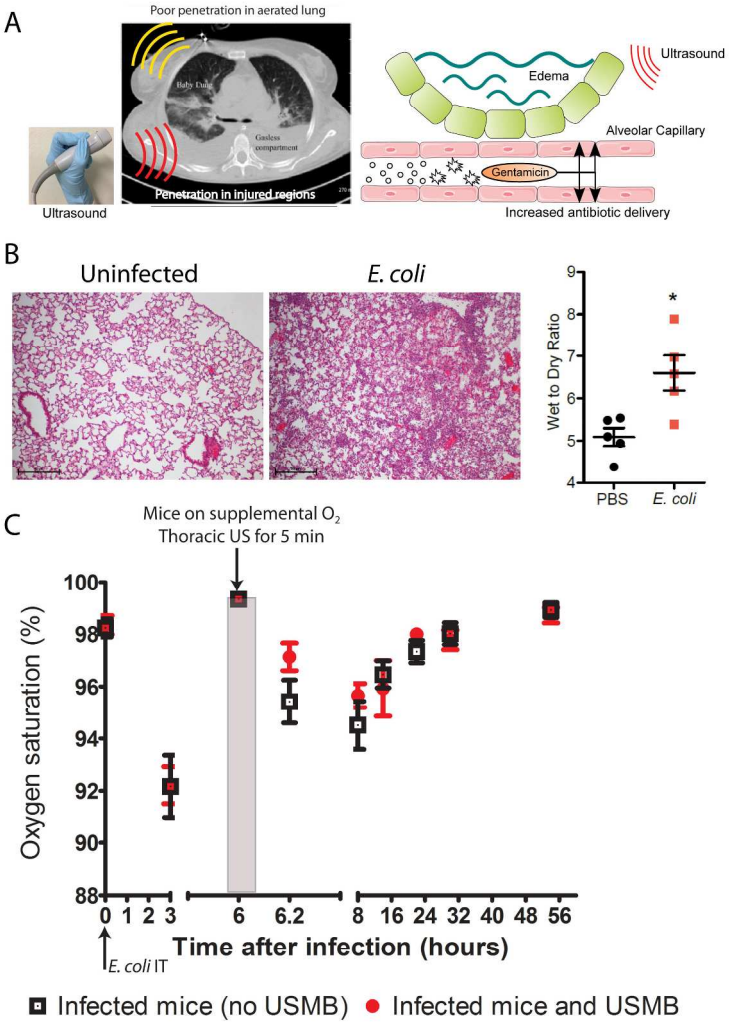
Figure Legends

Figure 1. Concept and safety of ultrasound-microbubble (USMB) therapy to increase drug delivery to the injured lung. **A)** *Left-hand panel*: an ultrasound probe is applied to the surface of the chest. In the normal regions, air-filled regions of lung cause scattering of the ultrasound waves (yellow waves). In contrast, damaged areas of the lung with ARDS are atelectatic or filled with fluid and allow penetration of the ultrasound beam (red waves). Note the marked heterogeneity of the lung in ARDS, with black (air-filled) areas of the lung interspersed with white (fluid-filled or atelectatic) regions. *Right-hand panel*: Cavitation of circulating microbubbles caused by the ultrasound pulse leads to enhanced cellular uptake of drugs in the vicinity of the microbubbles. The effect is similar whether the drugs are bound to or encapsulated by the microbubble or merely in close proximity. **B)** Mice were infected with *E. coli* intratracheally (IT). Representative H&E images from lung sections of uninfected and *E. coli*-infected mice. Note the alveolar infiltrate and neutrophil recruitment. *Right-panel*: Mice develop significant lung edema as measured by lung wet-to-dry ratio 6 hours after infection (*p<0.05). **C)** Safety study: Arterial oxygen saturation (SpO_2) during and after the experiment. Mice are infected IT with *E. coli* as in B) and develop hypoxemia within 3 hours. Mice are anesthetized with inhaled isoflurane and receive supplemental O_2 during USMB (grey rectangle) - this accounts for the rise in SpO_2; afterwards, animals are placed on room air and monitored for 48 hours. Control mice were infected but did not receive USMB. Note the comparable profile of O_2 saturation levels in USMB-treated (red circles) and control mice (hollow squares); data are mean and SEM (n=13 (control) or 14 (USMB) mice per group from 0-8 h and n=8 per group from 14-54 hours). No antibiotics are administered in this safety experiment and all mice survived.

Figure 2. Effect of ultrasound-microbubble (USMB) treatment on the delivery and efficacy of gentamicin for *E. coli* pneumonia. **A)** Male C57BL/6 mice were infected intratracheally with $5X10^7$ colony forming units (cfu) of *E. coli*. Six hours later, mice received 1.5 mg/kg gentamicin (Gent) or no antibiotics (Abx) by intraperitoneal injection; thirty minutes later, mice were then injected with $1 X 10^9$ microbubbles (MB; Definity™) by tail vein followed by thoracic ultrasound administration. 2 hours later, mice were euthanized and lungs homogenized for cfu. Control groups are as indicated. Data are mean plus SEM with n=7 mice per group. Note the almost 1-log reduction in cfu in mice receiving USMB with gentamicin (red triangle in hatched box) compared to controls, including animals that received ultrasound and microbubbles but no antibiotics; *p=0.0013 by one-way ANOVA. By Dunn's Multiple Comparison Test, p<0.05 for Gent/USMB *vs* Gent alone and p< 0.01 for Gent/USMB *vs* all other groups. **B)** Bronchoalveolar lavage (BAL) levels of gentamicin by ELISA in infected mice treated with 5 mg/kg gentamicin alone or in combination with USMB. Levels were measured 30 minutes after USMB treatment; data are mean and SEM, p=0.04 by two-tailed t-test, n=3 mice per group. C) Corresponding lung homogenates probed for gentamicin, per gram of lung; p = 0.03 by two-tailed t-test.



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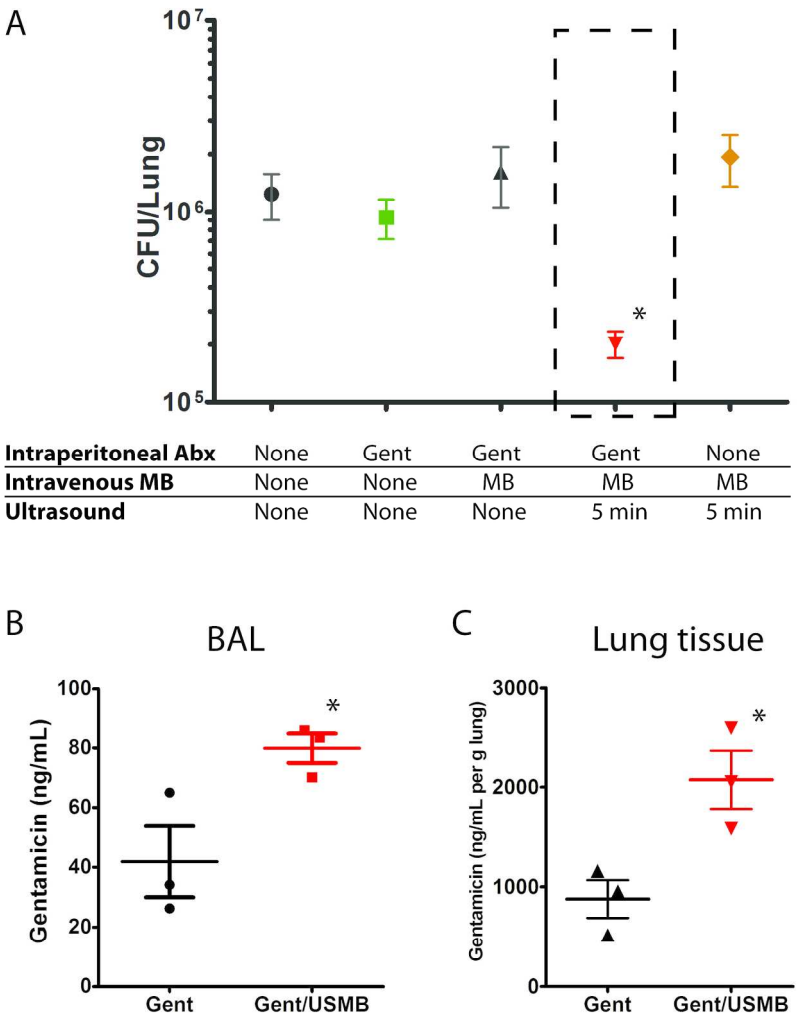

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