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Low-frequency ultrasound increases non-viral gene transfer to the mouse lung

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The aim of the study was to assess if low-frequency ultrasound (US), in the range of 30–35 kHz, increases non-viral gene transfer to the mouse lung. US is greatly attenuated in the lung due to large energy losses at the air/tissue interfaces. The advantages of low-frequency US, compared with high-frequency US are: (i) increased cavitation (responsible for the formation of transient pores in the cell membrane) and (ii) reduced energy losses during lung penetration. Cationic lipid GL67/plasmid DNA (pDNA), polyethylenimine (PEI)/pDNA and naked pDNA were delivered via intranasal instillation and the animals were then exposed to US (sonoporation) at 0.07 or 0.1 MPa for 10 min. Under these conditions, US did not enhance GL67 or PEI-mediated transfection. It did, however, increase naked pDNA gene transfer by approximately 4 folds. Importantly, this was achieved in the absence of microbubbles, which are crucial for the commonly used high-frequency (1 MHz) sonoporation but may not be able to withstand nebulization in a clinically relevant setup. Lung hemorrhage was also assessed and shown to increase with US pressure in a dose-dependent manner. We have thus, established that low-frequency US can enhance lung gene transfer with naked pDNA and this enhancement is more effective than the previously reported 1 MHz US.

Keywords low-frequency ultrasound; sonoporation; gene transfer; non-viral vector; lung; cystic fibrosis

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Introduction

Our aim is to enhance gene transfer to the airway epithelium for the purposes of cystic fibrosis (CF) gene therapy. In particular, we are focusing on non-viral vectors because of their relative safety, lack of immunogenicity and ability to be repeatedly administered [1,2]. Non-viral gene transfer agents have already shown proof-of-principle for transfecting airway epithelia in human clinical trials [3,4]. However, transfection efficiency was modest, with only partial correction of the chloride electrical defect in CF patients.

Approaches to enhance non-viral transfection efficiency have included the use of mucolytics [5], peptides to target vectors to apically localized receptors, e.g. integrins [6], as well as nanoparticles to overcome the nuclear membrane barrier [7,8]. Furthermore, physical methods such as magnetofection [9] and sonoporation [10,11], have recently been applied to enhance gene transfer of both non-viral and viral vectors in several organs. We have shown that sonoporation using 1 MHz ultrasound (US) can increase naked plasmid DNA (pDNA) transfection efficiency in the mouse lung [12]. However, this effect was dependent on the addition of Optison, an US contrast agent that thought to enhance US-mediated gene transfer by lowering the cavitation threshold [13]. Cavitation, the formation and oscillation of gas bubbles, is thought to be the main mechanism behind sonoporation. The US wave causes the bubbles to collapse, thus inducing transient pores in the cell membrane [14]. Importantly, the addition of Optison alone (no US) reduced the efficiency of GL67 and PEI/pDNA complexes, as well as naked pDNA. Ideally, we would like to achieve sufficient cavitation to increase gene transfer without the need for Optison.

In addition, we are interested in improving US penetration in the lung. Generally, US attenuation is caused by: (i) reflection, (ii) scattering and (iii) absorption (generation of heat). Although reflection, which is the main contributor to US attenuation in the lung, depends only on the properties of the medium and not on the frequency of the sound wave, scattering and absorption increase with increasing frequency [15]. We thus, turned our attention to

low-frequency US, which by definition ranges from 5 to 100 kHz.

Low-frequency US has been used to enhance the drug delivery to rat skin *in vitro* [16] and *in vivo* [17], as well as to human skin *in vitro* [18]. It has also been shown to improve the penetration of antibiotics to biofilm-forming bacteria [19]. These effects are thought to be mediated by an increase in cell permeability caused by US-induced cavitation. Compared with 1 MHz US, low-frequency US offers many advantages for lung gene transfer. Firstly, it allows more energy to penetrate the tissue by decreasing the energy losses due to wave scattering and absorption [15]. In addition, reducing the frequency increases the cavitational effects, since cavitation is inversely proportional to frequency [20]. A study assessing the effects of US exposure on skin *ex vivo* has also shown that for skin permeability to increase a certain threshold of US intensity or energy has to be exceeded and that this threshold decreases with lower frequencies [21]. This implies that by lowering the US frequency we might still be able to enhance gene transfer but at lower intensities, thus also reducing any possible side-effects such as hemorrhage.

In this study we evaluated the effect of low-frequency sonoporation on non-viral gene transfer in the air-filled mouse lung. In particular, we assessed if low-frequency US could enhance the transfection efficiencies of cationic lipid GL67/pDNA, polyethylenimine (PEI)/pDNA complexes and plasmid DNA (pDNA), in the absence of Optison.

Materials and Methods

Vectors

The gene transfer vectors used in these studies were: cationic lipid 67 (GL67) (Genzyme Corporation, Framingham, USA) and 25 kDa branched PEI (Sigma-Aldrich, Poole, UK) complexed to plasmid DNA and naked pDNA. The plasmid DNA was carrying a luciferase reporter gene under the control of a cytomegalovirus immediate early promoter. Equal volumes of GL67 (1.2 mM) and pDNA (4.8 mM, 1.6 mg/ml) were mixed at a molar ratio of 1:4 (GL67:pDNA) in sterile water for injection (Arnolds Veterinary Products, Shrewsbury, UK) and incubated at 30°C for 15 min. Similarly, equal volumes of PEI and pDNA were mixed at nitrogen (N): phosphate (P) ratio of 10:1, in sterile water for injection. The solution was vortexed and incubated for 20 min at room temperature. The complexing conditions for both GL67 and PEI have been previously optimized [22,23]. In some cases, our gene transfer agents were mixed with Optison (Amersham Health, Oslo, Norway), an US contrast agent, at a 1:1 (*v/v*, DNA:Optison) ratio, in a total volume of 100 μl. All the solutions were made up in sterile water for injection.

US equipment

The US transducer was taken from a commercial therapeutic machine Phys-Assist (Orthosonics Ltd, Devon, UK). An Agilent 33250A signal generator (Agilent, Palo Alto, USA) along with a PV2600 power amplifier (Peavey Electronics Corporation, Meridian, USA) and coupling transformer was used to drive the transducer. The diameter of the convex probe was 27 mm with a curvature radius of 15 mm and a length of 57 mm. The US output was characterized using a beam-plot facility at 30.7 kHz. A calibrated hydrophone (Bruel and Kjaer, model 8103) and digital oscilloscope were used to measure the acoustic output. The US field was unfocused. To avoid cavitation effects, the hydrophone measurements were performed at a low output setting of the ultrasonic apparatus. The resulting acoustic pressures were linearly extrapolated to the values at the pressure of 100 kPa used in the sonoporation experiments.

The US parameters used in these studies are summarized in **Table 1**. The frequency, exposure time and pulse repetition frequency were kept constant while changing the peak negative acoustic pressure. The duration of exposure was optimized in preliminary studies assessing animal viability after low-frequency sonoporation (data not shown). The waves generated by our equipment were not homogenous and the average acoustic pressures for conditions A and B were 0.0182 and 0.026 MPa, respectively.

In vivo transfection and sonoporation

Male Balb/c mice (6–10 weeks old, weighing 20–25 g) were anaesthetized by intraperitoneal injection of Ketaset/Dormitol (76 and 1 mg/kg, respectively) (National Veterinary Services Ltd, Stoke, UK) and the gene transfer vectors were delivered to the lungs by nasal instillation. Briefly, the animals were positioned upright, with their mouth held closed between the thumb and forefinger. A total of 100 μl of solution was delivered drop-wise onto the tip of the nose and was inhaled.

The animals that were exposed to US had their back and chest shaven, prior to the nasal instillation. Immediately after the vector was delivered Aquasonic, a water-based contact gel (Sonora Medical Systems Inc., Longmont, USA), was applied onto the shaven area and the lungs were exposed to US according to the conditions shown in **Table 1**. The US probe covered most of the chest but during exposure it was moved around in a circular motion to cover both the right and left lobes. During the exposure the animals were placed onto heated boards. At the end of the procedure, Antisedan (National Veterinary Services Ltd, Stoke, UK), an antidote to the anaesthetic, was administered (1 mg/kg), also by intraperitoneal injection. All

Table 1 The US parameters used in the present study

	Frequency range (kHz)	Peak negative pressure (MPa)	Average pressure (MPa)	Exposure time (min)	Pulse repetition frequency (ms)	Intensity (mW/cm^2)
A	30.5–35.5	0.07	0.0182	10	100	163
B	30.5–35.5	0.1	0.026	10	100	333
C	30.5–35.5	0.0175	0.005	10	100	11
D	30.5–35.5	0.035	0.009	10	100	41

procedures were approved by the Home Office under the Animals (Scientific Procedures) Act 1986.

The gene transfer vectors were administered at the following doses: 80 μg/100 μl for GL67/pDNA, 20 μg/100 μl for PEI/pDNA and 100 μg/100 μl for naked pDNA. All the vectors were made up in the sterile water for injection. The animals were culled 24 h after transfection by cervical dislocation and the trachea and lungs were removed. The left lobe was fixed in 10% formalin and paraffin embedded for histological analysis. The remaining tissues were snap-frozen in liquid nitrogen and homogenized (Ultra-Turrax homogenizer, Science Laboratory, Houston, USA) in 300 μl Reporter Gene Assay Lysis Buffer (Roche Diagnostics GmbH, Mannheim, Germany). The samples were then freeze thawed three times, spun at 13,000 *g* for 5 min and the supernatant was stored at −80°C to be used in the reporter gene expression assay.

Reporter gene expression assays

Luciferase activity was measured using the Luciferase Assay System (Promega, Southampton, UK) according to the manufacturer's instructions. The total protein content of the samples was quantified using the BioRad protein assay kit (BioRad, Hemel Hempstead, UK). Each sample was assayed in triplicate. Luciferase expression was then presented as relative light units (RLU)/mg of total protein.

Histology

Transverse sections (5 μm) were taken from the centre of the tissue to visualize the airways and stained with H&E. The tissues were then scored for area of hemorrhage using an arbitrary scoring system (0.5, less than a quarter of the area is affected; 1, less than a third of the area is affected, 2, between one- and two-thirds of the area are affected, 3, more than two-thirds of the area are affected). In some cases where a sample was given a double score, e.g. 1/2, an average score was used.

Statistical analysis

Data are expressed as mean ± SEM. Independent sample *t*-test or one-way analysis of variance followed by a Bonferroni *post hoc* correction were carried out. Where necessary a $\log_{10}$ transformation of the raw values was carried out to ensure normal distribution of the data and equal variance between groups. If normal distribution and equal variance were not achieved after the log transformation, a non-parametric Mann–Whitney or a Kruskal–Wallis plus a Dunns *post hoc* correction, were carried out. The null hypothesis was rejected at $P < 0.05$.

Results

Low-frequency US does not increase GL67/pDNA or PEI/pDNA gene transfer in the lung

GL67/pDNA complexes were delivered to the mouse lung via nasal instillation at the previously optimized dose of 80 μg DNA/100 μl [24]. The animals were subsequently exposed to low-frequency US for 10 min, at a negative pressure of either 0.07 or 0.1 MPa, but sonoporation does not enhance gene expression compared with GL67/pDNA alone (**Fig. 1**). Importantly, mice did not show any outward signs of toxicity after US exposure and 100% of mice survived the treatment.

Similar experiments were carried out with PEI/pDNA complexes. US did not increase the luciferase expression but led to a significant reduction ($P < 0.05$, $n = 5–8$) in gene expression after exposure to 0.07 MPa [**Fig. 2(A)**]. In

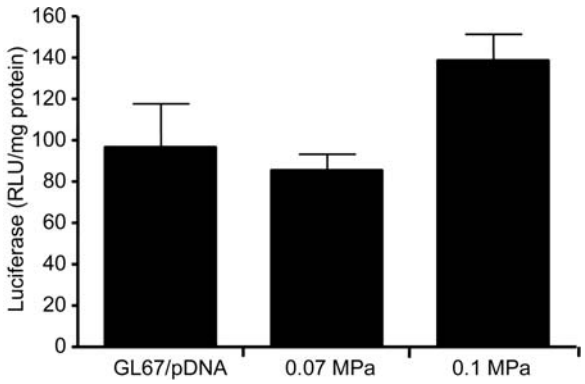

Figure 1 Low-frequency sonoporation of GL67/pDNA complexes Animals were instilled with GL67/pDNA complexes at a previously optimized dose of 80 μg pDNA/100 μl and exposed to US for a total of 10 min. Luciferase expression was measured in the trachea and right lobes and expressed as mean ± SEM ($n = 6–8$ mice/group).

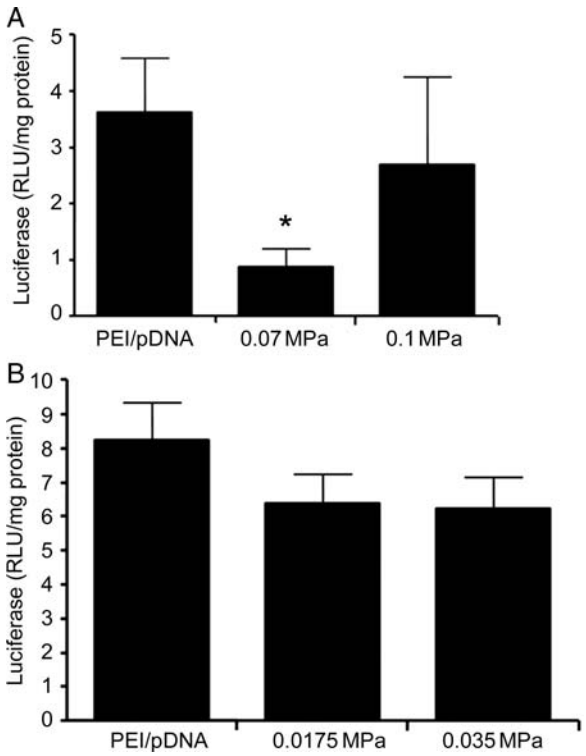

Figure 2 Low-frequency sonoporation of PEI/pDNA complexes PEI/pDNA complexes were delivered by nasal instillation at a dose of 20 μg pDNA/100 μl. The animals were then exposed to US for a total of 10 min at (A) 0.07 or 0.1 MPa negative pressure and (B) 0.0175 or 0.035 MPa. The data (mean ± SEM) represent the levels of luciferase expression measured in the trachea and right lobes. $*P < 0.05$ compared with the PEI/pDNA control; $n = 6–8$ mice/group.

addition, all the animals exposed to US looked unwell (hunched, piloerection), indicating toxicity that may explain the reduced reporter gene expression and only 60% of the treated mice survived. We did not analyze this group of animals further but instead focused on optimizing conditions with a more acceptable survival profile. In an attempt to overcome this problem we decreased the US pressure to 0.035 and 0.0175 MPa. Under these conditions animals did not display any outward signs of toxicity and 100% of mice survived. However, US failed to increase PEI/pDNA-mediated transfection [**Fig. 2(B)**]. Individual control groups were included for each set of studies.

Low-frequency US increases naked pDNA transfection efficiency

Next we assessed the effects of low-frequency US on naked pDNA. Importantly, a 10 min exposure to 0.1 MPa significantly increased gene expression by approximately 4 folds compared with the naked pDNA control group ($P < 0.05$, $n = 7–8$) (**Fig. 3**). Exposure to 0.07 MPa also enhanced gene expression, but these milder conditions did not reach statistical significance.

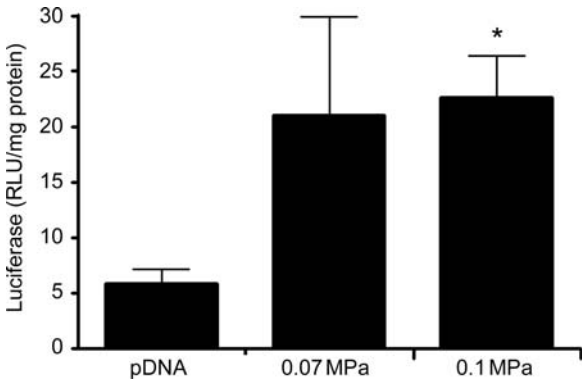

Figure 3 Low-frequency sonoporation of naked pDNA Animals instilled with naked pDNA (100 μg pDNA/100 μl) were exposed to US at 0.07 or 0.1 MPa for 10 min. Luciferase expression in the trachea and right lobes was measured and expressed as mean ± SEM. $*P < 0.05$ compared with the naked pDNA control group; $n = 6–8$ mice/group.

The efficiency of lung sonoporation with 1 MHz high-frequency US is dependent on Optison, which promotes cavitation. Here, we also assessed if Optison further enhances naked pDNA gene transfer after sonoporation with low-frequency US. For this purpose, animals were instilled with a mixture of naked pDNA (100 μg/100 μl) and Optison (1:1, *v*/*v*) and sonoporated as above. As shown previously [12] addition of Optison reduced naked pDNA gene transfer by more than one log. Exposure to low-frequency US at 0.1 MPa significantly increased gene expression approximately 4 fold compared with the pDNA + Optison control group ($P < 0.05$, $n = 7–8$ animals/group) (**Fig. 4**). However, similar to what we have previously reported with high-frequency US, this did not exceed the levels obtained with naked pDNA alone, due to the inhibitory effect of Optison. Although gene expression

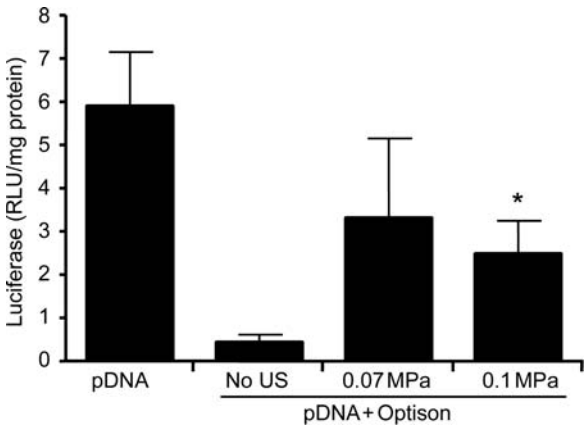

Figure 4 Low-frequency sonoporation of naked pDNA, in the presence of Optison Animals were dosed with a mixture of naked pDNA (100 μg pDNA/100 μl) and Optison (1:1, *v*/*v*) immediately prior to US exposure. The bars represent luciferase expression in the trachea and right lobes (mean ± SEM). $*P < 0.05$ compared with the pDNA + Optison control group (No US); $n = 6–8$ mice/group.

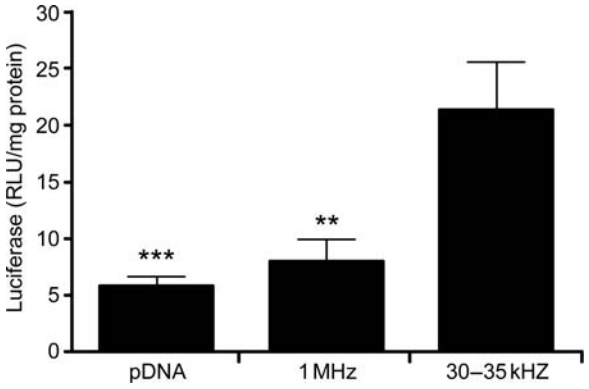

Figure 5 Comparison of high- and low-frequency sonoporation of naked pDNA Each type of US was tested at its optimal conditions. For high-frequency conditions a mixture of naked pDNA (100 μg pDNA/100 μl) and Optison was used (1:1, *v*/*v*) and the animals were exposed to 1 MHz, 3 W/cm^2 for 20 min. For low-frequency conditions naked pDNA was delivered at the same dose (100 μg pDNA/100 μl) but in the absence of Optison, and the animals were exposed to 30–35 kHz, 0.1 MPa for 10 min. The data show luciferase expression in the trachea and right lobes (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the low-frequency US group (30–35 kHz); $n = 6–8$ mice/group.

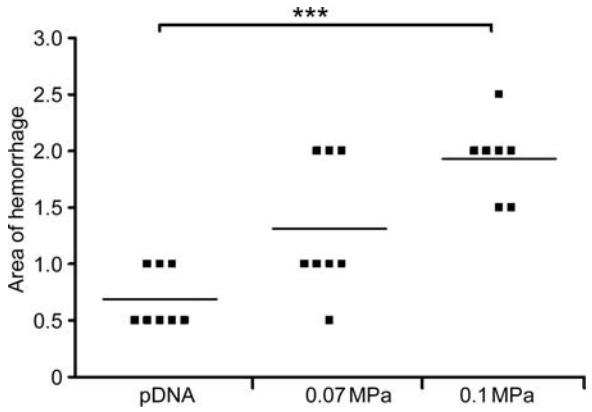

Figure 6 Histological score of US-induced lung hemorrhage with naked pDNA The lung area affected by hemorrhage was assessed 24 h after delivery of naked pDNA (100 μg pDNA/100 μl). Each symbol represents one animal with the mean score for the group shown as a horizontal bar. $^{***}P < 0.001$ compared with the naked pDNA control group; $n = 7–8$ animals/group.

was increased after exposure to 0.07 MPa, this did not reach statistical significance.

Low-frequency US is more effective than 1 MHz US for lung gene transfer

In an attempt to establish the most efficient sonoporation conditions for gene transfer to the lung we compared low-frequency (30–35 kHz) and high-frequency (1 MHz) sonoporation with naked pDNA, using 'optimal' conditions. Low-frequency US was applied at 0.1 MPa for 10 min to animals transfected with naked pDNA alone. High-frequency US at a peak pressure of 0.74 MPa was applied for 20 min to animals transfected with a mixture of naked pDNA and Optison (1:1, *v*/*v*) (**Fig. 5**). Low-frequency US significantly increased gene transfer, whereas high-frequency sonoporation did not enhance expression over the naked pDNA levels. Therefore, in the lung low-frequency US appears to be the more efficient process.

US-induced hemorrhage increases with pressure

Microvessel rupture is a known side-effect of US exposure. We therefore, assessed lung hemorrhage. Hemorrhage after naked pDNA transfection occurred throughout the lung and increased with US pressure in a dose-dependent manner (**Fig. 6**). Importantly, however, this did not affect survival or induce external signs of illness. Although there was a trend for increase with 0.07 MPa it did not reach statistical significance. However, even at 0.07 MPa hemorrhage is visible at hilar areas of the lung not just at the subpleura (**Fig. 7**), indicating wave penetration. Some hemorrhage was also seen in the control group treated with naked pDNA alone but at very low levels. However, this was most likely caused by the culling procedure, as similar levels of hemorrhage were also seen in untreated controls (data not shown).

Discussion

In an attempt to improve non-viral gene transfer to the airway epithelium we have turned our attention to physical

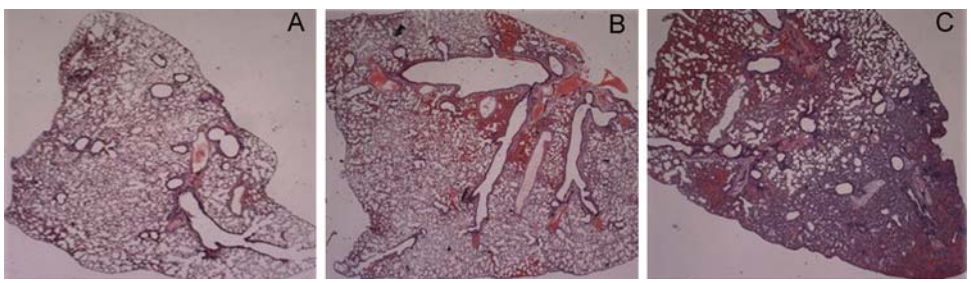

Figure 7 US-induced hemorrhage in the air-filled mouse lung after naked pDNA delivery Mice were treated with (A) naked pDNA (100 μg pDNA/100 μl) alone, (B) naked pDNA + 0.07 MPa and (C) naked pDNA + 0.1 MPa. Lung hemorrhage was assessed 24 h after gene delivery. Minimal hemorrhage is seen with pDNA alone but it increased after sonoporation in a dose-dependent manner. Each picture is representative of the group.

methods and in particular sonoporation. We have previously reported that high-frequency (1 MHz) US increased naked plasmid DNA gene transfer [12] but this required Optison. Here we assessed if low-frequency US can further improve lung sonoporation. Indeed, low-frequency US increased naked pDNA gene transfer approximately 4 fold. Importantly, this did not require Optison, which has been shown to reduce naked pDNA-mediated transfection in the lung.

Similar to what we have previously reported for high-frequency US, low-frequency did not increase PEI/pDNA and GL67/pDNA transfection efficiency. It therefore, appears that the efficacy of sonoporation in the mouse lung is vector dependent. There have been reports of sonoporation enhancing the transfection efficiency of PEI on mouse skeletal muscle *in vivo* [25] and cationic liposomes in several cell lines [26,27]. However, the US frequency in those studies was higher, in the MHz range, and none of them included GL67. The reasons for the vector-specific effect of sonoporation in the lung are unclear and may be related to the overall size or zeta potential of the non-viral vector. It is possible that the degree of cavitation is insufficient for PEI or liposome-complexed DNA.

Low-frequency sonoporation increased naked pDNA transfection efficiency by approximately 4 folds thus proving that despite US being attenuated by the air in the lungs the penetrating energy is sufficient to increase the gene transfer. However, naked DNA did not reach the levels of GL67 and thus currently does not offer any advantages over cationic lipid-based formulations. Nonetheless, these studies provide proof-of-principle that low-frequency sonoporation enhances non-viral transfections. Similar to other studies the effect of US on gene transfer increased with increasing negative pressure [28]. This is not surprising given the fact that it is during this part of the pressure 'cycle' that cavitation nuclei expand, gaining most of their energy [29]. Importantly, sonoporation on naked pDNA gene transfer was achieved in the absence of microbubbles. This is in contrast to high-frequency US, which was dependent on Optison, and supports the notion that low-frequency US can induce greater cavitation than high-frequency US. From a clinical point of view low-frequency US, therefore, offers significant advantages for gene transfer over high-frequency US.

We also histologically assessed US-induced lung hemorrhage, a common US bioeffect caused by damage to the lung vasculature and showed that it increases with negative pressure. Hemorrhage was also seen throughout the lung indicating that US does penetrate the tissue. Lung damage in mice has been reported with 30 kHz continuous wave US at 0.15 MPa [30], which is similar to the pressures applied in our experiments. These conditions are lower than the reported 1 MPa threshold for US-induced lung hemorrhage in mice, which was however, established with 1.15 MHz US [31]. This further highlights the importance of frequency on energy penetration and cavitation potential, and the difficulties in making comparisons between different studies, particularly as the US conditions are not always reported in detail.

Studies looking at the factors that determine the degree of susceptibility to US-induced bioeffects in the lung have shown that mice are more susceptible to such damage than other species [30]. This is attributed to differences in the structure and 'architecture' of the lung between different organisms [32]. In particular, differences in the thickness of the visceral pleura seem to be important, with mice, rats, dogs and monkeys (classified as the thin group) being more affected than sheep, pigs and humans (thick group). In addition, the airways in mice are shorter, thinner and less flexible than humans and may thus be more vulnerable to US-induced damage [30]. Interestingly, in the 30 kHz study mentioned above [30] mice were more sensitive than rabbits, which in turn were more sensitive than pigs.

This evidence suggests that mice are perhaps not the best model to assess the effects of sonoporation on lung gene transfer. Although here we show that US exposure can increase naked pDNA transfection efficiency, this effect could be hampered by the damage caused under these conditions. The fact that in mice the window between efficacy and toxicity is so small could also account for the fact that sonoporation did not enhance PEI or GL67 gene transfer.

To further study the effects of low-frequency sonoporation research may have to be undertaken in a large animal model with similar lung structure to humans. Sheep are an attractive model for such studies as they also allow the use of more clinically relevant methods for the delivery of the gene transfer agents, such as nebulization [33]. Given the difference in lung size the distance that US would have to 'travel' is much greater in sheep than in mice. Thus, the US conditions (exposure time, acoustic pressure, duty cycle) will have to be optimized for gene transfer in this particular model. Importantly, these studies will also be able to address the question whether sonoporation can mediate lung gene transfer without detrimental side-effects, which will in turn determine the clinical applicability of this technique.

In summary, we have shown proof-of-principle that low-frequency US can enhance the naked pDNA gene transfer in the air-filled mouse lung. Importantly, this effect is not relying on the use of microbubbles such as Optison. Studies in large animal models would be required for further validation of this technique.

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