Effects of Low-Intensity Ultrasound on Gramicidin D-Induced Erythrocyte Edema

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Objectives—To determine whether low-intensity ultrasound (US) can reduce red blood cell (RBC) edema and, if so, whether the US activity is associated with aquaporin 1 (AQP-1), a water channel in the cell membrane.

Methods—Red blood cell edema was induced by gramicidin D treatment at 40 ng/mL for 20 minutes and evaluated by a hematocrit assay. Low-intensity continuous wave US at 1 MHz was applied to RBCs for the last 10 minutes of gramicidin D treatment. To determine whether US activity was associated with AQP-1, RBCs were treated with 40 μM mercuric chloride ($HgCl_2$), an AQP-1 inhibitor, for 20 minutes at the time of gramicidin D treatment. Posttreatment morphologic changes in RBCs were observed by actin staining with phalloidin.

Results—Red blood cell edema increased significantly with gramicidin D at 20 (1.8%), 40 (6.7%), 60 (16.7%), and 80 (11.3%) ng/mL, reaching a peak at 60 ng/mL, compared to the control group (20 ng/mL, $P = .019$; 40, 60, and 80 ng/mL, $P < .001$). No significant RBC hemolysis was observed in any group. Edema induced by gramicidin D at 40 ng/mL was significantly reduced by US at 30 (3.4%; $P = .003$), 70 (4.4%; $P = .001$), and 100 (2.9%; $P = .001$) mW/cm^2. Subsequent experiments showed that edema reduction by US ranged from 7% to 10%. Cotreatment with $HgCl_2$ partially reversed the US effect and showed a significantly different level of edema compared to gramicidin D-alone and US-cotreated groups ($P = .001$). These results were confirmed by microscopic observation of RBC morphologic changes.

Conclusions—Low-intensity US could reduce gramicidin D–induced RBC edema, and its effect appeared to at least partly involve regulation of AQP-1 activity. These results suggest that low-intensity US can be used as an alternative treatment to control edema and related disorders.

Key Words—aquaporin 1; basic science; gramicidin D; edema; low-intensity ultrasound; water transport

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Abbreviations

AQP-1, aquaporin 1; $HgCl_2$, mercuric chloride; PBS, phosphate-buffered saline; RBC, red blood cell; US, ultrasound

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Water is the major environment of all living cells, and living processes take place in water. Plasma membranes are to some extent permeable to water, with slow diffusion. Therefore, water exchange through plasma membranes is essential to maintain osmotic stress and the integrity of the cells. Cellular edema (ie, cell swelling) is a reversible process and not serious for cells by itself. However, cellular edema is the first change when cells have a problem, and it commonly accompanies various cellular pathologic

phenomena. If cells cannot manage cellular edema, they eventually die. When an organ encounters cellular edema, cells push each other, which results in mass effects.[1–3] It is thus important for clinicians to manage cellular edema; however, the clinical means to manage it are limited to some degree. The most powerful way ever developed is administration of diuretics, which act on the kidneys to increase urine output. When urine output increases, the hydrostatic pressure in the plasma is reduced, which stimulates cells to excrete intracellular fluid. In clinics, diuretics are used to treat a number of diseases involving edema, such as heart failure, liver cirrhosis, hypertension, and certain kidney diseases. However, diuretics depend on the disease type for their efficacy and paradoxically can cause edema symptoms with long-term use. Another way to make cells excrete their fluids is to increase the plasma oncotic pressure.[4] Regardless of the approach, current treatments are roundabout therapies with no clear efficacy, and novel therapies directly targeting the edematous tissues and edema mechanism are needed.

Ultrasound (US) is a noninvasive modality that is distinguished from irradiation in the medical field and causes no harm to the body.[5] Low-intensity US is a sonic wave with an intensity of less than 1 W/cm^2. It is thought to generate radiation forces, shear stresses, and cavitation without generating heat energy.[6] Many studies have suggested that low-intensity US can modulate diverse cellular activities and events. Low-intensity US induces expression of various genes such as hypoxia-inducible factor 1α in osteoblasts,[7] collagen type II and aggrecans in chondrocytes,[8] and proliferating cell nuclear antigen in tendon cells.[9] Low-intensity US also stimulates proliferation of tendon cells[9] and chondrocytes,[10] phagocytosis of macrophages,[11] nitric oxide and prostaglandin E_2 production in osteoblasts,[12] and chondrogenic differentiation of mesenchymal stem cells.[13] As a therapeutic tool, low-intensity US promotes bone fracture healing,[14] cartilage regeneration[10] and tendon repair.[10] These US activities probably involve activation of many cellular signal pathways, including integrin, Rho, and mitogen-activated protein kinases.[12,15,16] Therefore, low-intensity US is not a simple mechanical force but an active regulator of diverse cellular functions.

We have also shown previously that low-intensity US of less than 500 mW/cm^2 can reduce knee edema by decreasing synovial fluids in the joint space in rabbit osteoarthritis and rat adjuvant-induced arthritis models.[17,18] In these studies, low-intensity US also showed a potent anti-inflammatory and therapeutic effect on disease phenotypes. The mechanism of low-intensity US is not clear, but this result suggests the possibility that it can directly affect cellular or tissue edema. Ultrasound has long been used in clinics as physical therapy for musculoskeletal management, including tissue edema.[19] It has also been thought to affect the porosity of cell membranes and thereby had been applied to drug delivery.[20] In the former case, varying US conditions, including low-intensity US, have been used but are still controversial because of their action mechanisms and therapeutic benefits. For drug delivery, high-intensity US of greater than 1 W/cm^2 has commonly been used, but some studies used low-intensity US.[21,22] However, these studies mostly focused on the mechanical perturbation of the cell membrane and delivery of therapeutic drugs, and no information is available on the effect of low-intensity US on water transport across the cell membrane.

In this study, we investigated the effect of low-intensity US on cellular edema induced by gramicidin D in rat red blood cells (RBCs). We thought that it was better to use cellular edema than tissue models for determining the direct effect of low-intensity US on water transport. Red blood cells are widely used to study cellular edema because they are easily available and have the advantage that substantial changes under osmotic stress can be easily detected.[23] Our aim was to determine whether low-intensity US can reduce RBC edema and, if so, whether the US activity is associated with aquaporin 1 (AQP-1), a water channel in the cell membrane.

Materials and Methods

Preparation of the RBC Suspension and Hematocrit Measurement

A 10-mL volume of blood was taken by cardiac puncture from a male Sprague Dawley rat (250 g; Samtako, Osan, Korea). All animals were maintained in accordance with the policies of the Institutional Animal Care and Use Committee of Inha University. On the day of collection, blood samples were centrifuged at 3000 rpm for 10 minutes to remove the supernatant and the buffy coat. Red blood cells in the pellet were washed 3 times with normal saline (0.9% sodium chloride, wt/vol) by repeating the centrifugation each time. Finally, the pellet was resuspended in the equal volume of normal saline to make a 50% (vol/vol) hematocrit. To measure the hematocrit, RBCs in suspension were aspirated into capillary tubes. The tubes were sealed with a sealer and centrifuged at 3000 rpm for 5 minutes in a hematocrit centrifuge (VS-1200; Vision Scientific Co, Bucheon, Korea). The hematocrit was calculated by the volume ratio of centrifuged RBCs to the whole sample.

Measurement of RBC Hemolysis

The RBC samples were harvested by centrifugation at 3000 rpm for 3 minutes. Supernatants were collected and diluted 100 times for measuring the optical density at 560 nm. The results were compared with a standard hemolysis curve to determine the degree of hemolysis of the samples.

Red Blood Cell Edema Model

Red blood cell edema was induced with gramicidin D (Sigma-Aldrich, St Louis, MO). Gramicidin D is known to promote accumulation of intracellular sodium ions (Na^+) and rapid excretion of potassium ions (K^+) out of cells.[24,25] Gramicidin D was administered to a 50% (vol/vol) RBC suspension at the concentrations indicated in each experiment for 20 minutes.

Low-Intensity US Stimulation

A 2-mL volume of the RBC suspension in saline (50%, vol/vol) was placed in a 35-mm culture dish and treated with continuous wave US for 10 minutes at a frequency of 1 MHz and an intensity of 30, 70, or 100 mW/cm^2. The US equipment was custom made in cooperation with Korust, Ltd (Anyang, Korea), and contained 6 transducers of 35 mm in diameter and 10 mm in thickness for research use. The transducers were immersed in water, and cells in culture dishes were placed on top of the water from a 1-cm distance so that the US was transmitted through the water and the plastic bottom. The samples in a set of experiments were treated with US at the same time. The samples in the same group were treated separately. The transducers were calibrated regularly and assigned randomly to each sample. The US parameters were based on our previous experiments treating osteoarthritis using low-intensity US.[10,15,17] The US intensity was precalibrated with a power meter (UPM-DT10-100AV; Ohmic Instruments, St Charles, MO). The time point of US application was right before or during the gramicidin D treatment so that the analysis time was the same for all treatment groups.

Mercuric Chloride Treatment

Mercuric chloride ($HgCl_2$) is known as an AQP-1 inhibitor.[24] Where indicated, RBCs (50%, vol/vol) were treated with $HgCl_2$ (40 μM) for 20 minutes at the time of gramicidin D treatment.

Staining of Actin Filaments

Red blood cells were smeared onto a glass slide. Samples were fixed with 0.5% glutaraldehyde in a potassium chloride solution containing 130 mM potassium chloride, 20 mM potassium/sodium phosphate buffer, 10 mM glucose, and 1 mg/mL bovine serum albumin (pH 7.8) for 20 minutes. To quench excess aldehyde, RBCs were incubated in phosphate-buffered saline (PBS) containing 0.1 M glycine for 30 minutes at room temperature, followed by permeabilization with 0.05% Triton X-100 in PBS for 10 minutes. After washing 3 times in PBS, RBCs were stained for F-actin with rhodamine-phalloidin (Invitrogen, Carlsbad, CA) in PBS for 40 minutes. Fluorescence images were visualized by a confocal microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany). The size of the RBCs was determined by image analysis software (Axio-Vision Ver; Carl Zeiss).

Statistical Analysis

All experiments were performed 3 times, each of which consisted of 4 samples per group. The data are expressed as mean ± standard deviation. Statistical significance was analyzed by 1-way analysis of variance followed by the Scheffé test using SPSS version 12.0 software (IBM Corporation, Armonk, NY). $P < .05$ was considered statistically significant.

Results

Titration of Gramicidin D Concentrations and US Intensities

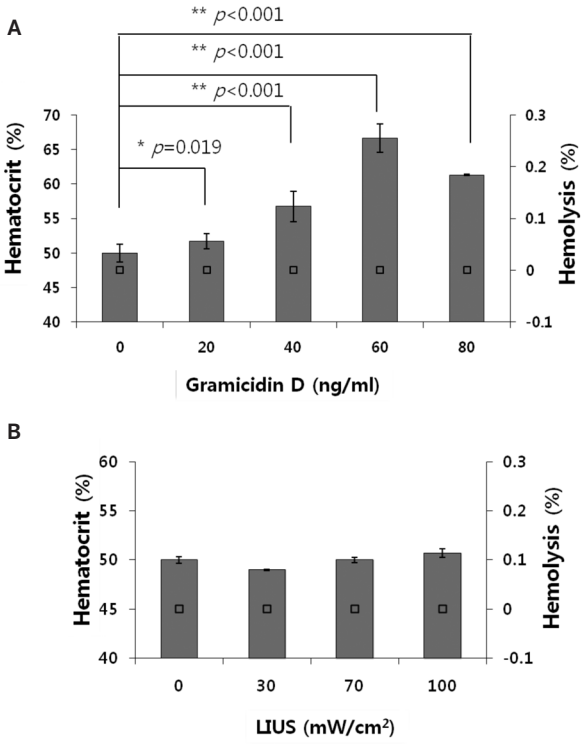
We used gramicidin D to induce RBC edema under an isotonic circumstance.[25] Red blood cells were incubated with gramicidin D for 20 minutes at 0, 20, 40, 60, and 80 ng/mL. As shown in Figure 1A, RBCs treated with gramicidin D at 60 ng/mL were the most edematous, reaching a peak value, where their volume (66.7% ± 2.0%) increased by 16.7% ($P < .001$) compared to the control group (50.0% ± 1.3%). Gramicidin D at 20 (51.8% ± 1.1%), 40 (56.7% ± 2.2%), and 80 (61.3% ± 1.0%) ng/mL also showed significant increases in RBC volume (20 ng/mL, $P = .019$; 40 and 80 ng/mL, $P < .001$). There was no significant RBC hemolysis at any gramicidin D concentration in our experiments (Figure 1A, open squares). To evaluate the effect of low-intensity US on RBC edema, we chose 40 ng/mL as the lowest gramicidin D dose with minimum toxicity and sufficient activity to induce edema. In parallel, the effect of low-intensity US alone on the hematocrit and hemolysis of fresh RBCs was assessed at varying intensities. Red blood cells were treated with US for 10 minutes at 30, 70, and 100 mW/cm^2. The hematocrit in the RBCs decreased about 1.9% ± 0.07% at 30 mW/cm^2 (control, 50.0% ± 10.3%; 30 mW/cm^2, 48.1% ± 1.0%) but did not change at all at higher US intensities (Figure 1B). No significant hemolysis was observed again at any US intensity (Figure 1B, open squares).

Effect of Low-Intensity US on RBC Edema Induced by Gramicidin D

We then evaluated the effect of low-intensity US on RBC edema induced by gramicidin D. Red blood cells were treated with gramicidin D at 40 ng/mL for 20 minutes. Low-intensity US was first applied at 0, 30, 70, or 100 mW/cm^2 for the last 10 minutes of gramicidin D treatment, when RBC edema occurred sufficiently. The hematocrit data showed that RBC edema induced by gramicidin D was clearly reduced by US at all intensities tested, with statistical significance (30 mW/cm^2, $P = .003$; 70 mW/cm^2, $P = .001$; 100 mW/cm^2, $P = .001$). The reduced values were 3.4% at 30 mW/cm^2, 4.4% at 70 mW/cm^2, and, 2.9% at 100 mW/cm^2; there was no significant difference among US intensities (Figure 2). Significant RBC hemolysis was not observed in any group, including the cotreated samples (Figure 2, open squares). These results suggest that low-intensity US could reduce gramicidin D-induced RBC edema without appreciable cell damage.

To further understand the mode of action of low-intensity US, the time point of US application was varied in the next experiment. Low-intensity US (70 mW/cm^2) was applied to the RBCs for 10 minutes right before (Pre), at the beginning (First), or at the end (Last) of gramicidin D treatment for 30 minutes. Red blood cell edema induced by gramicidin D (65.7% ± 1.0% hematocrit) was significantly decreased by low-intensity US only when it was applied for the first (57.4% ± 0.7% hematocrit; $P < .001$) or last (57.1% ± 3.2% hematocrit; $P = .009$) 10 minutes of gramicidin D treatment (Figure 3). Application of US before gramicidin D treatment showed no statistically significant difference in RBC volume from that of the gramicidin D-alone group. These results suggest that low-intensity US did not have any preconditioning effect on RBCs but, by any means, inhibited or reversed edema formation by gramicidin D.

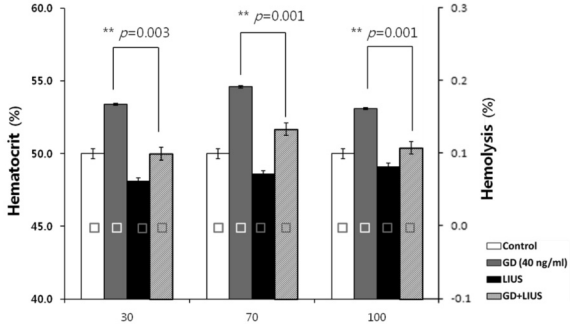
Figure 1. Effect of gramicidin D and low-intensity US (LIUS) alone on fresh RBCs. **A**, Red blood cells (50%, vol/vol) in PBS were treated with gramicidin D at 0, 20, 40, 60, and 80 ng/mL for 30 minutes. Hematocrit was measured to examine RBC edema (bars), and hemolysis was determined to examine cell lysis (open squares). **B**, Ultrasound was applied to RBCs (50%, vol/vol) in PBS for 10 minutes at intensities of 0, 30, 70, and 100 mW/cm^2 before examining the hematocrit (bars) and hemolysis (open squares). Data are presented as mean ± SD from 3 independent experiments.



Effect of $HgCl_2$ and Low-Intensity US on RBC Edema Induced by Gramicidin D

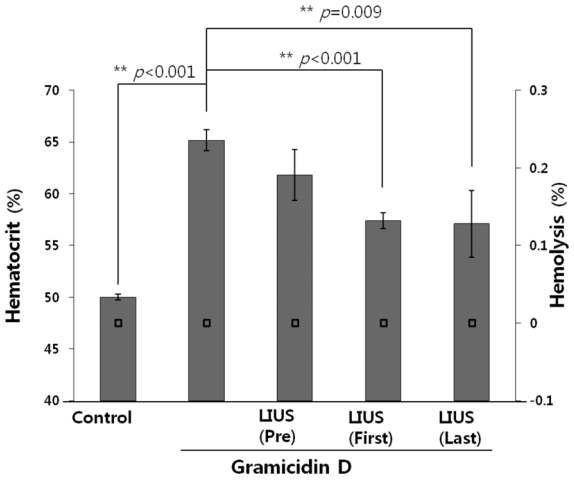
Water flow through the cell membrane is mainly mediated by a water channel called AQP. Red blood cells are known to express AQP-1. To investigate the role of AQP-1 in RBC edema induced by gramicidin D and the inhibitory effect of low-intensity US, we used $HgCl_2$, which is known to inhibit AQP-1 function by binding on its cysteine 189 residue.[26,27] Red blood cells were treated with gramicidin D (40 ng/mL) for 30 minutes in combinations with $HgCl_2$ (40 μM) for the first 20 minutes and low-intensity US (70 mW/cm^2)

Figure 2. Effect of low-intensity US (LIUS) on RBC edema induced by gramicidin D (GD). Gramicidin D was administered to RBCs (50%, vol/vol) in PBS at 40 ng/mL for 30 minutes. Ultrasound was applied alone or in combination with gramicidin D at 30, 70, and 100 mW/cm^2 for the last 10 minutes of gramicidin D treatment. Hematocrit was measured, and values are presented as mean ± SD from 3 independent experiments. Hemolysis is also presented (open squares).



for the last 10 minutes. As shown in Figure 4A, $HgCl_2$ alone decreased the gramicidin D-induced RBC edema by 5.0% (57.1% ± 3.8% hematocrit), and the combination of US and $HgCl_2$ resulted in a hematocrit value between those of each treatment (55.3% ± 1.3% hematocrit; $P = .001$). As above, gramicidin D induced RBC edema by 10.3% (60.3% ± 2.8% hematocrit) compared to the control group (50.0% ± 1.6% hematocrit). Ultrasound treatment again clearly decreased the gramicidin D-increased hematocrit by 7.5% (52.8% ± 1.5% hematocrit; $P < .001$). We then tried to observe the morphologic characteristics and size of the RBCs under a microscope after phalloidin staining of actin fibers (Figure 4B). The images collected were digitalized at a resolution of 32 bits into an array of 2048 pixels (Figure 4C). The results showed again that gramicidin D treatment induced RBC swelling, and low-intensity US and $HgCl_2$ alone ($P < .001$) and in combination ($P < .001$) significantly decreased the gramicidin D effect compared to the control group. The almost complete inhibition of the gramicidin D effect by $HgCl_2$ might be due to the limitations of 2-dimensional image analysis. The distribution of actin fibers appeared not to be changed substantially. Taken together with the hematocrit data, these results suggest that both low-intensity US and $HgCl_2$ had an inhibitory effect on gramicidin-induced RBC edema.

Figure 3. Effect of time points of low-intensity US (LIUS) treatment on gramicidin D-induced RBC edema. Ultrasound (70 mW/cm^2) was applied on RBCs (50%, vol/vol) for 10 minutes right before gramicidin D treatment for 30 minutes (Pre) or for the first (First) or last (Last) 10 minutes of GD treatment. Hematocrit values are presented as mean ± SD from 3 independent experiments. Hemolysis is also presented (open squares).



Discussion

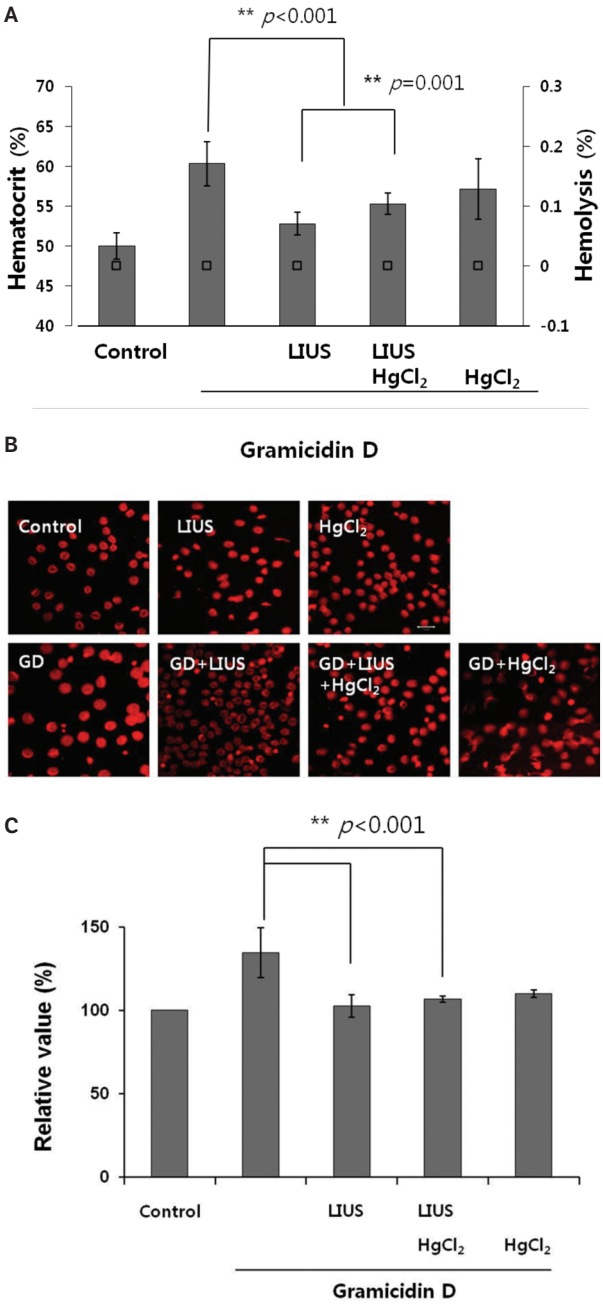
To our knowledge, a study investigating the effects of low-intensity US on cellular edema has not been reported previously. The results showed that low-intensity US could reduce RBC edema induced by gramicidin D. The frequency of the US was 1 MHz, and intensities of 30, 70, and 100 mW/cm^2 showed no significant differences in the reduction levels of RBC edema when US was applied for 10 minutes. The effect of low-intensity US was observed when it was applied at the same time or after gramicidin D treatment but not when the RBCs were preconditioned with US. The mechanism of low-intensity US is still unknown, but it appears to involve regulation of AQP-1 function because $HgCl_2$, an AQP-1 inhibitor, partially reversed the US effect. Taken together with previous reports, including ours on the reduction of joint edema by low-intensity US,[17,18] the results of this study suggest that low-intensity US could be a therapeutic tool for treating edema in many pathologic diseases. Further studies are needed to investigate the effect of low-intensity US with different parameters (pulse type, intensity, frequency, duty cycle, and treatment time) to understand its mode of action and find optimal conditions for various experimental models.

Cellular edema can be induced by several factors, such as impaired membrane permeability and osmotic imbalance. In this study, we induced RBC edema by using gramicidin D. An antibiotic compound, gramicidin D is a linear polypeptide made up of 15 hydrophobic amino acids in a levo-dextro sequence.[28] Gramicidin forms a cation-selective channel as a dimer stabilized by 15 intramolecular and 6 intermolecular hydrogen bonds. The gramicidin channel is gated by association and dissociation of dimers and is a good model for narrow, low-conductance channels (such as the selectivity filter region of K^+ channels).[29] The size of the gramicidin channel is approximately 0.4 nm in diameter, which is large enough to accommodate the passage of monovalent cations. Ion selectivity is observed in the order of $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$, and divalent cations such as Ca^{2+} block the channel.[18] Vitvitsky et al[30] reported that gramicidin D caused K^+ to rapidly leak and Na^+ to accumulate intracellularly within RBCs. A change in the cation concentration within the cell causes influx of water into the cell and, eventually, edema. The interval needed to reach the equilibrium between the intracellular and extracellular ion concentrations ranged from 30 minutes to several hours, depending on the gramicidin D concentration.[30] When gramicidin D was administered to RBCs for 30 and 60 minutes, RBC hemolysis was observed at 60 minutes but not at 30 minutes.

The two therapeutic mechanisms of US with diverse frequencies and intensities are thermal and nonthermal effects. This study used US of 100 mW/cm^2 or less in intensity. The cellular effect of low-intensity US generally involves nonthermal mechanisms such as radiation forces, shear stress, and cavitation.[6] These micromechanical forces can give rise to both physical and biochemical changes in target cells and tissues; thus, low-intensity US can reduce RBC edema. Practically, there were several possibilities for the low-intensity US mechanism in this study. First, the US might have physically perturbed the plasma membrane and thus increased its permeability to water molecules. We think, however, that the US mechanism did not involve this possibility because its intensity was very low to directly increase membrane permeability, and the US effect was deregulated to some extent by $HgCl_2$, an AQP-1 inhibitor. Second, the US might have directly regulated AQP-1 activity. We speculate that the mechanical force of low-intensity US could affect the AQP-1 structure and activity directly or via induction of changes in plasma membrane topology or other cell surface proteins such as integrins. It was recently shown that AQP-2 and AQP-4 activity could be regulated by integrins and extracellular matrix molecules.[31,32] Last, the US might have regulated other cell surface molecules such as an osmotic pump or a sodium-potassium channel, thereby indirectly affecting AQP-1 activity. Changes in the activity of these osmotic pumps and ion channels on the cell surface could disrupt osmotic or ionic balances and eventually cause changes in AQP-1 activity. The low-intensity US activity cannot be specific to APQs and could induce diverse changes in cells. Therefore, we think this process is the most likely mechanism of the low-intensity US effect.

There are several low-intensity US parameters such as intensity, frequency, exposure time, and waveform that can influence its edema-reducing effect. The parameters specific or optimal for low-intensity US effects were not identified in this study because we used fixed US conditions except intensity. In a previous study, treatment with low-intensity US at 500 mW/cm^2 and less than 100 kHz for 7 minutes induced porosity of and efficient gene delivery into rabbit RBCs without a significant loss of cell viability.[21] In another study, treatment with low-intensity US at 450 mW/cm^2 and 255 kHz for 30 minutes induced some structural changes in the membrane of HL60 leukemia cells.[33] The authors did not observe conspicuous pits or pores but did not rule out the possibility of small pores or ruptures occurring on the cell membrane. Although the US frequency was different, this study administered US at less than 100 mW/cm^2 for 10 minutes, which might have been sufficient to induce some changes on RBC mem-

Figure 4. Effect of $HgCl_2$ on low-intensity US (LIUS) activity to alleviate gramicidin D (GD)-induced RBCs edema. Gramicidin D (40 ng/mL) was administered to RBCs (50%, vol/vol) for 30 minutes. $HgCl_2$ was administered for the first 20 minutes of gramicidin D treatment, and then low-intensity US was administered for the last 10 minutes. **A**, Hematocrit was measured, and values are presented as mean ± SD from 3 independent experiments. Hemolysis is also presented (open squares). **B**, The actin cytoskeleton of RBCs was stained with phalloidin-Texas Red, and fluorescent images are presented. Scale bar indicates 10 μm. **C**, The area of stained RBCs was measured from the images, and relative values are presented as mean ± SD from 3 independent experiments.



branes or activate cell surface proteins. We tested but observed no significant differences among US intensities of 30, 70, and 100 mW/cm^2. It is not clear, but all intensities tested could have been strong enough to have the edema-reducing effect. We speculate that higher US intensities can generate too much mechanical energy, including heat, and cause highly nonspecific effects on cells. We could not test US intensities less than 30 mW/cm^2 and frequencies other than 1 MHz because of limitations of the US device used. In our previous study, low-intensity US showed some dose-dependent edema reduction in a rat inflammatory arthritis model when applied at 30, 100, and 200 mW/cm^2 for 10 minutes.[18] It may not be directly correlated in terms of biological mechanisms, but higher US intensities appeared to work in the animal study. This study used a continuous wave as a source of US, which might have caused standing waves by a reflection against the bottom of the culture plate. Comparisons among different US waveforms (continuous versus pulsed) and duty cycles also need to be performed in further studies.

Aquaporins are a family of transmembrane channels that allow water flow along osmotic gradients.[34] They are known to play key roles in both cellular and tissue edema. For example, AQP-4 is involved in cerebral edema, and its deletion in mice reduces brain edema after acute water intoxication and ischemic stroke.[35] Myocardial edema was alleviated by inhibiting the function of AQP-1.[36] $HgCl_2$ was known to inhibit AQP-1 activity reversibly by covalent modification of cysteine 187 located outside the plasma membrane.[24] Water permeability was shown to decrease 30% to 40% by 0.5 mM $HgCl_2$ in lung tissue.[37] In this study, $HgCl_2$ partially reversed gramicidin D-induced RBC edema in the hematocrit when administered at the same time as gramicidin D treatment. This partial inhibition might have been due to the competitive effect of $HgCl_2$ and gramicidin D on RBC edema and the reversible mode of $HgCl_2$ action. This study also showed that combined treatment with low-intensity US and $HgCl_2$ in the presence of gramicidin D resulted in an intermediary level of RBC edema in the hematocrit, which meant a partial inhibition of US activity to reduce gramicidin D-induced RBC edema by $HgCl_2$. Therefore, the US effect on RBC edema appears to be mediated at least in part by its action on AQP-1 activity. $HgCl_2$, an AQP-1 inhibitor, showed a similar inhibitory effect on the gramicidin-D induced RBC edema in this study. However, it is not clear at present whether low-intensity US also played an inhibitory role because of the complex mode of action of Hg^{2+} on RBC edema depending on the experimental context. The effect of Hg^{2+} on human RBCs has been shown to strongly decrease under swelling conditions, as in the case of gramicidin D-induced edema in this study.[38] In addition, Hg^{2+} can activate K^+-selective Gardos channels on RBCs to excrete K^+, causing eventual water efflux and cell shrinkage.[39] Although we could not detect any changes in RBC volume after $HgCl_2$ treatment alone (data not shown), these multiple actions of Hg^{2+} make our results very complicated and unpredictable.

Therapeutic benefits of US are commonly attributed to reduction of pain and edema. In a previous report, low-intensity US reduced postoperative pain and swelling and accelerates bone healing in rabbits.[14] It was also shown to increase intra-articular delivery of hyaluronan across the synovial membrane in rabbits.[40] The results of this study further indicate the effect of low-intensity US in regulating permeability and water transport across the cell membrane. It is not definitely clear, but it is plausible that these US activities are involved in its therapeutic benefit in reducing synovial edema in animal models, as shown in our previous studies.[17,18] Low-intensity US caused a 7% to 10% reduction in RBC edema at best, but we think it can have a critical influence on edema formation and disease progression in vivo, considering the complexity and signal amplification of physiologic systems. It could be particularly true when low-intensity US is administered repeatedly for a long time. Edema occurs when an excessive volume of fluid accumulates in tissues, either within cells (cellular edema) or within the collagen-mucopolysaccharide matrix distributed in the interstitial spaces (tissue edema). Cellular (cytotoxic) edema results primarily from dysregulation of the osmotic gradient across membranes, which is seen early in ischemic and toxic injuries.[1–3] Circulating RBCs are more prone to cellular edema than other cells because they are exposed to high oxygen concentrations. Oxidation of cell membrane components may lead to a nonselective increase in the membrane permeability to cations.[41] Impaired membrane permeability will result in an osmotic imbalance between the cell and the medium and, hence, in an increase in the cell volume.[30] The gramicidin D-induced model in this study may not be exactly the same but can mimic naturally occurring RBC edema. Gramicidin D induces a change in the cation concentration within RBCs and causes an influx of water into the cells and, eventually, edema. Tissue edema may occur as a result of aberrant changes in the pressures (hydrostatic and oncotic) acting across the microvascular walls, alterations in molecular structures, or alterations in the lymphatic outflow system.[42] It could be independent of AQP and in more complicated environments may involve many different cell types and the integrity of cell-to-cell junctions.[43] Therefore, the effect of low-intensity US on tissue edema might also depend on the tissue environment and disease cases.

In conclusion, this study indicates that low-intensity US has an inhibitory effect on gramicidin D-induced RBC edema, probably via the regulation of AQP-1 activity. Our results suggest therefore that low-intensity US could be an alternative to conventional therapy for reducing edema. Further studies are necessary to understand the exact mechanism of low-intensity US at various parameters and to examine whether it has therapeutic effects on other types of cellular and tissue edema and on edema-associated diseases in animal models.

References

1. Lang F. Mechanisms and significance of cell volume regulation. *J Am Coll Nutr* 2007; 26(suppl):613S–623S.
2. Strange K. Cellular volume homeostasis. *Adv Physiol Educ* 2004; 28:155–159.
3. Franco R, Panayiotidis MI, de la Paz LD. Autocrine signaling involved in cell volume regulation: the role of released transmitters and plasma membrane receptors. *J Cell Physiol* 2008; 216:14–28.
4. Guyton A, Hall J. The microcirculation and the lymphatic system. In: *Textbook of Medical Physiology*. 11th ed. Philadelphia, PA: Elsevier; 2006:117–190.
5. Mitragotri S. Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. *Nat Rev Drug Discov* 2005; 4:255–260.
6. Feril LB Jr, Kondo T. Biological effects of low intensity ultrasound: the mechanism involved and its implications on therapy and on biosafety of ultrasound. *J Radiat Res* 2004; 45:479–489.
7. Wang FS, Kuo YR, Wang CJ, et al. Nitric oxide mediates ultrasound-induced hypoxia-inducible factor-1α activation and vascular endothelial growth factor-A expression in human osteoblasts. *Bone* 2004; 35:114–123.
8. Choi BH, Woo JI, Min BH, Park SR. Low-intensity ultrasound stimulates the viability and matrix gene expression of human articular chondrocytes in alginate bead culture. *J Biomed Mater Res A* 2006; 79:858–864.
9. Tsai WC, Hsu CC, Tang FT, Chou SW, Chen YJ, Pang JH. Ultrasound stimulation of tendon cell proliferation and upregulation of proliferating cell nuclear antigen. *J Orthop Res* 2005; 23:970–976.
10. Min BH, Choi BH, Park SR. Low intensity ultrasound as a supporter of cartilage regeneration and its engineering. *Biotechnol Bioproc Eng* 2007; 12:22–31.
11. Zhou S, Bachem MG, Seufferlein T, Li Y, Gross HJ, Schmelz A. Low intensity pulsed ultrasound accelerates macrophage phagocytosis by a pathway that requires actin polymerization, Rho, and Src/MAPKs activity. *Cell Signal* 2008; 20:695–704.
12. Reher P, Harris M, Whiteman M, Hai HK, Meghji S. Ultrasound stimulates nitric oxide and prostaglandin E_2 production by human osteoblasts. *Bone* 2002; 31:236–241.
13. Cui JH, Park K, Park SR, Min BH. Effects of low-intensity ultrasound on chondrogenic differentiation of mesenchymal stem cells embedded in polyglycolic acid: an in vivo study. *Tissue Eng* 2006; 12:1275–1282.
14. Pilla AA, Mont MA, Nasser PR, et al. Non-invasive low-intensity pulsed ultrasound accelerates bone healing in the rabbit. *J Orthop Trauma* 1990; 4:246–253.
15. Choi BH, Choi MH, Kwak MG, Min BH, Woo ZH, Park SR. Mechanotransduction pathways of low-intensity ultrasound in C-28/I2 human chondrocyte cell line. *Proc Inst Mech Eng H* 2007; 221:527–535.
16. Zhou S, Schmelz A, Seufferlein T, Li Y, Zhao J, Bachem MG. Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. *J Biol Chem* 2004; 279;54463–54469.
17. Park SR, Park SH, Jang KW, et al. The effect of sonication on simulated osteoarthritis, part II: alleviation of osteoarthritis pathogenesis by 1 MHz ultrasound with simultaneous hyaluronate injection. *Ultrasound Med Biol* 2005; 31:1559–1566.
18. Chung JI, Barua S, Choi BH, Min BH, Han HC, Baik EJ. Anti-inflammatory effect of low intensity ultrasound (LIUS) on complete Freund's adjuvant-induced arthritis synovium. *Osteoarthiritis Cartilage* 2012; 20:314–322.
19. Wong RA, Schumann B, Townsend R, Phelps CA. A survey of therapeutic ultrasound use by physical therapists who are orthopaedic certified specialists. *Phys Ther* 2007; 87:986–994.
20. Mehier-Humbert S, Bettinger T, Yan F, Guy RH. Plasma membrane poration induced by ultrasound exposure: implication for drug delivery. *J Control Release* 2005; 104:213–222.
21. Wei W, Zheng-zhong B, Yong-jie W, Qing-wu Z, Ya-lin M. Bioeffects of low-frequency ultrasonic gene delivery and safety on cell membrane permeability control. *J Ultrasound Med* 2004; 23:1569–1582.
22. Greenleaf WJ, Bolander ME, Sarkar G, Goldring MB, Greenleaf JF. Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. *Ultrasound Med Biol* 1998; 24:587–595.
23. Dacie JV, Lewis SM. Basic haematological techniques. In: Briggs C, Bain B (eds). *Practical Haematology*. 6th ed. New York, NY: Churchill Livingstone; 1984:152–156.
24. Yang B, Kim JK, Verkman AS. Comparative efficacy of $HgCl_2$ with candidate aquaporin-1 inhibitors DMSO, gold, TEA^+ and acetazolamide. *FEBS Lett* 2006; 580:6679–6684.
25. Kelkar DA, Chattopadhyay A. The gramicidin ion channel: a model membrane protein. *Biochim Biophys Acta* 2007; 1768: 2011–2025.
26. Hasegawa H, Zhang R, Dohrman A, Verkman AS. Tissue-specific expression of mRNA encoding rat kidney water channel CHIP28k by in situ hybridization. *Am J Physiol* 1993; 264:C237–C245.
27. Zhang R, van Hoek AN, Biwersi J, Verkman AS. A point mutation at cysteine 189 blocks the water permeability of rat kidney water channel CHIP28k. *Biochemistry* 1993; 32:2938–2941.
28. Corry B, Chung SH. Mechanisms of valence selectivity in biological ion channels. *Cell Mol Life Sci* 2006; 63:301–315.
29. Sansom MS, Shrivastava IH, Ranatunga KM, Smith GR. Simulations of ion channels: watching ions and water move. *Trends Biochem Sci* 2000; 25:368–374.
30. Vitvitsky VM, Frolova EV, Martinov MV, Komarova SV, Ataullakhanov FI. Anion permeability and erythrocyte swelling. *Bioelectrochemistry* 2000; 52:169–177.

31. Tamma G, Lasorsa D, Ranieri M, Mastrofrancesco L, Valenti G, Svelto M. Integrin signaling modulates AQP2 trafficking via Arg-Gly-Asp (RGD) motif. *Cell Physiol Biochem* 2011; 27:739–748.
32. Tham DK, Moukhles H. Regulation of Kir4.1 and AQP4 expression and stability at the basolateral domain of epithelial MDCK cells by the extracellular matrix. *Am J Physiol Renal Physiol* 2011; 301:F396–F409.
33. Ogawa K, Tachibana K, Uchida T, et al. High-resolution scanning electron microscopic evaluation of cell-membrane porosity by ultrasound. *Med Electron Microsc* 2001; 34:249–253.
34. Agre P, King LS, Yasui M, et al. Aquaporin water channels: from atomic structure to clinical medicine. *J Physiol* 2001; 542:3–16.
35. Manley GT, Fujimura M, Ma T, et al. Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat Med* 2000; 6:159–163.
36. Ding FB, Yan YM, Huang JB, Mei J, Zhu JQ, Liu H. The involvement of AQP1 in heart oedema induced by global myocardial ischemia. *Cell Biochem Funct* 2013; 31:60–64.
37. Carter EP, Umenishi F, Matthay MA, Verkman AS. Developmental changes in water permeability across the alveolar barrier in perinatal rabbit lung. *J Clin Invest* 1997; 100:1071–1078.
38. Zolla L, Lupidi G, Bellelli A, Amiconi G. Effect of mercuric ions on human erythrocytes: relationships between hypotonic swelling and cell aggregation. *Biochim Biophys Acta* 1997; 1328:273–280.
39. Eisele K, Lang PA, Kempe DS, et al. Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. *Toxicol Appl Pharmacol* 2006; 210:116–122.
40. Park SR, Jang KW, Park SH, et al. The effect of sonication on simulated osteoarthritis, part I: effects of 1 MHz ultrasound on uptake of hyaluronan into the rabbit synovium. *Ultrasound Med Biol* 2005; 31:1551–1558.
41. Dwight JF, Hendry BM. The effects of tert-butyl hydroperoxide on human erythrocyte membrane ion transport and the protective actions of antioxidants. *Clin Chim Acta* 1996; 249:167–181.
42. Scallan J, Huxley VH, Korthuis RJ. Pathology of edema formation. In: *Capillary Fluid Exchange: Regulation, Functions, and Pathology*. 1st ed. San Rafaele, CA: Morgan & Claypool Life Sciences; 2010:47–62.
43. Papadopoulos MC, Manley GT, Krishna S, Verkman AS. Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. *FASEB J* 2004; 18:1291–1293.