The influence of octyl β-D-glucopyranoside on cell lysis induced by ultrasonic cavitation

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Octyl *β*-D-glucopyranoside (OGP) has been reported to completely inhibit cavitation-induced cell lysis *in vitro*, possibly by quenching critical free-radical effects. In this study, the influence of OGP on cell lysis in a 60 rpm rotating-tube exposure apparatus was assessed. HL-60 cell lysis was estimated with a Coulter Multisizer counter. Cavitation activity from the 2.3 MHz, 30 s duration exposures were monitored at the 1.15 MHz subharmonic. Cavitation nucleation was accomplished by addition of an ultrasound contrast agent, or by using freshly dissolved culture media. For both nucleation methods, exposures were conducted for 0–0.7 MPa peak rarefactional pressure-amplitudes with and without 5 mM OGP, and for 0.5 MPa with 0-5 mM OGP. The addition of OGP to the cell suspension medium generally had little influence on cavitation-induced cell lysis. Exposures with no rotation had reduced subharmonic and lysis for added contrast agent, but essentially no cavitation for the fresh medium. Since the decreases or increases in cell lysis found for added OGP generally were accounted for by concomitant decreases or increases in cavitation activity, the changes in cell lysis could be explained by variation of the mechanical effects of cavitation without invoking a critical role for free-radical effects.
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I. INTRODUCTION

Cellular biological effects of ultrasonic cavitation are conveniently studied with *in vitro* suspensions. This method allows control of many factors, including the ultrasonic exposure, cavitation nucleation, medium composition, and evaluation of cellular bioeffects. Ultrasonic heating can be minimized and specific cavitational bioeffects isolated. The research on this topic has led to a substantial understanding of *in vitro* cellular bioeffects which can arise from both mechanical and sonochemical mechanisms. The literature in this topic is extensive, extending over at least 40 yr and hundreds of published papers (Miller, *et al*., 1996; NCRP, 2002).

Nucleation of the cavitation is a key factor for *in vitro* bioeffects research, which is determined by the exposure system and the composition of the medium. Typically, no effects are seen without cavitation. The rarefactional pressure amplitude (RPA) must exceed the cavitation threshold for the available nuclei population, which is often highly variable. The nucleation problem is particularly acute for sterile cell culture media in which the sterilization process tends to eliminate cavitation nuclei. For research at moderate RPAs relevant to medical applications, special exposure systems have been developed (Miller *et al*., 1996). One common scheme utilizes an ultrasound source aimed upward in a water bath at a vessel, such as a vial or well in a tissue culture plate, which contains the cell suspension with a free air–water interface. This vertical beam system can lead to surface agitation and nucleation of cavitation by entrainment of microbubbles in the suspension (Chen *et al*., 2004). An alternative scheme is a vertically oriented tube exposed in a water bath from the side with a horizontal beam of ultrasound. This system has the advantage of well-defined free-field acoustical conditions (depending on the tube material) by eliminating the air–water interface. Rotation of the tube promotes and maintains cavitation activity, even for low cavitation nucleation, by recycling cavitation microbubbles back into the medium with each revolution (Miller and Williams, 1989).

The mechanisms of *in vitro* cavitational bioeffects broadly include the direct mechanical perturbation of cells by cavities and the indirect activity of free radicals and sonochemicals, which are produced by inertial cavitation (NCRP, 2002). Mechanical perturbation induces effects such as sonoporation, membrane damage, cell lysis, and fragmentation. Free radicals and sonochemicals induce secondary bioeffects such as deoxyribonucleic acid (DNA) damage in surviving cells (free-radical or sonochemical effects might also occur in mechanically disrupted, dead cells but would be of no biological consequence) (WFUMB, 1998). For example, the sonochemical hydrogen peroxide (H_2O_2) generated in the rotating tube exposure system could produce DNA single strand breaks in surviving cells (Miller *et al*., 1991). However, the duration of exposure in the rotating tube system, which was sufficient to generate an effective H_2O_2 concentration, was sufficient to mechanically disrupt virtually all of the cells (Miller *et al*., 1995). Apparently, H_2O_2 produced at inertial cavities could migrate to effect surviving distant cells, or effect fresh cells added to a previously exposed medium. The direct mechanical action of cavitation has appeared to be the dominant mechanism of *in vitro* bioeffects on cell suspensions in several different studies (Fu *et*

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al., 1979; Kondo and Kano, 1988; Kondo *et al*., 1989; Miller *et al*., 1995; Worthington *et al*., 1997; Hiraoka *et al*., 2006).

The use of additives to the cell suspension in cellular bioeffects studies provides for rich but complex research opportunities. An additive might enhance or reduce the cavitation nucleation, the overall cavitation activity, the action of mechanical or sonochemical mechanisms, or the cellular sensitivity to the mechanisms. For any system operating in the frequency range of medical ultrasound, an ultrasound contrast agent can be used to augment the cavitation activity. The stabilized gas bodies in these agents can serve as optimum cavitation nuclei (e. g., Miller and Thomas, 1995). Because these injectable agents are sterile and uniform from one vial to the next, the cavitation activity can be regulated and reproducible even in sterile cell cultures.

Various chemical additives are capable of enhancing the bioeffects of cavitation, possibly by enhancing the sonochemical mechanism, including dimethlyformamide (Jeffers *et al*., 1995) and hematophorphyrin (Yumita *et al*., 1996). Free-radical intermediates have been suggested to be the effective agents, which may persist long enough to reach cell membranes (Misík and Riesz, 2000). Synergistic enhancement of cavitational bioeffects has been proposed as a means of "sonodynamic therapy" (Umemura *et al*., 1989; Rosenthal *et al*., 2004). Recently, the complete inhibition of ultrasound-induced cell lysis was reported with the addition of mild surfactants to the cell suspension (Sostaric *et al*., 2005). That research utilized a vertical beam exposure system designed for sonochemistry research (Sostaric *et al*., 2008). Suspensions of HL-60 cells (a human leukemia-derived cell line grown in suspension culture) were exposed to 1.057 MHz unfocused ultrasound for 5–15 s with various additions of alkyl glucopyranosides. For example, 2 mM octyl β-D-glucopyranoside (OGP) added to the medium resulted in 100% survival of the cells after 5 s exposure under conditions which produced 35%–100% cell lysis without the additive. In separate tests, using mechanical shaking (not ultrasound), the glucopyranosides could not protect the cells from mechanical shear stress. Separate experiments with argon-bubbled media indicated that the cavitation occurred for exposure with or without the glucopyranoside additives. The protective effect was thought to be due to accumulation of the surfactants at the cavity surface, leading to reduced production of reactive oxygen species (Sostaric *et al*., 2005; Cheng and Riesz, 2007). This quenching of cytotoxic radicals was proposed to be responsible for the elimination of the cell lysis, an hypothesis which assumes an exceptionally large role for free-radical effects (rather than mechanical effects) in the production of cell lysis.

In the present study the influence of OGP on cell lysis induced by ultrasonic cavitation was investigated using the rotating tube exposure system. The HL-60 cell line was used in suspensions with natural nucleation or with nucleation augmented by ultrasound contrast agent gas bodies. Continuous 2.3 MHz ultrasound exposure was applied for 30 s with monitoring of acoustic emissions from the tube to gauge the level of cavitation activity during each exposure. The cell suspension was evaluated post-exposure for cell lysis. In this exposure system, cavitation-induced cell lysis was not eliminated by the addition of OGP. However, small variations in cell lysis were detected for some conditions with surfactant added, which appeared to be the result of concomitant variations in cavitation activity.

II. MATERIAL AND METHODS

A. Ultrasound exposure system

The ultrasound exposure system has been described previously (Miller and Williams, 1989). Briefly, 1.6 cm diameter tubes were made of acoustically transparent plastic film (Saran Wrap, S. C. Johnson & Son, Racine, WI) and fitted with a plastic plug at each end. The top plug had a filling hole and was attached to a vertical holder. The bottom plug had a small magnet opposite another magnet in the base of the assembly to aid in maintaining the vertical axis of the flexible tube. The tube was 2.5 cm long and contained about 5 ml. The holder was motorized and was rotated around the tube axis at 60 rpm for most exposures. The rotating tube assembly was placed in a water bath maintained at 25 °C. This cell suspension exposure system was used because it allows for accurate field characterization of the free-field ultrasound beam, together with maintenance of cavitation activity by the rotation of the tube.

An air-backed 1.9 cm diameter ultrasound transducer was aimed horizontally at the tube from a distance of 12.5 cm. The transducer was energized by a signal from a function generator (model 3314A, Hewlett-Packard Co., Palo Alto, CA) set to generate a 2.3 MHz continuous sine wave, which was amplified (A-500, Electronic Navigation Industries, Rochester, NY). The 30 s duration exposures were controlled by a manual switch. The ultrasound field was measured at the tube position with a membrane hydrophone (model 805 PVDF Bilaminar Membrane hydrophone, Sonora Medical Systems, Longmont, CO). Desired RPAs were obtained by setting the suitable voltage on the function generator. The −6 dB beam width (half-pressure diameter) was 7.8 mm.

To provide a measure of cavitation activity during exposure, a 1.9 cm diameter receiving transducer (Panametrics model A314S-SU, Olympus NDT, Waltham, MA) was aimed at right angle to the exposure beam. The 3.7 cm focus of the receiver was placed at the center of the tube. The −6 dB beam width at the focus was 5.6 mm (based on transmit measurements), which limited reception of cavitation emissions to the portion of the 7.8 mm diameter exposure beam at the center of the 1.6 cm diameter tube. The received signal was analyzed with a spectrum analyzer (model 89410A, Hewlett Packard, Everett, WA). The analyzer was manually switched on to collect the root-mean-square average spectrum of the signal, and the final reading at 1.15 MHz (the one-half subharmonic signal) was recorded. The background level for no cavitation was 7.2–7.6 μV. The subharmonic signal is a useful gauge of cavitation activity and associated biological effects (e.g., Morton *et al*., 1983; Miller and Bao, 1998).

B. Cell suspension

The HL-60 human leukemia-derived cell line (ATCC, Rockville, MD) was used for this study. The cells were thawed from frozen storage and cultured in Iscove's

modified Dulbecco's medium (IMDM) with glutamine and antibiotics (ATTC, Mannasa, VA) supplemented with 20% fetal bovine serum in a humidified incubator at 37 °C with 5% CO_2 in air. These cells did not attach to the culture flasks, which minimizes the impact of harvesting and suspension on the cells (relative to monolayer cells). For exposure, cells were harvested and resuspended at 0.5×10^6 cells/ml in culture medium. A 5 ml sample was made up of 4.5 ml of suspension plus 0.5 ml of sterile saline with or without surfactant.

Two cavitation nucleation strategies were used. First, Definity® (perflutren lipid microsphere injectable suspension, Bristol Myers Squib Medical Imaging, N. Billerica, MA) ultrasound contrast agent was added to the suspension (5 μl into 5 ml) made up using sterile prepared IMDM (Gibco IMDM #12440, Invitrogen, Grand Island, NY) cell culture medium. Definity contained lipid-stabilized microbubbles of octafluoropropane gas with a mean diameter range of 1.1–3.3 μm and a concentration up to 1.2×10^{10} microbubbles/ml. which served as artificial (i.e., deliberately engineered) cavitation nuclei. The octafluoropropane gas may have reduced the relative magnitude of the free-radical mechanism for bioeffects, because it may have reduced the cavitation collapse temperature relative to air. The relative collapse temperature for different gases depends on unity minus the ratio of specific heats for the gas (Leighton, 1994). The ratio of specific heats is, for example, about 1.7 for argon, 1.4 for air, and even less for complex gases such as octafluoropropane (it is about 1.13 for propane) (Kutz, 2006).

Second, suspensions were made up without Definity using freshly prepared culture medium from dry powder IMDM (17633, Sigma-Aldrich, St. Louis, MO) at the recommended concentration with the *p*H adjusted with NaOH. Sterilization of culture media by autoclave appears to completely eliminate cavitation nuclei under the conditions of this experiment. The fresh medium, which was essentially identical to the sterile medium aside from the omission sterilization step, was used because it contained "natural" cavitation nuclei for comparison to the "artificial" cavitation nuclei from the contrast agent.

The surfactant OGP (O8001, Sigma-Aldrich, St. Louis, MO) was added from a 50 mM stock solution in saline. This additive changed the surface tension of the IMDM medium. The surface tension was measured with a DuNouy Tensiometer (CSC Scientific Co., Fairfax, VA) and found to be 72 dyn/cm for water, and 73, 59, 51, 43, and 38 dyn/cm for IMDM with 0, 1, 2, 5, or 10 mM OGP, respectively.

After exposure, the suspension was removed from the tube and stored on ice. The cell lysis was characterized by evaluating the suspensions with a Coulter cell counter (Multisizer 3, Beckman Coulter, Fullerton, CA). The suspension was diluted by adding 0.5 ml in 10 ml saline. The total count of intact cells was obtained within the 10–15 μm size range. The Coulter counter method was used to achieve greater precision in the cell counts than was possible for manual counting with a hemacytometer, and was similar to the method used by Sostaric *et al.* (2005). The percentage of cell lysis (L) in a suspension was calculated from the Coulter counts for sham C_S and exposed C_E suspensions as

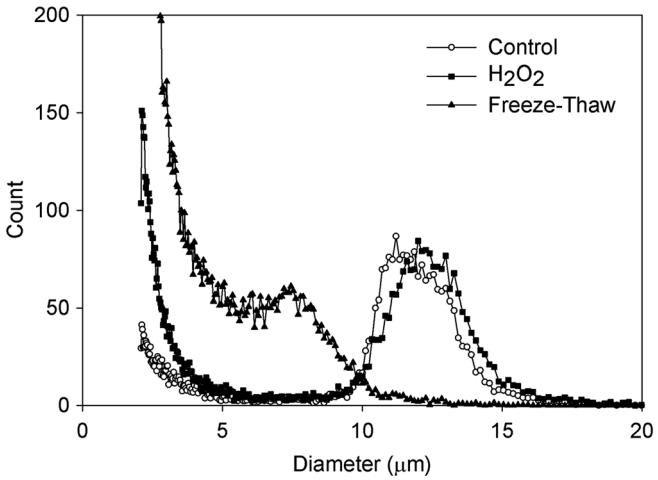

FIG. 1. Plots generated from Coulter Multisizer data for preliminary testing of the method. The control was a normal cell suspension while the treated suspensions had 3% added H_2O_2 or were frozen and thawed. The cells killed (i.e., stained by trypan blue) with H_2O_2 remained in the same size range of 10–15 μm as the controls. However, the cells lysed (also, stained by trypan blue) by mechanical disruption shifted to a smaller Coulter counter size range.

$$L = 100\left(1 - \frac{C_E}{C_S}\right). \quad (1)$$

This procedure was evaluated by comparison to the trypan blue dye exclusion test in preliminary experiments of cells killed by freezing and thawing the suspension or by addition of 3% H_2O_2. Examples of the Coulter counter results are shown in Fig. 1. The controls had no missing cells (by definition) by the Coulter method, but $3.4 \pm 1.6\%$ lysis (blue stained) by the trypan blue method. The freeze–thaw test, which mechanically kills the cells by ice crystal formation, had $88.7 \pm 11.6\%$ cell lysis by Coulter counting and $93.9 \pm 6.1\%$ lysis by the trypan blue test (stained plus missing cells). In contrast, the H_2O_2 treated cells had $5.5 \pm 10.9\%$ lysis by the Coulter counter method, and $99.8 \pm 0.6\%$ lysis by the trypan blue method. Similar disparate results were obtained for cells killed by heating (70 °C for 15 min) or by alcohol (70% ethanol), apparently because these dead cells remain intact and were counted in the normal size range. Because the 30 s ultrasound exposures would not produce lethal H_2O_2 concentrations or heating, the Coulter method was used to approximately assess cell lysis in this study (see Sec. III for an example of the Coulter results for sonicated suspensions). However, it should be noted that this method may underestimate cell lysis due to a few dead cells being counted as normal. This method (and the trypan blue method) would also not count other lethally injured cells, which die later by necrosis or apoptosis and require more elaborate methods for assessment (e.g., Miller and Dou, 2009).

C. Experimental plan and statistics

This study was planned to test the influence of OGP on cell lysis in the rotating tube system obtained with the two strategies of cavitation nucleation. The exposure response

was explored using 0 or 5 mM OGP for sham, 0.25, 0.35, 0.5, and 0.7 MPa RPAs. The OGP dose effect was tested using 0.5 MPa exposure with 0, 1, 2, and 5 mM. A concentration of 10 mM was also tried, but this produced a large fraction of dead cells (by the trypan blue test) even for sham exposure. In addition, the effect of not rotating the tube was tested for no OGP at 0.5 MPa, and for 5 mM OGP at 0.5 and 0.7 MPa exposure. Ten test repetitions were performed for each condition. Final results are given as the mean and standard deviation, or plotted as the mean with standard error bars. Statistical comparisons were made using the Student's t-test or Mann–Whitney rank sum test as appropriate. In addition, conditions, which produced hit-or-miss effects, were evaluated with the Z test for proportions.

III. RESULTS

The cell lysis induced by the different RPA exposures with Definity nucleation are shown in Fig. 2 (top panel) together with the mean subharmonic signals (bottom panel). The cell lysis and subharmonic show similar exposure-response trends. No hit-or-miss behavior, which can occur for poor nucleation, was evident. Judging by the trend in the data, the cell lysis would be expected to reach zero for exposure at about 0.2 MPa, but this RPA was not used. The addition of 5 mM OGP had little effect on the results except for the 0.25 MPa exposures for which the cell lysis ($P < 0.01$) and subharmonic ($P < 0.02$) were both reduced by the addition of the surfactant. The lack of variation of the cell lysis and subharmonic for 0.5 MPa exposure with different OGP concentrations is illustrated in Fig. 3.

The results were much more variable for nucleation from the fresh IMDM. The cell lysis induced by the different RPA exposures is shown in Fig. 4 (top panel) together with the mean subharmonic signals (bottom panel). Again, the trends in the subharmonic and the cell lysis were quite similar. The subharmonic was significantly reduced for added OGP at 0.5 MPa ($P < 0.05$) and 0.7 MPa ($P < 0.02$). However, the reduction in cell lysis was not statistically significant for any of the tested RPAs. The result with fresh IMDM tended to be hit-or-miss. Examples of the Coulter counter results for sham and exposure at 0.5 MPa with and without OGP are presented in Fig. 5. For these single exposure examples, the exposure without OGP produced substantial cell lysis, but the exposure example with OGP was virtually the same as the shams (other exposures with OGP did induce substantial cell lysis). The proportion of the exposures for which cavitation activity was detected (arbitrarily assumed for the subharmonic signal greater than 200 μV) varied with MPa. For 10 exposures at each condition, 0, 4, 9, and 10 had cavitation without and 0, 3, 4, and 8 had cavitation with OGP, for 0.25, 0.35, 0.5, and 0.7 MPa, respectively. The proportion of cavitation occurrence at 0.5 MPa was statistically significantly less with OGP (4/10 compared to 9/10, $P < 0.02$ for the Z test of proportions). However, the mean cell lysis was not significantly reduced owing to substantial cell lysis when cavitation did occur, such that cell lysis was $64 \pm 4\%$ ($n = 4$) with, or $43 \pm 14\%$ ($n = 9$) without added OGP. The results for varied concentrations of OGP showed a substantial influence on cell lysis for added surfactant, as

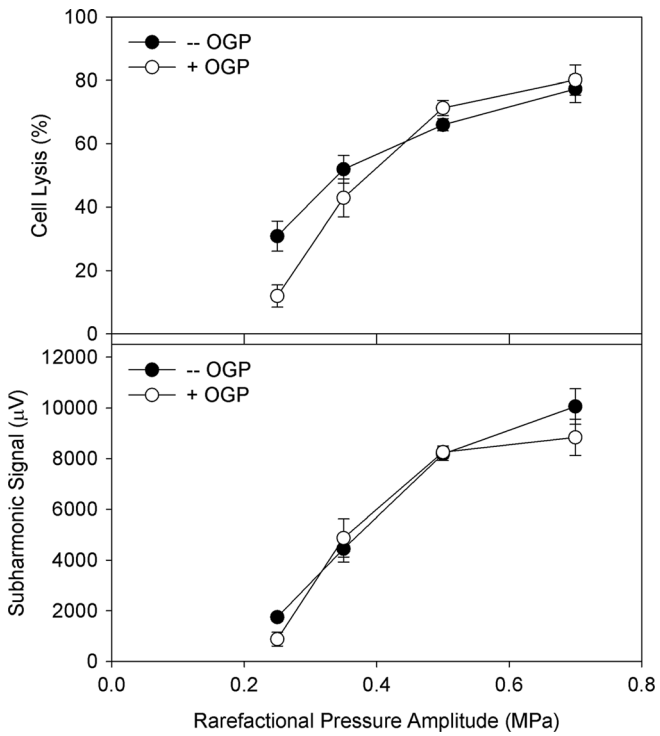

FIG. 2. Plots of the cell lysis (upper panel) and average subharmonic signal (lower panel) during exposure at a range of RPAs with added ultrasound contrast agent. The sham treatment (0 MPa) had zero cell lysis by definition [Eq. (1)]. The suspensions had additions of saline (−OGP) or 5 mM OGP (+OGP). The cavitation activity indicated by the subharmonic and the cell lysis was reduced significantly for added OGP at 0.25 MPa.

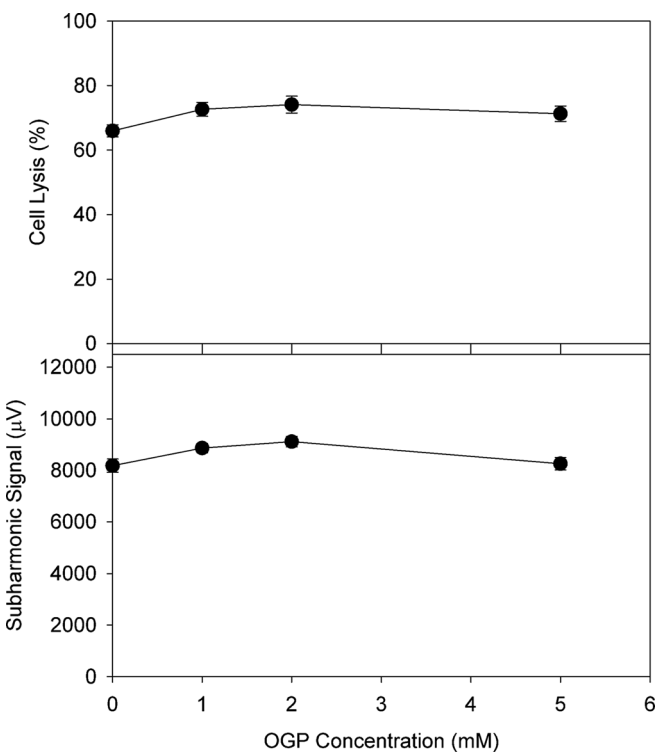

FIG. 3. Cell lysis (upper panel) and average subharmonic signal (lower panel) during exposure at 0.5 MPa for a range of OGP concentrations and added contrast agent. There was no significant effect of OGP addition.

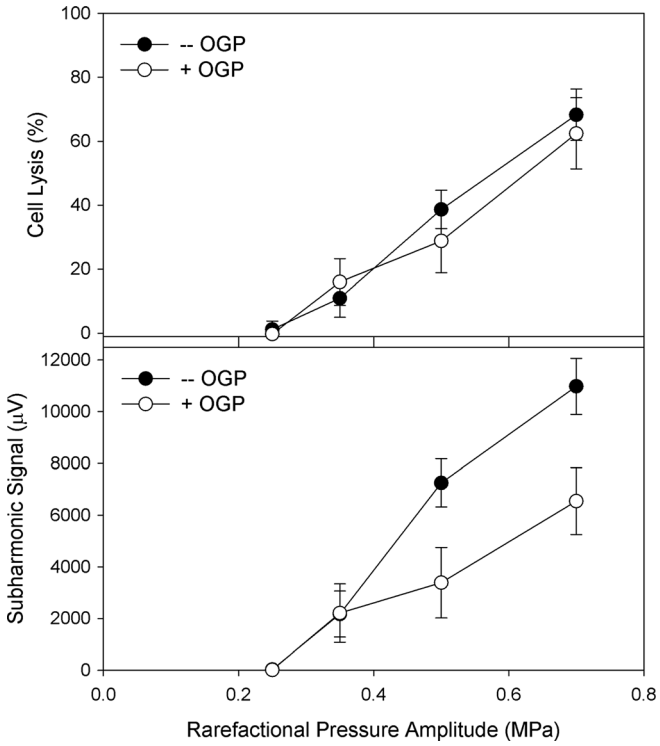

FIG. 4. Cell lysis (upper panel) and average subharmonic signal (lower panel) during exposure at a range of RPAs as for Fig. 2, but with nucleation by fresh culture medium. The sham treatment (0 MPa) had zero cell lysis by definition [Eq. (1)]. The suspensions had additions of saline (−OGP) or 5 mM OGP (+OGP). For the two highest megapascals, the mean cavitation activity indicated by the subharmonic signal was reduced, but the reduction in cell lysis was not significant for OGP addition.

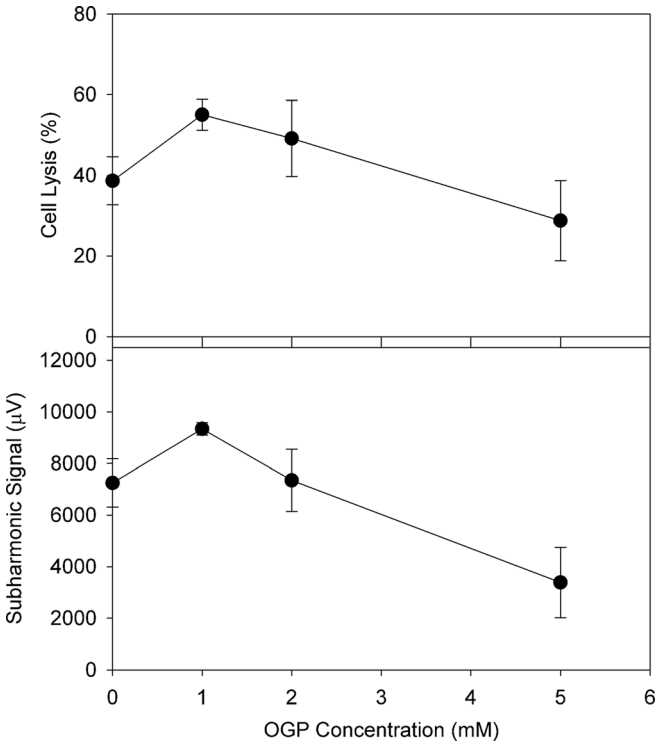

FIG. 6. Plots of the cell lysis (upper panel) and average subharmonic signal (lower panel) during exposure at 0.5 MPa for a range of OGP concentrations, as in Fig. 3, but with fresh culture medium. Addition of increasing concentrations of OGP initially significantly increased the cavitation activity and cell lysis, and then decreased them for the highest concentrations.

shown in Fig. 6. The subharmonic increased for 1 mM OGP ($P < 0.05$) and then decreased for 5 mM OGP ($P < 0.001$). Similarly, the cell lysis increased for 1 mM OGP ($P < 0.02$) and then decreased (not significant).

Tests were also performed for a stationary tube (not rotated during exposure) and the results are presented in Table I. Exposures with Definity nucleation at 0.5 MPa produced significantly less subharmonic signal and cell lysis with OGP (5 mM). For nucleation supplied by fresh IMDM, the cell lysis was not significantly different from shams with or without added OGP. The non-rotating condition was redone for fresh IMDM at 0.7 MPa, but again there were no significant differences in cell lysis, even though a significant decrease was detected with OGP for the subharmonic signal. These results illustrate both the excellent ability of Definity to nucleate cavitation activity and the importance of the tube rotation (for comparison see Figs. 2 and 4), especially for media with low nucleation.

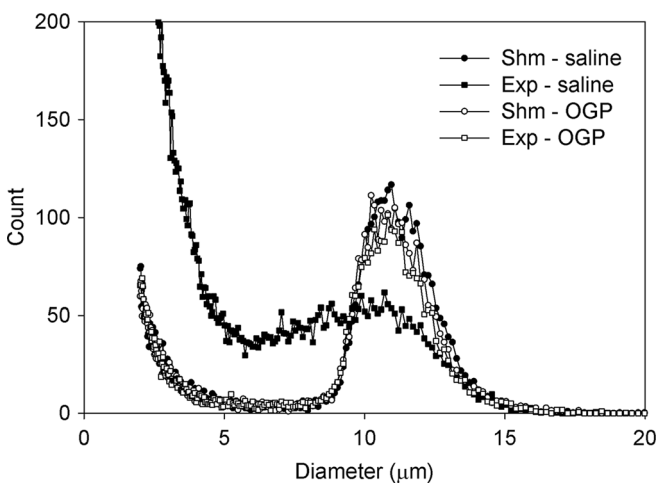

FIG. 5. Examples of the Coulter Multisizer histograms for single exposures at 0.5 MPa with fresh culture medium. For saline, the shift in cell size range was indicative of substantial cell lysis, while no lysis was evident for added OGP. This hit-or-miss behavior occurred with or without OGP, and other exposures (4 of 10) with added OGP had substantial cell lysis.

IV. DISCUSSION AND CONCLUSIONS

Cell suspensions were exposed to continuous wave ultrasound in a rotating tube apparatus to assess the influence of the surfactant OGP on cavitation and cell lysis. Cell lysis was estimated from counts with a Coulter Multisizer. Cavitation activity from the 2.3 MHz exposure was monitored at the 1.15 MHz subharmonic. For nucleation with ultrasound contrast agent, cell lysis increased steadily from 0.25 to 0.7 MPa (Fig. 2). OGP produced a significant reduction in cell lysis and in the subharmonic signal at 0.25 MPa, but not at higher megapascals. For 0.5 MPa exposure, there was no trend with OGP concentration from 0 to 5 mM (Fig. 3). However, if the tube was not rotated, a significant reduction was produced by OGP in cell lysis and the subharmonic signal (Table I).

For nucleation by freshly dissolved culture media, the 0.25 MPa exposure appeared to be at a threshold for

TABLE I. Results for subharmonic signals and cell lysis for a stationary (non-rotating) tube with and without 5 mM OGP.[a]

Nucleation	Additive	RPA (MPa)	Subharmonic (μV)	P	Lysis (%)	P
Definity	Saline	0.5	3 940 ± 1 560	< 0.01	59.2 ± 5.7	< 0.05
Definity	OGP	0.5	1 643 ± 1 390		29.4 ± 30.6	
Fresh IMDM	Saline	0.5	109 ± 78	NS	2.8 ± 11.7	NS
Fresh IMDM	OGP	0.5	75 ± 76		− 0.4 ± 10.4	
Fresh IMDM	Saline	0.7	601 ± 424	< 0.001	4.9 ± 7.8	NS
Fresh IMDM	OGP	0.7	117 ± 90		8.4 ± 13.9	

[a]Mean values are given with standard deviations. P values are given for significantly different results or NS for no significant difference.

cavitation (Fig. 4). Significant reductions in subharmonic occurred for 0.5 and 0.7 MPa exposures, but the small reduction in cell lysis was not significant. The cavitation activity was hit-or-miss for this nucleation strategy. This behavior is common for cavitation bioeffects research, due to low populations of cavitation nuclei, and was previously noted for a rotating chamber exposure system (Miller and Williams, 1992). At 0.5 MPa, cavitation occurred significantly less often (4/10) when OGP was added to the medium than when it was not added (9/10). However, the mean cell lysis was not significantly reduced, because substantial cell lysis occurred with added OGP when cavitation did occur. Variation of the concentration of OGP for 0.5 MPa exposure produced increased cavitation and lysis at 1 mM relative to 0 mM, but decreased cavitation at 5 mM (Fig. 6). If the tube was not rotated, no significant cell lysis occurred for 0.5 or 0.7 MPa (Table I).

These results are different from those of Sostaric *et al.* (2005). In that study, cavitation-induced cell lysis was completely inhibited by a concentration of about 2 mM OGP. This inhibition was attributed to a possible reduction in free-radical effects. In this study, small variations of cell lysis with added OGP were obtained under some conditions, but not others. In Figs. 2 and 3, 4 and 6, the cell lysis bioeffect was mirrored by the level of cavitation activity detected by the subharmonic signal. Furthermore, strong free-radical effects do not seem to be required for cell lysis, since the Definity nucleation was very effective, even though the free-radical mechanism might be expected to be reduced by the octafluoropropane filling gas. For ultrasound exposure, cell counts were shifted out of the normal range into smaller Coulter sizes (Fig. 5). This shift was similar to that for mechanically disrupted cells treated by freezing and thawing (Fig. 1), except freezing and thawing lysed essentially all the cells. This suggests that the cell lysis was caused by the mechanical action of cavitation.

Surfactants were expected to have several effects on ultrasonic cavitation activity. Air trapped in cracks or pits in dry microscopic particles can serve as cavitation nuclei (Leighton, 1994). Surfactants act as a wetting agents, which could lead to the modification or reduction of such nuclei and an increase in the cavitation threshold for some conditions (Crum, 1979). This consideration may explain the reduced number of times cavitation occurred for the fresh IMDM nucleation strategy. In addition, the surface tension is a critical factor in cavity dynamics (Leighton, 1994). The reduction of the surface tension from 73 to 43 dyn/cm for 0–5 mM OGP would have an appreciable effect on the pulsation of the cavities. The changes in surface tension would also likely have an influence on cavity stability, cavity break up. or bubble coalescence. For example, reducing the surface tension increased cavitation-microbubble dissolution times (Porter *et al.*, 2004), which might be expected to increase cavitation for the rotating tube exposure system. A slight increase in both the cavitation activity and cell lysis was noted for 1 mM OGP in Fig. 6, but not for higher concentrations.

In this study, addition of glucopyranoside to the cell suspension medium had very little influence on cavitation-induced cell lysis. This general result does not support a strong role for the free-radical mechanisms in the cavitational cell lysis reported here. For two conditions, cell lysis was reduced (Fig. 2, Table I), and for another cell lysis was increased (Fig. 6). For these conditions, the decrease or increase in cell lysis was accounted for by a concomitant decrease or increase in cavitation activity. Thus, the changes could be explained by variation of the mechanical effects of cavitation without invoking the critical free-radical effects. The contrast of this general result with that of Sostaric *et al.* (2005), which was the complete inhibition of cell lysis by glucopyranosides, is puzzling. The frequency was 2.3 MHz in this study compared to 1.057 MHz in Sostaric *et al.* (2005). However, the protective effect of OGP was found to be stronger for higher frequencies, for example, for 1.057 MHz compared to 0.354 MHz (Sostaric *et al.*, 2008). Given the complex processes involved in *in vitro* cell lysis by cavitation, the exact role of added OGP is uncertain. Possibly, the differences in results between this study and that of Sostaric *et al.* (2005) can be attributed to the different behavior of cavitation for added surfactant in the two very different exposure systems.

ACKNOWLEDGMENT

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Chen, W. S., Lu, X., Liu, Y., and Zhong, P. (**2004**). "The effect of surface agitation on ultrasound-mediated gene transfer in vitro," J Acoust Soc Am. **116**, 2440–2450.

Cheng, J. Y., and Riesz, P. (**2007**). "Mechanism of the protective effects of long chain n-alkyl glucopyranosides against ultrasound-induced cytolysis of HL-60 cells," Ultrason Sonochem. **14**, 667–671.

Crum, L. A. (**1979**). "Tensile strength of water," Nature (London) **278**, 148–149.

Fu, Y. K., Kaufman, G. E., Miller, M. W., Griffiths, T. D., and Lange, C. S. (**1979**). "Modification by cysteamine of ultrasound lethality to Chinese hamster V-79 cells," Radiat. Res. **80**, 575–580.

Hiraoka, W., Honda, H., Feril, L. B., Jr., Kudo, N., and Kondo, T. (**2006**). "Comparison between sonodynamic effect and photodynamic effect with

photosensitizers on free radical formation and cell killing," Ultrason Sonochem. **13**, 535–542.
Jeffers, R. J., Feng, R. Q., Fowlkes, J. B., Hunt, J. W., Kessel, D., and Cain, C. A. (**1995**). "Dimethylformamide as an enhancer of cavitation-induced cell lysis in vitro," J. Acoust. Soc. Am. **97**, 669–676.
Kondo, T., Fukushima, Y., Kon, H., and Riesz, P. (**1989**). "Effect of shear stress and free radicals induced by ultrasound on erythrocytes," Arch. Biochem. Biophys. **269**, 381–389.
Kondo, T., and Kano, E. (**1988**). "Effect of free radicals induced by ultrasonic cavitation on cell killing," Int. J. Radiat. Biol. **54**, 475–486.
Kutz, M. (**2006**). "Energy and Power," in *Mechanical Engineers' Handbook*, 3rd ed., edited by M. Kutz (Wiley, Hoboken, NJ.), Table 22.
Leighton, T. G. (**1994**). *The Acoustic Bubble* (Academic, London), Chap. 2, pp. 67–128.
Miller, D. L., and Bao, S. (**1998**). "The relationship of scattered subharmonic, 3.3-MHz fundamental and second harmonic signals to damage of monolayer cells by ultrasonically activated Albunex," J. Acoust. Soc. Am. **103**, 1183–1189.
Miller, D. L., and Dou, C. (**2009**). "Induction of apoptosis in sonoporation and ultrasonic gene transfer," Ultrasound Med. Biol. **35**, 144–154.
Miller, D. L., and Thomas, R. M. (**1995**). "Ultrasound contrast agents nucleate inertial cavitation in vitro," Ultrasound Med. Biol. **21**, 1059–1065.
Miller, D. L., Thomas, R. M., and Buschbom, R. L. (**1995**). "Comet assay reveals DNA strand breaks induced by ultrasonic cavitation in vitro," Ultrasound Med. Biol. **21**, 841–848.
Miller, D. L., Thomas, R. M., and Frazier, M. E. (**1991**). "Ultrasonic cavitation indirectly induced single strand breaks in DNA of viable cells in vitro by the action of residual hydrogen peroxide," Ultrasound Med. Biol. **17**, 729–735.
Miller, D. L., and Williams, A. R. (**1989**). "Bubble cycling as the explanation of the promotion of ultrasonic cavitation in a rotating tube exposure system," Ultrasound Med. Biol. **15**, 641–648.
Miller, D. L., and Williams, A. R. (**1992**). "Nucleation and evolution of ultrasonic cavitation in a rotating exposure chamber," J. Ultrasound Med. **11**, 407–412.
Miller, M. W., Miller, D. L., and Brayman, A. A. (**1996**). "A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective," Ultrasound Med. Biol. **22**, 1131–1154.
Misík, V., and Riesz, P. (**2000**). "Free radical intermediates in sonodynamic therapy," Ann. N.Y. Acad Sci. **899**, 335–348.
Morton, K. I., Ter Haar, G. R., Stratford, I. J., and Hill, C. R. (**1983**). "Subharmonic emission as an indicator of ultrasonically-induced biological damage," Ultrasound Med. Biol. **9**, 629–633.
NCRP (**2002**). "Exposure Criteria for Medical Diagnostic Ultrasound: II. Criteria Based on All Known Mechanisms," Report No. 140 (National Council on Radiation Protection and Measurements, Bethesda, MD), Chap.7, pp. 171–212.
Porter, T. M., Crum, L. A., Stayton, P. S., and Hoffman, A. S. (**2004**). "Effect of polymer surface activity on cavitation nuclei stability against dissolution," J. Acoust. Soc. Am. **116**, 721–728.
Rosenthal, I., Sostaric, J. Z., and Riesz, P. (**2004**). "Sonodynamic therapy—A review of the synergistic effects of drugs and ultrasound," Ultrason. Sonochem. **11**, 349–363.
Sostaric, J. Z., Miyoshi, N., Cheng, J. Y., and Riesz, P. (**2008**). "Dynamic adsorption properties of n-alkyl glucopyranosides determine their ability to inhibit cytolysis mediated by acoustic cavitation," J. Phys. Chem B **112**, 12703–12709.
Sostaric, J. Z., Miyoshi, N., Riesz, P., DeGraff, W. G., and Mitchell, J. B. (**2005**). "n-Alkyl glucopyranosides completely inhibit ultrasound-induced cytolysis," Free Radic. Biol. Med. **39**, 1539–1548.
Umemura, S., Yumita, N., Nishigaki, R., and Umemura, K. (**1989**). "Sonochemical activation of hematoporphyrin: A potential modality for cancer treatment," Proc. IEEE Ultrason. Symp. 1989, Vol. 2, pp. 955–960.
WFUMB. (**1998**). "WFUMB symposium on safety of ultrasound in medicine," Ultrasound Med. Biol. **24**, S29–S39.
Worthington, A. E., Thompson, J., Rauth, A. M., and Hunt, J. W. (**1997**). "Mechanism of ultrasound enhanced porphyrin cytotoxicity. Part I: A search for free radical effects," Ultrasound Med. Biol. **23**, 1095–1105.
Yumita, N., Umemura, S., Magario, N., Umemura, K., and Nishigaki, R. (**1996**). "Membrane lipid peroxidation as a mechanism of sonodynamically induced erythrocyte lysis," Int. J. Radiat. Biol. **69**, 397–404.