European Journal of Obstetrics & Gynecology and Reproductive Biology 193 (2015) 96–101

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Combined ultrasound-curcumin treatment of human cervical cancer cells

Kaylene R. Carr [a], Yevgeniya J. Ioffe [a], Maria Filippova [b], Penelope Duerksen-Hughes [b], Philip J. Chan [a,*]

[a] Department of Gynecology and Obstetrics, Loma Linda University School of Medicine, Loma Linda, CA 92354, USA
[b] Department of Basic Sciences, Center for Health Disparities and Molecular Medicine, Loma Linda University School of Medicine, Loma Linda, CA 92354, USA

ARTICLE INFO

Article history:
Received 23 December 2014
Received in revised form 13 July 2015
Accepted 23 July 2015

Keywords:
HPV
Ultrasound
Curcumin
Cervical cancer
HeLa
SiHa

ABSTRACT

Objectives: Human papillomavirus (HPV) is associated with cervical cancer. Studies showed curcumin inhibits HPV oncogenes expression but curcumin has low bioavailability. The objectives were: (1) to study ultrasound enhancement of curcumin effects on HeLa, SiHa and C33A, (2) to compare two frequencies for sonoporation and (3) to detect cell-free DNA released by the treatment.

Study design: HeLa, SiHa and C33A cells (non-HPV control) were processed and exposed to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz ultrasound, (4) control medium, or (5) 8 s of 7.5 MHz ultrasound. The five treated groups were incubated (48 h) and analyzed by dual fluorescence apoptosis/necrosis assay. DNA in spent media was analyzed by capillary analysis.

Results: Combined curcumin ultrasound resulted in 9-, 12- and 16-fold higher necrosis in HeLa, SiHa and C33A cells respectively. Increased necrosis correlated with higher ultrasound frequencies. There was increased apoptosis in HeLa or SiHa cells with the combined treatment. Curcumin alone resulted in a lesser 2–4-fold increase in necrosis in the groups. Cell-free DNA was detected in the spent media of HeLa and SiHa but not C33A cultures.

Conclusions: The results showed enhanced necrosis in cervical carcinoma cell lines after combined treatment and confirmed the ultrasound capacity to increase effectiveness of curcumin. Cancer cells were smaller post-treatment suggesting microtubule structural disruption. Cell-free DNA was low molecular weight consistent with lysed host cell.

Introduction

Cervical cancer is the fourth most common cause of death in women worldwide [1]. In 2013, 12,340 cases of invasive cervical cancer occurred in the United States with 4030 deaths [2,3]. With the advent of Cervarix® and Gardasil® vaccines [4], there has been a move to eradicate HPV type 16 and 18 infections which are the responsible for at least 70% of high grade squamous intraepithelial dysplastic lesions (HSIL) and frankly invasive carcinomas worldwide [5]. However, these vaccines are preventative only and do not treat pre-existing disease. There remains a need for a less-invasive treatment for cervical lesions.

Curcumin (diferulolyl methane) is a yellow pigment from the spice Tumeric (Curcuma longa linn) with antitumor, anti-inflammatory [8], anti-viral and microbicidal properties [6–13]. Treatment of HPV-infected cells from cervical carcinoma and high grade squamous intraepithelial lesions (HSIL) with curcumin inhibits the expression of a key oncogene known as HPV-E7 and restores cell cycle regulators including p53 and retinoblastoma protein (Rb) [11]. Increased expression of Rb and p53 has been linked to regression of cervical cancer [14–16]. While curcumin has promising anti-cancer properties, its clinical use is limited by its low bioavailability [17,18]. A variety of mechanisms, including nanoparticles [19] and micelles [20], have been studied to enhance cellular uptake of curcumin thereby increasing bioavailability. Ultrasound can increase uptake through increased permeability [21]. In one study of nasopharyngeal cancer, combined ultrasound and curcumin increased cytotoxicity resulting in membrane blebbing, microvillin disappearance, swollen mitochondria, and chromatin condensation [22].

Recently, vaginal curcumin cream became available but cell absorption was problematic [23]. The present study goal was to improve the efficacy and uptake of directly-applied curcumin into

* Corresponding author. Tel.: +1 909 5582851; fax: +1 909 5582450.
E-mail address: pchann@yahoo.com (P.J. Chan).

http://dx.doi.org/10.1016/j.ejogrb.2015.07.011
0301-2115/

cells using sonoporation. The objectives were: (1) to determine the effects of high frequency ultrasound on curcumin exposed cervical cancer cells, (2) to compare two different frequencies used in ultrasound-mediated sonoporation and (3) to study the release of cell-free DNA from the treated cells.

Materials and methods

Culture of cells

HeLa (containing HPV-18 integrated at chromosome 8), SiHa (HPV-16 integrated at chromosome 13) and C33A cells (control keratinocyte cells of non-HPV carcinoma origin) derived from human cervical squamous cell carcinoma (ATCC, American Type Culture Collection, Manassas, VA) were maintained in Eagle Minimal Essential Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/mL, Sigma–Aldrich Co., St. Louis, MO) and streptomycin (100 μg/mL, Sigma–Aldrich). After reaching approximately 80% confluency, passage of cells were performed and the resuspended cells divided into center-well organ culture dishes (Falcon 3037, Becton Dickinson Co., Franklin Lakes, NJ). The cells were cultured (24 h, 37 °C, 5%CO_2 in air mixture) and confirmed to have at least 60% confluency before initiating treatments.

The HeLa, SiHa or C33A cells were exposed to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound. The curcumin and/or high frequency ultrasound treatments were as described below. The five treated groups of cells for each of the three different cell types were further incubated for 48 h at 37 °C, 5% CO_2 in air. After incubation, the spent medium from each culture dish was removed and stored frozen for subsequent cell-free DNA testing. The remaining cells were analyzed for apoptosis, necrosis, viability and differences in cellular morphology.

Preparation of curcumin

The curcumin solution was prepared by dissolving curcumin powder (Cat. #C7727, Sigma–Aldrich Chemical Co., St. Louis, MO) in DMSO (Cat. # D2438, Sigma–Aldrich) vortex mixed and used within the hour. When the curcumin solution was added to each culture dish of cells, the final concentration was 10 μM in 1 mL of culture medium. This final concentration was based on studies in cultured cancer cells [26–28]. The curcumin-treated cells (groups 1, 2 and 3) were incubated for 1 h before beginning the high frequency ultrasound experiments as reported by Wang and colleagues [22]. The control group (group 4) and the ultrasound only group (group 5) also received the same amount of DMSO vehicle as the curcumin-treated groups (1, 2 and 3).

High-frequency ultrasound treatment protocol

The culture dishes with cells in groups 2, 3 and 5 were each separately placed under a vertically-positioned high-frequency vaginal transducer probe connected to the Sonoline Adara ultrasound generator (Siemens Medical Systems, Inc., Issaquah, WA). The probe was covered using a cell-culture tested sterile plastic sleeve and submerged into the culture medium at 2 mm above the layer of cells. The cells were exposed to either 5.0 or 7.5 MHz ultrasound frequency for the duration of 8 s [23,24]. After ultrasound treatment, the cells in all groups were further incubated at 37 °C, 5% CO_2 in air for an additional 48 h and analyzed as described below. To rule out temperature changes resulting from sonoporation, a certified thermometer was used to monitor temperature after 5, 8 and 11 s of ultrasound at 2 different frequencies, 5.0 and 7.5 MHz. The results showed no changes in culture media temperature.

Dual fluorescence stain analysis

After 48 h of incubation, the dual fluorescence stain method [25] was used to distinguish apoptotic or necrotic cells from viable cells. One drop of bisbenzimide (5 μL of 10 μM, Hoechst 33342, Sigma Chemical Co., St. Louis, MO) was added into each double-well culture dish containing the cells. Immediately, 5 μL of propidium iodide (32 μM, Sigma Chemical Co., St. Louis, MO dissolved in saline) was added to the same culture dish. After 1 min, the culture medium was discarded and the plated cells were washed in saline and cell viability assessed using an epifluorescence UV-microscope set at magnification of 500× (Nikon Optiphot, Nikon Instruments, Melville, NY). The percentages of viable, apoptotic and necrotic cells were determined and the fluorescent images were recorded. Viability was defined as the ability of the cells to exclude fluorescent dye, while apoptotic and necrotic cells were identified by their ability to take up bisbenzimide or propidium iodide stain, respectively.

Cell morphometric analysis

Cells plated on the culture dishes were fixed overnight in 4% formalin fixative, and stained using the Diff-Quik (Siemens Healthcare Diagnostics, Inc, Newark, DE) stain kit [26]. Briefly, the cells were rinsed and soaked in triarylmethane dye in methanol solution for 5 s. The solution was rinsed off and a xanthene dye based solution (Solution 1) added into each culture dish. After 5 s, the xanthene solution was rinsed off and the final solution (Solution 2) containing methylene blue was pipetted into the culture dish. After another 5 s, Solution 2 was rinsed off and the culture dishes were air-dried. Digital images of the stained cells were recorded, (250× magnification, Nikon Optiphot light microscope) and the area of each cell for the 5 groups of 3 different cell types were measured using an Adobe Photoshop image analysis system (Adobe Photoshop, San Jose, CA). The dimensions of 150 cells were recorded on Microsoft Excel spreadsheets and analyzed.

Detection of cell-free DNA in the spent media

The modified isocratic capillary electrophoresis method was used for the detection of trace amounts of cell-free DNA [27–29]. Briefly, spent culture medium (50 μL) was drawn into a glass micro-hematocrit capillary tube (internal diameter 1.2 mm, wall 0.2 mm, VWR Scientific Inc., San Francisco, CA). One end of the capillary tube was sealed closed with a 2.4% agarose gel plug while the other end was sealed with 10 μL of Liquasonic conducting gel (Chester Labs, Inc., Cincinnati, Ohio). The tubes were submerged in 1× TAE (0.04 M Tris-acetate with 0.001 M EDTA) buffer and a constant voltage of 100 V was applied for 24 min. After electrophoresis, each agarose plug was expelled on to a glass slide, held in position, stained with SYBR Gold fluorescent stain (Molecular Probes Inc., Eugene, OR), rinsed and examined using UV epi-fluorescent microscope at 40× magnification. Digital images were taken at 4 random points located 4 mm from the agarose tip (corresponding to low molecular weight fragments; 1 kilo-base pairs). A DNA ladder marker (HyperLadder, Bioline, Tauton, MA) was included in the experiments. Fluorescence intensities were determined using digital image analysis software (Adobe Photoshop, San Jose, CA). To control for intra-assay variation and low-level background staining, the data were expressed as a ratio of the fluorescence intensity of the stained gel to the background fluorescence.

Statistical analysis

The number of cells needed for each group was determined using the Kelsey calculation for unmatched sample size for case–control studies with 95% confidence and 80% power (OpenEpi statistics version 3.01, Atlanta, GA). Differences in each group were tested using the Mantel-Haenszel 2-tailed chi-square test statistics. Data from the morphometric analyses of cell dimensions and the ratios of cell-free DNA in spent media were tested using ANOVA and presented as mean ± standard error of the mean (S.E.M.) in the tables. Individual means were compared using the Students' 2-tailed *t*-test statistic adjusted for unequal variances. A value of $P < 0.05$ was considered significant.

Results

Necrosis in HeLa, SiHa and C33A cells (Fig. 1) in the combined 7.5 MHz high frequency ultrasound and curcumin group were 9-, 12- and 16-fold higher respective, when compared with the controls (Table 1). There was a dose-dependent effect of increased necrosis with higher ultrasound frequencies for the curcumin-treated SiHa and C33A cell lines.

In contrast to necrosis, the effect of different ultrasound frequencies and curcumin on cellular apoptosis was varied (Table 2). There was significant apoptosis in HeLa or SiHa cells only when the higher frequency (7.5 MHz) ultrasound was used in combination with the curcumin treatment. The low percent apoptosis in HeLa cells after ultrasound only (group 5) reflected

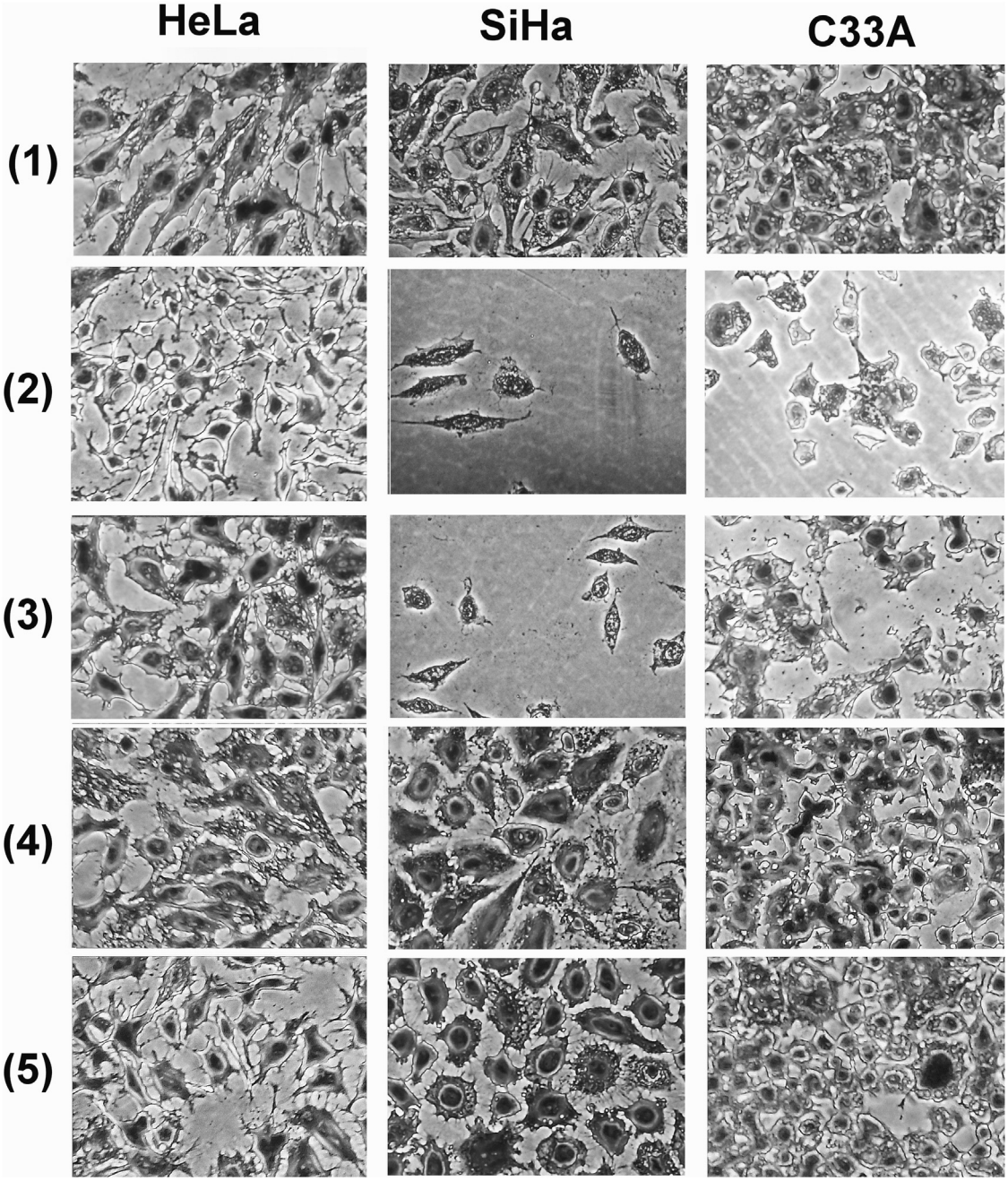

Fig. 1. Diff-Quik stained cells (HeLa, SiHa or C33A) after exposure to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound.

Table 1
The percentages of necrotic cervical cancer cells (HeLa, SiHa or C33A) after exposure to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound. Exposed cells were incubated at 37 °C, 5% CO_2 in air for 48 h. Bisbenzimide and propidium iodide dual-stain epifluorescence analyses were performed on the cells (n = number of cells). Data presented as mean ± S.E.M.

Treatment groups	% Necrosis HeLa (n, p-value*)	% Necrosis SiHa (n, p-value*)	% Necrosis C33A (n, p-value*)
(1) Curcumin only	12.4 ± 2.3 (79, 0.03)	16.3 ± 5.1 (84, 0.01)	24.3 ± 7.0 (65, <0.001)
(2) Curcumin + US 7.5 MHz	45.4 ± 5.0 (59, <0.001)	96.7 ± 4.1 (34, <0.001)	98.3 ± 2.0 (60, <0.001)
(3) Curcumin + US 5.0 MHz	40.0 ± 1.5 (67, <0.001)	76.7 ± 13.1 (73, <0.001)	67.0 ± 5.3 (108, <0.001)
(4) Control medium	5.0 ± 2.3 (161, na)	8.1 ± 3.4 (130, na)	6.3 ± 2.1 (248, na)
(5) US 7.5 MHz only	10.8 ± 2.8 (149, 0.06)	1.2 ± 0.2 (163, 0.06)	0.7 ± 0.7 (288, 0.01)

* Compared with Control medium group 4.

Table 2
The percentages of apoptotic cervical cancer cells (HeLa, SiHa or C33A) after exposure to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound. Exposed cells were incubated at 37 °C, 5% CO_2 in air for 48 h. Bisbenzimide and propidium iodide dual-stain epifluorescence analyses were performed on the cells (n = number of cells). Data presented as mean ± S.E.M.

Treatment groups	% Apoptosis HeLa (n, p-value*)	% Apoptosis SiHa (n, p-value*)	% Apoptosis C33A (n, p-value*)
(1) Curcumin only	15.3 ± 1.3 (79, 0.51)	28.5 ± 6.0 (84, 0.93)	25.8 ± 3.9 (65, <0.001)
(2) Curcumin + US 7.5 MHz	35.4 ± 10.6 (59, <0.001)	3.3 ± 10.1 (34, <0.001)	1.7 ± 9.6 (60, 0.27)
(3) Curcumin + US 5.0 MHz	12.9 ± 4.0 (67, 0.22)	20.2 ± 12.1 (73, 0.11)	33.0 ± 5.3 (108, <0.001)
(4) Control medium	18.5 ± 2.7 (161, na)	33.6 ± 8.4 (130, na)	5.1 ± 1.0 (248, na)
(5) US 7.5 MHz only	8.9 ± 4.3 (149, 0.01)	13.8 ± 3.2 (163, <0.002)	4.4 ± 1.5 (288, 0.71)

* Compared with Control medium group 4.

Table 3
The percentages of live viable cervical cancer cells (HeLa, SiHa or C33A) after exposure to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound. Exposed cells were incubated at 37 °C, 5% CO_2 in air for 48 h. Bisbenzimide and propidium iodide dual-stain epifluorescence analyses were performed on the cells (n = number of cells). Data presented as mean ± S.E.M.

Treatment groups	% Live HeLa (n, p-value*)	% Live SiHa (n, p-value*)	% Live C33A (n, p-value*)
(1) Curcumin only	72.2 ± 1.3 (79, 0.48)	55.2 ± 5.4 (84, 0.14)	49.9 ± 5.6 (65, <0.001)
(2) Curcumin + US 7.5 MHz	19.2 ± 13.3 (59, <0.001)	0.0 ± 0 (34, <0.001)	0.0 ± 0 (60, <0.001)
(3) Curcumin + US 5.0 MHz	47.1 ± 3.0 (67, <0.001)	3.1 ± 2.6 (73, <0.001)	0.0 ± 0 (108, <0.001)
(4) Control medium	76.5 ± 4.1 (161, na)	58.3 ± 10.1 (130, na)	88.6 ± 3.0 (248, na)
(5) US 7.5 MHz only	80.3 ± 5.0 (149, 0.38)	84.3 ± 3.1 (163, <0.001)	94.9 ± 1.6 (288, 0.02)

* Compared with Control medium group 4 ($p < 0.05$).

Table 4
A comparison of cell area dimension (HeLa, SiHa or C33A) after exposure to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 s of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 s of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound. Exposed cells were incubated at 37 °C, 5% CO_2 in air for 48 h and digitized images of individual cells analyzed. Data presented as mean ± S.E.M in micron squared (μ^2).

Treatment groups	HeLa (n, p-value*)	SiHa (n, p-value*)	C33A (n, p-value*)
(1) Curcumin only	444.7 ± 50.7 (10, 0.31)	496.3 ± 51.4 (10, 0.02)	386.0 ± 34.6 (10, 0.16)
(2) Curcumin + US 7.5 MHz	295.4 ± 32.2 (10, <0.001)	269.3 ± 52.0 (10, <0.001)	203.1 ± 12.6 (10, 0.003)
(3) Curcumin + US 5.0 MHz	370.0 ± 33.3 (10, 0.01)	312.1 ± 35.7 (10, <0.001)	312.0 ± 45.1 (10, 0.85)
(4) Control medium	506.0 ± 35.8 (10, na)	673.0 ± 51.1 (10, na)	321.9 ± 30.9 (10, na)
(5) US 7.5 MHz only	436.0 ± 32.0 (10, 0.14)	409.1 ± 31.6 (10, <0.001)	178.4 ± 11.7 (10, <0.001)

* Compared with Control Medium group 4.

rapid conversion of these cells into necrotic cells. In contrast, the SiHa cells in group 5 had higher survivability. The C33A cells had increased apoptosis when the combined lower frequency (5.0 MHz) ultrasound and curcumin was used suggesting delayed cell death when the lower 5.0 MHz ultrasound was used for these cells.

In terms of viability (Table 3), curcumin with either frequencies of ultrasound resulted in lower percentages of live cells. In the absence of curcumin, the use of high frequency ultrasound alone did not reduce viability in all 3 cell types. Curcumin only by itself did not reduce cell viability in the HPV-associated cervical cancer cells confirming the reports of ineffectiveness of curcumin treatment due to low bioavailability and rapid clearance [17,18].

Cell dimension, indicative of cytoskeletal disruption, was reduced in the HeLa or SiHa cell by the combined curcumin and high-frequency (5.0 and 7.5 MHz) ultrasound treatment (Table 4). However, C33A cell size was reduced only by the higher (7.5 MHz) frequency ultrasound treatment in the presence of curcumin. Interestingly, ultrasound treatment by itself reduced the SiHa and C33A cell size but not the HeLa cells. Most noteworthy was that curcumin treatment alone had no effect on HeLa and C33A cell size but had an effect on SiHa cells.

After 48 h of incubation, excessive low molecular weight cell-free DNA was detected in the spent media of cultured HeLa cells treated with curcumin with or without ultrasound treatment (Table 5). In this study, excessive cell-free DNA was defined as a detectable amount of DNA fragments greater than the control level (group 4). Excessive cell-free DNA was detected only in the spent media of the curcumin and higher (7.5 MHz) frequency ultrasound

Table 5
Detection of cell-free DNA in spent media of cultured HeLa, SiHa or C33A cells after exposure to either: (1) 10 μM curcumin only, (2) 10 μM curcumin with 8 secs of 7.5 MHz high frequency ultrasound, (3) 10 μM curcumin with 8 secs of 5.0 MHz high frequency ultrasound, (4) control culture medium, or (5) 8 s of 7.5 MHz high frequency ultrasound. Exposed cells were incubated at 37 °C, 5% CO_2 in air for 48 h, spent media collected (n = number of cultures) and analyzed for cell-free DNA presence using capillary gel electrophoresis. Results are presented as a ratio to background noise fluorescence (mean ± S.E.M.). Higher numbers indicate more cell-free DNA.

Treatment Groups	HeLa (n, p-value*)	SiHa (n, p-value*)	C33A (n, p-value*)
(1) Curcumin only	1.38 ± 0.15 (5, <0.001)	1.63 ± 0.09 (5, <0.001)	1.66 ± 0.20 (5, <0.001)
(2) Curcumin + US 7.5 MHz	2.10 ± 0.16 (5, <0.001)	2.66 ± 0.40 (5, <0.001)	2.32 ± 0.26 (5, 0.22)
(3) Curcumin + US 5.0 MHz	2.19 ± 0.22 (5, <0.001)	1.27 ± 0.03 (5, <0.001)	1.88 ± 0.20 (5, <0.002)
(4) Control medium	1.11 ± 0.06 (5, na)	1.45 ± 0.07 (5, na)	2.53 ± 0.24 (5, na)
(5) US 7.5 MHz only	1.14 ± 0.04 (5, 0.38)	1.26 ± 0.04 (5, <0.001)	2.43 ± 0.18 (5, 0.48)

* Compared with Control medium group 4.

treated SiHa cells group. In contrast, there was no excessive cell-free DNA released by the non-HPV associated C33A cells in all groups.

Comment

The results showed increased necrosis in cervical carcinoma cells by combined curcumin and high frequency ultrasound treatment. Cell necrosis was increased by 9-, 12- and 16-fold using combined 7.5 MHz ultrasound and curcumin treatment for the HeLa, SiHa and C33A cells respectively. In contrast, curcumin alone resulted in only a 2–4-fold increase in necrosis in the groups. This confirmed the low efficacy of curcumin only treatment for HPV-associated cervical cancer cells [30] and supported the use of enhanced drug-delivery techniques such as sonoporation. This suggested that the effectiveness of curcumin for the treatment of cervical cancer cells was enhanced by a brief pulse of high frequency ultrasound. Indeed, in a recent study, the combination of ultrasound sonoporation and curcumin was effective in destroying nasopharyngeal carcinoma CNE2 cells [22]. Another study showed the cytotoxic effect of curcumin on two oral squamous cell carcinoma cell lines when curcumin was combined with a brief pulse of ultrasound [31]. The postulated mechanism of action involved ultrasound acting as a sonoporation agent that increased cell membrane permeability to curcumin through cavitation. Enhanced internalization of curcumin resulted in an increased intracellular concentration of reactive oxygen species (ROS) which led to the induction of cell cycle arrest and apoptosis [32]. Characteristics of cell treated by combined ultrasonic insonation and curcumin showed specific membrane disruption and damaged mitochondria, cellular markers of apoptosis and necrosis.

Cucumin is a phenolic compound well-known for its anti-inflammatory and anti-oxidative activities properties [33]. Curcumin is isolated from the rhizome of tumeric or Cucumina longa Linn, a member of the Zingiberaceae ginger family. The mechanism of action of curcumin involved reduction of inflammation through down-regulated cyclooxygenase-2, lipoxygenase and inducible nitric oxide synthase enzymes [34]. However, its low solubility and poor stability in aqueous solutions [35] and its rapid systemic elimination [17] limits its use in clinical applications [36]. To overcome the shortcomings, a combination method using ultrasound and curcumin was used to internalize curcumin rapidly into the cancer cells to address stability concerns and to bypass systemic clearance. The present study provided supporting evidence of the effectiveness of this combination for the destruction of cervical cancer cells. The treated cancer cells were smaller in size when compared with the control suggesting altered cytoskeletal framework. Interestingly the cell size of the non-HPV cervical cancer cell line, C33A, was only affected by higher (7.5 MHz) frequency ultrasound treatment in the presence of curcumin suggesting: (1) resistance of specific cell types to sonoporation and (2) the need to determine the effective frequency for sonoporation of specific cell types. However, this is less likely to be clinically significant as the vast majority (90% or more) of cervical cancers are indeed HPV-mediated.

An interesting aspect of tumor cell death is the release of excessive cell-free DNA [37]. Low molecular weight cell-free DNA was detected in the spent media of cultured HeLa cells treated with curcumin and with or without sonoporation. In the cultured SiHa cells group, the cell-free DNA was detected only when higher (7.5 MHz) frequency ultrasound and curcumin were used. In contrast, excessive cell-free DNA was not detected in the C33A cells group. This suggested that during cellular necrosis, the extent of the breakdown of cell walls differed among the cell types. However, in this study, only low molecular weight cell-free DNA of about 1 kilo-base pairs consistent with lysed host cell DNA was detected. Cell-free HPV DNA was ruled out due to the fact that the HPV genome average size was about 8 kilo-base pairs and hence, much larger than the cell-free DNA detected in the spent media. A limitation of the present study was the use of in vitro cultured cells. To address the limitation, clinical studies are needed to study the effectiveness of the combined treatment and document systemic effects in vivo.

Conflict of interest

All the authors declare they have no financial and personal relationships with other people or organizations that could influence this research work.

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
[2] American Cancer Society. Cancer Facts & Figures; 2013.
[3] Cancer Trends Progress Report–2011/2012 Update. Bethesda, MD: National Cancer Institute, NIH, DHHS; August 2012, http://progressreport.cancer.gov.
[4] Stanely M. Potential mechanisms for HPV vaccine-induced long-term protection. Gynecol Oncol 2010;118:S2–7.
[5] Armstrong EP. Prophylaxis of cervical cancer and related cervical disease: a review of the cost-effectiveness of vaccination against oncogenic HPV types. J Manag Care Pharm 2010;16:217–30.
[6] Dhillon N, Aggarwal BB, Newman RA, et al. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res 2008;14:4491–9.
[7] Sharma RA, Euden SA, Platton SL, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 2004;10:6847–54.
[8] Strimpakos A, Sharma RA. Curcumin: preventive and therapeutic properties in laboratory studies and clinical trials. Antioxid Redox Signal 2008;10:511–45.
[9] Kanai M, Yoshimura K, Asada M, et al. A phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with gemcitabine-resistant pancreatic cancer. Cancer Chemother Pharmacol 2011;68:157–64.
[10] Cheng AL, Hsu CH, Lin JK, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or premalignant lesions. Anticancer Res 2001;21:2895–900.
[11] Maher DM, Bell MC, O'Donnell EA, Gupta BK, Jaggi M, Chauhan SC. Curcumin suppresses human papillomavirus oncoproteins, restores p53, Rb, and PTPN13 proteins and inhibits benzo[a]pyrene-induced upregulation of HPV E7. Mol Carcinog 2011;50:47–57.
[12] Singh RK, Rai D, Yadav D, Bhargava A, Balzarini J, De Clercq E. Synthesis antibacterial and antiviral properties of curcumin bioconjugates bearing dipeptide, fatty acids and folic acid. Eur J Med Chem 2010;45:1078–86.

[13] Naz RK, Lough ML. Curcumin as a potential non-steroidal contraceptive with spermicidal and microbicidal properties. Eur J Obstet Gynecol Reprod Biol 2014;176:142–8.
[14] Baak JPA, Kruse A-J, Garland SM, et al. Combined p53 and retinoblastoma protein detection identifies persistent and regressive cervical high-grade squamous intraepithelial lesions. Am J Surg Pathol 2005;29:1062–6.
[15] Øvestad IT, Gudlaugsson E, Skaland I, et al. The impact of epithelial biomarkers, local immune response and human papillomavirus genotype in the regression of cervical intraepithelial neoplasia grades 2e3. J Clin Pathol 2011;64:303–7.
[16] Øvestad IT, Gudlaugsson E, Skaland I, et al. Local immune response in the microenvironment of CIN2-3 with and without spontaneous regression. Mod Pathol 2010;23:1231–40.
[17] Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol Pharm 2007;4:807–18.
[18] Begum AN, Jones MR, Lim GP, et al. Curcumin structure–function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. J Pharmacol Exp Ther 2008;326:196–208.
[19] Lee Wing-Hin, Loo Ching-Yee, Young PM, Traini Daniela, Mason Rebecca S, Rohanizadeh Ramin. Recent advances in curcumin nanoformulation for cancer therapy. Expert Opin Drug Deliv 2014;11(8):1183–201. http://dx.doi.org/10.1517/17425247.2014.916686.
[20] Li X, Chen T, Xu L, Zhang Z, Li L, Chen H. Preparation of curcumin micelles and the in vitro and in vivo evaluation for cancer therapy. J Biomed Nanotechnol 2014;10:1458–68.
[21] Domenici F, Giliberti C, Bedini A, et al. Structural and permeability sensitivity of cells to low intensity ultrasound: infrared and fluorescence evidence in vitro. Ultrasonics 2013;54:1020–8.
[22] Wang X, Xia X, Leung AW, et al. Ultrasound induces cellular destruction of nasopharyngeal carcinoma cells in the presence of curcumin. Ultrasonics 2011;51:165–70.
[23] Debata PR, Castellanos MR, Fata JE, et al. A novel curcumin-based vaginal cream Vacurin selectively eliminates apposed human cervical cancer cells. Gynecol Oncol 2013;129:145–53.
[24] Lin HY, Thomas JL, Chen HW, Shen CM, Yang WJ, Lee MH. In vitro suppression of oral squamous cell carcinoma growth by ultrasound-mediated delivery of curcumin microemulsions. Int J Nanomed 2012;7:941–51.
[25] Rowland SC, Jacobson JD, Patton WC, King A, Chan PJ. Dual fluorescence analysis of DNA apoptosis in sperm. Am J Obstet Gynecol 2003;188:1156–7.
[26] Gramlich TL1, Fritsch C, Shear S, Sgoutas D, Tuten T, Gansler T. Analysis of epidermal growth factor receptor gene expression in stained smears and formalin-fixed, paraffin-embedded cell pellets by reverse transcription intron differential polymerase chain reaction. Anal Quant Cytol Histol 1993;15:317–22.
[27] Chou JS, Jacobson JD, Patton WC, King A, Chan PJ. Modified isocratic capillary electrophoresis detection of cell-free DNA in semen. J Assist Reprod Genet 2004;21:397–400.
[28] Checa ML, Dunner S, Canon J. Prediction of X and Y chromosome content in bovine sperm by using DNA pools through capillary electrophoresis. Theriogenology 2002;58:1579–86.
[29] Hart EA, Patton WC, Jacobson JD, King A, Corselli J, Chan PJ. Luteal phase serum cell-free DNA as a marker of failed pregnancy after assisted reproductive technology. J Assist Reprod Genet 2005;22:213–7.
[30] Sreekanth CN, Bava SV, Sreekumar E, Anto RJ. Molecular evidences for the chemosensitizing efficacy of liposomal curcumin in paclitaxel chemotherapy in mouse models of cervical cancer. Oncogene 2011;30:3139–52.
[31] Lin HY, Thomas JL, Chen HW, et al. In vitro suppression of oral squamous cell carcinoma growth by ultrasound-mediated delivery of curcumin microemulsions. Int J Nanomed 2012;7:941–51.
[32] Weir NM, Selvendiran K, Kutala VK, et al. Curcumin induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by modulating Akt and p38 MAPK. Cancer Biol Ther 2007;6:178–84.
[33] Xu YX, Pindolia KR, Janakiraman N, Noth CJ, Chapman RA, Gautam SC. Curcumin, a compound with anti-inflammatory and anti-oxidant properties, down-regulates chemokine expression in bone marrow stromal cells. Exp Hematol 1999;7(25):413–22.
[34] Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE, Rimbach G. Curcumin – from molecule to biological function. Angew Chem Int Ed 2012;51:5308–32.
[35] Wang YJ, Pan MH, Cheng AL, et al. Stability of curcumin in buffer solutions and characterization of its degradation products. J Pharm Biomed Anal 1997;15:1867–76.
[36] Zhang C, Li B, Zhang X, Hazarika P, Aggarwal BB, Duvic M. Curcumin selectively induces apoptosis in cutaneous T-cell lymphoma cell lines and patients' PBMCs: potential role for STAT-3 and NF-kappaB signaling. J Invest Dermatol 2010;130:2110–9.
[37] Li CN, Hsu HL, Wu TL, Tsao KC, Sun CF, Wu JT. Cell-free DNA is released from tumor cells upon cell death: a study of tissue cultures of tumor cell lines. J Clin Lab Anal 2003;17:103–7.