

High Energy Shock Waves (HESW) for Sonodynamic Therapy: Effects on HT-29 Human Colon Cancer Cells

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Abstract. *Background:* Whether high energy shock waves (HESW), generated by a piezoelectric generator, were able to activate a sonosensitizer, 5-aminolevulinic acid (ALA) and induce inhibition of cell growth in HT-29 human colorectal cancer cells was investigated. *Materials and Methods:* Cell survival and cell death pathways were investigated by cell growth curves, flow cytometry analysis and ELISA nucleosome evaluation. HT-29 cells were exposed to ALA and different HESW treatments: E1 (energy flux density = 0.22 mJ/mm²; 500 and 1000 shots) and E2 (energy flux density = 0.88 mJ/mm²; 500 and 1000 shots). *Results:* A significant reduction of HT-29 cell growth with respect to untreated cells was observed only after treatment with ALA and HESW E2, 500 shots. In particular, HESW E2, 500 shots, was able to induce an arrest of HT-29 cells exposed to ALA in the G₀/G₁-phase of the cell cycle. *Conclusion:* HESW is proposed for the sonodynamic treatment of cancer cells.

Photodynamic therapy (PDT) for cancer patients has developed into an important new clinical treatment modality in the past 25 years. PDT involves administration of a tumor-localizing photosensitizer or photosensitizer pro-drug, such as 5-aminolevulinic acid (ALA), a precursor in the heme biosynthetic pathway, and the subsequent activation of the photosensitizer by light (1). However, the low penetration depth of light through the skin and tissues, limiting PDT to the treatment of superficial endoscopically reachable tumors, is the main drawback. Sonodynamic therapy is an analogous approach based on the synergistic effect of ultrasound and

chemical compound referred to as "sonosensitizer", but the attractive feature of this modality for cancer treatment emerges from the ability to focus the ultrasound energy on malignancy sites placed deep in tissues (2).

Ultrasound is a mechanical wave with periodic vibration of particles in a continuous, elastic medium at frequencies equal to or greater than 20 kHz. The interaction of ultrasound with bulk liquid is accompanied by quite unique phenomenon of cavitation that leads to an enormous concentration and conversion of the diffuse sound energy. Cavitation has been classified into two types, non-inertial cavitation and inertial cavitation. Non-inertial cavitation bubbles oscillate about an equilibrium radius and often persist for many acoustic cycles. As a result of these oscillations, streaming of surrounding liquid occurs and mechanical stresses create mixing of the medium. Inertial cavitation are gas bubbles that grow to near resonance size and may expand to a maximum before violently collapsing. These conditions may induce a multitude of chemical reactions within and surrounding the bubble, including a concentration of energy sufficient to generate light, an emission known as sonoluminescence, able to cause electronic excitation of porphyrins by energy transfer and initiate a photochemical process resulting in the formation of the cytotoxic singlet oxygen (2). Several studies have been conducted *in vitro* and *in vivo* to investigate the cytotoxic effect of the combination of hematoporphyrin, the most common photodynamic sensitizer, and ultrasounds showing encouraging results (3-7). Nevertheless, it has been reported that when the human colorectal adenocarcinoma cell line, HT-29, was used to test the cytotoxicity of the hematoporphyrin derivative and ultrasounds *in vitro*, no significant difference was found with or without sonosensitizer (3).

In this study, the *in vitro* effects of high energy shock waves (HESW), generated by a piezoelectric device, on HT-29 cells exposed to ALA were investigated and the mechanisms induced by pulse HESW were found to activate the photosensitizer and produce a cytotoxic effect.

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Materials and Methods

Cell culture. The human colon adenocarcinoma cell line, HT-29, was obtained from the European Collection of Cell Cultures (ECCAC, Wilshire, UK). The HT-29 cells were grown as a monolayer culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 UI/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) (Sigma, St. Louis, MO, USA), at 37°C in a humidified atmosphere of 5% CO₂-air. Once a week, the cells were detached with 0.05% trypsin-0.02% EDTA solution (Sigma) and re-seeded at a dilution 1:4.

Shock wave exposure. A piezoelectric shock wave generator (Piezoson 100, Richard Wolf, Knittlingen, Germany) by Med & Sport 2000 S.r.l., Torino, Italy, was provided. The device generates focused underwater shock waves at various frequencies (1–4 shocks/s) and intensities (0.05–1.48 mJ/mm²). The energy at the focal point is defined as energy flux density (EFD) per impulse (8) recorded as joules per area (mJ/mm²). The focal area is defined as the area in which 50% of the maximum energy is reached (9): with regard to Piezoson 100 it has a length of 10 mm in the direction of the axis of the shock wave and a diameter of 2.5 mm perpendicular to this axis. Aliquots of 1 ml of cells suspension adjusted to 1x10⁶ cell/ml were placed into 2 ml polypropylene tubes (Corning, New York, USA) which were then completely filled with culture medium. Subsequently, cells were gently pelleted by centrifugation at 250 xg in order to minimize the motion during shock wave treatments. The experimental set-up was performed as previously described (10). Briefly, each cell-containing tube was placed in vertical alignment with the focal area and was adjusted so that the central point of the focal area corresponded to the centre of the tube bottom. The shock wave unit was kept in contact with the cell-containing tube by means of a water-filled cushion. Common ultrasound gel was used as a contact medium between cushion and tube. Different HESW treatments were investigated: E1, characterized by EFD of 0.22 mJ/mm² and peak positive pressure of 31 MPa (500 or 1000 shots; frequency: 4 shocks/s) and E2, characterized by EFD of 0.88 mJ/mm² and peak positive pressure of 90 MPa (500 or 1000 shots; frequency: 4 shocks/s). Cell viability after HESW treatment was assayed with trypan blue dye exclusion. Hydrophone measurements showed that peak pressure and pressure profile were only slightly altered inside the tubes (data not shown).

ALA treatment. HT-29 cells in the exponential growth phase were pre-incubated in RPMI 1640 medium supplemented with 1% FCS containing ALA (50 µg/ml) (Sigma) for 24 h, were then removed from the flask with 0.05% trypsin-0.02% EDTA solution and treated with HESW E1, 1000 shots and HESW E2, 500 shots, respectively.

Cell growth. Untreated cells (*i.e.*, cells receiving no ALA and HESW treatment), cells treated with ALA (*i.e.*, preincubated with ALA 50 µg/ml for 24 h), cells treated with HESW (E1, EFD = 0.22 mJ/mm², 1000 shots; and E2, EFD = 0.88 mJ/mm², 500 shots) and their combination were normalized at 25,000 cells/ml, seeded in 24 wells/plate in RPMI 1640 medium with 10% FCS and subcultured in standard culture conditions for 9 consecutive days. At days 3, 6 and 9 the cells were detached with trypsin/EDTA and viable cell growth was determined by trypan blue dye exclusion.

Measurement of intracellular reactive oxygen species. Reactive oxygen species (ROS) were measured using 2',7'-dichloro-fluorescein (DCFH) diacetate as a probe. DCFH diacetate is a stable, nonfluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent DCFH, which is rapidly oxidized in the presence of peroxides to highly fluorescent 2',7'-dichlorofluorescein (DCF), which is then measured fluorometrically (11). The DCF fluorescence intensity is proportional to the amount of reactive oxygen species formed intracellularly (12). Cells in the exponential growth phase were incubated in RPMI 1640 medium supplemented with 1% heat inactivated FCS containing ALA (50 µg/ml) for 24 h then harvested in PBS and treated with HESW, E1 and E2, respectively. After HESW treatments, cells were gently pelleted by centrifugation at 250 xg and the reactions were performed in the supernatant containing DCFH diacetate (5 µM, final concentration). After 15 min incubation at 37°C in the dark, ROS levels were measured spectrofluorimetrically at 525 nm with an excitation wavelength set at 488 nm in a stirred quartz cuvette.

Analysis of DNA content by flow cytometry. Perturbations of cell cycle and antiproliferative effects were investigated on HT-29 cells at 12 and 24 h after treatment with ALA and HESW E1, 1000 shots, and E2, 500 shots, respectively. Single staining with propidium iodide (PI) enables the identification of apoptotic cells and the definition of the distribution of the whole population in the different cell cycle phases. Apoptotic nuclei show a diminished PI staining and can be identified as a distinct 'sub-G₀/G₁' peak. Cells (10⁶) were pelleted, washed twice with Ca²⁺/Mg²⁺-free PBS, fixed in 70% ethanol and stored at 4°C. Prior to analysis, they were incubated with PI (20 µg/ml) (Sigma) in the presence of ribonuclease A (0.2 mg/ml) (Sigma) for 1 h at room temperature. Analysis of the whole cell population, as well as that of the viable fraction alone, was performed using an EPICS XL flow cytometer (Coulter Corp., Hialeah, FL, USA).

ELISA nucleosome evaluation. For apoptosis studies, the cells were treated with ALA and HESW, as described above. The HT-29 cells were then seeded in 96 multiwell microtitre plates, incubated in drug-free medium and processed after 24 h. Apoptosis was evaluated using Cell Death Detection ELISAPLUS (Roche, Basel, Switzerland) following the manufacturer's instructions. This assay is based on a quantitative sandwichenzyme-immunoassay-principle using monoclonal antibodies directed against DNA and histones, respectively. This allows for the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. Before apoptosis determination, the medium was removed and the cells were washed three times with fresh medium to remove necrotic cells. Apoptosis was expressed as enrichment factor, calculated as fraction of the absorbance of treated cells with respect to the untreated control.

Data analyses. All experiments were carried out three times and each condition was performed in triplicate. Data are expressed throughout as means±SD, calculated from three different experiments. Statistical comparisons between treatment groups were performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated according to Bonferroni's test. Statistical significance was attained for values of *p*<0.05.

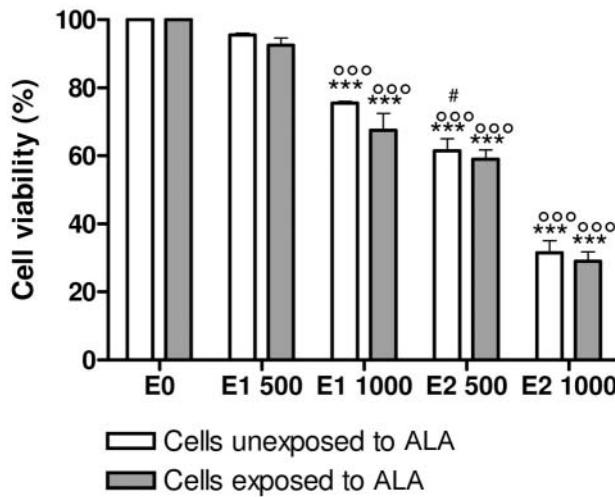


Figure 1. *HT-29 cell viability after 5-aminolevulinic acid (ALA) and high energy shock wave (HESW) treatment. Cell viability was determined by trypan blue dye exclusion and viability is expressed as the ratio between cells treated with ALA (preincubated for 24 h with ALA 50 µg/ml), HESW (E0=unshocked cells; E1=0.22 mJ/mm², 500 and 1000 shots; E2=0.88 mJ/mm², 500 and 1000 shots) and their combination with respect to untreated cells (i.e., cells unexposed to ALA and unshocked). Results are expressed as means ± SD, n=3. Significance versus untreated cells: ***p<0.001. Significance versus cells exposed to ALA alone: **p<0.001. Significance of cells exposed to E2, 500 shots, versus cells exposed to E1, 1000 shots: #p<0.05.*

Results

Effect of HESW on cell viability. The viability of HT-29 cells was assessed after HESW treatment at EFD of 0.22 mJ/mm² (E1) and of 0.88 mJ/mm² (E2), both at 500 and 1000 shots, respectively. As shown in Figure 1, a significant reduction in cell viability was observed with HESW E1 1000 shots, HESW E2 500 and 1000 shots. Cell exposure up to 1000 shots at 0.22 mJ/mm² and up to 500 shots at 0.88 mJ/mm² decreased cell viability to 60% with respect to untreated cells. Exposure up to 500 shots at 0.22 mJ/mm² led to an almost ineffectiveness of the treatment, while exposure up to 1000 shots at 0.88 mJ/mm² decreased cell viability to 30% with respect to untreated cells. Thus, the effects of HESW E1, 1000 shots and HESW E2, 500 shots were investigated on HT-29 cells even considering that the decrease in cell viability determined by these two conditions was slightly different (p<0.05). Moreover, after HESW treatment no difference in HT-29 cell viability was observed between the cells not exposed to ALA and those exposed to ALA (50 µg/ml) for 24 h. The concentration of ALA, 50 µg/ml, was chosen because cell exposure up to 50 µg/ml did not decrease cell viability with respect to untreated cells and the cellular uptake of ALA after 24-h exposure was confirmed by flow cytometry analyses (data not shown).

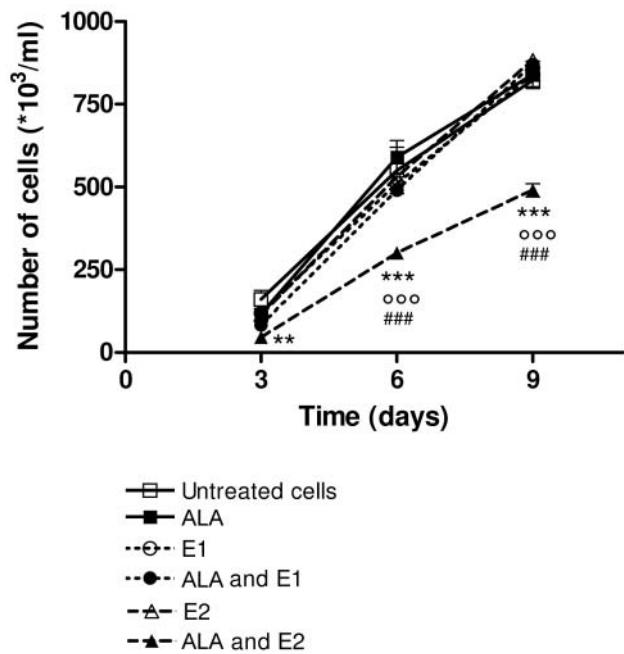


Figure 2. *HT-29 cell growth after 5-aminolevulinic acid (ALA) and high energy shock wave (HESW) treatment. Cell growth was determined by trypan blue dye exclusion at day 3, 6 and 9. Untreated cells and cells treated with ALA (preincubated for 24 h with ALA 50 µg/ml), HESW (E1=0.22 mJ/mm², 1000 shots; E2=0.88 mJ/mm², 500 shots) and their combination. Results are expressed as means ± SD, n=3. Significance versus untreated cells: *** p<0.001. Significance versus cells exposed to ALA alone: **p<0.001. Significance of cells exposed to ALA and E2, 500 shots, versus cells exposed to ALA and E1, 1000 shots: #p<0.05.*

Effect of ALA and HESW treatment on cell growth. The effect of exposure to HESW E1, 1000 shots, or E2, 500 shots, in the absence or presence of ALA (50 µg/ml) for 24 h on HT-29 cells is shown in Figure 2. At days 3, 6 and 9 in presence of ALA, only the viability of cells treated with HESW E2 500 shots, was significantly lower with respect to that of cells not subjected either to shock waves or to ALA. A significant reduction of HT-29 growth rate to about 30% (p<0.01), 50% (p<0.001) and 60% (p<0.001) was observed.

Increase of reactive oxygen species by ALA and HESW treatment. We examined whether HESW alone and combined with ALA increased the level of ROS in HT-29 cells using DCF fluorescence, as described in the Materials and Methods. HESW treatment markedly enhanced the generation of ROS in the cells, as shown in Figure 3. This effect was greater when cells were exposed to HESW E2 500 shots, than to E1 1000 shots, with an increase in fluorescence intensity of 10 and 5 folds with respect to untreated cells. Moreover, when cells were exposed to ALA and HESW E2 500 shots, or to ALA and HESW E1 1000

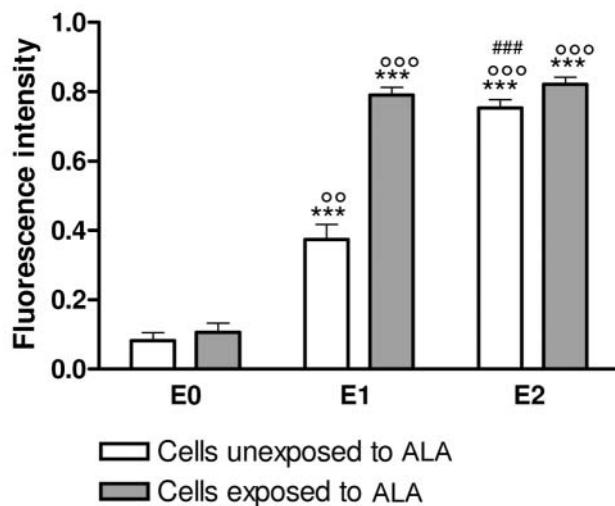


Figure 3. DCF fluorescence intensity in HT-29 cells. Untreated cells and cells treated with 5-aminolevulinic acid (ALA) (preincubated for 24 h with ALA 50 µg/ml), high energy shock wave (HESW) (E0=unshocked cells; E1=0.22 mJ/mm² 1000 shots; E2=0.88 mJ/mm² 500 shots) and their combination. The reaction mixture contained 1 x 106 cells and 5 µM DCFH-DA. The fluorescence intensity was monitored after the reaction mixture was incubated for 15 min at 37°C. Results are expressed as means ± SD; n=3. Significance versus untreated cells: *** p<0.001 Significance versus cells exposed to ALA alone: ** p<0.01; ** p<0.001. Significance of cells exposed to E2, 500 shots, versus cells exposed to E1, 1000 shots: # # p<0.001.

shots, a similar level of ROS was observed, with an increase in absorbance values of 10.8- and 10-fold compared to untreated cells, respectively.

Effect of ALA and HESW treatment on cell cycle. The proportion of cells in each phase of the cell cycle did not change appreciably in cells exposed either to ALA alone or to HESW (E1 or E2) with respect to that observed in untreated cells at 12 and 24 h. On the contrary, an increase in G₀/G₁ cells, as well as a reduction of the S population, following treatment with ALA and HESW, E2 500 shots were observed, at 12 h. Moreover, an increase in the percentage of cells with sub-G₀/G₁ DNA content, which correlated with the internucleosomal DNA fragmentation typical of apoptosis, was observed at 24 h in cells exposed to combined treatment with ALA and HESW (E1 or E2) in comparison with untreated cells (Table I).

Increase of apoptosis by ALA and HESW treatment. Apoptosis induction was explored at 24 h after ALA (50 µg/ml) or HESW (E1 1000 shots or E2 500 shots) treatment, as well as after the combined treatments [ALA and HESW (E1 1000 shots or E2 500 shots)]. Results were

Table I. Cell cycle distribution of HT-29 cells. Cells were treated with ALA (preincubated for 24 h with ALA 50 µg/ml), HESW (E1=0.22 mJ/mm², 1000 shots; E2=0.88 mJ/mm², 500 shots) and their combinations. Cells were stained with propidium iodide as described in the Materials and Methods and were analyzed by flow cytometry. Results are expressed as means±SD, n=3.

Conditions	Percentage of HT-29 cells			
	G ₀ /G ₁	S	G ₂ /M	sub-G ₀ /G ₁
Untreated cells				
12 h	69.6±2.6	21.7±0.9	6.9±0.2	1.7±0.1
24 h	47.8±1.7	25.1±1.2	25.7±1.0	1.7±0.0
ALA				
12 h	68.3±3.1	22.3±1.5	7.6±0.7	1.9±0.2
24 h	43.1±1.6	27.8±1.2	27.7±1.4	1.8±0.1
HESW E1				
12 h	68.9±2.1	22.0±1.2	7.3±0.3	1.7±0.0
24 h	47.5±1.6	26.2±0.7	24.2±0.8	2.4±0.2
ALA and HESW E1				
12 h	69.4±3.4	21.3±1.5	7.9±1.0	1.6±0.4
24 h	41.9±2.0	25.1±1.5	24.4±1.1	9.1±0.2
HESW E2				
12 h	68.6±3.2	22.7±1.6	7.7±0.8	1.1±0.1
24 h	47.0±1.4	27.3±0.9	23.6±1.2	2.5±0.0
ALA and HESW E2				
12 h	80.0±1.4	11.5±1.2	6.7±0.9	1.7±0.2
24 h	42.5±1.2	27.4±1.1	22.3±0.9	8.1±0.4

ALA=5-aminolevulinic acid;
HESW=high energy shock waves.

compared with those observed in control condition (*i.e.*, cells unshocked and unexposed to ALA). As shown in Figure 4, shock wave treatment alone did not elicit any significant increase in apoptosis when cells were exposed either to E1 1000 shots or to E2 500 shots. ALA treatment alone induced apoptosis (*p*<0.01). Apoptosis was also evident after the combined treatment with ALA and both E1 1000 shots, (*p*<0.001) and E2 500 shots, (*p*<0.001). The combined treatment with ALA and HESW (E1 1000 shots or E2 500 shots) elicited a statistically significant increase of apoptosis with respect to ALA exposure alone.

Discussion

Sonodynamic therapy might be suggested as an innovative modality for cancer treatment, able to enhance the cytotoxic activities of compounds defined as sonosensitizers. Nevertheless, its clinical applications are still under debate, since the specific mechanisms of sonosensitization are influenced by multiple factors, including the nature of the biological model, the sonosensitizer and the ultrasound parameters (2). The main assumption about the sonodynamic therapy is to generate ultrasound energy which

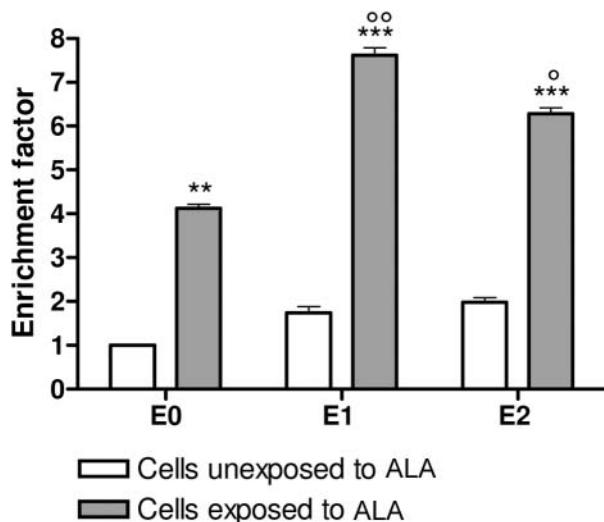


Figure 4. ELISA detection of DNA-histone complex in the cytoplasm of HT-29 cells after 5-aminolevulinic acid (ALA) and high energy shock wave (HESW) treatment. Untreated cells and cells treated with ALA (preincubated for 24 h with ALA 50 µg/ml), HESW (E0=unshocked cells; E1=0.22 mJ/mm² 1000 shots; E2=0.88 mJ/mm² 500 shots) and their combination were processed at 24 h. The enrichment factor is calculated as the ratio between absorbance measurement of dying cells and of corresponding control (unexposed to ALA and unshocked cells: Abs=0.045±0.002). Results are expressed as means ± SD; n=3. Significance versus untreated cells: ** p<0.01; ***p<0.001. Significance versus cells exposed to ALA alone: °p<0.05; °°p<0.01.

produces sonoluminescence to excite the hematoporphyrin derivative by energy transfer. This initiates a photochemical process which results in the formation of cytotoxic singlet oxygen (13). However, conflicting results have been reported regarding the synergic effect of low-energy ultrasound and porphyrins *in vitro*. Some authors showed that hematoporphyrin enhanced the killing from 30% to 95% of mouse sarcoma and rat ascites 130 tumor cells exposed *in vitro* to low-energy ultrasounds (14); conversely, when human colorectal adenocarcinoma cells (HT-29) were used to test the cytotoxicity of low-energy ultrasounds with or without hematoporphyrin derivatives, no significant difference was found by other authors (2, 3).

We investigated the effects on HT-29 cells exposed to ALA of a piezoelectric device able to generate pulse HESW with two basic effects: the direct generation of mechanical forces (non-inertial cavitation) and the indirect generation of mechanical forces by cavitation (inertial cavitation) (15). In particular, after a 24-h incubation with ALA 50 µg/ml, the HT-29 cells were exposed to HESW E1 (EFD = 0.22 mJ/mm²) 1000 shots and HESW E2 (EFD = 0.88 mJ/mm²) 500 shots.

A significant reduction of HT-29 cell growth after ALA exposure was observed only after HESW E2 500 shots,

treatment (Figure 2). Our results indicate that HT-29 cells are sensitive towards ALA and HESW treatment, even though the data reported by Meunier *et al.* (3) show that the growth inhibition of HT-29 cells, exposed to ultrasounds and sensitizer, was not modified through the cavitational effect. We suggest that our piezoelectric device is able to generate a different combination of the two basic effects. Indeed, the different characteristics either in the amount of energy applied with each HESW shot or in the number of shots, suggest that HESW E1 and E2 schedules stood for a dissimilar mechanism involved in the synergism between ultrasounds and ALA.

A divergence in viability between HT-29 cells exposed to different schedules of HESW treatment (E1 or E2; 500 or 1000 shots), with or without sonosensitizer, was observable immediately after HESW treatment (Figure 1). In spite of the reduced cell viability observed immediately after HESW treatment, cell growth, after normalization at 25,000 cells/ml, was not adversely influenced. This is in line with previous studies showing that the number of viable cells returned to control levels after few days, even when up to 90% of exposed cells had been destroyed by HESW treatment (16).

As cell viability differed immediately after HESW exposure, we investigated whether a difference in the ROS production was observable. Cells treated with HESW E1, 1000 shots, after ALA exposure showed an amount of ROS higher than observed in the cells not treated, treated with ALA alone or exposed only to HESW E1. In cells treated as above (HESW, E1 1000 shots and ALA) ROS levels did not significantly differ with respect to cells exposed only to HESW E2 or to HESW E2 and ALA (Figure 3). This measurement gave us direct information about the ability of our shock waves to produce free radicals by cavitation bubbles presumably *via* the two different routes. Our data suggest that after ALA treatment only HESW E1 1000 shots are able to produce intracellular sensitizer radicals, whereas HESW E2 500 shots are able to induce extracellular production of free radicals by the pyrolysis of the water vapor inside the bubble. Indeed, inertial cavitation is an extremely violent process of bubble activity that can result in pyrolysis of the water vapor inside the bubble, generating the very reactive hydroxyl radical and hydrogen atom. Inertial cavitation can also be followed by energy transfer to oxygen to generate the highly reactive singlet molecular oxygen in the presence of hematoporphyrin derivatives (2).

Otherwise, the lack of inhibition in cell growth after ALA treatment and HESW E1 exposition might be accounted for a resistance of HT-29 cell line to apoptosis *via* singlet oxygen (3). More difficult to explain is the cell growth inhibition after exposure to HESW E2 with sonosensitizer (Figure 2), since, after HESW E2 treatment, there was no difference in ROS production either in the presence or in

the absence of ALA (Figure 3). To investigate the mode of cell death, we evaluated cell cycle by flow cytometry as well as nucleosome generation by ELISA.

Flow cytometry analysis revealed a significant increase of HT-29 cells in the G₀/G₁-phase and a concomitant reduction of cells in the S-phase of the cell cycle after the combined treatment with ALA and HESW E2 (Table I). Moreover, nucleosome generation, evaluated by ELISA, showed that treatment with HESW E2 after ALA exposure elicited apoptosis induction. This result is in line with cell growth inhibition (Figure 2), as well as with increased sub G₀/G₁ cells (Table I).

A similar apoptosis induction with the combined treatment with ALA and HESW E1 (Figure 4 and Table I) was detected, although we did not observe cell growth inhibition (Figure 2). This observation further supports that HESW characterized by different EFD (namely, E1 and E2) may exert their effects through distinct pathways.

HESW E1 (0.22 mJ/mm²) through inertial cavitation, is able to induce apoptosis by intracellular activation of protoporphyrin IX via singlet oxygen. The lack of cell growth inhibition is in accordance with the observation that this cell line is refractory to the hematoporphyrin enhancement induced by sonication (2). HESW E2 (0.88 mJ/mm²), through a mix of non-inertial cavitation and inertial cavitation, is able to determine ROS generation by the pyrolysis of the water vapor inside the bubble and inhibition of cell growth by an increase of the G₀/G₁ population through the intracellular activation of protoporphyrin IX.

In conclusion, HESW may be proposed for the sonodynamic treatment of cancer cells, as they are able to overcome some drawbacks encountered during conventional ultrasonic and/or photodynamic treatment.

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