

High energy shock waves enhance the cytotoxic effect of doxorubicin and methotrexate to human osteosarcoma cell lines

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Received July 6, 2005; Accepted August 30, 2005

Abstract. Despite recent advances, the prognosis of relapsed osteosarcoma patients remains very poor. Application of high energy shock waves may change the tumour cell growth and increase the cytotoxic effect of *in vivo* and *in vitro* chemotherapeutic agents. We studied the effect of their association with doxorubicin or methotrexate on three *in vitro* osteosarcoma cell lines. The effect of these combinations on SJSA-1, MG-63 and HOS human osteosarcoma cell lines were evaluated through incubation with doxorubicin or methotrexate and high energy shock wave treatment with 1000 shots at 0.22 mJ/mm² and an evaluation of the cell number, cell proliferation and apoptosis at days 1, 3 and 6 from the start of treatment. The combination of high energy shock waves and doxorubicin induced a statistically significant advantage in terms of a decrease in cell number on the SJSA-1 and HOS cell lines, a decrease of cell proliferation on all three cell lines and an increase of apoptosis only on the SJSA-1 cell line. The combination of high energy shock waves with methotrexate induced a decrease of the cell number only in the SJSA-1 and in the HOS cell lines, of the cell proliferation in the SJSA-1 and in the MG-63 cell lines, and an increase of apoptosis only on the SJSA-1 cell line. In conclusion, these experiments show an interesting effect of high energy shock waves on osteosarcoma cell lines, which could lead to future studies of the *in vivo* effects of high energy shock waves in the murine model as well.

Introduction

Osteosarcoma (OS) is the most frequent bone tumour and is most common between the ages of 15 and 25. A high number

of patients have a high-grade tumour, which prognosis has dramatically improved from 10-15% to 65-70% in the last 25 years, thanks to the use of adjuvant and neoadjuvant chemotherapy (1,2). Nevertheless, despite continuing scientific research into the treatment, the prognosis of the patients with OS in metastatic relapse, especially to bones (3), is still very poor; even with a recently concluded Italian and Scandinavian phase II protocol including high-dose chemotherapy the 3-year overall survival and the 3-year disease free-survival rates were only 20% and 12% respectively (4).

The search of new treatments for improving the survival of the patients without increasing the collateral effects of the chemotherapy has led us to study new ways to increase the chemo-sensitivity of the OS cells.

High energy shock waves (HESW) are routinely used in clinical practise in orthopaedic and in urology to treat a number of different diseases, such as urinary stones, non-unions of the bone fractures, tennis elbow, calcifying tendonitis, pseudoarthrosis and necrosis of the femoral head (5,6).

In the past, many studies of HESW were conducted on different human and animal tumour cell lines, describing an alteration of the cell growth (7-11). The *in vitro* effects of shock waves are due to a suppression of cell proliferation correlated with an apoptotic cell death process; moreover, HESW treatment has been shown to cause a transient increase in cell membrane permeability by opening micropores (such as electroporation), allowing higher intracellular drug concentration (12-16). This effect has been shown as being capable of increasing the cytotoxicity of various chemotherapeutic agents on different tumour types, allowing treatments with lower doses of drugs *in vitro* (17-19), *in vivo* in animal models (12,20) and in one patient with prostate cancer (21).

This evidence led us to study the effects of HESW on OS cell lines and how to enhance cytotoxicity with a combined therapy with HESW and doxorubicin (DOXO) or methotrexate (MTX). These cytotoxic agents have been chosen since their well-known activity against OS and their routine use in clinical protocols for the treatment of OS patients (22-27).

Here, we show how HESW treatment enhances the cytotoxicity of two drugs against three OS cell lines by reducing cell viability and cell proliferation and by increasing the apoptosis levels.

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Key words: high energy shock waves, osteosarcoma, chemotherapy

Materials and methods

Cell cultures. We analyzed three human OS cell lines: SJSA-1, MG-63 and HOS, obtained from the American Type Culture Collection. The SJSA-1 cell line was maintained in Roswell Park Memorial Institute Medium (RPMI, Sigma), while MG-63 and HOS cell lines were maintained in Eagle's minimum essential medium (EMEM, EuroClone), both supplemented with 10% foetal bovine serum (FBS) and 0.5 mg/ml penicillin/streptomycin. Once a week, cells were detached with trypsin/EDTA (Cambrex, Verviers, Belgium), counted and re-seeded at 1×10^6 cell/flask.

Shock wave exposure. The shock wave generator used for the *in vitro* experiments is a piezoelectric device (Piezoson 100, Richard Wolf, Knittlingen, Germany) especially designed for clinical use in orthopaedics and traumatology. The instrument, kindly provided by Med & Sport 2000 S.r.l., Turin, Italy, generates focused underwater shock waves at various frequencies (1-4 shocks/sec) and intensities (0.05-1.48 mJ/mm²). The device has a high voltage electric current generator and a reflector set in a water-filled container. On the surface of the reflector, piezoelectric elements arranged to form a part of a sphere, are stimulated with high energy electrical pulses. This causes vibration or the rapid expansion of the crystals, leading to a shock wave, which can be propagated through the water and focused at the centre of the sphere. The pressure on the focal area is proportional to the voltage applied. The energy at the focal point is defined as the energy flux density (EFD) per impulse (28), recorded as joules per area (mJ/mm²). For medical use, in orthopaedics, shock waves of approximately 0.01-0.6 mJ/mm² are applied (29). The focal area, which is peculiar to each kind of generator, is defined as the area in which 50% of the maximum energy is reached (30): 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 1 ml of cell suspension adjusted to 5×10^6 cell/ml in of medium were placed into 2 ml polypropylene tubes (Corning, New York, NY, USA), which were then completely filled with culture medium. Then, cells were slowly pelleted by centrifugation at 250 x g in order to minimize the motion during shock wave treatment.

The experimental set-up was performed as previously described (17). Briefly, each 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 the cushion and the tube. Cells were treated as follows: control cells received no shock wave treatment, while treated cells received 1000 shots (frequency: 4 shocks/sec) at EFD = 0.22 mJ/mm² (E0.22-1000) (peak positive pressure 31 MPa; peak negative pressure 4.3 MPa). Each experiment was carried out in independent triplicate of three separate experiments.

Drug treatment. After shock wave treatment, treated and untreated cells were seeded in triplicate in complete medium

in 96-multi-well plates (Greiner Bio-One, Longwood, FL, USA) for the cell proliferation test, or in 6-well plates (Greiner Bio-One, Longwood, FL, USA) for cell viability and apoptosis evaluations in absence or presence of 0.1 μ M DOXO (Pharmacia Italia S.p.a., Milan, Italy) or 0.03 μ M MTX (Teva Pharma B.V., Mijdrecht, The Netherlands). Each experiment was set up in triplicate of three separate experiments.

Cell viability. Before and after HESW treatment, cell viability was assayed with trypan blue dye exclusion. On day 0, treated and untreated cells were plated in triplicates in 6-well plates at 0.5×10^6 cells/well (for the days 1 and 3 evaluations) or at 0.15×10^6 cells/well (for the day 6 evaluation), and then incubated at 37°C in 5% CO₂. At days 1, 3 and 6 after treatment, cells were detached with trypsin/EDTA and viable cell growth was determined by a microscopy count with trypan blue dye exclusion (3 counts for each sample).

Cell proliferation. On day 0, treated and untreated cells were plated in triplicates in 96-well plates at 5×10^3 cells/well (for the days 1 and 3 evaluations) or at 2.5×10^3 cells/well (for the day 6 evaluation), then incubated at 37°C in 5% CO₂ till days 1, 3 and 6 after treatment. All conditions were set up in triplicate. Then cells were pulsed with 0.5 μ Ci/well of [³H]-thymidine (Amersham Biosciences, Buckinghamshire, UK) for 18 h and then frozen. The day after, the plates were defrosted to detach adherent cells and then cells were harvested using a micro cell harvester (Filtermate harvester, Packard, Perkin Elmer, Boston, MA, USA) and counted with a Beta-counter (RackBeta 1219, Wallac, Evry, France). Percentage specific inhibition was calculated by the formula (CPM, counts per minute):

$$\% \text{ inhibition} = \frac{\text{Sample CPM} - \text{Control CPM}}{\text{Control CPM}} \times 100$$

Apoptosis determination. On day 0, treated and untreated cells were plated in triplicate in 6-well plates at 0.5×10^6 cells/well (for days 1 and 3 evaluations) or at 0.15×10^6 cells/well (for the day 6 evaluation), and then incubated at 37°C in 5% CO₂. At days 1, 3 and 6 after treatment the cells were detached with trypsin/EDTA and analysed with FACScan cytometer (Beckman Coulter, Fullerton, CA) using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit 1 (BD PharMingen, San Diego, CA). In brief, at least 2×10^5 cells were washed with phosphate buffer saline (PBS, Cambrex Bioscience, Verviers, Belgium), incubated for 15 min at room temperature in 100 μ l of Annexin binding buffer with 5 μ l of Annexin V and 5 μ l of propidium iodide (PI) staining solution and then, after the addition of 300 μ l of Annexin binding buffer, analyzed by flow cytometry within 30 min.

Statistical analysis. Each condition was carried out in three independent experiments. Data were expressed as means \pm standard deviation (SD) for each group. Data were analyzed for statistical significance between each condition and their controls using the two-sided Student's t-test; statistical significance was set at $p < 0.05$.

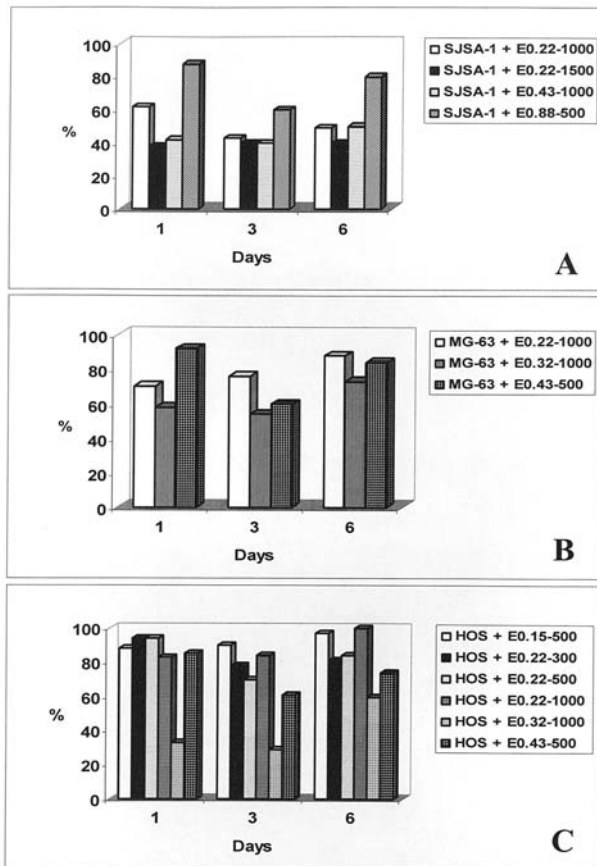


Figure 1. The effect of a different combination of energies and number of shots of HESWs on the cell number of SJSA-1 (A), MG-63 (B) and HOS cell lines (C).

Results

Preliminary experiments were carried out to set up the optimal experimental conditions. E0.22-1000 were chosen after all the cell lines had been treated with different combinations of 300, 500, 1000 or 1500 shots at 0.15, 0.22, 0.32, 0.43 or 0.88 mJ/mm² (peak positive pressure respectively at 26, 31, 38, 50 and 90 MPa) and evaluated for cell viability and cell apoptosis: a cell exposure up to 1000 shots at 0.22 mJ/mm² left enough viable cells for further drug treatments in all three evaluated cell lines. A higher number of shots (1500) decreased the cell viability with respect to untreated cells, while a smaller number of shots (500) was almost ineffective as treatment, even with an higher energy such as 0.88 mJ/mm². A low viability was also obtained with a combination of higher energies and 1000 shots. For all three lines we performed all the different combinations (Fig. 1).

DOXO concentrations of 0.1 μ M for SJSA-1 and of 0.01 μ M for MG-63 and HOS were chosen after *in vitro* examinations of cell viability and cell apoptosis after 1, 3 and 6 days of culture with different drug concentrations (0.01, 0.05, 0.1, 1 and 10 μ M), showing a different sensitivity between the three cell lines: lower concentrations were not tried since previous studies have shown the efficacy of DOXO on OS cell lines to be around 0.02-0.6 μ M (31,32). In our experiments, higher concentrations of those chosen were too toxic for the cells (Fig. 2).

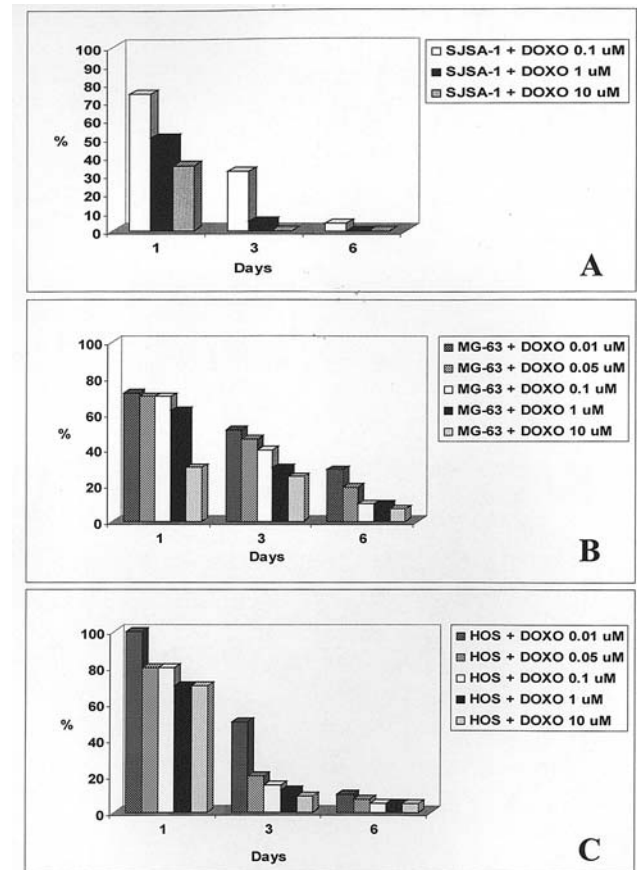


Figure 2. The effect of different concentrations of DOXO on the cell numbers of SJSA-1 (A), MG-63 (B) and HOS cell lines (C).

MTX concentration of 0.03 μ M for all the three lines was chosen after *in vitro* examinations of cell viability and cell apoptosis after 1, 3 and 6 days culture with different drug concentrations (0.0003, 0.003 and 0.03 μ M). As described elsewhere, OS cell lines have a different sensitivity to MTX: MG-63 and HOS cell lines are among the most responsive to this drug, even at a very low concentration such as 0.01 μ M (33). In our preliminary experiments, concentrations lower than 0.03 μ M proved inefficient, while a higher concentration was too toxic and did not leave enough viable cells for the evaluation of the HESW treatment (Fig. 3).

For all the experiments, combined treatment (HESW-DOXO cells or HESW-MTX cells) was compared with controls (untreated cells, DOXO cells, MTX cells or HESW cells). Data are expressed as mean of three values.

SJSA-1 cell line (Figs. 4 and 5)

Cell viability. HESW alone significantly reduced cell viability of 40.5% ($p=0.00004$), 64% ($p=0.00003$) and 53.5% ($p=0.0000008$) at days 1, 3 and 6 in comparison to untreated cells. With the HESW-DOXO cells we had a reduction of the cell viability at days 1 [of 17%, p -value not significant (NS)] and 3 (of 29%, $p=0.02$) in comparison to the drug alone, while at day 6 all the cells were dead. With the HESW-MTX cells we obtained a statistically significant reduction of the cell viability of 40% ($p=0.03$), 31% ($p=0.05$) and 52% ($p=0.03$) at days 1, 3 and 6 in comparison with the MTX alone.

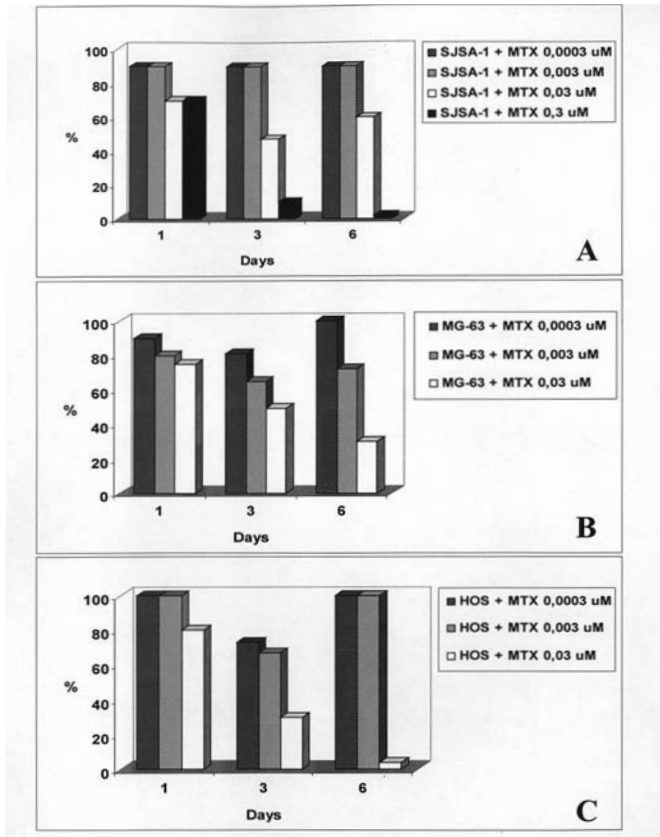


Figure 3. The effect of different concentrations of MTX on the cell numbers of SJS-1 (A), MG-63 (B) and HOS cell lines (C).

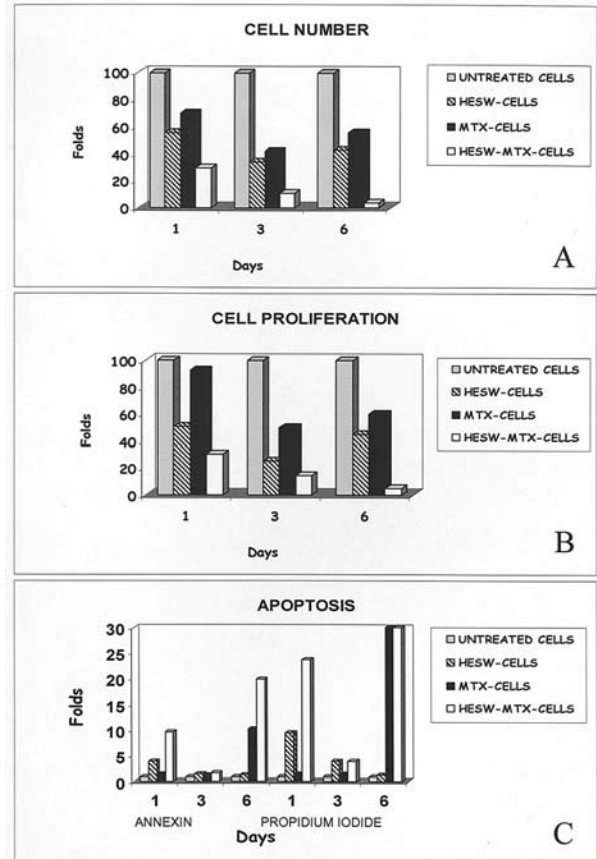


Figure 5. Effect of the association of HESW and MTX on SJS-1 cell line on cell number (A), cell proliferation (B) and apoptosis (C).

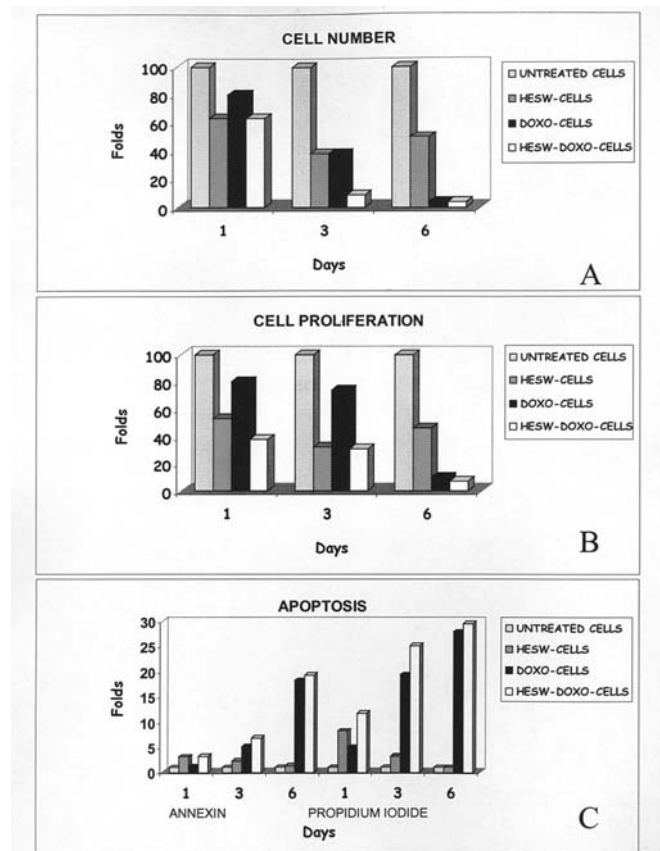


Figure 4. Effect of the association of HESW and DOXO on SJS-1 cell line on cell number (A), cell proliferation (B) and apoptosis (C).

Cell proliferation. The effect of HESW on the cell proliferation is shown by a reduction of 48% ($p=0.00006$), 71% ($p=0.000003$) and 53.5% ($p=0.003$) at days 1, 3 and 6, respectively, compared to untreated cells. As for the cell number, the combination of HESW and DOXO caused a decrease of 42% ($p=0.008$), 43% ($p=0.02$) and 3% ($p=0.03$) at days 1, 3 and 6 compared to DOXO cells. Also the association HESW and MTX caused a reduction of 63% ($p=0.01$), 36% ($p=0.049$) and 55% ($p=0.03$) at days 1, 3 and 6 in comparison to MTX-cells.

Apoptosis determination. HESW gave rise to an increase of 3.5- ($p=0.0001$), 1.9- ($p=0.005$) and 1.3-fold ($p=NS$) of Annexin positive (+) cells and of 8.8- ($p=0.00002$), 3.5- ($p=0.0002$) and 1.2-fold ($p=NS$) of PI⁺ cells at days 1, 3 and 6. With the HESW-DOXO cells we saw an increase of the Annexin⁺ cells of 2.1- ($p=0.0004$), 1.7- ($p=NS$) and 1.1-fold ($p=NS$); also the PI⁺ cells increased of 6.7- ($p=0.0008$), 5.7- ($p=0.03$) and 1.6-folds ($p=NS$) at days 1, 3 and 6 compared to DOXO-cells. In the HESW-MTX cells we obtained an increase of 8.2- ($p=0.04$), 0.4- ($p=NS$) and 9.6-fold ($p=0.01$) of the Annexin⁺ cells and of 22.8- ($p=0.04$), 2.5- ($p=NS$) and 11.7-fold ($p=0.02$) of the PI⁺ cells at days 1, 3 and 6 compared to MTX cells.

MG-63 cell line (Figs. 6 and 7)

Cell viability. HESW had a small effect on the MG-63 cell line, causing a reduction of the cell number of 26% ($p=0.0005$),

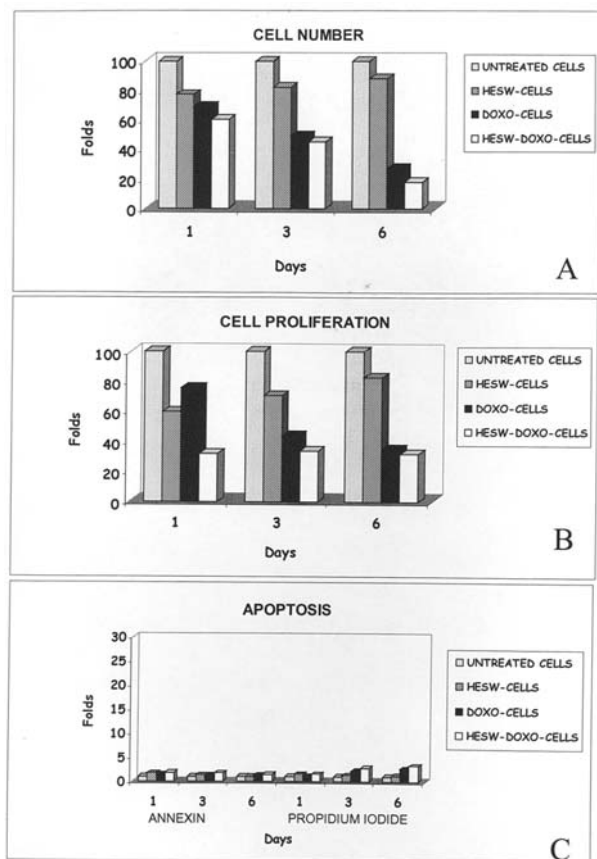


Figure 6. Effect of the association of HESW and DOXO on MG-63 cell line on cell number (A), cell proliferation (B) and apoptosis (C).

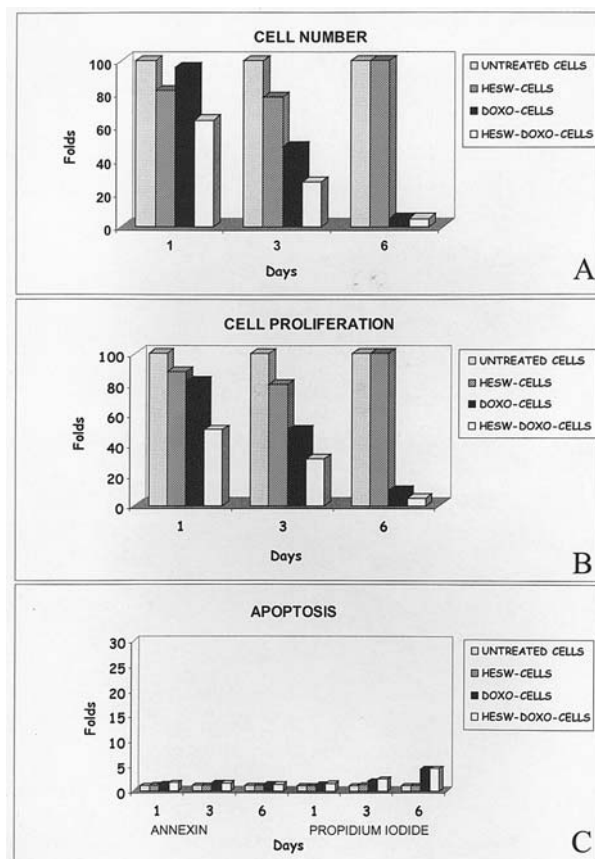


Figure 8. Effect of the association of HESW and DOXO on HOS cell line on cell number (A), cell proliferation (B) and apoptosis (C).

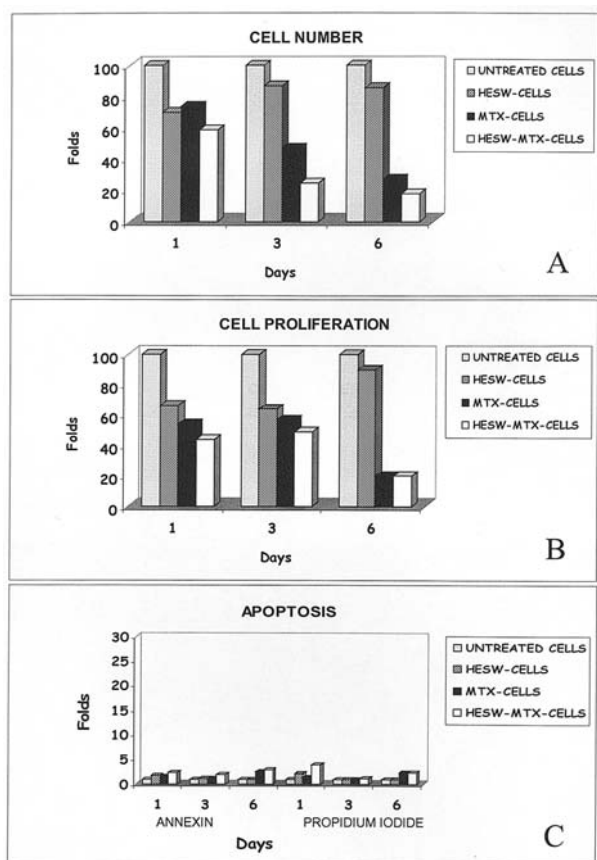


Figure 7. Effect of the association of HESW and MTX on MG-63 cell line on cell number (A), cell proliferation (B) and apoptosis (C).

15.5% (p=NS) and 13% (p=NS) at days 1, 3 and 6. When the HESW and DOXO combination was applied, no enhanced toxicity was found compared to DOXO cells. The combination HESW and MTX caused an increase of the effect of the 15% (p=NS) at day 1, 22% (p=0.02) at day 3 and 9% (p=NS) at day 6, in comparison to MTX cells.

Cell proliferation. As regards to the cell proliferation, HESW caused a reduction of 37% (p=0.0004) at day 1, of 32.5% (p=0.00007) at day 3 and of 13.5% (p=NS) at day 6. The cell proliferation of the HESW-DOXO cells decreased by 44% (p=0.002), 10% (p=NS) and 3% (p=NS) at days 1, 3 and 6 in comparison to DOXO cells. The HESW-MTX combination did not decrease the cell proliferation at any time and may be compared to MTX cells.

Apoptosis determination. HESW alone caused an Annexin⁺ cell increase at days 1, 3 and 6 of 1.7- (p=NS), 1.2- (p=NS) and 1.1-fold (p=NS) respectively, and a PI⁺ cell increase of 1.8- (p=NS), 1.2- (p=NS) and 1.1-fold (p=NS), compared to untreated cells. With HESW-DOXO cells we did not obtain a statistically significant increase of the Annexin and PI positivity. With the MTX addition we achieved only a significant PI⁺ cell increase of 2.5-fold (p=0.008) at day 1.

HOS cell line (Figs. 8 and 9)

Cell viability. HESW treatment alone induced a cell number reduction at day 1 of 18% (p=0.004), at day 3 of 19.5% (p=0.02) and at day 6 of 22% (p=NS) in comparison to the

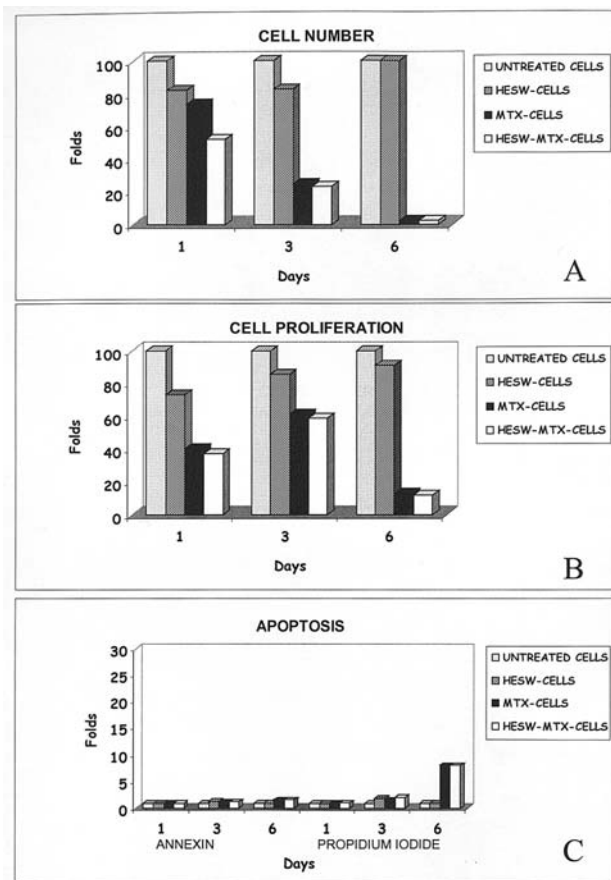


Figure 9. Effect of the association of HESW and MTX on HOS cell line on cell number (A), cell proliferation (B) and apoptosis (C).

untreated cells. The combination of HESW and DOXO caused a cell number reduction of 30% ($p=0.005$) and 21% ($p=0.02$) at days 1 and 3; at day 6, all the cells were already dead even with DOXO alone. With the HESW-MTX cells we obtained a cell number reduction of 22% ($p=NS$) at day 1 and of 2% ($p=NS$) at day 3; no viable cells were found in any wells at day 6.

Cell proliferation. An analysis of cell proliferation showed the same trend: HESW alone caused a decrease of 19.5%, 17% and 0% at days 1, 3 and 6 respectively, compared to untreated cells. The HESW-DOXO combination decreased the cell proliferation by 32% ($p=0.02$), 19% ($p=0.02$) and 5% ($p=NS$) at days 1, 3 and 6 compared to DOXO cells, while the HESW-MTX combination did not decrease the cell proliferation compared to MTX cells.

Apoptosis determination. HESW alone did not have an effect on the Annexin and PI positivity; neither did the addition of DOXO and MTX.

Discussion

Patients affected by relapsed OS have a very poor prognosis. To improve the chemo-sensitivity of OS cells, the effects of HESW have been evaluated. In this series of experiments we show how the HESW treatment seems to have a beneficial role on the enhancement of drug cytotoxicity. Three major points have been evaluated in this study.

The first was aimed at the study of the cell viability reduction following HESW treatment. In the SJSA-1 cell line the greatest effect was on day 3 from the start of treatment with HESW-DOXO treatment and on day 6 with HESW-MTX treatment. Cell viability difference (between drug-treated cell and HESW-drug-treated cells) increased for DOXO from 17% at day 1 to 29% on day 3, but the effect was annulled on day 6. However, when we compared the cell viability following MTX treatment, the difference was 40% at day 1, of 31% at day 3 and 52% at day 6. This may be due to different mechanisms of cytotoxicity of the two drugs. With the MG-63 cell line, we observed that both DOXO and MTX are slightly efficacious at days 1, 3 and 6. For the HOS cell line, the increase of efficacy with the combined treatment of HESW and drugs is evident on days 1 and 3 for DOXO but only on day 1 for MTX; then the effect is annulled by the high cell mortality given by the drugs alone. These differences might be due to a different sensitivity or resistance of the different OS cell lines to the same cytotoxic agent.

The second point was the cell proliferation, measured by the [3 H]-thymidine assay. In all three OS cell lines evaluated, we found that the addition of HESW to DOXO enhanced the reduction of cell proliferation from day 1 to day 6. This was not confirmed with the combination of HESW and MTX, where the MG-63 cell line had a reduction in the cell proliferation only on days 1 and 3 and the HOS cell line seemed unaffected by the HESW treatment.

The third point evaluated was on the pro-apoptotic effect of the HESW treatment. In these series of experiments, a significant increase of apoptosis was documented with the addition of the HESW to the DOXO or MTX only on the SJSA-1 cell line. The data might be due to a necrotic death of cells due to the opening of micropores in the cell membranes, producing higher drug concentration in cells.

Taken together the data open new perspectives for the study of both the biological properties of OS cells and for the future *in vivo* applications, especially in localized OS patients in whom the increasing of drug concentration might increase the necrotic cell death by the local administration of the drugs.

These data are promising for a better understanding of the optimal treatment for advanced or resistant OS tumours, but future work, including *in vivo* mouse experiments, needs to be carried out before further conclusions can be made.

Acknowledgements

This work was partially supported by the Italian Association for Cancer Research (AIRC-Milan, grant to E.M.) and by Compagnia di S. Paolo-Torino. We are grateful to Mr. Andrew Martin Garvey for editorial assistance.

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