

Effect of Hyperbaric Oxygenation and Gemcitabine on Apoptosis of Pancreatic Ductal Tumor Cells *In Vitro*

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Abstract. *Background:* Gemcitabine is first-line therapy for advanced pancreatic ductal adenocarcinoma (PDAC) with a poor survival and response rate. Hyperbaric oxygenation (HBO) enhances delivery of oxygen to hypoxic tumor cells and increases their susceptibility to cytotoxic effects of chemotherapy. We hypothesized that the anticancer activity of gemcitabine (GEM) may be enhanced if tumor cells are placed in an oxygen-rich environment. The present study evaluated the effects of gemcitabine, HBO and their combination on apoptosis of tumor cells. *Materials and Methods:* PANC-1 and AsPc-1 PDAC tumor cell lines were used. Cultured tumor cells were treated with GEM at its growth-inhibitory concentration (IC_{50}) and HBO at 2.5 ATA for 90 min or a combination of both (HBO then GEM and GEM then HBO). Twenty-four hours later, apoptotic cells in each group were analyzed and the apoptotic index (AI) was calculated. *Results:* PANC-1 cell line: HBO alone had no effect on AI: 6.5 ± 0.1 vs. 5.9 ± 0.1 . HBO before and after gemcitabine did not further increase AI: 8.2 ± 0.1 (HBO-GEM), 8.5 ± 0.1 (GEM-HBO) vs. 8.1 ± 0.1 (GEM). The combination of HBO and gemcitabine significantly increased AI: 10.7 ± 0.02 ($p < 0.001$ vs. all groups). AsPc-1 cell line:

HBO-alone had no effect on AI: 5.9 ± 0.1 vs. 5.9 ± 0.1 . HBO before and after gemcitabine did not further increase AI: 8.2 ± 0.1 (HBO-GEM), 8.4 ± 0.1 (GEM-HBO) vs. 8.0 ± 0.1 (GEM). The combination of HBO and gemcitabine significantly increased AI: 9.7 ± 0.1 ($p < 0.001$ vs. all groups). *Conclusion:* HBO-alone, whether administered before and after gemcitabine has no effect on apoptosis of PDAC cells in vitro. HBO significantly enhanced gemcitabine-induced apoptosis when administered during gemcitabine. Our findings suggest that the time window would be critical for using HBO as adjuvant to chemotherapy.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies and the fourth leading cause of cancer-related death in the United States (1). Systemic chemotherapy, radiotherapy or a combination of both have been utilized following surgery in order to improve outcome. However, there has been no significant improvement in the median survival time (less than six months) and 5-year survival rate (3%-5%) despite clinical and research efforts (2). Although adjuvant and neoadjuvant therapy has been widely used in recent years, the best choice for treatment modality remains highly controversial (3).

The searching for new adjuvant treatments targets characteristic properties of the cells. Hypoxia is a common feature of solid tumors. They usually grow so rapidly to exceed their blood supply, leaving portions of the tumor with regions where oxygen availability is considerably limited. Tumor hypoxia is also due to a high degree of cell proliferation which causes higher cell density and thus depletes local oxygen. Hypoxic tumor cells are usually

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resistant to radiotherapy and chemotherapy because oxygen is essential for the cytotoxic activity of these therapies. Nevertheless, hypoxic tumors can be made more susceptible to treatment by increasing the amount of oxygen inside (4).

Hyperbaric oxygen therapy (HBO) is one of the modalities to temporarily alleviate or eliminate hypoxic status in growing tumor cells. By providing 100% oxygen at elevated atmospheric pressure, HBO increases the partial pressure of the oxygen gas and thus forces more oxygen to be dissolved in the plasma, which allows the extra oxygen to be diffused or transported to the body tissues. HBO has been documented by The Undersea and Hyperbaric Medicine Society to be beneficial in 13 different diseases (5). The effects of HBO used as adjuvant to radiotherapy or chemotherapy has been reviewed (6). *In vitro* studies have demonstrated the beneficial effects of HBO used adjuvant to chemotherapy in the treatment of osteosarcoma, nasopharyngeal carcinoma CNE-2Z cells, and murine model of PC-3 prostate cancer cell line and glioma U251 cell lines (7-10). The use of HBO as adjuvant therapy for pancreatic tumors has not been reported. Gemcitabine is a pyrimidine anti-metabolite that with good clinical activity in pancreatic, breast, ovarian, non-small-cell lung, and bladder tumors (11). Gemcitabine is a first-line therapy for locally advanced pancreatic cancer; however, severe resistance is responsible for a response rate less than 20% and median survival less than six months of (2). Although efforts to overcome gemcitabine resistance have been made, the only combination treatment that has shown a small but statistically significant effect was gemcitabine with erlotinib, an epidermal growth factor receptor inhibitor (12). We hypothesized that a combination of HBO and gemcitabine may significantly enhance the efficacy of gemcitabine for pancreatic tumors. PANC1 is an epithelioid carcinoma cell line derived from human pancreas and AsPc-1 is a human pancreatic adenocarcinoma cell line (13, 14). Both cell lines are relatively sensitive to gemcitabine *in vitro* (15). These two cell lines were used in the present study to investigate for the efficacy of gemcitabine-alone, HBO-alone and the combination of both on the apoptotic index (AI) in these two cell lines.

Materials and Methods

Cell cultures. PANC-1 cell line and AsPc-1 cell line were used in the present study. Tumor cells were derived from pancreatotomy samples from patients with infiltrating ductal and intra-ductal carcinoma. Cells from these two lines were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Laborchemikalien, Seelze, Germany) with 10% fetal calf serum and a mixture of 100 IU/ml penicillin and 100 µg/ml streptomycin (all from GIBCO, Grand Island, NY, USA).

Hyperbaric chamber. A hyperbaric chamber, model Costruzioni Riunite Moro; Quinto di Treviso, Italy, located in Padova's ATIP center was used for HBO therapy. The tumor cells were treated at 2.5 atmosphere absolute (ATA) and 100% oxygen for 90 min, with five minutes for compression and five minutes for decompression.

Chemotherapy. Gemcitabine (Sigma-Aldrich Laborchemikalien) was dissolved in Phosphate buffered saline (PBS) and used its 50% growth-inhibitory concentration (IC₅₀) for cultured tumor cells. Its IC₅₀ value for PANC-1 cell is 3.25×10⁻⁸ ng/ml and for AsPc-1 cell is 1.27×10⁻⁷ ng/ml (16).

Sample analysis. Apoptosis was measured quantitatively with the use of the TUNEL assay. The ApopTag In situ Apoptosis Detection kit (Chemicon, Temecula, CA, USA) was used according to the manufacturer's instructions. A cell was considered TUNEL-positive if it was stained brown (from light to dark) and if it exhibited apoptotic bodies, chromatin condensation and membrane blebbing. The total number of TUNEL-positive tumor cells were counted in five different high-power fields with a microscope (Nikon Eclipse 80i, magnification ×20, NIKON INSTRUMENTS INC, Melville, NY, USA) and the average was calculated.

The total number of cells (both TUNEL-negative and -positive) was also counted and the apoptosis index was calculated.

Treatment. Tumor cells from the PANC-1 cell line and the AsPc-1 cell lines were prepared by seeding them in 35 mm Petri dishes at a density of 4000 cells/cm² 72 h before the beginning of the treatment. The tumor cells were divided into the following treatment groups.

GEM. The tumor cells were treated with gemcitabine alone at the IC₅₀ value. After 24 h, the culture media were replaced. The culture media were replaced again after 24 h and after a further 48 hours, the apoptotic index was analyzed with a TUNEL test.

HBO. The tumor cells were treated with a quantity of PBS (as placebo) equal to that used for dissolving gemcitabine for administering to the experimental samples. After 24 h, the culture media were replaced and the tumor cells were treated with HBO alone for 90 min at 2.5 ATA. The culture media were replaced again after 24 h and after a further 48 h, the apoptotic index was analyzed with a TUNEL test.

Control. The tumor cells were treated with PBS (as placebo) equal in quantity to that used to dissolve gemcitabine for administering to the experimental samples. After 24 h, the culture media were replaced. The culture media were replaced again after 24 h and after another 48 h the AI was analyzed with a TUNEL test.

GEM-HBO. Gemcitabine, at the IC₅₀ value for the tumor cells was administered the tumor cells cultured for 24 h. The drug was then eliminated by replacing the culture media. The tumor cells were then treated with HBO at 2.5 ATA for 90 min. The culture media were replaced 24 h later. The apoptotic index was analyzed with a TUNEL test after 48 h. The AI was calculated as: AI=total number of apoptotic cells counted/ total number of live cells counted.

HBO-GEM. The tumor cells were first treated with HBO at 2.5 ATA for 90 min. After 24 h, the culture media were replaced and the tumor cells were treated with gemcitabine at the IC₅₀ value. After 24 h, the culture media were replaced again and after another 48 h, the AI was analyzed with a TUNEL test.

GEM+HBO. Gemcitabine at the IC₅₀ value for the tumor cells was administered and HBO was administered for 90 min at 2.5 ATA at the same time. After 24 h, the culture media were replaced and after a further 48 h, the apoptotic index was analyzed with a TUNEL test.

Table I. Effect of hyperbaric oxygen (HBO) treatment and gemcitabine (GEM) on apoptosis of PANC-1 and AsPc-1 pancreatic ductal adenocarcinoma cells. Apoptotic indices are presented as mean±SD. HBO *per se* had no significant effect on apoptosis of tumor cells. Gemcitabine significantly induced apoptosis of tumor cells. HBO significantly enhanced gemcitabine-induced apoptosis only when administered during chemotherapy. Multiple comparisons among groups were analyzed as described in Materials and Methods. (The number of tumor cells for each group were 4×10^3 cells/cm² in Petri dish).

Cell lines	Treatment					
	Control	HBO	GEM	HBO-GEM	GEM-HBO	HBO+GEM
PANC-1	5.9±0.1	6.5±0.1	8.1±0.1* #	8.2±0.1* #	8.5±0.1* #	10.7±0.1** ## !!
AsPc-1	5.9±0.1	5.9±0.1	8.0±0.1* #	8.2±0.1* #	8.4±0.1* #	9.7±0.1** ## !!

Control: Without treatment; HBO: treated with HBO alone for 90 min at 2.5 ATA; GEM: treated with GEM alone; HBO-GEM: treated with HBO at 2.5 ATA for 90 min then after 24 h treated with GEM; GEM-HBOT: treated with GEM for 24 h then placed in drug-free culture media and treated with HBO; GEM+HBO: HBO was administered for 90 min at 2.5 ATA during therapy with GEM. * $p < 0.01$ vs. control; # $p < 0.01$ vs. HBO; !! $p < 0.01$ vs. GEM, HBO-GEM and GEM-HBOT.

Each treatment was repeated three times and for each sample, the cells in 23 randomly selected fields of 0.157 mm² within a 5 cm² area were counted for apoptotic and living cells. During the measuring of apoptotic cells, the number of all tumor cells in each group was counted to determine the tumor cell growth under different treatments.

Statistical analysis. Results are presented as the mean±SD. Multiple comparisons among groups were analyzed by one-way analysis of variance followed by the Tukey–Kramer method for post-hoc analysis after confirmation of normal distribution of the data. Tukey 95% simultaneous confidence intervals were applied, or p -value <0.05, when equality assumptions were rejected simultaneously. The individual confidence level was 99.54%, or with p -value <0.0046.

Results

The apoptotic indices are summarized in Table I. HBO *per se* had no significant effect on the induction of apoptosis in either cell line. HBO when administered before or after gemcitabine administration also had no significant effect on gemcitabine-induced apoptosis in either tumor cell line. HBO enhanced gemcitabine-induced apoptosis in both tumor cell lines only when administered concurrently with gemcitabine.

There was no significant change in the total number of tumor cells with therapy using HBO-alone or the control group (data not shown).

Discussion

The main findings of the present study are that: i) HBO-alone had no significant effect on inducing tumor cell apoptosis; ii) gemcitabine-alone significantly increased apoptosis of tumor cells; iii) administration of HBO before or after gemcitabine treatment did not further increase apoptosis; and iv) administration of HBO concurrently with

gemcitabine treatment significantly enhanced gemcitabine-induced apoptosis of the tumor cells. The AI was used to evaluate antitumor activity of Cox inhibitor in a canine model of human invasive urinary bladder cancer (21). The AI was used in the present study to evaluate the efficacy of HBO, gemcitabine and their combination in the induction of apoptosis of pancreatic tumor cells.

Solid tumors survive and proliferate in a hypoxic environment. These slowly-proliferating hypoxic tumor cells are less susceptible to chemotherapy and radiotherapy because molecular oxygen and reactive oxygen species (ROS) are essential for such therapies (6). Therapeutic efficacy may be improved if the hypoxic state of the tumor cells is eliminated. HBO is one of the modalities to significantly increase oxygen availability around tumor cells and thus has been evaluated as adjuvant to radiotherapy and chemotherapy (6). It has been a concern that HBO induces re-oxygenation of hypoxic tumor cells and an increase of angiogenesis may potentially promote tumor cell growth and recurrence of malignancy (6). Comprehensive investigations are mandatory before HBO can be safely used in clinical cancer therapy.

Tang *et al.* investigated the effect of HBO on tumor growth in an *in vivo* murine model of indolent prostate cancer (9). Mice with induced tumors were randomized to undergo 20 sessions of either therapy with HBO or air, under standardized conditions and were observed for 4 weeks before histological assessments of any palpable tumors that had developed. It was found that exposure to HBO had no effect on prostate cancer volume, tumor microvessel density, proliferative index, and apoptosis markers in comparison to the non-HBO group. Their study showed that HBO had no tumor-stimulatory effect on prostate cancer. They suggested that HBO may potentially be used safely in conjunction with other therapeutic modalities (9). Lu *et al.* also reported that

HBO inhibited proliferation in glioma U251 cells *in vitro* (10). The natural doubling time for the tumor cells used in the present study was approximately 52 h. The proliferation of tumor cells was not examined in the present study because treatment caused changes that may not be detectable 24 h after treatment (9). Nevertheless, we did record tumor cell numbers in the group treated with HBO-alone and control group and found there was no significant difference in the cell numbers in both groups, therefore ruling out a possible stimulating effect of HBO on growth. As mentioned by Schonmeyer *et al.*, conflicting results on the effects of HBO on tumors have been presented (22). Thus, some studies have shown suppressed tumor growth and reduced metastatic rates after HBO (23, 24); others have demonstrated enhanced tumor progression or no effects at all (25, 26). Schonmeyer *et al.* data showed that HBO does not accelerate squamous cell proliferation or promote tumor growth. Furthermore, their data showed that although HBO may decrease hypoxia within the tumors, there is no evidence of HBO altering angiogenesis or vascular invasion in squamous cancer cell tumor deposits (22). Our study is in agreement with the above findings that HBO has no tumor-stimulating effect in the pancreatic tumor cell lines studied.

Lu *et al.* reported that HBO-alone inhibited cell proliferation and induced apoptosis of glioma U251 cells *in vitro* (10). In our study, HBO-alone had no significant effect on the AI for pancreatic tumor cells. It is known that HBO increases the free oxygen radical level, which may cause tumor cell damage. In their study, HBO was given three times at 12 h intervals. Multiple HBO sessions may exceed the tumor cells' endogenous cellular antioxidant capacity, thus creating oxidative stress leading to cell damage. Consistent with the evidence that most chemotherapy agents cause tumor cell death primarily by inducing apoptosis, resistance to anticancer treatment is widely believed to involve mutations that lead to de-regulated cellular proliferation and suppression of mechanisms that control apoptosis. It has been observed that tumors with exhibiting AI after one cycle of chemotherapy are more likely to achieve pathological regression. Increased apoptosis after chemotherapy may also predict which patients will have a good pathological response (27). The utility of HBO as adjuvant therapy in tumor treatment is still under investigation. Bradfield *et al.* observed four patients with head and neck cancer who apparently had rapid progression of clinically-occult disease during or soon after undergoing HBO (28). However, other studies have reported on the beneficial effect of HBO as adjuvant therapy in tumor treatment. Haffty *et al.* conducted a randomized clinical trial to evaluate the role of HBO as adjuvant to radiotherapy in the treatment of locally advanced squamous cell carcinoma of the head and neck (29). The patients were randomized to radiation-alone or radiation under HBO over 21 days to a total of 23 Gy. There was a highly significant

difference in complete clinical responses between the two groups, with 21/25 complete clinical responses in the HBO treated group compared with 13/25 complete clinical responses in the control group, and a statistically insignificant trend towards improved 5-year local control in the HBO treated group (29% vs. 16%). There were no significant differences between the two groups with respect to 5-year survival, distant metastasis, or second primary tumors. They concluded that the long-term outcomes from their randomized trial demonstrated substantial improvements in response rate after use of HBO. McDonald *et al.* conducted a study using chemical carcinogen-induced squamous cell carcinoma in Golden Syrian hamsters to determine the effects of HBO on tumor management (30). Twenty hamsters underwent 30 HBO sessions for 60 min each to 2.81 ATA, while 20 untreated served as controls. At necropsy, animals receiving HBO had significantly smaller tumors and had a trend toward fewer cervical metastases. Ohgami *et al.* also demonstrated that HBO enhanced anticancer activity of artemisinin in Molt-4 human leukemia cells resulting in an additional 22% decrease in growth (31). The present study shows that HBO, only when simultaneously administered with gemcitabine significantly increased apoptosis of pancreatic tumor cells *in vitro*. Time may be an important factor to determine the efficacy of therapeutic management. A recent clinical trial showed that radiotherapy delivered immediately after HBO with chemotherapy was safe, with virtually no late toxicities, and seemed to be effective in patients with high-grade gliomas (32). An *in vitro* study using highly metastatic murine osteosarcoma cell lines found that HBO-alone significantly suppressed cell proliferation, and HBO-plus carboplatin exhibited significant synergism in suppression of cell proliferation. Authors also reported that concomitant HBO clearly enhanced the chemotherapeutic effects of carboplatin on both tumor growth and lung metastasis in osteosarcoma-bearing mice (6). Further study is needed to find an ideal HBO regime to maximize its enhancing effect for chemotherapy and minimize its adverse effect. In the present study, there was no effect on apoptosis if HBO was administered either before or after chemotherapy. Since our study was *in vitro*, the tumor cells were placed under an oxygen-rich environment only when HBO was provided. Enhanced antitumor activity of gemcitabine occurred only when tumor cells were under hyperbaric hyperoxic condition. Our study supports the notion that HBO may be useful as adjuvant to chemotherapy in the management of cancer treatment (5). Our study also suggests that the time window may be critical for using HBO during chemotherapy.

Although is still not fully-understood, multiple mechanisms may be responsible for the enhancement of gemcitabine induced apoptosis by HBO. HBO has been reported to directly increase the uptake of 5-fluorouracil in dimethyl- α -benzanthracene-induced mammary tumors *in vivo* (33). It is known that some chemotherapeutic drugs require oxygen to

generate free oxygen radicals that in turn induce cytotoxicity. HBO may disturb the membrane components of cells following oxidation phenomena caused by ROS overproduction. A change in the properties of any one membrane components is anticipated to change the conductance of membrane-spanning ion channels and thus cell function. HBO therefore may facilitate drug access to the cell, if the drug is already close to the cell. HBO may also slow down the elimination of the drug due to inhibition of protein response, increase membrane thickness, or oxidation of the amino acids of transport proteins, in particular of multidrug resistance proteins, thanks to increasing intracellular ROS (10, 34). Multidrug resistance arises primarily due to the up-regulation of proteins from the adenosine triphosphate binding cassette transporter family. Sensitivity to chemotherapy is strongly dependent on the expression of multidrug-resistance related transporters (35). HBO has been observed to inhibit multi drug-resistance-related transporters therefore enhancing anticancer activity of chemotherapy (9). Finally, TNF- α is implicated in the processes of tumor growth, survival, differentiation, invasion, metastasis, secretion of cytokines and pro-angiogenic factors. HBO has been reported to inhibit TNF- α production in ischemia reperfusion tissue injury (36, 37). HBO was also reported to reduce interleukin-1 production of macrophage (38). Since interleukin-1, in particular, is able to stimulate metastasis and growth of the cell lines we used here, HBO might enhance apoptosis by inhibiting its production (39).

In summary, HBO-alone was found to have no effects on apoptosis of pancreatic tumor cells *in vitro*. HBO did not enhance gemcitabine-induced apoptosis of pancreatic tumor cells when administered before or after chemotherapy. HBO significantly enhanced gemcitabine-induced apoptosis of pancreatic tumor cells when administered concomitantly with chemotherapy. Our study suggests that the time window of therapy is critical for effectively using HBO as adjuvant to chemotherapy.

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