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Effect of hyperbaric oxygenation and gemcitabine on apoptosis of pancreatic ductal tumor cells *in vitro*

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies [1], ranking 4th among causes of cancer-related death in the Western world [2]. Unlike most of the more frequent causes of cancer mortality (lung, colon, prostate and breast cancers) whose death rates are declining, the death rate for pancreatic cancer is increasing (figure 1).

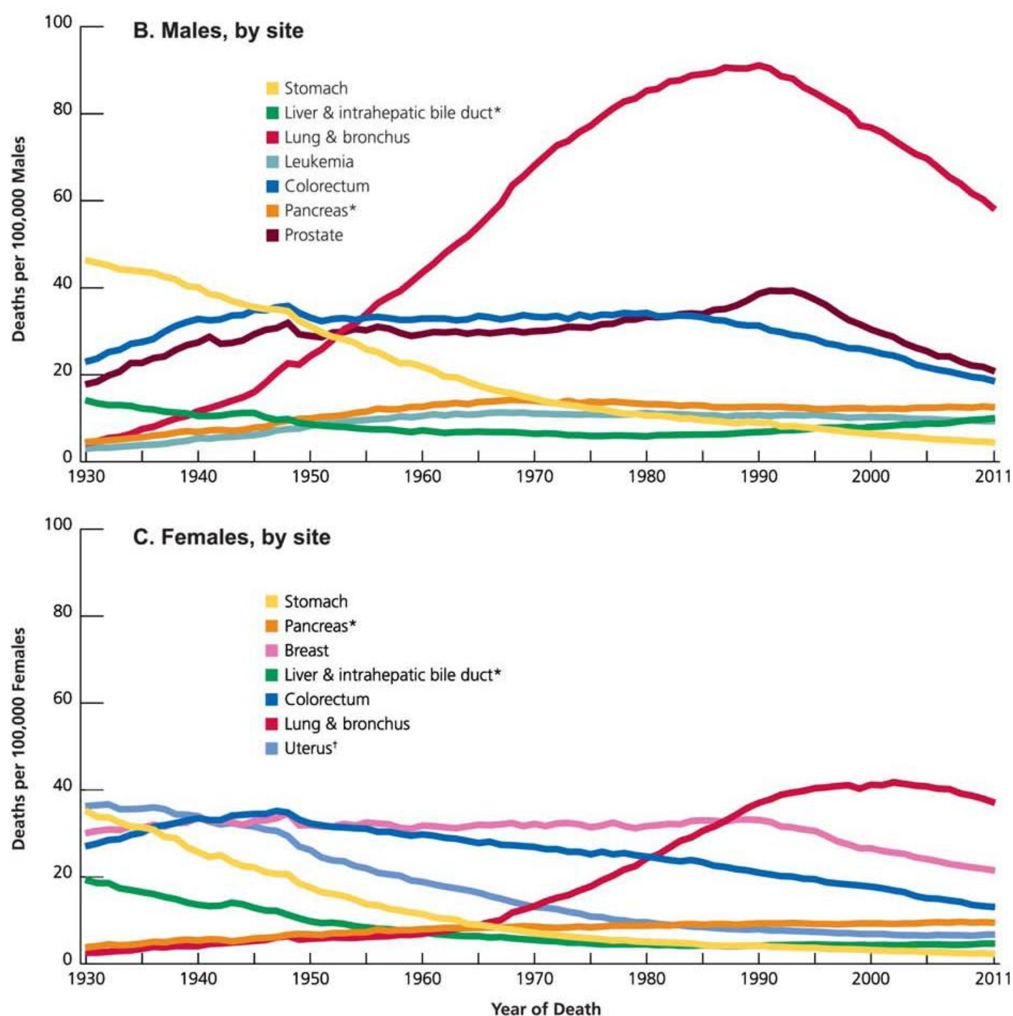

Figure 1: Trends in death rates overall and for selected sites by sex. United States, 1930 to 2011. Rates are age adjusted to the 2000 US standard population. Due to changes in International Classification of Diseases (ICD) coding, numerator information has changed over time. Rates for cancers of the lung and bronchus, colorectum, liver, uterus, and ovary are affected by these changes.
***Mortality rates for pancreatic and liver cancers are increasing.**
†Uterus includes uterine cervix and uterine corpus [2].

Exocrine pancreatic cancer is rarely curable and has an overall survival (OS) rate of less than 6% [1]. The highest cure rate occurs if the tumor is truly localized to the pancreas; however, this stage of disease accounts for less than 20% of cases. For patients with localized disease and small cancers (<2 cm) with no lymph node metastases and no extension beyond the capsule of the pancreas, complete surgical resection is associated with a 5-year survival rate of 18% to 24% [3]. The poor prognosis is reflected by a median survival of 5-8 mo and a 5-year survival of less than 5% when all stages are combined [4-6]. PDAC is characterized by a rapid disease progression and absence of specific symptoms, largely precluding an early diagnosis and curative treatment [6,7]. In most cases, PDAC is already locally advanced at time of diagnosis and only approximately 10%-20% [4,8] of patients are considered candidates for curative resection. The majority of patients (50%-60%) present with metastatic disease, and thus palliative chemotherapy remains the only option for almost all of these patients [9]. Owing to the high recurrence rate, surgical PDAC patients require adjuvant chemotherapy with or without radiotherapy providing a 5-year survival rate of 15%-25% [10]. There is no consensus on what constitutes 'standard' adjuvant therapy. This controversy derives from several studies, each fraught with its own limitations. Standards of care also vary depending on which side of the Atlantic you are on: chemo-radiotherapy followed by chemotherapy is considered the optimal therapy in North America (Gastrointestinal Tumor Study Group: GITSG; Radiation Therapy Oncology Group: RTOG) while chemotherapy alone is the current standard in Europe (European Study Group for Pancreatic Cancer: ESPAC-1; Charité Onkologie: CONKO) [11]. Hyperbaric oxygen therapy (HBO) is a complementary therapy that consists in intermittent delivery of 100% oxygen, at

elevated atmospheric pressures 2,5 ATA, for a short period of time (90 minutes). The therapy's main effect is to elevate the oxygen dissolved within plasma and completely saturate hemoglobin. In cells, this means a ROS (Radical Oxygen Species) and RNS' (Reactive Nitrogen Species) raised level [12], balancing by the contemporary increment of antioxidant enzymes [13], and increasing thickness and blebbing formation in cell membrane [14]. HBO is commonly used in the treatment of decompression sickness, carbon monoxide intoxication, arterial gas embolism, necrotizing soft tissue infections, chronic skin ulcers, severe multiple trauma with ischemia and ischemic diabetic foot ulcers [15-26]. 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 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 [27]. 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. 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 [28-31]. 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 [32]. 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 [5]. 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 [33]. We hypothesized that a combination of HBO and gemcitabine may significantly enhance the efficacy of gemcitabine for pancreatic tumors. In the study PANC-1 and AsPc-1 cells were used 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.

PANC-1 is a cellular line very sensitive to gemticabine with a IC_{50}, that represents the concentration of an inhibitor that is required for 50% inhibition of its target, of $3.25x10^{-8}$ ng/mL [34]. It derived from a 56-years old man with an unserectable head pancreatic cancer (stage III sec. AJCC) and it was studied for the first time by Lieber et al in 1975 [35]. Cells presented a duplication time of 56 h [36].

AsPc-1 is a cellular line with moderate sensibility to gemcitabine with a IC_{50} of $1.27x10^{-7}$ ng/mL [34] derived from a 62-years old man affected by an unresectable head pancreatic cancer stage IV sec. AJCC. This linear cell was studied for the first time in 1982 by Chen et al [37]. Cells presented a duplication time of 56 h [36].

Tumor cells were derived from pancreatectomy samples from patients with pancreatic adenocarcinoma. 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

(IC_{50}) for cultured tumor cells. Its IC_{50} value for PANC-1 cell is 3.25×10^{-8} ng/ml and for AsPc-1 cell is 1.27×10^{-7} ng/ml [34].

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/cm^2 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 IC_{50} 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.

The composition of PBS solution (Phosphate Buffered Saline) is reported below:

NaCl	(SIGMA S7653)	0.138 M
KCl	(SIGMA P9333)	0.0027 M
Na_2HPO_4	(SIGMA S0876)	0.015M
KH_2PO_4	(SIGMA P9791)	0.0015 M

Table 1: Composition of PBS solution.

GEM-HBO. Gemcitabine, at the IC_{50} 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.

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 IC_{50} value. After 24 h, the culture media were replaced again and after another 48 h, the AI was analyzed with a TUNEL test.

HBO+GEM. Gemcitabine at the IC_{50} 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.

Each treatment was repeated three times and for each sample, the cells in 23 randomly selected fields of 0.157 mm^2 within a 5 cm^2 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.

Apoptotic Index (AI). The AI was calculated as: AI=total number of apoptotic cells counted/ total number of live cells counted.

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 2. HBO *per se* had no significant effect on the induction of apoptosis in either cell line. HBO when administrated before or after gemcitabine administration also had no significant effect on gemcitabine-induced apoptosis in either cell line. HBO enhanced gemcitabine-induced apoptosis in both tumor cell lines only when administrated 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).

Treatment						
Cell lines	**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*#!

Table 2: 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 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 administrated during chemotherapy. The number of tumor cells for each group was $4x10^3$ cells/cm^2 in Petri dish. 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-HBO: treated with GEM for 24 h then placed in drug-free culture media and treated with HBO; HBO+GEM: HBO was administrated 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-HBO.

Discussion

Despite the improved survival rates noted in numerous cancers, including breast [38-40], prostate [41] and colon cancer [42], the overall survival rates for patients diagnosed with pancreatic cancer have shown little improvement over the past thirty years [43-45]. PDAC remains one of the most rapidly progressive and deadly malignancies worldwide [2]. The prevention of pancreatic cancer is difficult to assess, due to limited studies identifying potential risk factors compounded with the multifactorial, heterogeneous nature of the disease. Cigarette smoking has been noted to double the risk of pancreatic cancer, yet only accounts for 20%-25% of the cases [46,47]. Additionally, family history may also contribute a significant role as 5%-10% of individuals with pancreatic cancer report an incidence of pancreatic cancer in a close family member [48]. This risk is further substantiated when there is a larger number of family members with pancreatic cancer and a decrease in age of onset in kindred [49]. Other noted risk factors include alcohol abuse [50], a high-fat diet [51,52], and certain trace elements [53]. The challenge of diagnosing PDAC at an early stage further contributes to the dismal five-year survival rate that is projected for patients. Located in the retroperitoneum of patients who present with non-specific symptoms, PDAC is not diagnosed until it has reached an advanced clinical stage in 80% of patients [54]. In addition, lack of effective screening and early biomarker detection methods have prevented clinicians from identifying this cancer in a pre-malignant stage. Ideally, visual evaluation via computerized tomography (CT) and magnetic resonance imaging (MRI) should be incorporated upon suspicion of pancreatic cancer for detection and respectability assessment [55]. Although CT scan

has often been utilized to detect pancreatic cancer [56,58], reliance on MRI, particularly in regard to assessing local invasion and metastasis, has increased [59]. Other imaging may also provide certain benefits, such as endoscopic ultrasound for investigating vascular invasion [60], fludeoxyglucose-positron emission tomography scanning for recurrent tumors [61], and laparoscopy for more accurate staging [62]. While the use of these techniques remains helpful to determine prognosis and treatment regimen for patients diagnosed with pancreatic cancer, none have been validated as effective screening tests for general or high risk populations. Once diagnosed, a number of chemotherapy, radiation and combination therapy regimens have been used to treat patients with ductal pancreatic tumors. Unfortunately, the dynamic molecular and cellular makeup of individual pancreatic tumors, renders many of them resistant to the majority of these therapies. Although surgical resection has been shown to increase patient survival by 10 mo [63], the majority of patients who undergo these procedures experience comorbidities and recurrence.

Current research has identified additional sources of therapeutic resistance in the microenvironment of these tumors. Characterized by stromal proliferation, reduced angiogenesis and a unique subset of cells known as cancer stem cells (CSCs), the tumor microenvironment has become a target of new therapeutic agents.

While improved understanding of pancreatic cancer biology has lead to several therapeutic breakthroughs in the treatment of PDAC, major progress toward improving survival rates in patients has been extremely slow. However, as our understanding of this tumor's therapeutic resistant nature improves, so will future progress in treating pancreatic cancer. There is no consensus on what constitutes

'standard' adjuvant therapy. This controversy derives from several studies, each fraught with its own limitations [64].

The GITSG trial was the first prospective randomized trial suggesting survival advantage with postoperative chemo-radiotherapy using bolus 5-FU (median survival: 20 months vs. 11 months and 5-year survival:18% vs. 8%) [65]. However this study was criticized for poor patient accrual, early termination, and small patient numbers, and some maintained that the radiotherapy dose was suboptimal (some authors advocate 50 Gy as a total effective dose). Multiple authors have attempted to confirm its findings. The European Organization of Research and Treatment of Cancer (EORTC) compared 5-FU (25 mg/kg/day continuous infusion for 5 days every 4 weeks) with concurrent radiotherapy using a split course (40 Gy) with observation only in patients with resected pancreatic and periampullary cancer [66]. Klinkenbijl et al were able to show a trend toward benefit in terms of median survival (24.5 months vs. 19.0 months; p=0.208). The subgroup analysis looking only at pancreatic cancer patients showed a trend toward benefit in median survival (17.1 months vs. 12.6 months; p=0.099) [66]. This study too was criticized for suboptimal dose of radiotherapy and split courses. Lower radiotherapy dose and split courses that may have allowed cancer repopulation between courses thereby under-estimating the benefit of chemo-radiotherapy. Although not conclusive, these results showed a trend toward benefit of adjuvant therapy and led to the ESPAC-1 trial, the largest reported randomized study to date investigating the role of combination chemo-radiotherapy in pancreatic cancer [67]. This study, in fact, has sparked a new debate over the role of radiotherapy in the adjuvant therapy of pancreatic cancer. ESPAC-1 trial was 2x2 factorial designed study comparing adjuvant concurrent chemo-

radiotherapy (bolus 5-FU/split course radiotherapy), chemotherapy alone (5-FU/leucovorin), chemo-radiotherapy followed by chemotherapy, and observation. Chemotherapy only arm had statistically significant benefit over observation arm in median survival (20.1 months vs. 15.5months; p=0.009). However, chemoradiotherapy arm showed worse median survival compared with patients who did not receive chemo-radiotherapy (15.9 months vs.17.9 months; p=0.05) [66]. Interpretation of this study is complicated slightly because two different study designs are used: a 2x2 factorial design and direct head-to-head comparisons (chemotherapy vs. no chemotherapy and chemo-radiotherapy vs. no chemo-radiotherapy). Eligible patients were pre-enrolled in one of the above strategies. The authors then reported their findings for each of the separate study designs as well as for the pooled data. Therefore, major criticism was made on this study for possible selection bias as both patients and clinicians were allowed to select which trial to enter, a concern of suboptimal radiotherapy, and for allowing the final radiotherapy dose to be left to the judgment of the treating physicians. Moreover, the treatment for patients in the chemo-radiotherapy group did not include post-radiotherapy adjuvant chemotherapy, making direct comparison to the GITSG trial difficult. The ESPAC-1 study uses only a 5-FU-based chemotherapy regimen; and certainly, a gemcitabine-based approach is the most logical place to start, which was evaluated in the RTOG 9704 study. RTOG 9704 study randomized 538 resected pancreatic cancer patients to evaluate benefit of adding gemcitabine to infusional 5-FU combined with radiotherapy [68]. One arm received 5-FU plus radiotherapy and the other arm was treated with gemcitabine before and after 5-FU plus radiotherapy. Patients with pancreatic head tumors (No. 380) showed benefit in median survival

(18.8 months vs.16.7 months; p=0.047) by the incorporation of gemcitabine before and after 5-FU plus radiotherapy. However, there was no significant difference when pancreatic body and tail cancers were all included. While benefit of radiation therapy was inconclusive in randomized trials, Oettle et al published the results of CONKO-001 study in JAMA in 2007 [69]. CONKO-001 study randomized 368 patients with resected pancreatic cancer to gemcitabine or observation for 6 months. Tumor prognostic characteristics were similar in both arms. This trial showed statistically significant disease free survival benefit (13.4 months vs. 6.9 months; p<0.001) of gemcitabine over observation. Gemcitabine rendered a trend toward overall benefit (22.1 months vs. 20.2 months; p=0.06). This benefit of chemotherapy was consistent with the result from ESPAC-1 trial which showed benefit of 5-FU/leucovorin over no adjuvant therapy in pancreatic cancer patients (median survival of 19.7 months vs. 14.0 months) who had complete resection [67]. The CONKO-001 study has many worth mentioning points. Gemcitabine, the current standard of care in first line treatment, has clearly showed superiority over 5-FU in patients with advanced pancreatic cancer, both in terms of dramatic improvement in clinical benefit response as well as a modest improvement in median survival [70]. Therefore, ESPAC-1 (in which 5-FU was the chemotherapy agent of choice) and the Burris et al study both provide a rationale for choosing gemcitabine arm in CONKO-001 study [67,70]. CONKO-001 study also reconfirmed that single-agent chemotherapy with gemcitabine was generally well-tolerated in this study and most of the patients were able to complete the full six cycles of treatment [69]. On the other hand, the median disease free survival of patients in the observation-only was dismal (less than 7 months), underlying the fact in addition to further improve the adjuvant treatment

regimens, specialized surgeries such as Whipple's procedure should preferentially be carried out at high volume centers by experienced surgeons, where outcomes are known to be better [71]. Recent studies demonstrated that neoadjuvant chemoradiation is safe with respect to toxicity, perioperative morbidity, and mortality [72,73].

The cellular mechanisms of therapeutic resistance in pancreatic cancer are not clear, but the mutations for the KRAS2 oncogene, resulting in the constitutive production of the Ras protein [74-77] maybe is important. Occurring early in tumorogenesis, these point mutations are essential for maintaining the malignant phenotype because once activated, Ras initiates a signal transduction cascade that activates proliferative and cell survival pathways and increases cell invasion [78,79]. The majority of the point mutations occur on codon 12 of the ras protein and give rise to pancreatic tumor-specific neo-antigens. The mutations of K-ras stimulate the extracellular proliferation of leukocytes, fibroblasts, endothelial cells, neuronal cells, collagen and hyaluron. This extracellular proliferation of cells is known as a desmoplastic reaction. The desmoplastic reaction not only provides a mechanical barrier to the pancreatic cancer cells, but it is also thought to contribute to the anti-angiogenic environment that is characteristic of PDAC.

Rather than being activated like the mutated KRAS2 oncogene, the p53 tumor suppressor gene is inactivated in 75%-90% of pancreatic tumors. As a result, there is an impaired response to DNA damage in pancreatic epithelial cells, impaired apoptosis and impaired cell cycle control. Two other tumor suppressor genes, *p16Ink4a* and *p15ARF* are encoded by the cdkn2a locus. Inactivation mutations in these genes are present in about 90% of human PDAC. A fourth common mutation

seen in more than half of pancreatic cancers causes an alteration in DPC4 (Deleted Pancreatic Cancer, locus 4 cromosome 18). DPC4 is a gene that codes for a protein important in the TGF-β superfamily of signaling pathways. The knock out of the TGF-β gene in a cancer cell, also inactivated the TGF-β pathway. These pathways usually act to promote differentiation (maturation to normal adult forms) and to slow the growth of cells. TGF-β has a role in humans similar to that in other species, and most normal cells stop proliferating when exposed to TGF-β. So, the mutations of DPC4 confer a metastatic phenotype [64].

In this study we analyzed the role of a single treatment with hyperbaric oxygen in pancreatic ductal adenocarcinomas cells, wondering if oxygen plays a role as promoter the tumor cells growing, as inhibitor the tumor cell growing or as adjuvant a chemotherapeutic drug's activity, in our case, gemcitabine. 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;

iv: administration of HBO concurrently with gemcitabine treatment significantly enhanced gemcitabine induced apoptosis of the tumor cells.

A possible mechanism of HBO mediating beneficial effects has been described as attenuation of the production of proinflammatory cytokines in response to an inflammatory stimulus such as surgery [80-82] and modulation of the immune response [82-84]. In fact, HBO is known to promote new vessel growth into areas with reduced oxygen tension due to poor vascularity, and therewith promotes

wound healing and recovery of radiation-injured tissue. Hyperbaric oxygen therapy applies, according to HUMS guide lines [85], can be use to treat all pathologic conditions where hypoxia or ischemia are clinically relevant. In the last years, many studies evaluate HBO efficacy in treating tumors [86]; in fact in solid tumors there are extended hypoxic areas [87] so it may be rational to use hyperbaric oxygen therapy. There are evidence that HBO therapy could elevate oxygen delivery to hypoxic cells and so increase their susceptibility to radiotherapy or chemotherapy [86,88]. The correlation between hypoxia and tumor growth is unclear, but there is a correlation between hypoxia and Hypoxia Inducible Factor (HIF)-1α. HIF-1α is a transcription factor that regulates the transcription of genes associated with cell proliferation and vascular development. In various cancer tissues, HIF-1α is associated with clinicopathological factors, such as the tumor size, histological grade, and lymph node status. Although HIF-1α plays a critical role in tumor growth by inducing vascular endothelial growth factor (VEGF), it is unclarified whether HIF-1α affects lymphatic metastasis. As dimostrated by Katsuta et al [89] in esophageal squamous cell carcinoma (ESCC), the expressions of HIF-1α and VEGF-C, which is one of the main lymphangiogenic factors, were expressed in all ESCC cell lines. Immunohistochemically, 34 of the 48 patients (70.8%) were positive for HIF-1α and 29 patients (60.4%) were positive for VEGF-C. Clinicopathologically, HIF-1α expression correlated with lymphatic invasion and VEGF-C expression ($p=0.003$ and $p=0.01$, respectively). Furthermore, HIF-1α expression tended to correlate with lymph node metastasis ($p= 0.09$). These findings suggest that HIF-1α plays a role in lymphatic invasion and lymph node metastasis through the induction of VEGF-C in ESCC. In a very recent paper, Wang et al demonstrated that Reactive Oxygen Species

(ROS) can drive the de-differentiation of tumor cells leading to the process of epithelial-to-mesenchymal transition (EMT) to enhance invasion and metastasis by the HIF-1α/LOX/E-cadherin via. The invasive and metastatic phenotype of malignant cells is often linked to loss of E-cadherin expression, a hallmark of EMT. The hypoxic exposure causes HIF-1α -dependent repression of E-cadherin. Wang et al found that ROS accumulation in ovarian carcinoma cells upregulated HIF-1α expression and subsequent transcriptional induction of lysyl oxidase (LOX) which repressed E-cadherin. Loss of E-cadherin facilitated ovarian cancer (OC) cell migration in vitro and promoted tumor growth in vivo [90].

In a very interesting paper, Zhu et al in 2013 [91] demonstrated that HIF-1α plays a crucial role in the maturation process from Cancer staminal cells (CSCs) *naïve* in CSCs $CD133^{+}$. CD133 is a marker protein typically used to identify and isolate human pancreatic CSCs [92]

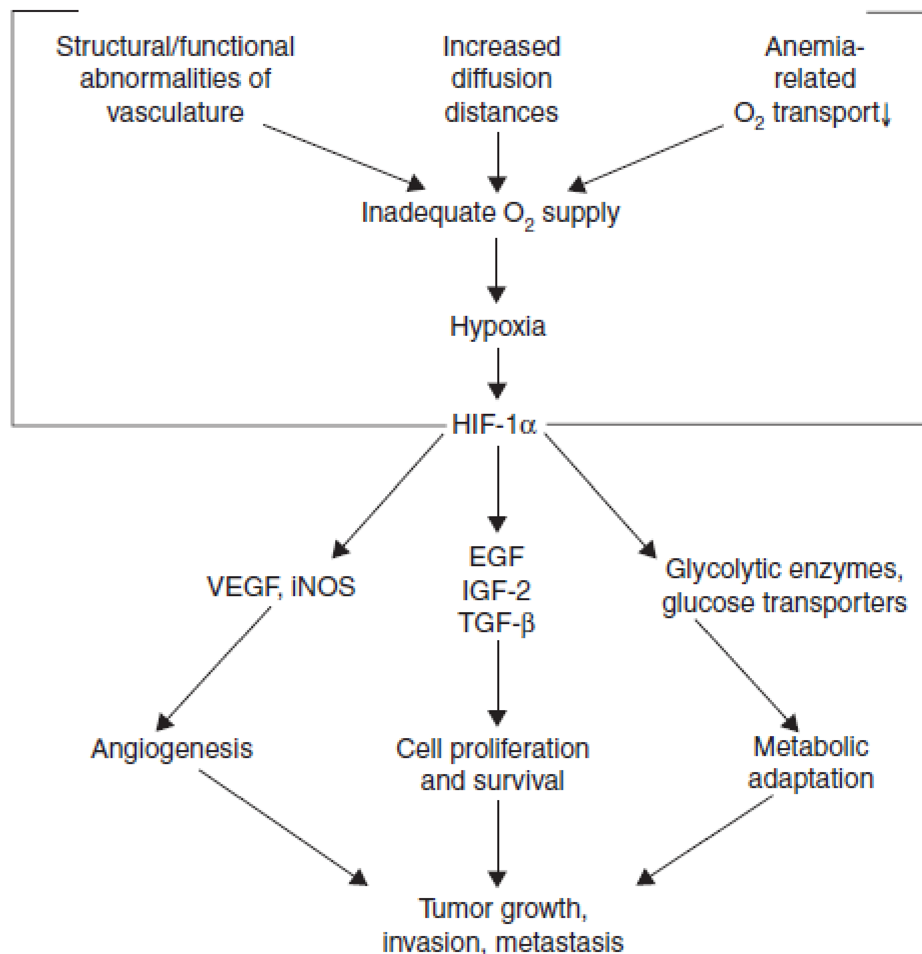

Figure 2: Expression of HIF-1 in human cancer: causes and consequences [87].

In fact, under hypoxic conditions, HIF-1α subunits translocate to the nucleus, where they heterodimerize with HIF-1β subunits. The resultant product is an active HIF-1 protein that binds to specific hypoxic response elements present in target genes, ultimately activating transcription of these genes. In particular, Vaupel [87] demonstrated that under hypoxic conditions, HIF-1β dimerizes with HIF-1α and the active HIF-1 dimer binds to hypoxia response elements containing the core recognition sequence 5'-RCGTC-3' and then recruits coactivator molecules, resulting in the formation of an increased transcription initiation complex and mRNA

synthesis, leading ultimately to the biosynthesis of proteins that mediate responses to hypoxia.

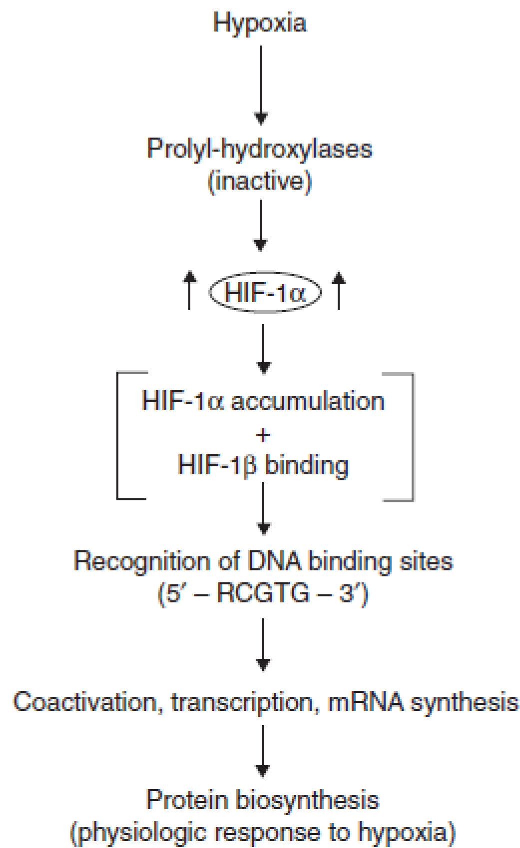

Figure 3: Regulation of HIF-1α by cellular O_2 level [87].

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. 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. 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 [86]. 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 [93]. 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 [93]. Lu et al also reported that HBO inhibited proliferation in glioma U251 cells in vitro [94]. 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 [93]. 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 Schonmeyr et al, conflicting results on the effects of HBO on tumors have been presented [95]. Thus, some studies have shown suppressed tumor growth and reduced metastatic rates after HBO [96,97]; others have demonstrated enhanced tumor progression or no effects at all [98,99]. Schonmeyr et al 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 [95]. 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 [94]. 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 [100]. 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 [101]. 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 [102]. The patients were randomized to radiation-alone or radiation under HBO over 21 days to at 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 [103]. Twenty hamsters underwent 30 HBO seasons 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 [104]. 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 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 [105].

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 [86]. 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 [106]. 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 dimetyl-α- benzantracene-induced mammary tumors in vivo [107]. It is known that some chemotherapeutic drugs require oxygen to enerate 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 [94,108]. 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 [109]. HBO has been observed to inhibit multi drug-resistance-related transporters therefore enhancing anticancer activity of chemotherapy [93].

Conclusions

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 [110,111]. HBO was also reported to reduce interleukin-1 production of macrophage [112]. 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 [113]. The HBO is also important in the regulation of HIF-1α level [91].

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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