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The effect of hyperbaric oxygen therapy on tumour growth in a mouse model of colorectal cancer liver metastases

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ABSTRACT

Background and aims: Hyperbaric oxygen (HBO) therapy involves the administration of 100% oxygen at high pressure. It has been used to treat a variety of conditions including non-healing wounds, carbon monoxide poisoning, and as an adjuvant to radiotherapy or chemotherapy. The effect of HBO alone on the growth of malignancy remains controversial. This study investigates the impact of HBO on tumour growth, kinetics and microcirculation of colorectal cancer liver metastases in an experimental model.

Methods: Male CBA mice were induced with colorectal liver metastases *via* an intrasplenic injection of a murine derived colorectal cell line. Tumours were examined using quantitative stereological analysis, histology and scanning electron microscopy of microvascular resin casts. The effect of HBO on tumour proliferation and apoptosis was quantified using immunohistochemistry.

Results: Daily exposure to HBO at 2.4 atm for 90 min had no effect on the volume of liver metastases. At day 13, HBO caused a significant reduction in tumour necrosis and proliferation compared to the non-HBO group ($p = 0.002$ and $p = 0.008$, respectively). By day 25 however, no differences were observed ($p > 0.05$). No differences in apoptosis or microvascular architecture were observed.

Conclusion: HBO did not have a tumour stimulatory effect on colorectal liver metastases and may potentially be used safely in conjunction with other therapeutic treatment modalities.

1. Introduction

Hyperbaric oxygen (HBO) has been applied clinically for the treatment of ischemic or non-healing wounds and radiation-injured tissue.[1–5] It has also been applied in conjunction with radiotherapy or chemotherapy for the treatment of malignancy. The effect of HBO on the natural history of malignancy is uncertain. Minimal evidence exists regarding the direct effect of HBO on tumour cell kinetics. HBO has been previously contraindicated in the treatment of malignancy due to concern that an increased oxygen pressure may stimulate tumour growth *via* re-oxygenation of hypoxic tumour cells and increased neovascularization. Alternatively, HBO may remove the hypoxic stimulus that drives tumour angiogenesis and other adaptations that facilitate tumour growth in a hypoxic environment.

Tumour hypoxia plays a significant role in the treatment of malignancy. Tumour angiogenesis, evasion of apoptosis and an increased glycolytic rate are all adaptations made by tumours in a hypoxic microenvironment.[6] To improve therapeutic efficacy, recent efforts have concentrated on the concept of eliminating the hypoxic state of tumours in order

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to remove the driving force behind these adaptations. HBO therapy is one means of altering tumour hypoxia and possibly improving treatment outcome.

Hyperbaric oxygen therapy involves the administration of pure oxygen under a pressure greater than 1 atmosphere (atm). The additional pressure coupled with inspiration of 100% oxygen substantially increases the amount of oxygen dissolved in plasma. Short-term effects of hyperoxia include enhanced oxygen delivery to ischemic tissues,[7] vasoconstriction, reduction of edema and immunomodulatory properties.[8,9] Long-term effects include neovascularization[4,10,11] and fibroblast proliferation.[4] The effect of HBO administration on tumour growth has been inconclusive.[3,12–15] Some studies report an increase or early onset of metastases with HBO therapy.[16,17] Johnson and Lauchlan first reported a tumour stimulatory effect with HBO, with increased metastases in patients with cervical cancer.[16] This has been supported by other experimental studies[15,18,19] and clinical trials.[16,17,20] Other studies report a tumour inhibitory[14,21–23] or negligible effect with HBO.[24–27]

HBO has been shown to enhance the effects of chemotherapy, photodynamic therapy and radiotherapy. This is based on the rationale that HBO may alter oxygen levels *in vivo* sufficiently to increase the sensitivity of tumours to radiotherapy,[28] photodynamic therapy (PDT)[29–31] and some forms of chemotherapy.[4,32,33] This may be due to several factors: HBO greatly improves tumour perfusion and cellular sensitivity. Altering hypoxia within tumours may remove the stimulus for the angiogenic switch. Hypoxia is also a major source of reactive oxygen species (ROS) production. At low levels, ROS function as stimulants of tumour growth but become toxic at high levels. HBO may indirectly increase intratumoural ROS to toxic levels and induce tumour cell destruction by overriding tumour antioxidant defences. This has been confirmed in both *in vitro*[34] and *in vivo*[35] studies.

Since HBO has been used clinically as an adjuvant it is important to rule out any potential adverse effects. This study investigates the effect of HBO therapy on tumour growth, proliferation, apoptosis and the tumour microvasculature in an animal model of colorectal cancer liver metastases.

2. Materials and methods

2.1. Animals

Six–eight-week-old inbred CBA (Aghooti) male mice (Adelaide University Animal Facility, Australia) were housed in cages with access to irradiated food and water *ad libitum* and exposed to a 12-h light–dark cycle. All experiments were conducted at the Austin Hospital Department of Surgery (Heidelberg, Australia) in accordance and with ethics approval from Austin Health Animal Ethics Committee.

2.2. Liver metastases model

A dimethyl hydrazine (DMH) induced primary colon carcinoma murine derived cell line was used for the study. A tumour cell suspension was prepared as described previously.[36] Liver metastases were induced *via* an 0.05 ml intrasplenic injection of the cell suspension (1×10^6 cells/ml) following which a splenectomy was performed.[37] Macroscopic tumours in the liver are evident by day 10 following induction of tumours. This is followed by a rapid exponential tumour growth phase with fully established tumours by day 21.[37]

2.3. HBO protocol

Animals exposed to HBO were placed in cages in a specifically designed animal hyperbaric chamber (Donated by the Alfred Hospital Hyperbaric Service, Vic., Australia). HBO therapy was administered at a pressure of 2.4 atm for 90 min. A minimum of 15 min pressurisation and depressurization was allowed for animals to adjust to the changes in pressure. HBO therapy was administered daily and animals were killed at four time points on days 7, 13, 19 and 25 post-tumour induction. At each end point, livers were excised for quantitative stereological analysis.[38] Mean cross-sectional tumour area was also calculated.

2.4. Histological analysis

The histological features of liver metastases in the various treatment groups were observed with haematoxylin and eosin (H&E) staining using a standard protocol. Serial sections were examined using light microscopy to detect changes in tumour cell morphology, necrosis and any tumour vessel changes caused by HBO therapy. The percentage tumour necrosis was quantified using image analysis software.

2.5. Microvascular resin casting

Microvascular resin casting was performed to observe changes in microvascular architecture according to previously described techniques.[36] Casts were viewed with scanning electron microscopy (Philips XL30 field emission scanning electron microscope, School of Botany, The University of Melbourne, Australia). Micrographs of tumour and normal liver vasculature were captured at magnifications ranging between 30× and 200×.

2.6. Immunohistochemistry

The effect of HBO therapy on tumour cell proliferation was performed using immunohistochemistry of paraffin embedded sections with the commercially available monoclonal rat anti-mouse Ki-67 antibody (M7249) (DakoCytomation, Denmark). Immunohistochemical detection of apoptosis was conducted using caspase-3 (anti-human/mouse caspase-3 active AF835) staining (R&D Systems, USA). Detection was conducted using the Envision + HRP detection system (K4011) (DakoCytomation, Denmark).

For Ki-67, antigen retrieval was performed using citrate buffer (1 mM, pH 6) at 99 °C for 20 min and endogenous peroxidase activity was quenched with a 1:10 dilution of hydrogen peroxide in phosphate buffered solution. Sections were incubated with the primary antibody for 90 min at room temperature (1:100). The secondary antibody, a polyclonal rabbit anti-rat (E0468) was applied at 1:200 dilution. Following incubation with horseradish peroxidase (HRP)-complex, sections

were incubated with DAB (diaminobenzidine) substrate-chromagen for 10 min. Sections were counterstained with haematoxylin, dehydrated in ascending ethanols and mounted for viewing with light microscopy.

A similar protocol as described above was used to detect caspase-3. Endogenous peroxidase activity was quenched after incubation with the primary antibody (1:1000 dilution for 30 min at room temperature). The secondary antibody step was not required. Presence of the respective antigens was denoted by brown staining of nuclei. A representative sample of each liver was imaged for analysis using image analysis software. Expression was quantified in a blinded manner by counting the number of positively stained nuclei divided by the number of unstained nuclei.

2.7. *Statistical analysis*

All data was expressed as the means ± standard error of the mean, (SEM) using a Statistical Package for the Social Sciences (SPSS 11.5®, Chicago, IL, USA). Differences between the various treatment groups was compared using non-parametric -Mann-Whitney *U* test, where $P < 0.05$ was considered statistically significant (All *P*-values were two sided). Animal numbers were based on preliminary studies, estimating a minimum requirement of 10 animals per study group to detect a 20–30% reduction in percentage metastases or 2–3% reduction in mean cross-sectional tumour area, each with a power of at least 0.8.

3. Results

3.1. *Effect of HBO on tumour growth*

HBO therapy was well tolerated by all animals without toxicity. Daily exposure to HBO at 2.4 atm for 90 min caused no significant reduction in percentage liver metastases at any of the observed time points D7, 13, 19 and 25 ($P > 0.05$), (Fig. 1). Although not statistically significant, tumours were consistently smaller at days 7, 13 and 25 with the exception of day 19, where HBO treated tumours were significantly larger than untreated tumours ($P = 0.023$) (Fig. 2).

3.2. *Tumour necrosis*

No evidence of necrosis was observed in tumours at day 7. At day 13 there was a 36% reduction in the percentage of tumour necrosis in HBO treated tumours (4.43% ± 1.6 *versus* control, 6.9% ± 1.1, $P = 0.002$). Reduced necrosis was evident at days 19 and 25 but was not statistically significant ($P > 0.05$, Fig. 3).

3.3. *Immunohistochemistry*

Ki-67 staining within tumours was heterogeneous, with features observed common to both control and HBO treated tumours. Immunoreactivity within each specimen was variable with some tumours being highly proliferative, whist

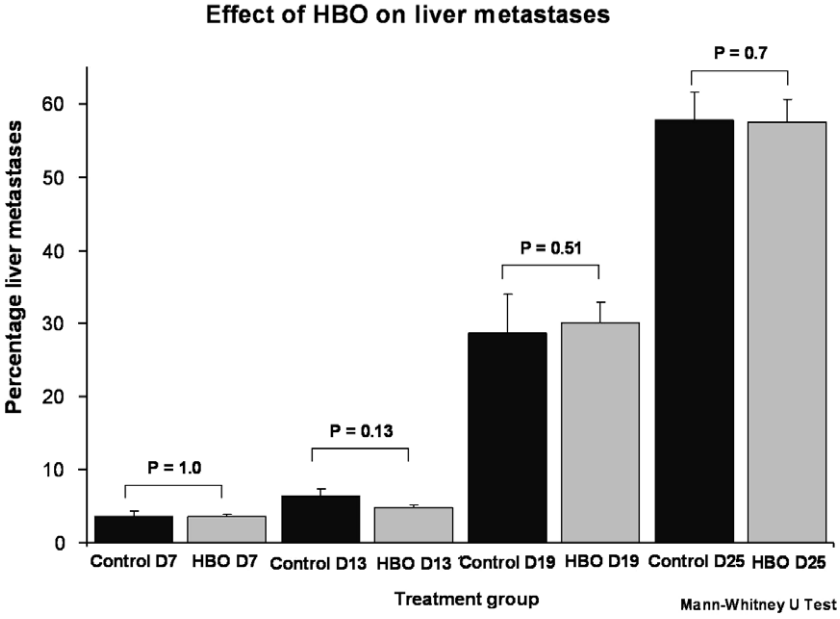

Fig. 1 – Effect of HBO therapy on colorectal liver metastases. Animals were exposed daily to HBO at 2.4 atm for 90 min. Control animals remained at ambient pressure. The percentage liver metastases was quantified on animals at days 7, 13, 19 and 25 post-tumour induction using stereological analysis. Briefly, livers were sectioned and images of a representative sample were captured and the amount of tumour measured using image analysis software. HBO treatment had no significant effect on liver metastases at any time point. (NB: $n = 10$ for each treatment group except Control day 25, $n = 22$ and HBO day 25, $n = 18$).

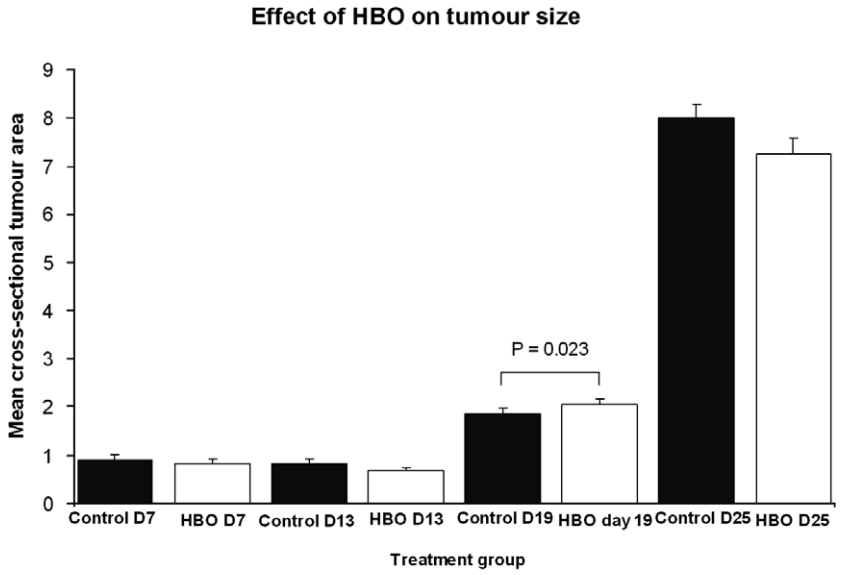

Fig. 2 – Effect of HBO therapy on tumour size. The mean cross-sectional tumour area was measured to detect more discrete differences between the treatment groups. Using image analysis software the mean tumour area was measured. HBO treated tumours at day 13 were significantly larger area (2.064 mm^2 ± 0.12) compared to untreated tumours (1.86 mm^2 ± 0.12) ($P = 0.023$) HBO did not have a significant impact on tumour size compared to control tumours at any of the other time points ($P > 0.05$). (NB: $n = 10$ for each treatment group except Control day 25, $n = 22$ and HBO day 25, $n = 18$).

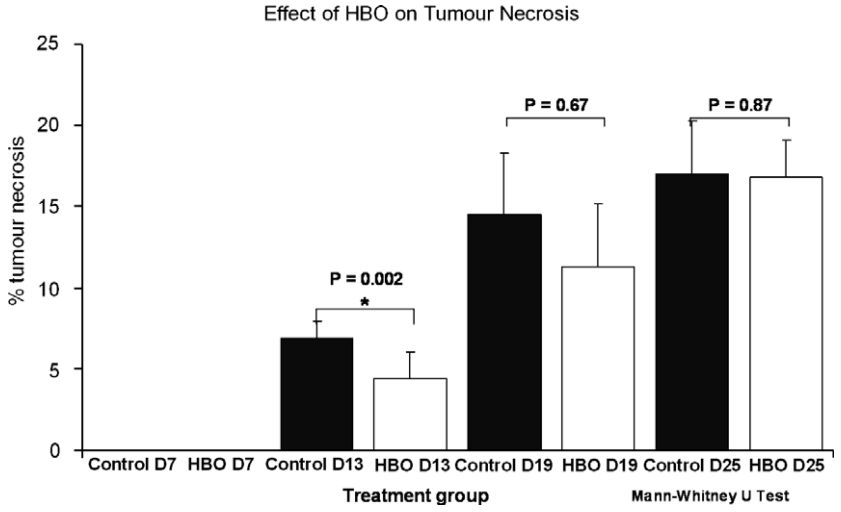

Fig. 3 – Effect of HBO therapy on the percentage of tumour necrosis. A representative sample of H&E stained sections was used to measure the percentage of tumour necrosis amongst treatment groups. The percentage tumour necrosis increased as tumours progressed. HBO treated tumours at day 13 had significantly less necrosis compared to untreated tumours (4.43% ± 1.6 *versus* control, 6.9% ± 1.1) ($P = 0.002$). (NB: Day 7 Cont and HBO group, $n = 11$, D 13–25 Cont and HBO groups, $n = 20$–30 tumours per group).

others had minimal proliferation. Poorly differentiated tumours (identified from serial H&E sections) demonstrated a higher percentage of proliferating cells compared to well differentiated tumours. Small sized tumours (1–200 μm in diameter) demonstrated the highest degree of proliferation. The same was observed in poorly differentiated large tumours (>500 μm in diameter). Proliferation of the normal liver was unaltered by HBO therapy (data not shown).

Quantification of the percentage of proliferating cells showed no difference in tumours treated with HBO therapy compared to matched controls at days 7, 19 and 25 ($P > 0.05$, Table 1). Again the exception was day 13, where HBO treated tumours demonstrated reduced tumour proliferation ($P = 0.008$).

Both control and HBO treated tumours exhibited highly proliferative tumours with minimal apoptosis. HBO had no

Table 1 – Effect of HBO on tumour cell proliferation and apoptosis

Treatment group	Rate of proliferation	Rate of apoptosis
Control day 7	37.45 ± 5.5	0.017 ± 0.00
HBO day 7	33.09 ± 4.5	0.020 ± 0.01
Control day 13	51.39 ± 2.9[a]	0.070 ± 0.04
HBO day 13	31.7 ± 5.3[a]	0.241 ± 0.14
Control day 19	51.39 ± 4.9	0.029 ± 0.06
HBO day 19	45.73 ± 5.4	0.286 ± 0.08
Control day 25	56.58 ± 3.3	0.152 ± 0.05
HBO day 25	56.09 ± 3.4	0.217 ± 0.08

Tumour proliferation and apoptosis were assessed by immunohistochemistry on 4 μm paraffin embedded sections using Ki-67 and Caspase-3, respectively. The percentage of positively stained cells was calculated. At day 13, HBO treatment significantly reduced tumour proliferation compared to untreated tumours ($P = 0.008$). However, it had no effect on tumour proliferation or apoptosis at any of the other time points assessed. (NB: $n = 10{,}000$ cells in total counted for each treatment group). All values are percentages ± SEM

a $P = 0.008$.

effect on the percentage of apoptosis at any of the time points (Table 1).

3.4. *Microvascular architecture*

Changes in tumour microvasculature were studied at day 25 by scanning electron microscopy (SEM) of microvascular resin casts. The highly dense microvascular architecture in both the control and treated groups indicated the advanced stage of tumours which gave rise to coalesced and flattened vessels forming vascular lakes. Dilated vessels at the tumour periphery gradually tapered toward areas of complete vascular occlusion at the tumour centre. Filling defects were identified as an absence of vasculature or regions of necrosis. The features described above were similar amongst specimens with and without HBO therapy, suggesting HBO had minimal impact at the microvascular level (Fig. 4).

4. Discussion

The effect of HBO administration on tumour growth has been contradictory[3,12–15] with some researchers reporting that HBO

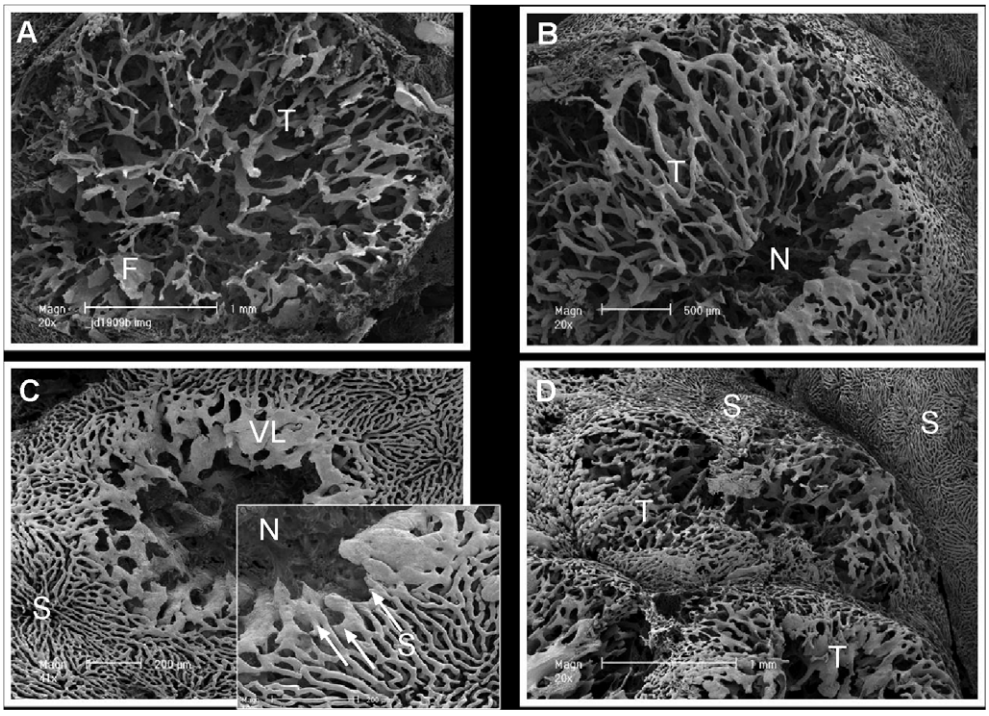

Fig. 4 – Effect of HBO therapy on microvascular architecture of liver metastases. The vasculature of animals was perfused with an acrylic resin. Upon polymerisation, the surrounding tissue was eroded and the remaining microvascular cast was prepared and viewed with scanning electron microscopy. Scanning electron micrographs of control tumours with and without HBO (B,D and A,C, respectively), demonstrated typical tumour vasculature consisting of well-established tumours with a dense network of tumour vessels (T) which were dilated and flattened (F) forming vascular lakes (VL) and had an abnormal structure compared to the surrounding normal liver sinusoids (S). There was also evidence of direct sinusoidal angiogenesis (insert C, white arrows). Regions with an absence of filling were indicative of regions of necrosis (N). There were no evident differences between HBO treated versus untreated tumours. (NB, magnifications vary as indicated on micrographs).

has a tumour stimulatory effect in experimental[15–19] and clinical studies.[16,17,20] In 2003, Feldmeier and colleagues extensively reviewed experimental and clinical data and concluded that HBO exposure had no primary or metastatic tumour stimulatory effect.[1] Our review of the literature over the past 50 years drew a similar conclusion.[6]

Our aim was to firstly determine the effect of daily HBO administration in mice induced with colorectal liver metastases. Secondly to investigate the mechanisms in terms of tumour necrosis, kinetics (apoptosis and proliferation) and microcirculatory changes induced by HBO administration. Tumour growth was investigated at various time points post-tumour induction (days 7, 13, 19 and 25). The effects of HBO may indeed be dependent on a multiplicity of factors including tumour type, stage and size, or the duration, atmospheric pressure and number of HBO exposures. HBO was therefore administered daily to determine the maximum effect.

Indeed the atmospheric pressure would influence the effect of HBO on tumours. In this study HBO was administered daily at 2.4 atm for 90 min and had no effect on the volume of liver metastases at any of the time points investigated. This finding is in accordance with Dettmer and colleagues who demonstrated that HBO had no effect on Walker carcinocarcoma in rats at 1 or 3 atm.[21] However, in another similar study conducted this time on lung metastases, HBO had a tumour inhibitory effect at 3 atm (but not 1 atm).[23] Collectively, these findings demonstrate that the effect of HBO is not only dependent on the partial pressure of oxygen but may also be tumour specific. This is reflected in the literature where patients with head and neck cancers tend to be most responsive to HBO therapy and patients with cervical and bladder cancer, the least responsive.[6]

The effects of HBO are also dependent on the stage of tumour growth and timing of HBO administration. The model of colorectal liver metastases used in this study has an initial lag phase (days 1–6) followed by an inductive growth phase (days 7–20) and a plateau in growth from day 21 onwards. HBO was administered daily (from the day of tumour induction) and tumour growth was observed at different time points (days 7, 13, 19 and 25). HBO had no effect on colorectal cancer liver metastases at any time point. However, HBO treated tumours at day 19 (inductive growth phase) were significantly larger compared to untreated tumours. These observations are similar to another study where HBO was shown to have a tumour stimulatory effect during the proliferative phase of oral carcinoma.[15]

In another study, Mestrovic *et al.* showed significantly improved survival and reduced tumour deposits after HBO administration at 3 atm on days 1–6 or 7–12. No significant difference was seen with HBO administered from days 13 to 18.[23] This suggests that the timing of HBO administration may also play a role. Our protocol was however different to that adopted by Mestrovic in that HBO was administered continuously from day 1 in order to determine the maximum effect. Although HBO treated tumours were larger at day 19, this effect was negated by day 25. We found no histological evidence of extrahepatic metastases with HBO treatment. This is in contrast to a study in C3H mice bearing spontaneous mammary tumours where HBO stimulated pulmonary metastases.[18] The majority of studies however agree with our results that HBO does not stimulate distal metastases.[39]

There are two rationales for using HBO to treat malignancy and both involve tumour hypoxia. Hypoxia is the main regulator of HIF-1α and subsequent VEGF expression. Induction of HIF-1α promotes VEGF-induced angiogenesis.[40,41] HBO may alter the hypoxic state and in doing so remove the hypoxic stimulus that drives angiogenesis. Due to the hypoxic microenvironment, tumours are under constant oxidative stress and have elevated levels of ROS and are tightly regulated by tumour antioxidants. Under these conditions, ROS are at a sub-lethal level and conducive to tumour growth by causing DNA damage and genomic instability. If elevated further however, these ROS become toxic. This is known as the threshold concept and has been reported previously[42] and demonstrated *in vitro*[34] and *in vivo*.[35,43] The second rationale is that HBO may overwhelm tumour antioxidant defences *via* excessive ROS production. Based on these two rationales we measured the effect of HBO on tumour necrosis and apoptosis and proliferation.

Using haematoxylin and eosin staining the percentage of tumour necrosis was quantified. We found that HBO had no overall effect (at day 25) on tumour necrosis, although HBO treated tumours at day 13 demonstrated reduced necrosis. The percentage of tumour proliferation was quantified using immunohistochemistry for the nuclear proliferation factor, Ki-67. HBO treatment reduced tumour proliferation at day 13 (inductive growth phase). Other researchers have shown reduced tumour proliferation with HBO treatment and suggest that it is dependent on the phase of growth[15] and increases with prolonged exposure.[9] McMillan and colleagues showed that HBO reduced tumour proliferation during the inductive phase of tumour growth in experimental oral carcinoma. In our model this *inductive* phase occurs during days 10–19. Our findings were in agreement with Macmillan where HBO showed a consistent trend for reduced tumour proliferation at all time points investigated and this was significant at day 13. Again this effect was nullified after 25 days of continual HBO treatment.

The degree of apoptosis was calculated using immunohistochemistry for active caspase-3. We found that HBO did not significantly alter the apoptotic rate of tumours at any time point investigated although there was a trend towards increased apoptosis in HBO treated tumours. Although these findings are in disagreement with other studies[34] and the overall rationale that HBO induces tumour cell destruction *via* enhanced ROS production. Other researchers have also shown that HBO has the ability to reduce proliferation of tumour cells without affecting tumour necrosis or apoptosis.[9]

If HBO was to inhibit tumour growth through reduced hypoxia and subsequently reduced angiogenesis, one may speculate this would be evident upon examination of the microvascular architecture. Microvascular architecture was observed using scanning electron microscopy of microvascular resin casts of tumours with and without HBO treatment. This technique has been reported previously and is effective in highlighting disturbances in changes in microcirculation or microvascular structure and density. Control tumours exhibited aberrant vascular architecture characteristic of advanced tumours. HBO treated tumours demonstrated similar

features and no differences were evident with treatment. This is contrary to other experimental studies that have shown increased microvascular density and neovascularization following HBO therapy in ovarian cancer xenografts in mice[11] and irradiated tissue.[4] HBO pre-treatment could potentially render chemotherapy more effective and has been investigated in a pilot study in patients with locally advanced breast cancer.[44]

Our results confirm that HBO does not have a tumour stimulatory effect and does not promote distal metastases. The study also expands on the mechanisms contributing to the effect or rather lack thereof of HBO. Although there are transient effects such as increased tumour size at day 19 or reduced proliferation and necrosis at day 13 there are no overall significant changes with prolonged treatment (at day 25). Furthermore no adverse effects of HBO were observed and it was well tolerated. The clinically relevant question from these findings is whether HBO may provide some benefit in an adjuvant setting.

HBO therapy has been shown to enhance the efficacy of several chemotherapies including taxol,[9,45] doxorubicin,[22,33,46,47] 5FU[48,49] and bleomycin[15] by increased cellular uptake and ROS induced tumour destruction. It has also improved the sensitivity of cells to PDT in experimental models of adenocarcinoma,[29] mammary carcinoma[31] and carcinoma of the esophagus.[50] The most promising effects of HBO have been demonstrated in combination with radiotherapy for head and neck tumours and other primary tumours (breast, bowel, bladder and uterus) in terms of tumour inhibition or improved local tumour control.[12,13,51,52] A recent Cochrane review stated that HBO reduced mortality and improved survival for patients with head and neck and cervical cancer treated with radiotherapy.[53] Since HBO was well tolerated and did not cause stimulation of liver or extra-hepatic metastases, it warrants further investigation for its use in combination with other therapies-in particular therapies where treatment efficacy is limited by the hypoxic tumour microenvironment.

Conflict of interest statement

There is no conflict of interest to be declared for any of the authors.

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