# Lung metastatic load limitation with hyperbaric oxygen.

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Haroon TMY, Patel M, Al-Mehdi AB. Lung metastatic load limitation with hyperbaric oxygen. Undersea Hyperb Med 2007; 34(2): 83-90. Despite some theoretical concern about cancer-enhancing effects of hyperbaric oxygen ($HBO_2$) therapy, it is frequently administered to cancer patients. We evaluated the growth of murine breast cancer cells in the lung after hyperbaric oxygen treatment in an experimental metastasis assay. Young nu/nu mice were injected intravenously with $3 \times 10^3$ 4T1-GFP tumor cells per g body weight followed by lung isolation, perfusion, and intact organ epifluorescence microscopy 1 to 37 days after injection. A group of animals (n=32) was exposed once daily for 5 days a week to 45 min of 2.8 ATA hyperbaric oxygen ($HBO_2$) in a research animal $HBO_2$ chamber. Control animals (n=31) were not subjected to $HBO_2$ treatment, but received similar intravenous administration of $3 \times 10^3$ 4T1-GFP tumor cells. Single tumor cells and colonies were counted in the subpleural vessels in areas of about 0.5 $cm^2$ of lung surface. $HBO_2$ treatment did not lead to an increase in the number of the large or small colonies in the lungs. Rather, a significant reduction in the number of the large colonies was observed at 1 and 16 to 21-day periods of measurements after hyperbaric treatment. However, most importantly, there was a significant decrease in large colony size in the $HBO_2$ group during all periods of observation. The results indicate that $HBO_2$ is not prometastatic for breast cancer cells; rather it restricts the growth of large tumor cell colonies.

## INTRODUCTION

Hyperbaric oxygen ($HBO_2$) therapy is used clinically for a variety of conditions, including treatment of decompression sickness and chronic wounds, carbon monoxide poisoning, and for radiosensitization of tumors to improve the effectiveness of radiotherapy (1,2). However, the notion of 'tumor-promoting' effects of $HBO_2$ limits enthusiasm for its use in cancer patients by some clinicians (3). Therefore, it is important to address the concerns about cancer-promoting effects of $HBO_2$ (4). The notion of pro-carcinogenic effect of $HBO_2$ stems from the postulated mechanisms such as improved nourishment of tumors due to stimulation of angiogenesis and growth stimulation due to improved oxygenation of tumor mass (5).

The first report suggesting the metastasis-causing effect of $HBO_2$ was about a group of patients with advanced cervical cancer (6). Cade et al. (7) showed that $HBO_2$ did not lead to increase in metastasis in patients with bronchogenic carcinoma, but doubled the incidence of metastasis in case of bladder carcinoma. However, almost all other published reports since 1967 have not demonstrated any cancer-causing or -promoting effects. Van DenBrenk et al. (8) showed significant decrease in metastasis with $HBO_2$. Disease-free survival in 104 patients with head and neck cancer improved with $HBO_2$ (9). No increased metastasis was seen in a controlled trial involving 1,500 patients with head and neck, bladder, bronchus, or cervical cancer (10). Lower incidence of new tumors or local

 

recurrence was seen with $HBO_2$ compared with matched for stage historic controls (11). Rapid progression of squamous cell carcinoma of the cervix after hyperbaric oxygenation has been noted (12).

In animal studies, $HBO_2$ increased survival time and reduced tumor growth in mice implanted with S-180 sarcoma (13). No correlation between intensity of $HBO_2$ treatment and development of radiation-induced osteosarcoma of the mandible was demonstrated in a rabbit model (14). A recent study found no evidence for persistent changes in tumor microenvironment or tumor growth promotion caused by hyperbaric oxygen exposure in mice (15). $HBO_2$ was also shown to decrease growth-rate and increase chemosensitivity of metastatic prostate cancer cells *in vitro* (16). Synchronization of cell cycle and dose-dependent accumulation in G2/M was shown to be the mechanism of the $HBO_2$ effect (17). $HBO_2$ inhibited benign and malignant mammary epithelial cell proliferation, but did not enhance cell death (18).

Therefore, despite some earlier studies suggesting the tumor-promoting potential of $HBO_2$, most subsequent studies show an anti-tumor effect of $HBO_2$ in clinical studies, in animal tumor models, and with tumor cells *in vitro*. To help resolve this controversy at an experimental level, our studies evaluated the effect of $HBO_2$ on murine breast carcinoma cell growth in a natural, metastatic environment of the pulmonary circulation *in vivo* in mice.

## MATERIALS AND METHODS

### Tumor Cell Injection

A syngeneic breast cancer model employing an established experimental metastasis assay was used in the study. The 4T1-GFP cell line was derived from the 4T1 mouse mammary adenocarcinoma cell line (# CRL-2539, ATCC , Manassas, VA) by stable transfection with enhanced green fluorescent protein gene (eGFP, Clontech, Mountain View, CA). We injected young nu/nu mice (Charles River) into the tail-vein with single-cell suspensions 4T1-GFP cells at $30 \times 10^3$ cells per g body weight followed by lung isolation, perfusion, and intact organ epifluorescence microscopy 1 to 37 days after injection. The volume of injected medium with cells was 100 µL. Animals were monitored daily for signs of respiratory distress. The research was conducted under a protocol reviewed and approved by the University of South Alabama Institutional Animal Care and Use Committee. The animals were divided into 2 groups: $HBO_2$ group (n=32) was exposed once daily for 5 days a week to 45 min of 2.8 ATA hyperbaric oxygen ($HBO_2$) in a research animal $HBO_2$ chamber. Control animals (n=31) were not subjected to $HBO_2$ treatment, but received similar administration of $3 \times 10^3$ 4T1-GFP tumor cells intravenously.

### Hyperbaric Oxygen Treatment

After tail-vein injections of tumor cells, a group of animals was exposed once daily 5 days a week to 45 min of 2.8 ATA (equivalent to 1.8 atmospheres or 26.5 psi or 182 kPa) hyperbaric oxygen ($HBO_2$) in a research animal $HBO_2$ chamber (Model 20220, Life Support Associates, Baltimore, MD). This hyperbaric exposure regimen is commonly used in research involving small rodents (19). The research animal $HBO_2$ chamber is 30 cm in diameter and 75 cm in length with a 10 cm acrylic viewport and having a maximum pressure rating of 80 psi (552 kPa). After the animals in their cage were placed in the chamber, pressurization was done over 1 min. At the end of the exposure to $HBO_2$, decompression was carried out over 2 min. Animals received $HBO_2$ treatment for up to 5 weeks.

**Tumor cell and colony counting in the lung**

An established intact organ microscopy method was utilized to observe and image subpleural pulmonary vessels *in situ* in the isolated, ventilated, blood-free lungs in real time using an epifluorescence microscope (20, 21). In brief, for lung isolation, the animal was anesthetized with intraperitoneal injection of 60 mg/kg sodium pentobarbital. A tracheostomy was performed and artificial ventilation with 95% air + 5% $CO_2$ was started through a cannula. The abdomen was opened and the animal was exsanguinated by transection of major abdominal vessels. A cannula was inserted into the main pulmonary artery via a puncture in the right ventricle. The lung was cleared of blood by gravity perfusion via the pulmonary artery with an artificial medium (Hanks' solution with 5% dextran and 10 mM glucose at pH 7.4). The flow-through perfusate exited the lung via the transected left ventricle. Once the lung became visibly cleared of blood, the heart-lung preparation was dissected en-bloc and was placed in a specially designed Plexiglas chamber. The lung was suspended sideways over a coverslip-window at the bottom of the chamber with the posterior surface of the lung gently touching the coverslip. The subpleural space of the lungs was directly visualized at high magnification (600×) by epifluorescence microscopy. For imaging 4T1-GFP cancer cell colonies in the subpleural pulmonary circulation, we used a high-resolution digital fluorescence video microscopy system consisting of a Nikon TE-2000 inverted fluorescence microscope, 60× water-immersion (N.A. 1.2) and 60× oil immersion (N.A. 1.4) objectives, automated 10-position filter wheels for both excitation and emission (Sutter Instruments, Lambda 10-2), automated dichroic filter cube changer (Nikon), XY axis automated stage (Prior Scientific, Inc.), Z-axis motor (Prior), a high resolution 12-bit C4742-95-12ERG IEEE 1394 digital CCD camera (Hamamatsu Inc.), and MetaMorph image acquisition, processing and analysis software with 3-D reconstruction and point spread function (PSF) -based deconvolution capabilities (Molecular Devices Corp, Downingtown, PA). For 3-D reconstruction, images of the same area were acquired along 40 μm of z-axis at 0.5 μm intervals (optical slicing). The deconvolved stacks were used to create noise-free 3-D reconstructions to determine the relationship of the tumor cells to their surrounding structures. The excitation light source was a 120 watt metal halide lamp (X-cite 120, Exfo Photonics Solutions, Mississauga, ON). A high quality GFP filter set (Chroma Technology Corp, Brattleboro, VT) was used. Single tumor cells and colonies were counted in the subpleural space by taking 100 pictures from contiguous areas of 0.5 $cm^2$ lung surface using automated stage movement in a 10 × 10 grid fashion. Pictures of each stack were stitched using MetaMorph software to create a large overview picture where cells and colonies were counted visually and using software automation after properly thresholding the images. All images were calibrated dimensionally and colony sizes were measured. Colonies were classified as large (with 75 μm or more in diameter in any direction) and small (less than 75 μm in any direction).

**Statistical Analysis**

Data analysis was done by SigmaStat (Systat Software Inc., San Jose, CA) using one way analysis of variance and Bonferroni's test. Data are expressed graphically as means ± SE using SigmaPlot (Systat). Differences were considered significant with $P<0.05$.

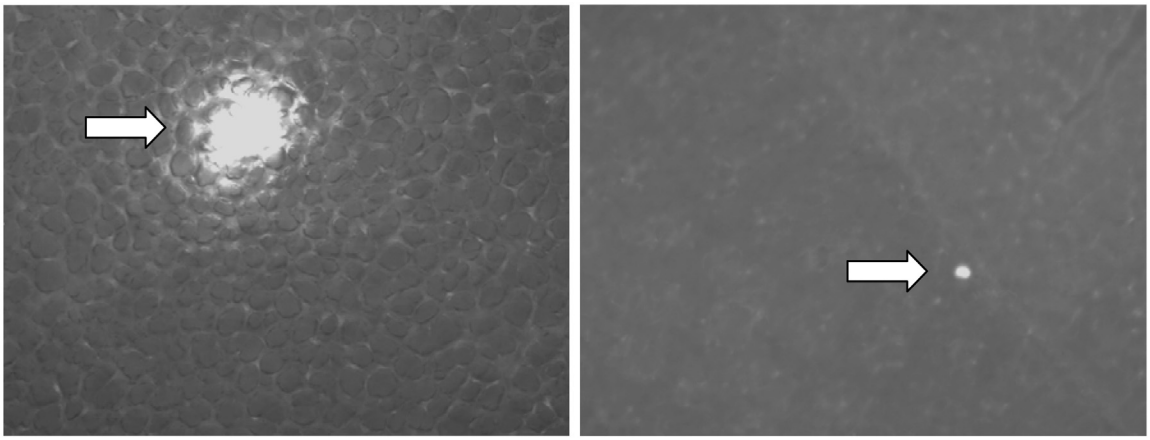

**Fig. 1.** Representative colonies (arrows) in the lung of nude mice 13 days after tail-vein injection of 4T1-GFP cells in the Control (left panel) and $HBO_2$(right panel). Images were acquired in the GFP channel by transpleural imaging in the intact mouse lung.

## RESULTS

The number of tumor cells injected intravenously for a 20 g mouse was 600,000. Although all of these cells encountered the pulmonary vascular bed on the first pass, only 4,825 ± 225 cells were found attached in the subpleural vessels one hour after injection. The number of single cells and small colonies one day after injection was 247 ± 234, indicating low efficiency of the metastatic process.

Fig. 1 shows representative colonies (arrows) in the lung of nude mice 13 days after tail-vein injection of 4T1-GFP cells in the Control (left panel) and $HBO_2$ (right panel). Note the significantly reduced size of the colony in the $HBO_2$ mouse lung. The green background color reflects lung tissue autofluorescence.

$HBO_2$ treatment did not lead to an increase in the number of the large (Fig. 2) or small (Fig. 3) colonies in the lungs of nude mice. Rather, a significant reduction in the number of the large colonies was observed in the 1 and 16-21 day periods of the $HBO_2$ group (Fig. 2; $^{*}$ $p < 0.05$ compared with Control). Data combining all colony counts (Fig. 4) demonstrate no statistically significant changes in the colony numbers with $HBO_2$ treatment after any period of observation.

On the other hand, a significant early increase in the number of the single cells was observed in the 5-7 day period of the $HBO_2$ group (Fig. 5; $^{*}p < 0.05$ vs. Control). An increase in the combined number of the small colonies and the single cells was observed during the 1, 11-13, 16- 21 and 30-37 day periods in the $HBO_2$ group while a decrease is noted in the 22-29 day period (Fig. 6).

No statistically significant changes in the combined number of large colonies, small colonies and the single cells were observed in the lung after $HBO_2$ treatment (Fig. 7). In contrast, a significant decrease in large colony size was observed in all time periods in the $HBO_2$ group (Fig. 8; $^{*}$ $p < 0.05$ vs. Control). The results indicate significant reduction in the metastatic load with $HBO_2$ treatment in an experimental metastasis assay.

## DISCUSSION

The most significant new finding from this study is that the total metastatic load in the lung is reduced after $HBO_2$ treatment. Total metastatic load is the combined mass of large colonies, small colonies and the single cells

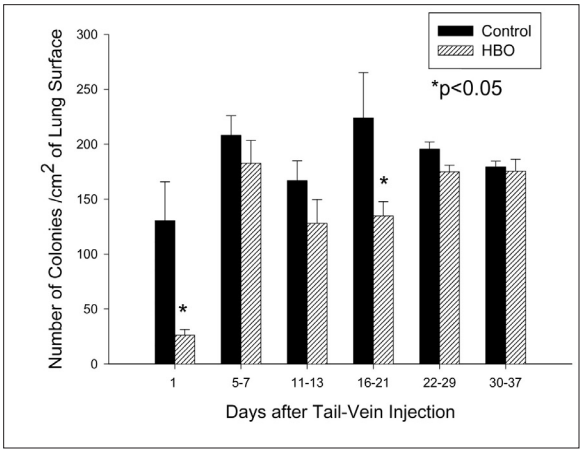


**Fig. 2.** Effect of hyperbaric oxygen ($HBO_2$) on the number of the large colonies in the lungs of nude mice. Data are means ± SE and represent the number of large colonies per mouse in the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 days period in the Control (black bars) and $HBO_2$ (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 respectively in the $HBO_2$ group.

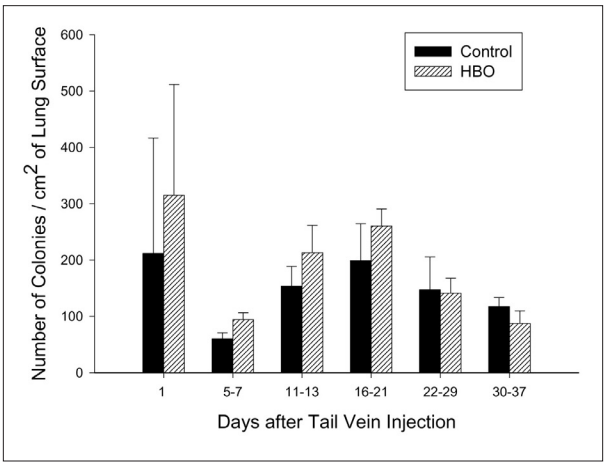


**Fig. 3.** Effect of $HBO_2$ on the number of the small colonies in the lungs of the nude mice 1, 5-7, 11-13, 16-21, 22-29 and 30-37 days after tail-vein injection of 4T1-GFP tumor cells. Data are means ± SE of the number of colonies in the lungs of each mouse in the Control (black bars) and $HBO_2$ (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 respectively in the $HBO_2$ group.

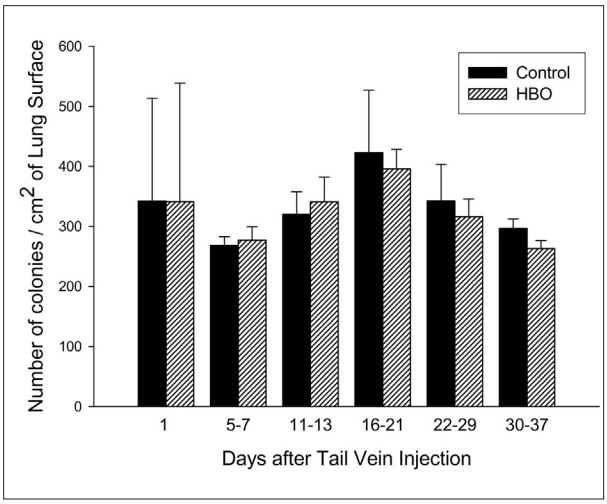


**Fig. 4.** Effect of $HBO_2$ on the number of the colonies (large and small) in the lungs of the nude mice. Data are means ± SE and represent the number of both large and small colonies per mouse in the Control (black bars) and HBO2 (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 respectively in the $HBO_2$ group.

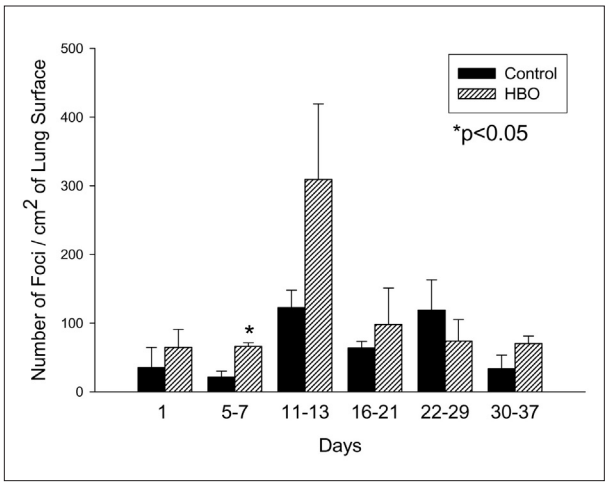


**Fig. 5.** Effect of $HBO_2$ on the number of the single tumor cells in the lungs of the nude mice after tail vein injection. Data are means ± SE and represent the number of single cells per mouse in the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 days periods in the Control (black bars) and $HBO_2$ (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 respectively in the $HBO_2$ group. For the 11-13 day period, the difference between the $HBO_2$ and the Control groups was not statistically significant.

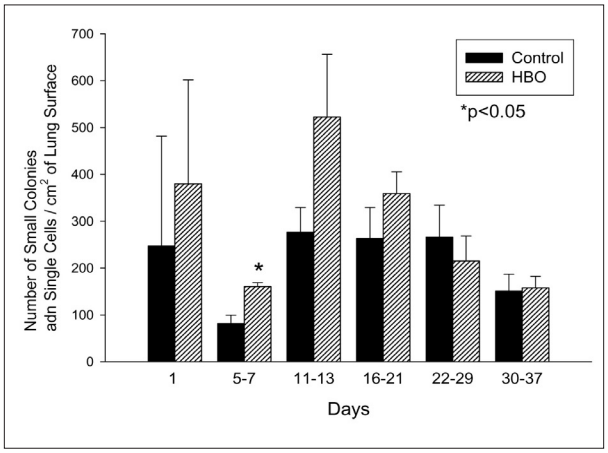


**Fig. 6.** Effect of $HBO_2$ on the combined number of small colonies and single cells in the experimental metastasis assay. Data are means ± SE and represent the combined number of the small colonies and the single cells per mouse in the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods in the Control (black bars) and $HBO_2$ (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 respectively in the $HBO_2$ group. For the 11-13 day period, the difference between the $HBO_2$ and the Control groups was not statistically significant.

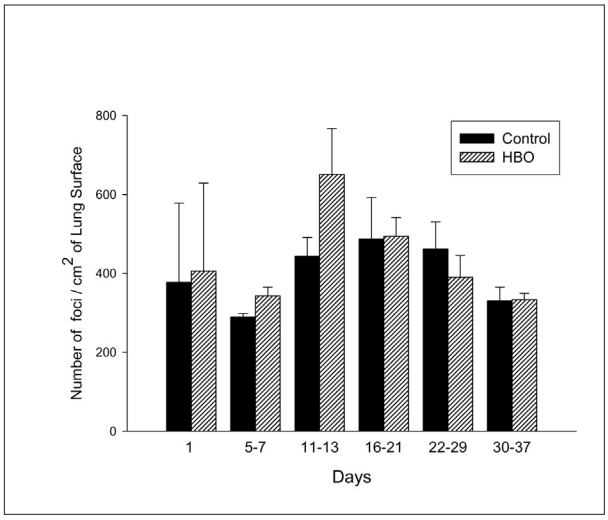


**Fig. 7.** Effect of $HBO_2$ on the number of foci (large colonies, small colonies and the single cells) in the lungs of nude mice. Data are means ± SE and represent the number of foci per mouse in the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 days periods in the Control (black bars) and $HBO_2$ (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 respectively in the $HBO_2$ group.

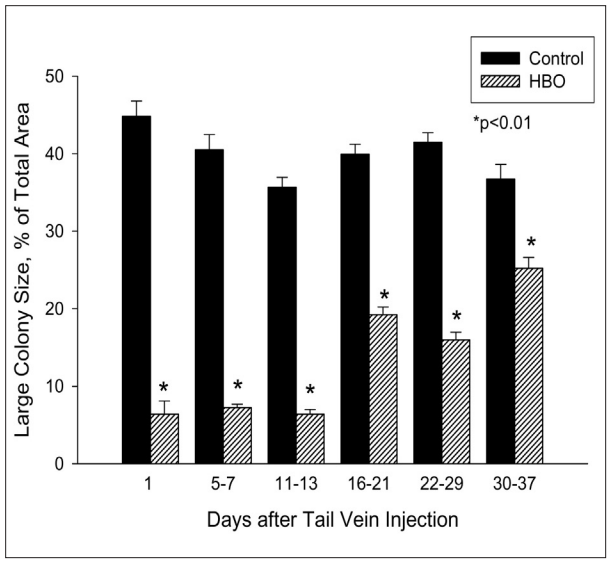


**Fig. 8.** Effect of $HBO_2$ on large colony size in the lungs of the nude mice. Data are means ± SE and represent percentage of area occupied by large colonies in the transpleural images of the lungs of the nude mice during the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 days periods in the Control (black bars) and $HBO_2$ (striated bars) groups. For the 1, 5-7, 11-13, 16-21, 22-29 and 30-37 day periods, n = 4, 3, 6, 7, 8 and 3 mice respectively in the Control and n=4, 5, 5, 9, 5 and 4 mice respectively in the $HBO_2$ group.

in the target organ. $HBO_2$ treatment did not lead to an increase in the combined number of all the metastatic foci in the lung. The load reduction was accomplished because the size of the colonies was limited.

It is interesting to note relatively high variability in the initial number of metastatic foci in the lungs one day after tail vein injection of tumor cells (standard error of the mean bars in Figs. 3, 4, 6, and 7), suggesting a high biological variability of the initial attachment process and tumor cell survival in the pulmonary circulation. However, the subsequent growth of the foci is reproducibly predictable. The number of foci one day after injection was significantly less than the number of attached cells one hour after injection, indicating that the majority of attached cells did not survive.

The number of single cells during the 5 weeks of observation exhibited a cyclic pattern, with a periodicity of 2 weeks. The variable number of single cells suggests formation of secondary metastases in the lung. The number of colonies also exhibited a cyclic pattern, but the periodicity was 4 weeks. This would suggest that micrometastases are susceptible to spontaneous resolution and a tissue threshold exists for the number of metastatic foci the lung can tolerate or support. This could explain the decrease in the number of small colonies in the 22-29 day period.

These studies are comparable to others in literature (15) showing that there is no adverse effect of $HBO_2$ on tumor growth, and suggesting $HBO_2$ may have an anti-cancer effect with breast cancer cells. Use of $HBO_2$ in human breast cancer patients did not have any adverse effects in a recent long term follow up study (22). $HBO_2$ is even considered for treating lymphedema associated with breast cancer surgery (23).

The efficiency of hematogenous metastasis depends on the attachment, apoptosis, dormancy, and growth of blood-borne tumor cells. However, our findings raise the interesting possibility that restrictions imposed by the local microenvironment may be key determinants of metastatic efficiency. The number of foci one day after injection was about 20-fold less than the number of attached cells one hour after injection, indicating that the majority of attached cells did not survive in the lung. The total metastatic load of the target organ (lung) was not only minimized by the foci number restriction, but also by the size-limitation of the large colonies. The results indicate that although the tumor cells can manifest organotropism for metastasis and select their site of secondary growth, the progression of the secondary or tertiary tumors is controlled by the local microenvironment. Hyperbaric oxygen enhanced the metastatic load limitation by keeping colony sizes smaller than in control by altering the local environment. The mechanism of the $HBO_2$ effect needs to be further investigated.

## ACKNOWLEDGMENT

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