

## Research Article

# Hyperbaric Oxygen Therapy (HBO), DNA Damage and Tumor Progression - A Survival Study on a Mice Tumor Model

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## Keywords

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## Abstract

HBO has been used as an adjuvant to chemo (CT) or radiotherapy (RT) in cancer; however, in our previous work on survival study in small animal mice model, we reported cancer enhancing effect after cessation of the HBO therapy. The present study was designed to understand the underlying molecular mechanism that may be linked to cancer enhancing phenomenon. Tumor bearing C3H mice were subjected to hyperbaric hyperoxia (1.1 bar & 1.2 bar) therapy consequently for 6 days with a day off in between for 3 weeks. Each session comprised of daily 120 minutes of HBO exposure prior to and after being subjected to RT or CT. Another group was subjected to one, two and three weeks of HBO therapy to observe graded response and severity of DNA damage. We observed proportionate increase in the severity of DNA damage and histone modification in the tumor samples when subjected to longer duration of HBO therapy as compared to tumor control. Thus, we suggest that HBO therapy should be given under highly dose controlled condition and with caution, else dual effect of oxygen toxicity and molecular effects may lead to tumor enhancement or recurrence and/or metastasis.

## INTRODUCTION

Several studies have shown that tumors in invasive or advanced stage are hypoxic as compared to the normal tissues, while oxygenation of tumor improves the sensitivity of tumor cells to the chemotherapy and radiotherapy. Thus, modulating tumor microenvironment with oxygenation may be a useful therapeutic modality in cancer. HBO (hyperbaric oxygen therapy) has been used to treat various conditions like radiation injury, non-healing wounds and also as an adjuvant to chemotherapy and radiotherapy [1-5]. Hypoxia in tumor is known to drive angiogenesis, increases glycolysis, acidosis and facilitate tumor growth by up regulating the genes involved in apoptosis/cell survival while at the same time may put some cancer cells in dormancy state in order to adapt to low oxygen microenvironment [1]. These are all adaptive mechanisms enabling cancers cell to survive any hostile environment. HBO elevates the levels of dissolved oxygen coupled with the higher pressure applied during the therapy which can eliminate tumor hypoxia [2-4]. It makes tumor cells more radio or chemo sensitive due to generation of high free radicals and by increasing the uptake of anticancer drug by tumor rendering tumor cells for damage, apoptosis, reduced vascularity and number of metastasis and probably mutations [5-26]. It also modulates tumor microenvironment with respect to aerobic glycolysis and change the harmful acidosis milieu. Some investigations have studied direct tissue polarography which indicates that external

HBO effectively increases oxygen levels [2]. With 4 atmospheres absolute of oxygen, a 12 fold rise in tumor pO<sub>2</sub> occurred with a 15-50 fold increase in the P<sub>O2</sub> of normal tissues [26]. Moreover, we also know that HBO >2ATM for a minimum of 60 minutes duration is the protocol for human being to treat severe burn injuries and associated infection, decompression sickness, CO poisoning, post radiation injuries, ulcer etc [27]. However, in divers exposed to multiple carcinogens was shown to cause more morbidity and/or even cancer [15]. Thus, long term or uncontrolled exposure to HBO has the ability to potentially damage the cells though DNA damage, chromosomal or epigenetic changes and direct oxygen toxicity. The detrimental effects of exposure at high concentration are due to the reactive oxygen species (ROS). These free radicals are known to increase the damage and this situation is termed as oxidative stress. In order to re-assess the extent of ill effects and the survival advantage of HBO therapy, we conducted a pre-clinical study involving cancer therapeutic experimental model to assess the effect of hyperbaric oxygen therapy in cancer progression, survival and metastasis. It involved the administration of pure oxygen at pressure optimally higher than the normal atmospheric pressure. Inspiration of 100% oxygen coupled with the pressure increases the amount of oxygen dissolved in plasma. In our survival study, we observed some undesirable clinical outcome in terms of survival and quality of life [27]. The lifespan of HBO treated mice was found to be less {(median value 62 SD, 91 days (86 D to 95 D))} than control group i.e., tumor induced mice who did not receive therapy

{median value 62 SD, 104 days (52 D to 143 D)} in absolute terms, however, statistically the difference was insignificant. We did not find any cancer enhancing effect during the HBO therapy. However, acceleration in tumor growth was observed following completion of HBO therapy. At the same time, mice subjected to HBO therapy lived shorter as compared to those not exposed to HBO therapy (survival period from induction of tumor cells until deaths). The effect of HBO in terms of causing DNA damage due to exposure of proliferating cells to free radicals (ROS) as well as compensating tumor hypoxia thus making cancer cells more chemo or radio sensitive is well studied. The mutagenic effect of HBO exposure on proliferating cells causing aneuploidy and the mechanism of its action on tumor vascular remodeling and micro-environment has not been well studied. There is also a need to understand DNA damage and repair pathways [27]. The findings led us to further investigate the extent of DNA damage & epigenetic changes that may occur during HBO therapy and also report the end survival duration of the mice subjected to chemo & radiotherapy. This study helped us to bring about more clarity on the subject of HBO therapy in disease condition. We observed not only severe degree of DNA damage due to free radicals as expected but also histone modification in the tumor samples excised from the tumor bearing mice exposed to different time duration of HBO therapy (one week, two week, and three weeks). Thus, there is a possibility of HBO or re-oxygenation causing further genetic mutation or instability by inducing epigenetic changes followed by induction of chromosomal alteration. It could have probably led to rebound acceleration in the growth of tumor post HBO therapy. We may conclude that our assumption of careful and cautious use of HBO as an adjuvant therapy in cancer was correct. HBO should be used under highly monitored and controlled situation, where patient should be watched for side effects and relapses post therapy.

## MATERIALS AND METHODS

### Epigenetic and DNA study

The present study mainly focused on the epigenetic and DNA study post HBO therapy and end point survival outcomes of the mice subjected to radiotherapy & chemotherapy which was one of the arms of the main study. Details of the outcome study of HBO therapy was reported in the paper published in 2012 [27].

Experimental Study was carried out in Laboratory Animal Facility (ACTREC) in strict accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Tata Memorial Centre-Advanced Center for Treatment Research and Education in Cancer (Proposal No. 19/2010). All efforts were made to minimize suffering during the study. An inbred C3H strain with agouti coat color was used for this study. C3H strain has high incidence (70%) of mammary tumors and does not carry mouse mammary tumor virus (MMTV). Six to eight week old inbred C3H (Agouti) female mice were housed in cages with access to food and water ad libitum and exposed to 12 hour light dark cycle. The breeding system adopted in our animal facilities was 1:1 brother sister mating till 20 generation to achieve isogenicity. The

isogenicity has been further verified by regular quality control monitoring with biochemical markers (Hbb, Car-2, Idh, Mod), skin grafting, molecular markers (microsatellite) apart from regular health monitoring. A tumor cell suspension was prepared from aseptically excised spontaneous mammary tumor.

Mammary tumors were induced using 0.1 ml subcutaneous injection of cell suspension (2.56106 cells/ml) in mammary fat pad. Daily inspection of the induced mice was done for food, water, bedding, comfort and appearance of the tumor nodule under all aseptic precaution by the same research fellow to minimize the error and reduce inter personal variation. The tumor measurements were noted from the day the nodule was palpated. The mice were subjected to experimental intervention from the time the nodule size measured between 5-10 mm in diameter. Macroscopic tumors were evident by day 60 (median value/mean value) following tumor induction – latent period.

### Measurements of tumor growth

Tumor size was measured using calipers at first tumor nodule appearance, that is considered as day 1 and they were followed with daily measurements till the study end point i.e., natural demise or when a decision was taken by the investigator to euthanize due to heavy tumor burden with associated morbidity. Tumor growth was calculated/estimated according to the formula:  $V = L \times (W)^2 / 2$  and volume by  $4\pi r^3$  (plot is same). Daily weight measurements of the animal were done until they survive. Statistical analysis was carried out by finding out the median and mean values of the tumor volume over the period of time and the survival duration. The level of significance was taken as  $P < 0.05$ , by Mann Whitney test and results are expressed as median & means  $\pm 2SE$  (25<sup>th</sup> to 75<sup>th</sup> percentile). For understanding the mathematical model and the growth pattern following HBO therapy, one may refer to [27].

### Animal distribution and HBO protocol to observe relationship between severity of DNA damage and epigenetic i.e., H2AX phosphorylation

Animals were divided into two groups, A (N=6), B (N=6) and kept in two separate cages. Animals were exposed to hyperbaric oxygen therapy at 1.2 bar daily for 120 minutes for 7, 14 & 21 days in a specifically designed Hyperbaric Chamber in our facility (ACTREC). They were further followed up for tumor growth, body weight, survival period and metastasis. After death tissues (tumor, lung, brain, liver and heart) were collected for histological analysis. The tumor size were allowed to grow maximum to the size of 3x3cm<sup>2</sup> before subjecting them to the experimentation in order to get adequate samples for the histone extraction and DNA studies. Both the groups were subjected to HBO therapy as per the protocol for 7 days (A=2, B=2), 14 days (A=2, B=2) and 21 days (A=2, B=2) at 1.2 bar daily for 120 minutes. Tumor samples were excised after euthanizing the mice immediately following completion of the HBO therapy in group A (N=6) & 48 hours post therapy (N=6) in group (B) of mice. In addition, samples from normal control-NC (N=2) i.e., mammary pad of normal mice, normal HBO control-NHC (N=2) i.e., mammary pad of normal mice exposed to HBO and for mammary tumor control - TC (N=2) i.e., mammary tumor of non HBO exposed mice were taken from the tumor repository of the main study arm as reported in paper

[27] kept for the study. A preliminary analysis of the tumor pool collected from the mice (exposed to HBO, N=20 or more) and Control group not exposed to HBO (Tumor bearing, N=20 & normal mice, N=3) was already done where we observed DNA changes as normally expected with free radicle injury or HBO exposure but also some histone modification, which led us to do the present specific study to test whether, increase in observed DNA damage also resulted in increase of H2AX phosphorylation at Serine 139, western blot studies were carried out with site-specific antibody in minimum of 4 mice each at 7, 14 & 21 days by pooling the tumor samples of 3x3 cm size to get adequate amount of samples for analysis, and large sample size was not a concern. Large well defined study can be done by anyone interested based upon our finding.

**Experimental protocol for HBO:** Experimental conditions for the mice exposed to HBO remained the same as explained above in (a) and the paper published in 2012 [27]. The tumor bearing mice with tumor size of approximately 10mm x 10mm size were exposed to 120 minutes of HBO continuously at 1.2 bar for 3 weeks. Total number of mice exposed to HBO for whom the mean length of survival was calculated was 29.

**Experimental protocol for radiotherapy:** Total number of tumor bearing mice exposed to radiotherapy was 28 & 10 mice in control group (no radiotherapy); A preliminary trial expt. for adequate dose selection was done at 5mm x 5mm tumor size with 2 mice each at 2, 4, 8 Gy dose (N=6) and at 10x10mm tumor size with 2 mice each at 2, 4, 8 Gy dose (N=6); The final experiment done at tumor size 10x10mm size with RT dose of 4Gy (N=8) & 8 Gy dose (N=8) and combined mean length of survival was calculated. Experimental conditions for the mice remained the same as explained above for HBO groups. Animals were subjected to RT only after the tumor size reached the size between 5 to 10 mm. Animals were transferred from the animal holding area of the radiotherapy unit in a specially designed cage fitted with HEPA filter to avoid any infection and exposure to the open air. Cocktail of Ketamine (100mg/Kg) and Xylazine (10mg/Kg) IM anesthesia was used for sedation. The anesthetized mice were prepared for radiotherapy in a SARP (small animal radiation Platform) made of acrylic (Figure 1). Short anesthesia lasting 30-60 minutes was given only to those mice getting exposed to RT for temporary immobilization and we don't expect that such as shot anesthesia will induce any change in the tumor cellularity or morphology as such.

Their hands and legs are sealed and kept inside the chamber. The protection of the tissues located at other parts other than the tumor is very important. For that the parts are shielded with the metal, lead to avoid radiation damage to the tissues. Lead is one of the main metals used for the radiological protection. After the shielding is done in a proper manner they are given the radiation (Figure 2).

After the radiation is given the mice were put under warmer, and were allowed to come out of sedation. Finally put the animal back into respective cage. Mice were followed for weight, tumor regression and recurrence as per the protocol explained above.

Dose of 4 Gy (N=8) & 8 Gy (N=8) X rays were delivered to mice in five fractions using Linear accelerator (LINAC) in normobaric



**Figure 1** Small Animal Radiation Platform (SARP).

conditions daily for 5 days as per the protocol with the tumor shielded by commercially available standard lead shield. Dose of 4 Gy was selected after a trial experiment subjecting 2 mice each at 5x5 mm and 10x10 mm size at 2 Gy, 4 Gy & 8 Gy, 2 Gy showed no response in size reduction while 4 Gy showed good response but the tumor regrew while 8 Gy showed very good response in reducing the size by more than 50 percentile and remained so. We noted hair loss and some local skin burn (darkening of skin color) in mice subjected to 8 Gy radiations. We calculated mean length of survival in days in 16 mice treated with 4 Gy & 8 Gy X rays.

Type of radiation	Radiation Energy	Radiation dose	Dose / Fraction
Electrons	9 MeV	20 Gray (Gy)	4 Gray (Gy)

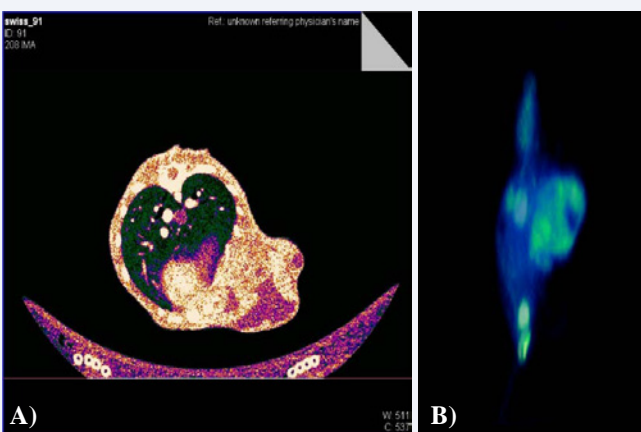
**Experimental protocol for chemotherapy:** Response of 5 FU was noted in 20 tumor bearing mice at tumor size of 10mm x 10mm but not exposed to HBO to compare the mean length of survival & tumor size vis a vis mice exposed to HBO & Radiotherapy alone. Initial dose of 150 mg/Kg was injected intraperitoneally and tumor size was measured daily using caliper till survival and estimated using formula  $V = L \times (W)^2 / 2$ . Growth was calculated as change in tumor growth as compared to Day 1. Before the experimentation, weight of the mice was taken and recorded using weighing balance, dose of 5FU Chemotherapy drug for 150 mg/Kg was calculated, the ampoule of 5 FU was broken in Biosafety cabinet and stored in falcon tubes at room condition, calculated dose was prepared in dilution of 5FU in Phosphate Buffer Saline for intraperitoneal injection. We wiped the surface with spirit before injecting. Animals were strictly followed up daily for symptoms, toxicity and size of the tumor in the isolation area of animal facility. Some lethargy post chemo was observed in all of them and aggressive behavior (hyperactive) in some of them during the next few days.

**Imaging protocol for tumor size measurements and metastasis:** We measured the size of the tumor daily with Vernier caliper and confirmed our caliper measurement in 4 randomly





**Figure 2** Radiotherapy set up showing the placement of SARP under the beam.



**Figure 3** a: Tumor image obtained of Mice on small animal micro CT Imaging. b: Tumor image obtained of Mice on small animal PET Imaging.

selected small animal after the tumor size reached 1cm<sup>2</sup> size at different period of therapy with MRI and micro CT-PET studies [28-30] in our small animal imaging facility as per the standard protocol followed in the facility (Figure 3a, Figure 3b and Figure 4). We performed MRI, micro CT and PET in two different sittings. For micro-CT images and MRI procedure, we used cocktail of IM ketamine (100mg/ml) + xylene (20mg/ml) solution in ratio of 3:1 after placing the animal in anesthetic platform. We used heat lamp to keep the animal heated while injecting the anesthetic agents. Animals were placed in prone position on a multimodality bed post anesthesia while it maintains temperature at 37 degree. For PET procedure, after 8-12 hours of fasting, General anesthesia was induced with 4% Forane @ (Isoflurane) inhalation anesthesia and later maintained with 2% throughout the imaging procedure. After the process is completed the animals are kept back in their cages. Number of literatures is available on small animal PET.

**Micro-CT:** Images were acquired on Flex Triumph GE Trimodality PET/SPECT/CT platform using Triumph XO software version 4.1.1.0 (Gamma Medica-Ideas). The X-ray voltage was set at 80 KVp with anode current 500mA, exposure time 100ms. The scan was completed over 360 of rotation (number of acquisition) with 256 projections (views) and 1.3 x magnifications. The whole body images were acquired with a field of view (FOV) set at 110 mm, with reconstructed voxel size 170 m (matrix size 512 x 512 x 512) without respiratory gating. The total scan time was 12 minutes. The reconstructed image data were visualized and analyzed in VIVID @ (Volumetric Image Visualization, Identification and Display) software based on AMIRA version 4.1 platform (Visage Imaging, San Diego, CA). This scanner offered an excellent spatial resolution and allowed dynamic acquisition while contrast is being administered (Figure 3a).

**PET (Positron emission tomography):** Images were acquired on FLEX triumphtrimodality PET/SPECT/CT platform from WIPRO GE, India. 18F-FDG, 18F-FLT, 18F-NaF in normal and tumor bearing animals were performed. An intra peritoneal injection of 8-10 MBq (18F)-2-fluoro-2-deoxyglucose in a total volume of 100 ml sterile isotonic saline solution was given using 25 gauge needles and data acquisition was started 45 minutes after the administration and a static acquisition was performed for 60 minutes (Figure 3b).

**MRI (Magnetic resonance imaging):** The MRI was done after injecting intraperitoneally gadolinium (0.2ml/kg body weight) intraperitoneally and then the mice was secured in a RF coil; Images were acquired after setting parameters for T1/T2 measurement by spin echo sequence at SL2; TE 20-35ms (multiple), TR 700-2260 ms, FOV 100-140x100-140, image 256x526, T1 - 0, T2- as per the protocol using 3D tesla imaging of GE. T1- and T2-weighted dynamic, contrast- Highlighted the tumors to monitor size and growth (Figure 4).

### Comet assay

Alkaline comet assay was performed as described previously [31]. Briefly, single cell suspension was mixed with low-melting agarose in PBS and was layered on slides pre-coated with normal



**Figure 4** MRI of small animal mice showing liver metastasis of mm size.

agarose. After solidification, the cells were incubated in a pre-cooled lysis buffer. Subsequently, the slides were incubated with chilled electrophoresis buffer and electrophoresis was performed at 25V, 300mA, 40C for 20min. After electrophoresis, slides were washed with neutralization buffer and stained with Propidium iodide (50µg/ml). DNA of individual cells was viewed using a Zeiss upright fluorescence microscope connected to a CCD camera.

### Histone isolation and immunoblot analysis

Histones were extracted and purified as described by [31] with slight modifications. The tissue (400 mg) was homogenized in chilled buffer A (0.34M Sucrose, 50mM Tris-Cl pH 7.5, 25mM KCl, 5mM MgCl<sub>2</sub>, 1mM DTT, 0.1% TritonX100, 0.1mM EDTA pH 8.0, 0.15mM EGTA, 0.1mM PMSF, 0.5mM spermidine). The homogenate was centrifuged at 3000g for 15min at 4°C. The pellet was suspended in chilled buffer A followed by centrifugation at 2,000g, 15min at 4°C. The pellet obtained was further used for histone extraction by addition of 0.2N H<sub>2</sub>SO<sub>4</sub>. Intermittent vortexing was done for 2-3hr to extract the chromatin bound histone followed by high speed centrifugation at 14,000g for 20min at 4°C. The supernatant containing histone protein was precipitated with four volumes of chilled acetone and left overnight at -20°C. The histone pellet obtained was washed twice with acidified acetone (50mM HCl) followed by two washes of chilled acetone. The histone pellet was dried and dissolved in 0.1% βME. Histones were stored at -20°C. Histones resolved on 18% SDS-polyacrylamide gel were transferred to PVDF membrane and probed with gamma H2AX antibody. The signal was detected by using ECL plus western blot detection kit (Millipore; Catalog no.WBKLS0500) (Figure 5, Figure 6).

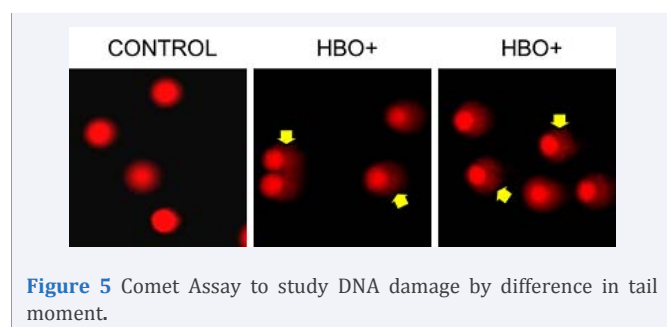
Data availability statement - The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## RESULTS AND DISCUSSION

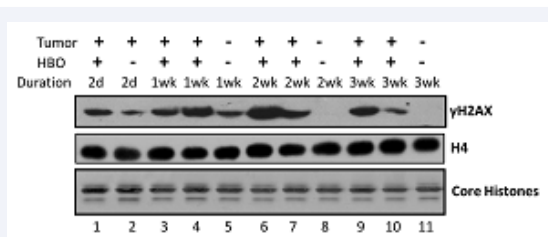
In our first paper in this series, we showed tumor regression during HBO therapy where end point tumor size/area was also correspondingly less compared to the size attained at the specified time in the untreated control group. It was probably due to reduced cell proliferation or cell turnover rate (slow regenerative potential) and /or increased cellular damage as result of HBO therapy. The results also showed accelerated tumor growth and poor survival benefit post HBO therapy compared to a control group [27].

In this paper, we compared the end point survival duration

of mice subjected to HBO with the mice subjected to chemo & radiotherapy. The length of survival in the HBO treated mice (N=29) was 78. Significant survival benefits were also not observed in post chemotherapy group (N=20) & radiotherapy group (N=16) where mean survival days were 146 & 130 days respectively when compared to 127 days in the control group (N=20). The shortest length of survival in HBO group was possibly due to high oxidative injury as a result of uninterrupted & longer exposure to HBO, or metastasis and/or rebound growth post therapy or some unknown underlying molecular changes. We tried to search for underlying molecular changes that may be linked to rebound growth and poor survival benefit in HBO group. It is a known fact that exposure to high concentrations of oxygen or free oxygen radical induces cell death or injury by causing DNA damage and the preliminary results obtained by us also showed DNA damage as such in tumor samples exposed to HBO with some evidence of histone modification. The comet assay clearly showed increased DNA damage after HBO exposure compared to control. Thus, we specifically extended our study to investigate the DNA-damaging effect of HBO with the alkaline version of the single cell gel test (comet assay) and analyzing the phosphorylation status of H2AX in tumor samples exposed to increasing duration of HBO exposure. To test whether, increase in observed DNA damage also resulted in increase of H2AX phosphorylation at Serine 139, western blot studies were carried out with site-specific antibody. The data suggested significant increase in the level of γH2AX in HBO treated animals compared to control. Therefore, we observed that the exposure to higher levels of oxygen under hyperbaric conditions caused oxidative stress *in vivo* and induced DNA damage & increased level of γH2AX, which increases the probability of mutation and chance of aneuploidy. The tumor growth acceleration observed post HBO therapy was most likely due to the epigenetic effects (histone modification) following severe DNA damage during HBO therapy. Therefore based upon the initial results obtained in this study, we proposed that in any pathological conditions, where DNA instability exists one should be careful in manipulating or modulating oxygen milieu or metabolism or tumor microenvironment. The re-oxygenation with 100% oxygen or HBO or on the contrary inducing any kind of hypoxia could be potentially dangerous or disturbing to the existing phenotypical modified cancer cells that has adapted to aerobic glycolysis and acidosis. Modulating tumor microenvironment either by creating hypoxia or hyperoxia or hyper-baria should be considered carefully and with utmost seriousness where outcome should be weighed critically keeping in mind the chances of enhancing aneuploidy, mutation and metastasis. Whenever any treatment modality is planned to modulate tumor microenvironment, the primary objective should be to ensure that such therapy should achieve maximum cell kill in tumor mass both in non invasive localized stage or when invasion through the basement membrane has already taken place. We also followed up the tumor growth progression and metastasis with small animal PET/CT/MRI *vis a vis* manual measurement with Vernier caliper. Additionally, we also noted complications, injuries, side effects during experimental period and performed histopathology corroboration of metastasis, cellular necrosis & degenerations following their death after conducting post mortem studies.



**Figure 5** Comet Assay to study DNA damage by difference in tail moment.



**Figure 6** The data suggested significant increase in the level of  $\gamma$ H2AX in HBO treated animals compared to control. It showed response to HBO over 1 to 3 weeks period.

The results obtained from the study fits well with our hypothesis i.e., creating further state of instability or perturbation in a newly acquired equilibrium or steady state condition as achieved by any physiological or pathological systems, cellular milieu or tumor microenvironment may potentiate or aggravate the pre-existing state by disturbing the cellular milieu resting in the state of dormancy. Thus, HBO therapy which is known to modulate the tumor microenvironment and metabolic pathways should be considered judiciously as an adjuvant to radiotherapy, chemotherapy or prior to surgery or as palliative therapy. One should keep in mind the treatment outcome in terms of length of survival and better quality of life.

## CONCLUSION

Most of the tumor modulators or cancer adjuvant therapy leads to tumor size reduction however patients expect long term survival benefits with improved quality of life. The therapeutic goal of any such therapy is non-emergence of a mutant variety of cancer cells that usually leads to recurrence and/or accelerated growth following a period of remission or a state of dormancy. Our study clearly showed the need to exercise optimal caution while manipulating or modulating cancer micro environment using modalities capable of altering the metabolic internal milieu such as inducing hypoxia or hyperbaric hyperoxia (HBO).

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## AUTHORS CONTRIBUTIONS

ASG – Wrote & edited the full manuscript, designed the study, conducted the HBO experiments, analyzed the data; overall supervision of the work SG- wrote protein analysis part of the manuscript, designed & conducted protein studies, analyzed

the data; supervised the work AI-designed and advised upon the animal studies and analysis; JG- Designed and conducted radiotherapy part of the study.

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