

## Original Article

## Ozone Therapy for Tumor Oxygenation: a Pilot Study

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Tumor hypoxia is an adverse factor for chemotherapy and radiotherapy. Ozone therapy is a non-conventional form of medicine that has been used successfully in the treatment of ischemic disorders. This prospective study was designed to assess the effect of ozone therapy on tumor oxygenation. Eighteen subjects were recruited for the study. Systemic ozone therapy was administered by auto-hemotransfusion on three alternate days over one week. Tumor oxygenation levels were measured using polarographic needle probes before and after the first and the third ozone therapy session. Overall, no statistically significant change was observed in the tumor oxygenation in the 18 patients. However, a significant decrease was observed in hypoxic values  $\leq 10$  and  $\leq 5$  mmHg of  $pO_2$ . When individually assessed, a significant and inverse non-linear correlation was observed between increase in oxygenation and the initial tumor  $pO_2$  values at each measuring time-point, thus indicating that the more poorly-oxygenated tumors benefited most ( $\rho = -0.725$ ;  $P = 0.001$ ). Additionally, the effect of ozone therapy was found to be lower in patients with higher hemoglobin concentrations ( $\rho = -0.531$ ;  $P < 0.034$ ). Despite being administered over a very short period, ozone therapy improved oxygenation in the most hypoxic tumors. Ozone therapy as adjuvant in chemo-radiotherapy warrants further research.

**Keywords:** cancer – hypoxia –  $pO_2$  measurement – polarographic probe

### Introduction

Tumor hypoxia can cause an increase in radio-resistance by up to 2.5–3 times (1) and predisposes a physiologic selection of tumor cells with decreased apoptosis. This results in additional resistance to radiotherapy and chemotherapy (2) and further increase in angiogenesis and a more aggressive tumor potential (3–5).

Tumor hypoxia, when assessed by polarographic probes, is an independent prognostic factor for response to treatment and/or survival of head and neck tumors (6–9) and uterine cervical tumors (10,11) as well as sarcomas (12,13). The polarographic probe technique was designated as ‘gold standard’ for tumor  $pO_2$  measurement in a special workshop sponsored by the National Cancer Institute (14), at which the importance of developing methods to overcome tumor hypoxia was emphasized. Since then, meta-analyses have

demonstrated that hypoxia modification during radiotherapy can improve treatment outcomes (15).

Ozone therapy has been shown to be beneficial to patients with ischemic disorders, particularly of the lower limbs (16–18). In our previous studies we had found that ozone therapy increases oxygenation in the most poorly-oxygenated tissues of the anterior tibialis muscles (19) and that oxygenation in these muscles might be related to tumor oxygenation (20).

The objective of the present preliminary (and prospective) study is to evaluate the effect of ozone therapy on tumor oxygenation, using the polarographic probe measurement technique.

### Subjects and Methods

#### Patients

Eighteen patients with accessible metastases or advanced tumors were enrolled in the study (14 with head and neck tumors, 2 with gynecological tumors and two bone metastases in chest wall region). Patients comprised 15 males and 3 females with mean age of 64 years (range, 50–91). The selection criteria included the following: a minimum age of 18

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**Table 1.** Characteristics of the patients and their tumors

Patient	Age	Location	Stage	Size	Hb	pO <sub>2</sub> pre-1	pO <sub>2</sub> post-1	pO <sub>2</sub> pre-3	pO <sub>2</sub> post-3
1	70	Maxillary bone relapsed chondrosarcoma		4 * 4	13.4	1	N.A.	33.9	21.9
2	60	Vulva, relapsed			NA	36	47.3	11.5	11.9
3	56	Tongue	T4 N2c M0	4 * 2	13.5	70	68.7	76.7	65.3
4	63	Hypopharynx	T4 N2a M0	5.5 * 5.5	12.1	17	N.A.	29.0	29.5
5	91	Hodgkin disease	II-A	9 * 6	10.8	3	9.1	18.1	9.7
6	51	Thyroid Medullar carcinoma	T4 N1 M1	10 * 7	13.4	29	38.8	31.5	34.1
7	52	Nasopharynx	T4 N2c M0	5 * 5	15.6	64	56.3	48.3	45.7
8	50	Vagina	T3 N0 M0		13.2	2	4.7	24.2	19.3
9	60	Oropharynx, relapsed		3 * 3	14.0	38	39.6	39.0	32.0
10	76	Chest wall metastases clear cell carcinoma	Tx Nx M1	6 * 6	13.3	43	48.5	39.6	18.9
11	69	Chest wall metastases hepatocellular carcinoma	T3 N0 M1	10 * 8	NA	29	42.9	34.4	34.0
12	65	Oesophagus, relapsed		3 * 3	10.8	1	31.8	3.1	30.9
13	68	Hypopharynx	T3 N2b M1	3 * 3	11.8	38	47.3	35.9	27.1
14	53	Hypopharynx	T4 N3 M1	12 * 10	11.7	15	38.0	31.1	45.0
15	74	Oropharynx	T4 N2c M0	5 * 3.5	15.3	7	8.6	12.0	6.8
16	63	Supraglottis	T2 N3 M0	8.5 * 5.5	13.6	3	9.9	13.2	4.3
17	67	Oral cavity	T4 N3 M0	8.5 * 5.5	14.7	1	2.2	1.3	.9
18	71	Cervical metastases from UPT	Tx N3 M1	12 * 7.5	15.6	17	17.1	9.0	13.9

Stage; T = Tumor; N = Node; according to AJCC cancer staging manual, 5th edition. Size = largest diameter clinically measured (in cm). All pO<sub>2</sub> measurements were in the largest node. UPT = unknown primary tumor. pO<sub>2</sub> pre-1 = pO<sub>2</sub> before session #1 = basal. pO<sub>2</sub> post-1 = pO<sub>2</sub> after session #1. pO<sub>2</sub> pre-3 = pO<sub>2</sub> 48 h after session #2 and before session #3. pO<sub>2</sub> post-3 = pO<sub>2</sub> after session 3. N.A. = not available.

years, Karnofsky performance status of >70%, cancer diagnosis histologically confirmed with metastases or advanced tumors accessible to physical examination and not being suitable for surgical resection. The mean of measured tumors/nodes was 6.5 cm across the greatest diameter (range, 3–12 cm). The exclusion criteria included the following: unwillingness to participate in the study, treatment with experimental or evaluation drugs during the planned study or not fulfilling all of the selection criteria described above. The experimental nature of the study was explained in detail and informed consent was obtained from all patients prior to study. The study was approved by the Institutional Ethical Committee.

### Ozone Therapy

Ozone therapy was administered by autohemotransfusion on three alternate days over one week. The procedure involved the extraction of 200 ml venous blood into heparin (25 IU/ml) and CaCl<sub>2</sub> (5mM). Using clinical-grade O<sub>2</sub>, the O<sub>3</sub>/O<sub>2</sub> gas mixture was prepared with an OZON 2000 device (Zotzmann + Stahl GmbH, Plüderhausen, Germany) and sterilized by passing it through a sterile 0.20- $\mu$ m filter. The blood was mixed with 200 ml of the O<sub>3</sub>/O<sub>2</sub> gas mixture at a concentration of 60  $\mu$ g/ml, in a single-use sterile container with a capacity of 300 ml. Following this, it was slowly re-introduced into the patient's body. The blood had been extra-corporeal for about 15–30 minutes but no adverse reactions were observed. Table 1 summarizes some of the most relevant clinical characteristics of the patients.

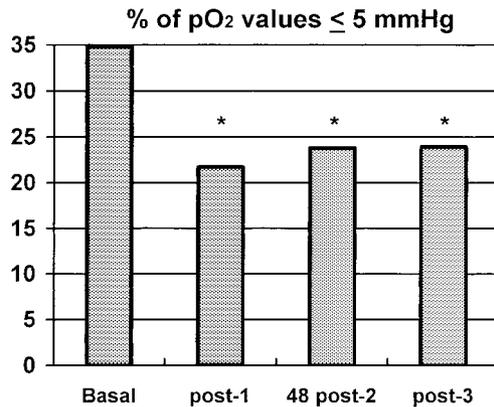
### Tumor pO<sub>2</sub> Measurement

Tumor oxygenation was measured using a polarographic probe system: the 'pO<sub>2</sub> Histogram' (Eppendorf AG, Hamburg, Germany). The details of this technique have been described previously (21). Briefly, a 0.5 mm diameter electrode (0.3 mm diameter at the tip) is inserted into the tumor while the patient is under subcutaneous anesthesia. The movement is computer controlled and consists of a 1 mm forward motion and a 0.3 mm backward motion to avoid tissue compression at the measurement site. A pO<sub>2</sub> value is obtained at every 0.7 mm. For each set of measurements obtained, 150–200 single pO<sub>2</sub> values were automatically recorded using at least six different electrode tracks. To determine tumor oxygenation, median pO<sub>2</sub> and the percentage of pO<sub>2</sub> values  $\leq$ 10 mmHg and  $\leq$ 5 mmHg were obtained from the pooled data for each individual.

Tumor oxygenation values were obtained on four occasions: First, before session #1; second, after session #1; third, 48 h after session #2 and before session #3; fourth, after session #3.

For each tumor, the change in oxygenation ( $\Delta$ pO<sub>2</sub>) was calculated as the pO<sub>2</sub> value at each time-point relative to the pre-session #1 ('baseline') pO<sub>2</sub> value.

The measurements were carried out on accessible, clinically palpable lymph nodes or subcutaneous metastases without using an imaging technique.



**Figure 1.** Change in percentage of pO<sub>2</sub> values ≤ 5 mmHg. During ozone therapy, a decrease in percentage of pO<sub>2</sub> values ≤ 5 mmHg at each measurement time-point was observed in the tumors of patients: *Baseline* = before ozone therapy; *post-1* = after session #1 ( $P = 0.002$ ); *48 post-2* = 48 h after session #2 ( $P = 0.045$ ); *post-3* = after session #3 ( $P = 0.033$ ). Significant differences ( $P < 0.05$ ) are indicated with an asterisk (\*)

### Statistical Analysis

The SPSS 11.0 for Windows software package was used for this study. The distribution of data was assessed by the Kolmogorov–Smirnov test. Two-tailed tests were applied for significance. The paired t-test was used to compare means of all the median tumor values and all the percentages of the ≤10 and ≤5 mmHg measurements. These data are expressed as means ± SD. The Mann–Whitney U test was used to compare the  $\Delta pO_2$  between tumors above and below the median baseline pO<sub>2</sub>. These data are expressed as median and 25%–75% inter-quartile interval. Linear correlation was assessed by Pearson's  $r$  test and non-linear correlation by Spearman's  $\rho$  test. Differences were considered significant at the  $P < 0.05$  level.

## Results

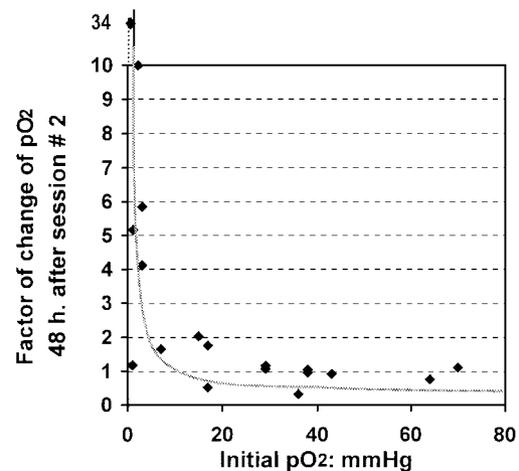
### Tumor Oxygenation

The patient's individual data for hemoglobin levels and pO<sub>2</sub> values at each measurement time-point are shown in Table 1. Initial tumor oxygenation was  $23 \pm 5.1$  mmHg, and was not related to sex, age, hemoglobin levels, clinical status or tumor size.

After session #1 tumor oxygenation was  $31.9 \pm 5.1$  mmHg, and this difference was significant,  $P = 0.009$ . However, no statistically significant differences were found in the other two measurement time-point: 48 h after session #2 ( $27.3 \pm 4.3$  mmHg) and after session 3 ( $25.1 \pm 3.9$  mmHg).

### Hypoxic Values

The percentage of values ≤10 mmHg at the baseline proceeded to decrease significantly during ozone therapy from  $40.8 \pm 7.3\%$  to  $27.4 \pm 7.3\%$  ( $P = 0.002$ ) after session #1 and to  $29 \pm 6.2\%$  ( $P = 0.039$ ) 48 h after session #2. The decrease



**Figure 2.** Factor of change in pO<sub>2</sub> ( $\Delta pO_2$ ) and initial pO<sub>2</sub> For each participant, the  $\Delta pO_2$  was calculated as the pO<sub>2</sub> value at each time-point relative to the baseline pO<sub>2</sub> value measured before the start of the ozone therapy. A non-linear correlation was found between baseline pO<sub>2</sub> and  $\Delta pO_2$  at each measurement time-point. The figure shows an inverse correlation ( $\rho = -0.798$ ) after session #3 of ozone therapy, which indicates that the highest therapy-associated changes in tumor pO<sub>2</sub> occurred in tumors with the poorest baseline oxygenation. A  $\Delta pO_2$  value  $< 1$  signifies decrease in oxygenation and  $\Delta pO_2 > 1$  signifies an increase in tumor oxygenation after session #3.

to  $31 \pm 5.1\%$  after session #3 did not qualify as statistical significance ( $P = 0.058$ ).

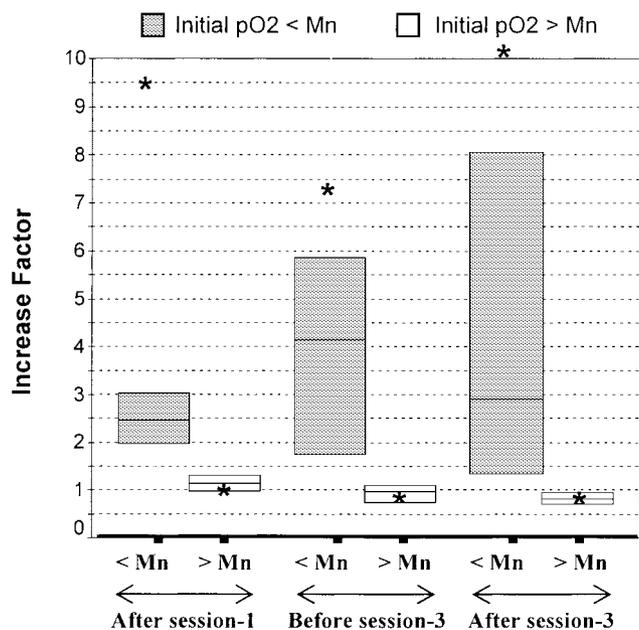
The percentage of values ≤5 mmHg at the baseline proceeded to decrease significantly during ozone therapy from  $34.8 \pm 7.5\%$  to  $21.7 \pm 6.9\%$  ( $P = 0.002$ ) after session #1, to  $23.8 \pm 5.9\%$  ( $P = 0.045$ ) 48 h after session 2 and to  $23.9 \pm 4.9\%$  ( $P = 0.033$ ) after session #3 (Fig. 1).

### Factor of Change of pO<sub>2</sub> ( $\Delta pO_2$ ):

At each measurement time-point, an inverse and non-linear correlation was found between individual  $\Delta pO_2$  and initial pO<sub>2</sub> values. A higher  $\Delta pO_2$  was observed in those tumors that had had lower initial pO<sub>2</sub> values. Significant changes were observed after session #1 ( $\rho = -0.812$ ,  $P < 0.001$ ), 48 h after session #2 ( $\rho = -0.798$ ,  $P < 0.001$ ) and after session #3 ( $\rho = -0.725$ ,  $P = 0.001$ ) (Fig. 2).

This was corroborated by the comparison of  $\Delta pO_2$  between tumors above and below the median pO<sub>2</sub> prior to ozone therapy (baseline), at each measurement time-point. While the initially well-oxygenated tumors (those above the median) showed oxygenation decrease, the initially most poorly-oxygenated tumors (those below the median) showed an increase in oxygenation after the ozone therapy. The changes recorded were a factor of 2.5 (range, 2–3.1;  $P = 0.002$ ) after session #1, a factor of 4.1 (range, 1.7–8;  $P < 0.001$ ) 48 h after session #2, and a factor of 2.9 (range, 1.1–15;  $P = 0.002$ ) after session #3 (Fig. 3).

Further, at each measurement time-point, an inverse, non-linear correlation between individual  $\Delta pO_2$  and hemoglobin levels was found. The  $\Delta pO_2$  in tumors was lower in patients with higher hemoglobin levels after session #1 ( $\rho = -0.650$ ,



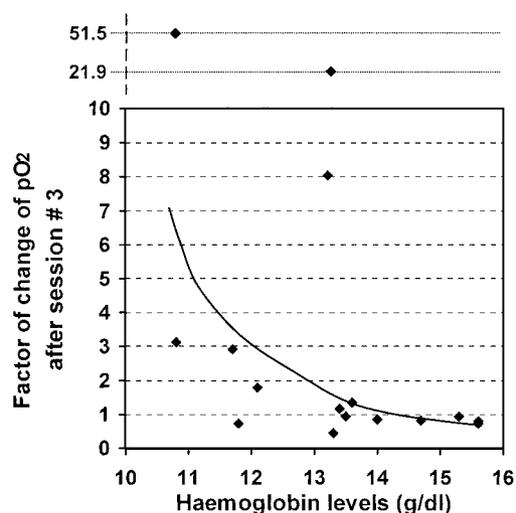
**Figure 3.** Factor of change of  $pO_2$  ( $\Delta pO_2$ ) segregated with respect to the initial median  $pO_2$ . The figure shows the  $\Delta pO_2$  at each measurement time-point following ozone therapy and segregated with respect to baseline  $pO_2$  value above or below the median  $pO_2$  value (17 mmHg) of the overall study group. The boxes show the 25%–75% inter-quartile interval, which includes the 50% values. The horizontal lines in the boxes represent the median and the \* represents the mean of  $\Delta pO_2$  for both groups of tumors at each measurement time-point. During ozone therapy, well-oxygenated tumors (baseline  $pO_2$  above the median) showed no change ( $\Delta pO_2$  approximately 1) or even decrease after session #3 ( $\Delta pO_2 = 0.8$ ). However the most ‘poorly-oxygenated’ tumors (baseline  $pO_2$  below the median) showed increase in tumor oxygenation ( $\Delta pO_2 > 1$ ). These differences were significant at all the three measurement time-points ( $P = 0.002$ ,  $0.001$  and  $0.002$ , respectively). < Median = tumors with baseline  $pO_2$  values below the median value; > Median = tumors with baseline  $pO_2$  values above the median value.

$P = 0.012$ ), 48 h after session #2 ( $\rho = -0.531$ ,  $P = 0.034$ ) and after session #3 ( $\rho = -0.579$ ,  $P = 0.019$ ) (Fig. 4).

## Discussion

Ozone ( $O_3$ ) is the allotropic form of oxygen with three atoms and two unpaired electrons, which has a higher oxidizing capacity than oxygen. In order to avoid lung toxicity, medical applications of ozone require to preclude airways involvement. Autohemotransfusion fulfils this requirement. In appropriate concentrations, this technique leads to a transient oxidative stress that can stimulate blood antioxidants by up-regulation (22–24). This mechanism has been ascribed to ozone therapy’s protection against free radical damage of heart (22), and prevention of renal (25) and hepatic (26) disorders. Hemolysis of <2.5% and an acceptable level of lipid peroxide formation has been described in autohemotransfusion at  $O_3/O_2$  concentrations of 60  $\mu g/ml$  (23).

The objective of the present study was to assess whether changes in tumor oxygenation occurred during ozone therapy. Each patient served as his own control and elective non-ozonated autohemotransfusion was not performed in a sepa-



**Figure 4.** Factor of change of  $pO_2$  ( $\Delta pO_2$ ) after session #3 and hemoglobin levels. There was an inverse and non-linear correlation between hemoglobin levels and the  $\Delta pO_2$  at each measurement time-point following ozone therapy, i.e., a lower effect of ozone therapy was observed in patients with higher hemoglobin levels. The figure shows the correlation with the  $\Delta pO_2$  after session #3 ( $\rho = -0.579$ ,  $P = 0.019$ ).

rate control group. It was not considered ethical for these advanced cancer patients to undergo invasive study-manipulations over several days in a control group which, theoretically, did not offer any potential benefit (transfusion of oxygenated blood is not a therapeutical approach). On the other hand, several studies have already demonstrated that the administration of ozone-free oxygen in a control group does not produce the ‘prooxidant/antioxidant’ response necessary to mediate the clinical effects of ozone therapy. This reaction was produced only when ozone was added to oxygen in equimolar amounts (18, 24 and 26).

In the course of ozone therapy by autohemotransfusion, ozone, *per se*, does not enter the organism, and its effects are mediated by rapid (a matter of seconds) oxidation of blood components in the transfusion recipient. The oxidized molecules and the specific antioxidant generated would vary according to the levels of ozone therapy. The vascular effect of ozonated blood transfusion is explained by an increase of malonyldialdehyde and lipid peroxidation leading to leading to activation of the hexose monophosphate shunt with an increased production of 2,3-diphosphoglycerate in erythrocytes (27). This results in a displacement of the oxyhemoglobin dissociation curve to the right and an increase in the release of oxygen to the tissues. A pH decrease in erythrocytes may also shift the oxyhemoglobin dissociation curve to the right (Bohr effect) without modification of 2,3-diphosphoglycerate (28). Furthermore, a charge modification in red cell membranes results in an improvement in membrane flexibility and a decrease in blood viscosity and resistance (18,29). Adenosine, prostaglandins and, especially, nitric oxide release could collaborate in affecting the micro-circulation and lead to a decrease in vascular resistance (30).

Overall, ozone therapy decreased the percentage of values  $\leq 10$  and  $\leq 5$  mmHg at each measurement time-point. How-

ever, no increase was observed in tumor  $pO_2$ , as has been reported in an animal study (31). In the present study, the oxygenation decreased in tumors with  $pO_2$  concentrations above the median. Based on the oxygen radio-sensitivity curve, it can be inferred that this is not of clinical relevance in well-oxygenated tumors. However, in tumors with baseline  $pO_2$  below the median, i.e. tumors in which the radio-resistance could increase in relation to this 'adverse' value, ozone therapy actually increased the tumor  $pO_2$ . This effect is similar to that observed by us (19) in anterior tibialis muscle tissues following the administration of ozone therapy.

The mechanisms underlying this effect in tumors have yet to be defined. Based on previously described effects, we hypothesize that the inverse correlation between initial oxygenation and  $\Delta pO_2$  in tumors and tissues during ozone therapy is secondary to blood flow redistribution, i.e., a drop in blood flow in well-oxygenated tissues in favor of less well-oxygenated tissues. Tumor vessels have structural and functional abnormalities with decreased or absent auto-regulatory mechanisms (32). Hence, an improvement in blood rheologic parameters, as described by other authors (18,29), could play an important role in the effect of ozone therapy in high-resistance systems such as in tumors; this could apply to at least the areas of the tumor that are most hypoxic. Congruent with this concept is the improvement we observed with ozone therapy in patients with lower hemoglobin levels and, as a consequence, with lower blood viscosity. This vascular effect is further supported by our preliminary studies with Doppler techniques, indicating a lasting blood flow increase following three alternating ozone therapy sessions (B. Clavo, personal communication). However, our hypothesis of an increase in tumor perfusion resulting from ozone therapy needs further confirmation with studies specifically addressing the effect on tumor blood flow using, for example, multi-channel laser Doppler.

Techniques such as hyperbaric chambers or carbogen breathing plus nicotinamide can increase arterial  $pO_2$ , with secondary tumor  $pO_2$  increase. Usually, however, this is less effective in modifying hypoxic areas and, as well, the effect is of a very short duration; of the order of 10–15 minutes (33). Furthermore, if applied for more than 15–30 min, these therapies can lead to vaso-constriction resulting in a potential blood-flow decrease, secondary to hyperoxia, in most organs (34) as well as in tumors (33). Our results show that, in the most hypoxic tumors, ozone therapy leads to an improvement in tissue  $pO_2$  for at least 48 h after the second session of therapy. Similarly, it should be noted that the hypoxic fraction was decreased for protracted periods. Nevertheless, better results could probably be achieved using combined therapies, principally, techniques to increase blood oxygenation.

On the other hand, metastatic or large-size tumors are probably not the best situations in which to evaluate oxygen delivery or the vascular effect of ozone therapy, as observed in anemia-modification studies (35). However, for the pur-

pose of the present study, the patients selected were those with advanced cancer or with large affected nodes that were easily accessible to physical examination so as to facilitate the tumor  $pO_2$  measurements.

Tumor hypoxia predisposes to a physiologic selection of tumor cells with decreased apoptotic potential, which results in resistance to radiotherapy and chemotherapy (2), higher angiogenesis and a more aggressive tumor potential (3–5). If ozone therapy successfully decreases tumor hypoxia in some patients, it could be useful as an adjuvant in the treatment of these patients by improving tumor oxygenation, by reducing radio-resistance and improving local control. Survival could be improved by decreasing tumor hypoxia, as shown by Overgaard's meta-analyses (15). The results of the present study indicate that tumor  $pO_2$  modification could support the anecdotal clinical reports of an improved effect of radiotherapy in advanced tumors when ozone therapy is included in the schedule (36).

Radio-mimetic (37) and synergistic (38) effects of radiotherapy as well as growth inhibition of human cancer cells by ozone (39) and increase in chemo-sensitivity in colon carcinoma cells resistant to 5-fluorouracil (40) have been described; albeit, these effects of ozone are not directly applicable to human ozone therapy. However, from a clinical oncology point of view, further research needs to be conducted on the effects of ozone-enriched blood. The effects described in relation to increasing antioxidant (22–26) and cytokine production (41,42) are particularly relevant. A review on the potential role of ozone therapy as a biological response modifier in oncology has been published by Bocci (43), and we concur with the view that the appropriate controlled clinical trials would be particularly valuable.

In conclusion, many aspects regarding the bio-medical application of ozone therapy remain unexplored. In the present prospective study, the effect of ozone therapy on human tumor  $pO_2$  has been measured using the polarographic probe technique, and the results indicate that ozone therapy could increase oxygenation in the most hypoxic tumors. This suggests the potential use of this therapy as adjuvant in chemo-radiotherapy schedules, and would warrant further investigation.

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